Development of Antibody-based Strategies for the Detection of Mastitis and its Causes in Dairy Cattle

A thesis submitted for the degree of Ph.D.

by

Sushrut Arora M.V.Sc.,

Based on the research carried out at the
School of Biotechnology
and the
Biomedical Diagnostics Institute,
Dublin City University,
Dublin 9, Ireland.

Under the supervision of Professor Richard O'Kennedy

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Declaration:

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _____ (Candidate) ID No.: 56124066 Date:_____

Dedicated to

My Parents

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ABSTRACT

Mastitis is the major disease affecting cattle worldwide and there is a requirement for specific, highly sensitive and easy-to-detect biomarkers for mastitis detection. *N*-acetyl-β-D-glucosaminidase (NAGase) is such a marker, however, currently available assays for NAGase detection lack reproducibility and are not sufficiently sensitive. The major aim of this project was to generate antibodies and immunoassays to NAGase using polyclonal, monoclonal and recombinant antibodies.

Purified NAGase was unavailable commercially. Therefore, purification of NAGase from bovine spleen tissue, using fast protein liquid chromatography, and bacterial expression, were used to generate sufficient quantities of the enzyme, and associated antigenic peptides of the required purity, for antibody and assay generation. A bioinformatics-based approach was also used to compare NAGases from a variety of species to help elucidate their similarities, differences and variations in selective pressure. Difficulties were encountered in antibody generation and this appears to be due, at least in part, to lack of immunogenicity.

A bovine scFv library from the spleens of cows with a history of chronic mastitis was generated and, subsequently, used in attempts to select antibodies to the major aetiological agent responsible for causing mastitis. This is a novel approach and there is no previous report of the generation of such a bovine scFv library.

Progesterone has the potential to act as a biomarker for mastitis detection. Anti-progesterone antibodies were generated and incorporated into an inhibition ELISA.

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ABBREVIATIONS AND ACRONYMS

Abbreviation / Acronym	Full form
2D-GE	Two-dimensional gel electrophoresis
Abs	Antibodies
Abs	Absorbance
aLTR	Approximate Likelihood Ratio Test
AP	Alkaline phosphatase
APP	Acute phase protein
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BEB	Bayes Empirical Bayes
BHV	Bovine herpes virus
BIV	Bovine immunodeficiency virus
BLAST	Basic Local Alignment Search Tool
BLV	Bovine leukaemia virus
BS	Bootstrap support
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhoea virus
CD	Compact disc
cDNA	Complementary DNA
CDR	Complementary determining region
cfu	Colony forming units
C_H	Constant heavy chain
C_L	Constant light chain
CL	Corpus luteum
CMT	California Mastitis Test
CT	C-terminal
C-terminal	Carboxyl terminal
CV	Column volume
DNA	Deoxyribonucleic acid
dt	Different oligo
DTT	1,4-Dithio-DL-threitol
EC	Electrical conductivity
e.g.	*exempli gratia* (Latin for "for example")
EK	Enterokinase
ELISA	Enzyme-linked immunosorbent assay
et al.	*et alia* (Latin for 'and the rest')
etc.	*et cetera* (Latin for 'and so on')

Fab	Antibody binding fragment
FACS	Fluorescence activated cell sorting
Fc	Fragment crystallisable (of an antibody)
FCS	Foetal calf serum
FG	Fish gelatine
FMD	Foot-and-mouth disease
FPLC	Fast protein liquid chromatography
FR	Framework region
FF	Fast Flow
GalNAc	β-D-*N*-acetylgalactosamine
GlcNAc	β-D-*N*-acetylglucosamine
HA	Haemagglutinin
HAT	Hypoxanthine, Aminopterin, and Thymidine
Hb	Haemoglobin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hex	Hexosaminidase
HEXA	Gene coding for hexosaminidase α-subunit / α-polypeptide
HexA	Hexosaminidase A
HEXB	Gene coding for hexosaminidase β-subunit / β-polypeptide
HexB	Hexosaminidase B
HexS	Hexosaminidase S
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
HIC	Hydrophobic interaction chromatography
His	Histidine
Hp	Haptoglobin
HP	High Performance
HRP	Horseradish peroxidase
HSA	Human serum albumin
HT	Hypoxanthine and Thymidine
HV	Hypervariable
i.e.	*id est* (Latin for 'that is')
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IMAC	Immobilised metal affinity chromatography

IMS	Industrial methylated spirit
IPTG	Isopropyl-β-D-thiogalactoside
KLH	Keyhole Limpet Haemocyanin
LB	Luria-Bertani
LC-Biotin	Long chain biotin
LDH	Lactate dehydrogenase
LTR	Likelihood test ratio
mAbs	Monoclonal antibodies
ML	Maximum-likelihood
MOPS	3 (N-Morpholino) propane sulfonic acid
MS	Mass spectroscopy
MWCO	Molecular weight cut off
NAGase	*N*-acetyl-β-D-glucosaminidase / β-*N*-acetylglucosaminidase / β-hexosaminidase
NASBA	Nucleic Acid Sequence Based Amplification
NEB	Naïve Empirical Bayes
N-terminal	Amino terminal
OD	Optical density
OVA	Albumin from chicken egg white; ovalbumin
P4	4-Pregnen-3, 20-dione; progesterone
pAbs	Polyclonal antibodies
PAML	Phylogenetic Analysis by Maximum Likelihood
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-Tween
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGF2α	Prostaglandin F2α
PGN	Peptidoglycan
PI	Parainfluenza
PIPES	Piperazine-N,N′-bis (2-ethanesulfonic acid)
PMN	Polymorphonuclear neutrophil
pNPP	p-Nitrophenyl phosphate
Prep	Preparation
Progesterone-3-CMO	4-Pregnen-3, 20-dione 3-O-carboxymethyloxime
Progesterone-3-CMO-BSA	4-Pregnen-3, 20-dione 3-O-carboxymethyloxime - bovine serum albumin
rAbs	Recombinant antibodies
rEK	Recombinant Enterokinase
SAA	Serum amyloid A

SAATK	*Staphylococcus aureus* antibody test kit
SCC	Somatic cell count
scFv	Single chain variable fragment
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOC	Super optimal catabolities
SOE	Splice by overlap extension
SPCE	Screen-printed carbon electrode
SPF	Specific pathogen free
ssDNA	Single-stranded DNA
sub	Substitution
TEA	Triethanolamine
TEMED	Tetramethylethylenediamine
TK	Thymidine kinase
TMB	Tetramethylbenzidine dihydrochloride
TNFα	Tumour necrosis factor-α
Trx	Thioredoxin
UV	Ultraviolet
V_H	Variable heavy chain (of antibody)
V_L	Variable light chain (of antibody)
vs.	Versus
VMRD	Veterinary Medical Research and Development

UNITS

Unit	Full form
µF	Microfarad
µg	Microgram
µL	Microlitre
µm	Micrometre
cm	Centimetre
Da	Daltons
FU	Fluorescence Unit
G	Gram
kDa	Kilodaltons
kV	Kilovolt
L	Litre
M	Molar
mg	Milligram
mL	Millilitre
mM	Millimolar
ng	Nanogram
nm	Nanometer
^{o}C	Degrees Celcius
pfu	Plaque-forming unit
rpm	Révolutions per minute
U	Unit
V	Volts
v/v	Volume per unit volume
w/v	Weight per unit volume
x g	Centrifugal acceleration
Ω	Ohm

PUBLICATIONS AND PRESENTATIONS

Research Publications

Arora, S., Morgan, C.C., Lynch, M.G., Loscher, C.E., O'Kennedy, R. and O'Connell, M.J., 2011. Evidence for positive selection in sites neighboring TaySachs and Sandhoff disease implicated regions. (Manuscript in preparation).

Gilmartin, N., Guo, W.L., Viguier, C., Welbeck, K., **Arora, S.** and O'Kennedy, R., 2011. The relevance of lactate dehydrogenase, *N*-acetyl-β-D-glucosaminidase, lactation stage and storage conditions in the diagnosis of mastitis. Journal of Dairy Research (Submitted).

Welbeck, K., Leonard, P., Gilmartin, N., Byrne, B., Viguier, C., **Arora, S.** and O'Kennedy, R., 2011. Generation of an anti-NAGase single chain antibody and its application in a biosensor based assay for the detection of NAGase in milk. Journal of Immunological Methods **1-2**:14-20.

Reviews

Dixit, C.K., **Arora, S.**, McCraith, B.D. and O'Kennedy, R., 2011. Immobilising antibody fragments: a comprehensive review. (Manuscript in preparation).

Ayyar, B.V., **Arora, S.**, Murphy, C.S. and O'Kennedy, R., 2011. Affinity chromatography as a tool for antibody purification. Methods (Submitted).

Viguier, C., **Arora, S.**, Gilmartin, N., Welbeck, K. and O'Kennedy, R., 2009. Mastitis detection: current trends and future perspectives. Trends in Biotechnology **27**, 486-493.

Book Chapter

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Arora, S., Hearty, S., Ayyar, B.V. and O'Kennedy, R., 2010. Generation of novel immunoreagents for antibody-based detection of NAGase in mastitic milk. 5th International Dairy Federation (IDF) International Mastitis Conference, Christchurch, New Zealand (March 21-24th, 2010).

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Invention Disclosure

O'Kennedy, R., Viguier, C., Gilmartin, N., Welbeck, K. and **Arora, S.**, 2008. Development of novel reagents, approaches and principles for the isolation and analysis of *N*-acetyl-β-D-glucosaminidase (A and B isomeric forms) and its relevance in the diagnosis of disease states such as mastitis. (Submitted to Invent, DCU in September, 2008).

Chapter 1
Introduction

1. INTRODUCION

1.1 Mastitis

1.1.1 Introduction

Bovine mastitis (mast-breast; itis-inflammation), a major disease affecting dairy cattle worldwide, results from the inflammation of the mammary gland (Harmon, 1994). The severity of the inflammation can be classified into sub-clinical, clinical and chronic forms, with its degree dependent on the nature of the causative pathogen and on the age, breed, immunological health and lactation state of the animal. Sub-clinical mastitis is difficult to detect, due to the absence of any visible indications, and it has major cost implications (Harmon, 1994). Chronic mastitis is a more rare form of the disease but results in persistent inflammation of the mammary gland. Currently, milk quality payments are based on somatic cell counts (SCC) with elevated levels resulting in reduced payments. This, in addition to reduction in milk volume and treatment costs, significantly affects farm incomes (Yalcin, 2000). Table 1.1 gives a brief overview of the effects of mastitis.

Table 1.1. ***Impact of mastitis.***

Direct effects (Yalcin, 2000; Hillerton and Berry, 2005)
o Temporary or permanent loss in milk production.
o Poor milk quality, *e.g.* reduction in milk fat content, resulting in dairy products with less favourable organoleptic properties.
o Reduction in price due to high somatic cell count.
o Loss due to discarding of milk after the antibiotic treatment.
o Additional treatment costs, *e.g.* drugs, veterinary costs.
o Increased labour costs, *e.g.* extra labour required for husbandry of cattle and for application of preventive measures.
o Increased costs for surveillance of milk quality and disease status among the rest of the herd.
o Premature culling or reduced productive-life of cattle.
o Lower value for culled cattle meat as the carcass yield and quality is reduced.
Financial Costs (Donovan *et al.*, 2005; Hillerton and Berry, 2005; Huijps *et al.*, 2008)
o In the United States the projected annual losses due to mastitis are $2 billion.
o In UK mastitis causes an annual loss of approximately £300 million to dairy farmers.
o In Northern Ireland the cost of clinical mastitis for an average 100-cow herd is £5,000 per year, with total mastitis infections costing £14 million, annually.
o In the Republic of Ireland the cost of clinical mastitis is approximately €693 per year for every infected cow.
o In the Netherlands average costs per infected cow vary between €164 and €235.

1.1.2 Aetiology

Mastitis may be infectious, caused by microbial organisms, or non-infectious, resulting from chemical, thermal or mechanical injury to the gland (Kuang *et al.*, 2009; Zhang *et al.*, 2009). Over 150 mastitis-inducing species have been identified (Kuang *et al.*, 2009) that utilise nutrient-rich milk to multiply and, subsequently, synthesise toxins, enzymes and metabolites that ultimately damage the milk-secreting tissues of the mammary gland. Aetiological agents of mastitis are discussed below.

1.1.2.1 Bacteria

The primary cause of mastitis is a wide spectrum of invasive Gram-positive and Gram-negative bacterial strains (Watts, 1988), and a comprehensive list of the known mastitis-causing prokaryotes is presented in Table 1.2. These species are classified as 'major', *e.g. Staphylococcus aureus* or *Escherichia coli*, or 'minor' pathogens, which include coagulase-negative staphylococcal strains and the facultatively anaerobic pathogen *Corynebacterium bovis*, according to their prevalence and the severity of infection caused (Harmon *et al.*, 1986).

Furthermore, these pathogens may be described as being contagious or environmental (Blowey and Edmondson, 1995). Contagious pathogens may spread from one cow to another *via* poor farming practices, such as improper milking techniques or by using contaminated farming equipment. Some infection sites may be sequestered on the udder or in the teat lesions of the cow (Blowey and Edmondson, 1995; Radostits, 1984). Contagious pathogens include *Mycoplasma* spp., *S. aureus* (Akineden *et al.*, 2001; Hillerton and Berry, 2005; Zschock *et al.*, 2005) and various streptococcal strains such as *S. agalactiae*, *S.*

dysgalactiae and *S. uberis* (Watts and Owens, 1988). In contrast, environmental pathogens originate from faecal matter, soil, animal bedding or water and typically include coliforms and pseudomonads. A report by Bradley and Green (2001a) suggests that these strains can exhibit pathogenicity traits similar to their contagious counterparts in that they are able to proliferate and survive in the udder of the infected animal. This is unusual as these organisms tend to decrease in numbers after a relatively short time post infection. For instance, strains of the facultative anaerobe *E. coli* may also invade and survive in mammary epithelial cells and, in so doing, evade the immune response elicited by the host (Dogan *et al.*, 2006). These persistent intra-mammary infections (Dopfer *et al.*, 1999) occur in 5-24% of all *E. coli*-related mastitis infections (Bradley and Green, 2001b; Dogan *et al.*, 2006).

Further complexity arises from the fact that between 20 and 35% of all mastitis cases arise from unidentified sources (Miltenburg *et al.*, 1996; Wedderkopp, 1997). A contributory factor is the difficulty in cultivating all of the causative agents, such as *Mycoplasma* spp. and certain yeasts and moulds. An alternative argument is that these so-called 'difficult to cultivate' micro-organisms cannot be responsible for all of the culture-negative milk samples isolated from mastitic cows as these pathogens are not prevalent udder pathogens (Pfutzner, 1994; Wendt, 1994).

Mycoplasma spp. are often overlooked as causative agents of mastitis (Nicholas and Ayling, 2003) and several reports have outlined the increased incidence of mycoplasma-related mastitis (Wilson *et al.*, 1997; Burnens *et al.*, 1999; Ghadersohi *et al.*, 1999; Brice *et al.*, 2000; Kusiluka *et al.*, 2000;

Feldmann *et al.*, 2003; Thomas *et al.*, 2003; Al-Abdullah and Fadl, 2006; Kampa *et al.*, 2009; Hertl *et al.*, 2010). The most frequently encountered strain of this bacterium that has infected cattle in the US, Australia and throughout Europe is *Mycoplasma bovis* (Gonzalez and Wilson, 2003; Ayling *et al.*, 2004). Eleven other *Mycoplasma* and *Acholeplasma* species have been also isolated from infected milk (*Mycoplasma alkalescens*, *M. arginini*, *M. bovigenitalium*, *M. bovirhinis*, *M. californicum*, *M. canadense*, *M. dispar*, *Mycoplasma* species group 7, *Mycoplasma* F-38, *Acholeplasma laidlawii* and *A. axanthum*) (Gonzalez and Wilson, 2003) with the severity of illness caused differing with each strain.

Several strains of *Chlamydiales* have also been shown to cause mastitis. These include *Chlamydia psittaci* (Kaltenboeck *et al.*, 1991)*, Chlamydophila abortus* and *C. pecorum* (Biesenkamp-Uhe *et al.*, 2007).

The wide range of strains responsible for mastitis suggest that the monitoring of bacterial strains and numbers is of paramount importance for determining the onset of mastitis.

Table 1.2. ***Bacterial causative agents of mastitis.***

Genus	Species / Serotypes	References
Gram positive bacteria		
Aerococcus	*A. viridans*	Jayarao *et al.*, 1992; Jayarao and Oliver, 1994
Arcanobacterium	*A. pyogenes* obsolete names *Actinomyces pyogenes* and *Corynebacterium pyogenes*	Shukla *et al.*, 1998; Turutoglu and Mudul, 2002; Deutz *et al.*, 2005; Ya Dong *et al.*, 2005; Lakew *et al.*, 2009; Thorberg *et al.*, 2009; Garmo *et al.*, 2010; Hertl *et al.*, 2010
Arcobacter	*A. cryaerophilus*	Pianta *et al.*, 2007
	A. butzleri	Pianta *et al.*, 2007
Bacillus	*B. cereus*	Hussain *et al.*, 2006; Varma and Dorairajan, 2006
	B. licheniformis	Nieminen *et al.*, 2007
	B. pumilus	Nieminen *et al.*, 2007
	B. subtilis	Hussain *et al.*, 2006
	Unidentified	Nagal *et al.*, 1999; Turutoglu and Mudul, 2002; Deutz *et al.*, 2005; Lakew *et al.*, 2009
Corynebacterium	*C. amylocolatum*	Watts and Rossbach, 2000; Watts *et al.*, 2000; Hadimli *et al.*, 2006
	C. bovis	Watts and Rossbach, 2000; Watts *et al.*, 2000; Hadimli *et al.*, 2006; Lakew *et al.*, 2009; Thorberg *et al.*, 2009; Hertl *et al.*, 2010
	C. minutissimum	Hommez *et al.*, 1999
	C. pseudotuberculosis	Hommez *et al.*, 1999; Hadimli *et al.*, 2006
	C. ulcerans	Hommez *et al.*, 1999
	C. xerosis	Hadimli *et al.*, 2006
	Unidentified	Nagal *et al.*, 1999; Bueno *et al.*, 2003; Sudhan *et al.*, 2005; Fadlelmula *et al.*, 2009;

		Garmo *et al.*, 2010
Enterococcus	*E. avium*	Jayarao *et al.*, 1992
	E. faecalis	Watts *et al.*, 1993; Petersson-Wolfe *et al.*, 2007; Rysanek *et al.*, 2009
	E. faecium	Petersson-Wolfe *et al.*, 2008; Rysanek *et al.*, 2009
	E. saccharolyticus synonym *Streptococcus saccharolyticus*	Jayarao *et al.*, 1992
	Unidentified	Gianneechini *et al.*, 2002; Park *et al.*, 2007
Listeria	*L. monocytogenes*	Winter *et al.*, 2004; Rawool *et al.*, 2007; Varma *et al.*, 2007
Micrococcus	Unidentified	Dego and Tareke, 2003; Ozdemir, 2005; Sudhan *et al.*, 2005; Hussain *et al.*, 2006; Malinowski and Smulski, 2007; Lakew *et al.*, 2009
Peptostreptococcus	*P. indolicus*	Jonsson *et al.*, 1991; Madsen *et al.*, 1992; Jousimies-Somer *et al.*, 1996
Staphylococcus	*S. albus*	Hussain *et al.*, 2006
	S. aureus	Kim, 1990; Bes *et al.*, 2000; Gianneechini *et al.*, 2002; Chhabra and Arora, 2006; Bradley *et al.*, 2007; Kumar *et al.*, 2007; Fadlelmula *et al.*, 2009; Kampa *et al.*, 2009; Lakew *et al.*, 2009; Rysanek *et al.*, 2009; Thorberg *et al.*, 2009; Fessler *et al.*, 2010; Garmo *et al.*, 2010; Hertl *et al.*, 2010
	S. capitis	Kim, 1990; Martin and Bergmann, 1993
	S. chromogenes	Bes *et al.*, 2000; Kirkan *et al.*, 2005; Malinowski *et al.*, 2006; Thorberg *et al.*, 2009
	S. cohnii	Kim, 1990; Martin and Bergmann, 1993; Bes *et al.*, 2000; Kirkan *et al.*, 2005; Thorberg *et al.*, 2009
	S. epidermidis	Kim, 1990; Martin and Bergmann, 1993; Bes *et al.*, 2000; Kirkan *et al.*, 2005; Thorberg *et al.*, 2009
	S. equorum	Bes *et al.*, 2000

	S. haemolyticus	Kim, 1990; Martin and Bergmann, 1993; Bes *et al.*, 2000; Malinowski *et al.*, 2006; Thorberg *et al.*, 2009
	S. hominis	Kim, 1990; Martin and Bergmann, 1993
	S. hyicus	Kirkan *et al.*, 2005; Malinowski *et al.*, 2006; Thorberg *et al.*, 2009
	S. intermedius	Chaffer *et al.*, 1998; Reyes *et al.*, 2005
	S. kloosi	Thorberg *et al.*, 2009
	S. lentis	Kirkan *et al.*, 2005
	S. saprophyticus	Kim, 1990; Martin and Bergmann, 1993; Ozdemir, 2005; Thorberg *et al.*, 2009
	S. sciuri	Martin and Bergmann, 1993; Kirkan *et al.*, 2005; Malinowski *et al.*, 2006; Thorberg *et al.*, 2009
	S. simulans	Martin and Bergmann, 1993; Kirkan *et al.*, 2005; Malinowski *et al.*, 2006; Thorberg *et al.*, 2009
	S. uberis	Bradley *et al.*, 2007
	S. warneri	Martin and Bergmann, 1993; Bes *et al.*, 2000; Thorberg *et al.*, 2009
	S. xylosus	Kim, 1990; Martin and Bergmann, 1993; Bes *et al.*, 2000; Malinowski *et al.*, 2006; Thorberg *et al.*, 2009
	Unidentified	Kim, 1990; Martin and Bergmann, 1993; Bueno *et al.*, 2003; Deutz *et al.*, 2005; Hertl *et al.*, 2010
Streptococcus	*S. acidominimus*	Jousimies-Somer *et al.*, 1996
	S. agalactiae	Bueno *et al.*, 2003; Deutz *et al.*, 2005; Kivaria and Noordhuizen, 2007; Pianta *et al.*, 2007; Piepers *et al.*, 2007; Rakotozandrindrainy and Foucras, 2007; Fadlelmula *et al.*, 2009; Kampa *et al.*, 2009; Lakew *et al.*, 2009; Nam *et al.*, 2009
	S. bovis	Nam *et al.*, 2009

	S. canis	Hassan *et al.*, 2005
	S. dysgalactiae	Benites *et al.*, 2002; Gianneechini *et al.*, 2002; Turutoglu *et al.*, 2002; Borkowska *et al.*, 2006; Haltia *et al.*, 2006; Hameed *et al.*, 2007; Kivaria and Noordhuizen, 2007; Moatamedi *et al.*, 2007; Pianta *et al.*, 2007; Riekerink *et al.*, 2007; Fadlelmula *et al.*, 2009; Lakew *et al.*, 2009; Rysanek *et al.*, 2009; Thorberg *et al.*, 2009; Garmo *et al.*, 2010
	S. equi subsp*. Ruminatorum*	Fernandez *et al.*, 2004
	S. equinus	Jayarao *et al.*, 1992; Watts *et al.*, 1993
	S. intermedius	Nam *et al.*, 2009
	S. mitis	Jayarao *et al.*, 1992
	S. oralis	Nam *et al.*, 2009
	S. parauberis	Watts *et al.*, 1993
	S. pyogenes	Nagal *et al.*, 1999
	S. saccharolyticus	Jayarao *et al.*, 1992
	S. salivarius	Jayarao *et al.*, 1992; Nam *et al.*, 2009
	S. uberis	Turutoglu *et al.*, 2002; Bueno *et al.*, 2003; Kivaria and Noordhuizen, 2007; Fadlelmula *et al.*, 2009; Nam *et al.*, 2009; Rysanek *et al.*, 2009; Thorberg *et al.*, 2009; Garmo *et al.*, 2010
	S. zooepidemicus	Sharp *et al.*, 1995
	Unidentified	Bueno *et al.*, 2003; Deutz *et al.*, 2005; Thorberg *et al.*, 2009; Garmo *et al.*, 2010; Hertl *et al.*, 2010
Gram negative bacteria		
Acinetobacter	*A. baumannii*	Nam *et al.*, 2010

Aeromonas	*A. hydrophila*	Bergmann *et al.*, 1981
	A. sobria	Kumar *et al.*, 2001
	Unidentified	Costa *et al.*, 1986
Bacteroides	*B. fragillis*	Jousimies-Somer *et al.*, 1996
	B. melaninogenicus	Jonsson *et al.*, 1991; Madsen *et al.*, 1992
Campylobacter	*C. jejuni*	Gudmundson and Chirino-Trejo, 1993; Orr *et al.*, 1995; Varma *et al.*, 2007
Chromobacterium	*C. violaceum*	Watts, 1988
Citrobacter	*C. freundii*	Ghadersohi *et al.*, 1999; Turutoglu and Mudul, 2002; Nam *et al.*, 2010
	Unidentified	Klossowska *et al.*, 2005; Hertl *et al.*, 2010
Clostridium	*C. perfringens* type A	Goncalves *et al.*, 2006
Enterobacter	*E. aerogenes*	Turutoglu and Mudul, 2002; Kivaria and Noordhuizen, 2007
	E. agglomerans	Wenz *et al.*, 2001
	E. cloacae	Varma and Dorairajan, 2006; Chockalingam *et al.*, 2007; Nam *et al.*, 2010
	Unidentified	EunSil *et al.*, 2007; Vijayalakshmi and Prathaban, 2007; Fadlelmula *et al.*, 2009; Garmo *et al.*, 2010; Hertl *et al.*, 2010
Escherichia	*E. coli*	Nagal *et al.*, 1999; Bradley and Green, 2001a; Turutoglu and Mudul, 2002; Bueno *et al.*, 2003; Bean *et al.*, 2004; Bradley *et al.*, 2007; Kivaria and Noordhuizen, 2007; Fadlelmula *et al.*, 2009; Lakew *et al.*, 2009; Rysanek *et al.*, 2009; Thorberg *et al.*, 2009; Garmo *et al.*, 2010; Hertl *et al.*, 2010
	Verotoxin-Producing *E. coli* VTEC	Stephan and Kuhn, 1999
Fusobacterium	*F. nectrophorum*	Jonsson *et al.*, 1991; Jousimies-Somer *et al.*, 1996
Hafnia	*H. alvei*	Klossowska *et al.*, 2005
Klebsiella	*K. pneumoniae*	Kivaria and Noordhuizen, 2007; Munoz *et al.*, 2007; Nam *et al.*, 2010

	K. oxytoca	Ghadersohi *et al.*, 1999; Opsomer *et al.*, 2001; Fadlelmula *et al.*, 2009
	Unidentified	Hussain *et al.*, 2006; Thorberg *et al.*, 2009; Hertl *et al.*, 2010
Mannheimia haemolytica	*M. haemolytica* obsolete name *Pasteurella haemolytica*	Daignault *et al.*, 1996; Wenz *et al.*, 2001
Morgenella	*M. morganni*	Ghadersohi *et al.*, 1999
Neisseria	Unidentified	Klossowska *et al.*, 2005
Pasteurella	*P. multocida*	Nagal *et al.*, 1999; Wenz *et al.*, 2001
	Unidentified	Deutz *et al.*, 2005
Prevotella	Unidentified	Jousimies-Somer *et al.*, 1996
Proteus	*P. mirabilis*	Shukla *et al.*, 1998
	Unidentified	Nagal *et al.*, 1999; Deutz *et al.*, 2005; Hussain *et al.*, 2006; Dutta *et al.*, 2007; Garmo *et al.*, 2010
Pseudomonas	*P. aeruginosa*	Nagal *et al.*, 1999; Turutoglu *et al.*, 2002; Kivaria and Noordhuizen, 2007; Fadlelmula *et al.*, 2009; Rysanek *et al.*, 2009; Nam *et al.*, 2010
	Unidentified	Deutz *et al.*, 2005; Hussain *et al.*, 2006; Hertl *et al.*, 2010
Salmonella	*S. enterica* serotype Typhimurium	Wenz *et al.*, 2001
Shigella	Unidentified	Fadlelmula *et al.*, 2009
Serratia	*S. liquefaciens*	Bowman *et al.*, 1986
	S. marcescens	Kamarudin *et al.*, 1996; Di Guardo *et al.*, 1997; Reugg and Reinemann, 2002; Chockalingam *et al.*, 2007; Nam *et al.*, 2010
	Unidentified	Deutz *et al.*, 2005; EunSil *et al.*, 2007
Yersinia	*Y. pseudotuberculosis*	Sampimon *et al.*, 2005; Shwimmer *et al.*, 2007
Spirochetes		

Leptospira	*L. interrogans* serotype Hardjo	Kocabiyik and Cetin, 2003; Vidic and Bobos, 2003
Acid fast bacteria		
Mycobacterium	*Mycobacterium avium* subsp. *Paratuberculosis*	Raizman *et al.*, 2007
	M. smegmatis	Schultze and Brasso, 1987; Thomson *et al.*, 1988
	M. fortuitum	Salman *et al.*, 1982
	M. cheloni	Salman *et al.*, 1982
	Runyon group IV	Schultze and Brasso, 1987
Nocardia	*N. asteroides*	Schultze and Brasso, 1987; Cook and Holliman, 2004)
	N. caviae	Salman *et al.*, 1982
	N. farcinica	Brown *et al.*, 2007
	N. neocaledoniensis	Pisoni *et al.*, 2008
	Unidentified	Bueno *et al.*, 2003; Deutz *et al.*, 2005; Hussain *et al.*, 2006

1.1.2.2 Viruses

While bacterial strains are the predominant etiological causes of mastitis, the incidence of viral, algal and fungal-related illness should not be overlooked. A certain proportion of the unidentified causes of clinical and subclinical mastitis may arise from viral sources and shed viral particles may be detected in milk (Fuchs, 1994). These include bovine herpes virus (BHV1) (Gourlay *et al.*, 1974), and BHV4 (Wellenberg, 2002), foot-and-mouth disease (FMD) virus (Burrows *et al.*, 1971; Fuchs, 1994) and parainfluenza 3 (PI3) virus (Kawakami *et al.*, 1966).

While the participation of viruses in mastitis pathogenicity is the subject of debate (Wellenberg, 2002), one theory is that teat lesions that arise from viral infections affect the integrity of the bovine udder and this may inadvertently contribute to the onset of mastitis. Furthermore, ulcerations resulting from local dermatitis induced by bovine herpesvirus type 2 (*Bovine Herpes mammillitis*) virus (BHV2), vaccinia, cowpox, pseudocowpox, FMD viruses and vesicular stomatitis virus may be prone to secondary bacterial infections in the mammary gland (Francis, 1984).

Finally, in addition to viruses that cause teat lesions, other viral infections may induce or enhance bovine mastitis due to their immunosuppressive effects. These viruses include BHV1 virus (Straub, 1991), bovine viral diarrhoea virus (BVDV) (Waage, 2000), bovine leukaemia virus (BLV) (Rusov *et al.*, 1991; Emanuelson *et al.*, 1992; Jacobs *et al.*, 1995) and bovine immunodeficiency virus (BIV) (Snider *et al.*, 1996).

1.1.2.3 Fungi

Fungal intra-mammary infections result from various genera of yeast and moulds. The extensive administration of antibiotics and the performance of intra-mammary procedures in the absence of aseptic protocols are the primary reasons for mycotic mastitis (Elad *et al.*, 1995; Moretti *et al.*, 1998). The isolation of fungal species from bovine milk was first reported in 1901 (Beck, 1957). Mycotic mastitis tends to occur sporadically, with enzootic forms occurring less frequently (Krukowski, 2001). The most frequent causes of fungal mastitis are outlined in Table 1.3 and these include *Candida* spp., *Aspergillus* spp., *Trichosporon* spp., *Cryptococcus* spp. and *Saccharomyces* spp. (Costa *et al.*, 1993; Ebrahimi and Nikookhah, 2005; Vimalraj *et al.*, 2006; Seker, 2010). The most severe mastitis infection is caused by *Cryptococcus neoformans* which causes permanent udder damage in the infected cow (Schalm *et al.*, 1971).

The monitoring of the occurrence of fungal mastitis tends to vary between countries, with incidences ranging from between 1.3% and 29% (Costa *et al.*, 1993; Krukowski *et al.*, 2006; Kivaria and Noordhuizen, 2007). There is also a higher incidence of fungal mastitis in tropical countries than in temperate countries (Pal and Randhawa, 1976; Lagneau, 1996; Lagneau *et al.*, 1996). Studies have also shown that the incidence of fungal mastitis has increased significantly in several other countries (Costa *et al.*, 1993; Moretti *et al.*, 1998). The results of a recent survey demonstrated a steady increase in the occurrence of clinical *Candida albicans* mastitis in a region of Tanzania, with a significantly higher prevalence (17%) than for a similar study made in 1971 (less than 1%) (Kivaria and Noordhuizen, 2007).

Table 1.3. ***Fungal causative agents of mastitis.***

Genus	Species	References
Yeast		
Aureobasidium	*A. pullulans*	Costa *et al.*, 1993
Blastoschizomyces	*B. capitatus*; obsolete name -*Trichosporon capitatum*	Krukowski *et al.*, 2001; Benites *et al.*, 2002; Krukowski *et al.*, 2006
Candida	*C. albicans*	Ognean *et al.*, 1992; Costa *et al.*, 1993; Kuo and Chang, 1993; Shukla *et al.*, 1998; Chhabra *et al.*, 1999; Das *et al.*, 1999; Krukowski *et al.*, 2001; Casia dos Santos and Marin, 2005; Ebrahimi and Nikookhah, 2005; Krukowski *et al.*, 2006; Senthilvelan *et al.*, 2006; Kivaria and Noordhuizen, 2007; Seker, 2010
	C. catenulate	Watts, 1988; Costa *et al.*, 1993
	C. ciferrii	Costa *et al.*, 1993; Krukowski *et al.*, 2001; Krukowski *et al.*, 2006
	C. diversa	Lagneau *et al.*, 1996
	C. famata; obsolete name - *Torulopsis candida*	Costa *et al.*, 1993; Kuo and Chang, 1993; Casia dos Santos and Marin, 2005; Seker, 2010
	C. firmetaria; obsolete name - *C. lambica*	Lagneau *et al.*, 1996; Krukowski *et al.*, 2006
	C. freyschussii	Costa *et al.*, 1993
	C. glabrata	Lagneau *et al.*, 1996; Klossowska and Malinowski, 2001; Ebrahimi and Nikookhah, 2005; Krukowski *et al.*, 2006; Seker, 2010
	C. glaebosa	Costa *et al.*, 1993
	C. globosa	Costa *et al.*, 1993
	C. guilliermondii	Watts, 1988; Kuo and Chang, 1993; Lagneau *et al.*, 1996; Casia dos Santos and Marin, 2005; Kivaria and Noordhuizen, 2007; Seker, 2010
	C. haemulonii	Costa *et al.*, 1993
	C. inconspicua	Krukowski, 2001; Krukowski *et al.*, 2006
	C. intermedia	Costa *et al.*, 1993
	C. kefyr; obsolete name – *C. pseudotropicalis*	Ognean *et al.*, 1992; Lagneau *et al.*, 1996; Das *et al.*, 1999; Klossowska and Malinowski, 2001; Krukowski *et al.*, 2001;

		Malinowski *et al.*, 2001; Krukowski *et al.*, 2006; Seker, 2010
	C. krusei	Singh *et al.*, 1989; Costa *et al.*, 1993; Kuo and Chang, 1993; Elad *et al.*, 1995; Sheena and Sigler, 1995; Chhabra *et al.*, 1999; Das *et al.*, 1999; Klossowska and Malinowski, 2001; Krukowski *et al.*, 2001; Malinowski *et al.*, 2001; Casia dos Santos and Marin, 2005; Fadlelmula *et al.*, 2009; Gaudie *et al.*, 2009; Seker, 2010
	C. lipolytica	Kuo and Chang, 1993
	C. lusitaniae	Watts, 1988; Kuo and Chang, 1993; Krukowski *et al.*, 2006
	C. membranifaciens	Watts, 1988
	C. mogii	Costa *et al.*, 1993
	C. norvegensis	Watts, 1988; Krukowski *et al.*, 2006
	C. parapsilosis	Singh *et al.*, 1989; Costa *et al.*, 1993; Kuo and Chang, 1993; Lagneau, 1996; Moretti *et al.*, 1998; Chhabra *et al.*, 1999; Krukowski *et al.*, 2001; Casia dos Santos and Marin, 2005; Krukowski *et al.*, 2006; Fadlelmula *et al.*, 2009; Seker, 2010
	C. pelliculosa	Watts, 1988; Kivaria and Noordhuizen, 2007
	C. rugosa	Watts, 1988; Costa *et al.*, 1993; Kuo and Chang, 1993; Krukowski *et al.*, 2001; Casia dos Santos and Marin, 2005; Krukowski *et al.*, 2006; Seker, 2010
	C. shehatae	Costa *et al.*, 1993
	C. solani	Watts, 1988
	C. sorbosa	Costa *et al.*, 1993
	C. tenuis	Costa *et al.*, 1993
	C. tropicalis	Watts, 1988; Ognean *et al.*, 1992; Costa *et al.*, 1993; Kuo and Chang, 1993; Lagneau *et al.*, 1996; Moretti *et al.*, 1998; Chhabra *et al.*, 1999; Das *et al.*, 1999; Klossowska and Malinowski, 2001; Krukowski *et al.*, 2001; Ebrahimi and Nikookhah, 2005; Kivaria and Noordhuizen, 2007; Seker, 2010

	C. variabilis	Costa *et al.*, 1993
	C. vini	Das *et al.*, 1999; Casia dos Santos and Marin, 2005
	C. zeylanoides	Costa *et al.*, 1993; Casia dos Santos and Marin, 2005; Seker, 2010
	Unidentified	Costa *et al.*, 1993; Eulalia Garcia and Blanco, 2000; Krukowski *et al.*, 2006; Vimalraj *et al.*, 2006
Cryptococcus	*C. albidus*	Watts, 1988; Costa *et al.*, 1993; Lagneau *et al.*, 1996
	C. curvatus	Lagneau *et al.*, 1996
	C. flavus	Costa *et al.*, 1993
	C. humicolus; obsolete name - *Candida humicola*	Kuo and Chang, 1993; Krukowski *et al.*, 2006
	C. laurentii	Costa *et al.*, 1993; Lagneau *et al.*, 1996
	C. luteolos	Costa *et al.*, 1993; Lagneau *et al.*, 1996
	C. neoformans	Sharma *et al.*, 1977; Watts, 1988; Bada *et al.*, 1992; Daignault *et al.*, 1997; Klossowska and Malinowski, 2001
	Unidentified	Eulalia Garcia and Blanco, 2000; Vimalraj *et al.*, 2006
Debaryomyces	*D. hansenii*	Lagneau *et al.*, 1996
Geotrichum	*G. candidum*	Misra and Panda, 1986; Kuo and Chang, 1993; Chahota *et al.*, 2001; Klossowska and Malinowski, 2001
	G. klebahnii; obsolete name – *Trichosporon penicillatum*	Kuo and Chang, 1993
	Unidentified	Watts, 1988; Singh *et al.*, 1989; Costa *et al.*, 1993; Costa *et al.*, 1998; Ebrahimi and Nikookhah, 2005
Pichia	*P. angusta*; obsolete name – *Hansenula polymorpha*	Watts, 1988
	P. anomala; obsolete name – *Hansenula anomala*	Watts, 1988; Kuo and Chang, 1993
	P. fabianii; obsolete name – *Hansenula fabianii*	Watts, 1988
	P. holstii; obsolete name – *Hansenula holstii*	Watts, 1988
	P. membranifaciens	Lagneau *et al.*, 1996

	P. ohmeri	Costa *et al.*, 1993
Rhodotorula	*R. glutinis*	Watts, 1988; Costa *et al.*, 1993; Krukowski *et al.*, 2001; Krukowski *et al.*, 2006
	R. minuta	Costa *et al.*, 1993
	R. mucilaginosa; obsolete name - *R. rubra*	Costa *et al.*, 1993; Das *et al.*, 1999
	Unidentified	Cuci and Matraku, 1987; Ebrahimi and Nikookhah, 2005
Saccharomyces	*S. cerevisiae*	Ognean *et al.*, 1992; Moretti *et al.*, 1998; Das *et al.*, 1999; Klossowska and Malinowski, 2001; Malinowski *et al.*, 2001; Krukowski *et al.*, 2006
Trichosporon	*T. asahii*	Lagneau *et al.*, 1996; Moretti *et al.*, 1998; Klossowska and Malinowski, 2001; Fadlelmula *et al.*, 2009
	T. beigelii	Kuo and Chang, 1993; Gonzalez *et al.*, 2001; Benites *et al.*, 2002
	T. cutaneum	Watts, 1988; Costa *et al.*, 1993; Krukowski, 2006
	Unidentified	Cuci and Matraku, 1987; Vimalraj *et al.*, 2006
Moulds		
Absidia	Unidentified	Mehrotra and Rawat, 1989
Acremonium	Unidentified	Cuci and Matraku, 1987; Mehrotra and Rawat, 1989
Alternaria	Unidentified	Cuci and Matraku, 1987; Mehrotra and Rawat, 1989; Singh *et al.*, 1989; Costa *et al.*, 1993
Aspergillus	*A. amstelodami*	Misra and Panda, 1986
	A. chevalieri	Misra and Panda, 1986
	A. ficuum	Sharma *et al.*, 1977
	A. flavus	Misra and Panda, 1986; Reddy and Khan, 1994; Chhabra *et al.*, 1999; Das *et al.*, 1999
	A. fumigates	Schallibaum *et al.*, 1980; Singh *et al.*, 1989; Ognean *et al.*, 1992; Reddy and Khan, 1994; Chhabra *et al.*, 1999; Das *et al.*, 1999; Ebrahimi and Nikookhah, 2005
	A. nidulans	Schallibaum *et al.*, 1980
	A. niger	Misra and Panda, 1986; Singh *et al.*, 1989;

		Reddy and Khan, 1994; Chhabra *et al.*, 1999; Das *et al.*, 1999
	A. sydowii	Misra and Panda, 1986
	A. terreus	Sharma *et al.*, 1977
	Unidentified	Mehrotra and Rawat, 1989; Costa *et al.*, 1993; Klossowska and Malinowski, 2001; Vimalraj *et al.*, 2006
Cladosporium	Unidentified	Singh *et al.*, 1989
Curvularia	*C. lunata*	Sharma *et al.*, 1977
	C. verruculosa	Das *et al.*, 1999
	Unidentified	Cuci and Matraku, 1987; Mehrotra and Rawat, 1989
Epicoccum	Unidentified	Costa *et al.*, 1993
Fusarium	Unidentified	Singh *et al.*, 1989; Reddy and Khan, 1994
Monilia	Unidentified	Mehrotra and Rawat, 1989
Mortierella	*M. wolfii*	Mac Donald and Corbel, 1981
Mucor	Unidentified	Mehrotra and Rawat, 1989; Chhabra *et al.*, 1999; Das *et al.*, 1999
Paecilomyces	Unidentified	Cuci and Matraku, 1987
Penicillium	Unidentified	Misra and Panda, 1986; Mehrotra and Rawat, 1989; Costa *et al.*, 1993; Reddy and Khan, 1994; Chhabra *et al.*, 1999; Das *et al.*, 1999
Phoma	Unidentified	Costa *et al.*, 1993
Pseudallescheria	*P. boydii*; obsolete name – *Petriellidium boydii*	Thompson *et al.*, 1978
Rhizopus	Unidentified	Mehrotra and Rawat, 1989; Singh *et al.*, 1989; Vimalraj *et al.*, 2006
Trichophyton	*T. verrucosum*	Kivaria and Noordhuizen, 2007
Trichoderma	*T. koningii*	Cuci and Matraku, 1987
	T. viride	Cuci and Matraku, 1987
Trichothecium	*T. roseum*	Cuci and Matraku, 1987

1.1.2.4 Algae

The first incidence of bovine mastitis caused by algae was reported in 1952 with *Prototheca zopfii*. *P. wickerhamii* was subsequently also associated mastitis (Dion, 1982; Mc Donald *et al.*, 1984). Recently, a new species, *Prototheca*

blaschkeae, was also implicated as mastitis-causing algae (Marques *et al.*, 2008; Thompson *et al.*, 2009). Infection was characterised by reduced production of milk which was found to be thin, watery and flaky (Lerch, 1952).

Over the years, sporadic cases of algal mastitis were observed. However, algal mastitis is now becoming endemic in most countries (Aalbaek *et al.*, 1994; Aouay *et al.*, 2008). Algal mastitis is usually prevalant during months of warm weather with high rainfall, reflecting poor managemental and hygienic conditions (Matsuda and Matsumoto, 1992). During these periods colourless algae may be found in housing pens and pastures used in dairy farms, with poor environmental conditions and insufficient milking hygiene contributing towards infection (Costa *et al.*, 1996; Schlenstedt *et al.*, 1997; Tenhagen *et al.*, 1999). Studies have shown that the *P. zopfii* genotype 2 is exclusively associated with mastitis infection (Blaschke-Hellmessen *et al.*, 1985; Moller *et al.*, 2007). Sporadic cases and outbreaks of protothecal mastitis have subsequently been reported worldwide (Mc Donald *et al.*, 1984; Wilson *et al.*, 1997; Krukowski *et al.*, 2006).

1.1.3 Pathogenesis

A comprehensive understanding of the pathogenicity of mastitis is key for the development of appropriate detection techniques. The primary cause of mastitis is a wide spectrum of bacterial strains, however, incidences of viral, algal and fungal-related mastitis were also reported (Pyorala, 2003). The causative agents are listed in Tables 1.2 and 1.3.

Normally, the teat canal is tightly closed by sphincter muscles preventing the entry of pathogens. It is lined with keratin, a waxy material, derived from stratified squamous epithelium that obstructs the migration of bacteria and contains antimicrobial agents, such as long chains fatty acids, which assist in combating the infection. However, the efficiency of keratin is restricted (Craven and Williams, 1985; Capuco *et al.*, 1992; Paulrud, 2005). Fluid accumulates within the mammary gland, as parturition approaches, resulting in increased intramammary pressure (Paulrud, 2005) and mammary gland vulnerability due to the dilation of the teat canal and leakage of mammary secretions (Sordillo and Streicher, 2002). Additionally, during milking, the keratin is flushed out and there is distention of the teat canal (Rainard and Riollet, 2006). The sphincter requires about 2 h to return back to the contracted position (Capuco *et al.*, 1992).

Once inside the teat, bacteria must also elude the cellular and humoral defence mechanisms of the udder (Sordillo and Streicher, 2002). If not eliminated, multiplication occurs in the mammary gland (Figure 1.1). The bacteria liberate toxins and induce leukocytes and epithelial cells to release chemoattractants including cytokines, such as tumour necrosis factor-α (TNFα), interleukin 8 (IL-8) and interleukin (IL-1), eicosanoids (like prostaglandin F2α (PGF2α)), oxygen radicals and acute phase proteins (APP) (*e.g.* haptoglobin (Hp), serum amyloid A (SAA)). This attracts circulating immune effector cells, mainly polymorphonuclear neutrophils (PMNs), to the site of infection (Giri *et al.*, 1984; Sordillo and Daley, 1995; Paape *et al.*, 2003; Zhao and Lacasse, 2008)

PMNs act by engulfing and destroying the invading bacteria *via* oxygen-dependent and oxygen-independent systems. They contain intracellular granules that store bactericidal peptides, proteins, enzymes, such as myeloperoxidase, and neutral and acidic proteases, such as elastase, cathepsin G, cathepsin B and cathepsin D (Owen and Campbell, 1999; Bank and Ansorge, 2001). The released oxidants and proteases destroy the bacteria and some of the epithelial cells resulting in decreased milk production and release of enzymes, such as *N*-acetyl-β-D-glucosaminidase (NAGase) and lactate dehydrogenase (LDH) (Figure 1.1). Destruction of most of the PMNs, takes place by apoptosis, once their task is fulfilled. Subsequently, macrophages engulf and ingest the remaining PMNs (Paape *et al.*, 2002; Paape *et al.*, 2003). The dead and sloughed-off mammary epithelial cells, in addition to the dead leukocytes, are secreted into the milk resulting in high milk somatic cell counts (SCCs).

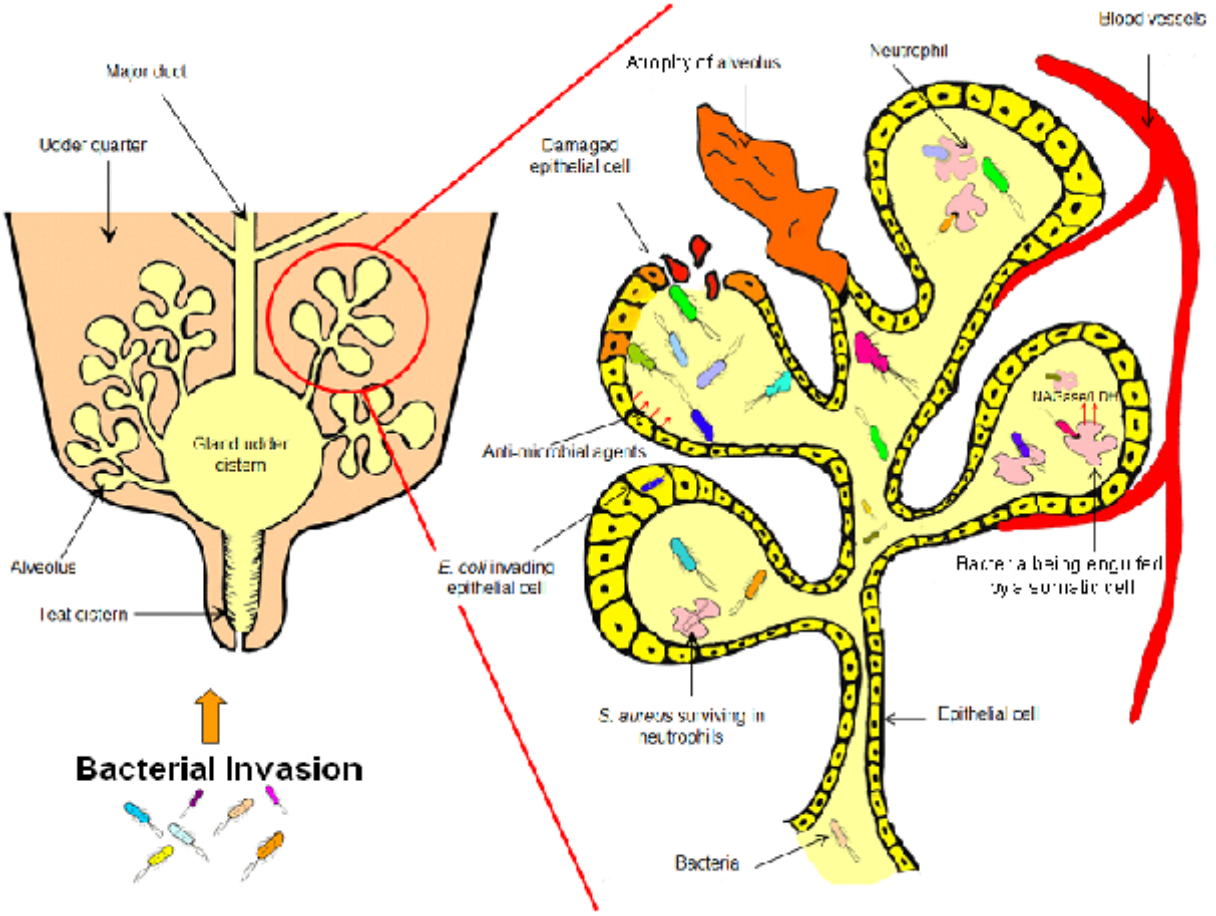

Figure 1.1. ***Schematic representation of mastitis development in an infected udder.***
Environmental and contagious microorganisms invade the udder through the teat cistern. They then multiply in the udder where they are attacked by neutrophils while damaging the epithelial cells lining the alveoli, with subsequent release of enzymes like NAGase and LDH. The epithelial cells also secrete anti-microbial compounds. Considerable tissue damage is observed once the immune effector cells begin to combat the invading pathogens (Viguier *et al.*, 2009).

If the infection persists, internal swelling within the mammary epithelium, not normally detectable by an external examination, can occur. The mammary gland alveoli become damaged and start losing anatomical integrity (Figure 1.1). The blood-milk barrier is breached causing extra-cellular fluid components such as chloride, sodium, hydrogen, potassium and hydroxide ions to enter the gland and mix with the milk (Zhao and Lacasse, 2008).

Blood may also be detected in milk when extensive damage to the blood-milk barrier has occurred. This leads to visible changes on the udder, such as enhanced external swelling and reddening of the gland. Changes also occur in milk including increased conductivity, increased pH, raised water content and the presence of visible clots and flakes (Lee *et al.*, 1980; Kitchen, 1981; Milner *et al.*, 1996; Zhao and Lacasse, 2008). This marks the initial stage of clinical symptoms and the most severe infections might ultimately result in the death of the animal.

1.1.4 Current Approaches for Diagnosis of Mastitis

Early diagnosis is of the utmost importance due to the high costs of mastitis. European Union legislation (Regulation 853/2004) stresses that milk selected for human consumption must originate from healthy animals. Diagnostic methods have been developed to check the quality of the milk through detection of mammary gland inflammation and diagnosis of the infection and its causative pathogens. Currently assays often used include measurement of SCCs, enzymatic analysis and the California milk clotting test (Pyorala, 2003). In Europe elevated SCCs above 200,000 cells/mL are widely used as an indicator of mastitis (Schukken *et al.*, 2003) and are determined using haemocytometers or cell counters. Colorimetric and fluorometric assays have been developed for measuring the concentrations of enzymes elevated in milk during mastitis (*e.g. N*-acetyl-β-D-glucosaminidase (NAGase) and lactate dehydrogenase (LDH)). Use of culturing techniques for the detection of mastitis-causing microorganisms is still the gold standard, although it is very labour-intensive and, therefore, expensive.

Mastitis may also be detected using ‘cow-side’ or ‘on-site’ tests, which can be used by both farmers and veterinarians and require relatively little training. One of the oldest and best known is the California Mastitis Test (CMT) (Schalm and Noorlander, 1957). It is based on the principle that the addition of a detergent to a milk sample with a high cell count will lyse the cells, release nucleic acids and other constituents and lead to the formation of a ‘gel-like’ matrix consistency. However, the interpretation can be subjective and this may result in false positives and negatives. Mastitis can also be detected using changes in conductivity or pH (Table 1.4). While these effects are easy to monitor they are relatively insensitive. Thus, there is a major need for new biomarkers that are specific for mastitis, easy to detect, occur at a very early stage and that can be measured ‘on-site’.

Table 1.4. ***Current somatic cell count and alternative methods for detection of mastitis.***

California mastitis Test (CMT)
Assay indirectly measures SCC in milk samples using a bromocresol purple-containing detergent to break down the cell membrane of somatic cells. The subsequent release and aggregation of nucleic acid forms a gel like matrix proportional to the leukocyte number. o Advantages: cost effective (~$12 for 350 tests), rapid, and user-friendly and can be used ‘on-site’ or in the lab. o Disadvantages: can be difficult to interpret and has low sensitivity.
Portacheck
Assay uses an esterase-catalysed enzymatic reaction to determine the SCC in milk. o Advantages: cost effective (~$3 per test), rapid and user friendly. o Disadvantage: low sensitivity at low SCC.
Fossomatic SCC
The counter operates on the principle of optical fluorescence. Ethidium bromide penetrates and intercalates with nuclear DNA and the fluorescent signal generated is used to estimate the SCC in milk. o Advantages: rapid and automated. o Disadvantages: device is expensive (~$7,000) and complex to use.

Delaval Cell Counter
Counter operates on the principle of optical fluorescence, whereby propidium iodide is used to stain nuclear DNA to estimate the SCC in milk. o Advantages: rapid and easily transportable device. o Disadvantage: relatively expensive (~$7,400).
Electrical Conductivity (EC) Test
Test measures increase in conductance in milk due to the elevation in levels of ions such as sodium, potassium, calcium, magnesium and chloride during inflammation. o Advantage: can be used 'on-site'. o Disadvantage: non-mastitis-related variations in EC can present problems for diagnosis.
Culture Tests
Laboratory-based tests use selective culture to identify different microorganisms involved in causing mastitis. o Advantage: identifies specific pathogens causing mastitis. o Disadvantages: laboratory-based assays and waiting time for results can be days.
pH Test
The rise in milk pH, due to mastitis, is detected using bromothymol blue. o Advantages: 'user-friendly', cost effective and rapid. o Disadvantage: not as sensitive as other tests.
Enzymes
Assays are used to detect enzymes, such as *N*-acetyl-beta-D-glucosaminidase (NAGase) and lactate dehydrogenase (LDH). o Advantage: assays are rapid. o Disadvantage: assays may currently be laboratory-based.

1.1.5 Development of New Biomarkers for Mastitis

Earlier detection of mastitis, and the identification of the associated causative agents, will improve the well-being of animals by allowing timely and efficient treatment. Advances in relevant proteomics techniques, such as two-dimensional gel electrophoresis (2D-GE) and mass spectroscopy (MS) (Lippolis and Reinhardt, 2005; van Leeuwen *et al.*, 2005; Smolenski *et al.*, 2007), have led to the identification of a number of new proteins involved in mastitis.

Smolenski and colleagues (Smolenski *et al.*, 2007) compared a mastitic milk sample to a non-mastitic milk sample using direct liquid chromatography-tandem MS and 2D-GE followed by matrix assisted laser desorption ionisation-time-of-flight MS analysis of individual protein spots. Six chaperonins, with a role in pathogen recognition, were identified only in mastitic samples, and therefore, have potential as new markers for mastitis. This study also reported the presence of some neutrophil-associated proteins, cathelicidin, peptidoglycan recognition protein and lymphocyte cytosolic protein 1 and the macrophage scavenger receptor, types I and II, for the first time in milk samples (Smolenski *et al.*, 2007). The whey protein patterns of mastitic milk were also studied. Baeker and co-workers (Baeker *et al.*, 2002) reported the potential use of prostaglandin D synthase as a new marker up-regulated in mastitic milk but as yet there have been no significant advances in its use. Hogarth *et al.* (2004) also demonstrated that the whey from cows affected with mastitis inflammation had increased levels of proteins of blood origin, such as serotransferrin and bovine serum albumin, and reduced concentrations of many of the major milk proteins.

Proteomic analysis of bovine neutrophils has resulted in the identification of over 250 proteins of which 19 are known to be involved in the immune response of the host. They could potentially be used as markers for its detection as reduced neutrophil function has been correlated with mastitis (Lippolis and Reinhardt, 2005).

A recent study investigated the protein patterns of mammary tissues from healthy and mastitis-infected animals to identify new markers for the disease.

The results showed that there is an up-regulation of kappa-casein and a down-regulation of cytochrome C oxidase and annexin V in animal's tissues that are mastitis-infected (Yang *et al.*, 2009b).

The development of proteome profiles of mastitis-causing pathogens, combined with available information on enzymes, toxins and metabolites produced in the udder, could assist in their identification in milk. To that end, Taverna *et al.* (2007) carried out proteomic characterisation of an *S. aureus* strain isolated from a mastitis case and presented a 2D-GE reference map of surface proteins known to contribute to bacterial adhesion to mammary tissues and to increase bacterial resistance to phagocytosis.

The proteomics studies mentioned above resulted in information on the different protein expression pattern obtained from the milk of mastitis infected animals and proteins expressed by invading pathogens. This information can be applied for the discovery of new therapeutic targets but also in the search for new diagnostic biomarkers. The successful application for these new biomarkers in a detection device, however, still remains a challenge.

1.1.6 Recent Laboratory Developments in the Detection of Mastitis

Technological advances, together with increased proteomic and genomic information have resulted in improvements in the sensitivity of assays used for the detection of mastitis. Immunoassays, such as enzyme-linked immunosorbent assay (ELISA), can provide a reliable and inexpensive approach provided that suitable antibodies are available against specific inflammation-related biomarkers or the causative microorganisms. There have

also been significant developments in nucleic acid-based testing for the identification of the latter.

1.1.6.1 Immunoassays

While more than one hundred known organisms can be responsible for causing mastitis (Radostits, 1984), ELISAs have only been developed for some of the most prevalent pathogens such as *Staphylococcus aureus*, *Escherichia coli* and *Listeria monocytogenes*. For example, an *S. aureus* antibody test kit (SAATK) (Veterinary Medical Research and Development (VMRD), Inc., Pullman, WA, U.S.A.) was assessed as a primary screen for cows suspected of having an *S. aureus* infection. However, microbial culture of the milk of ELISA-positive cows was required for confirmation (Fox and Adams, 2000). An ELISA to determine the level of antibodies produced against *L. monocytogenes* was also developed (Kalorey *et al.*, 2007).

A magnetic bead-based ELISA was developed for the detection of staphylococci using beads coated with an anti-*S. aureus* monoclonal antibody (Yazdankhah *et al.*, 1998). This approach has certain advantages over conventional ELISAs, having shorter incubation times, fewer manipulations, and it requires smaller volumes of reagents. Flow cytometry was also used to detect antibodies to *S. aureus* in milk. The method gave an earlier result in 25% of cases, when compared to bacteriological tests (Lannelli *et al.*, 1998).

Numerous immunoassays have been developed for detection of pathogens in milk (Barbuddhe *et al.*, 2002; Arimi *et al.*, 2005; Arora *et al.*, 2006; Vaidya *et al.* (ahead of print)), which are used for monitoring milk quality. However, very few

studies have been undertaken for the development of immunoassays which detect pathogens in milk as definitive causative agents of mastitis. Such assays may also be useful for mastitis detection. However, studies to validate this assumption are required.

Immunoassays can also be used to detect inflammation-related biomarkers present in the milk at different stages of subclinical mastitis. For example, haptoglobin (Hp) concentrations have been reported to increase significantly in plasma, as well as in milk, during mastitis and, thus, Hp was suggested as a potential marker for diagnosis (Gronlund *et al.*, 2003; Hiss *et al.*, 2004). Hiss and co-workers (Hiss *et al.*, 2004) developed an ELISA for its detection, with a limit of 0.07 µg/mL in both milk and serum.

SAA is another example of an acute phase protein marker that shows elevated levels in mastitic milk (Eckersall, 2007; Åkerstedt *et al.*, 2008; Molenaar *et al.*, 2009). Szczubial *et al.* (2008) were able to detect elevated concentrations of SAA, up to 322.26 µg/mL, in mastitic milk compared with normal levels of 11.67 µg/mL, using a commercially available solid phase-sandwich ELISA (Tridelta Phase^{TM} range SAA kit, Tridelta Development Ltd., Co. Wicklow, Ireland). This kit consists of an anti-SAA monoclonal antibody, which captures SAA from either test samples or standards. This binding event is detected by the addition of streptavidin-horse radish peroxidase conjugate and, subsequently, a TMB substrate, leading to a colour change, which is dependent on the concentration of SAA present.

The application of biomarker-based assays, developed within the last decade, has already shown considerable promise for mastitis detection. Nevertheless, additional studies on the validation of these assays for mastitis detection are required.

1.1.6.2 Nucleic acid testing

The genome sequences of many of the major mastitis-causing pathogens are now available and can be utilised to develop nucleic acid-based testing methods such as Polymerase Chain Reaction (PCR). Such tests are generally more expensive than, for example, immunoassays. They have the advantages of being highly sensitive and specific and can be performed rapidly (*e.g.* 'real-time' PCR) and are able to overcome the sensitivity and time-constraints sometimes encountered with culture-based tests (Studer *et al.*, 2008) and could thus complement or replace them in the long-term.

PCRs allow the identification of closely-related organisms within a few hours. Multiplex PCR and 'real-time' PCR assays that can simultaneously detect different mastitis-causing organisms in milk samples have been described (Phuektes *et al.*, 2003; Cai *et al.*, 2005; Cremonesi *et al.*, 2005; Gillespie and Oliver, 2005; Glynn *et al.*, 2006; O'Grady *et al.*, 2008), with the most recently developed assay capable of detecting 11 of the major mastitis-associated pathogens including *E. coli, S. aureus, Streptococcus agalactiae* and *Streptococcus uberis* (Koskinen *et al.*, 2009). Nucleic Acid Sequence Based Amplification (NASBA), used for quantification of RNA, has the advantage over PCR methods that it is capable of discriminating between dead and living organisms and real-time NASBA for the detection of *Bacillus cereus* in milk has

been reported (Gore *et al.*, 2003). The application of real-time PCR or NASBA could revolutionise veterinary diagnostics, by reducing sample analysis times significantly and allowing the simultaneous analysis of large number of samples for multiple organisms.

1.1.7 Recent Developments in 'Cow-Side' Tests for the Detection of Mastitis

Rapid, 'cow-side' mastitis tests could be used by farmers and veterinarians to diagnose and treat the inflammation at its early stages, thus, having the potential to stop the propagation of the disease in the herd.

An increase in temperature is one of the symptoms associated with mastitis. A thermal camera was used to diagnose experimentally-induced mastitis and could detect temperature changes of 1 to 1.5^{o}C (Hovinen *et al.*, 2008). Infrared thermography was also used to measure skin surface temperatures in infected cows and a strong correlation (R^2= 0.92) between skin surface temperature and SCC was observed (Colak *et al.*, 2008). This non-invasive approach can be used 'on-site'. However, the ambient temperature can affect this assay and a rise in temperature may only occur in some cases of mastitis and, therefore, temperature may only act as an indicator of infection.

Estimation of the levels of inflammation-related enzymes might also be used for the detection of mastitis as these show good correlation with SCC. For example, an LDH activity assay was carried out by Hiss and co-workers (Hiss *et al.*, 2007) using dry chemistry and a portable spectrophotometer with comparable variation coefficients to the assays performed in a lab environment.

Other enzymatic tests include the detection of an esterase secreted by somatic cells using an enzymatic assay on a dipstick (http://www.portacheck.com/guelph.php). Bioluminescence-determination assays, based on estimation the ATP concentrations in somatic cells (Frundzhyan *et al.*, 2008), or their DNA by fluorescent staining (http://www.delaval.com/Products/Milking/Cell-counter-DCC/DeLaval_cell-counter-DCC/default.htm), can also be used 'on-site' for the reliable determination of elevated SCC levels and, thus, the probable presence of mastitis.

1.1.8 Current and New Trends in Mastitis Detection During Automatic Milking Systems

Robotic milking has increased significantly over the last 15 years with 4% of Dutch farmers implementing this method (Kamphuis *et al.*, 2008). It provides an ideal format for 'on-line' mastitis monitoring and, therefore, reliable and sensitive methods are necessary (Figure 1.2).

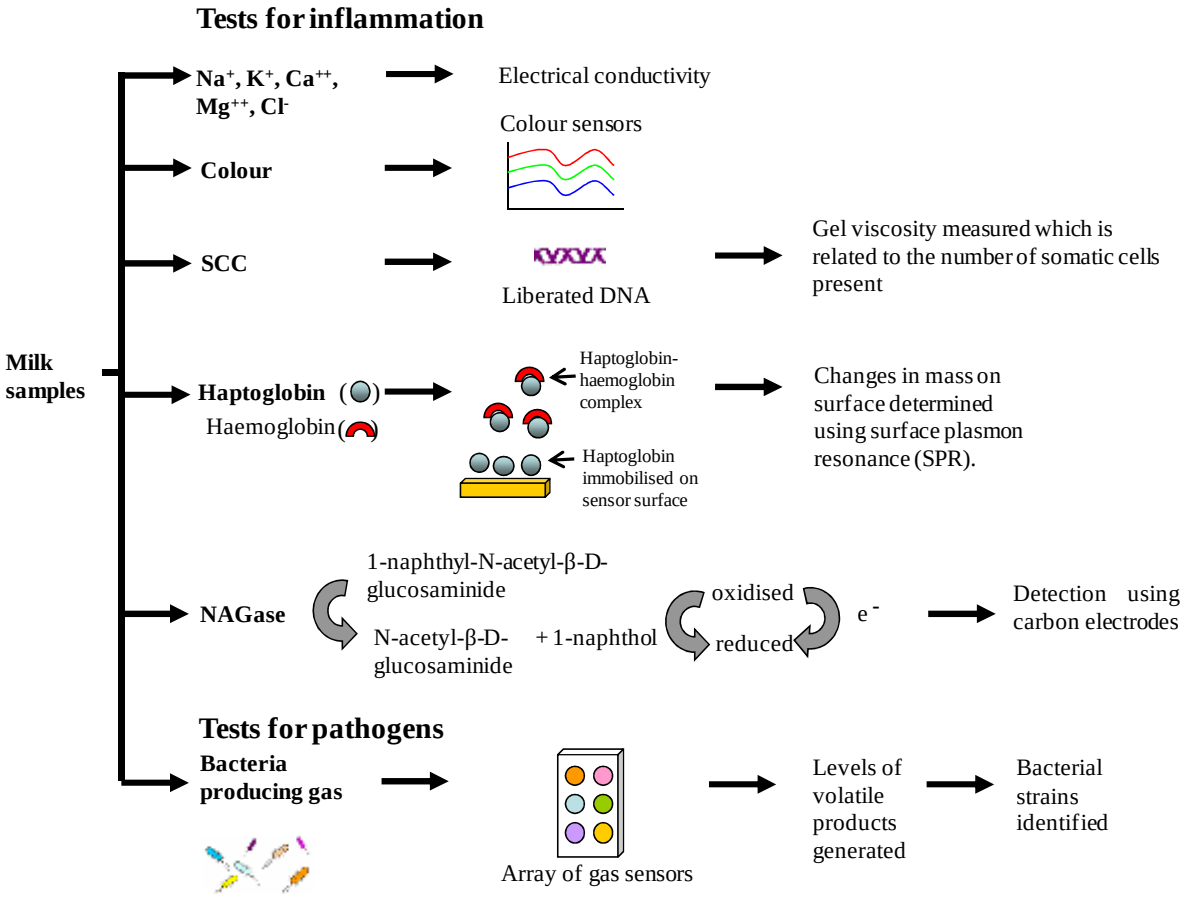

Figure 1.2. ***Current and potential 'on-line' assays for the detection of mastitis in milk.***
The colour, electroconductivity and the SCC sensors are currently used as 'on-line' assays, whereas the sensors for the detection of NAGase, haptoglobin and gases produced by bacteria have yet to be incorporated 'on-line'. These sensors show great potential for the accurate detection of mastitis (Viguier *et al*., 2009).

Any 'on-line' mastitis detection is currently performed using electrical conductivity (EC), SCC, or colour determination, with milk EC being the most commonly used 'on-line' test. However, although a change in conductivity might be a useful indicator, on its own, it is not a reliable or sensitive parameter for conclusive diagnosis (Norberg, 2005; Hovinen *et al.*, 2006). Milk colour analysis has also been used in automatic milking systems for the indication of mastitic infection (Rasmussen and Bjerring, 2005) as the presence of a yellow colour, or that of blood in the milk, might be highly indicative of mastitis. However, the milk

fat content can also influence colour and some colour sensors failed to detect subclinical mastitis (Hovinen *et al.*, 2006). Therefore, the development of novel sensors with higher sensitivities is the goal of many recent research efforts.

For example, Mottram *et al.* (2007) evaluated a chemical array-based sensor, termed an 'electronic tongue', that was able to detect chloride, potassium and sodium ions released during mastitis in addition to inorganic and organic cations and anions (Figure 1.2). This sensor could successfully discriminate between normal and mastitic milk samples, with a specificity and sensitivity of 96 and 93%, respectively. Eriksson *et al.* (2005) demonstrated that milk from mastitic and healthy cows could be distinguished using a gas-sensor array system, or 'electronic nose'. It consisted of a number of gas sensors that interact with volatile substances including sulphides, ketones, amines and acids (Figure 1.2). More recently, Hettinga and colleagues (Hettinga *et al.*, 2008) were able to identify different pathogens such as *S. aureus*, coagulase-negative staphylococci, streptococci and *E. coli* and to determine infection-free udder quarters, based on the detection of the patterns of volatile metabolites produced (Figure 1.2). Elevated levels of lactate can also be used for the detection of early stages of mastitis. Limitation of oxygen availability in the mammary glands will lead to increases in the levels of lactate which are directly proportional to the level of metabolic activity. Lactate concentrations detected during mastitis infection showed positive correlation with SCC (Davis *et al.*, 2004). A lactate screen-printed sensor that contained lactate oxidase printed onto the sensor surface has already been developed (Davis *et al.*, 2006). Here, lactate oxidase reduces lactate and produces electrons, which generates a current that is measured using a potentiostat and that can be correlated to the concentration

of lactate present. These sensors have particularly promising potential as 'on-line' sensors as they are more sensitive, but, their ability to detect subclinical mastitis is yet to be demonstrated.

Biosensors have also been developed to detect mastitis. They use a biological receptor molecule (*e.g.* antibody, enzyme, nucleic acid) in combination with a transducer to produce an associated signal allowing observation of a specific biological event (*e.g.* an antibody-antigen interaction). For example, Pemberton and co-workers developed an electrobiochemical sensor using a screen-printed carbon electrode (SPCE) that could detect NAGase (Pemberton *et al.*, 2001) *via* its ability to convert the substrate 1-naphthyl *N*-acetyl-β-D-glucosaminidine to 1-naphthol, which was subsequently detected by the electrode (Day, 2005). The limit of detection of this NAGase assay is 10 mU/mL.

In another approach, Akerstedt *et al.* (2006) developed a competitive biosensor assay using surface plasmon resonance to monitor the interaction between Hp, which was immobilised onto the chip surface, and haemoglobin (Hb) to discriminate between subclinical mastitic and non-mastitic milk (Figure 1.2). Hp binds strongly to Hb. Therefore, by mixing the milk sample with Hb, any Hp present in the milk sample will bind to the Hb thus preventing the binding of the Hb to the immobilised Hp. In the absence of Hp (*i.e.* in an uninfected milk sample), Hb will bind to the immobilised Hp resulting in a positive signal, which is reduced in correlation to the levels of Hp present in the milk. Milk samples that contain any blood cannot be analysed using this method. However, blood in milk does not normally occur in the subclinical stage of mastitis, and this method could still be used in its detection.

Whyte *et al.* (2005) developed a method to automatically determine the SCC based on measuring the DNA content of somatic cells (Figure 1.2). After somal cell lysis, the liberated DNA and histones form a gel-like complex, with a viscosity proportional to the amount of DNA and histones released, and which can be measured and correlated to the SCC. In an alternative method, the DNA from somatic cells was incubated with PicoGreen and the resulting fluorescence was measured using an optical sensor. This assay showed good correlation to Fossomatic determination of SCC (R^2 = 0.918). However, the cell-lysis step must be automated before this sensor can be used 'on-line'.

These sensors described above have the potential to clearly discriminate between subclinical and non-mastitic milk. The combination of such sensor-based platforms with the development of novel biomarkers could thus allow the diagnosis of the pre-clinical stage of mastitis before significant loss in milk production occurs.

1.1.9 Advances in Microfluidics and Their Potential for Mastitis Detection

Recent advances in microfluidics and so-called 'biochips' or 'lab-on-a-chips' have the capacity to revolutionise diagnostics (Garcia-Cordero and Ricco, 2008) and these technologies have already been applied for the detection of mastitis.

Moon and colleagues (Moon *et al.*, 2007) developed disposable microchips to be used with a portable reader system to measure milk SCC. The milk sample is mixed with a lysis solution to burst the somatic cells and a fluorescent dye is added to stain the DNA. The sample is then applied to the microchip, which

uses a capillary flow to allow even distribution of the sample and the fluorescence is measured with the portable reader system. This assay showed good correlation with commercial tests for SCC. Similarly, another disposable device that can detect mastitis based on counting milk leukocytes was recently reported (Rodriguez and Galanaugh, 2008). Here, the milk is carefully mixed with a meta-chromatic substance in order to stain the leukocytes. The somatic cells are distributed evenly in the chip by capillary action and the stained cells are then visually identified using fluorescence microscopy. This device has the advantage of having different reaction chambers, allowing the milk to be mixed with the dye on the chip, thus, making the device even more user-friendly. Garcia-Cordero *et al.* (2008) developed a rapid, low-cost microfluidic CD-based assay device for determining SCC, in which milk samples are applied to a plastic disk with funnel-shaped channels. Following centrifugation on a conventional CD-player, the SCC can be measured based on the height of the cell pellet formed. This approach showed excellent correlation with SCC levels determined using conventional approaches.

Choi *et al.* (2006) designed a chip for simultaneously monitoring pathogens, somatic cells and pH in raw milk samples. Antibodies against pathogens and somatic cells were immobilised on the chip and antigen-antibody complexes formed were detected using fluorescence microscopy. The pH was measured utilising the fluorescence change of a hydrogel-entrapped pH indicator. Chip technologies could also be applied for the detection of causative pathogens. For instance, Lee *et al.* (2008) developed a biochip that incorporated DNA amplification of genes that are specific for seven known mastitis-causing pathogens. A similar microfluidic device that integrates solid-phase extraction

and NASBA has recently been reported for the identification of low numbers of *E. coli* (Dimov *et al.*, 2008).

