Exploration of Dipeptidyl Peptidase IV and Recombinant *Fasciola hepatica* Cathepsin L1 as Potential Biocatalysts

By

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A thesis submitted for the degree of

Doctor of Philosophy

Supervised by Dr. Ciarán Ó'Fágáin

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Dedicated to the memory of my best friend Boo
All dogs go to heaven

Think of the world like a big fruitcake
It wouldn't be complete
Without a few nuts in it!
(author unknown)

DECLARATION

I herby 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 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.

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The Lab

Goodbye!

Thank You

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They say diamonds are a girl's best friends, not true – friends are!

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The Von-Trapp Family Singers

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ABBREVIATIONS

GENERAL ABBREVIATIONS

ACN	acetonitrile
ADA	adenosine deaminase
AMC	7-amino-4-methycoumarin
BAA	*Bacillus amyloliquefaciens*
BCA	Bicinchoninic Acid
BLA	*Bacillus licheniformis*
t-Boc	tertiary-Butoxycarbonyl
BSA	bovine serum albumin
Bz	benzoyl
C_{50}	solvent concentration at which half-inactivation of enzyme is observed
CBZ	benzyloxycarbonyl
CD	cyclodextrin
CHES	2-[N-Cyclohexylamino]ethanesulfonic acid)
CLEC®	cross-linked enzyme crystal
CGTase	cyclodextrin glucanotransferase
Da	dalton
DC	Denaturation Capacity
DCC	N,N'-dicyclohexylcarbodiimide
DMF	dimethylformamide
DMSO	dimethylsulfoxide

DNA	deoxyribonucleic acid
cDNA	complementary DNA
DPP IV	Dipeptidyl Peptidase IV [EC 3.4.14.5]
DTT	dithiothreitol
E-64	trans-Epoxysuccinyl-L-Leucylamido(4-Guanidino)-Butane
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	diaminoethanetetra-acetic acid
EG-NHS	ethylene glycol-bis(Succinic acid N-hydroxysuccinimide ester)
EG-rFheCL1	modified rFheCL1
eu	enzyme units
F-moc	9-fluorenylmethoxycarbonyl
rFheCL1	recombinant *Fasciola hepatica* cathepsin L1 [EC.3.4.22.15]
HEPES	(N-[2-Hydroxyethyl]piperazine-N′-[2-ethane-sulfonic acid])
HIC	hydrophobic interaction chromatography
HPLC	high performance liquid chromatography
HR	high resolution
HRP	horseradish peroxidase
k_{cat}	catalytic constant
K_m	Michaelis-Menten constant
LC-MS	liquid chromatography mass spectrometry
Log	logarithm
Log *P*	partition coefficient of a solvent in water/octanol biphasic system
M	molar
MES	(2-[N-Morpholino]ethanesulfonic acid)

min	minute
mM	millimolar
MOPS	(3-[N-Morpholino]propanesulfonic acid)
MPEG	methoxypolyethylene glycols
mw	molecular Weight
nm	nanometre
OEt	ethoxy
OMe	methoxy
ONp	4-nitrophenyloxy
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
pHEMA-GMA	poly (2-hydroxyethyl methacrylate-glycidyl methacrylate)
PO	Prolyl oligopeptidase
PS	phenyl Sepharose
QAE	quaternary anion exchange
R_f	relative mobility
RNA	ribonucleic acid
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
Rpm	revolutions per minute
SA-NHS	suberic acid N-hydroxysuccinimide ester
SDS	sodium dodecyl sulphate
$t_{½}$	half-life

T_{50}	half-inactivation temperature
TEMED	N,N,N'N'tetramethylene-ethylenediamine
THF	tetrahydrofuran
TNBS	2,4,6-Trinitrobenzenesulfonic acid
Tris	Tris(hydroxymethyl) aminomethane
μM	micromolar
V_{max}	maximum velocity
v/v	volume per volume
w/v	weight per volume
Z	benzyloxycarbonyl

AMINO ACID ABBREVIATIONS

Name Abbreviation	Symbol	Name
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K

Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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ABSTRACT

The aim of this project was to study the stability characteristics of the serine protease dipeptidyl peptidase IV (DPP IV) and recombinant *Fasciola hepatica* cysteine protease cathepsin L1 (rFheCL1), with a view to using them in peptide synthesis.

DPP IV was purified from bovine serum. The chromatographic techniques used were phenyl-Sepharose hydrophobic interaction, Sephacryl S-300-HR gel filtration and quaternary-Sepharose anion exchange, producing an overall purification factor of 35. The enzyme was shown to have a subunit molecular weight of 77,224 Da, by SDS-PAGE; native weight was not determined. DPP IV activity remained stable up to 62°C and over a broad pH range, with a preference for neutral pH. The estimated T_{50} was 71°C. With regard to solvent tolerance DPP IV was most stable in acetonitrile, at all concentrations tested. THF was the most deleterious solvent with little or no activity exhibited above 40% (v/v) concentration. Michaelis-Menten kinetics of DPP IV were determined at different pH values. p*K* values were observed at pH 6.5 and 9.5. The lower p*K* value is likely due to the ionisation of the active site histidine side chain of soluble DPP IV; however, the upper p*K* value possibly belongs to the α-amino group of the Gly moiety of the Gly-Pro-AMC substrate.

rFheCL1 was characterised and compared with the enzyme purified from liver fluke (*Fasciola hepatica*). rFheCL1 was also stabilised by the reaction of the enzyme with ethylene glycol-bis(succinic acid N-hydroxy-succinimide ester) (EG-NHS). The enhanced stability of the modified enzyme is likely due to an intramolecular crosslink(s) being formed in the enzyme. The estimated T_{50} of modified rFheCL1 was 87°C as compared to native rFheCL1, which had an estimated T_{50} of 78°C. At 70°C modified rFheCL1 retained 100% amidase activity for up to 60 minutes compared to native rFheCL1, which showed an 80% loss in activity under the same assay conditions. Modified rFheCL1 also demonstrated enhanced stability in the solvents acetone and acetonitrile; however, modification did not enhance stability in THF. Kinetic studies indicate that the active site of the enzyme has not been affected by cross-linking and that stability gain has not been at a cost of poorer catalysis. Native rFheCL1 was used to catalyse the synthesis of the model tripeptide Z-Phe-Arg-SerNH_2. LC-MS analysis indicated that formation of the new tripeptide occurred within ten minutes.

CHAPTER 1

INTRODUCTION

1.1. Chemical Peptide Synthesis

Chemical synthesis involves joining together optically active amino acids in chains of known length and predetermined sequence. The side chain group, designated R, defines different structures of amino acids. These side chains may contain certain functional groups that can interfere with the formation of the peptide bond; therefore, it is important that functional groups other than those involved in peptide bond formation (i.e. amino side chains containing thiol, imidazole, amino, carboxylic and hydroxyl functional groups) be blocked by protecting groups. However, this protection must be reversible and not damage the assembled peptide chain. Typical protecting groups for the amino function include tertiary-Butoxycarbonyl, or t-Boc and 9-fluorenylmethoxycarbonyl or Fmoc (FIGURE 1.1.). Each protecting group has its own conditions for application to, and removal from, the amino acid.

The main types of chemical synthesis are solution-phase peptide synthesis and solid-phase peptide synthesis (Andersen *et al.*, 1991; Murphy & Ó'Fágáin, 1996a).

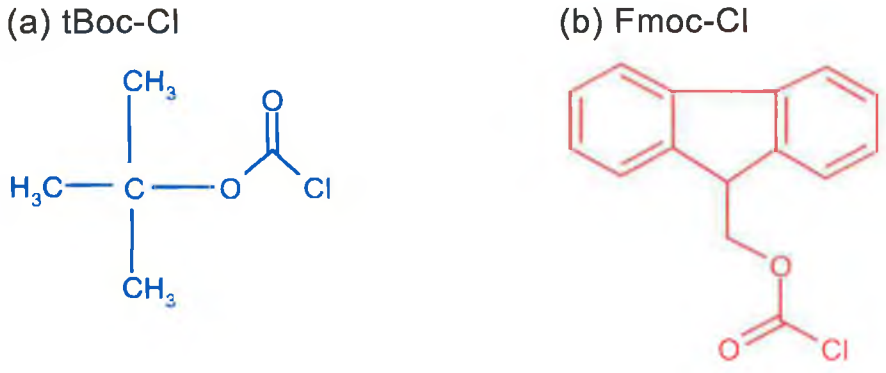


FIGURE 1.1. STRUCTURES OF PROTECTING GROUPS COMMONLY USED IN CHEMICAL PEPTIDE SYNTHESIS (Murphy & Ó'Fágáin, 1996a).

1.1.1. Solution-phase peptide synthesis

This method involves classical wet chemistry and the assembly of peptides in solution (FIGURE 1.2.). It consists of two steps: the first step requires the activation of a carboxyl group on an Nα-protected amino acid with an appropriate agent. The second step involves coupling this activated carboxyl group to the free amino group of a Cα-protected amino acid via a dehydration reaction promoted by an appropriate coupling reagent (e.g. DCC) leading to peptide bond formation (Andersen *et al.*, 1991; Murphy & Ó'Fágáin, 1996a).

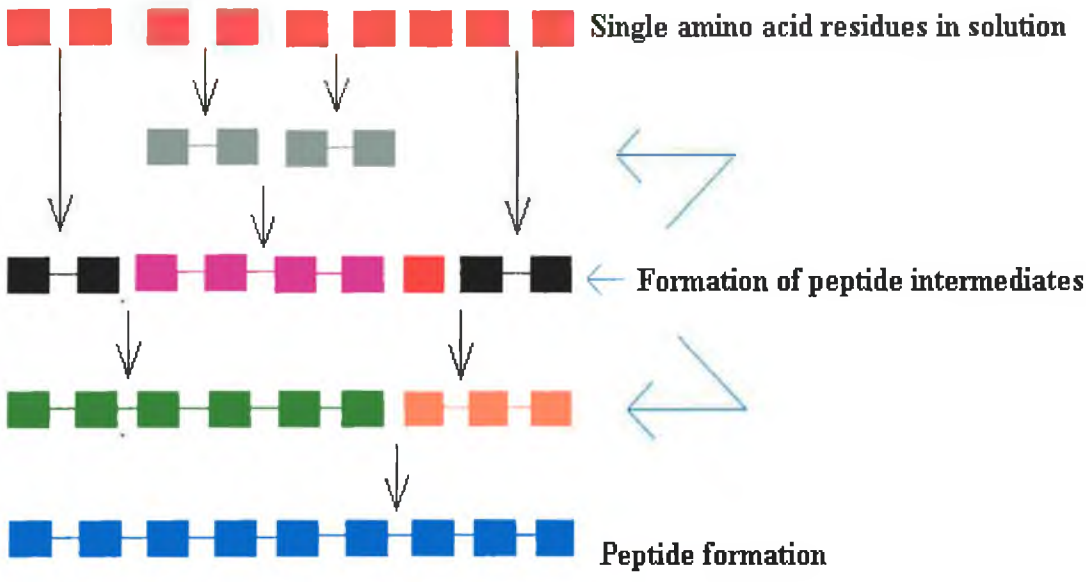


FIGURE 1.2. SCHEMATIC DIAGRAM OF FRAGMENT CONDENSATION IN SOLUTION (Kent, 1988).

1.1.2. Solid-phase peptide synthesis (SPPS)

Robert Bruce Merrifield developed this method in 1963. It involves the assembly of a peptide chain, starting with the carboxyl-group of an amino acid attached to a solid support. In a series of defined steps Nα-protected amino acids are deprotected and coupled to the attached amino acid until the desired peptide or polymer has been obtained (FIGURE 1.3.).

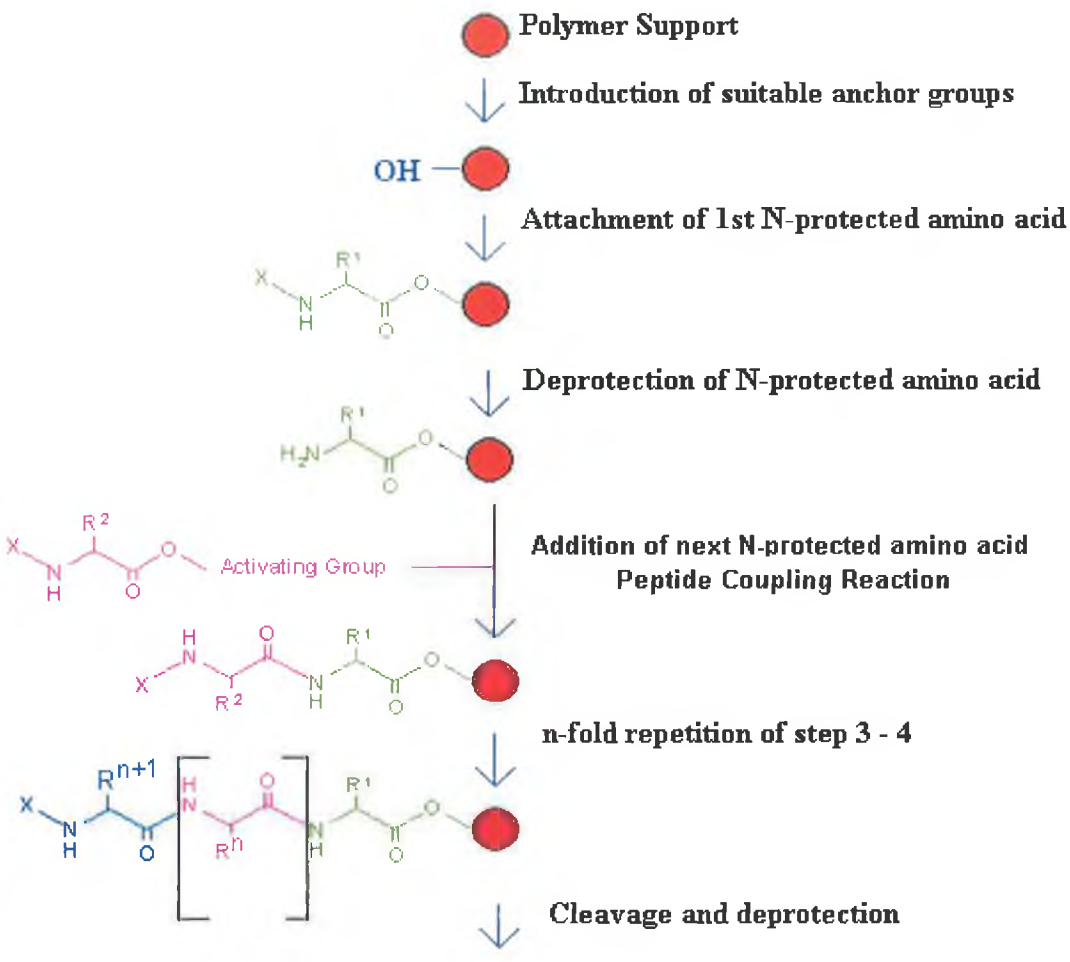


FIGURE 1.3. GENERAL SCHEME FOR SOLID-PHASE PEPTIDE SYNTHESIS
X = temporary protecting group, R^1, R^2, R^n & R^{n+1}, amino acid side chains (Sewald & Jakubke, 2002b).

1.2. Recombinant DNA Techniques

This is a preparative technique for ribosomal synthesis of polypeptides and proteins based on the principle of gene technology. Expression of peptides and proteins in organisms such as bacteria e.g. *Escherichia coli* is possible. This technique relies on the recombination of the microorganism's genetic material with the DNA fragment encoding for the desired protein and consists of a number of steps (Sewald & Jakubke, 2002c):

(1) Isolation of the encoding DNA fragment from the donor organism.

(2) Insertion of the DNA into a vector.

(3) Transfection of the vector into the host organism.

(4) Cultivation of host organism, leading to gene amplification, mRNA and protein synthesis.

(5) Isolation of the recombinant protein.

Lueking *et al.* (2000) used this technology to successfully develop a novel *Pichia pastoris*/*Escherichia coli* dual expression vector for the production of recombinant protein in both host systems.

1.3. Proteases and Enzymatic Peptide Synthesis

Proteolytic enzymes, or proteases, catalyse a single reaction, the hydrolysis of a peptide bond. This reaction is one of the most frequent and important enzymatic modifications of proteins. They can, however catalyse the reverse reaction, i.e. formation of the peptide bond leading to oligopeptide synthesis. Enzymatic peptide synthesis has been used to produce a number of peptides. Oyama *et al.* (1981) used the enzyme thermolysin to catalyse the condensation between L-phenylalanine methyl ester and N-CBZ-L-aspartic acid, to produce the well-known sweetener aspartame. There are a number of advantages associated with enzymatic peptide synthesis as opposed to chemical methods. These are:

(1) Enzymes catalyse reactions with high regiospecificity reducing the need for side chain protection of reactants

(2) Owing to the stereospecificity of enzymes, no racemization is observed

(3) Reactions take place under mild conditions

(4) No requirement of protection/deprotection steps; this simplifies the process (Gill, *et al.*, 1996; Sears & Wong, 1996).

Proteases are divided into two categories on the basis of where they cleave the protein exopeptidases and endopeptidases (FIGURE 1.4.). Exopeptidases require the presence of an unsubstituted N- or C- terminus in the substrate and remove a single amino acid, di- or tripeptides from one or other terminus, producing small peptides or amino acids. This mode of action is the basis for the classification of the exopeptidases. The same consideration of specificity cannot be applied to the endopeptidases, as they cleave within the protein chain, producing large peptides. Therefore, the classification of these peptidases is dependent on the catalytic mechanism of their active sites to yield four main classes: serine, cysteine, aspartic and metallo-endopeptidases. The same catalytic mechanisms are functional among

the exopeptidases; hence, some can be further subdivided on the same basis (Creighton, 1993b; Kenny, 1999; Barrett, 2001).

1.3.1. Serine proteases

Two distinct families are present in this group: the mammalian serine proteases e.g. Trypsin EC 3.4.21.4 and the bacterial serine proteases e.g. Subtilisin EC 3.4.21.14. They are characterized by the presence of a highly reactive serine residue. This is included in the active site arrangement of their catalytic triad Ser-His-Asp. Catalysis proceeds by covalent attack of polarised serine side-chain and the formation of an acyl-enzyme intermediate. The same type of mechanism underlies the action of all serine protease (Creighton, 1993b; Dunn, 2001).

1.3.2. Cysteine proteases

Included in this group are several mammalian lysosomal cathepsins and the plant proteases papain and actinidin. Cysteine proteases are the most comparable to the serine proteases, with a cysteine side chain playing the role of the serine. Catalysis proceeds by covalent attack of the polarised cysteine side chain and the formation of a thiol ester intermediate. The active site arrangement is Cys-His charge transfer (Creighton, 1993b; Dunn, 2001).

1.3.3. Aspartic proteases

Bacterial penicillopepsin EC 3.4.23.6. mammalian pepsin EC 3.4.23.1., chymosin EC 3.4.23.4. and certain fungal proteases belong to this family. The active site is composed of two aspartic acid residues, which lie in close proximity to each other. Catalysis proceeds via water molecule attack polarised by the enzyme's aspartic acid side chains. In the

enzymatically active pH range 2-3, one of these is ionised, the other unionised (Dunn, 2001).

1.3.4. Metalloproteases

Metalloproteases use bound metal in their active sites. Included in this family are the carboxypeptidases A and B, thermolysin and medically important enzymes such as angiotensin-converting enzyme. Catalysis proceeds via water molecule attack polarised by the enzyme's co-ordinated zinc. The active site components include zinc, with its three ligands of two glutamic acids and one histidine (Dunn, 2001).

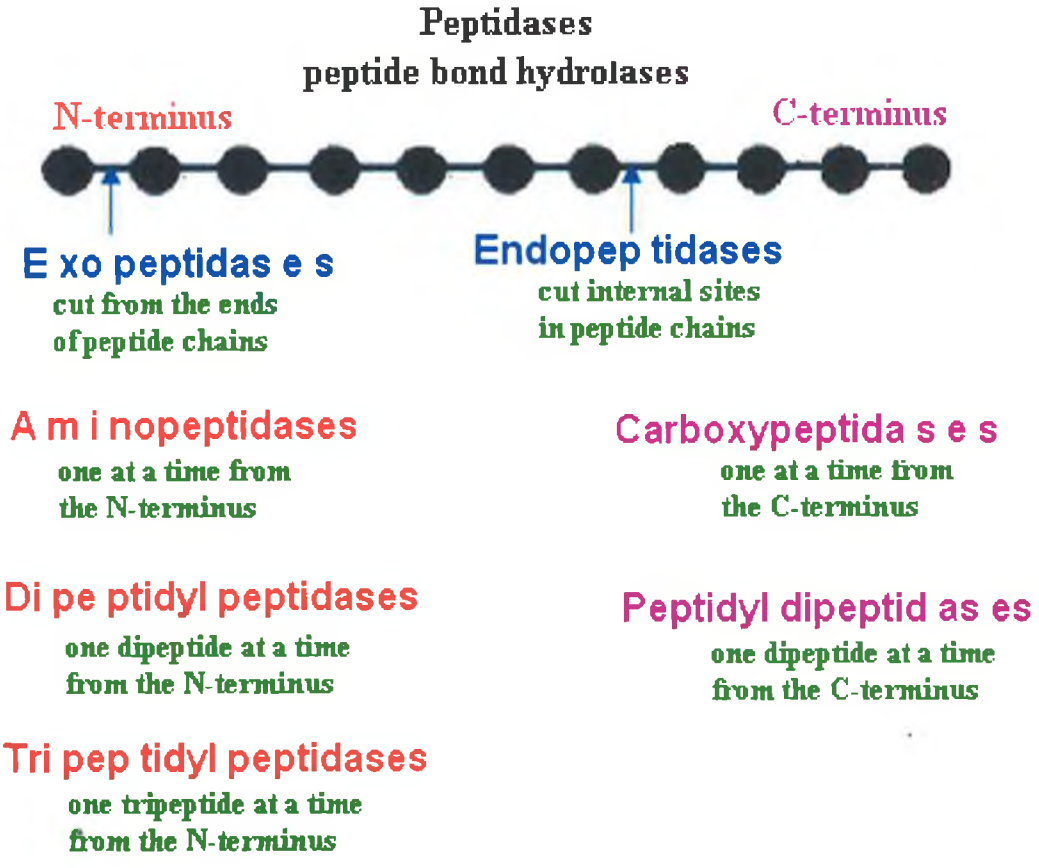


FIGURE 1.4. SCHEMATIC REPRESENTATION ILLUSTRATING CLEAVAGE SITES OF ENDO- AND EXOPEPTIDASES. (WEBSITE: burnham.org/labs/salvesen/classification)

1.4. Strategies for Enzymatic Peptide Synthesis

Two basic strategies exist for protease-catalysed peptide bond formation; (1) equilibrium-controlled synthesis and (2) kinetically-synthesis. The type of synthesis is dependent on the type of carboxyl component used in the reaction. In equilibrium-controlled synthesis the carboxyl component is free (RCOOH), whereas in kinetically-controlled synthesis the carboxyl component is used in an activated form (RCOOX) (FIGURE 1.5.). These strategies are fundamentally different because of the energy requirements for the conversion of the substrates into peptide products (Sewald & Jakubke, 2002d).

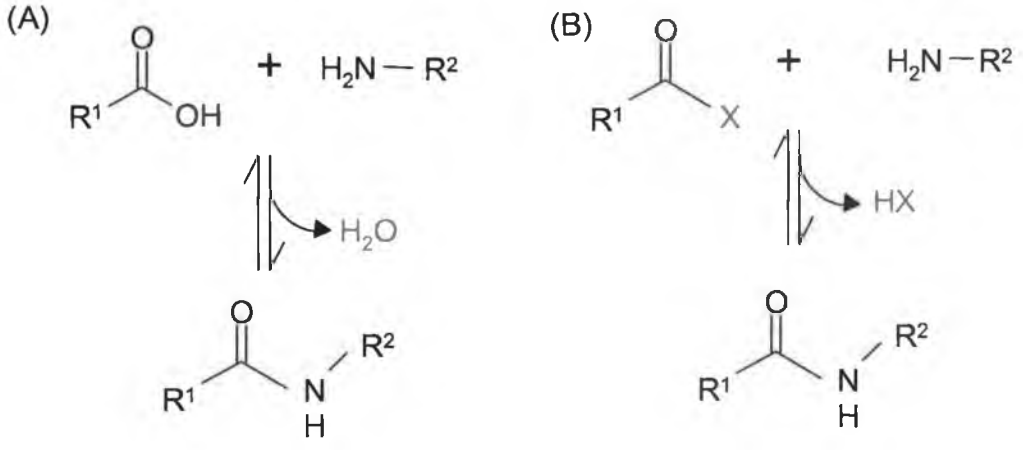


FIGURE 1.5. SCHEMATIC DIAGRAM TO COMPARE EQUILIBRIUM (A) AND KINETICALLY (B) CONTROLLED PEPTIDE SYNTHESIS. R^1-COOH = free carboxyl component, R^1-COX = activated carboxyl component (Sewald & Jakubke, 2002d).

1.4.1. Equilibrium-controlled peptide synthesis

This type of synthesis involves direct reversal of protease-catalyzed hydrolysis. It involves a condensation reaction between the free terminal α-carboxyl component and an unblocked nucleophilic α-amino component (FIGURE 1.6.). All mechanistic classes of proteases i.e. cysteine, serine, aspartic and metallo, can be used for this type of synthesis, as it is independent of their catalytic mechanisms. The enzyme is used to accelerate the rate with which equilibrium is reached and cannot influence this equilibrium (Jakubke, 1987; Kasche, 1989; Sewald & Jakubke, 2002).

FIGURE 1.6. EQUILIBRIUM-CONTROLLED PEPTIDE SYNTHESIS. Direct reversal of peptide hydrolysis (Barbas *et al.*, 1988).

1.4.2. Kinetically-controlled peptide synthesis

The rate of product formation relative to peptide hydrolysis is important in kinetically-controlled peptide synthesis. In this type of synthesis (FIGURE 1.7.), the enzyme rapidly reacts with the ester substrate forming an acyl-enzyme intermediate, with the loss of an amino acid or peptide fragment (HX). There is partitioning of the intermediate between aminolysis (k_4) and hydrolysis (k_3). Nucleophilic attack of the intermediate by water results in hydrolysis of the peptide. The resulting hydrolytic products possess very low acylation potential and are therefore, not available for formation of the desired product. Hence, the ratio between aminolysis and hydrolysis of the acyl-enzyme ester is of the utmost importance for successful peptide synthesis and, for maximum peptide synthesis, k_4 should be greater than k_3. Thus, if a more powerful nucleophile (H_2NR') than water is present then

the desired product will be preferentially formed (R-CO-NH-R'). Formation of the desired product is a function of time. After complete consumption of the substrate, the possibility of product hydrolysis is high; therefore, the reaction should be stopped at the kinetic optimum. Organic cosolvents may be included in the reaction, thereby limiting the concentration of water available and decreasing the potential for hydrolytic reactions.

In contrast to equilibrium-controlled synthesis, the kinetic approach requires an acyl donor ester (R-CO-X) as the carboxyl component and is limited to those enzymes forming acyl-enzyme intermediates i.e. serine and cysteine proteases. Ester substrates are conveniently used as the carboxyl components, allowing rapid formation of the acyl-enzyme intermediate: hence, a lower enzyme concentration can be used (Jakubke, 1987; Morihara, 1987; Sears & Wong, 1996; Sewald & Jakubke, 2001).

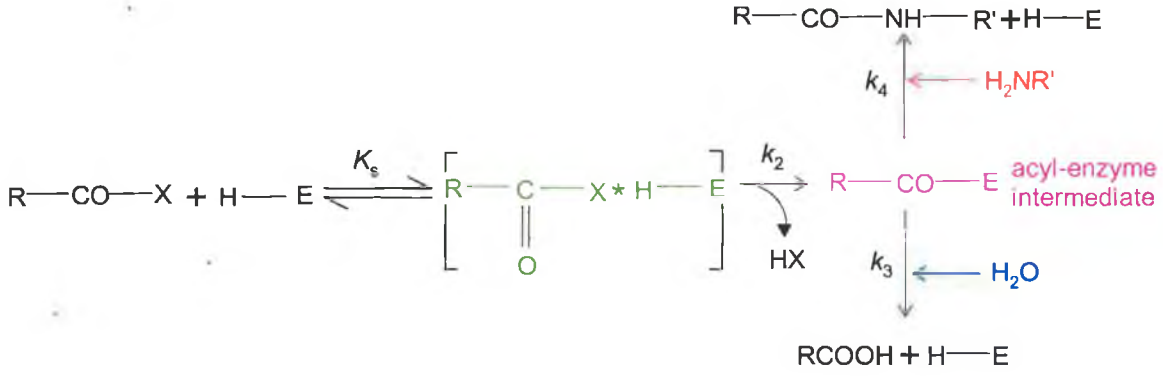


FIGURE 1.7. KINETICALLY-CONTROLLED PEPTIDE SYNTHESIS CATALYSED BY SERINE AND CYSTEINE PROTEASES (H-E). R-CO-X is carboxyl component, X is OAlk, R-CO-E is the acyl-enzyme complex, H_2NR' is amino component, R-CO-NH-RN' is peptide product and RCOOH is hydrolysis product. K_s is the equilibrium constant for E-S complex formation. k_2 is the rate constant for formation of the acyl-enzyme intermediate. k_3 and k_4 are the rate constants for hydrolysis of the acyl-enzyme intermediate and synthesis of the peptide product, respectively (Jakubke, 1987).

Haensler *et al.* (1999) used α-Chymotrypsin and papain to catalyse peptide synthesis in frozen aqueous systems. They successfully synthesised a number of dipeptides, using different nucleophiles, achieving yields up to 90%.

Getun *et al.* (2001) catalysed the synthesis of tetra-peptides containing multifunctional amino acid residues in ethanol using subtilisin. They obtained yields of up to 92%; this was dependent on the duration of assay and the acyl donor structure.

Zhou *et al.* (2003) succeeded in synthesizing the tripeptide Bz-RGD-OEt in a two step procedure, utilizing a chemo-enzymatic method, obtaining a 72.5% yield. The first step involved chemical synthesis of the Gly-Asp-diethyl ester $(OEt)_2$. Linkage of the third amino acid Bz-Arg-OEt to the previously synthesized dipeptide was completed by the enzymatic method in organic solvents. This formed tripeptide can act as both an acyl donor and as a nucleophile for peptide chain extension directly (without deprotection/activation).

1.5. Enzyme Catalysis in Aqueous and Non-Aqueous Systems

In previous years, conventional notions assumed that enzymes were active only in water. Indeed, it was more or less accepted that organic solvents destabilized protein molecules; however, this notion is fast becoming obsolete and in more recent years many solvent systems have been employed for non-aqueous enzymatic reactions. These may be divided into the following categories: (1) water:water-miscible (hydrophilic) organic cosolvents (2) water:water-immiscible (hydrophobic, biphasic) organic solvent systems and (3) nearly non-aqueous or anhydrous organic solvents (Krishna, 2002). There are a number of advantages to using enzymes in organic media, some of which are highlighted in TABLE 1.1.

1.	The catalysis of reactions unfavourable in water, e.g. reversal of hydrolysis reactions in favour of synthesis.
2.	Solubilization of hydrophobic substrates.
3.	Ease of recovery for some products.
4.	Enhanced biocatalyst thermostability.
5.	Suppression of water-induced side reactions.

TABLE 1.1. ADVANTAGES OF USING ENZYMES IN ORGANIC AS OPPOSED TO AQUEOUS MEDIA (Bell *et al.*, 1995)

In the natural environment, enzymes function in aqueous solutions and exhibit their catalytic properties to the full extent when they have a strictly defined conformation. In solution, a network of interactions determines the conformation of an enzyme e.g. hydrogen bonding, ionic interactions, van der Waals and hydrophobic forces. Water mediates all of the above non-covalent interactions and also acts as a lubricant, allowing the conformational mobility required for catalysis. Therefore, in order to maintain these interactions, the enzyme molecule requires a definite hydration shell and, thus, removal of this hydration shell will result in distortion of the enzyme structure and denaturation (Khmelnitsky *et al.*, 1988; Klibanov, 1997; Krishna, 2002). Numerous studies have investigated the use of enzymes in organic solvents and the effects of these solvents on conformation and function.

Gorman and Dordick (1992) demonstrated that organic solvents had the ability to strip water from enzymes (Subtilisin Carlsberg, α-chymotrypsin, HRP) a phenomenon known as "water stripping". In their study, the enzyme bound water exchanged with tritiated water (T_2O) and they monitored its desorption into the solvent system under study. Exposure to polar solvents resulted in the highest degree of T_2O desorption i.e. methanol desorbed 56-62% of the bound T_2O, whereas exposure to nonpolar solvents resulted in the lowest degree of desorption i.e. hexane desorbed 0.4-2% of the bound T_2O. They also concluded that at least two fractions of enzyme-bound water must exist, as methanol (most polar solvent used in this study) managed only to desorb 62% of T_2O. Indeed, in lyzozyme four distinct hydration levels have been shown to exist (TABLE 1.2.).

LEVELS	PROPERTIES
1. 0 - 0.07g H_2O/g protein	Hydration of ionisable (charged) groups
2. 0.07 - 0.25g H_2O/g protein	Shift in water distribution on protein surface from purely ionic sites to polar regions, enzyme becomes more mobile.
3. 0.25 – 0.38g H_2O/g protein	Enzyme activity is observed: water approaches monolayer coverage, polar sites hydrated first, followed by nonpolar regions.
4. Above 0.38g	Protein fully hydrated. (≈ 300 molecules of H_2O per enzyme molecule)

TABLE 1.2. ENZYME HYDRATION LEVELS (Reference: Dordick, 1991; Gupta, 1992)

As is clearly seen in the above table, very little water is required for native enzyme structure and function to be realised; even so, this bound water is crucial (Dordick, 1991). However, the amount of water required to retain activity in organic solvent systems is enzyme dependent e.g. α-chymotrypsin needs only 50 molecules of water per enzyme molecule to remain active, whilst polyphenol oxidase requires 3.5×10^7 molecules of water. Therefore, enzymes should be investigated at various levels of hydration in organic solvents, as it may be possible that an enzyme deemed previously to be inactive in an organic solvent may have been used at non-optimal levels of hydration (Krishna, 2002).

Fitzpatrick *et al.* (1993) compared the crystal structures of subtilisin Carlsberg in water and anhydrous acetonitrile. Subtilisin Carlsberg had an identical three-dimensional structure in either solvent. They concluded that out of the 119 enzyme-bound water molecules, 99 have such affinity to the subtilisin molecule that they are not displaced in anhydrous acetonitrile. They also observed that 12 acetonitrile molecules bound to the enzyme surface, displacing only 4 water-bound molecules and 8 binding where no water was observed before. Klibanov (1997 & 2001) suggested that enzyme stability and retention of catalytic activity

in anhydrous solvents is in part due to (1) the ability of the enzyme to strongly bind and retain some "essential water" and (2) the absence of bulk water (which acts as a molecular lubricant), thereby leading to a rigid enzyme structure. Thus, even though the drive to unfold in these solvents may be great, the pliability and conformational flexibility necessary to proceed is lacking.

All solvents are not the same and will exhibit differences in properties such as hydrophobicity, hydrogen-bonding capacity and miscibility in water; these in turn will have an effect on the enzyme's structural integrity and catalytic activity (Mattos & Ringe, 2001). A number of attempts have been made to establish the ideal parameter for the selection of organic cosolvents for use in enzymatic reactions. Log *P* (*P* is the partition coefficient of a solvent in a water/octanol biphasic system) is a common parameter used to correlate the denaturing efficiency of organic solvents with their hydrophobicity. This parameter revealed good correlation with denaturing strength: the higher the log *P* value, the stronger the denaturing effect. However, this parameter is only valid when applied to solvents of the same functionality e.g. alcohols. When solvents of a different functionality are included, the correlation breaks down (Mozhaev *et al.*, 1989). Khmelnitsky *et al.* (1991a) developed the thermodynamic model of protein denaturation by organic solvents in solution (FIGURE 1.8.). This model explained the molecular mechanism of the denaturation process, establishing a quantitative relationship between the physicochemical properties of organic solvents and their denaturing effect, allowing construction of the denaturation capacity scale (DC) of organic solvents. Organic solvents are arranged in accordance with their DC value, thus forming the scale. This scale can then be used to quantitatively predict the threshold concentration (C_{50}) of various organic solvents at which half-inactivation of the enzyme is observed.

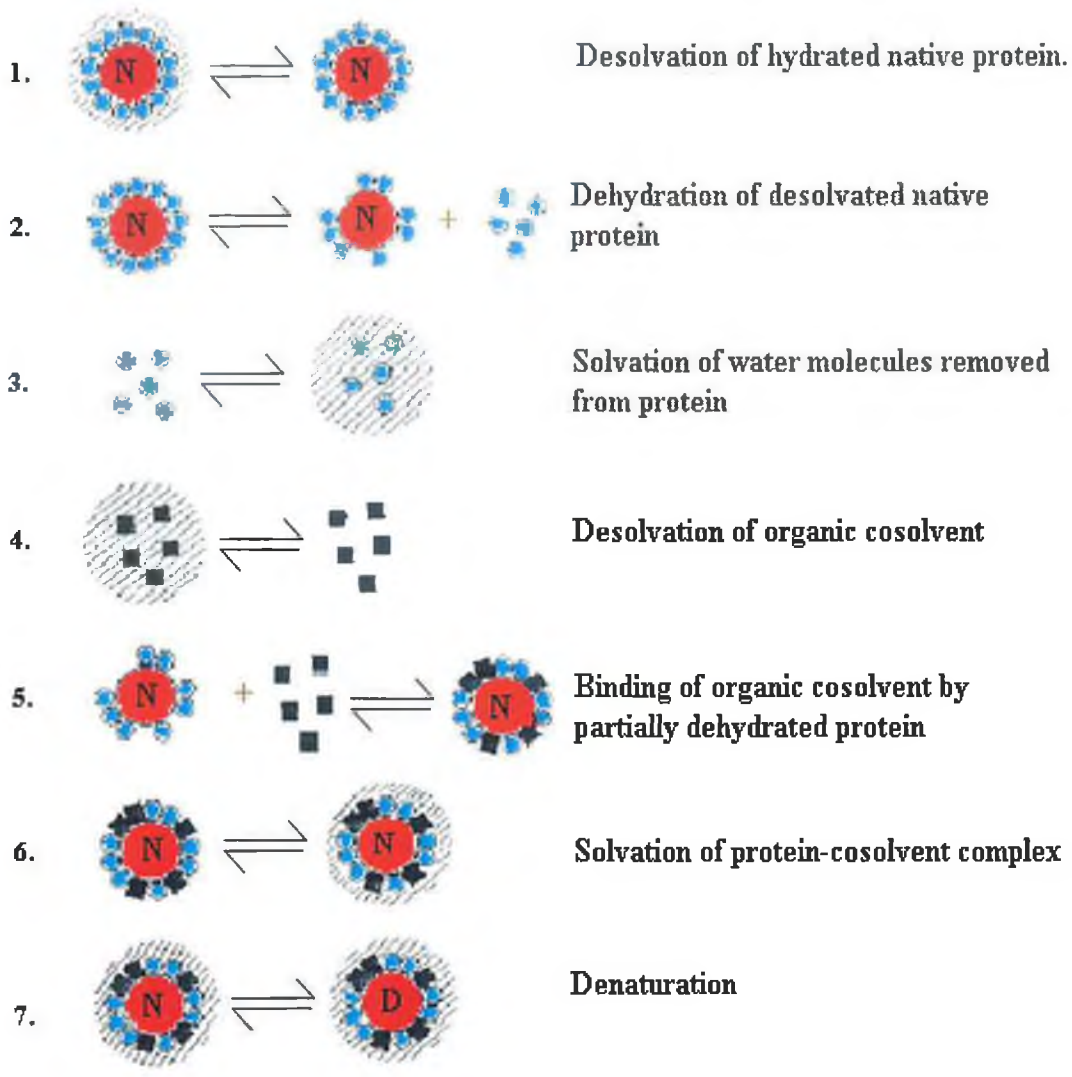


FIGURE 1.8. A SCHEMATIC REPRESENTATION OF THE MOLECULAR STEPS INVOLVED IN THE PROCESS OF REVERSIBLE PROTEIN DENATURATION BY ORGANIC SOLVENTS (Khmelnitsky *et al.*, 1991a).

water molecule, organic cosolvent molecule, surrounding solvent.

There are many advantages to employing enzymes as catalysts in organic as opposed to aqueous media (TABLE 1.1.) and the stability of certain enzymes is greater in the presence of organic media than aqueous media, e.g. the half-life of LST-03 lipase from *Pseusomonas aeruginosa* in *n*-decane was >100 days; in the absence of organic solvent the half-life decreased to 12.5 days. However, most enzymes are denatured and inactivated in the presence of organic media. Because of this several methods have been devised for enhancing the stability of enzymes e.g. immobilization and chemical modification, which will be discussed in the next section (Ogino & Ishikawa, 2001).

1.6. Stability of Proteases

The increasing use of proteins and their stability *in-vitro* remains a critical issue in biotechnology. In particular, the stabilization of enzymes without diminishing and diluting their activity is crucial for their use as industrial biocatalysts (Govardhan, 1999).

The complicated structure of proteins renders them subject to chemical and physical instability. Chemical instability refers to covalent modifications of the protein molecules resulting in the formation of a new chemical entity that can have different biological activities. Chemical instability occurs at high temperatures or at extreme pH values. The main degradation processes are hydrolysis, oxidation, racemization, elimination of Cys-Cys bridges and disulfide exchange. Chemical degradation processes may be controlled by adjusting environmental conditions or by adding stabilizers such as sugars or polyols.

The native structure of a protein is usually regarded as the conformation that is exhibited by proteins within the cellular environment or by isolated proteins at their maximum biological activity. Physical instability is mainly associated with processes that change the local spatial arrangement of (1) the protein backbone (2) subunits or (3) three-dimensional structures. These spatial changes can lead to denaturation, aggregation or precipitation; however, they do not involve covalent bond breakage, except in disulfide exchange. These processes may eventually lead to a loss of biological activity or inactivation (Gianfreda & Scarfi, 1991; Sowdhamini & Balaram, 1993; Wong & Parasrampuria, 1997).

