THE ISOLATION AND PARTIAL CHARACTERIZATION OF $\alpha_2$-ANTIPLASMIN AND PLASMINOGEN FROM OSTRICH PLASMA

by

Adele René Thomas

Submitted in partial fulfilment of the requirements for the degree of

Magister Scientiae

in the Faculty of Science at the University of Port Elizabeth.

February 2000

Supervisor: Prof. R. J. Naudé
CONTENTS

SUMMARY ................................................................................................................... ii

OPSOMMING ........................................................................................................ iii

ACKNOWLEDGEMENTS .......................................................................................... iv

LIST OF ABBREVIATIONS ....................................................................................... v

LIST OF FIGURES ..................................................................................................... vii

LIST OF TABLES ...................................................................................................... x

LIST OF DIAGRAMS ............................................................................................... xi

CHAPTERS

1 LITERATURE REVIEW ......................................................................................... 1

2 INTRODUCTION TO THE PRESENT STUDY .................................................. 35

3 MATERIALS AND METHODS ........................................................................... 36

4 ISOLATION AND PURIFICATION OF OSTRICH α2-ANTIPLASMIN, PLASMINOGEN AND PLASMIN ................................. 69

5 CHARACTERISATION OF OSTRICH α2-ANTIPLASMIN, PLASMINOGEN AND PLASMIN ........................................ 96

6 CONCLUSIONS .................................................................................................... 122

REFERENCES ........................................................................................................ 126
This study reports the isolation, purification and partial characterisation of the ostrich serpin, α₂AP, as well as its target enzyme, ostrich plasmin, in its active and inactive proenzyme, viz. plasminogen, forms.

Three different procedures were undertaken to isolate and purify ostrich α₂AP. The first one involved L-lysine-Sepharose chromatography, ammonium sulfate fractionation, ion-exchange chromatography on Toyopearl Super-Q 650S, and ostrich plasminogen-Sepharose affinity chromatography. The second procedure replaced the latter chromatographic step with gel filtration on Sephadex G-200 and hydroxylapatite chromatography, while the third one employed instead the theoretically more efficient LBSI-Sepharose chromatographic step. The third procedure yielded purified ostrich α₂AP, but the degree of purity and yield were relatively low. Ostrich plasminogen was highly purified after L-lysine-Sepharose chromatography and ostrich plasmin was obtained by the urokinase-activation of the purified ostrich plasminogen.

Ostrich α₂AP revealed an Mᵋ of 77-84 K and two isoelectric forms of pI 3.85 and 6.18. N-terminal sequence analysis showed ostrich α₂AP to have only 2 out of 11 residues in common with both those of human and bovine α₂AP. Ostrich α₂AP showed the largest inhibitory effects on ostrich plasmin, followed by comm. bovine chymotrypsin, trypsin and plasmin, in that order, and it appeared to be a much less potent plasmin inhibitor than bovine aprotinin, but a much more potent one than the synthetic inhibitors, DFP and EACA. Ostrich plasminogen showed an Mᵋ of 92 K and multiple isoelectric forms (~7) in the pI range 6.01-9.18, with a major one of pI 6.01. It showed a total of 775 amino acid residues and its N-terminal sequence showed ~53% identity with those of human, rabbit, cat, and ox plasminogens. Ostrich plasmin revealed an Mᵋ of 78 K, two isoelectric forms of pI 4.07 and 6.01, and a total of 638 amino acid residues. N-terminal sequence analysis showed that 2-4 residues are identical to the 5 of human, cat, dog, rabbit, and ox plasmins. The pH and temperature optima of ostrich plasmin were determined as 8.0 and 40 °C, respectively. The thermodynamic and kinetic parameters of ostrich plasmin were computed, and plasmin was shown to prefer Lys to Arg residues in the S₁ position.

In conclusion, ostrich α₂AP, plasminogen and plasmin showed definite similarities to their mammalian counterparts, but there were also significant differences.

**Keywords:** ostrich, serpin, α₂-antiplasmin, plasminogen, plasmin.
Hierdie studie behels die isolasie, suiwering en gedeeltelike karakterisering van die volstruis serpien, α2AP, asook sy teiken ensiem, volstruis plasmien, in sy aktiewe en onaktiewe voorganger, naamlik plasmienogeen, vorme.

Drie verskillende prosedures om volstruis α2AP te isoleer en suiwer was onderneem. Die eerste het ingesluit L-lisien-Sepharose chromatografie, ammoniumsulfat fraksionering, ionouitruil chromatografie op Toyopearl Super-Q 650S, en volstruis plasmienogeen-Sepharose affiniteitschromatografie. Die tweede prosedure het laasgenoemde chromatografie stap met gel-filtrasie op Sephadex G-200 en hidroksielapatiet chromatografie vervang, terwyl die derde een in plaas daarvan die teoreties meer bruikbare LBSI-Sepharose chromatografie stap gebruik het. Die derde prosedure het gesuiwerde volstruis α2AP gelewer, maar die graad van suiwerheid en opbrengs was betreklik laag. Volstruis plasmienogeen was hoog gesuiwer na L-lisien-Sepharose chromatografie en volstruis plasmien was deur die urokinase aktivering van die gesuiwerde volstruis plasmienogeen verkry.

Volstruis α2AP het h Mᵋ van 77-84 K en twee isoelektriese vorme van pI 3.85 en 6.18 gelewer. N-terminale volgorde analise het gewys dat volstruis α2AP net 2 uit 11 residue besit wat ooreenstem met albei mens en bees α2AP. Volstruis α2AP het die grootste inhiberende effek op volstruis plasmien getoon, gevolg deur kommersiële bees chimotripsien, tripsien en plasmien, in daardie volgorde, en dit blyk h veel swakker plasmien inhibeerder as bees aprotinien te wees, maar h veel sterker een as die sintetiese inhibideerdes, DFP en EACA. Volstruis plasmienogeen het h Mᵋ van 92 K en veelvoudige isoelektriese vorme (~7) in die pI gebied van 6.01-9.18 gelewer, met h hoofbestandeel van pI 6.01. Dit het h totaal van 755 aminosuur residue getoon en sy N-terminale volgorde het ~53% ooreenstemming met dié van mens, konyn, kat, en os plasmienogene gewys. Volstruis plasmien het h Mᵋ van 78 K, twee isoelektriese vorme van pI 4.07 en 6.01, en h totaal van 638 aminosuur residue gelewer. N-terminale volgorde analise het gewys 2-4 residue wat identies is met die 5 van mens, kat, hond, konyn, en os plasmiene. Die optimale pH en temperatuur van volstruis plasmien was 8.0 en 40 °C onderskeidelik. Die termodinamiese en kinetiese parameters van volstruis plasmien was bepaal, en plasmien het Lys bo Arg residue in die S₁ posisie verkies.

Ten slotte, volstruis α2AP, plasmienogeen en plasmien het groot ooreenstemming getoon met die vergelykende soogdier molekule, maar daar was ook betekenisvolle verskille.
Sleutelwoorde: volstruis, serpien, α₂-antiplasmien, plasmienogeen, plasmien.

ACKNOWLEDGEMENTS

The author wishes to extend her sincere gratitude and heart-felt appreciation to:

- Prof. R. J. Naudé for his invaluable input, willing assistance and guidance, for his genuine interest and support, and for his patience throughout this study.

- Staff and fellow post-graduate students of the department of Biochemistry and Microbiology for their support, help and encouragement.

- The Foundation for Research and Development and the University of Port Elizabeth for their financial support.

- The Grahamstown abbatoir and the farms in the vicinity of Port Elizabeth for their generous supply of ostrich blood.

- Prof. K. Muramoto and Miss. Naganuma from the University of Tohoku (Japan) for all the amino acid composition and N-terminal sequence analyses, and Prof. K. Muramoto for the kind gift of D-Val-Leu-Lys-pNA.

- Family and friends for their continued support, encouragement and understanding.

- Our Lord God and Saviour for proving himself so faithful and just throughout the course of this project.
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-AHA</td>
<td>6-Aminohexanoic acid</td>
</tr>
<tr>
<td>t-AMCHA</td>
<td>trans-4-(aminomethyl)cyclohexane-1-carboxylic acid</td>
</tr>
<tr>
<td>α₂AP</td>
<td>α₂-Antiplasmin</td>
</tr>
<tr>
<td>ATNA</td>
<td>N-acetyl-L-tyrosine-ρ-nitroanilide</td>
</tr>
<tr>
<td>BAPNA</td>
<td>Benzoyl-DL-arginine-ρ-nitroanilide-HCl</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair(s)</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribose nucleic acid</td>
</tr>
<tr>
<td>comm.</td>
<td>Commercial</td>
</tr>
<tr>
<td>DABS-Cl</td>
<td>4-(dimethylamino)azobene-4’-sulphonyl chloride</td>
</tr>
<tr>
<td>DFP</td>
<td>Diisopropyl fluorophosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>E</td>
<td>Enzyme</td>
</tr>
<tr>
<td>EACA</td>
<td>ε-Aminocaproic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Endo-H</td>
<td>Endo-β-N-acetylglucosaminidase H</td>
</tr>
<tr>
<td>FITC-PITC</td>
<td>Fluorescein isothiocyanate-phenylisothiocyanate</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxylapatite</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>hr</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>I</td>
<td>Inhibitor</td>
</tr>
<tr>
<td>IEC</td>
<td>Ion-exchange chromatography</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase(s)</td>
</tr>
<tr>
<td>KIU</td>
<td>Kallikrein inhibitory unit(s)</td>
</tr>
<tr>
<td>LBS(s)</td>
<td>Lysine-binding site(s)</td>
</tr>
<tr>
<td>LBSI</td>
<td>Lysine-binding site I</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>$M_r$</td>
<td>Relative molecular weight</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>PAG-IEF</td>
<td>Polyacrylamide gel isoelectric focusing</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>pNA</td>
<td>$\rho$-Nitroanilide</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>$r^2$</td>
<td>Correlation coefficient</td>
</tr>
<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>$R_m$</td>
<td>Relative mobility</td>
</tr>
<tr>
<td>RPC</td>
<td>Reversed-phase chromatography</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reversed-phase high performance liquid chromatography</td>
</tr>
<tr>
<td>RSL</td>
<td>Reactive site loop</td>
</tr>
<tr>
<td>R.T.</td>
<td>Room temperature</td>
</tr>
<tr>
<td>s</td>
<td>Second(s)</td>
</tr>
<tr>
<td>SAPNA</td>
<td>N-Succinyl-Ala-Ala-Ala-$\rho$-nitroanilide</td>
</tr>
<tr>
<td>serpin(s)</td>
<td>Serine proteinase inhibitor(s)</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminoxane</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE NO.</th>
<th>FIGURE TITLE</th>
<th>PAGE NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Multiple regulatory functions of serpins.</td>
<td>1</td>
</tr>
<tr>
<td>1.2</td>
<td>Organisation of the human $\alpha_2$AP gene.</td>
<td>8</td>
</tr>
<tr>
<td>1.3</td>
<td>Nucleotide and deduced amino acid sequence of human $\alpha_2$AP.</td>
<td>10</td>
</tr>
<tr>
<td>1.4</td>
<td>Alignment of the amino acid sequence of human $\alpha_2$AP from the sequences of CNBr fragments (CB), tryptic (T) and chymotryptic (C) peptides.</td>
<td>11</td>
</tr>
<tr>
<td>1.5</td>
<td>Homology of $\alpha_2$AP with 12 other serpins.</td>
<td>13</td>
</tr>
<tr>
<td>1.6</td>
<td>Complete nucleotide sequence and translated protein sequence of bovine $\alpha_2$AP.</td>
<td>14</td>
</tr>
<tr>
<td>1.7</td>
<td>Alignment of the protein sequences of bovine (b) and human (h) $\alpha_2$APs.</td>
<td>15</td>
</tr>
<tr>
<td>1.8</td>
<td>Proposed spatial structure of native, functional serpins.</td>
<td>17</td>
</tr>
<tr>
<td>1.9</td>
<td>Diagram of the cleavage sites in human $\alpha_2$AP caused by trypsin and chymotrypsin.</td>
<td>18</td>
</tr>
<tr>
<td>1.10</td>
<td>Reactive site sequence of bovine $\alpha_2$AP.</td>
<td>19</td>
</tr>
<tr>
<td>1.11</td>
<td>Amino acid sequence for human plasminogen.</td>
<td>21</td>
</tr>
<tr>
<td>1.12</td>
<td>Mechanism of activation of human Glu-plasminogen (Pg) to 2-chain disulfide-linked Lys$^{78}$-plasmin (Pm) by urokinase (UK).</td>
<td>21</td>
</tr>
<tr>
<td>1.13</td>
<td>Reaction model for the inhibition of plasmin by $\alpha_2$AP.</td>
<td>22</td>
</tr>
<tr>
<td>1.14</td>
<td>The normal pathway for the reaction between plasmin and $\alpha_2$AP.</td>
<td>26</td>
</tr>
<tr>
<td>1.15</td>
<td>Proposed suicide inhibitor mechanism for serpins.</td>
<td>27</td>
</tr>
<tr>
<td>1.16</td>
<td>Proposed mechanism for serpin inhibition of proteinases showing the structures of the serpin at each step of the reaction.</td>
<td>28</td>
</tr>
<tr>
<td>1.17</td>
<td>Acyl-transfer reaction between Gln$^{14}$ of $\alpha_2$AP and Lys$^{303}$ of fibrin(ogen), catalysed by activated Factor XIII, forming the $\varepsilon$-($\gamma$-glutamyl)lysine crosslinks.</td>
<td>32</td>
</tr>
<tr>
<td>1.18</td>
<td>Synthesis, oligosaccharide processing, and intracellular transit of $\alpha_2$AP.</td>
<td>34</td>
</tr>
<tr>
<td>3.1</td>
<td>Plasmin assay progress curves for various comm. bovine plasmin concentrations (microtiter plate assay).</td>
<td>41</td>
</tr>
<tr>
<td>3.2</td>
<td>Rate of hydrolysis of D-Val-Leu-Lys-pNA as a function of varying comm. bovine plasmin concentrations (microtiter plate assay) ($r^2=0.997$).</td>
<td>42</td>
</tr>
<tr>
<td>3.3</td>
<td>Inhibition of comm. bovine plasmin by comm. human $\alpha_2$AP as a function of incubation time and molar ratios of I:E.</td>
<td>43</td>
</tr>
</tbody>
</table>
3.4 Trypsin assay progress curves for various comm. bovine pancreatic trypsin concentrations. 45
3.5 Rate of hydrolysis of BAPNA as a function of varying comm. bovine pancreatic trypsin concentrations. 45
3.6 Chymotrypsin assay progress curves for various comm. bovine pancreatic chymotrypsin concentrations. 47
3.7 Rate of hydrolysis of ATNA as a function of varying comm. bovine pancreatic chymotrypsin concentrations. 48
3.8 Elastase assay progress curves for various comm. porcine pancreatic elastase concentrations. 49
3.9 Rate of hydrolysis of SAPNA as a function of varying comm. porcine pancreatic elastase concentrations. 50
3.10 BCA protein standard curve. 51
3.11 L-Lysine-Sepharose chromatography of the plasminogen fragments resulting from elastase digestion. 57
3.12 SDS-PAGE patterns of the elution profile of L-lysine-Sepharose chromatography of the plasminogen fragments resulting from elastase digestion. 57
3.13 Chemical structure of reactive yellow 86. 61
3.14 Chemical structure of cibacron blue F3G-A. 62
3.15 Calibration curve of the high molecular weight markers on a 10% SDS-PAGE (r²=0.962). 65
3.16 Calibration curve of the 3.6-6.6 IEF mix obtained from PAG-IEF (6% gel) (r²=0.990). 66

4.1 Gradient elution profile of Toyopearl Super Q-650S chromatography of sample 1B. 72
4.2 Ostrich plasminogen-Sepharose chromatography of sample 1C. 73
4.3 SDS-PAGE patterns of the samples after each purification step of the first isolation and purification procedure. 75
4.4 Sephadex G-200 chromatography of sample 2C. 78
4.5 Hydroxylapatite chromatography of sample 2D. 79
4.6 SDS-PAGE patterns of the three samples of the second isolation and purification procedure. 80
4.7 LBSI-Sepharose chromatography of sample 3C. 84
4.8 SDS-PAGE patterns of the final sample of the third isolation and purification procedure. 85
4.9 Reactive yellow 86-Toyopearl HW-65 chromatography of sample 3B. 87
4.10 Q HyperD 10 HPLC of sample 3C. 88
4.11 RP-HPLC of sample 3C on a Capcell Pak C18 ODS/AGS120 column. 89
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.12</td>
<td>Elution profile of L-lysine-Sepharose chromatography of ostrich plasma.</td>
</tr>
<tr>
<td>4.13</td>
<td>SDS-PAGE pattern of ostrich plasminogen.</td>
</tr>
<tr>
<td>4.14</td>
<td>Activation of ostrich plasminogen to plasmin with various urokinase:plasminogen concentrations at various incubation times.</td>
</tr>
<tr>
<td>4.15</td>
<td>SDS-PAGE patterns of the activation of ostrich plasminogen to plasmin with 600 µg urokinase/mg plasminogen at various incubation times.</td>
</tr>
<tr>
<td>4.16</td>
<td>SDS-PAGE patterns of ostrich and comm. bovine plasmin.</td>
</tr>
<tr>
<td>5.1</td>
<td>PAG-IEF patterns of ostrich α2AP, plasminogen and plasmin.</td>
</tr>
<tr>
<td>5.2</td>
<td>Inhibitory effects of ostrich and comm. human α2APs on ostrich plasmin.</td>
</tr>
<tr>
<td>5.3</td>
<td>Inhibitory effects of ostrich and comm. human α2APs on comm. bovine plasmin.</td>
</tr>
<tr>
<td>5.4</td>
<td>Inhibitory effects of ostrich and comm. human α2APs on comm. bovine pancreatic trypsin.</td>
</tr>
<tr>
<td>5.5</td>
<td>Inhibitory effects of ostrich and comm. human α2APs on comm. bovine pancreatic chymotrypsin.</td>
</tr>
<tr>
<td>5.6</td>
<td>Inhibitory effects of comm. bovine lung aprotinin on ostrich and bovine plasmins.</td>
</tr>
<tr>
<td>5.7</td>
<td>Inhibitory effects of DFP on ostrich and bovine plasmins.</td>
</tr>
<tr>
<td>5.8</td>
<td>Inhibitory effect of EACA on bovine plasmin.</td>
</tr>
<tr>
<td>5.9</td>
<td>pH profiles of ostrich and comm. bovine plasmins.</td>
</tr>
<tr>
<td>5.10</td>
<td>Effect of temperature on ostrich and comm. bovine plasmins.</td>
</tr>
<tr>
<td>5.11</td>
<td>Arrhenius plots of ostrich and comm. bovine plasmins.</td>
</tr>
<tr>
<td>5.12</td>
<td>Modified Arrhenius plots of ostrich and comm. bovine plasmins.</td>
</tr>
<tr>
<td>5.13</td>
<td>Hanes plots of ostrich and comm. bovine plasmins with four different substrates.</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE NO.</th>
<th>TABLE TITLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Amino acid compositions (mol %) of human and bovine $\alpha_2$APs.</td>
</tr>
<tr>
<td>1.2</td>
<td>Kinetic constants for the reaction of $\alpha_2$AP with plasmin, proteolytically modified forms of plasmin and other proteases.</td>
</tr>
<tr>
<td>3.1</td>
<td>Summary of chemical materials used and their respective suppliers.</td>
</tr>
<tr>
<td>3.2</td>
<td>Procedure for the microtiter plate assay of plasmin.</td>
</tr>
<tr>
<td>3.3 (i)</td>
<td>Procedure for the standard assay of trypsin.</td>
</tr>
<tr>
<td>3.3 (ii)</td>
<td>Procedure for the microtiter plate assay of trypsin.</td>
</tr>
<tr>
<td>3.4</td>
<td>Procedure for the standard and microtiter plate assays of chymotrypsin.</td>
</tr>
<tr>
<td>3.5</td>
<td>Procedure for the standard and microtiter plate assays of elastase.</td>
</tr>
<tr>
<td>3.6</td>
<td>Procedure for the assay of plasmin using bovine fibrinogen.</td>
</tr>
<tr>
<td>4.1</td>
<td>Summary of the first isolation and purification procedure of ostrich $\alpha_2$AP.</td>
</tr>
<tr>
<td>4.2</td>
<td>Summary of the second isolation and purification procedure of ostrich $\alpha_2$AP.</td>
</tr>
<tr>
<td>4.3</td>
<td>Summary of the third isolation and purification procedure of ostrich $\alpha_2$AP.</td>
</tr>
<tr>
<td>5.1</td>
<td>Comparison of N-terminal sequence of ostrich $\alpha_2$AP with those of human and bovine $\alpha_2$APs.</td>
</tr>
<tr>
<td>5.2</td>
<td>Summary of inhibitory effects of ostrich and comm. human $\alpha_2$APs on a few serine proteases.</td>
</tr>
<tr>
<td>5.3</td>
<td>Summary of inhibitory effects of a few inhibitors on ostrich and comm. bovine plasmins.</td>
</tr>
<tr>
<td>5.4</td>
<td>Amino acid composition (molar ratios) of ostrich plasminogen as compared to those of human and rabbit plasminogens.</td>
</tr>
<tr>
<td>5.5</td>
<td>Comparison of N-terminal sequence of ostrich plasminogen with those of four mammalian plasminogens.</td>
</tr>
<tr>
<td>5.6</td>
<td>Amino acid composition (molar ratios) of ostrich plasmin as compared to those of human and rabbit plasmins.</td>
</tr>
<tr>
<td>5.7</td>
<td>Comparison of N-terminal sequence of ostrich plasmin with those of five mammalian plasmins.</td>
</tr>
<tr>
<td>5.8</td>
<td>Summary of thermodynamic parameters for the reaction of ostrich and comm. bovine plasmins with D-Val-Leu-Lys-pNA.</td>
</tr>
<tr>
<td>5.9</td>
<td>Summary of kinetic parameters of ostrich and comm. bovine plasmins.</td>
</tr>
</tbody>
</table>
### LIST OF DIAGRAMS

<table>
<thead>
<tr>
<th>DIAGRAM NO.</th>
<th>DIAGRAM TITLE</th>
<th>PAGE NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Procedure for the preparation of L-lysine-Sepharose affinity resin.</td>
<td>54</td>
</tr>
<tr>
<td>3.2</td>
<td>Procedure for the preparation of ostrich plasminogen-Sepharose resin.</td>
<td>55</td>
</tr>
<tr>
<td>3.3</td>
<td>Procedure for the preparation of ostrich LBSI.</td>
<td>56</td>
</tr>
<tr>
<td>3.4</td>
<td>Procedure for the preparation of reactive yellow 86-Toyopearl HW-65 resin.</td>
<td>61</td>
</tr>
<tr>
<td>3.5</td>
<td>KCl protein precipitation method.</td>
<td>64</td>
</tr>
<tr>
<td>4.1</td>
<td>First isolation and purification procedure of ostrich α2AP.</td>
<td>70</td>
</tr>
<tr>
<td>4.2</td>
<td>Second isolation and purification procedure of ostrich α2AP.</td>
<td>76</td>
</tr>
<tr>
<td>4.3</td>
<td>Third isolation and purification procedure of ostrich α2AP.</td>
<td>82</td>
</tr>
<tr>
<td>4.4</td>
<td>Isolation and purification procedure of ostrich plasminogen.</td>
<td>90</td>
</tr>
<tr>
<td>4.5</td>
<td>Activation procedure of ostrich plasminogen to plasmin.</td>
<td>92</td>
</tr>
</tbody>
</table>
CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION .................................................................................. 3

1.2. PHYSICOCHEMICAL, BIOLOGICAL AND IMMUNOCHEMICAL PROPERTIES OF \( \alpha_2 \)AP ................................................................. 5
   1.2.1. MOLECULAR WEIGHT ................................................................. 5
   1.2.2. ULTRACENTRIFUGATION STUDIES ........................................ 5
   1.2.3. STABILITY ................................................................................. 5
   1.2.4. HETEROGENEITY ................................................................. 6
   1.2.5. ISOELECTRIC POINT(S) ...................................................... 6
   1.2.6. IMMUNOCHEMICAL STUDIES ........................................ 6
   1.2.7. ABSORPTION COEFFICIENT ........................................ 7
   1.2.8. PLASMA LEVELS ................................................................. 7
   1.2.9. HALF-LIFE .............................................................................. 7

1.3. GENETIC PROPERTIES OF \( \alpha_2 \)AP ............................................. 8

1.4. STRUCTURE OF \( \alpha_2 \)AP ................................................................. 9
   1.4.1. PRIMARY STRUCTURE ............................................................. 9
      1.4.1.1. Amino Acid Composition .............................................. 9
      1.4.1.2. Amino Acid Sequence ................................................. 10
      1.4.1.3. Carbohydrate Content and Attachment Sites ............... 16
      1.4.1.4. Cysteine Content and Disulfide Bridge Arrangement ... 16
   1.4.2. THREE-DIMENSIONAL STRUCTURE .................................... 17
   1.4.3. FUNCTIONAL SITES .............................................................. 18
1.5. FUNCTIONS OF $\alpha_2$AP ......................................................... 20

1.5.1. REACTION WITH PLASMIN ..................................................... 20
   1.5.1.1. Background Information on Plasmin(ogen) .................. 20
   1.5.1.2. Kinetics of Reaction ............................................... 22
      1.5.1.2.1. Reaction with intact plasmin ......................... 22
      1.5.1.2.2. Reaction with proteolytically modified forms of plasmin 23
      1.5.1.2.3. Summary of kinetic constants ....................... 24
      1.5.1.2.4. Effects of plasmin substrate and lysine analogues . 25
   1.5.1.3. Mechanism of Action ........................................... 26

1.5.2. REACTION WITH OTHER PROTEINASES ............................. 29
   1.5.2.1. Specificity of $\alpha_2$AP ......................................... 29
   1.5.2.2. Reaction with Trypsin and Chymotrypsin .............. 29

1.5.3. INTERACTION WITH PLASMINOGEN .................................... 30

1.5.4. CROSS-LINKING TO FIBRIN(ogen) .................................... 31
   1.5.4.1. $\alpha_2$AP and Fibrinolysis .................................. 31
   1.5.4.2. Cross-Linking Site in Fibrin(ogen) ...................... 31
   1.5.4.3. Mechanism of Action ........................................ 32

1.6. CLINICAL ASPECTS ............................................................... 33

1.6.1. $\alpha_2$AP LEVELS IN HEALTH AND DISEASE .......................... 33
1.6.2. $\alpha_2$AP DEFICIENCY ...................................................... 33
CHAPTER 1

1.1. INTRODUCTION

The regulation of proteolytic enzymes in tissues by endogenous inhibitors is crucial for the maintenance of homeostasis. These endogenous inhibitors are found primarily in blood plasma and account for more than 10% of the total protein content of plasma. The vast majority of these plasma protein inhibitors are targeted toward the serine proteinases which are known to be involved in phagocytosis, coagulation, complement activation, and fibrinolysis (Travis and Salvesen, 1983; Potempa et al., 1994).

There are several classes of plasma proteinase inhibitors, but one superfamily of structurally related inhibitors referred to as serpins (serine proteinase inhibitors) predominates, with its members being found in humans, plants and viruses. The primary function for most serpins is the regulation of proteolytic events associated with a myriad of biochemical pathways (figure 1.1), but many have alternate functions such as hormone transport or blood pressure regulation (Potempa et al., 1994).

Serpins are single chain proteins with molecular masses between 40 and 100 kDa, those present in plasma being variably glycosylated (Gettins et al., 1993; Potempa et al., 1994). They contain a conserved domain of 370-390 amino acid residues, usually flanked by amino- or carboxyl-terminal extensions (Potempa et al., 1994). In contrast to other simpler proteinase inhibitors, serpins may interact with proteinases as inhibitors, substrates, or both (Gettins et al., 1993). Inhibitory serpins interact with their target proteinase at a reactive site located within a loop structure 30-40 residues from the carboxyl terminus (Potempa et al., 1994), forming 1:1 complexes (Gettins et al., 1993).
$\alpha_2$AP is an example of an inhibitory serpin. It is the primary physiological inhibitor of plasmin, the serine proteinase responsible for the dissolution of fibrin clots, and is therefore a specific and effective inhibitor of fibrinolysis (Collen, 1976; Moroi and Aoki, 1976; Müllertz and Clemmensen, 1976). It was discovered in 1974 (Müllertz, 1974), before which it was believed that the serpins $\alpha_2$-macroglobulin and $\alpha_1$-proteinase inhibitor were the major plasmin inhibitors in plasma.

The rest of this chapter reports specifically on the serpin $\alpha_2$AP.
1.2. PHYSICOCHEMICAL, BIOLOGICAL AND IMMUNOCHEMICAL PROPERTIES OF $\alpha_2$AP

The first physicochemical characterisations of $\alpha_2$AP were performed by Moroi and Aoki (1976) and Wiman and Collen (1977) on human $\alpha_2$AP, and most of their results agreed well (Lijnen and Collen, 1986).

1.2.1. MOLECULAR WEIGHT

Human $\alpha_2$AP showed, according to SDS-PAGE, a $M_r$ of 70 K under non-reducing conditions (Wiman and Collen, 1977) and 67 K when reduced by $\beta$-mercaptoethanol (Moroi and Aoki, 1976; Wiman and Collen, 1977). However, its molecular weight deduced from the cDNA sequence and the carbohydrate content is approximately 58 kDa (Aoki et al., 1993).