The incorporation of microfluidics-based technologies into chip design has made it possible to significantly reduce reagent volumes, leading to lower assay costs and faster results, and also to determine several targets on one platform. This could improve assay efficiency, specificity and sensitivity and thus ultimately might lead to better mastitis detection and treatment. In theory, such assays could be carried out 'on-site', thus providing a rapid mastitis detection format.

1.2 Antibodies

1.2.1 Antibody Structure – An Introduction

Immunoglobulins (Ig), also known as antibodies, are unique, soluble host glycoproteins secreted by B-lymphocytes in response to the administration of a foreign antigen into the body. Antigens are defined as proteins, lipids, chemical compounds, *etc.* that when introduced into the body stimulates the production of an antibody (Copley *et al.*, 1996). From a biotechnology perspective, antibodies are some of the most valuable molecules for clinical diagnostics and disease therapy.

Mammalian immunoglobulins are classified into five classes based on their distinct structures and biological properties: IgA, IgD, IgE, IgG and IgM (Belov *et al.*, 2002; Arnold *et al.*, 2004). The most abundant immunoglobulin is the IgG class (Salmon and Smith, 1970; Larson *et al.*, 1980). The rate of synthesis of IgG is higher by comparison to the other classes of immunoglobulin and their half-life longer. They are also smaller than the other immunoglobulins and are

stable during isolation and purification processes and, hence, IgG is the most used immunoglobulin in antibody-based assay development.

The structure of immunoglobulins varies depending on the isotype (Table 1.5). However, typically immunoglobulin structure is depicted as a Y-shape and it contains two large heavy chains and two small light chains, connected by disulphide bonds, as shown in Figure 1.3. IgG antibodies have a molecular mass of approximately 150 kDa, with light chains and heavy chains having a molecular mass of 23 kDa and 50 kDa, respectively (Sun *et al.*, 2001). Heavy and light chains consist of both variable regions and constant regions. The heavy chain has one variable region and three constant regions, whereas, the light chain is composed of one variable and one constant region. There are five different types of heavy chains, α, γ, μ, δ and ε, which determine the class of antibody, and only two types of light chains, κ and *λ* (Table 1.5). Each antibody molecule only contains one type of light chain and one type of heavy chain (Copley *et al.*, 1996).

Table 1.5: ***Characteristics of different immunoglobulin isotypes.***

Type	Heavy chain	Light chain	Molecular weight	Structure	Sub-classes
IgA	α1, α2	κ or λ	150-600 kDa	Monomer to tetramer	IgA1, IgA2
IgD	δ	κ or λ	190 kDa	Monomer	-
IgE	ε	κ or λ	190 kDa	Monomer	-
IgG	γ1,γ2,γ3,γ4	κ or λ	150 kDa	Monomer	IgG1, IgG2a, IgG2b, IgG3, IgG4
IgM	μ	κ or λ	900 kDa	Pentamer	-

(Modified from Copley *et al.*, 1996).

Light chains of an antibody are composed of 220 amino acid residues while heavy chains are composed of 440-550 amino acids. The amino sequence in the variable region of the antibody varies greatly among different antibodies. The variable region is comprises of three hypervariable (HV) regions interspersed by four framework regions (FR). The FRs provides a backbone structure for the antibody and HV regions confer on each antibody, the ability to identify a specific epitope as an antigen and bind specifically to that epitope. The antigen-binding site of an antibody is formed from six HV region loops, 3 each from both heavy and light chains. Therefore, HV regions are also known as “complementary determining regions” (CDRs). It is worth noting that FRs can also have an influence on antigenic specificity.

Functionally, an antibody is divided into antigen-binding arms (Fabs) and a constant fragment (Fc). The Fab region serves as the antigen-binding site and is made of heavy and light variable chains. The Fc domain of the constant region determines the effector functions of antibodies. Fc domains are necessary for interactions with effector cells and the activation of the complement cascade (Copley *et al.*, 1996).

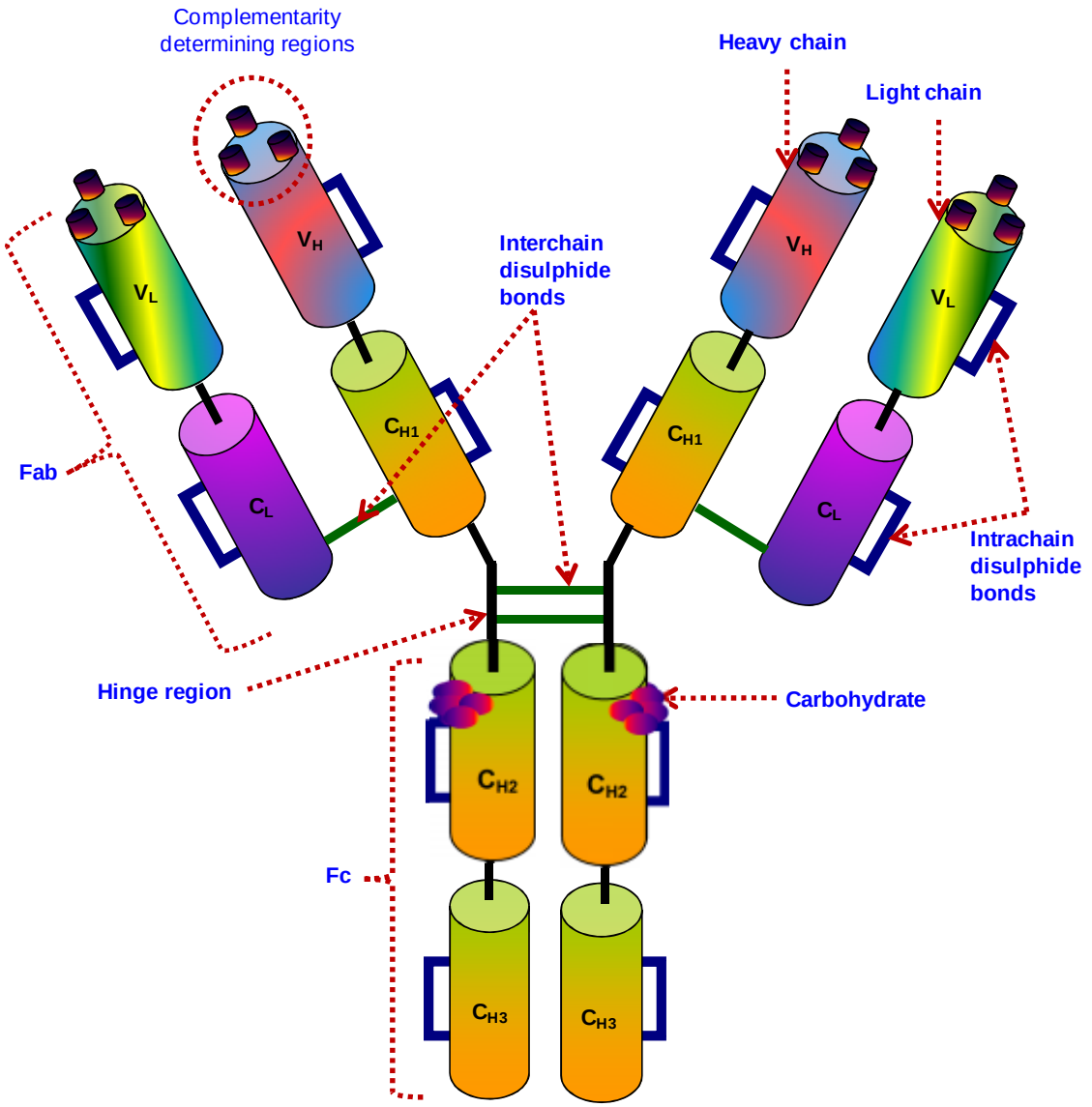

Figure 1.3. ***Structure of a typical immunoglobulin, IgG.***
IgG are large molecules of approximately 150-155 kDa containing 2 pairs of heavy and light chains composed of different domains. The heavy chain consists of a variable domain (V_H) and three constant domains (C_{H1}, C_{H2}, and C_{H3}). The two heavy chains are connected by disulfide bonds (SS) in the hinge region. The light chain has one variable domain (V_L) and only one constant domain (C_L) (Modified from Copley *et al.*, 1996).

1.2.2 Types of Antibodies Used in Diagnostic Assays

The natural function of antibodies is the protection of the body from foreign substances, however, antibodies have been well harnessed for use in various scientific and medical fields, over past few decades. This is due to their ability to bind specifically to a corresponding antigen (Lipman *et al.*, 2005). The most common exploitation of antibodies is as diagnostic tools in various formats. Antibody-based immunoassays represent one of the major sectors of diagnostics and are one of the fastest growing technologies (Borrebaeck, 2000; Hartmann *et al.*, 2009) owing to their cost-effectiveness, robustness, ease of operation and amenability for 'on-site' monitoring (Alcocer *et al.*, 2000). Immunoassays currently have a revenue share of more than 35% in the European clinical diagnostics market, with the earnings of $2.97 billion in 2008. These earnings are estimated to reach $4.20 billion in 2015 (http://engineers.ihs.com/news/2009/frost-immunoassay-diagnostics-1-09.htm; http://www.frost.com/prod/servlet/market-insight-top.pag?docid=141637486).

Three types and methods of generation of antibodies have been developed. Antibodies produced from different B-lymphocyte lines are known as polyclonal antibodies (pAbs). PAbs are, thus, a heterogeneous mixture of antibodies produced in an animal in response to an antigen, with different antibodies recognising different epitopes of the antigen. However, a monoclonal antibody (mAb) is a homogeneous antibody, specific to a single epitope, the product of a single B-lymphocyte clone (Pohanka, 2009). Recombinant antibodies / antibody fragments (rAbs) are the third type of antibodies. These are antibodies or antibody fragments generated in the laboratory using molecular techniques. RAbs are produced in various formats, including full-length, small and

conjugated antibodies (Carter, 2006). The most commonly used formats are scFv and Fab (Röthlisberger *et al.*, 2005; Townsend *et al.*, 2006) (Figure 1.4).

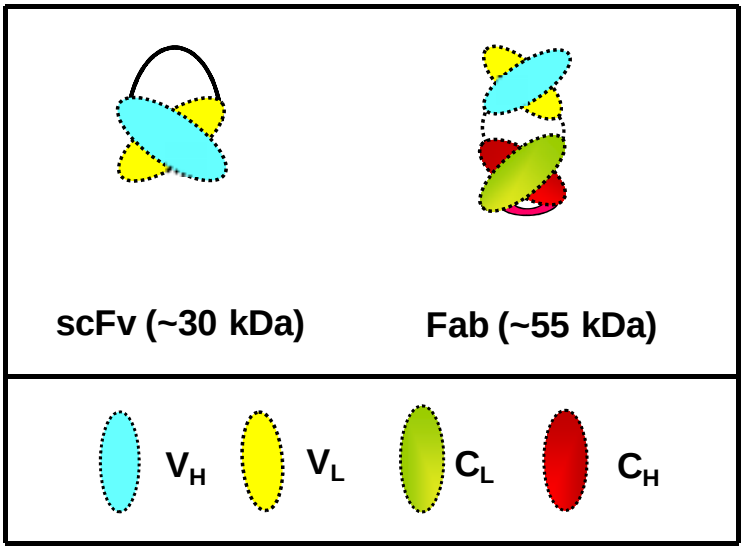

Figure 1.4. ***Commonly used antibody fragments.***
Two most commonly used antibody fragments are scFv and Fab. A scFv is an engineered antibody composed of IgG V_H and V_L domains tethered together by a peptide linker. The Fab fragment consists of the V_H domain and the first domain of the constant region (C_{H1}) paired with V_L and the light-chain constant (C_L) domains.

Each type of antibody has its advantages and disadvantages. Production of PAbs is achieved in a short duration and is simple, cheap and requires no specialised skill or equipment. Other advantages of pAbs include the ability to recognise multiple antigenic epitopes, stability over a broad pH and salt concentration, and frequently reported better specificity than mAbs (Lipman *et al.*, 2005). PAbs are more tolerant of minor changes in the antigen, *e.g.* polymorphism, heterogeneity of glycosylation, or slight denaturation. However, pAbs have numerous disadvantages as well. The native immunoglobulin pool of the host animal used can be sometimes give non-specific background effects. However, this can be overcome by use of 'specific pathogen free' (SPF) animals, which in turn increases cost. PAbs have a restricted supply, limited by

the size and lifespan of the animal. Also, their avidity might change over time. In addition, batch to batch variation is a major drawback associated with pAbs, which is more evident when multiple animals are used as a source of pAbs (Lipman *et al.*, 2005).

Similarly, mAbs have certain advantages over polyclonal antibodies. The high homogeneity of mAbs reduces or eliminates the risk of false positive reactions due to cross-reactivity, mAbs also provide great uniformity with no batch to batch variability (Gamble, 1984). The monospecificity and the defined composition of mAbs allow for detailed structural analysis, *e.g.* X-ray crystallography or gene sequencing which in turn are useful for structural and chemical analysis of antibody (Lipman *et al.*, 2005) and for studying mechanistic issues related to binding. Once the desired hybridoma has been generated for a mAb, it achieves immortality and can be frozen, thawed, and recultured *in vitro*. Consequently, for a given monoclonal line, there exists a steady and renewable source of antibodies. However, production of mAbs can take up to a year or longer in some cases, therefore, requiring considerably more expense and time. MAbs are of limited usefulness in case where the structure of an epitope is even minimally perturbed (*e.g.* as a consequence of genetic polymorphism, glycosylation, and denaturation). MAbs lack the ability to cross link antigens. Average affinities of mAbs are generally lower than pAbs and mAbs can be highly susceptible to changes in pH and salt concentrations (Lipman *et al.*, 2005). Additionally, the generation of mAbs against every antigen may not be possible (Polin, 1984). Furthermore, some immunoassays employing mAb have less sensitivity as compared to immunoassays using pAbs (Gamble, 1984).

Recombinant antibodies (rAbs), have distinct advantages over pAbs and mAbs, such as rapid growth, reduction of animal use, and production on large-scale from bacterial or other expression systems, at a low cost (Emanuel *et al.*, 2000; Smith *et al.*, 2004). RAb technology allows the generation of the antibodies of desired isotype. The antibodies can be genetically modified, making *in vitro* affinity maturation possible without the need of a new immunisation process (Siegel, 2002). Genetic modifications allow for generation of novel and rare functionalities (Alcocer *et al.*, 2000), *e.g.* tagging of fusion proteins, suitable for use in detection systems, to the antibody produced (Terpe, 2003). The disadvantages of rAb include, less stability, *in vivo*, inability to cross link antigens, and sometimes they may lack critical domains necessary for certain biological functions (Crowe *et al.*, 1994).

1.2.3 Antibody Production

1.2.3.1 Polyclonal antibody production

Polyclonal antibodies are typically produced by immunisation of an animal with the desired antigen. Adjuvants, substances that stimulate the immune system and increase the response, are often used to boost the immune response of the antigen used. Immunisation induces the B-lymphocytes to produce immunoglobulins specific for the antigen. The animal's serum is then harvested for pAbs.

The choice of animal for pAb production mainly depends on the amount of antibody required, genetic distance between the antigen species and the animal being used and other characteristics, such as the desired antibody class, isotype, *etc.* of the antibodies to be made (Cooper and Paterson, 1997). Many

species, including mouse, rat, rabbit, goat, sheep, horse, baboon, donkey, guinea pig, hamster, chicken, frog, fish, *etc.* have been employed for pAb production over the years (Dwyer *et al.*, 1987; Euhus *et al.*, 1990; Hanly *et al.*, 1995; Reynolds *et al.*, 1995; Cooper and Paterson, 1997; Hau and Hendriksen, 2005; Nuntaprasert *et al.*, 2005). Rabbits are usually the animal of choice in laboratories, because of their convenient size, ease of handling and bleeding, relatively long life-span, and adequate production of high-titre, high-affinity, precipitating antiserum (Stills, 1994), whereas, larger mammals, like sheep, goat and horse, are often preferred where larger amounts of sera are desired. Many investigators favour chickens because of their phylogenetic distance from mammals (Hau and Hendriksen, 2005).

1.2.3.2 Monoclonal antibody production

Production of monoclonal antibodies became possible after the discovery of hybridoma (*hybrid*-myel*oma*) technology by Georges Köhler and César Milstein (Kohler and Milstein, 1975), for which they were awarded the Nobel Prize in Physiology or Medicine in 1984, along with Niels Kaj Jerne. The underlying principle involves injecting a mouse with desired antigen for developing antibody-forming cells in the spleen. The next step is to fuse the single healthy antibody-producing spleenocyte, producing mono-specific antibody, with the immortal myeloma cell, which produced the single desired antibody and possess the immortality of the myeloma cells.

The production of mAbs is a time-consuming and multistage process, including antigen preparation, immunisation, fusion, screening, sub-cloning, and hybridoma expansion and mAb production (Figure 1.5). The mouse is usually

the animal of choice for mAb production and suitable mouse myeloma cells lines are widely available (Haaheim, 1991).

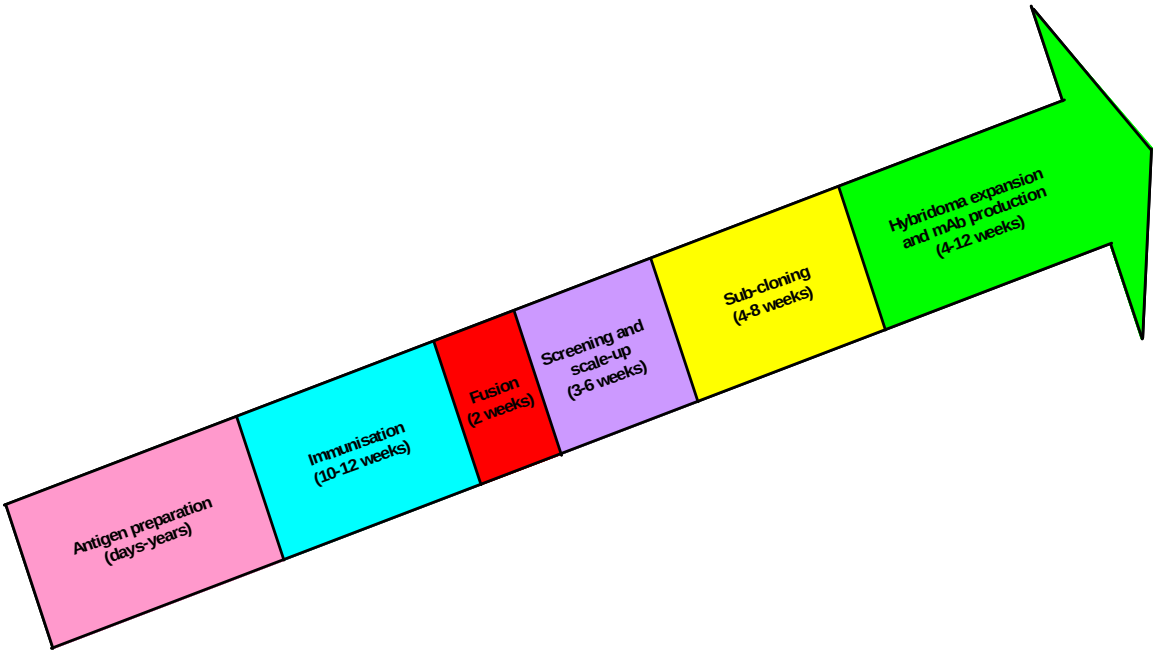

Figure 1.5. ***Time-line for monoclonal antibody generation.***
The generation of a monoclonal antibody is a multistage process. The stages and approximate time-lines are illustrated.

The generation of mAbs starts with preparation of pure antigen to be used for immunisation of mouse. When the antigen is ready mice are immunised and boosted every 2-3 weeks. However, the immunisation protocol may vary among investigators. The mouse is euthanised on reaching the desired titre, and its spleen is collected. At the same times, myeloma cells are grown in sufficient numbers in the laboratory.

The most critical stage of mAb production (Figure 1.6) is the fusion. The spleenocytes harvested from the immunised mouse are fused to myeloma cells. This fusion is achieved using polyethylene glycol (PEG), viruses (*e.g.* Sendai) or physical means (*e.g.* electrofusing) (Haaheim, 1991). However, fusion by PEG is most popularly used method. PEG is a polywax solution promoting the

adherence of the adjacent cells and, thus, facilitating the exchange of nuclei (Little *et al.*, 2000). Fusion is a relatively rare event. Hence, post-fusion the strategy involves selective enrichment of the hybridoma cells, as numerous unfused myeloma cells could quickly outgrow the relatively fewer hybridoma cells (Haaheim, 1991).

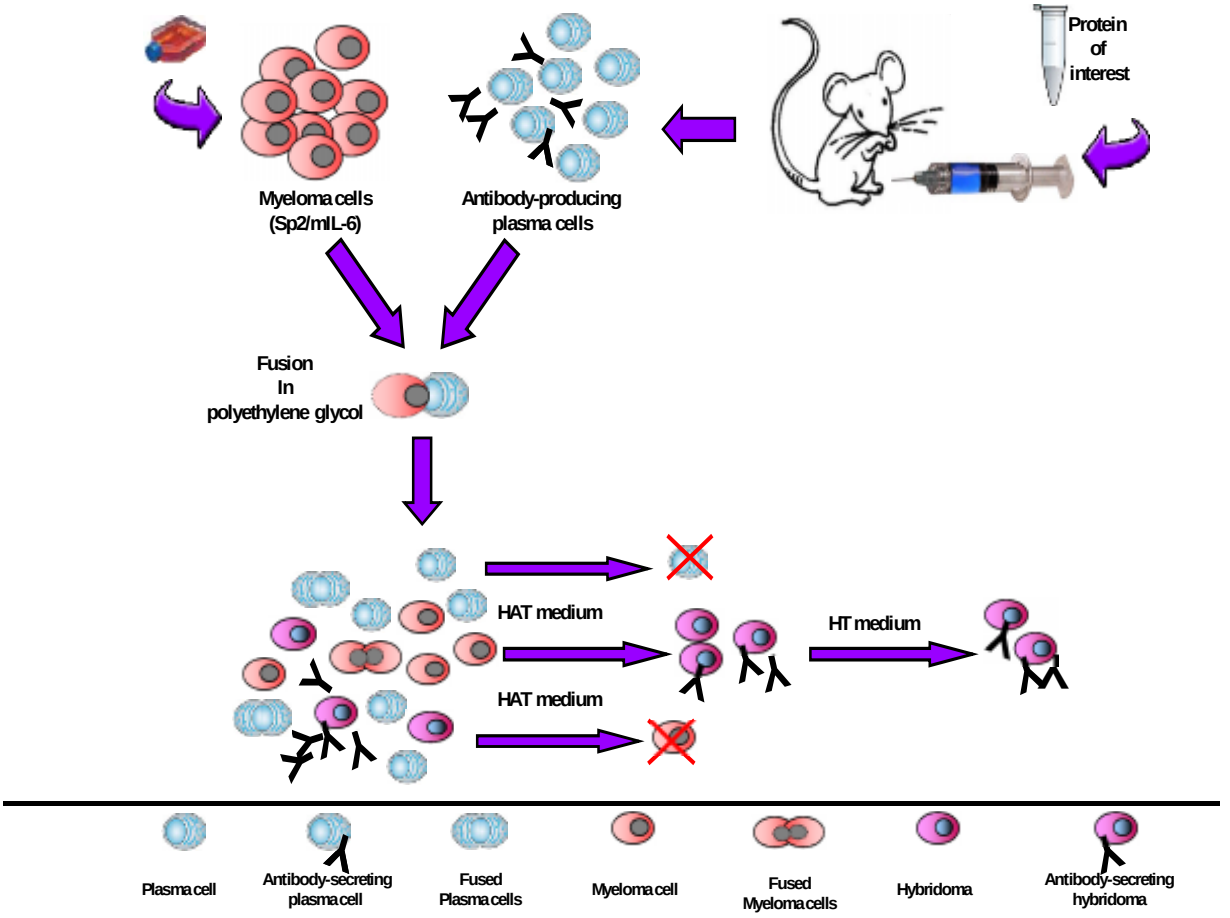

Figure 1.6. ***Production of monoclonal antibodies.***
Generation of monoclonal antibodies involves immunisation of mouse with the desired antigen. Once a sufficient titre is reached against the immunised antigen, the spleenocytes are harvested and fused with myeloma cells in the presence of polyethylene glycol (PEG). Hybriomas are subsequently selectively enriched in HAT medium, which does not allow unfused cells to grow. After 10-14 days, when only hybridoma cells remain viable, they are enriched by growing them in HT medium (Kohler and Milstein, 1975).

For the selection of hybridoma cells from the unfused cells a selective media known as HAT (so called as it contains Hypoxanthine, Aminopterin, and

Thymidine) medium is used. Aminopterin is a folic acid antagonist and it blocks the *de novo* pathways, the main pathways for purine and pyrimidine biosythesis. However, cells having the enzymes thymidine kinase (TK) and hypoxanthine guanine phosphoribosyl transferase (HGPRT) can still synthesise the DNA using salvage pathways, which involve recycling of pre-formed nucleotides, in the presence of thymidine and hypoxanthine. Aminopterin blocks the usage of *de novo* pathways for DNA synthesis for the unfused and hybridoma cells. HAT medium, containing aminopterin, blocks the *de novo* pathway for unfused cells as well as hybridoma cells (Figures 1.6 and 1.7). Myeloma cells lack HGPRT and TK enzymes, and so they cannot use the salvage pathway, and are unable to replicate their DNA. As a result, unfused myeloma cells fail to grow in HAT medium (Figures 1.6 and 1.7). Unfused spleenocytes have a definite life-span after which they do not survive (Figures 1.6 and 1.7). The hybridoma cells have HGPRT and TK enzymes, derived from spleenocytes, which allow them to replicate their DNA using salvage pathways, in presence of thymidine and hypoxanthine in the HAT medium. Additionally, the myeloma component of hybridomas confers immortality on these cells. As a result, hybridomas are able to grow indefinitely in HAT medium (Figures 1.6 and 1.7). After 10-14 days of selection in HAT medium only hybridoma cells are left. These hybridoma cells can then be maintained in a medium (HT medium) devoid of aminopterin (Goding, 1980; Haaheim, 1991; Nelson *et al.*, 2000). This is illustrated in Figure 1.6.

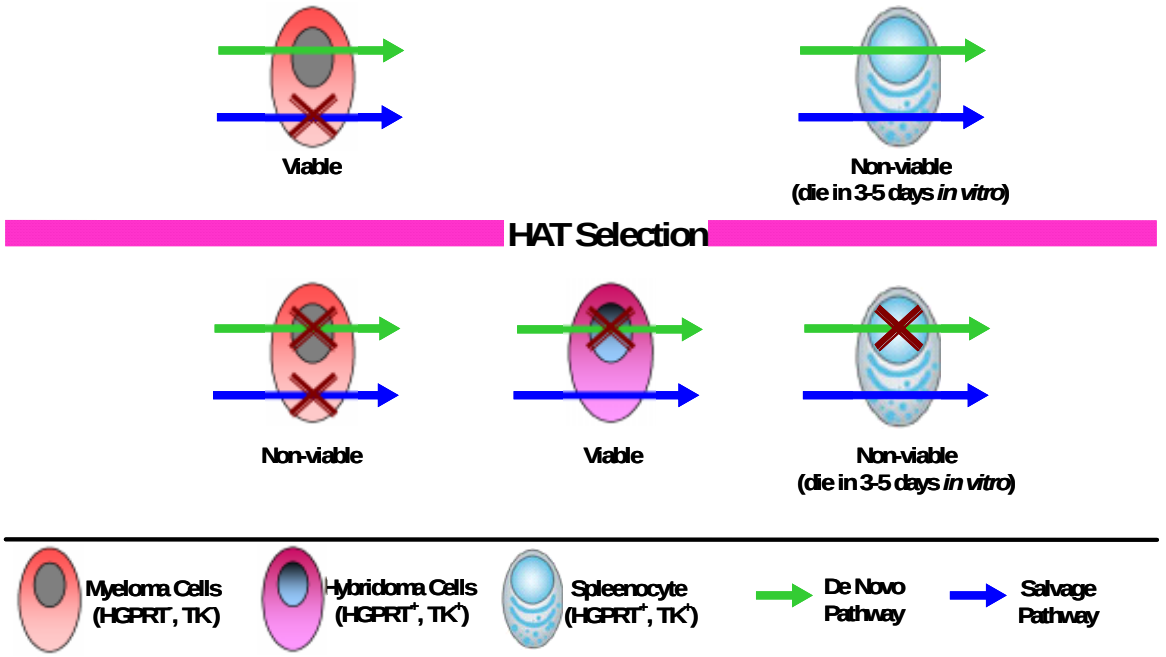

Figure 1.7. ***Selection of hybridomas.***
HAT (Hypoxanthine, Aminopterin and Thymidine) medium is used for selective growth of hybridoma cells. Aminopterin present in HAT medium blocks the *de novo* pathways for DNA synthesis. Thus, only the cells having HGPRT and TK enzymes can survive using the salvage pathways for DNA synthesis, utilising the hypoxanthine and thymidine in HAT medium. This allows only hybridoma and spleenocytes to grow. Spleenocytes have a limited life-span and so die in 3-5 days, leaving only hybridoma cells in the media.

Cultures containing hybridoma cells are then screened for the presence of antibodies with the desired antigen specificity (Figure 1.8). The specific clones of hybridoma cells are then sub-cloned using a limiting dilution approach (Figure 1.8) to ensure that a monoclonal antibody-producing cell line is obtained. A few sub-cloning steps can be used depending on the investigator. The sub-cloning steps allow the identification and selection of sub-clones that produce the largest amount of antibody, and it is necessary to ascertain that all antibodies produced by a hybridoma cell line are of the same desired specificity. It also helps in elimination of cells that are genetically unstable, or have lost the ability to produce antibody. The most productive and stable clone is then selected for

future use. The selected hybridoma cell line is then expanded. Generated cell lines can be grown indefinitely in a suitable cell culture media and the antibody can be purified from the media. The hybridoma cell line can be stored for an indefinite period in liquid nitrogen.

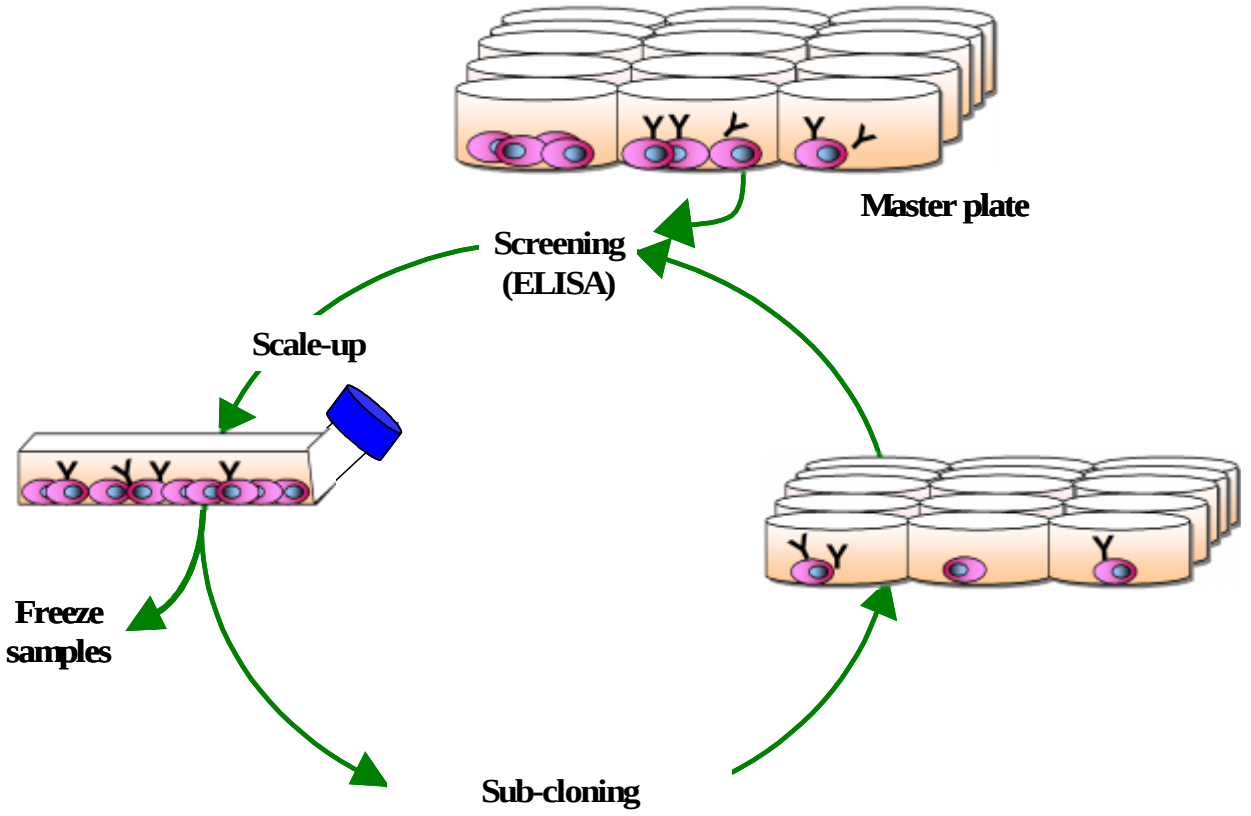

Figure 1.8. ***Steps involved in screening and sub-cloning of hybridomas.*** A sample is collected in each round of sub-cloning and is frozen. If a hybridoma cell line loses its 'antibody-producing' capability the frozen stock can be used to re-propagate the cell line.

1.2.3.3 Recombinant antibody production

Recombinant antibody technology uses a group of molecular approaches involving PCR amplification of the antibody genes, combining them together, and cloning them into a suitable vector. The vector is then transformed into a host for generating molecular display libraries. Antibody display technology is then employed for enrichment of specific antibodies from a vast number of

mostly antigen-irrelevant antibodies. Specific enrichment of antibody-expressing clones is carried out by employing a few rounds of screening against the antigen bound to a solid surface or in solution. Finally, the clones from the enriched pools are screened for antigen reactivity (Karn *et al.*, 1995; Siegel, 2002; Benhar, 2007; Weisser and Hall, 2009).

1.2.3.3.1 Antibody libraries

An antibody library is a repertoire, from which various antibodies can be obtained. Based on the source of antibody fragments used in construction of the library, four types of antibody libraries exist:

(i) Immunised libraries: The antibody genes used in constructing such a library are amplified from the B cells of an immunised animal or hybridoma cells generated from such an animal or human (Burton *et al.*, 1991; Clackson *et al.*, 1991).

(ii) Non-immunised or naïve libraries: The source for antibody genes is an animal or a human that was not intentionally immunised for the purpose of library construction (Vaughan *et al.*, 1996).

(iii) Semi-synthetic libraries: These are highly diverse antibody libraries containing genes not found *in vivo* and generated by causing random mutations in one or more CDR regions of one or more defined antibody(s) / antibody fragment(s) (Barbas *et al.*, 1992; Griffiths *et al.*, 1994; de Kruif *et al.*, 1995).

(iv) Synthetic libraries: These libraries are constructed artificially by *in vitro* assembly of heavy and light chain genes, after randomisation of the CDR regions. (Yang *et al.*, 2009a).

1.2.3.3.2 Antibody display technology

Several molecular display formats, such as phage-display (McCafferty *et al.*, 1990), ribosome display (Hanes and Plückthun, 1997; He and Taussig, 1997), eukaryotic cell-surface display (Russell *et al.*, 1993; Boublik *et al.*, 1995; Boder and Wittrup, 1997; Kieke *et al.*, 1997) and prokaryotic cell-surface display (Francisco *et al.*, 1993; Daugherty *et al.*, 1998; Du *et al.*, 2005), for selection and affinity maturation of antigen reactive antibody(s) / antibody fragment(s) are described. The common feature among all these systems is that a direct link is created between the genotype and the phenotype of the antibody/antibody fragment being displayed (Scott and Smith, 1990). The steps involved in each of these systems include generation of genotypic diversity, coupling of genotype with phenotype, clonal selection pressure and amplification. Among all these methods phage display is the simplest, robust and popularly used method for screening of an antibody library (Smith and Petrenko, 1997; Sidhu *et al.*, 2003; Bratkovič, 2010)

1.2.3.3.3 Phage display technology

George P. Smith introduced the principle of displaying foreign polypeptides on the surface of filamentous bacteriophage, *i.e.* phage-display, in 1985 (Smith, 1985). In early 1990s, phage display systems were developed for antibody fragments (McCafferty *et al.*, 1990). This approach combines the generation of billions of phage particles (termed a ‘library’), using recombinant DNA

technology, expressing a unique antibody / antibody fragment on their surface. These antibodies / antibody fragments have the potential to bind to the desired antigen. The phage library is allowed to infect male *E. coli* cells and a fast screening procedure, known as panning, is used to select and amplify phage particles expressing specific monoclonal antibody / antibody fragments towards the antigen of interest (Smith *et al.*, 2004; Weisser and Hall, 2009). Thus, phage display is a powerful technology that provides a convenient format for the production of large numbers of antibodies / antibody fragments (Azzazy and Highsmith, 2002).

1.2.3.3.3.1 Biology of filamentous phage

Filamentous phage are simple viruses having the capabilities to infect a variety of Gram-negative bacteria. The most characterised class of filamentous bacteriophages is the Ff class which infects *E. coli* cells through the bacterial F conjugative pilus. The M13, f1 and fd strains, used most commonly for phage display, are all included in Ff class. These three strains are more than 98% identical and their gene products are interchangeable (Russel *et al.*, 2004).

The filamentous phage genome consists of a circular single-stranded DNA (ssDNA), containing 11 genes (I-XI), encased in a capsid protein cylinder with a diameter of about 6.5 nm and a length of 900-2000 nm. The 11 genes encode 11 different proteins (pI-pXI). Five of these proteins are phage coat proteins (pIII, pVI, pVII, pVIII, and pIX), with pVIII being the major coat protein, with about 2700 copies per phage, and minor coat proteins (pIII, pVI, pVII, and pIX), having 3-5 copies per phage (Figure 1.9) (Webster, 2001; Russel *et al.*, 2004). Three proteins (pI, pIV and pXI) are involved with the assembly of the phage

particle and the three (pII, pV and pX) are involved in generation of the ssDNA. pIII comprises of 3 domains, N1, N2 and CT. The N1 domain is required during infection, the N2 domain is responsible for binding of phage to F-pilus and the CT domain is essential for forming stable phage particle. Infection of an *E. coli* cell is initiated by the attachment of the phage to the F-pilus of *E. coli* cell through N-terminal end of the pIII. The coat proteins are then disbanded on the cytoplamic membrane, and the single-stranded circular DNA of the phage is translocated into the cytoplasm of the *E. coli* cell. Phage DNA replicates in bacterial cytoplasm utilising the bacterial enzymes, and converts into a double-stranded replicative form. This replicative form serves as a template for synthesis of all the phage proteins. pV protein, on reaching a certain level, sequesters single stranded DNA preventing its conversion into the replicative form (Pini and Bracci, 2000). The viral particles are then assembled and extruded through the bacterial envelope at several hundred per cell per division cycle (Smith and Petrenko, 1997).

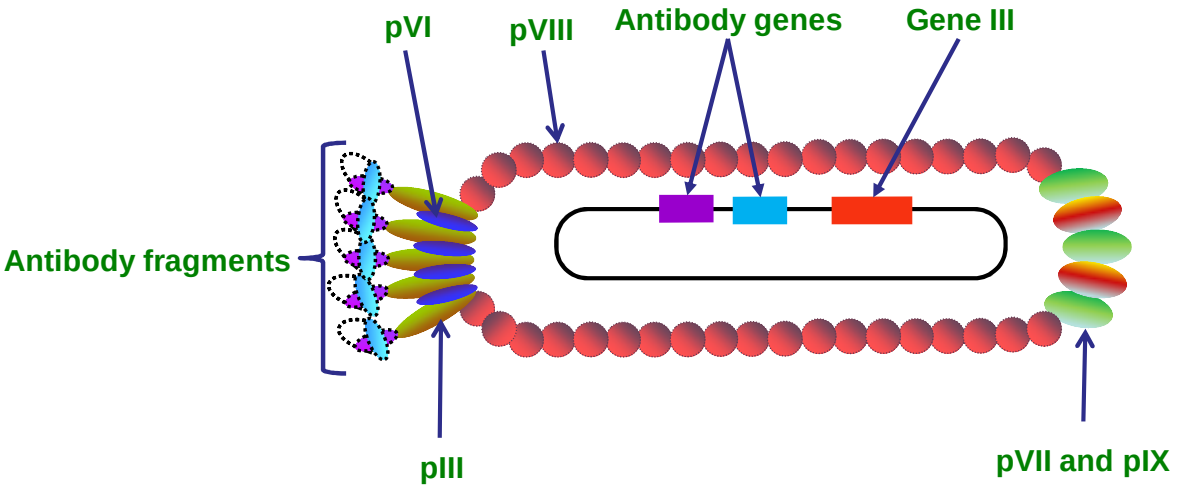

Figure 1.9. ***Schematic view of a filamentous phage particle displaying antibody fragments on pIII.***

The phage particle has a filamentous shape with approximately 6.5 nm in diameter and 900-2000 nm in length. It has a single-stranded DNA genome encased in a cylindrical protein coat (Barbas *et al.*, 2001).

1.2.3.3.3.2 Phage display vectors

Two different types of vectors are used for phage display. The vectors that are derived from the genome of a filamentous phage are known as true phage vectors. The vectors encode all the proteins needed for the replication and assembly of the filamentous phage (Russel, 1995; Marvin, 1998). The antibody gene sequence is cloned as a fusion with the coat protein of the phage genome (Scott and Smith, 1990; Petrenko *et al.*, 1996). This yields phage exclusively presenting the fusion coat protein. In another type of approach, the antibody gene can be inserted as fusion gene cassette with an additional copy of the coat protein (McLafferty *et al.*, 1993; Haaparanta and Huse, 1995). This produces phage that present the wild type and the fusion coat protein on the same phage particle.

The other types of vectors used are known as phagemid vectors. Phagemids are hybrids of plasmid and filamentous phage, containing a phage-derived origin of replication in addition to the plasmid origin of replication. Phagemids have the ability to grow like a plasmid and can also be packaged as ssDNA in the phage coat (Russel and Model, 1989). However, when phagemids are used, the presence of additional phage-derived proteins and enzymes are essential for the production of phages. These proteins and enzymes are provided by superinfecting the phagemid carrying bacterial cells with a “helper” phage, which activates the phage replication origin (Pini and Bracci, 2000; Paschke, 2006), thus, resulting in packaging of both the phagemid and the helper phage DNA into phage particles. This procedure is referred to as “phage rescue”. The helper phage genome also carries the kanaymcin resistance gene, which in addition to an ampicillin resistance gene on the phagemid, ensures the

selection of only those cells that are transformed by both genomes. Phagemids also have hemagglutinin (HA) and poly-histidine tags allowing detection and purification of the antibody fragments, respectively. Phagemids are smaller in size and their cloning is easier. Thus, phagemids serve as excellent cloning vehicles for antibody phage display (Scott and Barbas, 2001).

1.2.3.3.3.3 Selection of antibody libraries

Phage particles expressing the antigen-specific antibody fragment are selected from phage-displayed antibody libraries by a biopanning process (Figure 1.10). The selection normally consists of two vital steps, *i.e.* panning and screening (Kretzschmar and von Rüden, 2002). For panning, the phage library is incubated with the antigen to allow binding of antigen-specific phage antibodies to the antigen. Unbound phage particles are removed by washing, whereas antigen-bound phage particles are eluted and infected in *E. coli* for reamplification (Azzazy and Highsmith, 2002). Ideally, one round of panning is enough to obtain antigen-specific antibodies, however, a few consecutive rounds of panning are normally required as non-specific binding limits the enrichment. Antibody-displaying phage particles can be selected using either a solid phase (Clackson *et al.*, 1991), on an antigen in solution (Hawkins *et al.*, 1992), or can be directly selected against markers on cell surfaces (Azzazy and Highsmith, 2002).

After a few rounds of panning, a mixture of phage antibodies is enriched for antigen-specific binders. Monoclonal antibodies are subsequently screened individually from this mixture. The phage pool obtained after panning is used to infect *E. coli* cells and single colonies are picked. Consequently identification of

specific clones is performed using antibody-binding assays (such as ELISA and FACS) and sequencing (Hoogenboom, 2005).

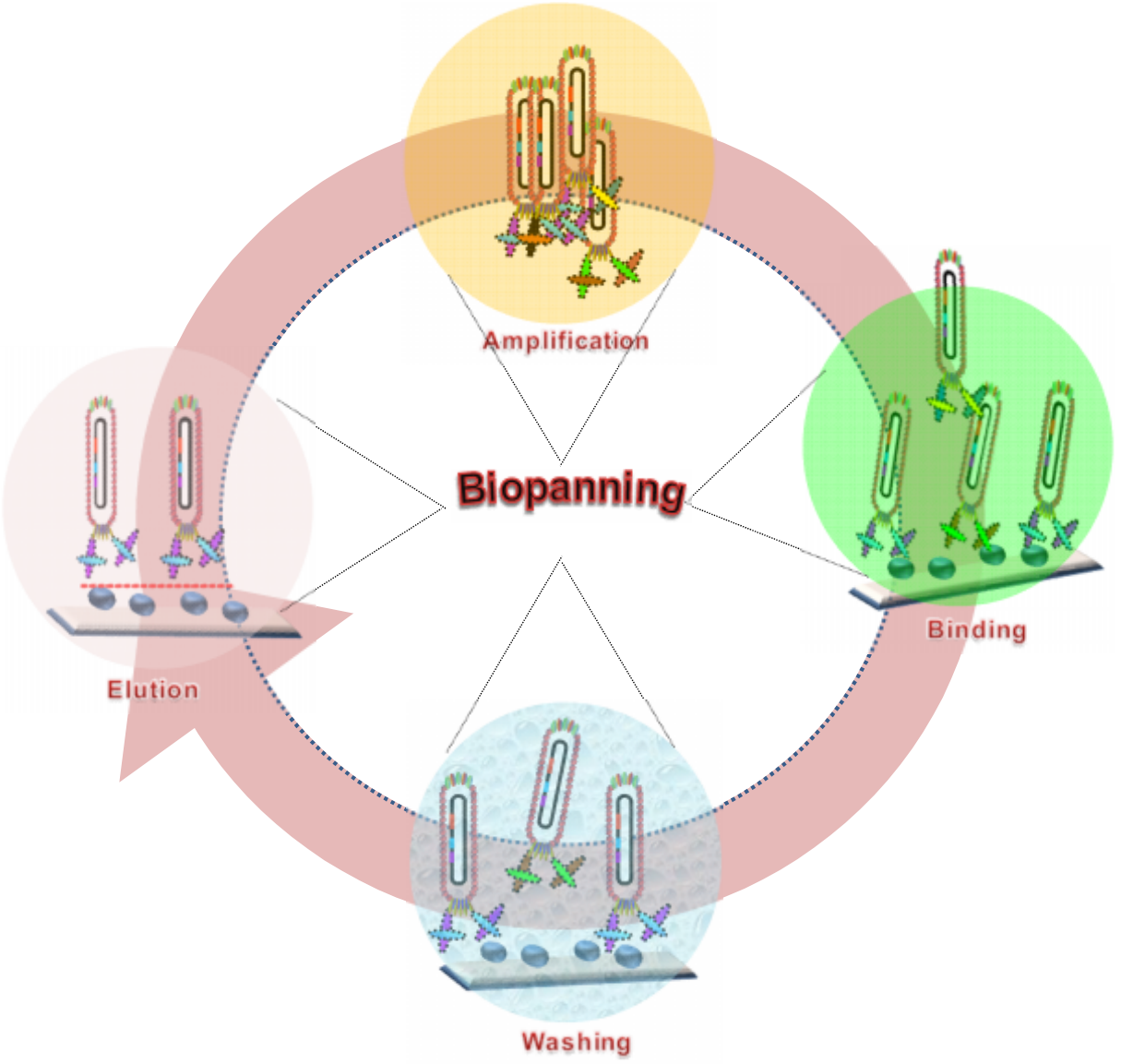

Figure 1.10. ***Biopanning for selection of antigen-specific antibodies from antibody libraries.***
Biopanning is a cyclic process of selecting and enriching the antibody, based on specificity from an antibody library. Phage bind *via* displayed antigen-specific antibodies to the antigen. Unbound phage are washed-off, whereas, bound phage are eluted and amplified. The phage eluents from the preceding round are used for a new selection. Several rounds of biopanning (usually 3 to 5) are carried out. This procedure amplifies the phage displaying the antibodies with high affinity to the selecting antigen.

1.3 Aims

The specific aims of this research project were:

(A) To generate antibodies against *N*-acetyl-β-D-glucosaminidase (NAGase), an important marker of bovine mastitis. This involved (a) generation and selection of antibodies to one or both isoforms of the enzyme (hexosaminidase A, HexA, and hexosaminidase B, HexB) of bovine NAGase, (b) purification of HexB from bovine spleen tissue, (c) cloning, expression and purification of Hex α- and β-subunits, and their application for the generation of antibodies, and (d) analysis of Hex α- and β-subunits from bovines and related mammals for evidence of selective pressure variations.

(B) To generate of a bovine scFv library from the spleens of cows that suffered from chronic mastitis and, subsequently, to utilise the library for selecting antibodies to *Staphylococcus aureus*, the major aetiological agent of bovine mastitis.

(C) To generate antibodies against the hormone progesterone, a key marker of reproductive status and a possible marker of mastitis. These antibodies will be applied in the development of rapid immunoassays for detection of progesterone in milk.

Chapter 2
Materials and Methods

2. MATERIALS AND METHODS

2.1 Materials

Equipment, reagents, culture media, bacterial strains, buffers, commercial kits, commercial antigens and antibodies, used in the study are listed below.

2.1.1 Equipment

The list of the standard equipment used in and their supplier information is presented in Table 2.1.

Table 2.1. ***Equipments used in the study along with their suppliers.***

Equipment	Supplier
ÄKTA™ purifier 100 and UV-900 monitor ÄKTA™ Frac-950 Fraction Collector	GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden.
Balances (Chyo JK-180) (Mettler PJ300)	Medical Supply Company Ltd, Damastown, Mulhuddart, Dublin 15, Ireland.
Bio Rad Gene Pulser Xcell SDS Bio-Rad Min-Protean® 3 Cell Trans-Blot® SD Semi-Dry Transfer cell Bio-Rad Powerpac Basic Gene Pulser Xcell™ electroporation system	Alpha Technologies, The Leinster Technology Centre, Blessington Industrial Estate, Blessington, Co. Wicklow, Ireland.
Biometra $T_{GRADIENT}$ PCR machine Orbital shaker Vibra Cell™ sonicator Eppendorf centrifuge 5810R	AGB Scientific Limited - A VWR International Company, Orion Business Campus, Northwest Business Park, Ballycoolin, Dublin 15, Ireland.
DNA gel apparatus Bio-Rad (Wide-Mini-Sub® CellGT)	Alpha technologies, The Leinster Technology Centre, Blessington Industrial Estate, Blessington, Co. Wicklow, Ireland.
Gelaire BSB4 laminar unit	Gelman Ltd, 71 Broomhill Road, Tallaght Industrial Estate, Tallaght, Dublin 24, Ireland.
HermLe Z233MK-2 refrigerated centrifuge	HermLe Labortechnik GmbH, 25 Siemensstrasse, Wehingen, 78564, Germany.

IKA® Ultra-Turrax® T-18 Homogenizer	Janke & Kunkel IKA-Werk Ultra-Turrax, Staufen, 79129, Germany.
New Brunswick Scientific-Excella E24 Incubator Shaker Series (plate shaker) Nanodrop™ ND-1000 Spectrophotometer Tomy Autoclave SX-700E High Pressure Steam Sterilizer	Mason Technologies, Greenville Hall, 228 South Circular Road, Dublin 8, Ireland.
pH meter (Orion 3 star)	Medical Supply Company Ltd, Damastown, Mulhuddart, Dublin 15, Ireland.
Safire 2 plate reader	Tecan Group Ltd., Seestrasse 103, CH-8708 Männedorf, Switzerland.
Sorvall RC-5B Plus	Unitech Ltd., Airton Business Park, Airton Road, Tallaght, Dublin 24, Ireland.
Tube Roller-Mixers, SRT1 (Stuart)	Lennox Laboratory Supplies Ltd., John F. Kennedy Drive, Naas Road, Dublin 12, Ireland.

2.1.2 Reagents

All reagents were purchased from Sigma-Aldrich Ireland Ltd., Vale Road, Arklow, Wicklow, Ireland except where otherwise stated in Table 2.2.

Table 2.2. ***Reagents used in the study along with their suppliers.***

Reagent	Supplier
Bacteriological agar Yeast extract Tryptone	Cruinn Diagnostics Ltd., Hume Centre, Parkwest Business Park, Nanogor Road, Dublin 12, Ireland.
DNA ligase Helper phage Restriction enzymes	ISIS Ltd., Unit 1 & 2, Ballywaltrim Business Centre, Boghall Road, Bray, Co. Wicklow, Ireland.
dNTP Mix GoTaq® DNA Polymerase	Medical Supply Company Ltd, Damastown, Mulhuddart, Dublin 15, Ireland.
FastDigest® Restriction Enzymes	Fermentas UK, Sheriff House, Sheriff Hutton Industrial Park, York Y060 6RZ, UK.

PageRuler™ Plus Prestained Protein Ladder	AGB Scientific Limited - A VWR International Company, Orion Business Campus, Northwest Business Park, Ballycoolin, Dublin 15, Ireland.
PCR primers	Eurofins MWG Operon, 318 Worple Road, Raynes Park, London, SW20 8QU, UK. Integrated DNA Technologies, Woodside House, 20-23 Woodside Place, Glasgow, G3 7QF, Scotland.
Platinum® Taq DNA Polymerase High Fidelity	Bio-sciences, 3 Charlemont Terrace, Crofton Road, Dun Laoghaire, Dublin, Ireland.
Hybricare™ (ATCC # 46-X)	LGC Standards. Queens Road, Teddington, Middlesex TW11 0LY, UK.

2.1.3 Culture Compositions

The recipie of various media used for culturing bacterial cells and in cell culture are presented in Table 2.3 and 2.4.

Table 2.3. ***Recipe of bacteriological media used in this study.***

Super Broth (SB) Media	MOPS	10 g/L
	Tryptone	30 g/L
	Yeast extract	20 g/L

100 X 5O5 media	Glycerol	50% (v/v)
	Glucose	5% (w/v)

Super Broth (SB) Media	Tryptone	10 g/L
	Yeast extract	5 g/L
	NaCl	0.5 g/L
	KCl	2.5 mM
	$MgCl_2$	20 mM
	$MgCl_2$	20 mM

Table 2.4. ***Constituents of cell culture media used in the study.***

Hybricare	Hybricare™	1 sachet
	Sodium bicarbonate	1.5 g

Mixed in 1 L of deionised water, filtered sterile by 0.2 µm filter and stored at 4^{o}C.

Hybricare regular growth medium (10% FCS) (200 mL)	Hybricare	176 mL
	Foetal calf serum	20 mL
	Penicillin/streptomycin	4 mL

Hybricare HAT medium (200 mL)	Hybricare	152 mL
	Foetal calf serum	40 mL
	Penicillin/streptomycin	4 mL
	HAT	4 mL

Hybricare HAT (x3) medium (200 mL)	Hybricare	164 mL
	Foetal calf serum	20 mL
	Penicillin/streptomycin	4 mL
	HAT	12 mL

Hybricare HT medium (100 mL)	Hybricare	72 mL
	Foetal calf serum	20 mL
	Penicillin/streptomycin	4 mL
	HT	4 mL

2.1.4 Bacterial Strains

The *E. coli* strains used for various purposes of expression are listed in Table 2.5.

Table 2.5. E. coli *strains used for expression.*

E. coli TOP10F′ strain: {*lac*I^{q}, Tn*10*(Tet^{R})} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ*ΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^{R}) *end*A1 *nup*G
E. coli XL1-Blue strain: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI^{q}ZΔM15* Tn*10 (*Tet^{R})].
E. coli BL21-CodonPlus(DE3)-RIPL strain: *B F^{-} ompT hsdS(r_B $^{-}$ $m_B$$^{-}$) dcm+ Tetr gal λ(DE3) endA Hte [argU proL Camr] [argU ileY leuW Strep/Specr]*

2.1.5 Buffer Composition

Compositions of commonly used buffers are shown in Tables 2.6 to 2.10.

Table 2.6. ***Make-up of buffers used in the study.***

150 mM Phosphate buffered saline (PBS), pH 7.2	**1 L**
NaCl	0.15 M
KCl	2.5 mM
Na_2HPO_4	10 mM
KH_2PO_4	18 mM

The constituents were dissolved in 800 mL of ultra pure H_2O, and the pH was adjusted to 7.2. The solution was brought to 1 L volume by adding ulra-pure water.

Phosphate buffered saline Tween (PBST) (150 mM, pH 7.2)	PBS
	0.05% (v/v) Tween 20 detergent

Skim Milk-PBS	PBS
	Specified % (w/v) milk marvel powder

BSA-Skim Milk-PBS	PBS
	Specified % (w/v) milk marvel powder
	Specified % (w/v) BSA

Table 2.7. ***Make-up of buffers for Fast Protein Liquid Chromatography.***

20 mM Tris-HCl	Trizma base	0.242 g/L
Trizma base was dissolved in 800 mL of ultra-pure water. The pH was adjusted to desired using 1M HCl. The solution was brought to 1 L volume by adding ulra-pure water. The solution was filtered through a 0.2 µm filter.		
20 mM PIPES	PIPES	6.08 g/L
PIPES was dissolved in 800 mL of ultra-pure water. The pH was adjusted to 7.4 using 1M NaOH. The solution was brought to 1 L volume by adding ulra-pure water. The solution was filtered through a 0.2 µm filter.		
20 mM HEPES	HEPES	4.766 g/L
HEPES was dissolved in 800 mL of ultra-pure water. The pH was adjusted to 7.4 using 1M NaOH. The solution was brought to 1 L volume by adding ulra-pure water. The solution was filtered through a 0.2 µm filter.		
20 mM Triethanolamine	Triethanolamine	2.984 mL/L
Triethanolamine was mixed in 800 mL of ultra-pure water. The pH was adjusted to 7.4 using 1M HCl. The solution was brought to 1 L volume by adding ulra-pure water. The solution was filtered through a 0.2 µm filter.		
20 mM MOPS	MOPS	4.185 g/L
MOPS was dissolved in 800 mL of ultra-pure water. The pH was adjusted to 7.4 using 1M NaOH. The solution was brought to 1 L volume by adding ulra-pure water. The solution was filtered through a 0.2 µm filter.		

20 mM Phosphate buffer	$Na_2HPO_4.7H_2O$	3.27 g/L
	NaH_2PO_4	0.94 g/L

Both ingredients were dissolved in 800 mL of ultra-pure water. The pH was adjusted to 7.4 using 1M HCl. The solution was brought to 1 L volume by adding ulra-pure water. The solution was filtered through a 0.2 µm filter.

20 mM Tris-HCl containing 500 mM NaCl	**1 L**
NaCl	29.22 g
20 mM Tris-HCl (desired pH)	to 1 L

The solution was filtered through a 0.2 µm filter and degassed for 1 h.

20 mM Tris-HCl, pH 7.8 containing 1.6 M ammonium sulphate	**1 L**
$(NH_4)_2SO_4$	211.42 g
20 mM Tris-HCl (pH 7.8)	to 1 L

The solution was filtered through a 0.2 µm filter and degassed for 1 h.

Table 2.8. ***Constituents of buffers for SDS-PAGE and Western blot.***

1 M Tris-HCl	Trizma base	121.14 g/L

Trizma base was dissolved in 800 mL of ultra-pure water. The pH was adjusted to pH 8.8 and 6.8 by adding 1M HCl. The solution was brought to 1 L volume by adding ulra-pure water. The solution was filtered through a 0.2 µm filter and used for SDS-PAGE preparations.

12.5% Separation gel	**1gel/6mL**
1 M Tris-HCl, pH 8.8	1.5 mL
30% (w/v) acrylamide	2.5 mL
2% (w/v) methylamine bisacrylamide (Bis-Acrylagel)	1.0 mL
Ulra-pure water	934 µL
10% (w/v) sodium dodecyl sulphate (SDS)	30 µL
10% (w/v) ammonium persulphate (APS)	30 µL
TEMED	6 µL

12.5% Stacking gel	**1gel/ 2.5 mL**
1 M Tris-HCl, pH 6.8	300 µL
30% (w/v) acrylamide	375 µL
2% (w/v) methylamine bisacrylamide (Bis-Acrylagel)	150 µL
Ulra-pure water	1.74 mL
10% (w/v) sodium dodecyl sulphate (SDS)	24 µL
10% (w/v) ammonium persulphate (APS)	24 µL
TEMED	2.5 µL

Electrophoresis buffer	**1 L**
Trizma base	3.0 g
Glycine	14.4 g
SDS	1.0 g
Ulra-pure water	to 1 L

Sample treatment buffer (4X)	10 mL
0.5M Tris, pH 6.8	2.5 mL
Glycerol	2.0 mL
2-mercaptoethanol	0.5 mL
20% SDS (w/v)	2.5 mL
Bromophenol blue	20 ppm
Ulra-pure water	2.5 mL
0.5M Tris, pH 6.8	2.5 mL

Coomassie blue stain dye	**1 L**
Coomassie blue R-250	2.0 g
Methanol	450 mL
Acetic acid	100 mL
Ulra-pure water	450 mL

Coomassie destain solution	**1 L**
Acetic acid	100 mL
Methanol	250 mL
Ulra-pure water	650 mL

Transfer buffer	**1 L**
Trizma base	3.0 g
Glycine	14.4 g
Methanol	200 mL
Ulra-pure water	to 1 L

Table 2.9. ***Constituents of solutions for*** **N-*acetyl-β-D-glucosaminidase enzymatic assay.***

200 mM Sodium carbonate	Na_2CO_3	21.2 g/L

200 mM Sodium bicarbonate	Na_2CO_3	16.8 g/L

250 mM Citrate buffer	$HOC(COONa)(CH_2COONa)_2.2H_2O_4$	6.025 g/L

Sodium citrate tribasic dihydrate was dissolved in 800 mL of ultra-pure water. The pH was adjusted to 4.4 using 1M HCl. The solution was brought to 1 L volume by adding ulra-pure water. The solution was filtered through a 0.2 µm filter.

Susbtrate solution	**10 mL**
4-Methylumbelliferyl *N*-acetyl-β-D-glucosaminide dihydrate	0.0042 g
250 mM Citrate buffer	10 mL

Development buffer (pH 10)	**50 mL**
200 mM Sodium carbonate	8 mL
200 mM Sodium bicarbonate	17 mL
Ulra-pure water	25 mL

Table 2.10. ***Constituents of buffers for Immobilised metal ion affinity chromatography (IMAC).***

100 mM NaOH	NaOH	4 g/L

Appropriate amount of NaOH was dissolved in 10 mL of ultra-pure water and the solution was filtered through a 0.2 µm filter.

100 mM Sodium acetate	CH_3COONa	8.2 g/L

Appropriate amount of CH_3COONa was dissolved in 45 mL of ultra-pure water. The pH was adjusted to 4.4 using 1M HCl and the solution was filtered through a 0.2 µm filter.

Lysis Buffer	**100 mL**
150 mM PBS	100 mL
NaCl	2.92 g
Imidazole	0.136 g

Running Buffer	**100 mL**
150 mM PBS	100 mL
NaCl	2.92 g
Imidazole	0.136 g
Tween-20	1 mL

Neutralisation Buffer	**1 mL**
100 mM NaOH	500 µL
10X PBS, pH 7.2	500 µL

2.1.6 Commercial Kits

Different commercial kits along with their suppliers are listed in Table 2.11.

Table 2.11. ***Commercial kits used and their suppliers.***

Kit	Supplier
Enterokinase Cleavage Capture Kit	Merck Chemicals Ltd., Boulevard, Industrial Park, Padge Road, Beeston, Nottingham, NG9 2JR, UK.
Superscript III reverse transcriptase kit	Invitrogen Corporation, 5791 Van Allen Way, Carlsbad, CA 92008, USA.
Perfectprep Gel Cleanup Kit	Eppendorf AG, Barkhausenweg 1, Hamburg 22339, Germany.
QIAGEN Plasmid Midi Kit	QIAGEN House, Fleming Way, Crawley West Sussex, RH10 9NQ, UK.
Wizard Plus SV Miniprep™ kit	Promega, 2800 Woods Hollow Road, Madison, WI 53711, USA.
EZ-Link Micro Sulfo-NHS-LC-Biotinylation Kit	Fisher Scientific Ireland, Suite 3, Plaza 212, Blanchardstown Corporate Park 2, Ballycoolin, Dublin 15

2.1.7 Fast Performance Liquid Chromatography (FPLC) Columns

All the FPLC columns used in this study (RESOURCE Q - 1 mL column, HiTrap™ HIC Selection Kit and HiLoad™ Superdex™ 200 prep grade column (25 mL) were obtained from GE Healthcare Life Sciences, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK.

2.1.8 Commercial Antigens and Antibodies

Different commercial antigens and antibodies along with their suppliers are listed in Table 2.12 and 2.13.

Table 2.12. ***Commercial antigens used and their suppliers.***

Antigen	Supplier
β-*N*-Acetylglucosaminidase from bovine kidney Peptidoglycan from *Staphylococcus aureus*	Sigma-Aldrich Ireland Ltd., Vale Road, Arklow, Wicklow, Ireland
N-acetyl-β-D-glucosaminidase (NAGase) (Pig NAGase)	Diazyme Laboratories - A Division of General Atomics, 12889 Gregg Ct., Poway, CA 92064, USA.
Progesterone-3-CMO Progesterone-3-CMO-BSA	Fitzgerald Industries International 30 Sudbury Road, Suite 1A North Acton, MA 01720 USA
Progesterone 3-biotin	Cambridge Bioscience Ltd. Munro House Trafalgar Way Bar Hill Cambridge, CB23 8SQ, UK

Table 2.13. ***Commercial antibodies used and their suppliers.***

Antigen	Supplier
Anti-chicken (HRP-labelled) pAb Anti-bovine (HRP-labelled) pAb	Gallus Immunotech Inc., 412 Waverly Hills Drive, Cary, NC 27519, USA.
Anti-HA (HRP-labelled), High Affinity mAb	Roche Diagnostics Ltd., Charles Avenue, Burgess Hill, West Sussex, RH15 9RY, UK.
Anti-HIS (HRP-labelled) mAb Anti-rabbit (HRP-labelled) pAb Anti-mouse (AP-labelled) pAb	Sigma-Aldrich Ireland Ltd., Vale Road, Arklow, Wicklow, Ireland.
Anti-M13 antibody (HRP-labelled)	GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden.

2.2 Methods

2.2.1 Production of Anti-NAGase Monoclonal Antibody

2.2.1.1 Immunisations

All the procedures involving animals were first approved by the ethics committees of the School of Biotechnology and Dublin City University and they then underwent the approval and licensing by the Department of Health and Children under license number B100/4256. The Laboratory Animal Science and Training (LAST), Ireland course was undertaken to ensure that legal aspects of animal usage in research are well understood and experimental procedures are performed with high standards of care. All the necessary precautions were taken to ensure minimal distress to the animals during the entire study.

Two Balb/c mice aged 8-10 weeks were immunised with the commercial bovine β-*N*-acetylglucosaminidase / *N*-acetyl-β-D-glucosaminide / β-hexosaminidase (NAGase) (Sigma-Aldrich, Ireland) for generation of anti-NAGase monoclonal antibodies. For primary immunisations, the antigen was diluted to a concentration of 50 µg in 150 µL of PBS and mixed with an equal volume of Freund's complete adjuvant. This mixture was vortexed until a stable emulsion was formed. For booster injections, the antigen was diluted to 25 µg in 150 µL of PBS and emulsified with an equal volume of Freund's incomplete adjuvant by vortexing. Mice were immunised intraperitoneally with 300 µL of the immunogenic preparation. Five boosts were given at three weekly intervals. A week after the every boost, the tail was bled to collect serum and verify the response of the animal to the immunisations. Serum separation was carried out by incubating the blood in a tilted position at 4^{o}C overnight. The next day the blood was centrifuged (Eppendorf centrifuge 5810R) at 805 g for 10 min

resulting in the separation of the straw coloured serum from the clotted remainder. The serum was removed carefully and 0.2% (w/v) sodium azide was added. The serum was then aliquoted into 1.5-mL tubes and stored in -20^{o}C. Ten µg of the antigen prepared in 100 µL 150 mM PBS (pH 7.2) was given intraperiteonally four days prior to the fusion as the final boost.

2.2.1.2 ELISA to check the titres of mice sera against NAGase

Bovine NAGase was diluted to 2 µg/mL in 150 mM PBS (pH 7.2) and 100 µL was coated onto wells of 96 well-plates (Nunc, USA), one plate for each mouse. Phosphate buffered saline (PBS) solutions are extensively employed in different laboratory protocols (Lichtenauer *et al*., 2009). PBS (150 mM) was selected for this study in order to mimic physiological conditions for optimum antigen-antibody interaction (Carr and Hinshaw, 1997; Prince and Dickinson, 2003). A pH of 7.2 was chosen as antigen-antibody interactions are stable at around pH 7.2 at 37^{o}C (Ramana *et al*., 1995). Additionally, PBS has high buffer capacity at pH 7.2 (Penalvo *et al*., 1996). Consequently, numerous studies have used PBS pH 7.2 for immunoassays (Miller and Kemp, 1979; Gerlach *et al*., 1993; Baltes *et al.*, 2001; Wang *et al.*, 2005). The plates were incubated for 1 h at 37^{o}C. All the wells of ELISA plates were then blocked with 200 µL 5% (w/v) skim milk-PBST for 1 h at 37^{o}C. Doubling dilutions of mice sera and pre-immune sera were prepared at dilutions of 1:1,000 to 1:512,000 and 100 µL of each was added in duplicate to wells. The plates were incubated for 1 h at 37^{o}C. One hundred µL of 1:2,000 dilution of the detection antibody (alkaline phosphatise-labelled anti-mouse IgG) in 150 mM PBS (pH 7.2) was then added and plates were again incubated for 1 h at 37^{o}C. The plates were washed 3 times with 0.05% (v/v) PBST and 3 times with PBS after each step. Finally, the enzyme

label was detected by addition of 100 µL of p-nitrophenyl phosphate (pNPP) (Sigma-Aldrich, Ireland). After 30 min the absorbances in the wells were read at 405 nm using a Safire 2 plate reader (Tecan, Switzerland).

2.2.1.3 Fusion protocol for monoclonal antibody production

An improved myeloma cell line, Sp2/mIL-6 (ATCC # CRL-2016™), was used for monoclonal antibody production. The Sp2/mIL-6 cell line had been engineered to produce interleukin-6 and was reported to be an improved myeloma fusion parent (Harris *et al*., 1992). The use of Sp2/mIL-6 cells was reported to produce an increased number of immunoglobulin-secreting clones and hybridoma cells with better immunoglobulin expression (Harris *et al*., 1992; Dessain *et al.*, 2004).

Sp2/mIL-6 were maintained between 2 x 10^5 cell/mL and 2 x 10^6 cell/mL in regular hybricare growth medium. The cells were fed and split every two to four days depending on the cell density. On the day of the fusion, samples of Sp2/mIL-6 flasks were counted and viability was determined using a Neubauer haemocytometer and trypan blue. An aliquot of the cell suspension was mixed at 1 in 2 with trypan blue (0.4% (w/v) Sigma-Aldrich, Ireland) and 20 µL was pipetted onto the counting chamber of the haemocytometer. The viable cell count was carried out within 5 min of the addition of trypan blue as the solution is cytotoxic. The trypan blue stains the dead cells blue and does not penetrate live cell membranes, which remain unstained.

Hemocytometers consist of 2 chambers, each of which is divided into 9 squares with the dimension of 1 x 1 mm. A cover glass is supported 0.1 mm over these

squares so that the total volume over each square is 1.0 mm^2 x 0.1 mm or 0.1 mm^3, or 10^{-4} cm^3. Since 1 cm^3 is equivalent to 1 mL, the cell concentration per mL will be the average count per square x 10^4.

Total and live cells in the 25 central squares of the grid were counted microscopically. The number of cells was multiplied by the dilution factor and by 10^4 to give the number of total or live cells/mL. The percentage viability was then calculated.

Flasks with the highest viability (> 95%) were combined to obtain a suspension containing 10^7 cells. The cells were transferred into a sterile 50-mL tube and placed in the incubator, at 37^{o}C, until needed. Polyethylene glycol (PEG) was used to induce fusion of myeloma cells with spleenocytes. PEG induces cell agglutination and cell-to-cell contact, leading to subsequent cell fusion. However, the detailed mechanisms underlying PEG-mediated cell fusion are unknown. PEG removed from the fridge and left to reach room temperature until the fusion.

The immunised mouse was killed by cervical dislocation and immersed in a beaker containing 70% (v/v) IMS and was placed in a laminar flow hood. The spleen was removed using sterile dissection instruments and transferred to a petri dish containing ‘serum-free’ medium. Excess fat was trimmed off from the spleen. The spleen was placed on a sieve and cells were gently forced out using a syringe and ‘serum-free’ medium. The spleen cell suspension was left standing for five min and decanted into a 50-mL sterile tube. The tube was topped up to 50 ml with serum-free medium. The 50-mL tube containing the

spleen cells and the 50-mL tube containing the myeloma cells were centrifuged (Eppendorf centrifuge 5810R) at 300 g for 5 min. The cell pellet in the 50-mL tube was loosened by scraping the 50-mL tube along the grid at the front of the laminar flow hood and re-suspended in 50 mL of serum-free medium prior to being spun down as before. The pellet was re-suspended in 25 mL of 'serum-free' medium. At this stage, 1 mL of each suspension was transferred to a vial, as controls, and moved into the incubator, at 37^{o}C, until needed. The spleen cells and the Sp2/mIL-6 cells were combined and spun down at 300 g for 5 min. The supernatant was decanted and the tube was placed in a beaker containing warm water (at 37^{o}C) in the laminar flow unit. The pre-warmed PEG (1.5 mL) was added to the pellet over a 1 min period, with constant mixing. Mixing was continued for another for 3 min, with tube still in the warm water. The cells were transferred to 400 mL of pre-warmed hybricare regular growth medium and the cells were plated out onto 20 x 96-well tissue culture plates at 200 µL per well. Twenty-four hours after the fusion, 100 µL of hybricare HAT (x3) was added to the wells for selection.

At day 4 post-fusion, the hybridoma growth was checked microscopically, and the cells were fed with HAT medium. A hundred µL of medium was carefully pipetted out of all the wells and replaced with 100 µL of HAT medium. Cells were fed with HAT medium until the control Sp2/mIL-6 cells were dead. At day 10 post-fusion a screening ELISA was carried out. A hundred µL of medium was carefully pipetted out of all the wells and used for screening. One hundred µL of HT medium was then added to all the wells. The wells were regularly checked after this stage any old HT media was replaced with fresh media wherever required. If sufficient viable cells were observed after feeding the cells

with HT medium for 2-3 times, the cells were switched to hybricare regular growth medium.

2.2.1.4 Screening ELISA

The ELISA was carried out as described in section 2.2.1.2. The exception was that for the screening ELISA, tissue culture supernatants from the 96-well tissue culture plates (Nunc, USA), diluted to 1:2 in 1% (w/v) skim milk in PBST, were used instead of the sera dilutions.

2.2.1.5 Inhibition ELISA

A 96-well plate was coated (half the wells) with 2 µg/mL bovine NAGase and with 5% (w/v) skim milk in 150 mM PBS for the remaining half (section 2.2.1.2). The plate was then blocked as described in section 2.2.1.2. Tissue culture supernatant from clone 10H5 (which was found to be positive on screening) was diluted to 1:64 and 1:100 in 150 mM PBS (pH 7.2). Both dilutions of tissue culture supernatant were mixed with bovine NAGase (diluted to 4 µg/mL in 150 mM PBS (pH 7.2)) in a 1:1 ratio and incubated for 30 min at 37^{o}C. A hundred µL of NAGase-tissue culture supernatant mixture was then added in duplicate to the NAGase-coated and skim milk-coated wells. The plate was incubated for 1 h at 37^{o}C. The bound antibody (contained in tissue culture supernatant) was detected by adding a 100 µL per well of a 1:2,000 dilution of alkaline phosphatase-labelled anti-mouse IgG secondary antibody with incubation for 1 h incubation at 37^{o}C. The plates were washed 3 times with 0.05% (v/v) PBST and 3 times with PBS after each step. Finally, 100 µL per well of p-Nitrophenyl phosphate (pNPP) (Sigma-Aldrich, Ireland) was added and plate was incubated

for 30 min at 37^{o}C. The absorbance was read at 405 nm on a Safire 2 plate reader (Tecan, Switzerland).