Mozhaev (1993) described the reversible and irreversible inactivation of enzymes as:

$$N \leftrightarrows D \rightarrow I$$

Where N, D and I represent the native, reversibly denatured and irreversibly inactivated forms of the protein, respectively. Reversible denaturation is a two state-transition unlike irreversible inactivation, which is a multi-step process. Reversible denaturation, also referred to as thermodynamic stability, is concerned with the resistance of the folded

protein conformation to denaturation, while irreversible inactivation measures the protein's long-term stability (Ó'Fágáin, 1997a).

Denaturation studies generally involve exposing the protein of interest to a variation of temperature (e.g. heat/cold denaturation) or the addition of chaotropic agents (e.g. urea, guanidine) as a means of inducing unfolding. Once the protein has unfolded, numerous factors may lead to irreversible damage to the protein, thereby rendering the protein inactive (Sowdhamini & Balaram, 1993).

By definition, stabilization of protein molecules means preventing these changes and preserving the native structure of the protein. Assuming that unfolding of the protein is the initial and essential step leading to inactivation, extensive research and experimental studies have been carried out on different chemical modification methods to approach the problem of enzyme stabilization (Gianfreda & Scarfi, 1991).

Chemical modification refers to any chemical alteration of the protein structure, with or without changes in biological function (Tyagi & Gupta, 1993). It is a valuable tool for altering and extending the activities of enzymes *in-vitro*. Chemically modifying specific functional groups of the protein can lead to an increase in the protein's stability, improve its solubility, alter patterns of inhibition and activation, and change its pH optimum or substrate specificity (Hilvert, 1991). A number of chemical modification techniques exist which will be discussed in detail including the following: modification with monofunctional reagents, cross-linking with bi- or multifunctional reagents, hydrophilization, immobilisation, coupling to polyethylene glycol (PEG) and aggregation.

1.6.1. Chemical modification with monofunctional reagents

These modifications occur when the free functional groups of the protein react with monofunctional (small molecular weight) reagents (Tyagi & Gupta, 1993).

Elsner *et al.* (2000) modified trypsin by succinylation of its lysine residues. This process did not influence the catalytic behaviour of trypsin; however, succinyl-trypsin displayed high stability against autodigestion. In particular, modified trypsin retained 100% amidase activity over a 5-hour period, while native trypsin showed a 95% loss in activity over the 5-hour period. Trypsin hydrolyses L-argininyl and L-lysyl bonds of polypeptides. It undergoes rapid autolytic inactivation at neutral pH due to hydrolysis of these bonds. However, autolysis may be prevented by chemically modifying the ε-amino group of lysine residues and the guanidine group of arginine residues (Nureddin & Inagami, 1975; & Ó'Fágáin, 1996b). Succinylation of trypsin results in a change in the surface charge of the protein. In native trypsin, the ratio of positively charged and negatively charged amino acid residues is 15/10, in succinyl-trypsin 2/23. Hence, Elsner *et al.* (2000) hypothesised that the high stability of succinyl-trypsin was as a consequence of the negatively charged protein surface. A negative electrostatic field exists in the substrate-binding pocket of trypsin. This negative field attracts the solvated arginine and lysine substrate side chains and helps to stabilize their postive charges in the enzyme-substrate complex. Therefore, changing the charge on the lysine residue by modification will prevent lysine binding in the negative substrate-binding pocket and hence, prevent autolysis (Briand *et al.,* 1999).

Khajeh *et al.* (2001) chemically modified lysine residues in two bacterial α-amylases, a mesophilic enzyme from *Bacillus amyloliquefaciens* (BAA) and a thermophilic enzyme from *Bacillus licheniformis* (BLA) using citraconic anhydride. They observed an enhancement in the thermostability of the modified mesophilic BAA. In particular, at 80°C, this modified enzyme retained 40% activity over a 10-minute assay period compared

with native BAA, of which activity fell to less than 5% over the same assay time. A dramatic enhancement of activity at 37°C was observed for the modified thermophilic BLA, resulting in a 3-fold increase in the k_{cat}/K_m (1523 mM/sec) when compared to native BLA (486mM/sec).

1.6.2. Chemical cross-linking with bi- or multifunctional reagents

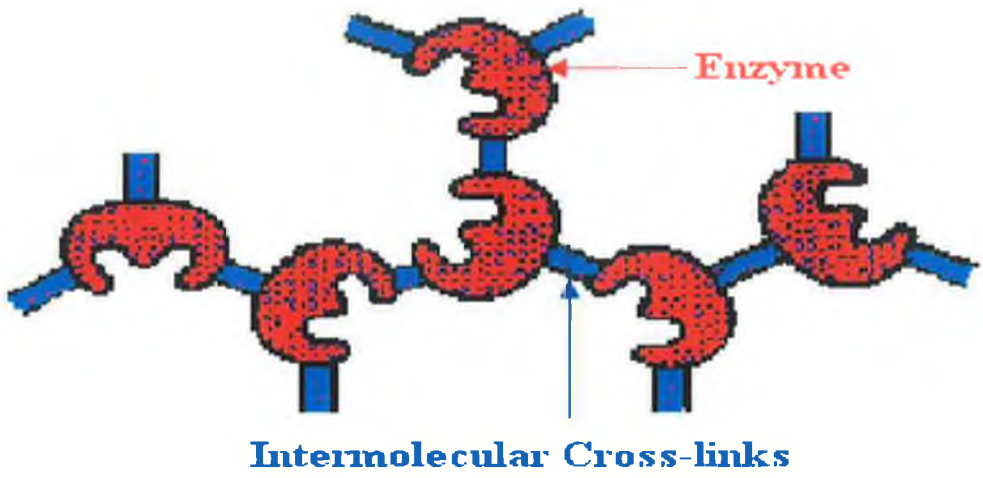


FIGURE 1..9. SCHEMATIC REPRESENTATION OF INTERMOLECULAR CROSS-LINKING (Katchalski-Katzir, 1993).

This is a support-free method involving covalent bond formation between proteins (same or different), or subunits of protein, by means of a bi- or multifunctional reagent (Bickerstaff, 1997). Bifunctional cross-linking reagents contain two reactive groups (Han *et al.*, 1984). They are classified into three categories: homobifunctional, heterobifunctional and zero-length (Ji, 1983). Homobifunctional reagents carry two identical functional groups. They have been shown to induce cross-linking between identical functional groups e.g. two thiols, two amines, two acids or two alcohols, and are predominantly used to form intramolecular cross-links. However, they have been used to conjugate two different

biomolecules e.g. an enzyme to an antibody (Ji, 1983). Examples of these reagents include: glutaraldehyde, succinimidyl esters, imidoesters, dianhydrides and diisocyanates (Gleich *et al.*, 1992). Heterobifunctional reagents contain two dissimilar functional groups e.g. photosensitive azide and conventional imidate, allowing the formation of cross-links between unlike functional groups (Ji, 1983). The zerolength reagents form a chemical bond between two chemical groups without being incorporated themselves into the product. A feature of their reaction is a shortening of the distance between the two reacted groups upon cross-linking. An example of this type of reagent/reaction is the water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). This reagent couples a carboxylic acid to a primary amino group, forming an amide bond (Ji, 1983).

Cross-linking reagents can be used to introduce both intra- and intermolecular bridges into proteins. Intramolecular bridges are principally used to stabilize the tertiary structure of proteins and are useful in determining the intramolecular distances between groups. Intermolecular cross-links may join similar molecules (homopolymers) or dissimilar (heteropolymers) together. The products can then be used as models for the study of protein interactions e.g. antibody-antigen interactions or hormone-receptor interactions (Ji, 1983: Han *et al.*, 1984). Ryan *et al.* (1994) chemically modified horseradish peroxidase (HRP) with the homobifunctional cross-linking reagents, suberic acid N-hydroxysuccinimide ester (SA-NHS) and ethylene glycol bis-succinimidyl succinate (EG-NHS). They observed a 6- to 23-fold increase in the thermostability of modified HRP (depending on the NHS ester used) as compared to native HRP. Native and succinimide derivatives of HRP were also studied by Miland *et al.* (1996). They investigated the stability properties of both native and modified HRP in various water/organic solvent mixtures. In each of the systems tested, increasing the solvent concentration resulted in a

loss of activity for native HRP; however, modification of HRP resulted in improved tolerance in these cosolvent systems, e.g. in a 50% (v/v) THF cosolvent system at 25°C, SA-NHS HRP retained 50% of its original activity in comparison to native HRP, where activity fell to less than 20%. Both modified peroxidases also exhibited improved resistance to the denaturant guanidine hydrochloride.

Cheon *et al.* (2000) observed from previous work that the homotetramer enzyme D-hydantoinase from *B. stearothermophilus* dissociated to a monomer under operational conditions and that the dissociated monomer was very unstable. They hypothesized that intersubunit cross-linking of tetrameric D-hydantoinase would reduce the dissociation of subunits, and hence stabilise the enzyme. Cross-linking was performed using EDC (1-ethyl-3-(3-di-methylaminopropyl) carbodiimide hydrochloride). The cross-linked enzyme was shown to exhibit maximum activity at 70°C, considerably higher than the native enzyme, where maximal activity was determined at 60°C. They investigated the stability of the cross-linked and native enzyme at 55°C, where the calculated half-life was 37 and 9.5 hours, respectively.

1.6.3. Hydrophilization

Structural analysis of proteins by X-ray crystallography suggests that about one half of the surface area of a protein is occupied by non-polar amino acids. These residues are very often organized as hydrophobic surface clusters, which play an important role *in-vivo*, enabling the protein to bind via hydrophobic interactions to other proteins forming multi-enzyme complexes, to lipids in biological membranes and to polysaccharides in cell walls. However, the contact of these non-polar residues with water is thermodynamically unfavourable and can affect protein stability *in-vitro*. Therefore, reducing the non-polar surface area should stabilize the protein (Mozhaev *et al.*, 1988). The process of hydrophilization can achieve this. It involves the preferential modification by alkylation or acylation of $-NH_2$ groups on the protein surface, e.g. transformation of protein $-NH_2$ groups into $-NHCH_2COOH$ groups, giving rise to an artificial hydrophilization of the surface area of the protein globule and resulting in increased enzyme stability. This effect can be explained by a decrease of non-polar clusters located on the protein surface, contact of which with water is thought to destabilize the enzyme (Gianfreda & Scarfi, 1991).

The modification can be performed in two ways: (1) hydrophobic amino acid residues are directly modified by the hydrophilic reagent or (2) any residue located near a nonpolar surface cluster is modified by the hydrophilic reagent, obtaining shielding of the cluster from the aqueous medium (FIGURE 1.10.).

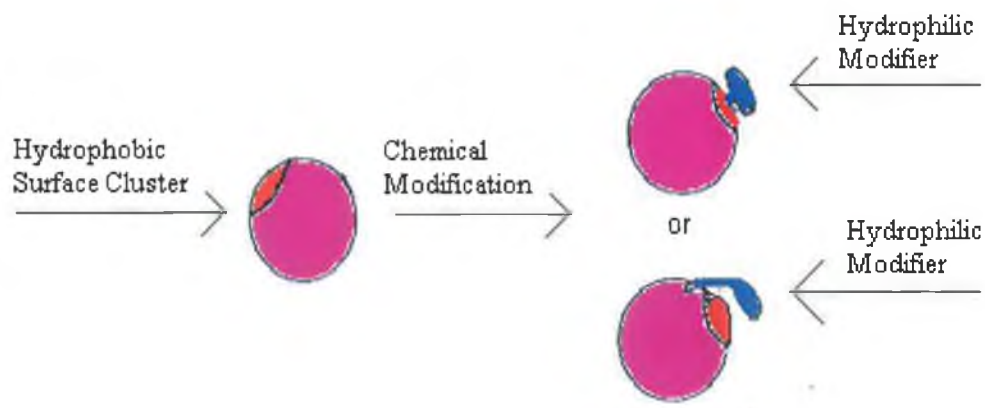


FIGURE 1.10. SCHEMATIC REPRESENTATION OF APPROACH TO HYDROPHILIZATION OF NON-POLAR SURFACE AREAS IN A PROTEIN (Mozhaev *et al.*, 1988).

Mozhaev *et al.* (1988) successfully used this method to artificially hydrophilize trypsin and α-chymotrypsin. In a two-step procedure the hydrophilic reagent directly modified the surface hydrophobic tyrosine residues of trypsin (FIGURE 1.11.). They determined that modified trypsin became up to 100 times more stable to the effects of thermoinactivation. In particular, at 56°C, modified trypsin showed 50% residual activity over a 2-hour period. In comparison, native trypsin was shown to have less than 10% residual activity over the same period. However, Mozhaev *et al.* (1988) concluded that the extent of stabilization depended exclusively on the number of aminotyrosines introduced; hence, the stabilizing effect increased with the number of aminotyrosine residues in trypsin. From spectral investigations it was deduced that only four tyrosines are entirely exposed to the solvent and can therefore undergo modification more readily than the other tyrosine residues in trypsin.

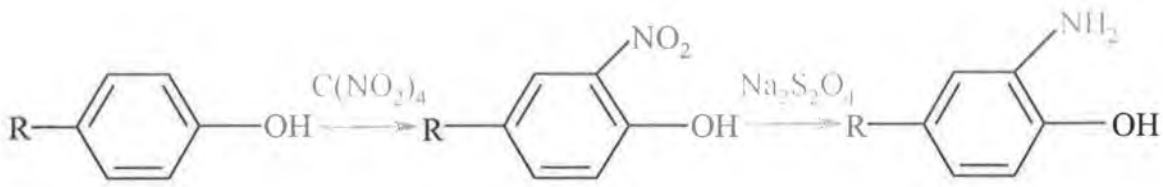


FIGURE 1.11. INTRODUCTION OF AMINO GROUP INTO THE TYROSINE RESIDUES OF TRYPSIN (Mozhaev *et al.*, 1988).

The same report also described the acylation of the amino groups of α-chymotrypsin by cyclic anhydrides of trimellitic, pyromellitic and mellitic acids. This allowed a variable number of carboxylic groups (up to 5) to be introduced into each lysine residue altered, hence bringing about considerable hydrophilization. Mozhaev *et al.* (1988) observed a 100-1000 fold increase in stability against thermoinactivation in modified α-chymotrypsin. Khmelnitsky *et al.* (1991b) hypothesized that hydrophilized enzymes had the ability to hold their hydration shell, resisting the denaturing stripping of water from the enzyme surface by organic solvents and consequently enhancing their stability in these solvents. They modified α-chymotrypsin with pyromellitic dianyhydride and determined that hydrophilized α-chymotrypsin could endure much higher concentrations of organic solvents without loss of catalytic activity as compared to the native.

Fernández *et al.* (2002) chemically modified trypsin with monoamino derivatives of α-, β-, γ-, cyclodextrin (CD) using EDC as a coupling agent. These cyclodextrin-enzyme complexes were found to be more stable against thermal incubation at temperatures ranging from 45°C to 60°C when compared to native trypsin; e.g. at 50°C the half-life of trypsin-γCD was 108 minutes, whilst native trypsin had a half-life of 16 minutes. These complexes were also more resistant to autolytic degradation at pH 9.0. Fernández *et al.* (2002) suggested that this could be associated with steric hindrance of residues to the cleavage sites in trypsin caused by the bulky cyclodextrin.

1.6.4. Immobilization

The term "immobilized enzyme" is used to denote a protein that is localized or physically confined in a defined region of space, with retention of catalytic activity and which can be used repeatedly and continuously. The immobilization of an enzyme closely resembles the state of the enzyme within the intracellular microenvironment of living cells and hence, provides a model system to study and solve basic problems in enzymology (Gianfreda & Scarfi, 1991; Katchalski-Katzir, 1993). Immobilization by multipoint attachment to a support may enhance stability and prevent inactivation of the enzyme. The support rigidifies the protein conformation, thus preventing unfolding and may also stabilize the protein by means of steric hindrance and diffusion restrictions (Wong & Parasrampuria, 1997). Enzymes can be immobilized by a number of methods, which result in: (1) enzyme-carrier conjugates, in which the enzyme is adsorbed or covalently bound to its carrier, (2) entrapment of the enzyme in gels, fibres or beads and (3) chemical aggregation, (Gianfreda & Scarfi, 1991; Katchalski-Katzir, 1993).

The chosen immobilization technique should allow retention of enzyme catalytic activity while diminishing processes that are detrimental to the enzyme, such as autolysis (Díaz & Balkus, 1996). There are a number of advantages associated with this technique. Because the enzyme is immobilized, contamination of the product with the immobilized enzyme can be avoided, permitting an easier recovery of the product. Better quality products are also obtained. Once the product has been recovered the biocatalyst can be re-used. Under optimum assay conditions both thermal and storage stability of an enzyme can be increased by this method, provided the tertiary and/or quaternary structure of the protein can be stabilized (Huckel *et al.*, 1996).

1.6.4.1. ENZYME IMMOBILIZATION BY CARRIER BINDING

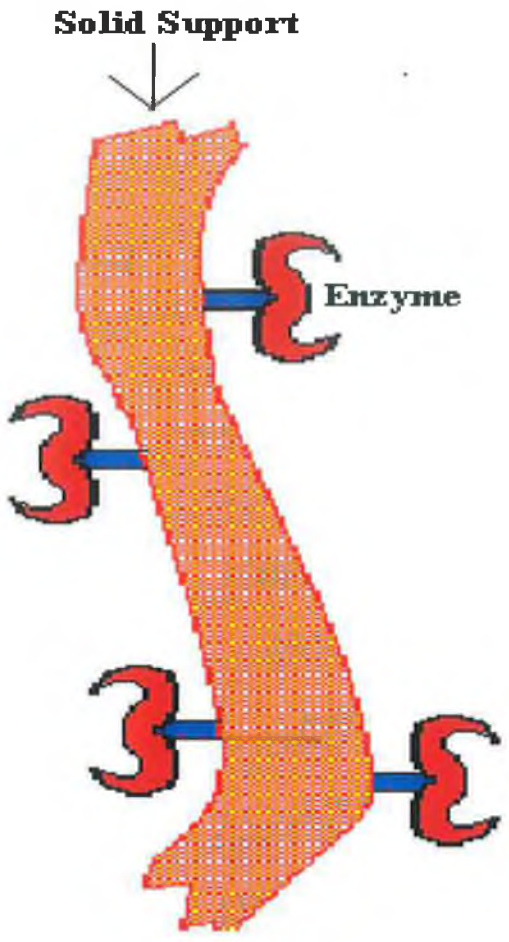


FIGURE 1.12. DIAGRAM SHOWING IMMOBILIZATION BY CARRIER BINDING (Katchalski-Katzir, 1993).

Yodoya *et al.* (2003) described the immobilisation of bromelain onto porous copoly (γ-methyl-L-glutamate/L-leucine) beads. They determined that immobilized bromelain is more stable than free bromelain at temperatures of between 60-80°C. Immobilized bromelain treated at 65°C for 60 minutes exhibited residual activity 3-4 times that of free bromelain. The storage stability of bromelain increased significantly upon immobilisation. Yodoya *et al.* (2003) observed that storage of immobilized bromelain at 4°C for 6 months resulted in no significant loss of activity, whereas free bromelain lost more than 50% of its

initial activity under the same conditions. They concluded that the increase in stability of immobilized bromelain could be attributed to the prevention of autolysis and thermal denaturation by covalent immobilisation of bromelain molecules on the surface of the beads.

Danisman *et al.* (2004) used this method to immobilise invertase onto a poly (2-hydroxyethyl methacrylate-glycidyl methacrylate) (pHEMA-GMA) membrane. The effect of immobilisation on thermal and pH stability was investigated. Differences were observed in the optimum temperature for immobilized and free invertase at 55°C and 45°C, respectively. Immobilized invertase incubated at 50°C for 90 minutes remained stable with no observed loss in activity; however, free invertase, lost 10% of its initial activity under the same assay conditions. Half-lives of the free and immobilized enzyme were 11 and 38 minutes at 70°C, respectively. The optimum pH value for free invertase was obtained at pH 5.0, whilst the optimum pH for immobilized invertase was shifted 1.5 pH units to the alkaline region.

1.6.4.2. ENZYME ENTRAPMENT

Enzyme entrapment differs from adsorption and covalent binding in that the enzyme molecules are free in solution but are restricted in movement by the lattice structure of the gel (Bickerstaff, 1997). The entrapment of an enzyme may be achieved by one of two methods, either a cross-linked polymeric network must be formed around the enzyme molecule or the enzyme is placed inside a polymeric material and then cross-linking of the polymer chains is performed (FIGURE 1.13.) (O'Driscoll, 1976).

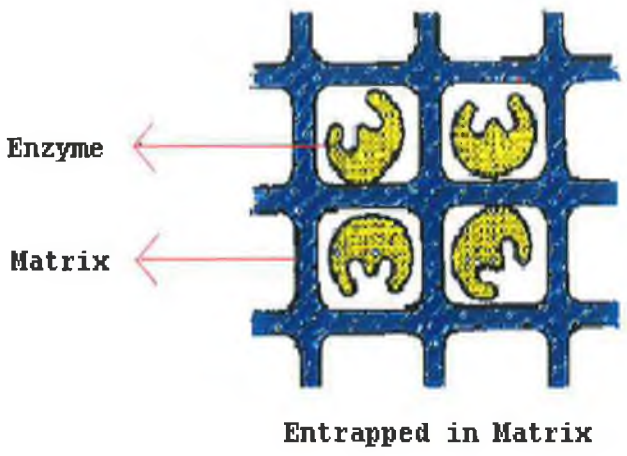


FIGURE 1.13. DIAGRAM SHOWING ENZYME ENTRAPMENT IN SEMI-PERMEABLE POLYMER MEMBRANE (Katchalski-Katzir, 1993).

Munjal and Sawhney (2002) used this method to immobilise mushroom tyrosinase in alginate, gelatin and polyacrylamide gels. The effect of immobilisation on thermal and pH stability was investigated. Differences were observed in the optimum temperature for immobilized and free tyrosinase. The maximum activity of the alginate- and gelatin-entrapped preparations was obtained at 35 and 40°C, respectively, as compared to 20°C for free enzyme. At 40°C, gelatine-entrapped tyrosinase retained 83% of its initial activity after 1 hour, while the free form retained only 58% of its initial activity during the same

period. The enzyme entrapped in polyacrylamide and alginate gels exhibited broader pH activity profiles, while a shift of pH optima toward the alkaline side was observed in the case of gelatin-entrapped tyrosinase.

Çetinus and Öztop (2003), immobilized catalase into chitosan beads prepared in a cross-linking solution. The formation of a cross-linked network decreases the enzyme's mobility. Various characteristics of immobilized catalase were evaluated including kinetic parameters. The value of K_m for free and immobilized catalase was found to be 35mM and 77.5mM, respectively, while the V_{max} was calculated as 32,000μmol (min mg protein)$^{-1}$ and 122μmol (min mg protein)$^{-1}$, respectively, for the free and immobilized enzyme. Çetinus and Öztop (2003) noted that the significant differences observed with these kinetic parameters before and after immobilisation of the enzyme could be attributed to a number of factors, namely, structural changes in the enzyme upon immobilisation and lower accessibility of the substrate to the active site due to steric hindrance by the support. Both enzymes showed an optimum pH of 7.0 at 35°C; however, immobilized catalase had a broader pH-activity range.

Chen and Lin (2003) investigated the effects of immobilization of lipase in a sol-gel matrix. The thermal stability of lipase increased 55-fold upon entrapment when compared to free lipase, the half-life ($t_{½}$) also increased from 49.9 to 2740 hours, at 35°C after immobilisation.

1.6.4.3. CHEMICAL AGGREGATION

The aggregation method involves using high concentrations of cross-linking agents to produce an insoluble aggregate of enzymes. The formation of this aggregate is attributed to both intramolecular and intermolecular cross-links in the protein molecule. This method has also been used to retain proteins on the surface of porous materials, thereby anchoring the protein onto its matrix. It has also been used together with inclusion to increase the mean size of enzyme molecules entrapped in a gel or microcell, allowing the use of lager gel pore sizes and reducing diffusion constraints (Broun, 1976; Khare *et al.,* 1991; Tyagi *et al.*, 1999).

Tyagi *et al.* (1999) used glutaraldehyde to prepare chemical aggregates of three enzymes, i.e. acid phosphatase, β-glucosidase and polyphenol oxidase. They investigated the thermal and solvent stability of the native and modified forms. An enhancement in thermal stability was observed for the modified forms. They also determined increased C_{50} values (concentration of organic cosolvent required to inactivate the enzyme by 50%) for the modified enzymes in the co-solvent systems tested (acetonitrile, DMF & THF), each at 50% (v/v).

A more recent technique known as cross-linked enzyme crystal technology (CLEC®), involves the cross-linking of enzymes in their crystalline state to produce particles which are insoluble in both water and organic solvents. This process consists of two major steps: first, the crystallization of the enzyme followed by the chemical cross-linking of the crystals with a suitable cross-linking reagent (e.g. glutaraldehyde) in such a manner that protein activity is maintained and the crystalline lattice is not disrupted. This process results in both stabilisation and immobilisation of the enzyme without dilution of activity. Channels formed within the CLEC® allow for easy diffusion of substrates and products. (Govardhan, 1999; Kim *et al.*, 2003; Ó'Fágáin, 2003).

Kim *et al.* (2003) used this method to synthesise CLECs of cyclodextrin glucanotransferase (CGTase). They studied the effect of temperature on the stability of CGTase-CLECs and soluble CGTase. At 70°C CGTase-CLECs retained more than 70% of its original activity as compared to soluble CGTase, where an 85% loss in activity was exhibited at the same temperature. Soluble CGTase exhibited no activity at 80°C whilst CGTase-CLECs retained 48% of its original activity at the same assay temperature. They also investigated the activity of both enzymes in 15% (v/v) solutions of water miscible organic solvents. The cross-linked enzyme was found to be stable in all of the tested solvents (2-butanol, DMSO, formamide, isopropanol) retaining more than 80% of its original activity, whereas soluble CGTase lost more than 50% of activity in the same solvents.

1.6.4.4. PEGYLATION OF ENYZMES

Enzymes are becoming the main tools for specific bioconversion in organic synthesis; however, they suffer from insolubility in most organic solvents (Veronese, 2001). Linking an enzyme to the amphipathic compound polyethylene glycol (PEG) has led to solubility in both aqueous solutions and organic solvents. The solubility of enzymes in organic solvents is due to the strongly hydrated PEG chains creating an aqueous shell around the enzyme molecule thus protecting it from denaturation by the organic solvent (Ogino & Ishikawa, 2001). It has been found that PEG-modified enzymes are also generally more stable against temperature that their native counterparts (Zhang *et al.*, 1999). Attachment of the long-chain PEG molecule to an enzyme molecule surface may make the protein less flexible, thus minimizing unfolding and thereby enhancing the stability of the enzyme at high temperatures (Yang *et al.*, 1996).

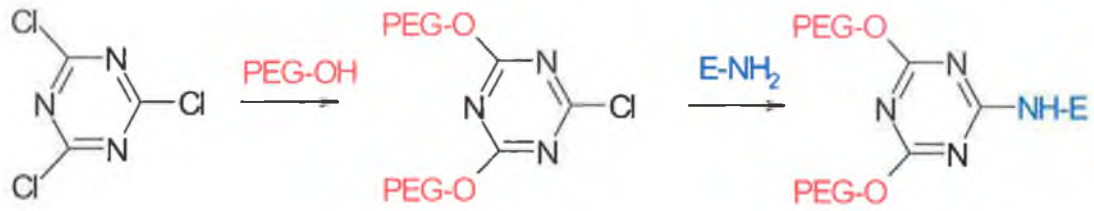


FIGURE 1.14. ENZYME [E] MODIFICATION WITH CYANURIC CHLORIDE ACTIVATED PEG (Yang *et al.*, 1996).

Zhang *et al.* (1999) used four activated methoxypolyethylene glycols (MPEG, molecular masses 350, 750, 2000 and 5000), to modify trypsin. They investigated the thermal stability of native and modified trypsin over the temperature range of 30°C to 70°C and found that all four MPEG-modified enzymes showed enhanced thermal stability as compared with the native enzyme. Thermal stability of the modified enzyme was found to increase with increasing molecular mass of the MPEG moiety. T_{50} (temperature required to inactivate enzyme by 50%) values were determined and ranged from 47°C for native trypsin up to 66°C for $MPEG_{5000}$-trypsin. A decrease in the rate of autolysis was observed for modified trypsin. While native trypsin retained only 20% of its initial activity at 60°C after 90 minutes, modified trypsin retained 28, 36, 57 and 63% of its initial activity for all four MPEG-trypsin, respectively.

1.7. Protein Engineering

Protein engineering involves using genetic manipulation techniques to alter the protein of interest in specific ways. This can be accomplished by site-directed mutagenesis, random mutagensis and directed evolution. Site directed mutagenesis allows the introduction of one or more defined changes at a time. Random mutagenesis is a broader method of introducing changes into the enzyme and involves the introduction of random mutations followed by screening the resultant mutants. These methods have been used to improve enzyme stability, substrate specificity and to create novel and efficient catalysts (Sears & Wong, 1996; Ó'Fágáin, 1997c). Sears and Wong (1996), Van Den Burg and Eijsink (2002) and Torrez *et al.* (2003) have all written reviews dealing with this area.

1.8. Application of Proteases

Enzymes have been used in the industrial field for many applications e.g. brewing, animal feeds and detergents. The composition of dirt and stains on most clothes includes lipids, carbohydrates, proteins, dyes, soil and small inert particles. Normal detergents do not remove proteinaceous stains. However, the addition of enzymes such as bacterial alkaline proteases e.g. subtilisins from *Bacillus licheniformis* and *Bacillus amyloliquifaceins* has led to better cleansing power of these detergents by hydrolytic degradation of difficult protein-containing solids, e.g. blood, egg-yolk and chocolate (Khaparde & Singhal, 2001).

Developments in molecular biology have led to the application of proteolytic enzymes as therapeutic agents. Proteases have been used as an alternative to mechanical debridement, to aid in the removal of dead skin in burns patients. Success of this treatment was variable, possibly due to the poor stability of the enzymes used. However, recombinant vibriolysin, a proteolytic enzyme from *Vibrio proteolyticus*, a marine micoorganism, was assessed topically in phase 1 clinical trials, for use as a debridement agent. Tolerability and

effectiveness of this topically applied enzyme for the debridement of burns has shown encouraging results (Vellard, 2003).

As industrialization of the planet has grown, so too has concern for the environment. Hence, finding more benign methods for the production and provision of services has led to further interest in enzymes. A number of examples exist where the use of enzymes has made a significant difference environmentally: laundry detergents where phosphates have been replaced with enzymes (proteases) and breadmaking where lipases have replaced chemical emulsifiers. This list is evergrowing and will continue to bring with it significant medical, social and environmental benefits (Cherry & Fidantsef, 2003).

This research describes the investigation and possible applications of two interesting candidate proteases, a proline-specific serine protease dipeptidyl peptidase IV (DPP IV) and a recombinant parasite cysteine protease cathepsin L1 (rFheCL1), neither of which undergo autolysis.

1.9. Mammalian Dipeptidyl Peptidase IV (DPP IV)

Dipeptidyl peptidase IV [DPP IV, EC 3.4.14.5] was first discovered by Hopsu-Havu and Glenner (1966) in rat homogenates and in commercial enzyme preparations as an activity liberating naphthylamine from Gly-Pro-2-naphthylamide and was initially termed glycylproline naphthamidase (Mentlein, 1999). It has also been known under different names such as "dipeptidyl aminopeptidase IV", "post-proline dipeptidyl aminopeptidase IV", "X-Pro dipeptidyl aminopeptidase" and "Gly-Pro naphthylamidase" (Sentandreu & Toldrá, 2001).

Since the amino acid sequence Gly-Pro is frequently found in collagens, a possible metabolic significance in collagen metabolism was proposed. However, the enzyme is unable to cleave Pro-Pro or Pro-Hyp bonds which mostly follow the Gly-Pro sequence in collagens and hence the physiological functions of dipeptidyl peptidase IV remained obscure for many years (Mentlein, 1999). Dipeptidyl peptidase IV has been isolated from various mammalian species and is present in most vertebrate tissues and body fluids; however, their activities often vary (Mentlein, 1999). The enzyme fulfils a variety of functions including the activation and inactivation of a number of bioactive peptides, e.g. β-casomorphin and substance P (Lambeir *et al.*, 2003). Inhibition of DPP IV activity increases insulin secretion, improving glucose control in diabetics. Thus, inhibition of DPP IV is a promising new strategy for treating type 2 diabetes (Rasmussen *et al.*, 2003)

The entire amino acid sequence of DPP IV for human, rat and mouse have been determined by cDNA cloning and sequencing. CD 26, a surface marker of T lymphocytes was found to be identical to DPP IV. DPP IV is a homodimeric type II transmembrane glycoprotein located on the surface of tissues. The enzyme is anchored to the membrane by a single hydrophobic helix with a short N-terminal cytoplasmic tail. The extracellular domain consists of a flexible stalk linking the membrane anchor to a highly glycosylated region

mostly in the N-terminal half, a cysteine rich region within the C-terminal half and the C-terminal catalytic domain (FIGURE 1.15.).

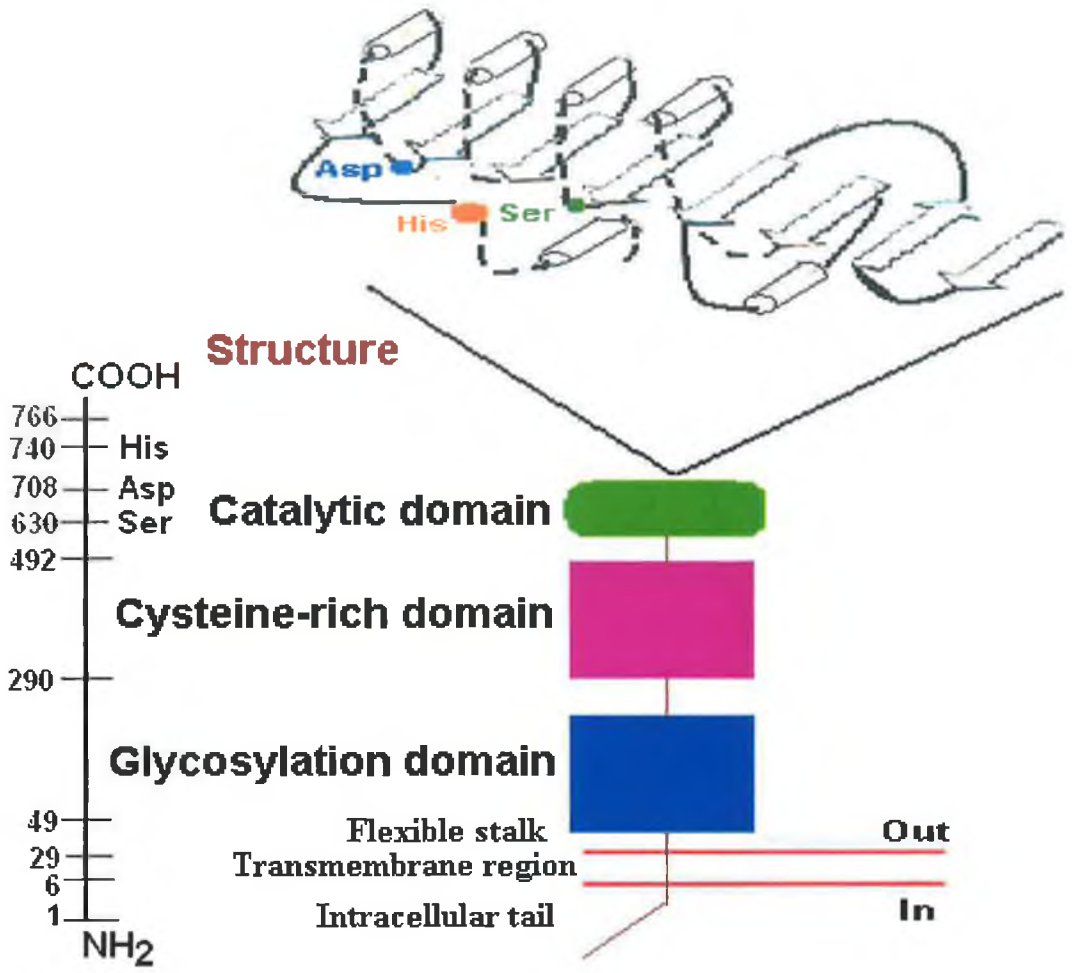


FIGURE 1.15. SCHEMATIC REPRESENTATION OF DPP IV/CD 26

This picture shows the main structural features along with the vertical bar, which highlights the primary structure and shows the positions of the active site residues as well as the borders of the extracellular domains. For the catalytic domain, an α/β protein fold is hypothesized (top): a core α/β-sheet comprising eight strands (arrows), linked by helices (cylinders) and loops (lines). The dotted lines indicate possible insertions and catalytic residues are situated on loops. Figure reproduced from DeMeester *et al.*, 1999.

Proteolytic cleavage of the membrane bound DPP IV results in a soluble protein migrating as a homodimer with a molecular weight range of 210-290kDa. Both membrane-bound and the soluble forms show identical enzymatic activity (Lambeir *et al.,* 1997; Engel *et al.,* 2003; Lambeir *et al.*, 2003; Rasmussen *et al.,* 2003).

The fully glycosylated crystal structure of human DPP IV has been solved and reported by Rasmussen *et al.* (2003). The subunits of the DPP IV homodimer consist of two domains; (1) α/β-hydrolase domain and (2) β-propeller domain. Between these two domains is a large cavity of ≈ 30-45 Å in width where the active site is situated. Accessibility to the active site is possible via two openings: (1) from the top of a central tunnel (4 Å) in the centre of the propeller domain and (2) from a large "cave" (more than 20 Å) located on the side between the hydrolase and propeller domain (Engel *et al.*, 2003; Hiramatsu *et al.*, 2003; Rasmussen *et al.*, 2003). Hiramatsu *et al.* (2003) hypothesised that substrates gain access to the catalytic site cavity via the large "cave". They consider that this is more probable due to its large size and also because the catalytic site centre is more exposed to the centre of the large "cave" than to entry via the central tunnel (FIGURE 1.16.). However, further experiments are needed to verify this.

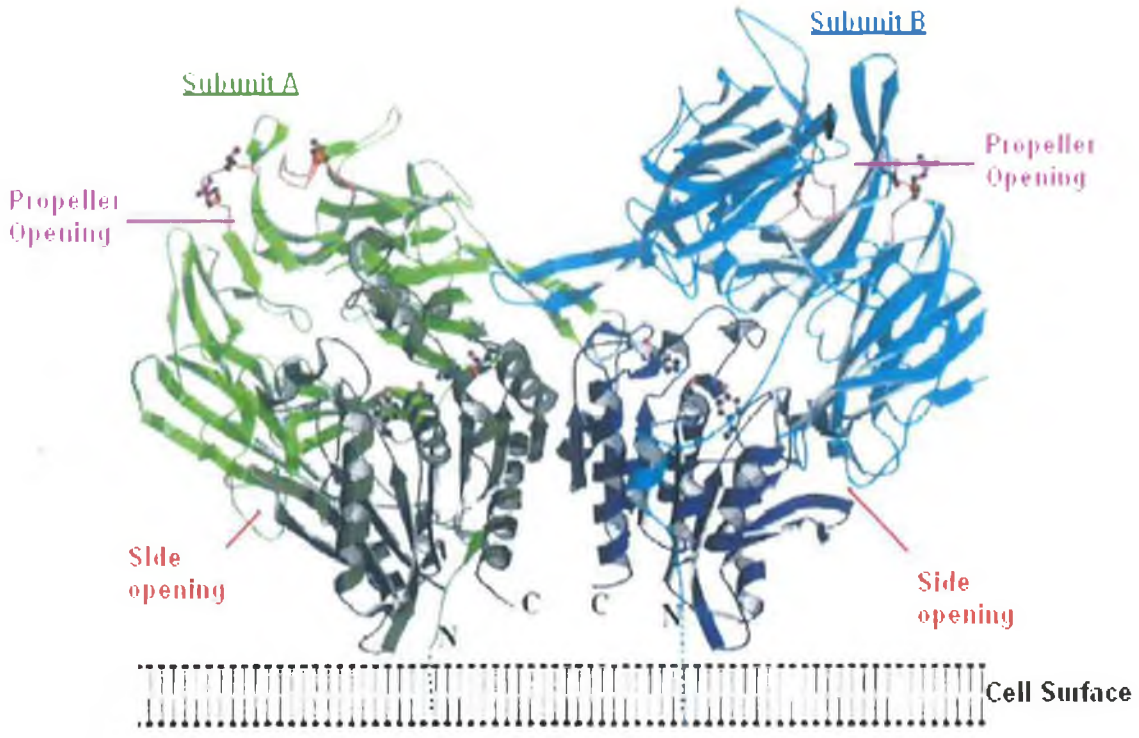


FIGURE 1.16. RIBBON DIAGRAM OF DPP IV

DPP IV forms a homodimer, (subunit A shown in green and subunit B in blue). Each subunit consists of two domains: an α/β-hydrolase domain and a β-propeller domain. The α/β-hydrolase domain located closest to the membrane contains the active site triad. N = amino termini and C = carboxyl termini. Figure reproduced from Rasmussen *et al.*, 2003; Thoma *et al.*, 2003).

Investigations into the structure of the active site have revealed how substrate specificity is achieved. DPP IV preferentially cleaves dipeptides with a penultimate Pro or Ala from the N-terminus of oligopeptides (FIGURE 1.17.). The hydrophobic pocket (S_1) is situated next to the active serine, as a consequence only amino acids with smaller side chains (proline, alanine and glycine) will be accommodated into this narrow pocket, limiting the possible residues at the P_1 position in substrates (Rasmussen *et al.*, 2003). This pocket is formed by a number of amino acids, which give a well-defined shape, and is better filled by proline than by alanine residues. Glycine is also accepted but with very low k_{cat}/K_m values.

Therefore, this is a feature in the determining the preference of DPP IV for a proline preceding the scissile bond. Side chains of the residues situated at P_2 and P_1' point into the large cavity and no specific interactions with DPP IV occur. This explains why DPP IV has no specific substrate requirements for amino acids in these positions; hence a large diversity of amino acids is possible at these positions (Engel *et al.*, 2003; Rasmussen *et al.*, 2003; Thoma *et al.*, 2003). Knowing the substrate preference of DPP IV enables one to choose the most suitable ester substrates and nucleophiles for peptide synthesis utilising this enzyme.

