The Purification and Characterisation of Prolyl Oligopeptidase from Human Saliva and Dipeptidyl Peptidase IV from Bovine Serum

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August 2001
DECLARATION

I, hereby certify that the material which I now submit for assessment on the program 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.

Signed [Signature]

Seamus Buckley

Date 14/08/01
I would like to thank the following

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ABSTRACT

Prolyl Oligopeptidase (PO) was purified from human saliva and partially characterised. The techniques employed during purification were Phenyl Sepharose Hydrophobic Interaction and Anion Exchange Chromatography, where the peptidase could be isolated from this low protein source.

The enzyme was shown to have a monomeric molecular weight of 81,000 Da, which was also believed to be the native molecular weight. DTT had a considerable enhancing effect on PO activity, suggesting the presence of thiol groups as part of cysteine residues at or near the enzyme's active site. A preference was shown for neutral pH's with an optimum at pH 7.5 in potassium phosphate buffer.

The peptidase's activity proved to be quite unstable at physiological temperature, even when stabilising agents were included. Similarly significant enzyme activity was lost at frozen storage conditions. Therefore it is probable that PO is secreted into saliva in an inherently unstable form.

Dipeptidyl Peptidase IV (DPPIV) was purified to homogeneity from bovine serum. The chromatographic techniques used were Phenyl Sepharose Hydrophobic Interaction, Sephacryl S300 Gel Filtration, Anion Exchange and G100 Gel Filtration, producing an overall purification factor of 257.

SDS PAGE resulted in a monomeric weight of 70,740 Da while Size Exclusion Chromatography generated an average native molecular weight determination of 302,000 Da. Therefore it is proposed that the enzyme was a tetramer. A glycoprotein stain of a SDS PAGE gel suggests that DPPIV is glycosylated, which adds to the estimated molecular weight.

DPPIV remains active over a broad pH range with a preference for neutral pH, while the isoelectric point was estimated at 4.7. Although the enzyme is generally classified as a serine protease, it was not inhibited by any serine protease inhibitors. The enzyme was inhibited by the phenanthrolines, which are metallo-protease inhibitors but remained unaffected by the metal chelators. Therefore it is not believed that DPPIV is a metallo protease but its activity could be reduced due to enzyme surface binding. This was confirmed by the minimal effect metal ions had on activity.

Substrate Specificity of DPPIV via HPLC proved that DPPIV is capable of sequential cleavage of β-Casomorphin, Substance P and Enterostatin Analogues of C-Reactive Protein (CRP), Insulin-like Growth
Factor (IGF) and Neuropeptide Y were also shown to be susceptible to DPPIV cleavage. The peptidase cleaved the standard DPPIV substrate, Gly-Pro-MCA with a $K_M$ of 38.4 $\mu$M, while Lys-Pro-MCA was hydrolysed with a $K_M$ value of 103 $\mu$M.

DPPIV was specifically inhibited by both Diprotin A and B, where the mode of inhibition was observed to be non-competitive, generating $K_i$'s of $1 \times 10^{-4} \text{M}$ for both inhibitors. Ile-Thiazolidide and Ile-Pyrrolidide both inhibited DPPIV competitively with estimated inhibition constants of $3.7 \times 10^{-7} \text{M}$ and $7.5 \times 10^{-7} \text{M}$ determined respectively. Inhibition studies of two proline containing peptides, $\beta$-Casomorphin and Substance P, generated two different modes of inhibition for the two peptides. $\beta$-Casomorphin inhibited competitively with an average $K_i$ of $1.1 \times 10^{-4} \text{M}$, while Substance P inhibited un-competitively generating an inhibition constant of $5.5 \times 10^{-4} \text{M}$.
**General Abbreviations**

ψ Indicates the peptide bond has been modified by the group in parenthesis immediately following

βNa β-naphthylamide
(NH₄)₂SO₄ Ammonium sulphate
2-NNap β-naphthylamide
Aβ Amyloid β-protein
A₂₈₀ Absorbance at 280nm
AA Amino acid
ACN Acetonitrile
AD Alzheimer's disease
ADH Alcohol dehydrogenase
AEBSF 4-(2-aminoethyl)-benzenesulfonyl fluoride
AMC 7-amido Methyl Coumarin
APMSF 4-amidino-phenyl methane-sulphonyl fluoride
APP Amyloid Precursor Protein
AVP Arginine vasopressin
BCA Bicinchoninic Acid
Bisacryl Bisacrylamide
BSA Bovine serum albumin
Bz Benzoyl
Ca Calcium
CAPS 3[cyclohexylamino]-1-propanesulphonic acid
Cd Cadmium
cDNA copy Deoxyribonucleic acid
CDTA 1,2-cyclo hexanediamnetetraacetic acid
Cl Chloride ion
CMK Chloromethylketone
CN 2-mtrile
CNS Central Nervous System
Co Cobalt
Conc Concentration
CPC Calcium cellulose phosphate
Cp
Cu
Da
DEAE
DFP
Dip A
Dip B
DMF
DMSO
DNP
DPP I
DPP II
DPP III
DPP IV
DPPX
DTNB
DTT
DXN
ed
EDTA
EGTA
EH
EtOH
Fmoc-
FPLC
FXGLM-NH₂
GPL
H
HCl
HEPES
HEXXH
Hg
HIC
HIV-1
HPLC
HRP  Horse radish peroxidase
H/W  Hanes-Woolf
I    Iodine
IC_{50}  Concentration at which enzyme is 50\% inhibited
IEF  Isoelectric focusing
JTP-4819 (S)-2-[(S)-2-(hydroxyacetyl)-1-pyrroldinyl]carbonyl]-N-[phenylmethyl]-1-
       pyrroldinecar-boxamide
K_{2}HPO_{4}  Potassium phosphate base
KCl  Potassium chloride
KH_{2}PO_{4}  Potassium phosphate acid
K_{i}  Inhibition constant
KI  Potassium iodide
K_{m}  Michaelis constant
LB  Lineweaver-Burk
LHRH  Lutensizing hormone-releasing hormone
Log  Logarithm
M W  Molecular weight
Mca  (7-methoxy-coumarin-4-yl) acetyl
MCA  7-amino-4-methyl coumarin
MeOH  Methanol
Meq  7-amino-4-methyl-2-quinoline
MES  2-[N-Morpholino]ethane-sulphonic acid
Mg  Magnesium
MM  Michaelis-Menten
Mn  Manganese
mRNA  Messenger ribonucleic acid
Na  Sodium
NaCl  Sodium chloride
NaOH  Sodium hydroxide
ND  Not determined
NEM  N-ethyl maleimide
Ni  Nickel
OH  Hydroxy-
P  Pellet
P_{n}  Substrate subsite located n positions from the scissile bond on the N-terminal side
P_{n}^{'}  Substrate subsite located n positions from the scissile bond on the C-terminal side
pAb  p-aminobenzoate
PAGE  Polyacrylamide gel electrophoresis
PBE  Polybuffer exchanger
PCMB  p-chloromercuribenzoate
PE  Perkin Elmer
PEG  Polyethylene glycol
pH  log of the reciprocal of the hydrogen ion concentration
pI  Isoelectric point
pKa  Negative log of the equilibrium constant for an acid
PMSF  Phenylmethylsulphonylfluoride
PO  Prolyl oligopeptidase
PPCE  Post Prolin Cleaving Enzyme
PS  Phenyl sepharose
PVDF  Polyvinylidene difluoride
Pyr  Pyrrolidide
Pz  Phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-d-Arg
Q  Quaternary
R²  Regression coefficient
RCO  Acyl
Rf  Relative mobility
RP  Reverse phase
Rpm  Revolutions per minute
S  Sulphur
S1  First supernatant
S2  Second supernatant
S9  Prolyl oligopeptidase family of serine proteases
SD  Standard Deviation
SDS  Sodium dodecyl sulphate (Lauryl sulphate)
SE  Standard error
SEC  Size exclusion chromatography
SEM  Standard Error Mean
SH  Thiol group
Sn  Enzyme subsite positioned n places from the active site on the N-terminal side
S'n  Enzyme subsite positioned n places from the active site on the N-terminal side
SO_4  Sulphate
S-S  Disulphide Bridges  
Suc-  Succinyl-  
TEMED  N, N, N, N'-tetramethyl ethylenediamine  
TFA  Trifluoroacetic acid  
Thia  Thiazolidide  
TLCK  Tosyl-L-lysylchloromethane  
TRH  Thyrotropin-releasing hormone  
Tris  Tris (hydroxymethyl) amino methane  
UV  Ultraviolet  
v/v  Volume per volume  
V_e  Elution volume  
V_o  Void volume  
wrt  With respect to  
Xaa  Any amino acid  
Yaa  Any amino acid  
Z-  N-benzyloxycarbonyl  
Zaa  Any amino acid  
ZIP  Z-Pro-Pro-linal insensitive Z-Gly-Pro-MCA degrading peptidase  
Zn  Zinc  
ZnCl  Zinc chloride  
ZPP  Z-Pro-Pro-linal  
ZTTA  Z-Thiopro-thioprolinal  

**UNITS**

A  Amp  
Da  Dalton  
g  Gram  
hr  Hour  
k  Kilo  
L  Litre  
m  Metre  
M  Molar  
min  Minute  
N  Normal  
sec  Second
PREFIXES

c centi (1x10^-2)
m milli (1x10^-3)
μ micro (1x10^-6)
n nano (1x10^-9)
p pico (1x10^-12)

AMINO ACID ABBREVIATIONS

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>A</td>
</tr>
<tr>
<td>Arg</td>
<td>R</td>
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</table>

Alanine                      Arginine                     Asparagine                    Aspartic acid                    Cysteine                        Glutamine                     Glutamic acid                    Glycine                            Histidine                        Hydroxyproline                     Isoleucine                         Leucine                           Lysine                           Methionine                        Phenylalanine                      Proline                           Serine                           Threonine                        Tryptophan                        Tyrosine                          Valine

X
TABLE OF CONTENTS

1 0. INTRODUCTION

1 1 PROTEOLYTIC ENZYMES

1 2 Dipeptidyl Peptidase I

1 2 1 Introduction 4
1 2 2 Distribution 4
1 2 3 Physiological Importance 4
1 2 4 Assay 6
1 2 5 Purification 6
1 2 6 Biochemical Characteristics 7
1 2 7 Catalytic Classification and Mechanism of Action 7
1 2 8 Inhibitors 9

1 3 Dipeptidyl Peptidase II

1 3 1 Introduction 11
1 3 2 Distribution 11
1 3 3 Physiological Importance 12
1 3 4 Assay 13
### PROLYL Oligopeptidase

1. **Introduction**

2. **Distribution**

3. **Physiological Importance**

4. **Assay**

5. **Purification**

6. **Biochemical Characterisation**

7. **Catalytic Classification**

8. **Mechanism of Action**

9. **Inhibitors**

### Materials and Methods

#### Materials

- **Materials**

#### Fluorescence Spectrometry using 7-Amino-4-Methyl-Coumarin

- **MCA Standard Curves**
- **Inner Filter Effect**
- **MCA Excitation and Emission Wavelengths**

#### Protein Determination

- **Absorbance at 280nm**
- **Biuret Assay**
- **Coomassie Plus Protein Assay**
- **Standard BCA Assay**
- **Enhanced BCA Assay**

#### Enzyme Assays

- **Quantitative Measurement of PO**
2.4.2 Quantitative Measurement of DPPIV
2.4.3 Non-Quantitative Measurement of PO
2.4.4 Non-Quantitative Measurement of DPPIV

2.5 Purification of Salivary PO
2.5.1 Saliva Preparation
2.5.2. Ammonium Sulphate Precipitation
2.5.3 Phenyl Sepharose Hydrophobic Interaction Chromatography
2.5.4 Quaternary Anion Exchange Chromatography
2.5.5 Alternative Chromatography Used for PO Purification
   2.5.6 1 DEAE Anion Exchange Chromatography
   2.5.6 2 Benzamidine Sepharose Chromatography

2.6 Purification of Bovine Serum DPPIV
2.6.1 Serum Preparation
2.6.2 Phenyl Sepharose Hydrophobic Interaction Chromatography
2.6.3 Hiprep S300 High Resolution Gel Filtration Chromatography
2.6.4 POROS 50 HQ Anion Exchange Chromatography
2.6.5 G100 Gel Filtration Chromatography
2.6.6 Alternative Chromatography Used in DPPIV Purification
   2.6.6 1 Concanavalin A Sepharose Chromatography

2.7 Purity Determination
2.7.1 Polyacrylamide Gel Electrophoresis
   2.7.1 1 Sample Preparation
   2.7.1 2 Preparation of SDS Gel
   2.7.1 3 Visualisation of Proteins
2.7.2 Fluorimetric Assays

2.8 Assay Development
2.8.1 Substrate Solvent Determination (PO/DPPIV)
2.8.2 Solvent Concentration Determination (DPPIV)
283 Linearity of Enzyme Assays with Respect to Time
   283.1 Discontinuous Assay
   283.2 Continuous Assay

284 Linearity of Enzyme Assays with Respect to Enzyme Concentration (DPPIV)

285 Optimum Assay Temperature (DPPIV)

286 Effect of DTT on Substrate Hydrolysis
   286.1 Effect of DTT on PO
   286.2 Effect of DTT on DPPIV

287 Effect of EDTA on Substrate Hydrolysis
   287.1 Effect of EDTA on PO
   287.2 Effect of EDTA on DPPIV

29 Stability Studies on PO
   29.1 Effect of 37°C Pre-Incubation
   29.2 Stability at -20°C

30 Characterisation
   30.1 Relative Molecular Mass Determination
      30.1.1 Size Exclusion Chromatography
         30.1.1.1 Void Volume Determination
         30.1.1.2 Column Calibration
         30.1.1.3 Estimation of Molecular Mass of DPPIV
      30.1.2 SDS Polyacrylamide Gel Electrophoresis
   30.2 DPPIV Glycosylation of SDS Gel
   30.3 Thermostability Studies
      30.3.1 Overnight Stability
   30.4 pH Effects
      30.4.1 pH Activation (PO)
      30.4.2 pH Activation (DPPIV)
      30.4.3 pH Inactivation (DPPIV)
   30.5 Isoelectric Point Determination

XV
2.10.5.1 Chromatofocusing
2.10.5.2 Isoelectric Focusing
2.10.6 Catalytic Classification of DPPIV
2.10.7 Effect of Other Functional Reagents (DPPIV)
2.10.8. Effect of Metal Ions (DPPIV)
2.10.9. Effect of General Salts on DPPIV Activity
  2.10.9.1 Effect of Ammonium Sulphate
    2.10.9.1.1 Ammonium Sulphate effect in the substrate
    2.10.9.1.2 Ammonium Sulphate effect with the enzyme
  2.10.9.2 Effect of Sodium Chloride
    2.10.9.2.1 Sodium Chloride effect in the substrate
    2.10.9.2.2 Sodium Chloride effect with the enzyme
  2.10.9.3 Effect of Potassium Chloride
    2.10.9.3.1 Potassium Chloride effect with the enzyme
2.10.10 Substrate Specificity
  2.10.10.1 Ion-Pair Reverse Phase HPLC Analysis
    2.10.10.1.1 Inhibition of β-Casomorphin Hydrolysis
  2.10.10.2 Kinetic Analysis
    2.10.10.2.1 $K_M$ Determination of Gly-Pro-MCA
    2.10.10.2.2 $K_M$ Determination of Lys-Pro-MCA
2.10.11 Inhibitor Studies
  2.10.11.1 $IC_{50}$ Determinations
  2.10.11.2 $K_i$ Determinations
  2.10.11.3 $K_i$ Determinations of β-Casomorphin and Substance P
3.0 RESULTS

3.1 MCA STANDARD CURVES AND THE INNER FILTER EFFECT

3.1.1 Optimum MCA Excitation and Emission Wavelengths

3.2 PROTEIN DETERMINATION

3.3 PURIFICATION OF HUMAN SALIVARY PO

3.3.1 Ammonium Sulphate Precipitation

3.3.2 Phenyl Sepharose Hydrophobic Interaction Chromatography

3.3.3 Quaternary Anion Exchange Chromatography

3.3.4 Alternative Chromatographies

3.4 PURIFICATION OF BOVINE SERUM DPPIV

3.4.1 Serum Preparation

3.4.2 Phenyl Sepharose Hydrophobic Interaction Chromatography

3.4.3 Hiprep S300 HR Gel Filtration Chromatography

3.4.4 POROS 50 HQ Anion Exchange Chromatography

3.4.5 G100 Gel Filtration Chromatography

3.4.6 Alternative Chromatography

3.5 PURITY DETERMINATION

3.5.1 SDS Polyacrylamide Gel Electrophoresis

3.5.2 Fluorimetric Assays

3.6 ASSAY DEVELOPMENT

3.6.1 Substrate Solvent Determination (PO/DPPIV)
3 6 2. Solvent Concentration Determination (DPPIV) 112
3 6 3 Linearity of Enzyme Assays with Respect to Time 115
3 6 3 1 Discontinuous Assay 115
3 6 3 2 Continuous Assay 115
3 6 4 Linearity of Enzyme Assay with Respect to Enzyme Concentration (DPPIV) 115
3 6.5 Optimum Assay Temperature 115
3 6 6 Effect of DTT 119
3 6 6.1 Effect of DTT on PO 119
3 6 6.2 Effect of DTT on DPPIV 119
3 6 7. Effect of EDTA 119
3 6 7 1 Effect of EDTA on PO 119
3 6 7.2 Effect of EDTA on DPPIV 119
3 7. STABILITY STUDIES ON PO 122
3 7 1 Thermostability 122
3 7 1 1 Effect of 37°C Pre-Incubation 122
3 7 1 2 Effect of -20°C storage 122
3 8 CHARACTERISATION 125
3 8 1 Relative Molecular Mass Determinations 125
3 8 1.1 SDS Polyacrylamide Gel Electrophoresis 125
3 8 1.2 Size Exclusion Chromatography (DPPIV) 127
3 8 2 Glycosylation Estimation of DPPIV 127
3 8 3 Thermostability Studies 130
3 8 3 1 Overnight Stability 130
3 8 4 pH Effects 132
3 8 5 Isoelectric Point Determination 135
3 8 5 1 Chromatofocusing 135
3 8 5 2 Isoelectric Focusing 135
3 8 6 Catalytic Classification of DPPIV 138
3 8 7 Effect of Other Functional Reagents on DPPIV 142

XVIII
3 8 8. Effect of Metal Ions on DPPIV 144
3 8 9 Effect of General Salts on DPPIV 144
  3 8 9 1 Effect of Ammonium Sulphate 144
    3 8 9 1 1 Ammonium Sulphate effect in the substrate 144
    3 8 9 1 2 Ammonium Sulphate effect with the enzyme 145
  3 8 9 2 Effect of Sodium Chloride 145
    3 8 9 2 1 Sodium Chloride effect in the substrate 145
    3 8 9 2 2 Sodium Chloride effect with the enzyme 145
  3 8 9 3 Effect of Potassium Chloride 145
    3 8 9 3 1 Potassium Chloride effect with the enzyme 145
3 8 10 Substrate Specificity of DPPIV 149
  3 8 10 1 Ion-pair Reverse Phase HPLC 149
  3 8 10 2 Inhibition of β-Casomorphin Hydrolysis 149
  3 8 10 3 Kinetic Analysis 163
    3 8 10 3 1 $K_m$ Determination on Gly-Pro-MCA 163
    3 8 10 3 2 $K_m$ Determination on Lys-Pro-MCA 163
3 8 11 Inhibitor Studies of DPPIV 169
  3 8.11 1 IC$_{50}$ of Specific Inhibitors 169
  3 8 11.2 $K_i$ Determinations of Specific Inhibitors 169
  3.8 11 3. $K_i$ Determinations of β-Casomorphin and Substance P 169
4.0. DISCUSSION

4.1 FLUORESCENCE SPECTROMETRY USING MCA
   4.1.1. MCA Standard Curves and the Inner Filter Effect

4.2 PROTEIN DETERMINATION

4.3 ENZYME ASSAYS

4.4 PURIFICATION
   4.4.1. Purification of Human Salivary Prolyl Oligopeptidase
      4.4.1.1. Saliva Preparation
      4.4.1.2. Ammonium Sulphate Precipitation
      4.4.1.3. Phenyl Sepharose Chromatography
      4.4.1.4. Q-Sepharose Fast Flow Ion Exchange Chromatography
      4.4.1.5. Alternative Chromatographies
   4.4.2. Purification of Bovine Serum Dipeptidyl Peptidase IV
      4.4.2.1. Serum Preparation
      4.4.2.2. Phenyl Sepharose H1 Chromatography
      4.4.2.3. Hi-Prep S300 HR Gel Filtration Chromatography
      4.4.2.4. POROS 50 HQ Anion Exchange Chromatography
      4.4.2.5. G100 Gel Filtration Chromatography
      4.4.2.6. Alternative Gel Filtration Chromatography

4.5 PURITY DETERMINATION
   4.5.1. SDS-Polyacrylamide Gel Electrophoresis
   4.5.2. Fluorimetric Substrate Analysis

4.6 ASSAY DEVELOPMENT
461 Substrate Solvent Studies

46.1.1 Substrate Solvent Determination for PO

46.1.2 Substrate Solvent Studies of DPPIV

46.2 Linearity Studies

46.2.1 Linearity of PO

46.2.2 Linearity of DPPIV

46.3 Optimum Assay Temperature

46.4 Effect of DTT and EDTA on Enzyme Activity

46.4.1 Effect of DTT and EDTA on PO

46.4.2 Effect of DTT and EDTA on DPPIV

47 Stability Studies of DPPIV

47.1 Thermostability of PO

47.1.1 Effect of 37°C Pre-incubation on PO activity

47.1.2 Effect of -20°C PO storage

48 Characterisation

48.1 Relative Molecular Mass Determination

48.1.1 SDS PAGE of PO

48.1.2 SDS PAGE and Size Exclusion Chromatography of DPPIV

48.2 Glycosylation Estimation of DPPIV

48.3 Overnight Thermostability of DPPIV

48.4 pH Effects

48.4.1 pH Effects on PO

48.4.2 pH Effects on DPPIV

48.5 Isoelectric Point Determination

48.6 Catalytic Classification and Effects of Other Functional Reagents on DPPIV

48.7 Effect of Metal Ions on DPPIV

48.8 Effect of Salts on DPPIV

48.9 Substrate Specificity
48.10 Inhibitor Studies 212

5.0. Bibliography 215

6.0. Appendices 240

6.1 Error Bars 240

6.2 Enzyme Quantitation 240

6.3 Purification Table Calculations 242

6.4 Kinetic Analysis 243
   6.4.1 K_M Determinations 243
   6.4.2 K_r Determinations 244
   6.4.3 Types of Reversible Inhibition 244

6.5 IC_{50} Determination 246

XXII
LIST OF FIGURES

INTRODUCTION

FIGURE 1 1  DPPI Inhibitor Structures  10
FIGURE 1 2  Schematic Representation of DPPIV/CD26  30
FIGURE 1 3  DPPIV Specificity  35
FIGURE 1 4  DPPIV Inhibitors  38
FIGURE 1 5  Representation of an Enzyme-Substrate Complex  51

RESULTS

FIGURE 3 1 1  MCA Standard Curve for Shl Widths 10 0, 10 0  87
FIGURE 3 1 2  MCA Standard Curve for Shl Widths 10 0, 2 5  87
FIGURE 3 1 3  Filtered MCA Standard Curve  88
FIGURE 3 1 4  Filtered MCA Standard Curve  88
FIGURE 3 1 5  MCA Excitation Scan  89
FIGURE 3 1 6  MCA Emission Scan  89
FIGURE 3 2 1  Biuret Standard Curve  91
FIGURE 3 2 2  BCA Standard Curve  91
FIGURE 3 2 3  Enhanced BCA Standard Curve  92
FIGURE 3 3 1 1  PO Activity in Supernatants After Salt Fractionation  94
FIGURE 3 3 1 2  PO Activity in Pellets After Salt Fractionation  94
FIGURE 3 3 2 1  Phenyl Sepharose Profile of Human Salivary Prolyl
    Oligopeptidase  95
FIGURE 3 3 3 1  Q-Sepharose Anion Exchange Profile of Salivary PO  96
FIGURE 3 3 4 1  DEAE Elution Profile of PO  98
FIGURE 3 3 4 2  Benzamidine Sepharose Profile of PO  99
FIGURE 3 4 2 1  Phenyl Sepharose Profile of Bovine Serum Dipeptidyl
    Peptidase IV  102
FIGURE 3 4 3 1  S300 Gel Filtration Profile of DPPIV  103

XXIII
**Discussion**

**FIGURE 4 1** BCA-Protein Reaction Scheme 179

**FIGURE 4 2** Sample-Cell Geometry 182

**FIGURE 4 3** Typical Progress Curve of an Enzyme Catalysed Reaction 193
## List of Figures

### Introduction

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>DPPI Inhibitor Structures</td>
<td>10</td>
</tr>
<tr>
<td>1.2</td>
<td>Schematic Representation of DPPIV/CD26</td>
<td>30</td>
</tr>
<tr>
<td>1.3</td>
<td>DPPIV Specificity</td>
<td>35</td>
</tr>
<tr>
<td>1.4</td>
<td>DPPIV Inhibitors</td>
<td>38</td>
</tr>
<tr>
<td>1.5</td>
<td>Representation of an Enzyme-Substrate Complex</td>
<td>51</td>
</tr>
</tbody>
</table>

### Results

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1.1</td>
<td>MCA Standard Curve for Slit Widths 100, 100</td>
<td>87</td>
</tr>
<tr>
<td>3.1.2</td>
<td>MCA Standard Curve for Slit Widths 100, 25</td>
<td>87</td>
</tr>
<tr>
<td>3.1.3</td>
<td>Filtered MCA Standard Curve</td>
<td>88</td>
</tr>
<tr>
<td>3.1.4</td>
<td>Filtered MCA Standard Curve</td>
<td>88</td>
</tr>
<tr>
<td>3.1.5</td>
<td>MCA Excitation Scan</td>
<td>89</td>
</tr>
<tr>
<td>3.1.6</td>
<td>MCA Emission Scan</td>
<td>89</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Biuret Standard Curve</td>
<td>91</td>
</tr>
<tr>
<td>3.2.2</td>
<td>BCA Standard Curve</td>
<td>91</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Enhanced BCA Standard Curve</td>
<td>92</td>
</tr>
<tr>
<td>3.3.1.1</td>
<td>PO Activity in Supernatants After Salt Fractionation</td>
<td>94</td>
</tr>
<tr>
<td>3.3.1.2</td>
<td>PO Activity in Pellets After Salt Fractionation</td>
<td>94</td>
</tr>
<tr>
<td>3.3.2.1</td>
<td>Phenyl Sepharose Profile of Human Salivary Prolyl Oligopeptidase</td>
<td>95</td>
</tr>
<tr>
<td>3.3.3.1</td>
<td>Q-Sepharose Anion Exchange Profile of Salivary PO</td>
<td>96</td>
</tr>
<tr>
<td>3.3.4.1</td>
<td>DEAE Elution Profile of PO</td>
<td>98</td>
</tr>
<tr>
<td>3.3.4.2</td>
<td>Benzamidine Sepharose Profile of PO</td>
<td>98</td>
</tr>
<tr>
<td>3.4.2.1</td>
<td>Phenyl Sepharose Profile of Bovine Serum Dipeptidyl Peptidase IV</td>
<td>102</td>
</tr>
<tr>
<td>3.4.3.1</td>
<td>S300 Gel Filtration Profile of DPPIV</td>
<td>102</td>
</tr>
</tbody>
</table>
RESULTS

TABLE 3 1  Slopes of Filtered MCA Standard Curves 86
TABLE 3 2  Purification Table for Human Salivary PO 97
TABLE 3 3  Purification Table for Bovine Serum DPPIV 106
TABLE 3 4  Molecular Weight Markers Used in SDS PAGE gels 110
TABLE 3 5  Purity Assessment of PO using Fluorimetry 106
TABLE 3 6  Purity Assessment of DPPIV using Fluorimetry 109
TABLE 3 7  Molecular Weight Determinations of Salivary PO 125
TABLE 3 8  Molecular Weight Determinations of Serum DPPIV 127
TABLE 3 9  Effect of Serine Protease Inhibitors 139
TABLE 3 10 Effect of Metallo-Protease Inhibitors 140
TABLE 3 11 Effect of Cysteine-Protease Inhibitors 141
TABLE 3 12 Effect of Cysteine Protease Activators 141
TABLE 3 13 Effect of Other Functional Reagents 142
TABLE 3 14 Effect of Metal Ions 144
TABLE 3 15 Amino Acid Sequences of Bioactive Peptides Susceptible to DPPIV Hydrolysis 149
TABLE 3 16 HPLC Substrate Specificity Analysis of Bioactive Peptides 150
TABLE 3 17 HPLC Substrate Specificity Analysis of Synthetic Peptides 151
TABLE 3 18 Kinetic Analysis of DPPIV using Gly-Pro-MCA 163
TABLE 3 19 Kinetic Analysis of DPPIV using Lys-Pro-MCA 164
TABLE 3 20 Effect of Specific Inhibitors on DPPIV Activity 169
TABLE 3 21 Kinetic Analysis of DPPIV with Proline-containing Peptides 170

DISCUSSION

TABLE 4 1  Comparison of Protein Determination Assays 180
TABLE 4 2  DPPIV Physiochemical Properties 204
1.0. INTRODUCTION
1.0. INTRODUCTION

11 PROTEOLYTIC ENZYMES

In mammalian systems, proteins play a crucial role in the life, death and general functionality of cells. Protein-Receptor interactions produce specific signals, instructing the fate of the cell. Similarly, the signal in the guise of endocrine molecules such as hormones, are proteins often with specific function. This function can be altered by the effect of proteolytic cleavage, which is the most frequent and important enzymatic modification of proteins. The resultant cleavage product can have a different, possibly more potent physiological effect, illustrating the powerful influence and necessity of enzymatic processes.

Proteases can roughly be divided into exopeptidases and endopeptidases. The specificities of exopeptidases often allow a high degree of synergism with endopeptidases, as well as with one another (McDonald and Barrett, 1986). The specificities may have evolved as part of a proteolytic mechanism that provides for the efficient retrieval of essential amino acids from dietary proteins and from intracellular proteins that are degraded as part of the dynamic process of protein turnover. This is achieved by hydrolysing peptide chains from either the amino or carboxyl terminals of the peptide. Initially proteases were classified according to their molecular size, charge or substrate specificity. A more rational system is now used, based on a comparison of active sites, mechanism of action and three-dimensional structure. Four mechanistic classes are recognised by the International Union of Biochemistry. These include serine and cysteine proteases (those that form covalent enzyme complexes) and aspartic and metallo-proteases (those that do not form covalent enzyme complexes) (Benyon and Bond, 1989).

Proteolytic enzymes dependent on a serine residue for catalytic activity are widespread and very numerous. Serine peptidases are found in viruses, bacteria and eukaryotes and they include exopeptidases, endopeptidases and oligopeptidases. Over 20 families of serine proteases have been recognised (Rawlings, 1994). Mainly based on the three-dimensional structure, most of these families can be grouped together into approximately 6 clans that may well have common ancestors. It is speculated there are at least four separate evolutionary origins of serine peptidases. However, there are similarities in the reaction mechanisms of several of the peptidases even with different evolutionary origins. They have a common 'catalytic triad' of three amino acids: serine (nucleophile), aspartic acid (electrophile) and histidine (base). The geometric orientations of these amino acids may vary slightly between
families, despite the fact that the protein folds are very different. It is this parameter that can often create a protein's individual functionality (See Table 11).

<table>
<thead>
<tr>
<th>Clan</th>
<th>Family</th>
<th>Representative Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA</td>
<td>S1</td>
<td>Chymotrypsin</td>
</tr>
<tr>
<td>SA</td>
<td>S5</td>
<td>Lysyl Endopeptidase</td>
</tr>
<tr>
<td>SB</td>
<td>S8</td>
<td>Subtilisin</td>
</tr>
<tr>
<td>SC</td>
<td>S9</td>
<td>Prolyl Oligopeptidase</td>
</tr>
<tr>
<td>SC</td>
<td>S10</td>
<td>Carboxypeptidase C</td>
</tr>
<tr>
<td>*</td>
<td>S15</td>
<td>* Lactococcus X-Pro-peptidase</td>
</tr>
<tr>
<td>*</td>
<td>S28</td>
<td>Lysosomal Pro-X Carboxypeptidase</td>
</tr>
<tr>
<td>SE</td>
<td>S11</td>
<td>Escherichia d-Ala-d-Ala peptidase A</td>
</tr>
<tr>
<td>SF</td>
<td>S26</td>
<td>Leader Peptidase</td>
</tr>
<tr>
<td>SG</td>
<td>S25</td>
<td>Multicatalytic endopeptidase complex</td>
</tr>
</tbody>
</table>

**Table 11 Clans and Families of Some Serine Peptidases**

* Represents Peptidases that are roughly grouped into the SC clan eventhough the catalytic residues have not been fully established.

Dipeptidyl Peptidases (DPP) are a group of enzymes that have not been grouped due to their catalytic similarity, molecular size nor charge. It is probable that the proteases have very little evolutionary link whatsoever. They are grouped due to their ability to hydrolyse dipeptides from the N-terminus of peptides. Therefore they are exopeptidases and may play a significant role in protein metabolism and their general turnover as mentioned earlier. It is possible too that they could be involved in the less reported process of peptide synthesis. Proteases are classified by their biological function as hydrolases, however as catalysts they must also be able to catalyse the reverse reaction, such as the formation of a peptide bond. Nevertheless, the four characterised DPPs are quite diverse. DPPI is a broad acidic, cysteine proteinase, DPPIII is generally recognised as a metalloprotease, while DPPII and IV resemble the greatest similarity, both classified as serine proteases and have almost identical unique substrate specificities. The only significant difference between both enzymes is their pH optimums, with DPPII enjoying acidic pH conditions while DPPIV favours more neutral pH. As a consequence, most of the research on the dipeptidyl peptidases have focused on both DPPII and IV. This work has grouped both proteases in the 'unclassical' serine protease SC clan, as part of the Prolyl Oligopeptidase family (See Table 11).
Other dipeptidyl peptidases have been discovered but await thorough characterisation and eventual classification. Most have been compared to DPPIV, in an attempt to ascertain a significant enzymatic difference and thus a possible novel, undiscovered protease.
12 Dipeptidyl Peptidase I

12.1 Introduction
Dipeptidyl Peptidase I (EC 3.4.14.1) is a lysosomal cysteine protease capable of sequentially cleaving dipeptides from the amino terminus of suitable substrates. The peptidase was first discovered in Porcine kidney (Gutman & Fruton, 1948) having chymotrypsin-like activity which was termed Cathepsin C (Tallan et al., 1952). The enzyme was named dipeptidyl transferase (Metnone et al., 1966) and later renamed dipeptidyl aminopeptidase I (DPPI) by McDonald et al., 1969a in recognition of its exopeptidase activity. The enzyme is predominantly found in cytotoxic T-cells, mast cells and myelomonocytic cells (Wolters et al., 1998).

12.2 Distribution
DPPI is widely distributed in mammalian tissues, spleen and kidney being rich sources (Kuribayashi, et al., 1993, Kominami et al., 1992). Recent work has demonstrated a high level of DPPI expression in bone marrow and bone marrow-derived cells. These include myeloid cells such as mast cells (Wolters et al., 1998) and lymphocytes with cytolytic potential (Brown et al., 1993). Within these cells, DPPI plays a requisite role in the post-translational processing and activation of granule serine proteases by removal of the activation dipeptide. Thus converting the pro-enzyme to the active mature enzyme. In blood it is predicted that DPPI activates thrombin cleaved urokinase-type plasminogen activator (Nauland & Rijken, 1994).

12.3 Physiological Importance
The main function of DPPI is protein degradation in the lysosome. Similarly, it has been found to activate neuraminidase and platelet factor XIII, highlighting its involvement in the regulation of the plasminogen-plasmin system. Maintenance of cell growth has been reported as another possible DPPI function.

Smyth et al., 1995, describes the common tissue expression of serine proteases and DPPI, their intracellular compartmentalisation and co-localisation, and the enzymatic characteristics of DPPI. These findings clearly suggest the enzyme is a potential candidate for the processing and activation of granzyme B. Granzyme B is a neutral serine protease found in cytotoxic lymphocytes. It is critically involved in delivering the rapid apoptotic signal to susceptible target cells. Active recombinant human granzyme B (hGrzB) was expressed in mammalian COS cells which was able to hydrolyse BOC-Ala-Ala-Asp-SBZL, which is
termed the Asp’ase assay. However unmodified hGrzB was inactive until DPPI was added. The inactive hGrzB differs from the active form only by the presence of an amino-terminal prodipeptide. Thus, it is believed that the proteolytic processing of the prodipeptide and subsequent activation of the enzyme is carried out by DPPI. Pham et al., 1998 found that at pH 5.0, bovine DPPI efficiently activated recombinant pro-GrzB. DPPI had no effect on the Asp’ase assay nor did it affect the activity of mature rGrzB, suggesting that once the prodipeptide is removed, mature GrzB is resistant to further proteolytic cleavage by DPPI. Kummer et al., 1996, and Mabee et al., 1998, report the activation of Granzyme A, another serine proteinase present in the granules of cytotoxic T lymphocytes and natural killer cells. Like GrzB, GrzA is synthesized as an inactive proenzyme with an amino-terminal prodipeptide, which is processed during transport to the cytotoxic granules, where it is stored as an active protease. DPPI’s involvement in cytotoxic lymphocytes and cells of myeloid origin is highlighted by the fact that human natural killer (NK) lymphocytes have approximately 20 times the DPPI activity as fibroblasts or B cells. This could also indicate that DPPI has a more specific role in immune effector or inflammatory cells, a subset of bone marrow derived cells.

Rao et al., 1997 discovered that high levels of DPPI were expressed in polymorphonuclear leukocytes (PMNL) and alveolar macrophages and their precursor cells. Treatment of lymphocytes with interleukin-2 (IL-2) resulted in a significant increase in DPPI mRNA levels, suggesting that the gene is subject to transcriptional regulation.

Dipeptidyl Peptidase I is also reported to activate chymase, a serine protease with chymotrypsin-like substrate specificity which is synthesized and stored in the secretory granules of mast cells. Chymase is released in response to allergens to defend against helminthic parasites, cardiovascular disease and chronic inflammatory disease (McEuen et al., 1998). Treatment with DPPI on recombinant prochymase resulted in enzymatically active chymase. Tryptase is also synthesized as an inactive zymogen and then activated by removal of the NH2-terminal dipeptide by DPPI.

Wolters et al., 1998, postulated that mast cell-DPPI (MC-DPPI) may have an extracellular role by degrading neuropeptides, cytokines or extracellular matrix proteins. As a consequence, the enzyme may influence cellular functions or matrix remodelling. In addition, MC-DPPI may be regulated by inflammatory mediators. As mast cells have a role in allergic and inflammatory diseases, inflammatory mediators may alter quantities in MC-DPPI and influence its role in these diseases.
Dolenc et al., 1995, report that DPPI serum levels are highest in hepatic diseases, followed by peripheral arterial disease, thromboembolism, myocardial infarction, diabetes mellitus and prostatic hypertrophy. In addition, it has been suggested that DPPI is also involved in several pathological events, such as Duchenne muscular dystrophy and basal cell carcinomas. Krepela et al., 1996, found that the mean specific activity of DPPI was higher in human squamous cell lung carcinoma (SQCLC) than in normal lung suggesting possible involvement of the DPPI enzyme.

124 Assay

DPPI preferentially cleaves dipeptides from the unsubstituted N-termini of polypeptide substrates with broad specificity. Most common assay substrates are derivatives of Gly-Phe-, Gly-Arg or Ala-Ala- dipeptides (McDonald & Schwabe, 1977, Kirschke et al., 1995, Wolters et al., 1998). Having unblocked N-termini, the substrates are susceptible to aminopeptidase activity and therefore may not be regarded as specific for the peptidase. Although widely classified as an exopeptidase, DPPI was shown to have an affinity for cleavage of Z-Phe-Arg, Pro-Phe-Arg and Suc-Leu-Leu-Val-Tyr-MCA (Kunbayashi et al., 1993) under optimal conditions, highlighting a possible secondary endopeptidase activity.

125 Purification

DPPI has been purified from many species, such as human, rat, bovine, porcine and goat as well as mammalian cells. The enzyme's molecular size and net charge appear to be the two main properties exploited while purifying the DPPI enzyme. One of the first documented purification of DPPI was by Mycek in 1970 from bovine spleen, employing Ammonium Sulphate precipitation and Sephadex G-200, DEAE Cellulose and the cation exchanger CM-Cellulose chromatography, resulting in an overall 92.5-fold increase in purity. Horn et al., 2000 also utilised this procedure when purifying from bovine spleen.

Kunbayashi et al., 1993, also purified DPPI from bovine spleen in an attempt to determine the enzyme's endopeptidase potential. A G3000SW gel filtration resin was used as well as a DEAE-Toyopearl 650M anion exchanger. However, it is not reported whether the enzyme was purified to homogeneity nor is a purification factor given.

Ishidoh et al., 1991 purified Cathepsin C from rat liver using a combination of Ammonium Sulphate salt precipitation, Ion Exchange, Gel Filtration and Hydroxyapatite chromatography. Although the percentage yield was quite low at 5%, a purification factor of 1580 was generated resulting in 1mg of pure Cathepsin C.
Wolter et al., 1998 purified DPPI to homogeneity from dog C2 mastocytoma cells. The enzymes glycosylation properties were exploited to bind to a lentil column. The subsequent pooled fraction was further purified via a Superose 6 gel filtration column connected to a Superose 12 column and finally applied to a Mono-Q anion exchange column. The procedure employed generated a 30-fold increase in purity.

Although this is not an exhaustive list of DPPI purification, it provides an ample survey of some of the techniques employed.

1.2.6 Biochemical Characteristics

DPPI exists as a 200kDa oligomer. Depending on the species, the cDNA generally encode a protein of three functional domains, a signal peptide, an unusually long pro-peptide and the mature region. DPPI is initially synthesised as a single-chain 55kDa pre-proenzyme and is converted rapidly to a two-chain form of the mature enzyme. Many reports have conflicting evidence on the subunit composition. Most regard DPPI as a tetramer, each subunit composed of three different polypeptide chains, a heavy chain, a light chain and a propeptide part. However, bovine DPPI was found to be composed of eight subunits in the form of two tetramers with two different types of subunits (Metrone et al., 1970).

The enzyme is generally characterised as a glycoprotein. Four potential glycosylation sites were found in rat DPPI (Ishidoh et al., 1991), three located in the propeptide region and one in the mature enzyme region.

The pI of the mature protein ranges from 5.0 to 6.0. It has a broad acidic pH optimum of between 4.5 and 7.0 (McGuire et al., 1992). This pH range correlates well to that of Wada et al., in 1992 who evaluated that Cathepsin C is located in lysosomes which exist in an acidic medium. Dolenc et al., 1995, reported a pH optimum of 6.0 for their lysosomal proteinase but believe it is extremely unstable at neutral pH. Wolters et al., 1998, found activity of mast cell DPPI over a broad pH range from 6.0-7.5, which agrees with the conditions required for the enzyme's ability to activate other mast cell proteases, tryptase and chymase. This may occur in either the Golgi apparatus or in secretory granules, which are both acidic organelles.

1.2.7 Catalytic Classification and Mechanism of Action

DPPI is universally classified as a lysosomal cysteine peptidase of the papain family. Nikawa et al., 1992 highlighted the enzyme to be inhibited by E-64 and leupeptin, two typical thiol...
Similarly, Green and Shaw, 1981, reported specific inactivation by Gly-Phe-diazomethyl ketone, another thiol blocking reagent. The enzyme was also inhibited by rat stefin A and chicken cystatin, two protein inhibitors of cysteine peptidases.

Ishidoh et al., 1991, deduced that the mouse DPPI sequence contains a distinctive tyrosine adjacent to the active site cysteine. This tyrosine residue appears to be unique in the papain family, of which DPPI is a member, whereby tryptophan usually occupies the position adjacent to the active site residue. This suggests that the tyrosine motif may play a role in the specificity of DPPI. In addition, while other cysteine proteases contain an Asn, Gin, Lys or Gly residue at position 64, mouse and rat DPPI contain Asp and human DPPI contain Glu at this position. It is possible that these acidic side-chains may directly interact with the amineterminus of the substrate and contribute to the substrate specificity of the enzyme and the requirement for the substrate to have an unblocked amino-terminus. All three species encode an Ile at position 205, which may reside in the cleft that interacts with the P2 amino acid side chain of the substrate. This could explain the exclusion of peptides with P2 Arg and Lys residues as potential DPPI substrates.

As the enzyme has four catalytically active subunits, it is a good candidate for allosteric regulation (Nikawa, et al., 1992). Dolenc et al., 1995, report for human DPPI that binding of the first substrate molecule to any of the four binding sites has little effect on the affinity for the second substrate molecule. However, increasing the substrate concentration enables the enzyme to bind additional substrate molecules. The binding of the second molecule reduces the catalytic activity of the first site and dramatically decreases the binding affinity for the third substrate molecule, exhibiting a large negative cooperativity effect.

Cigic and Pam, 1999, discovered that human kidney DPPI is activated by chloride (Cl) ions. This activation seemed to be specific and pH dependent. The ions involvement in substrate binding was confirmed by the decrease in the Michaelis-Menton constant, $K_m$, upon substrate hydrolysis without any significant change in $V_{max}$. Lower $K_m$'s for the hydrolysis of simple amide substrates show that $S$ sites are involved in catalysis. The ion appears to bind close to the S2 site while S sites do not seem to have any involvement.

However, Nagler et al., 1999, report that DPPI is able to hydrolyse substrates through the exopeptidase route even in the absence of preferred interactions in the S2 and S1 subsites. This is due to specific structural elements in DPPI which serve as an anchor for the C- or N-terminus of the substrate, allowing a favourable enzyme-substrate interaction independently of the P2-P1 sequence. Therefore, the nature of the residue at position P2 of a substrate, which
is usually the main factor determining the specificity of cysteine proteases of the papain family, does not have the same contribution towards exopeptidase activity.

Kuribayashi et al., 1993, report DPPI's unique endopeptidase ability. Cleavage of Z-Phe-Arg-MCA, Pro-Phe-Arg-MCA and Suc-Leu-Leu-Val-Tyr-MCA by DPPI required sulphydryl reagents and halide ions. The enzyme seems to favour less bulky residues such as Gly, Ala, Ser and acetyl groups at the S3 or S4 positions. The binding pockets at these sites may be small or absent due to steric hindrance in the enzyme structure, excluding the binding of peptides to these positions, so that the function of cathepsin C is largely as a dipeptidyl aminopeptidase and rarely as an endopeptidase. Similarly, it is apparent that a binding mode other than electrostatic between the enzyme and substrate is important for endopeptidase activity, whereas the electrostatic binding energy generated from the free α-amino group of the substrate is important for DAP activity.

In general, DPPI can hydrolyse most N\textsubscript{2}-terminal dipeptides except those with P1 or P1 proline or an N-terminal Arg or Lys. Another catalytic restriction exists when the N-terminus is hidden by a conformational change in the target protein.

1.2.8 Inhibitors

As previously described, DPPI is classified as a cysteine protease. As a consequence, thiol blocking agents or inhibitors, E64 and leupeptin have a potent effect on the enzyme's catalysis, although at times very weakly. Similarly, Smyth et al., 1994, report the inhibition of the activation of Granzyme B by DPPI via NEM, another thiol protease inhibitor. Complete inhibition was attained at 1mM while 1μM NEM achieved approximately 20% inhibition.