2.2.1.6 Dialysis of bovine NAGase

Dialysis of bovine NAGase was carried out using D-Tube™ Dialyzer Mini, MWCO 6-8 kDa columns (Merck Chemicals Ltd., UK). The protocol provided by the column manufacturers was used with a slight modification. The tube was removed and the tube was hydrated by adding 250 µL of filtered 150 nm PBS, pH 7.2. After 2 min the PBS from column was removed gently using a pipette. Bovine NAGase (200 µL) was added to the tube. The tube was placed in the floating rack provided with the kit. The rack was placed in a bucket containing 2 L 150 mM PBS, pH 7.2. It was set up so that the entire surface of membrane of the tube was submerged in PBS. A magnet was placed inside the bucket and the bucket was placed on a magnetic stirrer at 4^{o}C. The PBS in the bucket was allowed to stir gently for 24 h. After 24 h the contents of the tube were carefully removed and analysed for protein content using the Nanodrop™ ND-1000 Spectrophotometer. The purity of the protein was checked by SDS-PAGE.

2.2.1.7 SDS-PAGE analysis of dialysed bovine NAGase

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a technique for the separation of proteins based on their molecular weights. It is achieved by the migration of charged molecules in a gel matrix on application of an electrical field. The proteins are linearised by heating in presence of an anionic detergent sodium dodecyl sulphate (SDS) and a reducing agent β-mercaptoethanol. SDS disrupts the secondary, tertiary and quaternary structure of the protein to produce a linear polypeptide chain coated with negatively

charged SDS molecules. β-mercaptoethanol assists the protein denaturation by reducing inter- and intrachain disulfide bonds. This is achieved by heating the protein sample briefly in a boiling water bath in the presence of a reducing agent. The linearised proteins coated with a negative charge are then separated and resolved as discrete bands as they migrate in an electric field through the "sieving" action of the acrylamide gel matrix. Electric field causes the negatively-charged proteins to migrate across the gel towards the positive electrode. Smaller molecules face less resistance within the gel matrix and move faster. Whereas, larger molecules move slowly as they have to counter greater resistance. Thus, proteins are separated according to size.

Proteins were analysed for their purity and apparent molecular weight by separation on 12.5% (w/v) SDS-PAGE with a separation and stacking gel (Table 2.8). The separation gel was casted between two clean glass plates (provided with the apparatus) and left to polymerise. After the gel had polymerised, a stacking gel was poured and wells were prepared by using a 1 mm comb, for loading protein samples. The samples were prepared by adding appropriate volumes of 4X sample treatment buffer (Table 2.8) and ultra-pure water. Twenty µL of each protein sample was added into a well. The gels were placed in an electrophoresis apparatus and submerged in electrophoresis buffer. The gel was run at 100-150 V until the tracker dye had reached the bottom of the gel. The gels were taken out and stained using Coomassie blue (Table 2.8) for 1 h and then destained using destaining solution (Table 2.8) for 3-4 h until the protein bands are distinctively visible against a clear background.

2.2.1.8 Exploring the use of different NAGase-associated antigens for immunisations

Bovine NAGase exists in two predominant forms, hexosaminidase A (alpha polypeptide) (HEXA) and hexosaminidase B (beta polypeptide) (HEXB). Protein sequences of bovine HEXA and HEXB were analysed for homology with HEXA and HEXB of human, mouse, rabbit and chicken using Basic Local Alignment Search Tool (BLAST) tool, available at http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome. The protein sequences used in this study were available at Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/) (Altschul *et al*., 1997; Altschul *et al*., 2005) (Table 2.14).

Table 2.14: *Pubmed accession numbers of protein sequences used*.

Species	Pubmed accession no. for HEXA	Pubmed accession no. for HEXB
Cow (*Bos taurus*)	NP_001068632.1	NP_001069978.2
Pig (*Sus scrofa*)	ACA43012.1	NP_999086.1
Human (*Homo sapiens*)	AAH84537.1	NP_000512.1
Mouse (*Mus musculus*)	NP_034551.2	NP_034552.1
Rabbit (*Oryctolagus cuniculus*)	AAV34701.1	AAV34702.1
Chicken (*Gallus gallus*)	NP_001025561.1	XP_424791.2

Bovine HEXA and HEXB was aligned with HEXA and HEXB of the above mentioned species in order to identify possible short peptides sequences that are unique to bovines. ClustalX 2.0.11 (Thompson *et al.*, 1994; Larkin *et al.*, 2007) was used to align the sequences and aligned sequences were analysed using BioEdit v7.0.5. The selected peptides were analysed for net hydrophilicity by Hopp and Woods method (Hopp and Woods, 1981) using ProtScale an

online tool (available at http://www.expasy.ch/cgi-bin/protscale.pl) (Gasteiger *et al.*, 2005). The selected peptides were also analysed to determine any sequence homology with other bovine or mouse proteins. This was achieved using the BLAST tool available online.

Two BALB/c female mice aged 8-10 weeks were immunised with the two short peptides, conjugated to keyhole limpet hemocyanin (KLH), (Table 3.2) from bovine HEXA, pig NAGase and bovine NAGase. For primary immunisations, the antigens were diluted to a concentration of 50 µg in 150 µL of PBS and mixed with of an equal volume of Freund's complete adjuvant. This mixture was vortexed until a stable emulsion was formed. For booster injections, the antigens were diluted to 25 µg in 150 µL of PBS and emulsified with an equal volume of Freund's incomplete adjuvant by vortexing. Mice were immunised intraperitoneally with 300 µL of the antigen preparation. The mice were then boosted five times at three weekly intervals. A week every boost, the tail was bled to collect serum to verify the response of the animal to the immunisations.

2.2.1.9 ELISA to check the titre of mice sera against different NAGase-associated antigens

Ninty-six well-plates (Nunc, USA) (two plate for each mouse) were coated with NeutrAvidin (5 µg/mL in PBS), bovine NAGase (2 µg/mL in PBS), pig NAGase (2 µg/mL in PBS) and KLH (5 µg/mL in PBS) (three rows per plate were coated with NeutrAvdin and one row per plate was coated with each of the other antigens). The plates were incubated for 1 h at 37^{o}C and then blocked with 200 µL 5% (w/v) skim milk-BSA-PBST for 1 h at 37^{o}C. One hundred µL of 10 µg/mL of peptide 1 and peptide 2 were added to 1 row each per plate. A hundred µL of

PBS was added to the rest of the plate. Doubling dilutions of the serum from each mouse and the pre-immune serum were prepared at dilutions of 1:200 to 1:3,200 and 100 µL of each was added to ELISA plates wells to check response against both the peptides, bovine NAGase, pig NAGase, using NeutrAvidin, KLH and blocking solution as controls. The plates were incubated for 1 h at 37^{o}C. One hundred µL of 1:2,000 dilution of the detection antibody (alkaline phosphatise-labelled anti-mouse IgG) in 150 mM PBS was then added to add all the wells and plates were again incubated for 1 h at 37^{o}C. The plates were washed 3 times with 0.05% (v/v) PBST and 3 times with PBS after each step. Finally, the plates were developed for 30 min using 100 µL of p-Nitrophenyl phosphate (pNPP) (Sigma-Aldrich, Ireland). The absorbances in the wells were read at 405 nm using a Safire 2 plate reader (Tecan, Switzerland).

2.2.2 Generation of Avian Anti-NAGase scFv

2.2.2.1 Dialysis of NAGase

The dialysis of NAGase was carried out as outlined in section 2.2.1.6.

2.2.2.2 Immunisation of chicken with NAGase

For primary immunisations, the antigen was diluted to a concentration of 100 µg in 275 µL of PBS and emulsified by rigourous votexing following addition of an equal volume of Freund's complete adjuvant. For booster injections, the antigen was diluted to 50 µg in 275 µL of PBS and emulsified with an equal volume of Freund's incomplete adjuvant. A male leghorn chicken was injected with 500 µL antigen-adjuvant mixture. It was boosted 4 times at four weekly intervals. A bleed was taken after every boost and the serum was separated as described in section 2.2.1.1. The serum was assayed by ELISA to determine the antibody

titre against neat and dialysed NAGase. Once the titre had reached a satisfactory level, the chickens were again boosted 5 days prior to sacrifice.

2.2.2.3 RNA isolation and cDNA preparation from chicken spleen and bone marrow

A Gelaire BSB 4 laminar unit was prepared by spraying with both 70% (v/v) IMS and RNase™ ZAP, thus, ensuring an uncontaminated environment (free from contaminant RNA). The chicken was sacrificed by cervical dislocation and its spleen and bone marrow were harvested immediately using sterile dissection implements.

The bone marrow from chicken femurs was washed out with 10 mL of chilled TRIzol® reagent (Invitrogen, USA) using a needle and syringe. Ten mL of chilled TRIzol® reagent was added to the avian spleen and all samples were fully homogenised using a sterile (autoclaved and baked overnight at 180^{o}C) homogenizer. The tubes were incubated at room temperature for 5 min, to allow for the total dissociation of nucleoprotein complexes while maintaining the integrity of the RNA, and centrifuged (Eppendorf centrifuge 5810R) at 2,465 g for 10 min at 4^{o}C. The supernatants were carefully removed and transferred to fresh 'RNase-free' 50-mL Oakridge tubes (Thermo Fisher Scientific, USA). For each sample, 3 mL of 'RNase-free' chloroform were added and tubes were shaken vigorously for 15 s and incubated for 15 min at room temperature. Addition of chloroform leads to the separation of the homogenised spleen into an upper aqueous phase (containing RNA) and a lower organic phase (containing DNA and protein). Tubes were centrifuged (Eppendorf centrifuge 5810R) at 12,000 x g at 4^{o}C for 15 min producing 3 layers, a lower red phenol /

chloroform phase, a protein interphase and a colourless liquid upper phase with the RNA. The upper aqueous phase, containing the RNA, was carefully removed and transferred to a fresh 'RNase-free' 50-mL Oakridge tube. To each tube, 15 mL of propan-2-ol was added and contents were shaken vigorously for 15 s, stored at room temperature for 10 min and centrifuged (Eppendorf centrifuge 5810R) at 12,000 x g at 4^{o}C for 30 min. RNA was precipitated as a white pellet on the bottom and side of the tube. The supernatant was decanted carefully and the pellet was washed with 30 mL of 75% (v/v) ethanol and centrifuged (Eppendorf centrifuge 5810R) at 12,000 x g at 4^{o}C for 10 min. This step was repeated and after removal of the supernatant, the RNA pellet was allowed to air dry for 5 min. The pellet was then resuspended in 250 µL of 'RNase-free' water. The RNA concentrations were determined by spectrophotometric measurement at 260 nm with a NanoDrop^{TM} spectrophotometer ND-1000.

An aliquot of freshly isolated RNA was used for cDNA synthesis. The remaining RNA solution was precipitated at -20^{o}C with 1/10 the volume of 'RNase-free' sodium acetate, pH 5.2, (Applied Biosystems Inc., USA), and twice the total sample volume of 100% ethanol. To enhance RNA precipitation, 'nuclease-free' glycogen (Applied Biosystems Inc., USA) was added at a final concentration of 1 µg/µL.

2.2.2.4 Reverse transcription of total RNA to cDNA

A PCR reaction was set up for synthesizing first strand-cDNA from mRNA using oligo dT20 priming. A 20X reaction was set up for both the mixtures. Twenty 10 µL aliquots were made for mixture 1 (RNA, Oligo (dt) primer and dNTP mix)

(Table 2.15), in sterile 'RNase-free' PCR tubes and left to incubate at 65^{o}C for 5 min and then placed on ice. This allows initial denaturation of the sample. To this sample 10 µL of mixture 2 (RT Buffer, $MgCl_2$, DTT, RNase Out, Superscript™ enzyme) (Table 2.15) was added. This step allows the annealing of oligo (dt) to the RNA template containing poly (A) tail at the 3′ end. The reaction mixture was incubated at 50^{o}C for 50 min, during which cDNA strand was synthesised. The reaction was terminated by heating the mixture at 85^{o}C for 5 min. Finally, 2 µL of RNase™ H was added to each of the 20 x 10 µL reactions and incubated at 37^{o}C for 20 min. This step ensures the removal of residual RNA template which might decrease the sensitivity of PCR and other analysis carried out with the final cDNA product.

Table 2.15. ***Composition of cDNA synthesis reaction mixtures.***

Mixture 1 components	Concentration in 10 µL volume
Total RNA	5 µg
Oligo (dt) primer	0.5 µg
dNTP mix	1 mM
Molecular grade water	To make 10 µL

Mixture 2 components	Concentration in 10 µL volume
10X RT Buffer	2X
Magnesium chloride	2.5 mM
DTT	20 mM
'RNase-Out'	40 U
Superscript III enzyme	200 U

2.2.2.5 PCR primers for amplification of chicken scFv (pComb series)

The oligonucleotides utilised (Table 2.16) were obtained from Eurofins-MWG-Operon (Eurofins MWG Operon, Germany). The oligonucleotides obtained were used to generate a chicken scFv library from both the bone marrow and spleen,

as described in Barbas *et al.* (2001). The amplified heavy and light chains will contain a long linker sequence along with *Sfi*I sites compatible for cloning into pComb vector series.

Table 2.16. *Oligonucleotides for amplification of chicken scFv.*

Primer name	Sequence
CHICK-V_H-FOR	5′-GGT GGT TCC TCT AGA TCT TCC TCC GGT GGC GGT GGC TCC GGC GGT GGT GGC TCT TCC GCC CTG ACG TTG GAC GAG-3′
CHICK-V_H-REV	5′-CTG GCC GGC CTG GCC ACT AGT GGA GGA GAC GAT GAC TTC GGT CC-3′
CHICK-V_L-FOR	5′-GTG GCC CAG GCG GCC CTG ACT CAG CCG TCC TCG GTG TC-3′
CHICK-V_L-REV	5′-GGA AGA TCT AGA GGA ACC ACC TAG GAC GGT CAG G-3′
CHICK-SOE-FOR	5′-GAG GAG GAG GAG GAG GAG GTG GCC CAG GCG GCC CTG ACT CAG-3′
CHICK-SOE-REV	5′-GAG GAG GAG GAG GAG GAG GAG CTG GCC GGC CTG GCC ACT AGT GGA GG-3′

(Barbas *et al.*, 2001).

2.2.2.6 Amplification of antibody variable domain genes

For amplification of variable heavy and light genes the components for a 1X reaction are shown in Table 2.17.

Table 2.17. *Composition of PCR mix for chicken heavy and light antibody chain amplification.*

Components	Concentration in 50 µL volume
5X Colorless GoTaq® Flexi Buffer	1X
CHICK-V_H-FOR primer	60 pM
CHICK-V_H-REV primer	60 pM
cDNA	1.0 ng
dNTP mix	0.2 mM
Magnesium chloride	4 mM
GoTaq® Flexi DNA Polymerase	2.5 U
Molecular grade water	To make 50 µL

Components	Concentration in 50 µL volume
5X Colorless GoTaq® Flexi Buffer	1X
CHICK-V_L-FOR primer	60 pM
CHICK-V_L-REV primer	60 pM
cDNA	1.0 ng
dNTP mix	0.2 mM
Magnesium chloride	4 mM
GoTaq® Flexi DNA Polymerase	2.5 U
Molecular grade water	To make 50 µL

The PCR for the amplification of the antibody variable heavy and light genes was performed in the Biometra $T_{GRADIENT}$ PCR machine under the following conditions.

Temperature (°C)	Time (s)	No. of cycles
94.0	**600**	**1**
94.0	**30**	**30**
56.0	**30**	
72.0	**45**	
72.0	**600**	**1**

2.2.2.7 Purification of V_H and V_L variable gene fragments using Qiagen gel extraction kit

Gel extraction was carried out for purification of the amplified gene. The V_H and V_L chain gene amplicons were resolved on 0.8% (w/v) agarose gel. Bands of correct size were excised carefully from the gel by using sterile scalpels to avoid any cross-over of the DNA and transferred to sterile clean 1.5-mL micro-

centrifuge tubes. DNA binding buffer, provided by the manufacturer, was added to the gel fragment in 1:3 ratio (w/v). The gel-buffer mixture was dissolved by placing it in a water bath at 50^{o}C for 10 min. One volume of propan-2-ol, equal to the original weight of the gel slice, was then added and mixed for precipitation of the DNA. The resulting mixture was then added to the silica column placed on a collection tube. The column was centrifuged (HermLe Z233MK-2 refrigerated centrifuge) at 22,000 x g for 1 min to remove any residual buffer. The 'flow-through' was discarded and the column was washed with 750 μL wash buffer. The column was centrifuged as before and washed with 250 μL wash buffer. The column was centrifuged (HermLe Z233MK-2 refrigerated centrifuge) at 22,000 x g for 2 min and the DNA was eluted from the column using 30 μL molecular grade. The purified DNA absorbance was measured at 260 nm using Nanodrop™ ND-1000.

2.2.2.8 Splice by overlap extension (SOE) PCR

The V_H and V_L purified fragments were joined using an SOE-PCR *via* a glycine-serine linker $(G_4S)_3$, producing a fragment of approximately 750 bp. The composition of PCR mix was used for the SOE-PCR is shown in Table 2.18.

Table 2.18. ***Reaction mix for overlapping chicken heavy and light chains.***

Components	Concentration in 50 µL volume
10X High Fidelity PCR Buffer	1X
CHICK-V_L-FOR primer	60 pM
CHICK-V_L-REV primer	60 pM
Purified V_H chain from chicken spleen	100 ng
Purified V_L chain from chicken spleen	100 ng
dNTP mix	0.2 mM
Magnesium sulphate	4 mM
GoTaq® Flexi DNA Polymerase	2.5 U
Molecular grade water	To make 50 µL

Components	Concentration in 50 µL volume
10X High Fidelity PCR Buffer	1X
CHICK-V_L-FOR primer	60 pM
CHICK-V_L-REV primer	60 pM
Purified V_H chain from chicken bone marrow	100 ng
Purified V_L chain from chicken bone marrow	100 ng
dNTP mix	0.2 mM
Magnesium sulphate	3 mM
GoTaq® Flexi DNA Polymerase	2.5 U
Molecular grade water	To make 50 µL

The SOE-PCR was performed in the Biometra $T_{GRADIENT}$ PCR machine under the following conditions.

Temperature (°C)	Time (s)	No. of cycles
94.0	**600**	**1**
94.0	**30**	**30**
56.0	**30**	
72.0	**60**	
72.0	**600**	**1**

2.2.2.9 *Sfi*I restriction digest of purified SOE-PCR fragment and ligation into pComb3XSS vector

The SOE product and pComb3XSS vector (phagemid vector) were digested using the *Sfi*I restriction enzyme. The *Sfi*I sites on both forward and reverse SOE primers are different and similar to that of the *Sfi*I sites on the vector thus allowing unidirectional cloning of the scFv gene into the phagemid vector. Both the digestions (outlined in Table 2.19) were carried out for 5 h at 50^{o}C. The vector was further digested with *Xho*I and *Xba*I, cutting the stuffer released, and thus, eliminating the possibility of stuffer contamination in the resulting library. Finally the vector was treated with calf alkaline phosphatase to prevent vector re-ligation.

Table 2.19. ***Reaction mix for digestion of vector and chicken scFv SOE-product.***

Components	Concentration in 100 µL volume
10X NEB buffer 2	1X
pComb3XSS vector	50 µg
*Sfi*I enzyme	300 U
100X BSA	1X
Molecular grade water	To make 100 µL

Components	Concentration in 50 µL volume
10X NEB buffer 2	1X
SOE product	10 µg
*Sfi*I enzyme	300 U
100X BSA	1X
Molecular grade water	To make 100 µL

Both digests were resolved *via* electrophoresis on a 0.8% agarose gel and then gel-purified, as described in section 2.2.2.7. The restricted scFv gene was then

ligated into the pComb3XSS vector in a 2:1 (insert:vector) ratio, as outlined in Table 2.20, and incubated at room temperature overnight.

Table 2.20. ***Ligation mixture for cloning chicken scFv SOE-product into pComb3XSS vector.***

Components	Concentration in 50 µL volume
10X T_4 Ligase buffer	1X
Digested pComb3XSS vector	1.4 µg
Digested scFv gene	0.7 µg
T_4 DNA *Ligase* enzyme	400 U
Molecular grade water	To make 100 µL

The ligation mixture was then subjected to ethanol precipitation by adding 1/10nth the volume of 'RNase-free' sodium acetate (pH 5.2), twice the volume of 100% (v/v) ethanol and 1 µL of glycogen. After overnight precipitation at -20^{o}C, the sample was centrifuged (HermLe Z233MK-2 refrigerated centrifuge) at 22,000 x g for 20 min at 4^{o}C and the pellet was washed with 70% (v/v) ice-cold ethanol in molecular grade water. The mixture was centrifuged (HermLe Z233MK-2 refrigerated centrifuge) at 22,000 x g for 10 min at 4^{o}C and the pellet was allowed to air-dry briefly and resuspended in 5 µL of molecular grade water.

2.2.2.10 Preparation of Electrocompetent XL1-Blue *E. coli* cells

An overnight culture of XL1-Blue cells was prepared by inoculating a single colony (from a freshly streaked onto an agar plate) of commercial XL1-Blue cells (Stratagene, USA) in 15 mL SB media supplemented with 30 µg/mL tetracycline and shaking at 220 rpm at 37^{o}C. Next morning 500 mL of SB media supplemented with 10 mL of 20% (w/v) glucose and 5 mL of 1 M $MgCl_2$ were inoculated with 2.5 mL of the overnight culture. The culture was allowed to grow

until the OD reached ~ 0.6. When the optimal OD was reached the cultures were chilled on ice for 15 min and subsequently transferred to pre-chilled 250-mL centrifuge tubes. The cultures were centrifuged (Eppendorf centrifuge 5810R) at 4^{o}C for 20 min at 3,220 g. The supernatant was discarded and the pellet from each tube was re-suspended in 12.5 mL of 10% (v/v) glycerol. The re-suspended pellets were combined into two tubes and were subsequently filled with upto 250 mL of 10% (v/v) glycerol. Both tubes were again centrifuged (Eppendorf centrifuge 5810R) at 4^{o}C for 20 min at 3,220 g. The resultant pellets were again suspended in 250 mL of 10% (v/v) glycerol and centrifuged as before.

The resulting pellets were re-suspended in 12.5 mL of 10% (v/v) glycerol and transferred to sterile 50-mL tubes. The 50-mL tubes were centrifuged (Eppendorf centrifuge 5810R) for 15 min at 3,220 g at 4^{o}C. The supernatant was gently poured off until the pellet began to slide out and the pellet re-suspended in the remaining supernatant volume (~ 5 mL per tube). One hundred µL aliquots of the resulting cell preparation were prepared and used fresh for transformation.

2.2.2.11 Preparation of Helper Phage

A LB agar plate supplemented with 30 µg/mL tetracycline was streaked by commercial XL1-Blue cells (Stratagene, USA), and incubated overnight at 37^{o}C. Next day an overnight culture of XL1-Blue cells was prepared by inoculating a single colony (from the streaked agar plate) in 15 mL SB media supplemented with 30 µg/mL tetracycline and shaking at 220 rpm at 37^{o}C.

Next morning 4 mL of SB media 30 µg/mL tetracycline were inoculated with 4 µL of the overnight culture and incubated at 37^{o}C for 1 h while shaking at 220 rpm. The XL1-Blue cells were subsequently infected with 2 µL VCM13 helper phage (Stratagene, USA) and the culture was left static at 37^{o}C for 30 min for subsequent F-pilus conjugation to occur (an essential part of infection process) and then transferred into a 37^{o}C shaking incubator for 2 h at 220 rpm. Subsequently, kanamycin (70 µg/mL) was added and the culture was incubated overnight at 37^{o}C and 220 rpm. Kanamycin addition ensures the propagation of XL1-Blue cells infected with helper phage. On the following morning the cultures were transferred to sterile 250-mL centrifuge tubes and centrifuged (Eppendorf centrifuge 5810R) at 18,500 x g. The supernatants obtained were transferred to fresh 250-mL tubes and 4% (w/v) PEG 8000 and 3% (w/v) sodium chloride was added to them for precipitating out the phage. The tubes were kept chilled on ice for 1 h. The precipitated phage was centrifuged (Eppendorf centrifuge 5810R) for 20 min at 18,500 x g with the brakes off. The supernatant was discarded and the resulting phage pellet was re-suspended in 1 mL (per pellet) of 1% (w/v) BSA in 150 mM PBS, pH 7.2.

The suspended phages were serially diluted (10^{-1}-10^{-12}) in SB media. The phage titre was determined by infecting the XL1-Blue cells in exponential growth phase, with phage dilutions (10^{-8}-10^{-12}). Following a 15 min infection at 37^{o}C, the cells were plated on LB agar plates containing 100 µg/mL carbenicillin and incubated overnight at 37^{o}C.

2.2.2.12 Transformation of cloned gene into *E. coli* by electroporation

Electrocompetent XL1-Blue *E. coli* cells (section 2.2.2.10) were transformed with the ligated scFv vector construct, by electroporation, using the Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, USA). The program was set at 25 µF, 1.25 kV and the pulse controller at 200 Ω. The *E. coli* cells (100 µL) were thawed on ice just before transformation. The ligated product (10 µL) was added to the cells, mixed, left to incubate for 1 min and immediately transferred it to an ice-cold 0.2 cm electroporation cuvette (Bio-Rad Laboratories, USA). The cuvette was tapped gently to move the cell suspension at the base and was placed in the ShockPod and pulsed once. The cuvette was quickly removed from the chamber and 1 mL of SOC medium was added immediately to the cuvette, thus, resuspending the cells in medium. The resuspended cells were transferred to a 20-mL sterile tube containing 2 mL of SOC media and incubated for 1 h at 220 rpm at 37^{o}C shaking, to facilitate recovery of the cells. The transformants from both bone marrow and spleen libraries were pooled and inoculated in 500 mL sterile SB medium supplemented with 100 µg/mL carbenicillin. The culture was grown at 37^{o}C at 220 rpm until mid-exponential phase of growth (OD ~ 0.6) was achieved. Helper phage (2 x 10^{11} pfu/mL) was added and the culture was left static at 37^{o}C for 30 min and then transferred into a 37^{o}C shaking incubator for 2 h at 220 rpm. Subsequently, kanamycin (70 µg/mL) was added and the culture was incubated overnight at 37^{o}C and 220 rpm. The overnight culture was centrifuged (Eppendorf centrifuge 5810R) at 18,500 x g for 15 min at 4^{o}C and the supernatant was transferred to clean sterile centrifuge tubes (Thermo Fisher Scientific, USA), leaving the bacterial pellet.

Phage particles in the supernatant were precipitated by addition of 4% (w/v) of PEG 8000 and 3% (w/v) sodium chloride. The mixture was agitated at 220 rpm in a 37^{o}C shaking incubator until both additives were dissolved and then it was incubated on ice at 4^{o}C for 1 h for phage precipitation. The precipitated phage was harvested by centrifugation at 18,500 x g for 25 min at 4^{o}C with brakes off. After centrifugation, the supernatant was carefully decanted and the phage pellet was resuspended in 2 mL of 1% (w/v) BSA-PBS buffer solution. The resuspended phage pellet was then added to a clean 1.5-mLmicro-centrifuge tube and centrifuged (HermLe Z233MK-2 refrigerated centrifuge) at 22,000 x g for 10 min at 4^{o}C to remove any bacterial debris. Finally, the phage supernatant was transferred to a fresh micro-centrifuge tube and stored on ice at 4^{o}C.

2.2.2.13 Selection and enrichment of anti-NAGase scFv clones using biopanning

An immunotube (Nunc, USA) was coated overnight at 4^{o}C with 500 µL of 200 µg/mL bovine NAGase in PBS solution, and blocked for 2 h at room temperature with 3% (w/v) BSA in PBS (150 mM, pH 7.2). Following one wash with both PBST and PBS, 500 µL of rescued phage was added to the immunotube and incubated on a roller mixer for 2 h at room temperature. The immunotube was then washed 5 times with PBST and PBS, to remove non-binding phage. Specifically bound phage were then eluted by incubation with 500 µL of 10 mg/mL trypsin-PBS and incubating the tube at 37^{o}C for 30 min.

Eluted phage were added to 4 mL of mid-exponential phase XL1-Blue cells, allowing infection for 30 min (static) at 37^{o}C. Four mL of SB media supplemented with 2.4 µL of 100 mg/mL carbenicillin and 12 µL of 5 mg/mL

tetracycline were added. Two µL of infected culture was removed and diluted 1:100 in SB media. Ten and a hundred µL of the diluted culture was plated on LB agar plates containing 100 µg/mL carbenicillin for estimating the output titre. An input titre was performed by making serial dilutions (10^{-1}-10^{-12}) of the PEG-precipitated phage in in SB media. The XL1-Blue cells in exponential growth phase were infected with phage dilutions (10^{-8}-10^{-12}) for 15 min at 37^{o}C (static). The cells were plated on LB agar plates containing 100 µg/mL carbenicillin and incubated overnight at 37^{o}C. The 8 mL phage-infected culture was incubated at 37^{o}C for 1 h while shaking at 220 rpm. After 1h 2.4 µL carbenicillin was added and the culture was incubated at 37^{o}C for an additional hour. For subsequent rounds 250 mL sterile SB medium was used in contrast to 500 mL used in the first round. Other changes in panning parameters are outlined in Table 2.21.

Table 2.21. ***Parameters varied in subsequent rounds of panning of avian anti-NAGase scFv library.***

Biopanning round	Concentration of NAGae coated (µg/mL)	Number of washes	
		PBST	PBS
Round 1	200	5	5
Round 2	100	5	5
Round 3	100	7	7
Round 4	100	10	10
Round 5	100	15	15

2.2.2.14 Polyclonal phage ELISA

A 96-well ELISA plate, coated with 100 μL of 50 μg/mL bovine NAGase, was incubated at 37^{o}C for 1 h. It was then blocked with 200 μL of 5% (w/v) BSA-milk marvel in 150 mM PBS (pH 7.2) for 1 h at 37^{o}C. One hundred μL of phage inputs from each round of biopanning (diluted 1:5 in 1% (w/v) BSA PBST) were added in duplicate to the ELISA plate and incubated for 1 h at 37^{o}C. Following incubation of the phage, the plate was washed 3 times with both PBST and PBS. Subsequently, 100 μL of 1:2,000 dilution of a HRP-conjugated anti-M13 antibody (GE Healthcare Life Sciences, Sweden) in 1% (w/v) BSA-milk marvel-PBST was added to each well and incubated for 1 h at 37^{o}C. Detection of the antigen-antibody complex was achieved by the addition of 100 μL of TMB substrate. Colour was allowed to develop for 20 min at room temperature, after which the reaction was quenched using 50 μL of 10% (v/v) HCl. The absorbance values were then read at 450 nm on a Safire 2 plate reader (Tecan, Switzerland).

2.2.2.15 Infecting pan 4 and 5 output phage into TOP10F′ cells for soluble expression

Soluble antibody fragments, without the pIII protein, were produced by infecting phagemid DNA from round 4 and 5 of biopanning into different aliquots of *E. coli* TOP10F′ cells (Stratagene, USA) at mid-logarithmic growth phase. After incubation for 30 min at 37^{o}C, serial dilutions were prepared in SB media (10^{-2} to 10^{-10}), and plated on LB plates containing 100 μg/mL carbenicillin. Single colonies were inoculated into individual wells (inner 60) of a sterile 96-well plate containing 200 μL of SB media with 100 μg/mL carbenicillin and 1% (w/v) glucose. After an overnight incubation at 37^{o}C, master plates of the original

clones were prepared by adding glycerol (20% (v/v)) and storing at -80^{o}C. These plates were used as a backup stock for each putative clone of interest. Thirty µL from the overnight subculture plates were inoculated into fresh SB media (150 µL) supplemented with 1X 5O5 medium and 100 µg/mL carbenicillin. The sterile 96 well plates were propagated at 37^{o}C and 180 rpm until an OD of ~ 0.8 was achieved. A final concentration of 1 mM IPTG was added to each individual well and the plates were induced overnight at 220 rpm at 30^{o}C.

2.2.2.16 Screening of anti-NAGase clones by indirect ELISA

The overnight cultures were subjected to three cycles of freeze-thaw for periplasmic scFv extraction. Cell extracts were cleared by centrifugation for 10 min at 3,220 x g and the lysates were diluted 1:3 in 1% (w/v) BSA-milk-PBS. Indirect ELISA was carried out as described in 2.2.4.14, with the exceptions that a NAGase coating concentration of 10 µg/mL was used and a 1:2,000 dilution of horse radish peroxidase (HRP)-labelled rat anti-HA (hemagglutinin) antibody (in 1% (w/v) BSA-milk 1X PBST) was used to detect scFvs that were specific for bovine NAGase.

2.2.2.17 Direct ELISA of chicken anti-NAGase scFv clones against different NAGase-associated antigens

Anti-NAGase scFv clones (section 2.2.2.16) were checked against recombinant HexA (Chapter 4) and both Hex α-subunit peptides (Table 3.2). The ELISAs were carried out as previously outlined in sections 2.2.1.2, 2.2.1.9 and 2.2.1.16. For recombinant HexA a coating concenteration of 5 µg/mL (in PBS, 7.2) was used.

2.2.2.18 Chicken immunisations with different NAGase-associated antigens

Three chickens were individually immunised with with recombinant HexA (Chapter 4), HexA-1-KLH (Table 3.2) and HexA-2-KLH (Table 3.2). For primary immunisations, the antigen was diluted to a concentration of 100 µg in 275 µL of PBS and emulsified by rigourous votexing following addition of an equal volume of Freund's complete adjuvant. For booster injections, the antigen was diluted to 50 µg in 275 µL of PBS and emulsified with an equal volume of Freund's incomplete adjuvant. Chickens were injected with 500 µL antigen-adjuvant mixture. They were boosted 4 times at four weekly intervals. Bleed were taken after every boost and the serum was separated as described in section 2.2.1.1.

2.2.2.19 Chicken serum titre against different NAGase-associated antigens

A series of dilutions (ranging from 1:1,000 to 1:1,280,000) of the chicken sera, diluted in 1% (w/v) BSA-Milk-PBS (pH 7.2), were tested against the antigen they were immunised with in an indirect ELISA format (sections 2.2.1.2 and 2.2.1.9).

2.2.3 Generation of rabbit anti-NAGase polyclonal antibodies

2.2.3.1 Immunisations

Three New Zealand white rabbits were individually immunised with with recombinant HexA (Chapter 4), HexA-1-KLH (Table 3.2) and HexA-2-KLH (Table 3.2). For primary immunisations, the antigens were diluted to a concentration of 200 µg in 275 µL of PBS and mixed with an equal volume of Freund's complete adjuvant. This mixture was vortexed until a stable emulsion was formed. For booster injections, the antigen was diluted to a concentration of

100 µg in 275 µL of PBS and emulsified with an equal volume of Freund's incomplete adjuvant by vortexing. Rabbits were immunised intramuscularly with 500 µL of this preparation. Five boosts were given at three weekly intervals and a week after every boost, blood was collected from the rabbits and the serum was isolated.

2.2.3.2 Rabbit serum titre against different NAGase-associated antigens

Rabbit serum titres were checked to determine whether sufficient responses against the immunised antigens were achieved. The ELISAs were carried out as previously outlined in sections 2.2.1.2 and 2.2.1.9. For recombinant HexA a coating concenteration of 5 µg/mL (in PBS, 7.2) was used. A series of dilutions of immune and pre-immune sera, in 1% (w/v) BSA-Milk-PBS (pH 7.2), ranging from 1:1,000 to 1:1,280,000 were used.

2.2.4 Purification of Hexosaminidase B (HexB) from Bovine Spleen

2.2.4.1 Optimisation of protocol for preparation of spleen homogenate

All manipulations were carried out at 4^{o}C. After a brief washing in homogenisation buffer B (20 mM Tris-HCl, pH 7.4) bovine spleen tissue (provided by Enfer Scientific Ltd., Ireland) was skinned, cut into small pieces and suspended in an equal amount (v/w) of homogenisation buffer containing 1X protease inhibitor cocktail (Sigma-Aldrich, Ireland). The spleen was homogenised in a Warring blender (2 min at full speed). The homogenates were then centrifuged (Eppendorf centrifuge 5810R) at 18,500 g for 20 min. The supernatant was collected and filtered through Whatmann filter paper 1 and

subsequently through 0.45 µm sterile filter. The filtered homogenate obtained was assessed for NAGase activity (section 2.2.4.2).

Six different buffers (20 mM Tris-HCl, 20 mM MOPS, 20 mM PIPES, 20 mM HEPES, 20 mM Triethanolamine (TEA) and 20 mM potassium phosphate buffer) were evaluated to find the buffer which showing maximum retention of HEXB activity after homogenisation. Twenty millilitres of six different buffers chosen were used to prepare spleen homogenates using the same amount (w/v) of spleen tissue (20 g). The homogenates were processed as outlined above and the filtered homogenates obtained were analysed for NAGase active as outlined in section 2.2.4.2.

After assessment of appropriate buffer, *i.e.* Tris-HCl (section 4.2.1), experiments were conducted to assess the most appropriate pH of Tris-HCl allowing maximum retention of HEXB activity after homogenisation. Twenty millilitres of Tris-HCl buffer with seven different pHs ranging from 7.2 to 8.4, in increments of 0.2, were used to prepare spleen homogenates from 20 g tissue each. The homogenates were processed as outlined previously and the filtered homogenates obtained were analysed for NAGase active as outlined in section 2.2.4.2.

2.2.4.2 *N*-acetyl-β-D-glucosaminidase (NAGase) enzymatic assay

Enzymatic activity of NAGase was measured using a fluorometric procedure using 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide as a substrate. NAGase converts the substrate into a fluorescent product, 4-methylumbelliferone.

The NAGase enzymatic assay was performed using the method of Kitchen *et al.* (1976). Thirty µL volume of each sample was placed in a 1.5-mLtube containing 200 µL of the substrate solution (*i.e.* 1 mM 4-methylumbelliferyl-*N*-acetyl-β-D- glucosaminide). The contents were mixed by inverting the tubes 3-4 times and incubated at 37^{o}C for 5 min. One hundred µL of the incubated mixture was added to each well of a black 96-well microtitre plate (Nunc, USA) containing 100 µL of development buffer. The fluorescence intensity of each sample was measured on a Safire 2 plate reader (Tecan, Switzerland) at an excitation wavelength of 365 nm and an emission wavelength of 450 nm, gain as 53 and Z-position as 7500 µm. Substrate buffer or elution buffer used were employed as controls.

A standard curve using different concentrations of the end product (4-methylumbelliferone) of the enzymatic degradation of 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide was prepared. Appropriate dilutions of 4-methylumbelliferone (10 mM) were prepared in 250 mM citrate buffer pH 4.4 to obtain 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µM solutions of 4-Methylumbelliferone. Each dilution was prepared in triplicate. One hundred µl of each dilution was dispensed in wells of a black 96-well microtitre plate (Nunc, USA) containing 100 µL of development buffer. The fluorescence intensity of each standard was measured on a Safire 2 plate reader (Tecan, Switzerland) at an excitation wavelength of 365 nm and an emission wavelength of 450 nm, with gain as 53 and Z-position as 7500 µm. The enzymatic units were calculated using this standard curve.

2.2.4.3 Purification of HexB from spleen homogenate

The strategy to purify HexB encompassed two step chromatographic procedures, involving anion exchange chromatography followed by hydrophobic interaction chromatography or gel filtration chromatography. The filtered supernatant was purified using FPLC (ÄKTA™ Explorer 100 with UV-900 monitor and Frac-950 Fraction Collector) by anion exchange chromatography followed by hydrophobic interaction chromatography or gel filtration chromatography.

2.2.4.3.1 Anion exchange chromatography

Anion exchange chromatography was performed on a RESOURCE Q 1 mL column (GE Healthcare, UK) using 20 mM Tris-HCl, pH 7.8, (buffer A) for equilibration and 20 mM Tris-HCl, pH 7.8, containing 500 mM NaCl, (buffer B) for elution of the enzyme from the column. Ten mL of filtered supernatant was passed through the RESOURCE Q 1 mL column. Initially, a linear gradient was applied extending over 20 column volumes (CV) with increasing NaCl concentration (0%-100% (v/v) saturation) at a flow rate of 1 mL/min to elute the bound protein. One mL fractions were collected and fractions showing absorbance at 280 nm were analysed for NAGase activity (section 2.2.4.2).

The anion exchange protocol above was modified to include a five step gradient, from 0 to 10% buffer B with a hold at 20% (v/v) buffer B for 5 CVs, a second step from 20%-30% (v/v) with a hold at 30% (v/v) for 5 CVs, a third step from 30%-40% with a hold at 40% (v/v) for 5 CVs, a fourth step from 40%-50% (v/v) with a hold at 50% (v/v) for 5 CVs and a final step from 50%-100% (v/v) with a hold at 100% (v/v) for 10 CVs. Fractions of 3 mL were collected. The

NAGase activity assay was performed on all fractions showing an absorbance at 280 nm (section 2.2.4.2).

2.2.4.3.2 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) was performed using the HiTrap HIC Selection Kit (GE Healthcare, UK) consisting of seven hydrophobic interaction chromatographic media with different hydrophobic characteristics. Fractions showing NAGase activity (section 2.2.4.3.1) after anion exchange chromatography were pooled and ammonium sulphate, to a final concentration of 1.5 M, was added to the pooled fractions (refer section 4.2.2.2). One mL of this sample was applied to all the seven columns (HiTrap Phenyl Fast Flow (FF) (high substitution (sub)), HiTrap Phenyl FF (low sub), HiTrap Phenyl High Performance (HP), HiTrap Butyl FF, HiTrap Butyl-S FF, HiTrap Butyl HP and HiTrap Octyl FF) in the HiTrap HIC Selection Kit. Hydrophobic interaction chromatography was performed the same way for each column using 20 mM Tris-HCl, pH 7.8, containing 1.6 M ammonium sulphate, (buffer A) and 20 mM Tris-HCl, pH 7.8, containing 500 mM NaCl, (buffer B). A linear gradient was applied extending over 20 CVs with decreasing ammonium sulphate (50%-0% (v/v) saturation) at a flow rate of 1 mL/min to elute the bound protein. The NAGase activity assay was performed on all fractions showing an absorbance at 280 nm (section 2.2.4.2).

2.2.4.3.3 Gel filtration chromatography

Fractions (section 2.2.4.3.1) showing NAGase activity after anion exchange chromatography were pooled. Ten mL of this sample was applied to a HiLoad™ Superdex™ 200 prep grade column (25 mL) (GE Healthcare, UK) and gel

filtration chromatography was performed using 150 mM PBS, pH 7.2, at a flow rate 0.5 mL/min for a duration of 700 min. Fractions of 5 mL were collected and the NAGase enzymatic activity assay was performed (section 2.2.4.2) on all fractions showing an absorbance at 280 nm.

2.2.4.4 Analysis of purified enzyme fractions by SDS-PAGE

Fractions with NAGase activity obtained after FPLC were analysed (for purity and size of protein) using SDS-PAGE. SDS-PAGE was carried out as described in section 2.2.1.7.

2.2.5 Expression of Recombinant *Bos taurus* (cow) Hexosaminidase A (alpha polypeptide) and Hexosaminidase B (beta polypeptide)

2.2.5.1 PCR primers for amplification of HEXA and HEXB

Primers for recombinant *Bos taurus* (cow) hexosaminidase A (alpha polypeptide, α-subunit) (HEXA) were designed from the gene sequence pubmed accession no. NP_001068632.1 and primers for recombinant *Bos taurus* hexosaminidase B (beta polypeptide, β-subunit) (HEXB) were designed from the gene sequence pubmed accession no. NP_001069978.2 (Table 2.22). All the primers were checked for ORFs (open reading frames) and ordered from Integrated DNA Technologies after standard desalting.

Table 2.22. ***Oligonucleotides for amplification HEXA and HEXB.***

Primer name	Sequence
HEXAFOR	5′-CGC CGC GGA TCC GCA GGC TCC ACG CTC AGG TT-3′
HEXAREV	5′-CCT TTA GCG GCC GCG GTT TGT TCA AAC TCC ATG TCA-3′
HEXBFOR	5′-CGC CGC GGA TCC GGA GCA GCG CGG GCT-3′
HEXBREV	5′- CCT TTA GCG GCC GCC ATT CTG CCC TCA TGC TCA C -3′

2.2.5.2 Amplification of bovine HEXA and HEXB

HEXA and HEXB were amplified from cDNA extracted from one of the bovine spleen samples (sample no. 4) (section 2.2.7.1) by PCR using specific primers (section 2.2.5.1). The composition of the PCR mix used for HEXA and HEXB amplifications are shown in Table 2.23.

Table 2.23. ***Composition of reaction mix for HEXA and HEXB gene amplification for expression.***

Components	Concentration in 50 µL volume
10X High Fidelity PCR Buffer	1X
HEXAFOR primer	20 pM
HEXAREV primer	20 pM
cDNA	1.0 ng
dNTP mix	0.2 mM
Magnesium sulphate	3 mM
Molecular grade water	To make 50 µL

Components	Concentration in 50 µL volume
10X High Fidelity PCR Buffer	1X
HEXBFOR primer	20 pM
HEXBREV primer	20 pM
cDNA	1.0 ng
dNTP mix	0.2 mM
Magnesium sulphate	5 mM
Platinum® *Taq* DNA Polymerase High Fidelity	2.5 U
Molecular grade water	To make 50 µL

The PCR amplification of HEXA was performed in the Biometra $T_{GRADIENT}$ PCR machine under the following conditions.

Temperature (°C)	Time (s)	No. of cycles
94.0	**600**	**1**
94.0	**60**	**30**
61.9	**45**	
72.0	**90**	
72.0	**600**	**1**

The PCR amplification of HEXB was performed in the Biometra $T_{GRADIENT}$ PCR machine under the following conditions.

Temperature (°C)	Time (s)	No. of cycles
94.0	600	1
94.0	60	30
64.8	45	
72.0	90	
72.0	600	1

2.2.5.3 Purification of HEXA and HEXB gene fragments using Qiagen gel extraction kit

Gel extraction was carried out for purification of the amplified gene. The HEXA and HEXB gene amplicons were resolved on 0.8% (w/v) agarose gel. Bands of correct size were excised carefully from the gel by using sterile scalpels to avoid any cross-over of the DNA and transferred to sterile clean 1.5-mL micro-centrifuge tubes. Gel extraction was carried out as outlined in section 2.2.2.7.

2.2.5.4 Digestion and ligation of HEXA and HEXB gene into pET-32b(+) vector

Purified HEXA and HEXB genes (section 2.2.3.3) were digested along with the pET-32b(+) vector using *Bam*HI and *Not*I restriction enzymes. The digestion was carried out for 3 h at 37°C using the reaction mix shown in Tables 2.24 and 2.25.

Table 2.24. ***Composition of reaction mix for digestion of pET-32b(+) vector.***

Components	Concentration in 50 µL volume
10X FAST digest buffer	1X
pET-32b(+) vector	10 µg
*Not*I enzyme	10 µL
*Nco*I enzyme	10 µL
Molecular grade water	To make 50 µL

Table 2.25. ***Composition of reaction mix for digestion of HEXA and HEXB genes.***

Components	Concentration in 50 µL volume
10X FAST digest buffer	1X
HEXA	5 µg
*Not*I enzyme	5 µL
*Nco*I enzyme	5 µL
Molecular grade water	To make 50 µL

Components	Concentration in 50 µL volume
10X FAST digest buffer	1X
HEXB	5 µg
*Not*I enzyme	5 µL
*Nco*I enzyme	5 µL
Molecular grade water	To make 50 µL

The digested vector was treated with 2 µL of alkaline phosphatase for 15 min at 37^{o}C which was further inactivated by incubating the mix at 65^{o}C for 10 min. Alkaline phosphatase prevents self ligation of the vector by removing a phosphate group from the 5' end, keeping the vector linearised until ligation. The digested products were gel-purified and ligated overnight at room temperature. The composition of ligation mix is shown in Table 2.26.

Table 2.26. ***Ligation mix for HEXA and HEXB genes into pET-32b(+) vector.***

Components	Concentration in 50 µL volume
10X ligation buffer	1X
Digested pET-32b(+) vector	100 ng
Digested insert (HEXA)	140 ng
T_4 DNA *Ligase* enzyme	400 U
Molecular grade water	To make 50 µL

Components	Concentration in 50 µL volume
10X ligation buffer	1X
Digested pET-32b(+) vector	100 ng
Digested insert (HEXB)	140 ng
T_4 DNA *Ligase* enzyme	400 U
Molecular grade water	To make 50 µL

2.2.5.5 Transformation of cloned gene into *E. coli* cells

Cloned HEXA and HEXB genes were transformed into commercial BL21-CodonPlus®(DE3)-RIPL competent cells. BL21-CodonPlus®(DE3)-RIPL competent cells are specialised cells, producing an increased supply of rare *E. coli* tRNAs thus improving the expression of many proteins that are difficult to express in conventional *E. coli* due to codon bias.

An aliquot (100 µL) of cells was thawed on ice for each transformation. Two µL of β-mercaptoethanol mix (1:10 dilution) provided with the kit, was added to the cells followed by a 10 min incubation on ice. Addition of β-mercaptoethanol increases the efficiency of transformation. Five µL of ligation was added to the cells and the cells were further incubated on ice for 30 min. A heat pulse was given by transferring the cells to a water bath at 42^{o}C, for 20 s, followed by incubating the cells on ice for duration of 5 min. This step is critical as it allows entry of cloned DNA into the cells. SOC media (1 mL) was added to the cells and they were grown in 37^{o}C for 1 h. Finally 200 µL of cells were plated on LB

agar plates supplemented with 100 µg/mL carbenicillin and incubated overnight at 37^{o}C.

2.2.5.6 Growing transformants and protein expression

Single colonies were inoculated into 7 individual wells of a 12-well plate containing 1 mL of SB media supplemented with 100 µg/mL carbenicillin. After overnight incubation at 37^{o}C, 100 µL from these plates were subcultured into 10 mL of fresh SB media supplemented with1X 5O5 medium and 100 µg/mL carbenicillin. Stock plates were prepared by adding glycerol (20% (w/v)) to the overnight grown cultures and the clones were then stored at -80^{o}C. The subcultured cells were grown until an OD of ~ 0.8 was reached followed by addition of isopropyl-β-D-thiogalactoside (IPTG) to 1 mM final concentration and inducing them overnight at 220 rpm at 30^{o}C. The overnight cultures were spun down at 3,220 x g for 20 min and the pellets were resuspended in 500 µL PBS. The resuspended culture was transferred to 1.5-mLtubes and sonicated on ice for 90 s (with 3 s interval between each pulse) at amplitude of 50, using a microtip Vibra Cell^{TM} sonicator and the cell debris removed by centrifuging at 3,220 x g for 20 min at 4^{o}C. The lysate supernatant was then passed through a 0.2 µm sterile filter to remove any residual cell debris.

2.2.5.7 Western blot analysis of the expressed proteins

Expression of α- and β-subunits was checked by performing Western blot analysis. The samples were prepared by adding of 4X sample treatment buffer followed by denaturing protein at 95^{o}C for 10 min. The SDS-PAGE gel was run at 100 V for 1 h with 20 µL of each sample added to each well (section 2.2.1.7) and was processed further for Western blotting. Eight samples were analysed

which included 7 induced clones and negative controls (heart-fatty acid binding protein clone in the pET-26b(+) vector along with HEXA clones and cardiac troponin I clone in pET-32b(+) vector along with HEXB clones).

Four sheets of Whatman® Gel Blot Papers (Sigma-Aldrich, Ireland) and one sheet of 3 mm Protran BA 85 Nitrocellulose membrane (Carl Stuart Ltd., Ireland) were cut to the same dimensions of the SDS-PAGE gel. Each of the layers of filter paper and the nitrocellulose membrane were soaked in transfer buffer for 15-30 min along with the gels. Two layers of the soaked filter paper were placed between the electrodes of Trans-Blot® Semi-Dry Transfer cell (BioRad, Ireland) apparatus. The soaked nitrocellulose membrane was placed over the filter paper over which the SDS-PAGE gel was carefully placed. The gel was followed by two buffer-soaked filter papers to complete the set up. All air bubbles were removed by carefully rolling each of the layers with a disposable 1 mL serological pipette. Proteins were transferred from the gel to the nitrocellulose membrane by applying a constant voltage of 20 V for 20 min. The membrane containing the transferred protein from the SDS-PAGE gel was blocked overnight with 5% (w/v) BSA-milk-PBS, at 4^{o}C. Following 3 washes with PBST and PBS the membrane was incubated with anti-HIS (HRP-labelled) mAb (1:2,000 dilution), with gentle agitation, for 1.5 h at room temperature. The membrane was washed, as before, and developed by adding TMB substrate. The reaction was stopped by washing the membrane three times with water.

2.2.5.8 Time-course induction study of recombinant HEXA and HEXB expression in *E. coli*

A single clone of both HEXA (clone no. 3) and HEXB (clone no. 4) were grown and processed for protein expression as described in section 2.2.5.6. However, in this case 10 mL samples were collected every hour post-induction for 4 h. A final sample was collected following overnight incubation, post-induction. The samples were processed, as described in section 2.2.5.6, and analysed by Western blotting as described in section 2.2.5.7.

2.2.5.9 Purification of recombinant HEXA

A selected HEXA clone (clone no. 3) was grown overnight culture at 37^{o}C in 10 mL SB media, containing 100 µg/mL carbenicillin, by inoculating with 10 µL of the stock culture. A 5 mL volume of this culture was then inoculated into 500 mL SB media containing 100 µg/mL carbenicillin and 1X 5O5 media. The subcultured clone was incubated at 37^{o}C at 220 rpm until an OD ~ 0.8 was achieved. The cultures were then induced by adding IPTG to a final concentration of 1 mM and incubating overnight at 30^{o}C and 220 rpm.

The overnight expressed culture was then transferred to two 250-mL sorvals tubes and centrifuged (Sorvall RC-5B Plus) at 18,500 x g in a GSA rotor for 20 min (‘brake-on’) to pellet the bacterial cells. The supernatant was discarded and the excess media removed by inversion of the sorval tubes onto a paper towel. The cell pellet was thoroughly resuspended in 30 mL of lysis buffer and aliquoted into 5 mL volumes in 50-mL sterile tubes. Each aliquot was sonicated for periplasmic protein extraction (section 2.2.5.6). The cell debris was removed by centrifuging at 3,220 x g for 10 min at 4^{o}C, followed by transferring the

supernatant containing the protein to fresh tubes and storing them on ice at 4^{o}C.

Purification of recombinant HEXA using immobilised metal affinity chromatography (IMAC) was carried out using Ni^{+}-NTA agarose resin (QIAgen, USA). A 4 mL aliquot of Ni^{+}-NTA agarose resin (QIAgen, USA) was added to a 20-mL column and allowed to settle for 10 min. The column was equilibrated using 30 mL of running buffer. The filtered lysate was then applied to the equilibrated column and the 'flow-through' was collected in a 50-mL tube. The column was then washed with 30 mL of running buffer to remove any loosely bound non-specific proteins and the wash collected in a 50-mL tube. The protein was eluted using of 100 mM sodium acetate, pH 4.4, and collected in 24 x 400 µL aliquots in 1.5-mLmicro-centrifuge tubes containing 100 µL of filtered neutralisation buffer. The neutralised protein (12 mL) was then thoroughly buffer exchanged against filtered 20 mL PBS using a 5 kDa cut-off Vivaspin™ 6 column (AGB, Ireland). The buffer-exchanged protein was quantified using the Nanodrop™ ND-1000 and aliquoted into clean PCR tubes and stored at -20^{o}C.

2.2.5.10 Analysis of purified recombinant HEXA by SDS-PAGE

Purified HEXA was analysed for its purity and apparent molecular weight by separation on 12.5% (w/v) SDS-PAGE. Samples were prepared and SDS-PAGE analysis was carried out as described in section 2.2.1.7.

2.2.5.11 Enterokinase treatment of purified recombinant HEXA

IMAC-purified recombinant HEXA was subjected to enterokinase digestion to cleave-off the N-terminal fusion tag using the Enterokinase cleavage capture kit

(Merck Chemicals Ltd., UK). The manufacturer's protocol was followed for the digestion. Recombinant enterokinase (rEK) was diluted in rEK dilution/storage buffer (supplied with the kit) to produce solutions with 0.1, 0.2, and 0.5 U enzyme/µL. Five digestion reactions were set up as shown in Table 2.27. The reaction mixtures were incubated at room temperature, removing 10 µL aliquots after 2, 4, 8 and 16 h. A negative control without EK was also incorporated. The extent of cleavage of the samples was determined by SDS-PAGE analysis (section 2.2.1.7).

Table 2.27. ***Reaction mix for Enterokinase digestion of IMAC-purified recombinant HEXA.***

Reaction components	Concentration in 50 µL volume
10X rEK cleavage/capture buffer	1X
Purified recombinant HEXA	10 µg
Diluted rEK*	1 µL
Molecular grade water	To make 50 µL

**(Each tube received 1 µL of a different enzyme dilution. The fourth tube receives 1 µL dilution/storage buffer only as a negative control).*

Following enterokinase cleavage reaction, rEK was removed with EKapture agarose (supplied in the Enterokinase cleavage capture kit). The 10X rEK cleavage/capture buffer supplied in the kit was diluting to 1 X with sterile deionised water. The EKapture agarose (supplied as a 50% slurry) was mixed by inversion until fully resuspended. Hundred µL of resuspended slurry was transferred to a sterile 1.5-mLmicro-centrifuge tube. The slurry was centrifuge (HermLe Z233MK-2 refrigerated centrifuge) at 1,000 g for 5 min and the supernatant was discarded. The agarose was resuspended in 500 µL 1X rEK cleavage/capture buffer and centrifuged again at 1,000 g for 5 min. The supernatant was discarded. The agarose was finally resuspended in 50 µL 1X

rEK cleavage/capture buffer and transferred to the sample cup of a 2-mLspin filter (included with the kit). The entire volume of the cleavage reaction was added to the sample cup and incubated at room temperature for 5 min. The spin filter was centrifuge at 1000 g for 5 min to remove the EKapture agarose. Bound rEK was retained in the sample cup, and the cleaved protein flows into the filtrate tube. The cleaved protein was determined by SDS-PAGE analysis (section 2.2.1.7).

2.2.6 Adaptive Evolution of the Bovine Hexosaminidase A and Hexosaminidase B Genes

2.2.6.1 Sequence data and multiple sequence alignment

The species name, genome version and release dates used for the study are listed in Table 2.28. Protein coding sequences for the hexosaminidase A (alpha polypeptide chain) and hexosaminidase B (alpha polypeptide chain) (Tables 2.29 and 2.30) were retrieved by from Ensembl Genome Browser (Ensembl database version 57) (http://www.ensembl.org/). The HEXA and HEXB coding sequences were translated into their corresponding amino acid sequences using the 'Translate tool' available at ExPASy Proteomics Server (http://expasy.org/tools/dna.html). The amino acid sequences for HEXA and HEXB were aligned using ClustalX 2.0.11 (Thompson *et al.*, 1994; Larkin *et al.*, 2007), using default parameters. Putgaps (http://bioinf.may.ie/software/putgaps/index.html) was used to add gaps into the nucleotide alignment based on their position of occurrence in the protein alignment. The resulting amino acid alignments for HEXA and HEXB were 554 and 571 amino acid residues in length, respectively (refer Appendix I and II).

Table 2.28. ***The species name and genome sequence version used in the analysis.***

Species	Assembly	Database Version	Release Date
Cow	Btau_4.0	62.4k	October 2007
Cat	CAT	62.1k	March 2006
Chicken*	CanFam 2.0	62.2r	May 2006
Chimpanzee	CHIMP2.1	62.21q	March 2006
Dog	CanFam 2.0	62.2r	May 2006
Elephant	Loxafr3.0	62.3e	July 2009
Dolphin	turTru1	62.1h	July 2008
Gorilla	gorGor3	62.3e	December 2009
Guinea Pig	cavPor3	62.3f	March 2008
Horse	Equ Cab 2	62.2i	September 2007
Human	GRCh37.p3	62.37g	February 2009
Macaque	MMUL 1.0	62.10q	February 2006
Mouse	NCBIM37	62.37o	April 2007
Opossum	monDom5	62.5n	October 2006
Pig	Sscrofa9	62.9f	April 2009
Rabbit	oryCun2	62.3b	November 2009
Rat	RGSC 3.4	62.34d	December 2004
Zebrafish*	Zv9	62.9b	April 2010

** Zebrafish and chicken were included as outgroups to provide directionality to the analysis.*

Table 2.29. ***Protein coding sequences for the HEXA used in the analysis.***

Species		Accession ID	Length (bp)
Scientific name	Common name		
Bos taurus	Cow	ENSBTAT00000017261	1806
Felis catus	Cat	ENSFCAT00000000654	1581
Gallus gallus	Chicken	ENSGALT00000002999	2719
Pan troglodytes	Chimpanzee	ENSPTRT00000013376	2319
Canis familiaris	Dog	ENSCAFT00000028088	1596
Loxodonta africana	Elephant	ENSLAFT00000018075	1590
Tursiops truncatus	Dolphin	ENSLAFT00000018075	1590
Gorilla gorilla	Gorilla	ENSGGOT00000002926	2470
Cavia porcellus	Guinea Pig	ENSCPOT00000010825	1593
Equus caballus	Horse	ENSECAT00000012568	1760
Homo sapiens	Human	ENST00000457859	1383
Macaca mulatta	Macaque	ENSMMUT00000014947	2685
Mus musculus	Mouse	ENSMUST00000026262	1865
Monodelphis domestica	Opossum	ENSMODT00000002215	1593
Sus scrofa	Pig	ENSSSCT00000002156	1795
Oryctolagus cuniculus	Rabbit	ENSOCUT00000009193	1782
Rattus norvegicus	Rat	ENSRNOT00000013747	1788
Danio rerio	Zebrafish	ENSDART00000051291	1915

Table 2.30. Protein coding sequences for the HEXB used in the analysis.

Species		Accession ID	Length (bp)
Scientific name	Common name		
Bos taurus	Cow	ENSBTAT00000048411	1520
Felis catus	Cat	ENSFCAT00000018368	1371
Gallus gallus	Chicken	ENSGALT00000024086	1350
Pan troglodytes	Chimpanzee	ENSPTRT00000031497	1899
Canis familiaris	Dog	ENSCAFT00000013149	1540
Loxodonta africana	Elephant	ENSLAFT00000018571	1578
Tursiops truncatus	Dolphin	ENSTTRT00000005159	1362
Gorilla gorilla	Gorilla	ENSGGOT00000009338	1377
Cavia porcellus	Guinea Pig	ENSCPOT00000006389	1302
Equus caballus	Horse	ENSECAT00000012513	1559
Homo sapiens	Human	ENST00000261416	1901
Macaca mulatta	Macaque	ENSMMUT00000028104	1804
Mus musculus	Mouse	ENSMUST00000022169	1904
Monodelphis domestica	Opossum	ENSMODT00000002329	1590
Sus scrofa	Pig	ENSSSCT00000015373	1811
Oryctolagus cuniculus	Rabbit	ENSOCUT00000005342	1745
Rattus norvegicus	Rat	ENSRNOT00000048554	1806
Danio rerio	Zebrafish	ENSDART00000006205	2235

2.2.6.2 Phylogeny reconstruction

Phylogenetic trees were reconstructed using the amino acid sequences and PhyML 3.0 aLRT (approximate likelihood-ratio test) online tool (available at http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=phyml) (Guindon and Gascuel, 2003; Anisimova and Gascuel, 2006; Dereeper *et al*., 2008; Dereeper *et al.*, 2010) using standard parameters. The trees were visualised using TreeView 1.6.6 (available at http://taxonomy.zoology.gla.ac.uk/rod/treeview.html), selecting zebrafish as outgroup and rooting the tree appropriately.

2.2.6.3 Selective pressure analysis

Likelihood is the hypothetical probability that an event that has already occurred would yield a specific outcome and the maximum likelihood (ML) is the highest likelihood under the given model. Maximum likelihood-based estimates are

robust and can be developed for various analytical conditions. An ML method was used to calculate the *dN/dS* (ω) ratio for site-specific and lineage-specific models. For this calculation Codeml part of Phylogenetic Analysis by Maximum Likelihood (PAML) 4.3 package, was employed (Yang, 1997; Yang *et al.*, 2005; Yang, 2007). Codeml runs a variety of codon models (Table 2.31) for site-specific and lineage-specific analysis of the provided data. It is a nested model system, *i.e. e*ach model represents different distributions of ω, and each model is different to preceding one by the addition of more complex parameters. The simplest model with the fewest parameters is model 0 (M0). M0 assumes that there is only one ω value among all sites and across all lineages, *i.e.* all sites and lineages are evolving at the same rate. Model 1 (M1) assumes there are two classes of sites, and assumes that ω is either equal to unity (ω = 1) or zero (ω = 0). M1 represents the neutral theory of protein evolution. Model 2 (M2) is an extension of M1, it allows for positive selection by the additional class of ω which is free to take any value (and can be greater than 1). Model 3 (M3), an extension of M0, allows discrete ω values, with ω > 1 allowed. M3 has 2 different variants, M3(k = 2) and M3(k = 3) which allow 2 and three variable classes of ω, respectively, estimated from the data. Model 7 (M7) is a beta model and it allows for 10 different ω-site classes, between 0 and 1. Model 8 (M8) (beta and omega) adds an extra ω class to M7 allowing the sites to have ω > 1. M8a (beta and omega = 1) is the null hypothesis of M8. Model A (MA) and model A-null (MA-null) are the lineage-specific models that test differences in ω in different part of the tree. MA is an extension of M1 and calculates three ω values (ω = 0, 0 < ω < 1, ω ≥ 1). MA-null (also implemented as model A1, MA1) is a null model of A with the last ω value fixed at 1 (ω = 0, 0 < ω < 1, ω = 1). Proportions of the sites that fit into each ω category are calculated from the

data and are given “proportion or p” values, *e.g.* p_0 = proportion of sites with ω_0 value. See Table 2.31 shows sample output.

Model M0 was compared to M3(k = 2), M3(k = 2) with M3(k = 3), M1 with M2, M7 with M8, M8 with M8a, M1 with MA and finally MA with MA-null by performing likelihood ratio tests (LRTs) and assessing their significance using a chisquare (χ^2) tests (Table 2.32). If the test statistic (2Δl) is greater than critical values of the chisquare (χ2) distribution with the appropriate degree of freedom (df), (df corresponds to the number of free parameter in a given model), then that model was selected as a better fit for the data. Individual amino acid sites, for each gene, were considered to have undergone positive selection ($\omega > 1$), selection if they were identified from the Bayes Empirical Bayes (BEB) method with a posterior probability of greater than 0.50 (Yang *et al.*, 2005; Loughran *et al.*, 2008). In the event of BEB not producing a result, the less favourable NEB (Naive Empirical Bayes, NEB) estimates were taken.

Table 2.31. ***Description of models used in Codeml part of PAML 4.3*****.**

Model Name	Characteristics	Positive selection (ω>1)?
M0	One ω allowed across all sites	Allowed
Site-specific models		
M1: Neutral	Two classes, ω_1 fixed at 0 and ω_2 fixed at 1. Returns the proportion p_2 of sites in the second category. 1- p_2 returns p_1	Not allowed
M2: Selection	M1 plus an additional class where ω is estimated from the data and can be larger than 1.	Allowed
M3: Discrete K=2	Two classes of ω allowed without constraint on either value, these values are estimated as are their relative proportions and ω can be larger than 1	Allowed
M3: Discrete K=3	As M3(K=2) but with 3 unconstrained classes of ω	Allowed
M7: Beta	ω is assumed to have a beta distribution between values of 0 and 1 inclusive. 10 classes of ω allowed but none >1	Not allowed
M8: Beta and omega>1	As Model 7 but a further ω category is estimated from the data and can be larger than 1.	Allowed
M8a: Beta and omega=1	As Model 8 but with ω fixed to 1 – is the null of M8.	Not allowed
Lineage-specific models		
Model A	Lineage-specific extension of M1. Four ω classes allowed, two of which can vary between the foreground and background lineages, one which is unconstrained apart from equality for the foreground and background lineages, and one which is set ω=1	Allowed
Model A-null	As Model A but with both the ω classes that are allowed to differ between the foreground and background lineages set to 1	Not allowed

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Table 2.32. ***Likelihood ratio tests (LRTs) performed using all the evolutionary models used in selection analysis.***

Comparison	*df*	Δl	Critical χ^2 values
M0 *vs.* M3k2	2	χ^2	≥ 5.99
M3k2 *vs.* M3k3	1	χ^2	≥ 1.00
M1 *vs.* M2	2	χ^2	≥ 5.99
M7 *vs.* M8	2	χ^2	≥ 5.99
M8 *vs.* M8a	1	χ^2	≥ 2.71 ≥ 5.41
M1 *vs.* Model A	2	χ^2	≥ 5.99
Model A vs. Model A-null	2	χ^2	≥ 3.84

(*Reproduced with permission from Morgan et al., 2010).*

2.2.6.4 Analysis of positively selected sites

Empirical Bayes methods in the site-specific and lineage specific analysis were performed for the estimation of sites under positive selection (ω > 1), using Bayes empirical Bayes (BEB) (Yang *et al.*, 2005). Significant sites, under positive selection, were identified by careful examination of the multiple sequence alignment (MSA) and codeml output using SeaView 4.2.12 (available at http://pbil.univ-lyon1.fr/software/seaview.html), alignment visualisation software (Gouy *et al.*, 2010). These sites were compared with unaligned human (and bovine, for lineage-specific positive selection in HEXB) amino acid sequence taken from UniProt (http://www.uniprot.org/) and examined to see if they were located in the catalytically or functionally important regions of the protein.

2.2.7 Construction of a Bovine scFv Library

2.2.7.1 RNA isolation and cDNA preparation from bovine spleen tissue

The spleen tissues from 4 cows, slaughtered as a result of chronic mastitis, were kindly provided by Enfer Scientific Ltd., Ireland. The RNA was extracted

from these tissues as outlined in section 2.2.2.3. The RNA was reverse transcribed to cDNA (section 2.2.2.3) and these templates were used to generate a bovine scFv library.

2.2.7.2 PCR primers for amplification of bovine scFvs

The oligonucleotides used (Table 2.33) were obtained from Eurofins-MWG-Operon (Eurofins MWG Operon, Germany). The oligonucleotides obtained were used to generate a bovine scFv library. The amplified heavy and light (lamba) chains will contain a long linker sequence along with *Sfi*I sites compatible for cloning into pComb vector series. Oligonucleotides were designed only to amplify lamba fraction of variable light chains because lamba chain makes upto 91% of bovine light chains (Arun *et al.*, 1996).

Table 2.33. *Oligonucleotides for amplification of chicken scFv.*

Primer name	Sequence
Bov-V_H-F	5′-GGT GGT TCC TCT AGA TCT TCC GGT GGC GGT GGC TCC GGC GGT GGT GGC TCT TCC GGA CCG AGC CTG GTG AAG CCC TCA CAG ACC-3′
Bov-V_H-R	5′-CTG GCC GGC CTG GCC TGA GGA GAC GGT RAC CWS GAG TCC-3′
Bov-V_λ-F	5′-GTG GCC CAG GCG GCC TCC GTG TCC GTS WMY CTG GG-3′
Bov-V_λ-R	5′-GGA AGA TCT AGA GGA ACC ACC GGT CAC CGA AGG TGG GGA CTT GGG-3′
Bov-SOE-F	5′- GAG GAG GAG GAG GAG GAG GTG GCC CAG GCG GCC TCC GTG TCC G-3′
Bov-SOE-R	5′-GAG GAG GAG GAG GAG GAG GAG CTG GCC GGC CTG GCC TGA GGA GAC GG-3′

2.2.7.3 Amplification of antibody variable genes

PCRs were carried out to amplify variable heavy and light (lambda) chain for all 4 bovine cDNA samples separately. For amplification of bovine variable heavy and light genes the components for a 1X reaction are shown in Table 2.34.

Table 2.34. ***Composition of PCR mix for bovine heavy and light antibody chain amplification.***

Components	Concentration in 50 µL volume
5X Colorless GoTaq® Flexi Buffer	1X
Bov-V_H-F primer	60 pM
Bov-V_H-R primer	60 pM
cDNA	1.0 ng
dNTP mix	0.2 mM
Magnesium chloride	3 mM
GoTaq® Flexi DNA Polymerase	2.5 U
Molecular grade water	To make 50 µL

Components	Concentration in 50 µL volume
5X Colorless GoTaq® Flexi Buffer	1X
Bov-V_λ-F primer	60 pM
Bov-V_λ-R primer	60 pM
cDNA	1.0 ng
dNTP mix	0.2 mM
Magnesium chloride	3 mM
GoTaq® Flexi DNA Polymerase	2.5 U
Molecular grade water	To make 50 µL

The PCR for the amplification of the bovine variable heavy gene was performed in the Biometra $T_{GRADIENT}$ PCR machine under the following conditions.

Temperature (°C)	Time (s)	No. of cycles
94.0	**600**	**1**
94.0	**30**	**30**
63.4	**30**	
72.0	**45**	
72.0	**600**	**1**

The PCR for the amplification of the bovine variable light (lambda) gene was performed in the Biometra $T_{GRADIENT}$ PCR machine under the following conditions.

Temperature (^{o}C)	Time (s)	No. of cycles
94.0	600	1
94.0	30	
65.1	30	30
72.0	45	
72.0	600	1

2.2.7.4 Purification of V_H and V_λ variable gene fragments using Qiagen gel extraction kit

The V_H and V_λ chain gene amplicons were resolved on 0.8% (w/v) agarose gel and bands of correct size were excised carefully from the gel by using sterile scalpels to avoid any cross-over of the DNA and transferred to sterile clean 1.5-mLmicro-centrifuge tubes. Gel extraction was carried out as outlined in section 2.2.2.7. The purified DNA absorbance was measured at 260 nm using Nanodrop™ ND-1000.

2.2.7.5 Splice by overlap extension (SOE) PCR

The V_H and V_λ purified fragments from each of the various combinations were pooled into equimolar concentrations and were joined using an SOE-PCR. The composition of PCR mix was used for the SOE-PCR is shown in Table 2.35.

Table 2.35. ***Reaction mix for overlapping bovine heavy and light chains.***

Components	Concentration in 50 µL volume
10X High Fidelity PCR Buffer	1X
Bov-SOE-F primer	60 pM
Bov-SOE-R primer	60 pM
Purified V_H chain from bovine spleen	100 ng
Purified V_λ chain from bovine spleen	100 ng
dNTP mix	0.2 mM
Magnesium sulphate	3 mM
GoTaq® Flexi DNA Polymerase	2.5 U
Molecular grade water	To make 50 µL

The SOE-PCR was performed in the Biometra $T_{GRADIENT}$ PCR machine under the following conditions.

Temperature (°C)	Time (s)	No. of cycles
94.0	**600**	**1**
94.0	**45**	**30**
64.3	**45**	
72.0	**75**	
72.0	**600**	**1**

2.2.7.6 *Sfi*I restriction digest of purified SOE-PCR fragment and ligation into pComb3XSS vector

The SOE product was resolved *via* electrophoresis on a 0.8% (w/v) agarose gel and then gel-purified, as described in section 2.2.2.7. The purified SOE product and pComb3XSS vector (phagemid vector) were digested using *Sfi*I restriction enzyme (detailed in section 2.2.2.9). Both the digestions (outlined in Table 2.19) were carried out for 5 h at 50°C. The vector was further digested with *Xho*I and *Xba*I, cutting the stuffer released, and thus, eliminating the possibility of stuffer contamination in the resulting library. Finally the vector was treated with calf alkaline phosphatase to prevent vector re-ligation. The digested vector and the

SOE-PCR product were gel-purified (section 2.2.2.7) and ligated as outlined in Table 2.20.

Three ligations each were performed overnight at room temperature for each bovine spleen sample. On the following morning the ligation was inactivated, by heating the mix at 65^{o}C for 15 min and then ethanol precipitated (section 2.2.2.9) to concentrate the DNA.

2.2.7.7 Transformation of cloned antibody genes in *E. coli*

Each ethanol precipitated ligation mixture was resuspended by adding 10 µL of molecular grade water. The resuspended ligated mix was electroporated into 100 µL of commercial XL1-Blue cells (Stratagene, USA) as outlined in 2.2.4.12. After the transformation the cells were resuspended in 3 mL of SOC media and propagated for an hour at 37^{o}C while shaking at 220 rpm.

Following incubation, all the transformants were pooled and plated on LB agar plates supplemented with 100 µg/mL carbenicillin. Untransformed XL1-Blue cells (negative control) were plated out in parallel on LB agar plates supplemented with 100 µg/mL carbenicillin. The plates were incubated overnight at 37^{o}C. Next morning the transformant colonies were scraped-off the plates and were suspended in 20% (v/v) glycerol, snap frozen in liquid nitrogen and stored at -80^{o}C as stocks.