1.2.2. ULTRACENTRIFUGATION STUDIES

Sedimentation velocity analyses performed by Moroi and Aoki (1976) and Wiman and Collen (1977) on human $\alpha_2$AP resulted in sedimentation constant values ($s_{20,w}$) of 3.43 S and $3.45 \pm 0.05$ S, respectively. The partial specific volume was calculated from the amino acid and carbohydrate compositions as 0.718 ml/g, thus giving a calculated molecular weight of 70.4 kDa (Wiman and Collen, 1977), whereas Moroi and Aoki (1976) obtained a molecular weight of 63 kDa from sedimentation equilibrium studies. From the molecular weight data, the sedimentation constant and the partial specific volume, Wiman and Collen (1977) calculated the Stoke's radius to be 5.0 nm and the frictional ratio 1.8, thus indicating an asymmetrical or highly hydrated molecule.

1.2.3. STABILITY

Human $\alpha_2$AP is rapidly inactivated below pH 5.7, but is stable between pH 6.0 and 9.0 (Wiman and Collen, 1977; Lijnen and Collen, 1986) with an optimum at about pH 7.0 (Moroi and Aoki, 1976). All activity is lost upon complete reduction under denaturing conditions, but the activity is retained when the disulfide bridges of the molecule are broken by partial reduction with dithioerythritol (Lijnen and Collen, 1986). Moroi and Aoki (1976) found that the presence of 1 mM $\beta$-mercaptoethanol tends to stabilise human $\alpha_2$AP. $\alpha_2$AP preparations seem to be stable during lyophilisation (Wiman and Collen, 1977; Aoki et al., 1993).
1.2.4. HETEROGENEITY

Müllertz and Clemmensen (1976) concluded that human $\alpha_2$AP is heterogeneous in normal plasma, consisting of functionally active and inactive material. They found that complete activation of the plasminogen present in normal human plasma converted only about 70% of the $\alpha_2$AP molecule into a complex with plasmin, while 30% of the inhibitor-related molecule appeared to be functionally inactive. Later work showed that human plasma contains a form of the inhibitor which binds to plasminogen (the plasminogen-binding form) and another, constituting about 40% of the total, which does not bind, but is nevertheless an active plasmin inhibitor (the non-plasminogen-binding form).

The non-plasminogen-binding form was shown to lack a 26-residue peptide from the C-terminus (containing the plasmin(ogen)-binding sites), and conversion of the plasminogen-binding to the non-plasminogen-binding form in vitro hence occurs by cleavage of an Arg-Gly peptide bond (Lijnen and Collen, 1986). Clemmensen et al. (1981) found that, in vitro, the plasminogen-binding form ($M_r$ 67 K) loses its plasminogen-binding properties upon storage while the $M_r$ decreases by 2 K ($M_r$ 65 K), and that storage of this form at 4 °C results in a further decrease in $M_r$ of 5 K and conversion into a form which is functionally inactive ($M_r$ 60 K).

1.2.5. ISOELECTRIC POINT(S)

The plasminogen-binding form of $\alpha_2$AP in normal human plasma shows multiple isoelectric points with a major component at pI 4.69 and a minor one at pI 4.92, whereas the non-plasminogen-binding form shows only one component at pI 4.59 (Lijnen and Collen, 1986).

1.2.6. IMMUNOCHEMICAL STUDIES

Four distinct sets of antigenic determinants within human $\alpha_2$AP have been delineated and partially localised. The plasminogen-binding and the active non-plasminogen-binding forms are immunochimically indistinguishable, whereas the inactive form is recognised immunologically (Lijnen and Collen, 1986). Moroi and Aoki (1976) showed by immunoelectrophoresis that $\alpha_2$AP migrates as an $\alpha_2$-globulin and by double immunodiffusion that $\alpha_2$AP does not react with antisera against other serpins. Clemmensen et al. (1981) showed by crossed-immunoelectrophoresis that all three molecular forms of $\alpha_2$AP react with specific antibodies raised against the inhibitor and that the migration rate increases with decreasing $M_r$. 
1.2.7. ABSORPTION COEFFICIENT

The absorption coefficient \( A^{1\%}_{1\text{cm}} \) of human \( \alpha_2 \text{AP} \) at 280 nm is 7.03 (Moroi and Aoki, 1976) or 6.70 (Wiman and Collen, 1977).

1.2.8. PLASMA LEVELS

The mean human plasma \( \alpha_2 \text{AP} \) concentration is 65.7 ± 10.6 mg/l according to the enzymatic method using the chromogenic substrate D-Val-Leu-Lys-pNA (S-2251) (Collen and Wiman, 1979a). Similarly, the concentration of the inhibitor in normal human plasma determined by electroimmunoassay is about 7 mg/100ml (~1 \( \mu \text{M} \)) (Wiman and Collen, 1977; Lijnen and Collen, 1986).

1.2.9. HALF-LIFE

The half-life of purified and radiolabeled biologically intact human \( \alpha_2 \text{AP} \) was found to be 2.6 ± 0.32 days, whereas the plasmin-\( \alpha_2 \text{AP} \) complex reveals a half-life of approximately 0.5 days (Collen and Wiman, 1979).
1.3. GENETIC PROPERTIES OF $\alpha_2$AP

The human $\alpha_2$AP gene (genetic symbol, PLI) contains 10 exons and 9 introns distributed over ~16 kb of DNA (Aoki, 1990) (figure 1.2).

**FIGURE 1.2: Organisation of the human $\alpha_2$AP gene.** The first line shows the positions of exons as rectangles, and the numbers above the line indicate the amino acids at which intron-exon junctions occur. Untranslated regions (UT) are shown as hatched areas. A small 5'-untranslated region exists in the second exon. The second line indicates the positions of restriction endonuclease recognition sites (B, Bam HI; D, Dra I; E, Eco RI; H, Hind III; X, Xba I). (Taken from Aoki, 1990)

The number of introns is the highest among those of the serpin gene family ever reported. All introns are located in the 5'-half of the corresponding mRNA; the 5'-untranslated region and the leader sequence are interrupted by 3 introns totalling ~6 kb. The leader sequence (39 amino acids) is a pre-pro peptide containing an N-terminal hydrophobic prepeptide (signal peptide) of 27 amino acids followed by a hydrophylic propeptide of 12 amino acids, and it is encoded by a part of exon II, exon III and a part of exon IV. (So far $\alpha_2$AP is the only pre-pro type processing protein reported among serpin family members.) The NH$_2$-terminal region, which contains the crosslinking site, is encoded by a part of exon IV, while the COOH-terminal region, which contains the reactive and plasmin(ogen) binding sites, is encoded by exon X (Aoki, 1990).

The complete human and bovine cDNA $\alpha_2$AP sequences are shown in figures 1.3 and 1.6, respectively.

A RFLP was found in the human $\alpha_2$AP gene. It can be attributed to the presence of two alleles, A and B, the minor allele, B, being due to a deletion of about 720 bp in intron 8 (Aoki, 1990). Kato et al. (1993) previously assigned the human $\alpha_2$AP gene to chromosome 18p11.1-q11.2 by isotopic in situ chromosomal hybridisation. However, they subsequently obtained results via Southern blot analysis of somatic cell hybrids and in situ fluorescence hybridisation studies that indicated that the gene is located at 17p13. PLI is therefore reassigned to 17p13 (Kato et al., 1993).
1.4. STRUCTURE OF $\alpha_2$AP

1.4.1. PRIMARY STRUCTURE

1.4.1.1. AMINO ACID COMPOSITION
The amino acid compositions of human and bovine $\alpha_2$AP are very similar as shown in table 1.1.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Human $\alpha_2$AP$^1$</th>
<th>Bovine $\alpha_2$AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>10.1</td>
<td>7.7</td>
</tr>
<tr>
<td>Thr$^2$</td>
<td>4.2</td>
<td>4.9</td>
</tr>
<tr>
<td>Ser$^2$</td>
<td>7.3</td>
<td>7.7</td>
</tr>
<tr>
<td>Glx</td>
<td>13.2</td>
<td>13.7</td>
</tr>
<tr>
<td>Pro</td>
<td>8.5</td>
<td>8.2</td>
</tr>
<tr>
<td>Cyh</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Gly</td>
<td>5.3</td>
<td>5.8</td>
</tr>
<tr>
<td>Ala</td>
<td>7.3</td>
<td>6.4</td>
</tr>
<tr>
<td>Val$^3$</td>
<td>4.6</td>
<td>6.2</td>
</tr>
<tr>
<td>Met</td>
<td>2.5</td>
<td>2.2</td>
</tr>
<tr>
<td>Ile$^3$</td>
<td>2.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Leu</td>
<td>15.0</td>
<td>14.8</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Phe</td>
<td>5.5</td>
<td>6.2</td>
</tr>
<tr>
<td>His</td>
<td>2.4</td>
<td>2.7</td>
</tr>
<tr>
<td>Lys</td>
<td>5.0</td>
<td>4.2</td>
</tr>
<tr>
<td>Trp</td>
<td>n.d.</td>
<td>1.3</td>
</tr>
<tr>
<td>Arg</td>
<td>4.3</td>
<td>4.2</td>
</tr>
</tbody>
</table>

$^1$ Taken from the cDNA sequence.
$^2$ Values extrapolated to 0 hr.
$^3$ Values determined after 72 hr of hydrolysis.

From table 1.1 it can be seen that $\alpha_2$AP has a very low tyrosine and tryptophan content, thus its low $\varepsilon$ value (Wiman and Collen, 1977), and leucine and glutamic acid/glutamine are present in the highest concentrations. It can also be noted that the content of acidic residues (assuming Glu and Asp) is much greater than the content of basic residues (His, Lys and Arg), thus explaining its low pI values.
1.4.1.2. AMINO ACID SEQUENCE
The complete primary structure of human $\alpha_2$AP has been deduced from the cDNA sequence (Holmes et al., 1987) (figure 1.3) and from amino acid sequence analysis of $\alpha_2$AP purified from plasma (Lijnen et al., 1987) (figure 1.4).

**FIGURE 1.3:** Nucleotide and deduced amino acid sequence of human $\alpha_2$AP. Nucleotides are numbered on the right; amino acids are numbered every 10th one. Potentially glycosylated asparagine residues are overlined. The reactive site bond is marked by an arrow. (Taken from Holmes et al., 1987)
FIGURE 1.4: Alignment of the amino acid sequence of human α2AP from the sequences of CNBr fragments (CB), tryptic (T) and chymotryptic (C) peptides. Solid lines indicate residues of a peptide that were sequenced; dotted lines indicate amino acids present in the amino acid composition of the peptide but not confirmed by sequencing. Xaa indicates unidentified amino acids; the arrow shows the reactive site peptide bond Arg^{364}-Met^{365}; Asn-linked carbohydrate is indicated with CHO. Pli indicates the peptide released upon interaction of α2AP with plasmin. (Taken from Lijnen et al., 1987)
Of the 452 amino acids of mature human $\alpha_2$AP deduced from the cDNA sequence, only 444 residues were identified by amino acid sequencing. However, only two differences in the amino acids determined by these two techniques could not be resolved: peptide analysis showed Gly instead of Leu at position 10, and Gly instead of Ser at position 369 (Lijnen et al., 1987).

$\alpha_2$AP is homologous with 12 other proteins belonging to the serpin superfamily, with the overall amino acid homologies ranging from 23-60% (Lijnen et al., 1987). However, unlike the other serpins whose COOH-terminal portions all end in nearly the same place, $\alpha_2$AP continues for another 51 amino acids (Lijnen et al., 1987) (figure 1.5).

Later, it was discovered that the liver secretes mature $\alpha_2$AP with 12 more N-terminal residues than hitherto anticipated, the first residue being Met at position 28, downstream from the N-terminus of pre-$\alpha_2$AP, and that the previously reported inhibitor with N-terminal Asn at position 40 ($\text{Asn}^{13}$-$\alpha_2$AP) is actually a proteolytically modified form (Bangert et al., 1993). The signal peptide was, therefore, proposed to consist of 27 amino acids (residues Met$^{-27}$-Ala$^{-1}$) and not 39 amino acids (residues Met$^{-27}$-Pro$^{12}$) as previously believed. The mature inhibitor (464 residues) and the proteolytically modified form (452 residues) are present in plasma in about equal amounts (Bangert et al., 1993). The rates of inhibition of plasmin by these two forms of $\alpha_2$AP are very similar, but mature $\alpha_2$AP is significantly less cross-linked to fibrin by activated coagulation factor XIII than is the $\text{Asn}^{13}$-$\alpha_2$AP (Bangert et al., 1993), thus possibly explaining the purpose of this modification.
FIGURE 1.5: Homology of $\alpha_2$AP with 12 other serpins. These serpins include $\alpha_1$-antitrypsin (A1AT), C1 inhibitor (C1 inh), antithrombin III (AT III), heparin cofactor II (hc II), $\alpha_1$-antichymotrypsin (A1AC), angiotensinogen (AGTH), contrapsin (CTPSN), ovalbumin (OVA), barley protein Z (BPZ), plasminogen-activator inhibitor (PAI), rabbit ORF-1, human protein C inhibitor (PCI), and $\alpha_2$-antiplasmin (A2AP). A dash indicates a gap in the sequence; the arrow indicates the known or predicted P1-P'1 peptide bond cleaved upon interaction with target proteases. The degree of homology is indicated above the aligned sequences as follows: (*) identity in all proteins, (+) identity in 80% or more proteins, (-) identity in 50% or more proteins. The positions of helices and $\beta$-sheet strands in the A1AT structure are indicated and the numbering is that of A1AT. (Taken from Lijnen et al., 1987)
The primary structure of bovine $\alpha_2$AP was also determined using cDNA and partial peptide sequencing (Christensen et al., 1994) (figure 1.6).

**FIGURE 1.6: Complete nucleotide sequence and translated protein sequence of bovine $\alpha_2$AP.** The initiation codon (ATG) for translation, stop codon (TGA) for translation and the polyadenylation signal (AATAAAA) are underlined. Protein numbering is in bold. Stretches of bovine $\alpha_2$AP determined from amino acid sequencing of protein and peptides are indicated by arrows. (Taken from Christensen et al., 1994)
Mature bovine $\alpha_2$AP contains 470 amino acid residues (i.e. 6 residues more than mature human $\alpha_2$AP), which is preceded by a signal peptide of 22 residues upon secretion. Bovine $\alpha_2$AP shows 81% homology with human $\alpha_2$AP (Christensen et al., 1994) (figure 1.7).

**FIGURE 1.7:** Alignment of the protein sequences of bovine (b) and human (h) $\alpha_2$APs.

Two dots (:) indicate identical and one dot (.) chemically similar residues. The reactive site P$_1$-P$_1'$ residues are shown with (★). The glycosylation sites are shown with (●). The paired Cys residues in bovine $\alpha_2$AP are connected by a line, and the unpaired Cys residue is marked with an (★). The 4 Cys residues of human $\alpha_2$AP are shown with (%).

(Taken from Christensen et al., 1994)

The primary structure of mouse $\alpha_2$AP is apparently also known (Christensen et al., 1997).
1.4.1.3. CARBOHYDRATE CONTENT AND ATTACHMENT SITES

α₂ AP is a glycoprotein containing 11-14% carbohydrate: 10 mole sialic acid, 30 mole hexose, and 7 mole glucosamine per mole of human α₂ AP (Wiman, 1981). In human α₂ AP, four potential Asn-linked glycosylation sites occur at Asn residues 99, 268, 282 and 289 (Lijnen et al., 1987). Bovine α₂ AP contains 3.1% (w/w) glucosamine and has five potential N-glycosylation sites at Asn residues 105, 227, 274, 288 and 295 (Christensen et al., 1994) (figure 1.7). Apart from Asn¹²², these sites correspond with those in human α₂ AP.

The role of carbohydrates in serpins is to aid in inhibitor secretion, recognition for complex uptake, and retention of a reasonable half-life in the circulation (Travis et al., 1990).

1.4.1.4. CYSTEINE CONTENT AND DISULFIDE BRIDGE ARRANGEMENT

Human α₂ AP contains 4 Cys residues (positions 43, 76, 116 and 125), of which three are conserved in bovine α₂ AP (positions 49, 122 and 131) (figure 1.6) and two in mouse α₂ AP (Christensen et al., 1997). Human α₂ AP has only one disulfide bridge, Cys⁴³-Cys¹¹⁶ (Christensen et al., 1997), which corresponds to the disulfide bridge found in bovine α₂ AP (Cys⁴⁹-Cys¹²²) (Christensen et al., 1994) (figure 1.7) and the two conserved Cys residues in the mouse protein. Cys¹²⁵ in human α₂ AP was found to exist both as a free thiol and as a mixed disulfide with another Cys residue. Likewise, the corresponding Cys¹³¹ residue in bovine α₂ AP exists as a partially free Cys residue (Christensen et al., 1997). Cys⁷⁶ is not conserved in bovine or mouse α₂ AP and was identified in human α₂ AP as a mixed disulfide with a cysteinyl-glycine dipeptide, an uncommon modification among plasma proteins (Christensen et al., 1997).

Although the disulfide bridge appears to be evolutionarily conserved, it does not seem to influence the thermodynamic stability or the inhibitory capacity of human α₂ AP, nor does it seem to affect the recognition by the mouse SR2 receptor which specifically recognises and eliminates human α₂ AP-proteinase complexes from the circulation. In other words, it does not appear to have a profound effect on the structure and function of this serpin. However, it may be hypothesised that the Cys residues and the disulfide bridge influence events such as in vivo binding to plasminogen or factor XIII-mediated cross-linking of α₂ AP to fibrinogen. Also, the disulfide bridge may affect the stability of α₂ AP so that the serpin retains inhibitory activity after advantageous proteolysis of the scaffold (Christensen et al., 1997).
1.4.2. THREE-DIMENSIONAL STRUCTURE

Circular dichroism studies of human α₂ AP in the far ultraviolet region (200-250 nm) indicated a content of 16% α-helix, 18% β-structure and 66% random coil (Nilsson et al., 1982). However, the spatial structure of native, functional serpins, including α₂ AP, have not yet been determined, but three-dimensional analysis of several serpins that are either cleaved at the RSL, non-functional, in a latent form, or in an inactive, dimeric form has been described. All have the same highly ordered tertiary structure, from which a model of the canonical, active form of inhibitory serpins can be derived. The predominant feature of the model is an antiparallel six-stranded β-sheet (β-sheet A), with the central strand (strand s4A) partially reincorporated in β-sheet A, where strand s4A contains several residues upstream from the cleavage site, those downstream being located on the surface of the protein within a second β-sheet (Potempa et al., 1994) (figure 1.8). Wright and Scarsdale (1995), however, suggested that the reactive site of intact serpins adopts a helical conformation to protect it from adventitious cleavage (see figure 1.15, section 1.5.1.3.).

**FIGURE 1.8:** Proposed spatial structure of native, functional serpins. s1-s6 represent strands 1-6 and the arrow represents the insertion of strand s4A into β-sheet A. (Taken from Carrell et al., 1991).
1.4.3. FUNCTIONAL SITES

The $\alpha_2$AP molecule has three functional sites: (i) reactive site, (ii) plasmin(ogen)-binding site, and (iii) cross-linking site (Aoki et al., 1993).

(i) The reactive site loop of human $\alpha_2$AP encompasses a $P_1$-$P'_1$ $\text{Arg}^{376}$-$\text{Met}^{377}$ sequence (Shieh and Travis, 1987), with the reactive site, i.e. the peptide bond cleaved by reaction with its primary target enzyme, plasmin, being $\text{Arg}^{376}$-$\text{Met}^{377}$ (Holmes et al., 1987). This is also the cleavage site for trypsin, but a separate, overlapping, inhibitory site exists for chymotrypsin, $\text{Met}^{377}$-$\text{Ser}^{378}$ (Enghild et al., 1993) (figure 1.9).

![Diagram of the cleavage sites in human $\alpha_2$AP caused by trypsin and chymotrypsin. (Taken from Enghild et al., 1993)](image)

Chymotrypsin was found to have another cleavage site in the RSL of human $\alpha_2$AP, $\text{Met}^{374}$-$\text{Ser}^{375}$ (figure 1.9). However, cleavage at this site results in the inactivation of human $\alpha_2$AP. This is more apparent at 37 °C than at 0 °C, implying that human $\alpha_2$AP is a more efficient inhibitor of chymotrypsin at 0 °C than at 37 °C (Enghild et al., 1993).

Residues $P_{15}$-$P'_{15}$ of the RSL of bovine $\alpha_2$AP shows 87% homology with the corresponding site in human $\alpha_2$AP, indicating the requirement of a strictly specified structure for interaction with the active site of plasmin (Christensen et al., 1994). The reactive site of bovine $\alpha_2$AP is identical to that of human $\alpha_2$AP: $\text{Arg}^{382}$-$\text{Met}^{383}$. Subtilisin was shown to cleave at the $P'_1$ Met residue and an elastase-like enzyme, which inactivates bovine $\alpha_2$AP, cleaves 3 residues upstream of the reactive site (Christensen and Sottrup-Jensen, 1992) (figure 1.10).
FIGURE 1.10: Reactive site sequence of bovine $\alpha_2$AP. Bovine and human $\alpha_2$AP are aligned which introduces a gap between P$_1$ and P$_2$ in $\alpha_2$AP. Identical residues in bovine and human $\alpha_2$AP are shown by a pair of dots; residues different in bovine $\alpha_2$AP are shown in bold. The P and P' sites are numbered. C-$\alpha_2$AP$_1$, C-$\alpha_2$AP$_2$ and C-$\alpha_2$AP$_3$ represent C-terminal peptides from $\alpha_2$AP cleaved with an elastase-like enzyme, plasmin and subtilisin, respectively. (Taken from Christensen and Sottrup-Jensen, 1992)

(ii) The extra 51 carboxyl-terminal amino acid sequence that is specific to $\alpha_2$AP compared to other serpins contains the plasmin(ogen)-binding site (Sumi et al., 1986; Sasaki et al., 1986). In human $\alpha_2$AP, the residues appearing to be involved are Lys$_{448}$ and C-terminal Lys$_{464}$ (Sugiyama et al., 1988).

The C-terminal extension of bovine $\alpha_2$AP (starting at residue 414) have only 69% residues in common with human $\alpha_2$AP. However, Lys$_{448}$ and Lys$_{464}$ of human $\alpha_2$AP are conserved in bovine $\alpha_2$AP, as well as four other Lys residues of unknown function (Christensen et al., 1994).

(iii) The cross-linking site in human $\alpha_2$AP is located at Gln$^{14}$ (Tamaki and Aoki, 1982), the second residue from the N-terminus in the proteolytically modified form of the inhibitor. This site in bovine $\alpha_2$AP is possibly located at Gln$^{39}$, the presumed counterpart of Gln$^{14}$ of human $\alpha_2$AP (Christensen et al., 1994).
1.5. FUNCTIONS OF $\alpha_2$AP

1.5.1. REACTION WITH PLASMIN

1.5.1.1. BACKGROUND INFORMATION ON PLASMIN(OGEN)

Plasmin, a plasma serine protease with trypsin-like specificity in that it catalyses the hydrolysis of $\alpha$-amino substituted lysine and arginine esters (Robbins and Summaria, 1970), is formed from its precursor plasminogen by a variety of activators, either urokinase-like from urine and malignant or transformed cells, or nonurokinase-like from plasma, tissues, and bacteria (Robbins et al., 1981). Plasmin functions in fibrinolysis and fibrinogenolysis, but has also been shown to be active in other systems: it is capable of digesting factor XII into factor XII fragments, activating several complement zymogens (e.g. C1, C3, and C5), and inactivating C1 inhibitor. It has been shown to substitute for factor D in the alternate pathway and it has also been implicated in digesting proaccelerin, antihemophilic factor, ACTH, glucagon, and $\gamma$-globulin (Castellino and Powell, 1981).

Native human plasminogen is a single-chain protein consisting of 791 amino acids with a molecular mass of 90-94 kDa and multiple isoelectric points (Castellino and Powell, 1981). Figure 1.11 shows the primary structure of human plasminogen.

Plasminogen contains an N-terminal Glu$^1$-Lys$^{77}$ peptide, five homologous kringle domains and a C-terminal serine protease domain:

- Up to 10% of native Glu$^1$-plasminogen is converted to Lys$^{78}$-plasminogen by plasmin which cleaves at the Lys$^{77}$-Lys$^{78}$ peptide bond (Wiman and Wallén, 1973).
- Of the five kringles, kringles 1 and 4 each contain a lysine-binding site and kringle 5 contains an aminohexyl-binding site. Kringles 1 and 5 have been shown to be involved in fibrin binding via their LBSs, whereas this may not be the case for kringle 4 although it contains a LBS. Kringles 2 and 3 have not been assigned specific functions, but may be structurally important for spatial separation of binding sites. The interdomain disulfide bridge connecting kringles 2 and 3, Cys$^{169}$-Cys$^{297}$, may also serve to stabilise the protein in a conformation important for the presentation of its fibrin-binding sites (Linde et al., 1998).
- Plasminogen is activated to plasmin by cleavage of a peptide bond at Arg$^{561}$-Val$^{562}$, resulting in a heavy chain (residues 78-561, containing the kringle domains) and a light chain (residues 562-791, containing the catalytic serine protease domain) linked by two interdomain disulfide bridges (Cys$^{548}$-Cys$^{670}$ and Cys$^{558}$-Cys$^{566}$) (Linde et al., 1998).
FIGURE 1.11: Amino acid sequence for human plasminogen. The positions of the 18 introns (A-R) are indicated by solid arrows at or between specific amino acids. The amino acid residues are numbered starting with the N-terminal Glu residue as number 1 and ending with residue 791. The signal peptide (shown in a box with negative numbers) contains 19 amino acids and is cleaved by signal peptidase at the Gly-Glu peptide bond. The preactivation peptide (PAP) is generated primarily by the cleavage between Lys$^{77}$ and Lys$^{78}$ (shown with an open straight arrow) by plasmin. The conversion of plasminogen to plasmin occurs by the cleavage between Arg$^{561}$ and Val$^{562}$ (shown by an open curved arrow). K1-K5 refer to kringle 1-5 in the A chain, while the active site His, Asp, and Ser residues in the B chain are circled. Carbohydrate attachment sites (Asn$^{289}$, Thr$^{346}$) are shown by diamonds. (Taken from Petersen et al., 1990)

The mechanism of activation of plasminogen to plasmin by the activator, urokinase, can be summarised as in figure 1.12.

FIGURE 1.12: Mechanism of activation of human Glu-plasminogen (Pg) to 2-chain disulfide-linked Lys$^{78}$-plasmin (Pm) by urokinase (UK). (1), UK catalyses cleavage of the Arg$^{561}$-Val$^{562}$ peptide bond, from Glu-Pg, to form Glu-Pm. (2), Autocatalytic cleavage of the Lys$^{77}$-Lys$^{78}$ peptide bond, by Pm, results in formation of Lys$^{78}$-Pm. (3), Pm can catalyse the cleavage of the same Lys$^{77}$-Lys$^{78}$ peptide bond, from Glu-Pg, to form Lys$^{78}$-Pg. (4), Lys$^{78}$-Pg is readily activated to Lys$^{78}$-Pm, by UK. (Taken from Castellino and Powell, 1981).
1.5.1.2. KINETICS OF REACTION

Kinetic analyses revealed a two-step binding mechanism for the reaction of $\alpha_2$AP with plasmin:

(i) the rapid formation of an initial tight 1:1 complex, and (ii) a tightening of this complex in a slow reaction step (Christensen and Clemmensen, 1977; Wiman and Collen, 1978; Longstaff and Gaffney, 1991; Christensen et al., 1995) (figure 1.13).

$$
\begin{align*}
E + I & \rightleftharpoons EI^* \rightleftharpoons EI \\
& \\
\end{align*}
$$

$\textbf{FIGURE 1.13: Reaction model for the inhibition of plasmin by }\alpha_2\text{AP.}$

E, enzyme (plasmin); I, inhibitor ($\alpha_2$AP); EI*, first tight enzyme-inhibitor complex; EI, final, even tighter complex. (Taken from Christensen et al., 1996)

Both binding steps are reversible, but binding during the second reaction step is so tight that it can be considered to be pseudoirreversible (Longstaff and Gaffney, 1991; Christensen et al., 1995). The first step follows second-order kinetics, while the second step is a first-order reaction (Christensen and Clemmensen, 1977; Wiman and Collen, 1978; Christensen et al., 1996).

1.5.1.2.1. Reaction with intact plasmin

The rate constant for the first step ($k_1$) for the reaction between human $\alpha_2$AP and human plasmin, obtained by stopped-flow fluorescence kinetics, is $2.2 \times 10^7$ M$^{-1}$s$^{-1}$ at pH 7.4, 25 °C (Christensen et al., 1996), which is close to the values obtained by conventional kinetic methods: $(3.8 \pm 0.3) \times 10^7$ M$^{-1}$s$^{-1}$ for the reaction between human plasmin I and human $\alpha_2$AP and $(1.8 \pm 0.2) \times 10^7$ M$^{-1}$s$^{-1}$ for human plasmin II and human $\alpha_2$AP, where plasmin I and II are two forms of plasmin with different affinities for lysine-Sepharose (Wiman and Collen, 1978). The stopped-flow method is, however, more reliable in measuring the first rate constant since it allows for direct determination of values obtained after just 1 ms, in the absence of another interacting compound, e.g. a plasmin substrate (Christensen et al., 1996). The dissociation constant for this first association complex ($K_1$) was determined to be $(1 \pm 0.3) \times 10^{-10}$ M (Christensen et al., 1996) or $(1.9 \pm 0.3) \times 10^{-10}$ M for human plasmin I with human $\alpha_2$AP and $(1.8 \pm 0.3) \times 10^{-10}$ M for human plasmin II with human $\alpha_2$AP (Wiman and Collen, 1978).

$k_1$ for the reaction between bovine $\alpha_2$AP and bovine plasmin is $0.9 \times 10^6$ M$^{-1}$s$^{-1}$ at pH 7.4, 25 °C, according to stopped-flow fluorescence kinetics. $K_1$ was calculated to be $(5 \pm 0.5) \times 10^{-10}$ M, giving a $k_{-1}$ value of $5 \times 10^4$ s$^{-1}$. 
However, the bovine plasmin used contained all of the activation products from plasminogen, but no full-length plasmin, because kringle 1-3 of bovine plasminogen is cleaved off, probably autocatalytically by plasmin, during the urokinase-catalysed activation of bovine plasminogen (Christensen et al., 1995).