DPPI activity can be selectively inhibited by Gly-Phe-CHN\textsubscript{2}, a peptidyl diazomethane that readily penetrates cells and irreversibly inhibits DPPI while not directly impairing the function of other granule thiol or serine proteases (McGuire, et al., 1993) Pham et al., 1998, also inhibited the activation of recombinant murine Granzyme B by DPPI with Gly-Phe-CHN\textsubscript{2}. Although the mechanism of inhibition is not well documented, it may be speculated that Gly-Phe-CHN\textsubscript{2} could act as a competitive inhibitor.

Cigic and Pan, 1999, discovered that DPPI was competitively inhibited by the guanidinium ion (Gdm\textsuperscript{+}) which binds to the S sites (see Fig 11). They postulated two main findings as a consequence, (i) Gdm\textsuperscript{+} binds to the enzyme only in the presence of Cl and (ii) it exerts an opposite effect to Cl on the rate of irreversible inhibition of DPPI. It is possible that bound
Cl forms an essential part of the binding site for Gdm⁺, creating a favourable electrostatic interaction which supplements the hydrogen bonding of the inhibitor to neighbouring residues in the S site.

Hepain and histamine have also been shown to have an inhibitory effect on DPPI, with the latter generating an IC₅₀ of 2mM (M'Euen et al., 1998) Wolters et al., 1998, investigated the inhibition of Ala-Ala-p-nitroamidide hydrolysis by mast cell DPPI They discovered that 1mM Iodoacetic acid had a 100% inhibitory effect, while 50µg/ml Cystatin inhibited hydrolysis by 93% Cystatins are naturally occurring inhibitors of cysteine proteases They inhibit by forming tight, reversible complexes with the target enzyme.

Horn et al., 2000, investigated the inhibition qualities of the N-terminal cluster of L-arginine residues The inhibitory potency of oligoarginines increases with the elongation of the peptide chain, reaching a maximum for octyl-arginine The oligoarginines competitively interact with the active site of DPPI, resulting in Kᵢ's in the region of 1x10⁻⁵M.

Korver et al., 2001 recently developed dipeptide Vinyl Sulfones for intracellular DPPI inhibition Vinyl Sulfone (VS) compounds are irreversible inhibitors of several cysteine proteases and do not react with serine proteases The sulphur of the active site cysteine residue of the protease forms a covalent bond with the double bond portion of the VS Leu-Phe-VS-CH₃ inactivated DPPI in splenic T-cells with an IC₅₀ of approximately 5µM The inhibitor proved non-toxic with a half-life of 97min at pH 7.5 (extracellular medium) and 1302min at pH 5.5 (the intracellular environment of DPPI) The inhibitor showed no cross reactivity on granzyme nor chymase activity Therefore it appears that this Vinyl Sulfone compound may be suitable for investigation of the influence of DPPI in cellular processes The inhibitor is highly reactive, non-toxic and able to cross plasma and granule membranes to selectively inhibit intracellular DPPI.

**Figure 1.1 Dipeptidyl Peptidase I Inhibitors**
13 Dipeptidyl Peptidase II

13.1 Introduction
The serine protease dipeptidyl peptidase II (EC 3.4.14.2) was originally discovered from the purification of dipeptidyl peptidase I from bovine pituitary glands and was initially referred to as dipeptidyl arylamidase II (McDonald et al., 1968a). The purified enzyme was characterised using peptide substrates and its name was changed to dipeptidyl aminopeptidase II or DAP II (McDonald et al., 1968b). The name dipeptidyl peptidase II (DPP II) was recommended when the enzyme was included as the second member of the dipeptidylpeptide hydrolase subclass (3.4.14). The enzyme is generally classified as a serine protease with an acidic pH optimum.

13.2 Distribution
Dipeptidyl Peptidase II is expressed in many tissues, most commonly lysosomes. Although species variations occur, the highest levels in the rat occur in the thyroid gland where it is localised to the secretory epithelial cell lysosomes of the thyroid follicles (McDonald et al., 1971). DPP II has been detected cytohistochemically in rat peritoneal and alveolar mast cells and macrophages. The enzyme is exclusively found in granules or lysosomes in both cell types. However, distribution seems to be restricted to a subtype of mast-cell granules (Struckhoff and Heymann, 1986).

Astrocytic glial cells in primary culture from neonatal rat brain express prominent DPP II activity levels (Stevens et al., 1987), as well as histologically in rat brain neurons. Mentlem and Struckhoff in 1989, purified two enzymes, a soluble and membrane bound form from rat brain homogenate which they named DPP II-S and DPP II-M respectively. Both enzymes have a pH optimum of 5.5 for the hydrolysis of Gly-Pro and Arg-Pro-nitroanilidides. Fukasama et al., 1983 isolated DPP II from rat kidney, suggesting a possible source of origin as DPP II is normally present in both T and B lymphocytes.

As well as rat, both mouse and guinea pig were shown to have a wide tissue distribution of DPP II. The epididymis of the guinea pig was a particularly rich source. Similarly, DPP II has been localised histochemically to the acrosomes of guinea pig washed sperm (McDonald et al., 1987). Huang et al., 1996 isolated DPP II from porcine seminal plasma where it co-exists with DPP IV. Vanha-Perttula, 1984, report that human seminal plasma DPP II activity occurs in both soluble and membrane-bound forms. Therefore, it is possible that DPP II in seminal plasma is derived from the epididymis and seminal plasma.
Despite initially being reported as DPPIV, Eisenhauer and McDonald, 1986 discovered a novel DPPII from porcine ovary due to its marked ability to hydrolyse prolyl bonds and its optimum activity at pH 5.5. Sentandreu and Toldra, 2001, report the first purification of DPPII from porcine skeletal muscle, although it is not particularly abundant there. While DPPII was initially discovered in bovine pituitary glands, it has also been prepared from bovine connective tissue (McDonald and Schwabe, 1977).

13.3 Physiological Importance

As a consequence of dipeptides, in contrast to tripeptides, having the ability to easily cross the lysosomal membrane, DPPII together with DPPIV possibly plays a critical role in returning the products of proteolysis to the metabolic pool. The acidic pH optimum of DPPII points to a localisation in acidic compartments such as lysosomes, secretory granules, and/or parts of the Golgi apparatus.

DPPII is believed to be involved in the ingestive, intrasomal process of collagen digestion. Eisenhauer and McDonald, 1986, demonstrated the ability of porcine ovary DPPII to complement the action of tripeptidyl peptidase I (TPPI), in the breakdown of collagen's $\alpha$-chain. TPPI produced poly Gly-Pro-Ala tripeptides, which were subsequently degraded by DPPIII.

Huang et al., 1996 report high levels of bovine DPPII activity in reproductive tissues including epididymides, testis, and ampulla as well as seminal vesicles and seminal plasma. It is proposed that DPPII, in the epididymal epithelial cells especially, plays an important role in digestion of the absorbed polypeptides derived from the maturing spermatozoa.

High levels of DPPII in some human carcinoma cells have suggested that the enzyme could be a useful marker for malignancy. Kamon et al., 1991, found that DPPII serum levels were higher than normal from patients with cancer, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), while DPPIV levels decreased. Therefore the resulting increase in the serum DPPII/DPPIV ratio may also be used as a potential diagnostic marker. Similarly, DPPII activity levels in synovial fluid from patients with rheumatoid arthritis were significantly higher than in patients with osteoarthritis. As before, a reverse effect was experienced for DPPIV, so again the DPPII/DPPIV ratio may be of particular interest. That ratio is also elevated in the cerebrospinal fluid of patients with Parkinson's disease, which is primarily as a result of significant increases in the levels of DPPII, which may be derived from the brain. (Hagihara et al., 1987) In conjunction with the presence of DPPII in the connective tissue,
synovial fluid and osteoblasts, there is a significant lack of detectable DPPII in osteoclasts, multinucleate cells which break down the calcified intercellular matrix of bone. This may reflect a higher rate of secretion from this cell type. It still remains elusive why DPPII is found to be 6-24 fold higher in various human carcinoma cells than that in normal human fibroblasts. As many cytokines have N-terminal Xaa-Pro or Xaa-Ala sequences, identification of endogenous substrates in each cell type remains to be accomplished for DPPII as reported for DPPI (Pham and Ley, 1999).

High levels of DPPII activity have also been reported in cases of muscular dystrophies and polymyositis, which are auto-immune disorders in the skeletal muscle, which is damaged by the inflammatory process. This is probably due to an increase of lysosomal activation observed in these diseases.

1.3.4 Assay

DPPII preferentially releases N-terminal dipeptides from oligopeptides, especially tripeptides, and from 2-naphthylamide, amides and methyl esters of dipeptides, provided their N-termini are unsubstituted (McDonald et al., 1968a, b). Almost any residue may reside at the terminal P2 position, even though acidic residues are not favourable. In general, Ala- and Pro- are the preferred residues in the P1 position.

Lys-Ala-NHNap and Lys-Pro-NHNap appear to produce the highest rates of hydrolysis of the naphthylamides by bovine pituitary DPPII. However, Phe-Pro, Arg-Pro and Ala-Pro are also cleaved but at a lower rate (McDonald et al., 1968b). DPPII from porcine ovaries optimally hydrolyse Phe-Pro at an 8-fold higher rate than Lys-Ala, while Lys-Pro is hydrolysed 4-times greater (Eisenhauer and McDonald, 1986). A similar preference for substrates with a P1 prolyl residue is displayed from porcine seminal plasma as described by Huang et al., 1996 where the rate on Gly-Pro-NHMec is twice that for Lys-Ala-NHMec.

Activity assays can be performed at pH 5.5 with Lys-Ala-NHNap or Lys-Ala-NHMec as the fluorogenic substrate. 1,10 phenanthroline is often used to inhibit any interfering aminopeptidase activity without affecting DPPII (Nagatsu et al., 1995). Assays have also been performed on a tripeptide substrate such as Ala-Ala-Ala but only at pH 4.5. The C-terminal, single amino acid is liberated and can be detected colorimetrically with a trinitrobenzene sulfonic acid reagent. Copper is added to prevent any reaction between the reagent and the peptides, thus significantly increasing the assay’s sensitivity (McDonald et al., 1968b).
1 3.5. Purification

As DPPII was initially detected in bovine pituitary glands, it was also purified from that source (McDonald et al., 1968b). After fractionation with Ammonium Sulphate, the dialysed fraction was applied to a DEAE cellulose column. The eluted, pooled fraction was dialysed, freeze-dried and applied to an IRC-50 column. 'Dipeptidyl Arylamidase II' did not bind to the column but was separated from 95% of the protein. This 'run-through' peak was subsequently applied onto a G200 gel filtration column, where it was purified further and resulted in an 1125-fold increase in purity.

Imai et al., 1983, purified 'dipeptidyl aminopeptidase' from rat brain. The chromatographies used were, CM-Cellulose column at pH 4.2, and Hydroxyapatite. A Gly-Pro-AH-Sepharose column was used and the eluted enzyme was dialysed and reapplied to the equilibrated column. This produced a purification factor of 2,600, however the affinity chromatography steps contributed greatly to the enzyme's overall purity.

Mentlein and Struckhoff, 1989, also purified dipeptidyl aminopeptidase II from rat brain but discovered both a soluble (DPPII-S) and a membrane-bound fraction (DPPII-M). The two fractions were separated after centrifugation, whereby the membrane-bound fraction was treated with Triton X-100 for 12 hours and re-centrifuged. Both 'solubilitates' were purified via a similar protocol using a combination of (i) the anion exchanger, DEAE-Sephacel, (ii) Affinity resin, Gly-Pro-AH-Sepharose, (iii) Sephacryl S-300 gel filtration column, (iv) Chromatofocusing with the use of polybuffer 74 and (v) Mono Q, another anion exchanger. The only difference between the two purification protocols was that the affinity chromatography step was omitted for the soluble fraction, even though a reason why is not reported. However, the soluble fraction was purified 10,490 times, while the membrane-bound fraction produced a similarly significant purification factor of 7,720. It must be noted that the Mono-Q step seemed to contribute considerably to the purity rate for both fractions.

Eisenhauer and McDonald, 1986, purified DPPII from porcine ovary. After a 60% ammonium sulphate precipitation step, the sample was dialysed and freeze-dried before being purified further using various chromatographic techniques. The freeze-dried sample was reconstituted and applied to an Ultrogel AcA 34 (LKB) column, followed by concentration and then a chromatofocusing resin. A Con-A Sepharose resin bound any glycosylated proteins while a Mono S cation exchanger column as part of an FPLC system was the last column employed. This protocol generated a final purification factor of 1,400.
13 6 Biochemical Characteristics

The molecular mass of native DPPII purified from a variety of sources varies considerably. Values range from 100 kDa in porcine ovary (Eisenhauer and McDonald, 1986) to 185 kDa in porcine seminal plasma (Huang et al., 1996). The latter enzyme proved to be trimeric, however all other reports showed DPPII to be a homodimer, with the two protein chains non-covalently associated. Imai et al., 1983, found a rat brain dipeptidyl aminopeptidase whose properties were similar to bovine pituitary DPPII but the molecular weight was quite different at 220 kDa. Nevertheless, DPPII normally exist as 60 kDa subunits that are glycosylated. This explains the use of Con-A Sepharose described previously during the purification. As a consequence, the protein is approximately 2% w/w glycosylated containing mannose and glucosamine, but little or no sialic acid (Mentlein and Struckoff, 1989).

DPPII is widely reported as an acidic protease. Its pH optimum ranges from 5.5 to 6.3 with maximal activity being observed at pH 5.5, therefore allowing possible purification via either anion or cation exchange chromatography without largely affecting activity.
The first 41 residues have been documented (Huang et al., 1996) suggesting DPPII is homologous with lysosomal Pro-X carboxypeptidase and belongs to the S28 peptidase family. Fukasama et al., 2001 cloned and expressed rat kidney DPPII and from their predicted primary structure, the protein contained six potential N-glycosylation sites. Thus they believe that the mature protein could contain approximately 14% carbohydrate, however it is probable that the COS-7 cells employed for enzyme expression could have affected this percentage.

137 Catalytic Classification and Mechanism of Action

Dipeptidyl Peptidase II is universally classified as a serine protease and DPPII from all sources is sensitive to the classical inhibitors. Eisenhauer and McDonald, 1986 achieve 100% inhibition on porcine ovary DPPII with 10mM DFP while 1mM PMSF inhibited activity by 91%, both serine protease inhibitors. Mentlein and Struckhoff, 1989, report 75% inhibition from 0 1mM DFP on their membrane bound rat brain, DPPII-M while DPPII-S was inhibited by over 90%. Similarly 1 0mM PMSF reduced DPPII-M and DPPII-S activities by 84% and 80% respectively. Porcine seminal plasma DPPII was affected by DFP and AEBSF significantly where 0 5mM of each functional reagent inhibited activity by 93% and 90% respectively (Huang et al., 1996). Sentandreu and Toldra, 2001, report similar inhibitory profiles with the sulphonyl fluoride, Pefabloc SC exerting a 76% reduction in activity at 0 5mM.

DPPII is not inhibited by cysteine peptidase inhibitors such as E-64 and p-CMB, indicating that there is no -SH group involved in the catalytic mechanism. Similarly, enzymatic activity is not effected by chelating agents EDTA or EGTA, suggesting that the presence of metal cations is not necessary (Huang et al., 1996).

It is evident that the serine residue is necessary for DPPII catalytic activity. The enzyme seems to possess a catalytic trad integrated by Ser/Asp/His, which is different to classical serine peptidases with a His/Asp/Ser trad. The active site of serine proteases classified in clan SC have the unclassical trad and they also show an α/β hydrolase fold (Rawlings and Barrett, 1994). Fukasawa et al., 2001 report both Ser 136 and His 418 are in the α/β hydrolase fold but Asp 192 is not. Active site Ser 136 exists between the fifth β-sheet and the third α-helix region from the N-terminus.

Although the substrate specificity and the amino acid residue preference for hydrolysis is well documented, little has been reported on the protease’s specific mechanism of action. One may only predict similar catalytic properties to other enzymes assigned to the same clan SC, such as serine carboxypeptidase (EC 3.4.16.1).
13.8. Inhibitors

Specific inhibitors of DPPII have not been well documented in the literature. McDonald and Schwabe, 1980, appears to be the only report of an active-site directed inhibitor, Lys-Ala-CH₂Cl which provides specific irreversible inhibition. This is understandable as the inhibitor may serve as a substrate. In addition, Mentlein and Struckhoff, 1989, discovered that Z-Lys-Pro reduced DPPII-M and DPPII-S activity from rat brain by 62% and 79% respectively, suggesting another possible active-site effect. Stockel-Maschek et al., 2000, synthesized proline specific peptidase inhibitors, utilizing the influence of the thiooxamide bond, incorporated into amino acid pyrrolidides (Pyr) and thiazolidides (Thia). All compounds proved to competitively inhibit DPPII. Xaa-Thia was more effective than Xaa-Pyrr on DPPII inhibition, however the introduction of a thiocarbonyl bond (ψ[CS-N]) between the P₂ and P₁ amino acids increased the degree of inhibition by approximately, one order of magnitude.

DPPII is uniquely sensitive to cations, especially those of high molecular weight e.g., Tris, Puromycin and Hyamine 10-X, which all surprisingly inhibit competitively (McDonald et al., 1968a). The effect of small cations on DPPII in mast cells, is of interest, seeing as the mast cell intragranular concentration of histamine could be 0.4M and that of Zn²⁺ 4-20mM (Struckhoff and Heymann, 1986). These concentrations may inhibit the enzyme completely from this source.

Heavy metals, especially Hg²⁺, are potent inhibitors of DPPII. 0.01mM HgCl inhibited glial DPPII from neonatal rat brain by 94% (Stevens et al., 1987). The effect was shown to be reversible once the metal chelator, EDTA was added. Other papers have also reported that Cd²⁺ and Cu²⁺ inhibited DPPII activity (Fukasawa et al., 1983). These findings indicate that a metal-binding region(s) on the DPPII may also be important for regulation of enzyme activity.
<table>
<thead>
<tr>
<th>Properties</th>
<th>Brain (R)</th>
<th>Kidney (R)</th>
<th>Kidney (H)</th>
<th>Ovary (P)</th>
<th>Seminal Plasma (P)</th>
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<tr>
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<td>61 000</td>
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<tr>
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<td>6.1</td>
</tr>
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<td>Lys-Ala-MCA</td>
<td>Phe-Pro-Nnap,</td>
<td>Lys-Ala-MCA</td>
</tr>
<tr>
<td>Specificity</td>
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<td></td>
<td></td>
<td>Lys-Ala-NNap</td>
<td>Gly-Pro-MCA</td>
</tr>
<tr>
<td>$K_M$ (µM)</td>
<td>87 (S, Lys-Ala-)</td>
<td>555</td>
<td>250</td>
<td>45 (Phe-Pro-)</td>
<td>360 (Lys-Ala-)</td>
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<tr>
<td></td>
<td>210 (M, Lys-Ala)</td>
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<td></td>
<td>99 (Lys-Ala-)</td>
<td>1330 (Gly-Pro-)</td>
</tr>
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<td>5.5</td>
<td>5.5-6.0</td>
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<td>PMSF</td>
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<td>AEBSF</td>
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<tr>
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<td></td>
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Table 1.2 Comparison of Dipeptidyl Peptidase II from various sources

R, Rat, H, Human, P, Porcine, S, Soluble, M Membrane-bound
14 **Dipeptidyl Peptidase III**

14.1 **Introduction**

The enzyme was initially discovered in extracts of bovine anterior pituitary glands when a range of aminoacyl and dipeptidyl 2-Naphthylamide fluorogenic substrates were employed to ascertain the type of peptidases in that tissue (Ellis and Nuente, 1967). Its distinctive action on a restricted number of unsubstituted dipeptidyl-2-NHNap substrates, identified it as the third in a series of dipeptidyl arylamidases. Its name was changed to dipeptidyl aminopeptidase III when peptide substrates confirmed its specificity. In 1984, IUB recommended the name dipeptidyl peptidase III and assigned the EC number 3.4.14.4. This name was hyphenated to dipeptidyl-peptidase III in 1992 and abbreviated it to DPPIII. Reports of its action before its current name was assigned included names such as 'red cell angiotensinase' (Kokubu et al., 1969) and 'enkephalinase B' (Inaoka and Tamaoki, 1987) which gives an insight into the enzyme's potential functionality.

14.2 **Distribution**

The enzyme is localised generally in the cytosol of many mammalian tissues such as bovine pituitary gland, human placenta, lens, erthrocytes and seminal plasma, rat and guinea pig brains and porcine spleen.

Immunohistochemical staining revealed the widespread distribution of DPPIII in rat organs (Ohkubo et al., 2000). The enzyme was intensely stained in the cytosol of many tissues such as hepatocytes in the liver, epithelial cells of distal and collecting tubules in the kidneys, transitional cells in the urinary bladder, red pulp in the spleen, cortical and medullary cells in the adrenal gland and choroid epithelium in the cerebrum.

14.3 **Physiological Importance**

The enzyme's somewhat broad specificity on peptides, as well as its wide distribution in the cytosol of mammalian cells, suggests a general role for this peptidase in the terminal stages of intracellular protein degradation and red cell maturation.

The ability of DPPIII to cleave peptides such as Substance P, β-lipotropin, Angiotensin II and leu-enkephalin from brain suggests that the enzyme may serve to modulate their levels in the brain (Hazato et al., 1984).
In general, enkephalin-degrading enzymes in the human neutrophils play a vital role in inflammation (Hashimoto et al., 2000) During pregnancy, DPPIII levels increase 12-fold in retroplacental serum (Shimamori et al., 1986) It is possible that DPPIII is synthesized in placental cells and released into the maternal circulation As a result, it is speculated that this peptidase may contribute to the elevated level of plasma angiotensin-hydrolysing activity that exists during pregnancy and may also regulate the level of angiotensin in the cells of origin However the data indicates that DPPIII expressed in many tissues may represent one of the housekeeping proteins

14.4 Assay
Substrates employed for DPPIII activity is very restricted The enzyme from bovine pituitary is limited to Arg-Arg-NH\textsubscript{N}ap (Ellis and Nuenke, 1967) Rat skin DPPIII is capable of cleaving Leu-Arg-NH\textsubscript{N}ap but at 44% that of the corresponding Arg-Arg- derivative (Hopsu-Havu et al., 1970) Similarly, the rat liver enzyme showed a moderate rate on Ala-Arg-NH\textsubscript{N}ap (Ohkubo et al., 2000) Interestingly no action occurs on any of the DPPI, II or IV substrates Therefore its potential to contaminate any of the other dipeptidyl peptidase activities is eliminated

In general, the enzyme preferentially cleaves dipeptide residues (Arg-Arg, Ala-Arg, Asp-Arg or Tyr-Gly) from the amino termini of oligopeptides or proteins at pH 6-8 (McDonald and Barrett, 1986) Its enkephalinase B activity has also been utilised as a substrate The enzyme from both rat brain cytosol and membrane can cleave enkephalins at pH 7.4, with Met- or Leu-enkephalin employed as the substrate (Inaoka and Tannoki, 1987)

14.5 Purification
DPPIII has been purified from a number of the sources in which it is distributed Hazato et al., 1984, purified two DPPIII-like enzymes from monkey brain with an average purification factor of 162 Inaoka and Tamaoki, 1987, discovered and purified rat brain enkephalinase B with an impressive 9,429-fold increase in purity It is worth noting that both Mono P HR and Toyopearl HW-55 appear to contribute significantly to the purification factor achieved DPPIII from human placenta was purified 1,775-fold where the majority of contaminating protein was eliminated by a hydroxyapatite chromatography step (Fukasawa et al., 1998) Similarly rat liver cytosolic DPPIII was separated by Ohkato et al., 2000, with a 240-fold purification factor Correspondingly recombinant rat liver DPPIII was purified without impurities by Li et al., 2000 They utilised a 1ml GSTrap column with elution achieved by
incubation with 20 units of α-thrombin while on the column for 16 hours at room temperature. However, a purification factor was not reported for this method.

Other sources of DPPII purification include human erythrocytes and rat spleen. Similarly, a mammalian DPPII-like enzyme was purified 91-fold from *Saccharomyces* (Watanabe *et al.*, 1990).

### 1.4.6 Biochemical Characteristics

Most molecular weight estimates of DPPIII are approximately 80kDa (Imaoka and Tamaoki, 1987; and Ohkubo *et al.*, 2000). Hazota *et al.*, 1984, estimated that the two dipeptidyl aminopeptidases in monkey brain to have a slightly greater molecular weight of 100kDa. However, all reports believe DPPIII to be monomeric with a single polypeptide chain.

The enzyme's optimum pH is on average pH 9.0 but depends on the source. The pH range at which the enzyme is functional is quite wide, pH 6.0-6.5 for rat brain enkephalinase (Imaoka and Tamaoki, 1987) to pH 10 for the enzyme from the slime mould, *Dictyostelium discoideum* (Huang *et al.*, 1992). An isoelectric point range of 4.4-4.6 is reported from human erythrocytes (Abramic *et al.*, 1988). This appears to be the only documentation of the enzyme's pI value.

### 1.4.7 Catalytic Classification and Mechanism of Action

Dipeptidyl Peptidase III has been assigned to the SX clan and the S99 family, suggesting that the enzyme is a serine protease. This may be due to its potential inhibition by DFP and PCMB, two known serine protease inhibitors (Hazato *et al.*, 1987). However, no reports describe it as such. On the contrary, DPPIII has been demonstrated to be a metallo-enzyme by inhibition and restoration experiments on the activity utilizing metal chelators and metal ions (Ohkubo *et al.*, 2000). Potently inhibited DPPIII with EDTA and 1,10 phenanthroline. They deduced from rat liver cDNA that 738 amino acids are encoded. Classical metallo-peptidases contain the sequence 'HEXXH' which provides two ligands for the zinc atom, however rat liver DPPIII was found to have a novel zinc-binding motif, 'HEXXXH.' The enzyme has only one Zn$^{2+}$ ion in its active site, proved by a zinc-binding study (Fukasawa *et al.*, 1998).

Fukasawa *et al.*, 1999 investigated the HELGH motif (residues 450-455) in the sequence of rat liver DPPIII by replacing the Glu$^{451}$ with an alanine or aspartic acid residue and by replacing His$^{450}$ and His$^{455}$ with a tyrosine residue by site-directed mutagenesis. None of the expressed mutated proteins exhibited DPPIII activity. Furthermore, Glu$^{451}$ seemed to
constitute the active site as Glu\textsuperscript{451} mutants had the same zinc contents as the wild-type enzyme but the activity was completely abolished Li \textit{et al}, 2000 report that the mutation of Cys\textsuperscript{176} to Ala reduced activity by 65-75\% that of the wild-type, while the same mutation to Gly or Glu eliminated activity completely Therefore, they report that Cys\textsuperscript{176} is essential for DPPIII regulation Nevertheless, it is probable that DPPIII is a metalloprotease regulated by SH modification and belongs to family 1 of the clan MA

14.8 Inhibitors

A specific, synthesized DPPIII inhibitor has not been reported and possibly has not been successfully attempted However, Ac-L-leucyl-L-argininal, a naturally occurring DPPIII inhibitor has been isolated from a bacterium culture filtrate 0.04µg/ml of this compound inhibited rat pancreas DPPIII by 50\% (Nishikiori \textit{et al}, 1986) A similar microbiological product, Propioxatins A and B had a potent inhibitory effect on enkephalinase B, with $K_i$'s reported as $1.3 \times 10^{-8}$M and $1.1 \times 10^{-7}$M respectively (Inaoka and Tamaoki, 1987) In addition, spinorphin and tynorphin had a potent inhibitory effects on human neutrophil DPPIII's enkephalinase activity with IC\textsubscript{50}'s of 4.2 and 0.029µg/ml respectively (Hashimoto \textit{et al}, 2000)

All other inhibitors tested only inhibit the enzyme's functionality and are not substrate analogues Some of these inhibitors include EDTA and phenanthroline as mentioned previously as well as DCI and NEM
15 Dipeptidyl Peptidase IV

15.1 Introduction

Dipeptidyl Peptidase IV (DPPIV) activity was first reported as glycylproline naphthylamidase in rat liver during a commercial preparation of acylase I by Hopsu-Havu and Glenner (1966) and has been named dipeptidyl aminopeptidase IV (DAPIV) and/or post proline dipeptidyl peptidase IV in early work (Yoshimoto et al., 1978). Since the amino acid sequence Gly-Pro is frequently found in collagen, a possible metabolic significance in collagen metabolism was proposed. However, the enzyme is unable to cleave Pro-Pro or Pro-Hyp bonds which usually follow the Gly-Pro sequence in collagens, and therefore the physiological functions of DPPIV remained obscure. By virtue of the enzyme's predominant localization in the brush border of small intestine and kidney proximal tubules, it was initially suggested that DPPIV participates in the metabolism and the uptake of proline-containing peptides in tissues (Tiruppathi et al., 1990). DPPIV has been reported to be identical to CD26, the surface marker on T- and B-lymphocytes and as an adenosine deaminase (ADA) binding protein and is often referred to as such. DPPIV is a member of the peptidase family S9b, which along with prolyl endopeptidase (PEP, S9a) and acylaminoacyl peptidase (S9c) form the prolyl oligopeptidase family. It is also the fourth member of the Dipeptidyl Peptidase sub-group and is assigned the enzyme classification number EC 3.4.14.5.

15.2 Distribution

Most vertebrate tissues contain the enzyme, but the activities often vary. As the enzyme properties and the therapeutic potential are increasingly being investigated, the reported sources of the enzyme are similarly increasing. In addition, the protease has been cloned and expressed in many bacterial and cellular systems.

DPPIV is exceptionally concentrated in the kidney. It is located primarily in the cortex and is reportedly abundant in both brush-border and microvillus-membrane fraction in its membrane-bound form. As a result, many researchers have isolated the enzyme from kidney and found a Gly-Pro-X cleaving peptidase that could also hydrolyze Z-Gly-Pro-Leu-Gly-Pro. Yoshimoto and Walter in 1979 utilized lamb cortical kidney tissue as their source, while Tiruppathi et al., 1990, compared the hydrolysis of proline-containing peptides in the kidney of DPPIV positive and DPPIV-negative rat. De Meester et al. in 1997 employed rabbit kidney cortex to isolate DPPIV for inhibition studies. Even Wagner et al., 1999, isolated the enzyme from ostrich kidney.
In the liver, the enzyme is primarily located in cell membranes around the bile canalicular domain of hepatocytes and in the bile duct epithelia. Wang et al., 2001, investigated the effect of an anti-inflammatory drug on an enzyme from rat liver which proved to be DPPIV. Rat liver was also the source for Dobers et al., 2000 during their investigations into DPPIV's proteolytic activity. They used transfected Chinese hamster ovary cells to express 'modified' DPPIV. Ohta et al., 1992 examined the specific activity of DPPIV in both the uterus and ovary of mice during the estrous cycle. They noticed a change in activity only in the uterus with it low at estrus and high at diestrus.

One research group has carried out significant work on highlighting the presence of a soluble form of DPPIV in guinea-pig brain. O'Connor, McGovern and O'Cuinn in 1985, initially identified a particulate 'post-proline dipeptidyl aminopeptidase', while Smyth and O'Cuinn in 1994, confirmed DAPIV in the synaptosomal membrane and microsomes. However DPPIV was also found in the cytoplasm of guinea-pig brain indicating that the enzyme can exist in a soluble form (Gilmartin and O'Cuinn, 1999). As a consequence, DPPIV can also be found in many mammalian body fluids, such as plasma, urine and seminal fluid as well as on a variety of epithelial and endothelial cells. In 1982, Puschel et al., reported the presence of DPPIV in human placenta, where they found particularly high enzymatic levels in the microsomes. In the hematopoietic system, DPPIV was identified as the activation antigen, CD26. Using this knowledge, De Meester et al., 1992 isolated DPPIV/CD26 from human peripheral blood mononuclear cells. Duke-Cohen et al., 1995, obtained a homogeneous preparation of soluble serum DPPIV but found that it has no homology with recombinant soluble CD26. However Iwaki-Egawa et al., in 1998 also investigated human serum DPPIV and discovered no difference enzymatically with human kidney membrane-bound DPPIV. This includes its ability to bind adenosine deaminase (ADA) which Duke-Cohen's group claim otherwise. This may be explained by the fact that only 95% of serum DPPIV activity is associated with a protein with ADA-binding properties. DPPIVβ was found on certain human T-cell lines and is similar to DPPIV but lacks an ADA-binding capacity (Blanco et al., 1998). In addition, DPPT-L, which is rapidly expressed on activated T-cells, can mediate an interaction with monocytes and has been called attractin. Attractin also can not bind ADA.

Despite reports on this DPPIV-like activity, the DPPIV enzyme continues to be released into body fluids where it can have a potent effect, such as seminal plasma. Ohkubo et al., 1994 analysed the enzyme from porcine seminal plasma while De Meester et al., 1996 and Lambeir et al., have reported on the corresponding human form.
Although not as frequently reported as that from mammalian sources, DPPIV has also been analysed in bacterial systems such as *Flavobacterium meningosepticum* although its biochemical properties are different to the mammalian enzyme (Yoshimoto and Tsuru, 1981). However Koreeda *et al.*, in 2001 report the isolation of DPPIV from oral bacteria *Prevotella loeschei* ACTCC 15930 and found the enzyme's characteristics very similar to the corresponding mammalian enzyme.

1.5.3 Physiological Importance

Dipeptidyl peptidase IV has a dual function as a regulatory protease and as a binding protein. Since its discovery over 30 years ago, due to its unique ability to liberate Xaa-Pro or Xaa-Ala dipeptides from the N-terminus of regulatory peptides, many more examples are emerging from both *in vitro* and *in vivo* studies. Despite the minimal N-terminal truncation by DPPIV, many mammalian peptides are inactivated, either totally or only differentially, for certain receptor subtypes. The modified peptides then generate a different physiological response once bound to the receptor.

DPPIV's considerable presence in the brush border localisation of the small intestine and kidney contributes to the absorption and/or recycling of proline containing peptides. Tiruppathi *et al.*, 1993 fed DPPIV deficient rats a high proline diet and discovered that they lost weight compared to normal rats, whose expression of intestinal brush border DPPIV increased dramatically. Wagner *et al.*, 1999, made a similar finding for the catalytic properties of ostrich kidney DPPIV, with higher $K_{cat}$ values than the mammalian enzyme. A possible explanation may be, due to the fact that re-absorption and assimilation of proline containing dipeptides in the kidney is very important because of the low nutritional values in the food of the ostrich diet in arid conditions.

DPPIV's immunological role has become obvious since its presence on the surface of human peripheral lymphocytes was demonstrated (Schon *et al.*, 1984). In addition, it is concluded that the T-lymphocyte surface antigen, designated CD26 and a leukocyte activation marker (Tn103) were in fact DPPIV (Hegen *et al.*, 1990). These activation properties cause mitogenic interaction and results in lymphocyte differentiation and increased cytokine production. Although it is known that activation molecules do not need to be targeted directly to the active site of DPPIV, it is suggested that enzymatic activity may be required for signal potentiation (Reinhold *et al.*, 1994). As well as activating cytokine production, DPPIV's regulatory functionality is also possibly involved in the hydrolysis of susceptible cytokines. Hoffmann *et al.*, in 1993 demonstrated that oligopeptides with sequence analogous to the N-
terminal part of human IL-1β, IL-2, TNF-β and murine IL-6 were hydrolysed by DPPIV. However the intact recombinant cytokines remained stable in vitro due to the longer chain length. However, it is speculated that possibly the hydrolysis requires one or more preceding endo-proteolytic steps. Guthel et al., 1994 highlighted the binding ability of DPPIV to the HIV-1 Tat protein which suppresses antigen induced activation of T-cells. The protein bound to both cell surface and soluble forms of DPPIV. The high affinity of Tat for DPPIV indicates its influence in antigen-specific T-cell activation and makes the enzyme a plausible receptor for Tat's immunosuppressive activity. DPPIV/ADA binding has already been discussed, however the physiological significance of this process is of potential importance. Severe depression is accompanied by immune-inflammatory alterations, such as higher levels of positive acute phase proteins and increased production of some interleukins and soluble interleukin receptors. Serum DPPIV and ADA levels were measured in patients with minor and major depression. DPPIV activity was significantly lower in major depressive than minor or normal controls, thus reflecting an involvement in an immune system disturbance in depressed patients (Elgun et al., 1999). In addition, serum DPPIV activity is coupled to an increased number of CD4+ T-cells in depressed subjects (Maes et al., 1997). Maes et al., 2000, explored serum DPPIV levels in cancer patients receiving treatment with interleukin-2 (IL-2) and interferon alpha (IFNα). They discovered that DPPIV activity in serum was suppressed significantly when treatment with IL-2 with or without IFNα was carried out. During these treatments, IL-6 and IL-2R levels were elevated. Similarly, Van West et al., 2000, found significantly lower DPPIV activity in patients with anorexia nervosa and bulimia nervosa than in normal controls. It is hypothesised that a combined dysregulation of DPPIV and neuroactive peptides, which are substrates of DPPIV, could be an integral component of eating disorders. In contrast, DPPIV activity levels were seen to increase in the sera of osteoporotic patients (Kamori et al., 1991). The increase in activity paralleled the severity of osteoporosis. DPPIV is also implicated in the degradation of peptides derived from collagen. Thus, the results obtained are interpreted as being an indication of increased collagen breakdown in the bones of patients with osteoporosis. It should be noted that DPPIV activity decreases significantly with age and the enzymes activity is lower in women than in men which should be accounted for in any clinical trial employed (Durinx et al., 2001).

Dipeptidyl peptidase IV has been widely reported as being expressed in healthy, benign and malignant cells. Thus has led to the enzyme being described as a useful tumour burden marker. Squamous-cell lung carcinomas showed significantly higher DPPIV levels (Sedo et al., 1991). The enzyme was investigated to see if it could be useful to assess the pattern of heptocellular surface polarity in liver sections with various carcinomas (Stecca et al. 1997).
In normal liver, DPPIV was confined to the bile canalicular plasma membrane. However, the distribution pattern of DPPIV was altered in all hepatocellular carcinomas. Three different patterns were observed, (i) canaliculi were distorted and convoluted and contained abnormally high DPPIV activity, (ii) canalicular activity was lost and enzymatic activity was restricted to isolated spots, and (iii) pseudoacinar structures of hepatocytes with both basolateral and apical DPPIV expression appeared. Therefore, DPPIV is a useful bile canalicular enzyme to assess the functional polarization of hepatocytes. Cordero et al., 2000, measured the serum soluble CD26 levels in preoperative colorectal carcinoma patients. They found over a 2-fold decrease in CD26 activity in the cancer patients, which suggests that sCD26 may be a prognostic variable of early carcinoma patients. In contrast, Wilson et al., 2000, measured a 2-fold increase in DPPIV activity in primary human prostate cancer. A similar increase was observed in benign prostatic hyperplasia (BPH). This indicates that there may be some local factors produced by cancer cells that influence adjacent BPH epithelial cells to positively affect the immediate growth environment of the cancer. Plasminogen (Pg) activation is involved in the proteolytic degradation of extracellular matrix components during malignant cell metastasis. Gonzalez-Gronaw et al., 2001, reports that the Pg type-2 receptor in prostate tumour cells is compared primarily of DPPIV, adding another possible role for the enzyme not only in cancer but also indicates a significant general physiological role.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>N-Terminus</th>
<th>Amino Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide YY</td>
<td>Tyr-Pro-Ile-Lys-Pro-Glu-Ala-</td>
<td>36</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>Tyr-Pro-Ser-Lys-Pro-Asp-Asn-</td>
<td>36</td>
</tr>
<tr>
<td>Substance P</td>
<td>Arg-Pro-Lys-Pro-Gln-Phe-Phe-</td>
<td>11</td>
</tr>
<tr>
<td>β-Casomorphin</td>
<td>Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH</td>
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</tr>
<tr>
<td>GIP</td>
<td>Tyr-Ala-Glu-Gly-</td>
<td>42</td>
</tr>
<tr>
<td>GLP-1 (7-36) amide</td>
<td>His-Ala-Glu-Gly-</td>
<td>30</td>
</tr>
<tr>
<td>Endomorphin 2</td>
<td>Tyr-Pro-Phe-Phe-NH2</td>
<td>4</td>
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<tr>
<td>CRP(201-206)</td>
<td>Lys-Pro-Gln-Leu-Trp-Pro-OH</td>
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</tr>
<tr>
<td>IGF</td>
<td>Gly-Pro-Glu-</td>
<td>70</td>
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<tr>
<td>Enterostatin</td>
<td>Val-Pro-Asp-Pro-Arg-OH</td>
<td>5</td>
</tr>
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</table>

**TABLE 1.3. Some Peptides that can be cleaved by DPPIV**

Abbreviations used: GIP, glucose-dependent insulinhlike polypeptide, GLP-1, glucagon-like peptide, CRP, corticotrophin releasing peptide, IGF, insulin-like growth factor.
DPPIV can cleave N-terminal dipeptides from substrates consisting of three or more amino acid residues or dipeptides linked to C-terminal chromogenic or fluorogenic compounds such as 2-naphthylamides or methylcoumarin amides. The enzyme preferentially cleaves substrates containing the sequence Xaa-Pro-Yaa. The Pro residue can be substituted by an Ala or a hydroxyproline although the rates of hydrolysis for these substrates are often 100 to 1000-fold lower than that for the corresponding Pro containing substrate (Hems et al., 1988). The identity of the N-terminal residue (P2) of the substrate is not important for enzymatic activity, although it must have a free amino group (Yoshimoto et al., 1978). The restrictions on DPPIV substrate hydrolysis is that Pro or hydroxyproline can not be situated at the P1 position and that the P1-P1 bond must be in the trans configuration (Puschel et al., 1982).

Gly-Pro- is normally the dipeptide attached C-terminally to a compound that can be measured and quantified either spectrophotometrically or fluorometrically. Hopsu-Havu and Glenner, 1966 employed naphthylamide (NNap) while Natatsu et al., 1976 measured liberated para-nitroaniline (NHPhNO₂) photometrically. Para-nitroaniline has subsequently been abbreviated to NH-Np or pNA. Both compounds are still utilised but with the advance of analytical technology, 7-amido-methyl coumarin (AMC/MCA) is being increasingly employed to provide a very sensitive fluorimetric assay.

Modification of the backbone of biologically active peptides has become ever more important in the design of analogues possessing greater potency and enzymatic stability. Some researchers have attempted to synthesize modified substrates to possibly create better active site binding affinities. Schutkouski et al. in 1994 synthesized substrates containing a thioxoaminoacyl (C=S) -prolyl peptide bond. They compared the kinetics of DPPIV from porcine kidney on two substrates, the normal Ala-Pro-NH-Np and Ala-[CS-N]-Pro-NH-Np. They discovered that the modified derivative generated a specificity rate constant of 100-1000-fold lower than that compared to the unmodified substrate. This may be due to the fact that the thioxo-carbonyl bond (C=S) is longer than the amide (C=O), where = indicates a double bond. Similarly, thioxo peptides display a higher barrier of rotation about the C-N bond. This may be a result of the larger covalent and van der Waals radius of sulphur, restricting the φ,ψ angles in the vicinity of the thioxo amides.

The hydrolysis of the assay substrate selected is dependent on the source of enzyme and the substrate choice should be optimised as such. To highlight this, rat liver DPPIV catalysis was tested on some prolyl and alanyl dipeptide naphthylamides. The relative hydrolysis rates are...
1.5.5 Purification

DPPIV is widely distributed in a variety of aforementioned species and tissues. Since it is most enriched in kidney, liver and blood, it has been purified to homogeneity from these tissues. The intact membrane form is usually separated from soluble form by centrifugation, if both co-exist in that source. The membrane-bound form is then prepared by solubilisation with Triton X-100 and low pH. This stage itself may generate a 25-fold increase in purity when purifying from kidney (Seidel and Schaefer, 1991). The anion exchanger, DEAE is the most common resin employed when manipulating the enzymes pI and surface charge. However, the cation exchange resin CM-Cellulose has also been used in tandem with anion exchanger to good effect (Kenny et al., 1976 and Yoshimoto and Walter, 1977). Interestingly, a metal chelate column with Cu$^{2+}$ as the metal ligand was employed to purify ostrich kidney resulting in a 10-fold increase in purity from that resin alone (Wagner et al., 1999). Another resin that could be ‘unique’ to DPPIV at the correct pH is Gly-Leu affinity and Gly-Pro-EAH-Sepharose, although the increase in purity was not significant (Shibuya-Saruta et al., 1996 and Seidel and Schaefer, 1991). The latter research group also utilised the lectin affinity, Concanavalin A-Sepharose resin, which is used widely for both membrane-bound and soluble DPPIV (Duke-Cohan et al., 1995).

Hydrophobic Interaction Chromatography is not used often as a purification tool, but when it is, it proves very effective. Gilmartin and O’Cuinn, 1999, utilised Phenyl Sepharose as part of the purification process of soluble DPPIV from guinea-pig brain. Likewise Iwaki-Egawa et al., 1998, employed Butyl-Topearl 650S to significant effect by achieving an overall purification factor of 14,400 for human serum DPPIV. Gel Filtration Chromatography is another technique that is utilised less than expected. The resin pore sizes employed range from G150 for F. meningosepticum (Yoshimoto and Tsuru, 1981) to S300HR for human serum DPPIV (Iwaki-Egawa et al., 1998).

Although the standard chromatographic techniques mentioned previously contribute considerably to the overall purification of the enzyme, the affinity resins report the greatest purification factors. De Meester et al., in 1996 used adenosine deaminase (ADA) immobilised onto CNBr-activated Sepharose and manipulated DPPIV-ADA binding properties from human seminal plasma to purify the enzyme Shibuya-Saruta et al., 1996, achieved the highest purification observed of 18,000 with use of a monoclonal antibody, Ta1 bound to...
EAH-Sepharose 4B, when purifying human serum DPPIV. However, a resulting yield of 2.2% might suggest that active-site binding may reduce the lifetime of the enzyme.

**Figure 1.2. Schematic representation of DPPIV/CD26.**

The main structural features are listed on the left side along with a vertical bar, which highlights the primary structure and shows the active site residues as well as the speculated 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 the catalytic residues are situated on loops. Figure reproduced from De Meester et al., 1999.
Biochemical Characteristics

As expected, DPPIV's biochemical characteristics and properties vary depending on the species and/or the tissue of origin. However, all reports agree that DPPIV cDNA predicts a 766 amino acid type II transmembrane protein anchored to the lipid bilayer by a single hydrophobic segment located at the N-terminus with a cytoplasmic tail of only 6 amino acids. (Figure 12) The extracellular enzyme consists of 738 amino acids (Lambeir et al., 1997). The extracellular portion can be divided into three sections, a heavily glycosylated membrane proximal region (residues 47-323), a cysteine-rich region which contains 10 of the 12 cysteines present in the enzyme (residues 290-552), and a catalytic domain at the C-terminus (residues 553-766).

The enzyme is generally reported as a dimer of varying molecular weight but an average of 240kDa for membrane-bound DPPIV is observed with a subunit, monomeric weight of 100-130kDa. Soluble DPPIV consists of a similar native and monomeric weight, however, porcine seminal plasma DPPIV has been reported as a 310kDa trimer (Ohkubo et al., 1994). Bacterial DPPIV appears to be a considerably smaller enzyme with a native molecular mass of 160kDa compared to 75-80kDa monomers (Yoshimoto and Tsuru, 1981, Ogasawara et al., 1996 and Koreeda et al., 2001).

