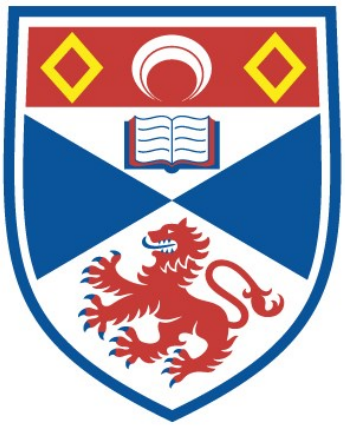
A FIBRINOLYTIC ACID PROTEINASE IN HUMAN PLASMA

Nooshabeh Pejhan

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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A FIBRINOLYTIC ACID PROTEINASE IN HUMAN PLASMA

by

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A Thesis submitted for the Degree of Doctor of Philosophy.

March 1984.

DECLARATION

I hereby declare that this thesis is my own composition, and that the experimental work was performed by me. None of the material in this thesis has been submitted for any other degree.

The research was carried out in the Department of Biochemistry of the University of St. Andrews, under the supervision of Dr. G.D. Kemp.

Nooshabeh Pejhan

CERTIFICATE

I certify that Nooshabeh Pejhan has spent eleven terms engaged in research work under my supervision, and that she has fulfilled the conditions of General Ordinance No. 12 (Resolution of the University Court No. 1, 1967), and that she is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

Dr. G.D. Kemp,
Department of Biochemistry.

ACKNOWLEDGEMENTS

I would like to thank my Supervisor, Dr. G.D. Kemp, for his help and encouragement throughout the course of this thesis. Also I would like to thank Mrs. M. Wilson for typing this thesis.

ABSTRACT

An attempt has been made to purify an acid proteinase from human plasma, which is capable of dissolving fibrin clot in 1% (w/v) monochloroacetic acid.

On SDS gel electrophoresis the enzyme isolated from plasma contains two protein bands with molecular weights of approximately 90,000 and 54,000.

Different substrates were used to evaluate the proteolytic activity of the plasma enzyme, including fibrin clot, azo-casein, acid denatured haemoglobin (Hb), haemoglobin polyacrylamide gel (insoluble Hb), haemoglobin electrophoresis (soluble Hb) and the synthetic substrate N-acetyl-L-phenylalanyl-L-diiodotyrosine.

The plasma enzyme seems to belong to the group of aspartyl proteinases as its activity against the above substrates was maximal at low pH, and was inhibited by pepstatin which is a powerful inhibitor of aspartyl proteinases.

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Abbreviation

α_2 M	alpha$_2$-macroglobulin
EDTA	ethylenediaminetetraacetate
EACA	epsilon aminocaproic acid
MCA	monochloroacetic acid
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyle sulphate

CHAPTER 1

GENERAL INTRODUCTION

1.1. PROTEINASES

Proteinases were originally divided into exopeptidase and endopeptidases.

Endopeptidases cleave bonds distant from the end of the polypeptide chain while exopeptidases cleave bonds only from the one or the other terminus of the polypeptide chain. Generally proteins are first broken down by endopeptidases (proteinase), then followed by removal of amino acid residues from the ends of the fragments (one, two or three at a time) by exopeptidases (peptidases), (Barrett, 1977a). Exopeptidases are classified on the basis of the substrate specificity, while the endopeptidases are classified according to their essential catalytic groups.

Hartley (1960) divided the proteinases into four classes according to the amino acid or ion which is involved in the catalytic process.

The four groups are serine, thiol, acid and metallo proteinases. Tang (1970) used the term carboxyl protease rather than acid protease for two reasons, one was that the active site of the enzymes contains two carboxyl groups, and the other that some proteases like renin have pH optima near neutrality and the name acid protease is not appropriate.

Barrett and McDonald (1980a) introduced the terms "cysteine proteinases" and "aspartic proteinases" instead of "thiol proteinase" and "acid" or "carboxylproteinase" respectively. Also they added a fifth class to the existing

four classes; it contains proteinases which cannot positively be classified. However, the terms aspartic, acid, carboxyl plus protease or proteinase all refer to the same class of enzyme and are categorised as 3.4.23 in the list of the Commission on Biochemical Nomenclature (1972).