The rate constant for the second step (k_2) for the reaction between human α₂AP and human plasmin was determined to be 4.2 x 10^{-3} s^{-1} (from a half-time of about 165 s) for both plasmin forms (Wiman and Collen, 1978), 6 x 10^{-3} s^{-1} (in the presence of 15-50 mM 6-AHA, but which, as will be seen later, has no effect on the second reaction step) (Longstaff and Gaffney, 1991), and 2 x 10^{-3} s^{-1} (accuracy ~5%) (Christensen et al., 1996). Longstaff and Gaffney (1991) calculated k_2 to be (1.7 ± 0.3) x 10^{-6} s^{-1} (in the presence of 15-50 mM 6-AHA), which corresponds to a half-life of 113 hr (4.7 days) for the complex. This is longer than the half-time of clearance from human plasma, which is 0.5 day (Collen and Wiman, 1979).

For the reaction between bovine α₂AP and bovine plasmin, k_2 was determined to be 9 x 10^{-3} s^{-1} (accuracy ~5%), and the half-time of the slow step, 78 s (Christensen et al., 1995).

The overall inhibition constant for the binding of human plasmin and α₂AP, K_{I, AP}, is 0.3 pM (Longstaff and Gaffney, 1991) and an estimate of an upper limit of the overall inhibition constant for the reaction of bovine α₂AP with bovine plasmin is 5 pM (Christensen et al., 1995).

1.5.1.2.2. Reaction with proteolytically modified forms of plasmin

The k_1 of the reaction of human α₂AP with human low-M_r, plasmin, obtained by limited elastase digestion of plasmin and composed of an intact B chain and a small A chain lacking the LBSs, is (6.5 ± 0.5) x 10^{5} M^{-1}s^{-1}, ~30-60 times smaller than that for normal human plasmin and human α₂AP (Wiman et al., 1978). K_1 is 1.9 x 10^{-9} M (Wiman et al., 1978), which is 10 times higher than that obtained by Wiman and Collen (1978) for normal human plasmin and human α₂AP. k_2 is (4.2 ± 0.2) x 10^{-3} s^{-1} (Wiman et al., 1978), which is the same for normal human plasmin and human α₂AP as determined by Wiman and Collen (1978).

Human miniplasmin, obtained by the removal of kringle 1-4 from plasminogen with elastase (Sottrup-Jensen et al., 1978) before conversion with plasminogen activator, shows catalytic properties very similar to that of intact human plasmin (Christensen et al., 1979), but its rate of association, 9 x 10^{5} M^{-1}s^{-1} (Christensen et al., 1979), is 20-42 times slower than that of intact human plasmin.
The reaction of bovine $\alpha_2$AP with bovine midiplasmin (plasmin lacking kringle 1-3) shows essentially the same kinetic behaviour as the reaction with bovine plasmin, the only difference being the fast association rate, $k_1 = (1.7 \pm 0.2) \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, which is ~2-fold greater than the value obtained for the reaction with bovine plasmin (Christensen et al., 1996).

The above results show the importance of the LBSs on the rate of the fast association step and its lack of involvement in the second step. These results also support the recent finding that the LBS of kringle 1 is of little importance compared with that of kringle 4 in regulating the inhibition reaction of plasmin with $\alpha_2$AP (see section 1.5.1.2.3.) (Christensen et al., 1995; Christensen et al., 1996).

### 1.5.1.2.3. Summary of kinetic constants

All the kinetic constants obtained for the binding of $\alpha_2$AP to plasmin and its proteolytically modified forms, as well as to a few other proteases mentioned in section 1.5.2., are summarised in table 1.2.

**TABLE 1.2: Kinetic constants for the reaction of $\alpha_2$AP with plasmin, proteolytically modified forms of plasmin and other proteases.** (Unless otherwise stated, the enzyme refers to the human enzyme.)

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>$k_1$ (M$^{-1}$s$^{-1}$)</th>
<th>$K_1$ (M)</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$K_{I, AP}$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmin$^a$</td>
<td>$2.2 \times 10^7$</td>
<td>$1 \times 10^{-10}$</td>
<td>$2 \times 10^{-3}$</td>
<td>$1.7 \times 10^{-6}$</td>
<td>$3 \times 10^{-13}$</td>
</tr>
<tr>
<td>Bovine Plasmin$^b$</td>
<td>$9 \times 10^5$</td>
<td>$5 \times 10^{-10}$</td>
<td>$9 \times 10^{-3}$</td>
<td>$\leq 4.5 \times 10^{-6}$</td>
<td>$\leq 5 \times 10^{-12}$</td>
</tr>
<tr>
<td>Low-Mr Plasmin$^c$</td>
<td>$6.5 \times 10^5$</td>
<td>$1.9 \times 10^{-9}$</td>
<td>$4.2 \times 10^{-3}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miniplasmin$^d$</td>
<td>$9 \times 10^5$</td>
<td>$1.21 \times 10^{-8}$</td>
<td>$6.25 \times 10^{-3}$</td>
<td>$1.21 \times 10^{-8}$</td>
<td></td>
</tr>
<tr>
<td>Bovine Midiplasmin$^b$</td>
<td>$1.7 \times 10^9$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin$^e$</td>
<td>$1.8 \times 10^6$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chymotrypsin$^f$</td>
<td>$6.6 \times 10^9$</td>
<td>$9 \times 10^{-3}$</td>
<td>$1.1 \times 10^{-4}$</td>
<td>$1 \times 10^{-10}$</td>
<td></td>
</tr>
<tr>
<td>Urokinase$^f$</td>
<td></td>
<td></td>
<td></td>
<td>$4.2 \times 10^{-8}$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Christensen et al., 1996  
$^b$ Christensen et al., 1995  
$^c$ Wiman et al., 1978  
$^d$ Christensen et al., 1979  
$^e$ Wiman and Collen, 1978  
$^f$ Longstaff and Gaffney, 1991.
1.5.1.2.4. Effects of plasmin substrate and lysine analogues

The substrate D-Val-Leu-Lys-pNA bound to plasmin, as well as to low-Mₐ plasmin, before reaction with \( \alpha_2 \)AP was found to decrease the value of \( k_1 \) to about 10% of the value obtained without substrate at a concentration of 1.8 mM. It was thus concluded that plasmin and low-Mₐ plasmin with substrate bound to their active sites do not react or react only very slowly with \( \alpha_2 \)AP, thus proving the significance of the active site for the inhibition reaction (Wiman and Collen, 1978; Wiman et al., 1978).

Christensen and Clemmensen (1977, 1978) found that L-lysine influences the first reaction step, but not the second, and that the inhibitory effect of L-lysine and 6-AHA, a lysine analog, on the inhibitor-plasmin reaction occurred at concentrations much too low to affect the active site of plasmin. Wiman et al. (1978) showed that 6-AHA in concentrations up to 1 mM decreases the reaction rate of normal plasmin ~50-fold, but only slightly influences the reaction rate of low-Mₐ plasmin with \( \alpha_2 \)AP. These results already showed the importance of the LBSs of plasmin for the rate of its reaction with \( \alpha_2 \)AP.

In 1991 Longstaff and Gaffney found that plasmin binding kinetics are changed in the presence of lysine analogues such that equilibrium is reached slowly following a lag phase after mixing of enzyme and inhibitor, and therefore argued that lysine analogues do not act by blocking a second binding site for the inhibitor on the enzyme, but rather cause a conformational change in the enzyme. However, Christensen et al. (1995, 1996) claimed that, according to their experiments, the active-site inhibition cannot at all account for the effect of 6-AHA or \( t \)-AMCHA on \( k_1 \), which must therefore be explained in terms of binding of 6-AHA or \( t \)-AMCHA to a secondary site (LBSs).

For the reaction of bovine \( \alpha_2 \)AP with bovine plasmin and midiplasmin, Christensen et al. (1995) found that 10 mM 6-AHA causes an 11-fold and a 17-fold reduction in \( k_1 \), respectively, while the association rate for the reaction of human \( \alpha_2 \)AP with human plasmin showed up to a 20-fold reduction in the presence of 1 mM \( t \)-AMCHA (Christensen et al., 1996). The dissociation constant, \( K_{\text{LBS}} \), for the interaction between the LBS and 6-AHA was therefore estimated to 0.2-0.6 mM (Christensen et al., 1995), while the \( K_{\text{LBS}} \) for the interaction of the LBS with \( t \)-AMCHA was estimated to 34 \( \mu \)M (Christensen et al., 1996).
From the results of the effect of 6-AHA on the reaction of bovine $\alpha_2$AP with bovine midiplasmin and plasmin, it was concluded that, since kringle 5 alone, as in miniplasmin, reveals no kinetic effects on the inhibition by $\alpha_2$AP (Christensen et al., 1979), and because the active site is inhibited only at high concentrations of 6-AHA ($K_i = 42$ mM), kringle 4 is of importance in the regulation of the inhibition of midiplasmin by $\alpha_2$AP (Christensen et al., 1995). Furthermore, the effect of $t$-AMCHA on the reaction of human $\alpha_2$AP with human plasmin proved that, since the rate reduction is only 2-fold in the range 5-25 µM, when the LBS of kringle 1 is saturated, whereas saturating the LBS of kringle 4 with $t$-AMCHA resulted in a further 10-fold reduction, and since the active site is inhibited only at very high concentrations of $t$-AMCHA ($K_i = 16$ mM), kringle 4 is indeed of importance in the regulation of the inhibition of plasmin by $\alpha_2$AP (Christensen et al., 1996). All in all, these data proved that the LBS of kringle 1 is of little importance compared with that of kringle 4 in regulating the inhibition reaction of plasmin with $\alpha_2$AP, as opposed to what was previously believed (Christensen et al., 1995, 1996).

1.5.1.3. MECHANISM OF ACTION
The first tight complex between plasmin and $\alpha_2$AP is formed via the reaction of the LBSs in plasmin A-chain with the corresponding plasmin(ogen)-binding sites in the $\alpha_2$AP molecule, followed by an additional interaction of the plasmin active site with the $\alpha_2$AP reactive site (Wiman and Collen, 1979a), the purpose of binding to the LBSs being to optimally position the susceptible areas of the inhibitor at the active site of plasmin (Müllertz, 1979). However, a small percentage of the molecules might first form a complex via the active site of plasmin (figure 1.14) (Wiman and Collen, 1979a).

**FIGURE 1.14:** The normal pathway for the reaction between plasmin and $\alpha_2$AP. P, plasmin; A, $\alpha_2$AP; PA_{LBS}, plasmin-$\alpha_2$AP complex formed via LBSs; PA_{AS}, plasmin-$\alpha_2$AP complex formed via active site; PA, reversible, inactive plasmin-$\alpha_2$AP complex; PA', pseudoirreversible, much more stable, inactive plasmin-$\alpha_2$AP complex. (Taken from Wiman and Collen, 1979a)
The results of Moroi and Aoki (1977a) suggested that a covalent bond is formed between the inhibitor and plasmin and it is an ester, the proposed mechanism being that at the complex formation an acyl bond is formed between the active serine of plasmin (Ser\textsuperscript{741}) and the carbonyl of the reactive site residue of the inhibitor (Arg\textsuperscript{376}). Wiman and Collen (1979b) were unsure whether this bond is of tetrahedral intermediate type or an ester bond, while it was later suggested that an ester bond is more likely (Nilsson and Wiman, 1982), and still later that the reversibility of binding actually argues against a covalent bond (Longstaff and Gaffney, 1991). Matheson et al. (1991) however found evidence for a tetrahedral intermediate complex during serpin-proteinase interactions.

The mechanism of formation and structure of the $\alpha_2$AP-plasmin complex, or of the serpin-inhibitor complex in general is still, unfortunately, not completely understood, and only a proposal given by Wright and Scarsdale (1995) for serpins in general, which is in accordance with that described by Potempa et al. (1994), is currently available (figure 1.15).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.15.png}
\caption{Proposed suicide inhibitor mechanism for serpins.}
\end{figure}

\textit{FIGURE 1.15: Proposed suicide inhibitor mechanism for serpins.}
EI\textsubscript{M}, Michaelis complex; EI\textsubscript{TI}, tetrahedral intermediate; EI', acyl enzyme; EI*, stable complex of proteinase with fully inserted s4A.
(Taken from Wright and Scarsdale, 1995)
As figure 1.15 shows, serpins undergo several steps before reaching a final, highly stable, covalent serpin-proteinase complex. The reaction is initiated by the target proteinase that recognises the reactive site bond as part of normal substrate attack. However, the interaction of the enzyme with this peptide bond induces the RSL to shift from the $\alpha$-helix to an extended $\beta$-sheet, causing the initiation of $s4A$ insertion into $\beta$-sheet A at $P_{15}$. This shift would alter the packing ratio of the RSL, releasing over 15 Å of potential $\beta$-sheet, corresponding to 4 residues. This released polypeptide chain will be available for insertion at the hinge region, and permits insertion of $P_{15}$ to $P_{12}$ of $s4A$ into $\beta$-sheet A, without requiring any major movement of the proteinase covalently linked and protected from solvent hydrolysis at the $P_1$ end of the RSL. The release of this segment of $s4A$ disrupts a number of hydrogen-bond constraints on the chain causing relaxation which, with the formation of a stable acyl linkage between serpin and proteinase, and the directionality on $s4A$ movement imposed by the Glu$^{342}$-Lys$^{290}$ ion pair, permit reptation of the proteinase to the opposite end of the molecule, probably driven by cooperativity in the insertion of the remaining residues ($P_3$-$P_{11}$) of $s4A$ into $\beta$-sheet A. This carries the proteinase to a new position at the opposite pole of the serpin molecule, where it is again protected from solvent attack on the acyl linkage to the proteinase (figure 1.16) (Wright and Scarsdale, 1995).

**FIGURE 1.16:** Proposed mechanism for serpin inhibition of proteinases showing the structures of the serpin at each step of the reaction. This mechanism is the same as the one in figure 1.14, except that the tetrahedral intermediate (EI$_{10}$), assumed to have the same structure as EI$_{M}$, has been omitted. Proteinase is shown as coil, serpin as ribbon and coil. (Taken from Wright and Scarsdale, 1995)
1.5.2. REACTION WITH OTHER PROTEINASES

1.5.2.1. SPECIFICITY OF $\alpha_2$AP
$\alpha_2$AP only binds plasmin and, to a lesser extent, trypsin in normal plasma, but its physiological role as an inhibitor of proteinases other than plasmin is negligible (Lijnen and Collen, 1986). However, it has a broad inhibitory spectrum in vitro. Besides reacting very rapidly with plasmin, it reacts rapidly with trypsin (Wiman and Collen, 1978), moderately with chymotrypsin (Wiman, 1981), slowly with kallikrein, factor Xa and thrombin (Wiman, 1981; Lijnen and Collen, 1986), and very slowly with urokinase (Moroi and Aoki, 1976) and tissue-plasminogen activator (Wiman, 1981).

1.5.2.2. REACTION WITH TRYPsin AND CHYMOTrypsIN

The first order reaction rate for the reaction of human $\alpha_2$AP with trypsin is $(1.8 \pm 0.2) \times 10^6$ M$^{-1}$s$^{-1}$ (Wiman and Collen, 1978), which is at least 10-fold smaller than $k_1$ for the reaction with plasmin (Wiman and Collen, 1978; Christensen et al., 1996) (table 1.2). Moroi and Aoki (1977a) proposed that binding of $\alpha_2$AP to trypsin was due to a covalent, carboxylic ester linkage at the active site serine of trypsin as well as to a sum of weak forces in a complementary fit between the inhibitor and the enzyme.

The reaction of $\alpha_2$AP with chymotrypsin was shown to follow the same reaction mechanism as the one for the reaction with plasmin (figure 1.13) The kinetics of the reaction of human $\alpha_2$AP with chymotrypsin are: $K_1 = 6.6$ nM, $k_2 = 9 \times 10^{-3}$ s$^{-1}$ and $k_{-2} = 1.1 \times 10^{-4}$ s$^{-1}$, giving $K_{I,AP} = 0.1$ nM (Longstaff and Gaffney, 1991) (table 1.2). Comparing these values with the kinetics of the reaction with human plasmin (section 1.5.1.2.3.; table 1.2), it can be seen that all the kinetic constants are similar, except for $k_{-2}$. $k_{-2}$ is 65-fold greater for the reaction with chymotrypsin and it is therefore this kinetic constant that determines the much weaker binding of $\alpha_2$AP with chymotrypsin than with plasmin (the overall rate constant, $K_{f,AP}$, for the reaction with chymotrypsin is 333-fold greater than that for the reaction with plasmin). As mentioned in section 1.4.3.(i), chymotrypsin binds to $\alpha_2$AP at a different P1 residue than for plasmin and, therefore, as Longstaff and Gaffney (1991) pointed out, it would be surprising if a different mechanism was involved for binding of the two enzymes.
As mentioned in section 1.4.3.(i), chymotrypsin has another cleavage site in $\alpha_2$AP, cleavage of which results in the inactivation of $\alpha_2$AP and which is more apparent at 37 °C than at 0 °C. Therefore, since the locations of the chymotrypsin-interaction sites vary with temperature and since $\alpha_2$AP have overlapping inhibitory sites for trypsin and chymotrypsin (section 1.4.3.(i)), it was suggested that $\alpha_2$AP have a mobile RSL: some conformations of the loop favour trypsin binding while others favour chymotrypsin binding; some facilitate an inhibitory function while others allow proteolysis leading to inactivation, depending on the environmental conditions. When a proteinase binds to an inhibitory conformation, the RSL most probably becomes locked in the canonical conformation (Enghild et al., 1993).

A mobile reactive centre of serpins was previously described (Carrell et al., 1991), and the proposal for the mechanism of serpin action presented above in section 1.5.1.3. was based on this characteristic.

The dissociation of $\alpha_2$AP-chymotrypsin or $\alpha_2$AP-trypsin complexes usually does not yield functionally active inhibitors, but in the presence of $\alpha_2$-macroglobulin the released inhibitor is active and capable of inactivating trypsin and chymotrypsin, respectively (Shieh et al., 1989).

1.5.3. INTERACTION WITH PLASMINOGEN

About 15% of $\alpha_2$AP occurs reversibly complexed with plasminogen (Lijnen et al., 1980), the proenzyme of plasmin, the interaction being mainly due to binding of the plasmin(ogen)-binding sites in $\alpha_2$AP (Lys$^{448}$ and C-terminal Lys$^{464}$) (Wiman et al., 1979) with the high-affinity LBSs in the plasminogen molecule (kringles 1 and 4) (Linde et al., 1998). This binding is, however, weaker than the binding of $\alpha_2$AP with plasmin since it does not involve binding of the active and reactive sites. Intact Glu-plasminogen was found to bind less strongly to $\alpha_2$AP ($K_D = 4.0 \times 10^6$ M) than Lys-plasminogen ($K_D = 6.3 \times 10^7$ M), the partially degraded form of plasminogen lacking the preactivation peptide region (residues 1-78). This was attributed to a non-covalent interaction between the preactivation peptide of Glu-plasminogen and kringle 1 (Wiman and Collen, 1979c). This phenomenon further proves the importance of the LBSs in the binding of $\alpha_2$AP to plasminogen.
1.5.4. CROSS-LINKING TO FIBRIN(OGEN)

1.5.4.1. α₂AP AND FIBRINOLYSIS

α₂AP is an efficient inhibitor of fibrinolysis, the plasmin-catalysed dissolution of thrombi (Moroi and Aoki, 1976). It exerts its inhibitory effect on fibrinolysis in three ways: (i) rapid inactivation of plasmin, (ii) interference with the adsorption of plasminogen to fibrin, and (iii) cross-linking to fibrin(ogen) (Sakata and Aoki, 1980, 1982; Tamaki and Aoki, 1981).

The third function is most important in inhibition of endogenous fibrinolysis that occurs subsequent to fibrin formation and is caused by fibrin-associated plasminogen activation (Sakata and Aoki, 1982). Also, the cross-linking of α₂AP to fibrin(ogen) not only stabilises the fibrin clot, but also inhibits binding of plasminogen to fibrin, and therefore plays a vital role in the inhibition of fibrinolysis.

1.5.4.2. CROSS-LINKING SITE IN FIBRIN(OGEN)

α₂AP is cross-linked exclusively with the fibrin α-chain or with the fibrinogen Aα chain by activated coagulation Factor XIII, a plasma transglutaminase, in a reaction which rapidly proceeds to a maximum and then plateaus (Tamaki and Aoki, 1981; Ichinose and Aoki, 1982) with only about 25% of the α₂AP being cross-linked to fibrin in plasma clots (van Giezen et al., 1993). It was found that less than 1% of circulating fibrinogen is complexed to α₂AP (Greenberg et al., 1990).

The site in the fibrinogen Aα chain that was found to form a cross-link with the corresponding site in α₂AP, Gln^{14}, was Lys^{303} (Kimura and Aoki, 1986). The latter authors were unable to study the cross-linking of α₂AP to fibrin because of the tight structure of fibrin caused by cross-linked polymerisation of fibrin monomers. However, it appears that Lys^{303} is also involved in the cross-linking to fibrin (Kimura and Aoki, 1986).
Lys\textsuperscript{303} is located in the single-chain region that emerges from the terminal domain (residues 161-206) and extends the carboxyl two-thirds of the A\textalpha chain (Doolittle \textit{et al.}, 1979). The C-terminal portion of the two A\textalpha chains folds back from the terminal domain to interact at the centre of the molecule and to form a central \alpha domain (Weisel \textit{et al.}, 1985). The single-chain region between the terminal domain and the central \alpha domain is rich in polar residues and lacks helical structure, except for the region close to the carboxyl terminus (central a domain) (Doolittle \textit{et al.}, 1979). The middle region (zone ZM, composed of residues 240-42), in particular, is rich in polar residues and contains a series (9-10 times) of a 13-residue repeat, of which the fourth repeat is the location of Lys\textsuperscript{303}. Lysine residues are scarce in zone ZM and Lys\textsuperscript{303} is the only lysine residue found in the region of residues 230-413 (Kimura and Aoki, 1986). The predicted peptide conformations in the vicinity of Lys\textsuperscript{303} is predominantly \(\beta\)-turns with some \(\beta\)-structures, the presence of helical structure being most unlikely (Chou and Fasman, 1978).

1.5.4.3. MECHANISM OF ACTION

The reaction involved is an acyl transfer reaction, catalysed by activated Factor XIII, in which the \(\gamma\)-carboxamide group of the glutamine residue of \(\alpha_2\)AP is the acyl donor (amine acceptor) and the \(\varepsilon\)-amino group of the lysine residue in fibrin(ogen) serves as the acyl acceptor (amine donor). The reaction proceeds through the formation of acyl-enzyme intermediates and subsequent transfer of acyl groups to the acceptor amine (Folk and Finlayson, 1977; figure 1.17).

![Acyl-transfer reaction between Gln\textsuperscript{14} of \(\alpha_2\)AP and Lys\textsuperscript{303} of fibrin(ogen), catalysed by activated Factor XIII, forming the \(\varepsilon\)-(\(\gamma\)-glutamyl)lysine crosslinks.](image-url)
1.6. CLINICAL ASPECTS

1.6.1. α\textsubscript{2}AP LEVELS IN HEALTH AND DISEASE

The level of α\textsubscript{2}AP in healthy subjects varies between 80 and 120% of the normal plasma α\textsubscript{2}AP level (Lijnen and Collen, 1986). α\textsubscript{2}AP appears to be a weak acute-phase reactant. It has been reported that patients with liver disease and with severe intravascular coagulation have decreased α\textsubscript{2}AP levels, while those undergoing thrombolytic therapy may have immediately but temporarily exhausted α\textsubscript{2}AP levels, resulting in severe fibrinogenolysis (Wiman, 1981; Lijnen and Collen, 1986).

1.6.2. α\textsubscript{2}AP DEFICIENCY

Only a few patients with α\textsubscript{2}AP deficiency have so far been reported. They seem to suffer from severe lifelong haemorrhagic (bleeding) tendencies due to premature lysis of haemostatic plugs before the restoration of injured vessels because, in the absence of α\textsubscript{2}AP, plasmin molecules generated on the fibrin surface of the haemostatic plug persist much longer than normal (Wiman, 1981; Lijnen and Collen, 1986; Aoki, 1990).

Aoki (1990) determined the molecular basis for α\textsubscript{2}AP deficiency by analysing the α\textsubscript{2}AP genes from two Japanese families with congenital α\textsubscript{2}AP deficiency. Both families were found to have changes of nucleotide sequence(s) in an exon coding for plasma α\textsubscript{2}AP, resulting in productions of variant proteins (mutant α\textsubscript{2}APs). The precursor forms of the mutant α\textsubscript{2}APs are retained within the endoplasmic reticulum and, instead of being secreted into the media, most of the mutant molecules should have undergone degradation within the cells, thus retarding their transport in the intracellular transport pathway (figure 1.18). This therefore results in a deficiency of α\textsubscript{2}AP in circulating blood plasma (Aoki, 1990).
FIGURE 1.18: Synthesis, oligosaccharide processing, and intracellular transit of $\alpha_2$AP.
The proteins synthesized in the RER are glycosylated by the addition of a high mannose-type carbohydrate side-chain which is Endo H-sensitive. During its transit to the Golgi apparatus, the carbohydrate side-chain is converted to a complex type of oligosaccharides which is Endo H-resistant. Most of the mutant $\alpha_2$AP are retained in the RER as a Endo H-sensitive form, and their transit to the Golgi apparatus is blocked. (Taken from Aoki, 1990)
CHAPTER 2

INTRODUCTION TO THE PRESENT STUDY

As mentioned in the previous chapter, \( \alpha_2 \)AP is the primary physiological inhibitor of the enzyme responsible for the dissolution of fibrin clots, namely plasmin, and hence is a potent inhibitor of fibrinolysis. A deficiency of \( \alpha_2 \)AP therefore results in severe hemorrhagic tendencies due to premature lysis of haemostatic plugs before the restoration of injured vessels. The physiological importance of this inhibitor is thus well known.

Previous inhibition studies undertaken in our laboratory from ostrich blood include \( \alpha_1 \)-antitrypsin (Kuhn et al., 1994), \( \alpha_2 \)-macroglobulin (Van Jaarsveld et al., 1994) and \( \alpha_1 \)-antichymotrypsin (Frost et al., 1997). Frost et al. (1999) also reported on blood coagulation studies in the ostrich. Therefore, to obtain more information about the physiological role of these proteinase inhibitors in ostrich blood, the study of \( \alpha_2 \)AP was initiated.

To date, human \( \alpha_2 \)AP has been well characterised, as well as bovine, and apparently also mouse \( \alpha_2 \)AP. However, no attention has been given to avian \( \alpha_2 \)AP. The ostrich (Struthio camelus) belongs to the class Avis, although biochemical investigations of proteases in our laboratory revealed a phylogenetic relationship closer to that of the class Reptilia (Oelofsen et al., 1991). Research on this primitive flightless bird has become very important for South Africa due to the rapid increase in the ostrich industry over the past few years. For these reasons too the study of ostrich \( \alpha_2 \)AP was undertaken. During the study of \( \alpha_2 \)AP a certain amount of attention was also paid to its target enzyme, plasmin, both in its inactive proenzyme form (viz. plasminogen) and its active form.

The objectives of this study were therefore to:

1. Isolate and purify \( \alpha_2 \)AP from ostrich plasma;
2. Isolate plasminogen from ostrich plasma;
3. Activate ostrich plasminogen to plasmin;
4. Partially characterise ostrich \( \alpha_2 \)AP, plasminogen and plasmin physically and chemically, and in the cases of \( \alpha_2 \)AP and plasmin, kinetically, using the activated ostrich plasmin.
# CHAPTER 3

**MATERIALS AND METHODS**

### 3.1. MATERIALS ................................................................. 38
- 3.1.1. BIOLOGICAL MATERIALS ........................................ 38
- 3.1.2. CHEMICAL MATERIALS ............................................ 38

### 3.2. ENZYME ASSAYS ......................................................... 40
- 3.2.1. PLASMIN .............................................................. 40
- 3.2.2. TRYSIN ............................................................... 43
- 3.2.3. CHYMOTRYPSIN .................................................... 46
- 3.2.4. ELASTASE ........................................................... 48

### 3.3. PROTEIN DETERMINATION ............................................ 51

### 3.4. PROTEIN PRECIPITATION TECHNIQUES ......................... 52
- 3.4.1. AMMONIUM SULFATE PRECIPITATION ..................... 52
- 3.4.2. POLYETHYLENE GLYCOL PRECIPITATION .............. 53

### 3.5. COLUMN CHROMATOGRAPHY ...................................... 53
- 3.5.1. AFFINITY CHROMATOGRAPHY ................................... 53
  - 3.5.1.1. L-Lysine-Sepharose ........................................ 53
  - 3.5.1.2. Ostrich Plasminogen-Sepharose .......................... 55
  - 3.5.1.3. Ostrich LBSI-Sepharose .................................. 56
- 3.5.2. ION-EXCHANGE CHROMATOGRAPHY .......................... 58
  - 3.5.2.1. Toyopearl Super Q-650S ................................... 58
  - 3.5.2.2. Q HyperD© 10 ............................................. 58
- 3.5.3. GEL FILTRATION .................................................... 59
  - 3.5.3.1. Sephadex G-200 ............................................. 59
  - 3.5.3.2. Superdex 200© 200 HR 10/30 .............................. 59
- 3.5.4. HYDROXYLAPATITE CHROMATOGRAPHY ................... 59
3.5.5. IMMOBILISED-DYE CHROMATOGRAPHY .............................. 60
  3.5.5.1. MIMETIC ligand A6XL adsorbents .......................... 60
  3.5.5.2. Reactive yellow 86-Toyopearl HW-65 ..................... 60
  3.5.5.3. Blue-Sepharose CL-6B .................................. 62
3.5.6. REVERSED-PHASE HPLC ...................................... 62

3.6. ELECTROPHORETIC METHODS .................................... 63
  3.7.1. SDS-PAGE ....................................................... 63
  3.7.2. PAG-IEF ......................................................... 65

3.7. AMINO ACID AND N-TERMINAL SEQUENCE ANALYSIS ... 66

3.8. KINETIC CHARACTERISATIONS ................................. 67
  3.8.1. KINETIC CHARACTERISATION OF OSTRICH $\alpha_2$AP .............. 67
     3.8.1.1. Inhibitory effect on different serine proteases ............ 67
     3.8.1.2. Comparison with other inhibitors ........................ 67
  3.8.2. KINETIC CHARACTERISATION OF OSTRICH PLASMIN .......... 68
     3.8.2.1. pH Optimum .............................................. 68
     3.8.2.2. Temperature optimum ..................................... 68
     3.8.2.3. Kinetic parameters ....................................... 68
CHAPTER 3

3.1. MATERIALS

3.1.1. BIOLOGICAL MATERIALS

Ostrich blood was collected from farms in the vicinity of Port Elizabeth directly from the veins, as well as from the Grahamstown abbatoir where the blood was collected from the neck of the ostrich immediately after killing. The former method is preferred since it yields blood containing minimal thromboplastin, however, it is the more laborious and time consuming method.