Eight single clones were randomly picked from the incubated LB agar plates before scrapping them off and were checked for the presence of inserts for assessing the success of transformation. Bovine SOE primers were used to

amplify inserts from the plasmids extracted from the clones using the PCR mix shown in Table 2.36. The amplified scFv fragments were then analysed *via* gel electrophoresis on 0.8% (w/v) agarose gel.

Table 2.36. ***Reaction mix for checking inserts for assessing the transformation success.***

Components	Concentration in 50 µL volume
10X High Fidelity PCR Buffer	1X
Bov-SOE-F primer	30 pM
Bov-SOE-R primer	30 pM
cDNA	1.0 ng
dNTP mix	0.2 mM
Magnesium sulphate	3 mM
Platinum® *Taq* DNA Polymerase High Fidelity	2.5 U
Molecular grade water	To make 50 µL

The SOE-PCR was performed in the Biometra $T_{GRADIENT}$ PCR machine under the following conditions.

Temperature (°C)	Time (s)	No. of cycles
94.0	**600**	**1**
94.0	**45**	**30**
64.3	**45**	
72.0	**75**	
72.0	**600**	**1**

2.2.7.8 Rescue and subsequent precipitation of scFv-displaying phage

The transformants were propagated by inoculating 150 µL of the library stock into 400 mL of SB media, supplemented with 100 µg/mL carbenicillin and 10 µg/mL tetracycline, followed by shaking at 220 rpm at 37°C. At mid-exponential phase of growth (OD ~ 0.6), the library was rescued by infecting with 400 µL of commercial M13K07 helper phage (ISIS, Ireland) and incubated for 30 min at 37°C without shaking, followed by incubation for 2 h at 37°C shaking (220 rpm).

Subsequently, kanamycin was added to a final concentration of 70 µg/mL and the culture was incubated overnight at 37^{o}C at 220 rpm. Phage precipitation was carried out as described in section 2.2.2.12.

2.2.7.9 Bio-panning the bovine scFv library against peptidoglycan from *Staphylococcus aureus* for enrichment of specific clones

An immunotube was coated with 500 µL of 1 mg/mL peptidoglycan from *Staphylococcus aureus* (*S. aureus*-PGN) antigen overnight at 4^{o}C. The tube was blocked for 2 h at room temperature with 3% (w/v) BSA-PBS followed by washing once with both PBST and PBS. Precipitated phage (500 µL) was added to the blocked tube and the tube was incubated on a roller mixer for 2 h at room temperature. The tube was washed 5 times with both PBST and PBS. Specific phage was eluted by adding 500 µL of 10 µg/mL trypsin solution in PBS and incubating the tube at 37^{o}C for 30 min. All of the eluted phage was then infected into 4 mL of XL1-Blue cells (OD ~ 0.6) and the cells were left static at 37^{o}C for 30 min. As mammalian clones are not very high expressers the infected cells were plated on LB agar plates containing carbenicillin and left to grow overnight at 37^{o}C. Plating out allows weak expresser clones with high affinity to grow and be enriched in subsequent rounds rather than facilitating the biased selection of clones owing to their high expression.

The following day the plates were scraped off and the phage was rescued. Five rounds of bio-panning were carried out with repeating cycles of selection, enrichment and rescue with few variations in the parameters used (Table 2.37).

Table 2.37. ***Parameters varied in subsequent rounds of panning of bovine scFv library against*** **S. aureus** ***peptidoglycan.***

Biopanning round	Concentration of NAGae coated (µg/mL)	Number of washes	
		PBST	PBS
Round 1	1,000	5	5
Round 2	400	10	10
Round 3	200	15	15
Round 4	100	15	15
Round 5	100	15	15

2.2.7.10 Infecting pan 5 output phage into TOP10F′ cells for soluble expression

Phagemid DNA from round 5 of biopanning was added to different aliquots of *E. coli* TOP10F′ cells (Stratagene, USA) at mid-logarithmic growth phase, as outlined in section 2.2.2.15.

2.2.7.11 Screening of anti-*S. aureus*-PGN clones by indirect ELISA

The induced cultures were subjected to three cycles of freeze-thawing and the lysates were collected by centrifugation. An indirect ELISA was carried out on a *S. aureus*-PGN coated (20 µg/mL) 96-well plate as described in section 2.2.2.14 with modifications outlined in section 2.2.2.16. HRP-labelled anti-bovine antibody (1:2,000) was used as secondary detection antibody.

2.2.7.12 Preparation of *S. aureus* crude extract

Two-hundred mL culture of an isolate of *S. aureus* from mastitic milk (Strain number 493102) was grown overnight in LB broth at 37^{o}C overnight in an orbital shaker. The resulting broth cultures were centrifuges at 4,000 g for 20 min at 4°C. The resultant pellet of cells was washed three times with ice-cold PBS (PBS; 137 mM NaCl, 4.7 mM KCl, 9.32 mM Na_2HPO_4, 0.68 mM NaH_2PO_4, pH

8.0) containing 1 mM $MgCl_2$ (PBS^{2+}) to remove amine-containing media and proteins from the cells. After washings, the cells were suspended at a concentration of approximately 25 × 10^6 cells/mL in PBS (pH 8.0). The prepared extract was stored at -80^oC until further use.

2.2.7.13 Biotinylation of *S. aureus* surface proteins

The *S. aureus* crude extract was prepared as outlined in section 2.2.7.12. Biotinylation of the surface proteins was carried out using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific Pierce) using the manufacturer's instructions, Breifly, 1 mg of Sulfo-NHS-LC-Biotin reagent per mL of cell suspension in PBS was added. The reaction mixture was incubated at room temperature for 30 min. The cells were washed c three times with PBS containing 100 mM glycine to quench and remove excess biotin reagent and byproducts. Surface proteins were solubilised by resuspending the washed cell pellet in 2% (w/v) SDS at 37°C for 45 min, while shaking at 125 rpm. The cell debris was deposited by centrifugation at 12,000 g for 30 min at 4°C and supernatant was preserved at -80°C till further use.

2.2.7.14 Bio-panning the bovine scFv library against *S. aureus* crude extract for enrichment of specific clones

Bio-panning the bovine scFv library against *S. aureus* crude extract was carried out as outlined in section 2.2.7.9 using *S. aureus* crude extract as antigen. The conditions used for biopanning are outlined in Table 2.38.

Table 2.38. ***Parameters used for bio-panning of bovine scFv library against* S. aureus *crude extract.***

Biopanning round	Concentration of NAGae coated (µg/mL)	Number of washes	
		PBST	PBS
Round 1	600	5	5
Round 2	400	10	10
Round 3	400	15	15
Round 4	200	15	15
Round 5	200	15	15

2.2.7.15 Polyclonal phage ELISA

Polyclonal phage ELISA was carried out as outlined in section 2.2.2.14. A coating antigen (*S. aureus* crude extract) concentration of 25 µg/mL was used.

2.2.7.16 Soluble expression and direct ELISA of bovine anti-*S. aureus* crude extract scFv clones

Soluble expression and screening of bovine anti-*S. aureus* crude extract scFv clones was carried out as decribed in section 2.2.7.10 and 2.2.7.11 using *S. aureus* crude extract as antigen.

2.2.7.17 Bio-panning the bovine scFv library against *S. aureus* surface proteins for enrichment of specific clones

An immunotube was coated with 500 µL of 5 µg/mL NeutrAvidin (in PBS, pH 7.2) and incubated overnight at 4^{o}C. The tube was blocked for 2 h at room temperature with 3% (w/v) BSA-PBS followed by washing once with both PBST and PBS. Five hundred µL of the dilution with appropriate concentration of biotinylated *S. aureus* surface proteins (Table 2.39) was then added and tubes were incubated for 2 h at room temperature. Thereafter, the protocol outlined in

section 2.2.7.9 was followed. The conditions used for biopanning are outlined in Table 2.39.

Table 2.39. ***Parameters used for bio-panning of bovine scFv library against*** **S. aureus** ***surface proteins.***

Biopanning round	Concentration of NAGae coated (µg/mL)	Number of washes	
		PBST	PBS
Round 1	600	5	5
Round 2	400	10	10
Round 3	400	15	15
Round 4	200	15	15
Round 5	200	15	15

2.2.7.18 Polyclonal phage ELISA

Wells of an ELISA plate were coated with 100 µL of 5 µg/mL NeutrAvidin (in PBS, pH 7.2) and incubated for 1 h at 37^{o}C. The plates were then blocked overnight with 200 µL 5% (w/v) skim milk-BSA-PBS followed by washing once with both PBST and PBS. A hundred µL of biotinylated *S. aureus* surface proteins (25 µL/mL) was then added. Rest of the ELISA was carried out as outlined in section 2.2.7.14.

2.2.7.19 Soluble expression and direct ELISA of bovine anti-*S. aureus* surface proteins scFv clones

Soluble expression of bovine anti-*S. aureus* crude extract scFv clones was carried out as decribed in section 2.2.7.10. For screening an indirect ELISA was carried out. Wells of the ELISA plates were coated with 100 µL of 5 µg/mL NeutrAvidin (in PBS, pH 7.2) and incubated for 1 h at 37^{o}C. The plates were then blocked overnight with 200 µL 5% (w/v) skim milk-BSA-PBS followed by washing once with both PBST and PBS. A hundred µL of biotinylated *S. aureus*

surface proteins (20 µL/mL) was then added. Rest of the ELISA was carried out as outlines in section 2.2.2.14.

2.2.8 Production of Anti-Progesterone Antibodies

2.2.8.1 Immunisations

A chicken was immunised with the commercial available progesterone-3-carboxymethyloxime-bovine serum albumin conjugate (Progesterone-3-CMO-BSA) (Fitzgerald Industries, USA) for generation of anti-progesterone antibodies. For primary immunisations, the antigen was diluted to a concentration of 100 µg in 275 µL of PBS and emulsified by rigorous votexing following addition of an equal volume of Freund's complete adjuvant. For booster injections, the antigen was diluted to a concentration of 50 µg in 275 µL of PBS and emulsified with an equal volume of Freund's incomplete adjuvant. The chicken was injected with 500 µL of an antigen-adjuvant mixture. It was boosted 4 times at three weekly intervals. A bleed was taken after every boost and serum was separated as described section 2.2.1.1.

Two New Zealand white rabbits were immunised with an 'in-house' progesterone-3-CMO-BSA conjugate (prepared in DCU) and a commercial progesterone-3-CMO-BSA conjugate (Fitzgerald Industries, USA) for the generation of anti-progesterone antibodies. For primary immunisations, the antigen was diluted to a concentration of 200 µg in 275 µL of PBS and mixed with an equal volume of Freund's complete adjuvant. This mixture was vortexed until a stable emulsion was formed. For booster injections, the antigen was diluted to a concentration of 100 µg in 275 µL of PBS and emulsified with an equal volume of Freund's incomplete adjuvant by vortexing. Rabbits were

immunised intramuscularly with 500 µL of this preparation. Five boosts were given at three weekly intervals to the rabbit immunised with the 'in-house' generated conjugate and two boosts were given at three weekly intervals to the rabbit immunised with the commercial conjugate. A week after every boost blood was collected from the rabbits and the serum was isolated (section 2.2.1.1).

2.2.8.2 ELISA to check the titre of chicken serum against progesterone

NeutrAvidin was diluted to 5 µg/mL in 150 mM PBS (pH 7.2) and 100 µL was coated onto the wells of a 96 well-plate (Nunc, USA). The plate was incubated for 1 h at 37^{o}C and then blocked with 200 µL of 5% (w/v) skim milk-PBS for 1 h at 37^{o}C. A hundred µL of progesterone 3-biotin, diluted to the concentration of 2 µg/mL in 150 mM PBS (pH 7.2), was then added to the wells. Doubling dilutions of immunised chicken serum and pre-immune chicken serum diluted from 1:1,000 to 1:512,000, were prepared in 1% (w/v) skim milk-BSA-PBS and 100 µL of each dilution was added in duplicate to wells. The plates were incubated for 1 h at 37^{o}C. The plates were washed 3 times with 0.05% (v/v) PBST and 3 times with PBS after each step. Subsequently, 100 µL of 1:2,000 dilution of a HRP-conjugated anti-chicken antibody (Sigma-Aldrich, Ireland) in 1% (w/v) skim milk-BSA-PBS was added to each well and incubated for 1 h at 37^{o}C. Detection of the antigen-antibody binding was achieved by the addition of 100 µL of TMB substrate. Colour was allowed to develop for 20 min at room temperature, after which the reaction was quenched using 50 µL of 10% (v/v) HCl. The absorbance values were then read at 450 nm on a Safire 2 plate reader (Tecan, Switzerland).

2.2.8.3 ELISA to check the titres of rabbit sera against progesterone

ELISA was performed, as described in section 2.2.8.2. For the rabbit immunised with the 'in-house' prepared progesterone-3-CMO-BSA conjugate a range of dilution (from 1:1000 to 1:64,000) of immunised rabbit serum and respective pre-immune rabbit serum were used. Whereas, for rabbit immunised with commercial progesterone-3-CMO-BSA conjugate (and its respective pre-immune serum) a series of different dilutions, *viz.* 1:1000, 1:10,000, 1:50,000, 1:100,000, 1:200,000, 1:400,000, 1:800,000, 1:1,600,000, 1:3,200,000, 1:6,400,000, were prepared. HRP-conjugated anti-rabbit antibody (Sigma-Aldrich, Ireland) was used as detection antibody.

2.2.8.4 Purification of anti-progesterone polyclonal antibody from rabbit serum

The rabbit anti-progesterone polyclonal was purified using Protein G Sepharose® Fast Flow resin (Sigma-Aldrich, Ireland). A two mL suspension of protein G immobilised on sepharose was equilibrated in a column with 40 mL of sterile-filtered PBS (150 mM, pH 7.2). Two mL of rabbit serum was diluted with 8 mL of sterile-filtered PBS and passed through the column. The eluant was collected and passed through the column one more time. The column was washed with 40 mL of sterile-filtered PBS. The retained protein, bound to the protein G, was eluted with 0.1 M glycine-HCl buffer (pH 2.5). Fractions of eluate were collected in micro-centrifuge tubes containing 150 µL of neutralisation buffer (2 M Tris-HCl, pH 8.5). The fractions were quantified on a NanoDrop™ ND-1000 using the pre-programmed 'IgG' option. The fractions containing high concentrations of IgG were pooled. The antibodies were buffer exchanged against PBS and analysed using SDS-PAGE (section 2.2.1.7). All purification

and desalting procedures were conducted at 4°C to minimise denaturing of antibody.

2.2.8.5 Checkerboard ELISA for estimation of the optimal progesterone-3-biotin coating concentration

NeutrAvidin was diluted to 5 µg/mL in 150 mM PBS (pH 7.2) and 100 µL was coated onto wells of a 96 well-plate (Nunc, USA). The plate was incubated for 1 h at 37^{o}C and then blocked with 200 µL 5% (w/v) skim milk-PBS for 1 h at 37^{o}C. Five different concentrations (*i.e.* 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL and 4 µg/mL) of progesterone 3-biotin in 150 mM PBS (pH 7.2) were each added to the wells and incubated for 1 h at 37^{o}C. Different dilutions (1:10,000, 1:50,000, 1:100,000, 1:500,000 and 1:1,000,000) of the purified rabbit anti-progesterone polyclonal antibodies, in 1% (w/v) skim milk-BSA-PBS, were added to the (duplicate) wells having different progesterone 3-biotin concentrations. Likewise, 1% (w/v) skim milk-BSA-PBS (negative control) was also added to the wells (in duplicate) having different progesterone 3-biotin concentrations. The plates were incubated for 1 h at 37^{o}C. The plates were washed 3 times with 0.05% (v/v) PBST and 3 times with PBS after each step. Subsequently, 100 µL of 1:2,000 dilution of a HRP-conjugated anti-rabbit antibody (Sigma-Aldrich, Ireland) in 1% (w/v) skim milk-BSA-PBS was added to each well and incubated for 1 h at 37^{o}C. Detection of labelled antibody binding was achieved by the addition of 100 µL of TMB substrate. Colour was allowed to develop for 20 min at room temperature, after which the reaction was quenched using 50 µL of 10% (v/v) HCl. The absorbance values were then read at 450 nm on a Safire 2 plate reader (Tecan, Switzerland).

2.2.8.6 ELISA to check the titres of purified rabbit polyclonal antibodies against progesterone

This ELISA was performed as described in section 2.2.8.2. However, in this case a coating concentration of 0.25 µg/mL of progesterone 3-biotin was used and a range of different dilutions, of purified polyclonal antibodies, were prepared and checked in the ELISA format. Purified antibodies from both the rabbits were titred using dilutions mentioned in section 2.2.8.3. HRP-conjugated anti-rabbit antibody (Sigma-Aldrich, Ireland) was used as the secondary detection antibody.

2.2.8.7 ELISA to assess the best blocking solution for use in the assay for detection of progesterone in milk

NeutrAvidin was diluted to 5 µg/mL in 150 mM PBS (pH 7.2) and 100 µL was coated onto wells of a 96 well-plate (Nunc, USA). The plate was divided in to 5 areas with each area blocked using 200 µL of different blocking agents, *viz.* 5% (w/v) skim milk-PBS, 5% (w/v) human serum albumin (HSA)-PBS, 5% (w/v) Albumin from chicken egg white (ovalbumin, OVA)-PBS, 5% (w/v) fish gelatine (FG)-PBS and 5% (v/v) PBS-Tween 20 (PBST). A hundred µL of progesterone 3-biotin, diluted to the concentration of 0.25 µg/mL in PBS, was then added to the wells. Different dilutions (1:10,000, 1:50,000, 1:100,000, 1:500,000 and 1:1,000,000) of the purified rabbit anti-progesterone polyclonal antibodies, in 1% (w/v) BSA-PBS, were added to the (duplicate) wells blocked using different blocking agents. Likewise, 1% (w/v) BSA-PBS (negative control) was also added to the wells (in duplicate) blocked using different blocking agents. The plates were incubated for 1 h at 37^{o}C and washed 3 times with 0.05% (v/v) PBST and 3 times with PBS after each step. Subsequently, 100 µL of 1:2,000

dilution of a HRP-conjugated anti-rabbit antibody (Sigma-Aldrich, Ireland) in 1% (w/v) PBS was added to each well and incubated for 1 h at 37^{o}C. Detection of labelled antibody binding was achieved by the addition of 100 µL of TMB substrate. Colour was allowed to develop for 20 min at room temperature, after which the reaction was quenched using 50 µL of 10% (v/v) HCl. The absorbance values were then read at 450 nm on a Safire 2 plate reader (Tecan, Switzerland).

2.2.8.8 Titration of purified rabbit polyclonal antibodies and inhibition ELISA

Titration ELISA was performed as outlined in section 2.2.8.6. However, 3% (w/v) OVA-PBS solution was used for blocking the wells. A graph was plotted by using antibody dilution on the X-axis and absorbance on the Y-axis. Midpoint was determined for purified rabbit pAbs, as it represents the dilution at which pAbs are most sensitive to the antigen. This dilution was used for inhibition ELISA.

Three plates were coated with NeutrAvidin, blocked with 3% (w/v) OVA-PBS as outlined in sections 2.2.8.6 and 2.2.8.7. A hundred µL of progesterone 3-biotin, diluted to the concentration of 0.25 µg/mL in PBS, was then added to the wells. Doubling dilutions of the progesterone-3-CMO conjugate were made from 8,000 to 3.9 ng/mL. The antigen dilutions were mixed with appropriate dilution of purified rabbit pAbs in a 1:1 (as determined by the titre) ratio and incubated for 30 min at 37^{o}C. A hundred µL of antigen-antibody mixture was then added to the wells. The plates were incubated for 1 h at 37^{o}C and washed 3 times with 0.05% (v/v) PBST and 3 times with PBS after each step. Subsequently, 100 µL

of 1:2,000 dilution of a HRP-conjugated anti-rabbit antibody (Sigma-Aldrich, Ireland) in 1% (w/v) PBS was added to each well and incubated for 1 h at 37^{o}C. Detection of labelled antibody binding was achieved by the addition of 100 µL of TMB substrate. Colour was allowed to develop for 20 min at room temperature, after which the reaction was quenched using 50 µL of 10% (v/v) HCl. The absorbance values were then read at 450 nm on a Safire 2 plate reader (Tecan, Switzerland).

Chapter 3
Generation of Anti-NAGase Antibodies

3. GENERATION OF ANTI-NAGASE ANTIBODIES

3.1 Introduction

Mastitis is an important disease owing to its effects on animal health and production coupled with its public health implications (Fadlelmula *et al.*, 2009). A plethora of diagnostic tests were developed for mastitis detection (discussed in Chapter 1). However, the quest for better tests continues due to the inadequecies of current approaches. Antibody-based immunoassays are preferred tools for the diagnostics industry (discussed in Chapter 1). In addition, the use of biomarkers for diagnosis of different human and animal diseases has increased significantly over the last few years and biomarker measurement is proving to be an extremely important tool in the diagnosis of inflammation (Hoffmann, 2006). Different biomarkers for mastitis were proposed, *e.g.* Lactate dehydrogenase (LDH) (Bogin and Ziv, 1973), lactose (Kitchen, 1981), *N*-acetyl-β-D-glucosaminidase (NAGase) (Kitchen *et al.*, 1984; Mattila and Sandholm, 1985), antitrypsin (Mattila and Sandholm, 1985), an unidentified 23.5-kDa bovine inflammatory antigen (Ball *et al.*, 1991), elevated polymorphonuclear granulocytes (PMN) (O'Sullivan *et al*., 1992), myeloperoxidase (Cooray, 1994), α_1-acid glycoprotein (Eckersall *et al.*, 2001), haptoglobin (Eckersall *et al.*, 2001), serum amyloid A (Eckersall *et al.*, 2001), adenosine triphosphate (ATP) (Eckersall *et al.*, 2001), lipocalin-type prostaglandin D (Baeker *et al.*, 2002) and L-lactate (Davis *et al.*, 2004). These have varying degree of diagnostic utility. However, this research focused on NAGase.

NAGase (3.2.1.52) is a high molecular-weight lysosomal enzyme, found in many tissues of the body (including kidney, spleen and liver) (Sellinger *et al.*, 1960). It catalyses the hydrolysis of terminal non-reducing *N*-acetyl-β-D-

glucosamine residues from glycoproteins. In bovines, it is secreted in large quantities in the mammary gland, from the damaged epithelial cells as well as from other somatic cells present in milk, during involution and inflammation (Kitchen *et al.*, 1978). The first report on NAGase in milk appears to be that of Mellors (1968), who suggested that NAGase could be a convenient index of mammary gland infection. The effectiveness of NAGase as an indicator of tissue damage and degree of udder inflammation during mastitis was later demonstrated (Kitchen, 1976; Kitchen and Midleton, 1976; Kitchen *et al.*, 1978).

NAGase provides a sensitive marker for the detection of mastitis (Pyorala, 2003), and there have been numerous studies on the reliability of NAGase as a mastitis marker (Kitchen, 1981; Mattila and Sandholm, 1985; Pyorala and Pyorala, 1997; Chagunda *et al.*, 2006). NAGase has also been reported to be a useful aid in discriminating between minor and major pathogen infections (Berning and Shook, 1992).

Kitchen and Masters (1985), isolated two isozymes, hexosaminidase A (HexA) and B (HexB), of bovine NAGase, differing in molecular mass (*i.e.* 118 and 234 kDa, respectively) and charge, from bovine mammary tissue. Each isoenzyme dissociates into two dissimilar subunits of mass 55 and 25 kDa, on treatment with 2-mercaptoethanol and sodium dodecyl sulphate. However, another study reported the molecular weight of HexA to be 109 kDa (a heterodimer with an α-subunit of 52 kDa and β-subunit of 57 kDa) and molecular weight of HexB to be 114 kDa (homodimer with 2 β-subunits) (Legler *et al*., 1991). A third isoform HexS (homodimer with 2 α-subunits) is also reported in humans. However, this isoform is usually produced in the absence of the β-subunit (O'Dowd *et al.*,

1986) and there is no report of this isoform in bovines. The α- and β-subunits are encoded by the HEXA and HEXB genes, respectively.

NAGase is an established marker of mastitis since the mid 1970s (Kitchen and Middleton, 1976; Mattila and Sandholm, 1985; Huszenicza *et al.*, 1997; Obara and Komatsu, 1997; Pyörälä and Pyörälä, 1997; Chagunda *et al.*, 2006). However, there was no specific antibody available against bovine NAGase or its subunits. Consequently, it was decided to generate antibodies to facilitate mastitis diagnosis by the generation of immunoassays for NAGase detection.

3.2 Production of Anti-NAGase Monoclonal Antibody

3.2.1 Immunisations

Two BALB/c mice aged 8-10 weeks were immunised with the commercial β-*N*-acetylglucosaminidase / *N*-acetyl-β-D-glucosaminide (NAGase) (Sigma-Aldrich, Ireland). The mice were given 5 booster doses with NAGase. When the immunisation programme was complete, bleeds were taken to determine the immune response. Sera were screened for the presence of anti-NAGase antibodies in an indirect ELISA, using plates coated with commercial NAGase at 2 µg/mL. Both mice showed poor response to the immunisations. A series of dilutions, in PBS (pH 7.2), of the sera from mice, were tested. Figure 3.1 shows antibody levels against commercial bovine NAGase in one of the mice. Subsequently, monoclonal antibody production (section 3.2.2) was undertaken.

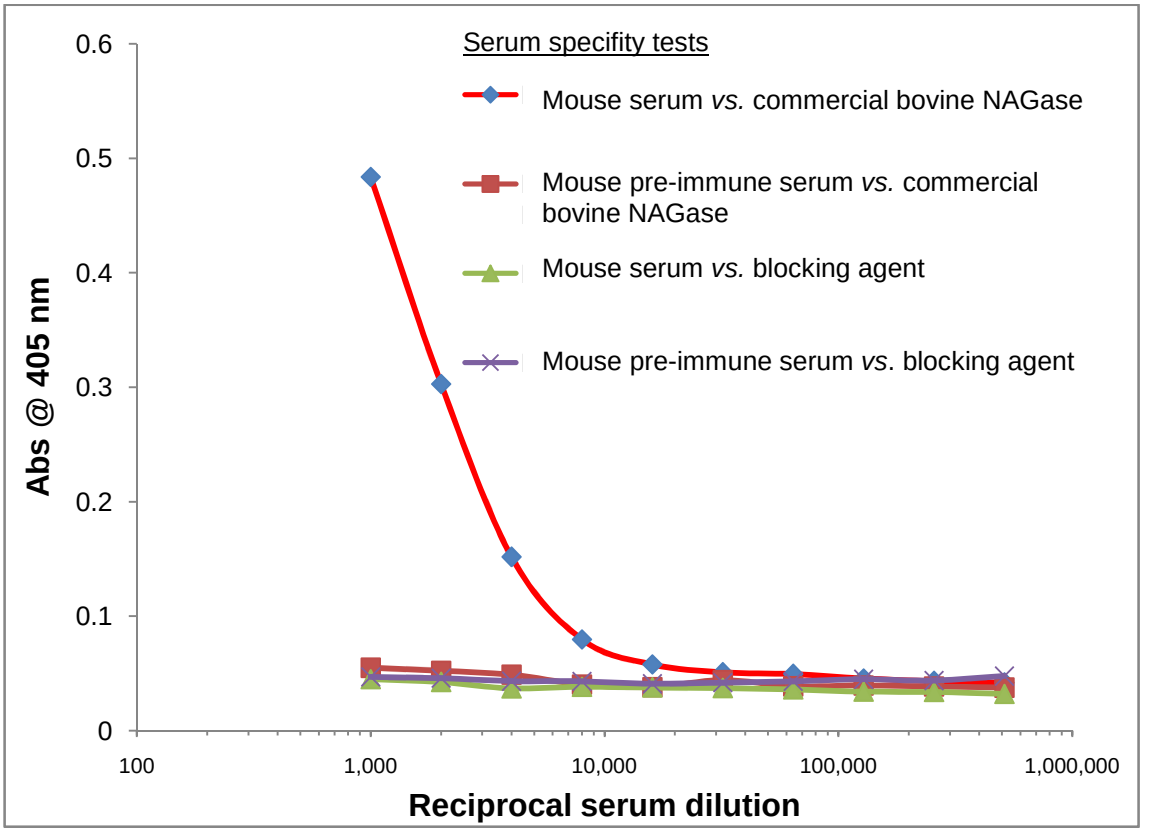


Figure 3.1. ***Evaluation of the response following administration of NAGase.***
Mouse serum antibody titre, after 5 boosts, was checked with bovine NAGase and blocking agent (5% (w/v) skim milk-PBS) using a series of dilutions ranging from 1:1,000 to 1:52,000. Similar dilutions of mouse pre-immune serum were analysed. Alkaline phosphatase-labelled anti-mouse IgG was used as detection antibody.

3.2.2 Cell fusions for monoclonal antibody production

Two fusions of Sp2/mIL-6 with spleenocytes from immunised mice were carried out (section 2.2.1.3). On day 7, post fusion, the tissue culture supernatant of each well was screened for the presence of anti-NAGase antibodies. In the first fusion only 27 wells (out of total 1,680) were positive in the screening assay. However, only 18 wells were found to be positive in the second screening on day 11. The cells in the additional 9 wells, found positive in the first screening, might have stopped secreting antibodies. After expansion in a 24-well plate, none of the hyridomas remained positive. In the second fusion, 60 wells were

positive in the first screen. However, all clones, but one, were found to be negative in the second screening. The positive clone, 10H5, was expanded in a 48-well plate and subsequently in a 24-well plate. The supernatants collected from both the 24 and 48-well plates for clone 10H5 were checked for anti-NAGase antibody in a competitive ELISA format along with appropriate controls. However, it was found that the supernatants were reacting non-specifically with the blocking agent (5% (w/v) skim milk-PBS). It was observed that the mice were not responding strongly to NAGase immunisations (section 3.2.1). As both fusions failed to generate any hybridoma secreting anti-NAGase antibody, it was decided to explore other NAGase-associated antigens which could be administered in mice to generate a better anti-NAGase immune response.

3.2.3 Exploring the use of different NAGase-associated antigens for immunisations

It was observed that the mice were not responding strongly to NAGase immunisations (section 3.2.1). NAGase is an omnipresent glycosidase found in animal and human tissues, microorganisms and plants. Thus, a possible reason could be high degree of structural similarity between mouse and bovine NAGase forms.

Commercially available NAGase was analysed on a SDS-PAGE gel. The commercial NAGase (Sigma-Aldrich, Ireland) preparation contains in 3.2 M ammonium sulphate and, therefore, was not suitable for direct analysis on SDS-PAGE gel. Thus, it was buffer exchanged with 150 mM PBS (pH 7.2) using the D-Tube™ Dialyzer Mini, MWCO 6-8 kDa columns. The gel (Figure 3.2) revealed

the presence of non-specific proteins and it was not possible to exactly identify the NAGase isoforms as there were numerous protein bands around the size of NAGAse isoforms (HexA – 109 kDa and HexB – 114 kDa) and their subunits (α-subunit – 52 kDa and β-subunit – 57 kDa).

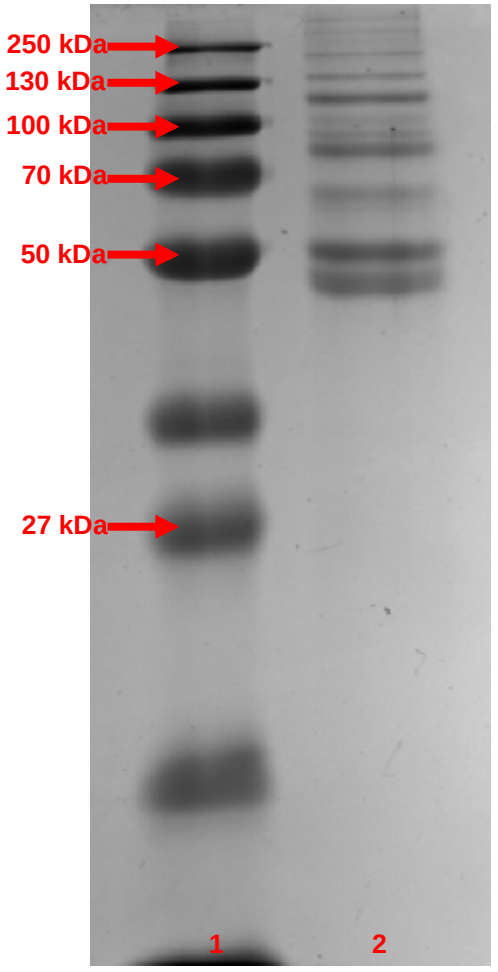


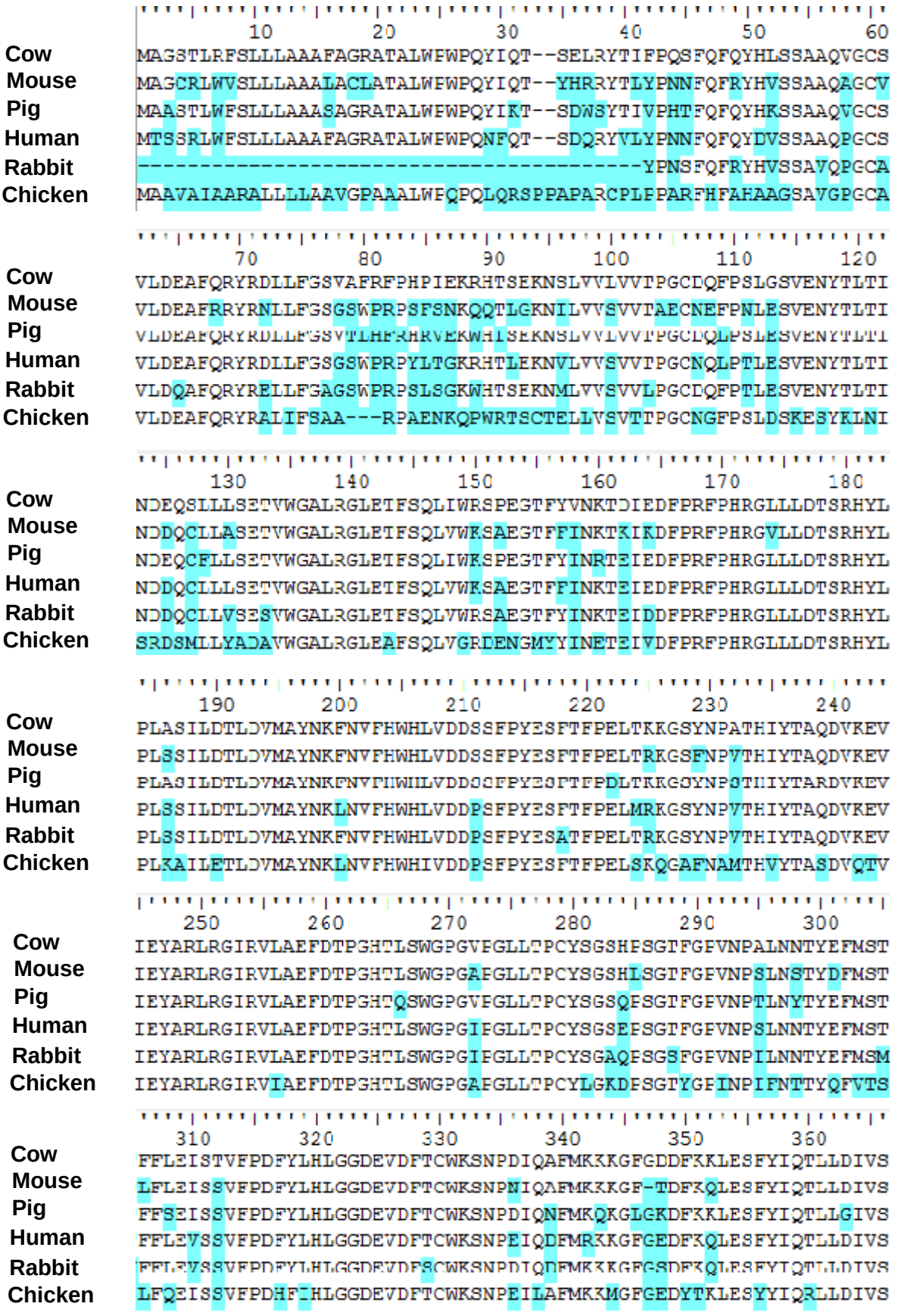
Figure 3.2. ***SDS-PAGE analysis of bovine NAGase.***
Commercial bovine NAGase was analysed on a SDS-PAGE gel. Lane 1: PageRuler™ Plus Prestained Protein Ladder; lane 2: dialysed commercial bovine NAGase. It is evident that there were several bands within the molecular weight range of bovine NAGase isoforms and their subunits, making it difficult to identify specific NAGase bands. Many bands of variable sizes, which could not be characterised, were also present, thus, making this antigen unsuitable for specific antibody selection.

Sequence identities between bovine NAGase and that from certain species (human and pig) that can be possibly utilised as a source of the NAGase antigen or as animal hosts (mouse, rabbit and chicken) for generating anti-

NAGase antibodies were analysed. Basic Local Alignment Search Tool (BLAST), available at http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch, was used for this analysis. NAGase has two predominant isoforms, HexA and HexB (composed of α- and β-subunits (section 3.1)). Both the subunits were analysed separately for possible similarity with α- and β-subunits of other species. The percentage of sequence identities is shown in Table 3.1. High sequence similarities were observed between bovine and mouse NAGase (Table 3.1 and Figure 3.3) and, thus, there is less chance of eliciting a good immune response on administration of bovine NAGase to mice. However, bovine NAGase was found to be relatively more distinct from chicken NAGase, based on the differences in their sequences (Table 3.1 and Figure 3.3), and, therefore, chickens could possibly be better hosts for generating anti-bovine NAGase antibodies. The chicken immune system will, supposedly, recognise and process the regions with different sequences (epitopes) as foreign antigens and should generate a strong immune response to them, thus, generating high antibody titres against these epitopes.

Table 3.1. ***Percentage sequence identities observed between bovine hexosaminidase α- and β-subunits and other species. The table illustrates the identities obtained by blasting the test protein sequences with the control protein sequence (bovine).***

Species	Percentage sequence identities	
	α-subunit	β-subunit
Pig	89%	71%
Human	84%	73%
Mouse	81%	70%
Rabbit	83%	73%
Chicken	66%	64%


Cow
Mouse
Pig
Human
Rabbit
Chicken

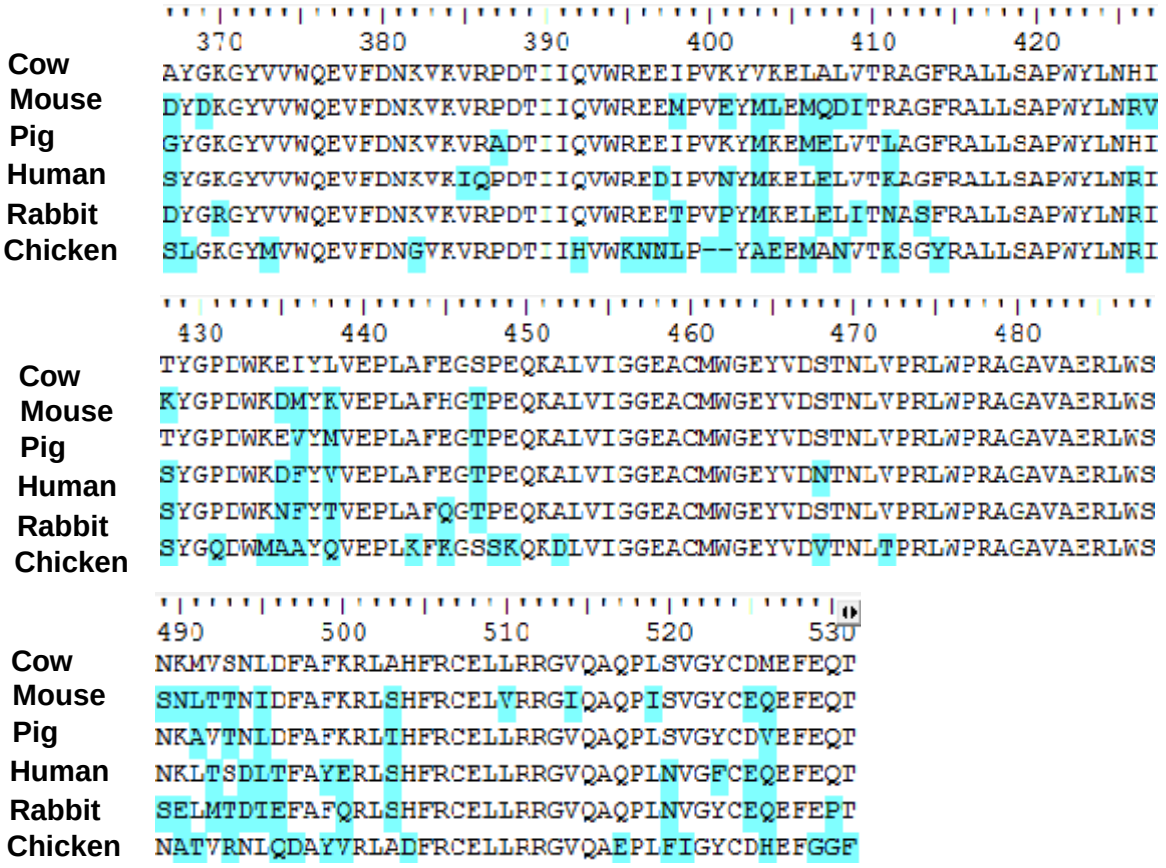


Figure 3.3. ***Alignment of bovine hexosaminidase α-subunit with α-subunit from other species.***
HexA sequences were aligned to identify phylogenetically distinct animals suitable for generation of antibodies against bovine α-subunit. Chicken was found to be most suitable because chicken α-subunit was least similar to bovine α-subunit in sequence.

Bovine hexosaminidase (Hex) α-subunit was aligned with α-subunits from other species (Figure 3.3), using CLUSTALX 2.0.11 (Thompson *et al.*, 1994; Larkin *et al.*, 2007), to identify possible short peptides sequences (with 12-16 amino acid residues) that are unique to bovines. Two short (13 amino acid residue each) peptides were identified and used as antigens for immunising mice (Table 3.2). A peptide was selected from both the N-terminal and C-terminal region of the protein. The selected peptides were analysed for net hydrophilicity, as a good immunogen should be hydrophilic. The selected peptides were also analysed to determine any sequence homology with other bovine or mouse proteins. This was achieved using BLAST. The locations of the peptides were also analysed

by visualising the peptides on the model of the complete HexA protein (Figure 3.4). The model was generated using $(PS)^2$: protein structure prediction server (Chen *et al.*, 2006). The peptides were visualised on the model using Swiss-PdbViewer.

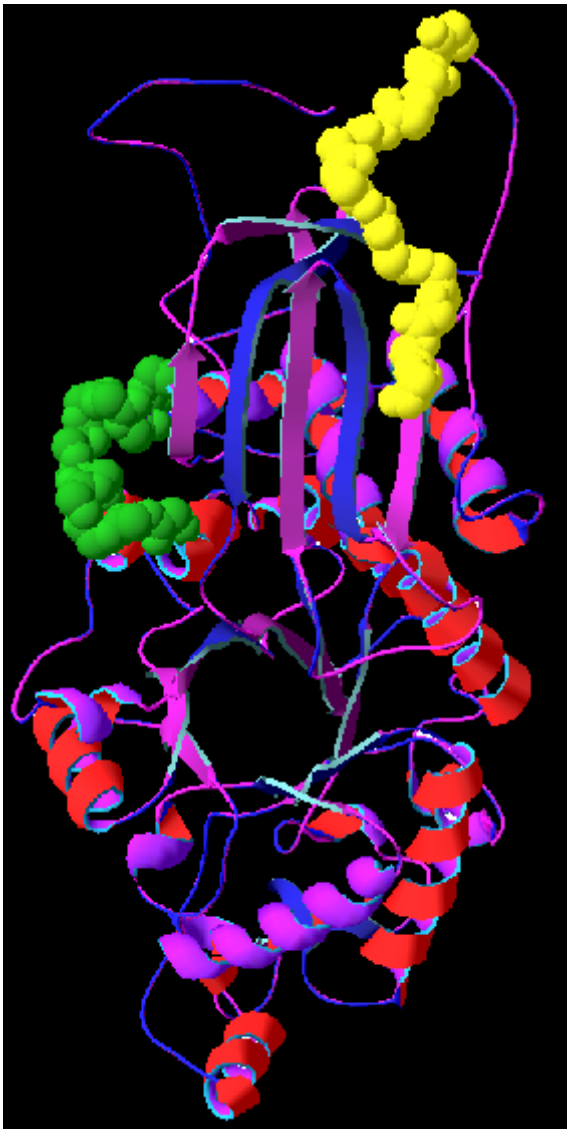


Figure 3.4. ***3-Dimensional structural model of bovine HexA.***
A 3-Dimensional structural model of bovine HexA was generated using $(PS)^2$: protein structure prediction server. The two Hex α-subunit peptides designed in this study are represented as yellow (HexA peptide 1) and green (HexA peptide 2).

The selected peptides (Table 3.2) were synthesised and conjugated with KLH and LC-biotin by Cambridge BioScience. The KLH-conjugated peptides were

used for immunisations and the LC-biotin-conjugated peptides were used in an ELISA to screen for antibodies generated against the peptides.

Table 3.2. ***The short peptide sequences selected for generation of antibodies against the HexA isoform of bovine NAGase.***

Name	No. of amino acid residues	Sequence	N-terminal conjugation	C-terminal conjugation
HexA-1-CT	13	PIEKRHTSEKNSL	Acyl	LC-BIOTIN
HexA-1-KLH	13	PIEKRHTSEKNSL	Acyl	KLH
HexA-2-NT	13	SNKMVSNLDFAFK	LC-BIOTIN	Amide
HexA-2-KLH	13	SNKMVSNLDFAFK	KLH	Amide

Pig NAGase (Diazyme Labs, USA) was also purchased for use as an antigen for immunising mice. It shares 89 and 71% sequence identity with bovine HexA and HexB, respectively. Thus, it was thought to share common epitopes with bovine NAGase, which could be used for generating antibodies to bovine NAGase.

3.2.4 Immunisations of mice with different NAGase-associated antigens

BALB/c female mice aged 8-10 weeks were immunised with the two short peptides (Table 3.2), commercial pig NAGase and commercial bovine NAGase. A mouse immunised with commercial bovine NAGase died after one boost. The remaining mice were boosted five times. The sera collected after the fifth boost and the immune response was checked against various antigens and the blocking agent (negative control). All the mice showed very poor immune responses to the bovine NAGase as demonstrated in Figures 3.5-3.11. The titres obtained were not adequate for mAb production considering that the past two failed fusions were performed with spleen cells from mice that had anti-NAGase antibody titres above 1:8,000. However, the titres against KLH in the

mice immunised with either of the peptides showed that the mice did respond to the immunisations. In some cases (Figures 3.5-3.8, 3.11) a low response was observed against commercial bovine NAGase. However, this reponse was not significant enough to attempt monoclonal antibody generation.

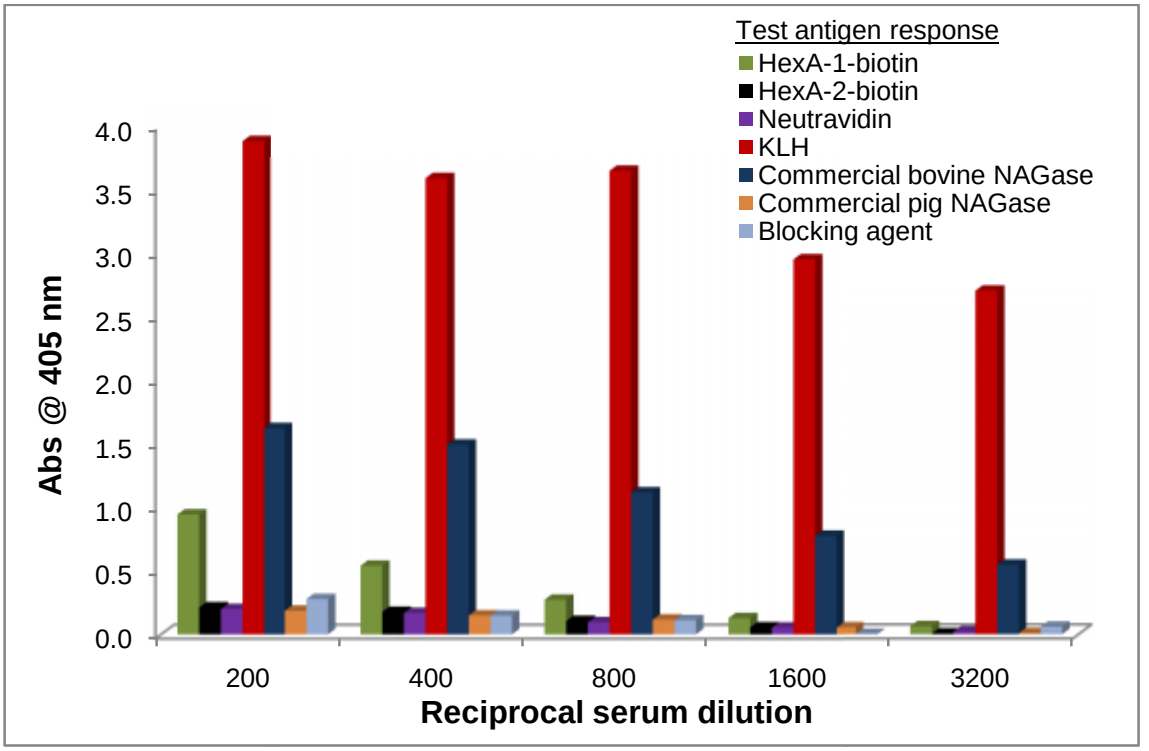


Figure 3.5. ***Antibody titre of serum from mouse immunised with HexA-1-KLH.***
After 5 boosts with HexA-1-KLH, mouse serum antibody titres were checked against HEX-A-1-biotin, HEX-A-2-biotin, bovine NAGase, pig NAGase, neutravidin, KLH and blocking agent (5% (w/v) BSA-milk-PBS) using a series of dilutions ranging from 1:200 to 1:3,200. Similar dilutions of mouse pre-immune sera were analysed. All the sera dilutions were assayed in duplicate. Pre-immune values were subtracted from all values. Alkaline phosphatase-labelled anti-mouse IgG antibody was used as the detection antibody.

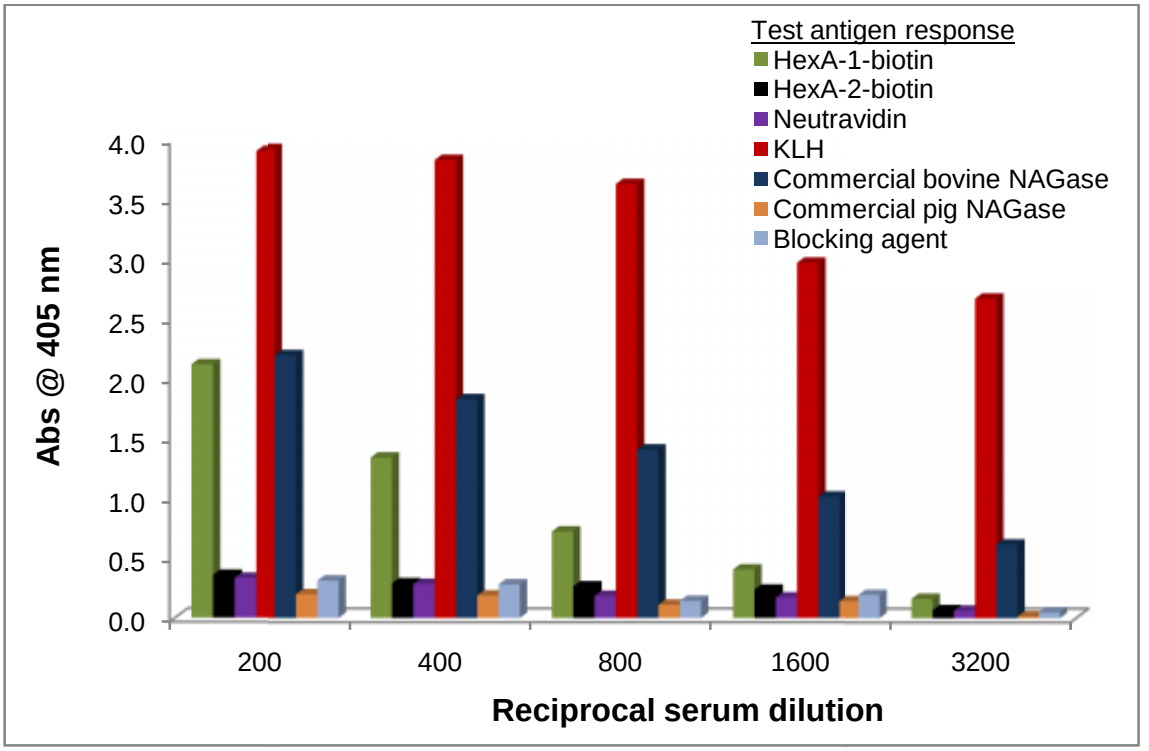


Figure 3.6. ***Antibody titre of serum from mouse immunised with HexA-1-KLH.***
After 5 boosts with HexA-1-KLH, mouse serum antibody titres were checked against HEX-A-1-biotin, HEX-A-2-biotin, bovine NAGase, pig NAGase, neutravidin, KLH and blocking agent (5% (w/v) BSA-milk-PBS) using a series of dilutions ranging from 1:200 to 1:3,200. Similar dilutions of mouse pre-immune sera were analysed. All the sera dilutions were assayed in duplicate. Pre-immune values were subtracted from all values. Alkaline phosphatase-labelled anti-mouse IgG antibody was used as the detection antibody.

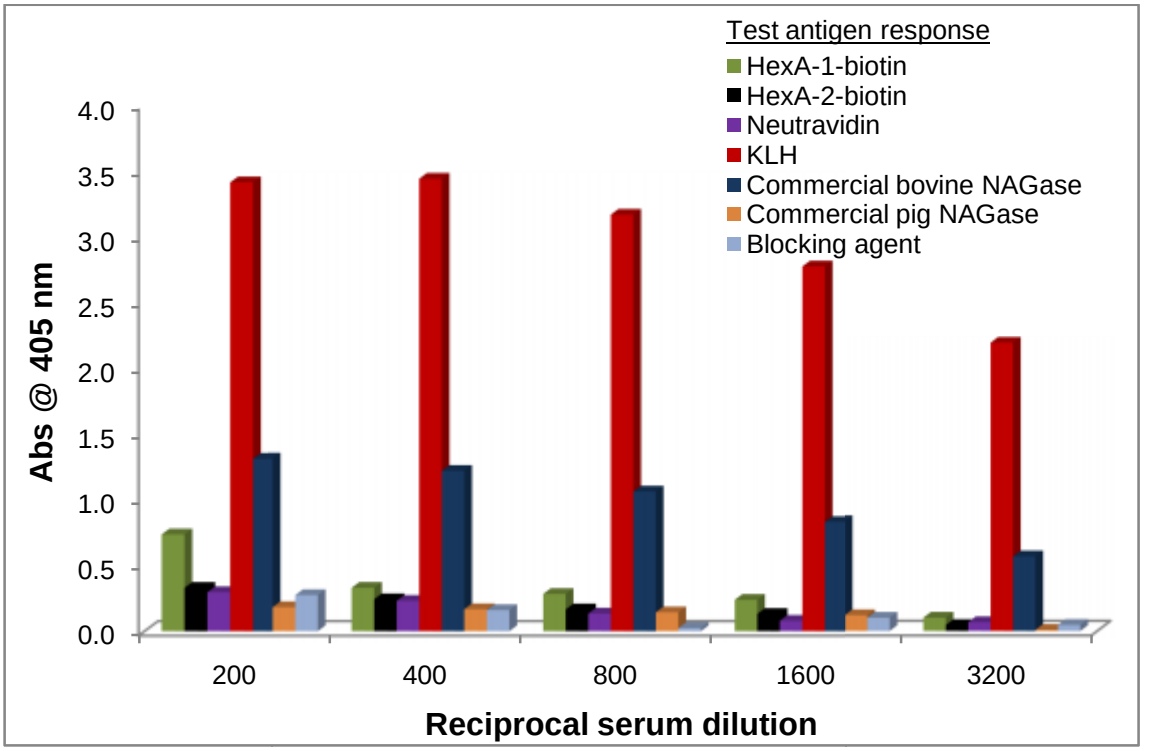


Figure 3.7. ***Antibody titre of serum from mouse immunised with HexA-2-KLH.***
After 5 boosts with HexA-2-KLH, mouse serum antibody titres were checked against HEX-A-1-biotin, HEX-A-2-biotin, bovine NAGase, pig NAGase, neutravidin, KLH and blocking agent (5% (w/v) BSA-milk-PBS) using a series of dilutions ranging from 1:200 to 1:3,200. Similar dilutions of mouse pre-immune sera were analysed. All the sera dilutions were assayed in duplicate. Pre-immune values were subtracted from all values. Alkaline phosphatase-labelled anti-mouse IgG antibody was used as the detection antibody.

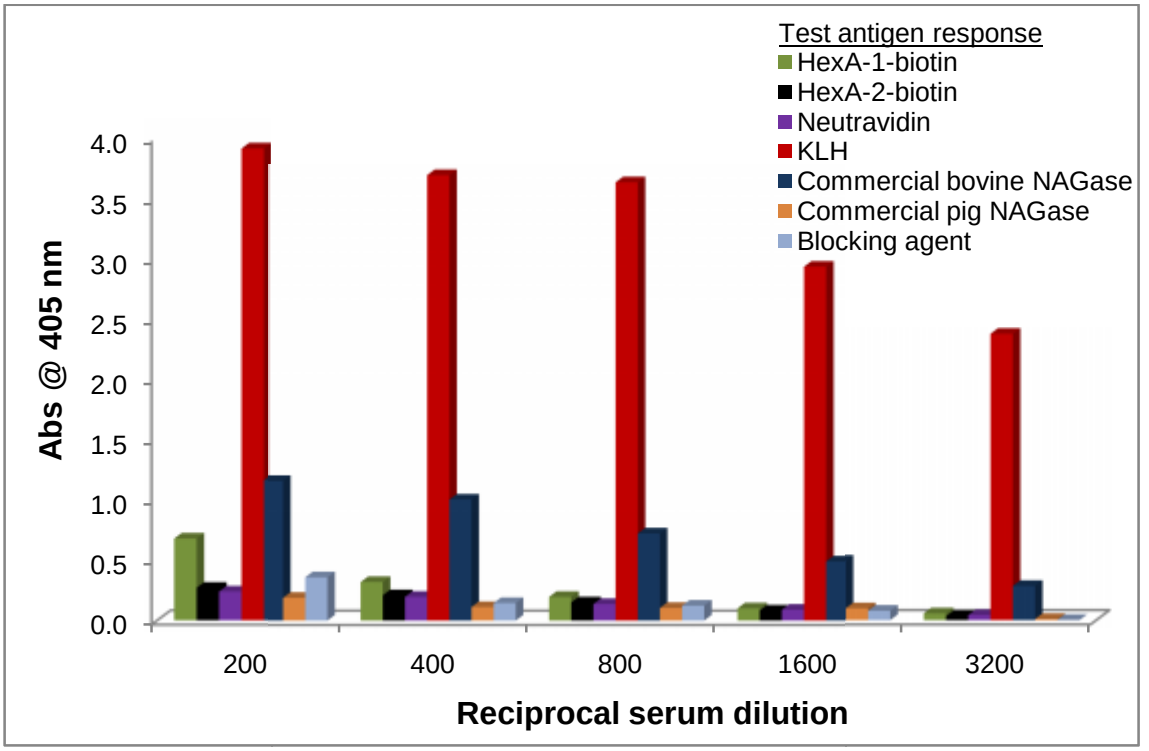


Figure 3.8. ***Antibody titre of serum from mouse immunised with HexA-2-KLH.***
After 5 boosts with HexA-2-KLH, mouse serum antibody titres were checked against HEX-A-1-biotin, HEX-A-2-biotin, bovine NAGase, pig NAGase, neutravidin, KLH and blocking agent (5% (w/v) BSA-milk-PBS) using a series of dilutions ranging from 1:200 to 1:3,200. Similar dilutions of mouse pre-immune sera were analysed. All the sera dilutions were assayed in duplicate. Pre-immune values were subtracted from all values. Alkaline phosphatase-labelled anti-mouse IgG antibody was used as the detection antibody.

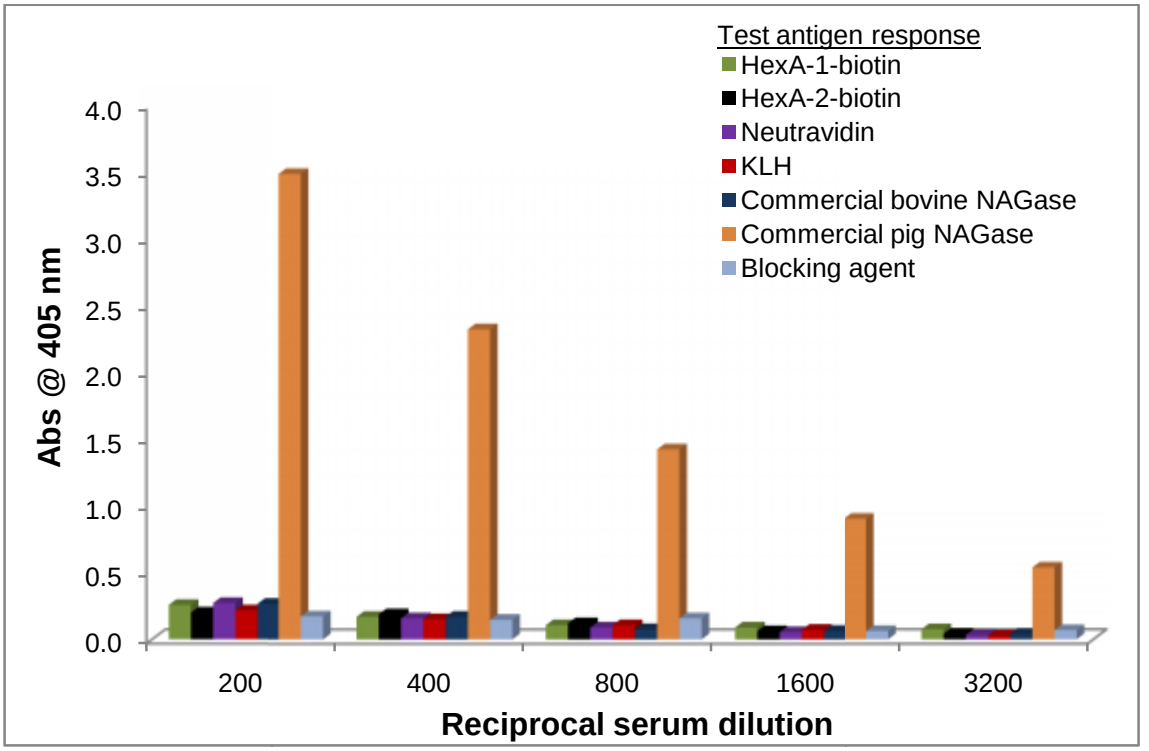


Figure 3.9. ***Antibody titre of serum from mouse immunised with pig NAGase.***
After 5 boosts with pig NAGase, mouse serum antibody titres were checked against HEX-A-1-biotin, HEX-A-2-biotin, bovine NAGase, pig NAGase, neutravidin, KLH and blocking agent (5% (w/v) BSA-milk-PBS) using a series of dilutions ranging from 1:200 to 1:3,200. Similar dilutions of mouse pre-immune sera were analysed. All the sera dilutions were assayed in duplicate. Pre-immune values were subtracted from all values. Alkaline phosphatase-labelled anti-mouse IgG antibody was used as the detection antibody.

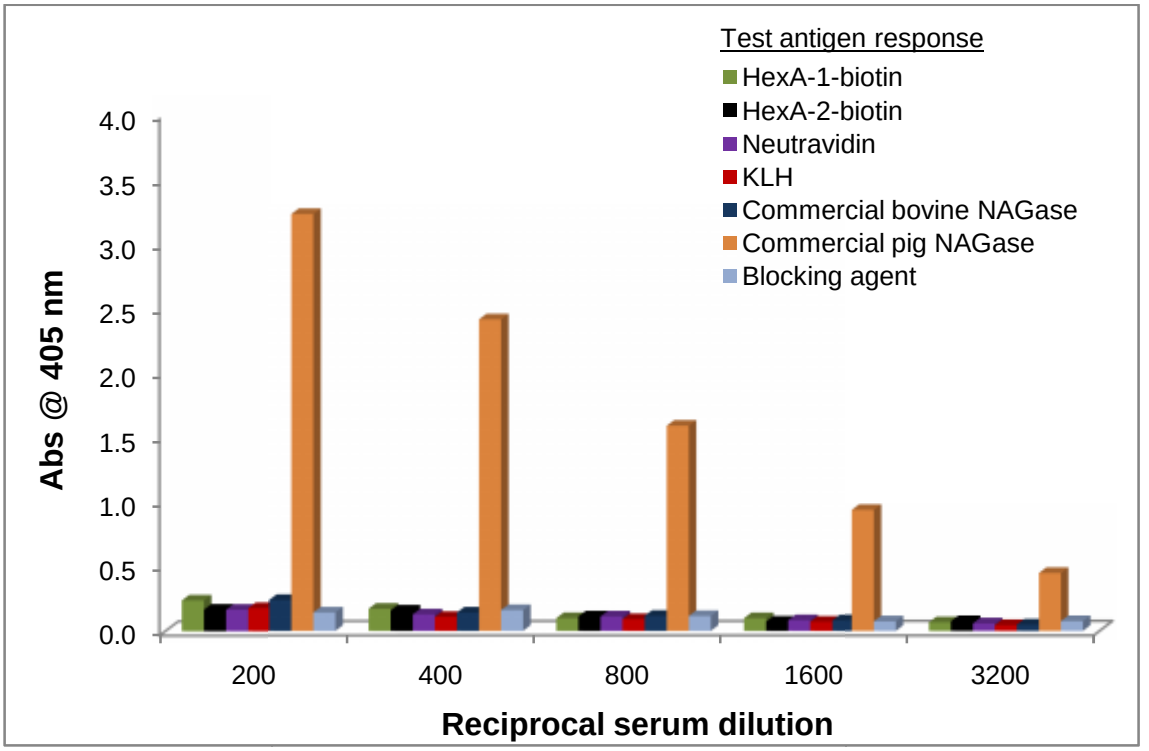


Figure 3.10. ***Antibody titre of serum from mouse immunised with pig NAGase.***
After 5 boosts with pig NAGase, mouse serum antibody titres were checked against HEX-A-1-biotin, HEX-A-2-biotin, bovine NAGase, pig NAGase, neutravidin, KLH and blocking agent (5% (w/v) BSA-milk-PBS) using a series of dilutions ranging from 1:200 to 1:3,200. Similar dilutions of mouse pre-immune sera were analysed. All the sera dilutions were assayed in duplicate. Pre-immune values were subtracted from all values. Alkaline phosphatase-labelled anti-mouse IgG antibody was used as the detection antibody.

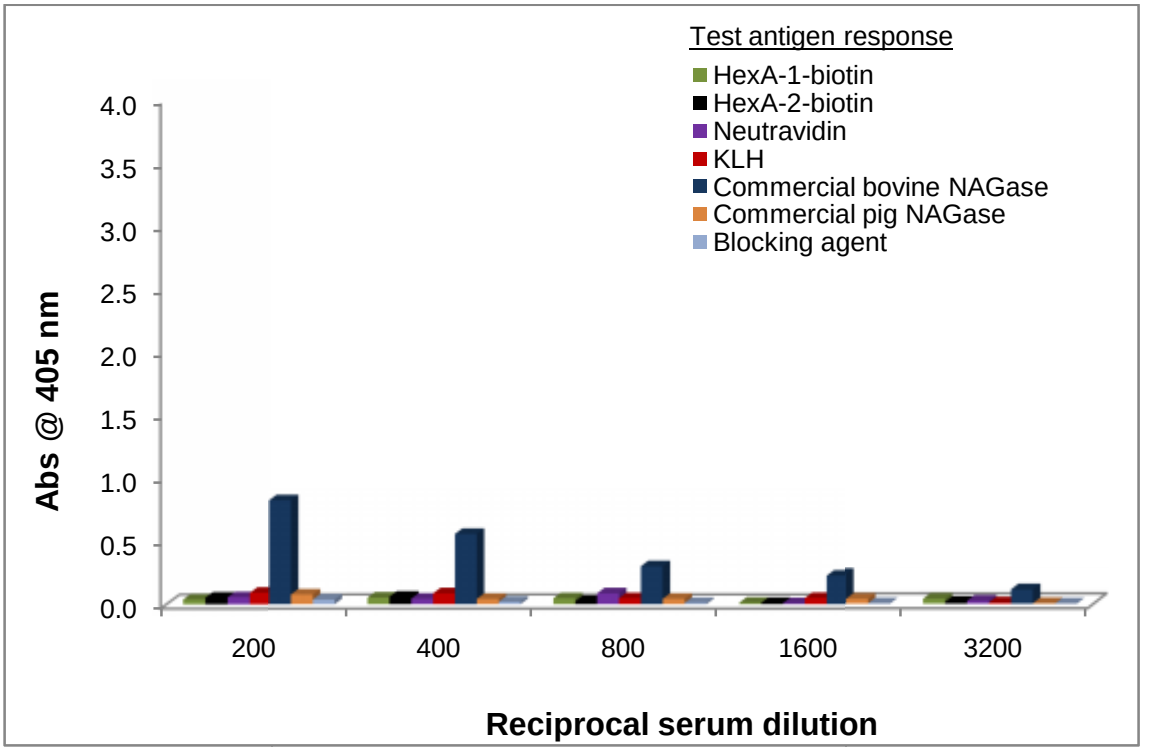


Figure 3.11. ***Antibody titre of serum from mouse immunised with bovine NAGase.***
After 5 boosts with bovine NAGase, mouse serum antibody titres were checked against HEX-A-1-biotin, HEX-A-2-biotin, bovine NAGase, pig NAGase, neutravidin, KLH and blocking agent (5% (w/v) BSA-milk-PBS) using a series of dilutions ranging from 1:200 to 1:3,200. Similar dilutions of mouse pre-immune sera were analysed. All the sera dilutions were assayed in duplicate. Pre-immune values were subtracted from all values. Alkaline phosphatase-labelled anti-mouse IgG antibody was used as the detection antibody.

3.3 Generation of Avian Anti-NAGase scFv

As no success was achieved in generating anti-NAGase monoclonal antibodies, efforts were made to generate anti-NAGase scFvs using chicken as the host. Chickens are phlogenetically distinct from mammals and, therefore, would be expected to generate better immune responses to most mammalian proteins.

3.3.1 Chicken serum titre against NAGase

A chicken was immunised with dialysed commercial NAGase. The chicken was sacrificed after 4 boosts. Prior to its sacrifice a serum titre was performed to determine whether a sufficient response against the commercial NAGase was achieved. A series of dilutions of the chicken serum, diluted in 1% (w/v) BSA-Milk-PBS (pH 7.2), were tested against neat (non-dialysed) and dialysed commercial NAGase in an indirect ELISA format. A titre in excess of 1:2,560,000 was observed for neat commercial NAGase (Figure 3.12) and the titre against dialysed commercial NAGase was in excess of 1:1,280,000 (Figure 3.13).

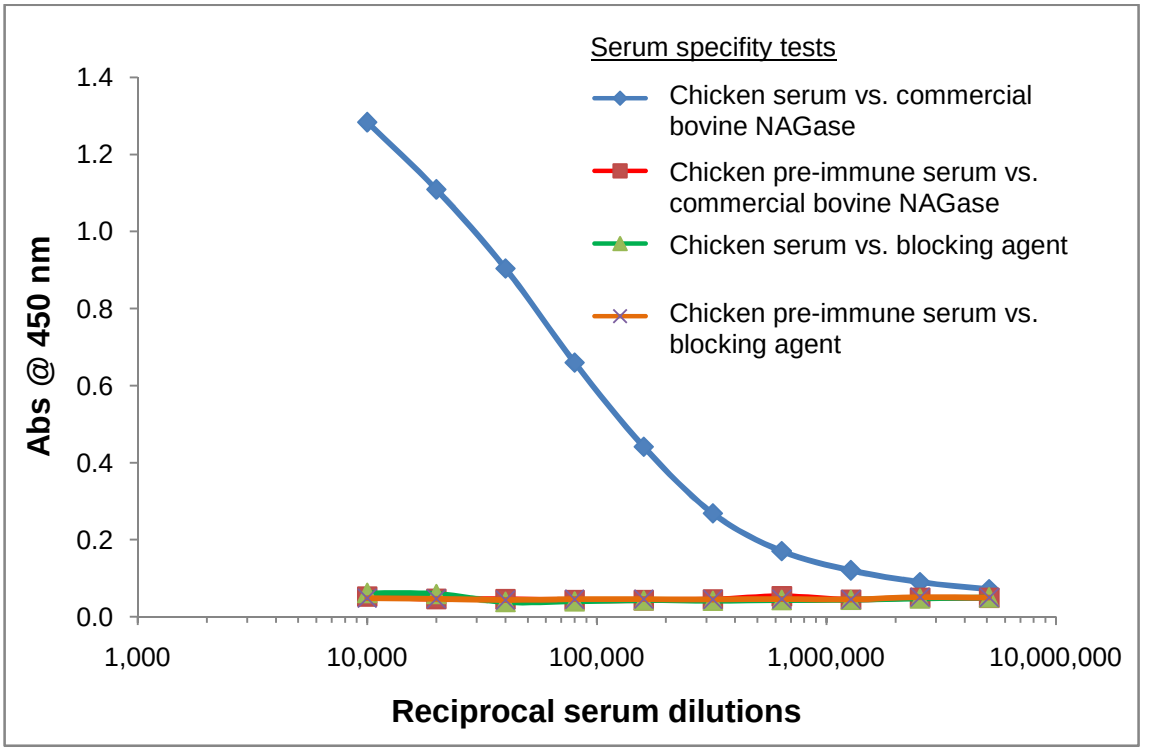


Figure 3.12. ***Titre of chicken serum against commercial bovine NAGase.*** After 4 boosts the chicken serum antibody titre was checked against commercial bovine NAGase and the blocking agent (5% (w/v) BSA-milk-PBS) using a series of dilutions of serum ranging from 1:10,000 to 1:5,20,000. Similar dilutions of chicken pre-immune serum were analysed. Anti-chicken IgY (H+L)-horseradish peroxidase-labelled antibody was used as detection antibody.

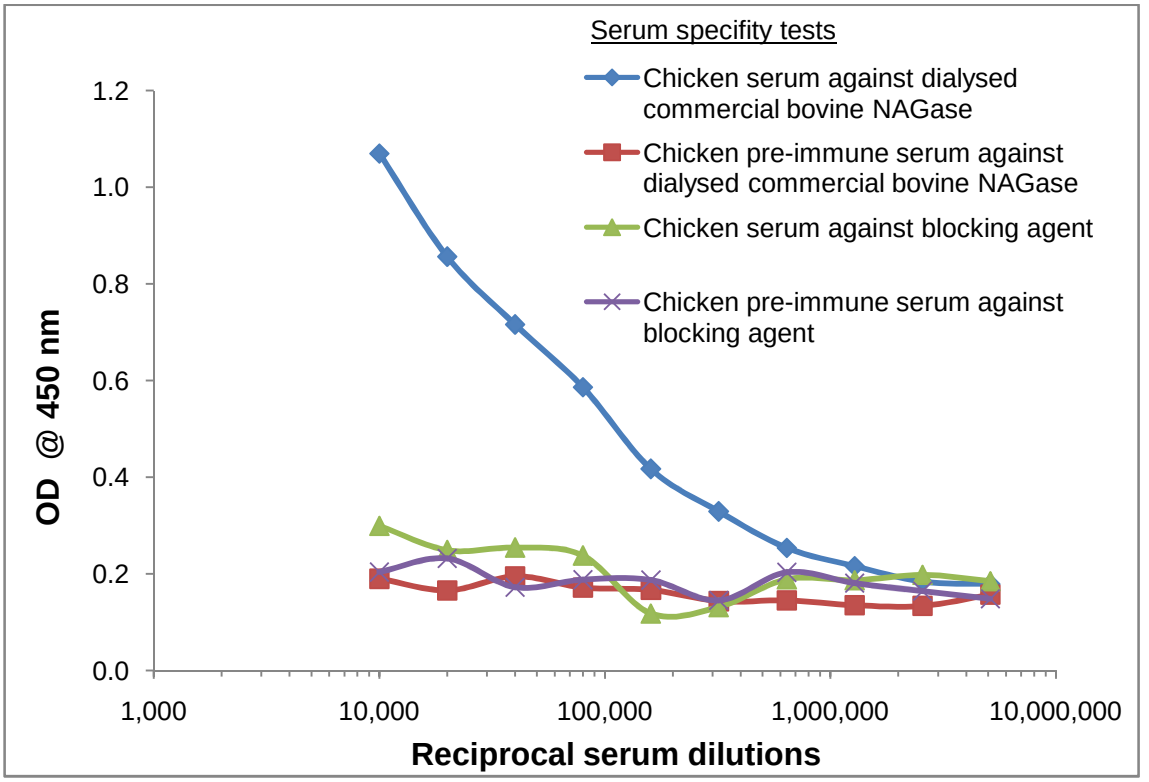


Figure 3.13. ***Titre of avian serum against the dialysed commercial NAGase.***
After 4 boosts the chicken serum antibody titre was checked against dialysed commercial bovine NAGase and the blocking agent (5% (w/v) BSA-milk-PBS) using a series of dilutions ranging from 1:10,000 to 1:5,20,000. Similar dilutions of chicken pre-immune serum were analysed. Anti-Chicken IgY (H+L)-horseradish peroxidase-labelled antibody was used as detection antibody.