1.2. ASPARTIC PROTEINASES

Aspartic proteinases generally have pH optima below 6.0 and they have two specially protective aspartic groups of which one can be labelled with active site directed diazo compounds, and the other with epoxy compounds. Both labelling procedures inactivate the enzyme. Aspartic proteinases are also inhibited by pepstatin (Foltman and Pedersen, 1977).

Tang (1979) suggested that precursors of carboxyl proteinases can be divided into two groups, on the basis of the way in which they are activated. One group is irreversibly converted into active enzyme at acid pH, during which process the NH_2-terminal segments of the peptide chains are released. The well known proteinases of this group are the gastric enzymes. The other group are not apparently converted to the active enzyme on acidification, but are activated by other proteases, e.g. renin is activated by several proteases, including kallikrein. No zymogens for microbial carboxyl proteases have been found to date.

1.3. FIBRINOLYSIS

Many proteolytic enzymes digest fibrin, but "fibrinolysis" is a more specific process in which the digestion of fibrinogen takes place, by splitting only a few peptide bonds (Astrup, 1978). Fibrinolysis is a very complex system. It is

involved in haemostasis, thrombolysis and spread of cancer. The mechanism by which thrombolysis take place is not well understood. Balkuvulutin (1978) reported that there is a homeostatic balance between coagulation and fibrinolysis. McNicol and Douglas (1976) however, believe that the two systems interact, but the concept of dynamic equilibrium between coagulation and fibrinolysis is not proven. Fibrinolysis has been extensively studied and different pathways have been suggested, including Factor XII dependent pathway, Factor XII independent pathway and complement mediated fibrinolysis.

Ratnoff (1977) reported that Hageman factor acts in many systems to defend the body against injury, initiating clotting, fibrinolysis, kinin formation, chemotaxis, and enhancement of vascular permeability. Moroz and Gilmore (1976) studied the fibrinolytic activity of normal plasma, using I^{125} fibrin solid phase assay. Plasma enzymes were compared with plasminogen-plasmin fibrinolytic system, Hagman factor activation pathway and complement system.

Removal of plasminogen did not change the plasma activity, and plasmin inhibitors in concentrations which would inhibit pure plasmin had no effect. Proteolytic activity was detected in globulin and pseudoglobulin fractions, and contact factor inhibitors and activators did not effect the plasma enzyme. From these and other experiments Ratnoff concluded that plasmin and Hageman factor dependent pathway play a minor role in the spontaneous or basal fibrinolytic activity of normal plasma. This activity therefore is due to proteinases other than plasmin

and might include proteinases from the complement system. However, the plasminogen-plasmin system has been the most extensively studied. Most investigators believe that plasminogen plays a major role in fibrinolysis. Many biological pathways leading to plasminogen activation have been suggested. Under normal conditions no plasmin is detected in the blood, but activators of plasminogen are present in blood and tissues. However, the sites and the normal mechanisms of fibrinogen catabolism are not certain.

Larrieu et al.(1978) suggested the theory of deposition of fibrin on the endothelial wall and dissolution of fibrin by fibrinolysis. They also mentioned that this pathway is not a major system for fibrinogen catabolism, and there are many other unknown factors. Sherman (1977) reported that fibrinogen may be catabolized in endothelial cells, fibrinogen fragments D and E in kidney, while the site of formation and degradation of fraction I-8 is unknown, although it is the result of a proteolytic pathway other than plasmin. Sherman also believes that the pathways which have been mentioned so far cause only a small turnover of fibrinogen and the major catabolite site is not known.

Moroz and Gilmore (1976) reported that several factors are involved in fibrinolytic activity in blood. These include plasma factors, platelets, plasminogen independent enzymes of leucocytes and the complement pathway. Plow and Edgington (1975) also reported that leucocytes contain a fibrinolytic pathway independent of the plasminogen system. Two fibrinolytic proteinases were purified from the granular fraction of leucocytes. One of the proteolytic enzymes,

responsible for 60-70% of fibrinolytic activity was similar to elastase, while the other was a chymotrypsin-like enzyme. Both enzymes digested fibrinogen fragments D and E as well as fibrinogen.