The blood was collected in 0.1 M trisodium citrate buffer in a ratio of 9:1 to prevent blood clotting, followed by centrifugation at 3000 rpm (Beckman benchtop) for 15 min at room temperature. The supernatant (plasma) was pipetted off and immediately frozen in liquid nitrogen, all within 30 min after collection.

3.1.2. CHEMICAL MATERIALS

The various chemical materials used and their suppliers are summarised in table 3.1. All other chemicals were of the best analytical grade available.
**TABLE 3.1:** Summary of chemical materials used and their respective suppliers.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzymes</strong></td>
<td></td>
</tr>
<tr>
<td>Plasmin, bovine</td>
<td>Sigma</td>
</tr>
<tr>
<td>Trypsin, bovine pancreas (Type III)</td>
<td>Sigma</td>
</tr>
<tr>
<td>α-Chymotrypsin, bovine pancreas (Type I-S)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Elastase, porcine pancreas (Type I)</td>
<td>Sigma</td>
</tr>
<tr>
<td><strong>Substrates</strong></td>
<td></td>
</tr>
<tr>
<td>D-Val-Leu-Lys-pNA-HCl</td>
<td>Sigma</td>
</tr>
<tr>
<td>N-ρ-Tosyl-Gly-Pro-Lys-pNA-acetate</td>
<td>Sigma</td>
</tr>
<tr>
<td>N-Benzoyl-Phe-Val-Arg-pNA-HCl</td>
<td>Sigma</td>
</tr>
<tr>
<td>BAPNA-HCl</td>
<td>Sigma</td>
</tr>
<tr>
<td>ATNA</td>
<td>Merck</td>
</tr>
<tr>
<td>SAPNA</td>
<td>Sigma</td>
</tr>
<tr>
<td>Fibrinogen, bovine</td>
<td>Sigma</td>
</tr>
<tr>
<td><strong>Inhibitors</strong></td>
<td></td>
</tr>
<tr>
<td>α₂-Antiplasmin, human</td>
<td>Athens Research Technology (ART) and Sigma</td>
</tr>
<tr>
<td>Trypsin inhibitor, bovine pancreas (Type I-P)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Aprotinin, bovine lung</td>
<td>Sigma</td>
</tr>
<tr>
<td>Trasylol, bovine lung</td>
<td>Sigma</td>
</tr>
<tr>
<td>DFP</td>
<td>Sigma</td>
</tr>
<tr>
<td><strong>Activators</strong></td>
<td></td>
</tr>
<tr>
<td>Urokinase, human kidney cells</td>
<td>Sigma</td>
</tr>
<tr>
<td>Cyanogen bromide</td>
<td>Sigma</td>
</tr>
<tr>
<td>Epichlorohydrin</td>
<td>Sigma</td>
</tr>
<tr>
<td><strong>Gel matrices, resins and columns</strong></td>
<td></td>
</tr>
<tr>
<td>Sepharose CL-4B</td>
<td>Sigma</td>
</tr>
<tr>
<td>Toyopearl HW-65</td>
<td>Tosohaas</td>
</tr>
<tr>
<td>Toyopearl Super Q-650S</td>
<td>Tosohaas</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>Bio-Gel HTP Hydroxylapatite</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Blue-Sepharose CL-6B</td>
<td>Sigma</td>
</tr>
<tr>
<td>Q HyperD® 10</td>
<td>Beckman</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td><strong>Superdex 200 200 HR 10/30</strong></td>
<td><strong>Pharmacia</strong></td>
</tr>
<tr>
<td><strong>Capcell Pak C₁₈</strong></td>
<td><strong>Shiseido</strong></td>
</tr>
<tr>
<td><strong>PIKSI® test kit</strong></td>
<td><strong>Affinity Chromatography Limited (ACL)</strong></td>
</tr>
</tbody>
</table>

**Miscellaneous**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BCA</strong></td>
<td><strong>Sigma</strong></td>
</tr>
<tr>
<td><strong>BSA</strong></td>
<td><strong>Miles Laboratories (Pty.) Ltd.</strong></td>
</tr>
<tr>
<td><strong>EACA</strong></td>
<td><strong>Sigma</strong></td>
</tr>
<tr>
<td><strong>Lysine monohydrochloride</strong></td>
<td><strong>Merck</strong></td>
</tr>
<tr>
<td><strong>Reactive Yellow 86</strong></td>
<td><strong>Sigma</strong></td>
</tr>
<tr>
<td><strong>Molecular weight markers, high range</strong></td>
<td><strong>Sigma</strong></td>
</tr>
<tr>
<td><strong>IEF mix, 3.6-6.6</strong></td>
<td><strong>Sigma</strong></td>
</tr>
<tr>
<td><strong>Ampholyte, pH 3-10</strong></td>
<td><strong>Bio-Rad</strong></td>
</tr>
<tr>
<td><strong>TCA</strong></td>
<td><strong>Merck</strong></td>
</tr>
</tbody>
</table>

### 3.2. ENZYME ASSAYS

*In vitro* $\alpha_2$AP is capable of reacting and forming stable enzymatically inactive complexes with many enzymes, but most notably with plasmin (reacting very rapidly), trypsin (rapidly) and chymotrypsin (moderately) (Wiman, 1981). Since inhibition of plasmin is presumably its only physiologically important reaction (Wiman, 1981), plasmin was used to test for $\alpha_2$AP inhibitory activity throughout the isolation procedure.

#### 3.2.1. PLASMIN

Plasmin activity was determined using the synthetic peptide substrate D-Valine-L-Leucine-L-Lysine-$\rho$-Nitroanilide (S-2251). Plasmin cleaves D-Val-Leu-Lys-$\rho$NA at the C-terminus of lysine, thus releasing the yellow product, $\rho$-nitroaniline. The increase in absorbance at 412 nm could therefore be used to monitor this continuous assay.

The assay procedure was adapted from Aiach *et al.* (1983) and is presented in table 3.2.
**TABLE 3.2: Procedure for the microtiter plate assay of plasmin.**

<table>
<thead>
<tr>
<th></th>
<th>Sample Blank</th>
<th>Enzyme Activity</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme (µl)</td>
<td>-</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Inhibitor (µl)</td>
<td>10</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Buffer (µl)</td>
<td>10</td>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>

*Mix and incubate for 2 min at R. T.*

<table>
<thead>
<tr>
<th></th>
<th>Sample Blank</th>
<th>Enzyme Activity</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate (µl)</td>
<td>190</td>
<td>190</td>
<td>190</td>
</tr>
<tr>
<td>Total Volume (µl)</td>
<td>210</td>
<td>210</td>
<td>210</td>
</tr>
</tbody>
</table>

*Follow increase in A_{412 \text{nm}} for 5 min at 10 s intervals*

**Substrate:** 0.6 mM D-Val-Leu-Lys-pNA-HCl: Per assay, 62.871 µg D-Val-Leu-Lys-pNA dissolved in 190 µl buffer (stored in the dark).

**Buffer:** 15 mM Tris-HCl (pH 8.4) containing 7.5 mM EDTA and 175 mM NaCl.

The assay was tested at various concentrations of comm. bovine plasmin and the progress curves are represented in figure 3.1.

![Graph showing progress curves](image)

**FIGURE 3.1: Plasmin assay progress curves for various comm. bovine plasmin concentrations (microtiter plate assay).**

- ◆, 50 µg/well; □, 25 µg/well; ▲, 12.5 µg/well; ○, 6.25 µg/well.
The slopes obtained from the linear portions of the progress curves were used to indicate the linear relationship between the rate of hydrolysis of the substrate and the enzyme concentration (figure 3.2).

**FIGURE 3.2:** Rate of hydrolysis of D-Val-Leu-Lys-pNA as a function of varying comm. bovine plasmin concentrations (microtiter plate assay) ($r^2 = 0.997$).

One unit of plasmin activity is defined as the change of 1.0 absorbance unit per minute at 412 nm.

To determine $\alpha_2$AP inhibitory activity, the decrease in enzyme activity after incubation with the inhibitor is expressed as a percentage of that of the enzyme activity blank, as follows:

$$%\text{ Inhibition} = \frac{\text{Enzyme Activity Control} - (\text{Test-Sample Blank})}{\text{Enzyme Activity Control}} \times 100$$

The optimum incubation time of plasmin with $\alpha_2$AP, viz. 2 min, was determined by measuring the % remaining enzyme activity after various incubation times of comm. bovine plasmin with comm. human $\alpha_2$AP (ART) for varying I:E molar ratios (figure 3.3).
FIGURE 3.3: Inhibition of comm. bovine plasmin by comm. human \( \alpha_2 \)AP as a function of incubation time and molar ratios of I:E.

- ◆, 1:1; □, 0.1:1; ▲, 0.05:1; ○, 0.01:1.

This plasmin assay was used throughout the isolation procedure of \( \alpha_2 \)AP to test for \( \alpha_2 \)AP inhibitory activity, using a constant 25 µg comm. bovine plasmin per well, since plasmin is specifically inhibited by \( \alpha_2 \)AP, unlike the other enzymes presented below. One unit of inhibitor activity (U) is defined as that causing 50% inhibition of plasmin under the defined assay conditions.

3.2.2. TRYPsin

Trypsin activity was determined using the synthetic substrate N\( \alpha \)-Benzoyl-DL-Arginine-\( \rho \)-Nitroanilide-HCl. Trypsin cleaves BAPNA at the C-terminus of arginine, releasing \( \rho \)-nitroaniline, a yellow product. The assay could thus be monitored as an increase in absorbance at 412 nm.

The procedure for the standard assay (table 3.3 (i)) was taken from Erlanger et al. (1961), while the microtiter plate assay procedure (table 3.3 (ii)) was adapted from Skidgel and Erdös (1988).
**TABLE 3.3 (i): Procedure for the standard assay of trypsin.**

<table>
<thead>
<tr>
<th></th>
<th>Sample Blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate (µl)</td>
<td>950</td>
<td>950</td>
</tr>
<tr>
<td>Buffer (µl)</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Enzyme (µl)</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>Total Volume (µl)</td>
<td>1000</td>
<td>1000</td>
</tr>
</tbody>
</table>

*Follow increase in A_{412 nm} for 4 min at 10 s intervals.*

**TABLE 3.3 (ii): Procedure for the microtiter plate assay of trypsin.**

<table>
<thead>
<tr>
<th></th>
<th>Sample Blank</th>
<th>Enzyme Activity Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme (µl)</td>
<td>-</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Inhibitor (µl)</td>
<td>10</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Buffer (µl)</td>
<td>25</td>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>

*Mix and incubate for 5 min at R. T.*

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate (µl)</td>
<td>265</td>
<td>265</td>
<td>265</td>
</tr>
<tr>
<td>Total Volume (µl)</td>
<td><strong>300</strong></td>
<td><strong>300</strong></td>
<td><strong>300</strong></td>
</tr>
</tbody>
</table>

*Follow increase in A_{412 nm} for 4 min at 10 s intervals.*

**Substrate:**
(i) 1 mM BAPNA-HCl: 10.9 mg BAPNA-HCl solubilised in 1.5 ml DMSO and made up to 25 ml with buffer.
(ii) 1 mM BAPNA-HCl (final).

**Buffer:**
50 mM Tris-HCl (pH 8.2) containing 20 mM CaCl₂.

The assays were tested at various concentrations of comm. bovine pancreatic trypsin and the progress curves for both the standard and microtiter plate assays are shown in figure 3.4.
FIGURE 3.4: Trypsin assay progress curves for various comm. bovine pancreatic trypsin concentrations. (i), standard assay: ●, 39 µg/well; ▲, 19.5 µg/well; ▲, 9.75 µg/well, (ii), microtiter plate assay (in duplicate): ●, 25 µg/well; ▲, 12.5 µg/well; ▲, 6.25 µg/well; ○, 3.125 µg/well.

The slopes obtained from the linear portions of the progress curves were used to indicate the linear relationship between the rate of hydrolysis of BAPNA and the trypsin concentration. The resultant BAPNA standard curves for both the standard and microtiter plate assays are shown in figure 3.5.

FIGURE 3.5: Rate of hydrolysis of BAPNA as a function of varying comm. bovine pancreatic trypsin concentrations. (i), standard assay ($r^2=0.999$); (ii), microtiter plate assay (results represent means of duplicates; $r^2=1.000$).
One unit of trypsin activity is defined as the change of 1.0 absorbance unit per minute at 412 nm.

α₂AP inhibitory activity is calculated as described in section 3.2.1. One unit of inhibitor activity is defined as that causing 50% inhibition of trypsin under the defined assay conditions.

### 3.2.3. CHYMOTRYPSIN

Chymotrypsin activity was determined using the chromogenic substrate N-Acetyl-L-Tyrosine-ρ-Nitroanilide. Chymotrypsin cleaves ATNA at the C-terminus of tyrosine to yield a yellow product, ρ-nitroaniline. This continuous assay could therefore be monitored as an increase in absorbance at 412 nm.

The procedure for the standard and microtiter plate assays, as described by Van Jaarsveld et al. (1994), is presented in table 3.4.

**TABLE 3.4: Procedure for the standard and microtiter plate assays of chymotrypsin.**

<table>
<thead>
<tr>
<th>Sample Blank</th>
<th>Enzyme Activity Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Standard</strong> (i) Microtiter (i)</td>
<td><strong>Standard</strong> (i) Microtiter (i)</td>
</tr>
<tr>
<td>Enzyme (µl)</td>
<td>- -</td>
<td>30 6</td>
</tr>
<tr>
<td>Inhibitor (µl)</td>
<td>10 4</td>
<td>- -</td>
</tr>
<tr>
<td>Buffer (µl)</td>
<td>290 56</td>
<td>270 54</td>
</tr>
</tbody>
</table>

**Mix and incubate for 5 min at R. T.**

<table>
<thead>
<tr>
<th>Substrate (µl)</th>
<th>1000 200</th>
<th>1000 200</th>
<th>1000 200</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Volume (µl)</strong></td>
<td><strong>1300 260</strong></td>
<td><strong>1300 260</strong></td>
<td><strong>1300 260</strong></td>
</tr>
</tbody>
</table>

(i) Follow increase in $A_{412\text{ nm}}$ for 30 min at 5 min intervals.

(ii) Follow increase in $A_{412\text{ nm}}$ for 10 min at 30 s intervals.

**Substrate:**

(i) 1.27 mM ATNA: 10.9 mg ATNA solubilised in 1.5 ml DMSO and made up to 25 ml with buffer (stored in the dark).

(ii) 1 mM ATNA (final).

**Buffer:**

50 mM Tris-HCl (pH 8.2) containing 20 mM CaCl₂.

The assays were tested at various concentrations of comm. bovine pancreatic chymotrypsin and the progress curves for both the standard and microtiter plate assays are shown in figure 3.6.
FIGURE 3.6: Chymotrypsin assay progress curves for various comm. bovine pancreatic chymotrypsin concentrations. (i), standard assay; (ii), microtiter plate assay (in duplicate). 
- ◆, 60 µg/well; □, 30 µg/well; ▲, 15 µg/well; ○, 7.5 µg/well.

The slopes obtained from the linear portions of the progress curves were used to indicate the linear relationship between the rate of hydrolysis of ATNA and the chymotrypsin concentration. The resultant ATNA standard curves for both the standard and microtiter plate assays are shown in figure 3.7.
FIGURE 3.7: Rate of hydrolysis of ATNA as a function of varying comm. bovine pancreatic chymotrypsin concentrations. (i), standard assay ($r^2=0.991$); (ii), microtiter plate assay (results represent means of duplicates; $r^2=0.999$).

One unit of chymotrypsin activity is defined as the change in absorbance at 412 nm of 1.0 absorbance unit per minute.

$\alpha_2$AP inhibitory activity is calculated as explained in section 3.2.1., with one unit of inhibitor activity being defined as that causing 50% inhibition of chymotrypsin under the defined assay conditions.

3.2.4. ELASTASE

Elastase does not react with $\alpha_2$AP at all, but is specifically inhibited by $\alpha_1$PI, the most prominent serpin in serum (Travis and Salvesen, 1983). The assay for elastase is hence important for testing for the presence of $\alpha_1$PI and is therefore included here.

Elastase activity was determined using the chromogenic substrate N-Succinyl-Ala-Ala-Ala-p-Nitroanilide. Elastase cleaves SAPNA at the C-terminus of alanine, releasing the yellow product, p-nitroaniline. The increase in absorbance at 412 nm could thus be used to monitor this assay.

The modified assay procedure of Beatty et al. (1980) was used, as described in table 3.5.
TABLE 3.5: Procedure for the standard and microtiter plate assays of elastase.

<table>
<thead>
<tr>
<th></th>
<th>Sample Blank</th>
<th>Enzyme Activity</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard (i)</td>
<td>Microtiter (ii)</td>
<td></td>
</tr>
<tr>
<td>Enzyme (µl)</td>
<td>-</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Inhibitor (µl)</td>
<td>10</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Buffer (µl)</td>
<td>290</td>
<td>270</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>54</td>
<td>2</td>
</tr>
</tbody>
</table>

Mix and incubate for 5 min at R. T.

|                     | 1000         | 1000            | 1000 |
| Substrate (µl)      | 1000         | 200             | 200  |
| Total Volume (µl)   | 1300         | 260             | 1300 |
|                     | 260          | 260             | 260  |

(i) Follow increase in A<sub>412 nm</sub> for 2 min at 10 s intervals.
(ii) Follow increase in A<sub>412 nm</sub> for 3 min at 10 s intervals.

**Substrate:** 1 mM SAPNA: 2.7 mg SAPNA solubilised in 1 ml DMSO and made up to 10 ml with buffer (stored in the dark).

**Buffer:** 0.1 M Tris-HCl (pH 8.0) containing 50 mM NaCl.

The assays were tested at various concentrations of comm. porcine pancreatic elastase and the progress curves for both the standard and microtiter plate assays are shown in figure 3.8.

**FIGURE 3.8:** Elastase assay progress curves for various comm. porcine pancreatic elastase concentrations. (i), standard assay; (ii), microtiter plate assay. ◊, 20 µg/well; □, 10 µg/well; ▲, 5 µg/well; ○, 2.5 µg/well.
The slopes obtained from the linear portions of the progress curves were used to indicate the linear relationship between the rate of hydrolysis of SAPNA and the elastase concentration, as shown in figure 3.9.

![Graphs showing rate of hydrolysis of SAPNA as a function of varying elastase concentrations.](image)

**FIGURE 3.9:** Rate of hydrolysis of SAPNA as a function of varying commercial porcine pancreatic elastase concentrations. (i), standard assay ($r^2=0.999$); (ii), microtiter plate assay ($r^2=0.995$).

One unit of elastase activity is defined as the change in absorbance at 412 nm of 1.0 absorbance unit per minute.

$\alpha_1$PI inhibitory activity is calculated as described for $\alpha_2$AP in section 3.2.1. One unit of $\alpha_1$PI inhibitory activity is defined as that causing 50% inhibition of elastase under the defined assay conditions.
3.3. PROTEIN DETERMINATION

Protein was determined using the *bicinchoninic acid method*. BCA, in its water-soluble sodium salt form, is a stable and sensitive reagent highly specific for the cuprous ion (Cu⁺). This protein assay method combines the well-known biuret reaction (protein reduces Cu²⁺ in an alkaline medium to produce Cu⁺) with the unique features of BCA (two molecules of BCA combines with one cuprous ion). The reaction product is water-soluble and exhibits a strong absorbance at 562 nm, thus allowing the spectrophotometric quantitation of protein in aqueous solutions (Smith *et al.*, 1985). Peptide bonds and four amino acids (cysteine, cystine, tryptophan, and tyrosine) have been reported to be responsible for the purple colour formation (Smith *et al.*, 1985; Wiechelman *et al.*, 1988).

The method used was that of Smith *et al.* (1985) and was followed according to the Pierce chemical product bulletin 23225 of the BCA protein determination kit (microtiter plate method). Figure 3.10 depicts a typical BCA protein standard curve.

![Figure 3.10](image)

*FIGURE 3.10: BCA protein standard curve.*

Results represent means of duplicates ($r^2=1.000$).
3.4. PROTEIN PRECIPITATION TECHNIQUES

3.4.1. AMMONIUM SULFATE PRECIPITATION

Proteins differ in their solubility in the presence of high salt concentrations and can therefore be separated from one another by precipitation. Ammonium sulfate is the precipitant most frequently used in the salting-out of proteins primarily because it does not significantly affect pH, it does not have a high heat of solution so that heat generated is easily dissipated, and it is very soluble and therefore does not destabilise proteins. Further advantages are that it is of sufficient high molarity at saturation that it causes the precipitation of most proteins, the density even of its saturated solutions is not so large that it interferes with the sedimentation of most precipitated proteins by centrifugation, and its concentrated solutions prevent or limit most bacterial growth. However, the purification achieved is usually only 2-5 times greater than the previous step, and the salt has to be removed (usually by means of dialysis) before continuing with the following purification steps (Englard and Seifter, 1990).

Ammonium sulfate precipitation was used as the second purification step in the isolation of α₂AP. The precipitate collected between 30 and 50% saturation (i.e. 1.2-2.0 M (NH₄)₂SO₄), according to Moroi and Aoki (1976), was used in the first isolation attempt, but the top of the range was thereafter increased to 67.5% (2.7 M), as adapted from Christensen and Clemmensen (1978). The precipitates were removed by centrifugation, usually at 17 700 g for 1 hr at 4 °C. The resultant precipitate was either solubilised in the buffer for the next purification step and dialysed against three changes of this buffer at 4 °C, or in the case of large quantities, solubilised in and exhaustively dialysed against distilled water and freeze-dried.
3.4.2. POLYETHYLENE GLYCOL PRECIPITATION

The main advantage of PEG over ammonium sulfate and other precipitating agents is its little tendency to denature or otherwise interact with proteins even when present at high concentrations and elevated temperatures. Another advantage is the shorter time required for the precipitated proteins to equilibrate and achieve a physical state suitable for large-scale centrifugation (Ingham, 1990).

PEG was used in comparison with ammonium sulfate in an attempt to determine the more suitable precipitant for use in the second purification step of $\alpha_2$AP. The precipitate collected at 6% (w/v) PEG was, after stirring for 1 hr, removed by centrifugation at 17 700 g for 1 hr at 4°C. The resultant precipitate was solubilised in and exhaustively dialysed against distilled water and freeze-dried.

3.5. COLUMN CHROMATOGRAPHY

3.5.1. AFFINITY CHROMATOGRAPHY

Affinity chromatography takes advantage of one or more biological properties of the molecule(s) being purified. These interactions are not due to the general properties of the molecule, but utilises the specific reversible interactions between biomolecules (Ostrove, 1990).

3.5.1.1. L-LYSINE-SEPHAROSE

Lysine-Sepharose chromatography is usually used as the first step in the isolation and purification of $\alpha_2$AP to remove plasminogen, a major contaminant during the isolation procedure, from plasma. Since lysine specifically binds plasminogen, this chromatographic step yields pure plasminogen. The ostrich plasminogen isolated as such proved useful in two ways: (i) it was used to make ostrich plasminogen-Sepharose and LBSI-Sepharose, theoretically important columns in the purification of $\alpha_2$AP, and (ii) it was activated to ostrich plasmin to be used in the kinetic studies of $\alpha_2$AP. This column was also used in the purification of the LBSI fragment (see section 3.5.1.3.).

The procedure for the preparation of L-lysine-Sepharose was adapted from the procedure outlined by Robbins and Summaria (1976a), and is presented in diagram 3.1.
**Diagram 3.1: Procedure for the preparation of L-lysine-Sepharose affinity resin.**

- **100 ml Sepharose 4B**
  - Wash with 1.67 l ice-cold water.
  - Remove water with water suction system.
- **10 g Cyanogen bromide**
  - Dissolve in 200 ml R.T. distilled water.
  - Mix and monitor pH continuously and adjust with 4 M NaOH (pH 10.5-11.0).
  - Maintain at R.T. by adding ice.

**Reaction complete within 30 min (pH 10.5).**

- Dry on büchner funnel under suction.
- Hydrate to 133 ml with 0.1 M NaHCO₃, pH 9.0.

**CNBr-activated Sepharose**

- 1.67 g L-Lysine monohydrochloride
  - Dissolve in 6.67 ml 0.1 M NaHCO₃, pH 9.0.
  - Mix in cold room for 48 hr.

**100 ml L-Lysine-Sepharose**

- Wash under suction with 0.5 l 0.1 M NaHCO₃, pH 9.0.
- Equilibrate with appropriate buffer (0.1 M sodium phosphate, pH 7.4, containing 3 mM EDTA).
- Pack into column (2.6 x 10.5 cm).
- Wash with equilibration buffer.

The resin was regenerated by simply washing with large volumes of distilled water and equilibrated with 0.1 M sodium phosphate buffer (pH 7.4) containing 3 mM EDTA. However, after a few uses the resin was first washed with 0.1 M acetic acid.
3.5.1.2. OSTRICH PLASMINOGEN-SEPHAROSE

Since plasminogen specifically binds $\alpha_2$AP, plasminogen-Sepharose is an important resin in the purification of $\alpha_2$AP. However, $\alpha_2$AP binds weakly to plasminogen, with the non-plasminogen-binding form not binding at all. This resin therefore only allows for the purification of the plasminogen-binding form.

The procedure for the preparation of ostrich plasminogen-Sepharose resin (diagram 3.2) was adapted from that described by Cuatrecasas (1970) for also coupling ligands containing an amino group to agarose.

**DIAGRAM 3.2: Procedure for the preparation of ostrich plasminogen-Sepharose resin.**

- **Cyanogen bromide (4g)**
- **Sepharose 4B (15 ml)**

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wash and mix with equal volume distilled water; stir.</td>
</tr>
<tr>
<td>2</td>
<td>Immediately raise pH to 11 and maintain with 3 M NaOH.</td>
</tr>
<tr>
<td>3</td>
<td>Maintain temperature at 20 °C by adding ice until reaction is completed within 8-20 min.</td>
</tr>
<tr>
<td>4</td>
<td>Add large amount of ice rapidly.</td>
</tr>
<tr>
<td>5</td>
<td>Quickly transfer to büchner funnel.</td>
</tr>
<tr>
<td>6</td>
<td>Wash under suction with cold 0.1 M NaHCO₃, pH 9.0 ($\geq 10-15 \times$ volume packed Sepharose).</td>
</tr>
<tr>
<td>7</td>
<td>Mix immediately on büchner funnel with glass rod.</td>
</tr>
<tr>
<td>8</td>
<td>Transfer to beaker and gently stir at 4 °C for 24 hr.</td>
</tr>
<tr>
<td>9</td>
<td>Wash with large volumes of distilled water and appropriate buffer (40 mM sodium phosphate, pH 7.0).</td>
</tr>
<tr>
<td>10</td>
<td>Pack into column (1.0 x 2.0 cm).</td>
</tr>
<tr>
<td>11</td>
<td>Wash with equilibration buffer.</td>
</tr>
</tbody>
</table>

The resin was regenerated by washing first with 0.1 M acetic acid and then with distilled water. It was equilibrated with 40 mM sodium phosphate buffer, pH 7.0.
3.5.1.3. OSTRICH LBSI-SEPHAROSE

The preparation of this affinity resin involved 3 major steps:
1. Selective cleavage of the purified ostrich plasminogen with porcine pancreatic elastase.
2. Purification of the LBSI fragment containing kringles 1-3.
3. Coupling of the purified LBSI fragment to the gel matrix.

Steps 1 and 2, portrayed in diagram 3.3, were adapted from the procedure described by Sottrup-Jensen et al. (1978).

**Diagram 3.3: Procedure for the preparation of ostrich LBSI.**

- **210 mg Ostrich plasminogen** (section 4.5.1.)
- **1.4 mg Trypsin inhibitor (bovine pancreas)**
- **0.63 mg Elastase** (porcine pancreas)

  - Dissolve in 14 ml 0.1 M NH₄HCO₃, pH 8.3.
  - Add solid NH₄HCO₃ to 0.3 M.
  - Incubate at R.T. for 3 hr with stirring.
  - Stop reaction by adding 3.5 µl DFP; incubate at R.T. for 30 min with stirring.
  - Add solid NH₄HCO₃ to ~0.55 M.
  - Stir for 18 hr in cold room.
  - Centrifuge: 27 000 g / 30 min / 4 °C.
  - Freeze-dry supernatant.

- **181.5 mg Plasminogen fragments**
  - Dissolve in 0.1 M NH₄HCO₃, pH 8.3.