3.3.2 Chicken variable heavy and light chain PCR amplifications

For generating an anti-NAGase chicken scFv library the variable heavy (V_H) and variable light (V_L) genes were amplified from chicken spleen and bone marrow cDNA. Optimisation of PCR reactions for amplification of heavy and light chains was carried out using different $MgCl_2$ concentrations (1 to 4 mM) in 1 mM increments. A concentration of 4 mM $MgCl_2$ was found to be optimum and was used for subsequent reactions (Figures 3.14 and 3.15 for avian spleen and bone marrow, respectively).

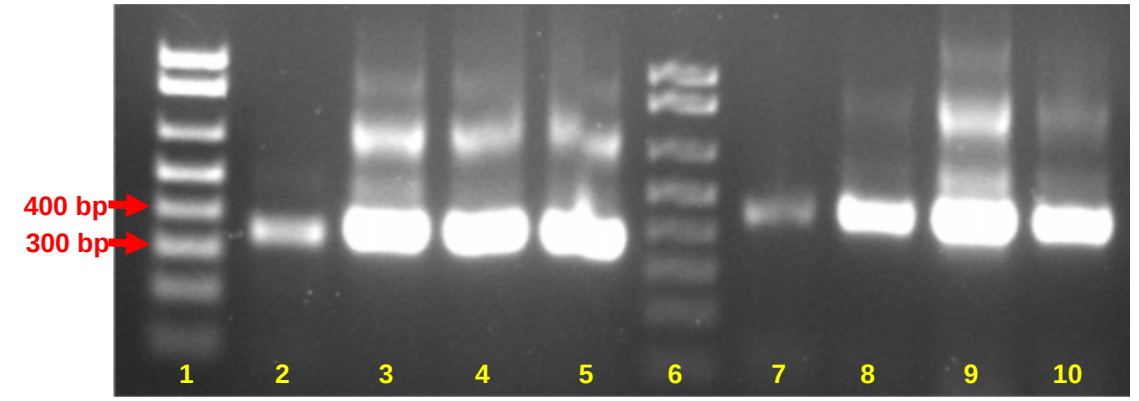


Figure 3.14. ***Optimisation of Mg^{2+} ion concentrations for variable heavy and light chain PCR amplifications from avian spleen.***
cDNA from the spleen of chicken immunised with dialysed commercial NAGase was used as template for amplification of V_H (~400 bp amplicon) and V_L (~350 bp amplicon) chains. Four different $MgCl_2$ concentrations (1 to 4 mM in 1 mM increments) were used to assess optimal concentrations required for amplifications. Lanes 1 and 6: 1 Kb plus DNA ladder; lanes 2-4: V_L amplifications with increasing (1 to 4 mM) $MgCl_2$ concentrations and lanes 7-10: V_H amplifications with increasing (1 to 4 mM) $MgCl_2$ concentrations.

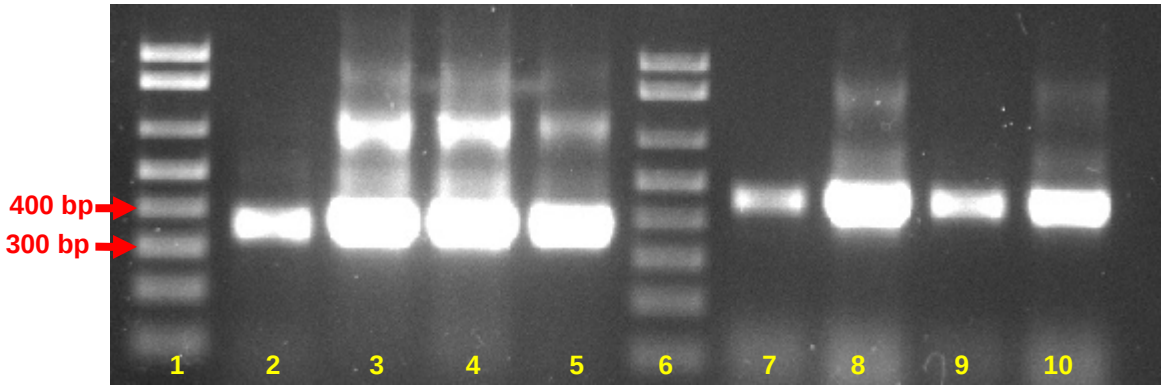


Figure 3.15. ***Optimisation of Mg^{2+} ion concentrations for variable heavy and light chain PCR amplifications from avian bone marrow.***
cDNA from the bone marrow of chicken immunised with dialysed commercial NAGase was used as template for amplification of V_H (~400 bp amplicon) and V_L (~350 bp amplicon) chains. Four different $MgCl_2$ concentrations (1 to 4 mM in 1 mM increments) were used to assess optimal concentrations required for amplifications. Lane 1 and 6: 1 Kb plus DNA ladder; lanes 2-4: V_L amplifications with increasing $MgCl_2$ concentrations (1 to 4 mM in 1 mM increments) and lanes 7-10: V_H amplifications with increasing $MgCl_2$ concentrations (1 to 4 mM in 1 mM increments).

3.3.3 Chicken SOE-PCR of variable heavy and light chains

Purified V_H and V_L chains from spleen were mixed in equimolar ratio for coupling them together using a glycine-serine linker. Similarly, V_H and V_L chains from bone marrow were also coupled together. The PCR conditions were optimised using different $MgCl_2$ concentrations (1 - 4 mM in 1 mM increments) to yield an amplicon of approximately 750 bp. The optimum concentration of $MgCl_2$, for SOE-PCR for joining V_H and V_L chains from spleen, was found to be 4 mM (Figure 3.16). Two mM $MgCl_2$ yielded best amplification (Figure 3.16) for SOE-PCR for joining V_H and V_L chains from bone marrow. However, non-specific bands were observed adjacent to the specific band and, thus, 3 mM $MgCl_2$ was used (Figure 3.16).

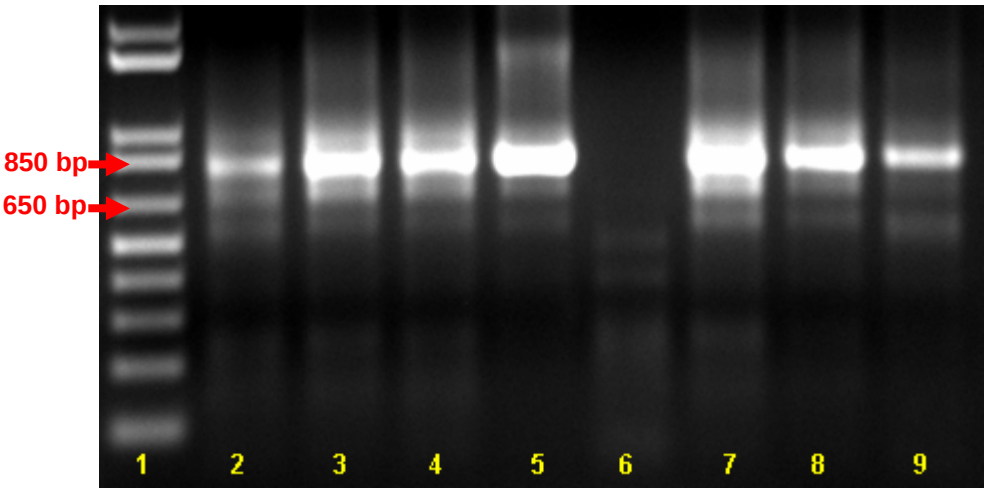


Figure 3.16. ***Optimisation of Mg^{2+} ion concentrations for chicken SOE-PCR.***
Purified V_H and V_L chains were mixed in equimolar ratios for SOE-PCR. The SOE product (~750 bp) yield was optimised using four different $MgCl_2$ concentrations (1 to 4 mM in 1 mM increments). Lane 1: 1 Kb plus DNA ladder; lanes 2-5: SOE amplifications, for coupled V_H and V_L chains from spleen, with increasing $MgCl_2$ concentrations (1 to 4 mM in 1 mM increments) and lanes 6-9: SOE amplifications, for coupled V_H and V_L chains from bone marrow, with increasing $MgCl_2$ concentrations (1 to 4 mM in 1 mM increments). No SOE amplification was observed for bone marrow using 1 mM $MgCl_2$ concentration (lane 6).

3.3.4 Chicken anti-NAGase scFv library construction and biopanning

Large-scale SOE-PCRs, from both spleen and bone marrow V_H and V_L chains, were performed. The amplicons were subsequently concentrated using ethanol precipitation and quantified using the Nanodrop ND-1000™. The SOE amplicons were then cloned into pComb 3XSS vector following *Sfi*I digest. The ligated products were transformed into XL1-Blue electroporation competent cells by electroporation. The transformation mix from spleen and bone marrow were pooled to generate a library having a size of 4.2×10^8 cfu/mL. The library was subject to phage display biopanning against immobilised dialysed commercial NAGase (Table 3.3).

Table 3.3. ***The phage input and output titres over the 5 rounds of biopanning of the avian anti-NAGase scFv library.***

Biopanning round	Input titre (cfu/mL)	Output titre (cfu/mL)
Round 1	2.4×10^{12}	3.3×10^6
Round 2	1.9×10^{12}	2.2×10^5
Round 3	6.3×10^{11}	1.7×10^5
Round 4	1.1×10^{12}	3.5×10^6
Round 5	1.3×10^{12}	1.9×10^6

3.3.5 Chicken polyclonal phage ELISA

The precipitated output phage from all the five rounds of biopanning was tested for enrichment against dialysed commercial NAGase using a polyclonal phage ELISA. Phage from an unpanned library and the helper phage used were also checked in parallel as negative controls. The scFv-displaying phage particles were detected using a HRP-conjugated mouse anti-M13 antibody (GE Healthcare Life Sciences, UK) and the absorbance read at 450 nm following 20 min incubation with TMB substrate. There was a definitive increase in signal in

round 4 and 5 of panning (Figure 3.17). However, there was a minor decrease in signal in round 5 when compared to round 4, which may be the result of increased stringency in round 5.

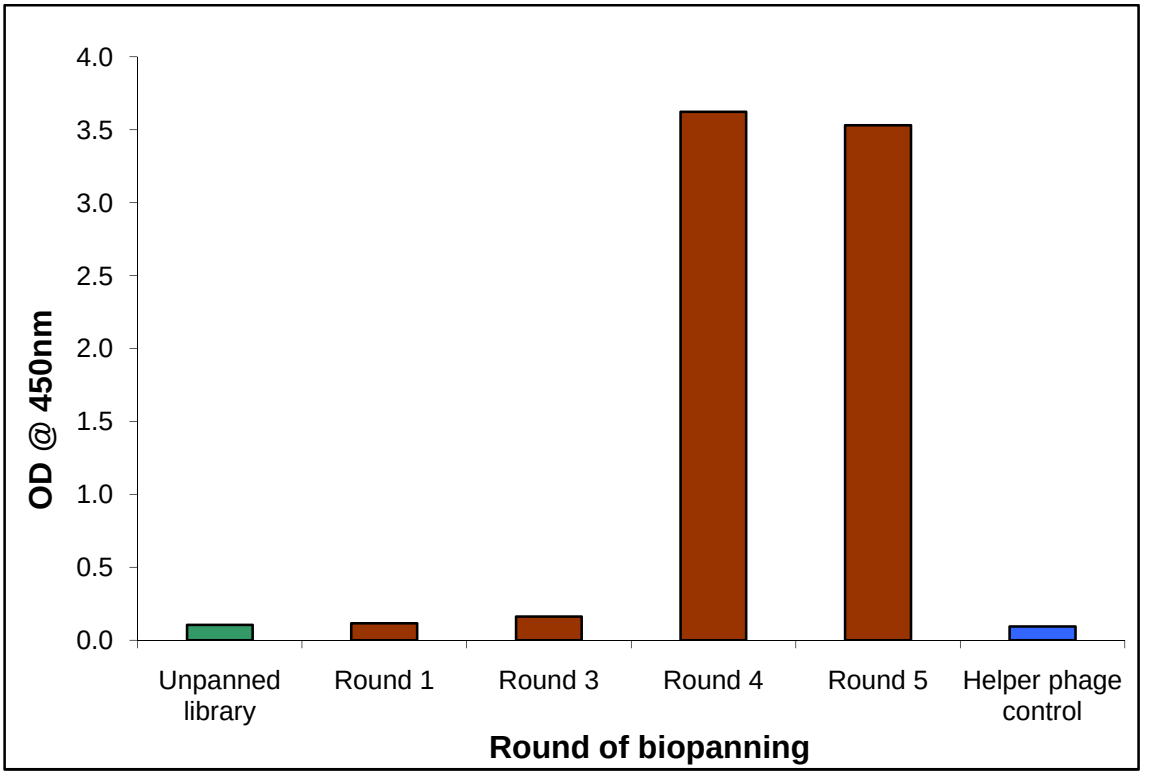


Figure 3.17. ***Polyclonal phage ELISA.***
The phage outputs from the successive rounds of biopanning and the helper phage used in biopanning were tested against bovine NAGase. The helper phage, used in the panning, was included as control to check for non-specific phage binding. The results show that only specific-phage were amplified against the antigen in consequetive rounds of panning, starting from the unpanned library.

3.3.6 Soluble expression and direct ELISA of chicken anti-NAGase scFv clones

The phage outputs from rounds 4 and 5 were infected into TOP10F´ cells for soluble expression. Eighty nine colonies each were picked from both round 4 and round 5, and were analysed using their expressed lysates in a direct soluble monoclonal ELISA format, to evaluate biding to dialysed commercial NAGase. A high percentage of clones, approximately 88%, showing specificity

to dialysed commercial NAGase were observed in round 4 (Figure 3.18). Approximately 30% NAGase-specific clones were observed in round 5 (Figure 3.19).

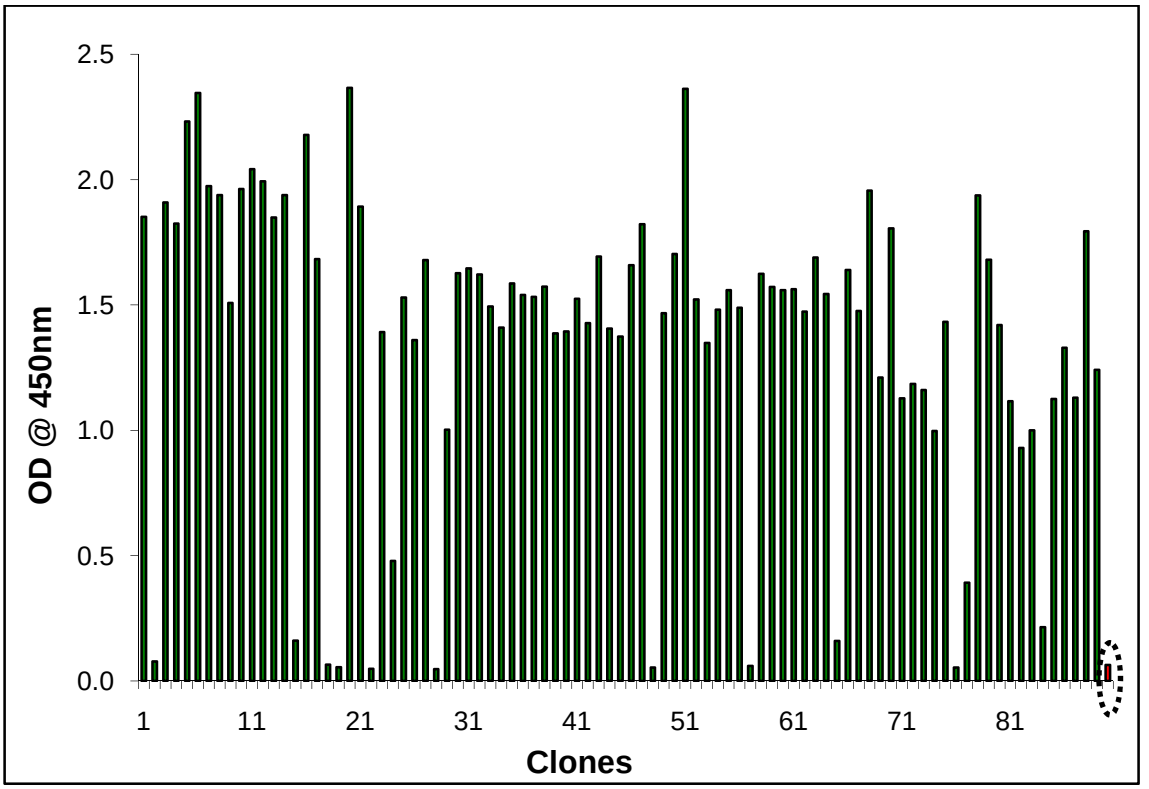


Figure 3.18. ***Monoclonal ELISA of solubly expressed scFvs from biopanning round 4.***
Direct soluble ELISA involving scFv-enriched lysate expressed from 89 chicken anti-NAGase scFv clones from biopanning round 4. A high percentage of clones, approximately 88%, showed specificity to dialysed commercial NAGase The red bar (circled) represents the reagent control (negative control).

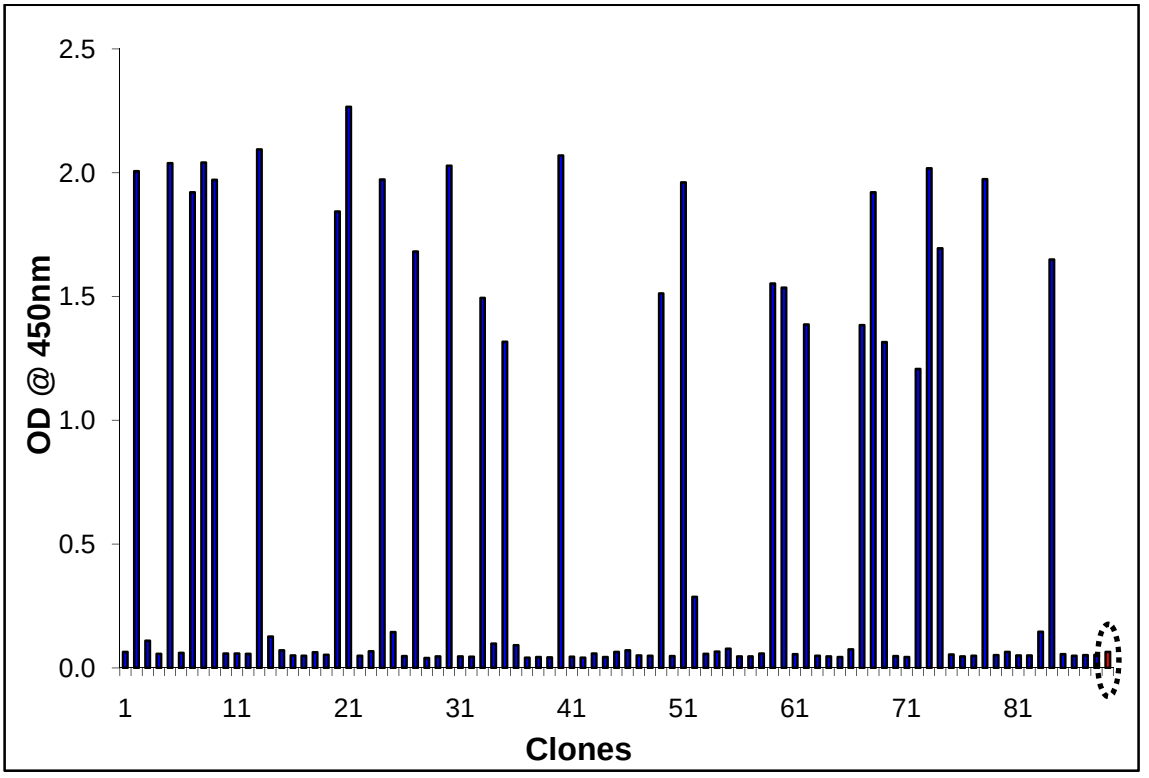


Figure 3.19. ***Monoclonal ELISA of solubly expressed scFvs from biopanning round 5.***
Direct soluble ELISA involving scFv-enriched lysate expressed from 89 chicken anti-NAGase scFv clones from biopanning round 5. Approximately 30% of clones showed specificity to dialysed commercial NAGase The red bar (circled) represents the reagent control (negative control).

3.3.7 Direct ELISA of chicken anti-NAGase scFv clones against different NAGase-associated antigens

The Sigma NAGase used for immunisation of the chicken, used for generation of anti-NAGase scFvs, was found to contain certain contaminating proteins, other than HexA and HexB. Consequently, these scFv clones (Figure 3.34 and 3.44) could not be clearly defined as anti-NAGase in specificity. Therefore, the clones were also checked against recombinant HexA (Chapter 4) and both Hex α-subunit peptides (Table 3.2). However, none of the clones were positive for any of the 3 antigens (*i.e.* recombinant HexA and both Hex α-subunit peptides) tested.

3.3.8 Chicken serum titre against different NAGase antigens

The genearation of anti-NAGase scFvs could not be accomplished from the chicken immunised with commercial NAGase. Therefore, chickens were individually immunised with recombinant HexA (Chapter 4), HexA-1-KLH (Table 3.2) and HexA-2-KLH (Table 3.2). The antibody titres to chicken sera were checked after 4 boosts. A series of dilutions of the chicken sera, diluted in 1% (w/v) BSA-Milk-PBS (pH 7.2), were tested against the antigen they were immunised with in an indirect ELISA format. None of the chickens responded to the immunisations and titres of less than 1:1,000 were observed for all the chickens. Consequently, none of these chickens were utilized for scFv generation. Chickens failed to generate adequate immune responses against recombinant HexA (Chapter 4), HexA-1-KLH (Table 3.2) and HexA-2-KLH (Table 3.2). Therefore, rabbits were immunised for polyclonal antibody generation.

3.4 Generation of rabbit anti-NAGase polyclonal antibodies

Three New Zealand white rabbits were immunised with recombinant HexA (Chapter 4), HexA-1-KLH (Table 3.2) and HexA-2-KLH (Table 3.2). The rabbits were given 5 boosts and sera titres were checked to determine whether sufficient responses against the immunised antigens were achieved. A series of dilutions in 1% (w/v) BSA-Milk-PBS (pH 7.2), of the rabbit sera, were tested against the antigen used for immunisation in an indirect ELISA format. A titre of less than 1:1,000 was observed for rabbits immunised recombinant HexA and HexA-2-KLH. The rabbit immunised with HexA-1-KLH gave a titre of 1:10,000 (Figure 3.20).

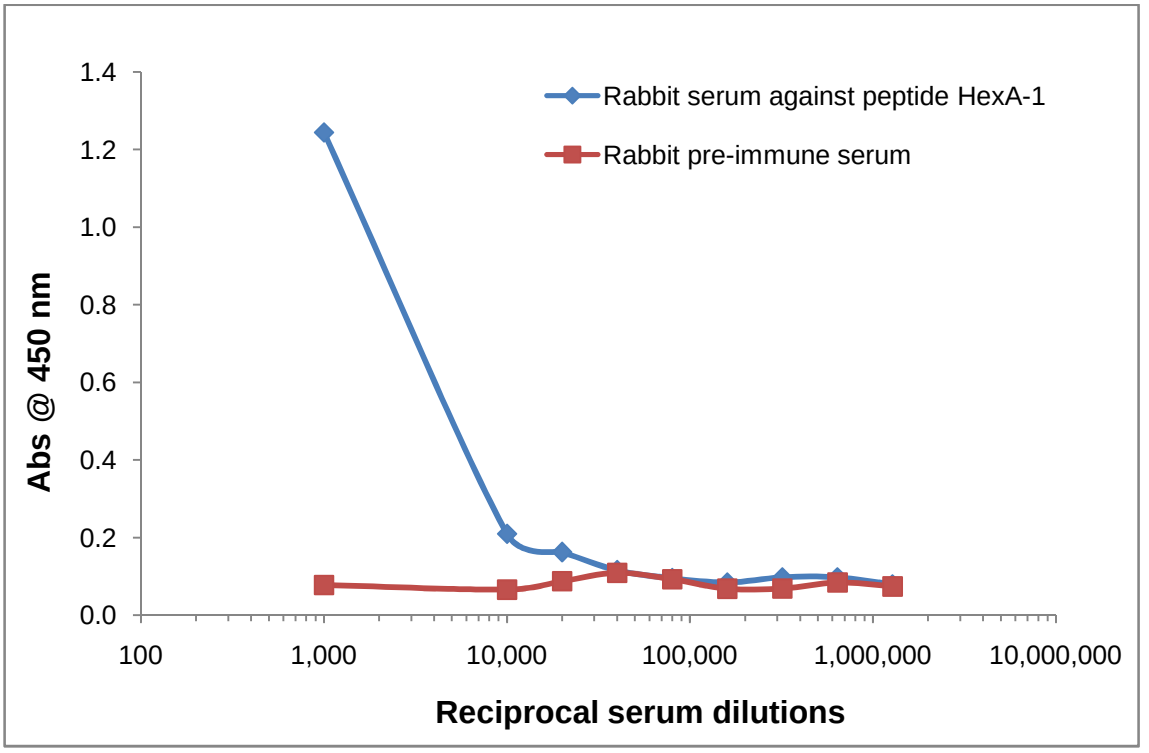


Figure 3.20. ***Evaluation of the rabbit antibody response following administration of HexA-1-KLH.***
After 5 boosts the rabbit serum antibody titre was checked against HexA-1-Biotin using a series of dilutions ranging from 1:1,000 to 1:1,000,000. Similar dilutions of rabbit pre-immune serum were analysed. Anti-rabbit IgG (whole molecule)-horseradish peroxidase-labelled antibody was used as the detection antibody.

The serum obtained from rabbit immunised with HexA-1-KLH was further titred against dialysed commercial NAGase (section 2.2.1.6) and recombinant HexA (in an indirect ELISA format), in order to ascertain if the generated polyclonal antibodies were detecting the complete HexA protein. Titres of less than 1:1,000 were observed. Therefore, these antibodies might not be useful in detecting HexA (NAGase), even though they had a titre of 1:10,000 against the peptide used for immunisation (Figure 3.20).

3.5 Discussion

Initial efforts to generate NAGase-specific antibodies involved the use of commercially available bovine NAGase as antigen. Attempts were made to

generate monoclonal antibodies (mAbs) against commercial bovine NAGase. However, poor immune responses in mice were observed. Analyses revealed high sequence homologies between both NAGase isoforms (HexA and HexB) from mouse and cow. This might explain the non-recognition of most antigenic determinants in bovine NAGase as foreign by the mouse immune system and consequently, failure to elicit a strong immune response. There is short evolutionary distance between mouse and other mammals (Hughes, 1994a) and, therefore, many proteins, such as enzymes, are highly conserved between them (Hughes, 1994a; Hofer *et al.*, 2007). As a result, mice are known to show weak or no response against some antigens, such as certain mammalian proteins (*e.g.* human galactosyltransferase and human tyrosinase-related protein-2) (Chatterjee *et al.*, 1984; Scheffer *et al.*, 2000; Narat, 2003; Yamano *et al.*, 2006).

However, some workers were successful in generated mAbs from mice with a weak antigenic response (Chatterjee *et al.*, 1984; Wagner *et al.*, 1994). Therefore, it was decided to continue with fusions even after getting a weak antigenic response in mice. Consequently, two different fusions of Sp2/mIL-6 with spleenocytes from immunised mice were carried out to select antibodies against immunodominant epitopes of NAGase. However, both attempts failed to generate a NAGase-specific antibody.

Subsequent analysis revealed that the commercial NAGase contained contaminating proteins, in addition to HexA and HexB (Figure 3.2). There were several bands in the size range of NAGase isoforms and its subunits (section 3.2.3) and, thus, it was not possible to charaterise them. Hence, the protein

sequences of the two NAGase subunits from cow were aligned with sequences of NAGase subunits from mouse, human, pig, rabbit and chicken, in order to, (a) assess the possibility of using commercial NAGase from other species as an immunogen and, (b) consider use of another species source for generation of NAGase-specific antibodies.

Amino acid sequences of pig and cow α- and β-subunits revealed a high degree of similarity. Therefore, it was decided to use commercial pig NAGase as a possible antigen to generate antibodies against conserved epitopes of pig and cow NAGase. High similarities in amino acid composition between the bovine and pig NAGase were also reported by Vehpoort (1972). High sequence homologies among HexA proteins from different species were also reported by Liu and Gao (2009). Sheep HexA showed 98%, 84%, 84%, 80% and 81% homologies with HexA from bovine, human, *Bornean orangutan*, rat and mouse, respectively (Liu and Gao, 2009). However, pig NAGase failed to generate a suitable immune response in mice. This might be as a result of high homology (79%) between mouse and pig NAGase.

Proteins from natural sources may serve as ideal antigens for antibody production in most cases, due to their conformation and post-translational modification options (Angeletti, 1999). These proteins may retain conformational epitopes due to the presence of appropriate tertiary folding structures. In addition, these proteins undergo post-translational modifications (such as glycosylation, phosphorylation, nitrosylation and acylation), which are generally useful for immunological recognition of the immunogen. However, peptides are extremely valuable in cases where protein is not available in an isolated or

purified form (Angeletti, 1999). Additionally, they are usually stable, can be produced in large quantities and in highly pure form (http://www.anaspec.com/html/antibody_notes.html) (Angeletti, 1999). Peptides are short amino acid sequences which cannot elicit a sufficient immune response on their own. Therefore, they were conjugated to carrier proteins, such as keyhole limpet haemocyanin (KLH), bovine serum albumin (BSA), and ovalbumin (OVA), to make them more potent immunogens. The use of peptides in antibody generation is not new and various antibodies against peptides were successfully generated in the past (Jacob *et al.*, 1983; Choi *et al.*, 2001; Tsurumi *et al.*, 2003; Giménez *et al.*, 2007; Sreepian *et al.*, 2009; Conroy, 2011). Antibodies against peptide sequences from bovine proteins were also successfully produced (Wohlfart *et al.*, 1992; Ivell *et al.*, 2000; Takahashi *et al.*, 2004; Chiu *et al.*, 2008), previously. Consequently, two short peptide sequences were synthesised as possible antigens from the conserved regions of bovine Hex α-subunit.

The peptides were selected based on the heterology of bovine Hex α-subunit from other animals. Peptide sequences that are exposed conformationally are desirable for antibody production. Such peptides sequences usually contain antigenic determinants owing to their availability for contacts with antigen-combining sites and are thus, necessary for development of useful antibodies (Ivey and Berg, 1985; Novotný *et al*., 1986). Therefore, a peptide sequence from both the N-terminal and C-terminal region of bovine Hex α-subunit was chosen (Table 3.2), as the terminals are usually exposed (Jacob and Unger, 2006). Bioinformatic analysis further confirmed that both peptides were exposed conformationally (Figure 3.4). Additionally, both peptides were 13 residues in

length which is within the range of 12-16 residues, usually recommended, since they contain several epitopes and are easy to synthesise (Angeletti, 1999). The peptides for immunisations were synthesised and conjugated to KLH as the carrier protein (Table 3.2) since KLH has no homology with vertebrate proteins (Angeletti, 1999). However, these conjugated peptides failed to generate a significant immune response in mice. The immunogenicity of the peptide fragment in a host animal is dependent on various host-related factors (*e.g.* host immunoglobulin repertoire, and host cellular and regulatory mechanisms) in addition to the properties of the peptide (Van Regenmortel, 2001). It was also observed that different animals within the same group frequently respond differently to the same peptide used as immunogen (Hancock and O'Reilly, 2005). The immunogenicity of different epitopes also varies, *i.e.* certain epitopes might fail to generate significant immune response (Lu *et al.*, 2000; Spencer and Braciale, 2000) and, likewise, the epitopes present in the choosen peptides might be weakly immunogenic. Therefore, it is impossible to predict the immunogenic effectiveness of a synthetic peptide (Van Regenmortel, 2001). Unfortunately, it is still not fully clear why some epitopes are more immunogenic than others (Van Regenmortel, 2009). Consequently, it was suggested to use numerous peptides from various regions of the protein sequence (Hancock and O'Reilly, 2005). Therefore, there is a need to synthesise other peptides from bovine Hex α-subunit and check them for their utility in antibody generation.

The greatest possible phylogenetic distance between the species of antigen and the host being immunised is desirable as it leads to a significantly increased immune response. Chicken are phylogenetically distinct from mammals and, therefore, widely used as hosts for antibody generation against

highly conserved mammalian proteins such as hormones and enzymes (Ingles *et al.*, 1973; Gassmann *et al.*, 1990; Rosol *et al.*, 1993; Narat, 2003). Chickens offer numerous additional advantages, over other hosts, in antibody production. In addition, mammalian proteins generally evoke a stronger immune response when administered, even in low doses, to chickens (Larsson *et al.*, 1998). Chicken antibodies do not activate the mammalian complement system, and there is no reactivity to bacterial Fc receptors, such as staphylococcal protein A or streptococcal protein G (Schade *et al.*, 1996; Zhang, 2003). They also offer stability over a wider range of pH and temperature (Shimizu *et al.*, 1992; Olovsson and Larsson 1993; Shimizu *et al.*, 1994; Zhang, 2003). Rearing chickens is easier and economical, which are extra advantages. Chicken IgY stock is derived from single V_H and V_L germ line sequences (McCormack *et al.*, 1993). Thus, for generating a recombinant chicken antibody library only a single set of primers are required for PCR amplification of chicken V genes (Yamanaka *et al.*, 1996; Andris-Widhopf et *al.*, 2000). This makes recombinant chicken antibody production faster and economical, and reduces the loss of rare transcripts due to differences in primer efficiencies (Barbas *et al.*, 2001). Thus, it was decided to use chickens as the host of choice for developing anti-NAGase antibodies.

A chicken was immunised with commercial bovine NAGase, concurrently with the purification and expression of NAGase being attempted. The chicken immunised with commercial bovine NAGase responded well to the immunisations. Spleen and bone marrow were harvested from the chicken on completion of the immunisation protocol. RNA was extracted from the bone marrow and spleen and was used for cDNA synthesis. Chicken V_H and V_L were

amplified from bone marrow and spleen cDNA and a scFv library was constructed using them. A library with a diversity of 4.2 x 10^8 cfu/mL was generated. This was good compared to other workers who constructed and successfully biopanned chicken immunse scFv libraries with diversities in the order of 10^5 to 10^8 (Sapats *et al.*, 2003; Finlay *et al.*, 2006; Ayyar *et al.*, 2010). This library was successfully panned against commercial bovine NAGase and approximately 88 and 30% positive clones were obtained from round 4 and 5 of the biopanning, respectively. However, as the commercial bovine NAGase was not pure, thus, it was not possible to know whether any clone will react specifically with bovine NAGase. These clones were further analysed to check if they were able to recognise recombinant HexA and Hex α-subunit peptides (Table 3.2). None of the clones reacted with any of the three antigens tested.

Subsequently, three different chickens were immunised with recombinant HexA (Chapter 2) and two Hex α-subunit peptides (Table 3.2), each. All 3 chickens failed to generate an immune response. A possible reason for the chicken not responding to the recombinant HexA immunisations might be due the lack of immunogenicity of the unconserved region, between bovine and chicken HexA, *i.e.* the epitopes present in the unconserved region might not weakly immunogenic. The immunogenicity of peptides is hard to predict (section 3.5).

Consequently, generation of polyclonal antibodies was attempted. Rabbits were used as host for polyclonal antibody production as they are reported to be an important source for stable, high affinity polyclonal antibodies (Rader, 2009). However, rabbits, immunised with recombinant HexA and HexA-2-KLH, also failed to generate a significant immune response. A low immune response was

observed in the rabbit immunised with HexA-1-KLH. However, the polyclonal antibodies generated failed to recognise whole antigen (recombinant HexA).

Different sources (such as native antigen, recombinant antigen and peptides) of NAGase were used in an attempt to generate anti-NAGase antibodies, however, these antigens failed to generate good antibody response. The reason for low immunogenicity of NAGase is probably its ubiquitous presence in a large variety of animal species, including mammals, lower vertebrates, invertebrates and plants, *etc.* (Walker, 1966; Caldwell *et al.*, 2005), coupled with the homology observed in the gene HEXA and HEXB gene sequences of different animals (Vehpoort 1972; Liu and Gao, 2009).

Chapter 4
Purification and Expression of Bovine NAGase

4. PURIFICATION AND EXPRESSION OF BOVINE NAGASE

4.1 Introduction

Availibility of sufficient amounts of high-quality antigen is one of the most important requirements in the generation of specific, high-affinity antibodies. Similarly, for generation of anti-NAGase antibodies, a significant amount of pure bovine NAGase enzyme was imperative. Traditionally, proteins and enzymes were purified from various plant and animal tissues (Takács, 2006). Antibodies generated using native antigens are advantageous when applied in diagnostic assays / platforms, as they recognise the surface-exposed epitopes when used for antigen detection in biological samples (Mishra *et al.*, 1991; Nair *et al.*, 1994). However, isolation of sufficient amounts of native antigens is not always feasible. Additionally, native antigens are often reported to have traces of contaminating host proteins (Hu *et al.*, 2008) which may cause problems in the production of specific required antibodies.

The advent of recombinant DNA technology facilitated the production of various proteins, either unavailable or difficult to obtain in sufficient quantities, in microorganisms (Glick and Whitney, 1983). Recombinantly expressed proteins are now regularly utilised in various diagnostic and therapeutic applications (Swartz, 2001; Srivastava *et al.*, 2006; Klubo-Gwiezdzinska *et al.*, 2010; Seismann *et al.*, 2010). Various expression systems, such as bacteria, fungi, insect cells, mammalian cell cultures, eggs, nematodes, and transgenic plants and animals, are well established (Nøhr *et al*., 2003; Corral *et al*., 2007; Vermasvuori *et al*., 2009). However, expression of recombinant proteins in *E. coli* is the simplest and most commonly used, as its molecular biology, biochemistry, and physiology is well understood (Baneyx, 1999; Swartz, 2001).

It also offers the advantages of speed, simplicity, high-level expression (Nøhr *et al*., 2003; Vermasvuori *et al*., 2009) and production is also economical (Corral *et al*., 2007).

Purification of protein, native or recombinant, can be achieved by a range of methodologies based on their exclusive physical, chemical and functional properties, such as size, shape, solubility, charge, isoelectric point, hydrophobicity and biological function (Figure 4.1) (Janson and Janson, 2011). Consequently, a large number of methodologies, including precipitation methods, dialysis, electrophoretic separations, filtration (and ultrafiltration) techniques and chromatography (adsorption), were suggested for protein purification (Ward and Swiatek, 2009). However, chromatography has the highest purification capability (Takács, 2006) and can be applied to a wide spectrum of compounds, making it one of the most widely employed techniques for protein purification.

Chromatography is the term used to describe a physical separation technique in which a mixture is dissolved in a mobile phase (gas or liquid) and this mobile phase is then forced to move over a selectively absorbent stationary phase, causing the components of a mixture to separate out. Different types of chromatography, classified on the basis of the mobile and the stationary phase used, are reported. For purification of proteins, liquid chromatography (where the mobile phase is an aqueous buffer and an insoluble matrix is used as stationary phase) is commonly employed (Janson and Janson, 2011). For liquid chromatography the stationary phase may be packed in a column, spread as a layer, or distributed as a film. However, column chromatography is one of the

most common physical configurations used. In order to facilitate the chromatographic separation of mixtures, high-resolution techniques, such as High Performance Liquid Chromatography (HPLC) and Fast Protein Liquid Chromatography (FPLC) were developed (Ersson *et al.*, 2011). HPLC is essentially an improved version of column chromatography where a mixture of compounds is forced through, under high pressures. It is an automated system, allowing use of particles of a much smaller size for the column packing material, and thus, resulting in the better separation of the components of the mixture by providing a much greater surface area for interactions between the stationary phase and the molecules flowing past it. FPLC is modified form of HPLC, offering even higher resolution of proteins being separated and allowing higher protein loadings (Madadlou *et al.*, 2011). FPLC offers various other advantages, such as cost-effectiveness, simplicity, automation (computerised operation), accelerated purifications, availability of a wider range of aqueous, biocompatible buffer systems and commercial chromatographic columns (of different types and sizes) (Baniel *et al.*, 1998; Ersson *et al.*, 2011; Madadlou *et al.*, 2011). Consequently, FPLC is often the most preferred method for protein purification.

4.2 Purification of Hexosaminidase B (HexB) from Bovine Spleen

As the commercial bovine NAGase was found to contain contaminating proteins (Figure 3.2), it was decided to purify NAGase ‘in-house’. A fast protein liquid chromatography (FPLC)-based purification strategy was devised for purification of hexosaminidase B (HexB) from bovine spleen (Figure 4.2) using an ÄKTA™ Explorer 100 with UV-900 monitor and Frac-950 fraction collector.

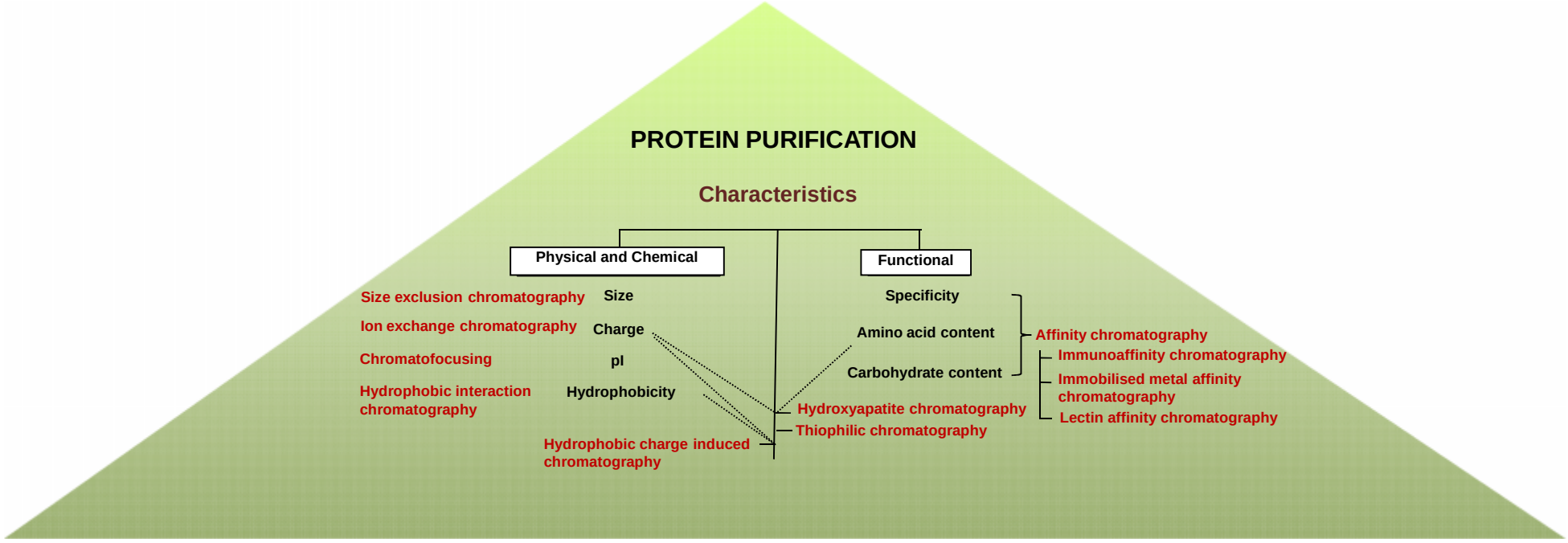


Figure 4.1. ***Protein purification by chromatography.***
Purification of proteins can be achieved by exploiting the physical, chemical and functional properties of proteins.

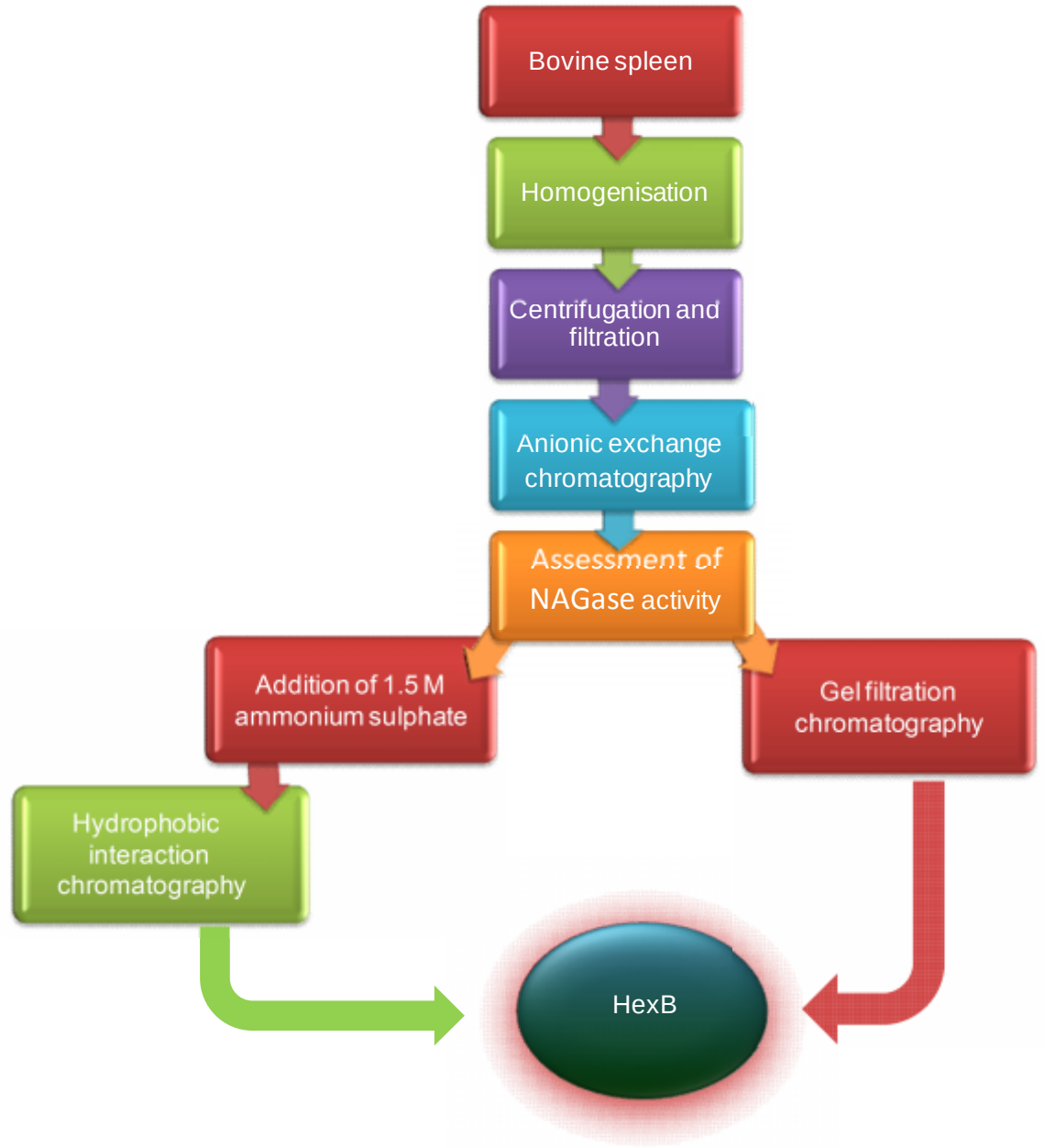


Figure 4.2. ***Schematic representation of HexB purification from bovine spleen.***
The protocol involved homogenisation of bovine spleen followed by centrifugation and filtration of the homogenate. The strategy to purify HexB encompassed two step chromatographic procedures, involving anion exchange chromatography followed by hydrophobic interaction chromatography or gel filtration chromatography. The latter approach was successful.

4.2.1 Optimisation of protocol for preparation of spleen homogenate

Bovine spleens, provided by Enfer Scientific Ltd., Ireland, were chopped into small pieces and homogenised in an equal amount (v/w) of buffer (20 mM Tris-HCl pH 7.4), in a Warring blender at 4^{o}C. The homogenate obtained was centrifuged at 18,500 g for 20 min and the supernatant was filtered through Whatmann filter paper 1, followed by filtration through a 0.45 µ filter. The centrifugation and filtration ensured that the homogenate was free of any solid debris that might block the tubing of the FPLC.

Buffer composition affects the activity of the enzyme (Jakoby, 1984). Evaluation of different buffers was carried out to establish the buffer which showed maximum HexB activity after homogenisation. Twenty millilitres of six different buffers were used to prepare spleen homogenates using the same amount of spleen tissue (20 g). The buffers used in the study were 20 mM Tris-HCl, 20 mM MOPS, 20 mM PIPES, 20 mM HEPES, 20 mM Triethanolamine (TEA) and 20 mM potassium phosphate buffer. All six buffers used had a pH of 7.4 which was close to the theoretical pI (7.2) of the HexB (Legler e*t al.*, 1991) and was within the range of their buffering capacity. Supernatants collected after preparing homogenates were assessed for hexosaminidase enzyme activity using a fluorimetric assay using 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide as substrate. Tris-HCl, TEA and potassium phosphate buffers gave comparable hexosaminidase activities. However, Tris-HCl was chosen for further use (Figure 4.3) due to advantages discussed in section 4.4.

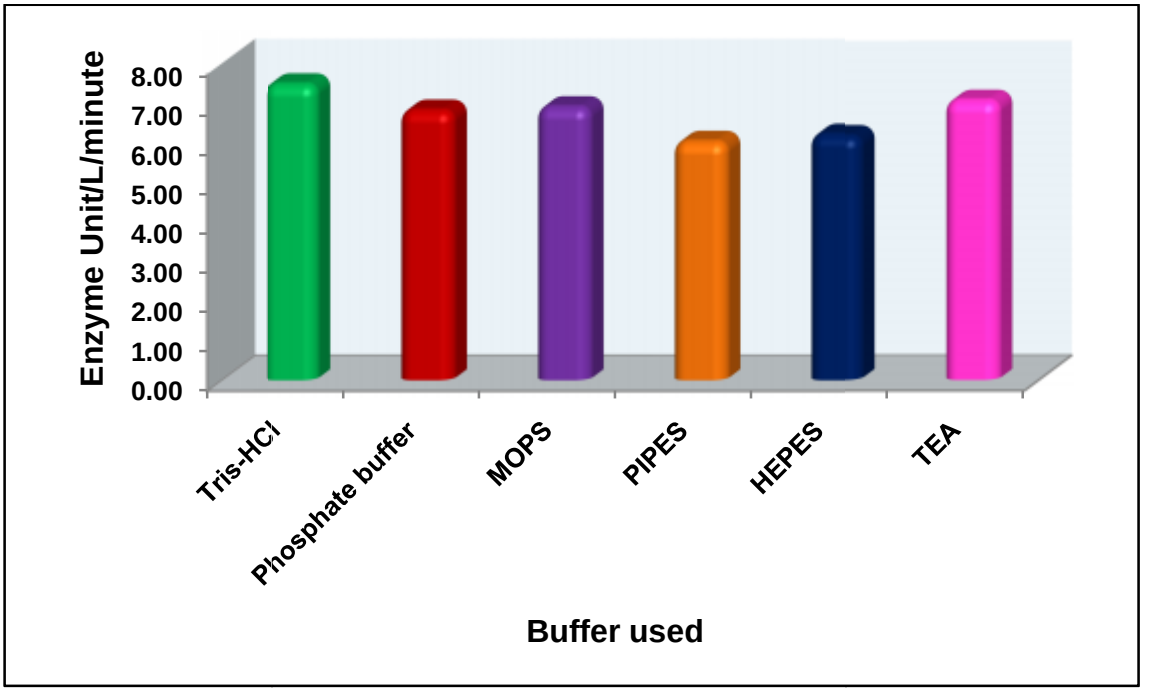


Figure 4.3. ***Evaluation of different buffers for maximum HexB enzymatic activity retention.***
Enzymatic activity of bovine hexosaminidase in the supernatants collected after homogenising spleen tissue in 6 different buffers. The substrate solution on its own was used as negative control. Tris-HCl was selected for futher studies. (All the samples were analysed in duplicate).

For anion exchange chromatography, the protein of interest should be negatively charged. The pI of HexB is 7.2 (Legler e*t al.*, 1991). Therefore, in order to make this protein negatively charged the pH of the buffer must be higher than the pI of the protein. Changes in pH affect the polar and non-polar intramolecular attractive and repulsive forces and alter the shape of the enzyme and the active site, thus, resulting in the substrate no longer fitting the active site (Jakoby, 1984). The enzymatic activity was, therefore, measured at different pHs above the pI of protein. Twenty millilitres of Tris-HCl buffer with seven different pHs ranging from 7.2 to 8.4, in increments of 0.2, were used to prepare spleen homogenates from 20 g tissue each, and enzymatic activity of each homogenate was measured. The pH range selected (7.2 to 8.4) was within the buffer range of Tris-HCl pH 7.0 to 9.0

(http://www.sigmaaldrich.com/life-science/core-bioreagents/biological-buffers/learning-center/buffer-reference-center.html). The enzyme activity was not significantly affected by changes in pH over the range tested (Figure 4.4). However, enzyme activity at pH 7.8 was marginally higher than enzyme activity at other pHs. Therefore, pH 7.8 was subsequently used for FPLC.

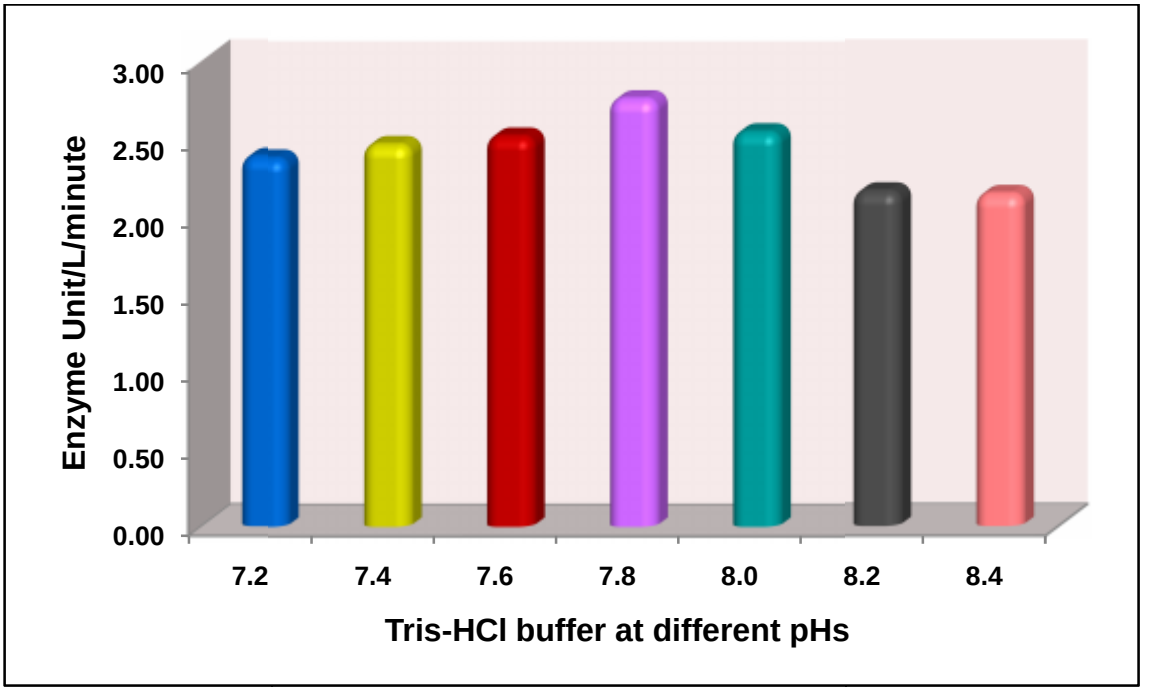


Figure 4.4. ***Evaluation of different pHs of Tris-HCl buffer for maximum HexB enzymatic activity retention.***
Enzymatic activity of bovine hexosaminidase in the supernatants collected after homogenising spleen tissue in tris-HCl at seven different pHs (7.2, 7.4, 7.6, 7.8, 8.0, 8.2, and 8.4). Substrate solution on its own was used as a negative control. (All the samples were analysed in duplicate).

4.2.2 Purification of HexB from spleen homogenate

4.2.2.1 Anion exchange chromatography

Ion exchange chromatography relies on electrostatic interactions between the charged amino acid side chains of proteins in the sample and the surface charge on the resin (Stanton, 2004). There are two general types of ion exchange chromatography, cation exchange chromatography, where positively

charged proteins bind to a negatively charged resin, and anion exchange chromatography, where the resin is positively charged and the proteins are negatively charged (Stanton, 2004). In this study, anion exchange chromatography using RESOURCE Q column was used.

The filtered supernatant sample was purified using FPLC (ÄKTA™ Explorer 100 with UV-900 monitor and Frac-950 Fraction Collector). Anion exchange chromatography was performed on a RESOURCE Q 1 mL column using 20 mM Tris-HCl, pH 7.8, (buffer A) and 20 mM Tris-HCl, pH 7.8, containing 500 mM NaCl (buffer B). Samples were eluted from the column, at a flow rate of 1 mL/min, using a gradient from 0 to 70% (v/v) buffer B and 1 mL fractions were collected. The NAGase enzymatic activity assay was performed on all fractions showing an absorbance at 280 nm. Two fractions showed high NAGase activity. A single peak was not observed (Figure 4.5). However, it was observed that NAGase was eluted at between 20%-40% (v/v) elution buffer gradient. Sometimes, the use of multi-step gradients results in better resolution of the proteins, using chromatographic applications (Johns *et al.*, 2009). Therefore, to obtain a single peak of HexB, which was being eluted between 20%-40% (v/v) elution buffer gradient, the protocol was modified to include a five step gradient, from 0 to 10% (v/v) buffer B with a hold at 20% (v/v) buffer B for 5 mL, a second step from 20%-30% (v/v) with a hold at 30% (v/v) for 5 mL, a third step from 30%-40% (v/v) with a hold at 40% for 5 mL 30%-40% (v/v), a fourth step from 40%-50% with a hold at 50% for 5 mL and a final step from 50%-100% (v/v) with a hold at 100% (v/v) for 10 mL. Fractions of 3 mL were collected. The gradient elution decreases the retention of the eluting components, making them elute faster, giving narrower peaks.

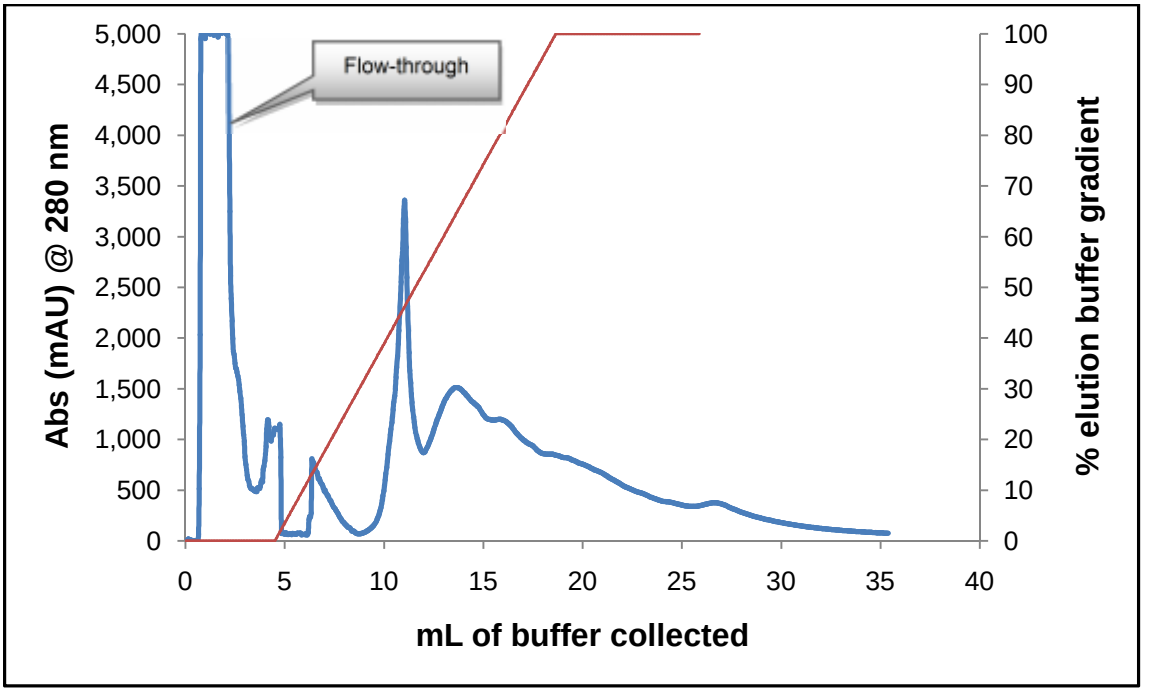


Figure 4.5. ***A typical chromatogram for HexB purification from filtered spleen homogenate using a linear gradient protocol on RESOURCE Q column on an ÄKTA™ Explorer 100 with a UV-900 monitor and a Frac-950 fraction collector.***
Ten mL filtered spleen homogenate was passed through the RESOURCE Q 1 mL column using 20 mM Tris-HCl, pH 7.8, (buffer A) for equilibration and 20 mM Tris-HCl, pH 7.8, containing 500 mM NaCl (buffer B) for elution of the enzyme from the column. A linear gradient was applied extending over 20 column volumes (CVs) with increasing NaCl concentration (0%-100% saturation) at a flow rate of 1 mL/min to elute the bound protein. One mL fractions were collected and fractions showing absorbance at 280 nm were analysed for NAGase. A clear single peak showing NAGase activity could not be indentified. (The blue line represents the protein and the red line represents the buffer gradient).

The NAGase enzymatic activity assay was performed on all fractions showing an absorbance at 280 nm. The HexB was contained in a distinct peak (Figure 4.6). Therefore, these conditions were used for anion exchange chromatography for the purification of HexB. However, the protein obtained after this step was not pure (Figure 3.23) and thus, another purification step was required.

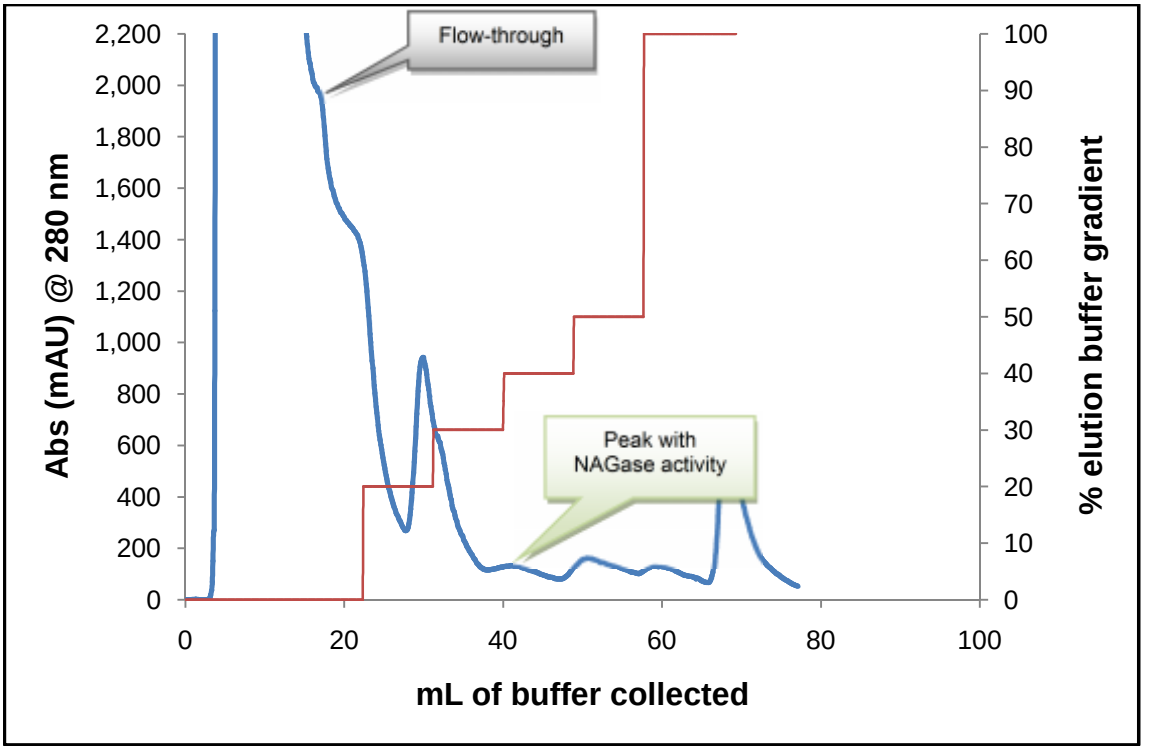


Figure 4.6. ***A typical chromatogram for HexB purification from filtered spleen homogenate using step gradient protocol on RESOURCE Q column on an ÄKTA™ Explorer 100 with a UV-900 monitor and a Frac-950 fraction collector.***

Ten mL filtered spleen homogenate was passed through the RESOURCE Q 1 mL column using 20 mM Tris-HCl, pH 7.8, (buffer A) for equilibration and 20 mM Tris-HCl, pH 7.8, containing 500 mM NaCl, (buffer B) for elution of the enzyme from the column. A step gradient was employed at a flow rate of 1 mL/min to elute the bound protein. One mL fractions were collected and fractions showing absorbance at 280 nm were analysed for NAGase activity. The peak with highest NAGase activity was indentified. (The blue line represents the protein and the red line represents the buffer gradient).

4.2.2.2 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) relies on the relative hydrophobicity for separation of peptides, proteins, and other biological molecules (Roettger and Ladisch, 1989; O'Farrell, 1996). A protein is composed of different amino acids which may be hydrophilic (water-attracting) or hydrophobic (water-repelling) and the variations in these amino acids give each protein its specific character (Pietzsch, 2002). The amino acids making up a protein interact differently in a given environment, and, thus, their binding affinities to different hydrophobic groups on the surface of a stationary matrix vary. HIC exploits these variations in interactions for separating out the protein of interest (O'Farrell, 1996). However, in a solution, hydrophobic groups on the stationary matrix and soluble proteins are shielded by water molecules and to expose these hydrophobic regions, water must be removed. On addition of lyotropic salts, such as high concentrations of ammonium sulphate, the hydrophobic regions of proteins are exposed (Wang and Ghosh, 2008; McCue, 2009). This promotes the binding of the proteins in solution to the resin by allowing the exposed regions to be adsorbed on the resin. This adsorbtion is then reversed by applying a descending salt gradient to elute the bound protein (Benedek, 2004).

Hydrophobic interaction chromatography (HIC) was performed to further purify HexB from the fractions obtained after anion exchange chromatography. HiTrap HIC selection kit (GE Healthcare, UK) consisting of seven hydrophobic interaction chromatography media with different hydrophobic characteristics, was used for this purpose. The seven different media contained in the kit were HiTrap Phenyl FF (high sub), HiTrap Phenyl FF (low sub), HiTrap Phenyl HP,

HiTrap Butyl FF, HiTrap Butyl-S FF, HiTrap Butyl HP, and HiTrap Octyl FF. All seven media were checked to select the best medium for purification of HexB. The protein of interest may pass through a certain column without any interaction and might bind strongly to another. The objective was to choose a column that releases the target protein somewhere in the middle of the salt gradient. If the protein elutes too early it means that the protein was loosely bound to the column matrix, and will not be successfully separated from other unwanted proteins in the sample. On the contrary, elution of the protein at the end of the gradient indicates a strong binding of protein to the column matrix which might prevent complete elution of the protein (http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/65E156E3A5FCB4B0C1257628001CCFDD/$file/18114321AC.pdf).

Anion exchange chromatography was carried out and 1.5 M ammonium sulphate was added to the NAGase fractions obtained. Ammonium sulphate, being a lyotropic salt, modulates the adsorption of proteins to HIC columns when added in high concentrations. The extract was then applied to different columns of HiTrap HIC selection kit. Hydrophobic interaction chromatography was performed using the buffers 20 mM Tris-HCl, pH 7.8, containing 1.6 M ammonium sulphate (buffer A) and 20 mM Tris-HCl, pH 7.8, containing 500 mM NaCl (buffer B). A linear gradient was applied extending over 200 mL with decreasing ammonium sulphate (50%-0 (v/v) saturation) at a flow rate of 1 mL/min to elute the bound protein. The NAGase assay was performed on all fractions showing an absorbance at 280 nm.

As evident in Figures 4.7, 4.8 and 4.9, HexB is eluted towards the end of gradient. Therefore, if these columns were used for purification, complete elution of the protein may not occur. No distinct protein peak is visible in Figure 4.10 and 4.11, indicating that the target protein may still be on the column and was not eluted. Figure 4.12 has a protein peak in the middle of the gradient, however, this peak has a large tail on the left hand side, indicating that other unwanted proteins are present. When the sample was analysed on the SDS-PAGE contaminating proteins were observed. Further attempts to reduce the broad range of this peak including reducing the flow rate, extending the gradient over more column volumes resulted in the same peak profile. The hydrophilicity of the NAGase and the contaminating proteins are similar. Purification using size exclusion was performed to further enhance purity.

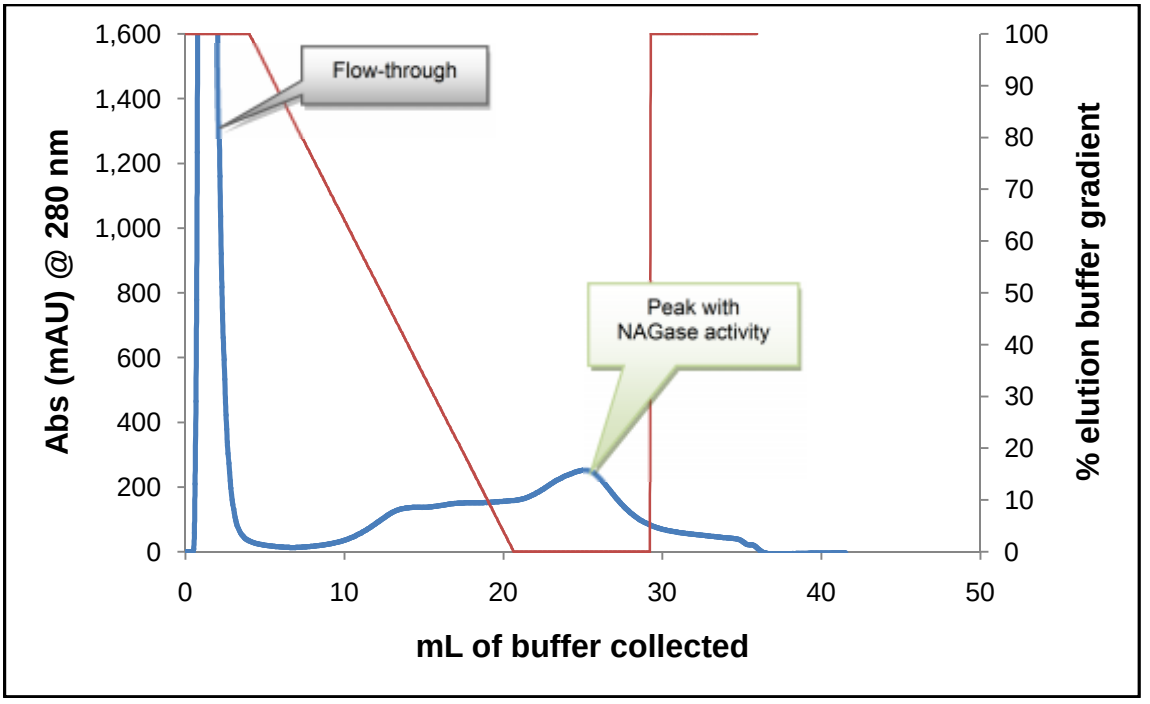


Figure 4.7. ***Chromatogram for second stage HexB purification using HiTrap Butyl FF column on an ÄKTA™ Explorer 100 with a UV-900 monitor and a Frac-950 fraction collector.***
One mL of pooled fraction (showing NAGAse activity) from the RESOURCE Q 1 mL column was passed through HiTrap Butyl FF 1 mL column, after addition of 1.5 M ammonium sulphate, using the buffers 20 mM Tris-HCl, pH 7.8, containing 1.6 M ammonium sulphate, (buffer A) and 20 mM Tris-HCl, pH 7.8, containing 500 mM NaCl, (buffer B). A linear gradient was applied extending over 20 CVs with decreasing ammonium sulphate (50%-0% saturation) at a flow rate of 1 mL/min to elute the bound protein. The NAGase assay was performed on all fractions showing an absorbance at 280 nm. The peak with highest NAGase activity was indentified. (The blue line represents the protein and the red line represents the buffer gradient).

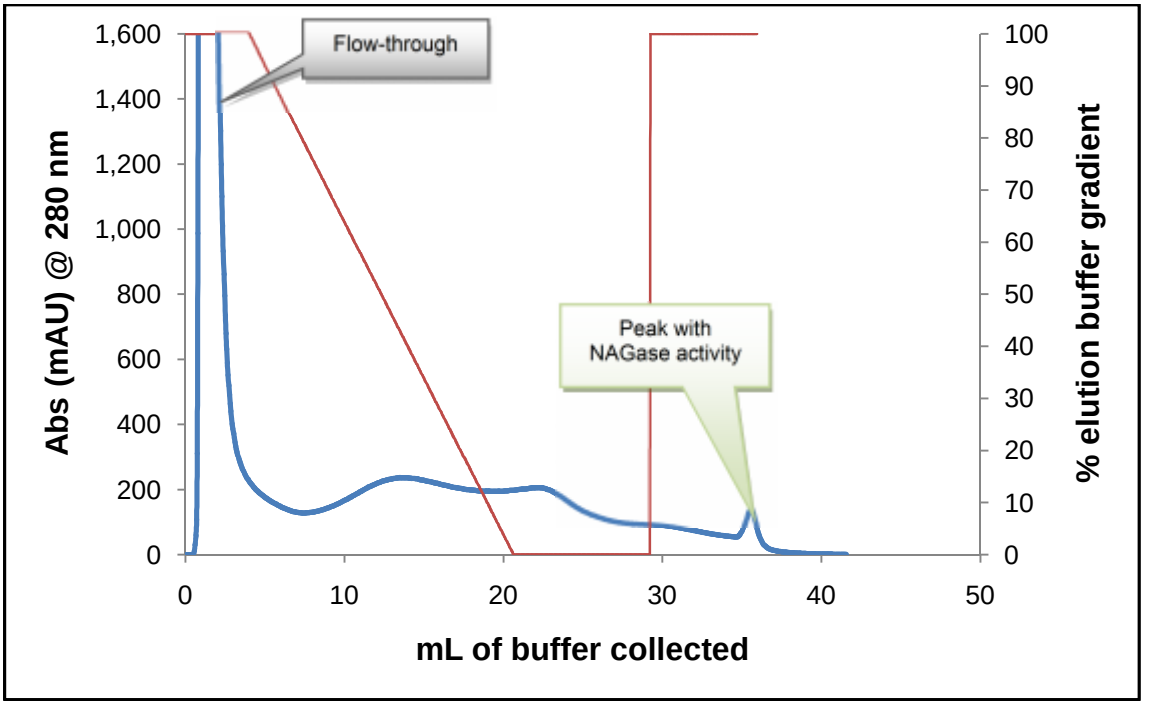


Figure 4.8. ***Chromatogram for second stage HexB purification using HiTrap Octyl FF column on an ÄKTA™ Explorer 100 with a UV-900 monitor and a Frac-950 fraction collector.***

One mL of pooled fraction (showing NAGAse activity) from the RESOURCE Q 1 mL column was passed through HiTrap Octyl FF 1 mL column, after addition of 1.5 M ammonium sulphate, using the buffers 20 mM Tris-HCl, pH 7.8, containing 1.6 M ammonium sulphate, (buffer A) and 20 mM Tris-HCl, pH 7.8, containing 500 mM NaCl, (buffer B). A linear gradient was applied extending over 20 CVs with decreasing ammonium sulphate (50%-0% saturation) at a flow rate of 1 mL/min to elute the bound protein. The NAGase assay was performed on all fractions showing an absorbance at 280 nm. The peak with highest NAGase activity was indentified. (The blue line represents the protein and the red line represents the buffer gradient).