Gottlob et al.(1978) reported that leucocyte enzymes which digest fibrin, elastase and collagen are present in the leucocyte's granules. The fibrinolytic action of leucocytes was studied by incorporation of leucocytes in plasma clots, and addition of streptokinase to washed clots. The enriched leucocytes clots were dissolved completely within 2-7 days. Addition of streptokinase enhanced the clot lysis, although very little plasminogen is present in the washed clot. From their work, Gottlob et al.(1978) deduced that the leucocytes proteinase differed from plasmin in that it is less inhibited by serum and is less active towards the substrate casein. The enzyme is not inhibited by EDTA, EACA or trasylol, and the inhibition by α_2^M is very weak.

Joist (1977) reported that platelets play an important role in the maintenance of vascular integrity, forming the basis of the haemostatic plug which facilitates the interaction of coagulation factors involved in the formation of fibrin. Platelets can also activate, as well as inhibit, fibrinolysis.

Watada et al.(1977) using gel filtered platelets, showed that platelets contain a fibrinolytic enzyme which lyses plasminogen free fibrin. The activity of the enzyme increased when sonicated platelets were used and no activity was detected when platelet rich plasma was used. From these observations Watada et al.(1977) concluded that the fibrinolytic activity of platelets is masked by inhibitors in the

plasma environment.

Aberg et al.(1975) analysed the coagulation and fibrinolytic components of wound secretions, and found high levels of fibrin degradation products and unclottable fibrinogen. Wound exudates also showed high fibrinolytic activity when tested on fibrin plates. As a higher activity was found when heated plates were used, and EACA had very little effect on the proteolytic activity, Aberg et al.(1975) deduced that the fibrinolytic activity was not due to plasmin but to some other proteinases.

Gaffney et al. (1976) compared fibrin subunits from venous and arterial thrombi. The SDS gel electrophoresis patterns showed not only β, γ-γ and α polymer, but subunits of molecular weight of 33,000. On further examination they reported that this subunit is a degradation fragment of β or γ chains, which may have been due to plasmin or other enzymes.

Apart from proteinases which act on fibrin(ogen) under physiological conditions, there is some evidence for the presence of acid proteinases in human plasma, which are capable of digesting fibrin(ogen), and their mode of action has been studied in vitro.

Shanberge et al.(1972) investigated the variation in fibrin clot stability in acid media between individuals and found that fibrin clots from females are more resistant to lysis in monochloroacetic acid (MCA). Fibrin clots made from the plasma of females on oral contraceptive are even more stable than those from males or females. They concluded that the increased fibrin clot stability in acid media

is not due to an increase in fibrinogen concentration or factor XIII level. In 1973, Shanberge et al. purified an enzyme with molecular weight of 44,000 and a pH optimum of 2.0, which they thought could be responsible for the dissolution of fibrin clot in acid media.

Ikemori et al.(1975) investigated the solubility of fibrin clots in acid media. From their observations, they concluded that the solubility of the clots in monochloroacetic acid is not only due to the lack of factor XIII, as previously thought, but other factors are involved. They observed that although newly formed fibrin clots will dissolve in MCA, if older clots were washed free from serum before the addition of MCA, they become completely resistant to lysis. They suggested that an enzyme was present in plasma and serum and this was pepsinogen, because (a) it was activated at pH 2.0, (b) the activity was destroyed by heating to 60°C for 10 min., (c) when pepsinogen was added to MCA it dissolved well cross-linked fibrin clots, (d) purified sample of the enzyme gave a reaction line with pepsinogen antiserum with slight crossover. Serum did not give a precipitation line due to low concentration.

Ikemori et al.(1975) also reported that fibrin clots formed from the plasma of a patient with pernicious anemia were resistant to lysis. They concluded that it was due to the low level of pepsinogen in patients' serum rather than abnormally stable clots, as their washed clots were dissolved in acid media once they were exposed to normal plasma.

Ragaz et al.(1976) reported an unusual bleeding disorder in a female patient which clinically resembled factor XIII

deficiency. The patient had a history of easy bruising from childhood, postoperative haemorrhage, delayed wound healing and abnormal scar formation. Laboratory results showed no coagulation abnormalities, except that her plasma fibrin clot dissolved completely in 1% (w/v) monochloroacetic acid (MCA) within 3 h, but remained insoluble in 5 M urea. Factor XIII deficiency or abnormal polymerization of her fibrinogen was ruled out. Patient plasma markedly enhanced dissolution, in acid media, of well cross-linked fibrin from normal plasma. Normal plasma and patient plasma both hydrolysed denatured haemoglobin, but the enzyme activity in patient plasma was twice that of normal plasma. They concluded that patient had increased pepsin-like proteinase activity in her plasma.