The pH optimum of DPPIV depends on the substrate used but is reported as approximately pH 8.0. It appears that whether the enzyme is soluble or membrane-bound has no apparent effect on its pH optimum. Lamb kidney DPPIV cleaved Gly-Pro-Nnap optimally at pH 7.8 (Yoshimoto and Walter, 1977) as did soluble DPPIV from guinea-pig brain when hydrolysing Gly-Pro-MCA (Gilmartin and O’Cunn, 1999). The highest pH optimum observed was from human lymphocytes at pH 8.7 (De Meester et al., 1992) when Gly-Pro-NA was the substrate. pH stability is another parameter considered as a useful enzymatic characteristic. Human placenta DPPIV maintained activity at room temperature from pH 6-12. However, under the same conditions, the enzyme lost 10% of activity at pH 4.0, while being completely inactivated at pH 3.0 (Puschel et al., 1982). Duke-Cohen et al., 1995, found that weak acidic conditions to be detrimental to human serum DPPIV activity. Porcine seminal plasma DPPIV experienced a similar effect with activity remaining stable from pH 5.5-9.5. pI values of 3-5 have been reported, pointing more to a heterogeneity of pI values for the enzyme. Puschel et al., 1982 report multiple pI forms of human placenta DPPIV of pH 3-4, however, after treatment with sialidase, the isoelectric points shifted to pH 5-5.5, indicating that the enzyme is a neuraminic-acid-containing glycoprotein. De Meester et al., 1992, determined a pI of 5.0 for human lymphocyte DPPIV however it must be noted that this pI value was generated via chromatofocusing which may lead to inaccuracies due to the volume of sample needed for pH.
measurement  One of the main biochemical distinctions between eukaryotic and prokaryotic proteases may be their isoelectric points. This is highlighted by the enzyme isolated from *Flavobacterium meningosepticum*, with a reported pI of 9.5 (Yoshimoto and Tsuru, 1981), which is a common trend among prokaryotes. However, the corresponding *Pseudomonas* enzyme showed a comparatively lower pI of 5.4, indicating that the distinction drawn between the mammalian and bacterial enzyme may also depend on the species that it is isolated from.

Temperature optimum is a parameter that is often utilised but it does not solely depend on the enzyme as substrate hydrolysis is also required to measure this property. Mammalian DPPIV would be expected to have a temperature optimum of approximately 37°C, while all assays are normally carried out at this temperature. However, the enzyme may remain stable at temperatures greater than this and often their optima may also be greater as a consequence, e.g., ostrich kidney DPPIV showed an optimum at 45°C for Gly-Pro-pNA-tosylate (Wagner et al., 1999). Lamb kidney DPPIV showed an even greater optimum of 60°C while the corresponding bacterial enzyme from *F. meningosepticum* hydrolysed optimally at 45°C also (Yoshimoto and Tsuru, 1981). This temperature for bacterial DPPIV was confirmed by Ogasawara et al., 1996 with 40-50°C being the optimum for *Pseudomonas* DPPIV.

Kinetic studies can often reveal important properties about an enzyme and allow accurate distinctions to be made. The chromophore or fluorophore attached to the dipeptide greatly influences the rate of hydrolysis. Human placenta DPPIV hydrolysed Gly-Pro-2-Nnap generating a Michaelis-Menton constant (K_M) of 0.2 mM. This rate was halved when the substrate was prepared with 2% ethanol. The K_M value decreased by almost an order of magnitude at 0.018 mM when Gly-Pro-4-methoxy-2-Nnap was used (Puschel et al., 1982). Ostrich kidney DPPIV produced similar kinetics for Gly-Pro-pNA with a K_M of 0.111 mM, however, when the dipeptide was changed to Arg-Pro, the constant decreased to 0.0784 mM, suggesting a potential greater affinity for the latter dipeptide (Wagner et al., 1999). Bacterial DPPIV show little differences in kinetics to its mammalian counterpart. The *Flavobacterium* enzyme hydrolysed Gly-Pro-pNA with a K_M of 0.1 mM (Yoshimoto and Tsuru, 1981). This was increased to 0.142 mM for the *Pseudomonas* enzyme also when using Gly-Pro-pNA (Ogasawara et al., 1996). *Prevotella* DPPIV cleaved Gly-Pro-MCA with a dissociation constant of 0.2 mM. This was considerably greater than that for Lys-Pro-MCA, which generated a K_M of 0.05 mM (Koreeda et al., 2001). This suggests that DPPIV hydrolyses X-Pro-Y peptides exclusively.
15 7 Catalytic Classification

DPPIV has been mechanistically classified as a serine protease due to its inhibition by DFP and its resistance to sulphhydril blocking agents and chelators. The enzyme is a member of the S9 family where the active site Ser, Asp and His residues has been identified. All members of this family contain the active site residue within 130 amino acids of the C-terminus with the membrane-spanning domains near the N-terminus (Rawlings and Barrett, 1994). The linear order of the catalytic triad in this family of peptidases is Ser-Asp-His (nucleophile-acid-base), an arrangement not common for 'classic' serine-type peptidases such as trpsin or chymotrypsin-like enzymes but characteristic of clan SC enzymes. The active site Ser of human CD26 is surrounded by Gly-Trp-Ser-Tyr-Gly-Tyr-Val, which corresponds to the motif Gly-X-Ser-Y-Gly proposed for these peptidases (De Meester et al., 1999). The exact positions of the catalytic triad residue may vary slightly for every species, however murine DPPIV displays a triad of Ser, Asp and His. The general catalytic triad reported is Ser, Asp and His.

DPPIV is inhibited by classical serine protease inhibitors especially DFP, an organophosphate. 0.1 mM DFP achieved an IC₅₀ on human placenta DPPIV while 0.5 mM completely inhibits the enzyme. Another organophosphate, Bis-(4-nitrophenyl) phosphate reduces activity to 36% at 0.1 mM (Puschel et al., 1982). DFP reduces human lymphocyte DPPIV activity to 3% at 1 mM while 1 mM PMSF inhibits by 47% (De Meester et al., 1992). Similarly 1 mM PMSF has a 55% inhibitory effect on porcine seminal plasma. 0.1 mM DFP reduces activity to 13% while 1 mM almost completely inhibits the enzyme to 1.4% activity remaining (Ohkubo et al., 1994). Surprisingly 14 mM DFP is required to reduce human serum DPPIV to 1%, while 1 mM PMSF only has 16% inhibitory effect (Shibuya-Saruta et al., 1996). Bacterial DPPIV follow a similar trend, with 0.1 mM DFP completely inhibiting Pseudomonas DPPIV, while 10 mM PMSF reduces activity by 40% (Ogasawara et al., 1996). Similarly 50 mM DFP has a 100% inhibitory effect on the Prevotella enzyme and 50 mM PMSF reduces activity by 70%. This may imply that phenylmethylsulphonyl fluoride inhibits serine proteases less potently or via a different mode to the organophosphate, DFP.

15 8. Mechanism of Action

In general the action of serine proteases follow a predefined mode. Firstly acylation results in the transfer of an acyl moiety (RCO-) of the substrate to the enzymes serine group. Secondly via deacylation, imidazole activates a water molecule by general-base catalysis leading to formation of a tetrahedral intermediate, which in turn breaks down by expulsion of the enzyme via imidazolium-catalysed protonation, leaving an acidified product (Fink, 1987).
As DPPIV belongs to the Prolyl Oligopeptidase subfamily of serine proteases, the α/β-hydrolase fold has been predicted for its catalytic domain. This α/β hydrolase domain is covalently bound to the upper face of a seven-blade β propeller domain of 50 residues and four-stranded anti-parallel β sheets. Substrates enter via the central pore of the propeller lower face to reach the large catalytic pocket formed by both domains at their interface. Although the exact mechanism of action for DPPIV substrate hydrolysis is not well documented, it is proposed to be similar if not identical to that of prolyl oligopeptidase. Therefore, the first and last of the 7-bladed propeller are not closed. This opening allows access via its central tunnel to the active site. Substrates may then enter and are selected by their size and the specificity of the enzyme's active site (Fulop et al., 1998).

The formation of cysteine bridges has an important impact on the correct folding of proteins. Incorrect folding leads to retention in the endoplasmic reticulum and the misfolded proteins are degraded. DPPIV has a cysteine rich domain that is responsible for the binding of collagen I and extracellular ADA. Dobers et al., 2000, carried out point mutations on all 12 cysteine residues in DPPIV, 10 of which are present in the cysteine rich domain. Five Cys residues were mutated to Glycine, i.e., C299, C326, C383, C455 and C650, while seven were mutated to serine, C337, C395, C445, C448, C473, C552 and C763. They discovered that C326G, C337S, C445S, C448S, C455G, C473S, and C552S are essential for correcting folding, intracellular trafficking, and therefore for normal biological functionality. Leu294 and Val341 was also found to be required for ADA binding (Abbott et al., 1999).

The specificity of DPPIV's substrate hydrolysis is quite conserved. Dipeptides are N-terminally liberated with Pro or Ala at position P1. Certain peptides with other small amino acids in the P1 position are cleaved at low rates (See Fig 13). In the P2 position, bulky, hydrophobic or basic amino acids with an obligate free amino group are preferred. Peptides with Pro or Hyp in the P1 position are not cleaved by DPPIV, while the preferential residues for this position are not fully known.
Specific inhibitors are often substrate analogues that possess a high affinity for the enzyme's active site, therefore binding reversibly or irreversibly and preventing enzymatic activity. Active site-directed inhibitors such as DFP and PMSF serve to ascertain the catalytic classification of the enzyme, however specific, substrate analogue inhibitors ultimately may play a role in identifying the physiological effect of the peptidase. Thus many DPPIV specific inhibitors have been synthesised and their effect measured both enzymatically and physiologically.

Demuth et al., 1988 investigated the inactivation of DPPIV by N-peptidyl-O-aroyl hydroxylamines, which can act as mechanism-based inhibitors of serine and cysteine proteases. Mechanism-based inhibitors operate via two possibilities, (i) Catalytic attack causes the formation of an acyl enzyme which deacylates more slowly than the natural enzyme-product complexes. The inhibitor-enzyme complex is formed covalently with the active site nucleophile, but without damaging the target protein or (ii) catalytic attack causes the formation of latent chemically-reactive intermediates, which form stable covalent bonds with different functional groups within or near the active site, thus leading to an irreversibly modified protein. A comparison of the turnover values for different Xaa residues in the P2 position demonstrated that the more hydrophobic or longer the side chain of the amino acid, the more successfully DPPIV was inactivated. For example, the rate of inactivation between the Gly-Pro- and Lys(Z)-Pro- derivatives enhanced by two orders of magnitude for the latter compounds.

Flentke et al., 1991 examined the role of DPPIV in T-cell function with the use of Xaa-boroPro dipeptides, where boroPro is the X-amino boronic acid analog of proline (See Fig 15.8 below). Pro-boroPro inhibited DPPIV with a Kᵵ of 3nM while Ala-boroPro generated
an inhibition constant of 2nM. As expected, blocking the N-terminus of Ala-boroPro completely abolished its affinity for DPP IV. Similarly, removal of the N-terminal residue to give boroPro reduced its affinity by 5 orders of magnitude. Gutheil and Bachoichin, 1993 investigated this molecule further by fractionating L-Pro-DL-boroPro into its component L-L and L-D diastereomers and analysed their binding to DPP IV. The L-L diastereomer is the most potent inhibitor with a Kᵢ of 16pM. The L-D isomer binds 1,000 times weaker than the L-L with a 200-fold weaker inhibition constant. The dipeptide boronic acids exhibit slow, tight binding kinetics.

Li et al., 1995 synthesised a series of aminoacyl pyrroloidine-2-nitriles (Xaa-Pyr-2-CN), in which the carbonyl group of proline is replaced by a nitrile group (Fig 14). They investigated the nitrile's effect on DPP IV and discovered that they are potent and stable inhibitors of the enzyme. Derivatives were tested with the amino acid at position P2 varied. Arg-Pyr-2-CN inhibits DPP IV with a Kᵢ of 0.37µm, however when the bulky PMC guanidino blocking group was introduced, Arg-(PMC)-Pyr-2-CN inhibits even further with a Kᵢ of 0.19µm, while PMC represents 2,2,5,7,8-pentamethyl chroman-6-sulfonyl. This increase in inhibition is expected due to the enzyme's preference for bulky, hydrophobic residues at position P2. The nitriles are presumed to react with the active site serine to form an imidate adduct, therefore highlighting its effect as a general serine protease inhibitor and possibly not specific for DPP IV.

Another set of compounds, Xaa-pyrrolidine or Xaa-thiazolidine have shown to be specific, competitive inhibitors of DPP IV (Fig 14). Although the mechanism of inhibition is not widely reported, these molecules have prevented many physiological processes that DPP IV appears to be involved in. Processing of neuropeptide Y and peptide YY was completely abolished in human serum when Lys-pyrrolidine was present (Mentlein et al., 1993). Similarly, Lys[Z(NO₂)]-thiazolidine prevented the truncation of procalcitonin (PCT), a marker for bacterial infection, in blood plasma by DPP IV (Wrenger et al., 2000a). Glucagon and glucagon-like peptide (GLP-1) has a direct involvement in type-2 diabetes. Their sequences make them potential targets for DPP IV degradation. Pospisilik et al., 2001, proved that glucagon lost a lot of its hyperglycemic activity when incubated with DPP IV. However, this could be prevented by isoleucyl-thiazolidine. In addition, valine-pyrrolidine improved glucose tolerance and insulin secretion in mice, possibly through the augmentation of GLP-1 levels by inhibiting DPP IV (Ahren et al., 2000).
A common limitation with inhibitors is their half-life in vivo. Often the molecule may potently inhibit the enzyme in vitro but could be rapidly degraded in the body. De Meester et al., 1997 explored the in vivo effect of Pro-Pro-diphenyl-phosphonate (Prodipine) on DPPIV in rabbit plasma. DPPIV levels decreased by 20% and remained unchanged for 24 hours. Although the mode of inhibition could not be ascertained, it was speculated from previous results that the inhibitor is slow-binding, irreversible and obviously very stable.

Wrenger et al., 2000b tested the N-terminal nonapeptide Tat (1-9) analogue peptides carrying exchanges in the first three amino acids, for DPPIV inhibition. Of all examined, Trp²-Tat(1-9) inhibits most potently with a $K_i$ of 2 12μM. Database analysis of this peptide disclosed an identity with the thromboxane-A2 receptor (TXA2-R), which is localised on the surface of monocytes which intensively interact with T-cells during antigen presentation. Therefore, TXA2-R could represent a natural, physiological inhibition of T-cell expressed DPPIV.

One of the most widely utilised and therefore regarded as specific DPPIV inhibitors are Diprotin A (Ile-Pro-Ile) and Diprotin B (Val-Pro-Leu). As a consequence of the direct, substrate-like structure of both compounds, they may inhibit competitively (Rahfeld et al., 1991). The inhibitors have been employed regularly to uncover many DPPIV potential physiological roles, such as endomorphin-1 degradation (Brd et al., 2001) and GLP-1 (7-36) amide degradation (O'Harte et al., 2001). They have also been used to characterise a purified protein from various sources, including human lymphocytes (De Meester et al., 1992), porcine seminal plasma (Ohkubo et al., 1994) and guinea-pig brain (Gilmartin and O'Cuin, 1999).

Although metal ions do not normally inhibit in a specific, competitive fashion, some still have an inhibitory effect on DPPIV. Thus, they also serve to allow increased accuracy when characterising the enzyme. At 1mM, HgCl₂ almost completely abolishes enzymatic activity. Similarly, the zinc ($Zn^{2+}$) ion also has a potent effect at 1mM (De Meester et al., 1992 and Puschel et al., 1982). Due to the many possible physiological effects of DPPIV, a considerable quantity of inhibitors have been synthesised. An even greater volume of research has been devoted to inhibiting the enzyme, both specifically and non-specifically.
**Figure 14 DPPIV Inhibitors**

A representation of some DPPIV inhibitors as they exist in their natural form.
Many reports have been published recently on DPPIV-like activity in an attempt to
distinguish between what is classified as Dipeptidyl Peptidase IV and that which has similar
enzymatic activity. Many of the 'DPPIV-like' enzymes may show different biochemical
properties via inhibitor effect, pH optimum etc.

Duke-Cohan et al., 1995 isolated and characterised a novel form of DPPIV in human serum.
The soluble enzyme has a monomeric molecular weight of 175kDa instead of 105-110kDa
for membrane-bound CD26. Therefore they believe that sDPPIV is not just the shedded or
proteolytic digested form of CD26. Another difference is the relative expression of epitopes
detected by monoclonal anti-CD26 antibodies, which binds stronger to CD26 than to sDPPIV.
This favours the possibility that DPPIV related enzymes may have arisen by divergent
evolution from an α/β-hydrolase ancestor, where the distinct secondary and tertiary structure
may be retained despite complete differences in sequence. In an attempt to distinguish
between this larger protein and CD26/DPPIV, it was termed DPPT-L (Duke-Cohen et al.,
1998). They confirmed that DPPT-L had DPPIV activity but they had no significant sequence
homology. To reflect this, the name was subsequently changed to Attractin.

Scanlan et al., 1994, cloned human fibroblast activation protein α (FAPα), a 95kDa cell
surface antigen. They found that the cDNA codes for type II integral membrane protein with a
large extracellular domain, transmembrane segment and short cytoplasmic tail. FAPα shows
48% amino acid sequence identity to CD26. Niedermeyer et al., 1998, proved that FAP has
DPPIV activity by expressing the protein in a baculovirus system as chimeric CD8-FAP
fusion proteins, which is able to cleave Ala-Pro-NH-Mec.

Blanco et al., 1998 discovered a CD26-like 82kDa protein expressed by the lymphocytic cell
line C8166 which, they refer to as DPPIV-β, and speculate to be solubilized from the plasma
membrane as a monomer. They discovered that there were important differences between the
affinity of DPPIV/CD26 and DPPIV-β for some substrates and inhibitors. This was illustrated
by CD26 hydrolysing Gly-Pro-NP with a $K_M$ of 0.11μM while DPPIV-β could only achieve a
$K_M$ of 0.27μM. Similarly, ICI-11a inhibits CD26 with an $IC_{50}$ of 0.011μM however 2.6μM is
required for DPPIV-β. These differences may reflect divergences in the spatial structure of
the enzymes catalytic site.
Abbott et al., 2000, cloned a 882-amino acid protein that has 27% identity and 51% similarity to DPPIV which they term DPP8. The cloned enzyme has similar biochemical characteristics to DPPIV such as a 100kDa monomeric weight but seems to exist as a monomer in the cytoplasm, thus has no transmembrane domain. An interesting difference between the two enzyme is that the cloned, recombinant DPP8 enzyme was not glycosylated. However, it is possible that the lack of glycosylation may be a result of the expression system employed.

Chiravuri et al., 1999 report that post-proline cleaving aminodipeptidases are potential regulators of quiescent cell survival. Use of L-valinyl-L-boroproline (VbP) was seen to cause lymphocyte cell death but this was not due to the inhibition of CD26/DPPIV but a novel serine protease, designated quiescent cell proline dipeptidase (QPP). QPP is predicted to be a 58kDa monomeric glycoprotein found in lysosomes but also secreted in an active form. Although QPP has similar substrate specificities at neutral pH to CD26/DPPIV, they can be functionally and biochemically distinguished. This was illustrated by Underwood et al., 1999, with the same research group as Chiravuri. They found a 492 amino acid protein with a similar sequence to prolylcarboxypeptidase. QPP activity occurred over an unusually broad pH range cleaving substrate molecules at acidic as well as neutral pH, indicating a role in lysosomes. Although both CD26/DPPIV and QPP have the same catalytic residue arrangement (Ser, Asp, His), differences in their inhibitor profiles indicate variation in the catalytic sites and/or the mode of inhibition. Chiravuri et al., 2000 added that QPP also differs to CD26/DPPIV when it oligomerizes. CD26/DPPIV forms homodimers through disulphide bridges, however, QPP dimerizes via leucine zipper motifs which are leucine residue repeats that form a coiled coil structure. This results in the initially reported 58kDa protein to dimerize to a 120-130kDa species as proved by gel filtration. Interestingly, QPP also bears a similarity to the limited sequence of DPPII available. This correlates with the affinity for acidic pH and localisation within acidic organelles, such as lysosomes. Similarly, as already reported, DPPII may exist as a 60kDa monomer and dimerize to a 120kDa native protein. In addition, the DPPII sequence has also been reported as homologous to lysosomal prolylcarboxypeptidase. Therefore, it is highly probable that QPP could be identical or similar to DPPII.

In brain tissue, DPPIV is detected only at a trace level. cDNA clones obtained from a bovine cDNA library encode a 33% identity protein with DPPIV (Kim et al., 2001). The predicted DPPIV-related protein (DPPX) is suggested to exist as two isoforms, DPPX-S and DPPX-L, which differ in the sequence and length of the N-terminus. Thus, it is assumed that DPPX and DPPIV are different gene products. The active site motif Gly-X-Ser-X-Gly and the other active residues Asp and His are conserved in DPPX, although the active site Ser is replaced.
by Asp. Despite this, the DPPX's do not have any DPPIV activity. Even when the active site Asp is substituted for Ser, it still fails to generate an enzymatically active form. This may be due to a conformational difference between DPPX and DPPIV, such as the same conformationally active triad structures differing. Therefore, it appears that the link between DPPX and DPPIV may be quite insignificant, none more than two 'similar' Serine proteases.
Both DPPIV and Prolyl Oligopeptidase (PO) have been assigned to the Carboxypeptidase clan SC. In addition, the two peptidases have been grouped in the same family, S9, which is also known as the Prolyl Oligopeptidase Family. A common characteristic that S9 family enzymes share is the unclassical serine protease catalytic triad arrangement of Ser, Asp and His. This similarity is furthered by the fact that the catalytic triad for both enzymes is within 130 residues of the C-terminus (Rawlings and Barrett, 1994). In comparison to other classified serine proteases, DPPIV and PO have a unique amino acid sequence around the reactive serine residue (TABLE 14).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sequence around the active site, Serine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin-like</td>
<td>Gly-Asn-Ser-Gly-Gly-Pro</td>
</tr>
<tr>
<td>Subtilisin-like</td>
<td>Gly-Thr-Ser-Met-Ala-Ser/Thr</td>
</tr>
<tr>
<td>Carboxypeptidase-like</td>
<td>Gly-Glu-Ser-Tyr-Ala-Gly</td>
</tr>
<tr>
<td>Dipeptidyl Peptidase IV</td>
<td>Gly-Trp-Ser-Tyr-Gly-Gly</td>
</tr>
<tr>
<td>Acylaminoacyl-Peptidase</td>
<td>Gly-Gly-Ser-His-Gly-Gly</td>
</tr>
<tr>
<td>Prolyl Oligopeptidase</td>
<td>Gly-Gly-Ser-Asp-Gly-Gly</td>
</tr>
</tbody>
</table>

**TABLE 14** Amino acid sequence around the active site serine of some serine protease families

DPPIV and PO are included in the same family. The reactive serines are bold and underlined.

Serine has as many as six potential nucleotide codons and all six have been found to encode the active site serines of different serine proteases. However, the same triplet (TCA) codes for the active site serine residue of both DPPIV and PO (Polgar and Szabo, 1992d). Similarly, the glycine residues 5 and 6 in TABLE 14, is also encoded by the identical triplet for both enzymes even though glycine has four possible codon representations. This suggests the existence of an evolutionary relationship between the peptidases.

Even though the whole amino acid sequence of both enzymes only generated a 16.5% identity, the homology of the C-terminal is somewhat higher at 21.7%. This correlates well with the previously described similarity during the catalytic process via their tertiary structure. The α/β hydrolase is a unique but definite characteristic, highlighting the similarity between DPPIV and PO. Such is this similarity that within the S9 or Prolyl Oligopeptidase family, PO is classified in subfamily A (S9a), while DPPIV is assigned to subfamily B (S9b).
1.8. PROLYL OLIGOPEPTIDASE

1.8.1. Introduction
Prolyl Oligopeptidase (PO) was first discovered in human uterus as an oxytocin-degrading enzyme (Walter et al., 1971). Due to its high affinity for hydrolysing the peptide bond on the carboxyl side of proline, it was originally named post-proline cleaving enzyme. Since its discovery and isolation from many sources, the enzyme was often named according to the bioactive peptide it cleaved, i.e. Kininase B from rabbit brain (Oliveira et al., 1976) and TRH deamidase from rat brain (Rupnow et al., 1979). The name post-proline endopeptidase was recommended in 1978 by IUBMB and then changed to prolyl endopeptidase in 1981. On the basis of the oligopeptidase nature of the reactions catalysed, the amino acid sequence homology with other oligopeptidases and the substrate-size limitation, Barrett and Rawlings proposed the name prolyl oligopeptidase in 1991 and the EC number, EC 3.4.21.26 was assigned.

1.8.2. Distribution
Prolyl Oligopeptidase is widely distributed in mammalian plant and microbial sources. In mammalian sources, many species have shown considerable levels of PO, such as human (Kato et al., 1980), rat (Yoshimoto et al., 1979) and rabbit (Orlowski et al., 1979). All species reveal ubiquitous tissue distribution with uniformly high levels found in the brain. Localisation studies carried out have shown substantial species-species variation. In humans, the highest PO levels are observed in skeletal muscle and testes with the lowest levels occurring in the heart and serum (Kato et al., 1980). In contrast, skeletal muscle shows the lowest levels in rabbit (Orlowski et al., 1979), while the small intestine and lung show comparatively higher levels. Consistently high levels have been reported from brain, however localisation studies have revealed species variation in the regional distribution within the brain. The frontal cortex in human brain has 5-fold higher PO levels than the hypothalamus (Kato et al., 1980). The piriform cortex has over 3 times higher levels that the mamillary body in rat brain (Dauch et al., 1993), while the entorhinal of rabbit brain has over 2.5 times enzyme levels than the medulla (Orlowski et al., 1979). Due to PO’s cytosolic nature, the enzyme is often released into mammalian body fluids. Yoshimoto et al., 1978 reported from human, that semen shows the highest bodily fluid PO levels followed by serum and saliva.

Plant PO sources usually consist of carrot and mushroom while bacterial sources tested have resulted in Halocynthia roretzi and especially F. meningosepticum among the most common bacteria producing the enzyme (Chevailler et al., 1992). The hyperthermophilic archaeon,
Pyrococcus furiosus is another reported microbial source (Harwood and Schreider, 2001). In this case, the *P. furiosus* structural gene was identified, sequenced and expressed in an *E. coli* strain. This is a common technique employed to analyse many different PO sources.

**1.8.3. Physiological Importance**

Due to Prolyl Oligopeptidase’s ability to cleave many proline-containing peptides, the enzyme is believed to have a significant physiological effect. Similarly the enzymes distribution in many tissues and biological fluids, indicates the potential function it may possess. However any *in vitro* studies carried out on the enzyme must be correlated with a measured *in vivo* effect, in both healthy and diseased states.

Perhaps the most striking example of rapid cell growth and proliferation is the growth of cancer cells and tumour development. It is now established that peptidases play a very important role in tumour development and progression. Studies have determined a link between elevated PO levels and cancerous tumours. Increased PO levels have been found in lung tumours, prostate and breast malignancies (Goosens *et al*., 1996a; Sedo *et al*., 1991). It is evident that from the investigations on cell growth and development, it is possible that PO may be intrinsically linked with DNA synthesis specifically.

Comparative PO activity studies have been extensively researched and have generated significant results. Maes *et al*., 1994 observed lower PO levels in patients suffering from major depression in comparison to controls and it was inversely related to the severity of depression. It has been established that major depression is characterised by considerable neuroendocrine and immunological disturbances. This includes (a) Increased concentration of certain neuropeptides like TRH and Substance P in cerebrospinal fluid, (b) Increased secretion of arginine vasopressin, CRH, β-endorphin, etc, in the Hypothalmic-Pituitary-Adrenal axis and (c) T-cell activation, B-cell proliferation and increased levels of phagocytic cells in peripheral blood. It is uncertain if lowered PO activity levels of major depressive patients is involved from the onset of this disease or even if the enzyme is a marker for the condition. However treatment of major depressed patients with anti-depressants resulted in a significant increase in PO activity (Maes *et al*., 1995). Therefore it is possible that PO may be involved in the modulation of some of the aforementioned neuroactive peptides, *in vivo* and may have a role in generating an immune response.

PO activity levels were also measured in the plasma of manic and schizophrenic patients and enzyme levels appear to be increased in comparison to normal controls (Maes *et al*., 1995).
PO's potential role in these disorders is unclear, however its involvement is strengthened by decreases in cerebrospinal fluid arginine vasopressin and neurotensin and post-mortem brain TRH in schizophrenic patients. Similarly, treatment with the anti-psychotic drug, Valproate reduced PO activity. In addition, higher PO levels were observed in the serum of patients with post-traumatic related (Maes et al., 1998), while the majority of alcohol-dependent patients showed a decrease in serum PO levels (Maes et al., 1996b).

A common trend observed in neurodegenerative disorders is abnormally low PO brain activity levels. Data obtained by Mantle et al., (1996) suggest a 65-70% reduction in activity in tissues from Parkinson's, Huntington's and Alzheimer's disease and it is speculated that this reduction may be a characteristic of a generalised process of neurodegeneration. It is unlikely that PO is simply a marker for cell damage as it was also observed that PO activity uniformly decreased in both grey and white matter of post-mortem Alzheimer disease patients. The link between PO and Alzheimer's disease (AD) has been investigated to establish the enzymes possible influence in the generation of amyloid protein, which is found in the deposits of the senile plaques, characteristic of AD. Amyloid protein (Aβ) is derived from a larger transmembrane amyloid precursor protein (APP) and Ishiura et al., (1990) identified PO as a possible γ-secretase that cleaves APP between residues Ala713 and Thr714, generating Aβ. As a result, a number of PO specific inhibitor have been analysed to ascertain possible prevention of Aβ production. Shinoda et al., in 1997 retarded Aβ formation in a neuronal cell line upon JTP-4819 addition, implicating PO in the pathogenesis of Alzheimer's disease. However a number of factors question PO's involvement in this process. As already discussed, PO is believed to be capable of cleaving peptides no greater than 25 residues long, yet APP is over 700 residues. However it is possible that APP is first cleaved by a β-secretase, which may produce an APP fragment short enough for PO to hydrolyse.

Several studies have established a potential link between PO and cerebral ischemia effects (blood supply reduction), memory and learning. TRH, Substance P and arginine vasopressin (AVP) are a number of substrates, in vivo, that can exert neuroprotective effects as well as improving the performance of animals in memory and learning tasks (Griffiths, 1987). The degradation of these peptides by PO is highly possible, therefore the effect of PO inhibitors were employed on peptide hydrolysis. Oral administration of Z-Pro-Prolinal cause a significant increase in AVP levels in rat brain (Mura et al., 1995) Similarly, JTP-4819 enhance memory processes in aged rats (Toide et al., 1995) and reverse an age-decrease of Substance P and TRH levels (Shinoda et al., 1995).
1.8.4. Assay

Oxytocin and arginine-vasopressin were initially employed as natural substrates for the enzyme (Walter et al., 1976). Koida and Walter (1976) reported the use of the Z-Gly-Pro-Leu-Gly as substrate, with the ninhydrin assay being employed to detect the cleavage product Leu-Gly. The development of spectrophotometric and fluorimetric substrates in the late 1970’s, led to the synthesis of a number of potential chromogens and fluorophores. The basic structure of PO substrates focuses on N-blocked proline containing dipeptides linked to a molecule that is readily detected on cleavage. The N-terminal blocking agents N-benzyloxycarbonyl- (Z-) and succinyl- (Suc-) are most commonly linked to Gly-Pro residues such is the case for Z-Gly-Pro-MCA.

Various reports of novel fluorogenic synthetic substrates have been published since, and the superior sensitivity of fluorimetric over spectrophotometric assays is well-documented (Willard et al., 1988). Knisatschek et al., 1980 reported a $K_M$ value of 14μM for Z-Gly-Pro-2-NNAp, an alternative fluorogenic substrate. Although PO assays incorporating 2-NNAp (β-naphthylamide) have been reported, MCA shows enhanced sensitivity over the naphthylamide derivative (Polgar, 1994). A ten-fold lower $K_M$ (1.4μM) was determined by Noula et al., 1997 using 7-amino-4-methyl-2-quinoline (Meq) as an alternative to MCA. The enhanced affinity of PO was as a direct result of replacement of the P₄ positioned Gly with a cysteine-benzyl residue. Spectrophotometric assays have also been described using Z-Gly-Pro-4-nitroanilide (Polgar, 1995) although they are generally less sensitive than their fluorescent counterparts. To date, Z-Gly-Pro-MCA or Z-Gly-Pro-NHMec has been the most widely used, commercially available substrate employed for the detection of PO activity (Rennex et al., 1991; Goossens et al., 1995; Cunningham and O’Connor, 1998). It should be noted however, that the identification of a Z-Pro-Proinal insensitive Z-Gly-Pro-MCA degrading peptidase (ZIP) by Cunningham and O’Connor (1997a) questions the classification of Z-Gly-Pro-MCA as a specific PO substrate.

1.8.5. Purification

As previously described, PO is distributed in many species and many tissues. Similarly the enzyme has been purified from many of these sources, incorporating a number of different chromatographic techniques. Depending on the source, the tissue samples are initially homogenised and then subjected to ammonium sulphate fractionation, of up to 80% saturation required to precipitate the enzyme (Yoshimoto et al., 1982). The anion exchanger, DEAE is often used with sephadex or sepharose employed as the stationary matrix. This resin manipulates a potentially low isoelectric point for the enzyme, in comparison to the pH of the
buffer used. Goosens et al., 1995 used this column when purifying PO from human lymphocytes but followed it with Q-Sepharose, another anion exchanger and employed exactly the same protocol for both columns. Molecular size is another property of proteins that can be utilised for purification purposes. Depending on the source the enzymatic molecular size will vary and the resin pore size used varies accordingly. They range from Sephadex G-150 (Moriyama and Sasaki, 1983) to S-300 (Chevallier et al., 1992), illustrating the wide molecular weight variety to be accounted for. Hydrophobic Interaction Chromatography is also employed with Phenyl Sepharose representing one of the resins (Goosens et al., 1995; Cunningham and O'Connor, 1998). However this technique is not as popular as the two aforementioned chromatographies.

Some of the better purification factors generated during PO purification, have been achieved by somewhat ‘unorthadox’ resins, as they may have been constructed specifically for PO. Yoshimoto et al., 1977 employed the affinity resin, Z-Pro-d-Ala-poly(Lys)-Sepharose 4B to bind the enzyme’s active site and provide a quick purification method. In 1982, Yoshimoto et al., incorporated trypsin onto Sepharose to purify ‘post-proline cleaving enzyme’. Goosens et al., 1995 immobilised albumin-reactive antibodies onto a resin and it provided a very effective method to purify PO. The purification factor increased from 348 to 2,550 after the anti-albumin step illustrating the resin’s potential.

The range of purification factors generated during Prolyl Oligopeptidase purification vary widely depending on the resin utilised as well as the source of enzyme origin. It should be noted too that sources of comparatively low protein content may not return as high a specific activity value as others, mainly due to the smaller fractional decreases in protein content.

1.8.6. Biochemical Characteristics

PO has been extensively characterised to date, however early reports return conflicting evidence. The enzyme was thought to be a dimer with a molecular weight of 115-140kDa (Koida and Walter, 1979). It is now widely known that mammalian, plant and microbial forms of PO are monomeric with an average molecular weight of 75kDa (Kanatani et al., 1993). Amino acid sequences have been obtained from cDNA clones of PO which verify a monomeric structure. Porcine brain PO revealed a 710 amino acid sequence (Rennex et al., 1991) which is a common characteristic of the human enzyme. PO from F. meningosepticum is a slightly smaller protein of 705 amino acids (Yoshimoto et al., 1991), which Chevailler et al confirmed in 1992. However they also discovered a signalling pro-peptide of 20 amino acids preceding the mature enzyme, which when removed reduced the molecular weight from 78.71kDa to 76.78kDa.
Mammalian and bacterial PO generally have a broadly neutral pH optimum, depending on the source. PO from mammalian species have an acidic isoelectric point of approximately 4.8 (Goosens et al., 1995). Bacterial PO isoelectric points are somewhat higher and are considerably dependent on the microbial source. This was illustrated by Yoshimoto et al., in 1988 when Lyophyllum cinerascens PO had a pI of 5.2, however previously in 1980, PO from \textit{F. meningosepticum} had a comparatively very high pI of 9.6.

Both mammalian and microbial PO have a physiological temperature optimum of 37-40°C (Kanatam et al., 1993, Yoshimoto et al., 1983). Most temperature stability studies involve carrying out the enzymatic reaction at different temperatures for 30 minutes to ascertain the temperature at which 50% of activity is reduced. For carrot PO, this proved to be 43°C (Yoshimoto et al., 1987), while the same temperature was observed for lamb kidney PO (Yoshimoto et al., 1981). However, Harwood and Schreier, 2001 report a temperature optimum and stability in the region of 85-90°C for the thermophile, \textit{P. furiosus}.

18.7. Catalytic Classification

PO is classified as a serine protease mainly based on its inhibition by DFP, proline-containing chlorometanes and to a lesser extent PMSF. This is achieved by the high specificity of DFP for the catalytic serine of PO. This was confirmed by Yoshimoto et al., in 1981 when 0.1 mM DFP completely inhibited PO activity from both lamb brain and kidney. PO is also surprisingly inhibited by cysteine protease inhibitors. The most potent reagent is p-chloromercuribenzoate (pCMB), when 0.1 mM completely abolished bovine brain PO activity and reduced carrot PO to 89%. Interestingly, pCMB had almost no effect on \textit{F. meningosepticum} (Yoshimoto et al., 1987). A study of two other cysteine protease inhibitors N-ethylmaleimide (NEM) and iodoacetamide was carried out on PO. The larger molecule NEM seemed to inhibit the enzyme effectively (85%) while iodoacetamide only reduced activity by 50%. This would suggest the presence of a cysteine residue that is close enough to the active site and exclusion of a substrate when complexed with a bulky molecule could result. A smaller reagent such as iodoacetamide may only be able to exert an incomplete steric hinderance (Polgar, 1991). The cysteines of the non-catalytic \( \beta \)-propeller domain was also proved to influence catalytic capability (Szeltner et al., 2000). The Cys255 residue was mutated to three different variants, one of which, Thr was not inhibited by NEM, indicating that Cys255 contributes to the catalysis of the peptidase domain. In contrast, it appears that this cysteine residue may not be adjacent to the active site for microbial PO and therefore not close enough to influence catalytic activity. A common agreement with all reports is the absence of a metallo residue near the enzyme’s active site. This was illustrated by the use of...
common metallo-protease inhibitors, where lamb brain and kidney PO were not affected by 10mM EDTA nor phenanthroline (Yoshimoto et al., 1981).

Confirmation of PO as a serine protease was ultimately achieved by cloning the PO and deducing its amino acid sequence. However it also shows no apparent similarity with other proteins in its class. As previously discussed, PO is classified as an ‘unclassical’ serine protease like DPP IV but dissimilar to trypsin or subtilisin, containing the catalytic triad arrangement of Ser-Asp-His. Also like DPP IV, the active site is located at the C-terminal end of the protein. The position of the active site serine may vary depending on the source, however the general consensus is that Ser554 represents the active site serine in the 710 amino acid mammalian enzyme. His680 is the common third residue of the catalytic triad, however the position of the Asp residue initially caused some confusion. Asp529 was concluded to be located in a neutral, hydrophilic segment and possibly a member of the active site (Barrett and Rawlings, 1992). However Goosens et al., in 1995 confirmed that in fact Asp641 was the final active site residue, resulting in a catalytic triad arrangement of Ser554, Asp641, His680.

PO’s similarity to DPP IV reveals an identical consensus sequence around the active site serine such as GXSXGG, which is significantly dissimilar to other serine proteases (Fig. 1.8.6.) This sequence may be of considerable benefit in identifying other members of this family. Members of the Prolyl Oligopeptidase Family (S9) are quite diverse. It includes PO, which is confined to action on oligopeptides (EC 3.4.21.26), DPP IV, an ectopeptidase that cleaves N-terminal dipeptides only when the N-terminal is unblocked (EC 3.4.14.5) and acyl-aminoacyl peptidase, an omega peptidase that preferentially cleaves N-terminal acetyl-aminoacyl residues from polypeptides (EC 3.4.19.1) (Rawlings et al., 1991).

1.8.8. Mechanism of Action

Prolyl Oligopeptidase catalytic mechanism has revealed further distinctions from other extensively characterised serine proteases, such as the chymotrypsin and subtilisin families, but has similarities with DPP IV. A common phenomenon for all serine peptidases is that the catalytically competent histidine residue facilitates both the formation and decomposition of the intermediate acyl-enzyme that is formed. It is the ionisation of this residue that determines the pH dependence on catalysis. In general, it is these acid/base reactions that are rate-limiting for chymotrypsin/subtilisin families while ionic strength has little or no effect. In contrast, PO catalysis displays remarkable sensitivity to ionic strength (Polgar, 1991). As a consequence, it is postulated that two catalytic active PO forms could exist. As expected, ionisation of the catalytic histidine exhibits a pKa of 8.0. However a second significant ionisation event in pH
dependent kinetics is observed at pKa 6.0. Analysis of the two PO forms revealed that the rate-determining step of the low pH form followed the general acid/base catalysis, while that of the high pH form was due to a physical step. This was confirmed by Polgar, 1992c, using substrates with different leaving groups which did not seem to affect the rate of PO catalysis.

Thus it was concluded that the rate was limited physically rather than chemically, as a substrate with a better leaving group is known to react with a peptidase at a higher rate, providing the rate-limiting step is chemical.

Like DPPIV, PO is a member of the α/β hydrolase fold family, which comprises of a catalytic or peptidase domain and a non-catalytic β-propeller domain (Goosens et al., 1995). This propeller domain contains a seven-fold repeat of four-stranded anti-parallel β-sheets radially arranged around a central tunnel, which leads to cavity that contains the catalytic triad residues. The entrance to the active site is via a narrow hole at the bottom of the central tunnel but is normally too narrow to allow access of peptide substrates (Fulop et al., 1998). The flexible side chains that partially cover this hole may allow movement, thus widening the entrance, which in turn could be assisted by the opening of the propeller between the first and seventh blade. This opening induced by a substrate may explain the rate-limiting physical step or conformational change and any substrate-size limitations.

The ability of PO to hydrolyse a substrate is dictated by the presence of a particular residue (proline or alanine), the location of the residue and the length of the substrate. Studies on PO from several sources indicate that the active site of the enzyme is composed of five binding subsites, S1, S2, S3, S1', and S2', showing high stereospecificity in the S2, S1 and S1' subsites (see Fig. 15). PO's ability to also hydrolyse alanyl bonds is significant but occurs at a much lower rate than prolyl bonds. However, PO is unable to hydrolyse Pro-Pro bonds or oligoproline while it can hydrolyse oligoalanine, suggesting that the mode of hydrolysis is different for both residues or the restrictions conferred by these residues may differ. The lack of hydrolysis of d-isomer oligoalanine in the P1 and P1' position has highlighted the high specificity of the interactions of the P1-S1 and P1'-S1' residues (Koide and Walter, 1976, Yoshimoto et al., 1977). It is accepted that the S1 subunit of PO is designed to specifically fit proline residues (P1) but can tolerate residues carrying substituent groups, provided they do not exceed the size of the proline pyrrolidine (Nomura, 1986). The rate of cleavage of the Pro-Xaa bond was observed to be fastest when Xaa (P1) was a hydrophobic residue with a decreased rate for basic and acidic residues. The smallest peptide cleaved is a tetrapeptide or an N-blocked tripeptide amide, illustrating that the enzymes S3, S2, S1 and S1' subsites (Fig.
need to be occupied for catalysis. Another characteristic of PO substrate hydrolysis is that low molecular weight peptides seem to be hydrolysed much quicker than larger molecules. In addition, PO is unable to act on larger proteins, such as albumin, collagen and myoglobin, and in general can not hydrolyse peptides/proteins greater than 25–30 amino acids long (Koida et al., 1976). This substrate-size limitation is believed to be influenced solely by the tunnel/β-propeller structure deduced.

**Figure 15** Schematic Representation of an Enzyme-Substrate Complex
Prolyl Oligopeptidase has 3 catalytic subsites at the amino terminal side of the cleavage site (S3, S2 and S1) and 2 at the carboxyl side (S1' and S2') (Nomenclature Committee, 1992)

189 Inhibitors

An enzyme's catalytic mode of hydrolysis as well as its physiological function may often be ascertained with the use of specific inhibitors. The compounds are typically substrate analogues that can bind to the enzyme's active site. Therefore many PO specific inhibitors have been synthesised and are used in vivo but ideally should be able to cross the blood-brain barrier.

The first compound that was synthesised and successfully inhibited PO specifically was Z-Pro-Pro-hnal (ZPP) (Wilk and Orlowski, 1983). This compound is very suitable structurally, as its chain length can fit the required three catalytic enzyme subsites, S1-S3. The compound was reported to be a non-competitive, transition state inhibitor with a $K_i$ of 14nM for rabbit brain PO. However in 1990, Bakker et al., proposed that ZPP is a competitive, slow-tight binding inhibitor of mouse and human brain PO, generating $K_i$ values of 0.35nM and 0.5nM.
respectively. It was assumed that this compound has such a potent effect by binding to the enzyme’s subsite and forming a hemiacetal with the active site of the enzyme.

As a consequence of the potency of ZPP, many analogues have been investigated as potential PO inhibitors. With the P2, prolyl residue substituted for other amino acids, Z-Val-Proinal proved most potent with a $K_i$ of 2.4nM for PO (Yoskosawa et al., 1984). Similarly, alternative studies changed the prolyl residue to thio proline and proline to thio proline or thiazolidine. The resulting compounds, Z-thiopropyl-thioprolinal and thioprolinal-thiazolidine, yielded improved $K_i$ values of 0.01nM and 36nM respectively (Tsuru et al., 1988). These reports acknowledge the significant effect of the benzyloxycarbonyl (Z) group at the P3 position, fulfilling the S3 subsite preference for bulky, hydrophobic residues. Joyeau et al., 2000 synthesised a number of pyrrolidinyl and thiazolidinyldipeptide derivatives as potential PO inhibitors from Trypanosoma cruzi, the agent for Chagas disease in Latin America. They introduced either (i) a vinyl sulfone, (ii) a 2-ketobenzothiazole, (iii) a nitrile or (iv) a benzimidazole as surrogate of the P1-P1' scissile bond in the substrate. Only the 2-ketobenzothiazole and nitrile derivatives have a significant potent effect on PO activity with $K_i$ values of 139 and 38nM respectively. The nitrile derivative is especially potent, possibly by mimicking the initial covalent enzyme adduct through an imidate function and it inhibits reversibly.

The N-terminal blocked inhibitors proved very potent when developing Fmoc dipeptide nitrile derivatives, for PO inhibition (Li et al., 1996). Both Fmoc-Ala-Pro-CN and Fmoc-Pro-Pro-CN inhibited in a similar potent manner with $K_i$’s of 5nM. In addition, the inhibitors are stable, cell permeable and capable of penetrating the blood-brain barrier. BOC is also another N-blocking group that when attached to particular di- or tripeptides, results in potent PO inhibitors with $K_i$ values in the sub μm range (Yoshimoto et al., 1985; Demuth et al., 1993; Stockel-Maschek et al., 2000). JTP-4819 contains a benzylaminocarbonyl blocked dipeptide carboxamide and has a strong resemblance to Z-Pro-Proinal (Toide et al., 1996). The inhibitor is orally active with a $K_i$ of 0.83nM and it has been found to cause no significant toxicity. It has been used for the treatment of Alzheimer’s disease by restoring TRH levels and therefore reducing memory impairment (Shinoda et al., 1996).

Many bacterial derived inhibitors have been analysed to ascertain potential PO inhibition both in vitro and in vivo. Postatin, produced by Streptomyces viridochromogenes is a significant PO inhibitor exhibiting an IC$_{50}$ value of 0.03μg/ml (Aoyagi et al., 1991). A variety of postatin derivatives have been synthesised in an attempt to obtain a greater inhibitory potency and
selectivity. The (s)-3-amino-2-oxovaleryl moiety analogue increases inhibition to an IC50 of 58ng/ml (Tsuda et al., 1996). However, it should be acknowledged that it is possible that the bacterial inhibitor may not be specific to PO.