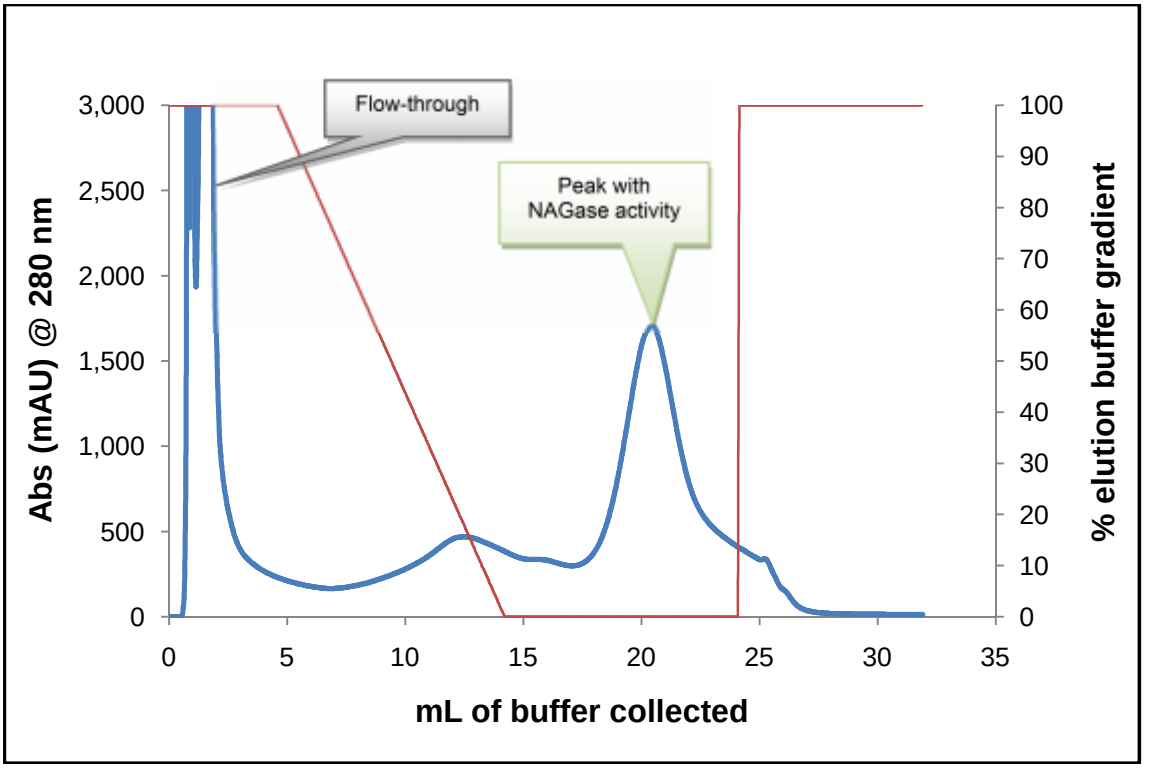


Figure 4.9. ***Chromatogram for second stage HexB purification using HiTrap Phenyl FF (high sub) column on an ÄKTA™ Explorer 100 with a UV-900 monitor and a Frac-950 fraction collector.***
One mL of pooled fraction (showing NAGAse activity) from the RESOURCE Q 1 mL column was passed through HiTrap Phenyl FF (high sub) 1 mL column, after addition of 1.5 M ammonium sulphate, using the buffers 20 mM Tris-HCl, pH 7.8, containing 1.6 M ammonium sulphate, (buffer A) and 20 mM Tris-HCl, pH 7.8, containing 500 mM NaCl, (buffer B). A linear gradient was applied extending over 20 CVs with decreasing ammonium sulphate (50%-0% saturation) at a flow rate of 1 mL/min to elute the bound protein. The NAGase assay was performed on all fractions showing an absorbance at 280 nm. The peak with highest NAGase activity was indentified. (The blue line represents the protein and the red line represents the buffer gradient).

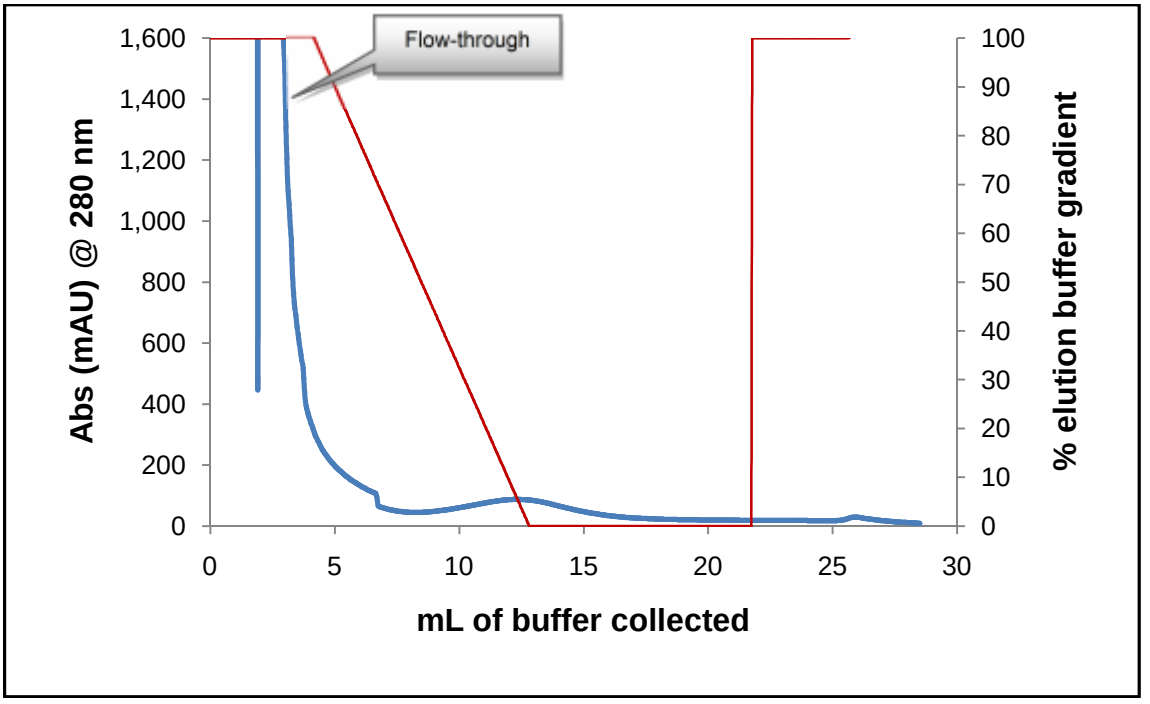


Figure 4.10. ***Chromatogram for second stage HexB purification using HiTrap Butyl-S FF column on an ÄKTA™ Explorer 100 with a UV-900 monitor and a Frac-950 fraction collector.***
One mL of pooled fraction (showing NAGAse activity) from the RESOURCE Q 1 mL column was passed through HiTrap Butyl-S FF 1 mL column, after addition of 1.5 M ammonium sulphate, using the buffers 20 mM Tris-HCl, pH 7.8, containing 1.6 M ammonium sulphate, (buffer A) and 20 mM Tris-HCl, pH 7.8, containing 500 mM NaCl, (buffer B). A linear gradient was applied extending over 20 CVs with decreasing ammonium sulphate (50%-0% saturation) at a flow rate of 1 mL/min to elute the bound protein. The NAGase assay was performed on all fractions showing an absorbance at 280 nm. No peak with NAGase activity was indentified. (The blue line represents the protein and the red line represents the buffer gradient).

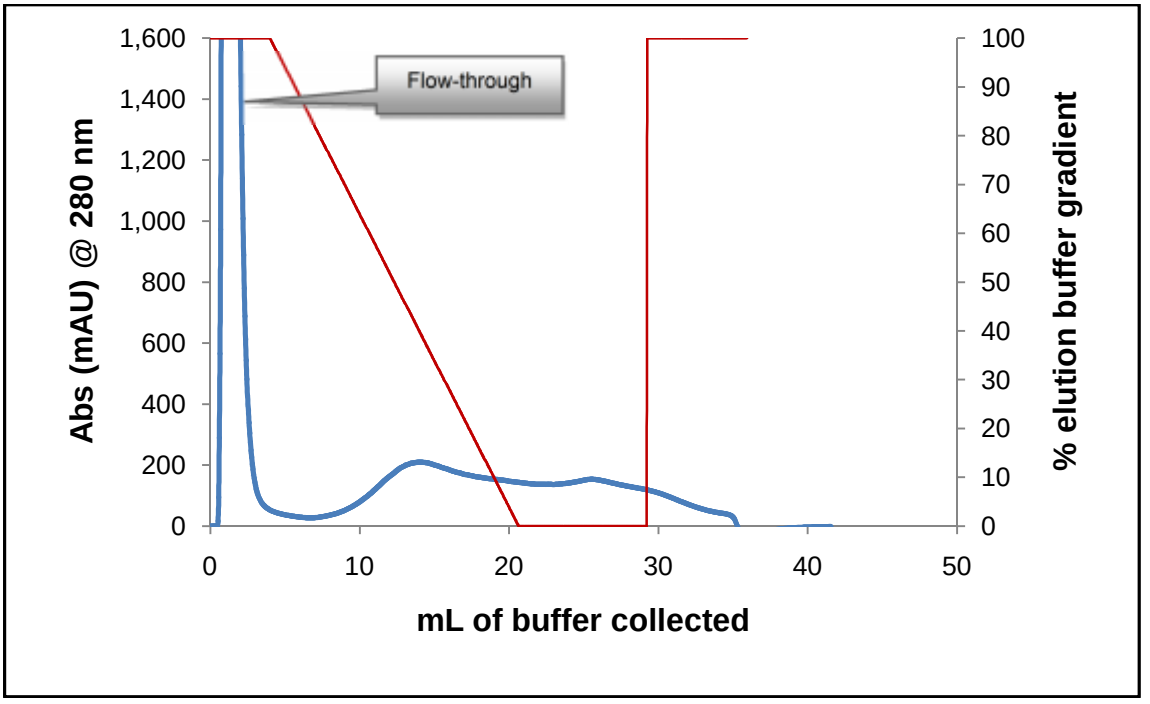


Figure 4.11. ***Chromatogram for second stage HexB purification using HiTrap Phenyl FF (low sub) column on an ÄKTA™ Explorer 100 with a UV-900 monitor and a Frac-950 fraction collector.***
One mL of pooled fraction (showing NAGAse activity) from the RESOURCE Q 1 mL column was passed through HiTrap Phenyl FF (low sub) 1 mL column, after addition of 1.5 M ammonium sulphate, using the buffers 20 mM Tris-HCl, pH 7.8, containing 1.6 M ammonium sulphate, (buffer A) and 20 mM Tris-HCl, pH 7.8, containing 500 mM NaCl, (buffer B). A linear gradient was applied extending over 20 CVs with decreasing ammonium sulphate (50%-0% saturation) at a flow rate of 1 mL/min to elute the bound protein. The NAGase assay was performed on all fractions showing an absorbance at 280 nm. No peak with NAGase activity was indentified. (The blue line represents the protein and the red line represents the buffer gradient).

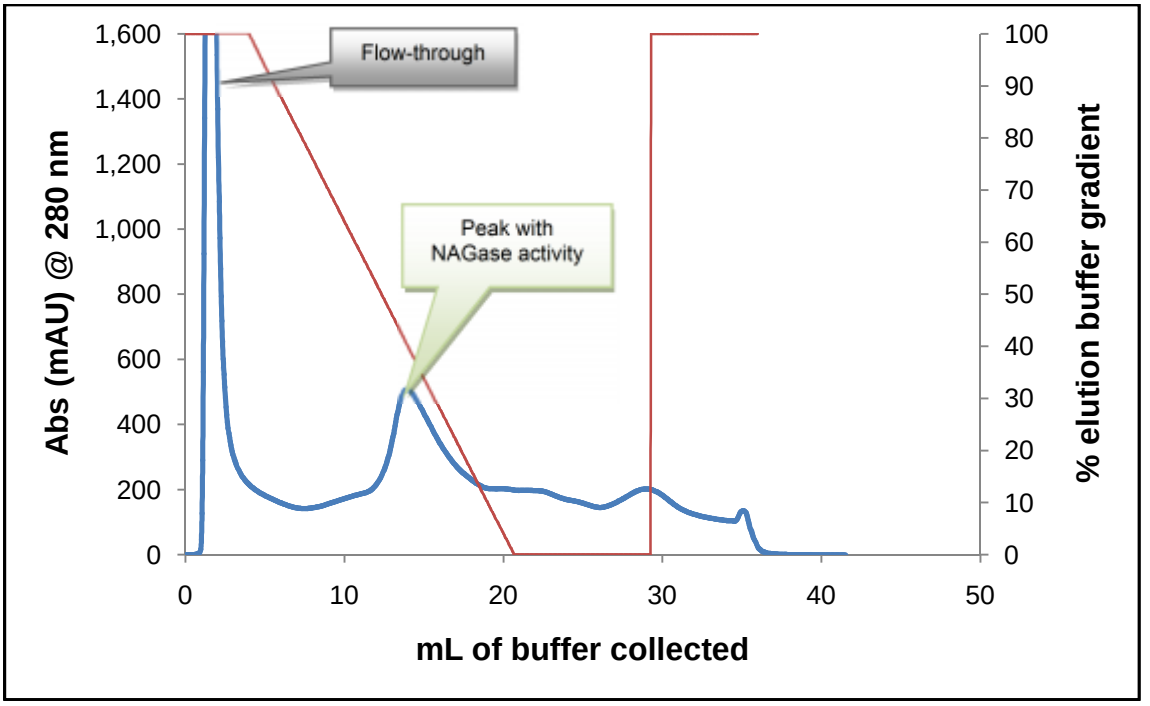


Figure 4.12. ***Chromatogram for second stage HexB purification using HiTrap Phenyl HP column on an ÄKTA™ Explorer 100 with a UV-900 monitor and a Frac-950 fraction collector.***
One mL of pooled fraction (showing NAGAse activity) from the RESOURCE Q 1 mL column was passed through HiTrap Phenyl HP 1 mL column, after addition of 1.5 M ammonium sulphate, using the buffers 20 mM Tris-HCl, pH 7.8, containing 1.6 M ammonium sulphate, (buffer A) and 20 mM Tris-HCl, pH 7.8, containing 500 mM NaCl, (buffer B). A linear gradient was applied extending over 20 CVs with decreasing ammonium sulphate (50%-0% saturation) at a flow rate of 1 mL/min to elute the bound protein. The NAGase assay was performed on all fractions showing an absorbance at 280 nm. The peak with highest NAGase activity was indentified. (The blue line represents the protein and the red line represents the buffer gradient).

4.2.2.3 Gel filtration chromatography

Gel filtration chromatography allows particles of different sizes to elute (filter) through at different rates. Thus, proteins are separated based on size (Bollag, 1994). The pooled NAGase fractions were applied to a HiLoad™ Superdex 200 preparation (prep) grade column and gel filtration chromatography was performed using phosphate buffered saline at a flow rate 0.5 mL/min for a duration of 700 min (Figure 4.13). Fractions of 5 mL were collected. The NAGase enzymatic activity assay was performed again on all fractions showing an absorbance at 280 nm. Two major enzyme-containing fractions, falling below

the NAGase peak and showing NAGase enzymatic activity, were analysed on SDS-PAGE, which showed the presence of a single band of HexB (Figure 4.14).

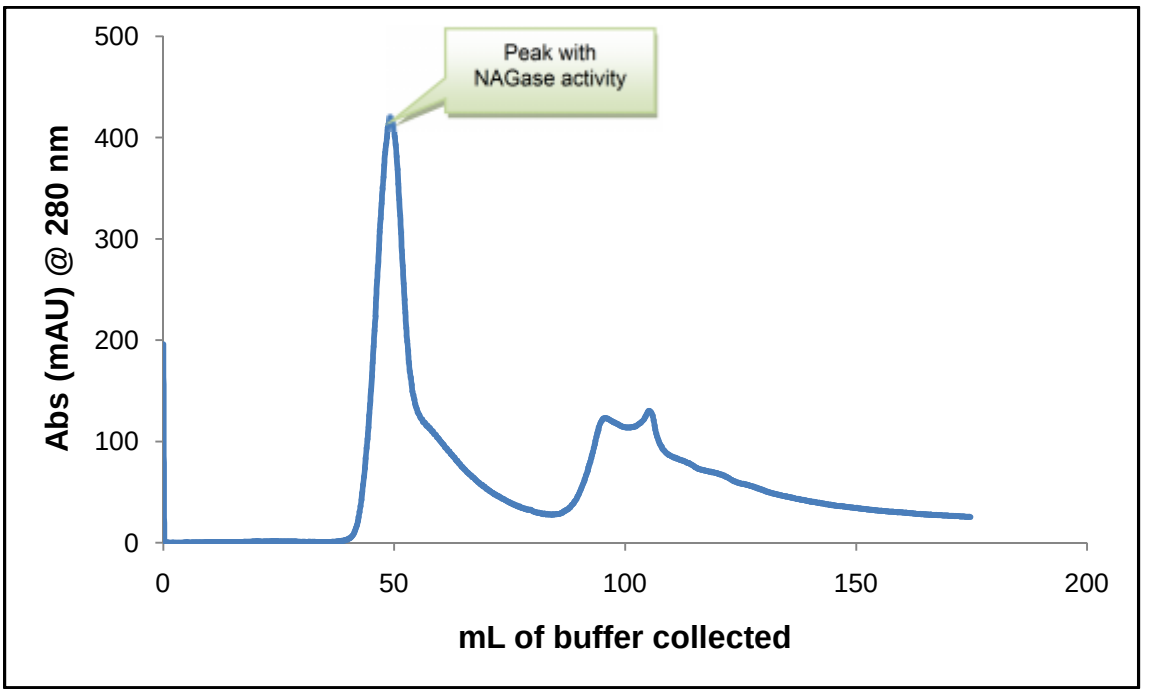


Figure 4.13. ***Chromatogram for second stage HexB purification using HiLoad™ Superdex 200 prep grade column on an ÄKTA™ Explorer 100 with a UV-900 monitor and a Frac-950 fraction collector.***
Ten mL of pooled fraction (showing NAGAse activity) from the RESOURCE Q 1 mL column were passed through HiLoad™ Superdex™ 200 prep grade 25 mL column and gel filtration chromatography was performed using 150 mM PBS, pH 7.2, at a flow rate 0.5 mL/min for a duration of 700 min. Fractions of 5 mL were collected and the NAGase enzymatic activity assay was performed on all fractions showing an absorbance at 280 nm. The peak with highest NAGase activity was indentified. (The blue line represents protein).

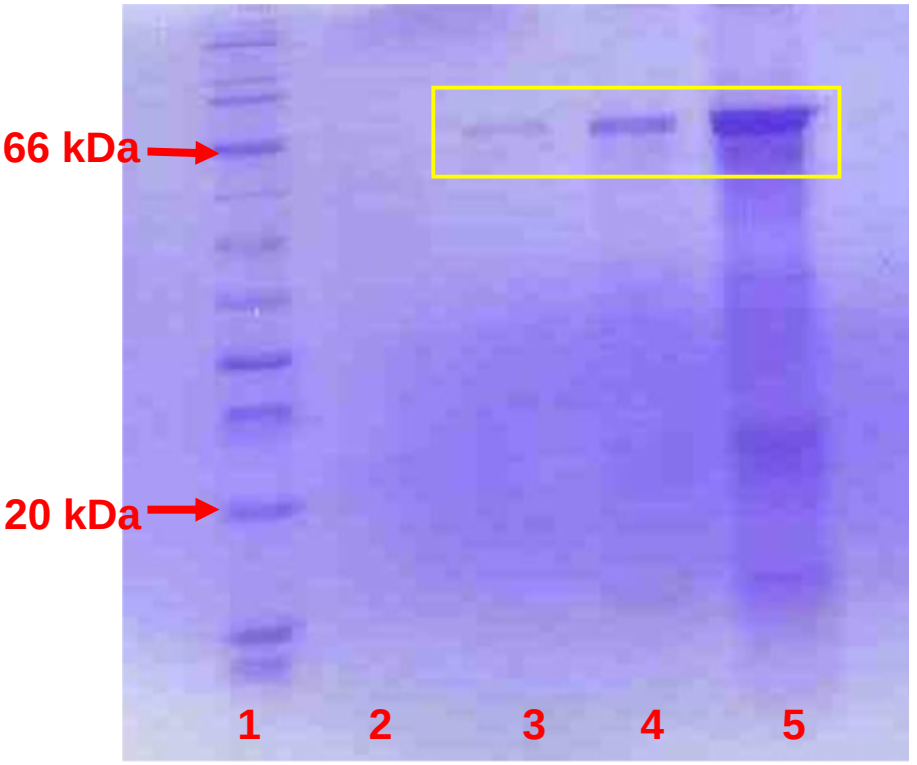


Figure 4.14. ***SDS-PAGE analysis of NAGase-containing fractions collected from RESOURCE Q and gel filtration columns.***
Lane 1: Sigma Wide Protein Marker; lanes 3 and 4: two different fractions (falling below the NAGase peak in Figure 4.13) collected after passing the extract from RESOURCE Q coloumn on to the gel filtration (HiLoadTM Superdex 200 prep grade) column; lane 5: the extract from RESOURCE Q column.

Table 4.1. ***Purification table of NAGase from bovine spleen.***

Purification step	Total Protein (mg)	Enzyme activity (U/L)	Specific activity (U/mg)	Purification factor
Crude extract	163	290.5	1.7	1.0
Anionic exchange	4.6	11.4	2.5	1.5
Gel filtration	0.4	8.8	21.9	12.9

A final purification factor of 12.9 was achieved (Table 4.1). The purification factor achieved in this study is low. Vehpoort (1972) also attempted purification of HexB from bovine (calf) spleen and received low yield (4.1%) of HexB. However, to achieve larger amounts of protein an alternative strategy involving cloning and expression of bovine HexA and HexB in *E. coli* was undertaken.

4.3 Expression of recombinant *Bos taurus* (cow) Hexosaminidase alpha polypeptide (HEXA) and Hexosaminidase beta polypeptide (HEXB)

4.3.1 Amplification of *Bos taurus* Hexosaminidase alpha polypeptide (HEXA)

Amplification of *Bos taurus* (cow) HEXA was attempted from bovine spleen sample. PCR optimisation for HEXA amplification was carried out using different annealing temperatures (Figure 4.15). The optimum annealing temperature was found to be 61.9^{o}C and was subsequently used as the annealing temperature in all further PCRs for HEXA amplifications.

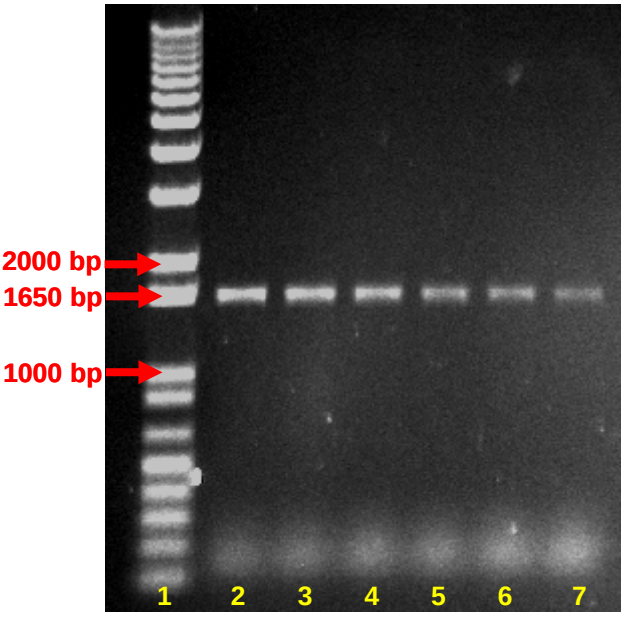


Figure 4.15. *Optimisation of annealing temperatures for amplification of* Bos taurus *HEXA.*
cDNA from the bovine spleen sample no. 4 was used as template for amplification of the HEXA (~1650 bp amplicon) gene. Six different annealing temperatures (55.3^{o}C, 56.8^{o}C, 57.9^{o}C, 59.2^{o}C, 60.5^{o}C and 61.9^{o}C) were tried to determine the optimal annealing temperature required for amplification of HEXA. Lane 1: 1 Kb plus DNA ladder; lanes 2-7: HEXA amplifications with decreasing annealing temperatures (61.9^{o}C, 60.5^{o}C, 59.2^{o}C, 57.9^{o}C, 56.8^{o}C, and 55.3^{o}C, respectively).

Further optimisation of PCR was carried out by varying the $MgCl_2$ concentrations (1 mM to 4 mM), using the annealing temperature at 61.9^{o}C. A concentration of 3 mM $MgCl_2$ concentration was found to be optimum and was used for subsequent reactions (Figure 4.16).

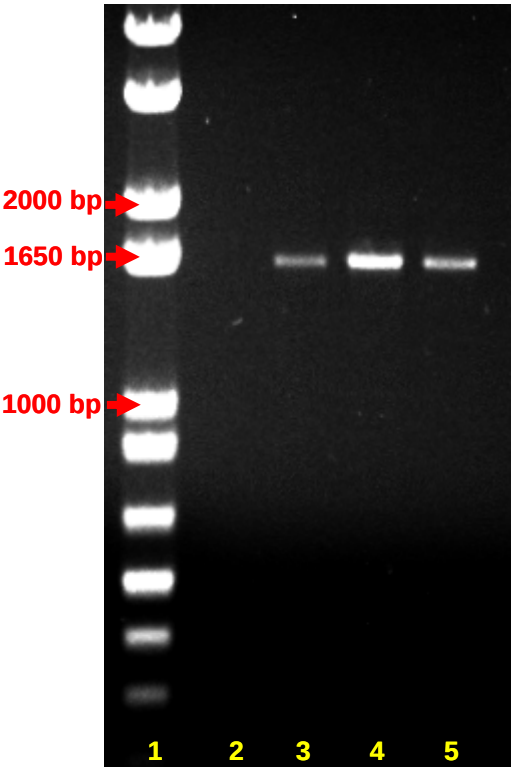


Figure 4.16. *Optimisation of Mg^{2+} ion concentrations for amplification of* Bos taurus *HEXA.*
Four different $MgCl_2$ concentrations (1 to 4 mM) were used to assess optimal $MgCl_2$ concentration required for amplification of the HEXA gene. Lane 1: 1 Kb plus DNA ladder; lanes 2-5: amplifications of HEXA gene with increasing $MgCl_2$ concentrations (1 to 4 mM in 1 mM increments).

4.3.2 Cloning of *Bos taurus* Hexosaminidase A (alpha polypeptide)

Six 50 µL PCR reactions for HEXA gene amplification were performed. The amplicons were ethanol-precipitated and quantified using the Nanodrop ND-1000™. The HEXA gene was then cloned into the pET-32b(+) vector following *Bam*HI and *Not*I digest. The digested HEXA amplicon (140 ng) was ligated with 100 ng of digested pET-32b(+). The ligated products were transformed into BL21-CodonPlus (DE3)-RIPL competent cells by heat shock. The

transformation mix was grown in SOC media for 1 h and plated on LB-carbenicillin plates.

Seven clones were picked randomly and analysed for HEXA expression by Western blotting using commercial monoclonal anti-polyhistidine-peroxidase-labelled antibody for detection. The size of bovine HEXA protein was estimated, using ProtParam tool (available at http://au.expasy.org/tools/protparam.html), to be approximately 60.5 kDa. The pET-32b(+) vector has a N-terminal fusion protein consisting of a Trx-Tag™, His-Tag, an enterokinase cleavage site, S-Tag and a thrombin cleavage site. The vector also has a C-terminal His-tag. These fusion proteins translate into a protein of approximately 8-10 kDa. Thus, the expected size of HEXA, along with fusion protein, on western blot, was approximately 70 kDa. Five clones, out of 7 checked, were positive (Figure 4.17). The remaining lysates were stored at 4^{o}C.

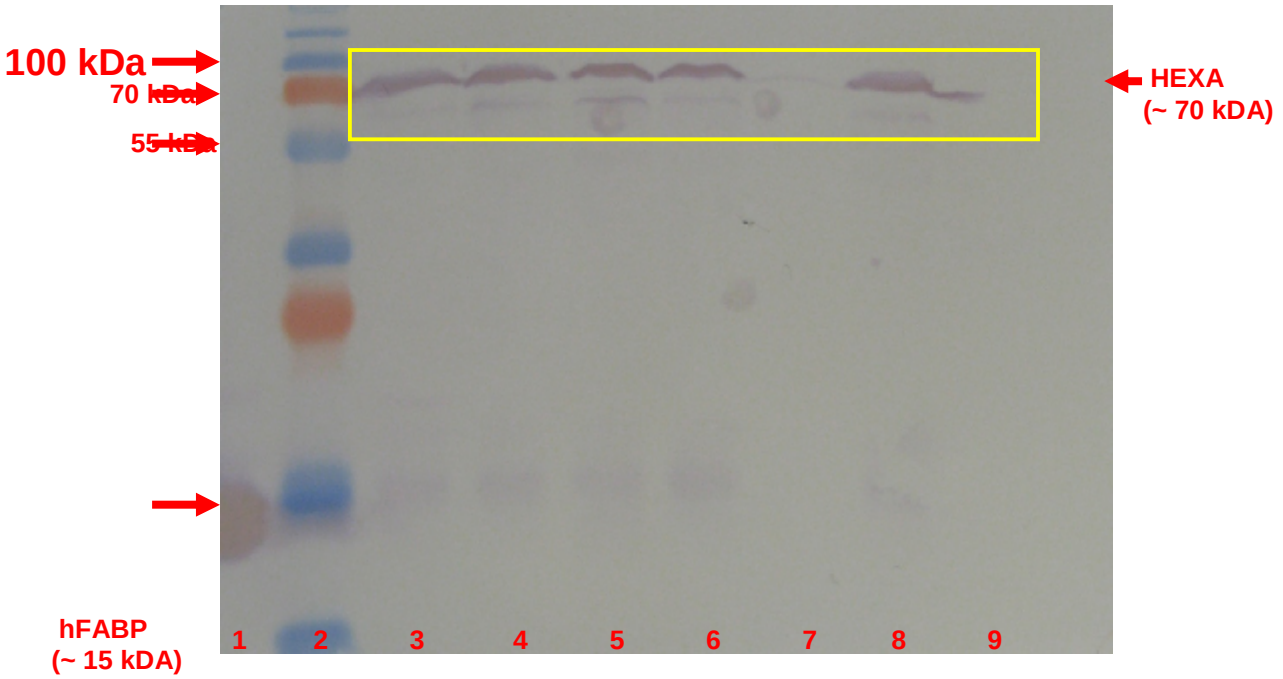


Figure 4.17. ***Screening of clones for HEXA expression by Western blotting.***
Seven random clones were picked. The presence of HEXA was determined by Western blotting, for HEXA expression. Lane 1: negative control (heart-fatty acid binding protein (hFABP, ~ 15 kDa) clone in the pET-26b(+) vector); lane 2: PageRuler™ Plus Prestained Protein Ladder; lanes 3-9: different HEXA clones. Lanes 3 to 6 and 8 show positive bands.

Experiments were carried out to estimate the optimal time required for HEXA expression. Samples were collected after 1, 2, 3, 4 h and overnight incubation of induced cultures and checked by western blot analysis using commercial mouse monoclonal anti-polyHistidine-peroxidase-labelled antibodyfor detection. A four hour incubation following induction was found to give optimal expression of HEXA (Figure 4.18).

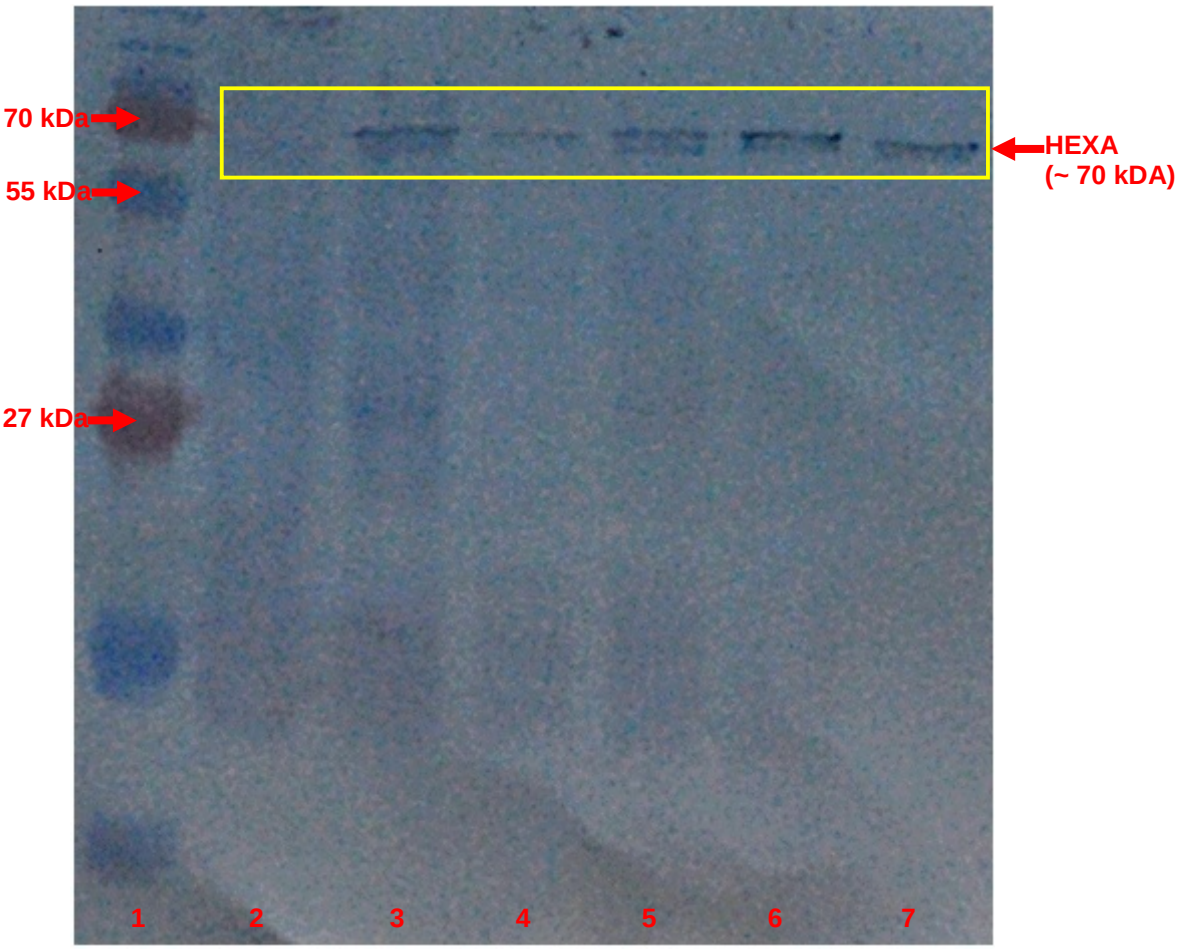


Figure 4.18. ***Time-course for optimal HEXA expression.***
Samples following 1, 2, 3, 4 h and overnight incubation, of induced HEXA cultures, were analysed by Western blotting. Lane 1: PageRuler™ Plus Prestained Protein Ladder; lane 2: uninduced control; lanes 3-7: expression levels obtained after 1, 2, 3, 4 h and overnight incubations, respectively.

4.3.3 Purification of recombinant HEXA

Purification of α-subunit was performed using immobilised metal affinity chromatography (IMAC). The purified protein was buffer exchanged to resuspend the protein in PBS. Filtered crude lysate, 'flow-through', washings and purified protein were analysed on a SDS-PAGE gel (Figure 4.19). The purified fraction revealed a band at approximately 70 kDa representing recombinant HEXA fused to N-terminal and C-terminal fusion proteins, and a band at approximately 61 kDa representing recombinant HEXA expressed along with C-terminal His-tag. Several impurities represented by a band at

approximately 25 kDa and few (minor) bands in between 10 and 15 kDa were also observed. There are few different methionine (ATG) residues in the vector sequence, and thus, possibly these band are products of partial translations. These bands were evident even after introducing urea and imidazole in the IMAC protocol.

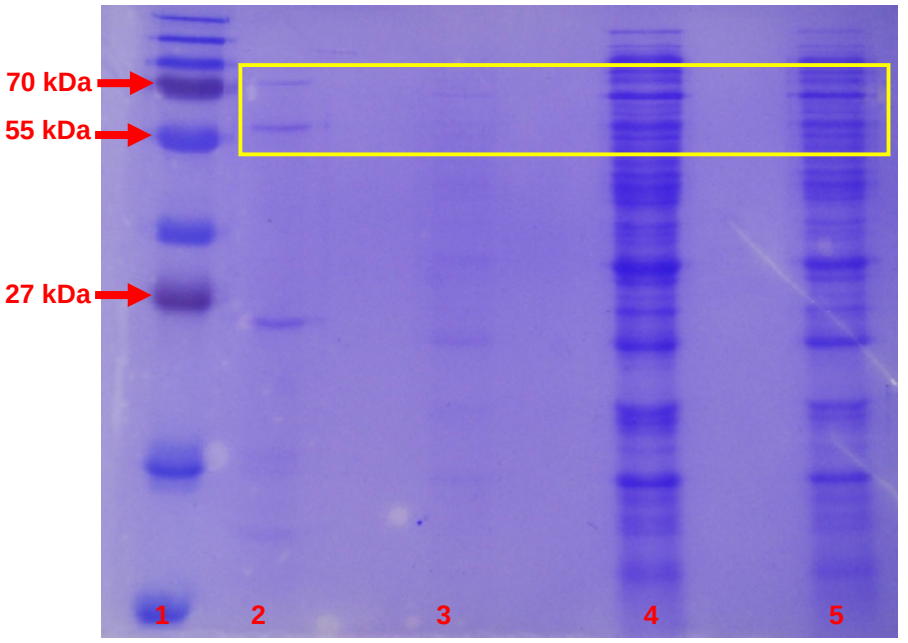


Figure 4.19. ***SDS-PAGE analysis of HEXA purification using IMAC.***
Lane 1: PageRuler™Plus Prestained Protein Ladder; lane 2: purified HEXA; lane 3: pooled washings collected during the IMAC purification procedure; lane 4: flow-through obtained after passing the lysate through IMAC; lane 5: filtered crude lysate. The purified fraction revealed HEXA bands at 70 kDa (with N- and C-terminal fusion proteins) and 61 kDa (with C-terminal His-tag). In addition, there was a non-specific band at approximately 25 kDa and few non-specific (minor) bands in between 10 and 15 kDa.

4.3.4 Enterokinase treatment of purified recombinant HEXA

Further studies were undertaken to cleave-off the N-terminal fusion protein from the expressed protein, in order to obtain pure recombinant HexA without the tag which can be used to isolate HEXA-specific antibodies. The IMAC-purified protein was treated with different concentrations of enterokinase (EK) enzyme for different periods of time to optimise both the concentration of enzyme and

duration to cleave-off the N-terminal fusion protein. Small scale reactions were carried out which were later scaled-up. No significant difference was observed between different enterokinase enzyme concentrations and time courses. Complete cleavage of N-terminal fusion protein could not be achieved using the enterokinase concentrations tested. Higher concentrations of enterokinase were not used as the enzyme was costly and use of higher concentration of the enzyme for large scale purification was not cost effective. Following enterokinase cleavage reaction, rEK was removed with EKapture agarose and analysed by SDS-PAGE (Figure 4.22). The EKapture agarose was effective in removing residual enterokinase (Figure 4.22).

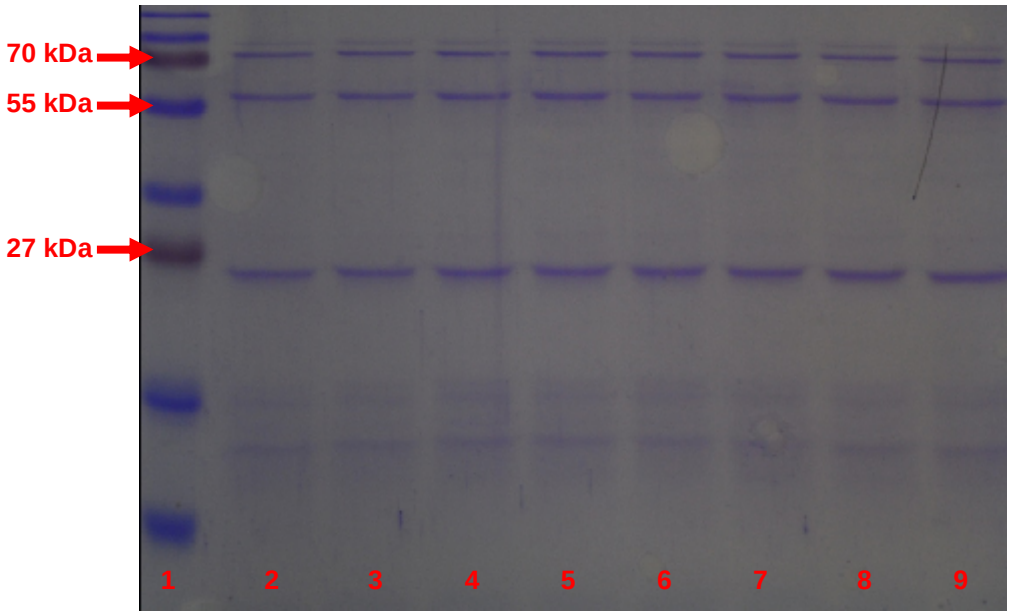


Figure 4.20. ***SDS-PAGE analysis of enterokinase-treated IMAC-purified HEXA.***
Lane 1: PageRuler™ plus Prestained Protein Ladder; lanes 2-5: purified protein control and purified protein treated with 0.1, 0.2 and 0.5 U/µL EK, respectively, after 2 h incubation; lanes 6-9: purified protein control and purified protein treated with 0.1, 0.2 and 0.5 U/µL EK, respectively, after 4 h incubation.

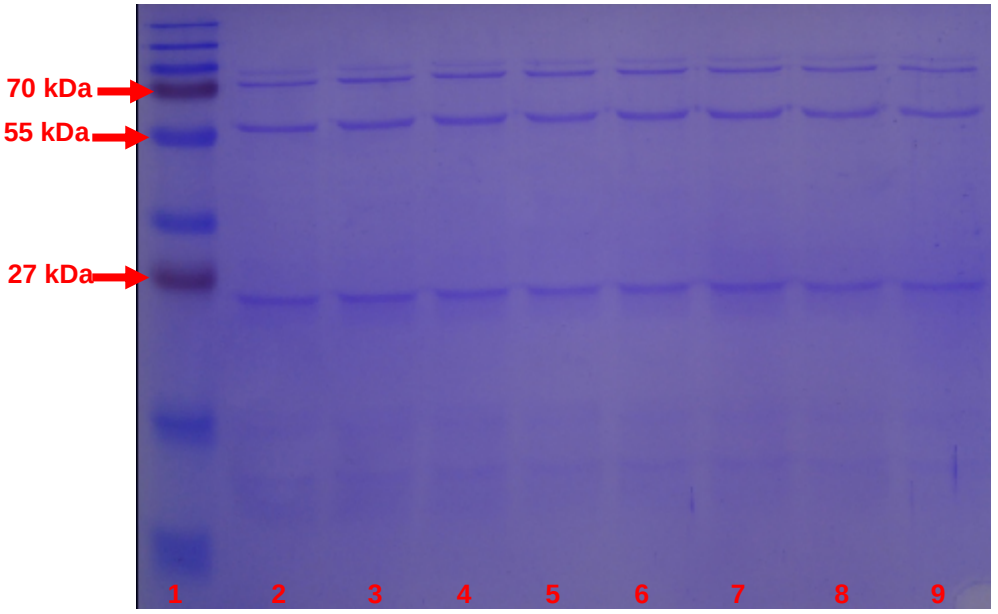


Figure 4.21. ***SDS-PAGE analysis of enterokinase-treated IMAC-purified HEXA.***
Lane 1: PageRuler™ plus Prestained Protein Ladder; lanes 2-5: purified protein control and purified protein treated with 0.1, 0.2 and 0.5 U/µL EK, respectively, after 8 h incubation; lanes 6-9: purified protein control and purified protein treated with 0.1, 0.2 and 0.5 U/µL EK, respectively, after 16 h incubation.

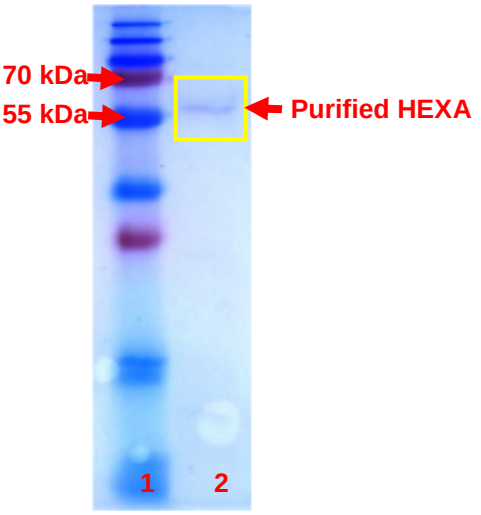


Figure 4.22. ***SDS-PAGE analysis of enterokinase-treated IMAC-purified HEXA following EKapture agarose purification.***
Lane 1: PageRuler™ plus Prestained Protein Ladder; lane 2: IMAC-purified HEXA following enterokinase-treatment and EKapture agarose purification. A band of approximately 55 kDa of purified HEXA was observed. (IMAC-purified HEXA and IMAC-purified HEXA following enterokinase-treatment are shown in Figure 4.19, 4.20 and 4.21.)

4.3.5 Amplification of *Bos taurus* Hexosaminidase beta polypeptide (HEXB)

Amplification of *Bos taurus* hexosaminidase beta polypeptide (HEXB) was attempted from a bovine spleen sample. Optimisation of PCR reaction for HEXB amplification was carried out using different annealing temperatures (Figure 4.23). The optimum annealing temperature was found to be 64.8^{o}C and was subsequently used as the optimal annealing temperature in all further PCRs for HEXB amplifications. This temperature was chosen as it provided a cleaner amplification without any non-specific bands (Figure 4.23). Further PCR optimisation was carried out by varying the $MgCl_2$ concentrations (1 to 5 mM) and using the optimised annealing temperature. A concentration of 5 mM $MgCl_2$ was optimal and was used for subsequent reactions (Figure 4.24).

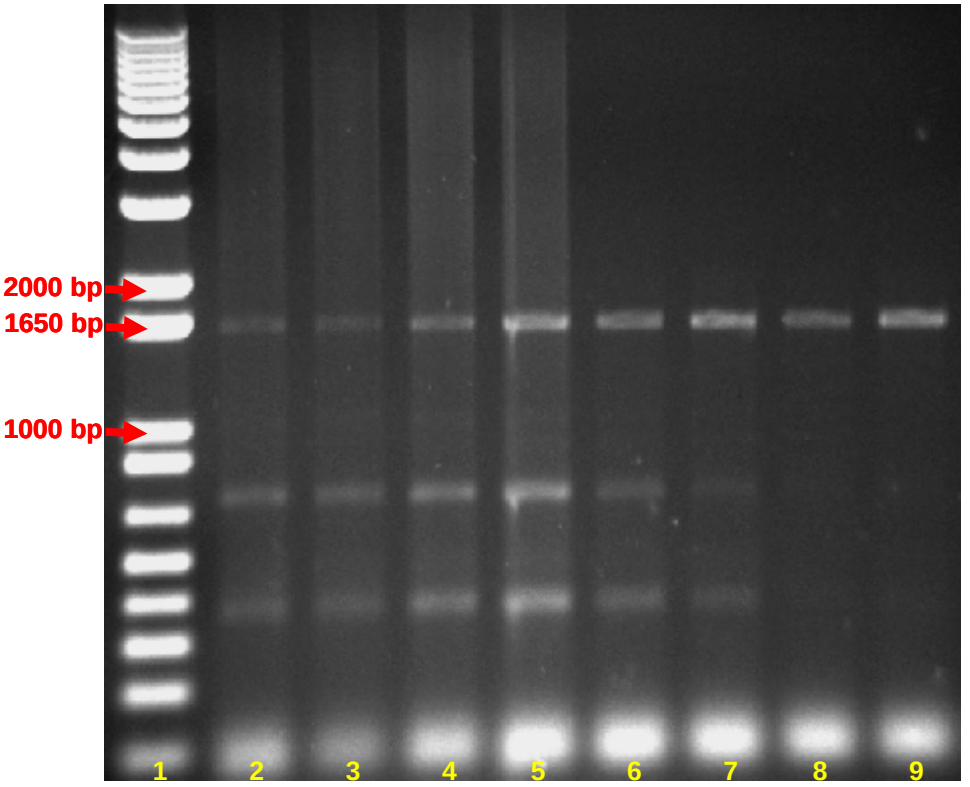


Figure 4.23. ***Optimisation of annealing temperature for amplification of*** **Bos taurus** ***HEXB.***

cDNA from the bovine spleen sample no. 4 was used as template for amplification of the HEXB (~1610 bp amplicon) gene. Eight different annealing temperatures (55.3^{o}C, 56.8^{o}C, 57.9^{o}C, 59.2^{o}C, 60.5^{o}C, 61.9^{o}C, 63.4^{o}C, and 64.8^{o}C) were tried to assess optimal annealing temperature required for amplification of HEXB gene. Lane 1: 1 Kb plus DNA ladder; lanes 2-9: HEXB amplifications with increasing annealing temperatures (55.3^{o}C, 56.8^{o}C, 57.9^{o}C, 59.2^{o}C, 60.5^{o}C, 61.9^{o}C, 63.4^{o}C, and 64.8^{o}C, respectively).

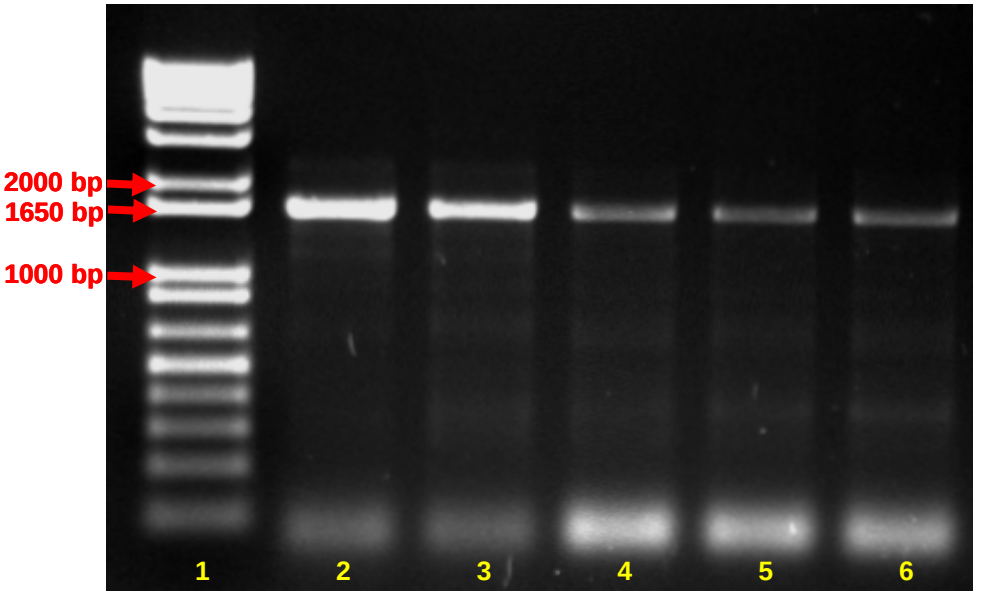


Figure 4.24. ***Optimisation of Mg^{2+} ion concentrations for amplification of* Bos taurus *HEXB.***
Five different $MgCl_2$ concentrations (1 to 5 mM) were used to assess optimal $MgCl_2$ concentration required for amplification of HEXB gene. Lane 1: 1 Kb plus DNA ladder; lanes 2-6: amplifications of HEXB gene with increasing $MgCl_2$ concentrations (5 to 1 mM in 1 mM decrements).

4.3.6 Cloning of *Bos taurus* Hexosaminidase B (beta polypeptide)

Six 50 µL PCR reactions for HEXB gene amplification were performed. The amplicons were ethanol-precipitated and quantified using the Nanodrop ND-1000™. The HEXB gene was then cloned into the pET-32b(+) vector following *Bam*HI and *Not*I digestion. The digested HEXB amplicon (140 ng) was ligated with 100 ng of digested pET-32b(+). The ligated products were transformed into BL21-CodonPlus (DE3)-RIPL competent cells by heat shock. The transformation mix was grown in SOC medium for 1 h and plated on LB-carbenicillin plates.

Seven clones were picked randomly and analysed for HEXB expression by Western blotting using commercial monoclonal anti-polyhistidine-peroxidase-labelled antibody for detection. The size of HEXB protein was estimated, using ProtParam tool (available at http://au.expasy.org/tools/protparam.html), to be

approximately 59 kDa. The vector fusion protein translates in to a protein of approximately 8-10 kDa. Thus, the expected size of HEXB, along with fusion protein, on Western blot, was approximately 69 kDa. Three clones, out of 7 checked, were positive (Figure 4.25).

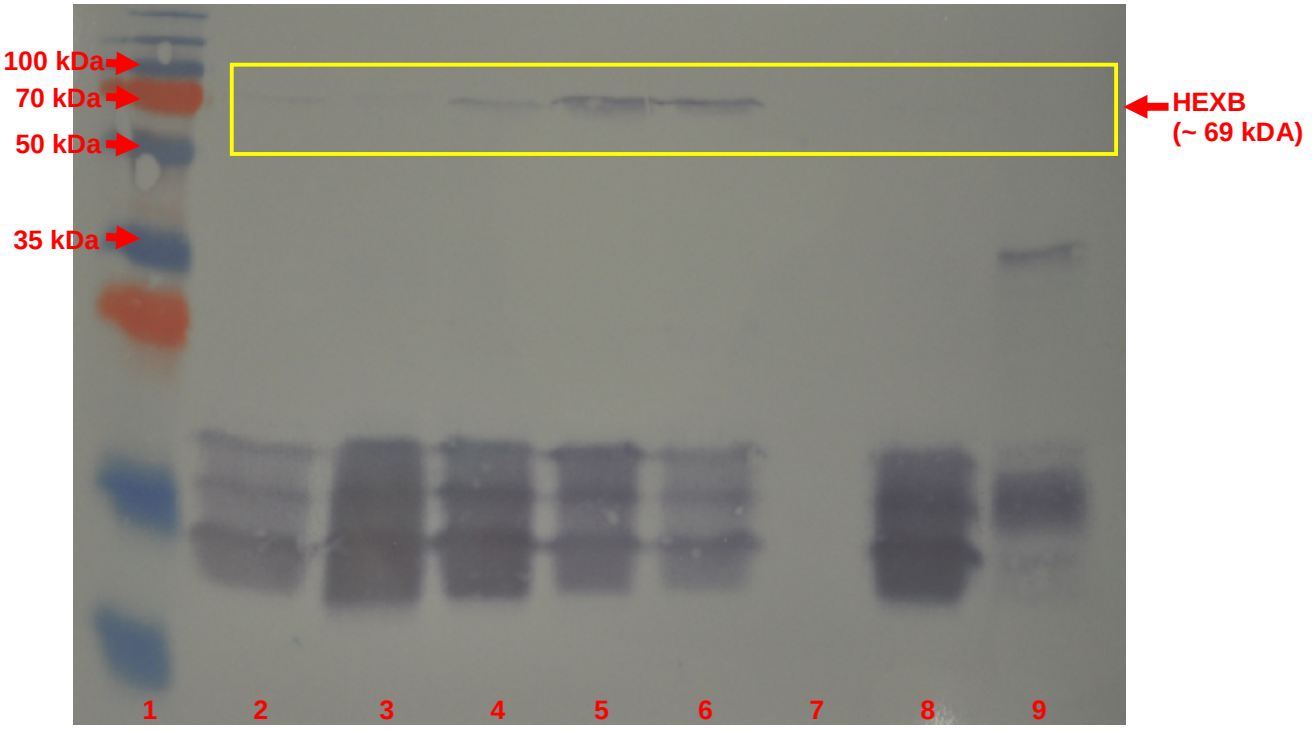


Figure 4.25. ***Screening of clones for HEXB expression by Western blotting.***
Seven random clones were picked and checked, by Western blotting, for HEXB expression. Lane 1: PageRuler™ Plus Prestained Protein Ladder; lanes 2-8: different HEXB clones; lane 9: negative control (cardiac troponin I clone in pET 32b(+) vector). Lanes 4 to 6 show positive bands. Lanes 2, 3, 7 and 8 show clones that were negative for HEXB.

Experiments were carried out to estimate the optimal time required for HEXB expression. Samples were collected after 1, 2, 3, 4 h and overnight incubation of induced cultures and checked by Western blot analysis using commercial mouse monoclonal anti-polyhistidine-peroxidase-labelled antibody for detection (Figure 4.26). A four hour incubation following induction was found to give optimal expression of HEXB. Non-specific proteins were also expressed after overnight incubation.

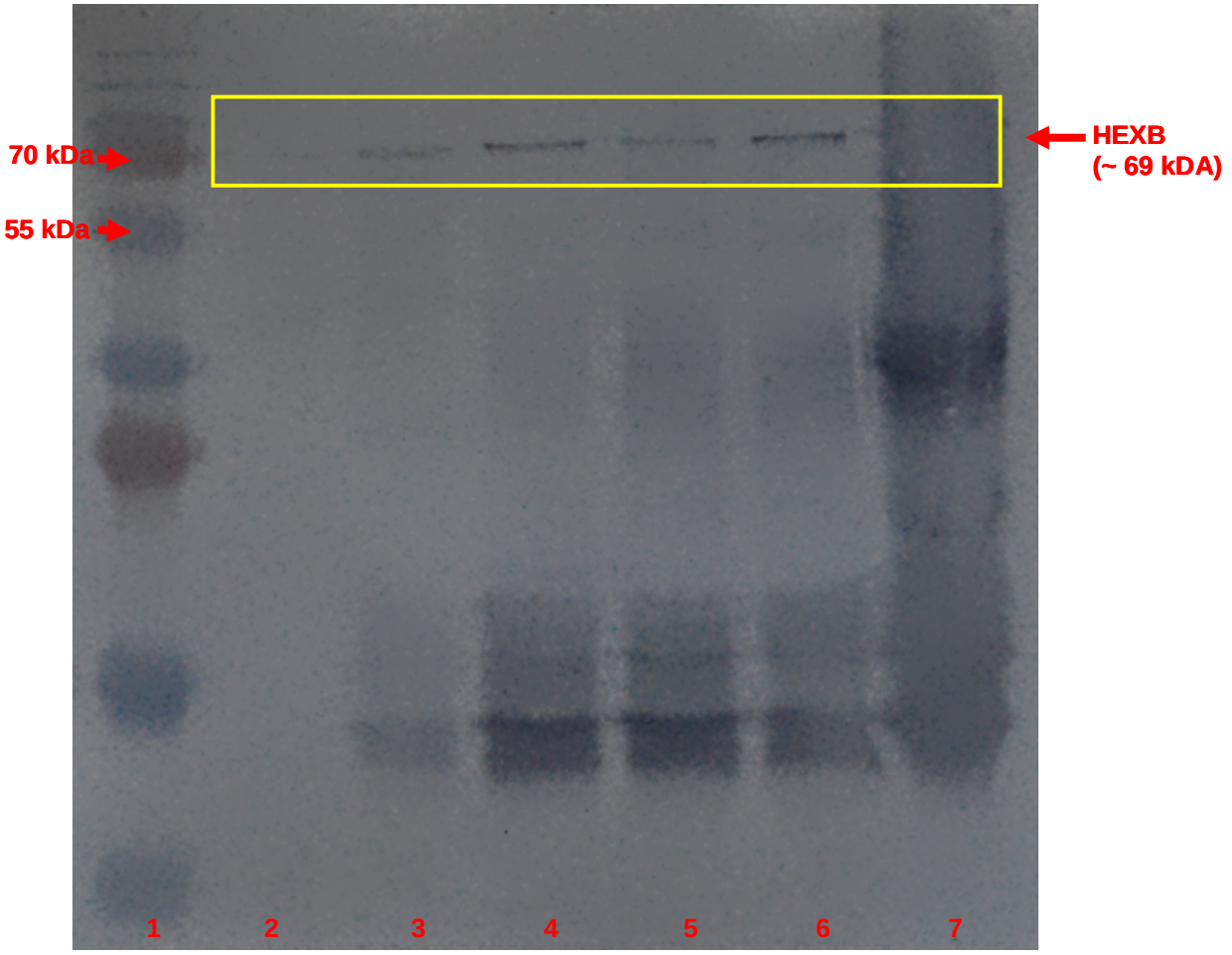


Figure 4.26. ***Time-course for optimal HEXB expression.***
Samples following 1, 2, 3, 4 h and overnight incubation, of induced HEXB cultures, were analysed by western blotting. Lane 1: PageRuler™ Plus Prestained Protein Ladder; lane 2: uninduced control; lanes 3-7: expression levels obtained after 1, 2, 3, 4 h and overnight incubations, respectively.

4.4 Discussion

Efforts were made to generate a method for 'in-house' purification of bovine HexA from bovine spleen tissue using FPLC. Previously it was reported that NAGase was purified from various bovine tissues including brain, spleen, epididymis, sperm, testes, mammary gland and kidney (Frohwein and Gatt, 1967; Verpoorte, 1972; Pokorny and Glaudemans, 1975; Khar and Anand, 1977; Sarber *et al*., 1978; Kitchen and Masters, 1985; Legler *et al*., 1991). A protocol for purification of HexB from bovine spleen tissue using FPLC was standardised (Section 4.2).

A 3-phase strategy is recommended for devising a protein purification protocol (http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/DC18E4B0B709603BC1257628001CCE3E/$file/18113229AD.pdf). This includes a protein capture phase for isolation, concentration and stabilisation of the desired protein, followed by an intermediate phase for removing most of the impurities and, finally, a polishing phase to remove the traces of residual impurities (http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/DC18E4B0B709603BC1257628001CCE3E/$file/18113229AD.pdf). These 3 phases are not always distinguishable and can overlap each other (http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/DC18E4B0B709603BC1257628001CCE3E/$file/18113229AD.pdf). Ion exchange chromatography (IEC) allows both concentration and purification of the desired protein (Jungbauer and Hahn, 2009). Therefore, in the current study, it sufficed for the first two phases of protein purification. Other reasons for choosing IEC included its simplicity, versatility, high-resolving and binding capacities (Jungbauer and Machold, 2004; Jungbauer and Hahn, 2009). In addition, numerous buffer systems are available for IEC and the biological activity of the protein is mostly preserved (Jungbauer and Machold, 2004).

Cation exchangers attract hydronium ions (H_3O^+) towards them and anionic exchangers attract hydroxyl ions (OH^-) towards them. As a result, the pH near the cation exchange matrix can be up to one pH unit lower than the buffer and similarly, it can be up to one pH unit higher than surrounding buffer in an anion exchange matrix (Scopes, 1994; Jungbauer and Machold, 2004; Jungbauer and Hahn, 2009). Usually, enzymes are more stable at mild alkaline pHs (*e.g.* pH 8-10), than in mild acidic pHs (*e.g.* pH 4-6) (Scopes, 1994). Consequently, the

use of cation exchange for enzyme purification might cause enzyme denaturation due to high acidic pH in the matrix. Anion exchange chromatography was reported to be successfully applied for purification of HexB from different sources (Sankaranarayanan *et al.*, 1987; Jordan and Barber, 1995; Cao *et al.*, 1997; Yuziuk *et al.*, 1998; Tassi *et al.*, 2001). Therefore, anion exchange was the preferred choice for HexB purification.

The buffer used for anion exchange chromatography should preferably consist of simple anions (*e.g.* Cl^-, CH_3COO^-, PO_4^{3-}) (Scopes, 1994; Williams and Frasca, 1999). One of the buffering species should also be uncharged and should not contribute to the ionic strength. Tris-HCl fits the aforementioned criteria as it contains Cl^- and $HTris^+$ as buffering species where $HTris^+$ does not contribute to the ionic strength (Scopes, 1994). Also, the pH of the selected buffer should be within ±1 pH unit of the pKa value (Jungbauer and Hahn, 2009). Tris-HCl satisfies this condition as it has a pKa of 8.06 (Williams and Frasca, 1999). Moreover, using Tris-HCl is economical (Blanchard, 1984; Williams and Frasca, 1999). Therefore, 20 mM Tris-HCl was used as the homogenisation buffer (Williams and Frasca, 1999; Grodzki and Berenstein, 2010) to avoid any buffer changes between homogenisation and anion exchange chromatography.

However, other buffers such as 20 mM Tris-HCl, 20 mM MOPS, 20 mM PIPES, 20 mM HEPES, 20 mM Triethanolamine (TEA) and 20 mM potassium phosphate buffer were subsequently tried as homogenisation buffers to assess the best buffer for retention of hexosaminidase activity. Tris-HCl, TEA and

potassium phosphate buffers gave comparable hexosaminidase activities. However, Tris-HCl was chosen due to the aforementioned advantages.

In order to bind to the anionic exchange resin, the target protein should be charged negatively and to achieve this, the pH of the buffer must be higher than the pI of the protein. However, alterations in the pH can lead to loss or reduction of enzyme activity (Jakoby, 1984). Therefore, pH 7.4 was used as starting pH which was close to the theoretical pI (7.2) of the HexB (Legler e*t al.*, 1991). However, it is better to use a buffer pH which is closer to its pKa (Jungbauer and Hahn, 2009). Therefore, seven different pHs ranging from 7.2 to 8.4, in increments of 0.2, were assessed for homogenisation of spleen. Enzymatic activity of each homogenate was measured and pH 7.8 was found to be marginally better, which was an added advantage considering the fact that this was significantly close to the pKa of Tris-HCl.

Elution of the absorbed protein from the anion exchange resin can be achieved by either lowering the buffer pH (pH gradient), which in turn increases the net positive charge on the protein and subsequently, weakens the binding between the protein and the resin (Rosenberg, 2005), or by increasing the ionic strength of the buffer by adding a salt (salt gradient), which causes displacement of protein ions with buffer ions from the resin (Scopes, 1994). However, lowering the pH is not a preferred elution strategy as the changes in pH are usually sudden and large, which results in poor separation of the eluted components (Scopes, 1994; Rosenberg, 2005). Salt gradients, using potassium or sodium chloride, are therefore, commonly used for elution. Consequently, for elution a

buffer with increased ionic strength (*i.e.* 20 mM Tris-HCl containing 500 mM NaCl) was used (Scopes, 1994; Grodzki and Berenstein, 2010).

The initial strategy of applying a linear salt gradient, for elution, failed to give a clear single protein peak, thus, indicating that the protocol was not suitable for HexB purification. A step salt gradient was subsequently successfully applied for elution. Step elutions are reported to give better separation with added advantages of shorter run-time and decreased buffer consumption (Johns *et al.*, 2009; Jungbauer and Hahn, 2009).

Hydrophobic interaction chromatography (HIC) was subsequently employed as another purification step to remove minor contaminants. Different HIC absorbents (resins) (HiTrap HIC selection kit (GE Healthcare, UK)) were tried as the hydrophobic properties of HexB were unknown. For HIC, the sample needs to be in a buffer containing lyotropic salts to promote binding of protein to the resin (section 4.2.2.2) (Scopes, 1994). Ammonium sulphate is most commonly employed for this purpose as it mostly retains the protein in its native configuration (Scopes, 1994; Rosenberg, 2005). HIC is an ideal next step in protein purification after employing IEC as the samples eluted from IEC are in high salt buffer, which is favourable for HIC. For eluting the bound protein from HIC resins a decreasing gradient of ammonium sulphate is usually sufficient (Benedek, 2004). However, it was observed that the hydrophilicity of the NAGase and the contaminating proteins was similar. Therefore, size exclusion (SE) / gel filtration chromatography was performed to enhance purification.

Buffer composition has no direct influence on the resolution in gel filtration chromatography. Therefore, buffer selection can be based upon other requirements (such as further purification or analysis, intended use, storage, *etc.*). However, an ionic strength equivalent to 150 mM NaCl or greater is recommended to avoid ionic interactions with the gel matrix (http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/DC18E4B0B709603BC1257628001CCE3E/$file/18113229AD.pdf) (Stellwagen, 2005). Consequently, 150 mM PBS was chosen. For gel filtration chromatography, the sample was passed at a lower flow rate (a flow rate 0.5 mL/min) than with IEC or HIC as lower flow rates are known to give better resolutions (Stellwagen, 2005). This strategy was successful in generating a single HexB protein peak.

The HexB purification protocol yielded HexB with a 12.9 fold purification which was very low when compared to Pokorny and Glaudemans (1975), who reported a much higher purification factor of 120 fold. However, Pokorny and Glaudemans (1975) did not recover pure hexosaminidase. Verpoorte (1972) also attempted purification of HexB from bovine (calf) spleen and received low yield (4.1%) of HexB. Additionally, the protocol was very labour intensive and time-consuming. The amount of HexB obtained from this protocol was also low. This may be due to spleen tissue having a limited amount of NAGase which was depleted with every additional purification step. Verpoorte (1972) reported that activity of HexA and HexB fractions decreased rapidly during chromatographic isolation.

Generation and characterisation of an antibody requires significant amounts of antigen (Hust and Dübel, 2004), *e.g.* for immunisations, antibody titrations,

antibody screening and characterisations, that could not be obtained by the FPLC protocol standardised in this current study. However, the isolated antigen was successfully used for selection of anti-HexB antibodies from naïve human antibody libraries (Welbeck *et al.*, 2011). These antibodies were then incorporation into an automated optical biosensor-based immunoassay to detect NAGase in milk (Welbeck *et al.*, 2011). Purification of additional HexB, from bovine spleens, was not further attempted, as very low yields were obtained following a very labour-intensive protocol. The protocol required weeks for a single purification cycle to complete and there were problems associated with the availability and the storage of the bovine spleens. Consequently, an alternate solution involving the cloning and expression of bovine HexA and HexB in *E. coli* was undertaken.

For cloning of HEXA and HEXB genes, the cDNA extracted from the spleen of a cow, that died following chronic mastitis, was used as template for amplification. HEXA has a hydrophobic N-terminal region. Thus, expression of HEXA in *E. coli* was toxic to the cells (Aggarwal and Mondal, 2006). The addition of an N-terminal fusion protein helps obliterate this problem. N-terminal fusion tags enhance solubility of the expressed protein by forcing the recombinant protein to pass through the bacterial chaperone pathway as a complete fusion protein (Ishihara *et al.*, 2005; Waugh, 2005; Xie *et al.*, 2009). Additionally, N-terminal tags are useful in efficient translation initiation of recombinant proteins (Waugh, 2005). It was decided to use the pET-32b(+) vector for expression, as it contained an N-terminal fusion protein. Additionally, pET series vectors allow tight regulation of protein expression in *E. coli* cells due to the presence of T7 phage RNA polymerase promoter (Sørensen and Mortensen, 2005).

When expressing mammalian genes in *E. coli*, few amino acid codons have been shown to be limiting as they are decoded by a low-abundant tRNA in its genome (Chen and Texada, 2006). These codons include arginine (AGA, AGG), isoleucine (AUA), leucine (CUA), and proline (CCC), and are termed as 'rare codons'. Both HEXA and HEXB sequences were observed to be interspersed with rare codons. Therefore, BL21-CodonPlus®(DE3)-RIPL competent cells, a special strain of *E. coli*, was used for protein expression as these cells are known to improve protein expression by supplying additional copies of specific tRNA genes that are rare in *E. coli* (rare codons) and also give higher expression levels than conventionally used strains (Assadi-Porter *et al.*, 2008). Consequently, both HEXA and HEXB were successfully cloned and expressed. However, overexpression of both the genes could not be achieved. A low level of protein expression was observed in the case of HEXB. In order to determine the optimal time for expression of HEXA and HEXB time course studies were carried out. Both genes were found to be expressing best at 4 h post induction. However, both genes failed to overexpress. Adequate purification of protein from HEXB clone could not be achieved as numerous bands were observed following IMAC purification. Different approaches (*i.e.* different induction times and temperatures) were tried to reduce non-specific bands, however, no improvements were observed. An alternative strategy involving the use novel N-terminal fusion protein is being pursued to improve HEXA and HEXB expression.

Chapter 5
Adaptive Evolution of the Bovine Hexosaminidase A and Hexosaminidase B Genes

5. ADAPTIVE EVOLUTION OF THE BOVINE HEXOSAMINIDASE A AND HEXOSAMINIDASE B GENES

5.1 Introduction

5.1.1 Significance of HexA and HexB

The presence of the enzyme *N*-acetyl-β-D-glucosaminidase was first reported in humans in 1936 (Watanabe, 1936). Subsequently, it was found that (NAGase) exists mainly in lysosomes. It was shown to cleave the glycosidic linkages of the non-reducing, terminal β-D-*N*-acetylglucosamine (GlcNAc) or β-D-*N*-acetylgalactosamine (GalNAc) residues on glycolipids, glycoproteins, proteoglycans, and glycosaminoglycans (Mahuran, 1999). Consequently, it was proposed that the enzyme should be called β-hexosaminidase or β-*N*-acetylhexosaminidase (EC 3.2.1.52) (Mahuran, 1999).

β-hexosaminidases are dimeric enzymes composed of two subunits, α and β. Three isoforms, Hexosaminidase (HexA) (structure, αβ), HexB (ββ) and HexS (αα), are reported. The β-subunit is again composed of 2 non-identical chains (β_a and β_b). Both the β chains are derived from a common pro-β precursor (Mahuran *et al.*, 1988). HexA is the major isoenzyme followed by HexB (Chavany and Jendoubi, 1998; Slámová *et al.*, 2010). However, HexB is more stable than HexA (Sinici *et al.*, 2004). HexS is an unstable isoenzyme with limited catalytic activity, not detectable in normal individuals (Chavany and Jendoubi, 1998; Slámová *et al.*, 2010). The α- and β-subunits have their own active sites, however, they are enzymatically active only in dimeric forms (Sinici *et al.*, 2004). The α- and β-subunits encoded by two closely related genes, HEXA and HEXB. These genes have 60% sequence similarity and share a large degree of primary structure homology (Sagherian *et al.*, 1994). The HEXA

and HEXB genes are present on chromosomes 15 and 10 in humans and chromosomes 5 and 20 in bovines.

Both, HexA and HexB can cleave β-linked GalNAc and GlcNAc from substrates such as neutral oligosaccharides, glycolipids, and glycoproteins. However, only HexA, owing to the presence of α-subunit, can hydrolyse the negatively charged substrates such as G_{M2} ganglioside and β-linked glucosamine-6-sulphate containing glycosaminoglycans (Svennerholm, 1962; Kresse *et al.*, 1981).

The interest in HexA and HexB is mainly derived from the discoveries that deficiencies in their activities (resulting from the mutations in the HEXA and HEXB genes) are associated with Tay-Sachs and Sandhoff diseases, respectively. Tay-Sachs disease is caused by HexA deficiency. HexA deficiency is caused by the defects in, or absence of, the α-subunit. Similarly, Sandhoff disease is caused defects in, or absence of, the β-subunit, which translates into deficiency in both HexA and HexB (Sinici *et al.*, 2004). Tay-Sachs disease and Sandhoff disease are reported primarily in humans. However, similar diseases were demonstrated in cats, dogs, deer, flamingos, pigs and sheep (Cork *et al.*, 1977; Cork *et al.*, 1978; Kosanke *et al.*, 1978; Cummings *et al.*, 1985; Singer and Cork, 1989; Fox *et al.*, 1999; Walkley *et al.*, 1990; Kolodny *et al.*, 2006; Paola *et al.*, 2010). To date, no work has been carried out to study these diseases in bovines.

5.1.2 Molecular evolution

Natural selection is the process of choosing the traits favourable for the survival and fecundity of a species and, subsequently, their fixation in the genome of the successive generations (Darwin, 1859; Sabeti *et al.*, 2006). Natural selection is an important phenomenon leading to the evolution of the genes, genome and species. At the molecular level the selective pressure on a given gene can be described in terms of ω, the ratio of the number of non-synonymous substitutions per non-synonymous site (*dN* or *Ka*), to the number of synonymous substitutions per synonymous site (*dS* or *Ks*) (*i.e.* ω = *dN/dS* or *Ka/Ks*) (Hurst, 2002; Yang and Swanson, 2002). Non-synonymous substitutions result in codons that code for different amino acids (Figure 5.1). Conversely, synonymous substitutions generate codons coding the same amino acids (Figure 5.1) (Yang, 2006). If the substitutions of nucleotides (*i.e.* mutations) cause deleterious or adverse effects on the survival and / or reproductive success of the organism, these mutations are removed from the population. This is known as “purifying selection” (Yang, 1998). Such a selective pressure has significantly higher number of synonymous substitutions (ω < 1), and is termed as negative or purifying selection (Anisimova *et al.*, 2002) (Figure 5.1). Rarely, mutations may confer a selective advantage to an organism by increasing its fitness for survival and / or reproductive success. Such mutations are preferentially selected for and this selective pressure is called positive / diversifying selection, or adaptive evolution, and is resultant from significantly higher number of non-synonymous substitutions (ω > 1) (Yang, 1998; Anisimova *et al.*, 2002) (Figure 5.1). However, the majority of mutations that occur do not affect the fitness of the organisms, *i.e.* they are selectively neutral. In these cases the number of non-synonymous and the number of synonymous

substitutions are not significantly different (ω = 1) and the absence of a selective pressure is termed neutral evolution (Kimura, 1968; Yang, 1998; Anisimova *et al.*, 2001) (Figure 5.1).