Although acid proteinase activity is reduced in women taking oral contraceptive (Shanberge et al.,1972) this patient had a high level of acid proteinase activity in her plasma despite being on oral contraception.

Laing (1975) partially purified the plasma enzyme(s) from the patient described by Ragaz et al.(1975) and normal plasma. From the elution pattern of column chromatography she concluded that there are three forms of acid proteinases in plasma, but was undecided as to whether they were entirely different enzymes or forms of the same enzyme. Taylor (1976) followed the work of Laing and on assaying the plasma enzyme using a dyed fibrin assay method found that the plasma enzyme had 10 times greater activity than pepsin. However, pepsin had higher activity on denatured haemoglobin, and on this basis Taylor suggested that the plasma enzyme was not pepsin.

Law and Kemp (1977) purified the plasma enzyme even further and reported that the semi-purified enzyme on SDS gel electrophoresis revealed a major band with molecular weight of 51,000 and a minor band of 86,000. Immuno-electrophoresis showed that the sample was contaminated with albumin.

Law (1978) followed the purification and characterization of the plasma enzyme further and concluded that the enzyme with highest activity had molecular weight of about 64,000, but was still contaminated with albumin. Law suggested that the enzyme digested the albumin and formed a complex with degraded albumin. The plasma enzyme had pH optimum of 2.4, and its activity against denatured haemoglobin was inhibited by pepstatin. Therefore Law classed it as a carboxyl (aspartyl) proteinase.

The aim of the project was to assess and develop assay systems suitable for the investigation of fibrin dissolution in acid environment. Such assay methods would then be used to attempt the purification and more significantly, the characterization of the enzyme system(s) responsible. The characterization will provide a rigorous test of the hypothesis (Ikemori et al., 1975) that the enzyme responsible is pepsin.