A number of naturally occurring inhibitors have been purified from a variety of mammalian tissues, including porcine pancreas (Yoshimoto et al., 1982) and bovine brain (Ohmori et al., 1994). Amino acid analysis on the purified inhibitor from bovine brain, revealed it to be identical to segment 38-55 of glial fibrillary acidic protein and generated a Ks of 8.6μM.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Potency</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Mammalian</td>
<td></td>
<td></td>
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<tr>
<td>Fmoc-Ala-Pro-CN</td>
<td>Ks = 5nM</td>
<td>Li et al., 1996</td>
</tr>
<tr>
<td>Fmoc-Pro-Pro-CN</td>
<td>Ks = 5nM</td>
<td>Li et al., 1996</td>
</tr>
<tr>
<td>JTP-4819</td>
<td>IC50 = 0.83nM</td>
<td>Toido et al., 1995</td>
</tr>
<tr>
<td>Z-Thioprol-thioprolinal</td>
<td>Ks = 0.01nM</td>
<td>Tsuru et al., 1988</td>
</tr>
<tr>
<td>Z-Pro-Proinal</td>
<td>IC50 = 0.74nM</td>
<td>Wilk and Orlowski, 1983</td>
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<td>Bacterial</td>
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</tr>
<tr>
<td>Erystatin A</td>
<td>IC50 = 0.004μg/ml</td>
<td>Toda et al., 1992</td>
</tr>
<tr>
<td>Postatin</td>
<td>IC50 = 0.03μg/ml</td>
<td>Aoyagi et al., 1991</td>
</tr>
</tbody>
</table>

**Table 1.5** PO Specific Inhibitors
2.0. MATERIALS AND METHODS
2.1. MATERIALS

**Sigma Chemical Company (Poole, Dorset, England)**

1, 10 Phenanthroline  
1, 7 Phenanthroline  
2-Iodoacetamide  
2-Mercaptoethanol  
4, 7 Phenanthroline  
7-amido-4-methylcoumarin  
8-Hydroxyquinoline  
Acetic Acid  
Adenosine Deaminase  
AEBSF  
Ala-MCA  
Ammonium Persulphate  
Ammonium Sulphate  
Antipain Hydrochloride  
Apoferitin  
Aprotinin  
Arg-MCA  
Bacitracin  
Benzamidine  
Bisacrylamide  
Biuret Reagent  
Blue Dextran  
Bovine Serum Albumin  
Brilliant Blue Concentrate  
Cadmum Chlorde  
Cadmum Sulphate  
Calcium Sulphate  
CDTA  
Cellulose Dialysis Tubing  
Cellulose type 50  
Coabalt Sulphate  
Dithiothreitol  
DTNB  
EDTA  
EGTA  
Glycine  
HEPES  
High Range Molecular weight markers  
Hydrochloride Acid  
Imidazole  
Iodoacetate  
Iodoacetic Acid  
Isopropyl Alcohol  
Leupeptin  
Magnesium Sulphate  
Manganese Sulphate  
Mercurc Sulphate  
MES  
Methanol  
Methyl-D-Mannoside  
MOPS  
N-Ethylmaleimde  
Nickel Sulphate  
pChloromercunbenzoate  
PEG-8000  
Pepstatin  
Phenylmethysulphonylfluonide  
Potassium Phosphate (dibasic)  
Potassium Phosphate (monobasic)  
Pro-MCA  
Puromycin  
SDS  
Sodium Chlorde  
Sodium Phosphate (dibasic)  
TEMED  
Thyroglobulin  
Trizma Base  
Zinc Sulphate
BDH Chemicals Ltd. (Poole, Dorset, England)
- Acrylamide
- Biuret Reagent
- Bromophenol Blue
- Calcium Chloride
- Citric Acid
- Copper Sulphate
- Dimethylformamide
- Dimethylsulphoxide
- Dioxane
- Glycerol
- Polyethylene Glycol 6000

Pharmacia Fine Chemical Company (Uppsala, Sweden)
- Benzamidine Sepharose 6B
- Chelating Sepharose Fast Flow
- CNBr-activated Sepharose
- Con-A Sepharose
- DEAE Sepharose Fast Flow
- G100 Sephadex
- Hiprep 16/60 Sephacryl S300
- Phenyl Sepharose CL-4B
- Polybuffer 74
- Polybuffer Exchanger 94
- QAE Sepharose HiLoad Fast Flow
- Sephacryl S300 HR

PerSeptive Biosystems (Cambridge, MA, USA)
- Poros 50 HQ Anion Exchanger

Bio-Rad Laboratories (Hercules, California, USA)
- Coomassie Blue Stain Solution
- Coomassie Detain Solution
- IEF standards (pl 4-6-9.6)

Kepak Meats (Clonee, Co Meath, Ireland)
- Bovine Whole Blood

Pierce Chemical Company (Illinois, USA)
- BCA Reagent
- Coomassie Protein Assay Kit
- Coomassie Protein Plus Assay Kit
- Gelcode Bluestain
- Glycoprotein Kit

Novex (San Diego, California, USA)
- Anode Buffer
- Cathode Buffer
- Precast IEF gels

Merck Chemical Company (Frankfurt, Germany)
- Potassium Chloride
- Sodium Hydrogen Phosphate
- Sodium Hydroxide
<table>
<thead>
<tr>
<th>NUI Galway (Courtesy of Dr G O’Cuinn)</th>
<th></th>
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<tbody>
<tr>
<td>Ala-Pro</td>
<td>Gly-Pro-Pro</td>
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<td>Ala-Pro-Gly</td>
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<tr>
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<td>Asp-Pro-Gln-Phe-Tyr</td>
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<td>Glu-Pro-Glu-Thr</td>
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</tr>
<tr>
<td>Gly-Pro</td>
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<td>Gly-Pro-Hyp</td>
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<th>Calbiochem-Novabiochem (UK) Ltd. (Nottingham, England)</th>
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<tr>
<td>Angiotensin I</td>
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<tr>
<td>ACTH</td>
<td>Lys-Ala-MCA</td>
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<tr>
<td>Ala-MCA</td>
<td>Lys-Pro</td>
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<td>Arg-MCA</td>
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<td>Arg-Pro</td>
<td></td>
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<tr>
<td>Bradykumin</td>
<td>Neuropeptide Y(1-24) amide</td>
</tr>
<tr>
<td>ß-Casomorphin</td>
<td>Neurotensin (9-13)</td>
</tr>
<tr>
<td>CRP(201-206)</td>
<td>pGlu-MCA</td>
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<tr>
<td>Enterostatin</td>
<td>Pro-MCA</td>
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<td>Gly-MCA</td>
<td>Substance P</td>
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<td>Gly-Pro-Arg</td>
<td>Tyr-Pro-Phe</td>
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<td>Gly-Pro-MCA</td>
<td>Val-Pro</td>
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<td>GRP (Human)</td>
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<td>IGF-I(1-3)</td>
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<td>Leu-MCA</td>
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</table>

<table>
<thead>
<tr>
<th>School of Biotechnology, Dublin City University</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
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</tr>
</tbody>
</table>

56
2.2 FLUORESCENCE QUANTIFICATION OF 7-AMIDO-4-METHYL-COUMARIN (MCA)

2.2.1. MCA Standard Curves

100μM of stock MCA solution was prepared in 100mM potassium phosphate (KPO₄), pH 7.4 when analysing PO activity or 50mM HEPES buffer, pH 8.0 for DPPIV analysis. This was stored in a light sensitive container at 4°C to minimise any fluorescence. Lower MCA concentrations were prepared using 100mM potassium phosphate, pH 7.4 or 50mM HEPES, pH 8.0 to form standard curves of 0-2.5μM 0-10μM and 0-20μM. All curves were prepared as follows;

- 100μl 100mM KPO₄, pH 7.4 or 50mM HEPES, pH 8.0
- 400μl appropriate MCA concentration
- 1ml 1.5M acetic acid

Fluorescent Intensities were measured and recorded 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 10 while the emission slit width was adjusted according to the extent of fluorescent intensity observed from each standard curve. The emissions slit widths used were as follows;

<table>
<thead>
<tr>
<th>MCA Range</th>
<th>Emission Slit Width</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2.5μM</td>
<td>10</td>
</tr>
<tr>
<td>0-10μM</td>
<td>5.0</td>
</tr>
<tr>
<td>0-20μM</td>
<td>2.5</td>
</tr>
</tbody>
</table>

All solutions were assayed in triplicate and the mean fluorimetric intensity was calculated.

2.2.2 Inner Filter Effect

The inner filter effect or ‘quenching’ was determined as 2.2.1. but 100μl of 100mM KPO₄, pH 7.4 or 50mM HEPES, pH 8.0 was substituted for 100μl of enzyme sample. These samples were also prepared and assayed in triplicate.

2.2.3. MCA excitation and emission wavelengths

The optimum excitation and emission wavelengths for MCA were investigated using the fluorimeter scan functions. 5μM MCA was made up in distilled water, 100mM potassium phosphate, pH 7.4 and 50mM HEPES, pH 8.0, containing 0.2%v/v EtOH. 1.5ml 5μM MCA was placed in a glass cuvette and a pre-scan was performed. 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 Optimal wavelengths were taken to be those at which fluorescence was at a maximum.

2.3. PROTEIN DETERMINATION

2.3.1 Absorbancy at 280nm

The absorbance of proteins based on the $\lambda_{\text{max}}$ of tryptophan residues was used as a qualitative method of determining protein levels in post column chromatography fractions during purification procedures where the protein levels were greater than 10mg/ml or when a high concentration of salt was present A Shimadzu UV 160A Spectrophotometer was used to determine this absorbance. The fraction or sample was placed in a quartz cuvette and inserted into the cuvette holder of the machine and the optical density was recorded UV light at 280nm.

2.3.2 Biuret Assay

The Biuret assay was used to quantify the protein concentration in samples of 2-10mg/ml protein. These samples include bovine serum and post phenyl sepharose pooled samples. Where necessary, the sample being analysed was dialysed against 100mM potassium phosphate, pH 7.4 or 50mM Hepes, pH 8.0 for a minimum of 3 hours at 4°C to remove interfering substances such as incompatible buffers or salts. Any sample that may have a higher protein concentration than 10mg/ml was diluted with 100mM potassium phosphate, pH 7.4 or 50mM Hepes, pH 8.0. In order to quantify the protein concentration, a BSA standard curve was included from 0-10mg/ml in triplicate. The assay was carried out on a 96 Well plate as follows,

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

Absorbancies from the incubation were measured at 570nm using a Tecan Spectra Classic Plate Reader and a standard curve was constructed where necessary and the concentration of protein of the unknown samples estimated.

2.3.3 Coomassie Plus Protein Assay

The Coomassie Plus Protein assay was performed on fractions and samples of 0-1.5 mg/ml protein content and when interfering substances such as DTT were present thus dialysis wasn’t always necessary. In order to quantify the protein concentration, a BSA standard curve was included of 0-1.5mg/ml in triplicate. The assay was carried out on a 96 Well plate.
by adding 200μl of Coomassie Plus Protein reagent to 50μl of fraction or standard and read immediately after shaking using the Tecan Spectra Classic Plate Reader using a wavelength of 595nm. A standard curve was constructed and the protein concentration of the unknown fractions could be calculated.

2.3.4 Standard BCA Assay
The standard biuret acid (BCA) assay was used to quantify protein concentrations of samples between 0-2mg/ml. As a result of DTT's interference with the assay, post column PO fractions could not be measured using this method. Where necessary, samples were dialysed against 50mM Heps, pH 8.0 to remove any interfering substances. BSA standards of 0-12mg/ml were made up in 50mM Heps, pH 8.0 and were included with the unknown fractions or post column pools. The assay was carried out as described in section 2.3.2. 200μl of BCA reagent was added to 50μl of sample or BSA standard in triplicate in a 96 well micro-plate and incubated for 30 minutes at 37°C. Absorbance of each well was recorded as described in 2.3.2 and standard curves were calculated.

2.3.5 Enhanced BCA Assay
The enhanced BCA assay was carried out to quantify the protein concentration of samples that could not be determined by the less sensitive standard BCA assay protocol. The assay was performed as described in 2.3.3 but with an incubation temperature of 60°C for 30mins. Alternatively, the assay could also be performed as described in section 2.3.3 with an incubation temperature of 37°C for 60mins. In addition, a microwave method was also employed. The micro-plate was placed inside the microwave with a 100ml beaker of water, which acted as a heat sink. The plate was microwaved for 15-20 second and turned to ensure even heating. This was repeated in 5 second intervals until adequate colour development was observed. Absorbancies were read at 570nm with a BSA standard curve of 0-100μg/ml.

2.4 Enzyme Assays

2.4.1 Quantitative Measurement of Prolyl Oligopeptidase Activity
Human salivary Prolyl Oligopeptidase activity was determined by the degradation of Z-Gly-Pro-MCA. 10mM Z-Gly-Pro-MCA stock was prepared in 100% Methanol. The stock solution was diluted to 100μM with 100mM potassium phosphate, pH 7.4 with 5mM DTT and 0.5mM EDTA and the methanol concentration was adjusted to 4%. 100μl of enzyme sample was added to 400μl of thermally equilibrated 100μM Z-Gly-Pro-MCA in trypsinate and incubated at 37°C for 60minutes. The reaction was terminated by the addition of 1ml of 1.5M acetic acid. Negative controls were included by the addition of 1ml of 1.5M acetic acid.
to the substrate prior to the addition of the sample. The controls were also incubated at 37°C for 60 minutes. Liberated MCA was fluorometrically determined as described in section 2.2.1. Fluorimetric intensities observed were converted as outlined in section 2.4.1. Total enzyme units could then be defined as nanomoles of MCA released per min at 37°C (section 6.2).

2.4.2 Quantitative Measurement of Dipeptidyl Peptidase IV Activity

Dipeptidyl Peptidase activity was determined by the degradation of Gly-Pro-MCA. 10 mM Gly-Pro-MCA stock was prepared in 100% Ethanol. The stock solution was diluted to 100μM with 50mM Hepes, pH 8.0 to a final Ethanol concentration of 1%. 100μl of enzyme sample was added to 400μl of thermally equilibrated 100μM Gly-Pro-MCA in triplicate and incubated at 37°C for 60 minutes. The reaction was terminated by the addition of 1ml of 1.5M acetic acid. Negative controls were included by the addition of 1ml of 1.5M acetic acid to the substrate prior to the addition of the sample. The controls were also incubated at 37°C for 60 minutes. Liberated MCA was fluorometrically determined as described in section 2.2.1. Fluorimetric intensities observed were converted to picomoles MCA released per mm per ml using the relevant quenched MCA standard curve as outlined in section 2.2.1. Total enzyme units could then be defined as nanomoles of MCA released per min at 37°C.

2.4.3 Non-Quantitative Measurement of Prolyl Oligopeptidase activity

The non-quantitative assay was developed to also assist in the rapid detection of Z-Gly-Pro-MCA degrading activity in post column chromatography fractions. 200μl of 100μM Z-Gly-Pro-MCA in 100mM potassium phosphate, pH 7.4 with 5mM DTT and 0.5mM EDTA was added to 100μl of sample in each well and the micro-plate was incubated at 37°C for 60 minutes. MCA released was determined fluorometrically as outlined in section 2.2.1 using the Perkin-Elmer LS50 Luminescence Spectrometer plate reader attachment.

2.4.4 Non-Quantitative Measurement of Dipeptidyl Peptidase IV activity

The non-quantitative assay was developed to assist in the rapid detection of Gly-Pro-MCA degrading activity in post column chromatography fractions. 200μl of 100μM Gly-Pro-MCA in 50mM Hepes, pH 8.0 was added to 100μl of sample in each well and the micro-plate was incubated at 37°C for 60 minutes. MCA released was determined fluorometrically as outlined in section 2.2.1 using the Perkin-Elmer LS50 Luminescence Spectrometer plate reader attachment.
2.5 **Purification of Human Saliva PO**

All the purification procedures were carried out at 4°C at 1ml/min and 3ml fractions were collected unless otherwise stated.

2.5.1 **Saliva Preparation**

Saliva was collected at least 1 hour after eating to maximise PO levels. The resulting volume was diluted with an equal volume of 200mM potassium phosphate, pH 7.4 with 10mM DTT and 1mM EDTA. This created a final buffered saliva solution of 100mM potassium phosphate, pH 7.4 with 5mM DTT and 0.5mM EDTA.

2.5.2. **Ammonium Sulphate Precipitation**

Solid ammonium sulphate was added to 20ml of buffered saliva with constant stirring at 4°C to achieve 80% saturation which is equivalent to 4 molar ammonium sulphate, (Harris, 1989), while adjusting the pH to 7.4 with 1M NaOH. After 1 hour of constant stirring, the aliquot was centrifuged at 27,200g for 45mins at 4°C using a Beckman J2-MC refrigerated centrifuge fitted with a JA-20 rotor. The resulting supernatants were decanted off, pooled and their volumes recorded. Residual PO activity and protein content in the supernatants was detected as described in sections 2.4.2 and 2.3.2 respectively. The pellets were re-suspended in a total volume of 4ml of 100mM potassium phosphate, pH 7.4, with 5mM DTT and 0.5mM EDTA to give a final ammonium sulphate concentration of 1M.

2.5.3 **Phenyl Sepharose Hydrophobic Interaction Chromatography**

A 25ml phenyl sepharose CL-4B hydrophobic interaction column (2.5cm x 7cm) was equilibrated with 100ml of 100mM potassium phosphate, pH 7.4 with 5mM DTT and 0.5mM EDTA containing 1M ammonium sulphate at 1ml/min. 4ml of post ammonium sulphate precipitation PO sample was applied to the equilibrated column followed by a 100ml wash of the equilibration buffer. Bound protein was eluted by a 50ml isocratic step of 25mM potassium phosphate, pH 7.4 with 5mM DTT and 0.5mM EDTA. A further 25ml wash of 25mM potassium phosphate, pH 7.4 with 5mM DTT and 0.5mM EDTA ensured all bound protein was eluted. Fractions were assayed for PO activity as outlined in section 2.4.5. Protein was determined using the UV 280nm Protein protocol as described in section 2.3.1. Fractions containing Z-Gly-Pro-MCA degrading activity was combined and the volume recorded to yield post-phenyl sepharose PO. The phenyl sepharose resin was regenerated with a 30% isopropanol wash and then equilibrated as described previously.
2.5 4  Q-Sepharose Fast Flow Anion Exchange Chromatography

A 20ml pre-packed Q-Sepharose column attached to a BioRad BioLogic Chromatography system was equilibrated with 50ml of 50mM Tris/HCl, pH 8.0 with 5mM DTT and 0.5mM EDTA. The post-phenyl sepharose PO sample was dialysed overnight against 3l of Tris/HCl, pH 8.0 at 4°C. The resulting dialysate was concentrated via reverse osmosis using Polyethylene glycol 6000 for 1 hour until the volume was reduced to a quarter of its initial volume. 1ml of the concentrated dialysate was applied to the column and washed with 50ml 50mM Tris/HCl, pH 8.0 with 5mM DTT and 0.5mM EDTA. Partially bound protein was eluted with a 50ml linear gradient of 50mM Tris/HCl, pH 8.0 with 5mM DTT and 0.5mM EDTA to 50mM Tris/HCl, pH 8.0 with 5mM DTT, 0.5mM EDTA and 1M NaCl. PO was eluted with a further 50ml wash of 50mM Tris/HCl, pH 8.0 with 5mM DTT, 0.5mM EDTA and 1M NaCl. Fractions were analysed for Z-Gly-Pro-MCA degrading activity as described in section 2.4.4 and the fraction protein content was determined using the Coomassie assay as outlined in section 2.3.3 as well as the online system monitoring of the 280nm UV detector. Fractions with PO activity were pooled and the volume noted. The rate of MCA hydrolysis from Z-Gly-Pro-MCA was quantified as described in section 2.4.2. The QAE resin was regenerated with 50ml 2M NaCl and re-equilibrated with 50mM Tris/HCl, pH 8.0 with 5mM DTT and 0.5mM EDTA. All the purification protocols were carried at room temperature.

Pooled post-QAE fractions represented partially pure human PO, which was employed for any initial characterisation as enzymatic instability didn’t allow for any further purification. Where necessary, dialysis was performed for over 3 hours to remove Tris buffer or salt prior to characterisation. The most commonly used dialysis buffer was 100mM potassium phosphate, pH 7.4 with 5mM DTT and 0.5mM EDTA. Future reference to purified human PO during the characterisation procedure indicates post-Q Sepharose PO.

2.5.5 Alternative Chromatographic Techniques Used to Purify Human Salivary PO

2.5.5.1 Diethylaminoethyl Sepharose Anion Exchange Chromatography

A 20ml DEAE Sepharose column was equilibrated with 100ml 50mM Tris/HCl, pH 8.0 with 5mM DTT and 0.5mM EDTA. Post Phenyl Sepharose PO sample was dialysed and concentrated as described in section 2.5.4. 2ml of the dialysate was applied onto the column followed by a 50ml wash of equilibration buffer. Protein and enzymatic elution was carried out by 50ml of 50mM Tris/HCl, pH 8.0 with 5mM DTT, 0.5mM EDTA and 0.5M NaCl. Fractions were analysed for Z-Gly-Pro-MCA degrading activity and protein using the
Coomassie assay as outlined in sections 2.4.4 and 2.3.3 respectively. Fractions that contained PO activity were pooled and the volume recorded. The resin was regenerated with 2 column volumes of 2M NaCl and re-equilibrated with equilibration buffer.

### 2.5.5.2 Benzamidine Sepharose Chromatography

The p-aminobenzamidine sepharose 6B resin was employed to purify trypsin or trypsin-like serine proteases. The 5ml column was equilibrated with 50ml 50mM Tris/HCl, pH 8.0 with 5mM DTT and 0.5mM EDTA. 2ml of dialysed post-phenyl sepharose was applied to the column and washed with 45ml of equilibration buffer. Elution was performed by a 50ml isocratic wash of 50mM Tris/HCl, pH 8.0 with 5mM DTT, 0.5mM EDTA and 0.5M NaCl. A further 25ml wash of 50mM Tris/HCl, pH 8.0 with 5mM DTT, 0.5mM EDTA and 0.5M NaCl ensured all bound protein was eluted. Fractions were analysed for PO activity and protein as described in sections 2.4.4 and 2.3.3 respectively.

Any fraction that contained Z-Gly-Pro-MCA degrading activity was pooled and its volume noted. The resin was regenerated with 20ml 100mM Tris/HCl, pH 8.0 with 1M NaCl followed by 20ml 100mM Sodium Acetate, pH 4.5 with 1M NaCl. This regeneration step was carried out 3 times and re-equilibrated with equilibration buffer.

### 2.6 Purification of Bovine Serum DPPIV

All the purification procedures were carried out at 4°C unless otherwise stated.

#### 2.6.1 Serum Preparation

Bovine whole blood was collected from a freshly slaughtered animal and kept in a 4°C cold room for 24 hours to allow a clot to form and shrink. The remaining unclotted blood was then decanted and centrifuged at 6,370g for 60mins at 4°C using a Beckman J2-MC refrigerated centrifuge fitted with a JL-10.5 rotor. The supernatant and loose cellular debris was decanted and re-centrifuged at 48,400g for 15mins at 4°C using a JA-20 rotor. The serum was collected, pooled and stored in 20ml aliquots at -17°C.

#### 2.6.2 Phenyl Sepharose Hydrophobic Interaction Chromatography

A 25ml phenyl sepharose CL-4B hydrophobic interaction column (2.5cm x 7cm) was equilibrated with 100ml 50mM Hepes, pH 8.0 with 5mM EDTA containing 1M ammonium sulphate. Crude serum was thawed at 37°C and solid ammonium sulphate was added to give a final ammonium sulphate concentration of 0.5M. 5ml of salted serum was applied to the equilibrated column followed by a 100ml wash of the equilibration buffer to pass through any unbound protein. Bound protein was eluted by a 50ml linear gradient of 50mM Hepes, pH 8.0 with 5mM EDTA containing 1M ammonium sulphate to 50mM Hepes, pH 8.0 with 5mM...
EDTA. A further 25ml wash of 50mM Hepes, pH 8.0 with 5mM EDTA ensured all bound protein was eluted. Fractions were assayed for DPPIV activity as outlined in section 2.4.4. Protein was determined using the Biuret assay as described in section 2.3.2. Fractions containing Gly-Pro-MCA degrading activity was combined and the volume recorded to yield post-phenyl sepharose DPPIV. The phenyl sepharose resin was regenerated with a 30% isopropanol wash and then equilibrated as described previously.

2.6.3. HiPrep 16/10 Sephacryl S-300 HR Gel Filtration Chromatography
The post-phenyl sepharose DPPIV pool was concentrated via reverse osmosis using PEG. Glycerol was added to the concentrated sample to give a final glycerol concentration of 10% (v/v). The 120ml Hiprep 16/10 Sephacryl S300 High Resolution Gel Filtration separated proteins by molecular size. It was equilibrated with 150ml 50mM Hepes, pH 8.0 with 5mM EDTA using the Bio-Rad Biologic system or until a steady conductivity baseline could be observed. 1.5ml of concentrated post-phenyl sepharose DPPIV was loaded onto the column and washed with 150mM of equilibration buffer. Fractions collected were assayed for DPPIV activity using the micro-plate assay outlined in section 2.4.4. The protein content was detected using the standard BCA assay described in section 2.3.4 parallel with online UV monitoring at 280nm. Fractions that contained DPPIV activity were pooled and stored at 4°C. All the purification steps were performed at room temperature.

2.6.4. POROS 50 HQ Strong Anion Exchange Chromatography
A 25ml Poros 50 HQ anion exchange column was equilibrated with 50ml 50mM Hepes, pH 8.0 with 5mM EDTA. 5ml of post-Sephacryl S300 HR DPPIV was applied onto the column followed by a 50ml wash with equilibration buffer. Any bound protein was eluted with a 50ml linear gradient of 50mM Hepes, pH 8.0 with 5mM EDTA to 50mM Hepes, pH 8.0 with 5mM EDTA and 0.5M NaCl. A further 25ml wash of 50mM Hepes, pH 8.0 with 5mM EDTA and 0.5M NaCl ensured that all bound protein was eluted. Fractions were assayed for DPPIV activity as described in section 2.4.4. and protein content was determined by use of the enhanced BCA assay as described in section 2.3.5. Fractions that contained DPPIV activity were pooled and labelled. The Poros resin was regenerated with 2M NaCl and re-equilibrated with equilibration buffer.

2.6.5. Desalting by G100 Gel Filtration Chromatography
A 50ml G100 Gel Filtration column was equilibrated with 100ml 50mM Hepes, pH 8.0 with 5mM EDTA. 10ml of the post POROS DPPIV pool was applied onto the column and the resin was washed with 150ml 50mM Hepes, pH 8.0 at 0.5ml/min with 5mM EDTA. 3ml fractions were collected and were analysed for Gly-Pro-MCA degrading activity as outlined
in section 2.4.3 Protein content of the fractions was measured using the enhanced BCA assay as described in section 2.3.5. The conductivity was measured by a Jenway 4071 Conductivity Meter which gave an accurate 'real time' conductivity reading in units of mS/cm which is directly proportional to the salt content. The fractions had to be individually poured into universals so that the conductivity electrode could be immersed in the solution and therefore give an accurate reading. Fractions with DPPIV activity were combined, labelled and stored at −17°C until required.

Pooled post-G100 fractions represented purified bovine serum DPPIV, which was used in all DPPIV characterisation studies. Future reference to purified DPPIV indicates post-POROS 50 HQ DPPIV which was desalted via a G100 column.

### 2.6.6 Alternative Chromatographic Regimes Employed to Purify DPPIV

#### 2.6.6.1 Con-A Sepharose Chromatography

A 5ml Concanavalin A resin was employed to purify glycoproteins from bovine serum. The column was equilibrated with 25ml 50mM Hepes, pH 8.0 with 0.1M NaCl. 2.5ml of post-Sephaseryl S300 HR DPPIV was applied onto the column and washed with 25ml of equilibration buffer. Bound protein was eluted with a 25ml linear gradient of 50mM Hepes, pH 8.0 with 0.1M NaCl to 50mM Hepes, pH 8.0 with 0.1M NaCl and 0.5M Methylmannoside. This was followed by a final 10ml wash of 50mM Hepes, pH 8.0 with 0.1M NaCl and 0.5M Methylmannoside. Fractions were assayed for DPPIV activity as described in section 2.4.4. The enhanced BCA assay was employed to detect protein content in the fraction as outlined in section 2.3.5. Fractions that contained DPPIV activity were pooled and stored at −17°C. The resin was regenerated by washing the gel alternatively with 3 column volumes of high pH and low pH buffer solutions containing 0.5M NaCl. The buffers and volumes were 20ml 50mM Hepes, pH 8.5 with 0.5M NaCl followed by 20ml 50mM Sodium Acetate, pH 4.5 with 0.5M NaCl. This step was repeated three times and the resin was finally regenerated with equilibration buffer.
2.7. **PURITY DETERMINATION**

2.7.1 Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was employed to determine the purity and monomeric molecular weight of both post-QAE Human Salivary PO and post-Poros 50 HQ Bovine Serum DPPIV pools based on the method of Laemmli, (1970)

2.7.1.1 Sample Preparation

Samples collected during the purification process, namely, (i) buffered saliva, post 80% salt precipitation, post-phenyl sepharose PO, post-QAE PO and (ii) crude serum, post-phenyl sepharose DPPIV, post-S300 DPPIV and post-Poros 50 HQ DPPIV were electrophoresed using SDS-PAGE. All samples were extensively dialysed for 18 hours at 4°C against 2 l 62.5 mM Tris/HCl, pH 6.8, with buffer changes after 3 and 6 hours. Dialysed samples were diluted with an equal volume of solubilisation buffer, which was composed of 62.5 mM Tris/HCl, pH 6.8, 20% v/v glycerol, 8% SDS, 10% 2-mercaptoethanol and 0.01% w/v bromophenol blue. Sigma Marker High Range molecular markers were used which consisted of Myosin (205,000 Da), β-Galactosidase (116,000 Da), Phosphorylase b (97,000 Da), Fructose-6-phosphate kinase (84,000 Da), BSA (66,000 Da), Glutamic Dehydoergencase (55,000 Da), Ovalbumin (45,000 Da) and Glyceraldehyde-3-phosphate Dehydrogenase (36,000 Da). Samples in solubilisation buffer and markers were denatured by immersion in a boiling water bath for 60 seconds and kept on ice until application onto the gel.

2.7.1.2 Preparation of SDS Gel

**Table 2.1** lists the solutions and their compositions used in the SDS gel. All solutions were prepared in ultra-pure water.

<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>COMPOSITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acryl/Bisacryl Stock</td>
<td>30% w/v Acrylamide, 0 8% w/v Bisacrylamide</td>
</tr>
<tr>
<td>Resolving Gel Buffer</td>
<td>3M Tris/HCl, pH 8 8</td>
</tr>
<tr>
<td>Stacking Gel Buffer</td>
<td>0.5M Tris/HCl, pH 6 8</td>
</tr>
<tr>
<td>Ammonium Persulphate</td>
<td>1.5% w/v ammonium persulphate</td>
</tr>
<tr>
<td>SDS</td>
<td>10% w/v SDS</td>
</tr>
<tr>
<td>Running Buffer</td>
<td>0.025M Tris/HCl, 0.192M Glycine, 0 1% SDS, pH 8 3</td>
</tr>
</tbody>
</table>

**Table 2.1** SDS PAGE BUFFER PREPARATION

A 10% w/v resolving gel with a 3.75% w/v stacking gel was poured with the volumes documented in **Table 2.2**
<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>10% v/v RESOLVING GEL (Volume)</th>
<th>3 75% v/v STACKING GEL (Volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acryl/bisacryl solution</td>
<td>5ml</td>
<td>1.5ml</td>
</tr>
<tr>
<td>Resolving Gel Buffer</td>
<td>1.8ml</td>
<td>-</td>
</tr>
<tr>
<td>Stacking Gel Buffer</td>
<td>-</td>
<td>2.5ml</td>
</tr>
<tr>
<td>Ammonium Persulphate</td>
<td>0.75ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td>SDS</td>
<td>0.15ml</td>
<td>0.1ml</td>
</tr>
<tr>
<td>Ultra-Pure Water</td>
<td>7ml</td>
<td>5.4ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>7.5μl</td>
<td>7.5μl</td>
</tr>
</tbody>
</table>

**TABLE 2.2 SDS PAGE GEL PREPARATION**

Gels were cast in a BioRad Mini Gel set and positioned in an ATTO vertical electrophoresis system. The gel box was filled with running buffer and 20μl of the prepared samples and markers were loaded into the wells under buffer head. Electrophoresis was carried out at a constant current of 25mA per gel and 300V for approximately 2.5 hours.

2.7.1.3 Visualisation of Proteins

Polyacrylamide gels were stained using a Brilliant Blue G Colloidal stain kit or a Gelcode Blue stain reagent. TABLES 2.3 and 2.4 outline the stages performed. An image of each stained gel was recorded using an Olympus digital camera coupled to Olympus C-2 //OW95E software. Gels were also scanned using a Microtek ScanMaker 330.

<table>
<thead>
<tr>
<th>STEP</th>
<th>REAGENT</th>
<th>VOLUME</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixing</td>
<td>30% v/v Ethanol</td>
<td>2 x 200ml</td>
<td>1 x 10mins</td>
</tr>
<tr>
<td></td>
<td>10% v/v glacial acetic acid</td>
<td>1 x 30mins</td>
<td></td>
</tr>
<tr>
<td>Rinsing</td>
<td>Ultra-pure water</td>
<td>3 x 300ml</td>
<td>3 x 15mins</td>
</tr>
<tr>
<td>Staining</td>
<td>80% v/v brilliant blue</td>
<td>300ml</td>
<td>Overnight</td>
</tr>
<tr>
<td></td>
<td>20% v/v Methanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>De-staining</td>
<td>10% v/v acetic acid</td>
<td>200ml</td>
<td>5mins</td>
</tr>
<tr>
<td></td>
<td>25% v/v Methanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rinsing</td>
<td>25% v/v Methanol</td>
<td>2 x 200ml</td>
<td>2 x 30mins</td>
</tr>
</tbody>
</table>

**TABLE 2.3 BRILLIANT BLUE G COLLOIDAL STAINING PROCEDURE**
**TABLE 2-4**  GELCODE BLUE STAINING PROCEDURE

**2.7.2. Fluorimetric Assays**

Post-column chromatography PO and DPPIV activities were assayed for the presence of various peptidase activities incubating the enzyme with different fluorimetric substrates. The substrates, their preparation and the peptidases they are commonly used to detect are listed in **TABLE 2-7.6**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity Detected</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala-MCA</td>
<td>Alanine Aminopeptidase</td>
<td>Buffer</td>
</tr>
<tr>
<td>Arg-MCA</td>
<td>Arginine Aminopeptidase</td>
<td>Buffer</td>
</tr>
<tr>
<td>Glu-Pro-MCA</td>
<td>Chymotrypsin</td>
<td>Buffer</td>
</tr>
<tr>
<td>Gly-Pro-MCA</td>
<td>Dipeptidyl Aminopeptidase IV</td>
<td>50mM Hepes, pH 8.0</td>
</tr>
<tr>
<td>Leu-MCA</td>
<td>Leucine Aminopeptidase</td>
<td>Buffer</td>
</tr>
<tr>
<td>Lys-Ala-MCA</td>
<td>Dipeptidyl Aminopeptidase II</td>
<td>Buffer</td>
</tr>
<tr>
<td>Lys-MCA</td>
<td>Aminopeptidase B</td>
<td>Buffer</td>
</tr>
<tr>
<td>Lys-Pro-MCA</td>
<td>Dipeptidyl Peptidase IV</td>
<td>Buffer</td>
</tr>
<tr>
<td>Na-benzoyl-Arg-MCA</td>
<td>Papain</td>
<td>4%v/v Ethanol</td>
</tr>
<tr>
<td>Pro-MCA</td>
<td>Proline Aminopeptidase</td>
<td>Buffer</td>
</tr>
<tr>
<td>Pyr-MCA</td>
<td>Pyroglutamyl Aminopeptidase I</td>
<td>4%v/v Ethanol</td>
</tr>
<tr>
<td>Pyr-His-Pro-MCA</td>
<td>Pyroglutamyl Aminopeptidase II</td>
<td>Buffer</td>
</tr>
<tr>
<td>Z-Arg-MCA</td>
<td>Trypsin, Papain, Soya-bean trypsin-like enzyme</td>
<td>4%v/v Ethanol</td>
</tr>
<tr>
<td>Z-Gly-Pro-MCA</td>
<td>Prolyl Oligopeptidase</td>
<td>4%v/v Ethanol</td>
</tr>
<tr>
<td>Z-Phe-Arg-MCA</td>
<td>Plasma kallikrein, Papain, Cathepsin B</td>
<td>Buffer and L, Oligopeptidase B</td>
</tr>
</tbody>
</table>

**TABLE 2-5. FLUORIMETRIC SUBSTRATES TESTED**
The buffer used for all assays was 100mM potassium phosphate, pH 7.4 for Prolyl Oligopeptidase and 50mM hepes, pH 8.0 for Dipeptidyl Peptidase IV. All substrates were prepared to 100μM and purified enzyme was incubated with each substrate and assayed as outlined in sections 2.4.1 and 2.4.2. Negative controls of each substrate were included which consisted of buffer being substituted for enzyme.

2.8. ASSAY DEVELOPMENT

2.8.1. Substrate Solvent Determination (PO/DPPIV)
In order to determine the most compatible solvent to prepare the substrate, 10mM Z-Gly-Pro-MCA in the case of PO and 10mM Gly-Pro-MCA for DPPIV stock solutions were dissolved in 100% Dimethyl Sulphoxide (DMSO), Dimethylformamide (DMF), Dioxane (DXN), Ethanol and Methanol. These solutions were diluted to 100μM (0.1mM) with 100mM potassium phosphate, pH 7.4 for PO and 50mM Hepes, pH 8.0 for DPPIV. Each substrate had the solvent concentration adjusted to 4%v/v. Both enzymes were assayed in triplicate with suitable negative controls as outlined in sections 2.4.1 and 2.4.2.

2.8.2. Solvent Concentration Determination (DPPIV)
0.1mM Gly-Pro-MCA was prepared in a range of increasing Ethanol concentrations to assess the optimum for DPPIV activity. 10mM Gly-Pro-MCA in 100% Ethanol was diluted to 0.1mM with 50mM Hepes, pH 8.0 and the ethanol concentration was adjusted to 1%, 2%, 4%, 6%, 8% and 10%. The assay was carried out as described in section 2.4.2 and the suitable negative controls included.

2.8.3. Linearity of Enzyme Assays with Respect to Time

2.8.3.1. Discontinuous Assay
In order to assess the effect of the purified enzyme in cleaving its corresponding substrate over the allocated assay time, the enzymes were assayed as described in sections 2.4.1 and 2.4.2. Reactions were terminated with 1.5M acetic acid after 10, 20, 30, 40, 50 and 60mins and the fluorescence measured. Plots of fluorescent intensity versus time were constructed.

2.8.3.2. Continuous Assay
In addition to the discontinuous assay, a continuous assay was also performed. 300μl of purified enzyme and 1.2ml of 0.1mM Z-Gly-Pro-MCA in 100mM potassium phosphate, pH 7.4 with 5mM DTT for PO and 1.2ml of 0.1mM Gly-Pro-MCA in 50mM Hepes, pH 8.0 for DPPIV were pre-incubated separately at 37°C for 15mins to allow thermal equilibrium.
Substrate and enzyme were combined in a pre-warmed cuvette in a holder attached to a water bath at 37°C. The fluorescence was continuously monitored by the Perkin Elmer LS-50 Luminescence Spectrometer for 60mins. Plots of fluorescent intensity versus time were prepared.

2.8.4. Linearity of Enzyme Assays with Respect to Enzyme Concentration (DPPIV)
A range of dilutions of purified DPPIV were prepared in 50mM Hepes, pH 8.0. The enzyme dilutions were assayed in triplicate with 0.1mM Gly-Pro-MCA as described in section 2.4.2. A plot of fluorescent intensity versus enzyme concentration (%) was constructed.

2.8.5. Optimum Assay Temperature (DPPIV)
Purified DPPIV was assayed at 4°C, 24°C, 30°C, 35°C, 37°C, 40°C, 50°C and 60°C by a modification of the method described in section 2.4.2. Both the substrate and enzyme were pre-incubated at the appropriate temperature for 15 minutes prior to the assay to ensure uniform thermal equilibrium had been reached.

2.8.6. Effect of DTT on Substrate Hydrolysis

2.8.6.1. Effect of DTT on PO activity
2ml aliquots of 0.1mM Z-Gly-Pro-MCA in 4% Methanol were prepared incorporating 0, 5, 10, 15, 20, 25 and 30mM of the cysteine protease activator, dithiothreitol (DTT) to assess its requirement for inclusion in the substrate in addition to the chromatography running buffers. Purified PO was assayed in triplicate as outlined in section 2.4.1.

2.8.6.2. Effect of DTT on DPPIV activity
1.6ml aliquots of 0.1mM Gly-Pro-MCA in 4% Ethanol were prepared incorporating 0, 2.5, 5, 10, 15, 20mM dithiothreitol (DTT). Purified DPPIV was assayed in triplicate as outlined in section 2.4.2.

2.8.7. Effect of EDTA on Substrate Hydrolysis

2.8.7.1. Effect of EDTA on PO activity
2ml aliquots of 0.1mM Z-Gly-Pro-MCA in 4% Methanol were prepared incorporating 0, 0.2, 0.4, 0.6, 0.8, 1, 1.5 and 2mM of the metal chelator, EDTA to explore its potential for inclusion in both the substrate and chromatography running buffers. Purified PO was assayed
in triplicate as outlined in section 2.4.2. The protocol was repeated including 5mM DTT to
assess the optimised conditions. Purified PO was assayed as described in section 2.4.1.

2.8.7.2 Effect of EDTA on DPPIV activity
1.6ml aliquots of 0.1mM Gly-Pro-MCA in 4% Ethanol were prepared incorporating 0, 1, 2.5,
5, 7.5, 10, 15 and 20mM EDTA. Purified DPPIV was assayed in triplicate as outlined in
section 2.4.2.

2.9 Stability Studies of PO

2.9.1 Effect of 37°C pre-incubation on PO activity
In order to determine the thermal stability of the Prolyl Oligopeptidase enzyme during the
assay, purified PO was incubated at 37°C over time and stored at 4°C until its activity was
monitored. 6ml of purified PO was placed in a container and immersed in a 37°C water-bath.
Over a period of 4 hours, 450μl aliquots of the sample were removed from the water-bath,
labeled and stored at 4°C. In addition, the stabilising effect of the protein, bovine serum
albumin was also investigated. 1% and 10% w/v BSA was added to the 'purified PO' and
both were maintained at the required temperature for 90mins. The activity of all fractions was
assayed as outlined in section 2.4.1.

2.9.2 Stability of PO when stored at -20°C
Post QAE PO was normally stored in a frozen state in the freezer. Therefore the stability of
the enzyme in these conditions was explored. The difference in stability when 1% BSA w/v
was added to the enzyme was also investigated. Two sets of five 0.5ml aliquots of post-QAE
PO were prepared. To one set of the aliquots, 1% BSA w/v was added (5mg BSA in 0.5ml).
Four of the five fractions were then labelled and frozen. 20ml of 100μM Z-Gly-Pro-MCA
with 5mM DTT and 0.5mM EDTA was also prepared, aliquoted into five 4ml fractions,
labeled and frozen. The remaining aliquot of PO and PO with 1% BSA was assayed as
outlined in section 2.4.1. The activity results were then labelled as Day 0 or Positive Controls.
After 24 hours (Day 1), one of each aliquot was removed from the freezer, thawed
and the PO enzymatic activity analysed and recorded. This was repeated for Days 2, 3 and 31
(1 month).

A similar study was carried out on buffered saliva. The saliva was prepared as described in
section 2.5.1. The buffered saliva was labelled, aliquoted and frozen as already outlined. One
set of buffered saliva samples had 20% glycerol added to determine any potential stabilising
effect. Both sets were then assayed as outlined in section 2.4.1 for Days 0, 3, 5, 6 and 22.
2.10 CHARACTERISATION

2.10 1. Relative Molecular Mass Determination
The native molecular mass of Bovine Serum Dipeptidyl Peptidase IV was determined by Gel Filtration Chromatography while its monomeric molecular weight was estimated by Polyacrylamide Gel Electrophoresis

2.10 1.1 Sephacryl S-300 HR Gel Filtration Chromatography
A 120ml Hiprep 16/10, Sephacryl S-300 High Resolution, column was used. The protocol used was a modification of that described in section 2.6.3

2.10 1.1.1 Void Volume Determination
The Sephacryl S-300 HR column was equilibrated with 200ml 50mM Hepes, pH 8.0 with 100mM NaCl. 1ml of 2mg/ml blue dextran solution was loaded via the Inject mode on the BioLogic system. The column was washed with equilibration buffer until the blue dextran was visibly eluted. The online absorbance at 280nm was monitored and the volume at which the absorbance reached a maximum was estimated to be the void volume (V₀).

2.10 1.1.2 Column Calibration
The S300 HR column was equilibrated as outlined in section 2.9.1.2. 1ml of each standard was applied at a concentration of 2mg/ml containing 10% v/v glycerol. TABLE 2.6 lists the standards used and their molecular weights. The elution volume (Vₑ) of each standard was monitored and calculated by the Biologic in situ 280nm UV detector as outlined in section 2.10 1.1. A plot of Log molecular mass versus Vₑ/V₀ of each standard, was prepared and a calibration curve for the column was constructed.

<table>
<thead>
<tr>
<th>STANDARD</th>
<th>MOLECULAR WEIGHT (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroglobulm</td>
<td>669</td>
</tr>
<tr>
<td>Apoferritin</td>
<td>443</td>
</tr>
<tr>
<td>Alcohol Dehydrogenase</td>
<td>150</td>
</tr>
<tr>
<td>BSA</td>
<td>66</td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td>29</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>12</td>
</tr>
</tbody>
</table>

**TABLE 2.6 MOLECULAR WEIGHT MARKERS USED IN S300 CALIBRATION**
2.10.1.3 Estimation of Relative Molecular Mass of Purified DPPIV

The sephacryl S300 column was prepared and equilibrated as described in section 2.9.1.2. 1ml of purified DPPIV with 10% v/v glycerol was loaded onto the column. 3ml fractions were collected and assayed for DPPIV activity as outlined in section 2.4.4. In addition, the protein content of the fractions was monitored online by the 280nm UV detector. Both protocols allowed for the determination of the elution volume ($V_e$), where the maximum DPPIV activity corresponded with the maximum protein peak. The molecular mass of the enzyme was determined using the calibration curve constructed from section 2.9.1.3.

2.10.1.2 SDS Polyacrylamide Gel Electrophoresis

The SDS PAGE of the standards and samples was carried out as described in section 2.7.1. The gel obtained for purity assessment was also used for molecular weight determination as well as the number of sub-units that are present. A standard curve of the Log Molecular Mass versus the Relative Mobility ($R_f$) of each standard was constructed. The $R_f$ value is defined as the distance migrated by the standard or sample divided by the distance of the bromophenol blue dye front. The Log molecular mass of the purified enzymes was thus calculated from the calibration curve and its relative molecular mass estimated.

2.10.2 DPPIV Glycosylation Estimation of SDS gel

Polyacrylamide gels were also stained with a Gelcode Glycoprotein Staining kit. The gels were prepared and run as described in section 2.7.2. No markers were included but were substituted for a Positive glycosylated control of Horseradish Peroxidase and a Negative glycosylated control of Soyabean Trypsin Inhibitor. Each control was applied onto the gel in separate lanes. When electrophoresis was complete, the gels were stained employing the method outlined in TABLE 2.7. The gels were scanned using a Microtek ScanMaker 330 and the image was recorded.