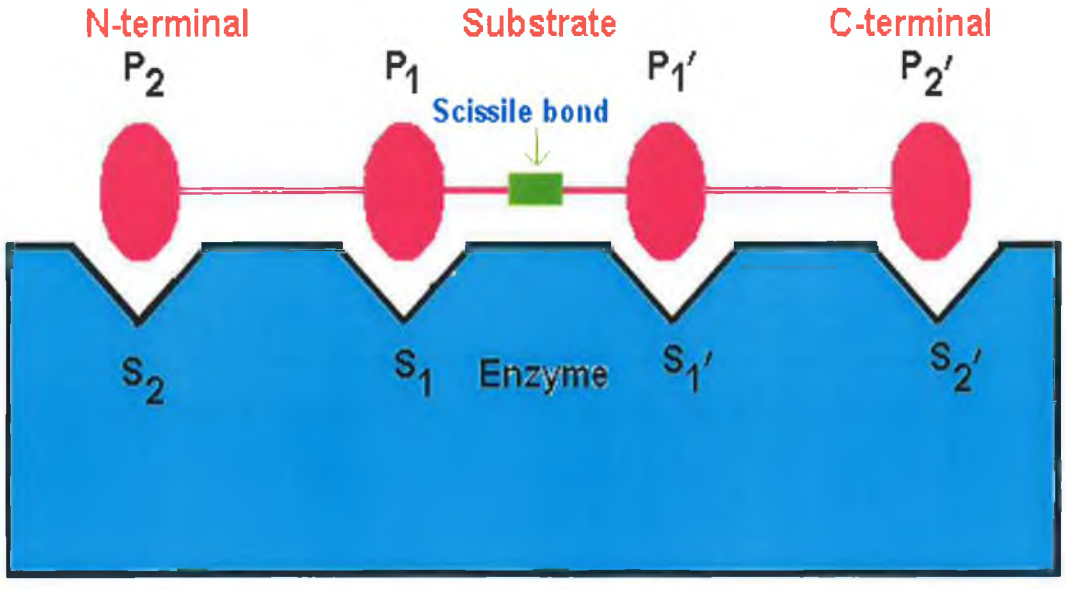


FIGURE 1.17. SCHEMATIC REPRESENTATION OF AN ENZYME-SUBSTRATE COMPLEX

Schechter-Berger notation for binding sites in serine proteases. Substrate residues are denoted according to their distance from the scissile bond and their location on the N-terminal or C-terminal side of the scissile bond (P_1, P_1', respectively), the subsites on the enzyme are similarly designated (S_1, S_1'). The scissile bond is located between the P_1 and P_1' (Fersht, 2002b).

1.10. Fluke (*Fasciola hepatica*) Cathepsin L1

The helminth parasite *Fasciola hepatica* is the causative agent of liver fluke disease in cattle and sheep and has recently emerged as an important pathogen of humans. This infection causes a loss of millions to the agricultural sector worldwide. The protease cathepsin L [3.4.22.15] a lysosomal cysteine protease belonging to the papain superfamily is secreted by this parasite. It plays an important role in many aspects of its pathogenicity, such as nutrient acquisition by catabolizing host proteins to absorbable peptides. Cathepsin L also cleaves matrix proteins such as fibronectin and collagen thereby facilitating the migration of this parasite through host tissue e.g. liver and intestine and has been implicated in the inactivation of host immune defences by cleaving immunoglobulins. (Dowd *et al.,* 1994; Dowd *et al.*, 2000; Dalton *et al.,* 2003; Collins *et al.*, 2004). Cathepsin L has been isolated from mammalian tissues such as rabbit, human and rat liver (Mason *et al.,* 1984; Mason *et al.,* 1985; Brömme *et al.,* 1993). The enzyme is also present in vertebrate tissues (fish) such as Carp hepatopancreas (Aranishi *et al.*, 1997).

Cathepsin L has only endopeptidase activity and preferentially cleaves peptide bonds with hydrophobic amino acid residues in P_2 and P_3 sites (Mort, 1998). It is synthesised as procathepsin L, and requires activation to the active monomer. Activation involves regeneration of the reactive thiol fuction (blocked as disulfide or metal complex) by addition of activators such as DTT together with chelate forming reagents e.g. EDTA (Creighton, 1993b). The crystal structure of human cathepsin L reveals that it contains two domains, (1) α-helical domain and (2) β-barrel domain. The domains separate on the top in a "V"-shaped active site cleft. The catalytic cysteine residue is situated at the top of the central α-helix and faces the catalytic histidine residue, forming the active site (FIGURE 1.18.) (Gunčar *et al.*, 1999; Turk *et al.*, 2002).

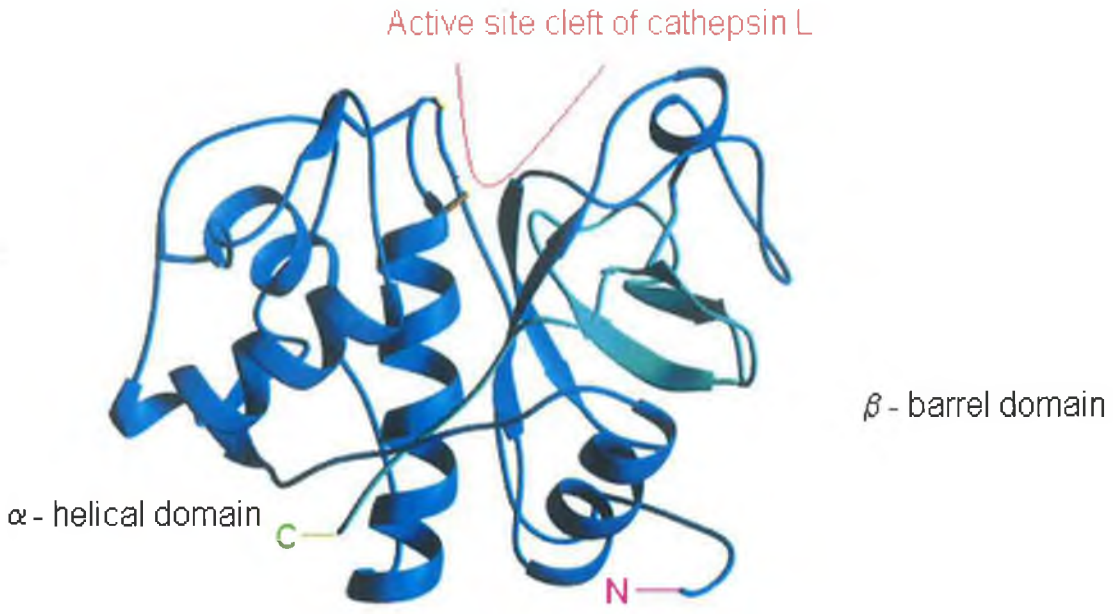


FIGURE 1.18. RIBBON-DIAGRAM OF HUMAN CATHEPSIN L

The picture shows the α-helical domain on the left side, the β-barrel domain on the right side of the picture and the active site opening at the top between the two domains. N = amino terminus and C = carboxyl terminus. Figure reproduced from Turk *et al.*, 2002.

Substrates bind along the active site cleft in an extended conformation. The S_2, S_1 and S_1' binding sites are well defined, whereas S_3 and S_2' ones are less well defined. The S_1' and S_2 binding sites are largely responsible for the specificity of the enzyme, with the S_2 site being the major determinant. This subsite is a deep hydrophobic pocket, which accommodates large hydrophobic residues at the P_2 position (Fujishima *et al.*, 1997; Turk *et al.*, 2002). Cathepsin L favours substrates with small amino acid side chains (Ala, Ser) or long but non-branched (Asn, Gln, Lys) at the P_1' position (Ménard *et al.*, 1993). Specificity of the enzyme for amino acids at this position is an important and deciding factor when considering suitable nucleophiles for use in peptide synthesis.

1.11. DPP IV and rFheCL1 as candidates for study

Considering the cheap and ample supply of the crude materials from which both dipeptidyl peptidase IV (DPP IV) and cathepsin L enzymes are sourced, investigation of their stability characteristics and classic enzyme kinetics *in-vitro* has been neglected to date. Due to the high selectivity of many proteases and their inability to cleave peptide bonds involving proline, it is not surprising that specific enzymes are involved in the cleavage of proline-containing peptide bonds, although they are few in number. Therefore, it was deemed a worthwhile project to investigate these stability and kinetic parameters for DPP IV, owing to its substrate specificity towards proline-containing bonds. This enzyme can also be considered as a suitable candidate to catalyse peptide synthesis, as the active site of many enzymes may not fit the bulky amino acid proline residue.

The development of a potential liver fluke vaccine targeting the fluke (*Fasciola hepatica*) cathepsin L1 protease is being undertaken at present. Yeast systems have been successfully employed for high-level expression of recombinant *Fasciola hepatica* cathepsin L1 (rFheCL1) with a view to targeting vaccines against the recombinant enzyme (rFheCL1). Therefore, elucidating the stability characteristics (pH, temperature, solvent) of this recombinant enzyme is essential for comparison to fluke (*Fasciola hepatica*) cathepsin L1 and also ascertaining its shelf-life as stability is a key attribute of any vaccine. The notable stability of fluke (*Fasciola hepatica*) cathepsin L1 makes rFheCL1 a useful candidate for chemical modification and peptide synthesis. Also, a review of the literature has indicated that very few cysteine proteases have been used for enzymatic peptide synthesis when compared with serine proteases, again making this enzyme a suitable candidate for enzymatic peptide synthesis.

The aims of the research described in this thesis involve exploring and elucidating the stability of bovine serum dipeptidyl peptidase IV (DPP IV) and recombinant *Fasciola*

hepatica cathepsin L1 (rFheCL1). DPP IV was fully investigated with regards to the stability of the enzyme at elevated temperatures (Results; section 4.1.2.), and in the presence of organic solvents commonly used in peptide synthesis (Results; section 4.1.3.). The effect of pH on the enzyme's stability and kinetic parameters was also determined at acidic, neutral and alkaline pH (Results; section 4.1.4.). The stability characteristics of both native and chemically modified rFheCL1 were studied. The effect of exposing both forms of the enzyme to increasing temperatures (Results; section 5.2.4 & 6.2.4., respectively) and organic solvents (Results; section 5.2.5. & 6.2.5., respectively) was fully elucidated and a comparison made. Hence, the direct effect of chemical modification upon the enzyme was determined. Chapter seven clearly outlines the usefulness of rFheCL1 with regards to enzymatic peptide synthesis.

CHAPTER 2

MATERIAL AND METHODS

2.1. EQUIPMENT

Centrifugations were performed using an ALC Multispeed PK 121 bench centrifuge (modification studies) and Beckman J2-MC high-speed centrifuge (serum isolation). Gel electrophoresis was performed using a Biorad Mini Gel Box with ATTO vertical electrophoresis system and power pac 1000. Images of gels were taken with Plustek OpticPro 482p scan maker (DPP IV) or Pharmacia Biotech Imagemaster® Video Documentation System for gel electrophoresis (rFheCL1). Enzyme and protein assays were performed using a Perkin Elmer LS-50 Luminescence Spectrometer, Unicam2 UV/Vis spectrophotomer and Labsystems Multiskan MS microplate reader. A Corning pH Meter 240 determined pH of buffers and solutions. LC-MS analysis utilised a Bruker Mass Spectrometer with electrospray ionisation and ion-trap detector linked to a Hewlett-Packard Esquire Liquid chromatograph 1100 with photo diode array detector.

2.2. MATERIALS

Suppliers of key chemicals used in these studies are listed below.

Amersham Biosciences UK Ltd., Bucks, England.

Sephadex™ G-25 Superfine

Bachem Feinchemikalein AG (Bebendorf, Switzerland)

Gly-Pro-AMC·HBr, Z-Lys-ONp·HCl, Z-Phe-Arg-AMC·HCl, Z-Phe-Arg-OMe·HCl, H-SerNH_2·HCl

BDH Limited Ltd., Poole, Dorset, England

Sodium sulphite

Fisher Scientific UK Ltd., Loughborough, England.

Acetic acid glacial, acetone, acetonitrile HPLC grade, dimethylformamide HPLC grade (DMF), diaminoethanetetra-acetic acid (EDTA), ethanol 100% (v/v), glycerol, hydrochloric acid (HCl 37%, (v/v)), sodium acetate anhydrous, sodium chloride (NaCl), sodium hydroxide (NaOH), tetrahydrofuran HPLC Grade (THF), Tris-(hydroxymethyl) aminomethane (Tris).

Kepak Meats (Clonee, Co. Meath, Ireland)

Bovine Whole Blood

Pierce Chemical Company, Illinois, USA.

BCA (Bicinchoninic acid) Protein Assay Kit, GelCode®, Blue Stain Reagent.

Riedel-de-Haen, Laborchemikalien, GmbH.

Acetonitrile CHROMASOLV®, water CHROMASOLV®.

Sigma Chemical Company (Poole, Dorset, England)

2-Mercaptoethanol, 7-amino-4-methylcoumarin (AMC), Acrylamide/bis-Acrylamide 30% (w/v) solution, Nα-Acetyl-L-Lysine, albumin bovine serum (BSA), ammonium acetate, ammonium persulfate, ammonium sulfate, borax, Bradford reagent, brilliant blue R, bromophenol blue, cellulose membrane dialysis tubing (33mm × 21mm), 2-[N-Cyclohexylamino] ethane-sulfonic acid (CHES), dimethylsulfoxide HPLC grade (DMSO),

DL-dithothreitol (DL-DTT), *trans*-epoxysuccinyl-L-leucylamido-(4-Guanidino)-butane (E-64), ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester) (EG-NHS), glycine, N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid] (HEPES), 2-[N-Morpholino] ethanesulfonic acid (MES), 3-[N-Morpholino] propanesulfonic acid (MOPS), picrylsulfonic acid (2,4,6-Trinitrobenzenesulfonic acid; TNBS), phenyl-Sepharose® CL-4B, polyethylene glycol (m.w 10,000), potassium phophate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4), quaternary-Sepharose fast flow anion exchanger, Sephacryl S-300-HR, sodium bicarbonate, sodium dodecyl sulfate (SDS), sodium phosphate monobasic (NaH_2PO_4), SigmaMarker™ low range (m.w. 6,500- 66,000), SigmaMarker™ wide range (m.w. 6,500-205,000), total protein reagent, trypsin (Bovine), N,N,N',N' –tetramethylene-ethylenediamine (TEMED).

All reagents were analytical reagent grade unless otherwise stated.

2.3. PURIFICATION AND ACTIVATION

2.3.1. PURIFICATION OF BOVINE SERUM DIPEPTIDYL PEPTIDASE IV (DPP IV)

The purification of dipeptidyl peptidase IV (DPP IV) was based on the method of Buckley *et al.,* (2004) and includes the following steps in sections 2.3.1.1, 2.3.1.2, 2.3.1.3 and 2.3.1.4.

2.3.1.1. Serum Preparation

Bovine whole blood was collected from a freshly slaughtered animal and transported to a 4°C cold room. A clot was allowed to form and shrink over 24 hours. The remaining unclotted whole blood was then decanted and centrifuged at 6000 rpm for 1 hour using a Beckman J2-MC refrigerated centrifuge fitted with a JL-10.5 rotor (radius 10cm). The supernatant and loose cellular debris was decanted and re-centrifuged at 20,000 rpm for 15 minutes at 4°C using a JL-20 rotor (radius 6cm). The serum thus produced was collected, pooled and stored in 20ml aliquots at -20°C.

2.3.1.2 Phenyl Sepharose Hydrophobic Interaction Chromatography (HIC)

A 30ml phenyl-Sepharose® CL-4B hydrophobic interaction column (2.5cm x 13cm) was equilibrated with 100ml 50mM HEPES, pH 8.0, containing 5mM EDTA and 1M ammonium sulphate at a flow rate of 2ml/min. A 20ml aliquot of crude serum was thawed at 37°C and solid ammonium sulphate was added to give a final ammonium sulphate concentration of 0.5M. Salted serum (20ml) was applied to the equilibrated column followed by a 100ml wash of the equilibration buffer to elute any unbound protein. Bound protein was eluted using a 50ml linear gradient of 50mM HEPES, pH 8.0, containing 5mM

EDTA and 1M ammonium sulphate to 50mM HEPES, pH 8.0, containing 5mM EDTA. A further 50ml wash of 50mM HEPES, pH 8.0, containing 5mM EDTA ensured all bound protein was eluted. Fractions (4ml) were collected throughout the run. Loading, washing and elution steps were carried out at a flow rate of 1.5ml/min. Fractions were assayed for DPP IV activity as described in section 2.4.3. Protein was determined using the Biuret assay as described in section 2.4.2.1. Fractions containing Gly-Pro-AMC degrading activity were combined and the volume recorded to yield post-phenyl Sepharose DPP IV. The phenyl-Sepharose resin was regenerated with a 100% ethanol (v/v) wash and then re-equilibrated with 100ml 50mM HEPES, pH 8.0, containing 5mM EDTA and 1M ammonium sulphate at a flow rate of 2ml/min.

2.3.1.3. Sephacryl S-300 High Resolution (HR) Gel Filtration Chromatography

The post-phenyl-Sepharose DPP IV (typically 35ml) was dialysed against 50mM HEPES, pH 8.0, containing 5mM EDTA (2 litres) for a minimum of 3 hours at 4°C to remove interfering salts. The dialysed sample was then concentrated via osmosis using solid polyethylene glycol. Glycerol was added to the concentrated sample to give a final glycerol concentration of 10% (v/v). The 100ml Sephacryl S-300-HR gel filtration column was equilibrated with 200ml 50mM HEPES, pH 8.0, containing 5mM EDTA at a flow rate of 2ml/min. The concentrated sample was loaded under the buffer head and the column washed with 150ml equilibration buffer at a flow rate of 1.5ml/min. Fractions of 5ml were collected and assayed for DPP IV activity as described in section 2.4.3. Protein was determined using the standard BCA assay as described in section 2.4.2.2. Fractions containing Gly-Pro-AMC degrading activity were combined and the volume recorded to yield post-Sephacryl S-300 DPP IV and stored at 4°C.

2.3.1.4. Quaternary (Q) Sepharose Strong Anion Exchange Chromatography

The post-Sephacryl S-300 DPP IV was concentrated via osmosis using solid polyethylene glycol. A 30ml Q-Sepharose strong anion exchange column (2.5cm x 13cm) was equilibrated with 100ml 50mM HEPES, pH 8.0, containing 5mM EDTA. The concentrated sample was applied onto the column followed by a 100ml wash with equilibration buffer. Bound protein was eluted using a 50ml linear gradient of 50mM HEPES, pH 8.0, containing 5mM EDTA to 50mM HEPES, pH 8.0, containing 5mM EDTA and 0.5M NaCl. A further 50ml wash of 50mM HEPES, pH 8.0, containing 5mM EDTA and 0.5M NaCl ensured all bound protein was eluted. Fractions (4ml) were collected throughout the run. Loading, washing and elution steps were carried out at a flow rate of 1.5ml/min. Fractions were assayed for DPP IV activity as described in section 2.4.3. Protein was determined using the standard BCA assay as described in section 2.4.2.2. Fractions containing Gly-Pro-AMC degrading activity were combined and the volume recorded. The post-Q-Sepharose DPP IV pooled fractions (typically 50ml) were dialysed against 50mM HEPES, pH 8.0, containing 5mM EDTA (2 litres) for a minimum of 3 hours at 4°C to remove interfering salts and stored at -20°C until required. The Q-Sepharose resin was regenerated with 2M NaCl and re-equilibrated with equilibration buffer. Future reference to purified DPP IV indicates post-Q-Sepharose DPP IV.

2.3.2. ACTIVATION OF r*Fasciola hepatica* Cathepsin L1 (rFheCL1)

Mr Peter Collins, Parasitology Research Group, formerly based at Dublin City University performed the purification step and generously supplied purified unactivated recombinant *Fasciola hepatica* Cathepsin L1 (rFheCL1) as a gift. rFheCL1 was activated to a protein concentration of 0.1mg/ml preparation based on a method by . Example of activation procedure:

Composition	Volume	Final Concentration
rFheCL1 (0.5mg/ml)	0.1ml	0.1mg/ml
DTT (100mM)	0.04ml	8mM
EDTA (500mM)	0.01ml	10mM

These solutions were combined and made up to total volume of 0.5ml with 0.1M sodium acetate, pH 5.0. The solution was then incubated at 37°C for 2 hours, when the preparation was stored at -20°C until use.

2.4. General Assays

2.4.1. Fluorescence Quantification of 7-Amino-4-Methyl-Coumarin

2.4.1.1. AMC Standard Curves

AMC (10mM in 100% DMSO (w/v)) was diluted to 100μM using 50mM HEPES, pH 8.0, containing 5mM EDTA when analysing dipeptidyl peptidase IV (DPP IV) or 0.1M sodium acetate, pH 5.0 containing 2mM DTT and 2.5mM EDTA when analysing recombinant *Fasciola hepatica* cathepsin L1 (rFheCL1). Stock solutions were stored in brown glass containers at 4°C to minimise any fluorescence. To form standard curves of 0-15μM, lower AMC concentrations were prepared using 50mM HEPES, pH 8.0, containing 5mM EDTA or 0.1M sodium acetate, pH 5.0, containing 2mM DTT and 2.5mM EDTA. All curves were prepared in a 96-well microplate in triplicate as follows:

- 25μl Buffer (HEPES or sodium acetate as above)
- 100μl appropriate AMC concentration
- 175μl 1.7.M acetic acid

Fluorometric analysis of these samples was achieved using a Perkin Elmer LS-50 Luminescence Spectrometer at excitation and emission wavelengths of 370nm and 440nm, respectively. The excitation slit width was maintained at 10nm while the emission slit width was adjusted according to the fluorescence intensity observed.

2.4.1.2. Inner Filter Effect

The inner filter effect or 'quenching' was determined as in section 2.4.1.1; however, 25μl buffer was substituted by 25μl crude bovine serum/enzyme sample. These samples were also prepared and assayed in triplicate.

2.4.1.3. AMC Excitation and Emission Wavelengths

The optimum AMC excitation and emission wavelengths were determined using the scan function option of the fluorimeter. A 5µM AMC solution was prepared by 1:20 dilution of the 100µM stock solution using 50mM HEPES, pH 8.0, containing 5mM EDTA or 0.1M sodium acetate, pH 5.0 containing 2mM DTT and 2.5mM EDTA. A pre-scan was performed on 1.5ml of 5µM AMC placed in a glass cuvette. The excitation wavelength obtained from the pre-scan was set and an emission scan was performed from 400-600nm at 5nm increments. Similarly an excitation scan was performed between 300nm and 500nm using the previously determined optimum emission wavelength. Optimum wavelengths were taken to be those at which fluorescence was at a maximum.

2.4.2. PROTEIN DETERMINATION

2.4.2.1. Biuret Assay

The Biuret assay was used to quantify the protein concentration in samples of 2-10mg/ml. These samples include bovine serum and post-phenyl Sepharose pooled samples (DPP IV purification). Samples (typically 20-35ml) were dialysed against 50mM HEPES, pH 8.0 containing 5mM EDTA (2 litres) for a minimum of 3 hours at 4°C to remove possible interfering substances such as incompatible buffers or salts. This assay was also used to determine the protein concentration of rFheCL1 samples. Samples with a protein

concentration higher than 10mg/ml were diluted with the appropriate buffer to achieve a suitable protein concentration with respect to the assay limits. To quantify the protein concentration, a BSA standard curve was included from 0-10mg/ml in triplicate. The assay was performed on a 96 well microplate as follows:

- 50µl Sample/BSA standard
- 200µl Biuret Reagent
- Incubation at 37°C for 30 minutes

The absorbance of each well at 560nm was determined using a Labsystems Multiskan MS microplate reader. Where necessary a standard curve was constructed and the protein concentration of the unknown samples was estimated.

2.4.2.2. Standard BCA Assay

The standard BCA assay protocol, based on the method of Smith *et al.* (1985), was used to quantify protein concentrations of samples that could not be determined accurately using the less sensitive Biuret assay. To determine the amount of protein present in samples, BSA standard curves in the range 0-2mg/ml were prepared. The assay was performed on a 96 well microplate in triplicate as follows:

- 50µl Sample/BSA standard
- 200µl BCA Reagent
- Incubation at 37°C for 30 minutes

The absorbance of each well was recorded as described in previous section and standard curves were plotted.

2.4.3. ENZYME ASSAYS

Quantitative and non-quantitative enzyme assays were performed based on a method by Buckley *et al.* (2004).

2.4.3.1. Quantitative Measurement of Dipeptidyl Peptidase IV (DPP IV) Activity

Dipeptidyl peptidase IV activity was determined by the degradation of Gly-Pro-AMC. A 10mM Gly-Pro-AMC stock solution was prepared in 100% ethanol (w/v). This stock solution was diluted 1:100 to 0.1mM with 50mM HEPES, pH 8.0, containing 5mM EDTA with a final ethanol concentration of 1% (v/v). One hundred microlitres of this solution was added to 25µl enzyme sample in triplicate and incubated at 37°C for 1 hour. Both enzyme sample and substrate were pre-incubated separately at 37°C for 15 minutes to allow them to reach thermal equilibrium. The reaction was terminated by the addition of 175µl 1.7 M acetic acid. Negative controls were prepared by adding 175µl 1.7M acetic acid to the substrate prior to the addition of the sample. The controls were also incubated at 37°C for 1 hour. Liberated AMC was determined fluorometrically from standard plots prepared as described in section 2.4.1.1. Enzyme units (defined as nanomoles of AMC released per minute at 37°C) were calculated using the relevant quenched AMC standard curve (section 2.4.1.2.).

2.4.3.2. Non-Quantitative Measurement of Dipeptidyl Peptidase IV (DPP IV) Activity

Gly-Pro-AMC degrading activity in post column chromatography fractions was detected using a non-quantitative assay. A 200µl solution of 0.1mM Gly-Pro-AMC in 50mM HEPES, pH 8.0, containing 5mM EDTA, was added to 100µl of sample in each well on a microplate. The plate was then incubated at 37°C for 1 hour. AMC released was determined fluorometrically as outlined in section 2.4.1.1 using the Perkin-Elmer LS-50 Luminescence Spectrometer.

2.4.3.3. Measurement of r*Fasciola hepatica* Cathepsin L1 (rFheCL1) Activity

rFheCL1 activity was determined by the degradation of Z-Phe-Arg-AMC. A 1mM Z-Phe-Arg-AMC stock solution was prepared in 100% DMSO (w/v). This stock solution was diluted 1:100 to 0.01mM with 0.1M sodium acetate, pH 5.0, containing 2mM DTT and 2.5mM EDTA with a final DMSO concentration of 1% (v/v). This solution (100µl) was added to 25µl enzyme sample in triplicate and incubated at 37°C for 30 minutes. Both enzyme sample and substrate were pre-incubated separately at 37°C for 15 minutes to allow them to reach thermal equilibrium. The reaction was terminated by the addition of 175µl 1.7M acetic acid. Negative controls were prepared by adding 175µl 1.7M acetic acid to the substrate prior to the addition of the sample. The controls were also incubated at 37°C for 30 minutes. Liberated AMC was determined fluorometrically as described in section 2.4.1.1. Fluorometric intensities observed were converted to nanomoles of AMC released per minute per ml using the relevant quenched AMC standard curve (section 2.4.1.2.). Enzyme units were defined as nanomoles of AMC released per minute at 37°C.

2.4.3.4. Esterase Activity of r*Fasciola hepatica* Cathepsin L1 (rFheCL1)

rFheCL1 esterase activity was determined by the degradation of Z-Lys-ONp, procedure taken from Shaw and Green (1981). A 10mM Z-Lys-ONp stock solution was prepared in 100% DMSO (w/v). Assays were performed in 0.1M sodium acetate, pH 5.0 (buffer). On a 96-well microtitre plate 12.5µl of substrate stock solution was combined with 250µl buffer. The reaction was initiated by the addition of 25µl activated rFheCL1 (0.1mg/ml). Immediately after initiation, the plate was placed on a Labsystems Multiskan MS microplate reader, programmed in kinetic mode, and read every minute for a period of 20 minutes at 405nm. Blank controls were prepared, where 0.1M sodium acetate, pH 5.0, containing 8mM DTT and 10mM EDTA was substituted for rFheCL1. All assays were carried out in triplicate.

2.5. ASSAY DEVELOPMENT

2.5.1. AUTODIGESTION ASSAY

The rate of autolysis was determined based on the method of Bickerstaff and Zhou (1993). Purified DPP IV (1ml) in 50mM HEPES, pH 8.0, containing 5mM EDTA was incubated in a test tube at 37°C. At set time intervals, a 10µl sample was removed and transferred to a 96 well microtitre plate. This sample was immediately mixed with 250µl Bradford dye reagent. After 15 minutes incubation at room temperature, the absorbance at 620nm was measured. Activated rFheCL1 was assayed similarly. Blank controls were prepared, where 50mM HEPES, pH 8.0, containing 5mM EDTA was substituted for DPP IV, while 0.1M sodium acetate, pH 5.0, containing 8mM DTT and 10mM EDTA was substituted for rFheCL1. Trypsin (0.1mg/ml) prepared in 0.2M sodium phosphate, pH 7.0, was used as a positive control. Controls were assayed as described above for enzyme-containing samples.

2.5.2. LINEARITY OF ENZYME ASSAY WITH RESPECT TO TIME

2.5.2.1. Dipeptidyl Peptidase IV (DPP IV)

Purified DPP IV (400µl) and 0.1mM Gly-Pro-AMC (1600µl) substrate were pre-incubated separately in a waterbath at 37°C for 15 minutes prior to assay to ensure that solutions had reached thermal equilibrium. Enzyme and substrate were then combined in a test tube held at 37°C to begin the reaction (t = 0). At appropriate time intervals, 125µl of the combined enzyme/substrate solution was removed and added to 175µl acetic acid (1.7M) on a 96 well microplate to terminate the reaction. Blank controls were prepared, where 400µl 50mM HEPES, pH 8.0, containing 5mM EDTA was substituted for enzyme. Fluorescence

intensity was measured as described in section 2.4.3.1. All assays were carried out in triplicate. A plot of fluorescence intensity versus time was constructed.

2.5.2.2. r*Fasciola hepatica* Cathepsin L1 (rFheCL1)

Activated rFheCL1 was diluted 1/50 with 0.1M sodium acetate, pH 5.0, and gently stirred to ensure equilibrium. This solution (700µl) and 2800µl 0.01mM Z-Phe-Arg-AMC substrate were pre-incubated separately in a waterbath at 37°C for 15 minutes prior to assay to ensure that solutions had reached thermal equilibrium. Enzyme and substrate were then combined in a test tube held at 37°C to begin the reaction (t = 0). At appropriate time intervals, 125µl of the combined enzyme/substrate solution was removed and added to 175µl 1.7M acetic acid on a 96 well microplate to terminate the reaction. Blank controls were prepared, where 0.1M sodium acetate, pH 5.0 containing 8mM DTT and 10mM EDTA was substituted for enzyme. Fluorescence intensity was measured as described in section 2.4.3.3. All assays were carried out in triplicate. A plot of fluorescence intensity versus time was constructed.

2.5.3. LINEARITY OF ASSAY WITH RESPECT TO ENZYME CONCENTRATION

2.5.3.1. Dipeptidyl Peptidase IV (DPP IV)

Purified DPP IV was diluted with 50mM HEPES, pH 8.0, containing 5mM EDTA to achieve enzyme concentrations ranging from 0-100% of initial stock solution. The enzyme at these different concentrations was assayed in triplicate with 0.1mM Gly-Pro-AMC as described in section 2.4.3.1. All assays were carried out in triplicate. A plot of fluorescenec intensity versus enzyme concentration (%) was constructed.

2.5.3.2. r*Fasciola hepatica* Cathepsin L1 (rFheCL1)

Activated rFheCL1 was diluted 1/50 with 0.1M sodium acetate, pH 5.0, and gently stirred to ensure equilibrium. From this 1/50 stock solution a range of concentrations (0-100%) was prepared in 0.1M sodium acetate, pH 5.0. The enzyme at these different concentrations was assayed in triplicate with 0.01mM Z-Phe-Arg-AMC as described in section 2.4.3.3. All assays were carried out in triplicate. A plot of fluorescence intensity versus enzyme concentration (%) was constructed.

2.6. KINETIC ANALYSIS

2.6.1. K_m and V_{max} determination for Gly-Pro-AMC

Gly-Pro-AMC (10mM in 100% ethanol (w/v)) was diluted to 0.5mM with 0.1M HEPES, pH 8.0, containing 5mM EDTA. This solution was further diluted in buffer to a range of Gly-Pro-AMC concentrations. Purified DPP IV was assayed at each substrate concentration as described in section 2.4.3.1. The Michaelis-Menten constant (K_m) and maximum velocity value (V_{max}) were determined using Michaelis-Menten plot analysis (Enzfitter Programme, Biosoft, Cambridge, UK).

2.6.2. K_m and V_{max} determination for Z-Phe-Arg-AMC

Z-Phe-Arg-AMC (10mM in 100% DMSO (w/v)) was diluted to 100μM Z-Phe-Arg-AMC with 0.1M sodium acetate, pH 5.0, containing 2mM DTT and 2.5mM EDTA. This solution was further diluted in buffer to a range of Z-Phe-Arg-AMC concentrations. Activated rFheCL1 (native and chemically modified; section 2.11.) was assayed at each substrate concentration, as described in section 2.4.3.3. The Michaelis-Menten constant (K_m) and maximum velocity value (V_{max}) were determined using Michaelis-Menten plot analysis (Enzfitter Programme, Biosoft, Cambridge, UK).

2.7. TEMPERATURE STUDIES

2.7.1. TEMPERATURE PROFILE

2.7.1.1. Dipeptidyl Peptidase IV (DPP IV)

Aliquots of purified DPP IV were incubated for ten minutes at temperatures ranging between 37-90°C. The samples were cooled rapidly on ice and residual activity was assayed as described in section 2.4.3.1. Blank controls were prepared, where 50mM HEPES, pH 8.0, containing 5mM EDTA was substituted for enzyme. All assays were carried out in triplicate. A plot of % residual activity versus temperature (°C) was constructed and the temperature of half–inactivation (T_{50}) determined by inspection.

2.7.1.2. r*Fasciola hepatica* Cathepsin L1 (rFheCL1)

Activated rFheCL1 was diluted 1/50 with 0.1M sodium acetate, pH 5.0 and gently stirred to ensure equilibrium. Aliquots of this 1/50 dilution were incubated for ten minutes at temperatures ranging between 37-90°C. The samples were cooled rapidly on ice and residual activity was assayed as described in section 2.4.3.3. Blank controls were prepared, where 0.1M sodium acetate, pH 5.0, containing 8mM DTT and 10mM EDTA was substituted for enzyme. All assays were carried out in triplicate. A plot of % residual activity versus temperature (°C) was constructed and the T_{50} determined by inspection.

2.7.2. THERMOINACTIVATION

2.7.2.1. Dipeptidyl Peptidase IV (DPP IV)

Purified DPP IV was maintained at a constant temperature in a heated waterbath (temperature-dependent on assay). At appropriate time intervals, aliquots were removed from the incubating solution and their activities assayed as described in section 2.4.3.1. Blank controls were prepared, where 50mM HEPES, pH 8.0, containing 5mM EDTA was substituted for enzyme. A plot of % residual activity versus time (minutes) was constructed. Data were fitted to a first-order exponential decay using the Enzfitter programme (Biosoft, Cambridge, UK) in order to estimate the rate constant (k) and, hence, the half-life ($t_{½}$).

2.7.2.2. r*Fasciola hepatica* Cathepsin L1 (rFheCL1)

Activated rFheCL1 was diluted 1/50 with 0.1M sodium acetate, pH 5.0 and gently stirred to ensure equilibrium. The enzyme was then maintained at a constant temperature in a heated waterbath (temperature dependent on assay). At appropriate time intervals, aliquots were removed from the incubating solution and their activities assayed as described in section 2.4.3.3. Blank controls were prepared, where 0.1M sodium acetate, pH 5, containing 8mM DTT and 10mM EDTA was substituted for activated enzyme. A plot of % residual activity versus time (minutes) was constructed. Data were fitted to a first-order exponential decay using the Enzfitter programme (Biosoft, Cambridge, UK) in order to estimate the rate constant (k) and, hence, the half-life ($t_{½}$).

2.8. ORGANOTOLERANCE STUDIES

2.8.1. SOLVENT STABILITY STUDIES

2.8.1.1. Dipeptidyl Peptidase IV (DPP IV)

Aliquots of purified DPP IV were incubated for 1 hour at room temperature with a range of solvents (acetonitrile, DMF, DMSO, THF) to achieve final solvent concentrations ranging from 0-90% (TABLE 2.1). The enzyme/solvent solution was adjusted to a final volume of 0.5 ml with 50mM HEPES pH 8.0, containing 5mM EDTA. Blanks containing no enzyme were included for each solvent, at each solvent concentration. Enzyme activity was assayed as described in section 2.4.3.1. All assays were carried out in triplicate. A plot of % residual activity versus solvent concentration (%) was constructed and the C_{50} determined by inspection. A temperature profile was performed as described in section 2.7.1.1. on each solvent at 50% (v/v) concentration. Thermoinactivation at 37°C was performed as detailed in section 2.7.2.1. on each solvent, also at 50% (v/v) concentration.

TABLE 2.1 PREPARATION OF ENZYME/SOLVENT SOLUTION (V/V)

Solvent Concentration (%)	0	10	20	30	40	50	60	70	80	90
Volume of Enzyme (µl)	50	50	50	50	50	50	50	50	50	50
Volume of Buffer (µl)	450	400	350	300	250	200	150	100	50	----
Volume of Solvent (µl)	----	50	100	150	200	250	300	350	400	450

2.8.1.2. r*Fasciola hepatica* Cathepsin L1 (rFheCL1)

Activated rFheCL1 (native and chemically modified; section 2.11.) was diluted 1/5 with 0.1M sodium acetate, pH 5.0 and gently stirred to ensure equilibrium. Aliquots of this stock solution were incubated for 1 hour at room temperature with a range of solvents (acetone, acetonitrile, DMF, DMSO, THF) to achieve final solvent concentrations ranging from 0-90% (TABLE 2.1). The enzyme/solvent solution was adjusted to a final volume of 0.5ml with 0.1M sodium acetate, pH 5.0. Blank controls containing no enzyme were included for each solvent, at each solvent concentration (%). Enzyme activity was assayed as described in section 2.4.3.3. All assays were carried out in triplicate. A plot of % residual activity versus solvent concentration (%) was constructed and the C_{50} determined by inspection. Thermoinactivation of rFheCL1 (chemically modified; section 2.11.) at 37°C and 50°C was performed as detailed in section 2.7.2.2. on acetonitrile and DMF, each at 50% (v/v) concentration.

2.9. pH Studies on Dipeptidyl Peptidase IV (DPP IV)

2.9.1. Effect of pH on Activity and Kinetics

Purified DPP IV (5ml) was dialysed for 12 hours against ultra-pure water (2 litres). A 500µl volume of dialysate was then further dialysed overnight into each of the buffers listed in Table 2.2. with buffer changes at 2 and 6 hours. The effect of pH and buffer system on enzyme activity was assayed in triplicate as described in section 2.4.3.1. at different pH values and in the different buffers to determine the pK_a of DPP IV. Liberated AMC was determined fluorometrically as described in section 2.4.1.1. Graphs of Log V/K_m (min^{-1}) versus pH, log V (µM/min) versus pH and log K_m (µM) versus pH were plotted.

Table 2.2. Buffer systems used in determining effect of pH on DPP IV activity

pH Range	Buffer	pH Adjuster
4.0 – 5.5	50mM Sodium acetate	5M HCl
5.5 – 6.5	50mM MES	5M NaOH
6.5 – 7.0	50mM MOPS	5M NaOH
7.0 – 8.0	50mM HEPES	5M HCl
8.0 – 9.0	50mM Tris	5M HCl
9.0 - 10	50mM CHES	5M NaOH

2.9.2. pH Temperature Profile

Purified DPP IV (5ml) was dialysed for 12 hours against 2L ultra-pure water. A 300µl volume of dialysate was then further dialysed overnight into each of the buffers listed in TABLE 2.2. (but at 20mM concentration instead of 50mM) with buffer changes at 2 and 6 hours. A temperature profile was performed as described in section 2.7.1.1.

2.10. ACTIVE SITE TITRATION

2.10.1. r*Fasciola hepatica* Cathepsin L1 (rFheCL1)

This procedure is based on a method by Barrett *et al.* (1982). *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64) (1mM initially dissolved in 0.1ml DMSO, brought to 10ml final volume) was diluted to a 20µM working solution using ultra-pure water. Lower E-64 concentrations were prepared (0-2.5μM) by dilution of the working solution with ultra-pure water. A titration of activated rFheCL1 with E-64 was prepared. At each point, 25μl of activated rFheCL1 (diluted 1/5 with 0.1M sodium acetate, pH 5.0) was reacted with an equal volume of 0-2.5µM E-64 and brought to a total volume of 100µl with 0.1M sodium acetate, pH 5.0, containing 8mM DTT and 10mM EDTA. Samples were incubated at 37°C for 15 minutes. Activity was then determined (section 2.4.3.3.). All assays were carried out in triplicate. A plot of fluorescence intensity versus E-64 concentration (µM) was plotted and the operational molarity of rFheCL1 determined.

2.11. CHEMICAL MODIFICATION

2.11.1. Cross-linking of r*Fasciola hepatica* Cathepsin L1 (rFheCL1) with EG-NHS

The protocol for the ester reaction was based on that of Ryan *et al.* (1994). Activated rFheCL1 (2ml) in 0.1M sodium acetate, pH 5.0, containing 8mM DTT and 10mM EDTA was dialysed extensively for at least 3 hours against 0.1M potassium phosphate, pH 7.5. Ethylene glycol-bis(succinic acid N-hydroxy-succinimide ester) (EG-NHS) (5mg) was dissolved in 0.125ml DMSO. Cross-linking was commenced by addition of the EG-NHS solution to rFheCL1 (2ml) in 0.1M potassium phosphate, pH 7.5. This reaction solution was stirred gently at room temperature for 20 minutes after which the reaction was terminated by removal of excess reagent as described in section 2.11.2. below. The modified enzyme was re-dialysed into 0.1M sodium acetate, pH 5.0, containing 8mM DTT and 10mM EDTA. Enzyme activity was determined pre- and post modification.