- **L-Lysine-Sepharose chromatography (22.3 x 1.6 cm)**
  - Wash to baseline A₂₈₀nm value.
  - Gradient elution: mixing chamber = 50 ml 0.1 M NH₄HCO₃, pH 8.3; reservoir = 50 ml 0.1 M NH₄HCO₃, pH 8.55 + 10 mM EACA.
  - Pool tubes containing the M, 36 K component (figure 3.11).
  - Dialyse exhaustively against running distilled water.
  - Freeze-dry.

- **28.5 mg Ostrich LBSI**
The L-lysine-Sepharose elution profile is shown in figure 3.11.

![Graph showing elution profile](image)

**FIGURE 3.11:** L-Lysine-Sepharose chromatography of the plasminogen fragments resulting from elastase digestion. \( A_{280 \text{ nm}} \); \( [\text{EACA}] \) (M).

The SDS-PAGE patterns of the resultant peaks are demonstrated in figure 3.12.

![SDS-PAGE patterns](image)

**FIGURE 3.12:** SDS-PAGE patterns of the elution profile of L-lysine-Sepharose chromatography of the plasminogen fragments resulting from elastase digestion. Lanes: 1, HMW markers; 2, ostrich plasminogen fragments resulting from elastase digestion; 3-7, tube nos. 17, 46, 137, 144, and 150, respectively; 8, gradient peak 2 (ascending tail); 9, gradient peak 2 (descending tail).

Figure 3.12 shows that the LBSI fragment (M, 36 K) eluted in the second peak in the EACA gradient (tubes 158-204, figure 3.11), which was expected considering that it is theoretically the fragment with the strongest affinity for lysine.
Step 3 was accomplished by employing the CNBr activation technique described in diagram 3.2, using the LBSI fragment and Sepharose 4B to make a 6 ml ostrich LBSI-Sepharose affinity resin.

3.5.2. ION-EXCHANGE CHROMATOGRAPHY

IEC, historically one of the most frequently utilised purification methods, is used to separate proteins from one another on the basis of their specific ionisation properties (pI) and also to distinguish localised charge differences in proteins with the same pI. It operates on the mechanism of the reversible binding of charged molecules to the matrix, with the binding strength being governed by the degree of charge on the protein, the pKₐ of the ion exchange matrix and the aqueous solution properties, pH and ionic strength (Rossomando, 1990)

3.5.2.1. TOYOPEARL SUPER Q-650S

Toyopearl Super Q-650S is a high capacity strong anion exchanger consisting of trimethylamino groups attached to a 1000 Å pore base resin of Toyopearl HW-65.

Regeneration of the matrix was performed as follows:
1. Wash matrix (120 ml) with 1 M HCl (1 l) for 30 min and decant the HCl.
2. Wash with distilled water (2 l) and decant (repeat at least 3 times).
3. Wash with 1 M NaOH (1 l) for 30 min and decant.
4. Wash with distilled water (2 l) and decant (repeat at least 3 times).
5. Equilibrate with equilibration buffer (75 mM sodium phosphate, pH 7.6).
6. Check pH and conductivity of wash buffer until it equals that of the equilibration buffer.

3.5.2.2. Q HYPERDIP 10

Q HyperDip 10 is a strong anion exchanger. However, its small bead sizes (10 µm) and HyperDiffusion properties provide very high resolution at high speed, while the rigid composite structure and proprietary hydrogel filled pores of the HyperD bead provide superior dynamic capacity at high speed (Beckman Instruction 015-142313).

Q HyperDip 10 was used with an HPLC system (Beckman System Gold) in an attempt to improve on Toyopearl Super Q-650S due to its higher resolution. The working conditions and cleaning of the column was followed as outlined in Beckman Instruction 015-142313.
3.5.3. GEL FILTRATION

Gel filtration, also known as size exclusion chromatography, separates proteins on the basis of their differences in size and/or shape by allowing smaller molecules to readily enter the pores of the gel whilst excluding larger ones. Gel filtration can also be used to desalt samples (Stellwagen, 1990a).

3.5.3.1. SEPHADEX G-200

Sephadex consists of branched dextran which is allowed to react with epichlorohydrin, an epoxide, which introduces glycerol side chains and cross linkages within and in between the dextran molecules. Sephadex G-200 has a fractionation range of 5-600 K and was used specifically to remove the very large contaminating proteins in the impure $\alpha_2$AP samples.

Regeneration of the gel suspension simply involved washing repeatedly with distilled water and it was equilibrated with a 40 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl. When necessary, the gel was first washed with 0.5 M NaOH to remove all possible contaminants.

3.5.3.2. SUPERDEX $\gamma$ 200 HR 10/30

Superdex 200 consists of dextran covalently bound to highly cross-linked porous agarose beads. It gives excellent resolution of proteins and peptides in the molecular weight range 10 - 600 K (Pharmacia LKB Biotechnology Superdex 200$\gamma$ 200 HR 10/30 instruction manual).

Superdex 200$\gamma$ 200 HR 10/30 was used with an HPLC system (Beckman System Gold) and the column operation was followed according to the Pharmacia LKB Biotechnology Superdex 200$\gamma$ 200 HR 10/30 instruction manual. It was used in an attempt to purify the LBSI fragment.

3.5.4. HYDROXYLAPATITE CHROMATOGRAPHY

Hydroxylapatite is a crystalline form of calcium phosphate. The adsorption of proteins to HA is due to (i) the primarily non-specific electrostatic interaction between the positively charged amino groups of proteins and the general negative charge on the HA column when equilibrated with phosphate buffers, (ii) the electrostatic repulsion of the carboxyl groups of proteins from the negative charge of the column, and (iii) the specific binding by complexation of the carboxyl groups to the calcium sites on the column (Gorbunoff, 1990).
Bio-gel HTP powder was used and was rehydrated as described in the Bio-Rad instructions. The hydroxylapatite resin was regenerated by first washing with a high salt concentration (150 mM sodium phosphate, pH 7.0) to remove all adsorbed materials and then with repeated volumes of deionised water. It was equilibrated with 10 mM sodium phosphate, pH 7.0.

3.5.5. IMMOLISIE DYE CHROMATOGRAPHY

A given immobilised reactive textile dye can function as an inexpensive stable affinity resin capable of purifying a large number of quite different proteins with impressive selectivity and capacity. This affinity is most likely due to the flexibility of the dye which can assume the polarity and geometry of the surface of a variety of competitive biomolecules (Stellwagen, 1990b).

3.5.5.1. MIMETIC LIGAND A6XL ADSORBENTS

MIMETIC\textregistered ligands, which have intermediate specificities, coupled to 6\% cross-linked agarose (A6XL) provide extremely stable affinity adsorbents which display high protein binding capacities for a wide range of proteins and exceedingly low ligand leakage (ACL data sheet: MIMETIC ligand A6XL adsorbents).

The PIKSI\textregistered module, a self-contained kit designed specifically for adsorbent screening studies, was used to identify the most appropriate adsorbent for the purification of ostrich \(\alpha_2\)AP. The screening procedure, as well as adsorbent regeneration and equilibration, was followed according to that outlined in the ACL data sheet for MIMETIC ligand A6XL adsorbents.

3.5.5.2. REACTIVE YELLOW 86-TOYOPEARL HW-65

Since both MIMETICs yellow-1 and yellow-2 A6XL gave the best purification of ostrich \(\alpha_2\)AP from plasma, a yellow dye column, viz. reactive yellow 86-Toyopearl HW-65 (structure of dye in figure 3.13), was made according to the method described by Stellwagen (1990b), as outlined in diagram 3.4.
**DIAGRAM 3.4: Procedure for the preparation of reactive yellow 86-Toyopearl HW-65 resin.**

**Toyopearl HW-65 (55 ml)**
- Add 46 ml distilled water.
- Add 46 ml 10 M NaOH.
- Add 69 ml epichlorohydrin.
- Shake: 105 shakes/min / 1 hr / 50 °C.

**Epoxy-activated Toyopearl**
- Remove liquid.
- Add 82.5 ml 25% ammonia.
- Shake: 105 shakes/min / 1.5 hr / 50 °C.

**Aminated Toyopearl**
- Wash: 257 ml distilled water.
- Wash: 110 ml 4 M NaCl.

**Reactive yellow 86 (3.667 g)**
- Dissolve in 73.34 ml distilled water.
- Add 1.83 ml 10 M NaOH.
- Stir: 105 shakes/min / 17 hr / 55 °C.

**Reactive yellow 86-Toyopearl HW-65 (45 ml)**
- Filter mixture with copious amounts of distilled water.
- Filter mixture with 1 M NaCl.
- Filter mixture with distilled water until filtrate is clear.
- Suspend resin in 2 M NH4Cl, pH 8.5.
- Stir: 105 shakes/min / 4 hr / R.T.
- Wash: 458 ml water.

**FIGURE 3.13: Chemical structure of reactive yellow 86.**
(Taken from a Sigma product information sheet for reactive dye resins.)
As for the MIMETIC ligand A6XL adsorbents, this dye resin was regenerated by washing with 1 M NaOH and then at least 4 times with distilled water before equilibrating with the starting buffer (25 mM sodium phosphate, pH 6.0).

3.5.5.3. BLUE-SEPHAROSE CL-6B

Blue-Sepharose CL-6B consists of the reactive dye cibacron blue F3G-A (structure in figure 3.14) covalently attached to the cross-linked agarose gel Sepharose by the triazine coupling method.

![Chemical structure of cibacron blue F3G-A](image)

**FIGURE 3.14: Chemical structure of cibacron blue F3G-A.**
(Taken from a Sigma product information sheet for reactive dye resins.)

It is known to specifically bind albumin from serum and was therefore used to determine if the major contaminant in the partially pure \(\alpha_2\)AP sample was indeed ostrich serum albumin, and if so, to simultaneously remove it. The component, however, did not bind to the resin but instead fell through the column, thus proving that it is not ostrich serum albumin.

Regeneration and equilibration were as for reactive yellow 86-Toyopearl HW-65 (section 3.5.5.2.).

3.5.6. REVERSED PHASE-HPLC

Reversed-phase chromatography depends on the native hydrophobicity of the protein and is carried out under conditions which expose nearly all hydrophobic groups to the matrix, i.e. denaturing conditions, as opposed to hydrophobic interaction chromatography which depends on surface hydrophobic groups and is carried out under non-denaturing conditions. The matrix is silica that has been substituted with long \(n\)-alkyl chains, usually \(C_8\) or \(C_{18}\), while the mobile phase is more polar (Kennedy, 1990). RPC combined with the high performance of HPLC makes RP-HPLC a widely practised technique for the separation of proteins and polypeptides (Chicz and Regnier, 1990).
A Capcell Pak C₁₈ ODS/AGS120 column (4.6 x 250 mm) was used in the purification of α₂AP and a smaller one (4.6 x 150 mm) was used for the amino acid analysis of ostrich α₂AP, plasminogen and plasmin (see section 3.8.). The column was regenerated by rinsing thoroughly with purified water, pure methanol, a 50:50 mixture of methylenechloride:methanol, and then reversing the solvent sequence back to water. It was equilibrated with 0.1% TFA.

### 3.6. ELECTROPHORETIC METHODS

#### 3.6.1. SDS-PAGE

SDS-PAGE is an electrophoretic method employing the detergent sodium dodecyl sulfate (SDS; CH₃(CH₂)₁₀CH₂OSO₃⁻Na⁺), which binds to most proteins in amounts roughly proportional to the molecular weight of the protein (~1 molecule SDS/2 amino acid residues). The bound SDS contributes a large negative charge which renders the charge of the protein insignificant. Also, when SDS is bound, the native conformation of the protein is altered and most proteins assume a similar shape, and thus a similar charge to mass ratio. Therefore, SDS-PAGE separates proteins almost exclusively on the basis of mass, or molecular weight, with smaller polypeptides migrating more rapidly. By comparing the position to which an unknown protein migrated to those migrated by proteins of known molecular weights, the Mr of the unknown protein can be determined (Lehninger et al, 1993). However, the molecular weight of glycoproteins are usually overestimated (Reynolds and Tanford, 1970). The sieving effect of the polyacrylamide is also important and the range of molecular weights that can be used depends on the 'pore size', or amount of cross-linking, of the gel (Plummer, 1987).

SDS-PAGE was used solely as an analytical tool to (i) monitor the purification of ostrich α₂AP and plasminogen, (ii) monitor the activation of ostrich plasminogen to ostrich plasmin, (iii) aid in the pooling of tubes from certain elution profiles, and (iv) estimate the relative molecular weights of the proteins of interest. It was performed according to the method of Laemmli (1970), using the reagents and gel preparation as outlined in the instruction manual of the Bio-Rad Mini-Protean II Dual Slab cell system. Generally it was performed on ~10 µg of protein per well, using gels containing 10% acrylamide, but when samples were in solution, the KCl protein precipitation method (diagram 3.5) was employed.
**Diagram 3.5: KCl protein precipitation method.**

100 µl sample
- Add 10 µl 5% SDS.
- Vortex and invert eppendorf tube.
- Add 10 µl 3 M KCl.
- Vortex and invert eppendorf tube.
- Centrifuge in a bench top centrifuge for 2 min.

**Protein precipitate**
- Add 10 µl sample buffer.

Where necessary, samples were reduced by adding less than 5% β-mercaptoethanol and then boiling for 5 min (unless otherwise stated, all samples shown are non-reduced). The gels were stained with Coomassie Brilliant Blue R-250 according to the Coomassie staining method; if the protein concentrations were too low, the silver staining method was used (instruction manual of the Bio-Rad Mini-Protean II Dual Slab cell system). The high range molecular weight marker mixture, which consists of the following proteins, were used to estimate the relative molecular weights of the proteins:

<table>
<thead>
<tr>
<th></th>
<th>Protein name</th>
<th>M_r (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Myosin, rabbit muscle</td>
<td>205</td>
</tr>
<tr>
<td>2</td>
<td>β-Galactosidase, E. Coli</td>
<td>116</td>
</tr>
<tr>
<td>3</td>
<td>Phosphorylase b, rabbit muscle</td>
<td>97</td>
</tr>
<tr>
<td>4</td>
<td>Fructose 6-phosphate kinase, rabbit muscle</td>
<td>84</td>
</tr>
<tr>
<td>5</td>
<td>Albumin, bovine serum</td>
<td>66</td>
</tr>
<tr>
<td>6</td>
<td>Glutamic dehydrogenase, bovine liver</td>
<td>55</td>
</tr>
<tr>
<td>7</td>
<td>Albumin, chicken egg</td>
<td>45</td>
</tr>
<tr>
<td>8</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase, rabbit muscle</td>
<td>36</td>
</tr>
</tbody>
</table>

The formula used to calculate the \( R_m \) values is:

\[
R_m = \frac{\text{Distance moved by protein}}{\text{Distance moved by tracking dye}}
\]

A typical calibration curve of the high molecular weight markers (excluding the highest molecular weight marker) on a 10% SDS-PAGE gel is given in figure 3.15.
3.6.2. PAG-IEF

Proteins, being amphoteric molecules, have net charges depending on the pH of their local environment. Thus, there is a specific pH for every protein at which its net charge is zero, termed pI. Isoelectric focusing is a high-resolution technique which separates proteins as they migrate through a pH gradient, on the basis of differences in their pI values (Garfin, 1990).

PAG-IEF was used analytically to determine the pI values of the proteins of interest. The method described by Robertson et al. (1987) was applied for a 6% gel in the Bio Rad Mini-Protean II Dual Slab cell system, using Bio-Lyte carrier ampholytes in the pH range 3-10. The pI markers used were the 3.6-6.6 IEF mix, which consists of the following proteins:

<table>
<thead>
<tr>
<th>Protein</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonic anhydrase I, human erythrocytes</td>
<td>6.57</td>
</tr>
<tr>
<td>Carbonic anhydrase II, bovine erythrocytes</td>
<td>5.85, 5.28</td>
</tr>
<tr>
<td>β-Lactoglobulin A, bovine milk</td>
<td>5.13</td>
</tr>
<tr>
<td>Trypsin inhibitor, soybean</td>
<td>4.55</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td>4.2</td>
</tr>
<tr>
<td>Amyloglucosidase</td>
<td>3.55</td>
</tr>
</tbody>
</table>

A calibration curve of the IEF mix obtained from PAG-IEF (6% gel) is presented in figure 3.16.
Amino acid and N-terminal sequence analysis were performed on the purified ostrich α_{2}AP, plasminogen and plasmin samples by Dr Koji Muramoto (Dept of Applied Biological Chemistry, Faculty of Agriculture, Aoba-Ku, Sendai, Japan). The samples were subjected to SDS-PAGE (10% gel) and the gels were electroblotted onto PVDF membranes, which were stained with 0.1% Coomassie Blue R-250 in water/methanol/acetic acid (5:4:1) and destained with water/methanol/acetic acid (3:2:1). The protein bands were excised and subjected to amino acid analysis and N-terminal sequence analysis. Unfortunately, the yield of ostrich α_{2}AP after electroblotting was too low to do amino acid analysis.

For amino acid analysis samples were hydrolysed in vacuo in 6 N HCl at 110 °C for 24 hours. The dried hydrolysates were derivatised with DABS-Cl and analysed by RP-HPLC on a Capcell Pak C_{18} ODS/AG120 column (5 µm, 4.6 x 150 mm, Shiseido, Tokyo, Japan) (Knecht and Chang, 1986).

N-terminal sequence analysis was performed on an automated Shimadzu PSQ-1 gas-phase protein sequencer using the FITC-PITC double coupling method (Muramoto et al., 1978; Muramoto et al., 1993).
3.8. KINETIC CHARACTERISATIONS

Due to the expense of the synthetic pNA substrates used to assay for plasmin, single values were determined for the kinetic characterisations where these substrates were used. In the case of the kinetic characterisation of ostrich α2AP, another limitation was the actual isolated ostrich α2AP, which was obtained in extremely low yields.

3.8.1. KINETIC CHARACTERISATION OF OSTRICH α2AP

3.8.1.1 INHIBITORY EFFECT ON DIFFERENT SERINE PROTEASES

The inhibitory effects of ostrich as well as comm. human α2APs were tested on ostrich and comm. bovine plasmins, comm. bovine trypsin and comm. bovine chymotrypsin. The assays were performed as described in sections 3.2.1., 3.2.2. and 3.2.3. for plasmin, trypsin and chymotrypsin, respectively. There were a few minor changes to the plasmin assay, however, as listed here:

i) The inhibitor and enzyme was incubated for 4 min instead of 2 min to ensure maximum inhibition.

ii) A 50 mM Tris-acetate buffer (pH 8.0) was used instead of the 15 mM Tris-HCl buffer (pH 8.4) containing 7.5 mM EDTA and 175 mM NaCl since the pH optimum of plasmin was found to be 8.0.

iii) The concentration of D-Val-Leu-Lys-pNA was increased to 1 mM to ensure substrate saturation.

The results were expressed graphically as % remaining enzyme activity (after the relevant incubation time) vs. I/E molar ratio. The linear portions of the graphs were used to calculate the resulting inhibitory effects, expressed as inhibitory activity (U)/(I/E) (mol/mol).

3.8.1.2. COMPARISON WITH OTHER INHIBITORS

The inhibitory effects of ostrich and comm. human α2APs on ostrich and comm. bovine plasmins were compared to those of other inhibitors and ligands (natural and synthetic) known to inhibit plasmin, viz. aprotinin (bovine lung), DFP and EACA. This was accomplished by performing the plasmin assay as described in section 3.8.1.1. The results were also expressed as described in section 3.8.1.1.
3.8.2. KINETIC CHARACTERISATION OF OSTRICH PLASMIN

3.8.2.1. pH OPTIMUM
The pH optima of both ostrich and comm. bovine plasmins were determined by performing the plasmin assay described in section 3.2.1., using different buffer systems for the different pHs, viz. 50 mM Na-citrate buffers for pHs 4, 5, and 6, and 50 mM Tris-acetate buffers for pHs 6, 7, 8, 9, and 10.1.

3.8.2.2. TEMPERATURE OPTIMUM
To determine the temperature optima of both ostrich and comm. bovine plasmins, the plasmin assay described in section 3.2.1. was scaled up 2.5-fold to a standard assay, using a 1 ml cuvette and a Perkin Elmer Lamda 3A spectrophotometer, which is fitted with a temperature regulator. The assays, using the Tris-acetate (pH 8.0) buffer, were performed at 20, 25, 30, 35, 40, 45, 50, and 60 °C by incubating the enzyme and substrate, and assaying at the respective temperatures.

3.8.2.3. KINETIC PARAMETERS
The kinetic parameters of ostrich as well as comm. bovine plasmins were determined using various concentrations of 3 synthetic pNA substrates, viz. N-\(p\)-Tosyl-Gly-Pro-Lys-pNA, D-Val-Leu-Lys-pNA, N-Benzoyl-Phe-Val-Arg-pNA, and a natural substrate, viz. bovine fibrinogen. The plasmin assays using the 3 synthetic pNA substrates were performed as described in section 3.2.1., using the Tris-acetate (pH 8.0) buffer. However, the assays with bovine fibrinogen employed the discontinuous TCA assay, as described in table 3.6.

**TABLE 3.6: Procedure for the assay of plasmin using bovine fibrinogen.**

<table>
<thead>
<tr>
<th>Sample Blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzyme (µl)</strong></td>
<td>-</td>
</tr>
<tr>
<td><strong>Buffer (µl)</strong></td>
<td>200</td>
</tr>
<tr>
<td><strong>Substrate (µl)</strong></td>
<td>600</td>
</tr>
</tbody>
</table>

*Mix and incubate for 30 min at R. T.*

| **10% (w/v) TCA (µl)** | 200 | 200 |
| **Total Volume (ml)** | 1 | 1 |

*Centrifuge at 3000 rpm (Beckman benchtop) for 15 min at R. T.*

*Read \(A_{280 \text{ nm}}\).*

The buffer used was the Tris-acetate (pH 8.0) buffer. The kinetic parameters were calculated as described in section 5.3.4.
CHAPTER 4

ISOLATION AND PURIFICATION OF OSTRICH α2-ANTIPLASMIN, PLASMINOGEN AND PLASMIN

4.1. FIRST ISOLATION AND PURIFICATION OF OSTRICH α2AP 70
  4.1.1. ISOLATION AND PURIFICATION PROCEDURE ........... 70
  4.1.2. RESULTS AND DISCUSSION ................................. 72

4.2. SECOND ISOLATION AND PURIFICATION OF OSTRICH α2AP 76
  4.2.1. ISOLATION AND PURIFICATION PROCEDURE ........... 76
  4.2.2. RESULTS AND DISCUSSION ................................. 78

4.3. THIRD ISOLATION AND PURIFICATION OF OSTRICH α2AP 82
  4.3.1. ISOLATION AND PURIFICATION PROCEDURE ........... 82
  4.3.2. RESULTS AND DISCUSSION ................................. 84

4.4. ADDITIONAL ATTEMPTS AT PURIFYING OSTRICH α2AP 86
  4.4.1. IMMobilised-DYE CHROMATOGRAPHY ..................... 86
    4.4.1.1. MIMETIC ligand A6XL adsorbents ................... 86
    4.4.1.2. Reactive yellow 86-Toyopearl HW-65 .............. 87
  4.4.2. HPLC .......................................................... 88
    4.4.2.1. Q HyperDイ 10 ........................................... 88
    4.4.2.2. RP-HPLC ................................................ 89

4.5. ISOLATION AND PURIFICATION OF OSTRICH PLASMINOGEN ........................................ 90
  4.5.1. ISOLATION AND PURIFICATION PROCEDURE ........... 90
  4.5.2. RESULTS AND DISCUSSION ................................. 91

4.6. ACTIVATION OF OSTRICH PLASMINOGEN TO PLASMIN 92
  4.6.1. ACTIVATION PROCEDURE .................................. 92
  4.3.2. RESULTS AND DISCUSSION ................................. 93
CHAPTER 4

4.1. FIRST ISOLATION AND PURIFICATION OF OSTRICH $\alpha_2$AP

4.1.1. ISOLATION AND PURIFICATION PROCEDURE

The first isolation and purification procedure of ostrich $\alpha_2$AP, presented in diagram 4.1, was adapted from that of Moroi and Aoki (1976), Wiman and Collen (1977) and Christensen and Clemmensen (1978).

**DIAGRAM 4.1:** First isolation and purification procedure of ostrich $\alpha_2$AP.
(Procedure performed at 4 °C, unless otherwise stated.)

Frozen ostrich plasma (244 ml)

- Thaw in 37 °C water bath.

L-Lysine-Sepharose chromatography (2.6 x 11 cm)

- Load plasma (flowrate ~5 ml/hr) onto column equilibrated with 0.1 M sodium phosphate buffer (pH 7.4) + 3 mM EDTA.
- Wash column with equilibration buffer to baseline $A_{280\ nm}$ value.

Fall-through peak: IA (325 ml)

Ammonium sulfate precipitation

- Add, with stirring, solid ammonium sulfate to 30% saturation.
- Stir for 1 hr.
- Centrifuge: 17 700 g / 15 min / 4 °C.

Supernatant (343 ml)
- Add solid ammonium sulfate to 50% saturation with stirring.
- Stir overnight.
- Centrifuge: 17 700 g / 15 min / 4 °C.
- Dissolve pellet in 72 ml 75 mM sodium phosphate buffer, pH 7.6.
- Dialyse against dissolving buffer.

**1B (81 ml)**

**Toyopearl Super Q-650S chromatography (96 ml)**
- Stir 1B with Toyopearl Super Q-650S resin, equilibrated with 75 mM sodium phosphate buffer (pH 7.6), overnight.
- Remove supernatant (unadsorbed material) and wash resin batch-wise with equilibration buffer until A_{280 nm} of supernatant is constant.
- Pack into column (2.6 x 18.1 cm) and wash further with equilibration buffer to ensure a A_{280 nm} baseline value.
- Elute with linear salt gradient (flowrate ~100 ml/hr):
  - reservoir chamber = 300 ml 0.1 M sodium phosphate buffer (pH 7.6) + 0.2 M NaCl
  - mixing chamber = 300 ml 0.1 M sodium phosphate buffer (pH 7.6).
- Continue eluting with buffer containing 0.2 M NaCl.
- Pool tubes containing α₂AP activity.
- Dialyse exhaustively against running distilled water.
- Freeze-dry.

**1C (117.1 mg)**

**Plasminogen-Sepharose chromatography (1 x 20 cm)**
- Apply 1C (50 mg) to column equilibrated with 40 mM sodium phosphate buffer, pH 7.0 (flowrate ~4 ml/hr).
- Wash to baseline A_{280 nm} value with equilibration buffer.
- Elute with 10 mM EACA (flowrate ~60 ml/hr).
- Pool A_{280 nm} peak.
- Dialyse exhaustively against running distilled water.
- Freeze-dry.

**1D (5.7 mg)**
4.1.2. RESULTS AND DISCUSSION

The general first step in the isolation and purification of α₂AP from plasma is lysine-Sepharose chromatography since it removes plasminogen, a major contaminant in the isolation and purification of α₂AP, from the plasma. α₂AP therefore does not interact with the resin and has to undergo further purification.

The second purification step is usually a bulk method. Ammonium sulfate vs. PEG precipitation (6% (w/v)) was tested, with ammonium sulfate being the preferred method since it gave better yields and specific activities, and ammonium sulfate was easily removed by dialysis, as opposed to PEG. The ammonium sulfate fractionation range used was 30-50% saturation. The α₂AP precipitate thus collected was subsequently subjected to anion-exchange chromatography, the resin used here being Toyopearl Super Q-650S. The gradient elution profile of Toyopearl Super Q-650S chromatography of sample 1B is shown in figure 4.1.

![Gradient elution profile of Toyopearl Super Q-650S chromatography of sample 1B.](image)

**FIGURE 4.1:** Gradient elution profile of Toyopearl Super Q-650S chromatography of sample 1B. ⬤-•, absorbance at 280 nm; ⬤⬤-\[\text{NaCl}\] (M); - - - , inhibitory activity (U). Sample 1C, tubes 77-111.

Tubes showing α₂AP activity was pooled (figure 4.1), yielding sample 1C which then underwent ostrich plasminogen-Sepharose chromatography (figure 4.2).
Plasminogen-Sepharose affinity chromatography (or LBSI-Sepharose chromatography – see section 4.3,) is the classical final purification step of $\alpha_2$AP, since $\alpha_2$AP reversibly binds plasminogen, yielding pure $\alpha_2$AP. However, ostrich plasminogen used to make the affinity resin, which was purified during the first isolation and purification step of ostrich $\alpha_2$AP, had undergone autocatalysis as a result of a large volume of plasma being loaded very slowly onto the lysine-Sepharose column in the absence of additional protease inhibitors. The ostrich plasminogen sample therefore consisted of a significant amount of plasmin, the disadvantage of this being that plasmin pseudoirreversibly binds $\alpha_2$AP, thus possibly explaining the very low yield obtained after ostrich plasminogen-Sepharose chromatography (see table 4.1).

The first isolation and purification attempt is summarised in table 4.1.
TABLE 4.1: Summary of the first isolation and purification procedure of ostrich \(\alpha_2\)AP.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Volume or Mass</th>
<th>Total Protein (mg)</th>
<th>Total Activity (U)</th>
<th>Specific Activity (U/mg)</th>
<th>Purification Factor</th>
<th>Yield* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>244 ml</td>
<td>11164.13</td>
<td>284127.11</td>
<td>25.45</td>
<td>1</td>
<td>58.00</td>
</tr>
<tr>
<td>1A</td>
<td>325 ml</td>
<td>9132.81</td>
<td>489883.93</td>
<td>53.64</td>
<td>2.11</td>
<td>100.00</td>
</tr>
<tr>
<td>1B</td>
<td>80.5 ml</td>
<td>1634.44</td>
<td>140006.13</td>
<td>85.66</td>
<td>3.37</td>
<td>28.58</td>
</tr>
<tr>
<td>1C</td>
<td>117.10 mg</td>
<td>49.77</td>
<td>18897.17</td>
<td>379.69</td>
<td>14.92</td>
<td>3.86</td>
</tr>
<tr>
<td>1D</td>
<td>13.38 mg</td>
<td>0.16</td>
<td>1785.63</td>
<td>11160.21</td>
<td>438.52</td>
<td>0.36</td>
</tr>
</tbody>
</table>

* Yield expressed as percentage of total activity of plasmin(ogen)-free plasma.