<table>
<thead>
<tr>
<th>STEP</th>
<th>REAGENT</th>
<th>VOLUME</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixing</td>
<td>30% Ethanol</td>
<td>100ml</td>
<td>30mins</td>
</tr>
<tr>
<td>Rinsing/Washing</td>
<td>3% Acetic Acid</td>
<td>100ml</td>
<td>10mins</td>
</tr>
<tr>
<td>Oxidising</td>
<td>Oxidation Solution</td>
<td>25 ml</td>
<td>15mins</td>
</tr>
<tr>
<td>Washing</td>
<td>3% Acetic Acid</td>
<td>100ml</td>
<td>3 x 5mins</td>
</tr>
<tr>
<td>Staining</td>
<td>Staining Solution</td>
<td>25 ml</td>
<td>15mins</td>
</tr>
<tr>
<td>Reducing</td>
<td>Reduction Solution</td>
<td>25 ml</td>
<td>5mins</td>
</tr>
<tr>
<td>Washing</td>
<td>3% Acetic Acid</td>
<td>100ml</td>
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</tr>
<tr>
<td>Rinsing/Washing</td>
<td>3% Acetic Acid</td>
<td>100ml</td>
<td>10mins</td>
</tr>
<tr>
<td>Oxidising</td>
<td>Oxidation Solution</td>
<td>25 ml</td>
<td>15mins</td>
</tr>
<tr>
<td>Washing</td>
<td>3% Acetic Acid</td>
<td>100ml</td>
<td>3 x 5mins</td>
</tr>
<tr>
<td>Staining</td>
<td>Staining Solution</td>
<td>25 ml</td>
<td>15mins</td>
</tr>
<tr>
<td>Reducing</td>
<td>Reduction Solution</td>
<td>25 ml</td>
<td>5mins</td>
</tr>
<tr>
<td>Washing</td>
<td>3% Acetic Acid</td>
<td>100ml</td>
<td>30mins</td>
</tr>
</tbody>
</table>
2.10.4.3 pH Inactivation (DPPIV)

5 ml of post Poros 50 HQ Sepharose DPPIV was dialysed overnight against 2 l distilled water at 4°C. 50 μl of dialysate was pre-incubated with 50 μl of each 50 mM buffer listed in Table 2.8 at 37°C for 15 minutes. 100 μM Gly-Pro-MCA was prepared in 50 mM Heps, pH 8.0. The effect of the pH was determined by assaying enzyme activities in triplicate as outlined in section 2.4.2.

2.10.5 Isoelectric Point Determination of DPPIV

2.10.5.1 Chromatofocusing Chromatography

A 25 ml polybuffer exchanger (PBE 94) column was equilibrated with 300 ml 50 mM Imidazole/HCl pH 7.8. Polybuffer 74 was diluted 8-fold in distilled water and the pH adjusted to 4.5 using HCl. 15 ml of the diluted polybuffer was applied to the column after equilibration. 5 ml purified DPPIV was dialysed overnight against 3 l imidazole/HCl, pH 7.8 at 4°C and applied onto the chromatofocusing resin. The resin was washed with 300 ml polybuffer, pH 4.5. 3 ml fractions were collected throughout and assayed for DPPIV activity as outlined in section 2.4.4. The pH of the fractions was also measured and a plot of both fluorescent intensity and pH versus fraction number was constructed.

2.10.5.2 Isoelectric Focusing

A vertical pre-cast isoelectric focusing (IEF) gel system was employed to determine the isoelectric point of purified DPPIV. The enzyme was dialysed overnight against 2 l 50 mM Heps, pH 8.0 to remove any IEF contaminants such as salt. The dialysed sample was diluted with an equal volume of sample buffer (Table 2.9). The gels were prepared in an ATTO vertical electrophoresis system (160 mm x 160 mm x 1 mm). After the bottom of the chamber was filled with 1 x anode running buffer, the gels were placed in position and the upper chamber filled with cathode buffer. 20 μl of each prepared sample and reconstituted markers were loaded into the wells under the cathode buffer. Electrophoresis was carried out at 100 V for 60 mins, 200 V for the second 60 mins and 500 V for the final 30 mins with the current running from 5 mA-6 mA/gel. Staining was performed on the gel using brilliant blue G colloidal stain as outlined in Table 2.3. The stained gel image was captured using a UVP white/UV transluminator camera unit driven by Image-Store 7500 software. A standard curve was constructed of the pI versus the Rf for each of the IEF markers electroforesed. The Rf represents the distance migrated by the standard or sample divided by the distance to the anodic lip of the gel. The pI of DPPIV was estimated using the calibration curve by calculating its Rf value from the stained gel.

75
Anode buffer (50X) | 4.7% w/v phosphoric acid
Cathode buffer (10X) | 3.5% w/v arginine, 2.9% w/v lysine
Sample buffer | 20% v/v 10X cathode buffer, 30% v/v glycerol

**TABLE 2.9**  IEF Buffers used for pH Determination

<table>
<thead>
<tr>
<th>MARKER</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome C (horse heart)</td>
<td>10.7</td>
</tr>
<tr>
<td>Ribonuclease A (bovine pancreas)</td>
<td>9.5</td>
</tr>
<tr>
<td>Lectin (Lens culinaris)</td>
<td>8.3, 8.0, 7.8</td>
</tr>
<tr>
<td>Myoglobin (horse muscle)</td>
<td>7.4, 6.9</td>
</tr>
<tr>
<td>Carbonic anhydrase (bovine erythrocytes)</td>
<td>6.0</td>
</tr>
<tr>
<td>β-Lactoglobulin (bovine milk)</td>
<td>5.3, 5.2</td>
</tr>
<tr>
<td>Trypsin inhibitor (soyabean)</td>
<td>4.5</td>
</tr>
<tr>
<td>Glucose oxidase (Aspergillus niger)</td>
<td>4.2</td>
</tr>
<tr>
<td>Amyloglucosidase (Aspergillus niger)</td>
<td>3.5</td>
</tr>
</tbody>
</table>

**TABLE 2.10**  IEF Markers used for pH Determination

### 2.10.6. Catalytic Classification of DPPIV

A range of functional reagents was prepared as presented in TABLE 2.9 to assess the catalytic classification of Bovine Serum DPPIV. 20 ml purified DPPIV was dialysed overnight at 4°C against 3 l of 50 mM Hepes/HCl, pH 8.0. All stock solutions were prepared in 50 mM Hepes and 1 M HCl was added to ensure a final pH of 8.0. Water insoluble reagents were first dissolved in a minimum volume of acetone and the volume's and pH's adjusted using HCl. Lower concentrations of each functional reagent were achieved using 50 mM Hepes/HCl, pH 8.0 ± acetone as appropriate (TABLE 2.11). 50μl of the dialysed enzyme was pre-incubated for 15 min at 37°C with an equal volume of each functional reagent under investigation. Positive controls were included where the functional reagent was replaced with 50 mM Hepes/HCl buffer, pH 8.0 containing the appropriate percentage v/v acetone as the corresponding reagent. DPPIV activity was determined fluorimetrically in triplicate as detailed in section 2.4.1. The fluorescent filtering effect of these functional reagents was assessed by the construction of unfiltered and filtered MCA standard curves as described in sections 2.2.1, 2.2.2, and 2.4.2.
<table>
<thead>
<tr>
<th>CLASS</th>
<th>REAGENT</th>
<th>STOCK CONC</th>
<th>PREPARATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine protease inhibitors</td>
<td>AEBSF</td>
<td>45mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>APMSF</td>
<td>20mM</td>
<td>5%v/v acetone</td>
</tr>
<tr>
<td></td>
<td>Aprotinin</td>
<td>2.5mg/ml</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>Benzamidine</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>Leupeptin</td>
<td>2mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>PMSF</td>
<td>5mg/ml</td>
<td>Buffer</td>
</tr>
<tr>
<td>Metallo-protease inhibitors</td>
<td>1,10-phenanthroline³</td>
<td>40mM</td>
<td>10%v/v acetone</td>
</tr>
<tr>
<td></td>
<td>1,7-phenanthroline³</td>
<td>30mM</td>
<td>10%v/v acetone</td>
</tr>
<tr>
<td></td>
<td>4,7-phenanthroline³</td>
<td>40mM</td>
<td>10%v/v acetone</td>
</tr>
<tr>
<td></td>
<td>8-hydroxyquinoline³</td>
<td>20mM</td>
<td>5%v/v acetone</td>
</tr>
<tr>
<td></td>
<td>CDTA²</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>40mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>EGTA²</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>Imidazole</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Cysteine protease inhibitors</td>
<td>DTNB</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>Iodoacetamide</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>Iodoacetic acid</td>
<td>40mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>NEM</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Cysteine protease activators</td>
<td>2-mercaptoethanol</td>
<td>2%v/v</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>DTT</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
</tbody>
</table>

**TABLE 2.11 REAGENTS USED IN THE CATALYTIC CLASSIFICATION OF DPPIV**

Buffer is 50mM Hepes/HCl, pH of 8.0

1 Concentration of the functional reagent before added to the enzyme (i.e., 2X)

2 Samples had to be heated at 37°C to achieve dissolution

3 Samples had to be maintained at 50°C until diluted

### 2.10.7 Effect of General Functional Reagents on DPPIV

The effect of a range of some general functional reagents on DPPIV activity was also tested. As in section 2.10.6, all stock solutions were prepared in 50mM Hepes/HCl pH 8.0. Addition of minimum volume acetone aided the dissolution of water-insoluble compounds and as before 50mM Hepes/HCl pH 8.0 ± acetone was used as diluant. 6ml of purified DPPIV was dialysed against 2l of 50mM Hepes/HCl pH 8.0 overnight at 4°C. 50μl dialysed...
enzyme was pre-incubated for 15 min at 37°C with an equal volume of each functional reagent to be tested. Positive controls and blanks were included and DPPIV activity was determined fluorimetrically in triplicate as detailed in section 2.4.1. The effect of fluorescent filtering by these functional reagents was assessed as outlined in section 2.2.2.

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>INHIBITS</th>
<th>STOCK</th>
<th>PREPARATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antipain</td>
<td>Papain, Trypsin</td>
<td>5mg/ml</td>
<td>Buffer</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>Protein proteases</td>
<td>2mg/ml</td>
<td>Buffer</td>
</tr>
<tr>
<td>Carnitine</td>
<td>PO</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>Pepsin, Renin</td>
<td>2mg/ml</td>
<td>5%v/v acetone</td>
</tr>
<tr>
<td></td>
<td>HIV-I protease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Puromycin</td>
<td>Protein proteases</td>
<td>2mg/ml</td>
<td>Buffer</td>
</tr>
<tr>
<td>Trypsin Inhibitor</td>
<td>Trypsin</td>
<td>2mg/ml</td>
<td>Buffer</td>
</tr>
</tbody>
</table>

Table 2.12 Preparation of Other Functional Reagents Tested

Buffer is 50mM Hepes/HCl, pH 8.0. Pepstatin A was initially dissolved in 100%v/v acetone and adjusted to stock concentration by addition of 50mM Hepes buffer.

2.10.8 Effect of Metal Ions on DPPIV Activity

The effects of metal salts on DPPIV activity were assessed using Ca, Cd, Co, Cu, Mg, Mn, Hg, Na, Ni, Zn sulphates. 5mM stock solutions were prepared in 50mM Hepes/HCl pH 8.0. Purified DPPIV was dialysed against 41 50mM Hepes/HCl, pH 8.0 for 5 hr at 4°C. 50μl metal salt and 50μl dialysed enzyme was pre-incubated in triplicate at 37°C for 15 min. Negative controls had 50μl buffer added to the metal instead of enzyme while positive controls consisted of 50μl buffer in place of the metal salt. Following pre-incubation, 400μl of 0.1mM Gly-Pro-MCA substrate was added to each triplicate and assayed as described in section 2.4.2.
<table>
<thead>
<tr>
<th>METAL</th>
<th>CONCENTRATION (mM)</th>
<th>PREPARATION AND COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaSO₄</td>
<td>5.0</td>
<td>Normal</td>
</tr>
<tr>
<td>CdSO₄</td>
<td>5.0</td>
<td>Normal</td>
</tr>
<tr>
<td>CoSO₄</td>
<td>5.0</td>
<td>Heating at 37°C required</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>5.0</td>
<td>Potassium phosphate</td>
</tr>
<tr>
<td>HgSO₄</td>
<td>5.0</td>
<td>Initially dissolved in 1M HCl then diluted with Hepes, pH 8.0</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>5.0</td>
<td>Readily falls out of solution</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>5.0</td>
<td>Normal</td>
</tr>
<tr>
<td>NaSO₄</td>
<td>5.0</td>
<td>Normal</td>
</tr>
<tr>
<td>NiSO₄</td>
<td>5.0</td>
<td>Normal</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>5.0</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Table 2.13. Preparation of Metal Salts Tested on DPPIV Activity

2.10.9. Effect of General Salts on DPPIV activity

2.10.9.1. Effect of Ammonium Sulphate on DPPIV

2.10.9.1.1. Ammonium Sulphate effect in the substrate

0.2mM Gly-Pro-MCA was prepared in 50mM Hepes/HCl, pH 8.0. 0, 200, 600, 1200, 1600 and 2000mM Ammonium Sulphate was also prepared in 50mM Hepes and the pH was adjusted to 8.0 with 1M NaOH. 1ml of the substrate was added to an equal volume of appropriate Ammonium Sulphate solution in triplicate and incubated at 37°C to reach thermal equilibrium. This gave a final Ammonium Sulphate concentration of 0, 100, 300, 600, 800 and 1000mM. 100μl of purified DPPIV was added to the triplicates and assayed as outlined in section 2.4.2. Negative controls were included which consisted of substituting 100μl of enzyme with 100μl of 50mM Hepes/HCl. The positive control was included by the presence of the 0mM Ammonium Sulphate/substrate solution. The influence of the salt on fluorescent filtering was taken into account as described in section 2.2.2.

2.10.9.1.2. Ammonium Sulphate effect with the enzyme

0, 400, 800, 1200, 1600 and 2000mM Ammonium Sulphate was prepared in 50mM Hepes and the pH was adjusted to 8.0 with 1M NaOH. 200μl of the enzyme was added to an equal volume of appropriate Ammonium Sulphate solution, divided into triplicates and pre-incubated at 37°C to reach thermal equilibrium. This gave a final Ammonium Sulphate
concentration of 0, 200, 400, 600, 800 and 1000mM. 400µl of substrate was added to each triplicate and assayed as outlined in section 2.4.2. Negative controls were included which consisted of substituting 100µl of enzyme with 100µl of 50mM Hepes/HCl with the appropriate Ammonium Sulphate concentration. The positive control was included by the presence of the 0mM Ammonium Sulphate/enzyme solution. The influence of the salt on fluorescent filtering was taken into account as described in section 2.2.2.

### 2.10.9.2 Effect of Sodium Chloride on DPPIV activity

#### 2.10.9.2.1 Sodium Chloride effect in the substrate

0.2mM Gly-Pro-MCA was prepared in 50mM Hepes/HCl, pH 8.0. 0, 400, 800, 1200, 1600 and 2000mM Sodium Chloride was also prepared in 50mM Hepes/HCl, pH 8.0. 1ml of the substrate was added to an equal volume of the appropriate Sodium Chloride solution in triplicate and incubated at 37°C to reach thermal equilibrium. This gave a final Sodium Chloride concentration of 0, 200, 400, 600, 800 and 1000mM. 100µl of purified DPPIV was added to the triplicates and assayed as outlined in section 2.4.1. Negative controls were included which consisted of substituting 100µl of enzyme with 100µl of 50mM Hepes/HCl. The positive control was included by the presence of the 0mM Sodium Chloride/substrate solution. The influence of the Sodium Chloride salt on fluorescent filtering was taken into account as described in section 2.2.2.

#### 2.10.9.2.2 Sodium Chloride effect with the enzyme

0, 200, 600, 1200, 1600 and 2000mM Sodium Chloride was prepared in 50mM Hepes/HCl, pH 8.0. 200µl of the enzyme was added to an equal volume of appropriate Sodium Chloride solution, divided into triplicate and incubated at 37°C to reach thermal equilibrium. This gave a final Sodium Chloride concentration of 0, 200, 400, 600, 800 and 1000mM. 400µl of the substrate was added to each triplicate and assayed as outlined in section 2.4.2. Negative controls were included which consisted of substituting 100µl of enzyme with 100µl of 50mM Hepes/HCl with the appropriate Sodium Chloride concentration. The positive control was included by the presence of the 0mM Sodium Chloride/enzyme solution. The influence of the salt on fluorescent filtering was taken into account as described in section 2.2.2.

### 2.10.9.3 Effect of Potassium Chloride on DPPIV activity

#### 2.10.9.3.1 Potassium Chloride effect with the enzyme

0, 200, 600, 1200, 1600 and 2000mM Potassium Chloride was prepared in 50mM Hepes/HCl, pH 8.0. 200µl of the enzyme was added to an equal volume of appropriate
Potassium Chloride solution, divided into triplicate and incubated at 37°C to reach thermal equilibrium. This gave a final Potassium Chloride concentration of 0, 200, 400, 600, 800 and 1000mM. 400μl of the substrate was added to each triplicate and assayed as outlined in section 2.4.2. Negative controls were included which consisted of substituting 100μl of enzyme with 100μl of 50mM Hepes/HCl with the appropriate Potassium Chloride concentration. The positive control was included by the presence of the 0mM Potassium Chloride/enzyme solution. The influence of the salt on fluorescent filtering was taken into account as described in section 2.2.2.

2.10.10 Substrate Specificity

2.10.10.1 Reverse Phase HPLC Analysis

HPLC analysis was carried out in order to ascertain the substrate specificity of DPPIV towards a number of synthetic and bio-active peptides. A Beckman Chromatographic system was used incorporating a Programmable module (dual pump), a 526 Photo-Diode array Detector and a 507 autosampler served by Beckman 32 Karat HPLC software. A Waters Spherosorb ODS-2 C-18 analytical column (250mm x 4.6mm) and a Supelguard LC-18 guard column was employed for all analysis. TABLES 2.15 and 2.16 lists the peptides analysed and the mode of preparation required. 400μl of each peptide was incubated with 100μl of purified DPPIV at 37°C for 24 hours. Negative controls (no enzyme) and blanks were also included. 25μl 0.5% v/v TFA was added to terminate the reaction after 24 hours. 20μl of each post-incubation sample, control and blank was applied at 0.5ml/min to the column which has been equilibrated. A 10ml wash of buffer B was carried out followed by a 10ml linear gradient from 100% buffer B to 100% buffer A. A further 5ml wash of 100% buffer A eluted any strongly bound peptides from the column. The absorbance of the eluant was continuously monitored online at 214nm and 280nm. The column was equilibrated between injections with 100% buffer B.

<table>
<thead>
<tr>
<th>Component</th>
<th>Buffer A %v/v</th>
<th>Buffer B %v/v</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultra-pure Water</td>
<td>19.8</td>
<td>94.8</td>
</tr>
<tr>
<td>HPLC-grade Acetonitrile</td>
<td>80</td>
<td>5</td>
</tr>
<tr>
<td>TFA</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**Table 2.14 Buffer Composition Employed for HPLC Analysis**

Buffer were filtered and degassed through a 0.45μm membrane filter prior to use.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Concentration (mM)</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala-Pro-Gly</td>
<td>5mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Asp-Pro-Gln-Phe-Tyr</td>
<td>3mM</td>
<td>Buffer*</td>
</tr>
<tr>
<td>Glu-Pro-Glu-Phe-Tyr</td>
<td>3mM</td>
<td>Buffer*</td>
</tr>
<tr>
<td>Gly-Pro-Ala</td>
<td>5mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Gly-Pro-Arg</td>
<td>5mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Gly-Pro-Gly-Gly</td>
<td>5mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Gly-Pro-Hyp</td>
<td>5mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Gly-Pro-Pro</td>
<td>5mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Phe-Pro-Ala</td>
<td>5mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Tyr-Pro-Phe</td>
<td>5mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Z-Pro-Ala</td>
<td>1 3mM</td>
<td>10%v/v ACN</td>
</tr>
<tr>
<td>Z-Pro-Gly</td>
<td>1 3mM</td>
<td>10%v/v ACN</td>
</tr>
<tr>
<td>Z-Pro-Leu</td>
<td>1 3mM</td>
<td>10%v/v ACN</td>
</tr>
<tr>
<td>Z-Pro-Pro</td>
<td>1 3mM</td>
<td>Buffer*</td>
</tr>
</tbody>
</table>

**Table 2.15 Preparation of Synthetic HPLC Peptides**

Buffer refers to 50mM Hepes, pH 8.0, was also the diluent for water insoluble peptides.

* Sonication was required to achieve complete dissolution.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Concentration (mM)</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>APi U 02 50%v/v Acetic Acid</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Ap20 29 05 Buffer</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Aß29 40 0.4%v/v TFA</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>2</td>
<td>Buffer*</td>
</tr>
<tr>
<td>(Arg)⁸ –Vasopressin</td>
<td>1</td>
<td>Buffer</td>
</tr>
<tr>
<td>β-Casomorphin</td>
<td>3</td>
<td>Buffer</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>2</td>
<td>Buffer</td>
</tr>
<tr>
<td>CRP 201 206</td>
<td>3</td>
<td>Buffer</td>
</tr>
<tr>
<td>Enterostatin</td>
<td>3</td>
<td>Buffer</td>
</tr>
<tr>
<td>IGF1 3</td>
<td>5</td>
<td>Buffer</td>
</tr>
<tr>
<td>LHRH</td>
<td>2</td>
<td>10%v/v ACN</td>
</tr>
<tr>
<td>Neuropeptide Y (1-24)</td>
<td>0.2</td>
<td>Buffer*</td>
</tr>
<tr>
<td>Neurotensin9 13</td>
<td>3</td>
<td>Buffer</td>
</tr>
<tr>
<td>Substance P</td>
<td>3</td>
<td>Buffer*</td>
</tr>
<tr>
<td>TRH</td>
<td>1.3</td>
<td>10%v/v ACN</td>
</tr>
</tbody>
</table>

**Table 2.16 Preparation of Bioactive HPLC Peptides**

Buffer refers to 50mM Hepes, pH 8.0, which was also used for water insoluble peptides

*Sonication was employed to achieve complete dissolution

2.10.10.11 Inhibition of β-Casomorphin hydrolysis

2mM of β-Casomorphin was prepared as described in Table 2.16. 2 x 10⁻⁴ M H-Ile-Pyro-hidide, a specific DPPIV inhibitor was also prepared as outlined in Table 2.17. 200μl of β-Casomorphin was pre-incubated with 200μl of the inhibitor for 15 minutes at 37°C. 50μl of purified DPPIV was added and the reaction was carried out and analysed as outlined in section 2.10.8.1

2.10.10.2 Kinetic Analysis

2.10.10.2.1 Km Determination for Gly-Pro-MCA

A stock solution of 0.5mM Gly-Pro-MCA in 50mM Hepes/HCl, pH 8.0 was prepared. The stock solution was diluted to a range of Gly-Pro-MCA concentrations with 50mM Hepes/HCl, pH 8.0. The DPPIV enzyme activity was assayed on each substrate concentration in triplicate as described in section 2.4.2. The Michaelis-Menten constant, Km and the
maximum velocity value, Vmax for the substrate was determined using Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf plot analysis

2 10 10 2 2 KM Determination for Lys-Pro-MCA
As outlined in the previous section 2 9 8 1 1, a stock solution of 0.5mM Lys-Pro-MCA was prepared and analysed as described. The KM and Vmax constants were also calculated using the aforementioned plot analyses.

2 10 11 Inhibitor Studies of DPPIV

2 10 11 1 IC50 Determination of DPPIV Specific Inhibitors
A range of DPPIV specific inhibitors were assayed to investigate its potency against DPPIV activity. TABLE 2 17 outlines the preparation and stock concentration of each inhibitor investigated. Stock solutions were prepared in 50mM Hepes/HCl, pH 8.0 (+/- solvent where necessary) Dilutions of each inhibitor were made up in 50mM Hepes/HCl, pH 8.0 to produce a range of serial dilutions 1ml of each dilution was added to double strength substrate resulting in a final concentration of 0.1mM Gly-Pro-MCA. 100µl of purified DPPIV was incubated in triplicate with 400µl of appropriate substrate/inhibitor solution for 1 hour at 37°C. Suitable blanks and controls were included. The IC50 for each inhibitor was determined for DPPIV.

2 10 11 2 KI Determination of DPPIV Specific Inhibitors
The effect of specific inhibitors on the cleavage of DPPIV on Gly-Pro-MCA was evaluated. A 1,000µM stock solution of Gly-Pro-MCA A range of substrate dilutions was prepared from 0-1,000µM using 50mM Hepes, pH 8.0 as diluant. 800 µl of a predetermined inhibitor concentration was added to an equal volume of each substrate dilution resulting in a final substrate range of 0-500µM. DPPIV was assayed in triplicate as described in section 2 4 1. The resulting data was applied to Michaelis-Menten and Lineweaver Burk plot analyses where the mode the inhibition could be ascertained and the inhibition constant, Ki, calculated.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Stock Solution (M)</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diprotin A</td>
<td>1x10⁻³</td>
<td>Hepes</td>
</tr>
<tr>
<td>Diprotin B</td>
<td>1x10⁻³</td>
<td>Hepes</td>
</tr>
<tr>
<td>H-Ile-Thiazolidide</td>
<td>1x10⁻⁴</td>
<td>10%v/v MeOH</td>
</tr>
<tr>
<td>H-Ile-Pyrrolidide</td>
<td>1x10⁻³</td>
<td>10%v/v MeOH</td>
</tr>
</tbody>
</table>

TABLE 2 17 PREPARATION OF SPECIFIC DPPIV INHIBITORS

84
Determination of β-Casomorphin and Substance P

The effect of two proline containing peptides on the cleavage of DPPIV on Gly-Pro-MCA was evaluated. A range of substrate dilutions was prepared from 0-1,000 µM using 50 mM Hepes, pH 8.0 as diluant. 800 µl of either 0.3 mM β-Casomorphin or 0.2 mM Substance P was added to an equal volume of each substrate dilution resulting in a final substrate range of 0-500 µM. DPPIV was assayed in triplicate as described in section 2.4.1. The resulting data was applied to a number of kinetic models where the mode of inhibition could be ascertained and the inhibition constant, K, calculated. (See Appendix, section 6.4.2.)
3.0. RESULTS
3.0 RESULTS

The majority of upcoming graphs illustrate data points that represent the average of triplicate fluorescence or absorbance readings, minus the blank measurement. All error bars represent the standard error of the mean of these triplicate values (see section 6.1).

3.1 MCA STANDARD CURVES AND THE INNER FILTER EFFECT

MCA standard curves were prepared as outlined in section 2.2.1. Plots of fluorescent intensity versus MCA concentration were constructed for both MCA ranges and the slope of the lines calculated. Figures 3.11 and 3.12 represent typical MCA standard curves at emission slit widths of 10nm and 2.5nm respectively. Demonstration of the inner filter effect was also performed as described in section 2.2.2 to assess the effect on fluorescence of including crude or post column fractions in the assay mixture. Figures 3.13 and 3.14 are plots of fluorescence versus MCA concentration for buffered saliva and crude serum. Table 3.1 lists the slopes calculated for each curve and the degree of filtering observed for enzyme samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$R^2$</th>
<th>Slope</th>
<th>Filtering %</th>
</tr>
</thead>
<tbody>
<tr>
<td>10nm, 2.5nm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>0.998</td>
<td>12.98</td>
<td>0.0</td>
</tr>
<tr>
<td>Serum</td>
<td>0.999</td>
<td>10.51</td>
<td>19</td>
</tr>
<tr>
<td>Post-phenyl sepharose DPPIV</td>
<td>0.999</td>
<td>12.55</td>
<td>3.3</td>
</tr>
<tr>
<td>10nm, 10nm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>0.998</td>
<td>14.95</td>
<td>0.0</td>
</tr>
<tr>
<td>Buffered Saliva</td>
<td>0.997</td>
<td>13.17</td>
<td>11.7</td>
</tr>
<tr>
<td>Post-phenyl sepharose PO</td>
<td>0.999</td>
<td>14.90</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Table 3.1 SLOPES OF FILTERED STANDARD CURVES

3.1.1 Optimum MCA Excitation and Emission Wavelengths

Scans of fluorescence versus wavelength were performed on free MCA as outlined in section 2.2.3 in order to determine the optimum excitation and emission wavelengths for this compound. Figures 3.15 and 3.16 show that the optimum excitation and emission wavelengths for MCA are 370nm and 440nm respectively.
Figures 3.11 and 3.12 MCA Standard Curves for slit widths 10, 10 and 10, 25 Plot of fluorescent Intensity versus free MCA concentration
FIGURES 3.1.3 AND 3.1.4. MCA QUENCHED STANDARD CURVES FOR SLIT WIDTHS 10, 10 AND 10, 2.5

Plots of Fluorescent Intensity versus free MCA concentration. The quenching agents used were Buffered Saliva and Bovine Serum respectively.
Figures 3.1.5 and 3.1.6. MCA Excitation and Emission Scans

Plot of fluorescent intensity versus wavelength for free MCA in solution. Figure 3.1.5 illustrates an optimum excitation wavelength of 370 nm for MCA while Figure 3.1.6 shows an emission wavelength of 440 nm as optimal.
3.2 PROTEIN DETERMINATION
BSA standard curves were prepared according to the methods given in section 2.3. Plots of protein absorbance versus BSA concentration are presented in FIGURES 3.2.1, 3.2.2, and 3.2.3 for the Biuret, standard BCA and enhanced BCA assays respectively.
**FIGURE 3.2.1 BIURET PROTEIN STANDARD CURVE**
Plot of absorbance at 570nm versus BSA concentration

**FIGURE 3.2.2 BCA PROTEIN STANDARD CURVE**
Plot of absorbance at 570nm versus BSA concentration
**Figure 3.2.3 Enhanced BCA Protein Standard Curve**

Plot of absorbance at 570nm versus BSA concentration
3.3 Purification of Human Salivary Prolyl Oligopeptidase

3.3.1 Ammonium Sulphate Precipitation
An 80% ammonium sulphate precipitation was carried out as described in section 2.5.2. PO activity was precipitated into the pellet after centrifugation. The pellet was re-suspended and analysed along with the supernatant for PO activity as described in section 2.4.1. The pellet was prepared and stored for phenyl sepharose application as outlined in section 2.5.2. Figures 3.3.11 and 3.3.12 show the enzymatic activity present in the supernatants and pellets respectively.

3.3.2 Phenyl Sepharose Hydrophobic Interaction Chromatography
4ml of post ammonium sulphate precipitation PO was applied to the 25ml phenyl sepharose column and partially purified as described in section 2.5.3. Post column fractions were analysed for PO activity as outlined in section 2.4.3. while the protein content of the fractions was also determined via UV absorbancy as described in section 2.3.1. Figure 3.3.2.1 shows the elution profile of PO, where one activity peak is observed when the ammonium sulphate concentration is reduced to zero. Contaminating protein was washed through the column during the 100ml wash of 100mM Potassium Phosphate, pH 7.4 with 5mM DTT and 0.5mM EDTA containing 1M ammonium sulphate. Fractions containing PO were combined to give the post Phenyl Sepharose PO pool. 1ml of the pool was retained for activity and protein analysis.

3.3.3 Q-Sepharose Fast Flow Anion Exchange Chromatography
1ml of dialysed and concentrated post phenyl sepharose PO sample was applied onto the Q-Sepharose column and purified as outlined in section 2.5.4. PO was found to bind and elute to the column while separated from the bulk contaminating protein as seen in Figure 3.3.3.1. All fractions were analysed for Z-Gly-Pro-MCA degrading activity and Coomassie plus protein assay as described in sections 2.4.3. and 2.3.3. respectively. Fractions containing PO were combined and labelled post QAE PO. 1ml was retained as before for activity and protein estimation. The post QAE PO pool was used as partially pure human salivary PO.
**Figure 3.3.1.1** PO Activity in Supernatants after Salt Fractionation

**Figure 3.4.1.2** PO Activity in the Pellets after Salt Fractionation
**Figure 3.3.2.1. Phenyl Sepharose Profile of Salivary Prolyl Oligopeptidase**
**Figure 3.3.1**

**Quaternary Anion Exchange Profile of Salivary PO**

[Graph showing Fluorescent Intensity and Sodium Chloride concentration against Fraction Number.]
A purification table was constructed to assess the overall effectiveness of the purification protocol for Human Salivary PO (Table 3.2). Calculations were performed as highlighted in the appendix.

<table>
<thead>
<tr>
<th>Purification Stage</th>
<th>Total Activity</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Purification Factor</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>unit</td>
<td>mg</td>
<td>Unit/mg</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>Buffered Saliva</td>
<td>2 241</td>
<td>23 78</td>
<td>0 094</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>80% Salt Cut</td>
<td>0 8752</td>
<td>5 173</td>
<td>0 1692</td>
<td>1 8</td>
<td>39 1</td>
</tr>
<tr>
<td>Post Phenyl Sepharose</td>
<td>0 1891</td>
<td>2 256</td>
<td>0 084</td>
<td>0 9</td>
<td>8 44</td>
</tr>
<tr>
<td>Post Q Anion Exchange</td>
<td>0 01686</td>
<td>1 72</td>
<td>0 00984</td>
<td>0 104</td>
<td>0 75</td>
</tr>
</tbody>
</table>

Table 3.2. Purification Table for Human Salivary PO
3.3.4 Alternative Chromatographic Techniques to Purify Human Salivary PO

3.3.4.1 DEAE Sepharose Anion Exchange Chromatography
Post phenyl sepharose PO was dialysed and concentrated as described in section 2.5.4. It was applied and eluted from a DEAE Sepharose column as outlined in section 2.5.5.1. Fractions were assayed for PO activity as described in section 2.4.3 while the protein content was measured via the Coomassie Plus Protein assay as outlined in section 2.3.3. Figure 3.3.4.1 illustrates the elution of PO activity where it was partially separated from the run through protein. Fractions containing PO were combined and its activity and protein content measured as described in sections 2.4.1 and 2.3.3 respectively.

3.3.4.2 Benzamidine Sepharose Chromatography
The benzamidine sepharose resin was prepared as described in section 2.5.5.2. Dialysed post phenyl sepharose was applied and eluted from the column at the start of the NaCl linear gradient while being separated from most of the protein that does not bind as illustrated in Figure 3.3.4.2. Any fractions containing PO activity was pooled and labelled. As before 1ml of the post-benzamidine pool was retained for evaluations on enzyme recovery and protein determination.
**FIGURE 3.3.4.1.** DEAE CHROMATOGRAPHY OF SALIVARY PO

Plots of fluorescent intensity, absorbance at 570nm and sodium chloride concentration versus fraction number.

**FIGURE 3.3.4.2.** BENZAMIDINE SEPHAROSE CHROMATOGRAPHY OF SALIVARY PO

Plots of fluorescent intensity, absorbance at 570nm and ammonium sulphate concentration versus fraction number.
3.4. **Purification of Bovine Serum DPPIV**

### 3.4.1 Serum Preparation

9l fresh bovine whole blood was collected and left to clot at 4°C as outlined in section 2.6.1, resulting in the formation of 1.8l unclotted serum and particulate matter. Centrifugation yielded 1.3l of serum.

### 3.4.2 Phenyl Sepharose Hydrophobic Interaction Chromatography

The crude serum containing 1M (NH₄)₂SO₄ was applied to a phenyl sepharose hydrophobic interaction chromatography column as outlined in section 2.6.2. An run-through activity peak was observed from this column as shown in FIGURE 3.4.2.1, while being separated from a significant proportion of bound protein. Any fractions with DPPIV activity were combined yielding a post phenyl sepharose DPPIV pool. 1ml of this pool was retained for both total activity and protein estimation.

### 3.4.3 HiPrep 16/10 Sephacryl S-300 HR Gel Filtration Chromatography

Following concentration by reverse dialysis, 1.5ml of the post phenyl sepharose chromatography pool was applied onto a S300 column as outlined in section 2.6.3. FIGURE 3.4.3.1 illustrates the molecular sieving effect of the resin on the enzyme's purification. Any fractions containing DPPIV activity were combined and labelled to give the post S300 DPPIV pool. 1ml of this pool was retained for quantitative activity and protein analysis as outlined in sections 2.4.2 and 2.3.4.

### 3.4.4 POROS Anion-Exchange Chromatography

5ml post-S300 DPPIV pool was applied to a POROS 50 HQ column as described in section 2.6.4. DPPIV activity was detected in the bound fractions from this anion-exchange column while no run-through protein could be visibly detected. The elution profile may be seen in FIGURE 3.4.4.1. 1ml was retained for estimation of enzyme recovery and protein determinations while the main pool was labelled and stored at -17°C.

### 3.4.5 Desalting by G100 Gel Filtration Chromatography

Post POROS DPPIV pool was passed through a G100 Gel Filtration column and washed with 150ml 50mM Hepes, pH 8.0 as outlined in section 2.6.5. FIGURE 3.4.5.1 illustrates the molecular sieving effect of the resin by the delayed elution of the salt. Any fractions containing DPPIV activity were combined, aliquoted, labelled as purified DPPIV and stored at -17°C until used for characterization studies.

100
3.4.6 Alternative Chromatographic Techniques used to purify DPPIV

3.4.6.1 Concanavalin-A Sepharose Chromatography

The Con-A column was prepared as described in section 2.6.1. 2.5 ml of post S300 DPPIV was applied onto the column and eluted with an isocratic step of 0.5M Methylmannoside. **Figure 3.4.6.1** illustrates the removal of contaminating protein but the inability to elute the enzyme sharply. Fractions containing DPPIV activity were combined and 1ml of the resulting pool was retained for both total activity and protein estimation.

For the Concanavalin A Sepharose Chromatography method, the DPPIV activity was deemed too low to be further purified or characterised. This may be due to over dilution or partial degradation as a consequence of inefficient binding and eluting from the resin.
**FIGURE 3.4.2.1**

**FIGURE 3.4.2.1 PHENYL SEPHAROSE CHROMATOGRAPHY OF BOVINE SERUM DPPIV**
**Figure 3.4.2.1**

Fluorescent Intensity

**Figure 3.4.3.2** S300 Gel Filtration Chromatography of DPPIV
Figure 34.1 POROS Anion Exchange Chromatography of DPPIV
Figure 3.4.5.1

**G100 Sepharose Gel Filtration of DPPIV**
A purification table was constructed to assess the overall effectiveness of the purification protocol (Table 3.3). Calculations were performed as highlighted in section 6.3.

<table>
<thead>
<tr>
<th>Purification Stage</th>
<th>Total Activity</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Purification Factor</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unit</td>
<td>mg</td>
<td>Unit/mg</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>Serum</td>
<td>1718</td>
<td>910</td>
<td>1.9</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Post PS</td>
<td>4512</td>
<td>49</td>
<td>91.3</td>
<td>48.1</td>
<td>26.3</td>
</tr>
<tr>
<td>Post S300</td>
<td>1695</td>
<td>12</td>
<td>141.25</td>
<td>74</td>
<td>9.9</td>
</tr>
<tr>
<td>Post QAE</td>
<td>495</td>
<td>0.104</td>
<td>475.96</td>
<td>250.5</td>
<td>2.9</td>
</tr>
<tr>
<td>Post G100</td>
<td>87</td>
<td>0.0178</td>
<td>488.8</td>
<td>257.3</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 3.3 Purification Table for Bovine Serum DPPIV
**Figure 34.61** CONCANAVALIN-A SEPHAROSE OF DPPIV

Plots of fluorescent intensity (●), absorbance at 570nm (□) and methyl mannoside concentration (▽) versus fraction number.
3.5 Determination of Enzyme Purity

3.5.1 SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed as described in section 2.7.1. Figures 3.5.11 and 3.5.12 represent images of gel-code stained gels of salivary PO and serum DPPIV respectively, showing molecular weight markers and the various post-column PO and DPPIV pools. Table 3.4 summarises the labels used to identify each of the markers on the gels and the relative molecular weight of each.

3.5.2 The Activity of ‘Purified Samples’ Using Fluorimetric Substrates

The presence of contaminating peptidase activities in the purified PO and DPPIV pools were determined by the detection of the hydrolysis of a number of fluorimetric substrates. These substrates were prepared and tested as outlined in section 2.7.2. Table 3.5 demonstrates the cleavage of many of these substrates by purified PO, indicating its impurity. Table 3.6 illustrates that no cleavage, other than those expected, was detected in the presence of purified DPPIV activity.

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>HYDROLYSIS</th>
<th>SUBSTRATE</th>
<th>HYDROLYSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala-MCA</td>
<td>No</td>
<td>Lys-Pro-MCA</td>
<td>Yes</td>
</tr>
<tr>
<td>Arg-MCA</td>
<td>Yes</td>
<td>Nα-benzoyl-Arg-MCA</td>
<td>Yes</td>
</tr>
<tr>
<td>Glu-Phe-MCA</td>
<td>Yes</td>
<td>Pyr-His-Pro-MCA</td>
<td>No</td>
</tr>
<tr>
<td>Gly-Pro-MCA</td>
<td>Yes</td>
<td>Pro-MCA</td>
<td>Yes</td>
</tr>
<tr>
<td>Leu-MCA</td>
<td>Yes</td>
<td>Z-Arg-MCA</td>
<td>No</td>
</tr>
<tr>
<td>Lys-Ala-MCA</td>
<td>Yes</td>
<td>Z-Gly-Pro-MCA</td>
<td>Yes</td>
</tr>
<tr>
<td>Lys-MCA</td>
<td>No</td>
<td>Z-Phe-Arg-MCA</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 3.5 Purity Assessment of PO Using Fluorimetry
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydrolysis</th>
<th>Substrate</th>
<th>Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala-MCA</td>
<td>No</td>
<td>Lys-Pro-MCA</td>
<td>Yes</td>
</tr>
<tr>
<td>Arg-MCA</td>
<td>No</td>
<td>Nα-benzoyl-Arg-MCA</td>
<td>No</td>
</tr>
<tr>
<td>Glu-Phe-MCA</td>
<td>No</td>
<td>Pyr-His-Pro-MCA</td>
<td>No</td>
</tr>
<tr>
<td>Gly-Pro-MCA</td>
<td>Yes</td>
<td>Pro-MCA</td>
<td>No</td>
</tr>
<tr>
<td>Leu-MCA</td>
<td>No</td>
<td>Z-Arg-MCA</td>
<td>No</td>
</tr>
<tr>
<td>Lys-Ala-MCA</td>
<td>Yes</td>
<td>Z-Gly-Pro-MCA</td>
<td>No</td>
</tr>
<tr>
<td>Lys-MCA</td>
<td>No</td>
<td>Z-Phe-Arg-MCA</td>
<td>No</td>
</tr>
</tbody>
</table>

**Table 3.6. Purity Assessment of DPPIV using Fluorimetry**
FIGURE 3.5.1.1. GELCODE STAIN OF SALIVARY PO SDS PAGE
The red arrow (→) within the gel represents purified salivary PO.

<table>
<thead>
<tr>
<th>Label</th>
<th>Marker</th>
<th>Molecular Weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Myosin</td>
<td>205</td>
</tr>
<tr>
<td>II</td>
<td>β-Galactosidase</td>
<td>116</td>
</tr>
<tr>
<td>III</td>
<td>Phosphorylase b</td>
<td>97</td>
</tr>
<tr>
<td>IV</td>
<td>Fructose-6-phosphate kinase</td>
<td>84</td>
</tr>
<tr>
<td>V</td>
<td>BSA</td>
<td>66</td>
</tr>
<tr>
<td>VI</td>
<td>Glutamic Dehydrogenase</td>
<td>55</td>
</tr>
<tr>
<td>VII</td>
<td>Ovalbumin</td>
<td>45</td>
</tr>
<tr>
<td>VIII</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>36</td>
</tr>
</tbody>
</table>

TABLE 3.4. MOLECULAR WEIGHT MARKERS USED IN SDS PAGE
**Figure 3.5.1.2. Gelcode Stained SDS PAGE of Bovine Serum DPPIV**

The green arrow (—→) within the gel represents the purified DPPIV band.

<table>
<thead>
<tr>
<th>Label</th>
<th>Marker</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Myosin</td>
<td>205 kDa</td>
</tr>
<tr>
<td>II</td>
<td>β-Galactosidase</td>
<td>116 kDa</td>
</tr>
<tr>
<td>III</td>
<td>Phosphorylase b</td>
<td>97 kDa</td>
</tr>
<tr>
<td>IV</td>
<td>Fructose-6-phosphate kinase</td>
<td>84 kDa</td>
</tr>
<tr>
<td>V</td>
<td>BSA</td>
<td>66 kDa</td>
</tr>
<tr>
<td>VI</td>
<td>Glutamic Dehydrogenase</td>
<td>55 kDa</td>
</tr>
<tr>
<td>VII</td>
<td>Ovalbumin</td>
<td>45 kDa</td>
</tr>
<tr>
<td>VIII</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>36 kDa</td>
</tr>
</tbody>
</table>

**Table 3.4. Molecular Weight Markers used in SDS PAGE**
3.6 ASSAY DEVELOPMENT

3.6.1 Substrate Solvent Determination (PO/DPP IV)
Stock substrates were prepared as outlined in section 2.8.1 in DMSO, DMF, DXN, EtOH and MeOH. The effects of using these solvents on both enzymes activities is illustrated in FIGURES 3.6.11 and 3.6.12. Methanol and ethanol was chosen to be the most suitable solvents for PO and DPP IV activities respectively and was thus used for subsequent substrate solubilisation.

3.6.2 Solvent Concentration Determination (DPPIV)
The optimum ethanol concentration was determined as described in section 2.8.2. FIGURE 3.6.2 shows the effect of increasing ethanol concentration in substrate preparations on purified DPPIV. Although 4%v/v EtOH yielded the highest activity of DPPIV, the substrate could be dissolved at lower ethanol concentrations while not interfering with the enzyme. Therefore 1%v/v EtOH was chosen as the most suitable final solvent concentration in the substrate for the assaying of DPPIV.
**Figure 3.6.11**

Bar chart of fluorescent intensity versus substrate solvent.

**Figure 3.6.11 Substrate Solvent Determination for PO**

Bar chart of fluorescent intensity versus substrate solvent.
**Figure 3.6.1.2.**

**Figure 3.6.1.2: Substrate Solvent Determination for DPPIV**

Bar chart of fluorescent intensity versus substrate solvent concentration.

**Figure 3.6.2**

**Figure 3.6.2: Solvent Concentration Determination for DPPIV**

Bar chart of fluorescent intensity versus substrate solvent concentration.
3 6 3 Linearity of Enzyme Assays with Respect to Time

3 6 3 1 Discontinuous Assay
The linearity of the Z-Gly-Pro-MCA and Gly-Pro-MCA degrading activity assays as a function of time was examined as described in section 2 8 3 1. Figure 3 6 3 1 1 illustrates that salivary PO activity in post QAE purified sample is linear over a period of 60 minutes ($R^2 = 0.996$). Similarly, DPPIV activity is linear ($R^2 = 0.97$) over the same time period (Figure 3 6 3 1 2).

3 6 3 2 Continuous Assay
Continuous assays of PO and DPPIV were also carried as outlined in section 2 8 3 2. PO was observed to be linear over the assay duration, 60 minutes (Graph not shown). Figure 3 6 3 2 1 illustrates DPPIV's enzymatic linearity over 120 minutes ($R^2 = 0.995$).

3 6 4 Linearity of Enzyme Assay with Respect to Enzyme Concentration (DPPIV)
Linearity of the Gly-Pro-MCA degrading activity assays with respect to enzyme concentration was determined as outlined in section 2 8 4. Figure 3 6 4 indicates that the purified DPPIV assay is linear ($R^2 = 0.991$) with respect to enzyme concentration.