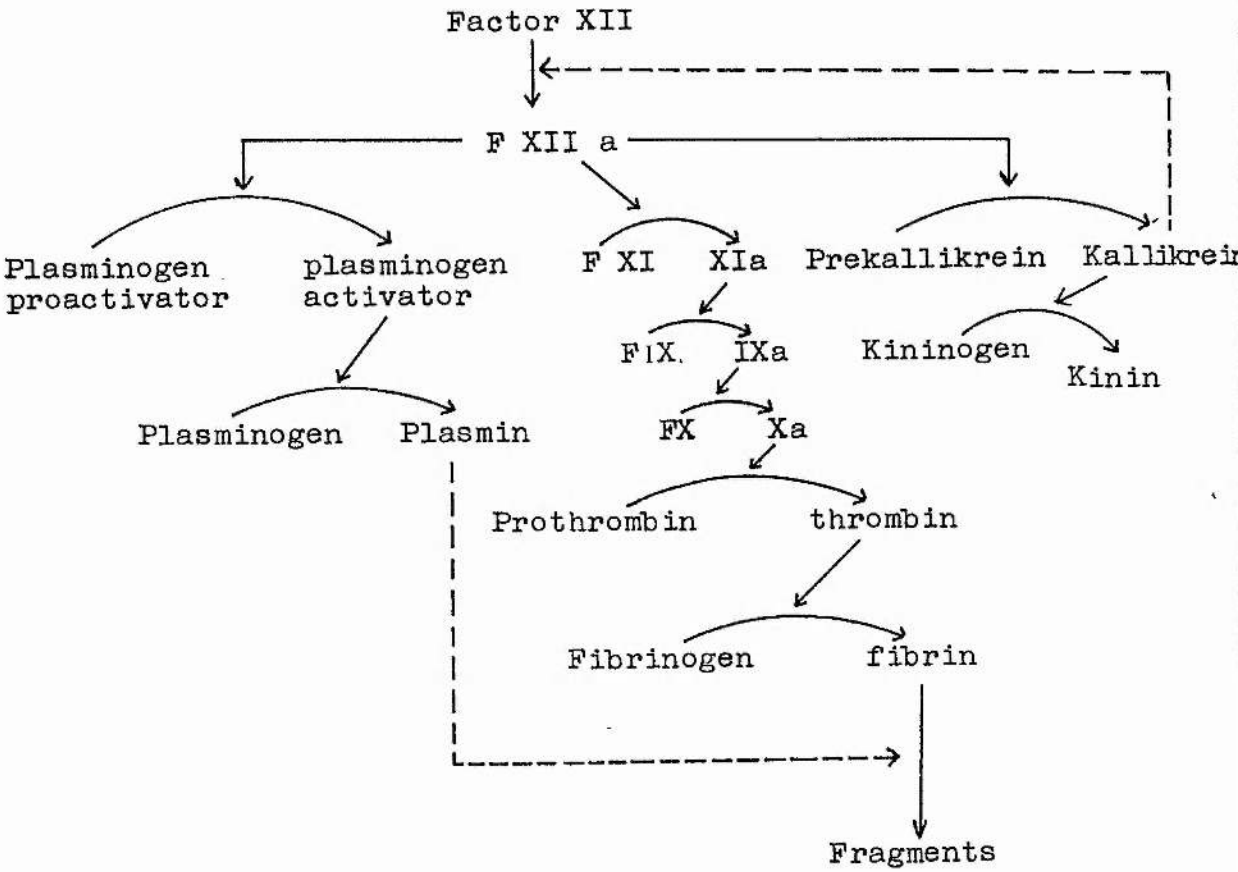


Fig. 1.1 Interrelationships among three plasma activation systems, i.e. the coagulation, fibrinolysis and kallikrein systems.
From Neurath and Walsh (1977).

CHAPTER 2

GENERAL METHODOLOGY

2.1. Sodium Dodecyl Sulphate (SDS) Disc Gel Electrophoresis

The method used was a modification of that described by Weber and Osborn (1969).

REAGENTS

2.1.1. Chamber buffer

0.1 M Tris-HCl, pH 7.4 containing 0.2% (w/v) SDS.

2.1.2. Gel buffer

0.1 M Tris-HCl, pH 7.4 containing 0.2% (w/v) SDS and 6 M urea

2.1.3. Acrylamide/N',N' methylbisacrylamide (bisacrylamide) stock solution

	5% gel solution
Acrylamide	9.5 g
Bisacrylamide	0.5 g
Gel buffer	200 ml

Stock solutions were stored at 4°C.

2.1.4. Ammonium persulphate

1.0% (w/v) in distilled water, made up fresh for use.

2.1.5. N, N, N',N', Tetra methyl ethylene diamine (TEMED)

2.1.6. Staining solution

Coomassie brilliant blue R250	2-3 g*
Distilled water	450 ml
Methanol	450 ml
Glacial acetic acid	100 ml

The solution was filtered before use.

* for reagent of 70% purity

2.1.7. Destaining solution

Methanol	250 ml
Glacial acetic acid	75 ml
Distilled water	675 ml

2.1.8. Tracking dye

0.05 g bromophenol blue in 100 ml distilled water

2.1.9. Standards

Suitable proteins such as:

Human serum albumin	(Sigma)
Egg albumin	(Sigma)
Myoglobin	(Sigma)
Phosphorylase	(Sigma)

Standard stock solutions

2.0 mg protein was dissolved in 1.0 ml of chamber buffer in thin walled glass tubes. The tubes were placed in a boiling water bath for 2 min. After cooling they were stored at -20°C.

Standard working solutions

50 µl of each stock solution of standards were mixed with 0.25 ml of chamber buffer in thin walled glass tubes. 0.25 ml solution of 8 M urea/3% (w/v) SDS/ 3% (v/v) mercaptoethanol was added and the tubes were placed in a boiling water bath for 2 min. 25 µl of each standard was used per run.

2.1.10. Gel preparation

19 ml of acrylamide/bisacrylamide stock solution was mixed with 1.0 ml of 1% (w/v) ammonium persulphate, and the mixture was degassed. 20 µl of TEMED was added,

and the gel was poured immediately into the 5 mm x 80 mm glass tubes, and layered the gels with distilled water. After the gels were polymerized, the water was replaced by gel buffer and the gels were stored at 4°C.

2.1.11 Sample preparation

Samples were dialysed against chamber buffer to ensure complete equilibration. They were then applied to the gels either reduced or non-reduced.

Preparation of reduced samples

To reduce the samples, equal volume of 3% (w/v) SDS/ 3% (v/v) mercaptoethanol/8 M urea was mixed with the sample and the mixture was boiled for 5 min.

Preparation of non-reduced samples

Equal volume of sample and 3% (w/v) SDS/8 M urea were mixed and incubated at room temperature for 5 min.