The false low total activity of \(\alpha_2\)AP in plasma is due to the presence of plasmin(ogen) in plasma which was subsequently removed by L-lysine-Sepharose chromatography, thus explaining the increase in total activity and yield after the first purification step. This is also the reason for expressing all yields of \(\alpha_2\)AP relative to that obtained after the first purification step instead of to that of plasma. The low protein content of sample 1D was due to the poor quality of the sample, it being extremely heavy for its physical size and very solid instead of fluffy, despite exhaustive dialysis.

Ostrich plasminogen-Sepharose gave a purification factor of 438.5 for ostrich \(\alpha_2\)AP, giving a 29.4-fold increase in purity from Toyopearl Super-Q 650S chromatography. Moroi and Aoki (1976), however, obtained a purification factor of 2897.6 for human \(\alpha_2\)AP after human plasminogen-Sepharose chromatography, but this chromatographic step gave a 63.7-fold increase in purity from the previous step, viz. DEAE-Sephadex A-50 chromatography, DEAE-Sephadex A-50 being a weaker anion-exchanger than Toyopearl Super-Q 650S. Wiman and Collen (1977), on the other hand, obtained a purification factor of only 147.7 for human \(\alpha_2\)AP after human plasminogen-Sepharose chromatography, but this chromatographic step was used immediately after lysine-Sepharose chromatography and the purification factor was expressed relative to the lysine-Sepharose fraction instead of to that of plasma. Also, DEAE-Sephadex A-50 chromatography was used after plasminogen-Sepharose chromatography, and this gave a purification factor of 815.4, a 5.5-fold improvement on the previous chromatographic step (Wiman and Collen, 1977).
The yield of ostrich $\alpha_2$AP using this procedure was extremely low (0.36%). Moroi and Aoki (1976), however, recovered 32.5% human $\alpha_2$AP activity, while Wiman and Collen (1977) recovered 50.6% human $\alpha_2$AP activity using only lysine-Sepharose and plasminogen-Sepharose chromatographies, and 40.6% after DEAE-Sephadex A-50 chromatography.

The SDS-PAGE patterns of the samples after each purification step are shown in figure 4.3.

**FIGURE 4.3: SDS-PAGE patterns of the samples after each purification step of the first isolation and purification procedure.** All samples were non-reduced, unless otherwise stated. Lanes: 1, HMW markers; 2, plasma; 3, 1A; 4, 1B; 5, 1C; 6, 1D; 7, comm. human $\alpha_2$AP; 8, 1D (reduced); 9, comm. human $\alpha_2$AP (reduced).

Aligning sample 1D with comm. human $\alpha_2$AP, which showed $\alpha_2$AP to have a monomer of $M_r$ 67 K and a dimer of $M_r$ 149 K, ostrich $\alpha_2$AP showed a monomer of $M_r$ ~80 K and a dimer of $M_r$ 137 K which reduced to an $M_r$ of only 63 K (not clear from figure 4.3 due to a very low yield of ostrich $\alpha_2$AP). However, there were still a number of contaminating proteins present in the final $\alpha_2$AP sample according to non-reducing SDS-PAGE (figure 4.3, lane 6), but they all seemed to reduce to either the ~80 K or the 63 K component (figure 4.3, lane 8). As a result of this inexplicable phenomenon, as well as the very low yield of $\alpha_2$AP obtained with ostrich plasminogen-Sepharose chromatography, a new isolation and purification procedure was attempted, with the aim of replacing the ostrich plasminogen-Sepharose chromatography step.
4.2. SECOND ISOLATION AND PURIFICATION OF OSTRICH $\alpha_2$AP

4.2.1. ISOLATION AND PURIFICATION PROCEDURE

The second isolation and purification procedure of ostrich $\alpha_2$AP, outlined in diagram 4.2, was basically also adapted from Moroi and Aoki (1976).

**DIAGRAM 4.2: Second isolation and purification procedure of ostrich $\alpha_2$AP.**
(Procedure performed at 4 °C, unless otherwise stated.)

Frozen ostrich plasma (170 ml)
- Thaw in 37 °C water bath.

L-Lysine-Sepharose chromatography (2.6 x 10.3 cm)
- Load plasma (flowrate ~5 ml/hr) onto column equilibrated with 0.1 M sodium phosphate buffer (pH 7.4) + 3 mM EDTA.
- Wash column with equilibration buffer to baseline A$_{280 \text{ nm}}$ value.

Fall-through peak: 2A (278 ml)

Ammonium sulfate precipitation
- Add, with stirring, solid ammonium sulfate to 30% saturation.
- Stir for 2 hr.
- Centrifuge: 30 100 $g$ / 20 min / 4 °C.

Supernatant (295 ml)
- Add solid ammonium sulfate to 67.5% saturation with stirring; stir overnight.
- Centrifuge: 12 800 $g$ / 30 min / 4 °C.
- Dissolve pellet in 43 ml 75 mM sodium phosphate buffer, pH 7.6.
- Dialyse against dissolving buffer.

2B (80 ml)
77

**Toyopearl Super Q-650S chromatography (1.6 x 14.9 cm)**

- Load 2B onto Toyopearl Super Q-650S column equilibrated with 75 mM sodium phosphate buffer, pH 7.6 (flowrate ~ 8 ml/hr).
- Wash to baseline $A_{280\ nm}$ value with equilibration buffer.
- Elute with linear salt gradient (flowrate ~ 100 ml/hr): reservoir chamber = 200 ml 0.1 M sodium phosphate buffer (pH 7.6) + 0.2 M NaCl; mixing chamber = 200 ml 0.1 M sodium phosphate buffer (pH 7.6).
- Continue eluting with buffer containing 0.2 M NaCl.
- Pool tubes containing $\alpha_2$AP activity.
- Dialyse exhaustively against running distilled water.
- Freeze-dry.

2C (336 mg)

**Sephadex G-200 chromatography (1.6 x 100.0 cm)**

- Load 2C (50 mg) onto column equilibrated with 40 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl (flowrate ~ 8 ml/hr).
- Wash with equilibration buffer at same flowrate.
- Pool tubes containing $\alpha_2$AP activity.
- Dialyse exhaustively against running distilled water.
- Freeze-dry.

2D (19 mg)

**Hydroxylapatite chromatography (1.1 x 16.5 cm)**

- Load 2D (flowrate ~ 4 ml/hr) onto column equilibrated with 10 mM sodium phosphate buffer, pH 7.0.
- Wash with 75 mM sodium phosphate buffer, pH 7.0.
- Elute with 110 and 150 mM sodium phosphate buffer, pH 7.0 (flowrate ~ 50 ml/hr).
- Pool $A_{280\ nm}$ peak containing $\alpha_2$AP activity and the M, 80 K component.
- Dialyse exhaustively against distilled water.
- Freeze-dry.

4.2.2. RESULTS AND DISCUSSION

2E (17.86 mg)
This procedure bypassed the usual plasminogen-Sepharose affinity chromatography step by employing hydroxylapatite chromatography, preceded by gel filtration. An additional, minor change in this procedure was that the ammonium sulfate fractionation range on the lysine-Sepharose fall-through peak was increased from 30-50% saturation to 30-67.5%, combining that used by Moroi and Aoki (1976) and Christensen and Clemmensen (1978).

The elution profiles of Sephadex G-200 chromatography of sample 2C and hydroxylapatite chromatography of sample 2D, which, according to activity assays and SDS-PAGE (result not shown), was the second ascending peak, are shown in figures 4.4 and 4.5, respectively.

FIGURE 4.4: Sephadex G-200 chromatography of sample 2C. ---, \( A_{280 \text{ nm}} \); ●, inhibitory activity (U). Sample 2D, tubes 30-43.
FIGURE 4.5: Hydroxylapatite chromatography of sample 2D.

\( A_{220\text{nm}} \); \( [\text{NaCl}] (\text{M}) \); \( \bullet \), inhibitory activity (U).

Sample 2E, tubes 172-186.

According to specific activity and SDS-PAGE results (not shown), \( \alpha_2 \)AP bound to hydroxylapatite eluted between 0.075 and 0.11 M sodium phosphate concentrations.

A summary of the second isolation and purification attempt is given in table 4.2.

**TABLE 4.2: Summary of the second isolation and purification procedure of ostrich \( \alpha_2 \)AP.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Volume or Mass</th>
<th>Total Protein (mg)</th>
<th>Total Activity (U)</th>
<th>Specific Activity (U/mg)</th>
<th>Purification Factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>170 ml</td>
<td>8321.24</td>
<td>300979.25</td>
<td>36.17</td>
<td>1.00</td>
<td>69.02</td>
</tr>
<tr>
<td>2A</td>
<td>278 ml</td>
<td>6750.27</td>
<td>436067.44</td>
<td>64.60</td>
<td>1.79</td>
<td>100.00</td>
</tr>
<tr>
<td>2B</td>
<td>80 ml</td>
<td>2790.45</td>
<td>345959.99</td>
<td>123.98</td>
<td>3.43</td>
<td>79.34</td>
</tr>
<tr>
<td>2C</td>
<td>336.00 mg</td>
<td>264.07</td>
<td>66099.36</td>
<td>250.31</td>
<td>6.92</td>
<td>15.16</td>
</tr>
<tr>
<td>2D</td>
<td>127.68 mg</td>
<td>84.48</td>
<td>28109.88</td>
<td>332.74</td>
<td>9.20</td>
<td>6.45</td>
</tr>
<tr>
<td>2E</td>
<td>120.02 mg</td>
<td>1.95</td>
<td>51627.07</td>
<td>26475.42</td>
<td>731.97</td>
<td>11.84</td>
</tr>
</tbody>
</table>
As for sample 1D in section 4.1.2., the low protein content of sample 2E was due to the poor quality of the sample. The unusual increase in total activity and yield of $\alpha_2$AP from sample 2D to 2E is due to the very high specific activity of sample 2E and the fact that total activity is calculated by extrapolating from the linear portions of specific activity vs. inhibitor concentration curves, whereas in actual fact the curves level off after a certain inhibitor concentration (i.e. they are hyperbolic).

Figure 4.6 depicts the SDS-PAGE patterns of three samples of the second isolation and purification procedure (the patterns of the other three samples are basically the same as those for the first isolation and purification procedure).

![SDS-PAGE patterns of three samples of the second isolation and purification procedure. All samples were non-reduced, unless otherwise stated. Lanes: 1, HMW markers; 2, 2C; 3, 2D; 4, 2E; 5, 2E (reduced); 6, HMW markers (reduced).](image)

According to the purification factors, yields and SDS-PAGE patterns, hydroxylapatite chromatography, in conjunction with gel filtration, proved to be a good substitute for plasminogen-Sepharose chromatography, with sample 2E having a 1.7-fold larger purification factor than sample 1D. Also, hydroxylapatite chromatography, in conjunction with gel filtration, gave a 3.6-fold larger increase in purity from Toyopearl Super-Q 650S chromatography than did plasminogen-Sepharose chromatography. However, Moroi and Aoki (1976) used hydroxylapatite chromatography (without gel filtration) after, and not in place of, plasminogen-Sepharose chromatography, and as a result obtained a 3.0-fold increase in the purification factor and a 2.2-fold decrease in yield of human $\alpha_2$AP activity.
Sample 2E was still not pure according to non-reducing SDS-PAGE, although the reduced sample revealed only two components, M, 77 and 63 K, which corresponds to the assumed α₂AP monomer and reduced dimer obtained in section 4.1.2., respectively. In a final attempt to purify α₂AP to homogeneity, a third isolation and purification procedure was employed, using a more sophisticated affinity column than the one used in the first isolation procedure. Affinity chromatography was revisited due to its potential success in separating diverse molecules, but this time the anticipated problem, which is the possible presence of the plasmin active site in the ligand, was eliminated, the ligand used here being a fragment of the plasminogen molecule containing the first three lysine-binding sites (LBSI), but lacking the light chain which contains the active site (see section 1.5.1.1.) (Wiman, 1980).
4.3. THIRD ISOLATION AND PURIFICATION OF OSTRICH $\alpha_2$AP

4.3.1. ISOLATION AND PURIFICATION PROCEDURE

The third isolation and purification procedure of ostrich $\alpha_2$AP, represented in diagram 4.3, was based on the procedure used by Wiman (1980).

**Diagram 4.3: Third isolation and purification procedure of ostrich $\alpha_2$AP.**
(Procedure performed at 4 °C, unless otherwise stated.)

- **Frozen ostrich plasma (503 ml)**
  - Thaw in 37 °C water bath.
  - Immediately add 100 000 KIU Trasylol.

- **L-Lysine-Sepharose chromatography (150 ml)**
  - Stir plasma overnight with L-lysine-Sepharose resin equilibrated with 0.1 M sodium phosphate buffer (pH 7.4) + 3 mM EDTA.
  - Remove supernatant by suction on Büchner funnel (R.T.).

- **Unadsorbed material: 3A (865 ml)**

- **Ammonium sulfate precipitation**
  - Add, with stirring, solid ammonium sulfate to 30% saturation.
  - Stir for 2 hr.
  - Centrifuge: 17 700 g / 1.5 hr / 4 °C.

- **Supernatant (905 ml)**
  - Add solid ammonium sulfate to 67.5% saturation with stirring: stir overnight.
  - Centrifuge: 17 700 g / 1 hr / 4 °C.
  - Dissolve pellet in small volume 0.1 M sodium phosphate buffer (pH 7.4) + 3 mM EDTA.
  - Dialyse exhaustively against running deionised water.
  - Centrifuge: 17 700 g / 1 hr / 4 °C.
  - Freeze-dry.
3B (6.67 g)

**Toyopearl Super Q-650S chromatography** (37 ml)

- Stir 1 g of 3B with Toyopearl Super Q-650S resin, equilibrated with 50 mM sodium phosphate buffer (pH 7.4), overnight.
- Remove supernatant (unadsorbed material) by suction on Büchner funnel at R.T.
- Wash with equilibration buffer on Büchner funnel to an A$_{280}$nm baseline at R.T.
- Pack resin into column (1.6 x 18.7 cm).
- Wash further with equilibration buffer to ensure an A$_{280}$nm baseline value.
- Elute with linear salt gradient (flowrate ~90 ml/hr):
  - reservoir chamber = 150 ml 50 mM sodium phosphate buffer (pH 7.4) + 0.2 M NaCl; mixing chamber = 150 ml 50 mM sodium phosphate buffer (pH 7.4).
- Continue eluting with buffer containing 0.2 M NaCl.
- Pool tubes according to SDS-PAGE patterns.
- Dialyse exhaustively against running distilled water.
- Freeze-dry.

3C (12.5 mg)

**LBSI-Sepharose chromatography** (6 ml)

- Apply 3C to column (1 x 7.6 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.4) (flowrate ~1 ml/hr).
- Wash to baseline A$_{280}$nm value with equilibration buffer.
- Wash with 20 mM sodium phosphate buffer (pH 7.4)
- Elute with 50 mM EACA in 20 mM sodium phosphate buffer (pH 7.4) (flowrate ~60 ml/hr).
- Dialyse exhaustively against running distilled water.
- Pool A$_{280}$nm peak.
- Freeze-dry.

3D (7.23 mg)
4.3.2. RESULTS AND DISCUSSION

This procedure is basically the same as the first isolation and purification procedure, except that the final affinity column used here (viz. LBSI-Sepharose) is different from the one formerly used (viz. plasminogen-Sepharose), as explained in section 4.2.2. The elution profile of sample 3C on the LBSI-Sepharose column is shown in figure 4.7.

![LBSI-Sepharose chromatography of sample 3C](image)

**FIGURE 4.7: LBSI-Sepharose chromatography of sample 3C.**
Start of elution with 20 mM sodium phosphate buffer (pH 7.4) and 50 mM EACA, tubes 34 and 60, respectively; sample 3D, tubes 60-67.

A summary of the third isolation and purification procedure is given in table 4.3.

**TABLE 4.3: Summary of the third isolation and purification procedure of ostrich α₂AP.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Volume or Mass</th>
<th>Total Protein (mg)</th>
<th>Total Activity (U)</th>
<th>Specific Activity (U/mg)</th>
<th>Purification Factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>503 ml</td>
<td>23817.83</td>
<td>733827.34</td>
<td>30.81</td>
<td>1.00</td>
<td>54.79</td>
</tr>
<tr>
<td>3A</td>
<td>865 ml</td>
<td>22655.44</td>
<td>1339389.61</td>
<td>59.12</td>
<td>1.92</td>
<td>100.00</td>
</tr>
<tr>
<td>3B</td>
<td>6.70 g</td>
<td>4742.60</td>
<td>297787.90</td>
<td>62.79</td>
<td>2.04</td>
<td>22.23</td>
</tr>
<tr>
<td>3C</td>
<td>83.55 mg</td>
<td>22.24</td>
<td>10525.75</td>
<td>473.28</td>
<td>15.36</td>
<td>0.79</td>
</tr>
<tr>
<td>3D</td>
<td>48.45 mg</td>
<td>7.27</td>
<td>3637.91</td>
<td>500.40</td>
<td>16.24</td>
<td>0.27</td>
</tr>
</tbody>
</table>
The relatively low yield obtained after ammonium sulfate fractionation in this isolation and purification procedure could possibly be due to the fact that the sample was dialysed and freeze-dried owing to its large quantity. This could also account for the relatively low purification factor. The reason for the large increase in purification fold and decrease in yield obtained after Toyopearl Super Q-650S chromatography, as compared to the other purification procedures, is that the tubes were not only pooled according to $\alpha_2$AP activity, as before, but more specifically according to SDS-PAGE patterns.

Sample 3D had a 27.0- and 45.1-fold lower purification factor than samples 1D and 2E, respectively. LBSI-Sepharose chromatography only resulted in a 1.1-fold improvement in the purification fold from Toyopearl Super-Q 650S chromatography, but the Toyopearl Super-Q 650S sample obtained in this isolation procedure was purer than those obtained from the other two isolation and purification procedures. The yield of active ostrich $\alpha_2$AP was the lowest of all the three isolation and purification procedures used (0.27%), but LBSI-Sepharose chromatography only caused a 2.9-fold decrease in the yield, the major loss occurring during Toyopearl Super Q-650S chromatography.

SDS-PAGE patterns of the final sample of the third isolation and purification procedure are depicted in figure 4.8 (the patterns of the other samples are similar to those of the first isolation and purification procedure and are therefore not shown).

**FIGURE 4.8:** SDS-PAGE patterns of the final sample of the third isolation and purification procedure. All samples were non-reduced, unless otherwise stated. Lanes: 1, HMW markers; 2, 3D; 3, comm. human $\alpha_2$AP; 4-6, fractions in lanes 1-3 (reduced), respectively.
According to the purification fold the LBSI-Sepharose column did not seem to be very effective, but SDS-PAGE contradicts this, showing it to purify ostrich $\alpha_2$AP to homogeneity ($M_t$ 84 K). A possible explanation for this discrepancy, and the low yield of active ostrich $\alpha_2$AP obtained, could be that plasma obtained from the ostrich neck, which is inferior to plasma obtained from venous blood (see section 3.1.1.), was used for this isolation and purification procedure. Also, as a result, $\alpha_2$AP was most probably present in ostrich plasma also as the non-plasmingen-binding form (see section 1.2.4.) since the LBSI-Sepharose fall-through peak (tubes 1-12 in figure 4.7), which contained two major bands with $M_s$ of ~63 and 55 K, also showed $\alpha_2$AP activity. Therefore, only the plasminogen-binding form was purified, resulting in the low purification factor and yield obtained, and the single band on SDS-PAGE.

4.4. ADDITIONAL ATTEMPTS AT PURIFYING OSTRICH $\alpha_2$AP

4.4.1. IMMOBILISED-DYE CHROMATOGRAPHY

Immobilised dye-chromatography was explored due to its impressive capability to separate diverse molecules (see section 3.5.5.) and recent popularity.

4.4.1.1. MIMETIC LIGAND A6XL ADSORBENTS

The PIKSI module, containing 10 different 1 ml mimetic ligand A6XL adsorbents, was used to identify the most suitable dye adsorbent for the purification of ostrich $\alpha_2$AP from plasma.

Plasma (2 ml) was loaded onto each adsorbent and thereafter was washed with the equilibration buffer (25 mM sodium phosphate, pH 6.0) to an $A_{280\text{ nm}}$ baseline. The pH of the wash was increased to 8.0, after which the adsorbed proteins were eluted with 1 M NaCl.

According to activity assays, MIMETICs yellow-1 and yellow-2 A6XL gave the best purification of all 10 adsorbents (purification factors of 89.5 and 71.5, respectively) and therefore a yellow dye resin, viz. reactive yellow 86-Toyopearl 650M, was made for preparative purposes.
4.4.1.2. REACTIVE YELLOW 86-TOYOPEARL HW-65

Sample 3B (1 g) was stirred with 45 ml reactive yellow 86-Toyopearl HW-65 overnight, the unadsorbed material was removed by suction on a Büchner funnel and the resin was washed with the equilibration buffer (25 mM sodium phosphate, pH 6.0) to an A$_{280}$ nm baseline. The resin was further washed with 25 mM sodium phosphate buffer (pH 8.0) on the Büchner funnel to remove non-specifically bound proteins. The resin was packed into a column (1.6 x 22.5 cm) and the bound proteins were eluted first with 0.25 M NaCl and then with 0.5 M NaCl.

Figure 4.9 represents the elution profile of sample 3B on reactive yellow 86-Toyopearl HW-65.

![FIGURE 4.9: Reactive yellow 86-Toyopearl HW-65 chromatography of sample 3B.](image)

According to activity assays, α$_2$AP was eluted between 0.25 and 0.5 M NaCl, giving a purification factor of 4.33 and a yield of 1.47%. However, SDS-PAGE (result not shown) did not show the presence of the M, 80 K component supposedly representing ostrich α$_2$AP and hence the component showing activity was unknown. On the whole, this chromatography step did not seem to be very effective and was therefore omitted.
4.4.2. HPLC
Owing to its high versatility and widespread use, HPLC was also exploited.

4.4.2.1. Q HYPERD\textsuperscript{10}
As mentioned in section 3.5.2.2., Q HyperD\textsuperscript{10}, a strong anion-exchanger, was used in an attempt to improve on Toyopearl Super Q-650S due its very high resolution.

Sample 3C (2.43 mg) was applied to a 1.7 ml column which was washed to an $A_{280\text{ nm}}$ baseline with the equilibration buffer (20 mM Tris-HCl, pH 7.4), all within a period of 10 min. Adsorbed proteins were eluted by a salt gradient of 0-1 M NaCl over 60 min at 1 ml/min. Tubes were collected by hand according to the elution profile (figure 4.10) and analysed by SDS-PAGE.

![FIGURE 4.10: Q HyperD\textsuperscript{10} HPLC of sample 3C.](image)

The elution profile showed a separation of peaks, but there was only a 1.1-fold increase in purification of $\alpha_2$AP from Toyopearl Super Q-650S, with a drastic decrease in yield (~48-fold). SDS-PAGE (not shown) confirmed the small improvement in purity of $\alpha_2$AP. This attempt was therefore not successful.
4.4.2.2. RP-HPLC
RP-HPLC was attempted since it is a widely used technique for the separation of a number of proteins (see section 3.5.6.).

Sample 2C (9.83 mg) was applied to a Capcell Pak C_{18} ODS/AGS120 column (4.6 x 250 mm) which was washed to an A_{280 nm} baseline with the equilibration buffer (0.1% TFA), all within 10 min. The adsorbed proteins were eluted by a 0-70% acetonitrile gradient in 0.1% TFA over a period of 70 min at 1 ml/min.

![RP-HPLC of sample 3C on a Capcell Pak C_{18} ODS/AGS120 column.](FIGURE 4.11)

The elution profile (figure 4.11) revealed two peaks. SDS-PAGE (result not shown) showed the first peak to contain a component of M_{r} ~63 K and the second one a doublet, consisting of the M_{r} ~63 K component and another component of M_{r} ~55 K, both of which, upon reduction, yielded a single component (M_{r} ~80 K) which aligned with the proposed ostrich \( \alpha_{2} \)AP component. These two components corresponded with those obtained in the LBSI-Sepharose fall-through peak, mentioned in section 4.3.2., which were proposed to be different forms of \( \alpha_{2} \)AP (see section 1.2.4.). Unfortunately, activity assays could not be performed on these two fractions since the buffer conditions used inactivated \( \alpha_{2} \)AP. The possibility of the two being different forms of \( \alpha_{2} \)AP was therefore investigated by performing amino acid and N-terminal sequence analyses on the two components, but no significant match was found with any part of the \( \alpha_{2} \)AP molecule (results not shown).
4.5. ISOLATION AND PURIFICATION OF OSTRICH PLASMINOGEN

4.5.1. ISOLATION AND PURIFICATION PROCEDURE

The isolation and purification procedure of ostrich plasminogen, an adaptation of that of Deutsch and Mertz, 1970), is outlined in diagram 4.4.

**DIAGRAM 4.4: Isolation and purification procedure of ostrich plasminogen.**
(Procedure performed at 4 °C, unless otherwise stated.)

- **Frozen ostrich plasma (503 ml)**
  - Thaw in 37 °C water bath.
  - Immediately add 100 000 KIU Trasylol.

- **L-Lysine-Sepharose chromatography (150 ml)**
  - Stir plasma overnight with L-lysine-Sepharose resin equilibrated with 0.1 M sodium phosphate buffer (pH 7.4) + 3 mM EDTA + 10 000 KIU/l Trasylol.
  - Remove supernatant by suction on Büchner funnel at R.T.
  - Wash with 0.3 M sodium phosphate buffer (pH 7.4) + 3 mM EDTA + 5 000 KIU/l Trasylol on Büchner funnel at R.T. until an A$_{280}$ nm baseline.
  - Re-equilibrate with equilibration buffer on Büchner funnel at R.T.
  - Pack resin into column (2.6 x 25.5 cm) and wash further with equilibration buffer to ensure an A$_{280}$ nm baseline.
  - Elute with equilibration buffer containing 10 mM EACA (flowrate ~80 ml/hr).
  - Pool A$_{280}$ nm peak, omitting ascending and descending tails of peak.
  - Dialyse exhaustively against running distilled water.
  - Freeze-dry.

**Ostrich plasminogen (226.16 mg)**
4.5.2. RESULTS AND DISCUSSION

The elution profile of L-lysine-Sepharose chromatography of ostrich plasma is given in figure 4.12.

![Elution profile of L-lysine-Sepharose chromatography of ostrich plasma. Ostrich plasminogen, tubes 12-26.](image)

**FIGURE 4.12:** Elution profile of L-lysine-Sepharose chromatography of ostrich plasma. Ostrich plasminogen, tubes 12-26.

Figure 4.13 shows the SDS-PAGE pattern of ostrich plasminogen and it is evident that a highly purified molecule was obtained.

![SDS-PAGE pattern of ostrich plasminogen. Lanes: 1, HMW markers; 2, ostrich plasminogen.](image)

**FIGURE 4.13:** SDS-PAGE pattern of ostrich plasminogen. Lanes: 1, HMW markers; 2, ostrich plasminogen.
4.6. ACTIVATION OF OSTRICH PLASMINOGEN TO PLASMIN

4.6.1. ACTIVATION PROCEDURE

The activation procedure of ostrich plasminogen to plasmin was adapted from Robbins and Summaria (1970) as outlined in diagram 4.5.

**Diagram 4.5: Activation procedure of ostrich plasminogen to plasmin.**

**Ostrich plasminogen (4 mg)**

- Dissolve in 120 µl buffer (50 mM Tris-20 mM lysine-100 mM NaCl-1 mM EDTA, pH 9.0).
- Adjust to 25% glycerol by adding 40 µl 99.5% glycerol.
- Add 4 µl human urokinase solution (600 µg/mg plasminogen).

Allow to stand at R.T. for 4 hr.

- Cool to 0 °C.
- Dilute 10-fold with ice-cold distilled water.
- Adjust to pH 6.2 with 1 M KH₂PO₄.
- Precipitate enzyme by adding 0.51 g ammonium sulfate.
- Allow to stand for 2 hr at 4 °C.
- Centrifuge at 3 000 g for 30 min at 4 °C.
- Dissolve precipitate in a small volume of ice-cold water.
- Clarify solution by centrifugation (3 000 g / 30 sec / 4 °C).
- Dialyse a few hours against a large volume of distilled water (4 °C).
- Freeze-dry.

**Ostrich plasmin**
4.6.2. RESULTS AND DISCUSSION

The activation procedure was at first optimised with regards the urokinase:plasminogen concentration and incubation time of plasminogen with urokinase. The activation of ostrich plasminogen to plasmin with various urokinase:plasminogen concentrations at various incubation times are shown in figure 4.14.

![Activation of ostrich plasminogen to plasmin with various urokinase:plasminogen concentrations at various incubation times.](image)

**FIGURE 4.14:** Activation of ostrich plasminogen to plasmin with various urokinase:plasminogen concentrations at various incubation times.

◆, 600 µg/mg; ▲, 10 µg/mg; □, 5 µg/mg; ○, 0 µg/mg.