3 6 5 Optimum Assay Temperature
The effect of incubating purified DPPIV at different temperatures was investigated according to section 2 8 5. An optimal temperature for the assay of bovine serum DPPIV was not fully determined. However, 37°C was used for all assays due to the enzyme's mammalian source, even though Figure 3 6 5 illustrates DPPIV's increased activity at temperatures higher than 37°C.
**Figure 3.6 4.1.1 PO Discontinuous Assay**

Plot of fluorescent intensity versus time illustrating linear enzyme activity

**Figure 3.6 4.1.2 DPPIV Discontinuous Assay**

Plot of fluorescent intensity versus time, indicating linear enzyme activity. Activity is expressed as percentage of the 60 minute value.
**Figure 36.2.2**

Plot of fluorescent intensity versus time, illustrating linear enzymatic activity.

**DPPIV Continuous Assay**
Figure 3.6.4 Linearity of DPPIV Concentration
Plot of fluorescent intensity versus enzyme concentration. Activity is represented by a percentage of the 100% enzyme value.

Figure 3.6.5 Effect of Assay Temperature on DPPIV Activity.
Plot of fluorescent intensity versus temperature. Activity is represented as a percentage of the 37°C value.
3.6.6 Effect of DTT

3.6.6.1 Effect of DTT on PO Activity
The incorporation of DTT into Z-Gly-Pro-MCA was carried out as detailed in section 2.8.6.1. Figure 3.6.6.1 illustrates the absolute requirement of salivary PO for DTT with a steady increase of activity up until 20mM DTT.

3.6.6.2 Effect of DTT on DPPIV Activity
The incorporation of DTT into Gly-Pro-MCA was carried out as detailed in section 2.8.6.2. Figure 3.6.6.2 illustrates the insignificant effect of DTT on purified DPPIV activity. DTT slightly inhibited DPPIV activity with 5% inhibition observed at 20mM DTT.

3.6.7 Effect of EDTA

3.6.7.1 Effect of EDTA on PO Activity
The influence of the metal chelator EDTA on PO was determined by its inclusion in the Z-Gly-Pro-MCA substrate as outlined in section 2.8.7.1. Figure 3.6.7.1 shows increased substrate catalysis up until 0.8mM EDTA with 113% activity.

3.6.7.2 Effect of EDTA on DPPIV Activity
The influence of EDTA on DPPIV was also determined by its inclusion in the Gly-Pro-MCA substrate as outlined in section 2.8.7.2. Figure 3.6.7.2 clearly shows increased substrate catalysis up to 7.5mM EDTA with 153% activity.
**Figure 3.6.1** Effect of DTT on PO Activity

Plot of fluorescent intensity versus DTT concentration. Activity is expressed as a percentage of 0mM DTT.

**Figure 3.6.2** Effect of DTT on DPPIV Activity

Plot of fluorescent intensity versus DTT concentration. Activity is expressed as a percentage of the zero DTT value.
**Figure 3.6.71: Effect of EDTA on PO Activity**

Plot of fluorescent intensity versus EDTA concentration. Activity is expressed as a percentage of 0mM EDTA.

**Figure 3.6.72: Effect of EDTA on DPPIV Activity**

Plot of fluorescent intensity versus EDTA concentration. Activity is expressed as a percentage of 0mM EDTA.
3.7 Stability Studies on PO

3.7.1 Thermostability of PO

3.7.1.1 Effect of 37°C Pre-incubation on PO activity
The effect of physiological temperature on PO activity was investigated as outlined in section 2.9.1. FIGURE 3.7.1 illustrates the significant detrimental effect the temperature has on enzymatic activity. After 60 minutes of 37°C pre-incubation, 60% of activity was lost which was even further degraded slowly to 20% activity after 4 hours. 1% BSA w/v had a minimal effect on stabilizing PO. However, on increasing the BSA concentration to 10% w/v, the enzymatic activity was still reduced but only by 46% after 90 minutes in comparison to a degradation of 60% when in the presence of 1% BSA w/v.

3.7.1.2 Stability of PO when stored at −20°C
The effect of frozen storage conditions of −20°C on PO with and without 1% BSA w/v included was carried out as described in section 2.9.2. FIGURE 3.7.2 illustrates that over the 31 days analyzed, there is a notable decrease in enzymatic activity (32%) when no BSA was present. The degradation process was reduced to only 9% when 1% BSA w/v was included with the partially purified enzyme during storage.

The effect of frozen conditions was also investigated for buffered saliva PO both with and without 20% glycerol. FIGURE 3.7.2 clearly highlights that a similar trend is observed for both buffered saliva and that with glycerol. Over the first 6 days, considerable activity is lost with buffered saliva being reduced to 40% activity. In comparison, glycerol seems to reduce the degradation with 64% activity remaining. The loss of activity appears to plateau off over the following 15 days for both frozen aliquots.
**Figure 3.7.1** Effect of 37°C Pre-incubation on PO after BSA Addition

Plot of fluorescent intensity versus time in minutes. Activities are expressed as a percentage of the 0 minutes values. Plot includes no BSA (●), 1% BSA w/v (△) and 10% w/v BSA (□).

**Figure 3.7.2** Stability of Post QAE PO when stored at −20°C

Plot of fluorescent intensity versus time in days. Activity is expressed as a percentage of the Day 0 value. The plot includes no BSA (●) and 1% BSA w/v (□).
Figure 3.7.2.2 Stability of Buffered Saliva PO at -20°C

Plot of fluorescent intensity versus time in days. Activity is expressed as a percentage of the Day 0 value. The plot includes buffered saliva (•) and buffered saliva with 20% glycerol (□).
3.8 Characterisation

3.8.1 Relative Molecular Mass Determinations

3.8.1.1 SDS PAGE

A non-native SDS gel was prepared as outlined in section 2.7.1 and employed to estimate the relative molecular weights of the purified PO and DPPIV activities. The distance migrated by the bromophenol blue dye front was calculated for the colloidal brilliant blue and gelcode stained gels respectively. A plot of log of molecular weight versus relative mobility ($R_f$) was constructed for the eight standards as described in section 2.10.2.1. Figures 3.8.1.1 and 3.8.1.2 show the calibration curves obtained from PO and DPPIV gels when stained with gelcode respectively. Table 3.7 lists the equations of the line for PO generated from the calibrated gels and was used to evaluate the molecular weight of Human Saliva PO. Weights such as 82,700 Da from the brilliant blue stain and 78,900 from the gelcode stain. It was speculated that the enzyme existed as a monomer.

From the equations of the lines shown in Table 3.8, the molecular weight of DPPIV was determined to be 67,100 Da (blue stain) and 74,300 Da (gelcode). It was deduced from this that bovine serum DPPIV is a tetramer.

<table>
<thead>
<tr>
<th>STAIN</th>
<th>EQUATION</th>
<th>MOLECULAR WEIGHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brilliant Blue</td>
<td>$\log MW = -0.9571(R_f) + 5.412$</td>
<td>82,668</td>
</tr>
<tr>
<td>Gelcode</td>
<td>$\log MW = -0.93(R_f) + 5.387$</td>
<td>78,862</td>
</tr>
</tbody>
</table>

Table 3.7 Molecular Weight Determination of Salivary PO

The average molecular weight of the enzyme was determined to be $80,765 \pm 1,903$ Da.
**Figure 3.8.1** SDS PAGE Standard Curve for Molecular Weight Determination of Salivary PO

Plot of log molecular weight versus Rf value, where the R value represents the distance migrated by the standard (•) or sample (○) divided by the distance migrated by the bromophenol blue dye front.

**Figure 3.8.2** SDS PAGE Standard Curve for Molecular Weight Determination of Serum DPPIV

Plot of log molecular weight versus Rf value, where the R value represents the distance migrated by the standard (•) or sample (○) divided by the distance migrated by the bromophenol blue dye front.
3 8 1 2 Size Exclusion Chromatography

The relative molecular mass of purified DPPIV was estimated using two different size-exclusion chromatography resins, namely sephacryl S-300 and a HiPrep sephacryl S-300 High Resolution. Each of these columns was calibrated with a range of standards as outlined in section 2 10 1 1. The void volume of the Sephacryl S-300 was 70ml while the HiPrep Sephacryl S-300 was 40ml. Thus allowed for the production of a calibration graph for each resin representing the log molecular weight versus $V/V_0$ as described in section 2 10 1 3. These graphs (FIGURES 3 8 1 2 1, and 3 8 1 2 2) were then used to estimate the molecular weight of purified DPPIV. TABLE 3 8 shows the equation of the lines obtained and the deduced molecular weights obtained using PAGE and size exclusion chromatography.

<table>
<thead>
<tr>
<th>METHOD</th>
<th>EQUATION OF LINE</th>
<th>$R^2$</th>
<th>MW Da</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS PAGE (blue stain)</td>
<td>$\log MW = -0.96 (R_d) + 5.504$</td>
<td>0.94</td>
<td>268,556*</td>
</tr>
<tr>
<td>SDS PAGE (gelcode stain)</td>
<td>$\log MW = -1.03 (R_d) + 5.34$</td>
<td>0.97</td>
<td>297,367*</td>
</tr>
<tr>
<td>Sephacryl S-300</td>
<td>$\log MW = -2.06(V_d/V_0) + 8.06$</td>
<td>0.97</td>
<td>275,966</td>
</tr>
<tr>
<td>HiPrep sephacryl S-300</td>
<td>$\log MW = -1.525(V_d/V_0) + 7.673$</td>
<td>0.97</td>
<td>328,010</td>
</tr>
</tbody>
</table>

**TABLE 3.8 MOLECULAR WEIGHTS DETERMINATION OF BOVINE SERUM DPPIV**

* These molecular weights are estimated based on the SDS PAGE indication that DPPIV is a hetero-tetramer

The average molecular weight of DPPIV was determined to be $292,475 \pm 23,083$ Da

3 8 2 Glycosylation Estimation of DPPIV SDS gel

SDS polyacrylamide gel electrophoresis was performed as described in section 2 10 2. FIGURE 3 8 2 represents the Gelcode Glycoprotein stained gel of serum DPPIV, illustrating magenta bands for the enzyme on a colourless background, indicating glycosylation.
**FIGURES 3.8.3 AND 3.8.4 SIZE EXCLUSION CHROMATOGRAPHY STANDARD CURVES FOR NATIVE MOLECULAR WEIGHT DETERMINATION OF DPPIV**

Plots of log molecular weight versus $V_e/V_o$, where $V_e$ is the elution volume of the standard (●) or sample (○) and $V_o$ is the void volume of the column used. **FIGURE 3.8.3** illustrates the standard curve from Sephacryl S-300 and **FIGURE 3.8.4** shows that of a HiPrep Sephacryl S-300 resin.
LANE SAMPLE
1. HRP
2. Post S300
3. Post QAE
4. Post G100

FIGURE 3.8.2. GLYCOPROTEIN STAIN OF DPPIV
3.8.3 Thermostability Studies of DPPIV

3.8.3.1 Overnight Stability

The stability of DPPIV at 37°C over 24hr was examined as outlined in section 2.10.3.1. Enzyme activity remained relatively stable over this time frame, with a maximum loss of 20% activity after 18.5hr. However, DPPIV activity was restored following further incubation for 24hr. Figure 3.8.3.1 illustrates the activity profile at 37°C overnight for purified DPPIV. This allows for accurate overnight or 24 hour assays such as that required for substrate specificity via HPLC analysis.
**Figure 3.83.1. Thermostability of DPPIV at 37°C**

Plot of fluorescent intensity versus time in hours. The enzymatic activity is expressed as a percentage of the 1 hour value.
3.8.4. pH Effects

The effects of pH on PO and DPPIV activities were monitored in two ways. Primarily, both enzymes were incubated at a range of pH values and buffers and then assayed for activity. The effect of pH on PO is described in section 2.10.4.1. FIGURE 3.8.4.1 clearly indicates that optimal PO activity occurs at pH 7.5 especially in Potassium Phosphate buffer. The enzyme was seen to be particularly inactive in low pH buffers.

The activity of DPPIV at various pH’s was also assessed as described in section 2.10.4.2 where the enzyme was incubated with substrates prepared in a range of pH values. FIGURE 3.8.4.2 illustrates this pH profile, suggesting activity is optimum between pH 7.0 and pH 8.5, while enzymatic activity is completely inhibited at pH’s below 5 and above pH’s 8.5. The enzyme exhibits a slight preference towards a potassium phosphate buffering system; however, all optimised assays were performed in Heps buffer as it was found that potassium phosphate buffer potentially resulted in the auto-catalysis of the Gly-Pro-MCA substrate.

The inactivation of DPPIV is graphically illustrated in FIGURE 3.8.4.3. A classical bell-shape curve was not observed as the enzyme remained active over the pH range. However, Gly-Pro-MCA catalysis was showed to be at its most optimum between pH 6.0 and pH 9.0.
**FIGURE 3.841.**

**PH ACTIVATION OF PO**

Plot of fluorescent intensity versus pH. Buffers tested were citrate (□), phosphate (●), tris (▼), glycine (△).
Figure 3.8.4.2 PH Activation of DPPIV
Plot of fluorescent intensity versus pH. The buffers tested were citrate (□), phosphate (●), hepes (▼), glycine (▲).

Figure 3.8.4.3 PH Inactivation of DPPIV
Plot of fluorescent intensity versus pH. The buffers tested were citrate (○), phosphate (●), hepes (■), glycine (▲).
3.8.5 Isoelectric Point Determination

3.8.5.1 Chromatofocusing
A PBE 94 chromatofocusing resin was employed in an attempt to determine the isoelectric point of DPPIV as described in section 2.10.5.1. The elution profile obtained from this column can be seen in Figure 3.8.5.1. DPPIV activity was eluted in a decreasing linear pH gradient and activity was detected in fractions corresponding to a pH of between 5.45 and 5.6. The pH of this pool was measured to be 5.52, which was taken to be the pI of bovine DPPIV.

3.8.5.2 Isoelectric Focusing
A vertical IEF acrylamide gel was run as outlined in section 2.10.5.2 and stained with brilliant blue G colloidal. Figure 3.8.5.2 represents the stained gel image showing the pI markers and purified DPPIV. The relative mobility (Rf) of each standard and DPPIV was calculated as described in section 2.10.5.2. The distance to the anodic edge of the plate was 30 cm. A plot of Rf versus pI was constructed (Figure 3.8.5.3) and the pI of DPPIV was estimated using the following equation of the line:

$$pl = -5.9932 \times Rf + 9.3232$$

The pI of DPPIV was calculated to be 4.71.
**Figure 385.1.** PI DETERMINATION OF DPPIV VIA CHROMATOFOCUSING

Plots of protein absorbance at 570nm and pH versus fraction number. The graph indicates a PI estimation of 5.2
**Figures 3.8.5.2 and 3.8.5.3.** PI Determination via Isoelectric Focusing

**Figure 3.8.5.2.** represents the IEF gel generated for pi estimation with the black arrow indicating the DPPIV band. **Figure 3.8.5.3.** is a plot of isoelectric point versus R$_f$, where R$_f$ is the distance migrated by the standard (•) or sample (○) divided by the length of the gel plate.
The effect of various functional reagents on DPPIV activity was investigated as outlined in section 2.10.6. TABLES 3.9, 3.10, 3.11, and 3.12 outline residual DPPIV activity following incubation with a range of serine, metallo- and cysteine protease inhibitors and cysteine protease activators respectively. Residual enzyme activity is expressed as a percentage of positive control activity where no functional reagent was added (represented as 100% activity). The concentration listed is the concentration of functional reagent in the pre-incubation mixture (100μl). These results suggest that DPPIV is inhibited by the metalloprotease inhibitors, 4,7-phenanthroline (53.8% residual activity at 200mM) and 1,10-phenanthroline, (75% activity at 200mM) Serine protease inhibitors had relatively no effect on DPPIV while the cysteine protease inhibitors mainly had an enhancing effect on DPPIV activity. Similarly, the cysteine protease activator, 2-mercaptoethanol had an enhancing effect on substrate hydrolysis.
<table>
<thead>
<tr>
<th>Serine Protease Inhibitor</th>
<th>Concentration</th>
<th>Residual DPPIV Activity</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEBSF</td>
<td>22.50 mM</td>
<td>99.2%</td>
<td>0.28%</td>
</tr>
<tr>
<td></td>
<td>11.25 mM</td>
<td>94.6%</td>
<td>4.26%</td>
</tr>
<tr>
<td>APMSF</td>
<td>10.00 mM</td>
<td>96.8%</td>
<td>9.78%</td>
</tr>
<tr>
<td></td>
<td>5.00 mM</td>
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<td>2.97%</td>
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<td></td>
<td>2.50 mM</td>
<td>100%</td>
<td>3.49%</td>
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<td></td>
<td>1.25 mM</td>
<td>97%</td>
<td>1.05%</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>1.25 mg/ml</td>
<td>107%</td>
<td>3.77%</td>
</tr>
<tr>
<td></td>
<td>0.63 mg/ml</td>
<td>100%</td>
<td>0.23%</td>
</tr>
<tr>
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<td>0.25 mg/ml</td>
<td>100%</td>
<td>1.13%</td>
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<td>100%</td>
<td>8.65%</td>
</tr>
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<td>Benzamidime</td>
<td>5.00 mM</td>
<td>97.7%</td>
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</tr>
<tr>
<td></td>
<td>1.00 mM</td>
<td>93.6%</td>
<td>5.42%</td>
</tr>
<tr>
<td>Leupeptin</td>
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<td>103%</td>
<td>2.01%</td>
</tr>
<tr>
<td></td>
<td>0.25 mM</td>
<td>98.1%</td>
<td>3.57%</td>
</tr>
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<td>PMSF</td>
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<td>95%</td>
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<tr>
<td></td>
<td>1.25 mg/ml</td>
<td>93.1%</td>
<td>1.58%</td>
</tr>
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<td></td>
<td>0.50 mg/ml</td>
<td>95.3%</td>
<td>3.67%</td>
</tr>
<tr>
<td></td>
<td>0.25 mg/ml</td>
<td>92.6%</td>
<td>3.37%</td>
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Table 3.9 Effect of Serine Protease Inhibitors on DPPIV Activity
<table>
<thead>
<tr>
<th>Metallo-protease Inhibitor</th>
<th>Concentration (mM)</th>
<th>Residual DPPIV Activity (%)</th>
<th>Standard Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,10-phenanthroline</td>
<td>20.00</td>
<td>75.2</td>
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<td>5.00</td>
<td>82.5</td>
<td>0.42</td>
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<td></td>
<td>1.00</td>
<td>87.6</td>
<td>1.67</td>
</tr>
<tr>
<td>1,7-phenanthroline</td>
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<td>91.8</td>
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<td>7.50</td>
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<td>5.38</td>
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<td>3.75</td>
<td>92.0</td>
<td>7.79</td>
</tr>
<tr>
<td>4,7-phenanthroline</td>
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<td>53.8</td>
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</tr>
<tr>
<td></td>
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<td>96.7</td>
<td>4.41</td>
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<td>8-hydroxyquinoline</td>
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<td></td>
<td>5.00</td>
<td>81.3</td>
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<td></td>
<td>1.0</td>
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<td>CDTA</td>
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<tr>
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<td>10.00</td>
<td>83.4</td>
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<td>5.00</td>
<td>81.4</td>
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</tr>
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<td>5.00</td>
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<tr>
<td></td>
<td>1.00</td>
<td>103.19</td>
<td>0.10</td>
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</table>

Table 3.10 Effect of Metallo-protease Inhibitors on DPPIV Activity
<table>
<thead>
<tr>
<th>Cysteine Protease Inhibitor</th>
<th>Concentration</th>
<th>Residual DPPIV Activity</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTNB</td>
<td>10.00</td>
<td>85.72</td>
<td>5.89</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>76.53</td>
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<tr>
<td></td>
<td>1.00</td>
<td>80.60</td>
<td>7.53</td>
</tr>
<tr>
<td>NEM</td>
<td>10.00</td>
<td>53.22</td>
<td>2.60</td>
</tr>
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<td></td>
<td>5.00</td>
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<td></td>
<td>1.00</td>
<td>77.66</td>
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<tr>
<td>Iodoacetamide</td>
<td>10.00</td>
<td>90.31</td>
<td>2.65</td>
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<td></td>
<td>5.00</td>
<td>85.63</td>
<td>2.71</td>
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<td></td>
<td>1.00</td>
<td>88.94</td>
<td>1.58</td>
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<tr>
<td>Iodoacetic Acid</td>
<td>20.00</td>
<td>113.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.00</td>
<td>122.59</td>
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<tr>
<td></td>
<td>5.00</td>
<td>120.57</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.11 Effect of Cysteine Protease Inhibitors on DPPIV Activity

<table>
<thead>
<tr>
<th>Cysteine Protease Activator</th>
<th>Concentration</th>
<th>Residual DPPIV Activity</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-mercaptoethanol</td>
<td>10.00</td>
<td>125.45</td>
<td>2.22</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>119.93</td>
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<td></td>
<td>1.00</td>
<td>113.56</td>
<td>4.11</td>
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<tr>
<td>DTT</td>
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<td>102.0</td>
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<td>5.00</td>
<td>105.4</td>
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<td>1.00</td>
<td>104.0</td>
<td>2.94</td>
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</table>

Table 3.12. Effect of Cysteine Protease Activators on DPPIV Activity
387 Effect of Other Functional Reagents

The effect of a range of other functional reagents on DPPIV activity was also examined as outlined in section 2.10.7. The residual activities and standard error of the means detected following incubation with these reagents are listed in Table 3.13. Trypsin inhibitor was the only reagent to have an inhibitory effect on enzyme activity with 19.60% inhibition at 0.05 mg/ml. 1.25 mg/ml Pepstatin A resulted in an enzymatic increase of 24% while 1.25 mg/ml Antipain increased activity by 17%. Bacitracin, Carnitine and Puromycin all had an insignificant effect on DPPIV activity.
<table>
<thead>
<tr>
<th>Functional Reagent</th>
<th>Concentration</th>
<th>Residual DPPIV Activity</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antipain</td>
<td>2.50 mg/ml</td>
<td>95.76%</td>
<td>4.37%</td>
</tr>
<tr>
<td></td>
<td>1.25 mg/ml</td>
<td>85.90%</td>
<td>0.74%</td>
</tr>
<tr>
<td></td>
<td>0.50 mg/ml</td>
<td>117.04%</td>
<td>3.56%</td>
</tr>
<tr>
<td></td>
<td>0.25 mg/ml</td>
<td>114.98%</td>
<td></td>
</tr>
<tr>
<td>Bacitracin</td>
<td>1.00 mg/ml</td>
<td>94.50%</td>
<td>2.48%</td>
</tr>
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<td></td>
<td>0.50 mg/ml</td>
<td>88.61%</td>
<td>0.89%</td>
</tr>
<tr>
<td></td>
<td>0.10 mg/ml</td>
<td>97.87%</td>
<td>1.24%</td>
</tr>
<tr>
<td></td>
<td>0.05 mg/ml</td>
<td>95.47%</td>
<td></td>
</tr>
<tr>
<td>Carnitine</td>
<td>10.00 mM</td>
<td>93.70%</td>
<td>8.53%</td>
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<tr>
<td></td>
<td>5.00 mM</td>
<td>96.21%</td>
<td>0.55%</td>
</tr>
<tr>
<td></td>
<td>1.00 mM</td>
<td>101.13%</td>
<td>4.97%</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>1.25 mg/ml</td>
<td>106.47%</td>
<td>1.70%</td>
</tr>
<tr>
<td></td>
<td>0.75 mg/ml</td>
<td>124.08%</td>
<td>3.72%</td>
</tr>
<tr>
<td></td>
<td>0.31 mg/ml</td>
<td>100.92%</td>
<td>1.15%</td>
</tr>
<tr>
<td>Puromycin</td>
<td>2.25 mM</td>
<td>99.74%</td>
<td>4.26%</td>
</tr>
<tr>
<td></td>
<td>1.00 mM</td>
<td>106.08%</td>
<td>1.13%</td>
</tr>
<tr>
<td></td>
<td>0.50 mM</td>
<td>101.79%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25 mM</td>
<td>106.42%</td>
<td>1.29%</td>
</tr>
<tr>
<td>Trypsin Inhibitor</td>
<td>1.00 mg/ml</td>
<td>85.30%</td>
<td>21.08%</td>
</tr>
<tr>
<td></td>
<td>0.50 mg/ml</td>
<td>84.74%</td>
<td>5.05%</td>
</tr>
<tr>
<td></td>
<td>0.10 mg/ml</td>
<td>84.87%</td>
<td>5.71%</td>
</tr>
<tr>
<td></td>
<td>0.05 mg/ml</td>
<td>80.40%</td>
<td>5.71%</td>
</tr>
</tbody>
</table>

**Table 3.13**  **Effect of Other Functional Reagents on DPPIV Activity**
3 8 8 Effect of Metal Ions on DPPIV Activity

The effect of metal salts on purified DPPIV activity was investigated by incorporating the metal salts into the fluorometric assay as outlined in section 2 10 8. The results are presented in TABLE 3 14. MgSO₄ had the most detrimental effect on activity, with a loss of 22% activity, followed by mercuric sulphate. All other metal salts had an insignificant effect on enzymatic activity.

<table>
<thead>
<tr>
<th>METAL SULPHATE</th>
<th>RESIDUAL DPPIV</th>
<th>STANDARD ERROR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACTIVITY</td>
<td>%</td>
</tr>
<tr>
<td>Control</td>
<td>100 00</td>
<td>8.5x10⁻⁴</td>
</tr>
<tr>
<td>Zinc</td>
<td>112 25</td>
<td>4.0x10⁻⁴</td>
</tr>
<tr>
<td>Manganese</td>
<td>105 82</td>
<td>5.1x10⁻⁴</td>
</tr>
<tr>
<td>Sodium</td>
<td>105 62</td>
<td>6.9x10⁻⁴</td>
</tr>
<tr>
<td>Calcium</td>
<td>102 00</td>
<td>7.6x10⁻⁴</td>
</tr>
<tr>
<td>Cadmium</td>
<td>101 04</td>
<td>5.5x10⁻⁴</td>
</tr>
<tr>
<td>Nickel</td>
<td>97 74</td>
<td>5.6x10⁻⁴</td>
</tr>
<tr>
<td>Cobalt</td>
<td>95 29</td>
<td>6.9x10⁻⁴</td>
</tr>
<tr>
<td>Copper</td>
<td>90 90</td>
<td>6.6x10⁻⁴</td>
</tr>
<tr>
<td>Mercury</td>
<td>79 67</td>
<td>6.2x10⁻⁴</td>
</tr>
<tr>
<td>Magnesium</td>
<td>77 74</td>
<td>2.1x10⁻⁴</td>
</tr>
</tbody>
</table>

TABLE 3 14 EFFECT OF METAL IONS

3 8 9 Effect of General Salts on DPPIV

3 8 9 1 Effect of Ammonium Sulphate

3 8 9 1 1 Ammonium Sulphate effect in the substrate

Ammonium Sulphate effect on DPPIV when included in the substrate was carried out as described in section 2 10 9 1 1. FIGURE 3 8 9 1 1 illustrates the significant effect the salt has on substrate catalysis. 300mM Ammonium Sulphate in the substrate resulted in a 60% decrease in activity which is further inhibited to 80% at 1M.
38912 Ammonium Sulphate effect with the enzyme
The effect of Ammonium Sulphate when pre-incubated with purified DPPIV was investigated as outlined in section 2.10.9.12. FIGURE 38912 highlights the inhibitory effect the salt has on enzymatic activity where even at 200mM Ammonium Sulphate a 21% decrease in activity was observed. This trend was continued sequentially to 50% enzymatic activity at 1M Ammonium Sulphate.

3892 Effect of NaCl on DPPIV Activity

38921 Sodium Chloride effect in the substrate
The effect of NaCl inclusion in the Gly-Pro-MCA substrate was carried out as outlined in section 2.10.9.21. It resulted in a significant decrease in purified DPPIV activity, where at a concentration of 600mM NaCl the activity of DPPIV was inhibited by 45% while substrate catalysis was further inhibited by almost 60% at 1 molar sodium chloride. FIGURE 38921 shows the pattern observed.

38922 Sodium Chloride effect with the enzyme
The direct effect of NaCl pre-incubation with purified DPPIV was experimented as described in section 2.10.9.22. It resulted in a significant decrease in purified DPPIV activity, up to a concentration of 600mM NaCl at which point the activity of DPPIV was inhibited by 25% and then the enzymatic activity began to plateau. This is shown in FIGURE 38922.

3893 Effect of Potassium Chloride on DPPIV Activity

38931 Potassium Chloride effect with the enzyme
The effect of KCl on DPPIV activity when pre-incubated was documented in section 2.10.9.3. A decrease in enzymatic activity was observed on increasing salt concentration with an eventual catalytic inhibition of 34% at 1M KCl. FIGURE 38931 illustrates this effect.
Figure 3.8.9.1.1 Effect of Ammonium Sulphate on DPPIV when included with the substrate
Plot of fluorescent intensity versus ammonium sulphate concentration. Activity is expressed as a percentage of the zero ammonium sulphate value.

Figure 3.8.9.1.2. Effect of Ammonium Sulphate on DPPIV activity when pre-incubated with the enzyme
Plot of fluorescent intensity versus ammonium sulphate concentration. Activity is expressed as a percentage of the zero ammonium sulphate value.
FIGURE 3.8.9.2.1 Effect of Sodium Chloride on DPPIV when included in the substrate.
Plot of fluorescent intensity versus sodium chloride concentration. Activity is expressed as a percentage of the zero sodium chloride value.

FIGURE 3.8.9.2.2 Effect of Sodium Chloride on DPPIV when pre-incubated with the enzyme.
Plot of fluorescent intensity versus sodium chloride concentration. Activity is expressed as a percentage of the zero sodium chloride value.
**Figure 3.89.3.1**

**Figure 3.89.3.1 Effect of Potassium Chloride on DPPIV when pre-incubated with the enzyme**

Plot of fluorescent intensity versus potassium chloride concentration. Activity is expressed as a percentage of the zero potassium chloride value.
3.8.10 Substrate Specificity

3.8.10.1 Ion-Pair Reverse Phase HPLC

The ability of purified DPPIV to hydrolyse a range of synthetic and bioactive peptides was investigated using ion-pair HPLC as outlined in section 2.10.10.1. Tables 3.16 and 3.17 lists the peptides which were tested and whether or not cleavage was detected while Table 3.15 indicates the sequences of some bio-active peptides that could be hydrolysed by DPPIV. The cleavage of Casomorphin, CRP (201-206), Enterostatin, Neuropeptide Y (1-24) and Substance P, can be seen in the chromatograms of absorbance versus retention time in Figures 3.8.10.1.1 to 3.8.10.1.20 respectively.

3.8.10.1.1 Inhibition of β-Casomorphin Hydrolysis

The ability of H-Ile-Pyrrolidide to inhibit DPPIV and prove that DPPIV is the enzyme responsible for β-Casomorphin hydrolysis was carried out as described in section 2.10.10.1.1. Figure 3.8.10.1.6 clearly illustrates the large, single β-Casomorphin peak and demonstrates the lack of peptide cleavage due to the specific inhibitor.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>N-Terminal Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Casomorphin</td>
<td>H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH</td>
<td>Bovine</td>
</tr>
<tr>
<td>CRP (201-206)</td>
<td>H-Lys-Pro-Gln-Leu-Trp-Pro-OH</td>
<td>-</td>
</tr>
<tr>
<td>Enteroctatin</td>
<td>H-Ala-Pro-Gly-Pro-Arg-OH</td>
<td>Human</td>
</tr>
<tr>
<td>Neuropeptide Y (1-24)</td>
<td>H-Tyr-Pro-Ser-Lys-Pro-Asp-Asn-</td>
<td>Human/Rat</td>
</tr>
<tr>
<td>Substance P</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.15 Amino Acid Sequences of Bio-Active Peptides Susceptible to DPPIV Hydrolysis
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Hydrolysis by DPPIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ_{11}</td>
<td>No</td>
</tr>
<tr>
<td>Aβ_{20-29}</td>
<td>No</td>
</tr>
<tr>
<td>Aβ_{29-40}</td>
<td>No</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>No</td>
</tr>
<tr>
<td>(Arg^8)-Vasopressin</td>
<td>No</td>
</tr>
<tr>
<td>β-Casomorphin</td>
<td>Yes</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>No</td>
</tr>
<tr>
<td>CRP_{201-206}</td>
<td>Yes</td>
</tr>
<tr>
<td>Enterostatin</td>
<td>Yes</td>
</tr>
<tr>
<td>IGF_{1-3}</td>
<td>Yes</td>
</tr>
<tr>
<td>LHRH</td>
<td>No</td>
</tr>
<tr>
<td>Neuropeptide Y (1-24)</td>
<td>Yes</td>
</tr>
<tr>
<td>Neuropeptide Y (9-13)</td>
<td>No</td>
</tr>
<tr>
<td>Substance P</td>
<td>Yes</td>
</tr>
<tr>
<td>TRH</td>
<td>No</td>
</tr>
</tbody>
</table>

**Table 3.16 HPLC Substrate Specificity Analysis of Bio-Active Peptides**
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Hydrolysis by DPPIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala-Pro-Gly</td>
<td>Yes</td>
</tr>
<tr>
<td>Asp-Pro-Gln-Phe-Tyr</td>
<td>No</td>
</tr>
<tr>
<td>Glu-ProGlu-Thr</td>
<td>No</td>
</tr>
<tr>
<td>Gly-Pro-Ala</td>
<td>Yes</td>
</tr>
<tr>
<td>Gly-Pro-Arg</td>
<td>Yes</td>
</tr>
<tr>
<td>Gly-Pro-Gly-Gly</td>
<td>Yes</td>
</tr>
<tr>
<td>Gly-Pro-Hyp</td>
<td>No</td>
</tr>
<tr>
<td>Gly-Pro-Pro</td>
<td>No</td>
</tr>
<tr>
<td>Phe-Pro-Ala</td>
<td>Yes</td>
</tr>
<tr>
<td>Tyr-Pro-Phe</td>
<td>Yes</td>
</tr>
<tr>
<td>Z-Pro-Ala</td>
<td>No</td>
</tr>
<tr>
<td>Z-Pro-Gly</td>
<td>No</td>
</tr>
<tr>
<td>Z-Pro-Leu</td>
<td>No</td>
</tr>
<tr>
<td>Z-Pro-Pro</td>
<td>No</td>
</tr>
</tbody>
</table>

**Table 3.17. HPLC Substrate Specificity Analysis of Synthetic Peptides**
Figure 3.8.9.1. HPLC Chromatogram of β-Casomorphin Incubation with DPPIV
Plot of absorbance at 214nm (___) and absorbance at 280nm (___) versus time in minutes. The graph shows the sequential hydrolysis of the peptide with the presence of Gly-Pro at 9.9 minutes.

Figure 3.8.9.2. HPLC Chromatogram of β-Casomorphin Standard
Plot of absorbance at 214nm (___) and absorbance at 280nm (___) versus time in minutes.
**Figure 3.8.9.3. HPLC Chromatogram of Tyr-Pro Standard**

Plot of absorbance at 214nm (___) and absorbance at 280nm (___) versus time in minutes.

**Figure 3.8.9.4. HPLC Chromatogram of Phe-Pro Standard**

Plot of absorbance at 214nm (___) and absorbance at 280nm (___) versus time in minutes.
**Figure 3.8.9.5.** HPLC Chromatogram of Gly-Pro Standard

Plot of absorbance at 214nm (__) and absorbance at 280nm (__) versus time in minutes.

**Figure 3.8.9.6.** HPLC Chromatogram of β-Casomorphin Incubation with Ile-Pro-Thiazolidide

Plot of absorbance at 214nm (__) and absorbance at 280nm (__) versus time in minutes. The graph shows inhibition of peptide hydrolysis.
**Figure 3.8.10.1.7.**

HPLC Chromatogram of Substance P Incubation

Plot of absorbance at 214nm (__) and absorbance at 280nm (__) versus time in minutes. The graph shows the two dipeptides that are cleaved off the bio-active peptide (See Table 3.15).

**Figure 3.8.10.1.8.**

HPLC Chromatogram of Substance P Standard

Plot of absorbance at 214nm (___) and absorbance at 280nm (___) versus time in minutes.
**Figure 3.8.9.9.** HPLC Chromatogram of Arg-Pro standard

Plot of absorbance at 214nm (__) and absorbance at 280nm (__) versus time in minutes.

**Figure 3.8.9.10.** HPLC Chromatogram of Lys-Pro standard

Plot of absorbance at 214nm (___) and absorbance at 280nm (___) versus time in minutes.
**Figure 3.8.9.11.** HPLC Chromatogram of Enterostatin Incubation with DPPIV.
Plot of absorbance at 214nm (___) and absorbance at 280nm (___) versus time in minutes. The graph illustrates the sequential of the pentapeptide.

**Figure 3.8.9.12.** HPLC Chromatogram of Enterostatin Standard
Plot of absorbance at 214nm (___) and absorbance at 280nm (___) versus time in minutes.
FIGURE 3.8.9.13

**HPLC CHROMATOGRAM OF ALA-PRO STANDARD**

Plot of absorbance at 214nm (____) and absorbance at 280nm (____) versus time in minutes.

FIGURE 3.8.9.14

**HPLC CHROMATOGRAM OF GLY-PRO STANDARD**

Plot of absorbance at 214nm (____) and absorbance at 280nm (____) versus time in minutes.
**FIGURE 3.8.9.15.** HPLC CHROMATOGRAM OF CRP (201-206) INCUBATION WITH DPPIV
Plot of absorbance at 214nm (__) and absorbance at 280nm (__) versus time in minutes. The chromatogram shows the presence of a cleavage product at 16.767 minutes.

**FIGURE 3.8.9.16.** HPLC CHROMATOGRAM OF CRP (201-206) STANDARD
Plot of absorbance at 214nm (___) and absorbance at 280nm (__) versus time in minutes.
FIGURE 3.8.9.17. HPLC CHROMATOGRAM OF LYS-PRO STANDARD

Plot of absorbance at 214nm ( ) and absorbance at 280nm ( ) versus time in minutes.
**Figure 3.8.9.18.**

HPLC Chromatogram of Neuropeptide Y (1-24) Incubation with DPPIV

Plot of absorbance at 214nm (__) and absorbance at 280nm (__) versus time in minutes. The figure illustrates the presence of the dipeptide Tyr-Pro at 16.683 minutes.

**Figure 3.8.9.19.**

HPLC Chromatogram of Neuropeptide Y (1-24) Standard

Plot of absorbance at 214nm (__) and absorbance at 280nm (__) versus time in minutes.
**Figure 3.8.9.20.**

Plot of absorbance at 214nm (red line) and absorbance at 280nm (blue line) versus time in minutes.

**Figure 3.8.9.20. HPLC Chromatogram of Tyr-Pro Standard**

Plot of absorbance at 214nm (red) and absorbance at 280nm (blue) versus time in minutes.
3.8.10.2 Kinetic Analysis

3.8.10.2.1 $K_M$ Determinations for Gly-Pro-MCA

The $K_M$ values for the reaction of DPPIV on Gly-Pro-MCA were determined experimentally as described in section 2.10.10.2.1. $K_M$ and $V_{max}$ values were determined using Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf analysis as described in section 6.4.1. Table 3.18 lists the $K_M$ and $V_{max}$ values deduced using these models. Figures 3.8.10.2.1 to 3.8.10.2.4 illustrate Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf plots.

<table>
<thead>
<tr>
<th>Model</th>
<th>$K_M$ (μM)</th>
<th>$V_{max}$ (Fluorescent units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Michaelis-Menten (MM)</td>
<td>40</td>
<td>142</td>
</tr>
<tr>
<td>Lineweaver-Burk (LB)</td>
<td>33 85</td>
<td>150 4</td>
</tr>
<tr>
<td>Eadie-Hofstee (EH)</td>
<td>34 65</td>
<td>151 02</td>
</tr>
<tr>
<td>Hanes-Woolf (HW)</td>
<td>45</td>
<td>143 2</td>
</tr>
<tr>
<td>Average</td>
<td>38 4</td>
<td>146 7</td>
</tr>
</tbody>
</table>

Table 3.18. Kinetic Analysis of DPPIV Using Gly-Pro-MCA

3.8.10.2.2 $K_M$ Determinations for Lys-Pro-MCA

The $K_M$ values for the reaction of DPPIV on Lys-Pro-MCA were determined experimentally as described in section 2.10.10.2.2. $K_M$ and $V_{max}$ values were determined using Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf analysis as described in section 6.4.1. Table 3.19 lists the $K_M$ and $V_{max}$ values deduced using these models. Figures 3.8.10.2.1 to 3.8.10.2.4 illustrate Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf plots.
<table>
<thead>
<tr>
<th>Model</th>
<th>K&lt;sub&gt;M&lt;/sub&gt;</th>
<th>V&lt;sub&gt;MAX&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Michaelis-Menten (MM)</td>
<td>95 μM</td>
<td>710 Fluorescent units</td>
</tr>
<tr>
<td>Lineweaver-Burk (LB)</td>
<td>103.9 μM</td>
<td>880.8 Fluorescent units</td>
</tr>
<tr>
<td>Eadie-Hofstee (EH)</td>
<td>106.7 μM</td>
<td>891.8 Fluorescent units</td>
</tr>
<tr>
<td>Hanes-Woolf (HW)</td>
<td>105 μM</td>
<td>881.6 Fluorescent units</td>
</tr>
<tr>
<td>Average</td>
<td>102.65 μM</td>
<td>841.1 Fluorescent units</td>
</tr>
</tbody>
</table>

**Table 3.19 Kinetic Analysis of DPPIV using Lys-Pro-MCA**
**Figure 3.8.10 2.1.1**

Represents the Michaelis-Menton plot (fluorescent intensity versus substrate concentration).

**Figure 3.8.10 2.1.2**

Is a Lineweaver-Burk plot (1/fluorescent intensity versus 1/substrate concentration).

**Figures 3.8.10 2.1.1 and 3.8.10 2.1.2: Kₘ Determination of DPPIV on Gly-Pro-MCA**

Figure 3.8.10 2.1.1 represents the Michaelis-Menten plot (fluorescent intensity versus substrate concentration). Figure 3.8.10 2.1.2 is a Lineweaver-Burk plot (1/fluorescent intensity versus 1/substrate concentration).
FIGURE 3.8.10.2.13

Fluorescent Intensity/Gly-Pro-MCA Concentration (fiM-1)

FIGURE 3.8.10.4.

Gly-Pro-MCA Concentration (μM)

Gly-Pro-MCA Concentration/Fluorescent Intensity

FIGURES 3 8 10 2 1 3 AND 3 8 10 2 1 4. K_M DETERMINATION OF DPPIV ON GLY-PRO-MCA

FIGURE 3 8 10 2 1 3 represents the Eadie-Hofstee plot (fluorescent intensity versus fluorescent intensity/substrate concentration) FIGURE 3 8 10 2 1 4 is a Hanes-Woolf plot (substrate concentration/fluorescent intensity versus substrate concentration)
**Figure 3.8.10.2.2.1**

Lys-Pro-MCA Concentration (µM)

**Figure 3.8.10.2.2.2**

1/Lys-Pro-MCA Concentration

**Figures 3.8.10.2.2.1** and **3.8.10.2.2.2** *K_m* Determinations of DPPIV on Lys-Pro-MCA

**Figure 3.8.10.2.2.1** represents the Michaelis-Menton plot (fluorescent intensity versus substrate concentration) **Figure 3.8.10.2.2.2** is a Line-Weaver Burk plot (1/fluorescent intensity versus 1/substrate concentration)
FIGURE 3810223 represents the Eadie-Hofstee plot (fluorescent intensity versus fluorescent intensity/substrate concentration).

FIGURE 3810224 is a Hanes-Woolf plot (substrate concentration/fluorescent intensity versus substrate concentration).

FIGURES 3810223 AND 3810224 KM DETERMINATION OF DPPIV ON LYS-PRO-MCA

FIGURE 3810223 represents the Eadie-Hofstee plot (fluorescent intensity versus fluorescent intensity/substrate concentration). FIGURE 3810224 is a Hanes-Woolf plot (substrate concentration/fluorescent intensity versus substrate concentration).
3811 Inhibitor Studies of DPPIV

38111 IC₅₀ Determination of Specific Inhibitors
The effect of specific peptidase inhibitors on DPPIV activity was determined (see section 210111). Plots of enzyme activity versus inhibitor concentration were constructed and are shown in FIGURES 38111 - 381114. TABLE 320 summarises the IC₅₀ values determined for DPPIV as described in section 65.

38112 Kᵢ Determination of Specific Inhibitors
The effect of the inclusion of various specific inhibitors on the kinetic interaction between DPPIV enzyme activity and Gly-Pro-MCA was determined as described in section 210112. A Kᵢ value was determined for DPPIV using the kinetic models described in section 381021. Kₚᵢ values were graphically deduced for each inhibitor and subsequently a Kᵢ value was estimated as outlined in the appendix, section 642. The Kᵢ values for DPPIV interactions studied are presented in TABLE 320. FIGURES 381123 and 381124 illustrate the competitive inhibition of DPPIV by Ile-Thiazolidide and Ile-Pyrrolidide respectively. FIGURES 381121 and 381122 show competitive-non-competitive mixed inhibition by Diprotin A and Diprotin B.

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>MODE</th>
<th>IC₅₀</th>
<th>Kᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diprotin A</td>
<td>Non-competitive</td>
<td>3.6x10⁻⁵</td>
<td>1.36x10⁻⁴</td>
</tr>
<tr>
<td>Diprotin B</td>
<td>Non-competitive</td>
<td>5x10⁻⁵</td>
<td>1.4x10⁻⁴</td>
</tr>
<tr>
<td>H-Ile-Thiazolidide</td>
<td>Competitive</td>
<td>7.5x10⁻⁷</td>
<td>3.7x10⁻⁷</td>
</tr>
<tr>
<td>H-Ile-Pyrrolidide</td>
<td>Competitive</td>
<td>1x10⁻⁶</td>
<td>7.5x10⁻⁷</td>
</tr>
</tbody>
</table>

TABLE 320 EFFECT OF SPECIFIC INHIBITORS ON DPPIV ACTIVITY
Concentrations as given in TABLE 216.

38113 Kᵢ Determinations for Proline-containing Peptides
The effect of the inclusion of various proline-containing peptides on the kinetic interaction between enzyme activities and Gly-Pro-MCA was determined as described in section 210113. A Kᵢ value was determined for DPPIV using the kinetic models described in section 381021. Kᵥ values were graphically deduced for each of the proline containing peptides and subsequently a Kᵢ value was estimated for each of the peptides tested (see appendix, section
The nature of the inhibition was determined as outlined in section 6.4.3. The $K_i$ values for DPP IV interactions studied are presented in Table 3.21. Figure 3.8.11.3.1 is a double reciprocal Lineweaver Burk plot which illustrates the competitive inhibition of DPP IV by β-Casomorphin. In contrast, Figure 3.8.11.2 shows uncompetitive inhibition by Substance P via a Lineweaver Burk plot.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Inhibition</th>
<th>$K_i$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LB</td>
<td>EH</td>
</tr>
<tr>
<td>β-Casomorphin</td>
<td>Competitive</td>
<td>9x10$^{-5}$M</td>
</tr>
<tr>
<td>Substance P</td>
<td>Un-competitive</td>
<td>4 5x10$^{-4}$M</td>
</tr>
</tbody>
</table>

**Table 3.21 Kinetic Analysis of DPP IV with Proline Containing Peptides**

Assay concentrations of inhibitors as given in Table 2.17.