2.11.2. Sephadex™ G-25 Gel Filtration

The method was based on that of Helmerhorst and Stokes (1980). Sephadex™ G-25 (5g) was swollen in 50ml 0.1M potassium phosphate, pH 7.5. This mixture was stirred slowly overnight. A slurry of the above mixture (20ml) was poured into a 10ml column and capped. The column was then centrifuged at 1800 rpm for 3 minutes in a bench centrifuge to remove excess buffer (rotor radius 12cm). Modified enzyme solution (2ml) was applied to the semi-dry Sephadex™ G-25 column and re-centrifuged as described above, to remove unreacted and excess ethylene glycol-bis(succinic acid N-hydroxy-succinimide ester) (EG-NHS).

2.11.3. Amino Group Estimation - TNBS Assay

The method was based on that of Fields (1971) using 2,4,6-trinitrobenzenesulphonic acid (TNBS) for the estimation of free amino groups in native and modified rFheCL1. A 20mM (w/v) Nα-acetyl-L-Lysine stock solution was prepared in ultra-pure water. Lower Nα-acetyl-L-Lysine concentrations were prepared in ultra-pure water to form standard curves in the range of 0-20mM. The TNBS assay was performed in test tubes as follows:

- 0.1ml rFheCL1 (native/modified)/Nα-acetyl-L-Lysine standard
- 0.5ml 0.1M borate buffer, pH 9.6
- 0.4ml ultra-pure water
- The reaction was initiated by addition of 0.02ml 0.5% (v/v) TNBS. Tubes were mixed by inversion and reaction proceeded at room temperature in the dark. After 5 minutes, the reaction was terminated by the addition of 2ml 0.1M monobasic sodium phosphate (NaH_2PO_4) containing 1.5mM sodium sulphite.

Absorbance at 420nm was determined on a Unicam2 UV/Vis spectrophotometer. Blank controls were included, substituting buffer for enzyme samples. All solutions were assayed in triplicate and their mean absorbance calculated.

2.12. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Denaturing electrophoresis was used to determine the success of purification based on the method of Laemmli (1970).

2.12.1. Sample Preparation

Activated rFheCL1 and samples collected during DPP IV purification (crude serum, post-phenyl-Sepharose DPP IV, and post-Q-Sepharose DPP IV) were combined with 5X sample buffer (60mM Tris/HCl pH 6.8, 25% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate, 5% (v/v) 2-mercaptoethanol and 0.1% (w/v) bromophenol blue) i.e. 20µl sample + 5µl sample buffer, and denatured by immersion in a boiling water bath for 5 minutes after which samples were kept on ice until application onto the gel. For DPP IV and rFheCL1 analysis Sigmamarker™ high and low molecular weight range markers were used as standards, respectively (TABLE 2.3.).

TABLE 2.3. MOLECULAR WEIGHT DISTRIBUTION OF SIGMAMARKER™

Proteins	**mw (Da)**	**High Range**	**Low Range**
Myosin	205,000	X	
β-Galactosidase	116,000	X	
Phosphorylase b	97,000	X	
Fructose-6-phosphate Kinase	84,000	X	
Albumin, Bovine Serum (BSA)	66,000	X	X
Glutamic Dehydrogenase	55,000	X	
Ovalbumin	45,000	X	X
Glyceraldehyde-3-phosphate Dehydrogenase	36,000	X	X
Carbonic Anhydrase	29,000		X
Trypsinogen	24,000		X
Trypsin Inhibitor	20,000		X
α-Lactalbumin	14,200		X

2.12.2. SDS Gel Preparation

TABLE 2.4. lists the composition of each solution used in the preparation of gels for SDS-PAGE electrophoresis. All solutions were prepared in ultra-pure water. Gels were prepared from the solutions in Table 2.4. with volumes documented in Table 2.5.

TABLE 2.4. SOLUTIONS AND THEIR COMPOSITIONS USED IN SDS-PAGE

SOLUTION	COMPOSITION
ACRYL/BIS-ACRYLAMIDE	30% (w/v) Acrylamide, 0.8% (w/v) bis-Acrylamide
AMMONIUM PERSULFATE	10% (w/v) ammonium persulfate
SODIUM DODECYL SULFATE	10% (w/v) sodium dodecyl sulfate (SDS)
4X RESOLVING GEL BUFFER	1.5M Tris/HCl, pH 8.8, with 0.4% (w/v) SDS
10X RUNNING BUFFER	0.25M Tris, 1.92M glycine, 1% (w/v) SDS, pH 8.3
4X STACKING GEL BUFFER	0.5M Tris/HCl, pH 6.8 with 0.4% (w/v) SDS

TABLE 2.5. SDS-PAGE GEL PREPARATION

(TEMED acts as a catalyst for gel polymerization so it is added last.)

STOCK SOLUTION	RESOLVING GEL 9% (V/V)	RESOLVING GEL 12% (V/V)	STACKING GEL 4% (V/V)
ACRYL/BIS-ACRYL SOLUTION	3.0ml	4.0ml	1.33ml
AMMONIUM PERSULFATE	0.1ml	0.1ml	0.1ml
RESOLVING GEL BUFFER	2.5ml	2.5ml	-------
STACKING GEL BUFFER	--------	--------	2.5ml
TEMED	50µl	50µl	50µl
ULTRA-PURE WATER	4.5ml	3.5ml	6.17ml

Gels were cast in a BioRad Mini Gel set and positioned in an ATTO vertical electrophoresis system. The gel box was filled with 1X running buffer and 10µl of the prepared samples and markers were loaded into the wells under buffer head. To avoid edge effects, 10µl of 5X sample buffer was loaded into unused wells. Electrophoresis was performed at room temperature at a constant voltage of 200V for approximately 1hour or until the dye front had migrated 5mm from the end of the gel.

2.12.3. Visualisation of Proteins

Polyacrylamide gels were stained using GelCode® Blue stain reagent. Table 2.6. outlines the stages performed. An image of each stained gel was recorded using a Plustek OpticPro 4821p scanmaker (for DPP IV) and Pharmacia Biotech Imagemaster® Video Documentation System for Gel Electrophoresis (for rFheCL1).

TABLE 2.6. GELCODE® BLUE STAINING PROCEDURE

Step	Reagent	Volume	Time
Rinsing	Ultra-pure water	3 × 100ml	3 × 15 minutes
Staining	GelCode® Blue Stain	1 × 200ml	1 × 60 minutes
Rinsing	Ultra-pure water	2 × 200ml	1 × 60 minutes

2.13. ENZYMATIC PEPTIDE SYNTHESIS

2.13.1. Synthesis of model tripeptide Z-Phe-Arg-SerNH$_2$

The enzymatic synthesis of Z-Phe-Arg-SerNH$_2$ using Z-Phe-Arg-OMe (ester) and SerNH$_2$ (amine) was performed based on similar methods described by Stehle *et al.* (1990) and Lozano *et al.* (1992). rFheCL1 was activated to a final protein concentration of 0.05mg/ml. Z-Phe-Arg-OMe was prepared as a 20mM stock solution in 100% acetonitrile (w/v). The nucleophile, SerNH$_2$, was prepared in 0.1M ammonium acetate, pH 9.0, giving a final concentration of 50mM. Enzyme, nucleophile and ester were separately pre-incubated in a waterbath at 37°C for 15 minutes prior to assay to ensure that solutions had reached thermal equilibrium. Into a 5ml volume test tube, 0.25ml of 20mM Z-Phe-Arg-OMe and 0.4ml SerNH$_2$ were combined. Reaction volume was adjusted to 2ml with 0.1M ammonium acetate, pH 9.0. The reaction was initiated by the addition of 0.25ml rFheCL1 (0.05mg/ml) and incubated at 37°C with gentle stirring. Aliquots (200µl) were removed at several time intervals from the reaction mixture and mixed with 25µl acetonitrile:water:formic acid (50:25:25 v/v/v) to quench the reaction. Analysis was performed by liquid chromatography mass spectrometry (LC-MS) (instrument – Bruker Mass Spectrometer with electrospray ionisation and ion-trap detector linked to a Hewlett-Packard Esquire Liquid chromatograph 1100 with photo diode array detector). The LC isocratic method used a Zorbax SB-C18, 50 × 2.1 mm, 3.5µm narrow bore column with a mobile phase 25:75:0.1(v/v/v) acetonitrile:water:formic acid at a flow rate of 0.2ml/min. Monitoring was performed using a photodiode array detector at 200nm and injection volume 2µl.

2.14. SEQUENCE ALIGNMENT

2.1.14. Sequence alignment of fluke and recombinant *Fasciola hepatica* cathepsin L1

Comparative analysis of the amino acid sequences for fluke (*Fasciola hepatica*) cathepsin L1 and recombinant *Fasciola hepatica* cathepsin L1 were aligned using ClustalW. The amino acid sequence for fluke (*Fasciola hepatica*) cathepsin L1, accession number Q24940, was obtained from Protein Data Bank. Sequence for recombinant *Fasciola hepatica* cathepsin L1 (rFheCL1) was obtained from Mr Peter Collins, Parasitology Research Group, formerly based at Dublin City University.

CHAPTER 3

PURIFICATION OF DIPEPTIDYL PEPTIDASE IV

3.1. INTRODUCTION

Dipeptidyl peptidase IV (DPP IV) is a serine protease enzyme with exopeptidase activity. It is specific for a Pro residue at the P_1 position hydrolysing, on the carboxyl side of the Pro (Burleigh & Andrews, 1998). The amide substrate, Gly-Pro-AMC when hydrolyzed by DPP IV releases AMC, which is monitored fluorimetrically, making this a sensitive and suitable substrate. The enzyme was purified from an ample supply of bovine serum, using a variety of techniques, based on a method by Buckley *et al.* (2004). Protein concentration, enzyme activity, and yield were determined after each purification stage. The extent of purity and subunit molecular weight of the enzyme were determined.

The undertaken studies differed from those of Buckley *et al.* (2004) who previously characterised the enzyme with regards to pH optimum, catalytic classification, substrate specificity and inhibition studies. This study focused on investigating the temperature and solvent stability of DPP IV. A temperature profile was determined for the enzyme and the temperature at half-inactivation (T_{50}) estimated. The effect of organic solvents on DPP IV activity was investigated following exposure to a range of organic solvents at various concentrations and the half-inactivation concentration (C_{50}) determined. The effect of pH on DPP IV activity and kinetics was also examined, with two p*K* values observed. The effect of changing the buffer on DPP IV activity was also investigated.

3.2. RESULTS – PURIFICATION OF DPP IV

3.2.1. OPTIMUM AMC EXCITATION AND EMISSION WAVELENGTHS

Scans intensity of fluorescence versus wavelength were performed on free AMC as described in section 2.4.1.3. to determine the optimum excitation and emission wavelengths for this compound. FIGURE 3.2.1.1. shows that the optimum excitation and emission wavelengths for AMC are 370nm and 440nm respectively.

FIGURE 3.2.1.1.

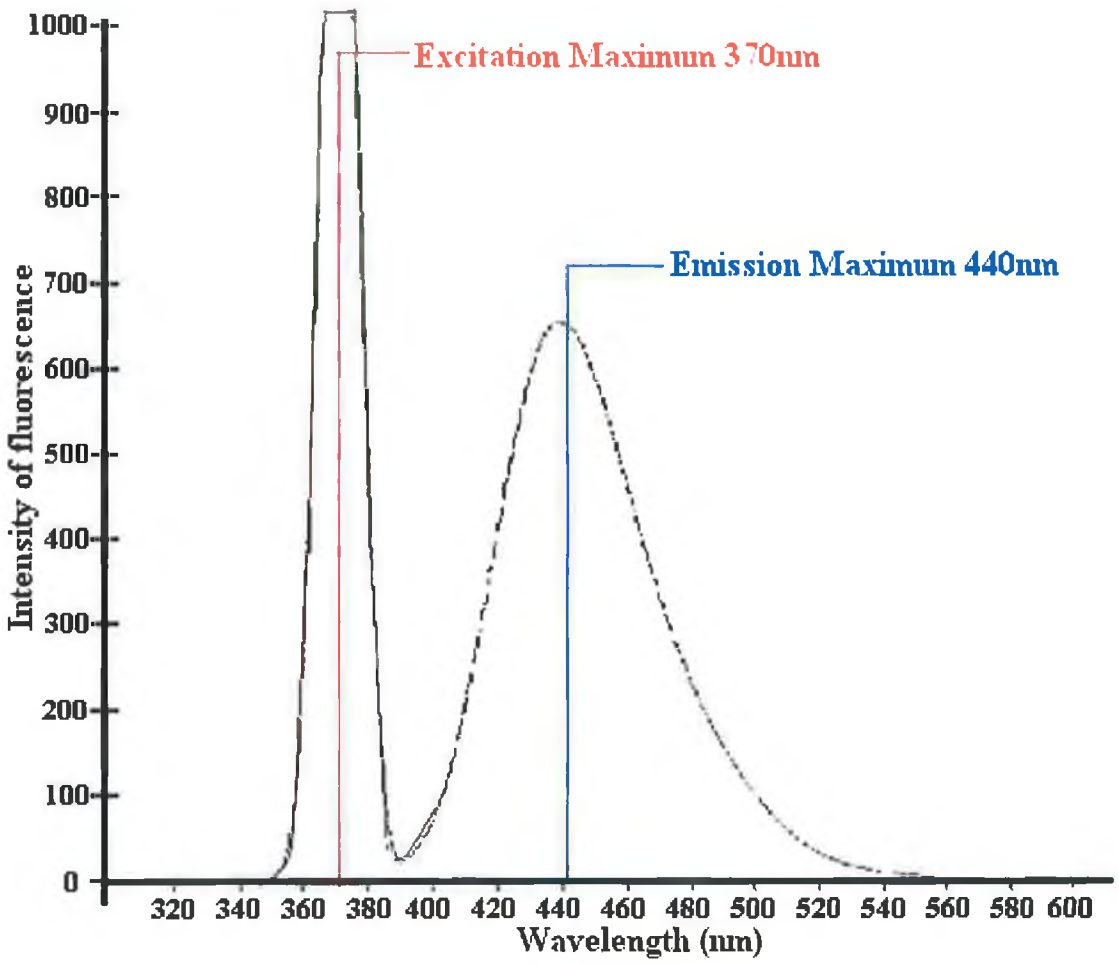


FIGURE 3.2.1.1. AMC EXCITATION AND EMISSION SCAN

Plot of intensity of fluorescence versus wavelength (nm) for free AMC in solution.

3.2.2. AMC Standard Curves and the Inner Filter Effect

AMC standard curves were prepared as outlined in Section 2.4.1.1. Plots of fluorescence intensity versus AMC concentration (micromol) were constructed and the slope of the lines calculated. FIGURE 3.2.2.1. represents a typical AMC standard curve at emission slit width 2.5nm. The inner filter effect was also determined as described in section 2.4.1.2. to assess the effect of including crude or post column fractions in the assay mixture on fluorescence intensity. FIGURE 3.2.2.2. is a plot of fluorescence intensity versus AMC concentration (micromol) each curve and the degree of filtering observed for enzyme samples.

TABLE 3.1. SLOPES OF FILTERED STANDARD CURVES

SAMPLE	R^2	SLOPE	FILTERING (%)
BUFFER	0.99	55.19	0
CRUDE SERUM	0.99	32.33	41
POST-Q-SEPHAROSE DPP IV	0.99	54.07	2

FIGURE 3.2.2.1.

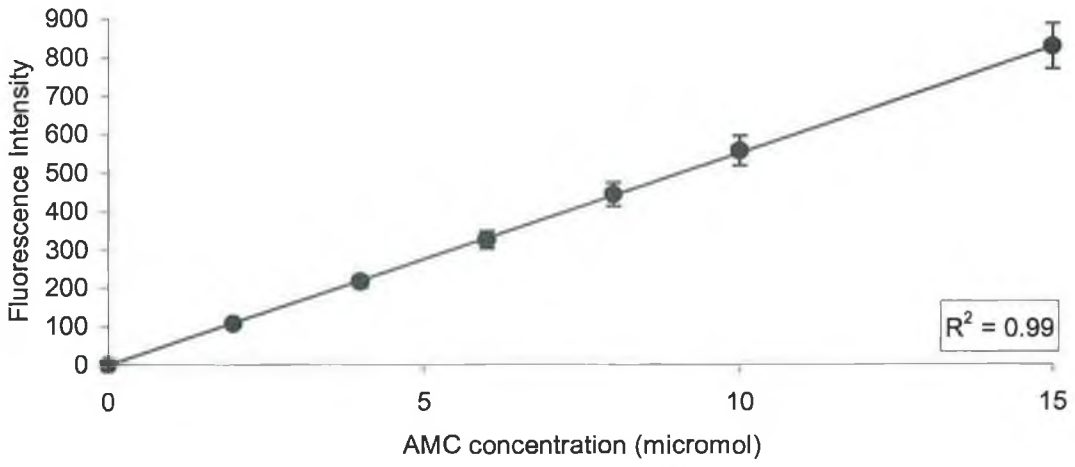


FIGURE 3.2.2.1. TYPICAL AMC STANDARD CURVE

Plot of fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus free AMC concentration (micromol). Values represent mean (n=3) and standard deviation (± 5%).

FIGURE 3.2.2.2.

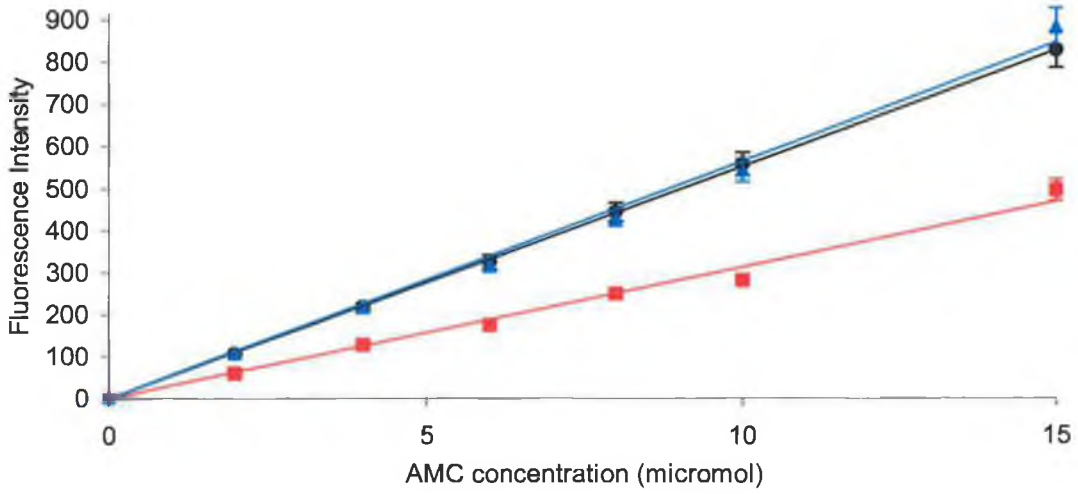


FIGURE 3.2.2.2. AMC QUENCHED STANDARD CURVE

Plot of fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width, 2.5nm) versus free AMC concentration (micromol) (•). The quenching agents used were crude bovine serum (▪) and post-Q-Sepharose DPP IV (▲). Values represent mean (n=3) and standard deviation (± 5%).

3.2.3. PROTEIN DETERMINATION

Bovine serum albumin (BSA) standard curves were prepared as outlined in section 2.4.2. Plots of protein absorbance at 560nm versus BSA concentration (mg/ml) are shown in FIGURES 3.2.3.1. and 3.2.3.2. for Biuret and standard BCA assays respectively.

FIGURE 3.2.3.1.

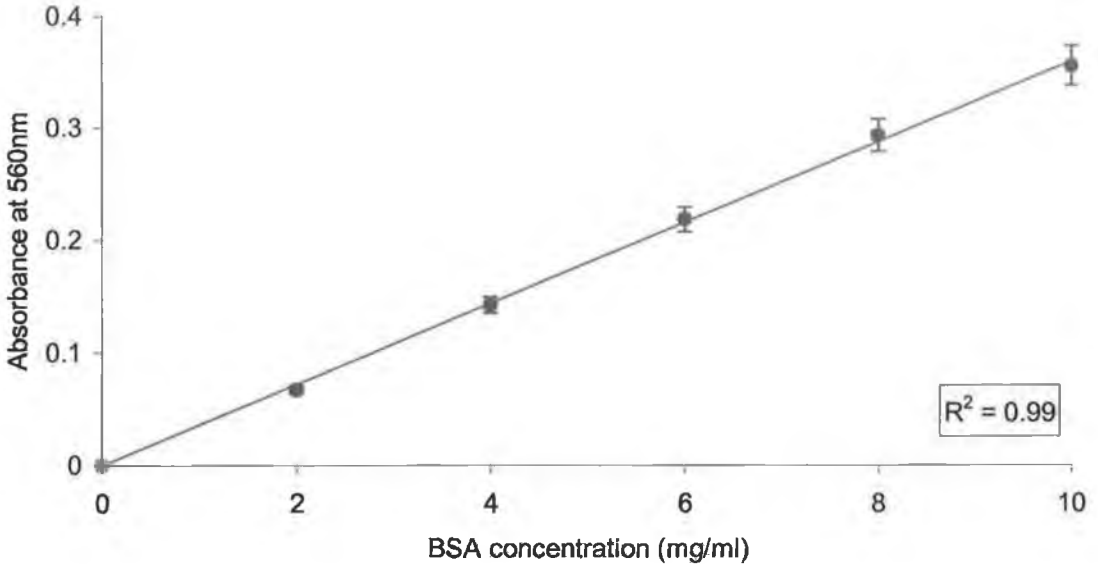


FIGURE 3.2.3.1. BIURET PROTEIN STANDARD CURVE

Plot of absorbance at 560nm versus bovine serum albumin (BSA) concentration (mg/ml). Values represent mean (n=3) and standard deviation (± 5%).

FIGURE 3.2.3.2.

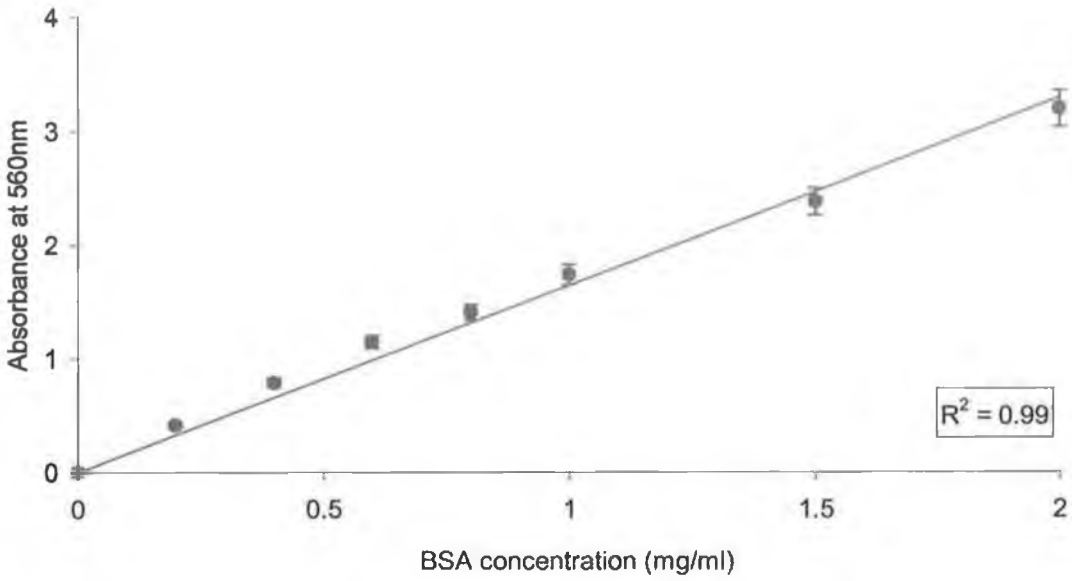


FIGURE 3.2.3.2. STANDARD BCA PROTEIN STANDARD CURVE

Plot of absorbance at 560nm versus bovine serum albumin (BSA) concentration (mg/ml). Values represent mean (n=3) and standard deviation (± 5%).

3.2.4. PURIFICATION OF BOVINE SERUM DPP IV

3.2.4.1. Serum Preparation

12 litres of fresh bovine whole blood was collected and allowed to clot at 4°C as described in section 2.3.1.1. This resulted in the formation of 2.5 litres unclotted serum and particulate matter. Centrifugation yielded 2 litres of serum.

3.2.4.2. Phenyl Sepharose Hydrophobic Interaction Chromatography

Crude serum containing 1M ammonium sulphate was applied to a phenyl Sepharose hydrophobic interaction chromatography column as outlined in section 2.3.1.2. A run-through DPP IV activity peak was observed from this column as shown in FIGURE 3.2.4.2.1. This activity was well separated from a significant proportion of bound protein. Fractions containing DPP IV activity were combined to yield a post-phenyl Sepharose DPP IV pool. A 1ml aliquot of this pool was retained to determine protein concentration and total enzymatic activity as described in section 2.4.2.1. and 2.4.3.1.

3.2.4.3. Sephacryl S-300 High Resolution (HR) Gel Filtration Chromatography

Following concentration of the post-phenyl Sepharose DPP IV pool by osmosis using solid polyethylene glycol, the concentrated sample was applied onto a Sephacryl S-300-HR gel filtration column as described in section 2.3.1.3. FIGURE 3.2.4.3.1. shows the molecular sieving effect of this column on the enzyme's purification. Fractions containing DPP IV activity were combined to yield a post-Sephacryl-S-300 DPP IV pool. A 1ml aliquot of this pool was retained to determine protein concentration and total enzymatic activity as described in section 2.4.2.2. and 2.4.3.1.

3.2.4.4. Quaternary (Q) Sepharose Strong Anion Exchange Chromatography

The post-Sephacryl S-300 DPP IV was concentrated via osmosis using solid polyethylene glycol. DPP IV activity was detected in the bound fractions from this column and no run-through DPP IV protein was detected. FIGURE 3.2.4.4.1. illustrates the elution profile from this column. Post-Q-Sepharose DPP IV fractions were combined, dialysed and stored at -20°C until required. A 1ml aliquot of the dialysed pool was retained for protein concentration and total enzymatic activity determination as described in section 2.4.2.2. and 2.4.3.1.

3.2.4.5. Effectiveness of Purification Procedure

A purification table (TABLE 3.2.) was constructed to determine the overall effectiveness of the purification protocol as outlined in section 2.3.

FIGURE 3.2.4.2.1.

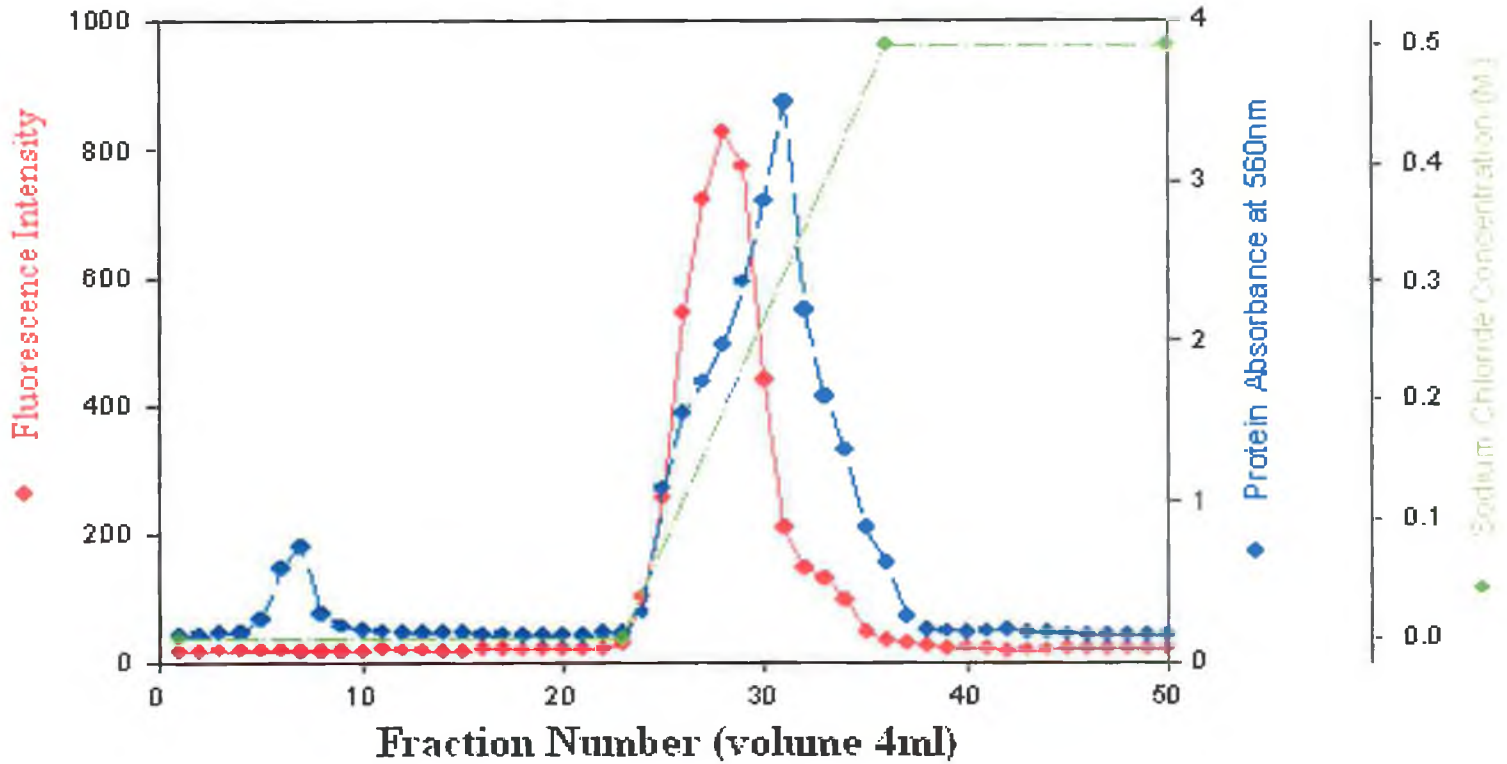


FIGURE 3.2.4.2.1. PHENYL SEPHAROSE CHROMATOGRAPHY OF BOVINE SERUM DPP IV (FRACTIONS 6-12 POOLED)

Fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width, 2.5nm) indicates DPP IV activity.

FIGURE 3.2.4.3.1.

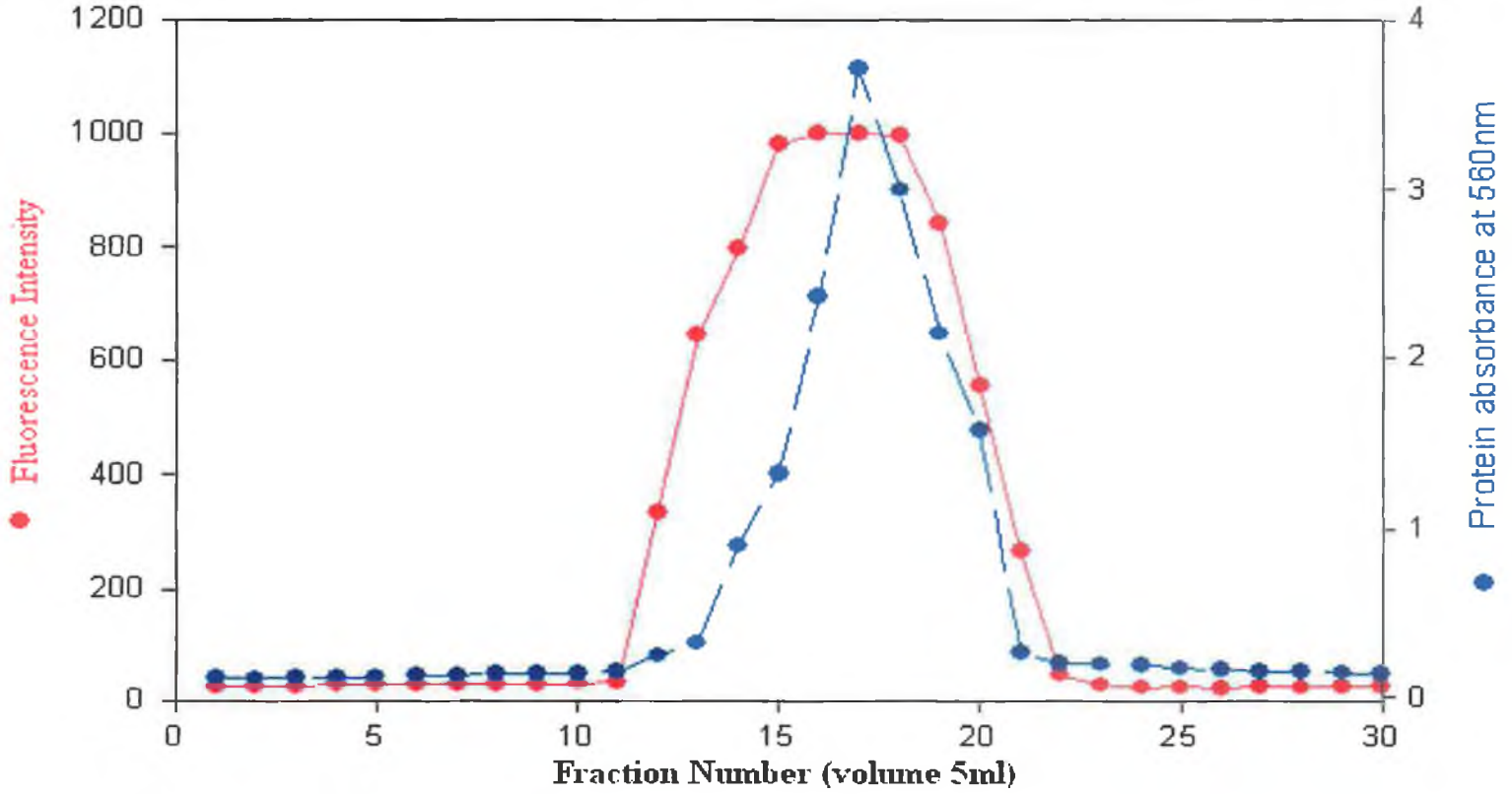


FIGURE 3.2.4.3.1. SEPHACRYL S-300-HR FILTRATION CHROMATOGRAPHY OF DPP IV (FRACTIONS 12-21 POOLED)

Fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width, 2.5nm) indicates DPP IV activity.

FIGURE 3.2.4.4.1.

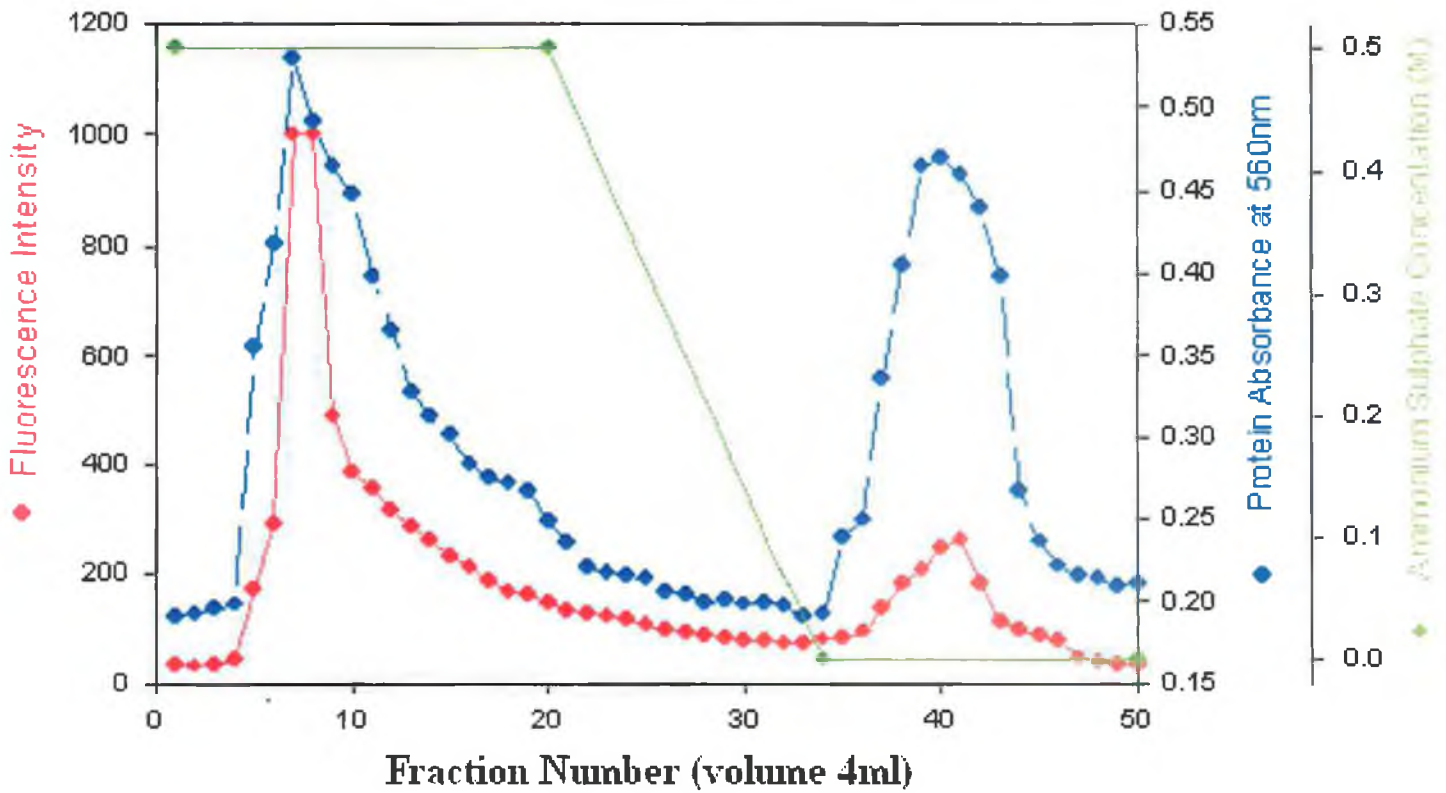


FIGURE 3.2.4.4.1. Q-SEPHAROSE STRONG ANION EXCHANGE CHROMATOGRAPHY OF DPP IV (FRACTIONS 25-31 POOLED)

Fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width, 2.5nm) indicates DPP IV activity.

TABLE 3.2. PURIFICATION TABLE FOR BOVINE SERUM DPP IV. Total activity (*eu*) defined as nanomoles/min/ml.

PURIFICATION STAGE	TOTAL PROTEIN *(mg)*	TOTAL ACTIVITY *(eu)*	SPECIFIC ACTIVITY *(eu/mg)*	PURIFICATION FACTOR	YIELD (%)
CRUDE SERUM	**10195**	**336**	**0.03**	**1**	**100**
POST-PS-DPP IV	**386**	**104**	**0.27**	**9**	**31**
POST S-300-HR DPP IV	**66**	**60**	**0.91**	**30**	**18**
POST-QAE-DPP IV	**41**	**43**	**1.05**	**35**	**13**

3.2.5. DETERMINATION OF ENZYME PURITY

3.2.5.1. SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed as outlined in section 2.12. FIGURE 3.2.5.1.2. represents an image of the GelCode® Blue stained serum DPP IV, illustrating high molecular weight markers and the various post-column serum DPP IV pools. The single peak of DPP IV activity post-Q-Sepharose (FIGURE 3.2.4.4.1) showed two major bands on SDS-PAGE (Lane 3); one band coincided with the position of bovine serum albumin among the molecular weight marker proteins, while the other was deemed to be DPP IV, with a subunit relative molecular mass of 77,224 Da estimated from a plot of log relative molecular mass versus relative mobility (R_f) (FIGURE 3.2.5.1.1.) standard proteins on the SDS gel.

FIGURE 3.2.5.1.1.

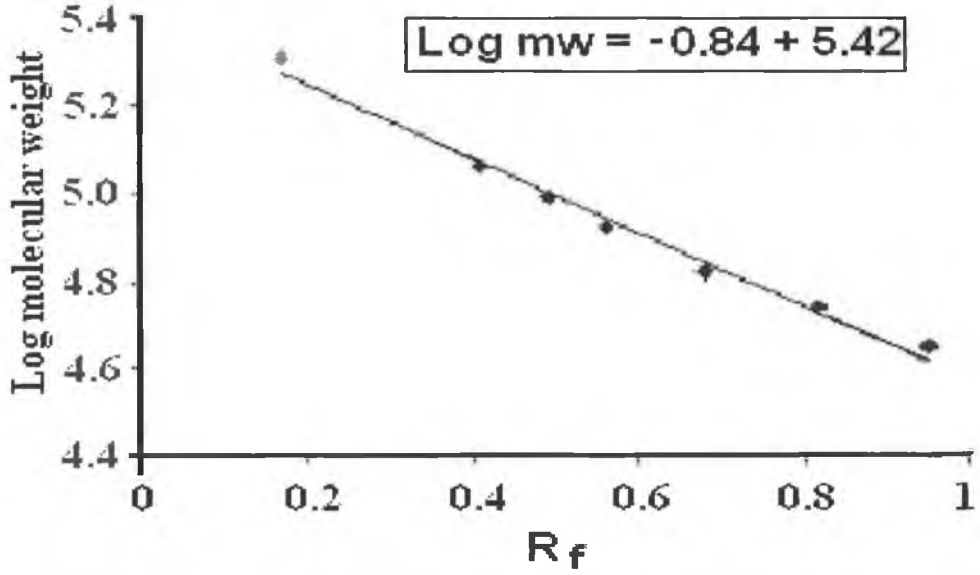


FIGURE 3.2.5.1.1. STANDARD CURVE FOR MOLECULAR WEIGHT DETERMINATION
Plot of log molecular weight versus R_f

FIGURE 3.2.5.1.2.