The activation of plasminogen with 600 µg urokinase/mg plasminogen was followed by SDS-PAGE at various incubation times (figure 4.15), the major component at 55 K being urokinase.
According to figure 4.14 the optimum urokinase:plasminogen concentration was 600 µg/mg and the optimum incubation time of plasminogen with urokinase at this concentration was 4 hr, with a decrease in plasmin activity after this time. On the other hand, figure 4.15 showed maximal activation with 600 µg urokinase/mg plasminogen only after an incubation time of 22.5 hr. A possible explanation for this discrepancy is that urokinase at a concentration of 600 µg/mg plasminogen was exhausted after 4 hr and autocatalysis of plasminogen by the resulting plasmin continued thereafter by removal of the preactivation peptide and not also by cleavage of the active site, as was the case with urokinase. Four hr was therefore used as the optimum incubation time for a urokinase concentration of 600 µg/mg plasminogen. The freeze-dried ostrich plasmin sample obtained after incubation with 600 µg urokinase/mg plasminogen for 4 hr is compared to comm. bovine plasmin on SDS-PAGE in figure 4.16.
Ideally, a much higher urokinase:plasminogen concentration should be used to achieve complete activation of ostrich plasminogen by human urokinase in the shortest possible time, but this was financially unfeasible. Robbins and Summaria (1970), on the other hand, used a urokinase:plasmingen concentration of only 0.25 µg/mg, but an incubation time of 20 hr, for the activation of human plasminogen with human urokinase. Activator concentration and incubation time do, however, depend on the specific activity of the activator and probably also the sources of activator used. Therefore, ostrich urokinase should have been used instead for the activation of ostrich plasminogen, but it was unavailable. Interestingly, it has been reported that some species require much higher concentrations of the activator streptokinase to achieve complete activation of plasminogen than other species, but that this is generally not the case for urokinase (Robbins and Summaria, 1970). The ostrich could, however, be an exception.
CHAPTER 5

CHARACTERISATION OF OSTRICH α₂-ANTIPLASMIN, PLASMINOGEN AND PLASMIN

5.1. CHARACTERISATION OF OSTRICH α₂AP ........................................ 98
  5.1.1. PHYSICOCHEMICAL CHARACTERISATION ............ 98
    5.1.1.1. Molecular weight determination ......................... 98
    5.1.1.2. pI Determination ........................................... 99
    5.1.1.3. N-Terminal sequence determination ...................... 100
  5.1.2. KINETIC CHARACTERISATION ...................................... 100
    5.1.2.1. Inhibitory effect on different serine proteases ......... 100
      5.1.2.1.1. Plasmin ............................................. 101
      5.1.2.1.2. Trypsin ............................................. 102
      5.1.2.1.3. Chymotrypsin .................................... 102
      5.1.2.1.4. Summary ........................................... 103
    5.1.2.2. Comparison with other inhibitors ....................... 104
      5.1.2.2.1. Aprotinin ....................................... 104
      5.1.2.2.2. DFP ............................................... 104
      5.1.2.2.3. EACA ............................................... 105
      5.1.2.2.4. Summary ......................................... 106

5.2. CHARACTERISATION OF OSTRICH PLASMINOGEN .............. 107
  5.2.1. PHYSICOCHEMICAL CHARACTERISATION ............ 107
    5.2.1.1. Molecular weight determination ......................... 107
    5.2.1.2. pI Determination ........................................... 107
    5.2.1.3. Amino acid composition .................................. 107
    5.2.1.4. N-Terminal sequence determination .................... 109
5.3. CHARACTERISATION OF OSTRICH PLASMIN ................. 110

5.3.1. PHYSICOCHEMICAL CHARACTERISATION ............ 110
  5.3.1.1. Molecular weight determination ......................... 110
  5.3.1.2. pI Determination ........................................ 110
  5.3.1.3. Amino acid composition .................................. 110
  5.3.1.4. N-Terminal sequence determination .................. 112

5.3.2. KINETIC CHARACTERISATION ............................ 113
  5.3.2.1. pH Optimum ............................................... 113
  5.3.2.2. Temperature optimum ..................................... 113
  5.3.2.3. Thermodynamic parameters ............................. 114
  5.3.2.4. Kinetic parameters ...................................... 117
CHAPTER 5

5.1. CHARACTERISATION OF OSTRICH $\alpha_2$AP

5.1.1. PHYSICOCHEMICAL CHARACTERISATION

5.1.1.1. MOLECULAR WEIGHT DETERMINATION

All molecular weight determinations were made using SDS-PAGE. However, $M_r$ values of glycoproteins are usually overestimated on SDS-PAGE and human $\alpha_2$AP has a carbohydrate content of 11-14% (Wiman, 1981). According to the SDS-PAGE patterns given in figure 4.8, non-reduced and reduced ostrich $\alpha_2$AP revealed an $M_r$ of 83.6 K, while comm. human $\alpha_2$AP showed an $M_r$ of 67.0 K. The non-homogeneous ostrich $\alpha_2$AP sample 2E (figure 4.6), however, revealed a monomer of $M_r$ 77.3 K. According to literature, human $\alpha_2$AP has an $M_r$ of 70 K before reduction (Wiman and Collen, 1977) and 67 K after reduction (Moroi and Aoki, 1976; Wiman and Collen, 1977), while bovine $\alpha_2$AP has an $M_r$ of 70 K after reduction (Christensen and Sottrup-Jensen, 1992). A possible explanation for the relatively large $M_r$ value for ostrich $\alpha_2$AP can be that ostrich $\alpha_2$AP has a larger carbohydrate content than both human and bovine $\alpha_2$APs. The molecular weight of human $\alpha_2$AP deduced from the cDNA sequence and carbohydrate content is approximately 58 kDa (Aoki et al., 1993), thus showing SDS-PAGE to overestimate by ~9 K for human $\alpha_2$AP.

Comm. human $\alpha_2$AP also revealed a dimer ($M_r$ 148.7 K) under non-reducing conditions (figure 4.8), in keeping with literature, but which is of a larger $M_r$ than reported (132 K; Wiman and Collen, 1977). However, the ostrich $\alpha_2$AP sample that has been purified to homogeneity, viz. 3D, did not show the presence of a dimer (figure 4.8), but samples 1D and 2E showed a component of $M_r$ 136.5 K which reduced to a component slightly smaller than the monomer, $M_r$ 63.2 K (figures 4.3 and 4.6, respectively; $M_r$ values according to figure 4.6).
5.1.1.2. pI DETERMINATION

The PAG-IEF patterns of both ostrich and comm. human $\alpha_2$AP are shown in figure 5.1, along with those of ostrich plasminogen and plasmin.

FIGURE 5.1: PAG-IEF patterns of ostrich $\alpha_2$AP, plasminogen and plasmin.
Lanes: 1, ostrich plasminogen; 2, ostrich plasmin; 3, comm. bovine plasmin; 4, pI markers (3.6-6.6); 5, ostrich $\alpha_2$AP; 6, comm. human $\alpha_2$AP; 7, pI markers.

According to figure 5.1, both ostrich and comm. human $\alpha_2$AP showed two major isoelectric forms which are comparable. The pI values for the two forms are 6.18 and 3.85 for ostrich $\alpha_2$AP and, for comm. human $\alpha_2$AP, 6.11 and 3.92. These values are, however, slightly different from those reported in literature for human $\alpha_2$AP; the plasminogen-binding form has multiple isoelectric points with a major component of pI 4.69 and a minor one of pI 4.92, whereas the non-plasminogen-binding form has only one component of pI 4.59 (Lijnen and Collen, 1986).
5.1.1.3. N-TERMINAL SEQUENCE DETERMINATION
The N-terminal sequence obtained for ostrich \( \alpha_2 \)AP (performed in the laboratory of Prof. Koji Muramoto, Sendai, Japan) is shown in table 5.1, aligned with those of human and bovine \( \alpha_2 \)APs (Christensen et al., 1994).

**TABLE 5.1:** Comparison of N-terminal sequence of ostrich \( \alpha_2 \)AP with those of human and bovine \( \alpha_2 \)APs. Amino acids identical to those of ostrich \( \alpha_2 \)AP are shaded.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ostrich</strong></td>
<td>Leu</td>
<td>Gln</td>
<td>Val</td>
<td>Asp</td>
<td>Tyr</td>
<td>Leu</td>
<td>Val</td>
<td>Leu</td>
<td>Glu</td>
<td>Val</td>
<td>Ala</td>
</tr>
<tr>
<td><strong>Human</strong></td>
<td>Asn</td>
<td>Gln</td>
<td>Glu</td>
<td>Gln</td>
<td>Val</td>
<td>Ser</td>
<td>Pro</td>
<td>Leu</td>
<td>Thr</td>
<td>Leu</td>
<td>Leu</td>
</tr>
<tr>
<td><strong>Bovine</strong></td>
<td>Ala</td>
<td>Gln</td>
<td>Gln</td>
<td>Lys</td>
<td>Leu</td>
<td>Pro</td>
<td>Pro</td>
<td>Leu</td>
<td>Ser</td>
<td>Leu</td>
<td>Leu</td>
</tr>
</tbody>
</table>

Of the 11 N-terminal amino acids determined for ostrich \( \alpha_2 \)AP, only 2 are identical to those of either human or bovine \( \alpha_2 \)AP, as compared to the 5 that human and bovine \( \alpha_2 \)APs have in common.

5.1.2. KINETIC CHARACTERISATION

5.1.2.1. INHIBITORY EFFECT ON DIFFERENT SERINE PROTEASES
As mentioned in section 1.5.2.1., *in vivo* \( \alpha_2 \)AP only binds plasmin and, to a lesser extent, trypsin (Lijnen and Collen, 1986), but *in vitro* it has a broad inhibitory spectrum, reacting very rapidly with plasmin, rapidly with trypsin (Wiman and Collen, 1978), moderately with chymotrypsin (Wiman, 1981), slowly with kallikrein, factor Xa and thrombin (Wiman, 1981; Lijnen and Collen, 1986), and very slowly with urokinase (Moroi and Aoki, 1976) and tissue-plasminogen activator (Wiman, 1981). The inhibitory effect of ostrich \( \alpha_2 \)AP on its three most important target enzymes, viz. plasmin, trypsin and chymotrypsin, was investigated *in vitro*. 
5.1.2.1.1. Plasmin
The inhibitory effects of ostrich and comm. human $\alpha_2$APs on ostrich plasmin are shown in figure 5.2, while their inhibitory effects on comm. bovine plasmin are shown in figure 5.3.

**FIGURE 5.2:** Inhibitory effects of ostrich and comm. human $\alpha_2$APs on ostrich plasmin. ▲, ostrich $\alpha_2$AP; ■, comm. human $\alpha_2$AP.

**FIGURE 5.3:** Inhibitory effects of ostrich and comm. human $\alpha_2$APs on comm. bovine plasmin. ■, ostrich $\alpha_2$AP; ▲, comm. human $\alpha_2$AP.
5.1.2.1.2. Trypsin
The inhibitory effects of ostrich and comm. human $\alpha_2$APs on comm. bovine pancreatic trypsin are shown in figure 5.4.

**FIGURE 5.4:** Inhibitory effects of ostrich and comm. human $\alpha_2$APs on comm. bovine pancreatic trypsin. ◆, ostrich $\alpha_2$AP; ▲, comm. human $\alpha_2$AP.

5.1.2.1.3. Chymotrypsin
The inhibitory effects of ostrich and comm. human $\alpha_2$APs on comm. bovine pancreatic chymotrypsin are shown in figure 5.5.

**FIGURE 5.5:** Inhibitory effects of ostrich and comm. human $\alpha_2$APs on comm. bovine pancreatic chymotrypsin. ◆, ostrich $\alpha_2$AP; ▲, comm. human $\alpha_2$AP.
5.1.2.1.4. Summary
The inhibitory effects of ostrich and comm. human \( \alpha_2 \)APs on ostrich and comm. bovine plasmins, comm. bovine trypsin and comm. bovine chymotrypsin are summarised in table 5.2.

\[ \text{TABLE 5.2: Summary of inhibitory effects of ostrich and comm. human } \alpha_2 \text{APs on a few serine proteases. Values represent inhibitory activity (U) } / \text{ (I/E) (mol/mol), corresponding to the slopes of the linear portions of the inhibition curves above, where 1 unit of inhibitory activity results in a 50\% loss in enzyme activity.} \]

<table>
<thead>
<tr>
<th>Serine Protease</th>
<th>Ostrich ( \alpha_2 )AP (r^2)</th>
<th>Comm. Human ( \alpha_2 )AP (r^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ostrich Plasmin</td>
<td>14.53 (0.819)</td>
<td>6.79</td>
</tr>
<tr>
<td>Comm. Bovine Plasmin</td>
<td>3.31 (1.000)</td>
<td>2.53 (0.961)</td>
</tr>
<tr>
<td>Comm. Bovine Trypsin</td>
<td>6.31 (1.000)</td>
<td>4.99 (1.000)</td>
</tr>
<tr>
<td>Comm. Bovine Chymotrypsin</td>
<td>13.19 (0.991)</td>
<td>6.34 (1.000)</td>
</tr>
</tbody>
</table>

Unfortunately, the active concentrations of the enzymes and inhibitors could not be determined due to the unavailability of the necessary chemicals, and therefore protein concentrations were used instead. However, judging from SDS-PAGE (figure 4.16), ostrich plasmin could not contain more than 50\% active enzyme since approximately half of the sample was non-activated plasminogen and there was also a minor urokinase component; comm. bovine plasmin was also highly unlikely to be 100\% active since it showed a few degradation products and/or contaminants. On the other hand, both ostrich and comm. human \( \alpha_2 \)APs did not appear to have any contaminants according to SDS-PAGE, but a 100\% active concentration was still doubted. Therefore, the values in table 5.3 are approximations, but if the active concentrations of the four enzymes are assumed to be constant within an experiment, as well as those of the two inhibitors, a few comparisons can still be made.

Table 5.2 shows ostrich \( \alpha_2 \)AP to be a more potent inhibitor than its comm. human counterpart, but this can simply be due to a difference in their active concentrations. Both ostrich and comm. human \( \alpha_2 \)APs has the strongest effect on ostrich plasmin, followed by bovine chymotrypsin, bovine trypsin, and finally comm. bovine plasmin. This is not in accordance with literature, where human \( \alpha_2 \)AP reacts most rapidly with plasmin, followed by trypsin and then chymotrypsin (see section 5.1.2.1.). Enghild et al. (1993) obtained an inhibitory activity/(I/E) value of ~2 U for bovine trypsin with human \( \alpha_2 \)AP and ~1.33 U for bovine chymotrypsin, which is much lower than that obtained in table 5.2, but they obtained complete inhibition of trypsin and chymotrypsin at I/E molar ratios of 1 and 1.5, respectively.
The inhibition of ostrich and comm. bovine plasmins were quite different, with both $\alpha_2$APs having a much higher inhibitory effect on ostrich plasmin than on comm. bovine plasmin (table 5.2). However, comparing figures 5.2 and 5.3, ostrich and comm. human $\alpha_2$APs inhibited ostrich plasmin maximally by only 10.2 and 10.5%, respectively, while ostrich and human $\alpha_2$APs maximally inhibited comm. bovine plasmin 38.9 and 42.1%, respectively.

5.1.2.2. COMPARISON WITH OTHER INHIBITORS

5.1.2.2.1. Aprotinin

The inhibitory effects of comm. bovine lung aprotinin, a natural protease inhibitor, on ostrich and bovine plasmins are shown in figure 5.6.

![Graph 1](image1)

![Graph 2](image2)

**FIGURE 5.6:** Inhibitory effects of comm. bovine lung aprotinin on ostrich and bovine plasmins. (ii) represents a magnified version of the initial portions in (i). ◻️: ostrich plasmin; ▲: comm. bovine plasmin.

5.1.2.2.2. DFP

The inhibitory effects of DFP, an active-site directed, irreversible, non-specific, synthetic inhibitor, on ostrich and bovine plasmins are shown in figure 5.7.
FIGURE 5.7: Inhibitory effects of DFP on ostrich and bovine plasmins. 

◦; ostrich plasmin; ▲; comm. bovine plasmin.

5.1.2.2.3. EACA

EACA, a lysine analogue that mimics the effect of fibrin, is an excellent non-competitive plasmin inhibitor at high concentrations (Robbins and Summaria, 1970), binding to a secondary site (LBS) on the plasmin molecule (Christensen et al., 1995). The inhibitory effect of EACA on bovine plasmin is shown in figure 5.8; it did not appear to have any inhibitory effect on ostrich plasmin.

FIGURE 5.8: Inhibitory effect of EACA on bovine plasmin.
5.1.2.2.4. Summary

The inhibitory effects of the different inhibitors on ostrich and comm. bovine plasmins are summarised in table 5.3.

**TABLE 5.3: Summary of inhibitory effects of a few inhibitors on ostrich and comm. bovine plasmins.** Values represent inhibitory activity (U) / (I/E) (mol/mol), corresponding to the slopes of the linear portions of the inhibition curves above, where 1 unit of inhibitory activity results in a 50% loss in enzyme activity.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Ostrich Plasmin (r²)</th>
<th>Comm. Bovine Plasmin (r²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ostrich α₂AP</td>
<td>14.53 (0.819)</td>
<td>3.31 (1.000)</td>
</tr>
<tr>
<td>Comm. Human α₂AP</td>
<td>6.79</td>
<td>2.53 (0.961)</td>
</tr>
<tr>
<td>Comm. Bovine Aprotinin</td>
<td>389.02</td>
<td>56.72</td>
</tr>
<tr>
<td>DFP</td>
<td>0.01 (1.000)</td>
<td>0.10 (0.994)</td>
</tr>
<tr>
<td>EACA</td>
<td>0</td>
<td>7.23 x 10⁻⁶ (0.986)</td>
</tr>
</tbody>
</table>

Table 5.3 clearly shows comm. bovine aprotinin to be the most potent plasmin inhibitor, followed by ostrich and then comm. bovine α₂AP. Theoretically, α₂AP should be the most potent plasmin inhibitor (Highsmith, 1979). The synthetic inhibitors are not as potent as the natural ones, with EACA being the weakest. However, at high concentrations EACA showed a maximum inhibition of 34.2% on comm. bovine plasmin. Interestingly, the natural protein inhibitors revealed a more potent effect on ostrich plasmin than on its comm. bovine counterpart, whilst the synthetic ones had the opposite effect.
5.2. CHARACTERISATION OF OSTRICH PLASMINOGEN

5.2.1. PHYSICOCHEMICAL CHARACTERISATION

5.2.1.1. MOLECULAR WEIGHT DETERMINATION
The M_r of ostrich plasminogen, as determined by SDS-PAGE (figure 4.13), is 92.1 K. This corresponds well with that of native human plasminogen (90-94 K) (Castellino and Powell, 1981) and the rabbit plasminogen forms (89-94 K), as determined by sedimentation equilibrium analysis (Robbins and Summario, 1976b).

5.2.1.2. pI DETERMINATION
The PAG-IEF pattern shown in figure 5.1 for ostrich plasminogen is not clear, but, relating it to an identical PAG-IEF pattern (result not shown due to unsatisfactory staining of most of the pI 3.6-9.3 markers), it indicates multiple isoelectric forms (~7) in the pI range of 6.01 to approximately 9.18, with a major one of pI 6.01. Human plasminogen also showed multiple isoelectric forms, but there were 11 with the following pI values: 6.2, 6.3, 6.4, 6.6, 6.7, 7.2, 7.5, 7.8, 8.1, 8.3, and 8.5 (Robbins and Summario, 1976a). The two rabbit plasminogen isoforms revealed only 5 isoelectric subforms, the pI values at 22 °C of form 1 being 6.20, 6.56, 6.85, 7.24, and 7.78, and that of form 2 being 6.95, 7.18, 7.89, 8.24, and 8.74 (Robbins and Summario, 1976b). The pI values of ostrich plasminogen therefore covers the range of those of human and rabbit plasminogens, but the major isoelectric form showed a pI value lower than both of the isoelectric forms of human and rabbit plasminogens with the lowest pI values.

5.2.1.3. AMINO ACID COMPOSITION
The amino acid molar ratios obtained for ostrich plasminogen (performed in the laboratory of Prof. Koji Muramoto, Sendai, Japan) are shown in table 5.4 and are compared to reported values for human plasminogen (Wallén and Wiman, 1972) and rabbit plasminogen forms 1 and 2 (Sodetz et al., 1972), which are two plasminogen major affinity chromatography-resolved forms (Robbins and Summario, 1976b).
### TABLE 5.4: Amino acid composition (molar ratios) of ostrich plasminogen as compared to those of human and rabbit plasminogens.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Ostrich Plasminogen</th>
<th>Human Plasminogen(^1)</th>
<th>Rabbit Plasminogen(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Form 1</td>
<td>Form 2</td>
</tr>
<tr>
<td>Asx</td>
<td>107.9 (108)</td>
<td>76</td>
<td>80</td>
</tr>
<tr>
<td>Thr</td>
<td>53.6 (54)</td>
<td>57</td>
<td>58</td>
</tr>
<tr>
<td>Ser</td>
<td>39.4 (39)</td>
<td>51</td>
<td>58</td>
</tr>
<tr>
<td>Glx</td>
<td>87.8 (88)</td>
<td>92</td>
<td>84</td>
</tr>
<tr>
<td>Pro</td>
<td>45.4 (45)</td>
<td>73</td>
<td>58</td>
</tr>
<tr>
<td>Cyh</td>
<td>15.6 (16)</td>
<td>38</td>
<td>48</td>
</tr>
<tr>
<td>Gly</td>
<td>54.3 (54)</td>
<td>58</td>
<td>56</td>
</tr>
<tr>
<td>Ala</td>
<td>45.4 (45)</td>
<td>38</td>
<td>49</td>
</tr>
<tr>
<td>Val</td>
<td>32.0 (32)</td>
<td>44</td>
<td>32</td>
</tr>
<tr>
<td>Met</td>
<td>7.4 (7)</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Ile</td>
<td>26.8 (27)</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td>Leu</td>
<td>54.3 (54)</td>
<td>43</td>
<td>40</td>
</tr>
<tr>
<td>Tyr</td>
<td>48.4 (48)</td>
<td>28</td>
<td>30</td>
</tr>
<tr>
<td>Phe</td>
<td>20.1 (20)</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>His</td>
<td>24.6 (25)</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td>Lys</td>
<td>54.3 (54)</td>
<td>50</td>
<td>53</td>
</tr>
<tr>
<td>Trp</td>
<td>0 (20)(^3)</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>Arg</td>
<td>39.4 (39)</td>
<td>39</td>
<td>55</td>
</tr>
<tr>
<td>Total</td>
<td>775</td>
<td>780</td>
<td>790</td>
</tr>
</tbody>
</table>

\(^1\) Wallén and Wiman (1972) (isoelectric form of pI 6.23).
\(^2\) Sodetz et al. (1972).
\(^3\) Assumed value.

The amino acid composition of ostrich plasminogen correlates well with those of its two mammalian counterparts. The only significant differences are increased values for Asx, Leu, and Tyr, and decreased values for Ser, Pro, and Cyh. The decreased Cyh value is, however, expected since Cyh was not protected during the acid hydrolysis procedure. From the amino acid composition data for ostrich plasminogen an \(M_{\text{min}}\) of 89.4 K was calculated.
5.2.1.4. N-TERMINAL SEQUENCE DETERMINATION

The N-terminal sequence of ostrich plasminogen (performed in the laboratory of Prof. Koji Muramoto, Sendai, Japan) is shown in table 5.5 and is aligned with reported N-terminal sequences of four mammalian plasminogens (Robbins and Summaria, 1976a; Robbins and Summaria, 1976b).

**TABLE 5.5: Comparison of N-terminal sequence of ostrich plasminogen with those of four mammalian plasminogens.** Amino acids identical to those of ostrich plasminogen are shaded.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ostrich</strong></td>
<td>Asn</td>
<td>Ile</td>
<td>Leu</td>
<td>Asp</td>
<td>Gly</td>
<td>Tyr</td>
<td>Val</td>
<td>Arg</td>
<td>X</td>
<td>Glu</td>
<td>Gly</td>
<td>Ala</td>
<td>Trp</td>
<td>Leu</td>
<td>Leu</td>
<td>Ser</td>
</tr>
<tr>
<td><strong>Human</strong></td>
<td>Glu</td>
<td>Pro</td>
<td>Leu</td>
<td>Asp</td>
<td>Tyr</td>
<td>Val</td>
<td>Asn</td>
<td>Thr</td>
<td>Gln</td>
<td>Gly</td>
<td>Ala</td>
<td>Ser</td>
<td>Leu</td>
<td>Phe</td>
<td>Ser</td>
<td></td>
</tr>
<tr>
<td><strong>Rabbit</strong></td>
<td>Glu</td>
<td>Pro</td>
<td>Leu</td>
<td>Asp</td>
<td>Tyr</td>
<td>Val</td>
<td>Asn</td>
<td>Thr</td>
<td>Glu</td>
<td>Gly</td>
<td>Ala</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cat</strong></td>
<td>Asp</td>
<td>Pro</td>
<td>Leu</td>
<td>Asp</td>
<td>Tyr</td>
<td>Val</td>
<td>Asn</td>
<td>X</td>
<td>Gln</td>
<td>Gly</td>
<td>Ala</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ox</strong></td>
<td>Asp</td>
<td>Leu</td>
<td>Leu</td>
<td>Asp</td>
<td>Tyr</td>
<td>Val</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Robbins and Summaria (1976a).
2 Robbins and Summaria (1976b).
X, unknown.

The N-terminal sequence of ostrich plasminogen shows 53% identity with that of human plasminogen (excluding residue 9), and similarly with those of cat and ox plasminogens (their sequences are just a little shorter); it has one more residue in common with rabbit plasminogen. The N-terminus of dog plasminogen is blocked (Robbins and Summaria, 1976a). This relatively low identity with mammalian plasminogens was expected since the ostrich belongs to a totally different class, viz. Avis.
5.3. CHARACTERISATION OF OSTRICH PLASMIN

5.3.1. PHYSICOCHEMICAL CHARACTERISATION

5.3.1.1. MOLECULAR WEIGHT DETERMINATION

The $M_r$ of ostrich plasmin determined by SDS-PAGE (figure 4.16) is 78.1 K, which correlates well with that of comm. bovine plasmin (78.1 K, figure 4.16) and human plasmin (75.4, 76.5 or 81.0 K, Robbins and Summaria, 1976a), but which is slightly smaller than that of rabbit plasmin (82.0-86.0 K, Robbins and Summaria, 1976b). The molecular weights of human and rabbit plasmins were, however, determined by sedimentation equilibrium methods.

5.3.1.2. pH DETERMINATION

The PAG-IEF pattern shown in figure 5.1 for ostrich plasmin is, once again, not as clear as an identical PAG-IEF pattern mentioned in section 5.2.1.2.; relating it to this gel, ostrich plasmin showed 2 major isoelectric forms, one of pH 6.01 and another of pH 4.07. Comm. bovine plasmin did not stain on the gel in figure 5.1 and was not loaded onto the other gel; therefore, no result is available. Literature showed human plasmin to have multiple isoelectric forms of the following pH values: 7.2, 7.4, 7.7, 7.9, 8.1, 8.2, and 8.5. It also showed the plasmin-derived heavy chain to have a pH value of 4.9, and the light chain pH values of 5.8, 5.9 and 6.0 (Robbins and Summaria, 1976a). The pH values for ostrich plasmin are therefore much lower than those for human plasmin, but instead correlate with those of the plasmin-derived heavy and light chains. However, it must be noted that the ostrich plasmin isoelectric form of pH 6.01 is the same as the major isoelectric form of ostrich plasminogen; therefore, considering also that the ostrich plasmin sample contained non-activated plasminogen (figure 4.16), this isoelectric form is most probably that of ostrich plasminogen.

5.3.1.3. AMINO ACID COMPOSITION

The amino acid molar ratios obtained for ostrich plasmin (performed in the laboratory of Prof. Koji Muramoto, Sendai, Japan) are shown in table 5.6 and are compared to reported values for human plasmin (Robbins and Summaria, 1976a) and rabbit plasmin forms 1 and 2, which are obtained by the urokinase-activation of the two rabbit plasminogen major affinity chromatography-resolved forms (Robbins and Summaria, 1976b).
### TABLE 5.6: Amino acid composition (molar ratios) of ostrich plasmin as compared to those of human and rabbit plasmins.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Ostrich Plasmin</th>
<th>Human Plasmin&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Rabbit Plasmin&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Form 1</td>
<td>Form 2</td>
<td>Form 1</td>
</tr>
<tr>
<td>Asx</td>
<td>71.2 (72)</td>
<td>60</td>
<td>81</td>
</tr>
<tr>
<td>Thr</td>
<td>41.6 (42)</td>
<td>50</td>
<td>53</td>
</tr>
<tr>
<td>Ser</td>
<td>49.0 (49)</td>
<td>40</td>
<td>58</td>
</tr>
<tr>
<td>Glx</td>
<td>75.2 (75)</td>
<td>59</td>
<td>76</td>
</tr>
<tr>
<td>Pro</td>
<td>58.7 (59)</td>
<td>61</td>
<td>71</td>
</tr>
<tr>
<td>Cyh</td>
<td>0 (40)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>37</td>
<td>45</td>
</tr>
<tr>
<td>Gly</td>
<td>63.8 (64)</td>
<td>53</td>
<td>57</td>
</tr>
<tr>
<td>Ala</td>
<td>43.8 (44)</td>
<td>29</td>
<td>39</td>
</tr>
<tr>
<td>Val</td>
<td>9.1 (9)</td>
<td>36</td>
<td>29</td>
</tr>
<tr>
<td>Met</td>
<td>0 (7)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Ile</td>
<td>8.5 (9)</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>Leu</td>
<td>16.5 (17)</td>
<td>36</td>
<td>38</td>
</tr>
<tr>
<td>Tyr</td>
<td>28.5 (29)</td>
<td>26</td>
<td>34</td>
</tr>
<tr>
<td>Phe</td>
<td>5.7 (6)</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>His</td>
<td>12.0 (12)</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>Lys</td>
<td>45.0 (45)</td>
<td>38</td>
<td>42</td>
</tr>
<tr>
<td>Trp</td>
<td>0 (20)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>16</td>
<td>25</td>
</tr>
<tr>
<td>Arg</td>
<td>41.0 (41)</td>
<td>36</td>
<td>46</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>638</strong></td>
<td><strong>635</strong></td>
<td><strong>755</strong></td>
</tr>
</tbody>
</table>

1 Robbins and Summaria (1976a).
2 Robbins and Summaria (1976b).
3 Assumed values.

The total number of amino acid residues of ostrich plasmin was similar to that of human plasmin, while rabbit plasmin displayed a substantially higher amino acid composition than both ostrich and human plasmins. The amino acid composition of the rabbit plasmin forms was, however, obtained by the direct addition of the amino acid compositions of the plasmin heavy and light chains, and this approach can give a slightly overestimated value, as was the case for human plasmin (Robbins and Summaria, 1976a). Most of the amino acid residues of ostrich plasmin fell within the range of those of human and rabbit plasmins, but the residues that gave a significantly lower value than both those of human and rabbit plasmins were Thr, Val, Ile, Leu, Phe, and His.
The values of those amino acids whose concentration were not determined were assumed, based on the fact that ostrich plasmin cannot have a greater concentration for a certain amino acid than ostrich plasminogen. However, in the case of Cyh, its value for ostrich plasminogen was underestimated since it was not protected during the acid hydrolysis procedure, and therefore its value for ostrich plasmin was based solely on those obtained for human and rabbit plasmins. From the amino acid composition data an $M_{\text{min}}$ of 75.5 K was calculated for ostrich plasmin.