Models used LB=Lineweaver-Burk, EH=Eadie Hofstee, HW=Hanes Woolff (see section 6.4 for calculations)
**Figures 3.8.11.1 and 3.8.11.2: Inhibitor Studies**

Log plots of DPP IV activity versus inhibitor concentration. Activity is expressed as a percentage of the no inhibitor value. **Figure 3.8.11.1** illustrates potency of Diprotin A on enzyme activity. **Figure 3.8.11.2** shows a similar effect of Diprotin B.
**FIGURE 3.8.11.3.**

![Graph showing Ile-Thiazolidide Concentration (M) vs. Enzyme Activity (%)]

**FIGURE 3.8.11.4.**

![Graph showing Ile-Pyrrolidide Concentration (M) vs. Enzyme Activity (%)]

**FIGURES 3.8.11.3 AND 3.8.11.4 INHIBITOR STUDIES**

Log plots of DPPIV activity versus inhibitor concentration. Activity is expressed as a percentage of the no inhibitor value. **FIGURE 3.8.11.3** highlights the potency of Ile-Thiazolidide on enzyme activity. **FIGURE 3.8.11.4** also shows a strong specific effect of Ile-Pyrrolidide on DPPIV.
**Figure 3.8.11.21**

Lineweaver-Burk reciprocal plots of fluorescent intensity versus Gly-Pro-MCA concentration.

**Figure 3.8.11.22**

Lineweaver-Burk reciprocal plots of fluorescent intensity versus Gly-Pro-MCA concentration.

**Figures 3.8.11.21 and 3.8.11.22 Kinetic Analysis of DPPIV Specific Inhibitors**

Lineweaver-Burk reciprocal plots of fluorescent intensity versus Gly-Pro-MCA concentration. **Figure 3.8.11.21** shows the mixed inhibition of Diprotin A (□). **Figure 3.8.11.22** illustrates the Competitive-non-competitive inhibition of Diprotin B at $2.5 \times 10^{-5}$M (▼) and $2.5 \times 10^{-4}$M (△). Both were compared to no inhibition (●).
**FIGURE 3 8.11.2 3**

Lineweaver-Burk reciprocal plots of fluorescent intensity versus Gly-Pro-MCA concentration. **FIGURE 3 8.11.2 3** highlights the Competitive inhibition of Ile-Thiazolidide at $1 \times 10^6 \text{M}$ (△) and $1 \times 10^5 \text{M}$ (△). **FIGURE 3 8.11.2 2** also illustrates the Competitive inhibition of Ile-Pyrrolidide at $1 \times 10^6 \text{M}$ (▽) and $1 \times 10^5 \text{M}$ (△). Both were compared to no inhibition (●).

**FIGURES 3 8.11 2 1 AND 3 8 11.2 2. KINETIC ANALYSIS OF DPPIV SPECIFIC INHIBITORS**

Lineweaver-Burk reciprocal plots of fluorescent intensity versus Gly-Pro-MCA concentration. **FIGURE 3 8.11.2 3** highlights the Competitive inhibition of Ile-Thiazolidide at $1 \times 10^6 \text{M}$ (▽) and $1 \times 10^5 \text{M}$ (△). **FIGURE 3 8.11.2 2** also illustrates the Competitive inhibition of Ile-Pyrrolidide at $1 \times 10^6 \text{M}$ (▽) and $1 \times 10^5 \text{M}$ (△). Both were compared to no inhibition (●).
FIGURE 3.8 11 3 1

Lineweaver-Burk reciprocal plots of fluorescent intensity versus Gly-Pro-MCA concentration.

- FIGURE 3.8 11 3 1 highlights the Competitive inhibition of $3 \times 10^{-3}$M $\beta$-Casomorphin (□).
- FIGURE 3.8 11 3 2 illustrates the Uncompetitive inhibition of Substance P at $2 \times 10^{-3}$M ($\triangle$). Both were compared to no inhibition ($\bullet$).
4.0. DISCUSSION
4.0. DISCUSSION

4.1 FLUORESCENCE SPECTROMETRY USING 7-AMINO-4-METHYL-COUMARIN

This work primarily focused on the study of two proline specific peptidases, namely PO and DPPIV from human saliva and bovine serum respectively. The employment of high affinity substrates is essential for accurate detection of low levels of these enzymes in biological tissues. The selection of substrates for the enzyme of interest has evolved over the years when the properties and functionality become clearer such as, Walter et al., 1971 identifying PO using radiolabelled oxytocin. Similarly, synthetic substrates have been specifically formulated for enzyme detection in recent years. The some of the main parameters ruling substrate selection are the specificity of the enzyme to the substrate and the chromophore employed. Hopsu-Havu and Glenner, 1966 first reported DPPIV as glycylproline naphthylamidase and many reports have utilised radiolabelled and spectrophotometric substrates. However, fluorimetric assays generally offer increased safety, sensitivity and specificity over colorimetric, spectrophotometric and radiometric assays. The high sensitivity from the wavelength difference between the exciting and fluorescence radiation results in minimal background, while the specificity is significantly increased due to a potentially unique excitation and emission spectra.

The substrates used for PO and DPPIV detection were Z-Gly-Pro-MCA and Gly-Pro-MCA respectively. These externally quenched fluorimetric substrates both allow for peptide cleavage on the carboxyl side of the proline residue liberating the fluorophore, MCA. Excitation to a higher energy state occurs at 370nm due to constant absorption of electromagnetic radiation by MCA and requires a significant quantity of energy to create this state. By the nature to fluorescence, the energised molecule returns to a lower state and results in a subsequent re-emission of radiation at 440nm. The assay’s sensitivity can be increased by widening of the excitation and emission slit widths used. This consequently broadens the bandwidth over which light is integrated. For instance, at an emission wavelength of 440nm and a slit width of 5nm, the fluorimeter integrates light radiated from 437.5nm to 442.5nm, while if the slit width is increased to 10nm, light radiated from 435nm to 445nm is integrated. Therefore, for samples of particularly low enzyme activity and hence low fluorescence, adaptation of the emission slit width ensures improved sensitivity. However, it is recommended to maintain the excitation slit width due to the high energy required to energise the MCA molecule. Thus, in turn will increase the accuracy and reproducibility of all subsequent assays.

176
4.1.1 MCA Standard Curves and the Inner Filter Effect

The excitation of the MCA molecule at 370nm and subsequent emission at 440nm is a very specific and well documented phenomenon. However, the selection of these wavelengths was investigated as described in section 2.2.3 and the subsequent scans reported in section 3.1.1. Figures 3.1.5 and 3.1.6 clearly demonstrate the fluorescent intensity peak heights at both 370nm for the excitation scan and 440nm for the emission scan. It is worth noting that a significant trend was observed during these experiments. On a number of occasions, the scan reported an excitation maximum at 340nm with the maximum emission wavelength remaining at 440nm. Preliminary analysis was undertaken by utilising these wavelengths when analysing enzymatic activity. The notable difference observed was that the fluorescent intensity of all fractions was significantly higher but no significant difference existed between blanks and active triplicates. This illustrates the presence of contaminating compounds that may fluoresce at 340nm and 440nm greater than MCA does at 370nm, 440nm. Therefore, the fluorometer searches for fluorescence maximum which is not attributed to free MCA and returns inaccurate optimised wavelengths. Therefore, the selection of 370nm as the excitation wavelength and 440nm as the emission wavelength agrees with other reports when MCA is employed as the fluorophore (Gilmartin and O’Cunn, 1999).

Other similar or almost identical disadvantages in fluorometric assays are called quenching and the inner filter effect. Both effects lead to an apparent alteration in fluorescent intensity and hence can be problematic where quantitation of fluorescence is important. Quenching involves the removal of energy from a molecule in the excited state by another molecule, the most common form (collisional impurity quenching) is due to the collision of molecules. This type of quenching may be attributed to the presence of heavy metals or dissolved oxygen in samples. The inner filter effect is a result of an overlap of the excitation or emission band of the fluorescing sample with the absorption band of a contaminating compound. These phenomena inevitably result in inaccurate false positives and negatives such as the inaccurate MCA wavelength scans.

To ascertain the extent of this effect and attempt to alleviate the problem, free MCA standard curves were constructed as outlined in section 2.2.1 and recorded as reported in section 3.1. A notable difference in fluorescence detection was observed when biological samples were added. Figures 3.1.3 and 3.1.4 demonstrate the effect buffered saliva and bovine serum can have on fluorescence and what is perceived as free MCA fluorescent intensity units. Both samples contribute to the inner filter effect with serum responsible for a significant 19% filtering. A notable decrease in filtering once purification commenced. Similarly, although buffered saliva did cause some filtering, none of the post column fractions contributed to any.
inner filter effect. This emphasises the importance of accounting for this effect, especially with coloured or turbid crude samples when quantifying the enzymatic activity present. This accountability is even more important when accurately, ascertaining purification performance and effectiveness.

It is worth noting too that, in addition to biological samples such as serum, many other compounds contribute to the inner filter effect. These included metal salts, functional reagents, inhibitors, and even buffers. Therefore, it was necessary to account for these effects during any characterisation studies to ascertain the effect the compound is having on the enzyme and not the fluorescence of the substrate.

4.2 Protein Determination

The enzymatic detection method employed is of significant importance and leads to much variability depending on the enzyme and the biological source. However, the accurate quantification of the total protein of a sample is of equal importance. As already described, correct enzyme activity is required for purification assessment. Similarly, this assessment can not be achieved without the knowledge to the total protein content. Many protein determination techniques and reagents have evolved, each having their own advantages and disadvantages depending on the assay required. The most non-specific, inexpensive method utilises ultraviolet spectrometry at 280 nm. This method exploits the ability of the aromatic amino acids, tyrosine and tryptophan, to absorb at 280 nm. Therefore, the assay will only be efficient when these amino acids are present in abundance. One must question the inaccuracy created from the analysis of proteins with an unusually high proportion of these amino acids. The assay is inaccurate unless the extinction coefficient of the protein is known, which is solely dependent on the precise amino acid composition which is not usually known. Similarly, the assay has many interferents that absorb at the same wavelength, giving an unusually high absorbance. These include compounds that contain purine and pyrimidine rings such as nucleic acids and nucleotides which is abundant in many biological samples (Dunn, 1989).

In contrast, the Biuret assay has little dependence on amino acid composition and has no interference from free amino acids. This is due to the binding of copper ions to the peptide chain, nitrogen of proteins and producing a purple colour with a maximum absorbance at 560 nm in alkaline conditions. This assay is utilised when the protein content is in the region of 2-10 mg/ml, however, at protein concentrations lower than this, the assay proves insensitive. Thus, this method was only employed for crude protein samples. Nonetheless, this technique

178
showed a notable reduction in interference substances, was linear up to 10mg/ml and requires less protein sample in comparison to the 280nm UV absorbance method.

The BCA assay combines the Biuret reaction of the reduction of Cu$^{2+}$ by protein to Cu$^{+}$, with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu$^{+}$) by bicinchoninic acid or BCA (FIGURE 4.1)

1. Protein (peptide bonds) + Cu$^{2+}$ $\xrightarrow{\text{OH}}$ tetradentate-Cu$^{+}$ complex

2. Cu$^{+}$ + 2 Bicinchoninic Acid (BCA) $\xrightarrow{\text{}}$ BCA-Cu$^{+}$ complex (purple coloured)

**FIGURE 4.1. BCA-PROTEIN REACTION SCHEME**

The BCA assay is generally linear up to 2mg/ml, however its linearity is significantly increased when the protein content is reduced to 1 2mg/ml or below (FIGURE 3.22). The BCA reagent is very sensitive and has few interfering compounds. In addition, the BCA assay’s adaptability is proven by simply adjusting the assay temperature and protein concentrations less than 100µg/ml can be detected, as outlined in section 2.3.5 to produce the enhanced BCA assay.

The Coomassie® Plus Protein assay has a similar linearity to the standard BCA assay of up to 2mg/ml. The Coomassie molecule binds to protein in an acidic medium causing a colour change from green/brown to blue which maximally absorbs at 595nm. The main advantage for utilizing this assay in comparison to the BCA method was its compatibility with the thiol reducing agent DTT. For all characterisation of human salivary PO, DTT was essential to maintain enzyme activity. However, its presence at even 5mM interfered significantly with the accuracy of the BCA reagent. Therefore, any protein profiles of post-column PO was carried out using the Coomassie Plus protein reagent. Nonetheless, when protein quantification was required, dialysis of the sample was employed and then subsequent BCA assay analysis. This was a result of the reduced linearity of the Coomassie reagent and the batch to batch variation of the results generated.
### Table 4.1 Comparison of Protein Determination Assays

The interferents listed are an indication and depend on the compounds concentration. Despite the disadvantage of the interferents, their effects can be compensated by the inclusion of an appropriate blank.

#### 4.3 Enzyme Assays

Enzyme assays employed for both PO and DPPIV were fluorimetric, with MCA the released fluorophore being detected. The linear relationship between fluorescence and MCA concentration allowed for subsequent accurate quantification of enzyme activity (see appendix, section 6.2).

Yoshimoto et al., (1979) classified Z-Gly-Pro-MCA as a specific substrate for PO. Since then, many researchers have utilised this substrate when trying to ascertain the influence of PO in biological systems. However, the existence of a second Z-Gly-Pro-MCA cleaving activity has been reported by this laboratory (Cunningham and O'Connor, 1997b). This activity proved unique to PO by its insensitivity to Z-Pro-Prohinal, a specific PO inhibitor and was therefore termed ZIP. Similarly, the relative ineffectiveness of the thiol reducing agent, on ZIP showed another significant difference to PO. Initial studies on salivary PO proved that the enzyme had an obvious dependence on DTT for enzyme stability and was included in all buffers and substrates when analysing the peptidase. More importantly, $1 \times 10^{-5}$M Z-Pro-Prohinal completely inhibited the enzyme even when buffered saliva was analysed. Thus, this would suggest that the Z-Gly-Pro-MCA cleaving activity observed is solely attributed to Prolyl Oligopeptidase and Z-Pro-Prohinal Insensitive Peptidase (ZIP) is not present. Therefore, the optimised assay conditions employed is described in section 2.4.1.
The selection of Gly-Pro-MCA for Dipeptidyl Peptidase IV analysis was comparatively less complicated. The use of the dipeptide, Gly-Pro, is widely reported and is subsequently deemed a specific DPPIV substrate when attached to a chromophore and mild basic conditions are employed. The choice of chromophore varies for many researchers; however, the superiority of fluorimetry has already been established. The choice of fluorophore utilised is somewhat more straightforward, with 7-amino-4-methyl-coumarin (MCA) being a very common selection, which is in tandem with many other recent reports (Ohkubo et al., 1994, Gilmartin and O’Cuinn, 1999). DPPIV does not show a preference for thiol reducing agents such as DTT nor does it require metals nor co-factors for activity. In addition, the enzyme has little requirement for the metal chelator, EDTA. Nevertheless, EDTA was often included in the substrate and running buffers when high salt concentrations were involved.

Both enzyme assays were highly sensitive, rapid and quantifiable. Another important parameter to account for is that the magnitude and characteristics of inner filter effects strongly rely on the sample-cell geometry. The use of a cuvette-based assay ensures perpendicular geometry between the sample and the incident beam. The fluorescence emitted is collected along an axis at right angles to the excitation beam. This is the preferred geometric orientation for fluorimetric assays as it avoids the effects of scattered and transmitted excitation, which can lead to inaccuracies (Figure 4.2).

The aforementioned assay conditions were also employed for microplate use as described in sections 2.4.2 and 2.4.4. This method was utilised for rapid identification of post-column fractions. The preferential perpendicular geometry is not obeyed but uses front surface geometry, the fluorescent signal is collected and measured through the illuminated surface (see Figure 4.2). This arrangement increased inner filter effects and light scattering, thus reducing the assay's sensitivity. Despite these factors, the microplate assay proved invaluable for non-quantification of both enzyme activities and was commonly utilised. It allowed for the use of less sample, substrate and acid than that of the cuvette assay and was subsequently quicker. Once activity could be identified, the assay was repeated employing the perpendicular geometry for accurate quantification.
**Figure 4.2. Sample-Cell Geometry**

MCA (®) absorbs electromagnetic radiation at 370nm (→) and emits fluorescence at 440nm (→).

**A:** Cuvette-based assay - Fluorescence is emitted along an axis at 90° to the excitation beam - Perpendicular geometry.

**B:** Microtitre plate assay - Fluorescence is emitted along the same axis as the excitation beam - Front surface geometry.
4.4 Purification

The purification of a protein incorporated a number of specialised techniques which exploit certain physiochemical and biological characteristics of the protein of interest. It would be considered that the purification of a protein is generally not the end-point but a means to carry out further studies. As a consequence, the maintenance of stability of the target protein is of vital importance. The nature of the further studies will dictate the necessary purity, activity and quantity of the protein. These parameters play a considerable role in the selection of the purification tools utilised and the extent to which they are implemented.

4.4.1 Purification of Human Salivary Prolyl Oligopeptidase

The purification of salivary PO was, from the outset, observed as potentially straightforward. The bovine brain and serum sources of PO contained a stable, functional protein. It was acknowledged that serum protein levels are often proportionally replicated in saliva without the large quantity of contaminating protein. Therefore, it was expected that a significant reduction in chromatographic techniques would be required to purify the salivary protein in comparison to its serum counterpart.

4.4.1.1 Saliva Preparation

Saliva was collected and prepared as outlined in section 2.5.1. Batch to batch variation was significant for PO levels, even though the time of collection was regulated. Eating habits had a big influence on the enzyme levels as they seemed to be proportional to the quantity of saliva produced. For instance, PO levels appeared to be particularly low just after food intake but rose steadily hours after. The immediate buffering of the saliva was of great importance in maintaining enzyme activity and allowing for reproducible results.

4.4.1.2 Ammonium Sulphate Precipitation

The salt fractionation of saliva was carried out in an attempt to begin the purification process but also concentrate PO activity. The technique operates according to the hydrophobic nature of the surface of the protein. Hydrophobic groups, by their very nature, predominate in the interior of the protein. When salt is added to the system, water surrounding the hydrophobic groups solvates the salt ions and as the salt concentration increases, water is removed from around the protein, eventually exposing the hydrophobic patches. Aggregation then results from the hydrophobic patches from one protein interacting with another. Proteins with more hydrophobic patches will then aggregate quicker than those with few patches. This
phenomenon is the basis upon which proteins can be sequentially salted out and thus separated from other proteins.

**FIGURES 3.3.11 and 3.3.12** highlight the success of the technique. Generally the removal of enzyme activity from the supernatants results in subsequent increase in the corresponding pellets. Despite the impressive quantity of activity observed in the pellets in comparison to the supernatants, it should be acknowledged that volume is not taken into account. All experiments involved salt addition to 10ml of buffered saliva while the pellets were re-suspended in 5ml of buffer. Nevertheless a considerable concentration of PO activity occurs and there is also a notable reduction in protein content generating a two-fold purification step (Table 3.2). However the table shows a 2.5-fold reduction in activity which may be attributed to many factors. According to Harris and Angel, 1989, 80% Ammonium Sulphate in 10ml of solution requires 5.23g of solid salt to be added. This is the equivalent of almost 4 Molar, which may easily have a detrimental effect on enzyme activity. Similarly, the addition of this quantity of this salt may cause localised unhomogenous decreases in pH, which may not be favourable for activity. As outlined in section 2.5.2 the method requires 1 hour of constant stirring at 4°C followed by a 45 minute centrifugation step both of which contribute significantly to the shear stress applied to the enzyme and may lead to protein instability. In addition the exposure of the enzyme’s hydrophobic patches may cause protein unfolding and subsequent partial denaturation.

4.4.1.3 Phenyl Sepharose Hydrophobic Interaction Chromatography

Like ammonium sulphate salt precipitation, hydrophobic interaction chromatography (HIC) exploits the presence of hydrophobic patches that can exist on the surface of a protein. This is due to the side chains of non-polar amino acids such as alanine, tryptophan and phenylalanine. The number and size of these patches varies for each protein and therefore can be used as a purification tool due to the specific interactions that result. A hydrophilic shell generally surrounds a hydrophobic core of the protein. This shell can be removed by salt to expose the patches and enforce binding to a non-ionic group such as phenyl.

Post 80% ammonium sulphate precipitation PO proved ideal for subsequent application to a Phenyl Sepharose column as the enzyme’s hydrophobic patches are already exposed and dialysis was not required. The chromatographic method was carried out also described in section 2.5.3. **Figure 3.3.21** illustrates the elution profile obtained from the HIC column where specific binding of PO separated the enzyme from most other contaminating proteins. The enzyme was eluted using an isocratic step, however a linear gradient was also employed. This step did not separate any more of the protein from the enzyme and appear to dilute the
PO elute peak Therefore the isocratic step was preferred However as TABLE 3 2 illustrates that despite a two-fold reduction in protein content, there is a considerable 4 6-fold decrease in enzymatic activity This reduced the purification factor to 0 9, which is undesirable It is worth noting that when purifying from a source of low protein, the purification factor can never be that high This is a result of the influence of protein content on the generation of the specific activity It is common to lose some enzyme activity during the course of a purification but this should be outweighed by the greater reduction in protein which would subsequently generate an increased specific activity factor and a greater purification factor When the protein level is low from the outset this occurrence is near impossible

4 4 1 4 Q-Sepharose Fast Flow Anion Exchange Chromatography
Proteins carry positive and negative charges on their surfaces, due mainly to the side chains of acidic and basic amino acids The net charge on a protein depends on the relative numbers of positive and negative charged groups, which varies with pH The pH at which a protein has an equal number of positive and negative charged groups is called the isoelectric point (pI) This point is often used to characterise a particular protein and similarly this property can also be used to purify proteins Ion exchange of proteins involves their adsorption to charged groups followed by their elution with a buffer of higher ionic strength

Q-Sepharose is an anion exchanger which is frequently employed to separate proteins of pI’s less than 7 Although the precise pI of salivary PO was unknown, bovine serum and brain PO had an isoelectric point in the range 4 5-5 0, which is common for most mammalian proteases Therefore this technique was selected to specifically bind and purify the enzyme FIGURE 3 3 3 1 illustrates the successful binding to the resin Its appears that almost all contaminating protein was eliminated in the run-through peak The linear gradient was selected as it did not lead to peak tailing and may have eluted other proteins undetected by the Coomassie protein method The purification table shows another reduction (11-fold) in activity compared to the previous phenyl sepharose column while the protein content decrease was only 1 3-fold This inevitably generated an extremely low overall purification factor

It would seem that binding of this peptidase to any resin results in denaturation None of the chromatographies employed exposed the enzyme’s active site and should not have resulted in such a decrease in total activity During the purification process the enzyme was subject to more disabling properties due to surrounding protein being removed However despite this low factor, if activity could be maintained to allow for subsequent analysis then the purification protocol would be acceptable
**4 4 1 5 Alternative Chromatographies for PO Purification**

After the Phenyl Sepharose step PO activity was moderately high but not sufficiently pure. This sample was subjected to a number of resins and techniques in an attempt to purify the protein while maintaining activity.

DEAE is a weak anion exchanger that separates similarly to the protocol outlined for QAE Sepharose in section 4 4 1 3. FIGURE 3 3 4 1 highlights the inability of the resin to separate the enzyme from contaminating protein. Although an isocratic step is reported, the linear gradient did not improve protein separation but diluted the enzyme further. As a consequence of the resin’s ability to bind too much protein, the efficient separation of enzyme from contaminating protein was restricted to the elution step which proved difficult.

Benzamidine is a serine protease inhibitor often used to classify classical, trypsin-like serine proteases. When the molecule is immobilised onto Sepharose 6B, it potentially creates a very specific purification tool. FIGURE 3 3 4 2 illustrates the effective binding and eluting of the enzyme from the resin. Similarly, the separation from other proteins was successful, however, the subsequent analysis of the enzyme showed significantly lowered activity. This could be due to the serine active site binding necessary for this method to work. Thus, obviously has a considerable effect on biological activity and maintenance of protein folding. Nevertheless, the enzyme was unfortunately too inactive to analyse when the method was employed.

**4 4 2 Purification Bovine Serum Dipeptidyl Peptidase IV**

**4 4 2 1 Serum Preparation**

A large volume of purified enzyme was required for optimisation of the purification protocol, necessitating a correspondingly large amount of starting material. Therefore, a reliable, commercially available, inexpensive source that was high in DPPIV activity was essential. Bovine serum fulfilled these requirements and was thus selected as an ideal source for the purification of DPPIV. Serum was prepared as outlined in section 2 6 1 by centrifugation of the non-clotted portion of bovine whole blood generating a large volume of bovine serum.

**4 4 2 2 Phenyl Sepharose Hydrophobic Interaction Chromatography**

The inclusion of this resin may seem confusing when FIGURE 3 4 2 1 is observed. This is because the protein of interest does not bind to the column but ‘runs through’, which is normally not favourable. Although the complete binding of DPPIV to Phenyl Sepharose could be achieved with 1.5M ammonium sulphate, the main function of this resin was to initially...
separate Gly-Pro-MCA degrading activity from Z-Gly-Pro-MCA activity (PO). Both enzymes evolve from the same serine protease family (S9) and have many shared properties. This includes the ability to hydrolyse identical peptides but at different cleavage sites. As a consequence, it was imperative to separate these activities which proved near impossible when Phenyl Sepharose was not employed. A small amount of the Gly-Pro-MCA degrading activity seems to bind which initially posed the question of a DPPIV isoform which would be in tandem with other reports from serum (Duke-Cohan et al., 1995 and Shibuya-Saruta et al., 1996). However, purification and initial analysis of this peak did not suggest any difference to the unbound peak. The protein separation profile shows significant contaminating protein binding in comparison to the run-through. This may be somewhat deceiving. It seems that when either the Buret assay or the BCA assay was used, inaccurate protein measurements were sometimes reported. The Buret assay often proved too insensitive to protein content and/or the presence of ammonium sulphate may have interfered with the assay even though the salt concentration was below the specified compatible concentration. Similarly, the BCA assay was significantly affected by the salt content when it was below the specified compatible concentration. Dialysis of the fractions increased the recorded protein absorbances but was rarely utilised as it was detrimental to enzyme activity. Either way a 186-fold reduction in protein resulted from this step, which aided the 48 1-fold increase in purity. However, a significant decrease in activity was also observed. This is surprising as the enzyme does not even bind to the resin but it was discovered that the high ionic effect of ammonium sulphate was considerably degrading for DPPIV activity. This caused the loss of enzyme recovery to 26%.

4.4.2.3 S-300 HR Gel Filtration Chromatography

All proteins have a characteristic molecular weight based on the number of amino acids, their size and conformation or folding of the protein. Size exclusion chromatography is a very popular non-binding technique employed in protein purification. The resin is composed of pores of a predefined size. When a mixture of differing molecular weights is applied to the resin, the small sized molecules become retarded as they enter the pore beads. In contrast, large molecules move through the column at a quicker rate. As a consequence, the rate of movement is directly proportional to the size of the molecule and it is on this basis that considerable protein separation can occur.

The S-300 resin has the capacity to retard up to 1,500 kDa sized globular proteins. Although the molecular size of DPPIV was unknown, it was initially assumed that it was less than 1,500 kDa. Therefore, Figure 3.4.3.1 clearly demonstrates the successful profile where the enzyme appeared to be separated from most of the contaminating protein. The second protein peak is
somewhat unusual but proves that there are a number of similar sized proteins present with DPPIV but are smaller. This non-binding column was effective in reducing the total protein content to sub mg level. However there is also a 2.6-fold decrease in activity compared to post-phenyl sepharose which led to a very low percentage yield of 9.9%. Thus it seems that the conformation of the S-300 resin is degrading to DPPIV activity where shear and frictional stress from entering the resin's pores may partially unfold the enzyme and reduce its functionality.

4.4.2.4 POROS Anion Exchange Chromatography

The POROS 50 HQ is a strong anion exchanger and can challenge the protein's pI property even more than a weak anion exchanger. If binding can be achieved then increased purification can be obtained mainly due to the differing extent of protein binding. The resin will be behave similarly to weak anion exchangers by immediately eliminating unbound proteins with high pI's but also proteins with mildly low pI's that can not bind strongly to the resin. However over the elution process a linear gradient is important as finite differences in protein surface charges can be exploited. **Figure 3.4.4.1** demonstrates the obvious separation of bound protein from unbound. In addition, there is expected if not immediately detectable protein separation over the sodium chloride positive linear salt gradient. There is noticeable protein peak, coinciding with the sharp activity peak. This suggests that this protein peak is mainly attributed to DPPIV. This is also reflected in the Purification table 3.3 whereby the first binding resin reduced the protein content 11.5-fold compared to post-S300 DPPIV. This is in tandem a smaller decrease in activity, which resulted in a significant increase in purity (250.5-fold).

4.4.2.5 G100 Gel Filtration Chromatography

Initially the G100 column was included solely to desalt the post-Anion Exchange sample as sodium chloride is detrimental to DPPIV activity. This resin restricts any molecules smaller than 100 kDa in a manner already described in section 4.4.2.3. The initial molecular weight determination of DPPIV along with its behaviour on the S300 resin suggested that the enzyme would not enter any of the resin's pores and elute very quickly. Similarly, due to the use of the S300 column it was assumed all molecules sized 100 kDa or smaller would have been separated prior to use of this last column. Protein analysis of the fractions showed no peaks but the assays sensitivity may be in question here. Nevertheless **Figure 3.4.5.1** clearly illustrates the removal of salt from the post-anion exchange preparation with an expected sharp DPPIV activity peak. However **Table 3.3** suggests an interesting phenomenon. The reduction in total protein was not expected to be in the region of almost 6-fold, nor was such an unfortunate decrease in enzyme activity (5.7-fold). The frictional effects enforced on the
enzyme should have been minimal but it appears that even when the protein is not retarded by the resin conventionally, the movement of the protein around the beads is sufficiently harsh on activity especially when very little other stabilising proteins surround it. The reduction in protein is confusing but may suggest the possible presence of smaller proteins that may attach temporarily to the enzyme over the purification protocol but is forced to separate during G100 chromatography. As a consequence, slight increase in purity is observed.

It should be acknowledged that for the generation of the purification table, both enzyme activity and protein had to be accurately quantified. This involved the removal of interfering compounds mainly salt by dialysis. This process undermined the importance of the purification table. For instance, the total activity of 49.5 units for post anion exchange has been applied to three columns but also had to be dialysed twice. Dialysis also appeared to be detrimental to enzyme activity, proportional to protein levels. This has a considerable effect on reducing the quantified enzyme activity as well as the purification factors. Therefore, it is presumed that the 'purified enzyme' is considerably more than 257 times purer than that in serum.

**4.4.2.6 Alternative DPPIV Chromatography**

Many reports have documented DPPIV as having a glycoprotein region. Similarly, Figure 3.8.2 indicates that bovine serum DPPIV is glycosylated. Therefore, the relative specificity of Lectin Chromatography was utilised. Concanavalin-A Sepharose was prepared and carried out as outlined in section 2.6.5.1. Figure 3.4.6.1 shows the separation of contaminating protein from DPPIV activity but considerable peak tailing resulted. It appeared that the enzyme bound very strongly to the column and was very difficult to remove. The recommended eluant was 0.2-0.3M Methyl-mannoside or Methylglucoside. However, even when these concentrations were increased to 1 Molar, the enzyme did not elute efficiently. As a consequence, the enzyme proved unstable after Con-A Sepharose. This is understandable as the glycosylation region appears to be quite near the catalytic active site (see CD26 diagram). Therefore, binding of the enzyme to lectin chromatographic columns would most occur deep within the protein’s structure, which could compromise its folding and ultimate functionality.

**4.5 Determination of Enzyme Purity**

The concept of the term purity in relation to proteins is not always straightforward. A pure protein would imply that the purified sample contains, in addition to water and buffer ions, only one population of molecules all with identical covalent and three-dimensional structures. This is often an unattainable and unnecessary goal. In addition, it is very near impossible to prove a protein’s complete purity. For the purpose of these studies, purity was assumed if the
sample was shown not to contain any species which would interfere with the experiments for which PO or DPPIV was intended. This was achieved using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and fluorimetry-based analysis.

4.5.1 SDS Polyacrylamide Gel Electrophoresis

FIGURE 3 5 11 represents a gelcode stained SDS PAGE gel completed using the protocol outlined in section 2.7.1. A number of protein bands are observed for both buffered saliva and post salt cut samples (lanes 5 and 4 respectively). Once column chromatography is undertaken, almost all of these contaminating proteins seem to be eliminated. The figure shows very little difference between the post-phenyl sepharose or post-anion exchange samples. This is understandable as there is only a slight reduction in protein after the last column. The mobility of the protein in the gel also allowed for accurate molecular weight determinations of the 'purified enzyme'.

FIGURE 3 5 12 represents a gelcode stained SDS PAGE gel completed using the protocol outlined in section 2.7.1. Like PO gel analysis, numerous protein bands were observed in the crude protein samples. In particular, even a dilution of crude serum had too much protein present to separate into sharp individual bands. Despite the initial perception that post-anion exchange DPPIV was pure, the gel suggests the elimination of a protein, approximately 150 kDa in the post-G100 sample. It is probable that the G100 Gel Filtration resin was successful in retaining this protein and separating it from DPPIV. No other protein bands were observed in the G100 sample, so it was assumed that the enzyme was pure.

4.5.2 Fluorimetric Substrate Analysis

Post anion exchange PO was incubated with other fluorimetric substrates as outlined in section 2.7.2, in attempt to ascertain the presence of other contaminating peptidases (TABLE 2.6). TABLE 3 5 lists the substrates that were hydrolysed and unfortunately it appears that post-anion exchange PO is far from pure. Most of the activities were quite low but may contribute to inaccurate characterisation studies. The presence of the aminopeptidases is significant, especially Proline Aminopeptidase. Due to the unique structure of the proline amino acid, the PO substrate, Z-Gly-Pro-MCA, becomes considerably more specific to the enzyme. However, if the Gly-Pro bond was cleaved then MCA could be released by Proline Aminopeptidase and what is perceived as PO activity would then be inaccurate. Both Gly-Pro-MCA and Z-Phe-Arg-MCA degrading activities were comparatively very high. Their contaminating influence would be observed most in specific inhibitor studies. The active site of these peptidases could be very similar to that of PO. Therefore calculated IC₅₀'s and Kₕ's
would be significantly inaccurate. In addition, substrate specificity studies would also be compromised by the presence of these peptidases, whereby hydrolysis may not attribute to the influence of just one enzyme. As a consequence, it would appear that the SDS PAGE gel observed is inaccurate. The gel does not suggest the presence of any other proteins but fluorometric analysis does. Therefore the sensitivity of the gel electrophoresis is not sufficient to detect low levels of proteins that may hydrolyse other substrates. It is worth noting that the stability of some of the substrates could be questioned. The difference between the blanks and the active triplicates is sufficient to suggest that a similar difference in stability could occur. The substrate may remain protected from auto-catalysis at an acid pH (blanks), whereby the substrate could be destabilised at neutral pH at 37°C during the 60 minute incubation (triplicates). If this was the case, then the fluorescent intensities could be considerably lower if taken into account and substrate hydrolysis could be negligible.

Like PO, purified DPPIV was incubated with other fluorometric substrates. Unlike PO, the results make for more favourable reading. All contaminating fluorometric substrates remained intact, which correlates well with the gel observed. High activity was recorded for both Gly-Pro-MCA and Lys-Pro-MCA, both of which are DPPIV substrates. Lower activity was seen from Lys-Ala-MCA analysis, which is a DPPII substrate. However DPPII can not hydrolyse this substrate at pH's other than acidic. Similarly the secondary affinity of DPPIV for Lys-Ala-MCA is well documented (Ohkubo et al., 1994 and Gilmartin and O’Cuinn, 1999). Therefore it is assumed that further characterisation studies could be undertaken whereby the experiments results would be attributed to the DPPIV enzyme alone.

4.6 ASSAY DEVELOPMENT

4.6.1 Substrate Solvent Studies

4.6.1.1 Substrate Solvent Determination for PO

The substrate employed for the detection of PO activity, Z-Gly-Pro-MCA, is water insoluble, so the addition of a non-aqueous organic solvent was essential for solubilisation. The majority of enzymes, which function in natural hydrophobic environments, did not evolve to function in non-aqueous environments and so the use of organic solvents is never favoured. It has been hypothesised that although water remains essential for catalysis, an enzyme molecule in aqueous solution becomes fully hydrated when surrounded by a few layers of water (Gorman and Dordick, 1992). Therefore, bulk water, other than tightly bound structural water, is not crucial for catalytic activity and replacement of the remaining water with an organic solvent should be possible without adversely affecting the enzyme. In fact, aqueous-organic solvent
mixtures may resemble the natural cellular environment more closely than pure aqueous media. The enhanced catalytic activity in aqueous-organic solvent may reflect the optimum exploitation of catalytic activity inherent in the natural structural design of the enzyme molecule.

A non-aqueous solvent was sought which proved optimal for both substrate solubility and enzyme activity. Z-Gly-Pro-MCA was prepared in a range of organic solvents and assayed with 'purified PO' as described in section 2.8.1. Figure 3.6.11 compares the effect on PO activity of MeOH, EtOH, DMF and DXN as substrate solvent to that of DMSO, the previously favoured solvent. Both alcohols increased enzyme activity but only slightly. Nevertheless, methanol contributed most to PO activity with a 22% increase. This, coupled to its effective solubilisation properties, resulted in MeOH being selected as the optimal solvent for Z-Gly-Pro-MCA preparation.

4.6.12 Substrate Solvent Studies of DPPIV

Although the substrate utilised for DPPIV detection, Gly-Pro-MCA is water-soluble and has no requirement for non-aqueous solvents, the potential of elevated catalysis in an organic-aqueous environment was investigated. Like PO, Figure 3.6.12 demonstrates the effect on DPPIV activity of EtOH, DMSO and DMF as substrate solvents, compared to MeOH. It is obvious how much of an effect ethanol has on substrate hydrolysis with a 2.7-fold increase. Surprisingly, methanol seemed to be the least favoured substrate and the large variation between both alcohols is significant.

Generally short chain aliphatics cause a lower degree of denaturation than solvents such as butanol (Harms, 1989), and this may explain why PO activity was least affected by the presence of MeOH in the substrate. Organic solvents effect both the denaturation and catalytic activity of enzymes, but not necessarily in a parallel manner. Aliphatic solvents (EtOH and MeOH) show only moderate effect on activity denaturation and loss of catalysis. DMF on the other hand, is a strong denaturant which also reduces catalytic activity, while dioxane does not induce denaturation but does diminish catalytic power. The difference between the effect of methanol compared to ethanol on DPPIV activity suggests that the ethyl alcohol has a specific effect on the enzyme. It is possible that the enzyme could be partially unfolded, exposing the active site and allowing for easier access of the substrate. It has also been suggested that the pH of a buffer and MeOH or EtOH mixture is more likely to remain at the pH value of the added buffer component, than in mixtures composed of DXN, DMSO, DMF and acetonitrile. This may offer another explanation as to why optimal enzyme activity was observed using MeOH or EtOH as the substrate solvent for PO and DPPIV respectively.
Once EtOH had been selected as the optimal solvent for DPPIV substrate preparation, the concentration at which it was most effective was then determined. Experiments were carried out according to section 2.8.2 in which the concentration of EtOH used in Gly-Pro-MCA preparation was varied. Enzyme activity increased slightly at 4% of the solvent (Figure 3.6.3) but eventually decreased when the ethanol concentration was increased further. Although the 10% increase in enzyme activity was notable, it was deemed too low to warrant change in the substrate and potentially high enough to interfere with the effect of other compounds. Therefore, as a consequence of the substrate's solubility without the influence of any solvent, 1% EtOH was decided as an acceptable concentration to aid enzyme activity.

4.6.2. Linearity Studies

A typical progress curve for an enzyme-catalysed reaction is presented in Figure 4.6. The time course is initially linear but the rate of product formation starts to decline after prolonged incubation. This departure from linearity may be due to a number of factors, most frequently, substrate depletion, enzyme or substrate instability, products inhibiting the reaction, or a change in assay conditions. It can be deduced from this that enzyme activity should only be quantified at a point where these factors are known not to be an issue. This point is known as the initial velocity of the reaction and can be calculated by drawing a tangent through the origin to the linear part of the progression curve as shown in Figure 4.6. Using a continuous assay system, this decrease in reaction velocity with respect to time is evident and generally due to substrate depletion.

![Figure 4.3. Typical Progress Curve of an Enzyme-catalysed Reaction](image-url)

Plot of product formation versus time.
The discontinuous assay can be treated in the same way. However, if slight deviation occurs, it may not be readily detected. Therefore, it is recommended that this assay be complemented by a continuous assay also.

### 4.6.2.1 Linearity of PO

The discontinuous assay was carried out as described in section 2.8.3.1 with the overall time decided 60 minutes as this was the time limit used for all standard assays. Figure 3.6.4.1.1 clearly illustrates the linearity of the assay even if the rate of MCA release is quite low. With a regression coefficient of 0.996, it may be assumed that the PO enzyme is stable enough over the 60 minutes to maintain hydrolysis and this assay time can be followed. Similarly, the continuous assay is linear over the same time frame using the protocol outlined in section 2.8.3.2. This involved the adaptation of the fluorimeter attached to the waterbath, providing a warmed cuvette. The graph is not shown as it proved difficult to convert the 'time-drive' file to one that could be imported and graphed.

### 4.6.2.2 Linearity of DPPIV

The discontinuous assay was carried initially and generally shows that DPPIV is linear over 60 minutes. However, there was some speculation over the actual 60 minute activity value. If this was an anomaly or was not included then the trend of the graph previous to this suggests a rate slowdown possibly due to substrate depletion or enzyme instability. In contrast, if the 50 minute value was excluded, the assay would appear to have greater linearity. Either way, the continuous assay was required to further confirm the enzyme’s linearity over time. Figure 3.6.4.2.1 shows the obvious direct linearity of DPPIV, not only over 60 minutes but also over 120 minutes (7200 seconds). This allowed for longer analysis if required and proved that the enzyme is stable and fully active over a wide time frame and that the assay is saturated with sufficient substrate over the selected time. This was vitally important if accurate enzyme activity quantification could be carried out for purification factor determination and general characterisation studies (See appendix section 6.3).

Another parameter to be considered in the quantification of DPPIV, is the linearity of the assay with respect to enzyme concentration. Figure 3.6.5 demonstrates that fluorescent intensity increases linearly and proportionally with increasing enzyme concentration. Therefore, the enzyme hydrolysed at the same rate depending on the quantity of enzyme present.
4 6 3. Optimum Assay Temperature

The effect of assay temperature on purified DPPIV activity was investigated as outlined in section 2 8 5. Samples were allowed to reach thermal equilibrium by pre-incubation for 15 minutes at the appropriate temperature prior to assaying. This ensured that the observed activity genuinely reflected the activity of the peptidase at the temperature under investigation. A typical bell-shape curve was not observed as Figure 3 6 6 illustrates. The rapid increase in fluorescent intensities observed in assays performed at temperatures greater than 37°C, reached a peak at the final temperature recorded, 60°C. It was expected that the fluorescent intensity values may increase even further if the temperature was increased accordingly. Although it is initially encouraging to suggest that DPPIV is thermostable to at least 60°C, it is more likely that the stability of the substrate has a significant part to play in these results. The blanks recorded for each temperature increase also rose steadily suggesting that even without enzyme influence, MCA is still being released possibly due to the gradual thermal degradation of the substrate. It is most likely that the substrate is partially destabilised by the high temperatures, the extent of which is directly proportional to the temperature but enzyme hydrolysis still occurs. Although most reports document the assay temperature as ambient or physiological temperature, the enzyme is reputed to be very thermostable (Yoshimoto et al., 1978). Despite the reports of DPPIV's thermostability, and the result of this experiment, it was deemed that accuracy and reproducibility could be maintained if the assay was kept at the physiological temperature of 37°C.

4 6 4. Effect of DTT and EDTA on Enzyme Activity

4 6 4 1. Effect of DTT and EDTA on PO

Numerous reports have shown PO to be substantially activated by dithiothreitol or DTT (Walter, 1976, Orlowski et al., 1979, Kalwant and Porter, 1991). Cunningham and O'Connor, 1998, reported a twelve-fold increase in PO activity in the presence of 10mM DTT. Therefore the stability of PO and the optimum assay conditions warranted a thorough investigation into this thiol reducing agent. Figure 3 6 6 1 clearly demonstrates the considerable influence DTT has on the enzyme's activity. A 3 3-fold increase in activity in the presence of 5mM DTT was seen to rise even further to 4 3-fold at 20mM. It is generally accepted that this marked increase is due to the reduction of disulphide bridges (S-S) to thiol groups (SH +SH) by DTT. This suggests the existence of a cysteine residue, situated near the active site, which when reduced increases the ability of PO to initiate a nucleophilic attack upon a bound substrate (Polgar, 1991). The results obtained suggest that PO, has a cysteine residue near its active site which in the presence of thiol-reducing agents, contributes to the activation and
stability of enzyme activity. Even though it is not immediately obvious, it was assumed that high concentrations of DTT could interfere with other characterisation studies, therefore the DTT concentration was maintained at 5mM in all substrates and purification running buffers. As a result, the enzyme was constantly stored in a known quantity of thiol reducing agent.

PO is not reported as a metallo-protease and the effect of the metal chelator, Ethylenediamine tetraacetate (EDTA) is minimal (Yoshimoto, et al., 1987). Nevertheless, many of compounds contain trace heavy metals that may have a detrimental effect on activity. Therefore, the enzyme was incubated with the substrate with differing concentrations of EDTA to ascertain its effect on the enzyme. Figure 3.6.7.1 illustrates the compound's effect on PO activity. Eventhough quite a small concentration was used, EDTA eventually had a detrimental effect. This suggests that the enzyme may have a metallo residue somewhere other than the active site. However, the 25% reduction in activity at 2mM may be attributed to partially blocking the enzyme from attacking the substrate and not related to the enzyme itself. Nonetheless, 0.5mM EDTA was selected to be included in all substrates and buffers in the hope to maintain stability from heavy metal toxicity.

4.6.4.2 Effect of DTT and EDTA on DPPIV

The schematic structure of DPPIV shows the presence of a cysteine rich domain close in proximity to the catalytic domain. Therefore, it could be assumed that cysteine residues may play a role in enzyme activity. Thus, the effect of DTT was of potential interest. However, Figure 3.6.6.2 demonstrates the in-effect the thiol reducing agent has on activity. This may be understandable seeing as even though the cysteine and catalytic domains are very close to each other, the active site is located near to the C-terminal side and further away from the cysteine residues than initially expected. It is possible too that the folding of the protein does not allow for adequate thiol reduction by DTT. This result correlates well with other reports of minimal DTT effects on DPPIV activity (De Meester et al., 1992 and Shibuya-Saruta et al., 1996). Therefore, it was not deemed necessary to include DTT in any of the buffers or substrate.

Like DTT effects, EDTA is reported to have a minor effect on enzyme activity. Nevertheless, heavy metal effects may destabilise the enzyme, therefore the level of compatibility was investigated as outlined in section 2.8.7.2. Figure 3.6.7.2 demonstrates the slight enhancing effect the metal chelator has on enzyme activity. An increase of 50% at 8mM EDTA was surprising but may be attributed to the removal of metals that may have co-existed in the substrate. It is possible that these metals destabilise the substrate, making easier for enzymatic hydrolysis. However, it is notable that the enzyme received increased activity. Therefore, 5mM EDTA was selected to be included in all substrates and buffers in the hope to maintain stability from heavy metal toxicity.
EDTA was included in all of the running buffers employed during purification but was not deemed necessary for substrate inclusion as that concentration may influence the effect of other compounds that are tested and the effect observed may not be attributed to the enzyme alone.

4.7 Stability Studies of PO

4.7.1 Thermostability of PO

Once a protein is released from its native environment, it will be subject to many inactivating conditions. Normally the pH of the native state is maintained at 6.5-7.5, the protein content is high (100mg/ml) and the reducing potential is considerable (Harris, 1989). Upon disruption into the buffer, the protein concentration is automatically reduced and the protein experiences an oxidising environment as well as acidification. Extremes in pH, temperature and organic solvents are the principal causes of denaturation. Correct buffer selection can minimise effect of pH extremes while exposure to organic solvents is often insignificant. However, temperature effects are a lot more prevalent. If a protein is exposed to an oxidising environment, then temperature extremes may not be necessary to destabilise it.