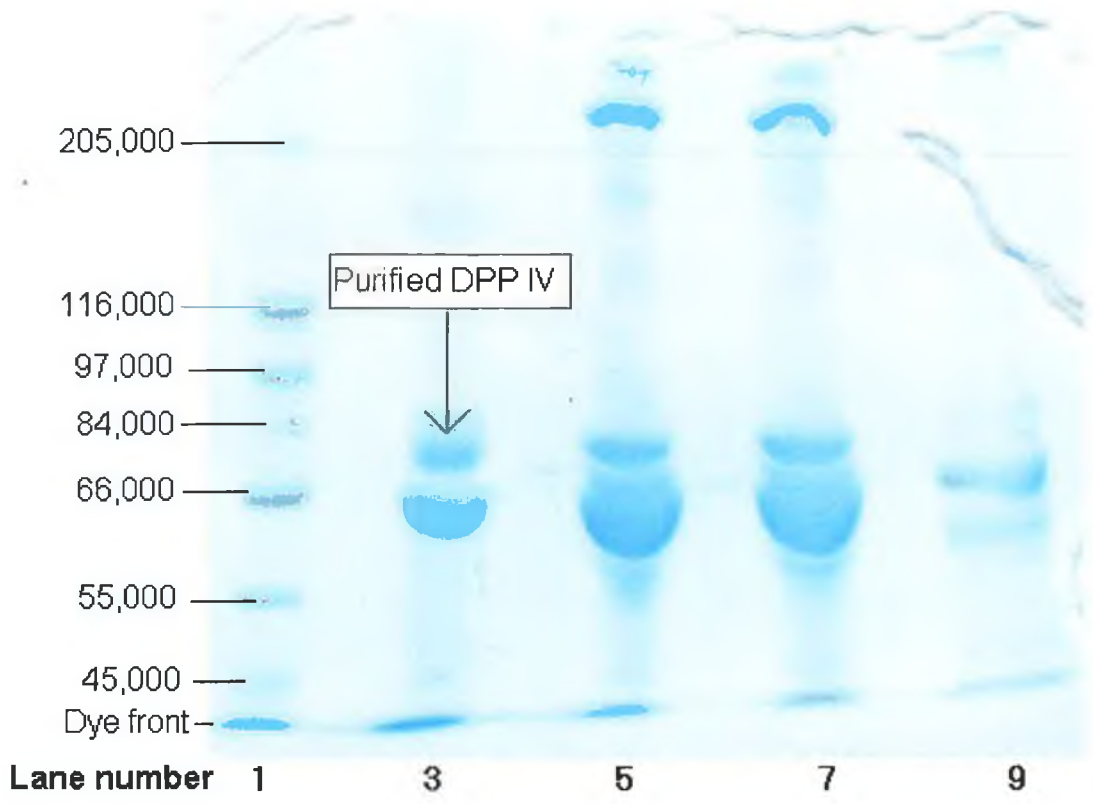


FIGURE 3.2.5.1.2. GELCODE® STAIN SDS-PAGE OF BOVINE SERUM DPP IV

Lane 1: molecular weight markers (from top), myosin (205,000 Da), β-Galactosidase (116,000 Da), phosphorylase b (97,000 Da), fructose-6-phosphate kinase (84,000 Da), bovine serum albumin (66,000 Da), glutamic dehydrogenase (55,000 Da) and ovalbumin (45,000 Da). Lane 3: post-Q-Sepharose anion exchange chromatography fraction. Lane 5: post-Sephacryl-S-300 gel filtration fraction. Lane 7: post-phenyl Sepharose chromatography fraction. Lane 9: crude serum fraction (1/100 dilution). Lanes between the samples and molecular weight markers were left empty.

3.3. Discussion

Fluorometric assays rely upon the excitation of a fluorophore by electromagnetic radiation at a specific wavelength, and the subsequent detection of emitted electromagnetic radiation at a higher specific wavelength. The observed fluorescence signal generally decreases as the solution becomes increasingly concentrated. This phenomenon is known as the inner filter effect and includes all light-attenuating processes – due not only to the fluorophore but also to any other chromophores that may be present (Lloyd, 1981). There are other concentration-dependent processes that reduce fluorescence intensities: intermolecular processes involving either ground or excited states of a fluorophore or both are a common cause. Quenching molecules may be present, e.g. oxygen in aerated solvents, or heavy atom perturbers such as residues of halogenated solvents (Lloyd, 1981). In crude biological samples, the effect of this phenomenon can be quite significant, leading to inaccurate determination of free AMC concentrations within a particular reaction mixture.

To determine the extent of this effect and overcome this problem, free AMC standard curves were constructed in a manner replicating standard assay conditions as outlined in section 2.4.1.1. Results were recorded in section 3.2.2. A significant difference in fluorescence detection was observed when biological samples were added; the effect of this on standard curves is clearly demonstrated in FIGURE 3.2.2.2. Both samples contributed to the inner filter effect and 41% of filtering was observed when crude bovine serum was added, yet only 2% filtering was demonstrated after the final step in the purification was performed. However, it is important to account for this effect when quantifying the enzymatic activity present in a sample and, therefore, the preparation of "filtered" standard curves is a useful method to ensure the accuracy of results obtained

from fluorometric assays and when ascertaining the effectiveness of a purification process.

Serum was prepared from freshly obtained whole bovine blood. This was a simple technique involving clotting, clot shrinkage and centrifugation. The source of starter material is a reliable, commercially available, and cheap source. Serum is also known to contain moderate amounts of DPP IV activity (Iwaki-Egawa *et al.*, 1998).

Hydrophobic interaction chromatography (HIC) depends on the affinity between a hydrophobic group on the matrix and regions of hydrophobicity on a protein (Bollag *et al.*, 1996a). Surface hydrophobic amino acids are usually arranged in patches, interspersed with more hydrophilic domains. The number, size and distribution of these non-ionic regions is a characteristic of each protein and can therefore be used as a basis for their separation. Salting-out decreases the availability of water molecules in solution, and enhances hydrophobic interaction (Roe, 1989). Hydrophobic interactions become stronger as the solution salt concentration increases. Hence, most HIC protocols call for protein loading at high salt and elution by lowering the salt concentration (Bollag *et al.*, 1996a).

Crude bovine serum was salted and applied to a phenyl-Sepharose hydrophobic interaction column. The elution profile demonstrates (FIGURE 3.2.4.2.1.) that two DPP IV-like activities were separated, one activity eluting in wash buffer, the second activity eluting in elution buffer. These results are comparable to results obtained by Shibuya-Saruta *et al.* (1996) who reported similar findings for human serum DPP IV, suggesting the possibility of a DPP IV isozyme. Lambeir *et al.* (1997) isolated different forms of DPP IV from seminal plasma by immobilised adenosine deaminase (ADA) and ion exchange chromatography, each form was distinguishable by net charge, and molecular

weight. They determined that the observed differences were primarily due to differing degrees of glycosylation. The elution profiles for ion exchange chromatography also differed, with some forms eluting at low salt concentrations, others eluting at high salt concentrations. Renal membrane-bound DPP IV of rat shows more that 12 isofoms each with different pI values ranging between 4.8 and 5.3 (Reutter *et al.*, 1995). The obtained results are also comparable to Buckley *et al.* (2004) who previously purified DPP IV from bovine serum. Further studies would be necessary to clarify if the second observed peak (eluting in elution buffer) is indeed a bovine serum DPP IV isozyme. This purification step provided a 27-fold reduction in protein, allowing a 9-fold increase in purity. Although DPP IV did not bind to the column (fractions eluted in wash buffer), a decrease in enzyme activity was observed. Recovery was reduced to 31%. Binding of DPP IV could be achieved by optimising the salting-out conditions; however, as is evident from the results, the salting-out step may be responsible for this loss in enzyme activity. The main object of this step was to separate DPP IV activity from PO activity, as both enzymes have similar properties.

Gel filtration chromatography is separation based on size. It is also a useful technique for transfering a protein into a different buffer (Bollag *et al.*, 1996b). The resin used for this separation, Sephacryl S-300-HR, has a fractionation range of 10-1500 kDa. This method is unique in fractionating without requiring protein binding, thus reducing the risk of protein loss through irreversible binding or protein inactivation. However, although this non-binding column was successful in providing a 6-fold reduction in the total protein content, a 42% reduction in enzyme activity was also observed, leading to a total yield of 18%. The poor sample recovery and loss of DPP IV activity, indicates that this step is possibly deleterious to DPP IV. This loss could be due to a number of

factors, including protein adsorption to the matrix as hydrophobic interactions with the matrix are possible; however, the elution profile does not fit this theory (Bollag *et al.*, 1996b).

Ion-exchange chromatography separates proteins on the basis of their charge. The resin quaternary ammonium was used for this procedure. This is classed as a strong anion exchange resin. FIGURE 3.2.4.4.1. illustrates the separation of bound from unbound protein. There is overlap between the protein peak and activity peak, suggesting that this protein peak can be mainly attributed to DPP IV. This is verified by only a slightly smaller reduction in total protein content and enzyme activity as compared to the previous purification steps. However, this final step resulted in an overall 35-fold increase in purity.

The aim of protein purification is to isolate one particular protein from all other proteins in the starting material. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins based primarily on their molecular weights (Laemmli, 1970) and is routinely used for determination of protein purity (Bollag *et al.*, 1996c). FIGURE 3.2.5.1.2. clearly illustrates that most of the contaminating albumin was separated from DPP IV activity, however a small amount of albumin was co-purified with DPP IV (Lane 3). Shibuya-Saruta *et al.* (1996) obtained similar results when they purified DPP IV from human serum. They successfully separated DPP IV from this contaminating albumin using a monoclonal antibody (Ta1) affinity chromatography column, thus producing a purer enzyme preparation. However, a 6-fold decrease in activity was observed suggesting that this co-eluting albumin may have a stabilizing effect on the enzyme. Albumin is a major component of serum, representing up to 60 to 70% of the total serum protein (Ung *et al.*, 2004). A number of methods are available

for depletion of albumin in serum samples. Dye affinity chromatography resins, employing chlorotriazine dyes, such as Cibacron Blue have been used for this purpose. Cibacron blue has three negatively-charged sulfonic acid groups and is known to bind at multiple sites on the surface of albumin. Although this dye can be used for the purification of many proteins e.g dehydrogenases and kinases, its most well-known application is its interaction with serum albumins (Hermanson *et al.*, 1992). Most albumin depletion protocols utilize buffered salt solutions to reduce non-specific binding to the resin, which may lead to a loss of the desired protein. However, an alternative is the use of immunoaffinity chromatographic methods, which can offer extreme specificity. The antibodies used for these columns can be targeted against either the albumin (anti-albumin) or the enzyme of interest.

Since dipeptidyl peptidase IV was first discovered by Hopsu-Havu and Glenner in 1966, the enzyme has been purified to homogeneity from a variety of tissues including kidney (Yoshimoto & Walter, 1977), pancreas (Yoshimoto *et al.*, 1982), human meconium (Caporale *et al.*, 1985) and human serum (Duke-Cohan *et al.*, 1995; Shibuya-Saruta *et al.*, 1996). Although the standard chromatographic techniques previously discussed contributed considerably to the overall purification of DPP IV, it is the affinity resins that have reported the greatest purification factors. Seidl and Schaefer (1991) utilised the resin Gly-Pro-EAH-Sepharose to successfully achieve a 2964-fold increase in purity. This resin exploits, and is based the affinity between the enzyme and its corresponding substrate, Gly-Pro-AMC. This group also utilised the lectin affinity resin Concanavalin A-Sepharose, which has been widely used for both membrane bound and soluble DPP IV (Duke-Cohan *et al.*, 1995).

DeMeester *et al.* (1996) utilised immobilized adenosine deaminase (ADA), thus manipulating the DPP IV-ADA binding properties to purify DPP IV from human seminal plasma, achieving an overall purification factor of 262. However, Shibuya-Saruta *et al.* (1996) achieved an 18,000 fold purification, the highest observed, using a monoclonal antibody, Ta1, bound to EAH-Sepharose when purifying human serum DPP IV, although the resulting yield of 2.2% suggests that active-site binding may reduce the lifetime of the enzyme.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) also allowed for estimation of the enzyme's monomeric molecular weight, which was calculated to be 77,224 Da. This is in contrast to data obtained for DPP IV isolated from other mammalian sources, were subunit molecular weight was 100kDa or higher (De Meester *et al.*, 1996; Shibuya-Saruta *et al.*, 1996). A dimeric structure has generally been proposed for this enzyme with reported weights for soluble DPP IV ranging between 210–290 kDa (Engel *et al.,* 2003). Therefore, bovine serum DPP IV would display a native molecular mass at about 150 kDa-160 kDa, this is lower than previously obtained for DPP IV isolated from bovine serum (Buckley *et al.*, 2004). However, these results are consistent with those of DPP IV isolated from some microorganisms, e.g. DPP IV isolated from *Flavobacterium meningosepticum* is composed of two subunits of 75 kDa (Yoshimoto & Tsuru, 1982). A trimeric form of DPP IV has also been isolated from porcine seminal plasma with a reported subunit molecular weight of 105 kDa and native molecular mass of 310 kDa (Ohkubo *et al.*, 1994). Differences in the molecular weight of DPP IV are influenced by the species, tissue and degree of glycosylation of the polypeptide chain of this glycoprotein (Sentandreu & Toldrá, 2001). The native molecular weight for this enzyme was not determined. This can be determined by either

native polyacrylamide gel electrophoresis or by gel filtration (Bollag *et al.*, 1996b). Performing this step would have established whether the enzyme has a dimeric or trimeric structure. However the main purpose of SDS-PAGE was to determine the purity of the purified enzyme preparation.

CHAPTER 4

STUDIES ON DIPEPTIDYL PEPTIDASE IV

4.1. RESULTS – ASSAY AND STABILITY STUDIES ON DPP IV

All of the upcoming graphs illustrate data points that represent the average of triplicate fluorescence intensity or absorbance readings (n=3), minus the blank measurement, standard deviation ± 5%. All error bars represent the standard error.

4.1.1. ASSAY DEVELOPMENT

4.1.1.1. Autodigestion Assay

The process of DPP IV autodigestion was examined as described in section 2.5.1. FIGURE 4.1.1.1.1. demonstrates that at 37°C no autodigestion was observed over a period of 240 minutes.

4.1.1.2. Linearity of Enzyme Assay with Respect to Time

The stability of DPP IV and its ability to cleave its corresponding substrate Gly-Pro-AMC with respect to time was examined as described in section 2.5.2.1. FIGURE 4.1.1.2.1. demonstrates that serum DPP IV activity is linear (R^2: 0.99) over a period of 120 minutes.

4.1.1.3. Linearity of Assay with Respect to Enzyme Concentration

Linearity of purified DPP IV degrading activity with respect to enzyme concentration was determined as outlined in section 2.5.3.1. FIGURE 4.1.1.3.1. illustrates that the enzyme assay is linear (R^2: 0.98) with respect to enzyme concentration.

FIGURE 4.1.1.1.1.

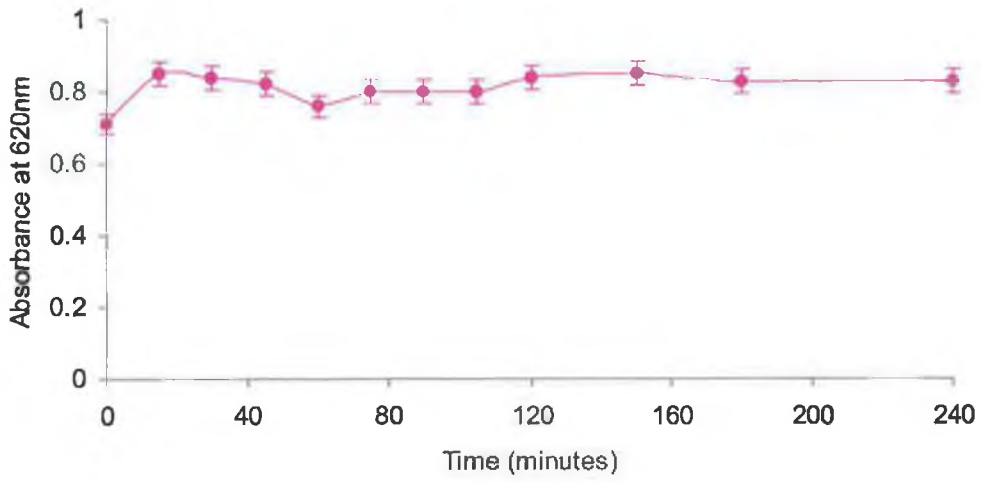


FIGURE 4.1.1.1.1. AUTODIGESTION OF DPP IV OVER TIME AT 37°C

Plot of absorbance at 620nm versus time (minutes).

FIGURE 4.1.1.2.1.

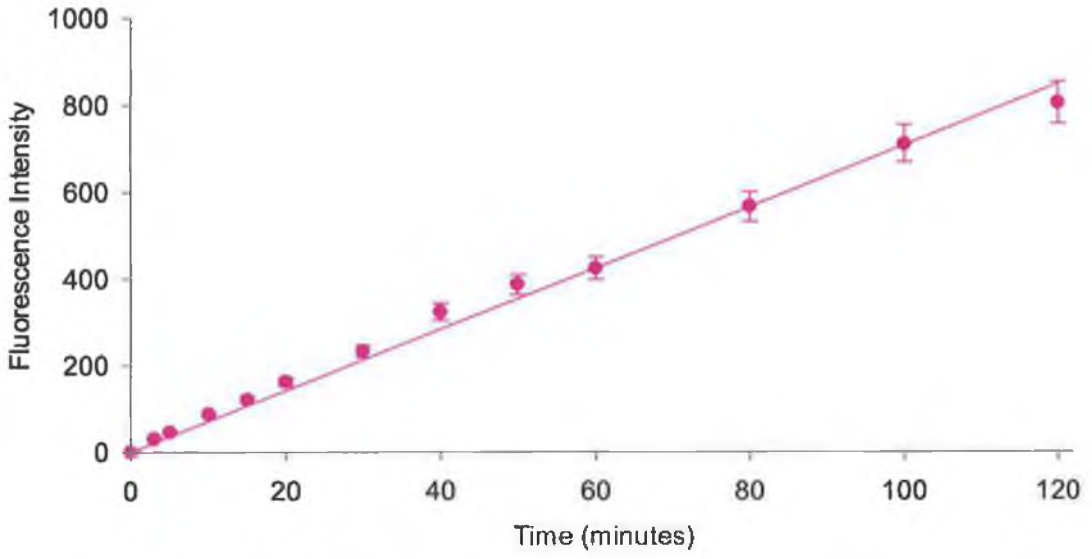


FIGURE 4.1.1.2.1. DPP IV LINEARITY WITH RESPECT TO TIME AT 37°C

Plot of fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus time (minutes).

FIGURE 4.1.1.3.1.

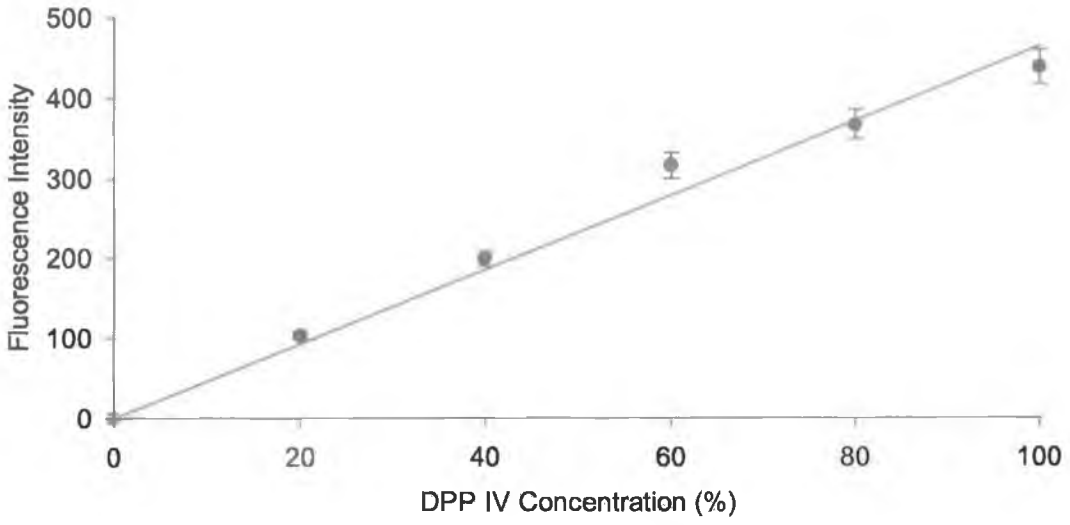


FIGURE 4.1.1.3.1. LINEARITY OF ASSAY TO DPP IV CONCENTRATION AT 37°C

Plot of fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus enzyme concentration (%). Value at 100% is equal to 43 enzyme units (refer: TABLE 3.2.).

4.1.2. TEMPERATURE STUDIES

4.1.2.1. Temperature Profile

A temperature profile of DPP IV was performed as outlined in section 2.7.1.1. Apparent activity increased to a maximum of 134% at 50°C. Activity decreased gradually above 58°C; however, activity at 64°C still equalled that at 37°C. The half-inactivation temperature T_{50}, where observed activity was 50% of 37°C rate after 10 minutes incubation in a waterbath, was estimated at 71°C ± 1°C. FIGURE 4.1.2.1.1. illustrates the effect of temperature on enzyme activity.

FIGURE 4.1.2.1.1.

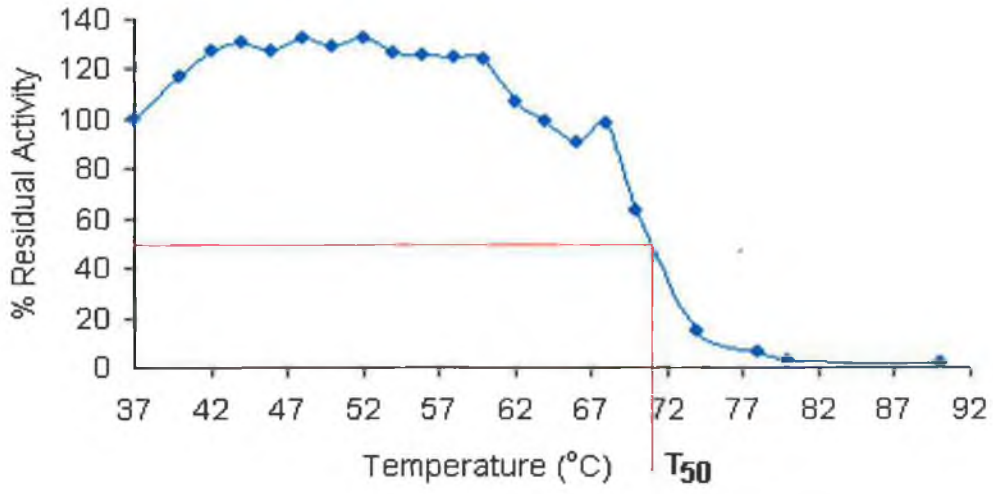


FIGURE 4.1.2.1.1. TEMPERATURE PROFILE OF DPP IV

Plot of % residual activity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus temperature (°C). Residual activity is represented as a percentage of that at 37°C.

4.1.2.2. Thermoinactivation of DPP IV

A thermoinactivation study was performed at 71°C (T_{50}) over a 60 minute period as outlined in section 2.7.2.1. FIGURE 4.1.2.2.1. illustrates the activity profile of DPP IV at 71°C over the 60-minute period. Data were fitted to first order exponential decay equation (FIGURE 4.1.2.2.2.). Data up to 28 minutes fitted well to a single exponential decay to give a *k*-value of 0.071 ± 0.003 min^{-1} and apparent half-life ($t_{½}$) of 10 minutes but, deviated above 30 minutes. After 60 minutes, 95% loss of original activity was observed. However, a double exponential decay plot at 71°C indicated that more than one molecular event was contributing to loss of activity at this temperature.

FIGURE 4.1.2.2.1.

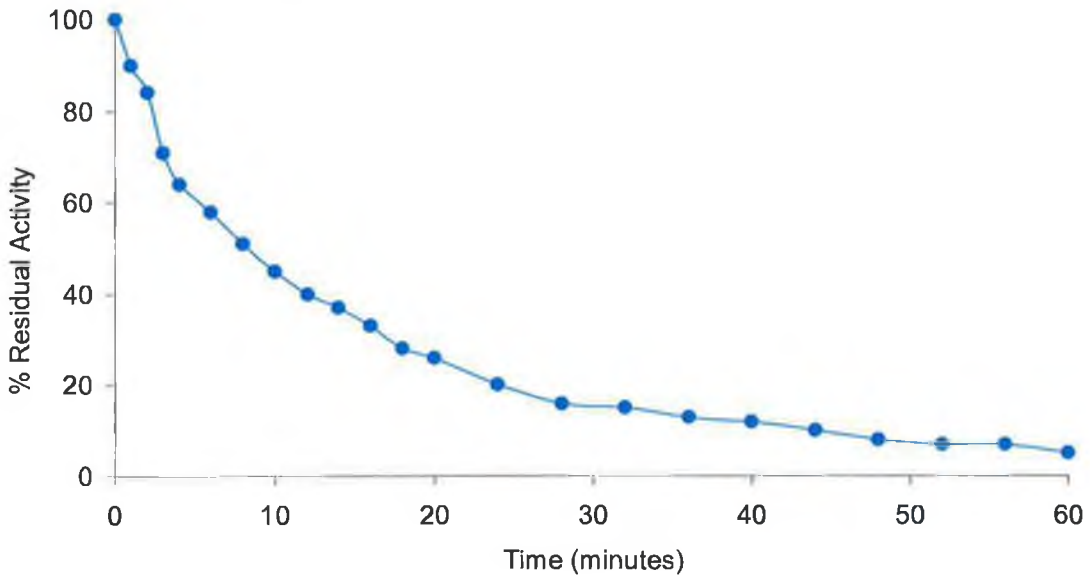


FIGURE 4.1.2.2.1. THERMOINACTIVATION OF DPP IV AT T_{50} (71°C)

Plot of % residual activity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus time (minutes). Residual activity is represented as a percentage of the t = zero value.

FIGURE 4.1.2.2.2.

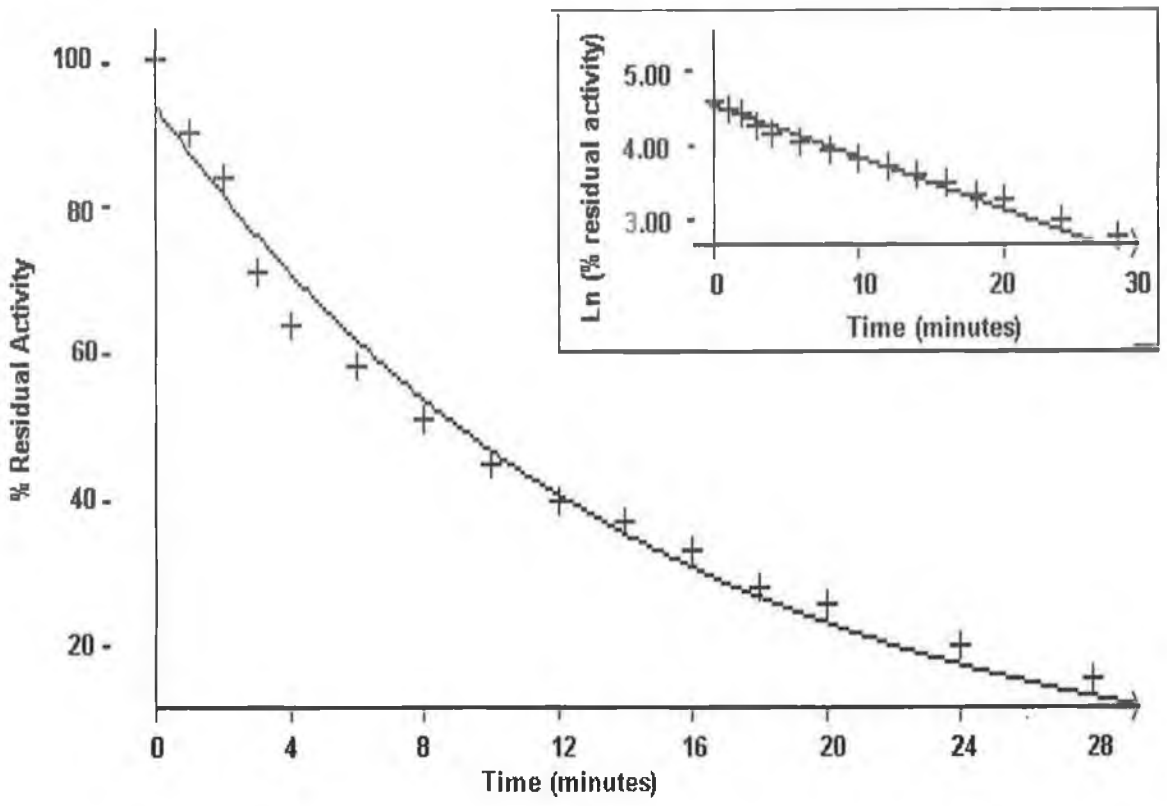


FIGURE 4.1.2.2.2. THERMOINACTIVATION DATA FITTED UP TO 28 MINUTES

Data for thermoinactivation at T_{50} (71°C) fitted to the first order exponential decay equation using the Enzfitter programme (Biosoft, Cambridge, UK).

4.1.3. ORGANOTOLERANCE STUDIES

4.1.3.1. Solvent Stability Studies

The stability of DPP IV in a range of solvents (0-90% (v/v)) was investigated as outlined in section 2.8.1.1. Threshold concentrations (C_{50}) at which half-inactivation is observed were determined for each solvent (TABLE 4.1.). FIGURE 4.1.3.1.1. illustrates the effect of solvent concentration on enzyme activity. Activation effects were observed in THF (up to 139%; between 10-20% (v/v)) and up to 118% in DMF (between 10-30% (v/v)). However, THF was the most potent denaturing solvent (observed C_{50} at 32% (v/v) solvent concentration). In acetonitrile, the enzyme retained 50% of its original activity at 70% (v/v) solvent concentration.

4.1.3.2. Temperature Profile of DPP IV in Organic Solvents

A temperature profile of DPP IV was examined in a range of solvents each at 50% (v/v) concentration as outlined in section 2.7.1.1. FIGURE 4.1.3.2.1. illustrates the effect of solvent on the temperature profile of DPP IV. At 37°C a dramatic loss of DPP IV activity was observed in each of the tested solvents (relative to aqueous DPP IV). Activation effects were observed in 50% (v/v) DMSO (at 45°C; relative to value at 37°C); however, activity declined dramatically above this temperature. No enhanced thermostability was observed in acetonitrile, DMF or THF, where activity was minimal at all temperatures examined.

4.1.3.3. Thermoinactivation of DPP IV in Organic Solvents

A thermoinactivation study was performed at 37°C over a 30 minute period as outlined in section 2.7.2.1. FIGURE 4.1.3.3.1. illustrates the activity profile of DPP IV in a range of organic solvents at 50% (v/v) at 37°C over 30 minutes. At time equals zero only DPP IV in acetonitrile had comparable activity to native enzyme, and a steady decline in activity was observed over the 30 minute time period. In contrast, placing DPP IV in DMSO resulted in an initial 20% loss in activity. However, 80% activity remained after 30 minutes. Minimal activity was observed in DMF and THF.

FIGURE 4.1.3.1.1.

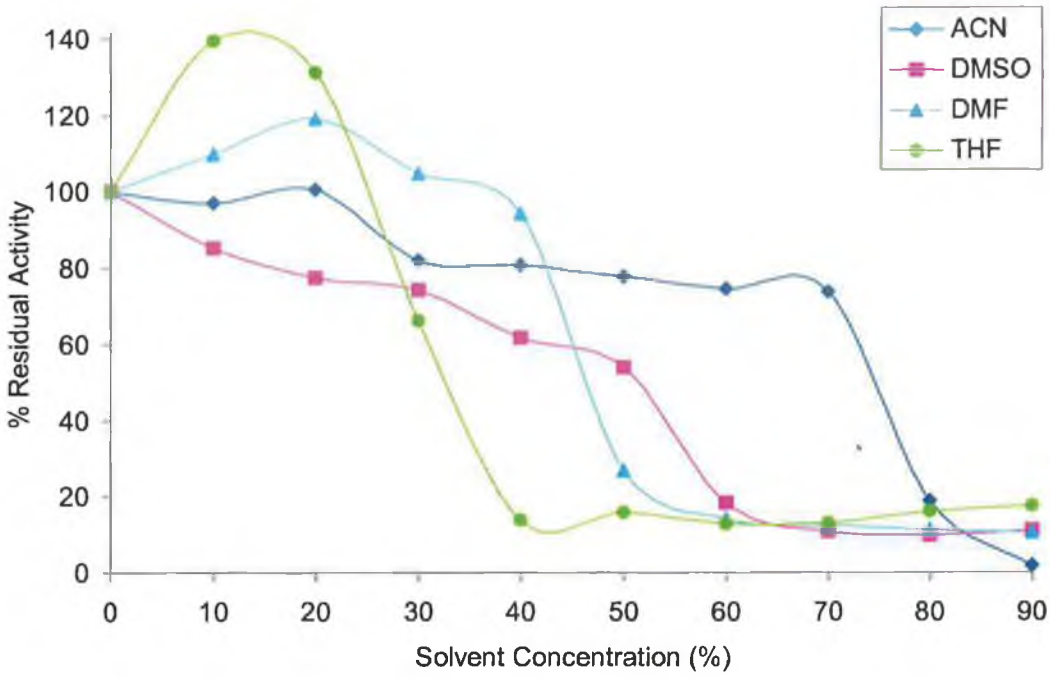


FIGURE 4.1.3.1.1. SOLVENT STABILITY OF DPP IV

Plot of % residual activity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus solvent concentration (%). Residual activity is represented as a percentage of that in aqueous buffer (C = zero value).

TABLE 4.1. OBSERVED C_{50} VALUES FOR DPP IV IN ORGANIC SOLVENTS

(Assay performed in triplicate)

SOLVENT	OBSERVED C_{50} VALUES
Acetonitrile	77% ± 0.5%
DMSO	54% ± 1%
DMF	47% ± 0.5%
THF	33% ± 0.5%

FIGURE 4.1.3.2.1.

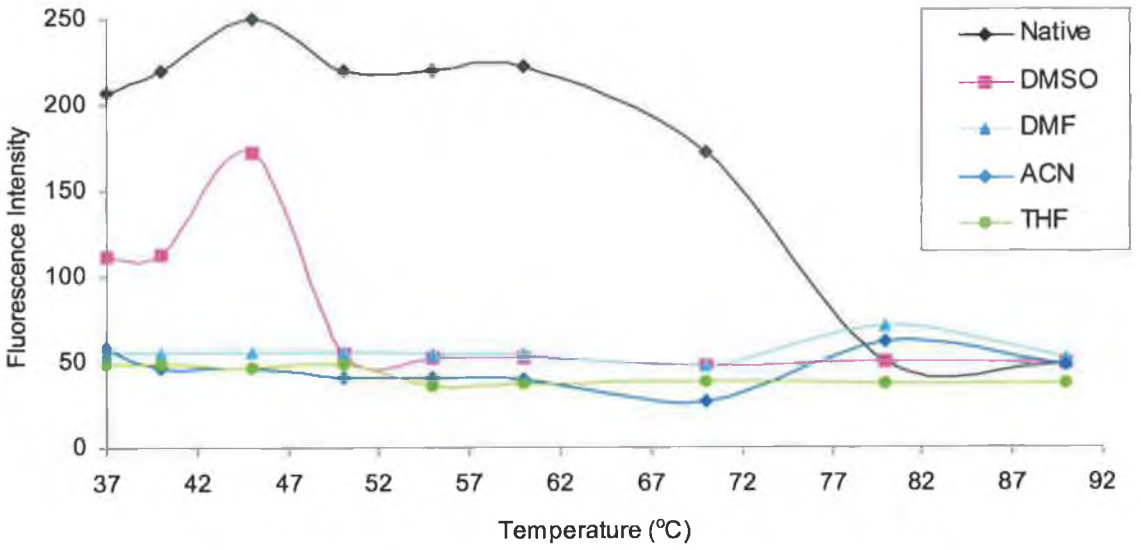


FIGURE 4.1.3.2.1. TEMPERATURE PROFILE IN 50% (V/V) ORGANIC SOLVENT

Plot of fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus temperature (°C).

FIGURE 4.1.3.3.1.

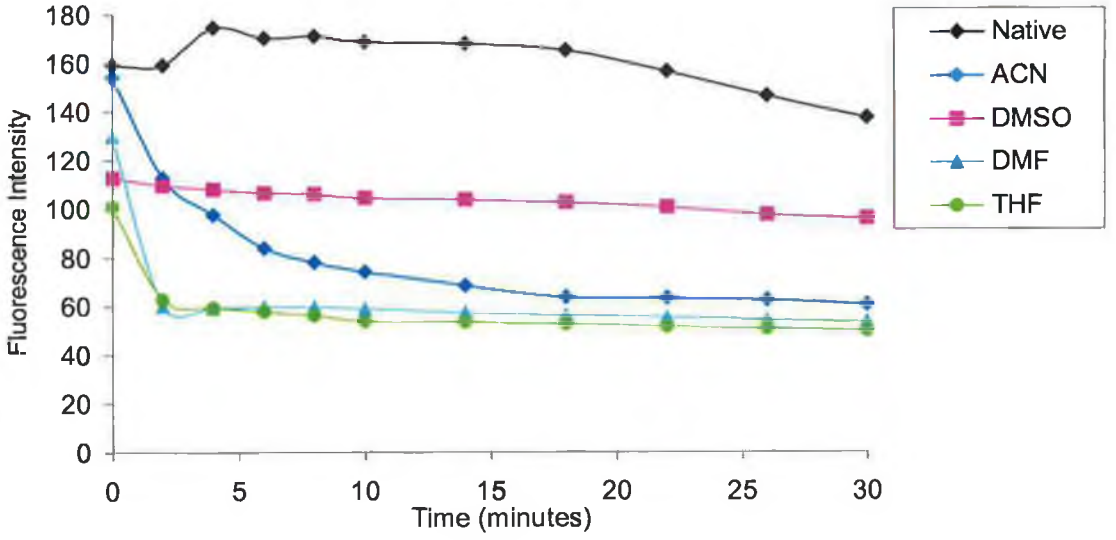


FIGURE 4.1.3.3.1. THERMOINACTIVATION IN 50% (V/V) ORGANIC SOLVENT AT 37°C

Plot of fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus time (minutes).

4.1.4. pH STUDIES

4.1.4.1. Effect of pH on DPP IV Activity

The effect of pH on DPP IV activity was determined as outlined in section 2.9.1. In order to determine if the effects on activity was due to buffer or pH, buffer systems with overlapping pH values were compared (TABLE 4.2.). FIGURE 4.1.4.1.1. illustrates the effect of both buffer and pH on DPP IV activity. TABLE 4.2. illustrates the direct effect of the buffer. Complete inactivation occurred at pH 4.0. At pH 5.5 a 25% decrease in enzyme activity was observed when the buffer system was changed from acetate to MES. Activity decreases of 65% and 67% were observed at pH 7.0 and 7.5, respectively, on changing from MOPS to HEPES. At pH 9.0 the enzyme was more active in CHES than in Tris. Optimum pH was observed between 7.5 and 9.0.

4.1.4.2. Effect of pH on DPP IV Kinetics

Michaelis-Menten kinetics were determined at each pH point as described in sections 2.6.1. and 2.9.1. In 50mM HEPES, pH 8.0, DPP IV displayed Michaelis-Menten kinetics giving V_{max} and K_m values of 0.00142 ± 0.00014 μM/min^{-1} and 173 ± 7.6 μM^{-1}, respectively. Two pK values were observed at pH 6.18 ± 0.07 and 9.7 ± 0.5 (Enzfitter software; data not shown). Both occurred in plots of log V_{max}/K_m and log $1/K_m$ versus pH; only the upper value occurred in a plot of log V_{max} against pH (FIGURES 4.1.4.2.1. and 4.1.4.2.2.). FIGURE 4.1.4.2.1. illustrates the classical bell-shaped curve and the pK values derived from the plot of log V/K_m versus pH.

4.1.4.3. pH Thermostability

A temperature profile of DPP IV was examined at various pH values in different buffer systems as outlined in section 2.9.2. The half-inactivation temperature T_{50}, was calculated for each buffer system at similar pH values (TABLE 4.3. and 4.4.). FIGURES 4.1.4.3.1. and 4.1.4.3.2. illustrate the effect of pH on the temperature profile of DPP IV. At temperatures above 72°C DPP IV demonstrated activity in only the following buffers; acetate, pH 5.5 and HEPES, pH 8.0; however, activity was minimal. DPP IV exhibited higher activity in acetate than MES buffer at pH 5.5. At pH 6.5, DPP IV was up to 70% more active in MES than in MOPS buffer (between 37-60°C). Activity was higher in HEPES than Tris, at pH 8.0. DPP IV demonstrated similar activity (between 64-72°C) when placed in either Tris or CHES, pH 9.0.

FIGURE 4.1.4.1.1.

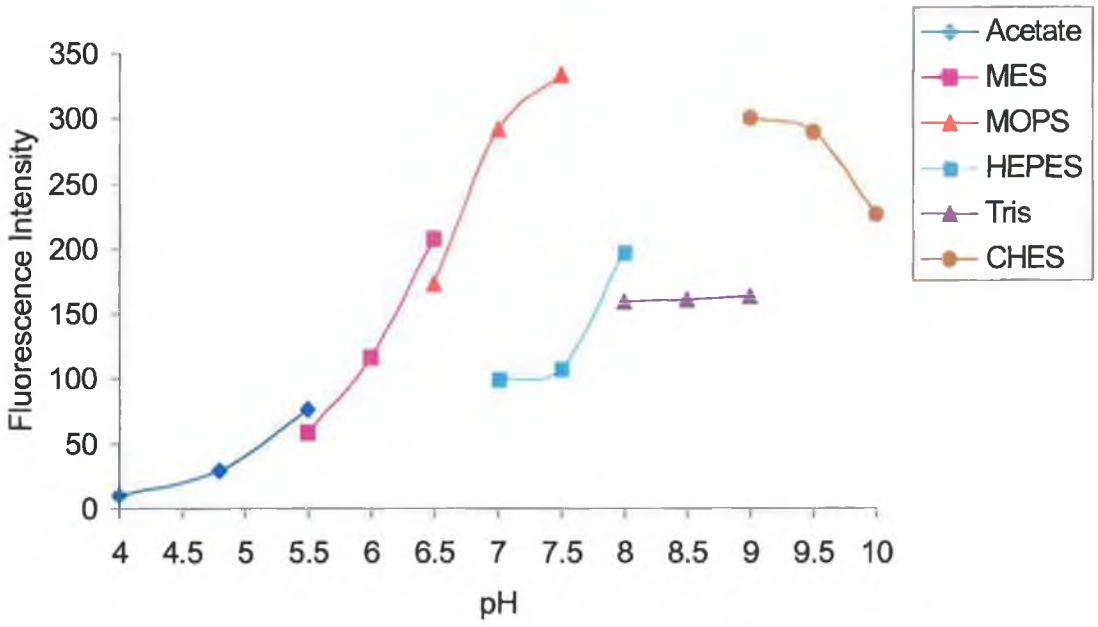


FIGURE 4.1.4.1.1. EFFECT OF pH/BUFFER ON DPP IV ACTIVITY

Plot of fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus pH.