5.3.1.4. N-TERMINAL SEQUENCE DETERMINATION

The N-terminal sequence obtained for ostrich plasmin (performed in the laboratory of Prof. Koji Muramoto, Sendai, Japan) is aligned with reported N-terminal sequences of five mammalian plasmins (Robbins and Summaria, 1976a; Castellino and Powell, 1981), as shown in table 5.7.

**TABLE 5.7**: Comparison of N-terminal sequence of ostrich plasmin with those of five mammalian plasmins. Amino acids identical to those of ostrich plasmin are shaded.

|       | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|-------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|
| Ostrich | Arg | Ile | Tyr | Leu | Asp | Glu | Val | Glu | Gly | Arg | Asp | Val | Val | Tyr | Tyr | Arg | Thr |
| Human¹ | Lys | Val | Tyr | Leu | Ser | Glu | Cys | Lys | Thr | Gly | Asp | Gly | Lys | Asn | Tyr | Arg | Gly |
| Cat²  | Lys | Ile | Tyr | Leu | Val |    |    |    |    |    |    |    |    |    |    |    |    |
| Dog²  | Arg | Ile | Tyr | Leu | Gly |    |    |    |    |    |    |    |    |    |    |    |    |
| Rabbit² | Lys | Val | Tyr | Leu | Gly |    |    |    |    |    |    |    |    |    |    |    |    |
| Ox² | Lys | Ile | Tyr | Leu | Val |    |    |    |    |    |    |    |    |    |    |    |    |

¹ Castellino and Powell (1981).
² Robbins and Summaria (1976a).

The N-terminal sequence obtained for ostrich plasmin shows only 35% identity with that of human plasmin (Lys⁷⁸-plasmin). Ostrich plasmin shows a better identity with those N-terminal sequences of cat and ox plasmins and the best with that of dog plasmin, but these sequences are too short to make significant comparisons.
5.3.2. KINETIC CHARACTERISATION

5.3.2.1. pH OPTIMUM
The pH profiles of ostrich and comm. bovine plasmins are illustrated in figure 5.9.

Ostrich and comm. bovine plasmins show similar pH profiles, both having a pH optimum of 8.0 (the pH optimum of comm. bovine plasmin is actually between 7.0 and 8.0), but ostrich plasmin is not as quickly inactivated at high pHs (i.e. above pH 8.0) as comm. bovine plasmin. Christensen and Ipsen (1979) obtained a pH optimum of 7.60 for human plasmin.

5.3.2.2. TEMPERATURE OPTIMUM
Figure 5.10 shows the effect of temperature on ostrich and comm. bovine plasmins.

Ostrich and comm. bovine plasmins show temperature optima of 40 and 45 °C, respectively, but the temperature optimum of comm. bovine plasmin is actually between 40 and 45 °C. Ostrich plasmin is much more quickly inactivated at temperatures below its optimum (between 20 and 35 °C) than its comm. bovine counterpart, having only 22% activity at 20 °C, whereas comm. bovine plasmin shows 56% activity. However, high temperatures, above the optima, inactivated both plasmins to a similar extent, with ostrich plasmin showing 44% activity at 60 °C and comm. bovine plasmin 49%.

5.3.2.3. THERMODYNAMIC PARAMETERS
Using the data obtained from the study of the effect of temperature on ostrich and comm. bovine plasmins, Arrhenius and modified Arrhenius plots could be plotted for both plasmins, from which their thermodynamic parameters could be calculated.

An Arrhenius plot is derived from the integrated Arrhenius equation:
\[ \log_{10} k = \log_{10} PZ - \left( \frac{E_a}{2.303RT} \right), \]
where \( k \)=velocity constant of reaction, \( P \)=constant (steric factor), \( Z \)=constant (collision frequency), \( E_a \)=activation energy, \( R \)=gas constant (8.31441 J/mol.K), and \( T \)=temperature (K) (Dixon and Webb, 1979). However, since \( k \) is difficult to obtain and since the initial velocity (\( v_o \)) reaction is a first-order reaction, i.e. \( k \) is directly proportional to \( v_o \), \( k \) can be substituted by \( v_o \) (Palmer, 1995). An Arrhenius plot can therefore be plotted as \( \log v_o \) vs. \( 1/T \) and \( E_a \) can be calculated from the slope as follows: \( E_a = -\text{slope} \times 2.303 \times R \).
A modified Arrhenius plot of \( \log (v_o/T) \) vs. \( 1/T \) is derived from the equation:

\[
\log_{10} \left( \frac{v_o}{T} \right) = \left( \frac{-\rho H^\ddagger}{2.303 R} \right) + \left( \frac{\rho S^\ddagger}{2.303 R} \right) + \log_{10} \left( \frac{R}{Nh} \right),
\]

where \( v_o \) substitutes for \( k \), \( \rho H^\ddagger \) = transition state enthalpy change, \( \rho S^\ddagger \) = transition state entropy change, \( N \) = Avogadro’s number \( (6.022 \times 10^{23} \text{ mol}^{-1}) \), and \( h \) = Planck’s constant \( (6.626 \times 10^{-34} \text{ J} \cdot \text{s}) \) (Dixon and Webb, 1979).

\( \rho H^\ddagger \) can therefore be calculated from the slope of the plot \( \left( \frac{\rho H^\ddagger}{2.303 R} = -\text{slope} \times 2.303 \times R \right) \) and \( \rho S^\ddagger \) from the y-intercept \( \left( \frac{\rho S^\ddagger}{2.303 R} = \frac{(y-intercept - \log_{10} \left( \frac{R}{Nh} \right)) \times 2.303 \times R}{2.303} \right) \).

\( \rho G^\ddagger \), the transition state free energy change, can be determined from the Gibb’s free energy equation:

\[
\rho G^\ddagger = \rho H^\ddagger - T\rho S^\ddagger.
\]

The transition state equilibrium constant, \( K_{eq}^\ddagger \), is related to \( \rho G^\ddagger \) as follows:

\[
-\rho G^\ddagger = RT \ln K_{eq}^\ddagger,
\]

and can therefore be calculated. The concentration of the transition state, which is formed during an enzymatic reaction: \( A + B \rightarrow X^\ddagger \rightarrow P + Q \), where \( A \) = enzyme, \( B \) = substrate, \( X^\ddagger \) = transition state (or activation complex), \( P \) = product(s), and \( Q \) = free enzyme, can therefore be calculated as follows:

\[
[X^\ddagger] = K_{eq}^\ddagger [A] [B].
\]

The Arrhenius and modified Arrhenius plots for both ostrich and comm. bovine plasmins are shown in figures 5.11 and 5.12, respectively.

**FIGURE 5.11: Arrhenius plots of ostrich and comm. bovine plasmins.**

- •, ostrich plasmin (slope, -3392.09 K; \( r^2 \), 0.984);
- ▲, comm. bovine plasmin (slope, -1242.37 K; \( r^2 \), 0.995).
A summary of the thermodynamic parameters obtained for the reaction of ostrich and comm. bovine plasmins with D-Val-Leu-Lys-pNA are given in table 5.8.

**TABLE 5.8: Summary of thermodynamic parameters for the reaction of ostrich and comm. bovine plasmins with D-Val-Leu-Lys-pNA.**

<table>
<thead>
<tr>
<th>Plasmin</th>
<th>$E_a$  (kJ/mol)</th>
<th>$\rho H^\parallel$ (kJ/mol)</th>
<th>$\rho S^\parallel$ (J/molK)</th>
<th>$\rho G^\parallel$ (kJ/mol)</th>
<th>$K_{eq}^\parallel$</th>
<th>$[X^\parallel]_{eq}$ (M$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ostrich</td>
<td>64.95</td>
<td>62.43</td>
<td>-51.87</td>
<td>78.67</td>
<td>7.54 x 10$^{-14}$</td>
<td>1.79 x 10$^{-21}$</td>
</tr>
<tr>
<td>Comm. Bovine</td>
<td>23.79</td>
<td>21.27</td>
<td>-185.52</td>
<td>79.37</td>
<td>5.78 x 10$^{-14}$</td>
<td>2.20 x 10$^{-24}$</td>
</tr>
</tbody>
</table>

The activation energy ($E_a$) for the reaction of ostrich plasmin with D-Val-Leu-Lys-pNA is almost 3 times larger than that for comm. bovine plasmin, implying that ostrich plasmin is less capable of lowering the $E_a$ for the reaction with D-Val-Leu-Lys-pNA, thus facilitating a slower reaction. Ostrich plasmin is therefore less efficient as an enzyme than its comm. bovine counterpart.

$\rho H^\parallel$ for the reaction with ostrich plasmin is also almost 3 times larger than for the one with comm. bovine plasmin, implying that the reaction with ostrich plasmin to form the transition state is more endothermic and therefore less efficient. However, $\rho S^\parallel$ is almost 4 times less negative for ostrich plasmin than for comm. bovine plasmin, which is more favourable since the increase in
the order of the molecules in moving from the substrate to the transition state is not so drastic for
the reaction with ostrich plasmin than for the one with comm. bovine plasmin, and therefore it
does not require as much energy. The combined effects of $pH^\parallel$ and $pS^\parallel$ produce a similar $pG^\parallel$
value for both ostrich and comm. bovine plasmins, but ostrich plasmin has a slightly lower value
and is therefore marginally more successful at lowering the free energy of the transition state.

$K_{eq}^\parallel$ values for both reactions are extremely small ($<<0$), implying that, in both cases, the reverse
reactions, i.e. the reactions away from transition state formation back towards the substrate and
enzyme, are much more favoured (as also indicated by the large positive $pG^\parallel$ value). $K_{eq}^\parallel$ is
slightly larger for ostrich plasmin than for comm. bovine plasmin which makes the reaction with
ostrich plasmin the more favourable one, at least until transition state formation. This is also
evident from the lower free energy ($pG^\parallel$) required by ostrich plasmin to form the transition state.
The extremely low $[X^\parallel]$ values, which are overestimated due to the fact that the $[A]$ does not take
into consideration the active concentrations, indicate that the transition states are very short-lived.
$[X^\parallel]$ for ostrich plasmin is approximately 8 times larger than that for its comm. bovine
counterpart, which implies that the transition state of ostrich plasmin with the substrate is much
more slowly decayed than that of its comm. bovine counterpart. However, since these values are
not accurate, definite conclusions cannot be made.

5.3.2.4. KINETIC PARAMETERS
The kinetic parameters were determined by Hanes plots ($[S]/v_o$ vs. $[S]$), since they seemed to be
the most reliable of the various linear plots available for the graphical representation of enzyme
kinetic results (Cornish-Bowden, 1976). The velocity of the reactions with the synthetic
substrates were measured as $\rho A_{412 nm}/min$. This, however, had to be converted to a rate of change
of concentration ($\mu mol.l^{-1}.min^{-1}$), i.e. absorbance (A) values had to be converted to concentration
(c). This was accomplished by implementing Beer-Lambert’s law as follows, using an $\varepsilon_{m}.l$ value
of 5514 M$^{-1}$ for p-nitroaniline, the product being measured at $A_{412 nm}$ in a microtiter plate
(Wagner, 1998):

\[
A = \varepsilon_{m} \times c \times l = \rho A_{412 nm}/min = v_o
\]

\[
\therefore c = v_o/(\varepsilon_{m}.l) \times 10^6 = v_o/5514 \times 10^6 = v_o/181.357 \mu mol.l^{-1}.min^{-1} = v_o \text{ i.t.o. concentration.}
\]

On the other hand, a discontinuous assay method was used to monitor the reaction of the plasmins
with a natural substrate, bovine fibrinogen, and therefore these results could not be compared
with those of the synthetic substrates, except for the $K_{m}$ values.

FIGURE 5.13: Hanes plots of ostrich and comm. bovine plasmins with four different substrates. (i) Gly-Pro-Lys-pNA: ◆, ostrich plasmin (r²=0.998); π, comm. bovine plasmin (r²=0.999). (ii) Val-Leu-Lys-pNA: ◆, ostrich plasmin (r²=1.000); π, comm. bovine plasmin (r²=0.992). (iii) Phe-Val-Arg-pNA: ◆, ostrich plasmin (r²=0.990); π, comm. bovine plasmin (r²=0.981). (iv) Bovine fibrinogen: ◆, ostrich plasmin (r²=0.939); π, comm. bovine plasmin (r²=0.987).
The kinetic parameters were calculated from the Hanes plots as follows:

Maximum velocity, \( V_{\text{max}} = \frac{1}{\text{slope}} \)

Michaelis constant, \( K_m \) ([S] at \( v = \frac{1}{2} V_{\text{max}} \)) = \( y\)-intercept \times V_{\text{max}} = \frac{\text{y-intercept}}{\text{slope}} \)

Catalytic rate constant, \( k_{\text{cat}} = \frac{V_{\text{max}}}{[E]_o} \)

Unfortunately, since the active concentrations of the plasmins are not available, as previously explained in section 5.1.2.1.4., \([E]_o\) is not accurate (it is overestimated), and, therefore, neither are \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) (both are underestimated). However, if the active concentrations of the two plasmins are assumed to be constant during experimentation (as in section 5.1.2.1.4.), then the degree of inaccuracy will be the same and comparisons between the two species can still be made.

The kinetic parameters obtained for the reactions of ostrich and comm. bovine plasmins with the various substrates are summarised in table 5.9.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Plasmin</th>
<th>( V_{\text{max}} ) (µM/s)</th>
<th>( K_m ) (mM)</th>
<th>( k_{\text{cat}} ) (s(^{-1}))</th>
<th>( k_{\text{cat}}/K_m ) (mM(^{-1}).s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly-Pro-Lys-pNA</td>
<td>Ostrich</td>
<td>1.230</td>
<td>0.333</td>
<td>2.953</td>
<td>8.854</td>
</tr>
<tr>
<td></td>
<td>Comm. Bovine</td>
<td>1.143</td>
<td>0.530</td>
<td>17.078</td>
<td>32.247</td>
</tr>
<tr>
<td>Val-Leu-Lys-pNA</td>
<td>Ostrich</td>
<td>0.591</td>
<td>0.379</td>
<td>1.419</td>
<td>3.748</td>
</tr>
<tr>
<td></td>
<td>Comm. Bovine</td>
<td>0.520</td>
<td>0.298</td>
<td>7.771</td>
<td>26.044</td>
</tr>
<tr>
<td>Phe-Val-Arg-pNA</td>
<td>Ostrich</td>
<td>0.060</td>
<td>0.186</td>
<td>0.143</td>
<td>0.769</td>
</tr>
<tr>
<td></td>
<td>Comm. Bovine</td>
<td>0.056</td>
<td>0.183</td>
<td>0.832</td>
<td>4.540</td>
</tr>
<tr>
<td>Bovine Fibrinogen</td>
<td>Ostrich</td>
<td>0.030(^{\dagger})</td>
<td>0.030</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Comm. Bovine</td>
<td>0.018(^{\dagger})</td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{\dagger}\) \( \rho A_{280 \text{ nm}}/30 \text{ min} \)
According to the $k_{\text{cat}}$ values, Gly-Pro-Lys-pNA is by far the best synthetic substrate for plasmin, followed by Val-Leu-Lys-pNA, whose values are more than half those of Gly-Pro-Lys-pNA for the respective plasmins. Phe-Val-Arg-pNA follows lastly, with $k_{\text{cat}}$ values more than 20 times smaller than those of Gly-Pro-Lys-pNA for the respective plasmins. This implies that plasmin prefers Lys to Arg as the $S_1$ residue and Gly-Pro to Val-Leu as the $S_3$-$S_2$ residues. Comparing the $V_{\text{max}}$ values of the two plasmin, ostrich plasmin has the higher value for all four substrates. $k_{\text{cat}}$ values, on the other hand, are higher for comm. bovine plasmin than for ostrich plasmin. However, the $k_{\text{cat}}$ values for the two plasmins cannot be accurately compared due to the inaccurate $[E]_0$ values. $k_{\text{cat}}$ values of $13.5 \pm 0.5$ and $8.5 \pm 0.3 \text{ s}^{-1}$ were reported for human plasmin with D-Val-Leu-Lys-pNA and Benzoyl-L-Phe-Val-Arg-pNA, respectively, at pH $8.00$ and $25 \degree C$ (Christensen and Ipsen, 1979). The former value is almost 10 x and 2 x higher than those for ostrich and comm. bovine plasmins, respectively, and, in the case of Phe-Val-Arg-pNA, it is almost 60 x and more than 10 x higher than that for ostrich and comm. bovine plasmins, respectively. However, as previously mentioned, the values for ostrich and comm. bovine plasmins are underestimated.

Considering the $K_m$ values, however, Phe-Val-Arg-pNA has the lowest values of all three synthetic substrates for both plasmins. Comparing the $K_m$ values for Gly-Pro-Lys-pNA and Val-Leu-Lys-pNA, ostrich plasmin has a slightly higher affinity for Gly-Pro-Lys-pNA, while comm. bovine plasmin has an almost 2 times higher affinity for Val-Leu-Lys-pNA. Therefore, the $K_m$ value for ostrich plasmin with Gly-Pro-Lys-pNA is 1.6 x lower than that for its comm. bovine counterpart, while Val-Leu-Lys-pNA has the opposite effect, but the difference in $K_m$ value is only 1.3 x. The $K_m$ values for both plasmins with Phe-Val-Arg-pNA are very similar. Christensen and Ipsen (1979) reported $K_m$ values of $0.22 \pm 0.02$ and $0.69 \pm 0.04 \text{ mM}$ for human plasmin with D-Val-Leu-Lys-pNA and Benzoyl-L-Phe-Val-Arg-pNA, respectively, at pH $8.00$ and $25 \degree C$. The former $K_m$ value is lower than those for both ostrich and comm. bovine plasmins, as well as for both with Gly-Pro-Lys-pNA, but the $K_m$ value for human plasmin with Benzoyl-L-Phe-Val-Arg-pNA is almost 4 x larger than those for both ostrich and comm. bovine plasmins. Bovine fibrinogen, however, has the lowest $K_m$ values of all the substrates used, which is to be expected since it is a natural protein substrate. The $K_m$ value is especially low for comm. bovine plasmin, which is its natural enzyme and which has an almost 2 x higher affinity compared to ostrich plasmin, as well as for human plasmin, which has the same $K_m$ value as for ostrich plasmin, i.e. $0.03 \text{ mM}$ (Robbins and Summaria, 1970).
Combining the $k_{cat}$ and $K_m$ values yield the specificity constant, $k_{cat}/K_m$, which gives the best indication of the efficiency of an enzyme. For all three synthetic substrates comm. bovine plasmin is between 3.6-6.9 x more efficient than ostrich plasmin. The $k_{cat}/K_m$ values of human plasmin with Val-Leu-Lys-pNA and Benzoyl-L-Phe-Val-Arg-pNA at pH 8.00 and 25 °C are 60 ± 4.1 and 12.4 ± 0.3 mM⁻¹.s⁻¹, respectively, which are much larger than both those of ostrich and comm. bovine plasmins, implying greatest efficiency (Christensen and Ipsen, 1979).

Comparing the $k_{cat}/K_m$ values for the different synthetic substrates, Gly-Pro-Lys-pNA is the synthetic substrate most favoured by both ostrich and comm. bovine plasmins, followed by Val-Leu-Lys-pNA and lastly by Phe-Val-Arg-pNA. This confirms that plasmin prefers Lys to Arg as the $S_1$ residue and Gly-Pro to Val-Leu as the $S_3$-$S_2$ residues.
CHAPTER 6

CONCLUSIONS

The objectives of this study, as outlined in chapter 2, were met, but unfortunately, due to a very poor yield of ostrich $\alpha_2$AP after numerous isolation and purification attempts, the characterisation of this ostrich inhibitor was limited to those aspects presented in section 5.1., and therefore it was not as extensively studied as is necessary to obtain a good understanding of this ostrich serpin.

Three different isolation and purification procedures of ostrich $\alpha_2$AP were undertaken. The first one followed the classical method used by Moroi and Aoki (1976), Wiman and Collen (1977), and Christensen and Clemmensen (1978). It involved L-lysine-Sepharose chromatography to remove plasminogen, the major contaminant in the isolation and purification of $\alpha_2$AP, from ostrich plasma, ammonium sulfate fractionation, ion-exchange chromatography on Toyopearl Super-Q 650S, and finally affinity chromatography on ostrich plasminogen-Sepharose (diagram 4.1). The latter column is supposedly specific for the plasminogen-binding form of $\alpha_2$AP, but non-reducing SDS-PAGE unfortunately revealed a number of components. However, only two components were apparent on reducing SDS-PAGE, one of $M_r \sim 80$ K and the other of $M_r$ 63 K, proposed to be the $\alpha_2$AP monomer and reduced dimer, respectively (figure 4.3). The purification fold was good (438.52), with the largest increase resulting from plasminogen-Sepharose chromatography, but the yield was very low (0.36%), with plasminogen-Sepharose chromatography also causing the largest decrease (table 4.1). The low yield was believed to be due to the irreversible binding of ostrich $\alpha_2$AP to ostrich plasmin that was present in the plasminogen sample used to make the ostrich plasminogen-Sepharose resin. Because of the heterogeneity of the ligand used to make the affinity resin, and therefore the inefficiency of the resin, another isolation and purification procedure was undertaken with the aim of bypassing the ostrich plasminogen-Sepharose chromatographic step.
The second isolation and purification procedure, adapted also from Moroi and Aoki (1976), replaced ostrich plasminogen-Sepharose chromatography with gel filtration on Sephadex G-200, followed by hydroxylapatite chromatography (diagram 4.2). Judging from the purification fold (731.97), yield (11.84%, but the value is overestimated) and SDS-PAGE patterns (less contaminants) (figure 4.6), this procedure proved to be much more successful, with hydroxylapatite chromatography being the key step (table 4.2). However, ostrich α2AP was not yet purified to homogeneity, and therefore, a third isolation and purification procedure was employed.

The third isolation and purification procedure basically replaced ostrich plasminogen-Sepharose chromatography with the theoretically more efficient ostrich LBSI-Sepharose chromatography (diagram 4.3), the major advantage of LBSI, which consists of plasminogen kringles 1-3, being that it does not contain a potential active site and therefore cannot bind α2AP irreversibly (Wiman, 1980). Ostrich LBSI was obtained from the specific elastase cleavage of ostrich plasminogen, followed by purification on a L-lysine-Sepharose resin. The third isolation and purification procedure successfully purified ostrich α2AP to homogeneity (figure 4.8), but it gave a very low final purification fold (16.24) and yield (0.27%), with ostrich LBSI-Sepharose chromatography giving only a 1.1-fold increase in purification (table 4.3), compared to the 29.4-fold increase that ostrich plasminogen-Sepharose gave. A reason for this low purification fold is that only the plasminogen-binding form was purified.

Ostrich plasminogen was highly purified from plasma by L-lysine-Sepharose chromatography (diagram 4.4, figure 4.13) and ostrich plasmin was obtained by the urokinase activation of purified ostrich plasminogen (diagram 4.5, figure 4.16). The optimum human urokinase concentration and incubation time were determined as 600 µg/mg plasminogen and 4 hr, respectively (figure 4.14).
Ostrich $\alpha_2$AP showed an $M_r$ of 77-84 K, while comm. human $\alpha_2$AP showed a smaller $M_r$ of 67 K (figure 4.8). The reported $M_r$ values for human and bovine $\alpha_2$APs are 67-70 K (Wiman and Collen, 1977) and 70 K (Christensen and Sottrup-Jensen, 1992), respectively. Ostrich and comm. human $\alpha_2$AP showed two similar isoelectric forms of pI 3.85 and 6.18, and pI 3.92 and 6.11, respectively (figure 5.1). However, it was reported that the plasminogen-binding form of human $\alpha_2$AP has multiple isoelectric points with a major component of pI 4.69 and a minor one of pI 4.92, while the non- plasminogen-binding form has only one component of pI 4.59 (Lijnen and Collen, 1986). N-terminal sequence analysis showed ostrich $\alpha_2$AP to have only 2 residues in common with the 11 N-terminal residues of human $\alpha_2$AP, as opposed to the 5 that bovine $\alpha_2$AP showed (table 5.1) (Christensen et al., 1994). Both ostrich and comm. human $\alpha_2$APs had the largest inhibitory effects on ostrich plasmin, followed by comm. bovine chymotrypsin, trypsin and plasmin, in that order (table 5.2). This is not in accordance with literature, where human $\alpha_2$AP reacts most rapidly with plasmin, followed by trypsin and then chymotrypsin (Lijnen and Collen, 1986). Ostrich and comm. bovine $\alpha_2$APs appeared to be much less potent plasmin inhibitors than bovine aprotinin , but much more potent ones than the synthetic inhibitors, DFP and EACA (table 5.3). Theoretically, however, $\alpha_2$AP should be the most potent plasmin inhibitor (Highsmith, 1979). Ostrich $\alpha_2$AP was more potent than its comm. human counterpart.

Ostrich plasminogen revealed an $M_r$ of 92 K (figure 4.13), which is in accordance with reported values for human (90-94 K; Castellino and Powell, 1981) and rabbit (89-94 K; Robbins and Summaria, 1976) plasminogens. It showed multiple isoelectric forms (~7) in the pI range of 6.01 to 9.18, with a major isoelectric form of pI 6.01 (figure 5.1). Human and rabbit plasminogens also showed multiple isoelectric forms, but human plasminogen had 11 in the pI range of 6.2 to 8.5 (Robbins and Summaria, 1976a) while the two rabbit plasminogen isoforms each had 5 subforms in the pI range of 6.2 to 7.78 (form 1) and 6.95 to 8.74 (form 2) (Robbins and Summaria, 1976b). The amino acid composition of ostrich plasminogen correlated well with those of human and rabbit plasminogens (table 5.4), showing a total amino acid composition of 775, which is slightly less than those of human plasminogen (780; Wallén and Wiman, 1972) and rabbit plasminogen forms 1 and 2 (790 and 792, respectively; Sodetz et al., 1972). The N-terminal sequence showed 53% identity with 16 N-terminal residues of human plasminogen, and similarly with the N-terminal sequences of rabbit, cat and ox plasminogens (table 5.5) (Robbins and Summaria, 1976a, Robbins and Summaria, 1976b).
Ostrich and comm. bovine plasmins showed M_r values of 78 K (figure 4.16), which correlated well with that of human plasmin (75.4, 76.5 or 81.0 K, Robbins and Summaria, 1976a), but which is slightly smaller than that of rabbit plasmin (82.0-86.0 K, Robbins and Summaria, 1976b). Ostrich plasmin revealed two isoelectric forms of pI 4.07 and 6.01 (figure 5.1), but human plasmin was reported to have 7 forms in the pI range of 7.2 to 8.5. However, literature also reported that the plasmin-derived heavy chain has a pI value of 4.9, while the light chain has pI values of 5.8, 5.9 and 6.0 (Robbins and Summaria, 1976a). The amino acid composition of ostrich plasmin showed a total of 638 amino acid residues, which is similar to that of human plasmin (635; Robbins and Summaria, 1976a), but much lower than those of rabbit plasmin forms 1 and 2 (755 and 757, respectively; Robbins and Summaria, 1976b) (table 5.6). The N-terminal sequence of ostrich plasmin showed 35% identity with 17 N-terminal residues of human plasmin (Castellino and Powell, 1981) and 2-4 identical residues with the 5 N-terminal residues of cat, dog, rabbit and ox plasmins (Robbins and Summaria, 1976a) (table 5.7). The pH optimum of ostrich plasmin was determined as 8.0, while that of comm. bovine plasmin was between 7.0 and 8.0 (figure 5.9), which correlated well with that of human plasmin (7.60; Christensen and Ipsen, 1979). The temperature optima of ostrich and comm. bovine plasmins were determined to be 40 and 45 °C, respectively (figure 5.10). Thermodynamic parameters were obtained from the temperature data for both ostrich and comm. bovine plasmins (table 5.8) and it showed comm. bovine plasmin to be the more efficient enzyme. Kinetic parameters obtained for both ostrich and comm. bovine plasmins (table 5.9) also showed comm. bovine plasmin to be the more efficient, but no definite conclusion could be made due to the lack of the active concentrations of the two enzymes. The kinetic parameters also showed plasmin to prefer Lys to Arg residues in the S1 position.

Physically, chemically and kinetically ostrich $\alpha_2$AP and plasmin(ogen) showed many properties similar to those of other known $\alpha_2$APs and plasmin(ogen)s, but there were a few significant differences.
REFERENCES