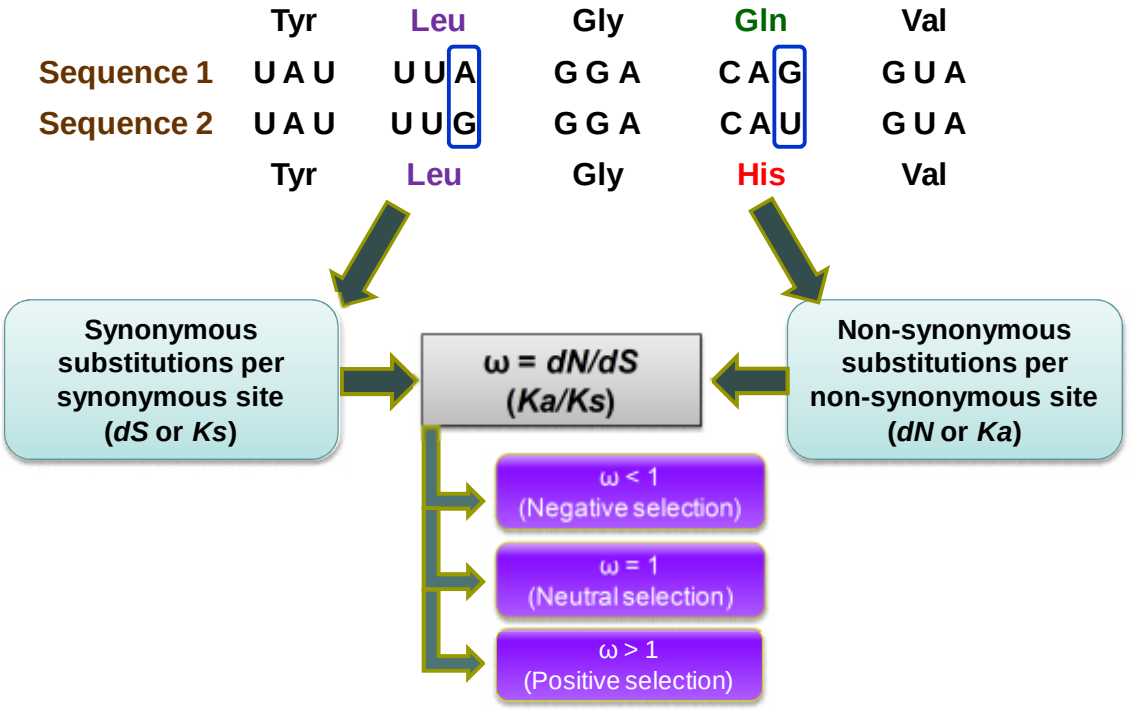


Figure 5.1. ***The process of natural selection.***
The ratio of the number of non-synonymous (*dN* or *Ka*) substitutions per non-synonymous site to the number of synonymous substitutions per synonymous site (*dS* or *Ks*) is denoted by ω. An ω value of unity signifies neutral evolution, whereas, ω values of less and more than unity signify negative and positive selection, respectively.

The molecular estimation of sites under positive selection can be further used for studying functional shifts. The identified sites can be mutated and analysed for change in function / structure (Dean and Golding, 1997; Benner *et al.*, 1998). For example, Levasseur *et al.* (2006) successfully demonstrated that positively selected sites in the fungal lipase-feruloyl esterase A family led to the functional diversification of the enzymes. The close relationship between positive selection and proven functional shift has resulted in these terms being used interchangeably in the field of molecular evolution. Using analysis of selective

pressures can therefore allow us to identify molecular reasons for functional inequality between proteins.

5.2 Adaptive Evolution of the Bovine Hexosaminidase A and Hexosaminidase B Genes

5.2.1 Phylogeny reconstruction

Phylogeny reconstruction was achieved using amino acid sequences of translated HEXA and HEXB sequences. Amino acid sequences were used as use of amino acids abate the effects of base and codon compositional biases (Morgan *et al.*, 2010). Trees were constructed using zebrafish as outgroup (Figures 5.2 and 5.3). The bootstrap support (BS) values for the different nodes, varied between 39-100 (HEXA) and 27-100 (HEXB). The BS values were low in most cases, however, they were still retained for the gene tree as they relationship of these species is well established (Murphy *et al.*, 2001).

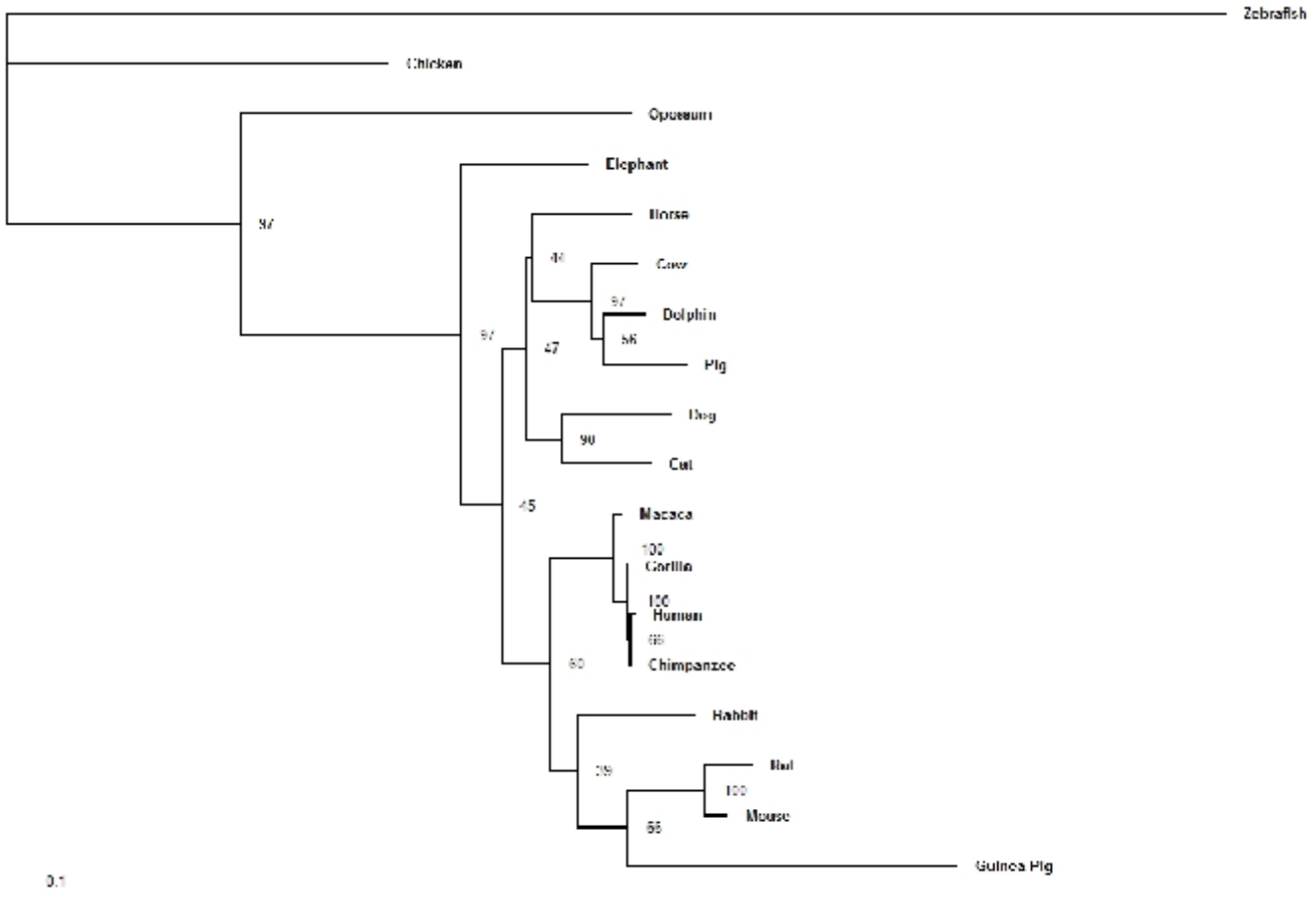


Figure 5.2. ***Phylogenetic tree constructed using HEXA amino acid sequences.***
The phylogeny of the species selected for analysis was reconstructed using HEXA amino acid sequences. The numbers on the tree indicate the bootstrap support (BS) values for each node. The BS varied from 39-100. The BS values were low in most cases, however, they were still retained for the gene tree as the relationship of these species is well established.

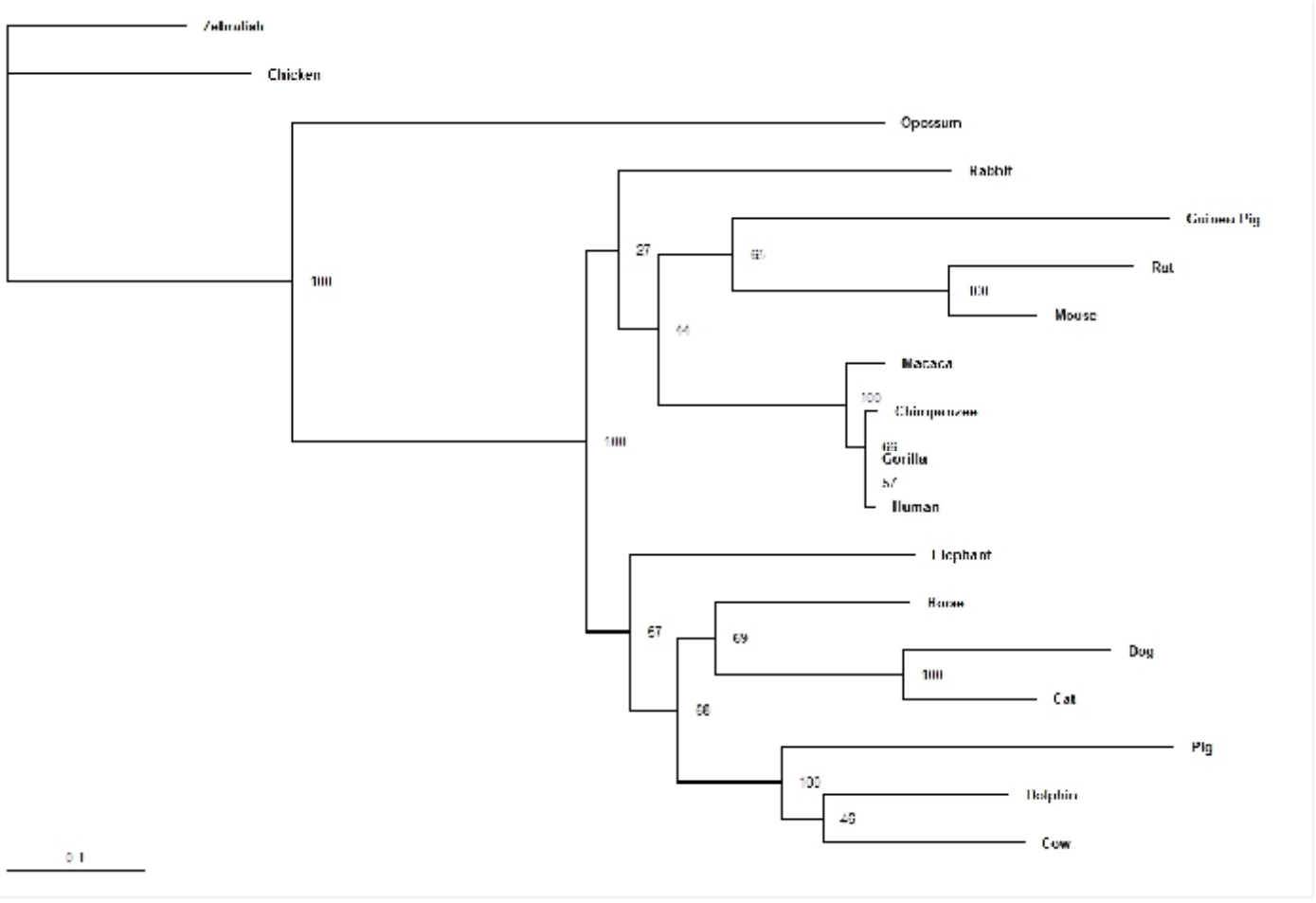


Figure 5.3. ***Phylogenetic tree constructed using HEXB amino acid sequences.***
The phylogeny of the species selected for analysis was reconstructed using HEXB amino acid sequences. The numbers on the tree indicate the bootstrap support (BS) values for each node. The BS varied from 27-100. The BS values were low in most cases, however, they were still retained for the gene tree as the relationship of these species is well established.

5.2.2 HEXA gene analysis

The ML approach uses the LRTs to compare two models, a model that account for sites with ω > 1, and a null model, that does not (Anisimova *et al.*, 2002). For each distribution, a likelihood value is calculated, and the best distribution is the one that maximizes this likelihood. The alignment of the HEXA gene resulted in 1615 aligned nucleotide positions. To examine whether there is evidence of site-specific evolution in HEXA gene, Codeml from the PAML 4.3 package was used. All models were run using 4 alternative starting omega values, so that the global minimum is obtained from the ML plane (Table 5.1). Several site-specific and lineage-specific models (M0 to M8 and MA and MA-null), allowing for various ω values (*dN/dS* ratios) among different codon sites were used to test alternative selection pressure (Table 5.2). Likelihood-ratio tests (LRTs) were conducted to establish the model that best fits the data. The LRTs compared the two (M0 *vs.* M3k2, M3k2 *vs.* M3k3, M1 *vs.* M2, M7 *vs.* M8 and M8 *vs.* M8a). The likelihood ratios of the two models test whether an alternative model fits the data better than the null model (Table 5.3). Positive selection can be inferred from this analysis when models M2, M3(k=2), M3(k=2) or M8 identify codon sites with an ω value greater than one. Model M8 was found to be the best fit model for the data. No lineage-specific positive selection was observed. The Bayes empirical Bayes (BEB) calculation of posterior probabilities for site classes was utilized to identify the codon sites under positive selection (Yang *et al.*, 2005). Model M8 had 15 codon sites that were positively selected with posterior probability of > 50% (Table 5.4). These sites identified were positions 5, 35, 50, 84, 96, 100, 101, 293, 314, 357, 407, 410, 420, 447 and 509 (renumbered as per protein sequence of humans (Table 2.29)). Position 5 is in signal peptide region of β-hexosaminidase α-subunit. The signal peptide directs

the insertion of β-hexosaminidase α-subunit into the membrane of the endoplasmic reticulum (ER), after which they are cleaved-off (Andrews *et al.*, 1988). The positions 35, 50 and 84 belonged to the propeptide region. The propetide regions undergo extensive proteolytic processing in the lysosome for biosynthesis of mature forms of HexA (Mahuran *et al.*, 1988). However, this propeptidic forms (pro-HexA) was shown to be active towards natural and artificial substrates (Hasilik *et al.*, 1982) and it was concluded that the propeptide fragment is a non-essential domain, which is cleaved-off just for the generation of mature forms (Mahuran *et al.*, 1988). A mutation at a site near position 35 (position 39) is known to cause Tay-Sachs disease (Akli *et al.*, 1993). A majority of the sites (positions 94, 95, 282, 303, 346, 396, 399, 409, 436 and 498) identified under positive pressure belonged to the actual enzymatic chain region. Interestingly, 4 of these sites (positions 282, 303, 396, and 498) were in close proximity (within 4 residues) to the mutated sites, known to cause Tay-Sachs disease (http://www.uniprot.org/uniprot/P06865) (Mules *et al.*, 1992; Drucker *et al.*, 1997; Tanaka *et al.*, 2003). Positions 399 and 436 are the sites for missense mutation and position 346 is the site for deletion mutations, causing Tay-Sachs disease (Mules *et al.*, 1992; Tomczak and Grebner, 1994) and all of these were positively selected.

5.2.3 HEXB gene analysis

The alignment of the HEXB gene resulted in 1705 aligned nucleotide positions. Like HEXA, all models were run using 4 alternative starting omega values (Table 5.1). The site-specific and lineage-specific models in codeml (Yang, 1997; Yang *et al., 2005*) were used to determine selective pressure at work (Table 5.2) and LRTs were conducted to establish the model that best fit the

data (Table 5.3). Again M8 was found to be the best fit model for the site-specific positive selection analysis. However, in this case there was also lineage-specific positive selection and model A was the best fit model. Using the BEB calculation of posterior probabilities for site classes model M8 identified 13 codon sites that were positively selected with posterior probability of > 95% (Table 5.4). These sites identified were positions 24, 28, 34, 41, 47, 51, 52, 53, 78, 92, 111, 118 and 119 (renumbered as per protein sequence of humans (Table 2.30)). In addition, a codon site (position 159) positively selected with posterior probability of > 99% was also identified (Table 5.4). The exact function of position 159 is currently unknown. Positions 24, 28, 34 and 41 are located in the signal peptide region and the positions 47, 51, 52, 53, 78, 92, 111, 118 and 119 belonged to the propeptide region of β-hexosaminidase β-subunit. The function of signal peptide and propeptide regions of β-hexosaminidase β-subunit are the same as per the α-subunit (Andrews *et al.*, 1988; Mahuran *et al.*, 1988). Pro-HexB was also shown to be active towards natural and artificial substrates (Hasilik *et al.*, 1982) and, thus, it was concluded that the propeptide fragment is a non-essential domain (Mahuran *et al.*, 1988). Positions 118 and 119 were in close proximity (within 3 residues) to the mutation sites, known to cause Sandhoff disease (http://www.uniprot.org/uniprot/P07686).

The branch specific analysis for HEXB gene identified 2 positions (positions 43 and 95, renumbered as per cow protein sequence) being positively selected, with posterior probabilities of > 50%, using BEB calculations (Table 5.4). Similarly, position 283 was identified to be positively selected, with a posterior probability of > 95% (Table 5.4). The exact function of position 283 is currently unknown.

Table 5.1. ***Alternative starting omega values for different models.***

Models	Omega 0	Omega 1	Omega 2	Omega 10
HEXA				
M0	-13165.781655	-13165.781655	-13165.781655	-13165.781655
Site-specific models				
M1: Neutral	-12597.339638	-12597.339638	-12597.339638	-12597.339638
M2: Selection	-12589.496883	-12597.339638	-12597.339638	-12597.339762
M3: Discrete k=2	-12575.435188	-12575.435188	-12575.435188	-12575.435188
M3: Discrete k=3	-12505.498988	-12505.498988	-12505.498988	-12575.435188
M7: Beta	-12516.609329	-12516.609329	-12516.609329	-12516.609329
M8: Beta and Omega>1	-12502.534936	-12502.534936	-12502.534936	-12516.612076
M8a: Beta and Omega=1		-12511.442516		
Lineage-specific models				
Model A	-12597.339638	-12597.339639	-12597.339639	-12597.339638
Model A-null		-12597.339639		
HEXB				
M0	-13994.708799	-13994.708799	-13994.708799	-13994.708799
Site-specific models				
M1: Neutral	-13406.522148	-13406.522148	-13406.522148	-13406.522148
M2: Selection	-13377.942153	-13377.942153	-13377.942153	-13377.942153
M3: Discrete k=2	-13403.283020	-13403.283020	-13403.283020	-13403.283020
M3: Discrete k=3	-13341.971714	-13341.971714	-13341.971714	-13341.971714
M7: Beta	-13382.426029	-13382.426029	-13382.426029	-13382.426029
M8: Beta and Omega>1	-13339.203921	-13339.203921	-13339.203921	-13339.203921
M8a: Beta and Omega=1	-	-13368.162101	--	-
Lineage-specific models				
Model A	-13403.420397	-13403.420397	-13404.355833	-13403.420397
Model A-null		-13405.519051	-	-

(The values highlighted in green are the ones selected).

Table 5.2. ***Complete set of estimates of HEXA and HEXB for all models.***

Model	Number of Parameters	Estimates of Parameters	Positively Selected Sites	Details of Positively Selected Sites
HEXA				
M0: One ratio	1	ω=0.21963	None	-
Site-specific				
M1: Neutral	2	p_0=0.66123, ω_0=0.05638	Not Allowed	-
M2: Selection	4	p_0=0.65403 , p_1=0.31497, (p_2=0.03100), ω_0=0.05665, ω_1=1.00000, ω_2=2.86054	NEB: 13>0.50, 0>0.95, 0>0.99 BEB: 13>0.50, 0>0.95, 0>0.99	BEB (>0.50): 9 T, 44 L, 59 L, 93 I, 105 E, 109 T, 110 S, 305 H, 369 D, 419 I, 422 K, 459 L, 522 K
M3: Discrete k=2	3	p_0=0.61170, (p_0=0.38830), ω_0=0.03607, ω_1= 0.66519	NEB: 0>0.50, 0>0.95, 0>0.99	-
M3: Discrete k=3	5	p_0=0.48874, p_1=0.34352, (p_2= 0.16774), ω_0=0.01214, ω_1=0.29166, ω_2= 1.22542	NEB: 40>0.50, 18>0.95, 27>0.99	NEB (>0.50): 11 R, 13 S, 28 T, 30 L, 54 Q, 56 Q, 75 Q, 83 G, 85 V, 88 R, 111 E, 118 L, 129 S, 133 V, 145 S, 169 R, 205 A, 237 F, 258 Q, 319 N, 407 R, 424 V, 427 L, 428 A, 429 L, 449 T, 464 A, 515 S, 518 D, 542 S, 552 Q NEB (>0.95): 8 S, 12 F, 26 R,47 T, 51 Q, 52 S, 86 A, 87 F, 89 F, 91 H, 108 H, 116 V, 121 T, 289 P, 293 V, 360 A, 457 I, 517 L, 525 A NEB (>0.99): 9 T, 20 F, 43 E, 44 L, 49 F, 59 L, 79 D, 92 P, 93 I, 105 E, 107 R, 109 T, 110 S, 114 S, 148 L, 305 H, 326 T, 369 D, 419 I, 422 K, 432 R, 456 E, 459 L, 466 E, 513 M, 521 F, 522 K
M7: Beta	2	p=0.22058, q=0.58529	Not Allowed	-
M8: Beta and omega>1	4	p_0=0.93371, p=0.25676, q=0.92274, (p_1= 0.06629), ω=1.79464	NEB: 26>0.50, 3>0.95, >0.99 BEB: 16>0.50, >0.95, >0.99	BEB (>0.50): 9 T, 44 L, 59 L, 93 I, 105 E, 109 T, 110 S, 305 H, 326 T, 369 D, 419 I, 422 K, 432 R, 459 L, 522 K

Lineage-specific models				
Model A	4	p_0=0.66124, p_1=0.33876, (p_2=0.00000, p_3=0.00000), ω_0=0.05638, ω_1=1.00000, ω_2=0.05638 (background) ω_0=0.05638, ω_1=1.00000, ω_2=1.00000 (foreground)	NEB: 0>0.50, 0>0.95, 0>0.99 BEB: 0>0.50, 0>0.95, 0>0.99	-
Model A-null	3	p_0=0.66124, p_1=0.33876, (p_2=0.00000, p_3=0.00000), ω_0=0.05638, ω_1=1.00000, ω_2=0.05638 (background) ω_0=0.05638, ω_1=1.00000, ω_2=1.00000 (foreground)	Not Allowed	-
HEXB				
M0: One ratio	1	ω=0.37835	None	
Site-specific				
M1: Neutral	2	p_0=0.61044, ω_0=0.09037	Not Allowed	
M2: Selection	4	p_0=0.58947, p_1=0.31899 , (p_2=0.09153), ω_0=0.09447, ω_1=1.00000, ω_2=2.52857	NEB: 38>0.50, 8>0.95, 0>0.99 BEB: 39>0.50, 6>0.95, 0>0.99	BEB (>0.50): 21 Q, 24 L, 28 R, 34 A, 41 A, 42 R, 53 G, 54 D, 62 V, 66 T, 79 F, 93 A, 113 W, 122 K, 123 I, 124 P, 125 S, 127 M, 139 M, 165 T BEB (>0.95): 48 R, 52 S
M3: Discrete k=2	3	p_0=0.63022 , (p_0=0.36978), ω_0=0.10367 , ω_1=1.15451	NEB: 39>0.50, 19>0.95, 141>0.99	NEB (>0.50): 20 E, 30 P, 36 L, 47 P, 50 S, 51 A, 60 L, 71 F, 90 P, 94 V, 96 Q, 118 H, 120 H, 129 L, 130 Q, 141 P, 161 G, 167 T, 189 S, 194 T, 195 A, 204 P, 220 F, 224 V, 250 Q, 294 L NEB (>0.95): 38 A, 72 Y, 74 S, 77 N, 83 S, 84 P, 91 S ,111 Y, 115 *, 116 *, 150 T, 208 P, 257 I NEB (>0.99): 21 Q, 22 R, 23 G, 24 L, 25 A, 26

				R, 27 L, 28 R, 29 L, 31 G, 32 L, 34 A, 37 A, 40 A, 41 A, 42 R, 43 T, 48 R, 49 V, 52 S, 53 G, 54 D, 56 N, 62 V, 63 S, 65 K, 66 T, 67 T, 69 R, 70 L, 76 G, 79 F, 80 F, 81 G, 85 T, 93 A, 105 D, 113 W, 117 H, 119 G, 121 N, 122 K, 123 I, 124 P, 125 S, 126 E, 127 M, 133 E, 136 S, 137 V, 138 I, 139 M, 145 S, 148 S, 149 I, 156 T, 158 L, 165 T, 169 N, 170 R, 198 S, 199 N, 201 V, 203 S, 217 S, 226 T, 228 L, 229 K, 258 S, 261 E, 264 N, 270 L, 281 T, 299 S, 304 E
M3: Discrete k=3	5	p_0=0.39886 , p_1=0.36334 , (p_2= 0.23779), ω_0=0.02277 , ω_1=0.38982 , ω_2= 1.55249	NEB: 52>0.50, 22>0.95, 48>0.99	NEB (>0.50): 22 R, 23 G, 25 A, 26 R, 27 L, 29 L, 31 G, 32 L, 38 A, 40 A, 43 T, 49 V, 56 N, 63 S, 65 K, 67 T, 70 L, 85 T, 119 G, 121 N, 133 E, 136 S, 137 V, 138 I, 145 S, 149 I, 158 L, 170 R, 201 V, 217 S, 226 T, 258 S, 270 L, 281 T, 304 E NEB (>0.95): 21 Q, 37 A, 42 R, 69 R, 81 G, 105 D, 126 E, 139 M, 148 S, 156 T NEB (>0.99): 24 L, 28 R, 34 A, 41 A, 48 R, 52 S, 53 G, 54 D, 62 V, 66 T, 76 G, 79 F, 93 A, 113 W, 122 K, 123 I, 124 P, 125 S, 127 M, 165 T, 199 N
M7: Beta	2	p=0.24894, q=0.37706	Not Allowed	
M8: Beta and omega>1	4	p_0=00.84178 , p=0.34040, q=0.86081, (p_1= 0.15822), ω=1.86054	NEB: 69>0.50, 17>0.95, 1>0.99 BEB: 84>0.50, 29>0.95, 4>0.99	BEB (>0.50): 21 Q, 22 R, 23 G, 25 A, 26 R, 27 L, 29 L, 31 G, 32 L, 37 A, 38 A, 40 A, 42 R, 43 T, 49 V, 56 N, 62 V, 63 S, 65 K, 66 T, 67 T, 69 R, 70 L, 76 G, 81 G, 85 T, 105 D, 119 G, 121 N, 122 K, 125 S, 126 E, 127 M, 133 E, 136 S, 137 V, 138 I, 139 M, 145 S, 148 S, 149 I, 156 T, 158 L, 170 R, 199 N, 201 V, 226 T, 258 S, 270 L, 281 T, 304 E BEB (>0.95): 24 L, 28 R, 34 A, 41 A, 48 R, 52 S, 53 G, 54 D, 79 F, 93 A, 113 W, 123 I, 124 P

				BEB (>0.99): 165 T
Lineage-specific models				
Model A	4	p_0=0.59754, p_1=0.38148, (p_2=0.01281, p_3=0.00818), ω_0=0.08903, ω_1=1.00000, ω_2=0.08903 (background) ω_0=0.08903, ω_1=1.00000, ω_2=8.49711 (foreground)	NEB: 1>0.50, 1>0.95, 0>0.99 BEB: 2>0.50, 1>0.95, 0>0.99	BEB (>0.50): 64 L, 120 H BEB (>0.95): 397 W
Model A-null	3	p_0=0.58263, p_1=0.37178, (p_2=0.02783, p_3=0.01776), ω_0=0.08930 , ω_1=1.00000, ω_2=0.08930 (background) ω_0=0.08930 , ω_1=1.00000, ω_2=1.00000 (foreground)	Not Allowed	-

(The values highlighted in green are the ones selected).

Table 5.3. ***Summary of LRTs for HEXA and HEXB.***

HEXA						
Comparison	***df***	**$\ln L_1$**	**$\ln L_2$**	**Critical χ^2 values**	**Significance**	**Choice of Model**
M0 v M3k2	2	-13165.781655	-12575.435188	1180.692934	Significant	M3k2
M3k2 v M3k3	1	-12575.435188	-12505.498988	69.936200	Significant	M3k3
M1 v M2	2	-12597.339638	-12589.496883	15.685510	Significant	M2
M7 v M8	2	-12516.609329	-12502.534936	28.148786	Significant	M8
M8a v M8	1	-12502.534936	-12511.442516	8.907580	Significant	M8
M1 v Model A	2	-12597.339638	-12597.339638	0.000000	Not significant	M1
Model A v Model A-null	1	-12597.339638	-12597.339639	-	Not significant	-
HEXB						
Comparison	***df***	**$\ln L_1$**	**$\ln L_2$**	**Critical χ^2 values**		**Choice of Model**
M0 v M3k2	2	-13994.708799	-13403.283020	1182.851558	Significant	M3k2
M3k2 v M3k3	1	-13403.283020	-13341.971714	61.311306	Significant	M3k3
M1 v M2	2	-13406.522148	-13377.942153	57.159990	Significant	M2
M7 v M8	2	-13382.426029	-13339.203921	86.444216	Significant	M8
M8a v M8	1	-13339.203921	-13368.162101	28.958180	Significant	M8
M1 v Model A	2	-13406.522148	-13403.420397	6.203502	Significant	Model A
Model A v Model A-null	1	-13403.420397	-13405.519051	2.098654	Significant	Model A null

(The values highlighted in green are the ones selected).

Table 5.4. ***List of sites identified to be positively selected with their posterior probabilities.***

Positions Under Selection Pressure	Posterior Probabilities using BEB Method
Site-specific analysis	
HEXA	
5	0.711
35	0.800
50	0.816
84	0.792
85	0.893
89	0.786
90	0.662
282	0.732
303	0.557
346	0.908
396	0.924
399	0.908
409	0.524
436	0.651
498	0.859
HEXB	
24	0.981
28	0.970
34	0.953
41	0.960
47	0.990
51	0.987
52	0.982
53	0.965
78	0.987
92	0.982
111	0.977
118	0.960
119	0.971
159	0.991
Lineage-specific analysis (cow branch)	
HEXB	
43	0.519
95	0.806
283	0.990

5.3 Discussion

Phylogenetic analyses of HEXA and HEXB genes were carried out before examining site and lineage-specific positive selection. The generated gene trees for both the genes revealed that the HEXA and HEXB genes evolved similarly. The trees resembled the typical mammalian phylogeny (Murphy *et al.*, 2001).

The site-specific analysis for HEXA gene identified 15 sites. Similarly, 14 sites were identified in the case of HEXB. The ω and p values were low for HEXA as compared to HEXB, signifying that there is a stronger selective pressure in HEXB as compared to HEXA. All these sites were analysed using the functional and mutational information available for humans as the β-hexosaminidases are relatively well studied in humans (Slámová *et al.*, 2010).

At least, 5 identified sites (4 in case of HEXA and 1 in case of HEXB) were in close proximity to the reported mutation sites known to cause Tay-Sachs and Sandhoff diseases. This can be attributed to hitchhiking effect (or selective sweeps). Hitchhiking is a phenomenon in which the sites experiencing positive selection might influence (positively or negatively) the nearby neutral regions (Smith and Haigh, 1974; Kaplan *et al.*, 1989; Barton, 2000; Andolfatto, 2001) by fixing them in the population, even though they themselves are not beneficial.

The site-specific analysis of the HEXA gene revealed that selection was occurring at three different mutation sites associated with Tay-Sachs disease (positions 346, 399 and 436). Position 346 is a site for deletion mutation (Tomczak and Grebner, 1994). Examining the physicochemical properties of

this mutation we find that the amino acid residues at position 346 of the mammalian species were all polar. However, the residues varied in charge, with cows, dolphins, humans, chimpanzees, gorillas, macaques and elephants containing negatively charged residues. Conversely, pig had a positively charged amino acid residue at position 346. The remaining mammalian species had uncharged polar residues at this position. In theory, all these polar amino acid residues may be substituted by other polar residues (Betts and Russell, 2003). Thus, there might not be a major change associated with the selection of this site. However, the presence of charged residues might alter the function and / or stability of the protein. For example, the presence of positively charged lysine in pigs may provide more structural stability to the porcine HEXA (Betts and Russell, 2003).

Position 399 in the α-subunit was reported to be associated with a missense mutation where asparagine is substituted with aspartic acid (N → D) and, thus, causes Tay-Sachs disease (Mules *et al.*, 1992). Substitution of this asparagine to other amino acids was observed among different mammals studied. Similar to position 346, the residues at position 399 were mostly polar amino acid residues (positively or negatively charged or neutral) which can be substituted within themselves without significant effect on the protein (Betts and Russell, 2003). However, in the rabbit and opossum α-subunit a radical substitution was observed. They both had a proline residue at position 399. Proline is a non-polar amino acid with an aliphatic side chain. Proline, unlike other amino acid residues at position 399, is generally not associated with the active or binding sites and is very non-reactive (Betts and Russell, 2003).

Position 436 in the α-subunit is also associated with missense mutations (I → V) leading to Tay-Sachs disease (Mules *et al.*, 1992). The amino acid residues at this position were hydrophobic in most of species studied, *i.e.* humans, macaques, cats, chimpanzees, gorillas, cows, dogs, elephants, dolphins, horses, pigs and rabbits. These residues are usually buried inside the proteins hydrophobic core and are non-reactive (Betts and Russell, 2003). However, in case of rabbit and opossum a radical substitution was observed. They had polar uncharged residues at position 436, which are usually present exposed on the protein surface and are associated with active / binding sites (Betts and Russell, 2003). Interestingly, in guinea pigs, rats and mice a positively charged polar residue (lysine) was present, which is again associated with active / binding sites. Lysine is generally found with its charged portion on the protein surface and the remaining part buried in the protein hydrophobic core (Betts and Russell, 2003).

The lineage-specific (branch-specific) analysis of HEXA did not reveal any positively selected site in bovine. However, 3 sites were identified as positively selected in bovine HEXB. At position 43, leucine was present in cows as opposed to valine in the other mammals studied. Both these amino acids are non-polar hydrophobic and are, thus, usually buried in the protein. They both are non-reactive. The substitution of valine to leucine generally does not lead to a significant difference in terms of protein structure and / or stability. Similarly, at position 283, tryptophan was substituted in bovine as compared to tyrosine in other mammals studied. Both these residues have aromatic side chains. Tyrosine is polar and uncharged, whereas tryptophan is non-polar and hydrophobic. Tyrosine is generally involved in phosphorylation, a

posttranslational modification (PTM), whereas, tryptophan is not. Generally, tryptophan is less reactive than tyrosine.

Another site that was being selected in bovines is position 95. In most of the selected species, *i.e.* cats, chimpanzees, dogs, elephants, gorillas, horses, human, macaques, mice, rats and rabbits, proline, a small amino acid is present at this position. Proline is not usually involved in active or binding sites of the proteins (Betts and Russell, 2003). However, radical substitutions were observed in dolphins, pigs and cows, where this site was occupied by polar, uncharged residues, known to be involved in active / binding sites (Betts and Russell, 2003). In dolphins and pigs a small amino acid, serine, was present. However, histidine, a larger residue is present in cows, at this position.

In conclusion, it can be said that the β-subunit was evolving at a higher rate or more radically than the α-subunit. Certain sites in the α- and β-subunits are being selected and some of them are in close proximity to the disease-causing mutations. No major difference in the α-subunit of cows was observed when compared to related species. However, a small number of changes were observed in the case of the β-subunit, especially position 95 for the cow lineage alone.

The amino acids can be classified into various groups based on their physicochemical properties, *e.g.* based on charge, polarity and volume (Zhang, 2000). Amino acid substitutions within a certain physicochemical group are called conservative substitutions, whereas those between different physicochemical groups are radical substitutions. In summary, the substitutions

observed at positively selected sites in site-specific analysis were all radical substitutions (based on net charge and polarity of the amino acids). Significantly higher rates of radical non-synonymous substitutions have been taken as evidence of positive selection (Hughes 1992; Hughes and Hughes, 1993; Hughes, 1994b). Radical substitutions are usually selected against as they are often associated with a major physicochemical change in protein, *i.e.* may alter the functionality and / or structure of the protein (Zhang, 2000; Smith, 2003; Popadin *et al.*, 2007). However, radical changes have associated merits as well, *i.e.* a major change in physicochemical properties offers a greater chance for a new advantageous protein function to be achieved resulting from a single substitution (Nielsen and Huelsenbeck, 2002; Smith, 2003). However, only one lineage-specific substitution (in the cow β-subunit) was a radical substitution (position 95). A higher percentage of radical substitutions in the cow lineage of hexosaminidase α- or β-subunits would have been desirable as they would have altered the immunogenicity (Nielsen and Huelsenbeck, 2002; Hanada *et al.*, 2006) of the bovine NAGase.

Chapter 6
Construction of a Bovine scFv Library

6. CONSTRUCTION OF A BOVINE SCFV LIBRARY

6.1 Introduction

Detection of mastitis-causing agents is of the utmost importance to devise efficient prevention policies as well as for efficient treatment. However, more than 150 mastitis-inducing species are reported (Kuang *et al.*, 2009). Culture or molecular biological investigations, routinely used in microbiology, are generally time-consuming and cumbersome when it comes to identifying the causative agents. Besides, it is not readily feasible to check for all causative agents using cultur-based or molecular assays. Recently, developments in novel analytical platforms, such as microarrays and biochips, along with significant advances in microfluidics have occurred. Hence, it is now possible to develop immunoassays to detect numerous antigens simultaneously on such platforms (Delehanty and Ligler, 2002; Anderson *et al.*, 2008) and this is now accepted as the ideal approach.

In order to take full advantage of such platforms specific antibodies to relevant targets for incorporation on these platforms must be developed. Production of monoclonal antibodies using classical methods is both laborious and time-consuming (Du *et al.*, 2010). Recombinant antibodies are a valuable potential alternative to monoclonal antibodies, and have advantages such as shorter generation-time, ease of production and the possibility for genetic modifications (section 1.2.2). Recombinant antibodies from different animal species were previously developed. The animal species used included humans (Jacobin *et al.*, 2002), mice (Dillon *et al.*, 2003), chickens (Hof *et al.*, 2008), pigs (Li and Aitken, 2004), rats (Sepulveda and Shoemaker, 2008), sheep (White *et al.*, 2001), llamas (Alvarez-Rueda *et al.*, 2007), guinea pigs (Hawlisch *et al.*, 2000),

macaques (Pelat *et al.*, 2007), alpacas (Maass *et al.*, 2007), sharks (Dooley *et al.*, 2003), rabbits (Chi *et al.*, 2002), and cows (O'Brien *et al.*, 2000; Koti *et al.*, 2010).

Among the various recombinant antibody formats the scFv is the most common format used due to its specificity and ease of manipulation (Du *et al.*, 2010). However, to date, there is no report on the generation of bovine scFvs using phage display. Consequently, this work was designed to generate a bovine scFv library and utilise it for selecting antibodies with specificities to different mastitis-causing pathogens.

The bovine immunoglobin repertoire is made up of IgG (IgG_1, IgG_2 and IgG_3 subtypes), IgA, IgM and IgE (Butler, 1998), with no evidence of IgD expression (Naessens, 1997). IgG1 is the predominant form of bovine Ig followed by IgG2, IgM and IgA (Butler, 1998). The bovine antibody light chain repertoire is dominated by lambda (λ) chains, even though a small population of kappa (κ) light chains are also expressed (Arun *et al.*, 1996). Bovine heavy chains are assumed to be responsible for antigen recognition. Exceptionally long CDRH3s in bovine antibodies are reported and they contribute significantly to antibody diversity (Saini *et al.*, 1999). Generally the length of CDRH3 ranges from 4 to 26 amino acids, however, CDRH3s, up to 61 amino acid residues have also been reported with restricted light chains (Saini and Kaushik, 2002). Long CDR's are reported to contain multiple cysteine residues which form intra-chain disulphide bonds forming loops which binds to the antigen, which is analogous to camelids (Desmyter *et al.*, 1996; Aitken *et al.*, 1999).

Where high rate somatic hypermutation (SHM) contributes to heavy chain Ig diversity, gene conversion is suggested to be the phenomena responsible for generating diversity in the bovine Ig light chain repertoire (Parng *et al.*, 1996; Lucier *et al.*, 1998). However, due to the high sequence similarity of the expressed light chains, it is presumed that they play a minimal role in antigen recognition (Sinclair *et al.*, 1995). Selection of particular sequences of light chains with long CDRH3s suggests they play a part as a supporting scaffold for antigen-antibody interactions mediated by bovine variable heavy chains.

6.2 Construction of a Bovine scFv Library

6.2.1 mRNA isolation and cDNA preparation from bovine spleens

The spleens from 4 cows, slaughtered as a result of chronic mastitis, were collected and the RNA extracted. The RNA was reverse transcribed to cDNA and these templates were used to generate a bovine scFv library (Figure 6.1).

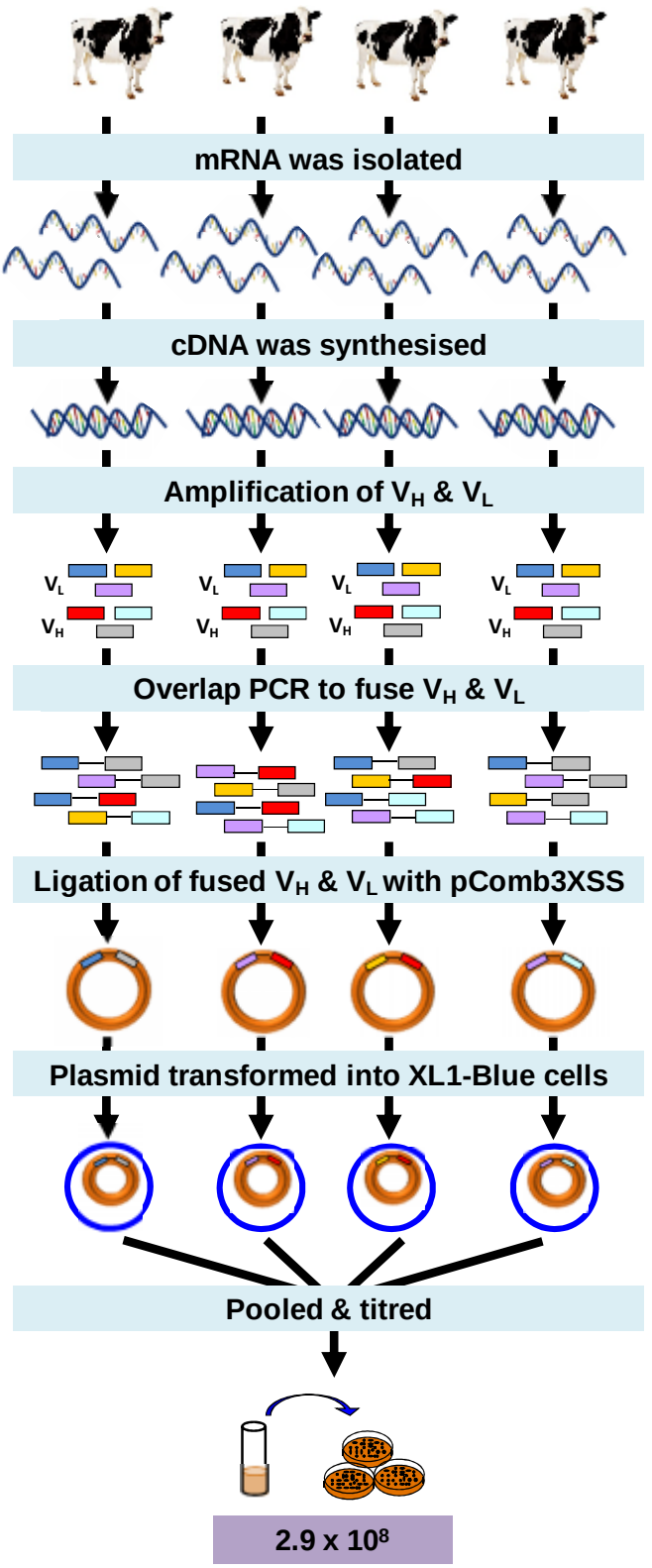


Figure 6.1. ***Strategic representation of procedures involved in constructing a bovine scFv library.***
A bovine scFv library was constructed using spleen tissues from 4 cows slaughtered as a result of chronic mastitis. A library with a diversity of 2.9 x 10^8 cfu/mL was generated.

6.2.2 Bovine variable heavy chain PCR amplifications

Optimisation of PCR reactions for amplification of variable heavy (V_H) chain from bovine spleen sample was carried out using different annealing temperatures. Non-specific bands were observed in the amplifications, which were subsequently reduced at higher temperatures (Figure 6.2). Thus, 63.4^{o}C was chosen to be used for further reactions. Further PCR optimisations were carried out by varying the $MgCl_2$ concentrations (0.5 to 4.5 mM) and using the annealing temperature at 63.4^{o}C. A concentration of 3 mM $MgCl_2$ was found to be optimal and was used for subsequent reactions (Figure 6.3).

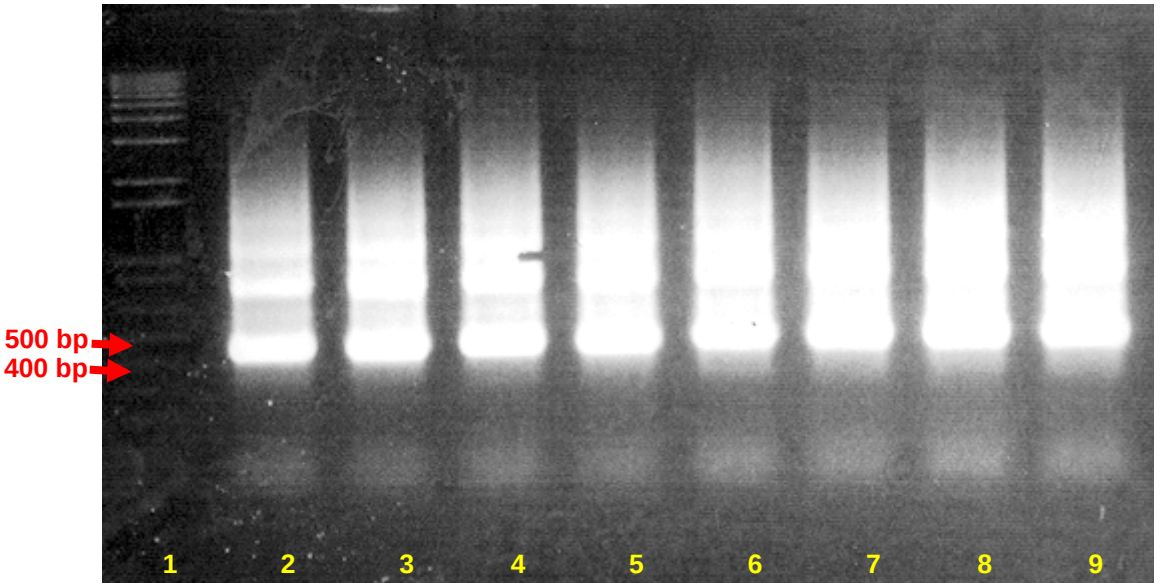


Figure 6.2. ***Optimisation of annealing temperature for amplification of bovine variable heavy chains.***
The cDNA from bovine spleen sample no. 1 was used as template for amplification of the V_H (~400 bp amplicon) chain. Eight different annealing temperatures (55.3^{o}C, 56.8^{o}C, 57.9^{o}C, 59.2^{o}C, 60.5^{o}C, 61.9^{o}C, 63.4^{o}C, and 64.8^{o}C) were tested to assess the optimal annealing temperature required for amplification of the V_H chain. Lane 1: 1 Kb plus DNA ladder; lanes 2-9: V_H amplifications with decreasing annealing temperatures (64.8^{o}C, 63.4^{o}C, 61.9^{o}C, 60.5^{o}C, 59.2^{o}C, 57.9^{o}C, 56.8^{o}C, and 55.3^{o}C, respectively).

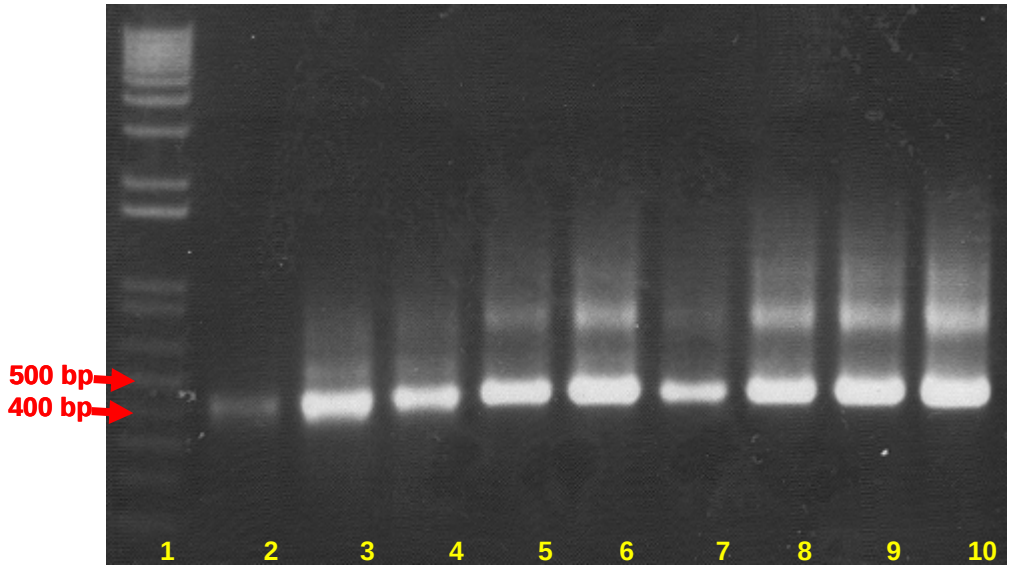


Figure 6.3. ***Optimisation of Mg^{2+} ion concentrations for amplification of bovine variable heavy chains.***
Nine different $MgCl_2$ concentrations (0.5 to 4.5 mM) were used to assess the optimal $MgCl_2$ concentration required for V_H amplification with minimum non-specific bands. Lane 1: 1 Kb plus DNA ladder; lanes 2-10: V_L amplifications with increasing $MgCl_2$ concentrations (0.5 to 4.5 mM in 0.5 mM increments).

6.2.3 Bovine variable light chain PCR amplifications

The cDNA synthesised from bovine spleens were used as templates for amplification of variable light (V_L) chains. Optimisation of PCR reactions for amplification of V_L regions was carried out using different annealing temperatures. cDNA from bovine spleen sample was used as template and 3 mM $MgCl_2$ concentration was used. The optimum annealing temperature was found to be 65.1^{o}C (Figure 6.4) and was subsequently used as the annealing temperature in all further PCRs for V_L amplification.

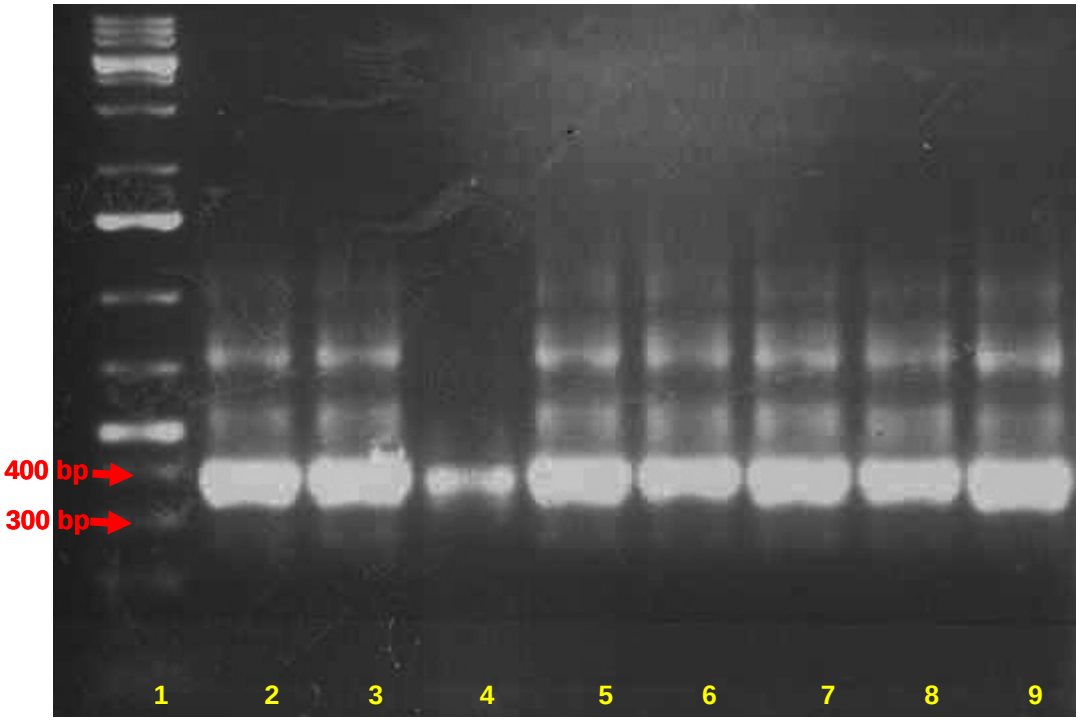


Figure 6.4. ***Optimisation of annealing temperature for amplification of bovine light heavy chains.***
cDNA from the bovine spleen sample no. 1 was used as template for amplification of V_L (~350 bp amplicon) chains. Eight different annealing temperatures (55^{o}C, 55.9^{o}C, 57.9^{o}C, 59.2^{o}C, 60.5^{o}C, 61.9^{o}C, 64.3^{o}C, and 65.1^{o}C) were tested to assess optimal annealing temperature required for amplification of V_L chain. Lane 1: 1 Kb plus DNA ladder; lanes 2-9: V_L amplifications with decreasing annealing temperatures (65.1^{o}C, 64.3^{o}C, 61.9^{o}C, 60.5^{o}C, 59.2^{o}C, 57.9^{o}C, 55.9^{o}C, and 55.0^{o}C, respectively).

6.2.4 Bovine SOE-PCR for assembling variable antibody genes

Purified V_H and V_L chains from respective spleen samples were mixed in equimolar ratio for coupling them together using a glycine-serine linker. The PCR conditions were optimised using eight different annealing temperatures (55^{o}C, 55.9^{o}C, 57.9^{o}C, 59.2^{o}C, 60.5^{o}C, 61.9^{o}C, 64.3^{o}C, and 65.1^{o}C) to yield an amplicon of approximately 750 bp (Figure 6.5). An annealing temperature of 64.3^{o}C was used for further SOE-PCR reactions.

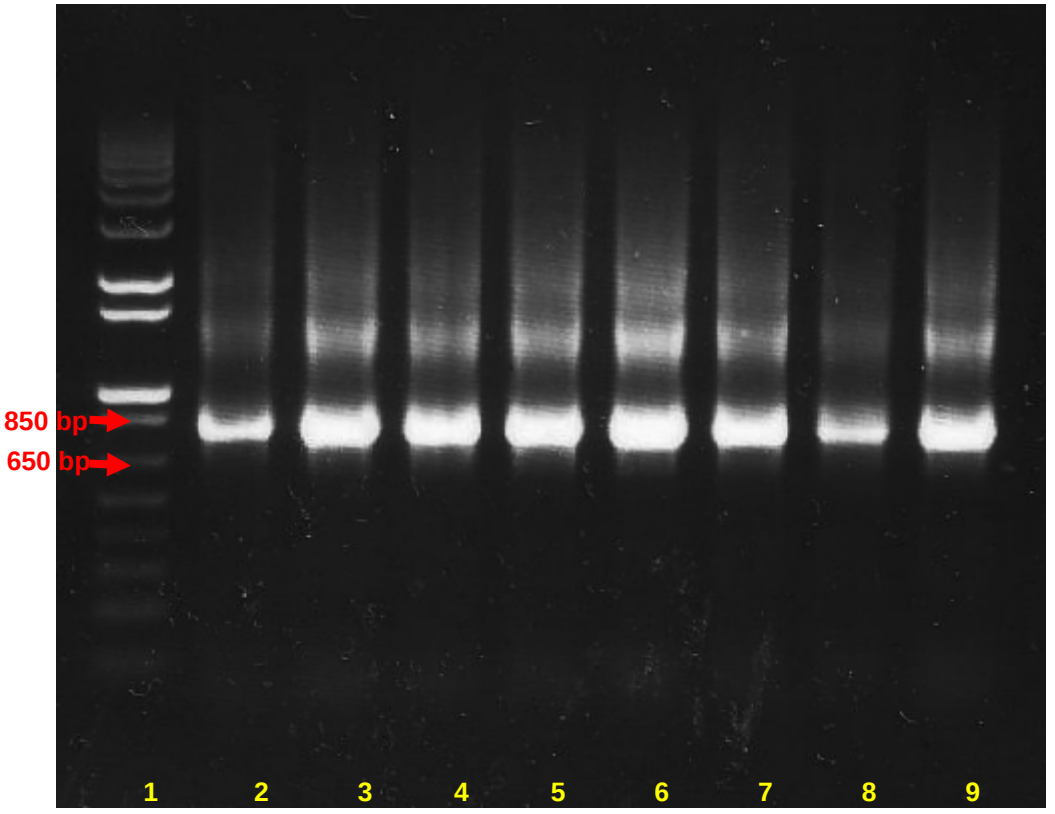


Figure 6.5. ***Optimisation of annealing temperature for bovine SOE-PCR.*** Purified V_H and V_L chains from bovine spleen sample no. 1 were mixed in equimolar ratios for SOE-PCR. The SOE product (~750 bp) yield was optimised using eight different annealing temperatures (55^{o}C, 55.9^{o}C, 57.9^{o}C, 59.2^{o}C, 60.5^{o}C, 61.9^{o}C, 64.3^{o}C, and 65.1^{o}C). Lane 1: 1 Kb plus DNA ladder; lanes 2-9: SOE amplifications with increasing annealing temperatures (55^{o}C, 55.9^{o}C, 57.9^{o}C, 59.2^{o}C, 60.5^{o}C, 61.9^{o}C, 64.3^{o}C, and 65.1^{o}C).

6.2.5 Bovine antibody library construction and biopanning for reactivity with peptidoglycan (PGN) from *Staphylococcus aureus*

Large-scale SOE-PCRs, from V_H and V_L chains amplified from all 4 bovine spleen samples, were performed. The amplicons were subsequently concentrated using ethanol precipitation and quantified using the Nanodrop ND-1000™. The SOE amplicons were then cloned into pComb 3XSS vector following *Sfi*I digest. The ligated products were transformed into XL1-Blue electroporation competent cells by electroporation. The transformation mixes

from all four bovine spleen samples were pooled to generate a library having a size of 2.9 x 10^8 cfu/mL.

Eight different clones were randomly picked from the constructed library and were checked for the presence of inserts for assessing the success of transformation. Bovine SOE primers were used to amplify inserts from the plasmids extracted from the clones. All eight clones showed the presence of inserts (Figure 6.6). The library was subsequently subjected to phage display biopanning against immobilised commercial peptidoglycan (PGN) from *Staphylococcus aureus* (Sigma-Aldrich, Ireland) (Table 6.1). *S. aureus*-PGN was chosen as there are several reports of it inducing significant immune responses in humans and animals (Verbrug *et al.*, 1980; Verbrug *et al.*, 1981; Wheat *et al.*, 1983; Kumar *et al.*, 2005).

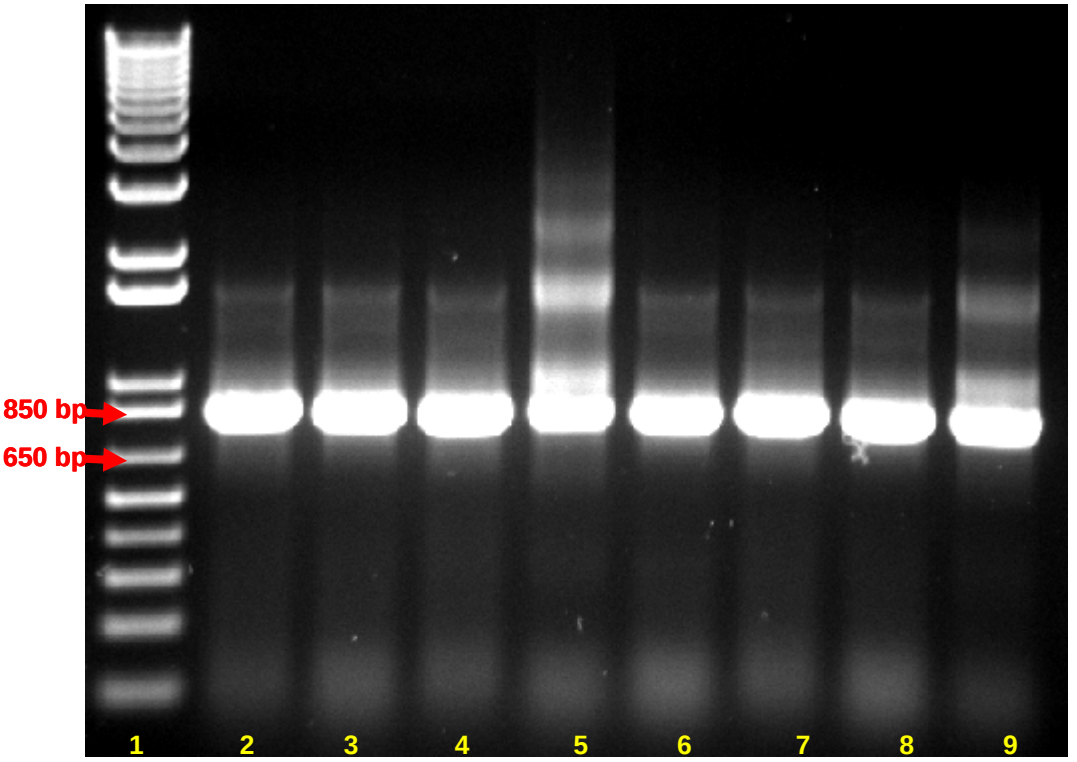


Figure 6.6. ***PCR for checking scFv inserts in the transformed bovine antibody library.***
Eight random clones from the transformed bovine antibody library were picked and plasmids were extracted from them. They were checked for scFv insert (~750 bp) using bovine SOE primers. Lane 1: 1 Kb plus DNA ladder; lanes 2-9: SOE from plasmids extracted from the clones.

Table 6.1. ***The phage input and output titres over the 5 rounds of biopanning of the bovine scFv library against*** **S. aureus-*PGN.***

Biopanning round	Input titre (cfu/mL)	Output titre (cfu/mL)
Round 1	2.1×10^{10}	9.12×10^{6}
Round 2	2.5×10^{10}	3.04×10^{5}
Round 3	4.2×10^{10}	3.76×10^{5}
Round 4	2.3×10^{11}	4.80×10^{5}
Round 5	8.2×10^{10}	6.1×10^{5}

6.2.6 Soluble expression and direct ELISA of bovine anti-*S. aureus*-PGN scFv clones

The precipitated output phage from all the five rounds of the biopanning (section 6.2.5) were tested for enrichment of anti-*S. aureus*-PGN scFvs using a polyclonal phage ELISA. Phage from an unpanned library and the helper phage

used were also checked in parallel as negative controls. No significant increase in signal was observed in any round. Subsequently, the phage outputs from round 5 were infected into TOP10F´ cells for soluble expression. One hundred and seventy seven colonies were picked from round 5, and were analysed using their expressed lysates in a direct soluble monoclonal ELISA format to evaluate biding to *S. aureus*-PGN. None of the clones showed specificity to *S. aureus*-PGN. Consequently, it was decided to use two different antigens, *S. aureus* crude extract and biotinylated *S. aureus* surface proteins, for biopanning (discussed in section 6.3).

6.2.7 Biopanning of bovine scFv library with *S. aureus* crude extract and biotinylated *S. aureus* surface proteins

The bovine scFv library (section 6.2.5) was subjected to phage display biopanning against immobilised *S. aureus* crude extract (Table 6.2) and biotinylated *S. aureus* surface proteins (Table 6.3).

Table 6.2. ***The phage input and output titres over the 5 rounds of biopanning of the bovine scFv library against a* S. aureus *crude extract.***

Biopanning round	Input titre (cfu/mL)	Output titre (cfu/mL)
Round 1	1.8×10^8	7.32×10^5
Round 2	2.2×10^8	1.76×10^5
Round 3	2.5×10^8	9.01×10^4
Round 4	1.3×10^8	4.80×10^5
Round 5	3.7×10^8	5.14×10^5

Table 6.3. ***The phage input and output titres over the 5 rounds of biopanning of the bovine scFv library against biotinylated*** **S. aureus** ***surface proteins.***

Biopanning round	Input titre (cfu/mL)	Output titre (cfu/mL)
Round 1	2.2×10^8	8.02×10^5
Round 2	3.1×10^8	2.94×10^5
Round 3	2.2×10^8	3.98×10^5
Round 4	1.8×10^8	2.56×10^5
Round 5	4.0×10^8	2.64×10^5

6.2.8 Bovine polyclonal phage ELISA

The precipitated output phage from all the five rounds of both the biopannings (section 6.2.7) were tested for enrichment using a polyclonal phage ELISA. Phage from an unpanned library and the helper phage used were also checked in parallel as negative controls. The scFv-displaying phage particles were detected using a HRP-conjugated mouse anti-M13 antibody (GE Healthcare Life Sciences, UK) and the absorbance read at 450 nm following 20 min incubation with TMB substrate. There was a definitive increase in signal in round 4 and 5 of pannings (Figure 6.7 and 6.8).

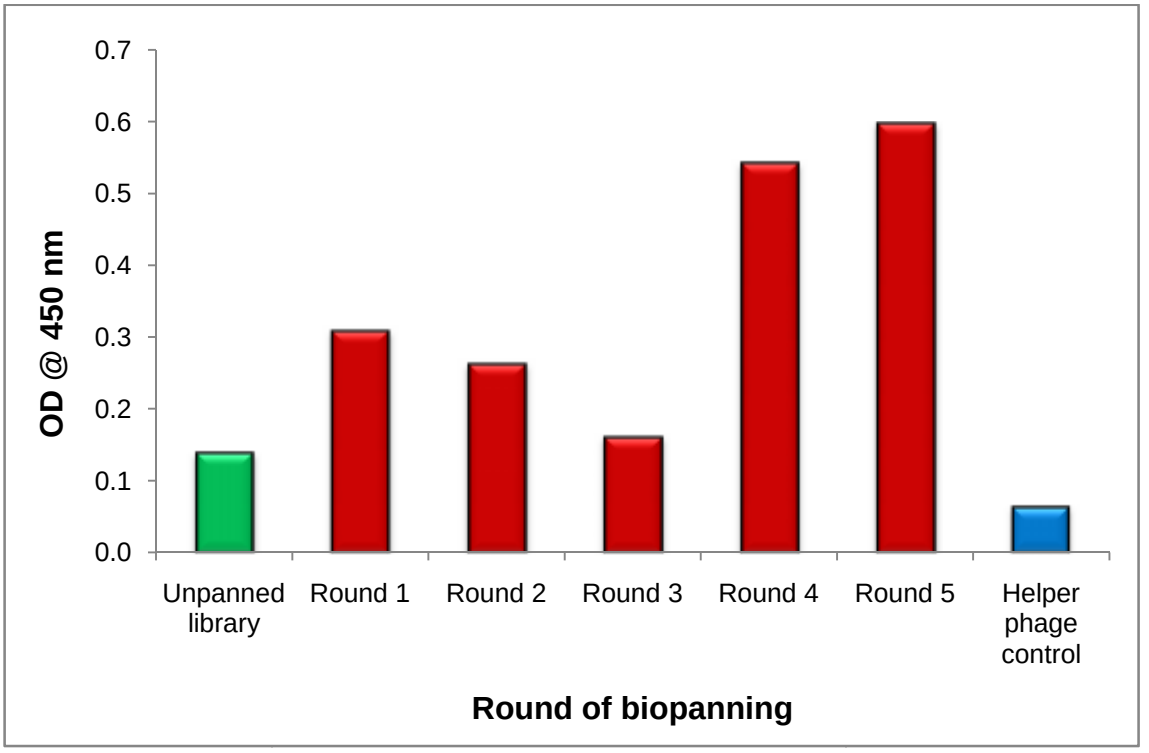


Figure 6.7. ***Polyclonal phage ELISA for biopanning against a*** **S. aureus** ***crude extract.***
The phage outputs from the successive rounds of biopanning, and the helper phage used in biopanning were tested against a *S. aureus* crude extract. The helper phage, used in the panning, was included as control to check for non-specific phage binding. The results show that only specific-phage were amplified against the antigen in consecutive rounds of panning, starting with the unpanned library.

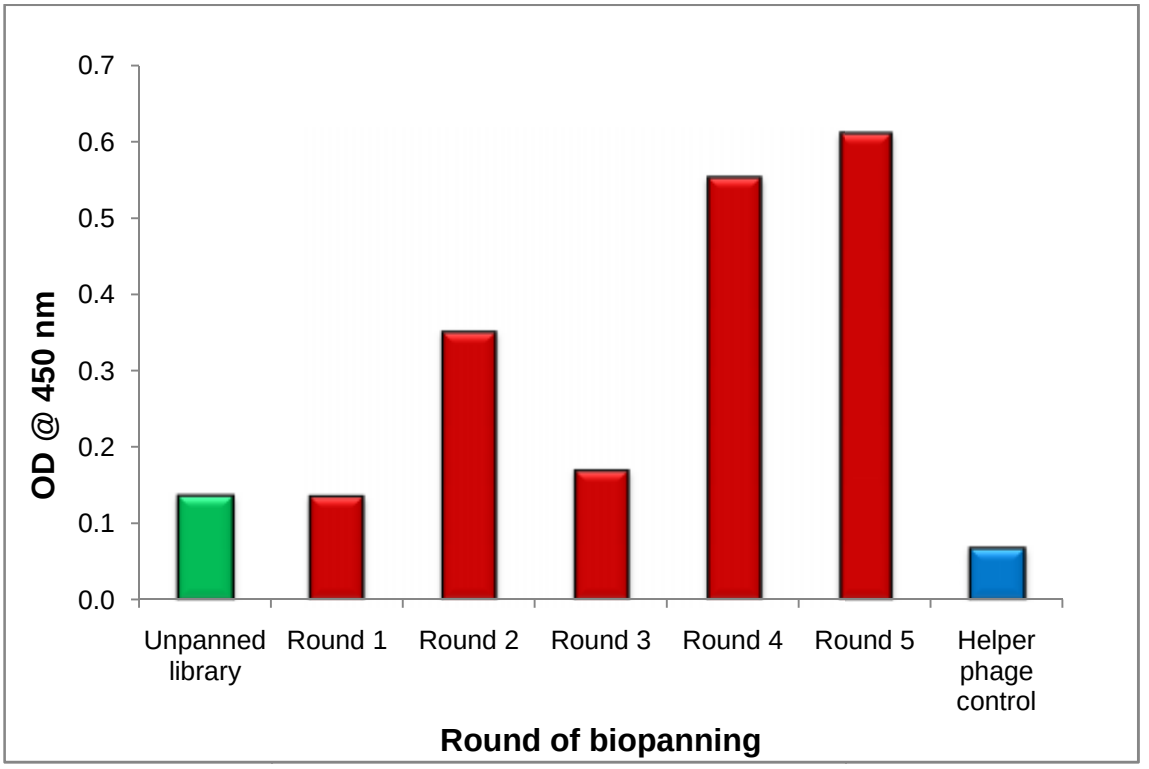


Figure 6.8. ***Polyclonal phage ELISA for biopanning against*** **S. aureus** ***surface proteins.***
The phage outputs from the successive rounds of biopanning and the helper phage used in biopanning were tested against *S. aureus* surface proteins. The helper phage, used in the panning, was included as control to check for non-specific phage binding. The results show that only specific-phage were amplified against the antigen in consecutive rounds of panning, starting from the unpanned library.

6.2.9 Soluble expression and direct ELISA of bovine scFv clones to *S. aureus* crude extract and *S. aureus* surface proteins scFv

The phage outputs from fifth rounds of bovine scFV library biopanings against an *S. aureus* crude extract and anti-*S. aureus* (biotinylated) surface proteins were infected into TOP10F´ cells for soluble expression. Eighty nine colonies were picked from both round 4 and 5 of each biopanning, and were analysed using their expressed lysates in a direct soluble monoclonal ELISA format to evaluate biding to a *S. aureus* crude extract and anti-*S. aureus* (biotinylated) surface proteins. None of the clones showed specificity to the antigens selected.

6.2.10 Analysis of anti-*S. aureus* crude extract and anti-*S. aureus* surface proteins scFv clones using PCR

None of the clones were found to be positive in monoclonal ELISAs against the anti-*S. aureus* crude extract or the anti-*S. aureus* surface proteins. Therefore, it was decided to check some clones with PCR to determine whether or not the clones contained the genome for the scFv or not. Ten random clones were picked from the round 5 of the biopanning with a *S. aureus* crude extract and *S. aureus* surface proteins (section 6.2.9) and alalysed by PCR using SOE primers (Figure 6.9 and 6.10). Thirty percent of clones were found positive for the scFv genome.

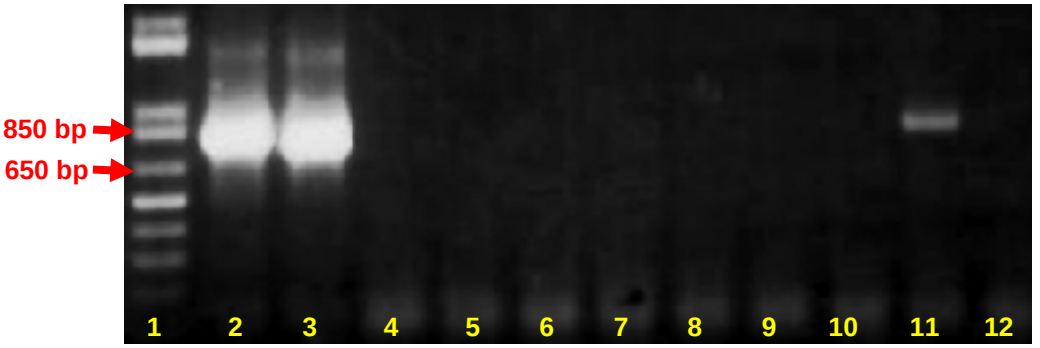


Figure 6.9. ***Colony pick PCR of round 5 biopanning, with*** **S. aureus** ***crude extract, for checking scFv gene.***
Ten random clones from round 5 biopanning, with *S. aureus* crude extract, were picked and analysed by PCR. They were checked for insert of ~ 750 bp using bovine SOE primers. Lane 1: 1 Kb plus DNA ladder; lanes 2-11: SOE-PCR analysis of 10 random clones; lane 12: negative control. Three clones (lane 2, 3 and 11) were positive for the scFv genome.