TABLE 4.2. COMPARISON OF DPP IV ACTIVITY IN VARIOUS BUFFER SYSTEMS AT SIMILAR pH. (FI = FLUORESCENCE INTENSITY)

Buffer ↓	**pH →**	**5.5 (FI)**	**6.5 (FI)**	**7.0 (FI)**	**7.5 (FI)**	**8.0 (FI)**	**9.0 (FI)**
Sodium acetate		76.5	--------	--------	--------	--------	--------
MES		58.1	207.7	--------	--------	--------	
MOPS		--------	173.2	291.7	333.8	--------	--------
HEPES		--------	--------	99.9	108.2	197.5	--------
Tris		--------	--------	--------	--------	159.5	163.4
CHES		--------	--------	--------	--------	--------	300.1

FIGURE 4.1.4.2.1.

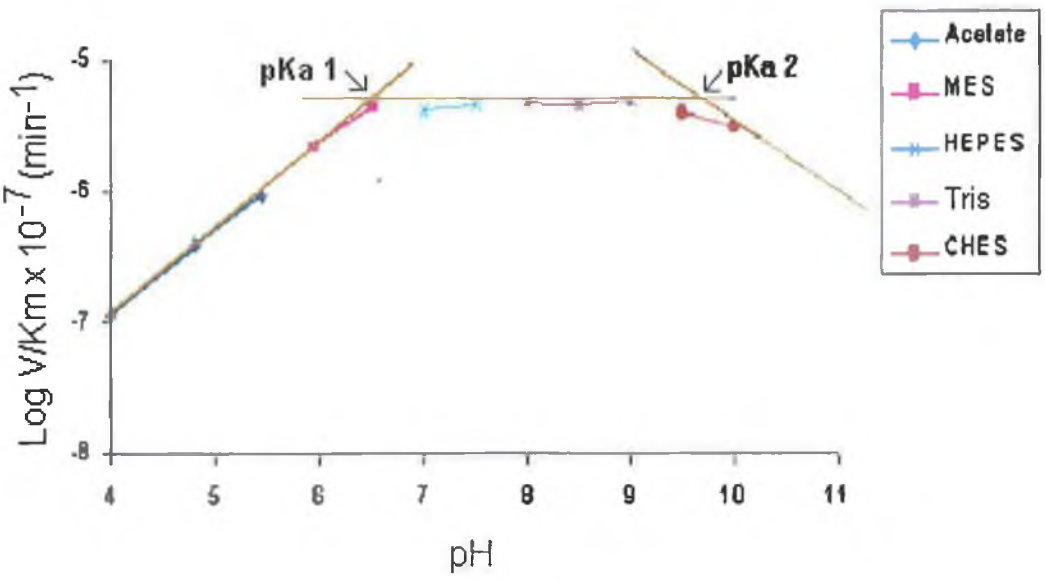


FIGURE 4.1.4.2.1. EFFECT OF pH ON THE KINETICS OF DPP IV

Plot of Log V/K_m x 10^{-7} (min^{-1}) versus pH

FIGURE 4.1.4.2.2.

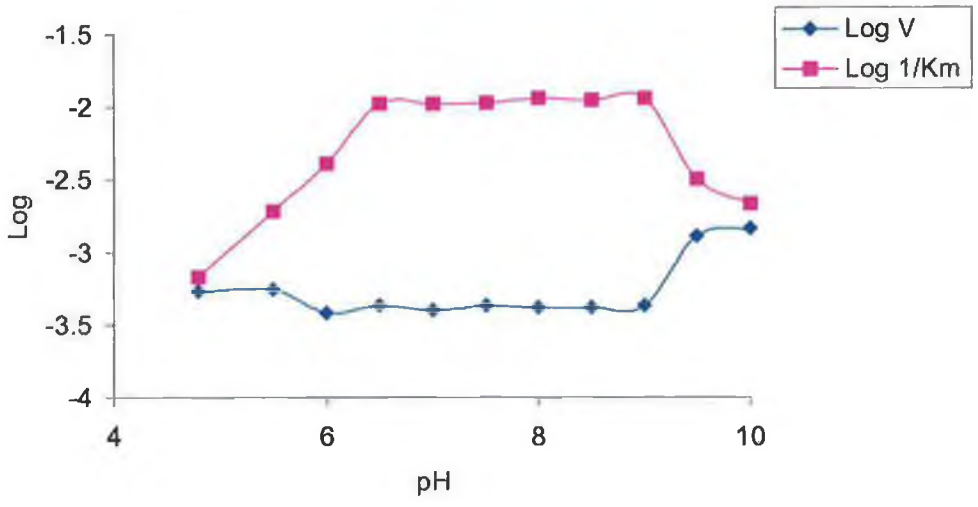


FIGURE 4.1.4.2.2. EFFECT OF pH DPP IV KINETICS

Plot of Log V(μM/min) versus pH and plot of Log $1/K_m$ (μM) versus pH

FIGURE 4.1.4.3.1.

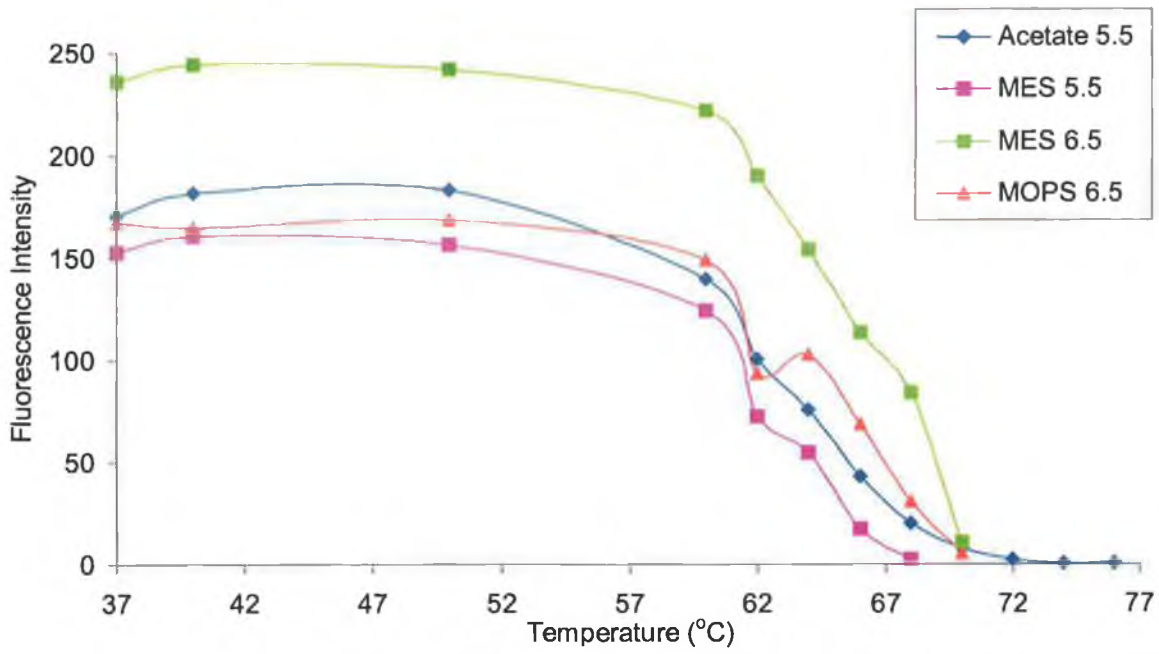


FIGURE 4.1.4.3.1. pH TEMPERATURE PROFILE OF DPP IV (pH 5.5-6.5)

Plot of fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus temperature (°C).

TABLE 4.3. OBSERVED T_{50} VALUES IN DIFFERENT BUFFER SYSTEMS (pH 5.5-6.5)

(Assay performed in triplicate)

Buffer ↓	pH →	5.5 T_{50}	6.5 T_{50}
Sodium acetate		63°C ± 0.5°C	--------
MES		62°C ± 0.5°C	66°C ± 0.5°C
MOPS		--------	65°C ± 0.5°C

FIGURE 4.1.4.3.2.

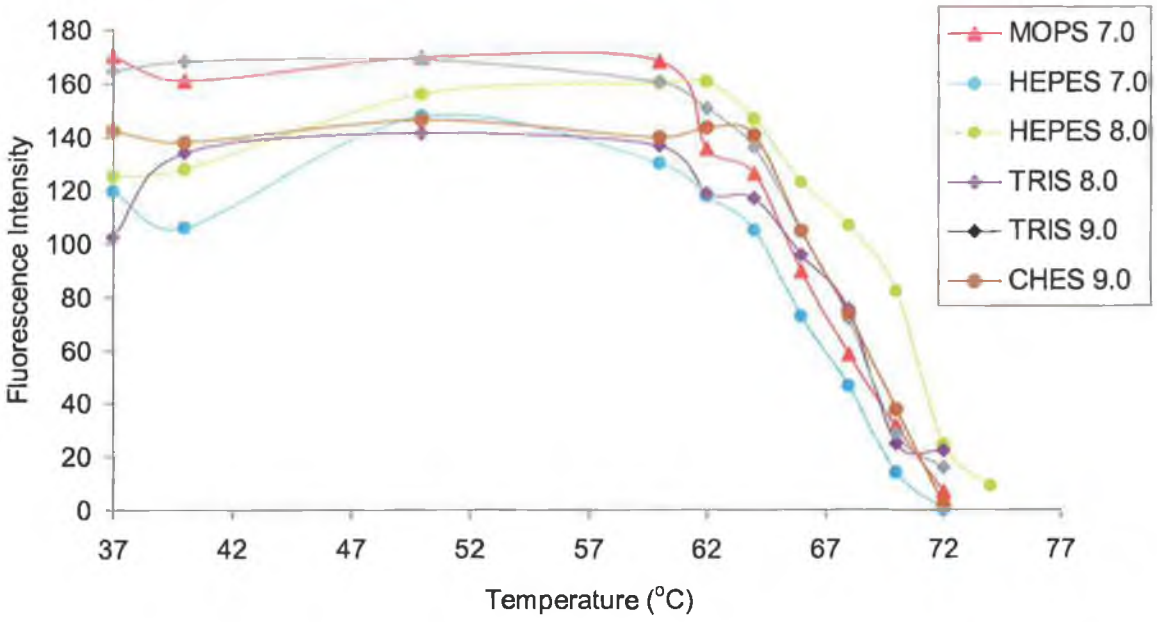


FIGURE 4.1.4.3.2. pH TEMPERATURE PROFILE OF DPP IV (pH 7.0-9.0)

Plot of fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus temperature (°C).

TABLE 4.4. OBSERVED T_{50} VALUES IN DIFFERENT BUFFER SYSTEMS (pH 7.0-9.0)

(Assay performed in triplicate)

Buffer ↓	**pH →**	**7.0** T_{50}	**8.0** T_{50}	**9.0** T_{50}
MOPS		66°C ± 0.5°C	--------	--------
HEPES		68°C ± 0.5°C	71°C ± 0.5°C	--------
Tris		--------	69°C ± 0.5°C	68°C ± 1°C
CHES		--------	--------	68°C ± 0.5°C

4.2. Discussion

Bickerstaff and Zhou (1993) developed a method to determine directly autolytic processes of a protease. This method gives a direct indication of the loss of enzyme protein with time and eliminates the need for gel electrophoresis (used to determine autolytic products/protein reduction). It employs the Coomassie blue dye reagent and enzyme alone, enabling the protein content to be monitored, while providing a direct measurement of autolytic activity. No autolysis was observed over the assay period at 37°C (FIGURE 4.1.1.1.1.). The resistance of DPP IV to proteolysis has been documented (Lambeir *et al.,* 1997).

FIGURE 4.1.1.2.1. illustrates that bovine serum DPP IV catalysed the linear release of AMC over the standard 60-minute assay period and up to 120 minutes, thus allowing for longer analysis if required. This shows that DPP IV is not only stable but also, fully active and saturated with sufficient substrate over this longer time.

Under given conditions, two enzyme molecules acting independently in solution will transform twice as much substrate in a given time as one molecule, hence, the velocity should be proportional to the enzyme concentration. This is found in a majority of cases and is the basis of nearly all enzyme assay methods. However, deviations can occur, and a linear relationship between velocity and enzyme concentration in the range used should be established before any further attempt at kinetics, or before attempts to calculate specific activities are made. Departures from linearity are due to an artefact of the assay system, but there are cases where such behaviour is a property of the enzyme itself (Dixon & Webb, 1979a).

FIGURE 4.1.1.3.1. illustrates that fluorescence intensity increases linearly and proportionally with the increase in enzyme concentration at 37°C. Hence, the enzyme hydrolyses at the same rate depending on the quantity of enzyme present.

The rate of inactivation of an enzyme in solution increases rapidly with the temperature. In most cases, inactivation becomes virtually instantaneous at temperatures well below 100°C and, in some cases, below 70°C (Dixon & Webb, 1979b). However, there are a number of enzymes that can withstand more extreme temperatures and remain catalytically active. The growth optimum of citrate synthase from the thermophilic Archaea *Pyrococcus furiosus* was found at 100°C (Danson & Hough, 1998). An extremely thermoresistant serine protease from *Staphylothermus marinus* maintains catalytic activity after 10 minutes at 135°C. A subtilisin-type serine protease detected in *Pyrococcus furiosus* called pyrolysin was found to retain 50% of its activity after a 20-minute 105°C challenge (Eichler, 2001).

An enzyme denatures at an elevated temperature due to unfolding of the protein backbone. At high temperatures, the enzyme loses its "regular" non-covalent interactions that support the native structure at moderate temperatures, and acquires high temperature 'non-native' non-covalent bonds. As the temperature decreases, these "irregular" non-covalent interactions, although thermodynamically unstable, remain and the molecular mobility of the polypeptide chain decreases with decreasing temperature. Hence, the protein remains denatured and cannot spontaneously reacquire the native form at low temperature (Mozhaev & Martinek, 1982). However, it has been shown that bonds and interactions at secondary and tertiary structural levels may indeed be broken without loss of any activity. Moreover, enzymes may be "stabilized" or "protected" against deactivation. Therefore, in these situations, it is important to know

not only the amount of denaturation required to abolish enzyme activity but also the order of denaturation stages, which often plays a significant role. Hence, it is reasonable to expect that more than one bond of a particular type or more than one "sensitive structure" must be broken before significant changes in conformation leading to loss of activity occur (Henley & Sadana, 1985). Bovine serum DPP IV demonstrated an increase in catalytic activity up to 62°C (maximal activity observed at 50°C, T_{50} at 71°C), showing an apparent activation, as opposed to the expected thermal inactivation. This is well above normal mammalian body temperature (37°C). Durinx *et al.* (2000) investigated the thermostability of human serum DPP IV over the range 25-80°C. They determined optimum temperature between 50-60°C. Similar results were obtained for porcine seminal plasma DPP IV (Ohkubo *et al.*, 1994). Yoshimoto *et al.* (1978) have reported an optimum temperature of 60°C for lamb kidney DPP IV with 50% of activity retained up to 72°C. Despite these reports, all routine and kinetic assays were performed at physiological temperature, 37°C.

Activity loss with time was investigated at 71°C (T_{50}). Double exponential decay at this temperature indicated that more than one molecular event was contributing to loss of activity at this temperature; however, data (up to 28 minutes) fitted well to single exponential decay, allowing the estimation of an apparent half-life (10 minutes). The first step in the inactivation of an oligomeric enzyme is the dissociation into nonactive subunits (Trubetskoy & Torchilin, 1985). Kurganov *et al.* (2000) suggested that denaturation proceeds by a dissociative mechanism, as determined by their investigation of the thermal denaturation of rabbit skeletal muscle glycogen phosphorylase b. This mechanism involves the reversible dissociation of the active dimer into the corresponding inactive monomers, followed by the irreversible denaturation of the

monomer. As demonstrated by the inactivation kinetics of DPP IV at 71°C, one inactivation process seems to dominate over a short period of time, after which a second process is apparent at longer times. This mechanism of dissociative unfolding could therefore be applied to explain the inactivation kinetics of dimeric bovine serum DPP IV. This unfolding could be monitored by gel electrophoresis, as the unfolded protein migrates less rapidly than the compact folded forms (Creighton, 1993c). Structural changes could be verified by LC-MS protein characterization analysis. This method gives an accurate mass of the intact protein, observing the loss of one or more amino acids. Denaturation can also be measured by fluorescence spectroscopy. This process causes an abrupt increase in the fluorescence intensity and wavelength of maximal emission (λ_{max}) as a result of contact between the aromatic chromophores (buried within the hydrophobic core of the protein) with the polar (solvent) environment e.g. fluorescence intensity increased and λ_{max} shifts from 333nm to 340nm were observed upon thermal denaturation of α-chymotrypsin (Mozhaev *et al.*, 1989).

For enzymes to exhibit their full catalytic properties they must have a strictly defined or native conformation. A network of hydrogen bonds, electrostatic and hydrophobic interactions, determines this conformation, which is maintained by an essential hydration layer of tightly bound water. Replacement of this water shell with an organic solvent may affect the nature of biocatalysis in a number of ways, solvents may; (1) interact with the essential hydration layer of the enzyme (2) disrupt the hydrogen bonds, ionic or hydrophobic interactions, altering the native conformation by direct interaction with the protein (Khmelnitsky *et al.,* 1988; Dordick 1991). The concentration at which half inactivation (C_{50}) is observed was determined for each solvent (TABLE 4.1). THF was found to be the most deleterious solvent. This corresponds with it having the

highest denaturing capacity (DC = 100) correlating with the findings of Mozhaev *et al.* (1989). However, an activation effect was observed up to 20% (v/v) concentration, after which activity greatly decreased where little or no activity was exhibited above 40% (v/v) concentration. This activation effect may be explained in two ways (1) changes in the conformation of the enzyme molecule caused by introducing the solvent into the system or (2) the solvent participating in the enzymatic reaction by acting as an additional nucleophile in the deacylation step (Khmelnitsky *et al.*, 1988). The enzyme was most stable in acetonitrile, where at 70% (v/v) the enzyme still retained up to 70% initial activity. Enzyme activity in DMSO steady declined to a minimum observed between 60-90 % (v/v). As FIGURE 4.1.3.1.1. illustrates, increasing solvent concentration (DPP IV in DMF) did not affect the catalytic activity; however, after a critical concentration of solvent had been reached, further addition resulted in an abrupt decrease in enzyme activity. The effect of solvent on protein conformation in organic media can be determined by means of high-resolution x-ray crystallography. This is a useful method to elucidate the three-dimensional structure of the enzyme in solvent and compare it to that in aqueous solution. Fitzpatrick *et al.* (1993) used this method to determine changes in the structure of subtilisin Carlsberg in anhydrous acetonitrile. Their findings concluded that the three-dimensional structure in anhydrous acetonitrile was essentially identical to that in water.

TABLE 4.5. CHARACTERISTICS OF ORGANIC SOLVENTS (Gupta *et al.* 1997)

SOLVENT	DENATURATION CAPACITY (DC)
Acetonitrile	64.3
DMF	63.3
DMSO	60.3
THF	100

No enhancement of thermostability was observed at any temperature when DPP IV was placed in any of the given solvents (50% (v/v)): only minimal activity was present as compared to DPP IV in aqueous buffer. Activity in DMSO was higher relative to other solvents (up to 47°C) after which a dramatic decrease in activity was observed. Similar results were obtained for thermoinactivation studies in 50% (v/v) solvent, at 37°C. DPP IV in aqueous buffer remained stable over the duration of the experiment. When DPP IV was placed in DMF or THF no appreciable activity was observed after two minutes (relative to DPP IV in aqueous buffer). In contrast only a minimal loss in activity was observed over the duration, when the enzyme was placed in DMSO. This diminished activity is not uncommon in aqueous-organic mixtures and can occur as a result of the enzyme being driven to denature by the organic component and being enabled to succumb to this denaturation by the aqueous component. Therefore, in such mixtures activity may drop below that seen when the enzyme is in pure water (Klibanov, 2001). This is possibly the reason why no enhanced stability was observed.

pH influences the secondary, tertiary and quaternary structure of proteins. Hence, the rate of inactivation of an enzyme, like other protein denaturation, is in most cases greatly dependent on the pH of the solution. pH inactivation of an enzyme involves

unfolding of the protein caused by changes in the balance of electrostatic and hydrogen bonding. These changes occur due to pH-induced alterations in the ionisation states of functional groups on the protein. The effect of pH may vary greatly from one enzyme to another. However, in general there is a zone of maximum stability, and the inactivation increases on the acid and alkaline side. In most cases a definite optimum pH is observed. The effect of pH on enzyme-catalyzed reactions is separated into two categories (1) pH influence on binding and catalysis (2) pH influence on enzyme stability. (Dixon & Webb, 1979c; Sadana & Henley, 1988).

The effect of pH on the thermostability of DPP IV was investigated. To determine if irreversible destruction occurred, enzyme activity was tested after readjustment to the standard pH 8.0 in 50mM buffer (having been previously exposed to range of pH values in 20mM buffer). The activity of DPP IV between 5.5-6.5 (acetate, MES) decreased steadily after 60°C, indicating decreased thermostability at non-neutral pH values. The highest activity was observed at pH 6.5 in MES (FIGURE 4.1.4.3.1.) The T_{50} value obtained for DPP IV in 20mM Tris, pH 8.0 was only 2°C less than that in 20mM HEPES, pH 8.0; therefore, changing the buffer did not greatly influence DPP IV thermostability. However, comparison of observed T_{50} values at each pH indicates that DPP IV is more thermostable in the neutral-alkaline pH region, with optimum stability observed at pH 8.0 in HEPES buffer. Irreversible destruction was observed in most cases at temperatures above 70°C. It should be mentioned that the pH of buffer solutions are influenced by changes in temperature e.g. pH 7.0 Tris buffer prepared at 4°C would have a pH of 5.95 at 37°C, this occurs because of temperature-dependent changes in the dissociation constant (pK_a) of ions in solution (Boyer, 2000). Therefore, further investigation is required to determine if possible changes in the pH of buffer

occurred with increasing temperature. These changes maybe assessed and accounted for by following theoretical procedures for calculating the practical p*K* values that apply to buffers under chosen experimental conditions and for determining the ionic strength of buffers at various pH values (Ellis & Morrison, 1982).

The effect of pH on DPP IV activity was investigated. Sodium acetate, MES, MOPS, HEPES, Tris and CHES buffer systems were used. FIGURE 4.1.4.1.1. illustrates a non-classical bell-shaped curve, with neutral pH preferred. These results are comparable to Sentandreu and Toldrá (2001) and Buckley *et al.* (2004), who found similar results when they studied DPP IV from porcine skeletal muscle and bovine serum, respectively. Complete inactivation was noticed at pH 4.0. This is similar to the findings of Duke-Cohan *et al.* (1995), who reported complete inhibition of human serum DPP IV at acidic pH 4.5-5.0. FIGURE 4.1.4.1.1 shows a broad, stable pH range 7.0-10 at 37°; however, this depends on the buffer system used. The objective of these pH studies was to ascertain the direct effect of pH on DPP IV activity. This is achieved by altering the pH of the buffer species within the limits of its buffering capacity, i.e. 1.0 pH unit either side of its designated p*K*a. Hence, the buffer species also changes as pH is altered, creating two variables to study, i.e. pH effect and buffer effect. This problem can be minimised if overlapping pH values are used between various buffer systems. However, some buffers can themselves have a specific effect on enzymatic activity regardless of pH, e.g. phosphate inhibits certain enzymes, including kinases, phosphatases, and dehydrogenases (Bollag *et al.*, 1996d). TABLE 4.2. demonstrates the direct effect of changing the buffer on DPP IV activity. Activity was higher at pH 8.0 in HEPES than in Tris and was greatest at pH 7.5 in MOPS buffer. These results are similar to Yoshimoto and Walter (1977), who reported a pH optimum of 7.8 for lamb

kidney DPP IV. However, all routine assays were performed at pH 8.0, in HEPES buffer.

The effect of pH on DPP IV kinetics with Gly-Pro-AMC as substrate was investigated. Sodium acetate, MES, MOPS, HEPES, Tris and CHES buffer systems were used. To determine pK values, a plot of Log V/K_m versus pH was constructed, giving the pK values for the free enzyme [E] and free substrate [S] (Fersht, 2002a). FIGURE 4.1.4.2.1. illustrates the classical bell-shaped curve, which indicates that at least two ionising groups change their state of protonation as the pH is raised from pH 4 to more alkaline values. In FIGURE 4.1.4.2.1. it is clearly demonstrated that the pK_B is far above the pK_A giving rise to a bell shaped curve with a flat top. Two pK values are observed at 6.18 and 9.7. These values occur in plots of Log 1/K_m and log V/K_m versus pH as downward bends, indicating that they belong to the free enzyme [E] and free substrate [S] (Dixon & Webb, 1979c; Fersht, 2002a). The 9.7 value alone occurs as an upward bend in the plot Log V versus pH. This represents the value affecting the enzyme-substrate [ES] complex. The upper pKa value of 9.7 possibly belongs to the α-amino group of the Gly moiety on the substrate Gly-Pro-AMC, as this value is common to plots indicating pK values in both the [ES] complex and also in the free [E] and free [S]. The unique arrangement of the DPP IV catalytic triad renders this a non-classical serine protease. Its catalytic residues are arranged in the unique order Ser-Asp-His (nucleophile-acid-base) order, different from that found in classical serine proteases such as the trypsin and subtilisin families. The 200 C-terminal residues of DPP IV containing the catalytic triad have similarity with several other non-classical serine hydrolases including prolyl oligopeptidase (Abbott *et al.*, 1999). A general catalytic triad reported is Ser_{630}, Asp_{708}, His_{740}. However, the exact position of these residues may vary in different species, e.g.

murine DPP IV – Ser_{624}, Asp_{702}, and His_{734}, bovine DPP IV – Ser_{630}, Asp_{702}, and His_{734}, (Niedermeyer *et al.*, 1998; DeMeester *et al.*, 1999; Lee *et al.,* 2002). It is tempting to assign a measured p*K* value to a particular amino acid side chain, from tables of values pertaining to the p*K* for free amino acids, but one should bear in mind that the side-chain environment within an enzyme may differ from that of the free amino acid causing a shift in p*K* (Price & Stevens, 1999). With this in mind, the lower p*K* value (6.18) was attributed to that of His_{734} in the enzyme active site.

The results presented here reveal that DPP IV from bovine serum does not undergo autolysis at 37°C and is catalytically active up 62°C, with T_{50} determined at 71°C ± 1°C. Solvent stability studies indicate that the enzyme is most stable in acetonitrile up to 70% (v/v). In comparison, activity in DMF and DMSO declines above 40% (v/v) and THF is the most deleterious solvent to the enzyme. pH studies show that DPP IV displays maximum activity at pH 7.5, and is completely inactive at pH 4.0. The effect of pH on the thermostability of DPP IV indicates that the enzyme is most thermostable in the neutral-alkaline pH region, with optimum thermostability observed at pH 8.0. Kinetic studies indicate that DPP IV activity depends on the ionization of a group with a p*K* value of 6.18, which has been attributed to the active site histidine residue.

CHAPTER 5

RECOMBINANT *FASCIOLA HEPATICA* CATHEPSIN L1 STUDIES

5.1. INTRODUCTION

Cathepsin L is an endopeptidase. It preferentially cleaves peptide bonds with hydrophobic amino acid residues in P_2 and P_3 (Barrett *et al.,* 1998). The amide substrate, Z-Phe-Arg-AMC when hydrolyzed by cathepsin L releases AMC, which is monitored fluorometrically, making this a sensitive and suitable substrate. The ester substrate Z-Lys-ONp when hydrolyzed by cathepsin L releases ONp and is determined colorimetrically.

Recombinant *Fasciola hepatica* cathepsin L1 (rFheCL1) was investigated with regards to temperature and solvent stability prior to chemical modification of the enzyme. A temperature profile was determined and the apparent half-inactivation temperature (T_{50}) estimated. The effect of organic solvents on rFheCL1 was investigated following exposure to a range of solvents at various concentrations and the half-inactivation concentration (C_{50}) determined. The steady state kinetics of native and modified (section 6.2.3.) rFheCL1 were studied to compare the respective K_m and k_{cat} values and to observe the effects of modification on rFheCL1-catalyzed aminolysis.

5.2. RESULTS – ASSAY AND STABILITY STUDIES ON rFheCL1

All of the upcoming graphs illustrate data points that represent the average of triplicate fluorescence intensity or absorbance readings (n=3), minus the blank measurement; standard deviation ± 5%. All error bars represent the standard error.

5.2.1. AMC STANDARD CURVES AND THE INNER FILTER EFFECT

AMC standard curves were prepared as outlined in Section 2.4.1.1. Plots of fluorescence intensity versus AMC concentration (micromol) were constructed (section 3.2.2. FIGURE 3.2.2.1.) and the slope of the lines calculated. The inner filter effect was also performed as described in section 2.4.1.2. to assess the effect on fluorescence of including the post column fraction in the assay mixture. FIGURE 5.2.1.1. is a plot of fluorescence intensity versus AMC concentration (micromol) for purified rFheCL1. TABLE 5.1. lists the slopes calculated for each curve and the degree of filtering observed for the enzyme sample.

TABLE 5.1. SLOPES OF FILTERED STANDARD CURVES

SAMPLE	R^2	SLOPE	FILTERING (%)
BUFFER	0.99	59.99	0.0
ACTIVATED rFheCL1	0.99	59.74	0.5

FIGURE 5.2.1.1.

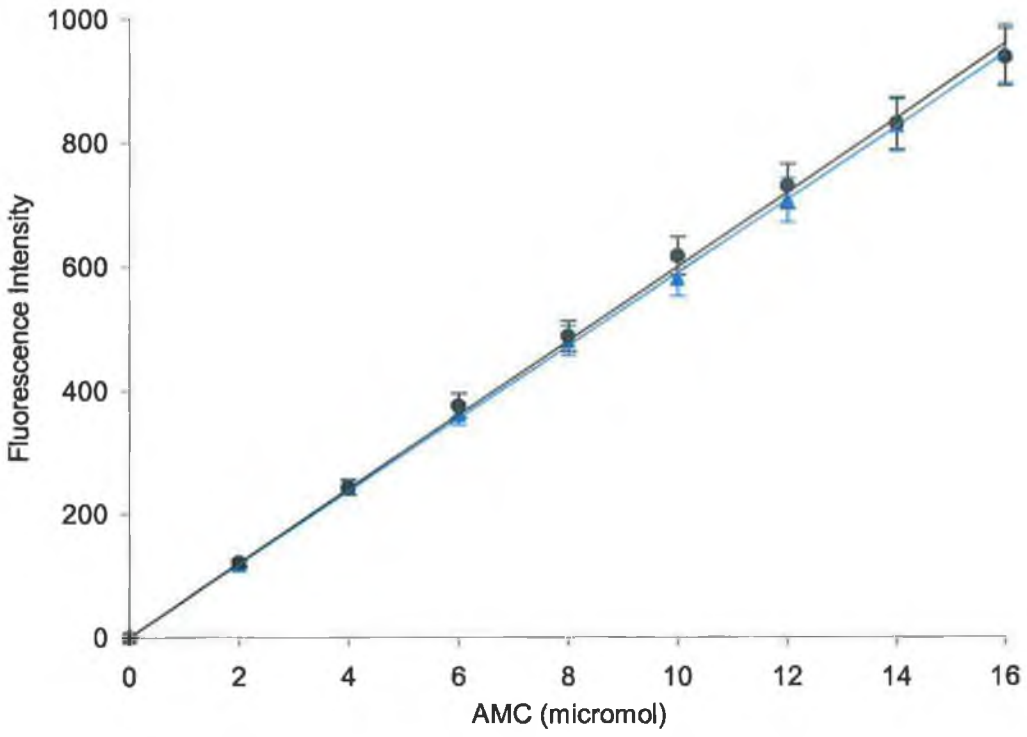


FIGURE 5.2.1.1. AMC QUENCHED STANDARD CURVE

Plot of fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width, 2.5nm) versus free AMC concentration (micromol) (•). The quenching agent used was purified rFheCL1 (▲).

5.2.2. PROTEIN DETERMINATION

Bovine Serum Albumin (BSA) standard curves were prepared as outlined in section 2.4.2. Plots of protein absorbance at 560nm versus BSA concentration (mg/ml) were constructed (section 3.2.3; FIGURE 3.2.3.2.) for standard BCA assays.

5.2.3. ASSAY DEVELOPMENT

5.2.3.1. Autodigestion Assay

The process of native rFheCL1 autodigestion was examined as described in section 2.5.1. FIGURE 5.2.3.1.1. demonstrates that at 37°C no autodigestion was observed over a period of 240 minutes.

5.2.3.2. Linearity of Enzyme Assay with Respect to Time

The stability of rFheCL1 and its ability to cleave its corresponding substrate Z-Phe-Arg-AMC with respect to time was examined as described in section 2.5.2.2. FIGURE 5.2.3.2.1. demonstrates that rFheCL1 activity is linear (R^2: 0.99) over a period of 30 minutes.

5.2.3.3. Linearity of Assay with Respect to Enzyme Concentration

Linearity of purified rFheCL1 degrading activity with respect to enzyme concentration was determined as outlined in section 2.5.3.2. FIGURE 5.2.3.3.1. illustrates that the assay is linear (R^2: 0.97) with respect to enzyme concentration.

FIGURE 5.2.3.1.1.

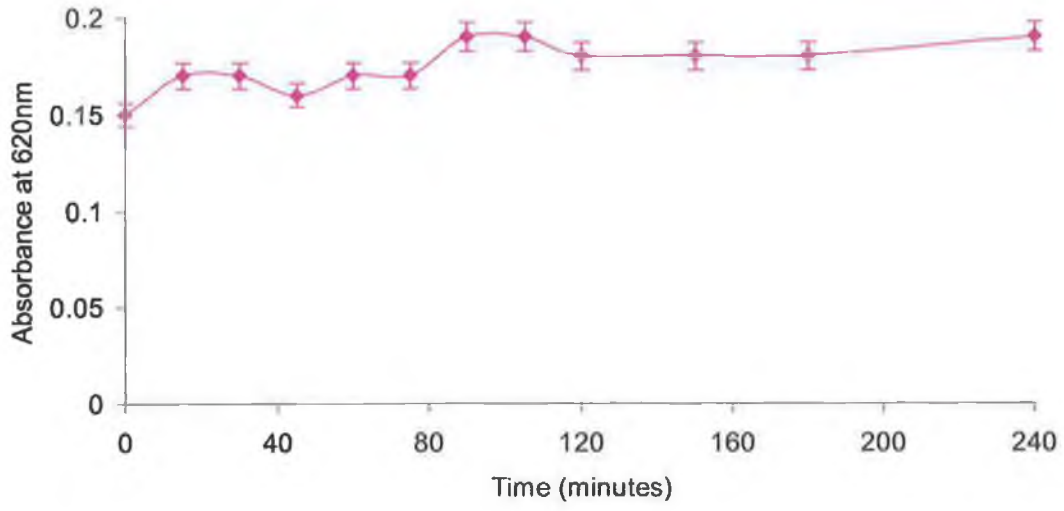


FIGURE 5.2.3.1.1. AUTODIGESTION OF rFheCL1 OVER TIME AT 37°C

Plot of absorbance at 620nm versus time (minutes).

FIGURE 5.2.3.2.1.

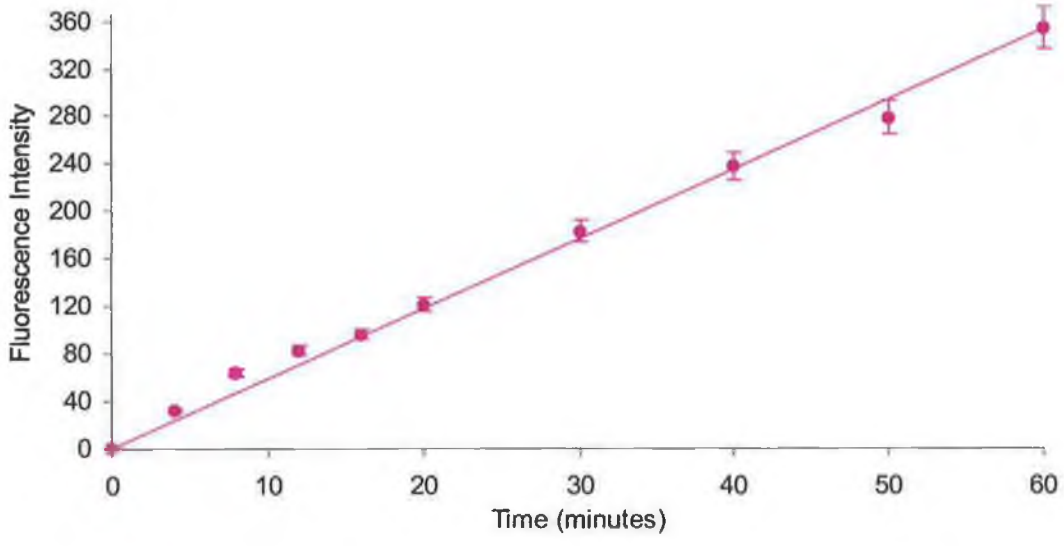


FIGURE 5.2.3.2.1. rFheCL1 LINEARITY WITH RESPECT TO TIME AT 37°C

Plot of fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus time (minutes).

FIGURE 5.2.3.3.1.

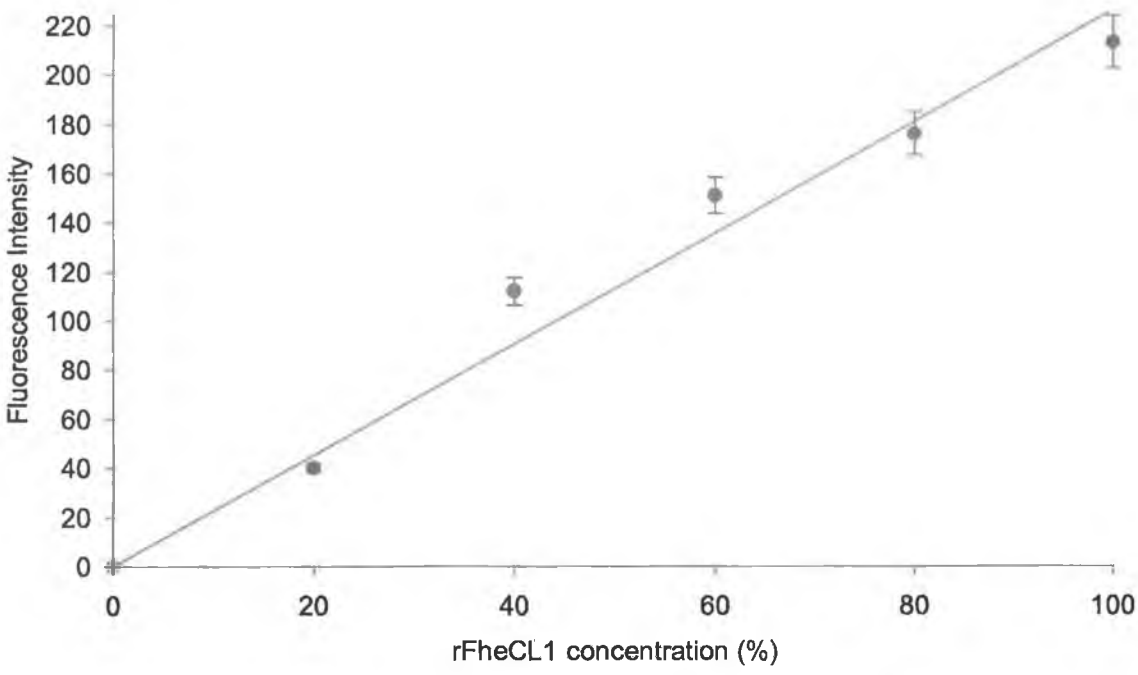


FIGURE 5.2.3.3.1. LINEARITY OF ASSAY TO rFheCL1 CONCENTRATION AT 37°C

Plot of fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus rFheCL1 concentration (%). Value at 100% is equal to an enzyme protein concentration of 2μg/ml.

5.2.4. TEMPERATURE STUDIES

5.2.4.1. Temperature Profile

A temperature profile of native rFheCL1 was performed as outlined in section 2.7.1.2. Apparent activity increased to a maximum of 265% at 65°C, above which activity decreased; however, at 70°C activity was still greater than that observed at 37°C. The T_{50}, where observed activity was 50% of maximal rate after 10 minutes incubation in a waterbath, was estimated as 78°C ± 1°C. FIGURE 5.2.4.1.1. illustrates the effect of temperature on enzyme activity.

5.2.4.2. Thermoinactivation of rFheCL1

A thermoinactivation study was performed at 78°C (T_{50}) over a 30 minute period as outlined in section 2.7.2.2. FIGURE 5.2.4.2.1. illustrates the activity profile of rFheCL1. Data fitted satisfactorily to a single exponential decay to give a *k*-value of 0.12 ± 0.01 min^{-1} and apparent half-life ($t_{½}$) of 5.4 minutes. After 30 minutes, less than 1% activity remained.

FIGURE 5.2.4.1.1.