4.7.1.1 Effect of 37°C Pre-incubation on PO activity

The inherent instability of salivary PO was very apparent and methods to try to stabilise enzymatic activity were undertaken. The assay utilised to monitor PO activity involved the incubation at 37°C for 60 minutes. Although it was established that activity was linear over the hour, the question was posed, how stable is the enzyme in these conditions and can they be improved upon? Therefore the behaviour of PO was investigated as outlined in section 2.9.1. The effect of pre-incubation time was observed and Figure 3.7.1 demonstrates the dramatic decrease in activity over time. This effect can be acknowledged by the 25% loss of PO only after 15 minutes of pre-incubation. This decrease continued steady to an almost 80% loss after 4 hours. It is worth noting that as well as a 15-minute pre-incubation, the enzyme was also subjected to another 60 minutes at the same temperature for analysis. Nevertheless, the activity loss is very significant. Although it is very unlikely that the enzyme would need to be pre-incubated for anytime longer than 30 minutes, the enzyme is considerably compromised and the results obtained from such a study would have to be analysed carefully.

It is obvious that the enzyme is very unstable under these conditions, therefore stabilising agents were employed. Although it may seem as a contradiction, the addition of protease-free BSA to a purified sample can often stabilise the protein of interest. As a consequence, the
previous study was also carried out in tandem with both 1% and 10% BSA added to the enzyme sample. Very similar trends are observed for both BSA additions compared to the untreated sample. However, the decrease in activity is considerably reduced when protein was added. The difference is quite alarming when after 90 minutes, 46% of PO activity was lost in the presence of 10% BSA, 60% was lost from the 1% sample while 67% was lost in the untreated sample. The treated purified samples were not continued after 90 minutes as the rate of activity loss was still sufficiently high to observe inevitable instability of the enzyme.

4.7.1.2 Effect of -20°C storage conditions on PO activity
Purified and post column samples were stored in a frozen state when not in use. The freeze-thaw process is known to be quite harsh on activity whereby the acid and conjugate basic species of buffers may have different freezing points. That inevitably leads to sudden rapid change in the pH of the solution, which could cause irreversible protein degradation. As well as the freeze-thaw process, the stability of PO over time at these conditions was evaluated. The thermostabilising effect of BSA was again incorporated with 1% also included and compared to the untreated sample. Figure 3.7.2.1 clearly shows a common trend between when the BSA is included and when not. Both observe decreases in activity over the first three days, which seem to plateau off for the following 28 days. 30% activity was lost from the no BSA sample while 1% inclusion reduced the loss considerably to only 9%. This would suggest that as well as aiding the enzyme during the thawing process BSA may also keep the enzyme protected during the frozen state. It is assumed that a certain amount of enzymatic loss should be expected from all samples with such a low protein content. Therefore, it was suggested to include 1% BSA when the purified PO enzyme was stored.

As a consequence of the purified PO sample behaviour under frozen conditions, the question was posed whether the purification has an irreversible, detrimental effect on activity or if the protein is unstable to begin with. Therefore, buffered saliva PO was investigated as it was assumed that in this environment, the protein would most stable. The same study was incorporated as previous with 20% glycerol also included for a comparison effect. Figure 3.7.2.1 demonstrates the surprising result. Protein degradation in untreated buffered saliva appears greater than in the purified sample mentioned previously. Enzymatic loss was in the region of 35% after 3 days to 58% after 5 days. It was hoped that glycerol would minimise the effects of freeze-thawing by possibly keeping the protein in a ‘semi-frozen’ state. Although it appeared to reduce the enzyme activity loss it still could not save a 32% loss after 5 days. It is worth noting that a general reduction in PO activity was observed when glycerol was added, suggesting that it may have restricted the enzyme mobility somewhat. It is also possible that the buffer capacity of buffered saliva is restricted due to the presence of oxidants. Similarly, it
is assumed that buffered saliva contains a number of proteases that may not be favourable to PO activity. The removal of these proteases and other contaminating proteins seem to allow for more reproducible results and decreased instability.

Therefore the results indicate an inherent instability of the enzyme despite the best efforts of stabilising agents. It is possible that the enzyme may be secreted in saliva without an N-terminal precursor or signalling peptide as part of its structure that is present in other mammalian PO species, and which maintains stability (Chevailler et al., 1992). This may cause the inevitable self-destruction of the enzyme before it reaches the acidic medium of the stomach. Thus it is expected that the half-life of salivary PO is extremely short while its ultimate physiological function still remains elusive. However, the presence of neuropeptides in saliva may suggest a specific, evolutionary role (Dawidson et al., 1997).

4.8. CHARACTERISATION

4.8.1 Relative Molecular Mass Determinations
The anionic detergent sodium dodecyl sulphate (SDS) readily binds to proteins in a ratio of 1:4:1, effectively masking the intrinsic charge of the polypeptide chains by coating the protein with a negative charge (Dunn, 1989). The net charge per unit mass becomes approximately constant, therefore separation of SDS-treated proteins is effectively based on molecular weight alone.

4.8.1.1 SDS PAGE of PO
SDS polyacrylamide gel electrophoresis (PAGE) was employed in the estimation of the molecular mass of purified PO as outlined in section 2.10.1.2. A 10% v/v gel was prepared since the molecular weight was expected to be in the range 15-200 kDa. Figure 3.5.1.1 represents the image of the PO gelcode stained gel. Measurement of the molecular weight marker Rf values allowed for the construction of standard curves (Figure 3.8.1.1). Using these curves, molecular masses of PO were determined to be 78,860 Da (gelcode stain) and 82,870 Da (blue stain). The interaction of SDS with proteins causes the irreversible denaturation of the 3-dimensional structure and proteins are thus broken down into individual polypeptide chains (monomers). Therefore SDS-PAGE yields no insight into the native molecular mass of a protein and so a second method for the determination of molecular mass is regularly performed. However, this was not employed for PO as all reports show the enzyme to be a monomer, therefore the determined molecular weight from SDS-PAGE is also the enzyme's native molecular weight.
4.8.1.2 SDS PAGE and Size Exclusion Chromatography of DPPIV

SDS-PAGE was also utilized for DPPIV molecular weight determination. The same method was followed as that of PO. Although the enzyme has been reported larger than 200kDa, a 10% gel was still employed as the enzyme’s monomeric weight was assumed to be considerably less. Figure 3.1 illustrates the gel code stained gel of DPPIV post-column fractions and molecular weight markers. The Rf value could be determined as described in section 3.8.1.1. Figure 3.1 allowed for the estimation of the enzyme’s monomeric molecular weight which was 67,140Da from the blue stain and 74,340Da for the gel code stain. As most reports of DPPIV’s molecular weight or structure are of a dimeric or trimeric enzyme, a second method was employed to ascertain the native molecular weight.

Size-exclusion chromatography (SEC) is frequently employed as a non-denaturing technique for molecular mass estimation. Separation is based on the hydrodynamic volume of a protein or simply its size, larger proteins eluting faster than smaller ones that interact with the pores of the column. Two SEC columns were run as described in section 2.10.11, namely a low pressure Sephacryl S-300 column and also a medium pressure HiPrep Sephacryl S-300 prepacked column. Figures 3.1.1 and 3.1.2 represent the standard curves of log molecular weight versus elution volume/void volume for each resin. Molecular masses of 276,000 and 328,000Da were estimated from these graphs for S-300 and HiPrep S-300 sephacryl resins respectively. Based on these results, it was assumed that the estimated 67kDa and 74kDa SDS PAGE bands corresponded to the mass of a subunit of the peptidase. It was deduced, due to the presence of a single band following PAGE analysis that DPPIV possibly exists as a tetramer of four subunits of equal or similar molecular weight and indicative molecular weights are reported in Table 3.7. Combination of SDS PAGE and SEC for the determination of molecular weight, compensated for the inadequacies of each technique and so an average molecular weight of 292,500Da can be confidently postulated for the enzyme.

4.8.2. Glycosylation Estimation of DPPIV

Many specialized cells secrete proteins produced by the rough endoplasmic reticulum. Most of these secretory proteins are glycoproteins. The carbohydrate side chains of glycoproteins are attached covalently during or after synthesis of the polypeptide chain. In some glycoproteins, the carbohydrate side chain is attached enzymatically to asparagine residues, in others serine or threonine residues (Lehnenger, 1982). The schematic structure of DPPIV shows a large glycosylation domain spanning almost 250 amino acids in the extracellular portion of the protease (De Meester, et al., 1999). This domain is located in the N-terminal as contains eight potential glycosylation sites. These sites account for the difference in molecular
mass between the predicted polypeptide and the actual mature protein. Although the sites are situated near the transmembrane domain of membrane-bound DPPIV, it is not part of it so would also be expected to be part of the soluble protein's structure.

This experiment was carried out in tandem with purity determination studies and/or molecular weight estimations as the test could be utilised on SDS-PAGE gels but simply stained differently (Table 2.6). Figure 3.8.2 illustrates the presence of a glycoprotein in the post-G100 sample. The mobility of the pink band coincided with a gelcode stained SDS-PAGE gel, indicating that the band is monomeric DPPIV. The gel further confirms the assumption that DPPIV is composed of similar sized subunits, with the presence of one band.

4.8.3 Overnight Thermostability of DPPIV

Although the stability of DPPIV has already been established at various temperatures, it was of interest to ascertain for what range of time the activity was stable for. In addition, any substrate specificity studies were recommended to be carried out over a 24 hour period at physiological temperature. Therefore it was deemed necessary to evaluate if the enzyme was capable of remaining active over this time frame. Figure 3.8.3.1 demonstrates the stability of DPPIV at 37°C over time. Even though the same substrate batch was used for analyses, there still was some fluctuation in the enzyme's activity. However this is expected and does not unduly inform of any significant physicochemical property of the enzyme. This study simply confirms the possibility of utilising 24 hour incubations, if required.

4.8.4 pH Effects

4.8.4.1 pH Effects on PO

The effects of pH on PO stability and activity was performed. Section 2.10.4.1 describes how the pH range over which the Z-Gly-Pro-MCA degrading peptidase remained stable was examined. This was achieved by pre-incubation at a range of pH values followed by assaying with substrate prepared in buffer at pH 7.4. Citrate, phosphate, Tris and glycine buffer systems (pKₐ values 4.76, 7.20, 8.06 and 9.78 respectively) were chosen to reflect a range of pKₐ values, since buffering capacity is maximal at the pKₐ. Figure 3.8.4.1 represents the bell-shaped inactivation curve constructed for PO. The enzyme showed a preference for neutral with a noticeable preference for potassium phosphate, pH 7.4, the buffer that had been used during all analyses. The enzyme was, not surprisingly, almost inactivated at low pH. This suggests that salivary PO does not have two forms that behave differently depending on
the pH that has been already reported (Polgar, 1991) Nevertheless the buffer selection was accurate for optimising enzymatic activity

4 8 4 2 pH Effect on DPPIV

Like PO, the effect of pH on DPPIV activity was investigated. Citrate, phosphate, Hepes and glycine buffer systems (pKₐ values 4.76, 7.20, 7.48 and 9.78 respectively) were utilised. Figure 3.8.4.2 demonstrates an un-classical bell-shaped curve with neutral pH generally being preferred. The highest enzyme activity was noticed in the phosphate buffer at pH 7.0 however the overall activities were considerably low so the margin of error is increased. The complete loss of activity at pH 9.0 to 10.5 is surprising seeing as DPPIV is reported to have a pH optimum at mild basic pH's. However it is very possible that the effect is due to the type of buffer used, i.e., glycine and not a pH effect.

The second study on the pH effects on DPPIV is termed 'pH Inactivation' and is expected to give a better insight into the optimal buffer and pH required for activity. As well as pre-incubation of the enzyme in the different buffer species and at different pHs, the substrate is also made up in the respective buffer. Figure 3.8.4.3 demonstrates some unusual behaviour. The effect of the individual buffers plays a more important part than before and suggests a general tendency for weak acidic pH in either citrate or phosphate buffer. The previous loss of activity at the high pH range is not observed, therefore a similar trend between both studies can not be drawn. Nevertheless the phosphate buffer at weak basic pH appeared to be the optimal buffer conditions to use. However the zwitteronic 'Good' buffer Hepes was selected despite its increased cost as the buffer of choice. This selection was made mainly due to the perceived auto-catalysis of the Gly-Pro-MCA substrate in phosphate buffer. It is unlikely that phosphate is the cause of this auto-catalysis but Hepes significantly reduced non-enzymatic substrate breakdown.

It is important to establish the significance or possible insignificance of these results. The objective of the studies was to ascertain the direct effect of pH on enzyme activity. This can only be achieved by altering the pH of a buffer species as far as its buffering capacity allows (usually 1.0 pH unit either side of the designated pKₐ). Therefore the buffer species has to the changed also. This creates two different variables and their effects can not be separated. Thus the decrease in activity from one buffer to the next may not be due to a change in pH but a change in buffer. This problem can be somewhat minimised if overlapping pHs are allowed but this may not always be the case. The buffer itself can have a specific effect on enzymatic activity, often regardless of pH. For instance, phosphate inhibits many kinases and dehydrogenases as well as enzymes with phosphate esters as substrates (Blanchard, 1984).
Therefore a certain amount of reserved judgement should be kept in mind when analysing these results.

4.8.5 Isoelectric Point Determination (DPPIV)

Chromatofocusing was employed in the determination of the isoelectric point (pI) of DPPIV. It differs from ion exchange in that the anion exchange matrix, pre-adjusted to a given pH, has distinct buffering properties that allows the creation of a pH gradient on the column rather than outside it. Chromatofocusing can be employed as either an isoelectric focusing or purification technique. Section 2.10.5.1 outlines the conditions at which a PBE 94 resin was run. Binding of DPPIV was achieved at the initial pH of 7.8 and eluted during a pH gradient between pH 5.2 and 5.3 (FIGURE 3.8.5.1). This implies that in this range DPPIV had no net charge and therefore is a good indication of its pI.

Chromatofocusing could have been applied in the purification protocol, but the low working pH range was not optimal for DPPIV activity, as well as the enzyme dilution that can occur. Coupled to this, polybuffer can be difficult to remove, requiring a further step such as ammonium sulphate precipitation, size-exclusion or affinity chromatography. As a consequence, chromatofocusing has not been widely reported in the purification of DPPIV.

In order to confirm the pI deduced using chromatofocusing, vertical isoelectric focusing (IEF) was performed which is based on the following theory: In the presence of an electric field and pH gradient, charged proteins migrate towards the anode or cathode until they reach their isoelectric point at which they have no charge and hence cannot further migrate. Vertical IEF systems are becoming more common due to their simplicity and there is no need for a separate IEF system as PAGE electrophoresis equipment can be employed. The relatively large pore size of agarose made it an ideal matrix for the large MW DPPIV. FIGURES 3.8.5.2 and 3.8.5.3 represent an image of the blue stained gel and the standard curve constructed as described in section 2.10.5.2 respectively. The isoelectric point of DPPIV was estimated to be 4.71 using this technique. This value does not correlate well with the chromatographic estimate of 5.2-5.3 but due to the superior resolving capabilities of IEF, the pI of DPPIV was concluded to be 4.71. This is acceptable as the chromatofocusing technique involved manually measuring the pH of each individual 3ml fraction. It is assume that of the 3ml, DPPIV constitutes a small fraction but the pH measurement is for the entire 3ml. The pI estimation correlates well with the enzyme’s behaviour on anion exchange resins as well as the literature (De Meester et al, 1992).
<table>
<thead>
<tr>
<th>SOURCE</th>
<th>ASSAY TEMP</th>
<th>MONOMERIC MW</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. meningosepticum</em></td>
<td>45</td>
<td>75</td>
</tr>
<tr>
<td>Lamb kidney</td>
<td>ND</td>
<td>115</td>
</tr>
<tr>
<td>Human placenta</td>
<td>37</td>
<td>120</td>
</tr>
<tr>
<td>Human lymphocytes</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Porcine seminal plasma</td>
<td>37</td>
<td>115</td>
</tr>
<tr>
<td>Human serum</td>
<td>37</td>
<td>100</td>
</tr>
<tr>
<td>Ostrich kidney</td>
<td>45</td>
<td>133</td>
</tr>
<tr>
<td>Guinea-pig bram</td>
<td>37</td>
<td>93</td>
</tr>
</tbody>
</table>

**Table 4.2 DPPIV Physiochemical Properties**

ND means not determined
<table>
<thead>
<tr>
<th>Native MW KDa</th>
<th>Optimum pH</th>
<th>pI</th>
<th>Catalytic Inhibition</th>
<th>Reference:</th>
</tr>
</thead>
<tbody>
<tr>
<td>160</td>
<td>7.4 - 7.8</td>
<td>9.5</td>
<td>DFP</td>
<td>Yohimoto and Tsuru, 1982</td>
</tr>
<tr>
<td>230</td>
<td>7.8</td>
<td>4.9</td>
<td>DFP</td>
<td>Yoshimoto and Walter, 1977</td>
</tr>
<tr>
<td>200</td>
<td>8.0</td>
<td>3.4</td>
<td>DFP</td>
<td>Puschel et al, 1982</td>
</tr>
<tr>
<td>264</td>
<td>8.7</td>
<td>5.0</td>
<td>DFP</td>
<td>De Meester et al, 1992</td>
</tr>
<tr>
<td>300</td>
<td>8.0</td>
<td>6.85</td>
<td>DFP</td>
<td>Ohkubo et al, 1994</td>
</tr>
<tr>
<td>250</td>
<td>8.5</td>
<td>ND</td>
<td>DFP</td>
<td>Shibuya-Saruta et al, 1996</td>
</tr>
<tr>
<td>270</td>
<td>8.0</td>
<td>4.7</td>
<td>PMSF</td>
<td>Wagner et al, 1999</td>
</tr>
<tr>
<td>194</td>
<td>7.8</td>
<td>ND</td>
<td>p-CMB</td>
<td>Gilmartin and O’Cuinn, 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NEM</td>
<td></td>
</tr>
</tbody>
</table>
4.8.5 Catalytic Classification and Effect of Other Functional Reagents

Sensitivity to specific active-site-directed inhibitors coupled to amino acid sequence analysis is frequently investigated to determine the catalytic class of an enzyme. In this study, a number of serine, metallo- and cysteine protease inhibitors were incubated with purified DPPIV to establish inhibition effects. Cysteine protease activators and some non-specific inhibitors were also examined as detailed in sections 2.10.6 and 2.10.7.

Table 3.9 lists the serine protease inhibitors and their percentage effect on DPPIV. Surprisingly, even though the enzyme is generally classified as a serine protease (Yoshimoto and Walter, 1977 and Iwaki-Egawa et al., 1998), none of the inhibitors had a significant effect on activity. This may be explained by the belief that DPPIV is not a classical serine protease (Rawlings and Barrett, 1991), so could be more resistant to serine protease inhibitors that may normally be potent to general serine proteases, such as, trypsin etc. Alternatively, the active site serine is the target for these studies and inaccessibility by steric hindrance may prevent any inhibition. This may explain the minimal inhibition observed due to PMSF, the classical serine protease inhibitor, which correlates with the literature (Shibuya-Saruta et al., 1996 and De Meester et al., 1992). These possibilities ultimately could have been answered with the availability of the potent serine inhibitor, Dnsopropyl Fluorophosphate (DFP), which is the standard reagent used. However, the hazardous nature of the compound suggested otherwise.

An interesting set of results came from the inhibitory effect of metallo-protease inhibitors on DPPIV activity. The phenanthrolines had a particular potent effect on activity with 20 mM 4,7-phenanthrolme decreasing DPPIV activity by 46% (Table 3.10). This may warrant the possible classification of a metalloprotease or the fact that a metallo residue is located near the catalytic site and is necessary for activity. However, the effect of the metal chelators does not indicate any significant reduction in activity compared to the phenanthrolines. If a metallo residue was present, it would be assumed that at least one of the chelators would have been more potent than observed. Therefore, this suggests that there may be more structural similarity between DPPIV and 4,7-phenanthrolme. The latter compound consists of an aromatic ring, which may interact with the hydrophobic patches on the surface of the enzyme and reduce its activity somewhat. This effect has also been observed in serum PO, which is classified as a serine protease (Cunningham and O’Connor, 1998).

The structure of DPPIV outlines a cysteine rich region located next to the catalytic domain (De Meester et al., 1999). Therefore, it is possible that cysteine protease inhibitors and activators could ascertain their influence in substrate catalysis both positively and negatively. Table 3.11 shows that 10 mM N-ethyl maleimide (NEM) had a 47% inhibitory effect on
DPPIV activity, while other inhibitors were insignificant. Similarly the cysteine protease activator, mercaptoethanol increased DPPIV activity by 25% at 10mM (TABLE 3.12). This suggests that the cysteine rich region near the catalytic site has some influence on enzyme activity. It is possible that the cysteine residue do not contribute to the catalytic process but aids the protease’s functionality. Despite this possibility, the standard thiol reducing agent, DTT did not aid activity at all. However that could be due to the differences in how both compounds reduce the thiol groups.

The effect of general functional reagents was explored on DPPIV. These reagents were expected to have a non-specific effect on activity by possibly partially binding to enzyme. None of the reagents had any significant effect on the activity of the enzyme however the effect of Trypsin Inhibitor is potentially interesting. Although the inhibitory effect of the reagent does not reach above 20%, it is possible that this could be due to binding to one of the catalytic triad residues. Trypsin is also a serine protease but the arrangement of the catalytic triad is different to that of DPPIV. However non-specific binding may occur, but is insufficient to make a mechanistic evaluation on the enzyme.

As a consequence of these results, no definite conclusions can made on the catalytic classification of DPPIV. It is likely that the protease has a serine in its catalytic domain but is not inhibited by any of the serine protease inhibitors. It was initially thought that a metallo residue was located near the enzyme’s active site that influences catalysis, however further evaluation may suggest otherwise. It can be assumed that cysteine residues are positioned near the active site but do not play a major role in substrate hydrolysis.

4.8.6. Effect of Metal Ions on DPPIV Activity

The effect of metal ions was investigated as described in section 2.10.8. To avoid any dual variable effects, all salts had the same counter-ion, sulphate, included in the controls and all active reactions. This ensured that the effect observed was due solely to the presence of the metal ion. Table 3.14 demonstrates the general ineffectiveness of the metals. The most significant effect was observed in both mercury and magnesium with a 20% and a 22% decrease in activity. The mercuric effect may be explained by the preparation technique that was required. Table 2.12 illustrates that mercuric sulphate required initial dissolution in 1 Molar HCl and then brought to volume by buffer. Although the pH was observed at 7.5, it is questionable the effect that this quantity of acid could have on the enzyme. Another possibility is that heavy metal cations such as mercury are known to react with histidine and tryptophan residues in proteins and polypeptides (Volkin and Klibanov, 1990). As DPPIV is classified as a serine protease, histidine forms part of the catalytic triad, therefore inhibitory
effects from mercury are most likely. However, their effects are not as potent as that previously reported, where almost complete inhibition is achieved (Puschel et al., 1982 and De Meester et al., 1992).

4.8.8 Effect of Salts on DPPIV Activity

During the purification of DPPIV, high concentrations of salt were often required, such as 500 mM NaCl to elute the enzyme from the ion exchange column. Analysis of post column fractions seemed to illustrate a decrease in enzyme activity. As a consequence of the lack of knowledge whether the salt effects were directed at the enzyme or at the substrate, it warranted a study of a number of salts and their effect on the substrate and the enzyme.

Ammonium sulphate, sodium chloride, and potassium chloride were pre-incubated with Gly-Pro-MCA and purified DPPIV, and the effect measured as outlined in section 2.10.9. Figure 3.8.9.11 illustrates the considerable effect ammonium sulphate has on DPPIV activity when included in the substrate. Enzyme activity seemed to be preserved up to 100 mM, but the enzyme activity was significantly reduced with an 80% decrease at 1 M ammonium sulphate. Figure 3.8.9.12 shows a similar effect when the salt was pre-incubated with the enzyme. A steady decline in enzymatic activity was observed to a maximum inhibition of 50% at 1 M salt, however, the salt concentrations listed are prepared in the required sample volume which is 4 times less than that in the substrate. Therefore, if a direct comparison was to be made between both studies, the equivalent 1 M salt concentration when in the enzyme is 250 mM when added to the substrate. Thus, the salt effects were very similar regardless of what mode of analysis was used which significantly lowered DPPIV activity. A common occurrence with ammonium sulphate is that it can lower the pH of the buffer. However, this was not a problem in Hepes buffer as the zwitterionic buffer maintained its buffering capacity. Nonetheless, the salt seemed to be capable of specific interaction with the enzyme.

The same study was investigated with sodium chloride in both the substrate and with the enzyme. Figure 3.8.9.21 illustrates a considerable reduction in activity on increasing salt concentration. The salt did not appear to affect as potently as that observed for ammonium sulphate but with a 59% decrease in DPPIV activity, it obvious that sodium chloride is having a unique effect on the enzyme. This is confirmed when the salt is added to the enzyme before addition to the substrate. Figure 3.8.9.22 highlights the potency of the salt on activity but when the volume factor is taken into account, it shows that 250 mM sodium chloride, comparative reaction concentration, reduces activity by 30%, 10% more than that observed when the salt was added to the substrate. Although sodium chloride does not affect buffer pH capacity, it must have a significant influence on the ionic interactions of enzyme-substrate.
catalysis In attempt to ascertain a substitute for the sodium chloride inclusion in relevant purification buffers, potassium chloride was investigated. FIGURE 38931 demonstrates the almost identical effect that this salt also has on the enzyme. It is assumed that both sodium and potassium chloride act in a similar way in reducing enzyme activity.

These results are surprising, not necessary for the effect the salts have on activity but the extent of the degradation. Timasheff and Arakawa, 1990 report that sodium chloride and especially ammonium sulphate are good protein stabilising agents by increasing surface tension. They also enhance the formation of water clusters around the protein, which causes a loss of total free energy. This 'preferential hydration' causes the protein to become more compact and therefore more stable. However they do acknowledge that there is potential complications due to the charged state of salts possibly interacting electrostatically with charged sites on proteins. This was explored by Polgar, 1995, when it was discovered that sodium chloride destabilised Prolyl Oligopeptidase by a mechanism opposing 'preferential hydration'. He proposes that the salt may penetrate the zone of preferential hydration and bind to the enzyme at specific sites. This binding could weaken electrostatic forces that stabilise the protein's conformation. Therefore it seems most likely that a similar phenomenon occurs in this study. It is not clear whether this destabilisation effect can be predicted due to some common conformation between protein, however it is important to be able to counteract the sensitivity that the enzyme has to increased ionic strength environments.

489. Substrate Specificity

The specificity of DPPIV for particular substrates is potentially the most important study that can undertaken to ultimately understand the functionality of the enzyme. It has not been established what the natural substrate of DPPIV is, in vivo, which has lead to much experimentation. Similarly the role the enzyme plays in protein degradation has many possibilities with the subsequent loss in the protein's physiological effect. However the cleavage of a dipeptide from a peptide often may activate the peptide or at least result in varied receptor binding and physiological effects.

The excellent resolving power of reverse phase high performance liquid chromatography (RP HPLC) has resulted in it becoming the predominant HPLC technique for peptide separation. In this study ion-pair RP HPLC was used to detect cleavage products following incubation of peptides with purified enzyme. A silica based analytical column containing octadecyl ligands (C18) was employed as the non-polar stationary phase. The polar mobile phase of water and MeOH contained the hydrophobic ion-pairing reagent trifluoroacetic acid (TFA). In the pH range dictated by the stability of the C18 column, basic amino acids and the terminal amino
group are fully ionised. It has been suggested that TFA complexes with basic residues that are positively charged and this interaction tends to increase the affinity of the peptide for the column and also results in unique differences in chromatography. Thus, ion-pair RP HPLC was chosen as the optimum analytical tool for this substrate specificity investigation.

A range of synthetic peptides were examined and the results are presented in Table 3.17. As described in section 1.5.7, the general preferences and restrictions for DPPIV activity is that the enzyme prefers bulky, hydrophobic residues but dislikes acidic groups in the P2 position, prefers proline in the P1 position and can not cleave peptides with proline or hydroxyproline in the P1' position. It was decided to maintain proline in the P1 position and thus vary the amino acids in the other position as well as the length of the peptides. The majority of the peptides analysed were tripeptides and a common trend was observed. Peptides with N-benzyloxycarbonyl (Z) at the P2 position were not cleaved. This is understandable as DPPIV can not cleave peptides when their amino terminus is blocked. Gly-Pro-Hyp or Gly-Pro-Pro were also uncleaved due to the residues at position P1. Phe-Pro-Ala was hydrolysed due to the hydrophobic nature of phenylalanine and the small size alanine at the P1' position. The most unexpected peptide cleaved was Tyr-Pro-Phe. The neutral, hydrophilic tyrosine residue is not favoured at that position while the bulky phenylalanine does not allow complete ease of access to the proline residue. This would explain the relatively small peak obtained as the rate of hydrolysis may have been restricted.

Table 3.16 lists the bioactive peptides that were examined and whether hydrolysis was achieved or not. It appears that bovine serum DPPIV does not have significant affinity to alanine when in position P1 as none of the amyloid β-proteins were hydrolysed. This could also be attributed to the size of the peptides analysed. Either way it would seem that the enzyme does not have any influence in Alzheimer's disease.

The first bioactive peptide to be cleaved was the opioid, β-Casomorphin. Opioids are polypeptides that influence nerve transmission in certain parts of the brain. They are called as such because they bind to specific receptors that bind opiate drugs such as morphine. Therefore they may be regarded as the brain's own 'pain killers' (Lehninger, 1982). Figure 3.8.1011 illustrates the chromatogram of the incubation of the peptide with the enzyme and shows multiple peaks. The sequence of Casomorphin (Table 3.15) has a proline residue at every second position of the seven amino acid peptide. Therefore it is a perfect candidate for DPPIV sequential hydrolysis. The peak at 9.9 minutes of Figure 3.8.1011, suggests the presence of the Gly-Pro dipeptide (Figure 3.8.1015). This dipeptide could not be produced.
unless the Tyr-Pro and then the Phe-Pro bonds were cleaved indicating complete hydrolysis. It is possible that the tyrosine residue could restrict hydrolysis due to the enzyme’s dislike for hydrophilic residues at the P2 position. As a consequence, the cleavage of the Tyr-Pro dipeptide could be the rate-limiting step. Therefore DPPIV plays a role in reducing pain prevention when in the brain. The serum enzyme could hydrolyse the peptide, which would subsequently pass the blood-brain barrier and have a physiological effect. This hydrolysis correlates with the literature (Mentlein, 1999), however the difference between the bovine peptide and that from human is that the latter does not have a second proline residue at the fourth position and therefore only one point cleavage could ever be achieved by DPPIV. Nevertheless the hydrolysis of the final Gly-Pro dipeptide has been reported as difficult or unattainable (Gilmartin and O’Cuinn, 1999). The peptide’s cleavage was confirmed as due solely to DPPIV by its inhibition when Ile-Pyrohydrodide a specific DPPIV inhibitor, was added.

**FIGURE 3 8 10 1 6** clearly illustrates the removal of the multiple peaks with a main peak eluted at a retention time of 18.90, which represents the intact Casomorphin peak. Two small peaks do appear and the peak at 16.783 correlates with the Phe-Pro dipeptide (**FIGURE 3 8 10 1 4**). However this dipeptide could not be produced unless the Tyr-Pro dipeptide was cleaved first. Therefore it is probable that this peak is due to the presence of the inhibitor.

Enterostatin is a five amino acid peptide with two proline residues at every second position. The peptide is released from the N-terminus of the pancreatic procolipase in the intestine and they act as specific satiety signals for fat intake. **FIGURE 3 8 10 1 11** demonstrates the peptide’s incubation with DPPIV and two cleavage peaks were observed which seemed to correlate with free Ala-Pro and Gly-Pro dipeptides (**FIGURES 3 8 10 1 13** and **3 8 10 1 14**). Therefore DPPIV could have an influence in obesity and fat intake. The pentapeptide is a perfect candidate for DPPIV hydrolysis due to the initial hydrophobic nature of the alanine residue, followed by Gly-Pro-Arg which DPPIV would also be expected to cleave.

**Substance P** is a neuropeptide from the tachykinin family. These peptides all have 10 or 11 residues with a common -FXGLM-NH2 ending. They elicit a wide range of responses from neurons, smooth muscle and cells of the immune system which are similar to bradykinin and serotonin. The peptide has many implications, some of which include its release during headache and migraine (Fuller, 1996) and scratching and biting behaviour when induced by intrathecal administration (Roman, 1995). Substance P Receptor binding results in specific physiological effects while the peptide’s analogues have different effects. Therefore the potential of hydrolysis by DPPIV was investigated. **FIGURE 3 8 10 1 17** illustrates the generation of two dipeptide peaks of Arg-Pro, and Lys-Pro, which would be expected due to the ability of DPPIV to hydrolyse peptides with basic residues in the P2 position.
remaining cleaved peptide may be hydrolysed further by other peptidases or could remain intact and be of physiological importance. Whatever pathway occurs, it is obvious that DPPIV contributes to this process (Harada, 1997).

Neuropeptide Y is widely distributed in central and peripheral neurons. The peptide is involved in food intake and anxiolysis with a significant role as a co-released transmitter (Roman, 1995). Figure 3 shows the chromatogram of the Neuropeptide Y analogue (1-24) incubation with DPPIV and the graph illustrates a cleavage product at a retention time of 16.683. This could be the dipeptide Tyr-Pro, which would correlate with Figure 3 or may be the corresponding 22 amino acid peptide. Either way the hydrolysis of Neuropeptide Y by DPPIV is very likely indicating the wide substrate specificity of the enzyme and its broad functionality. It should be noted that it may be unlikely that DPPIV could hydrolyse the native 36 amino acid peptide. However, Neuropeptide Y contains a proline-rich N-terminal sequence that would confer considerable resistance to the peptides degradation. Therefore, it is possible that DPPIV could play a major role in the hydrolysis of Neuropeptide Y, if the relevant proline containing amino dipeptide was exposed (Mentlein et al., 1993).

Michaelis-Menten constants (K_M) were determined for purified DPPIV activity as described in sections 2.10.10.1, and 2.10.10.2 using Gly-Pro-MCA and Lys-Pro-MCA respectively. Four kinetic models were applied to the data, namely the Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf models. Section 6.3 details how each model utilised the data to produce a K_M value for the two substrates. Tables 3.18 and 3.19 presents the calculated constants. It is obvious that the enzyme had a high affinity for Gly-Pro-MCA, since an average constant of 34.8μM was obtained. DPPIV appeared to have a lower affinity for Lys-Pro-MCA showing an average K_M of 103μM. However, the V_max constants obtained in both studies are considerably different. With an average V_max of 841 fluorescent units from Lys-Pro-MCA hydrolysis, the question may be asked whether the latter substrate would be better employed as the standard substrate for DPPIV. Although a higher concentration of substrate would be required to maintain saturation, a higher fluorescent signal could be achieved. This would correlate well with the preference of DPPIV for basic residues (lysine) than hydrophilic residues (glycine) at position P2. However, it was decided that this substrate could be used in addition to Gly-Pro-MCA, especially when the enzyme activity was quite low but the latter substrate was maintained as the characterisation substrate as it may have greater specificity, accuracy and reproducibility. The K_M determined was lower than that reported from other sources (Puschel et al., 1982, Shibuya-Saruta et al., 1996 and Wagner et
However the difference in the chromophore utilised for these studies may also influence the kinetics of the enzyme.

4.8.10 Inhibitor Studies

The in vivo effect of DPPIV is hard to elucidate but its potential generated from in vitro studies is widespread. One method to investigate the enzymes physiological effect in the body is the use of specific inhibitors. However the requirement for biocompatibility of these compounds has prohibited the introduction of some inhibitors into biological systems (De Meester et al., 1997). In general, specific inhibitors are synthesised to bind to the enzyme’s active site. Thus it can be speculated that good DPPIV substrates could also be adapted to become effective specific inhibitors and substrate specificity criteria could be applied to both.

Diprotin A (Ile-Pro-Ile) and B (Val-Pro-Leu) have been synthesised as specific DPPIV inhibitors but could also be regarded as good substrates. With isoleucine and valine at position P2, the amino acids hydrophobicity is preferred by DPPIV and should compete with the substrate for hydrolysis, creating a potent inhibitor. The effect of these compounds were investigated as described in section 2.10.11.1. Figures 3.8.11.1 and 3.8.11.2 show similar potency from both inhibitors resulting in complete inhibition of DPPIV. Although Diprotin A appeared to be most potent in inhibitory capacity, it is possible that both compounds behave in a similar fashion with IC50’s of 1x10^-5 M and 5x10^-5 M for Diprotin A and B respectively (Table 3.20). This correlates well with the literature (Ohkubo et al., 1994 and Gilmartin and O’Cuinn, 1999) even though the IC50 values were not estimated. The mode of inhibition was also determined as outlined in section 2.10.11.2. Lmeweaver-Burk plots were constructed and Figures 3.8.11.2.1 and 3.8.11.2.2 demonstrate the similar profiles obtained for both inhibitors. The curves all intersect above the negative portion of the y-axis suggesting that both inhibit by competitive-non-competitive mixed inhibition (see appendix section 6.4.3). This is slightly surprising as the structures of both compounds are very similar to the substrate and simple competitive inhibition was expected. The inhibition constants, K, were determined from these graphs as described in section 6.4.2 and were estimated as 1.36x10^-6 M and 1.93x10^-6 M for Diprotin A and B respectively.

An identical study was carried out on another two similar inhibitors, Ile-Thiazolidide and Ile-Pyrrolidide. Both have been reported as specific DPPIV inhibitors, which could prove an important tool in elucidating the structure and function of the proteolytic enzyme (Stockel-Maschek et al., 2000). The inhibitory studies carried out demonstrate the potency of the inhibitors to DPPIV activity as Figures 3.8.11.1.3 and 3.8.11.1.4 allow an IC50 estimation to be made. Ile-Thiazolidide appeared to be very potent to DPPIV activity with an IC50 of
7.5x10\(^{-7}\)M while the corresponding value for Ile-Pyrrolidide was 6x10\(^{-5}\)M (TABLE 3.20). However, the graphs obtained were considerably different to that of the Diprotins. The enzyme seemed to resist inhibition to a saturation point where the inhibitor concentration is such that reduction in activity is inevitable. This activity reduction occurs very quickly and complete inhibition is achieved at a lower inhibitor concentration. The mode of this inhibition was still unknown so both compounds were subjected to kinetic analysis and Lineweaver-Burk plots were constructed as outlined in section 2.10.11.2. Figures 3.8.11.2.3 and 3.8.11.2.4 clearly indicate the intersecting lines on the positive intercept of the y-axis. This is typical of competitive inhibition where the inhibitor competes with the substrate for the active site of the enzyme. The structures of both Thiazolidide and Pyrrolidide are almost identical, the only exception being the presence of a sulphur atom in the five-membered ring of Thiazolidide. Thus, it is expected that the mode of inhibition should be similar or the same. Despite the latter compound producing a very low IC\(_{50}\) value compared to Ile-Pyrrolidide, a less potent K\(_i\) was observed. Table 3.19 lists 3.7x10\(^{-7}\)M and 7.5x10\(^{-7}\)M as the inhibition constants of Ile-Thiazolidide and Ile-Pyrrolidide respectively. Although this correlates with reports of similar inhibition constants, Ile-Thiazolidide was seen to be the more potent (Stockel-Maschek et al., 2000).

The hydrolysis of some bioactive peptides by DPPIV has been successfully shown by HPLC. These peptides contain the proline residue and as a consequence of DPPIV cleavage it is possible that the peptides could also inhibit DPPIV's activity on the standard substrate, Gly-Pro-MCA. The effect of ^-Casomorphin and Substance P was investigated as described in section 2.10.11.3 and the kinetic models were applied to generate the corresponding Michaelis-Menton constant, K\(_M\) and the velocity maximum, V\(_{max}\). Depending on the inhibition obtained, the inhibition constant could be estimated. Figures 3.8.11.3.1 and 3.8.11.3.2 demonstrate Lineweaver-Burk plots for Casomorphin and Substance P respectively. It is clear that the two plots are very different with Casomorphin possibly inhibiting competitively. The Substance P plot is a clear example of Uncompetitive inhibition with the presence of two parallel lines. Casomorphin appeared to be the more potent inhibitor of the two peptides with an average K\(_i\) of 1.1x10\(^{-6}\)M, while Substance P inhibited DPPIV with an inhibitory constant of 5.5x10\(^{-6}\)M (TABLE 3.21). The difference in the mode of inhibition is potentially interesting as both peptides have a similar sequence with the second and fourth amino acid occupied by a proline residue. It is probable that sequential cleavage of the peptides is not required to inhibit the enzyme, however the size and the amino acid positions may play a major role in the mode of inhibition obtained. There still is much speculation about whether a substrate can be classified as an inhibitor and if an inhibitor is hydrolysed can it be regarded as a substrate. For all inhibitor studies carried out during this project, if a
decrease in the apparent rate of hydrolysis was observed then the compound was classified as an inhibitor. Similarly if all enzyme activity was eliminated by a specific compound then active site binding would be likely and the enzyme could be deemed as specifically inhibited.
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239
6.0. APPENDICES
60 APPENDICES

61 ERROR BARS

Error bars on all graphs represent the standard error of the mean of triplicate fluorescence or absorbance values. The standard error (SE) of mean is defined as

\[ SE = \frac{\sigma}{\sqrt{n}} \]

Where

\( \sigma \) is the standard deviation, i.e., \( \sqrt{\text{variance}} \) (variance is \( (a^2 + b^2 + c^2)/3 \), where \( a \), \( b \) and \( c \) are the triplicate values).

\( n \) is the number of repeat values measured (three in these studies).

62 ENZYME QUANTITATION

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

Derivation

Let MCA released = \( X \mu M \)

= \( X \) \mu moles/Litre

(assay uses 400\( \mu \)L MCA, 100\( \mu \)L enzyme)

= \( X \times 10^{-3} \times 0.4 \) \mu moles/min/100\( \mu \)L

\[ \frac{60}{60} \]

= \( X \times 10^{-3} \times 0.4 \times 10^{-2} \) nanomoles/min/100\( \mu \)L

\[ \frac{60}{60} \]

= \( X \times 0.4 \) nanomoles/minute/100\( \mu \)L

\[ \frac{240}{60} \]
= \(X \times 4\) nanomoles/minute/1000\(\mu\)L
\[\frac{60}{1} = \frac{X \times 4}{60}\]
\[= \frac{X}{15}\] nanomoles/min/mL

But

1 unit of activity is defined as amount of enzyme which releases 1 nanomole MCA/min.

Therefore

\[= \frac{X}{15}\] units/ml

And

MCA released \((X)\) is also defined as fluorescent intensity observed/slope of the appropriate MCA standard curve, so

\[
\text{Unit/mL} = \frac{\text{Fluorescent Intensity}}{\text{Slope of filtered standard curve} \times 15}
\]

Therefore

Unit of activity = \[
\frac{\text{Fluorescent Intensity}}{\text{Slope of filtered standard curve} \times 15}
\]
6.3 Purification Table Calculations

Total activity (unit) Units of enzyme activity, calculated from fluorescent intensity as described in section 6.2

Total protein (mg) mg/mL protein estimated from appropriate BSA standard curve (using Biuret or BCA assays) x volume of sample in mL

Specific Activity (unit/mg) Total activity/total protein

Purification factor Specific activity of sample/specific activity of starting sample (saliva or serum)

Yield (%) (Total activity of sample/total activity of starting sample (serum)) x 100
6.4 Kinetic Analysis

6.4.1 $K_M$ Determinations

The Michaelis constant ($K_M$) is defined as the dissociation equilibrium constant for the enzyme-substrate complex. It may be measured by the analysis of initial velocities at a series of substrate concentrations and one concentration of enzyme. The data points can then be fitted to a number of models as outlined below.

**Michaelis-Menten** – Plot of fluorescent intensity versus substrate concentration

$K_M$ is the substrate concentration at half the maximum fluorescent intensity.

**Lineweaver-Burk** – Plot of $1$/fluorescent intensity versus $1$/substrate concentration

The intercept of the line at the x-axis is given as $-1/K_M$ and the Y-Axis intercept represents $1/V_{max}$.

**Eadie-Hofstee** – Plot of fluorescent intensity versus fluorescent intensity/substrate concentration

The slope is given as $-K_M$ and the Y-Axis intercept represents $V_{max}$.

**Hanes-Woolf** – Plot of substrate concentration/fluorescent intensity versus substrate concentration

The intercept at the X-Axis is given as $-K_M$ and the slope represents $1/V_{max}$.
6.4.2 \( K_i \) Determinations

\( K_i \), the inhibition constant, is defined as the constant of dissociation of inhibitor from a reversibly bound enzyme-inhibitor complex for competitive inhibition

\[
K_i = \frac{K_M \times I}{K_{app} - K_M}
\]

Where

- \( K_i \) is the inhibition constant
- \( K_M \) is the Michaelis constant with no inhibitor present
- \( I \) is the inhibitor concentration
- \( K_{app} \) is the apparent Michaelis constant and is taken to be the 'new \( K_M \)' observed when a reversible inhibitor is incorporated into the assay. \( K_{app} \) is measured as for \( K_M \) (described in section 6.4.1).

When the inhibition is not competitive, the inhibition constant can be calculated as follows,

\[
K_i = \frac{V_{app_{\text{max}}} \times I}{V_{\text{max}} - V_{app_{\text{max}}}}
\]

Where

- \( K_i \) is the inhibition constant
- \( V_{\text{max}} \) is the maximum velocity with no inhibitor present
- \( I \) is the inhibitor concentration
- \( V_{app_{\text{max}}} \) is the apparent maximum velocity and is taken to be the 'new \( V_{\text{max}} \)' observed when an Un-competitive or Non-competitive inhibitor is incorporated into the assay.

6.4.3 Types of Reversible Inhibition

Reversible inhibition involves noncovalent forces that bind inhibitors to enzymes. The following are the common forms of reversible inhibition:

- \( E \) - enzyme
- \( I \) - inhibitor
- \( S \) - substrate
- \( P \) - product
**Competitive inhibition** involves binding of the enzyme to the inhibitor forming an enzyme-inhibitor complex. The inhibitor competes with the substrate for the enzyme active site

\[
E + S \leftrightarrow ES \leftrightarrow E + P \\
+ \\
I \\
\uparrow K_i \\
EI
\]

With competitive inhibition, the slope of the Lineweaver-Burk plot increases as does the $K_M$. Competitive inhibition is recognisable when plots intersect at a common point of intersection on the positive y-axis.

**Uncompetitive inhibition** occurs when the inhibitor binds to the enzyme-substrate complex, binding to a site other than the substrate binding site

\[
E + S \leftrightarrow ES \rightarrow E + P \\
+ \\
I \\
\uparrow K_i \\
ESI
\]

This type of inhibition is identified by the presence of parallel lines in the Lineweaver-Burk plot. $K_M$ decreases but slope values remain the same.

**Non-competitive inhibition** involves the random binding of inhibitor to either enzyme or enzyme-substrate complex

\[
E + S \leftrightarrow ES \leftrightarrow E + P \\
+ \\
I \\
\uparrow K_i \\
ESI \\
EI + S \leftrightarrow ESI
\]
Non-competitive inhibition may be identified by lines intersecting on the negative portion of the y-axis. $K_m$ remains the same but $V_{max}$ decreases.

**Mixed inhibition** occurs when inhibitor binds unequally to enzyme or enzyme-substrate complex. $V_{max}$ decreases and $K_m$ may increase or decrease. Non-competitive-uncompetitive mixed inhibition is evident when the type of inhibition lies between the two types and is identified by lines intersecting below the negative portion of the y-axis. Competitive-non-competitive inhibition is recognisable by lines intersecting above the negative portion of the y-axis.

### 6.5 IC$_{50}$ Determinations

The IC$_{50}$ is defined as the concentration at which an enzyme is half inhibited. It can be calculated graphically from a plot of enzyme activity versus inhibitor concentration, by locating 50% activity on the y-axis and calculating the corresponding concentration on the x-axis.