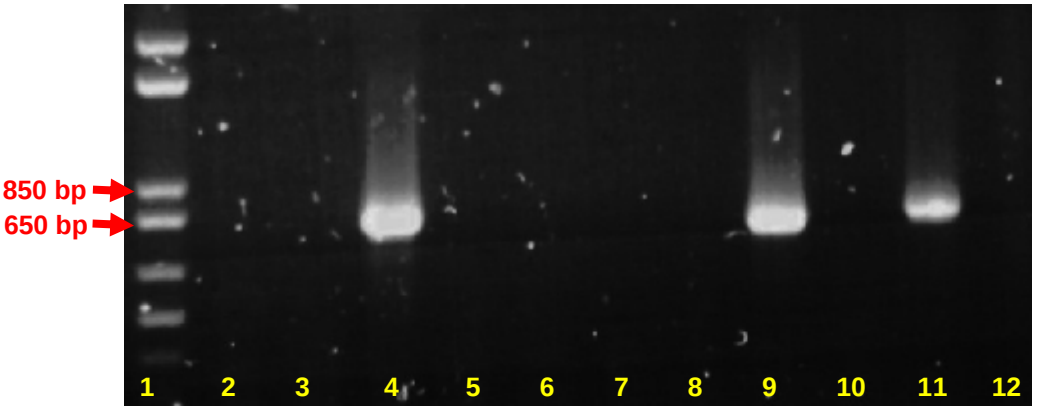


Figure 6.10. ***Colony pick PCR of round 5 biopanning, with*** **S. aureus** ***surface proteins, for checking scFv gene.***
Ten random clones from round 5 biopanning, with *S. aureus* surface proteins, were picked and analysed by PCR. They were checked for insert of ~ 750 bp using bovine SOE primers. Lane 1: 1 Kb plus DNA ladder; lanes 2-11: SOE-PCR analysis of 10 random clones; lane 12: negative control. Three clones (lane 4, 9 and 11) were positive for the scFv genome.

6.3 Discussion

Application of phage display libraries for selecting antibodies / antibody fragments offers an easier and effective approach for antibody generation compared to generation of monoclonal antibodies. For generation of phage display libraries, laboratory animals are usually chosen and are immunised with a single or a few antigens. However, there may be differences in the antigenic determinants recognised by the natural host immune system from those induced by immunisation of laboratory animals (O'Brien *et al.*, 1999). In addition, antibodies developed in natural hosts can be used as potential immunoprophylactic or immunotherapeutic agents. It is not always feasible to use the natural host, such as in the case of large animals, as they cannot be maintained in a laboratory. Therefore, spleen tissues from cattle reported to have died of mastitis were used, in this study, to generate recombinant antibody libraries.

The generation of bovine Fab libraries were previously attempted (O'Brien *et al.*, 1999; Kim *et al.*, 2004). However, there were issues with the bacterial expression of these Fabs (O'Brien *et al.*, 1999; O'Brien *et al.*, 2002; Kim *et al.*, 2004). Consequently, this study was planned to generate bovine scFvs, with a hope that they would have better expression characteristics, as reported previously (Rothlisberger *et al.*, 2005).

The bovine antibody library, generated in this study, offers the majority of the advantages of naïve antibody libraries. Like naïve antibody libraries, these libraries can be used to select antibodies against numerous antigens without *in vivo* immunisation of animals (Pansri *et al.*, 2009; Du *et al.*, 2010). Similarly, they require less time and are economical, as the costs for immunisations and maintaining animals are saved, when compared to libraries generated following *in vivo* immunisation of animals (Du *et al.*, 2010). However, it is a well known fact that unlike naïve antibody libraries, these libraries have the potential to produce antibodies of greater specificity and affinity, having undergone somatic mutation *in vivo* (O'Brien *et al.*, 1999). Such libraries can, in principle, be used for selecting antibodies to numerous antigens (such as various mastitis-causing pathogens, in this particular case). This is the first instance of use of phage display in generating bovine scFvs.

Spleens from four cows that died following chronic mastitis were obtained. It was highly likely that these cows had a diverse pool of antibodies against mastitis-causing pathogens. The total RNA was extracted from the spleens and converted to cDNA. Amplification of bovine antibody genes was attempted from the cDNA prepared, in order to obtain a generous antibody pool against

different mastitis-causing agents. Oligonucleotides were designed to amplify the variable heavy and the lambda fraction of variable light chains of bovine immunoglobulins, as the kappa fraction of variable light chains make up a very small fraction, *i.e.* 9%, of bovine light chains (Arun *et al.*, 1996). In addition, it is presumed that bovine variable light chains play a minimal role in antigen recognition (Sinclair *et al.*, 1995). Thus, single pairs of oligonucleotides were used for V_H and V_L, which was advantageous as any increase in the number of oligonucleotide sets decreases the amplification efficiency of antibody genes and may result in biased amplification of certain antibody genes (Imai *et al.*, 2006).

An scFv library with a diversity of 2.9 x 10^8 cfu/mL was generated using the selected oligonucleotides. The antibody repertoire of this library was as diverse as that of the other mammalian libraries, generated from hosts that were not immunised *in vivo*, which were reported to have diversities in the order of 10^8 to 10^9 (Vaughan *et al.*, 1996; Imai *et al.*, 2006; Pansri *et al.*, 2009). Eight random clones from the transformed bovine antibody library were picked and checked for the presence of the bovine scFv genome. All the clones checked were found to contain the scFv genome, signifying that the transformation was successful.

In order to demonstrate the utility of the generated bovine scFv library it was panned against commercial *S. aureus*-peptidoglycan. *S. aureus* was selected owing to the fact that it is the most prevalent contagious mastitis-causing pathogen worldwide (Fox *et al.*, 2001; Fox, 2009; Ote *et al.*, 2011). However, no anti-*S. aureus* PGN-specific clone could be selected by extensive biopanning. There is no report on the role of peptidoglycan in pathogenesis of *S. aureus*-

induced mastitis in bovines. Therefore, it is possible that there was no specific immune response against peptidoglycan itself. Thus, there was no antibody present against peptidoglycan in the generated antibody library pool or, even if there were any, the elution conditions were too stringent for their selection.

It was decided to use preparation(s) having a mixture of various *S. aureus* antigens, rather than using a very specific antigen, for biopanning. Two such antigens, a whole-cell *S. aureus* extract and *S. aureus* surface proteins, were selected and prepared. Whole-cell bacterial extracts were successfully used for anti-*S. aureus* antibody generation vaccine development (Cohen *et al.*, 1958; Oeding, 1960; Guidry *et al.*, 1994; Middleton, 2008) and are easy to prepare. Likewise, bacterial surface associated antigens are more likely to be more antigenic as they are first to come in direct contact to the host immune system (Ote *et al.*, 2011). These antigens are responsible for bacterial adherence to host cells and are crucial for infection (Ote *et al.*, 2011). Consequently, both, whole-cell *S. aureus* extract and biotinylated *S. aureus* surface proteins, were used for biopanning.

Five rounds of biopanning were carried out using immobilised whole-cell *S. aureus* extract and biotinylated *S. aureus* surface proteins. The polyclonal phage ELISA showed that there was a definitive increase in signals in round 4 and 5. However, no positive scFv clones were obtained in the monoclonal ELISAs. PCR analysis of 20 random clones (10 from round 5 biopan of both antigens) revealed that 30% of clones were carrying the genome for the bovine scFv. It seemed that the bovine scFv clones were not expressing in bacterial cells. Similar problems were encountered by certain research groups working

on generating and selecting bovine Fab fragments, using phage display technology (O'Brien *et al.*, 1999; O'Brien *et al.*, 2002; Kim *et al.*, 2004). Consequently, there is a need to determine the optimal growth conditions for maximal expression of bovine scFv clones, as was the case with bovine Fabs (O'Brien *et al.*, 2002).

Chapter 7
Production of Anti-Progesterone Antibodies

7. PRODUCTION OF ANTI-PROGESTERONE ANTIBODIES

7.1 Introduction

The bovine oestrous cycle is usually approximately 21 days in length, however, it can range from 17 to 24 days. The cycle consists of a luteal phase, from day 1 to day 17, and a follicular phase, from day 18 to day 21. The cycle starts with oestrus, caused by secretion of high levels of oestrogen. The duration of peak oestrogen secretion lasts between 4 to 27 h (average length 18 h), with ovulation happening 24 to 32 h after the initiation of oestrus. After the release of the ovum following the rupture of ovarian follicles, the follicle is modified to become the corpus luteum (CL). The CL act as a gland and releases the hormone progesterone (pregn-4-ene-3,20-dione; P4), which controls the luteal phase of the oestrous cycle (Hussein *et al.*, 1992). If fertilisation does not occur, regression of the CL (luteolysis) occurs at about day 17 of the cycle, allowing another follicle to mature and ovulate and, subsequently, another cycle starts. Alternatively, if fertilisation occurs, and the cow becomes pregnant, the CL is maintained and it continues the secretion of P4, which is essential for the establishment and maintenance of pregnancy.

This P4, produced by the CL, is secreted into the blood, and is subsequently found in different bovine fluids and tissues, such as the adrenal glands (Beall and Reichstein, 1938; Gorski *et al.*, 1958; Melampy *et al.*, 1959), non-luteal ovarian tissues (Gorski et al. 1958), ovarian follicular fluid (Edgar, 1953; Short, 1962), the placenta (Melampy *et al.*, 1959), amniotic fluid (Melampy *et al.*, 1959), allantoic fluid (Melampy *et al.*, 1959), body fat (McCracken, 1963) and milk (Pope and Swinburne, 1980).

P4 has an affinity towards milk fat because of the lipophilic character of steroids (Dobson *et al.*, 1975). Therefore, the concentration of P4 is higher in milk than blood. Concentrations in blood and milk of P4 change during the oestrous cycle (Simersky *et al.*, 2007). However, their relative values are closely correlated. Consequently, P4 concentrations in milk and blood are extremely helpful in monitoring ovarian activity, and, hence, the reproductive status of the cow (Nebel, 1987), *e.g.* for confirmation of oestrus in cattle (Pieterse and Van de Wml, 1981; Foulkes *et al.*, 1982). Changes in blood and / or milk progesterone concentrations are also used extensively for pregnancy detection (Shemesh *et al.*, 1973; Pennington *et al.*, 1985; Faustini *et al.*, 2007). Development of follicular or luteal cysts is characterised by typical changes in P4 concentrations. Consequently, estimation of P4 can be also used for the detection of such reproduction-affecting conditions (Hruska *et al.*, 1983; Nakao *et al.*, 1983; Sprecher *et al.*, 1988; Ruiz *et al.*, 1992).

Mastitis has always been associated with decreased fertility and associated conditions (Moore *et al.*, 1991; Barker *et al.*, 1998; Risco *et al.*, 1999; Schrick *et al.*, 2001; Santos *et al.*, 2004; Gunay and Gunay, 2008; Ahmadzadeh *et al.*, 2009). Likewise, the concentrations of P4 in milk are also reported to be affected by mastitis (Fayemi *et al.*, 1982; Anderson *et al.*, 1983; Laitinen, 1986). However, there are contradictory reports on the effects of mastitis on milk P4 levels. Decreased milk P4 levels were reported some researchers (Fayemi *et al.*, 1981; Kassa *et al.*, 1986). However, conversely, elevated milk progesterone levels were reported following the incubation of *Staphylococcus aureus* (in milk) with exogenous progesterone. However, similar incubations with

Staphylococcus epidermidis, alpha-hemolytic streptococcus or *Escherichia coli* had no effect (Fayemi *et al.*, 1982).

Currently, evidence on the effect of mastitis on progesterone concentrations in milk is inconclusive (section 7.1). However, studies done, to date, demonstrate its potential as a possible marker for mastitis detection along with its long standing uses in detection of pregnancy and in evaluation of reproductive status and associated pathological conditions.

7.2 Production of Anti-Progesterone Antibody

7.2.1 Immunisations

The ability of several hosts to generate antibodies to progesterone was examined. Following repeated immunisations chicken failed to generate significant response to the progesterone-3-CMO-BSA conjugate or the carrier BSA alone. The antibody serum titre of the rabbit immunised with 'in-house' progesterone-3-CMO-BSA conjugate (after boost 5) was found to be 1:32,000 (Figure 7.1). The antibody serum titre for rabbit immunised with commercial progesterone-3-CMO-BSA conjugate (after boost 2) was found to be 1:800,000 (Figure 7.2).

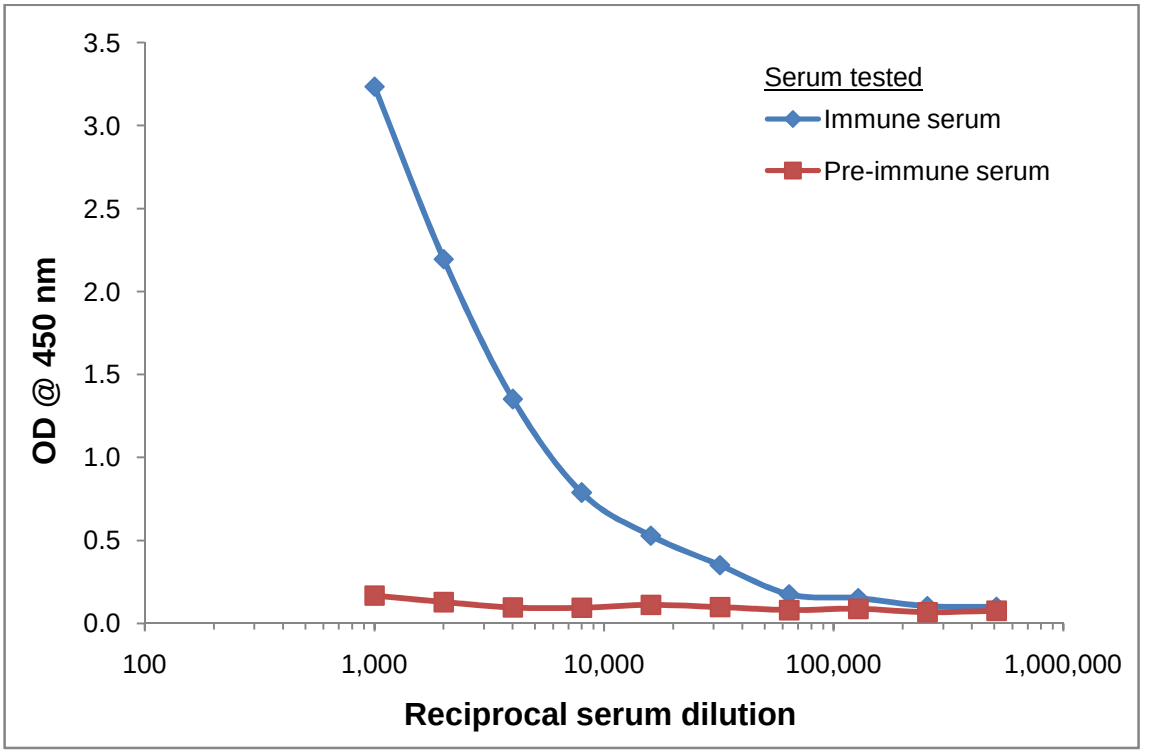


Figure 7.1. ***Evaluation of the antibody response in rabbit following administration of 'in-house' generated progesterone-3-CMO-BSA conjugate.***
After 5 boosts the rabbit serum antibody titre was checked against commercial progesterone-3-biotin using a series of serum dilutions. Similar dilutions of rabbit pre-immune serum were analysed. It is evident that a serum antibody titre of greater than 1:32,000 was achieved for the progesterone conjugate.

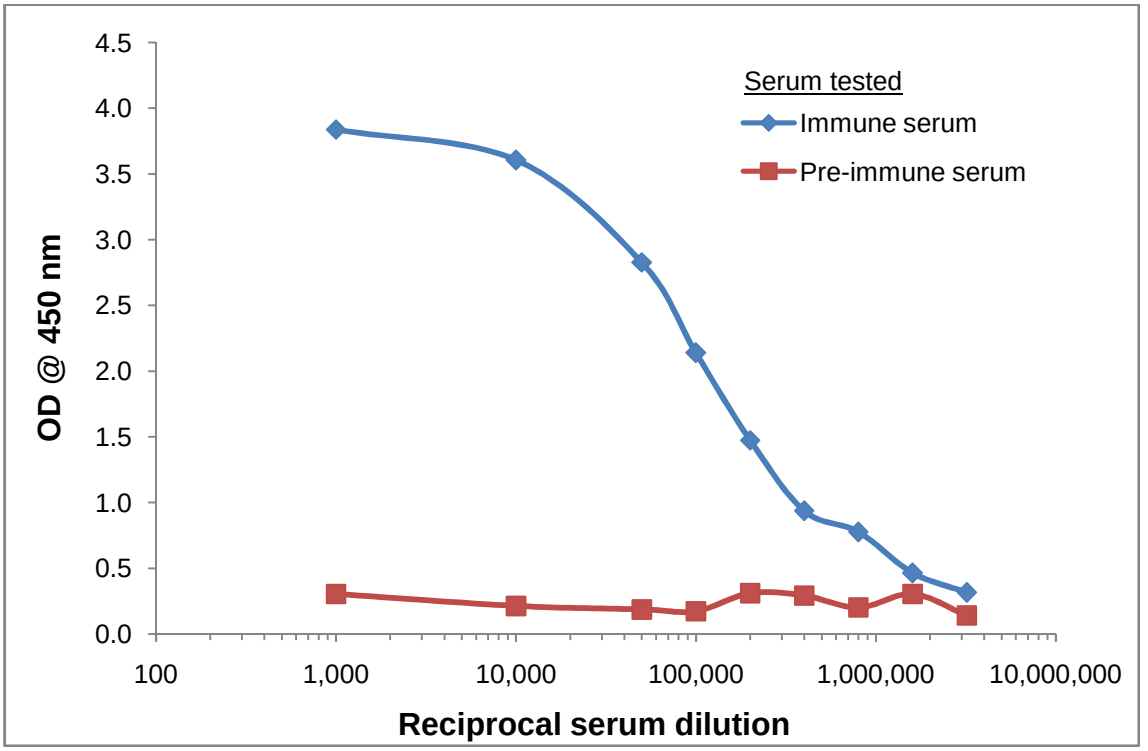


Figure 7.2. ***Evaluation of the antibody response in rabbit following administration of commercial progesterone-3-CMO-BSA conjugate.***
After 2 boosts the rabbit serum antibody titre was checked against commercial progesterone-3-biotin using a series of serum dilutions. Similar dilutions of rabbit pre-immune serum were analysed. It is evident that a serum antibody titre of greater than 1:800,000 was achieved for the progesterone conjugate.

7.2.2 Anti-progesterone polyclonal antibody purification from rabbit serum

Polyclonal antibodies generated by both rabbits were purified. The yield of purified antibody was found to be approximately 2 mg/mL. Purified antibodies were analysed on SDS-PAGE gel to determine purity. Two bands were obtained at ~ 50 kDa and ~ 23 kDa, for both the heavy and light chain of antibodies, due to reduction of the disulphide bridges (Figure 7.3).

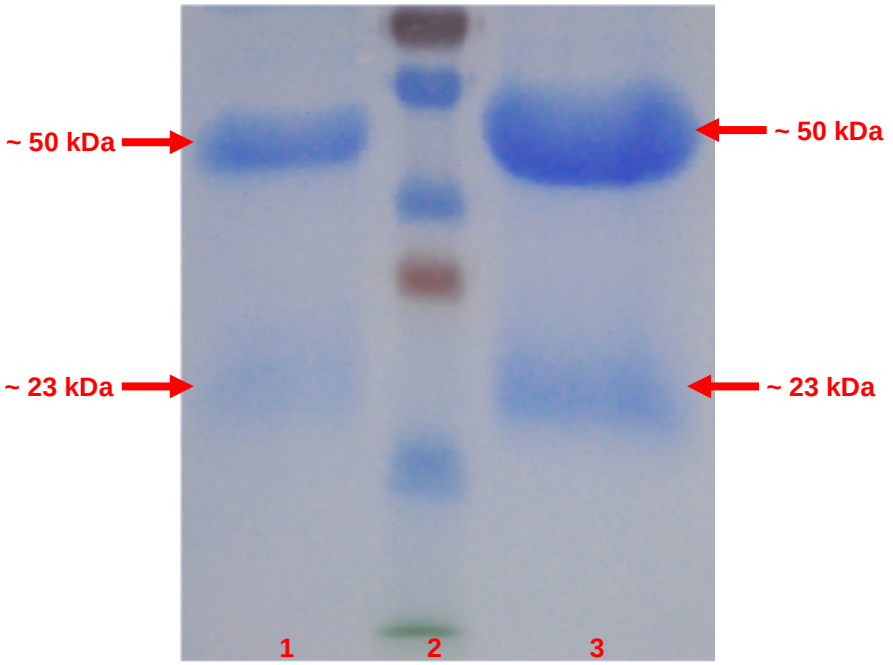


Figure 7.3. ***SDS-PAGE analysis of purified rabbit anti-progesterone polyclonal antibodies.***
Purified polyclonal antibodies from both the rabbits were analysed by SDS-PAGE electrophoresis for the presence of specific size bands. Lane 1: purified polyclonal antibody from rabbit immunised with 'in-house' generated progesterone-3-CMO-BSA conjugate; lane 2: PageRuler™ Plus Prestained Protein Ladder; and lane 3: purified polyclonal antibody from rabbit immunised with commercial progesterone-3-CMO-BSA conjugate. Two bands at approximately 50 kDa and 23 kDa were observed for heavy and light chains, respectively.

7.2.3 Checkerboard ELISA for estimation of the optimal progesterone-3-biotin coating concentration

A checkerboard ELISA was performed to assess the best coating concentration of progesterone-3-biotin for subsequent ELISA. No significant difference was observed for the different coating concentrations (section 2.2.8.5) tested (Figure 7.4). Subsequently, the lowest antigen concentration (0.25 µg/mL) was chosen for further study.

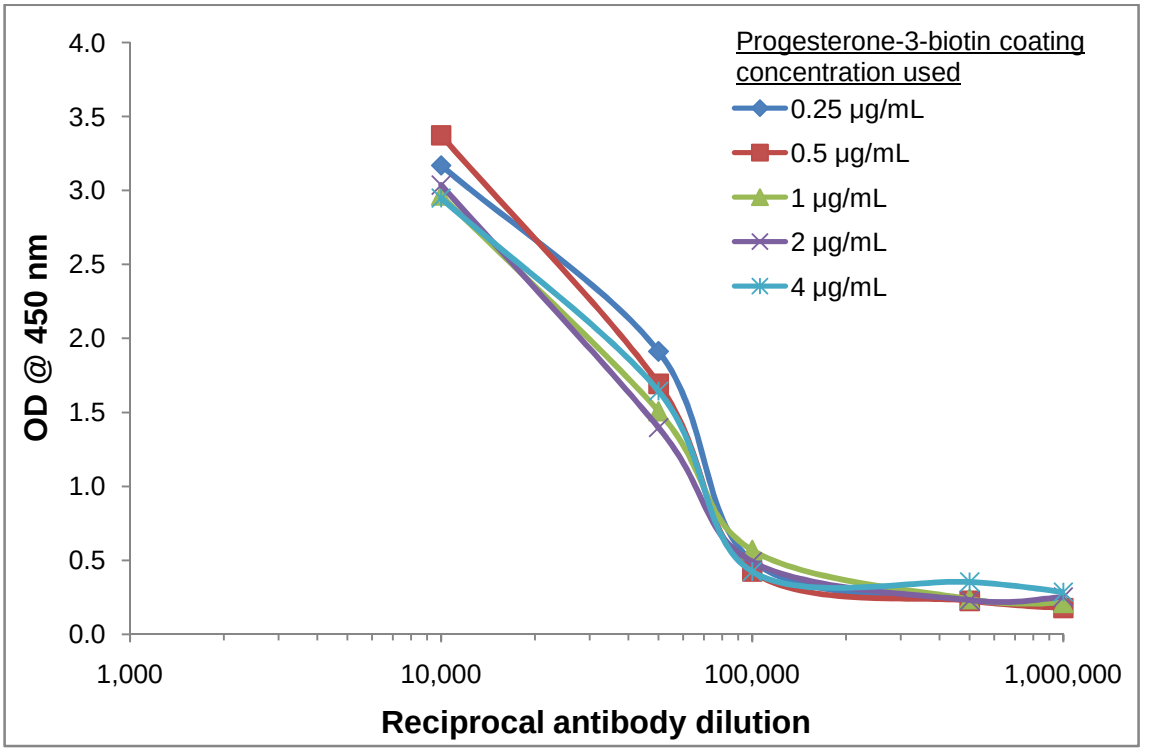


Figure 7.4. ***Checkerboard ELISA for assessing optimal antigen concentration of commercial progesterone-3-biotin conjugate.***
Five different concentrations of commercial progesterone-3-biotin were tested to assess the optimal antigen concentration for antibody binding. Five different antibody dilutions (1:10,000, 1:50,000, 1:100,000, 1:500,000 and 1:1,000,000) and a negative control (1% (w/v) skim milk-BSA-PBS) were tested against each antigen. The optimal coating concentration of progesterone-3-biotin was found to be 0.25 µg/mL.

7.2.4 ELISA to check titres of purified rabbit polyclonal antibody preparations against progesterone

Purified antibodies from both the rabbits were titred against progesterone-3-biotin. The use of progesterone-3-biotin enabled detection of antibody titres of only progesterone and not the carrier (BSA). The titre for antibodies purified from the rabbit immunised with the 'in-house' progesterone-3-CMO-BSA conjugate was found to be 1:32,000 (Figure 7.5), whereas, the titre for antibodies purified from the rabbit immunised with commercial progesterone-3-CMO-BSA conjugate was found to be 1:1,600,000 (Figure 7.6).

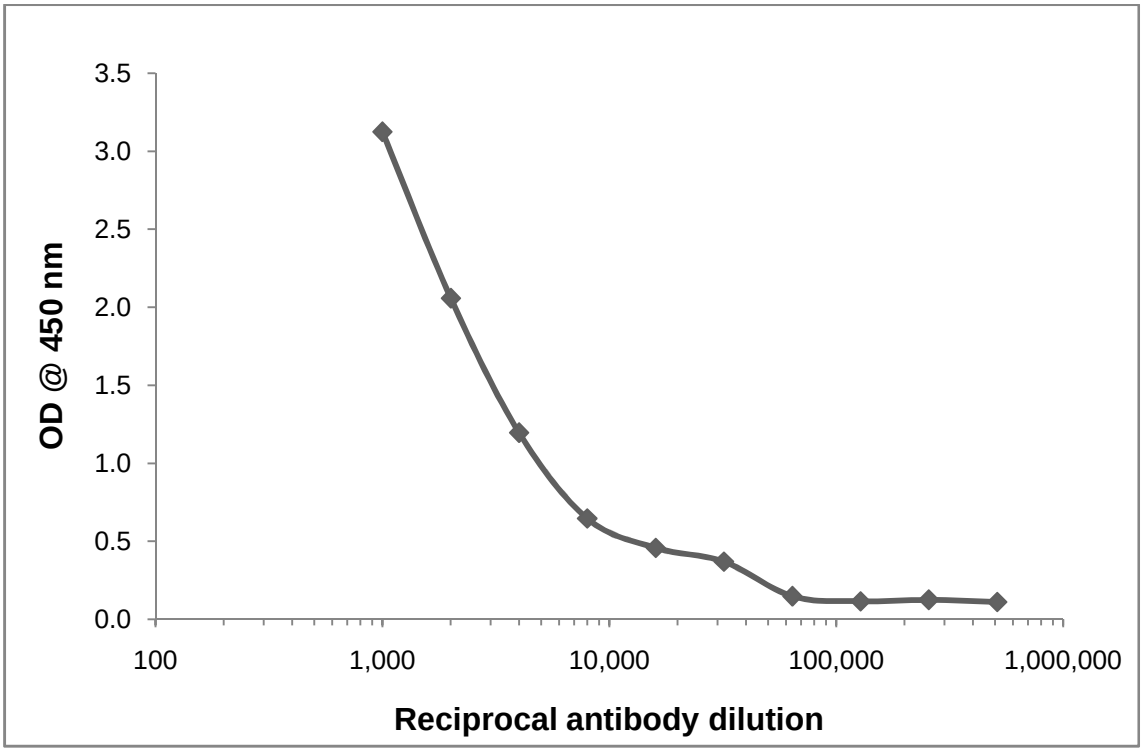


Figure 7.5. ***Titres of purified anti-rabbit polyclonal antibody (raised against 'in-house' generated progesterone-3-CMO-BSA conjugate) preparation.***
Anti-rabbit progesterone antibodies raised against the 'in-house' prepared progesterone-3-CMO-BSA conjugate were purified and titred, against progesterone-3-biotin, using a series of dilutions of sera. The titre was estimated to be 1:32,000.

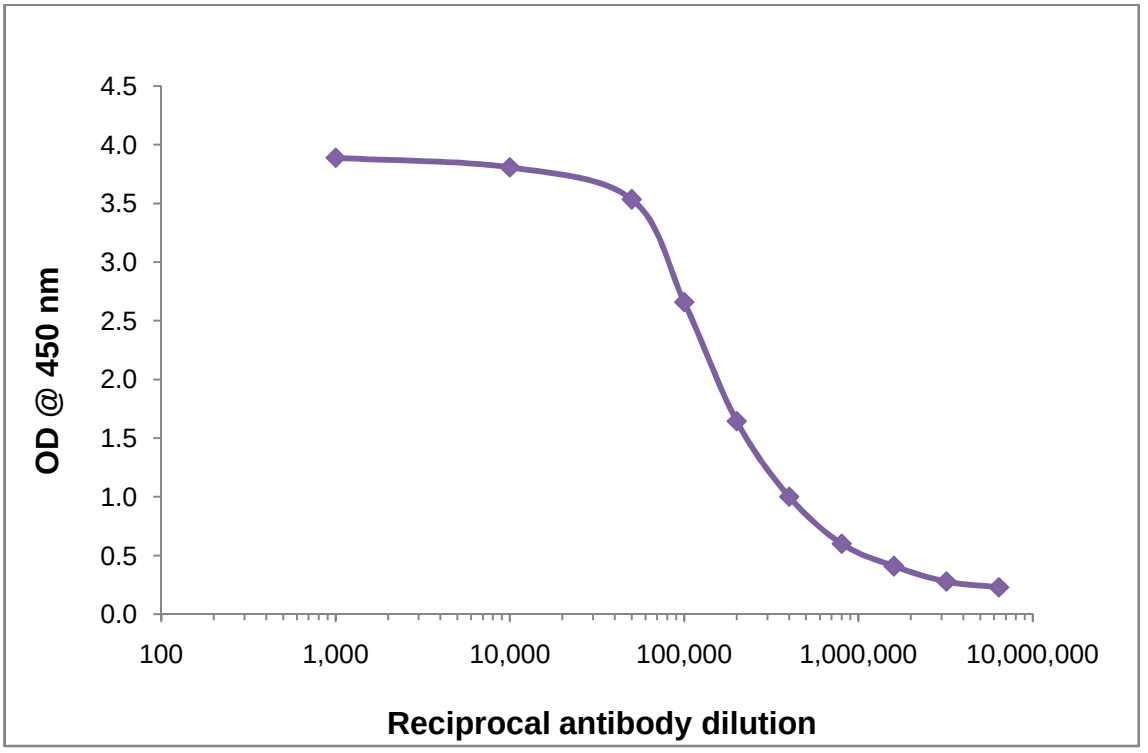


Figure 7.6. ***Titres of purified anti-rabbit polyclonal antibody (raised against commercial progesterone-3-CMO-BSA conjugate) preparation.***
Anti-rabbit progesterone antibodies, raised against commercial progesterone-3-CMO-BSA conjugate, were purified and titred against progesterone-3-biotin, using a series of dilutions of sera. The titre was estimated to be 1:1,600,000.

Antibodies purified from the rabbit immunised with commercial progesterone-3-CMO-BSA conjugate were used for further studies as their titre was found to be significantly higher than the titre for antibodies purified from the rabbit immunised with the 'in-house' progesterone-3-CMO-BSA conjugate.

7.2.5 ELISA to assess the best blocking solution to be used in the assay for detection of progesterone in milk

Five different blocking agents (section 2.2.8.7) were tested in ELISA format. A high background was observed for three of the blocking agents tested (5% (w/v) HSA-PBS, 5% (w/v) FG-PBT and 5% (v/v) PBST). Thus, these were not suitable for the assay (Figure 7.7). In addition, 5% (v/v) PBST was not an effective blocking agent (Figure 7.7). The remaining two blocking agents (5%

(w/v) skim milk-PBS and 5% (w/v) OVA-PBS) were found to be effective with reasonable background values. However, as the assay will be used to detect progesterone concentrations in milk, it was decided to use 5% (w/v) OVA-PBS.

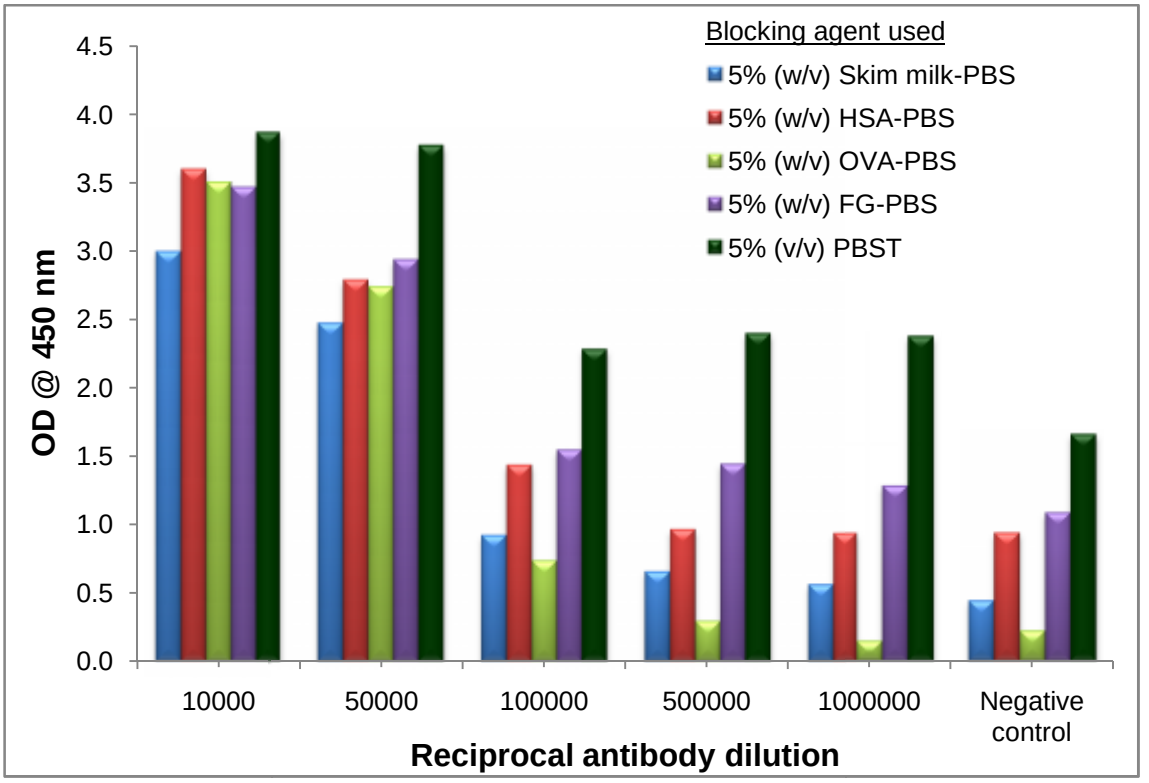


Figure 7.7. ***ELISA for assessing the best blocking solution to be used in the assay for detection of progesterone.***
Five different blocking solutions (5% (w/v) skim milk-PBS, 5% (w/v) HSA-PBS, 5% (w/v) OVA-PBS, 5% (w/v) FG-PBS and 5% (v/v) PBST) were tested to assess the best blocking solution to be used in the assay for detection of progesterone in milk. Five percent (w/v) OVA-PBS and 5% (w/v) skim milk-PBS were found to be the best blocking solutions.

7.2.6 Titration of purified rabbit polyclonal antibodies and inhibition ELISA

Titration ELISA of purified rabbit polyclonal antibodies was assessed using 5% (w/v) OVA-PBS as the blocking agent (section 2.2.8.6). A graph was plotted by using antibody dilution on the X-axis and absorbance on the Y-axis and the midpoint was determined for purified rabbit pAbs (Figure 7.8). The midpoint,

1:100,000 in this case, represents the dilution at which the pAbs are most sensitive to the antigen.

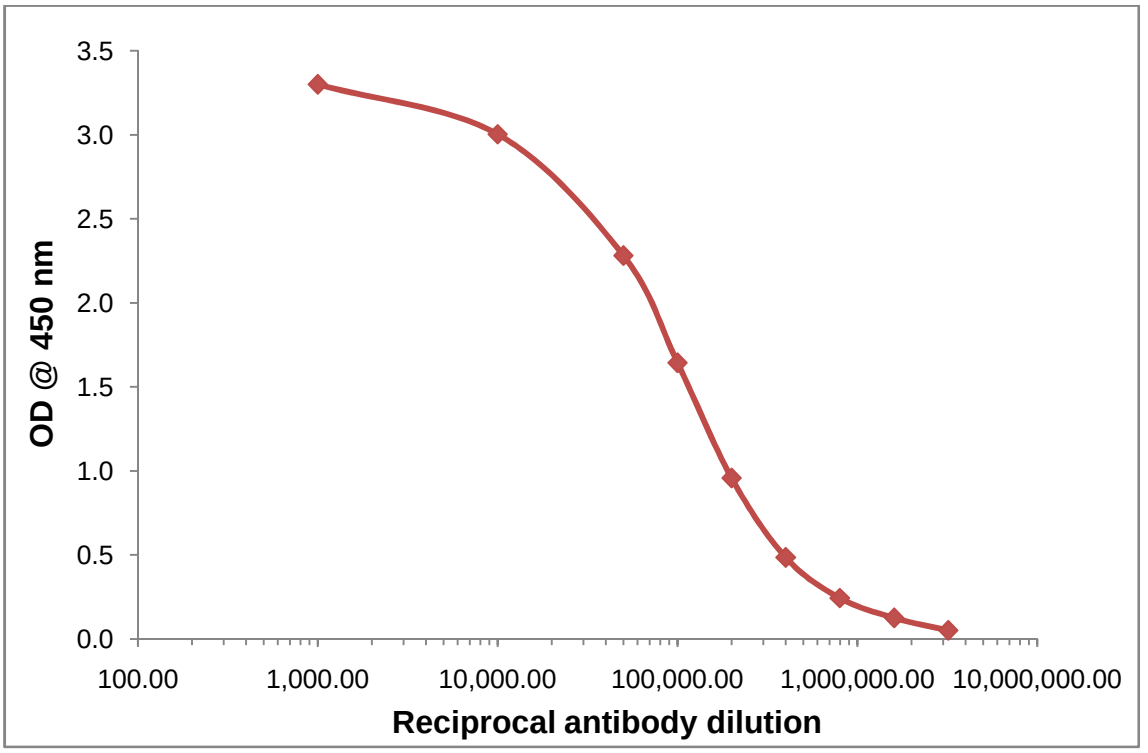


Figure 7.8. ***Titres of purified anti-rabbit polyclonal antibody (raised against commercial progesterone-3-CMO-BSA conjugate) preparation.***
Anti-rabbit progesterone antibodies raised against commercial progesterone-3-CMO-BSA conjugate were purified and titred, against progesterone-3-biotin, using a series of dilutions of sera. Five percent (w/v) OVA was used as blocking agent. The titre was estimated to be 1:1,600,000.

In an inhibition assay the free progesterone in solution competes with the immobilised progesterone, on the plate, for binding the antibody (in this case rabbit pAb). Different dilutions of antigen, ranging from 8,000 to 7.81 ng/mL, were used. Intraday (three assays on the same day) validations were carried out to evaluate experimental variations. The standard deviation was calculated for the A/A_0 value of each antigen concentration representing the inhibition of the rabbit pAb to free antigen in solution. Initial analysis shows the assay can detect levels down to 125 ng/mL of progesterone. However, further studies to accurately calculate the precision of the assay are ongoing.

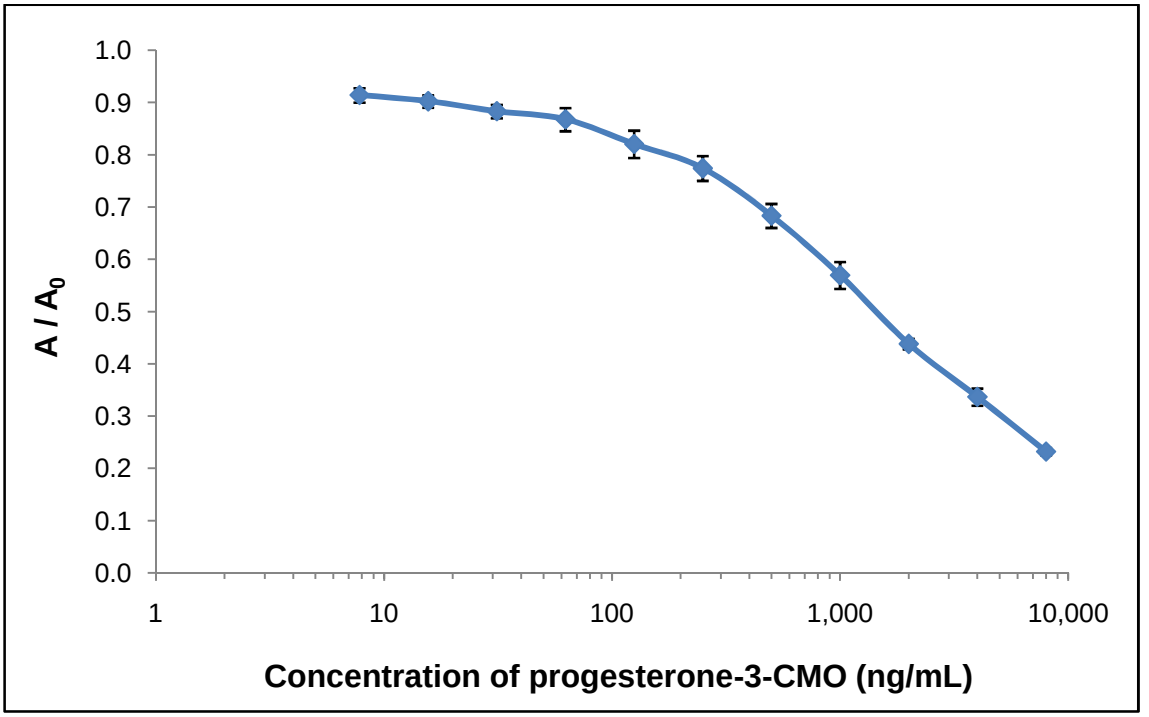


Figure 7.9. ***Inhibition ELISA of purified rabbit anti-progesterone polyclonal antibodies (raised against commercial progesterone-3-CMO-BSA conjugate).***
Three inhibition ELISAs were carried, in triplicate, on purified anti-rabbit polyclonal antibody (raised against commercial progesterone-3-CMO-BSA conjugate). Antibody was diluted to 1:50,000 and different concentrations of free progesterone-3-CMO, ranging from 8,000 ng/mL to 7.81 ng/mL, were added, in 1:1 ratio, to the antibody dilution (final dilution 1:100,000). The antibodies were found to be inhibiting, detecting up to 125 ng/mL of progesterone. The standard deviation (represented by the error bars on the graph) in the intraday analysis was within acceptable limits.

7.3 Discussion

Mastitis and inadequate reproductive performance are the two most common maladies affecting the dairy industry worldwide (Claycomb *et al.*, 1998; Viguier *et al.*, 2009). Progesterone is a well known marker for pregnancy diagnosis and detection of reproductive problems (section 7.1). In addition, fluctuations in milk progesterone concentrations were associated with mastitis (section 7.1). Milk contains higher progesterone concentrations than serum, and this makes it a good matrix to use for progesterone analysis (section 7.1). Consequently, progesterone concentrations in milk can aid in the timely detection of the two most commonly encountered pathological conditions in dairy cows (section 7.1).

Thus, the current study was undertaken to generate high-performance anti-progesterone antibodies, with the eventual aim of generating a very rapid assay.

Progesterone, being a small molecular weight hormone, is not a good immunogen. Therefore, it must be chemically conjugated to carriers, such as bovine serum albumin (BSA), ovalbumin (OVA) or keyhole limpet hemocyanin (KLH), to make it immunogenic. The progesterone-3-CMO-BSA conjugate was selected as the immunogen, in this study, as there are several reports of its successful use, as immunogen, for anti-progesterone antibody development (Basu *et al.*, 2006; Karir *et al.*, 2006; Shrivastav *et al.*, 2007; Khatun *et al.*, 2009; Shrivastav *et al.*, 2010). The chicken immunised with commercial progesterone-3-CMO conjugate failed to generate a significant immune response to the immunogen.

For antibody screening a different conjugate, with another carrier protein attached to the hapten, is always necessary, as screening antigen for hapten-specific antibody (Danilova, 1994; Branaa *et al.*, 1999). For screening of antibody response to small haptens, such as progesterone, it is beneficial to use a conjugate differing from the immunogen with respect to both the carrier protein and the chemical linkage with the hapten (Danilova, 1994). This was achieved using progesterone-3-biotin as screening antigen. Progesterone-3-biotin contains biotin directly conjugated to the progesterone molecule rather than through the CMO linker.

The rabbits immunised with the ‘in-house’ produced and commercial progesterone-3-CMO-BSA conjugates generated very high antibody titres to the conjugates (Figures 7.1 and 7.2) following immunisations, when compared to several other studies (Silvan *et al.*, 1993; Karir *et al.*, 2006). The anti-progesterone antibodies were successfully purified using protein G affinity chromatography. Analysis of purified antibodies revealed the presence of light and heavy chain bands at the reported sizes for rabbit IgG (Hatta *et al.*, 1993; Sun *et al.*, 2001). However, low yields of antibodies were recovered. Protein A/G resin will be used to further improve purification yields.

The blocking agents (BSA and skim milk) typically used for ELISA cannot be used for developing a progesterone inhibition ELISA. The conjugate used for immunisation contained BSA and, thus, BSA cannot be used. In addition, this assay will be finally employed to detect progesterone concentrations in milk, hence, milk cannot be used for blocking. Therefore, studies were conducted to assess the best blocking agent for use in the progesterone inhibition ELISA and 5% (w/v) OVA-PBS was selected.

The inhibition ELISA assay revealed that the polyclonal antibody were could detect 125 ng/mL of progesterone. This detection level is low when compared to certain reported assays, where detection levels of as low as 0.11 ng/mL were reported (Basu *et al.*, 2006; Shrivastav *et al.*, 2007; Shrivastav *et al.*, 2010). However, these antibodies were purified from rabbit serum collected after only 2 boosts. Further boosts will be given to the rabbit to ensure a better immune response and detection levels. In case, the polyclonal antibodies fail to achieve high sensitive, the spleen and bone marrow from this rabbit can be used to

generate an antibody library that can be utilised to select high-affinity anti-progesterone scFvs. Ayyar (2011) reported isolation of high-affinity scFvs from rabbit exhibiting high polyclonal antibody titre. In addition, mice are being immunised with commercial progesterone-3-CMO-BSA. These mice are responding well to the immunogen (data not shown) and will be used to generate mouse anti-progesterone scFvs.

Chapter 8
Conclusions

8. CONCLUSIONS

Mastitis is a major disease affecting cattle worldwide and is the costliest disease in the dairy sector. Mastitis causes losses in animal production and may lead to animal deaths and is also a threat to public health. Many tests for direct and indirect detection of subclinical mastitis are available. However, most of the available tests have their drawbacks (Chapter 1). Consequently, the proposed research, which was part of a multidisciplinary project, was aimed at developing a rapid immunodiagnostic kit for mastitis detection, using high-affinity antibodies against mastitis biomarkers and mastitis-causing pathogens. Two different mastitis biomarkers, *N*-acetyl-β-D-glucosaminidase (NAGase), a well established marker, and progesterone, a potential marker, were chosen for this study. For generating antibodies against mastitis-causing pathogens, a novel approach of developing bovine scFvs was tried.

NAGase is an important and sensitive marker for the detection of mastitis (section 3.1). It exists in two major isoforms, hexosaminidase A (HexA) and B (HexB) (section 3.1). Numerous studies have proved its reliability as a mastitis marker (section 3.1). The sensitivity of NAGase for the diagnosis of mastitis was 51.6-71.4% with 73.4-76.6% specificity (Gilmartin *et al.*, submitted). However, the currently available assays for NAGase detection are enzymatic. Comparatively, immunoassays are specific and sensitive, easy to perform and are relatively cheap. Consequently, one of the aims of this project was to generate antibodies against bovine NAGase.

Initially, generation of anti-NAGase mouse monoclonal antibodies was attempted using a commercially available bovine NAGase for immunisations

and antibody selection. The immunised mice failed to generate high antibody titres against the commercial antigen (Figure 3.1). Despite of the low titres, two fusions of Sp2/mIL-6 with spleenocytes from immunised mice were successfully carried out. However, both the fusions fail to generate any hybridoma secreting anti-NAGase antibody (section 3.5). The commercial bovine NAGase used for immunisation of mice was analysed by SDS-PAGE gel, in order to ascertain the cause of low immune response in mice. The gel revealed the presence of non-specific proteins and it was not possible to differentiate the bands of NAGase isoforms and their subunits (Figure 3.2).

Due to the unavailability of high-quality NAGase it was decided to explore other NAGase-associated antigens, which could be administered in mice to generate a better anti-NAGase immune response. Pig NAGase, sharing homology with bovine NAGase (Table 3.1), was used for generation of anti-NAGase antibodies (section 3.5). In addition, two peptides, from both the N-terminal and C-terminal region of the bovine Hex α-subunit, conjugated to KLH were synthesised as potential antigens (Table 3.2). However, pig NAGase, and these conjugated peptides, generated a low immune response in mice and the antibodies present failed to recognise the bovine NAGase (section 3.5).

The chicken NAGase was found to have divergent sequence from bovine NAGase (Table 3.1 and Figure 3.3). Therefore, it was decided to use chickens as the hosts of choice for generating anti-bovine NAGase antibodies. Different chickens were immunised with commercial bovine NAGase, recombinant HexA (Chapter 2) and two Hex α-subunit peptides (Table 3.2). Only the chicken immunised with commercial bovine NAGase responded well to immunisations,

giving a high antibody titre (1:1,280,000) (Figures 3.12 and 3.13). This chicken was used for construction of an scFv library. An antibody library with a good diversity of 4.2 x 10^8 cfu/mL was constructed and was successfully panned against commercial bovine NAGase, evident from the enrichment of specific phage in latter rounds of panning. A high percentage of scFv-expressing clones were obtained from round 4 and 5 of the panning. However, it was not possible to determine whether they had precise specificity to bovine NAGase, due to the impurity of the commercial bovine NAGase available (Figure 3.2). Therefore, these clones were further analysed against recombinant HexA and any of the two Hex α-subunit peptides (Table 3.2). However, none of the scFv clones reacted with any of the three antigens tested.

Subsequent attempts were made to generate rabbit polyclonal anti-bovine NAGase antibodies using recombinant HexA and the two Hex α-subunit peptides as the antigens. With the 3 rabbits immunised, a significant immune response was only observed in the rabbit immunised with HexA-1-KLH. However, the rabbit anti-HexA-1-KLH polyclonal antibodies failed to recognise the whole antigen (recombinant HexA). It was concluded that the low immunogenicity of bovine NAGase was a possible cause of its failure to generate good antibody responses (section 3.5).

Purification of NAGase, from bovine spleen, was attempted to generate high-quality NAGase for antibody generation and screening, A FPLC-based purification protocol for bovine HexB using ion exchange chromatography followed by gel filtration chromatography (Figure 4.2) was successfully standardised (Chapter 4). The purification protocol yielded HexB with a 12.9

fold purification. However, the purification yields were low and the protocol was labour intensive and time consuming (section 4.4). Consequently, to achieve higher amounts of antigens, recombinant expression of bovine HEXA (α-subunit) and HEXB (β-subunit) was attempted. Both HEXA and HEXB were successfully cloned and expressed using a vector containing an N-terminal fusion tag (pET-32b(+)) and BL21-CodonPlus®(DE3)-RIPL strain of *E. coli* (section 4.4). However, overexpression of both the genes could not be achieved even after optimising the time for their expression. In addition, adequate purification of protein from HEXB clone could not be achieved as numerous bands were observed following IMAC purification (section 4.4). An alternative strategy involving the use of a novel N-terminal fusion protein needs to be tried to improve HEXA and HEXB overexpression.

The bovine Hex subunits (α and β) and the Hex subunits from related mammals were analysed for identification of sites under positive selection. Both site-specific and lineage-specific analyses were performed (Chapter 5). Certain sites were identified by site-specific analysis to be under selection pressure in both the subunits and all these sites were associated with radical amino acid substitutions. It was observed that the rate of evolution was higher for the β-subunit, when compared with the α-subunit. Three lineage-specific sites were identified to be evolving in bovine the β-subunit. However, only position 95 could possibly play a role in the alteration of the protein structure and / or functionality (section 5.3). However, no regions with significant heterology between Hex subunits of bovines and other mammals were identified.

Detection of mastitis-causing agents is imperative for devising efficient prevention policies and efficient treatment strategies. However, with more than 150 mastitis-inducing species reported and the shortcomings of the currently available methodologies for the detection of mastitis, this task is not trivial (section 6.1). Immunoassays can be a useful aid in this regard, especially utilising the novel immunoassay-based platforms allowing simultaneous detection of various antigens (section 6.1). Consequently, another aim of this project involved the generation of an scFv library from spleen of cows with a history of chronic mastitis. A bovine scFv library with a diverse antibody repertoire (2.9×10^8 cfu/mL) was successfully generated (Chapter 6). This is the first instance of application of phage display for generation of bovine scFvs and the library can, potentially, be utilise for selecting antibodies to various mastitis-causing pathogens

To demonstrate the utility of the generated bovine scFv library in antibody selection, it was panned against different *Staphylococcus aureus* antigens, commercial *S. aureus* PGN, *S. aureus* crude extract and *S. aureus* surface proteins. *S. aureus* was chosen as it the major aetiological agent for contagious bovine mastitis (section 6.3). However, no anti-*S. aureus* specific clone could be selected by extensive biopannings (section 6.3). However, significant signals were obtained, in polyclonal ELISA, for the latter rounds of biopannings against *S. aureus* crude extract and *S. aureus* surface proteins and, therefore, few clones from both these biopannings were analysed for presence of the genome of bovine scFvs. The analysis revealed that 30% clones were carrying the bovine scFv genome, however, the clones were not expressing in bacterial cells

(section 6.3). Consequently, there is a need to determine the optimal growth conditions for maximal expression of bovine scFv clones.

The project was also aimed at generating anti-progesterone antibodies. Progesterone is a valuable marker for monitoring the reproductive status of the cow, *e.g.* ovarian activity, oestrus and pregnancy detection (section 7.1). In addition, it has the potential to be a marker for mastitis detection (section 7.1). A chicken, immunised with the commercial progesterone conjugate, failed to generate a significant immune response to the conjugate, possibly due to the failure of its immune system (section 7.3). Conversely, the rabbits, immunised with the commercial and 'in-house' progesterone conjugates, generated significantly high antibody titres (1:800,000 and 1:32,000, respectively) (Figures 7.1 and 7.2). Polyclonal antibodies from both the rabbits were successfully purified using protein G affinity chromatography (Figure 7.3). However, low antibody purification yields and detection limit (125 ng/mL) were obtained. This study is still in initial stages and a few strategies, such as using protein A/G affinity chromatography for antibody purification and boosting the rabbit immunised with the commercial progesterone conjugate need to be tried. In addition, generation of rabbit and / or mouse scFvs can be attempted (section 7.3).

The key outcomes from the study can be summarised as:

- Different sources (such as native antigen, recombinant antigen and peptides) of NAGase were used in an attempt to generate anti-bovine NAGase antibodies in different hosts (mice, chickens and rabbits), however, these antigens failed to generate good antibody response,

which can be attributed to a possible lack of immunogenicity of the bovine NAGase,

- An FPLC-based protocol was successfully standardised for purification of HexB from bovine spleen tissue. The protocol was labour intensive and time consuming. In addition, low purification yields were obtained. However, the antigen generated was used to isolate scFv from a naïve library in a collaboration,
- Recombinant expression of both α- and β-subunits of bovine hexosaminidase was successfully accomplished. However, overexpression of these proteins could not be achieved. In addition, problems were encountered in purification of the β-subunit,
- Selective pressure analysis revealed that certain sites in both hexosaminidase subunits (α and β) were under positive pressure. Many of these sites were in close proximity to the disease-causing mutation sites (of humans) and some were the disease site themselves. However, positive selection was occurring across all the mammalian species analysed and was not specific to bovines (expect at three positions in the β-subunit),
- The first bovine scFv library was successfully generated with significant antibody diversity. However, no positive scFv clones to *S. aureus* could be selected from this library after extensive biopannings. The scFv genome was found in 30% of the round 5 clones, suggesting that, possibly, there were issues with the expression of bovine scFv clones in bacterial cells, and

- Rabbit anti-progesterone antibodies were successfully generated and purified. The rabbit pAb was used in an inhibition ELISA for detection of progesterone.

In conclusion, it was observed that the immunogenicity of, bovine NAGase, was low and, thus, the animals immunised failed to generate significant antibody titres. Consequently, no useful antibody to NAGase could be selected in the current study. This research shows that NAGase is poorly immunogenic and future studies should target other biomarkers for the development of immunoassays for mastitis detection. The other objectives of (i) purifying HexB from bovine spleen tissue, (ii) cloning, expressing and purifying Hex α- and β-subunits and (iii) the analysis of Hex α- and β-subunits from bovines and related mammals for evidence of selective pressure variations, were successfully accomplished. The generation of a bovine scFv library was achieved and it was utilised to screen for antibodies to *Staphylococcus aureus*. However, it needs further optimisation before it can be used for selecting bovine antibodies to a variety of antigens. Finally, polyclonal antibodies to progesterone were successfully generated and used in an inhibition ELISA.

Chapter 9

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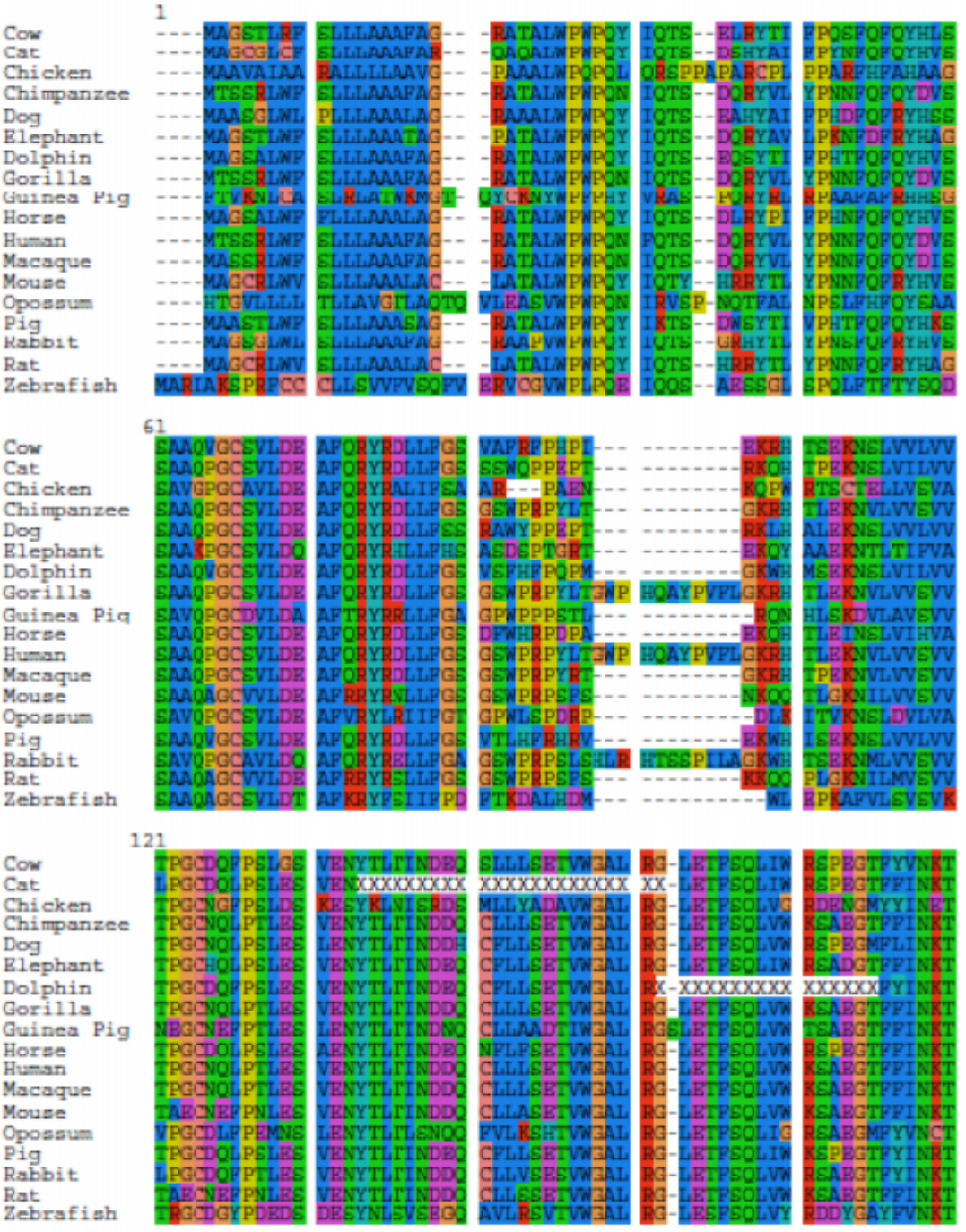
Appendix I

Hexosaminidase α-Subunit

Protein Alignments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Chicken
Chimpanzee
Dog
Elephant
Dolphin
Gorilla
Guinea Pig
Horse
Human
Macaque
Mouse
Opossum
Pig
Rabbit
Rat
Zebrafish
61
121


181
241
301
Cow
Cat
Chicken
Chimpanzee
Dog
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Dolphin
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Horse
Human
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Mouse
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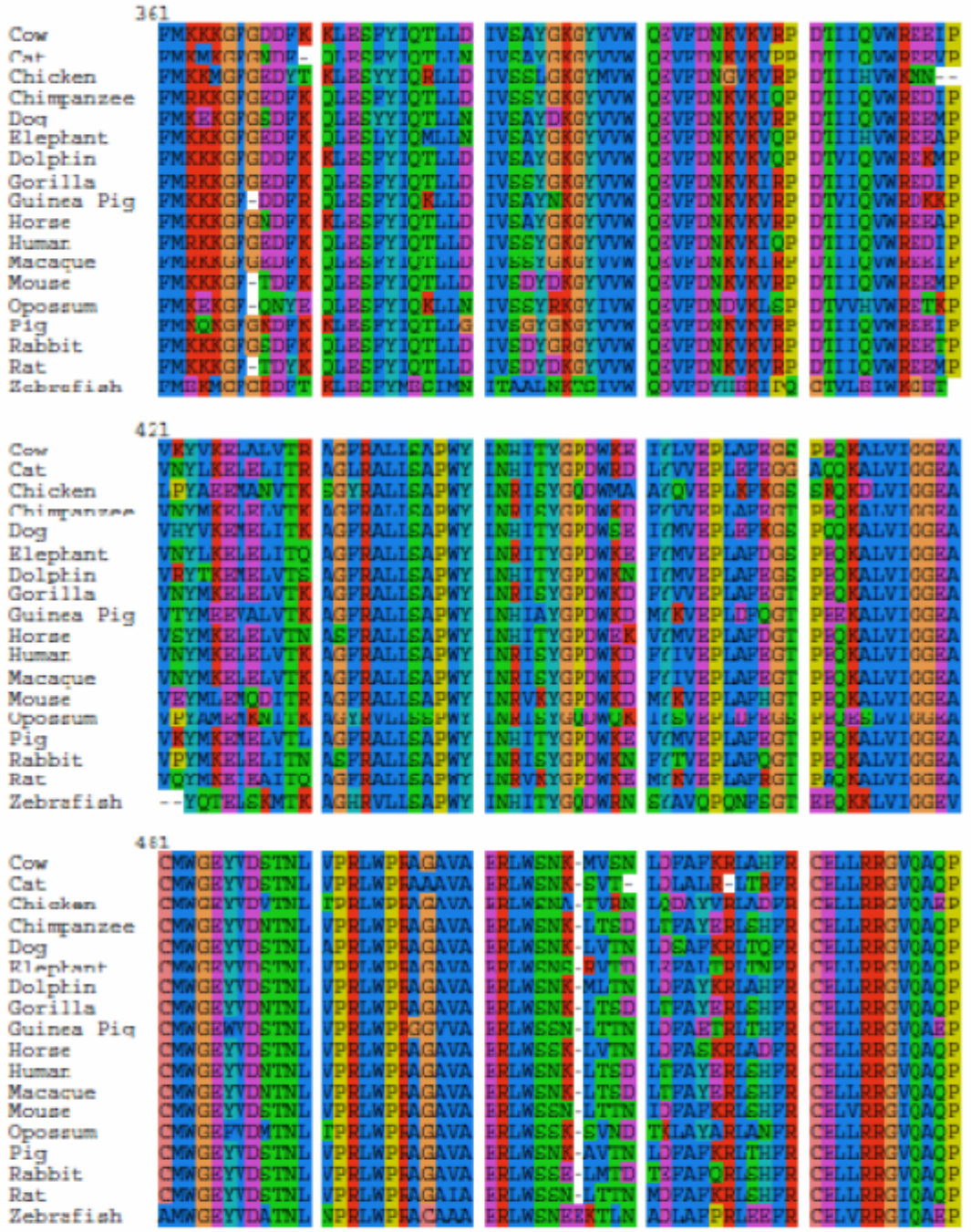
Rabbit
Rat
Zebrafish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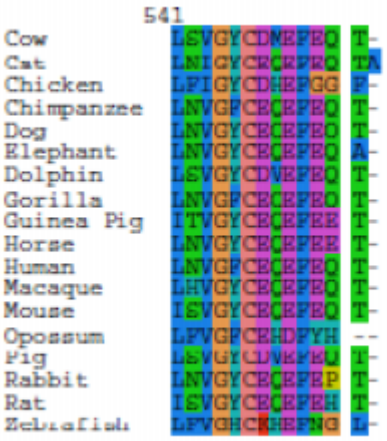
Macaque
Mouse
Opossum
Pig
Rabbit
Rat
Zebrafish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Cat
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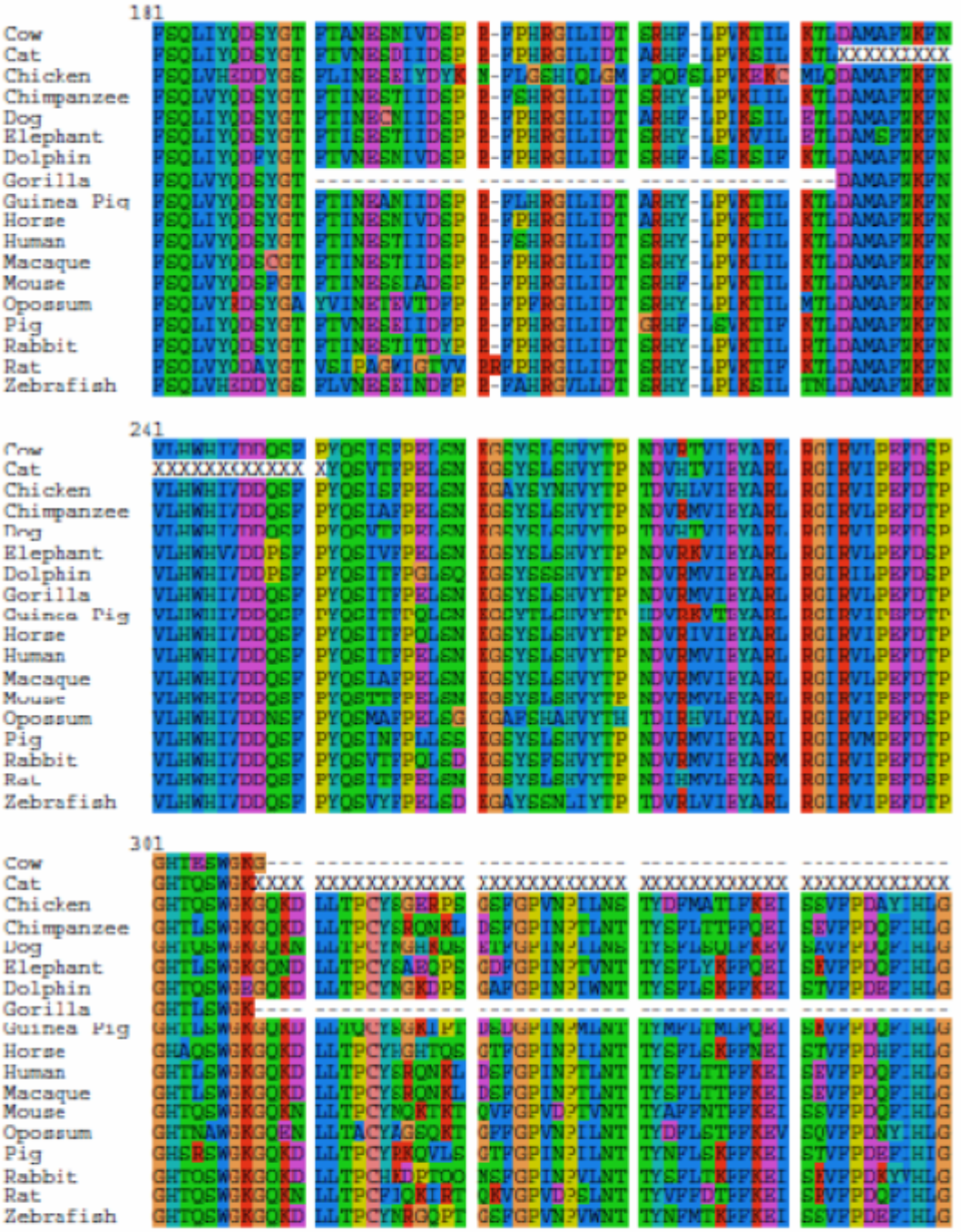
Chimpanzee
Dog
Elephant
Dolphin
Gorilla
Guinea Pig
Horse
Human
Macaque
Mouse
Opossum
Pig
Rabbit
Rat
Zebrafish

Appendix II

Hexosaminidase β-Subunit

Protein Alignments


1
Cow
Cat
Chicken
Chimpanzee
Dog
Elephant
Dolphin
Gorilla
Guinea Pig
Horse
Human
Macaque
Mouse
Opossum
Pig
Rabbit
Rat
Zebrafish
61
121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Dolphin
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Human
Macaque
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Pig
Rabbit
Rat
Zebrafish
241
Cow
Cat
Chicken
Chimpanzee
Dog
Elephant
Dolphin
Gorilla
Guinea Pig
Horse
Human
Macaque
Mouse
Opossum
Pig
Rabbit
Rat
Zebrafish
301
Cow
Cat
Chicken
Chimpanzee
Dog
Elephant
Dolphin
Gorilla
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Macaque
Mouse
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Rabbit
Rat
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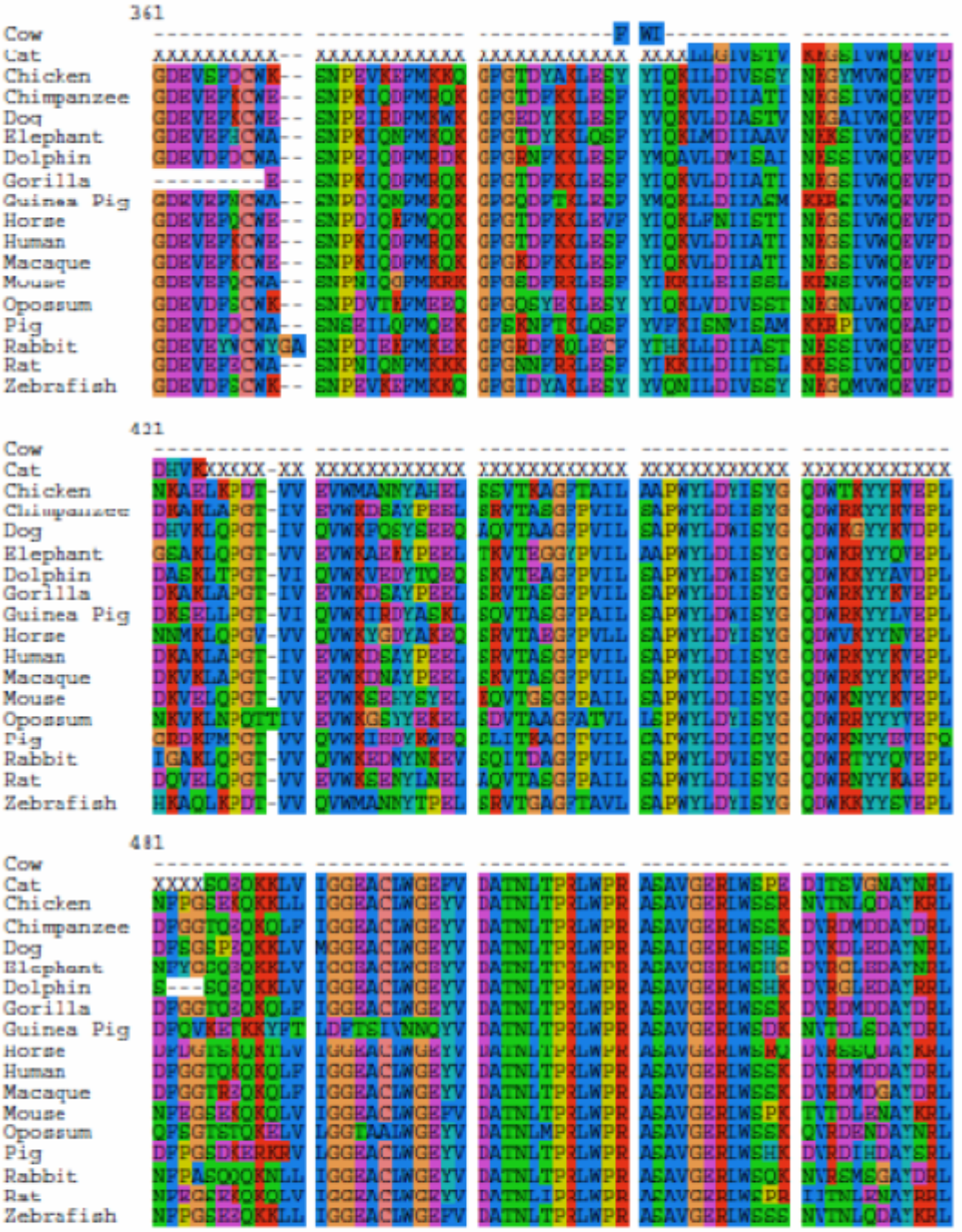
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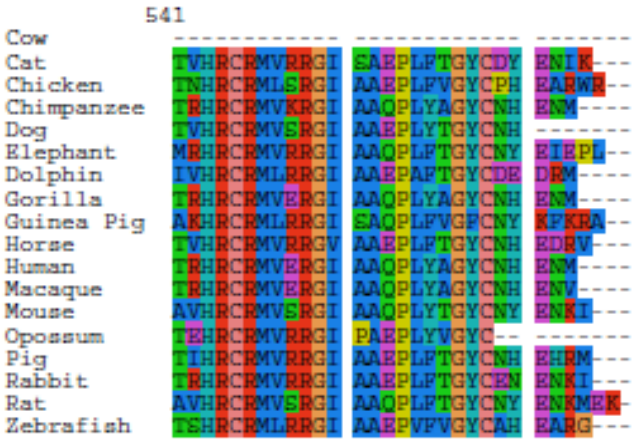
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Cow
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Pig
Rabbit
Rat
Zebrafish

Appendix III

List of Amino Acids, their Abbreviations and their Corresponding DNA Codons

Amino Acid	Abbreviation	Single Letter Codes	DNA Codon(s)
Isoleucine	Ile	I	ATT, ATC, ATA
Leucine	Leu	L	CTT, CTC, CTA, CTG, TTA, TTG
Valine	Val	V	GTT, GTC, GTA, GTG
Phenylalanine	Phe	F	TTT, TTC
Methionine	Met	M	ATG
Cysteine	Cys	C	TGT, TGC
Alanine	Ala	A	GCT, GCC, GCA, GCG
Glycine	Gly	G	GGT, GGC, GGA, GGG
Proline	Pro	P	CCT, CCC, CCA, CCG
Threonine	Thr	T	ACT, ACC, ACA, ACG
Serine	Ser	S	TCT, TCC, TCA, TCG, AGT, AGC
Tyrosine	Tyr	Y	TAT, TAC
Tryptophan	Trp	W	TGG
Glutamine	Gln	Q	CAA, CAG
Asparagine	Asn	N	AAT, AAC
Histidine	His	H	CAT, CAC
Glutamic acid	Glu	E	GAA, GAG
Aspartic acid	Asp	D	GAT, GAC
Lysine	Lys	K	AAA, AAG
Arginine	Arg	R	CGT, CGC, CGA, CGG, AGA, AGG