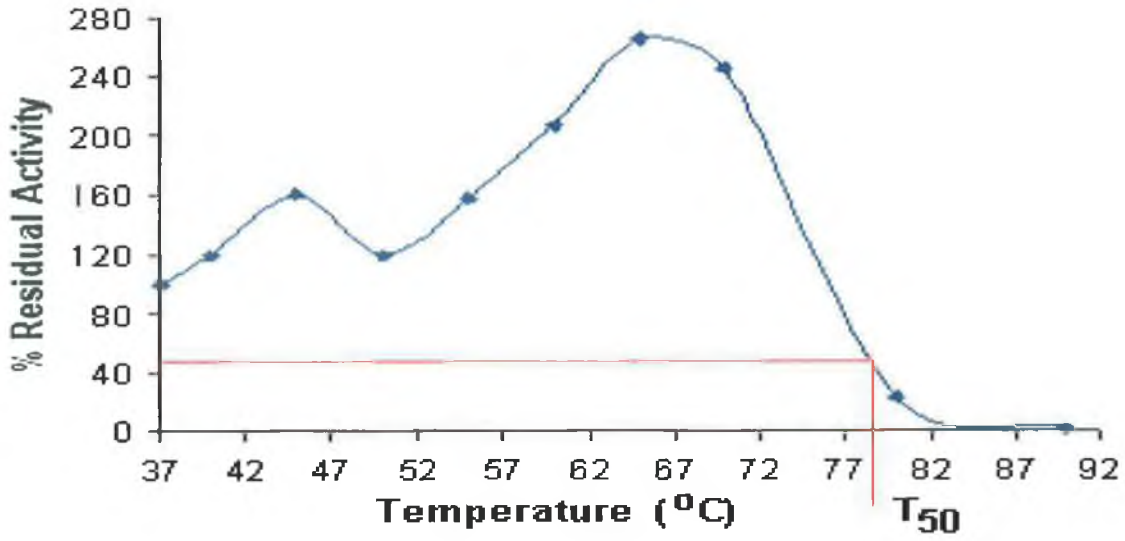


FIGURE 5.2.4.1.1. TEMPERATURE PROFILE OF rFheCL1

Plot of % residual activity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus temperature (°C). Residual activity is represented as a percentage of that at 37°C.

FIGURE 5.2.4.2.1.

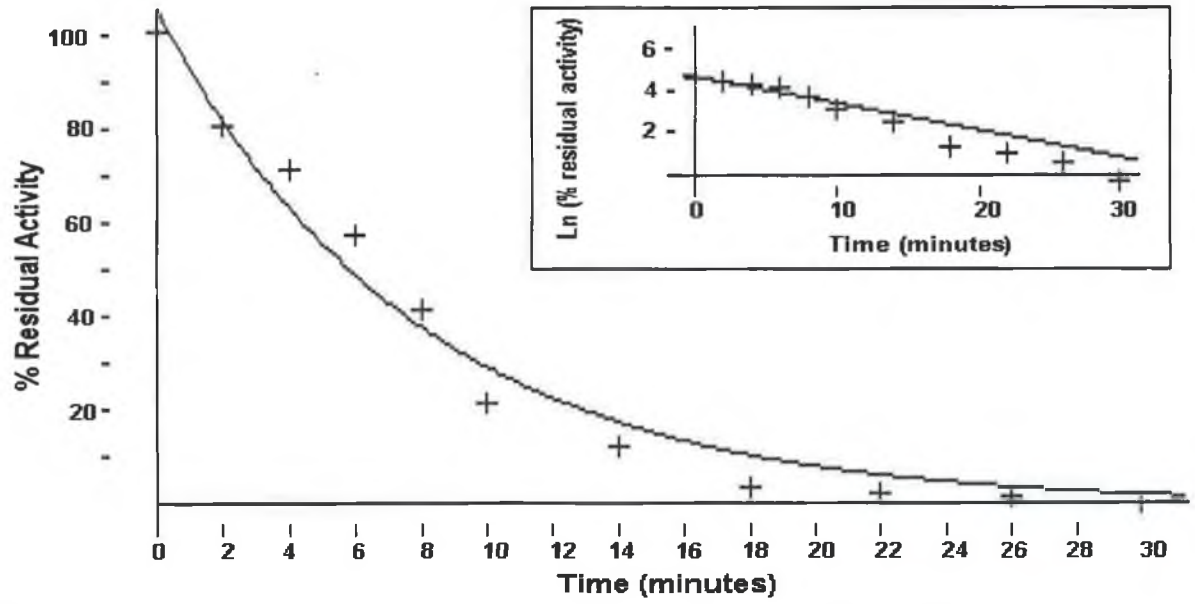


FIGURE 5.2.4.2.1. THERMOINACTIVATION DATA FITTED UP TO 30 MINUTES

Data for thermoinactivation at T_{50} (78°C) fitted to the first order exponential decay equation using the Enzfitter programme (Biosoft, Cambridge, UK).

5.2.5. ORGANOTOLERANCE STUDIES

5.2.5.1. Solvent Stability Studies

The organotolerance of rFheCL1 was investigated as outlined in section 2.8.1.2. for the following organic solvents: acetone, acetonitrile, DMF, DMSO and THF. Graphs of % residual activity versus solvent concentration (%) were plotted for each solvent and half-inactivation concentrations (C_{50}) were determined for each solvent (TABLE 5.2.). FIGURE 5.2.5.1.1. illustrates the effect of solvent concentration on enzyme activity. Activation effects were observed in all of the solvents studied with the exception of THF, which was found to be the most potent denaturing solvent (C_{50} at 26% (v/v) solvent concentration). In 70% (v/v) acetone and acetonitrile, enzyme activity was still greater than in the absence of solvent.

FIGURE 5.2.5.1.1.

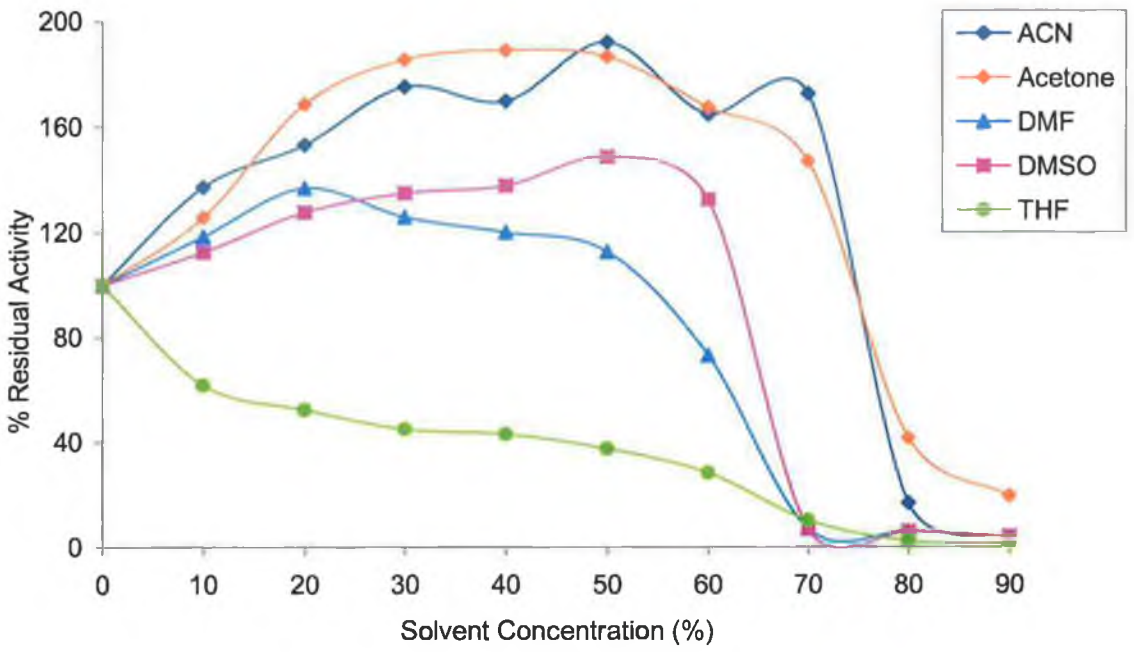


FIGURE 5.2.5.1.1. SOLVENT STABILITY OF rFheCL1

Plot of % residual activity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus solvent concentration (%). Residual activity is represented as a percentage of that in aqueous buffer (C=zero value).

TABLE 5.2. OBSERVED C_{50} VALUES FOR rFheCL1 IN ORGANIC SOLVENTS. (Assay performed in triplicate).

SOLVENT	OBSERVED C_{50} VALUES
Acetone	79% ± 2%
Acetonitrile	78% ± 2%
DMF	63% ± 2%
DMSO	66% ± 2%
THF	26% ± 1%

5.2.6. ACTIVE SITE TITRATION OF rFheCL1

Active site protease concentration was determined as described in 2.10.1. FIGURE 5.2.6.1. illustrates the titration of rFheCL1 with E-64. The concentration of active enzyme was determined from this plot, where the intercept on the x-axis (best line fit) is equal to the concentration of active enzyme. The enzyme was determined to have an operational molarity of 0.58μM, 78.4% of that expected on a protein basis.

FIGURE 5.2.6.1.

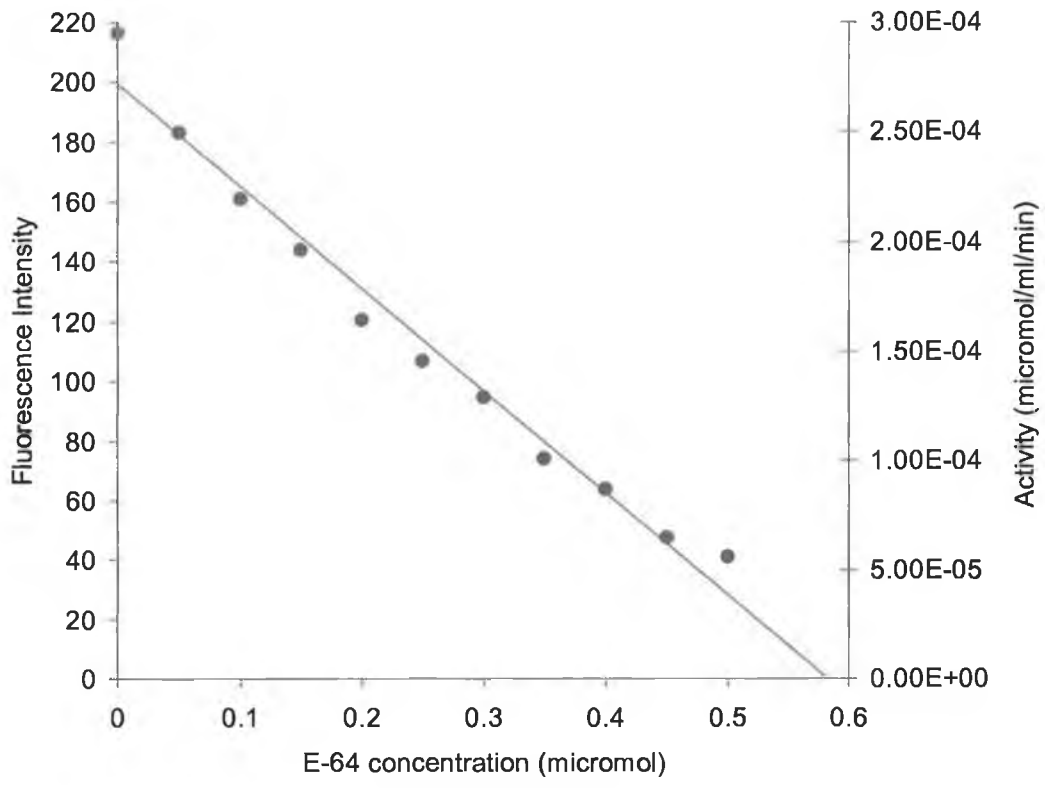


FIGURE 5.2.6.1. TITRATION OF rFheCL1 WITH E-64

Plot of fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) and activity (micromol/ml/min) versus E-64 concentration (micromol)

5.2.7. rFheCL1 KINETICS

Michaelis-Menten kinetics was determined for native enzyme as described in section 2.6.2. Native rFheCL1 displayed Michaelis-Menten kinetics giving k_{cat} and K_m values of 0.49 s^{-1} and 14.6 ± 0.6 μM^{-1}, respectively. Values of k_{cat} were calculated from the equation $V_{max}/[E] = k_{cat}$, where [E] is the active enzyme concentration (section 5.2.6). The k_{cat}/K_m was determined to be 33,562 s^{-1} M^{-1} for the amidase substrate Z-Phe-Arg-AMC.

5.2.8. ESTERASE ACTIVITY

The esterase activity of rFheCL1 was determined as described in section 2.4.3.4. FIGURE 5.2.8.1. demonstrates that the assay is linear up to 9 minutes (R^2 0.99). Optimum enzyme and substrate concentrations were determined at 0.1mg/ml and 0.5mM respectively for this assay. The k_{cat}/K_m was determined to be 1648 s^{-1} M^{-1} for the ester substrate Z-Lys-ONp.

FIGURE 5.2.8.1.

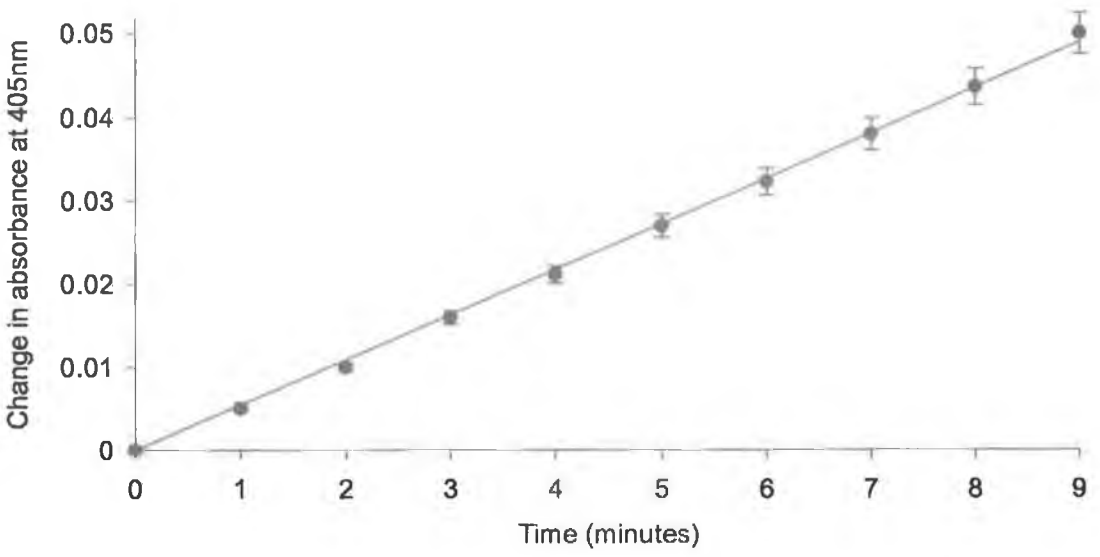


FIGURE 5.2.8.1. ESTERASE ACTIVITY DETEMINATION FOR rFheCL1 AT 37°C

Plot of change in absorbance at 405nm over time (minutes) for rFheCL1 using Z-Lys-ONp as substrate.

5.3. DISCUSSION

No autolysis was observed over the assay period at 37°C, pH 5.0 (FIGURE 5.2.3.1.1.). These results are comparable with Dowd *et al.* (2000), who demonstrated that fluke (*Fasciola hepatica*) cathepsin L1 remained fully active after incubation at 37°C for 20 hours. However, they performed the assay at pH 7.0. Human liver cathepsin L retained at least 70% of its initial activity when incubated for 24 hours at 37°C in the pH range 4.5-5.0. At pH 7.0, however, 85% of activity was lost after only 15 minutes incubation (Mason *et al.*, 1985). FIGURE 5.2.3.2.1. illustrates that rFheCL1 catalysed the linear release of AMC over the standard 30-minute assay period and up to 60 minutes, thus allowing for longer analysis if required. This demonstrates that rFheCL1 is not only stable but also is fully active and saturated with sufficient substrate over this longer time.

FIGURE 5.2.3.3.1. illustrates that fluorescence intensity increases linearly and proportionally with the increase in enzyme concentration at 37°C. Therefore, the enzyme hydrolyses at the same rate depending on the quantity of enzyme present.

Protein molecules are stable within a certain temperature range. However the subtle balance between the folded and unfolded state can be disrupted by a number of factors including temperature. The upper temperature for activity in enzymes appears to be governed by the limits of protein stability, since, there is no theoretical upper limit for conformational stability of proteins. It is possible, however, that irreversible reactions occurring when the protein has unfolded will set this temperature limit. Enzymes exist with optima above 100°C (previously discussed, section 4.2); indeed, some extremely thermophilic enzymes are known to have significant half-lives above 120°C (Daniel, 1996; Eichler, 2001). Dowd *et al.* (2000) investigated the thermostability of *Fasciola*

hepatica cathepsin L1 over the range 30-90°C. The enzyme retained 95-100% activity when incubated between 30-45°C. A 35% loss in activity was observed at 50°C and was substantially lost at 55°C or greater. They estimated T_{50} at 52.5°C however; activity assays were performed at pH 7.0. In contrast, rFheCL1 demonstrated an increase in catalytic activity up to 70°C (maximal activity observed at 65°C), showing an apparent activation, as opposed to the expected thermal inactivation. The temperature of half-inactivation (T_{50}) was estimated at 78°C. These results are much higher than previously reported for cathepsin L from other sources. Carp hepatopancreas cathepsin L lost activity above 40°C (Aranishi *et al.*, 1997). Despite these findings, all assays in this work were performed at physiological temperature, 37°C.

Activity loss with time was investigated at 78°C (T_{50}). Data fitted well to single exponential decay, allowing the estimation of an apparent half-life (5.4 minutes). This indicates that there is only one inactivation process under these assay conditions. Arrowtooth flounder muscle cathepsin L rapidly lost activity after incubation at 55°C for 2.5 minutes (Visessanguan *et al.*, 2003).

Enzymes and other proteins maintain their structural conformations through intramolecular interactions e.g. hydrophobic. In aqueous environments, they have both hydrophilic and hydrophobic regions. The hydrophilic regions form contacts with the essential layer of water, while hydrophobic regions fold inside the molecule. If the polarity of the medium surrounding the enzyme is reduced by introduction of an organic solvent, the hydrophobic core of the protein may disperse, resulting in unfolding of the molecule. As previously discussed, some solvents may replace the essential water layer surrounding the enzyme, affecting biocatalysis (Ogino & Ishikawa, 2001). The

concentration at which half-inactivation (C_{50}) is observed was determined for each solvent (TABLE 5.2). THF was found to be the most deleterious solvent. This corresponds with it having the highest denaturing capacity (DC = 100). However, while Dowd *et al.* (2000) reported a substantial loss in activity at concentration of THF greater than 15% (v/v) (at pH 7.0), rFheCL1 retained up to 30% of its original activity at 60% (v/v) solvent. Nevertheless, once this concentration had been reached, further addition of solvent resulted in an abrupt decrease in activity. Zaks and Klibanov (1988) determined that, at low solvent concentrations, the solvent cannot strip off the essential water layer; however, at increasingly higher concentrations, solvent molecules compete with water for the protein surface and replace some water molecules. Polar organic solvents can replace water in the essential water layer without damaging the protein structure. This was demonstrated by Fitzpatrick *et al.* (1993), who concluded that the three dimensional structure of subtilisin Carlsberg in anhydrous acetonitrile was essentially identical to that in water. The polarity index (I) is another guide among solvent parameters (DC, log *P*, etc.) for enabling the choice of solvent in which enzymes are relatively more stable. This indicates that solvents with polarity indexes of 5.1 and below interact with the protein surface differently from pure water. These particular solvents cannot substitute for water molecules in the essential water layer. This leads to the enzyme adopting a different conformation with consequential loss of biological activity, which is demonstrated by changes in emission spectra for the enzyme in solvent when compared to that in buffer (Gupta *et al.*, 1997). This may explain why THF (polarity index I = 4.0; TABLE 5.3) was determined to be the most deleterious solvent among those tested. Activation effects were observed in acetone, acetonitrile, DMF and DMSO. The enzyme retained up to 150% of its original activity

and was most stable in acetone and acetonitrile. Activation effects may be accounted for by conformational changes in the enzyme molecule cause by introducing the organic solvent into the system (Khmelnitsky *et al.,* 1988). The stability of the enzyme in these solvents may be accounted for by the ability of these solvents to replace water molecules in the enzyme maintaining the structure of the enzyme. However, at higher solvent concentrations this replacement may lead to deformation of the conformational structure and, hence, to denaturation of the enzyme (Ogino & Ishikawa, 2001).

TABLE 5.3. ORGANIC SOLVENT CHARACTERISTICS (Gupta *et al.,* 1997)

SOLVENT	POLARITY INDEX (I)	DENATURATION CAPACITY (DC)
Acetone	5.1	78.2
Acetonitrile	5.8	64.3
DMF	6.4	63.3
DMSO	7.2	60.3
THF	4.0	100

Organic solvents also affect the binding of the substrate to the active site by altering the apparent K_m values. rFheCL1 prefers non-polar amino acid residues in the P_2 and P_3 subsite positions of its substrate e.g. Z-Phe-Arg-. In polar solutions, these compounds will partition into the hydrophobic active site; again, this could be a possible reason for the activation effects seen when the enzyme in placed in these polar solvents excluding THF (Dordick, 1989).

Many strategies have been pursued to enhance enzyme stability e.g. immobilisation, chemical modification and genetic/protein engineering techniques. Site-specific mutagenesis allows a protein to be stabilized by substituting amino acid residues at specific sites in a protein, which may lead to enhanced stability by replacing chemical, thermal or solvent labile residues, or by increasing interior interactions such as hydrophobic ones, thereby increasing protein rigidity. A comparison was made between the amino acid sequence of fluke and recombinant *Fasciola hepatica* cathepsin L1. A number of differences were observed (FIGURE 5.3.1.). The Q24940 entry notes a lack of consensus at certain sites, some of which will be discussed thus giving a plausible explanation as to why the recombinant form of cathepsin appears to have enhanced stability when compared to fluke (*Fasciola hepatica*) cathepsin L1.

Hydrophobic interactions contribute greatly to the stability of the folded state of the protein and may be considered the driving force in protein folding. Investigations of structural differences observed between mesophilic and thermophilic proteins have suggested that the latter proteins are substantially more hydrophobic and have more surface area buried upon folding. It has also been suggested that higher stability observed in thermophilic proteins is due to better packing because of smaller and less numerous cavities. All of the substitutions at positions 165 (Gly for Arg), 293 (Thr for Leu) and 304 (Ala for Val) may enhance stability by increasing the compactness of the molecule, as all of these substitutions involve changing one smaller residue for a larger one, thereby decreasing the solvent accessible area. Substituting weakly polar threonine (T) at position 293 with the bulky hydrophobic amino acid leucine (L) may also strengthen the internal hydrophobicity of the protein. Glycine is a small amino acid, which is more destabilising because it has more conformational freedom in the unfolded

state. Changing this for arginine (with a considerably larger side chain) could possibly restrict its conformational freedom by reducing the entropy loss on unfolding and increase the rigidity of the protein. The guanidine group of this residue can form salt bridges; this formation has been implicated in the thermostability of proteins. Exchanging alanine for the more hydrophobic valine may help stabilise the protein. Substituting tyrosine for phenylalanine (position 118) with the loss of a hydroxyl group may contribute to increasing the hydrophobicity at this site (Nosoh & Sekiguchi, 1990; Fersht & Serrano, 1993; Kumar *et al.*, 2000).

Matsuura *et al.* (1998) demonstrated that the addition of peptide tails with random sequences to the C-terminal of the enzymes molecule enhanced thermostability. They suggested that peptide addition increases the dimension of the protein sequence space, thereby imposing constraints on the enzyme molecule. However, they concluded that a peptide tail providing beneficial effects to one enzyme may not provide the same effect to another. Eight amino acid residues are present at the C-terminal of rFheCL1 these include glycine, proline and a 6 histidine tag. The inclusion of this peptide "tail" is for purposes of purification; however, it may also be responsible for conferring some degree of stability upon the recombinant enzyme. Positively charged amino acid residues (such as histidine) at a protein terminus fortify the adjacent peptide bonds against acid hydrolysis, as they repel hydronium ions (Wong & Parasrampuria, 1997).

The substitution and addition of residues may be responsible for the enhanced thermostability and organic solvent tolerance demonstrated by this recombinant enzyme as compared to the fluke enzyme. However, the folding of an enzyme also dictates how an amino acid residue contributes to its stability. A crystal structure for the fluke enzyme is not yet available; hence the above reasoning involves assumptions. The

interesting results obtained for this recombinant enzyme would suggest that further studies should be performed to elucidate this phenomenon.

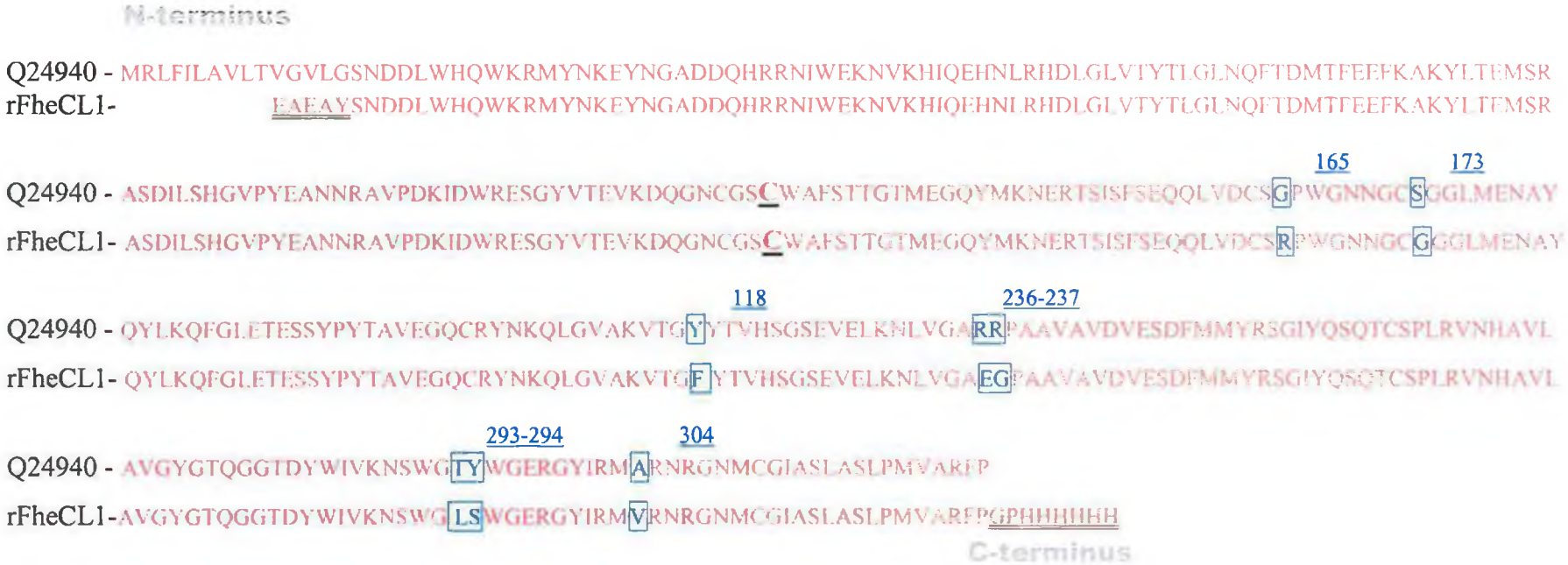


FIGURE 5.3.1. Amino acid sequence of fluke (*Fasciola hepatica*) cathepsin L1 accession number Q24940 and recombinant *Fasciola hepatica* cathepsin L1 (rFheCL1). The active site cysteine is bold and underlined. Differences in sequence appear in blue surrounded by border. Underlined sequence at N-terminus is the end of the yeast signal sequence. Underlined sequence at the C-terminal is the added 6 x His tag for purification. Sequence for fluke (*Fasciola hepatica*) cathepsin L1 obtained from Protein Data Bank, sequence for recombinant *Fasciola hepatica* cathepsin L1 obtained from Mr Peter Collins, Parasitology Research Group, formerly based at Dublin City University. Sequences aligned by ClustalW.

Kinetic constants for the hydrolysis of Z-Phe-Arg-AMC were determined for recombinant *Fasciola hepatica* cathepsin L1 (rFheCL1). The recombinant enzyme had similar affinity toward Z-Phe-Arg-AMC (K_m = 14.6 μM) as fluke (*Fasciola hepatica*) cathepsin L1, which reported a K_m value of 14.7μM (Dowd *et al.,* 1994). However, rFheCL1 had lower affinity toward Z-Phe-Arg-AMC than cathepsin L from other sources, which demonstrated K_m values in the range 1.8-8.2μM (Mason *et al.*, 1985; Visessanguan *et al.*, 2003). The k_{cat} value for rFheCL1 (0.49 s^{-1}) is lower than values reported for cathepsin L from rat liver (26 s^{-1}), human liver (17 s^{-1}), and arrowroot flounder (12.2 s^{-1}) (Kirschke *et al.*, 1982; Mason *et al.*, 1985; Visessanguan *et al.*, 2003). A difference was observed between the k_{cat} value obtained for the recombinant as opposed to the fluke (*Fasciola hepatica*) cathepsin L1 (1.08 s^{-1}) (Dowd *et al.*, 1994). Thus, resulting in a lower catalytic efficiency value (k_{cat}/K_m) for the recombinant enzyme (33,562 s^{-1} M^{-1}) when compared to the fluke enzyme (73,469 s^{-1} M^{-1}) (Dowd *et al.*, 1994). However, these observed differences in the kinetic constants may be explained by differences in the pH and temperature of assay conditions e.g. cathepsin L from other sources were routinely assayed at pH and temperature values ranging between pH 5.5-7.0, and 30°C-37°C, respectively (Brömme *et al.*, 1993; Dowd *et al.,* 2000; Visessanguan *et al.,* 2003).

The esterase activity of rFheCL1 was determined using the substrate Z-Lys-ONp. Literature values for esterase activity of cathepsin L are few. However, Kirschke *et al.* (1982) reported a k_{cat}/K_m value of 1980 × 10^3 s^{-1} M^{-1} for rat liver cathepsin L hydrolysis of Z-Lys-ONp. The result obtained for rFheCL1 demonstrated a 1000-fold lower value (1648 s^{-1} M^{-1}) for esterase activity, when compared to this value. A major disadvantage associated with the use of this substrate is its rapid spontaneous hydrolysis, especially in

the presence of thiol compounds e.g. DTT (Kirschke, 1981). While the amount present in the overall esterase assay was minimal, it may have been enough to cause some autolysis of the substrate, hence leading to erroneous results. Z-Lys-ONp has also been used as an active site titrant, inactivating thiol proteases that maybe present in crude samples and hence, maybe an unsuitable substrate to determine esterase activity (Shaw & Green, 1981). These factors may account for obtaining such a low k_{cat}/K_m value. To determine the overall esterase/amidase ratio for rFheCL1 a more suitable fluorescent ester substrate would be necessary i.e. Z-Phe-Arg-7HMC ester. In this case, both amidase/esterase measurement parameters would perhaps be more accurate, as both activities would be determined with similar Z-Phe-Arg peptides. A peptide synthesis company was engaged to synthesise this ester fluorescent substrate; however, they encountered numerous problems in this task and no further esterase studies could be undertaken.

CHAPTER 6

CHEMICAL MODIFICATION STUDIES

6.1. Introduction

N-Hydroxysuccinimide (NHS) esters are popular cross-linking reagents due to their mild reaction conditions, commercial availability and ease of synthesis. The reaction is rapid with the most accessible protein amino groups attacked with 10-20 minutes and proceeds most efficiently at pH 7-8, over a wide temperature range (4-25°C). A 10-fold molar excess of the NHS ester is usually sufficient to acylate amino groups. Their limited solubility in aqueous buffers requires that they be initially dissolved in organic solvents such as DMSO. The reaction involves the nucleophilic attack of an amine on the acid carboxyl of the NHS ester forming an amide and releasing the NHS (FIGURE 6.1.1.). Therefore, the product of the reaction is an amide with the loss of the positive charge from the original amino group. Reactions must proceed in buffers that do not contain extraneous amines e.g. Tris or glycine (Ji, 1983).

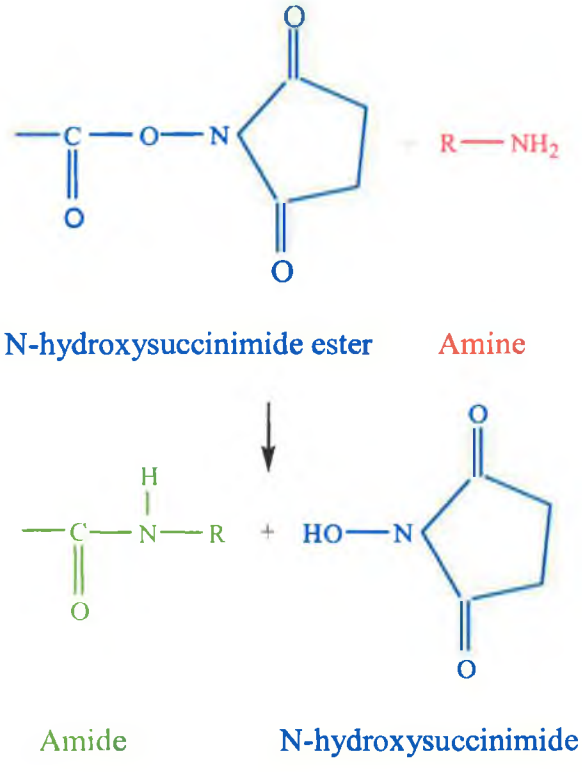


FIGURE 6.1.1. NHS ESTER REACTION SCHEME

Bis-N-hydroxysuccinimide esters are homobifunctional reagents with a reactive group at either end of the molecule. These reactive groups can be used to introduce both inter and intramolecular bridges into a protein. The N-hydroxysuccinimide (NHS) ester used for modification of rFheCL1 was an ethylene glycol succinic acid (EG) ester of NHS (FIGURE 6.1.2.) (Han *et al.*, 1984). Bis-N-hydroxysuccinimides cross-link the molecule and also neutralize the charge on the lysine residue. It has been shown that modification of enzymes with these cross-linking agents enhances thermostability, organic solvent tolerance and diminishes autolysis (Miland *et al.*, 1996; Murphy & Ó'Fágáin, 1996b).

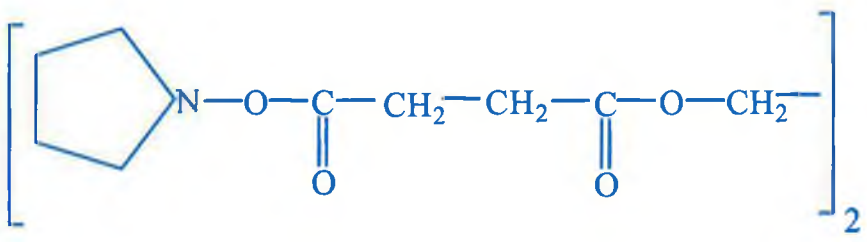


FIGURE 6.1.2. Ethylene glycol-bis(succinic acid N-hydroxy-succinimide ester) EG-NHS

6.1. RESULTS – ASSAY AND STABILITY STUDIES ON EG-rFheCL1

6.2.1. CROSS-LINKING OF rFheCL1

Modification of rFheCL1 was performed as described in section 2.11. A minimal loss (3%) in activity was observed following modification of the enzyme. TNBS assay indicated that approximately 62% of the lysine residues were modified, suggesting that approximately 8 of the 13-lysine residues per rFheCL1 molecule had been modified.

6.2.2. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-polyacrylamide gel electrophoresis was performed as described in section 2.12. FIGURE 6.2.1.1. illustrates that modified rFheCL1 did not show any higher molecular weight species as compared to native rFheCL1, suggesting that no intermolecular cross-linking occurred and that the product was free of higher molecular weight aggregates of rFheCL1. The band appearing above the native and modified samples corresponds to different processed forms of the monomer (Personal communication with Mr Peter Collins, Parasitology Research Group, formerly based at Dublin City University).

FIGURE 6.2.2.1.

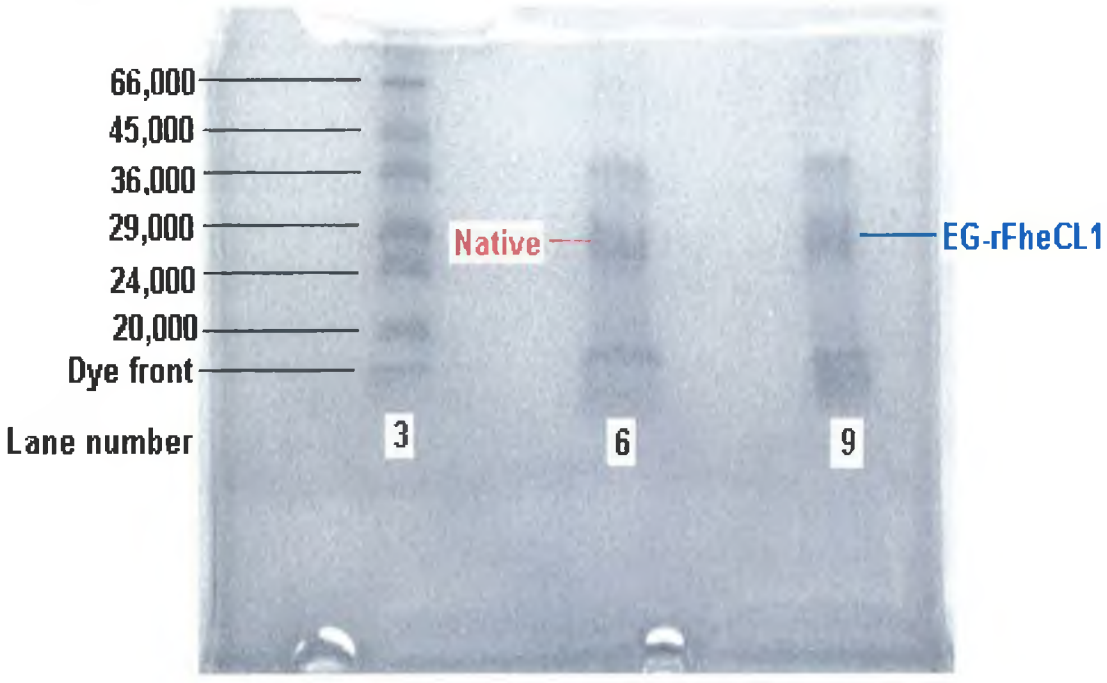


FIGURE 6.2.2.1. GELCODE® BLUE STAINED SDS-PAGE OF rFheCL1

Lane 3: molecular weight markers (from top), bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), glyceraldehyde-3-phosphate dehydrogenase (36,000 Da), carbonic anhydrase (29,000 Da), trypsinogen (24,000 Da) and trypsin inhibitor (20,000 Da). Lane 6: native-rFheCL1. Lane 9: EG-rFheCL1. Lanes between the samples and molecular weight markers were left empty.

6.2.3. EFFECT OF MODIFICATION ON rFheCL1 KINETICS

Michaelis-Menten kinetics was determined for native and EG-rFheCL1 as described in section 2.6.2.

TABLE 6.1. MICHAELIS-MENTEN PARAMETERS FOR NATIVE AND MODIFIED rFheCL1

rFheCL1	K_m (μM^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} M^{-1}$)
Native	14.6	0.49	33,562
Modified	12.5	0.41	32,800

6.2.4. TEMPERATURE STUDIES

6.2.4.1. Temperature Profile

A temperature profile of native and EG-rFheCL1 was performed as outlined in section 2.7.1.2. T_{50}, where 50% of initial activity remained after 10 minutes incubation in a waterbath, was estimated at 87°C ± 1% for EG-rFheCL1, a 9°C increase above that determined for native rFheCL1. Enhanced thermostability was demonstrated by EG-rFheCL1 at all of the investigated temperatures; however its greatest degree of thermostability was seen at higher temperatures (between 60-90°C).

6.2.4.2. Thermoinactivation of Native and Modified rFheCL1

A thermoinactivation study was performed at 70°C over a 60 minute period as outlined in section 2.7.2.2. FIGURE 6.2.4.2.1. illustrates the activity profile of native and EG-rFheCL1 at 70°C over 60 minutes. A 70% decrease in activity was observed for native rFheCL1 after the 60-minute incubation period. In contrast, EG-rFheCL1 retained 100% of its original activity after the same incubation period.

FIGURE 6.2.4.2.1.

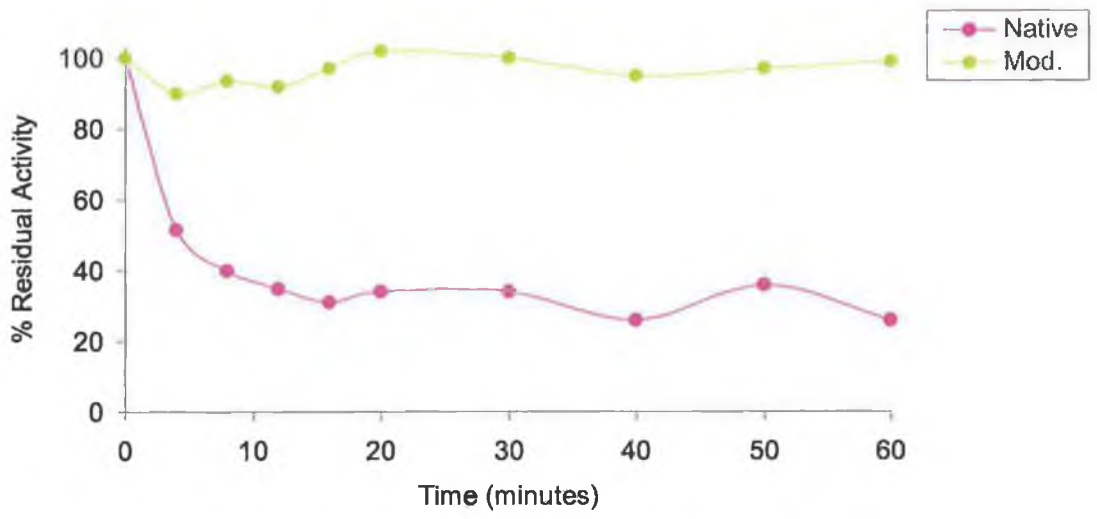


FIGURE 6.2.4.2.1. THERMOINACTIVATION OF NATIVE AND EG-rFheCL1 AT 70°C. Plot of % residual activity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus time (minutes). Residual activity is represented as a percentage of the t = zero value.

6.2.5. ORGANOTOLERANCE STUDIES

6.2.5.1. Solvent Stability Studies

Organotolerance studies on native and EG-rFheCL1 were investigated for the following solvents: acetone, acetonitrile, DMF, DMSO and THF as outlined in section 2.8.1.2. Graphs of % residual activity versus solvent concentration (%) were plotted for each solvent. Threshold concentrations (C_{50}) at which half-inactivation was observed were determined for native and modified rFheCL1 in each solvent (TABLE 6.2.). Activation effects were observed when native (results similar to section: 5.2.5) and modified rFheCL1 were placed in DMF; modified rFheCL1 demonstrating enhanced activity up to 60% (v/v) solvent concentration. However, threshold concentrations for native and modified rFheCL1 in DMF did not differ. Placing the enzyme in acetone and acetonitrile resulted in enhanced activity at higher concentrations (between 60-90%) as demonstrated by an increase in threshold concentrations. In contrast, THF was the most deleterious solvent to both native and modified rFheCL1; however, modified rFheCL1 demonstrated up to 15% higher activity at solvent concentrations between 70-90%.

6.2.5.2. Thermoinactivation of Native and Modified rFheCL1

A thermoinactivation study was performed at 37°C and 50°C over a 60 minute period as outlined in section 2.7.2.2. FIGURE 6.2.5.2.1. and 6.2.5.2.2. illustrate the activity profile of native and EG-rFheCL1 in 50% (v/v) organic solvent over 60 minutes. Both native and EG-rFheCL1 retained 100% of original activity after the 60-minute assay period when placed in 50% (v/v) DMF at 37°C. However, native rFheCL1 demonstrated higher activity in this solvent at 37°C. Similar results were obtained for rFheCL1 in

50% (v/v) acetonitrile at 37°C, with a slight activation effect being observed. Both forms of the enzyme demonstrated increased activity in 50% (v/v) acetonitrile as opposed to 50% (v/v) DMF. At 50°C the modified enzyme demonstrated higher activity in 50% (v/v) DMF than the native form; however, both native and modified forms retained up to 97% of original activity after the assay period. Both forms of the enzyme were deemed to be more stable in this solvent than in acetonitrile at the same concentration, where a steady decline in activity was observed over the assay period. However, EG-rFheCL1 was more active in this solvent than the native rFheCL1.

TABLE 6.2. C_{50} VALUES FOR NATIVE AND EG-rFheCL1 IN ORGANIC SOLVENTS
(Assay performed in triplicate)

SOLVENT	OBSERVED C_{50} VALUES native rFheCL1	OBSERVED C_{50} VALUES EG-rFheCL1
Acetone	81% ± 2%	85% ± 1%
Acetonitrile	76% ± 2%	88% ± 2%
DMF	65% ± 2%	65% ± 1%
DMSO	68% ± 2%	69% ± 1%
THF	15% ± 0.5%	17% ± 2%

FIGURE 6.2.5.2.1.

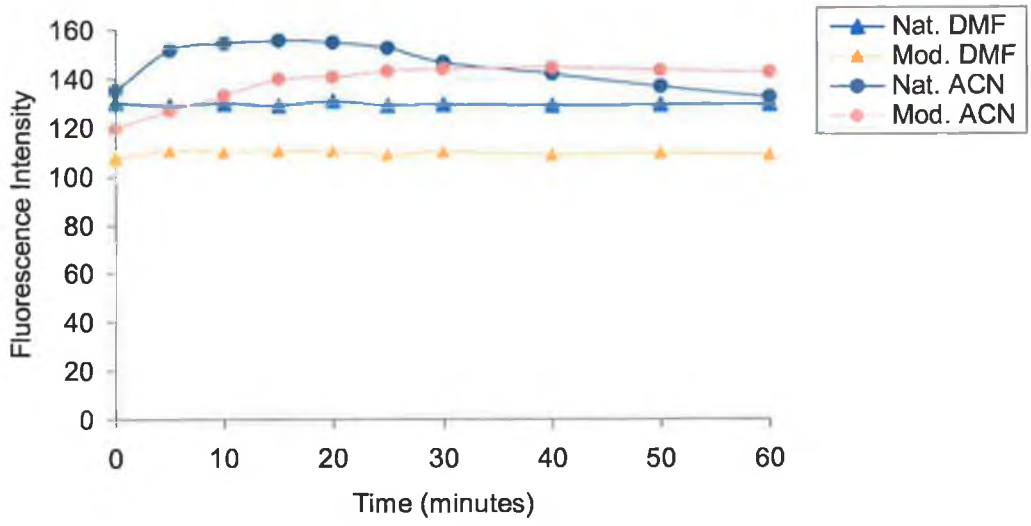


FIGURE 6.2.5.2.1. THERMOINACTIVATION OF NATIVE AND EG-rFheCL1 IN 50% (V/V) ORGANIC SOLVENT AT 37°C. Plot of fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus time (minutes).

FIGURE 6.2.5.2.2.

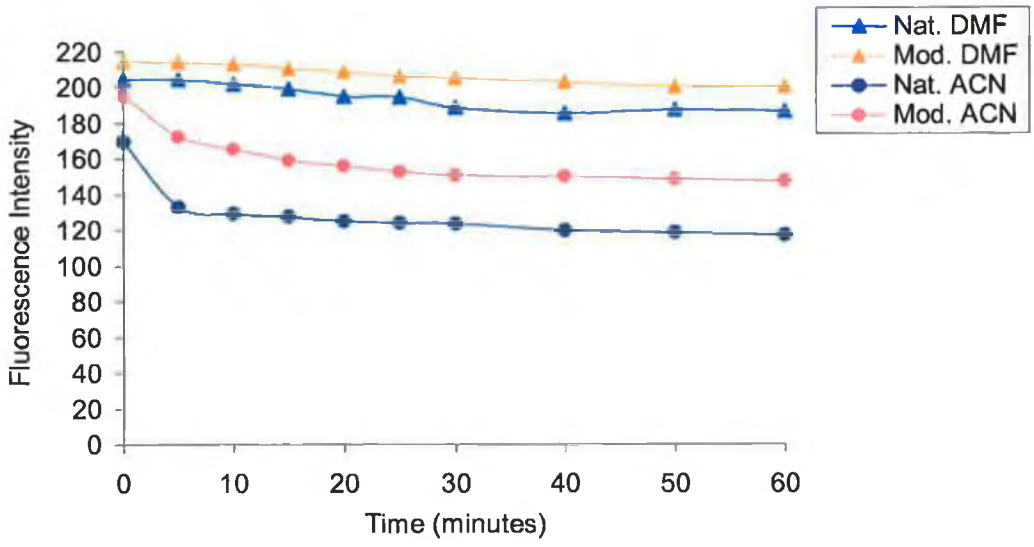


FIGURE 6.2.5.2.2. THERMOINACTIVATION OF NATIVE AND EG-rFheCL1 IN 50% (V/V) ORGANIC SOLVENT AT 50°C. Plot of fluorescence intensity (excitation 370nm, slit width 10nm; emission 440nm, slit width 2.5nm) versus time (minutes).

6.3. Discussion

There are a number of reasons for choosing to modify lysine residues. Lysine residues are polar and the ξ-amino groups of lysine residues are usually among the most abundant and most accessible of the potentially reactive groups. Many proteins have lysine residues that are not involved in the catalytic site (Guisán, 1987; Means & Feeney, 1990).

After chemical cross-linking a partial characterisation of the NHS derivative was performed. SDS-PAGE (FIGURE 6.2.2.1.) indicated no molecular weight differences between modified (EG-) and unmodified (native) rFheCL1. Hence, it appears that the observed NHS ester stabilization took place without intermolecular cross-linking. Approximately 8 lysine residues per rFheCL1 molecule were modified. A review of the literature did not reveal any reports in terms of chemical modification of rFheCL1.

The kinetics of native and modified rFheCL1 were studied to determine the effect of modification on rFheCL1-catalysed hydrolysis of its corresponding substrate Z-Phe-Arg-AMC and compare their respective K_m and k_{cat} values. The tabulated kinetic parameters (TABLE 6.1) show that the Michaelis constants for EG-rFheCL1 were similar. Although an insignificant difference was obtained between the native and modified K_m value, this can be explained by experimental variability. Overall, these results indicate that the active site of the enzyme has not been affected by cross-linking and that stability gain has not been at a cost of poorer catalysis.

EG-rFheCL1 showed increased resistance to elevated temperatures. The T_{50} value for EG-rFheCL1 was 9°C higher than that obtained for native cathepsin L1 (87°C and 78°C, respectively). Thermoinactivation of native and modified rFheCL1 is illustrated in FIGURE 6.2.4.2.1. Modified rFheCL1 demonstrated considerably enhanced

thermostability at 70°C over the duration when compared to native rFheCL1. Intramolecular cross-linking stabilises a protein's tertiary structure. The cross-linking agent acts as external braces that prevent unfolding of the tertiary structure. Therefore, the degree to which thermostability is observed may be dependant on the degree of cross-linking (Han *et al.*, 1984; Sheehan *et al.*, 1990). The positioning of lysine residues in the protein plays an important role in stability. The rate and extent of deamidation of asparagines residues is influenced by the nature of the carboxyl-flanking amino acid. Lysines positioned before asparagine residues in the protein have been found to contribute to the lability of asparagines and, therefore, its deamidation. Modification of these lysine residues could result in a lower extent of deamidation (Daniel, 1996; Khajeh *et al.*, 2001). Of the 13-lysine residues in rFheCL1, 4 are positioned immediately before asparagine residues. If these are among the 8 lysine residues which have been modified, this may hinder the deamidation reaction, giving a possible reason why EG-rFheCL1 demonstrated enhanced thermostability when compared to the native enzyme. Modification may also stabilise the protein by masking the hydrophobic methylene chain of the lysine from unfavourable contact with water, enhancing stability (Khaparde & Singhal, 2001). Enhanced stability may also be due to a simple cross-link stabilizing the tertiary structure (Han *et al.*, 1984).

The tolerance of EG-rFheCL1 in organic solvents (acetone, acetonitrile, DMF, DMSO) was enhanced. The C_{50} values for EG-rFheCL1 were higher than those for native rFheCL1 for all the tested solvents (exception DMF; TABLE 6.2); however, THF was still deemed the most deleterious solvent, with only a 2% difference between the native and modified C_{50} value. Transferring the enzyme to a solvent can be potentially destabilizing by leading to loss of solvation. This arises because the native

conformation of proteins in water tends to maximise the solvation contribution to the stability of charged residues. Therefore, removing the charge on these lysine residues should enhance the stability of the folded enzyme in solvents that are less capable than water of solvating charges. Removing the charge may also decrease the concentration of water required for enzyme activation (Arnold, 1990). Stability of EG-rFheCL1 may result from this factor and from increased conformational rigidity caused by introducing intramolecular cross-link into the enzyme, thereby preventing reversible unfolding of the molecule.

Many enzymes inactivate at high temperatures in aqueous environments, due to partial unfolding and covalent alterations in the primary structure (Dordick, 1991). Water is required as a reactant for a number of deleterious processes such as deamidation and peptide hydrolysis (Krishna, 2002). In reduced water environments, intra-molecular forces such as hydrogen bonding and ion pair interactions are stronger in organic solvents than in water. This leads to decreased flexibility, increased conformational rigidity and the absence of nearly all covalent reactions that cause irreversible thermoinactivation in water (Gupta, 1992; Klibanov, 2001; Colombo & Carrea, 2002).

Thermoinactivation of both native and EG-rFheCL1 in both 50% (v/v) acetonitrile and DMF was examined at 37° and 50°C. Both native and modified forms of the enzyme were stable at 37°C in these solvents, although the native enzyme was more active. Enhanced thermostability of the EG-rFheCL1 was observed in these solvents at 50°C. A combination of factors may contribute to this increase in thermostability namely: (1) the direct effect of the solvent/reduced water environment, discussed above; (2) modification of the lysine residues, reducing deleterious deamidation reactions or (3) the direct effect of cross-linking decreasing the flexibility of the enzyme.

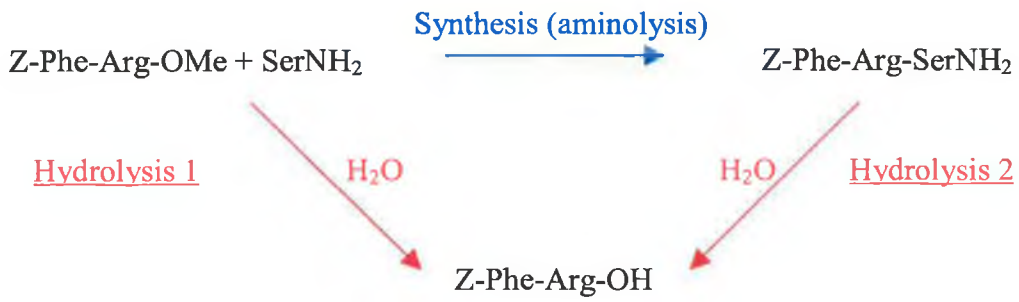
CHAPTER 7

PEPTIDE SYNTHESIS

7.1. INTRODUCTION

Over the last decade, proteases have been widely used to form peptide bonds. Enzymatic peptide synthesis offers an alternative to chemical methods, with numerous advantages (see section 1.3. for more details). The synthesis of the model tripeptide Z-Phe-Arg-SerNH$_2$ was performed. As previously discussed (section 1.4.), there are two strategies for enzymatic peptide synthesis: kinetically-controlled and equilibrium-controlled synthesis. The kinetic method making use of ester substrates was preferred due to its lower enzyme requirements and shorter reaction times. The chosen process can be summarized as follows:

SCHEME 7.1. KINETICALLY-CONTROLLED TRIPEPTIDE SYNTHSIS USING rFheCL1



7.2. RESULTS – PEPTIDE SYNTHESIS WITH NATIVE rFheCL1

7.2.1. LC Conditions

From the spectra obtained from the photodiode array detector the optimum wavelength for analysis was found at 200nm. Mobile phase concentration was adjusted: the most suitable concentration was a 25% (v/v) acetonitrile / 75% (v/v) water containing 0.1% (v/v) formic acid.

7.2.2. Concentration of Enzyme, Ester and Nucleophile

The enzymatic peptide synthesis of Z-Phe-Arg-SerNH_2 was performed as described in section 2.13. Initially, enzyme, ester and nucleophile concentrations were 0.5mg/ml, 25mM and 50mM, respectively, with an ester:nucleophile ratio of 1:2. Although formation of the desired tripeptide product was detected by mass spectrometry analysis, it was not detected on the LC chromatographs, as a peak pertaining to the ester was masking its detection. Therefore, concentrations of each constituent were reduced to 0.05mg/ml, 2.5mM and 10mM, yielding a 1:4 ester:nucleophile ratio.

7.2.3. Tripeptide Formation

FIGURE 7.2.3.2. (Time = 10 minutes) clearly demonstrates the formation of a new peak corresponding to the molecular weight of the desired tripeptide Z-Phe-Arg-SerNH_2 (mw: 542) as compared with FIGURE 7.2.3.1. (Time = 0 minutes). Formation of the new tripeptide occurred within ten minutes, while the ester substrate was slowly consumed over the sixty-minutes (FIGURE 7.2.3.4.). However, the formation of hydrolysis products, Z-Phe-Arg-OH, was noticed at an unexpectedly early stage of the reaction.

FIGURE 7.2.3.1.

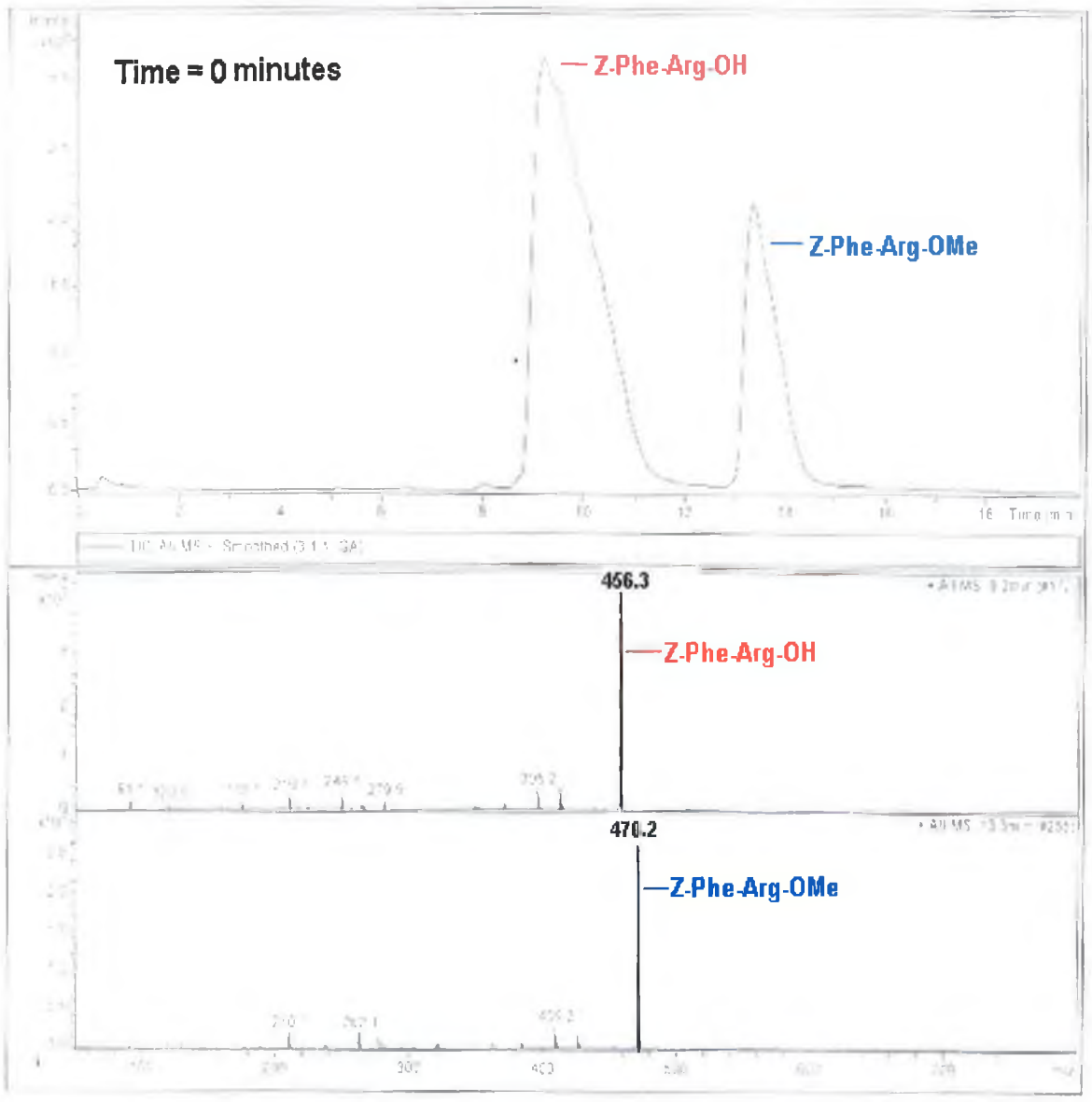


FIGURE 7.2.3.1. UV CHROMATOGRAM (UPPER) AND MASS SPECTROMETRY TRACES (LOWER) FOR rFheCL1 CATALYSED PEPTIDE SYNTHESIS, AT T = 0 MINUTES.

FIGURE 7.2.3.2.

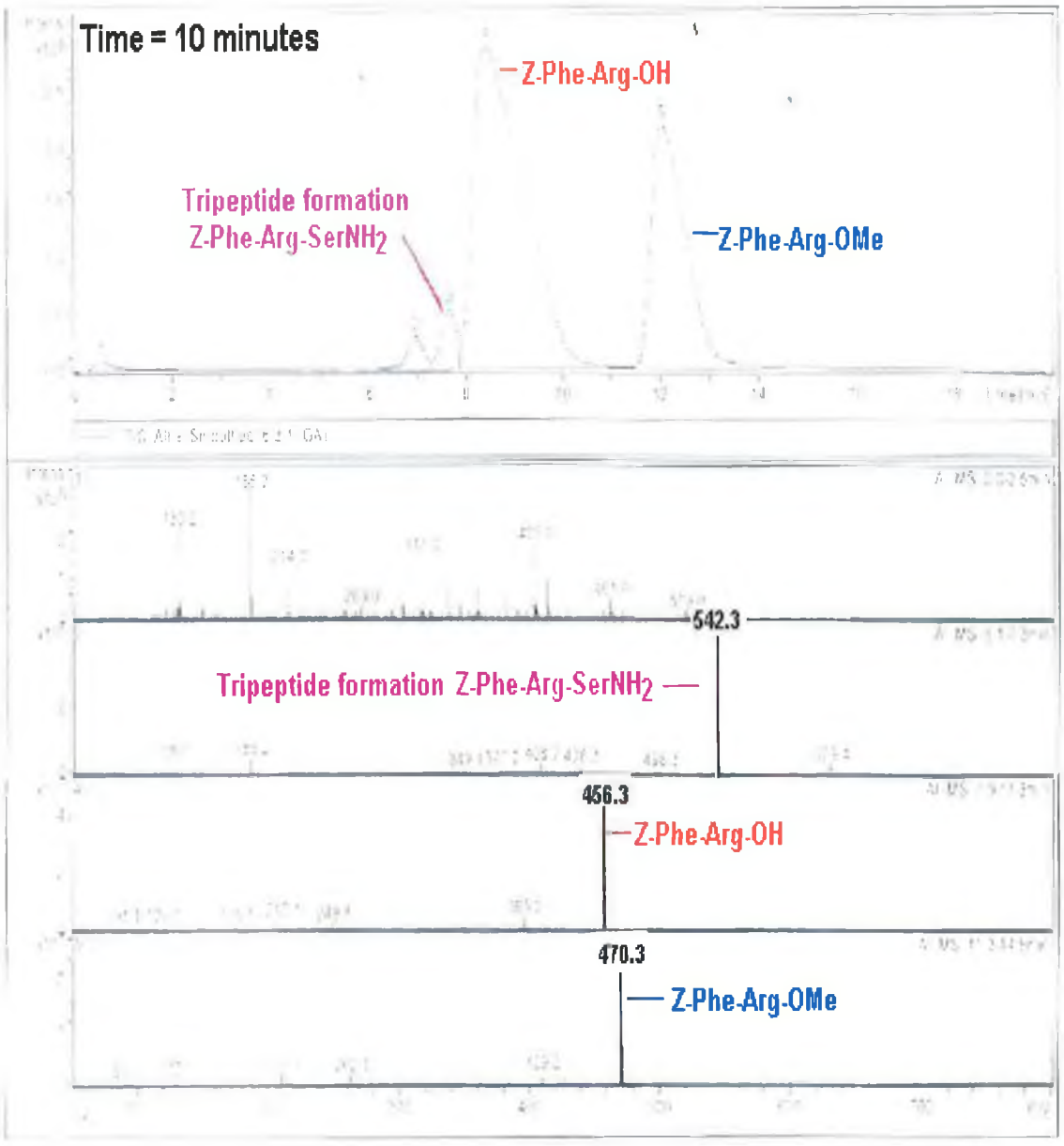


FIGURE 7.2.3.2. UV CHROMATOGRAM (UPPER) AND MASS SPECTROMETRY TRACES (LOWER) FOR rFheCL1 CATALYSED PEPTIDE SYNTHESIS, AT T = 10 MINUTES.

FIGURE 7.2.3.3.

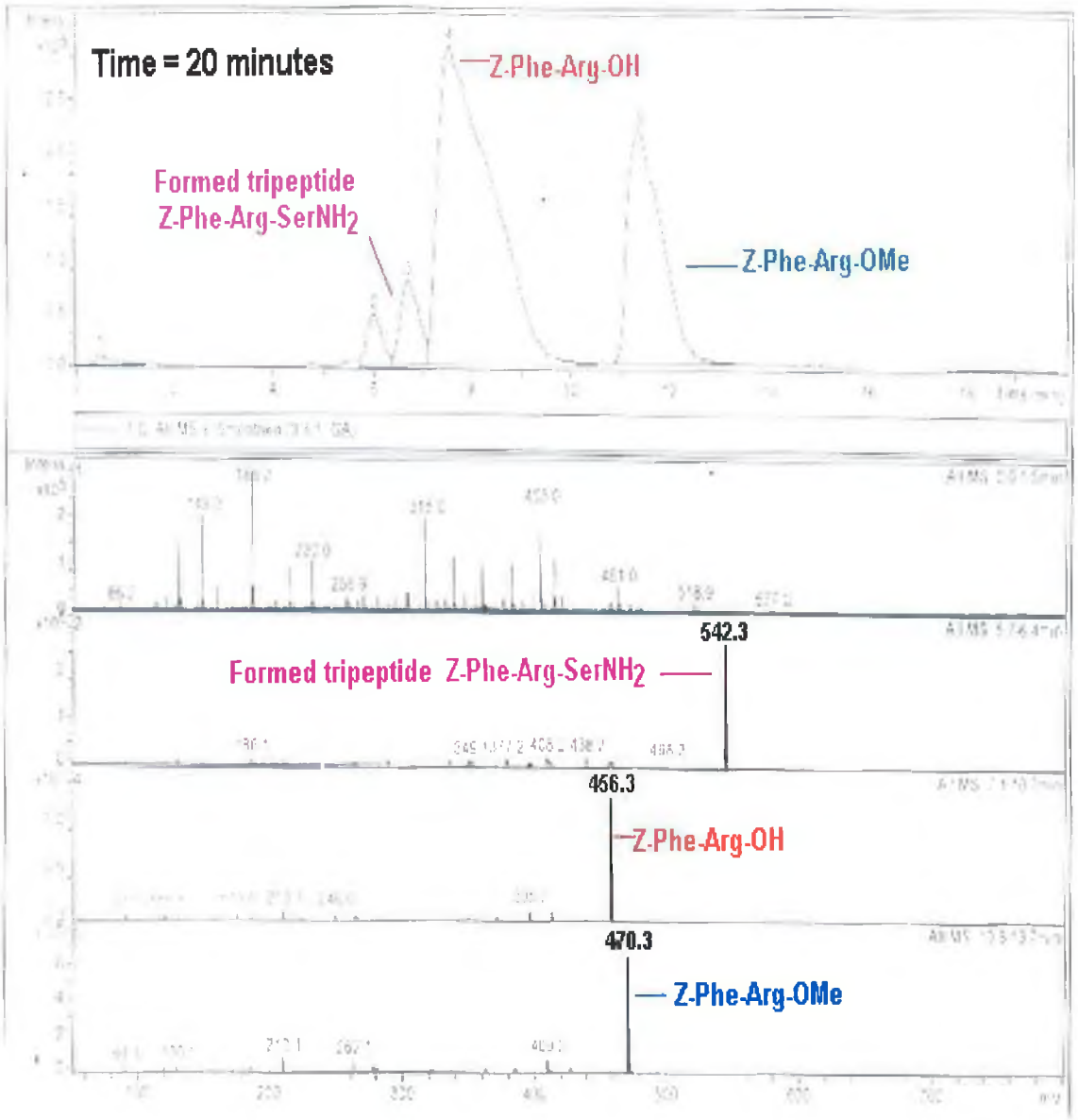


FIGURE 7.2.3.3. UV CHROMATOGRAM (UPPER) AND MASS SPECTROMETRY TRACES (LOWER) FOR rFheCL1 CATALYSED PEPTIDE SYNTHESIS, AT T = 20 MINUTES.

FIGURE 7.2.3.4.

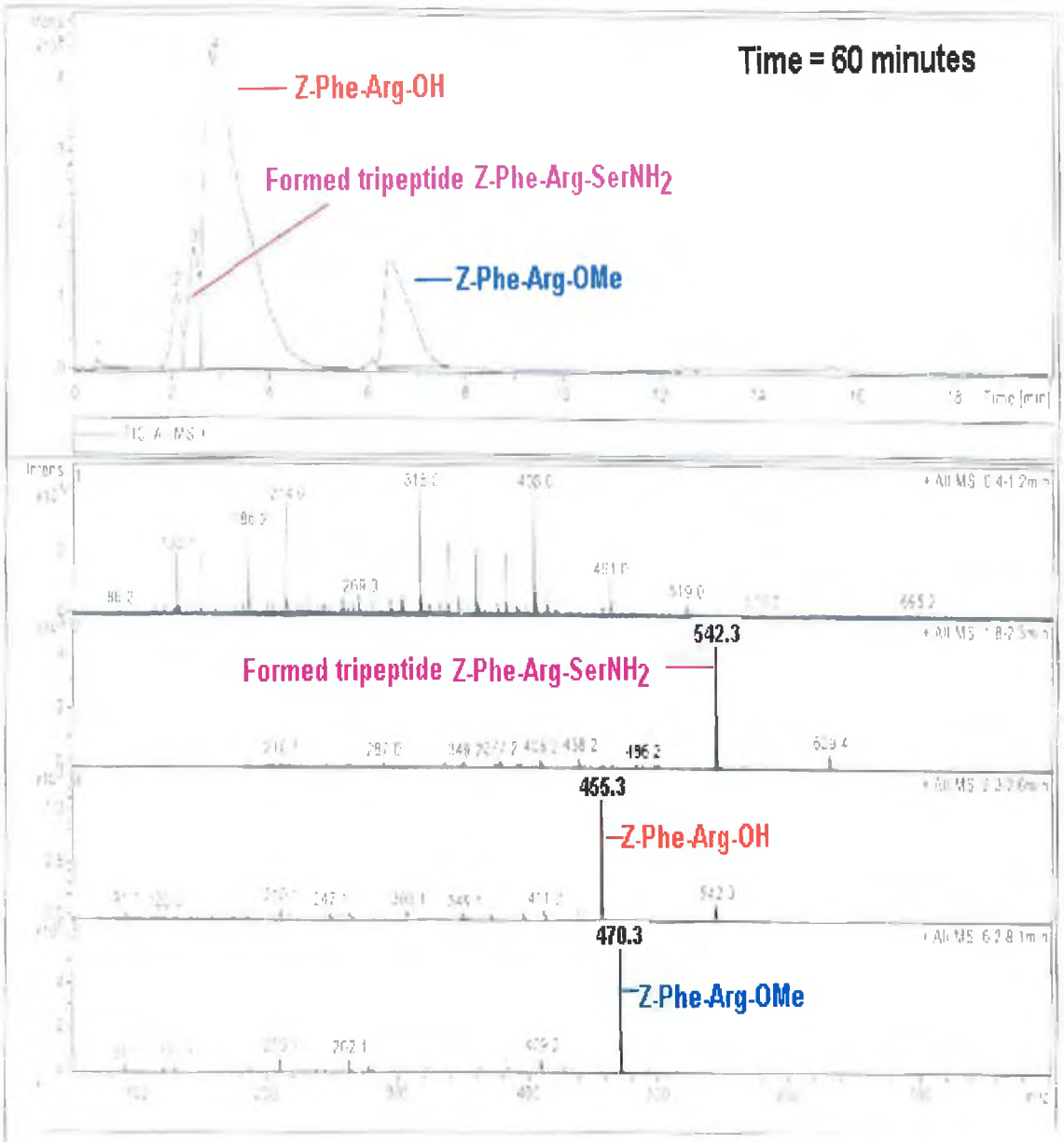


FIGURE 7.2.3.4. UV CHROMATOGRAM (UPPER) AND MASS SPECTROMETRY TRACES (LOWER) FOR rFheCL1 CATALYSED PEPTIDE SYNTHESIS, AT T = 60 MINUTES.

7.3. Discussion

A review of the literature indicated that cathepsin L has a preference for small amino acid side chains (Ala, Ser) or for long but non-branched (Asn, Gln, Lys) side chains at the P_1' position. It also indicated the order of preference among these residues at this site i.e. Ser > Ala >Lys > Asn > Gln (Ménard *et al.*, 1993). Hence, serine was chosen as a suitable nucleophile for this synthesis.

FIGURES 7.2.3.1.-7.2.3.4., clearly demonstrate that within ten minutes of reaction commencement a tripeptide was formed, while the ester was steadily consumed over the 60-minute period (although some ester still remained up to this point). However, formation of the ester hydrolysis product steadily increased over the reaction duration. Minimal optimisation of this reaction has taken place to date. Given time, many parameters could be investigated to ascertain the effects of varying enzyme, substrate and nucleophile concentrations, pH, temperature and reaction medium on the mechanistic features of the process.

Decreasing the water concentration in the reaction medium can minimize competing hydrolytic reactions. The behaviour of enzymes in solvent is variable: each enzyme has its own level of tolerance. Careful selection of the organic solvent for enzyme-catalysed peptide bond synthesis is essential as it affects not only the enzyme stability, but also solubility of the substrate and yield of peptide product (Zhou *et al.*, 2003). Synthesis in organic solvents prevents substrate ester hydrolysis as well as unwanted proteolysis (Jakubke, 1994). rFheCL1 demonstrated maximum activity in 50% (v/v) acetonitrile. The ester substrate is also soluble in this solvent; hence, this would be a good starting point for catalysing synthesis in a suitable organic cosolvent system.

Kinetically-controlled peptide synthesis has been performed at many temperatures ranging from frozen aqueous systems up to 40°C. Therefore, the optimisation of this reaction temperature would be worthwhile. Haensler *et al.* (1999) demonstrated that α-chymotrypsin and papain were capable of synthesising various peptides (kinetically-controlled synthesis) using unprotected amino acids as acyl acceptors in a frozen aqueous system, with yields ranging from 43-95% depending on the acyl acceptor used. Freezing is thought to increase the concentration of reactants in the unfrozen liquid phase that is in equilibrium with the ice crystals. Decreasing the temperature also reduces the extent of hydrolytic side reactions that take place at higher temperatures.

Optimising the concentration of reactants is an important step to achieve the highest rate of product formation. Decreasing the concentration of the reactants (as described in section 7.2.2.) enhanced the resolution of the LC chromatographs. This could be further optimised. It would be interesting to establish the highest ester:nucleophile ratio that would still allow product formation and to determine the saturation level where maximum tripeptide formation is achieved. Here, peptide synthesis was obtained at a 1:4 ratio. Lozano *et al.* (1992) used papain to catalyse the synthesis of a tripeptide using an ester:nucleophile ratio of 1:6. Varying the nucleophile acceptor would be an interesting experiment, to elucidate which nucleophile gives the highest rate of tripeptide formation. A comparison of results could be made with Ménard *et al.* (1993), who concluded that cathepsin L favoured serine (amongst other amino acids) at the P_1' position. Neither the rate nor the total amount of product synthesised by rFheCL1 was established in this reaction.

pH can influence kinetically-controlled synthesis in two ways: the binding of the ester substrate to the enzyme, leading to esterase activity and the deacylation of the acyl-

enzyme by the nucleophile, leading to synthesis. Therefore, investigating the optimum pH at which maximum synthetic activity is observed would be instructive (Lozano *et al.*, 1992). If not for the constraints of time, full optimisation of this reaction would have taken place. However, the results obtained have proved that rFheCL1 is capable of peptide synthesis.

The preference of DPP IV for proline residues at the P_1 position in substrates makes the investigation of using the enzyme for peptide synthesis an interesting concept. It is a difficult task to perform peptide coupling near a proline residue. The bulky size of the 5-membered ring may interfere with the coupling process (Tai, 2003). However, as previously discussed (section 1.9.), the side chains of residues at the P_1' position of DPP IV substrates point into the large active site cavity and no specific interactions with DPP IV occur. This explains why this enzyme has no specific preference for amino acids in substrates at this position. Therefore, peptide synthesis using a variety of nucleophiles should be possible. It had been hoped to investigate the potential of DPP IV in peptide synthesis; however, a suitable commercial ester substrate was not available preventing realisation of this ambition. A commercial peptide synthesis group was engaged to prepare a suitable ester substrate (Gly-Pro-7HMC). However, this has been unsuccessful to date, as many problems were encountered during its synthesis. Heiduschka *et al.* (1989) did achieve peptide synthesis using DPP IV, synthesising various peptides using unprotected amino acids (Ala, Gly) as acyl acceptors in 50% (v/v) glycerol. To date this is the only recorded synthesis using this enzyme.

CHAPTER 8

CONCLUSION

8.0 Conclusion

Initially, the stability properties of the serine protease dipeptidyl peptidase IV (DPP IV) were explored, the enzyme having been successfully purified from bovine serum with a final yield of 12% and an overall 32-fold increase in purity. DPP IV demonstrated no autolysis activity and was catalytically active up to 62°C, with a T_{50} value determined at 71°C ± 1°C. The apparent half-life ($t_{½}$) determined at 71°C was ten minutes. Organotolerance studies determined that DPP IV was most stable in acetonitrile up to 70% (v/v); activity declined in both DMF and DMSO above 40% (v/v). THF was deemed to be the most deleterious solvent to the enzyme. No enhancement of thermostability was observed when the enzyme was placed in acetonitrile, DMF or THF (50% (v/v)). pH investigations revealed complete inactivation of the enzyme at pH 4.0, but a broad stable pH range between 7.0-10. These studies also concluded that buffer species had an effect on the activity of the enzyme: activity in MOPS buffer at pH 7.0 was greater than in HEPES at the same pH. Two p*K* values were determined at 6.18 and 9.7, which were attributed to the ionisation of the active site histidine$_{734}$ and α-amino group of the glycine moiety on the substrate Gly-Pro-AMC, respectively. The effect of pH on DPP IV thermostability was investigated and revealed that activity sharply decreased above 60°C between pH 5.5-6.5. T_{50} values at each pH indicated that the enzyme was most thermostable in the neutral-alkaline pH region, with optimum stability observed at pH 8.0.

It had been hoped to use this enzyme for peptide synthesis; however, as discussed (section 7.3.) the lack of a suitable ester substrate to act as acyl donor impeded any such attempt.

The next part of this project involved investigating the stability properties of the cysteine protease, recombinant *Fasciola hepatica* cathepsin L1 (rFheCL1). The enzyme demonstrated no autolysis activity. rFheCL1 was catalytically active up to 70°C, with T_{50} at 78°C ± 1°C. Data fitted well to a single exponential decay and the apparent half-life ($t_{½}$) at 78°C was 5.4 minutes.

Solvent stability studies determined that little or no loss in activity occurred at lower concentrations of the solvents tested. Activity in acetone, acetonitrile, DMF and DMSO was actually enhanced. However, a dramatic decrease in activity occurred at a critical solvent concentration, which varied with solvent. Again, THF was found to be the most deleterious solvent to the enzyme.

Kinetic constants determined for native rFheCL1 were quite similar to reported values for fluke (*Fasciola hepatica*) cathepsin L1. Reported values for the esterase activity of this enzyme using the substrate Z-Lys-ONp are few. However, the enzyme demonstrated a k_{cat}/K_m 1000-fold lower value than that obtained by Kirschke *et al.* (1982).

Whether the observed stability associated with this enzyme is due to differences in amino acid sequence between the fluke and recombinant enzyme has yet to be elucidated.

Modification of rFheCL1 with the homobifunctional reagent EG-NHS yielded a catalytically active stable derivative probably with intramolecular cross-linked amino acid groups. A comparison of the kinetic constants for the native and modified enzyme indicated that the active site of the enzyme had not been affected by cross-linking, apparently no loss of catalytic performance upon modification. Modification yielded notable benefits. The EG-rFheCL1 demonstrated increased thermostability, with T_{50}

determined at 87°C, 9 degrees higher than that obtained for native rFheCL1. Thermoinactivation at 70°C showed that EG-rFheCL1 retained 100% activity over a 60-minute duration; in contrast, native rFheCL1 demonstrated a loss of up to 70% over the same period. Organotolerance studies revealed that the activity of EG-rFheCL1 in solvents (acetone, acetonitrile, DMF, DMSO) was enhanced: C_{50} values were higher than those obtained for the native enzyme. However, cross-linking did not improve the stability of the enzyme in THF. Thermoinactivation of native and EG-rFheCL1 in 50% (v/v) acetonitrile or DMF at 37°C and 50°C revealed that both forms of the enzyme were stable at 37°C, with the modified enzyme demonstrating enhanced thermostability at 50°C.

Successful synthesis of the tripeptide Z-Phe-Arg-SerNH_2 was achieved with native rFheCL1, although this reaction could be further optimised. A review of the literature did not reveal any reports in terms of peptide synthesis or chemically modifying fluke (*Fasciola hepatica*) cathepsin L1 or recombinant *Fasciola hepatica* cathepsin L1, so the present findings appear to be novel.

The original research aims of this project have been substantially achieved. Investigating the stability properties of both enzymes has revealed some interesting and potentially significant results. The thermostability of both mammalian DPP IV and rFheCL1 proved considerably higher than would be expected for these enzymes, as both are normally found existing in environments at the physiological body temperature of 37°C. Both enzymes exhibited enhanced tolerance in the present of organic solvents, proving their possible usefulness as biocatalytic reagents in industry, where most reactions are carried out in an organic environment. Enzymes in organic solvents can readily catalyze many transformations that are impossible in aqueous environments.

From a biotechnological aspect there are numerous potential advantages in employing enzymes in organic as opposed too aqueous media (section 1.5; TABLE 1.1.). Native rFheCL1 demonstrated that it could catalyse peptide synthesis, again proving a role for the enzyme in the biotechnology industry, where enzymes have been used to catalyse the synthesis of some well-known peptides e.g. aspartame. The successful modification of rFheCL1 and the enhanced thermostability demonstrated by the modified enzyme may allow this modified enzyme to be considered for use in detergents, where another cysteine protease papain, having also been successfully modified is currently being considered for application in detergent formulations (Khaparde & Singhal, 2001).

It is hoped that these results will be of use and interest to other researchers working in the field of modification, protein stability and enzymatic peptide synthesis and may in future be applied to a variety of biocatalytic applications, as both enzymes would appear to have considerable potential as biocatalysts.

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APPENDIX

Quantitation of enzyme activity:

One unit of enzyme activity is defined as the amount of enzyme, which releases 1 nanomole of AMC per minute at 37°C.

Derivation:

Let AMC released = Xμmoles/litre (assay uses 100μl AMC and 25μl enzyme sample)

$$= \frac{X \times 10^{-3} \times 0.1}{60} \text{ μmoles/min/25μl}$$

$$= \frac{X \times 10^{-3} \times 0.1 \times 10^{+3}}{60} \text{ nanomoles/min/25μl}$$

$$= \frac{X \times 0.1}{60} \text{ nanomoles/min/25μl}$$

$$= \frac{X \times 0.4}{60} \text{ nanomoles/min/1000μl}$$

$$= \frac{X \times 0.4}{60} \text{ nanomoles/min/1ml}$$

$$= \frac{X}{15} \text{ nanomoles/min/ml}$$

1 unit of activity is defined as the amount of enzyme, which releases 1 nanomole AMC/min, therefore $= \frac{X}{15}$ units/ml

AMC released (X) is also defined as observed fluorescence intensity/slope of the appropriate filtered AMC standard curve (section 2.4.1.1.-2.4.1.2.), hence:

Unit/ml or unit of activity = fluorescence intensity/ slope of filtered standard curve × 15