2.1.12 Gel electrophoresis

Samples and standards were mixed with a drop of a mixture of tracking dye and glycerol, and applied to the gels. The gels were electrophoresed at a current of 5-8 mA/gels from cathode to anode. The electrophoresis of the gels continued until the tracking dye reached about 5 mm from the bottom of the gel, which took approximately 1½-2 h. The amount of samples applied to the gels was usually between 5-25 µg. The gels were removed from the tubes and the length of the gels and the distance which the tracking dye had

travelled were measured, or the tracking dye bands were marked with Indian ink. The gels were stained for 30-60 min. and destained overnight with several changes of destaining solution.

2.1.13 Molecular weight estimation

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length of the gel after destaining}} \times \frac{\text{length of the gel before staining}}{\text{distance of the dye migration}}$$

or

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{distance of dye migration}}$$

The mobility of standards are plotted against their molecular weight on a semi logarithmic scale. The molecular weight of unknown is read from the graph.

2.2. Polyacrylamide Gradient Gel Electrophoresis

The gel gradients (3-27%) were very kindly provided by Dr. Kemp.

Gel electrophoresis

Gradiant pore Uniscil electrophoresis unit with a pump was used for the circulation of the buffer. Gels were electrophoresed at constant voltage of 150 for 2 h. in 0.1 M Tris-HCl/0.006 M boric acid, pH 9.2. The gels were removed, and 1% (w/v) molten agar (in chamber buffer) was layered on the top of the gel and an "inverted comb" was placed on the hot agar. After the agar had set, the comb was removed and samples were applied as 2.1.12. Samples were electrophoresed for approx. 18 h. then gels were removed, stained and destained as 2.1.12.

2.3. Immunoelectrophoresis

Method

0.24 g Agarose (BDH) was dissolved in 24 ml of 2% (w/v) sodium barbitone buffer, pH 8.2 by boiling. Six microscope alides were covered uniformly with the molten agarose, on a horizontal table, and allowed to set. Samples were placed in the well punched in the agarose along with tracking dye (Bromophenol Blue). The samples were run at a constant voltage of 150 v, until the tracking dye had run to the end (about 3-4 h.). Specific antisera were placed in each trough and allowed to diffuse into the agarose in a humid chamber for 18 h. at room temperature.

Slides were either (a) washed in 1% (w/v) NaCl for 48 h., followed with overnight wash in distilled water, before drying at 37°C or (b) dried by pressing with thick layers of filter paper between two heavy blocks. In this way time spent on washing in salt and water was eliminated. Slides were stained in Coomassie Blue (2.1.6) and destained (2.1.7) overnight.

2.4. Immunodiffusion Technique

Agar plates were prepared as (2.3). Rosette patterns were cut in the gels and the wells were filled with different concentrations of samples with the antiserum in the centre well. The plates were left at room temperature or incubated at 37°C for 24h. Antigen and antibody placed in wells diffuse towards each other and precipitate is formed as an opaque line in the region where they meet in optimal proportions.

2.5. Protein Estimation

The method used was based on that of Bradford (1976). Coomassie brilliant blue G-250 binds to protein and causes a shift of maximum absorption from 465 nm to 595 nm. The increase in absorption at 595 nm is monitored.

2.5.1. Preparation of protein reagent

100 mg of Coomassie brilliant blue (Sigma) was dissolved in 50 ml of 95% (v/v) ethanol. 100 ml of 85% (w/v) phosphoric acid was added, and the total volume was made up to 1 l. The solution was mixed and filtered through Whatman No. 1 filter paper.

2.5.2. Assay

A standard curve was constructed using solutions of bovine serum albumin (Sigma BSA). Varying concentrations (100-1000 µg/ml) of standard were prepared. To 0.1 ml of each concentration, 5 ml of protein reagent was added, mixed and left at room temperature for 2 min., for the colour to develop. The absorbance was measured at 595 nm against a blank which has been prepared the same way as the standard except BSA was replaced by 0.1 ml of buffer of choice. The test sample was prepared in the same way as the standard and the concentration of the test sample was determined from the standard curve. The response was linear up to 60 µg protein. Reagent supplied by Bio-Rad and reagent made in the laboratory gave very similar results, Fig. 2.1. The home-made reagent being cheaper, was preferred.

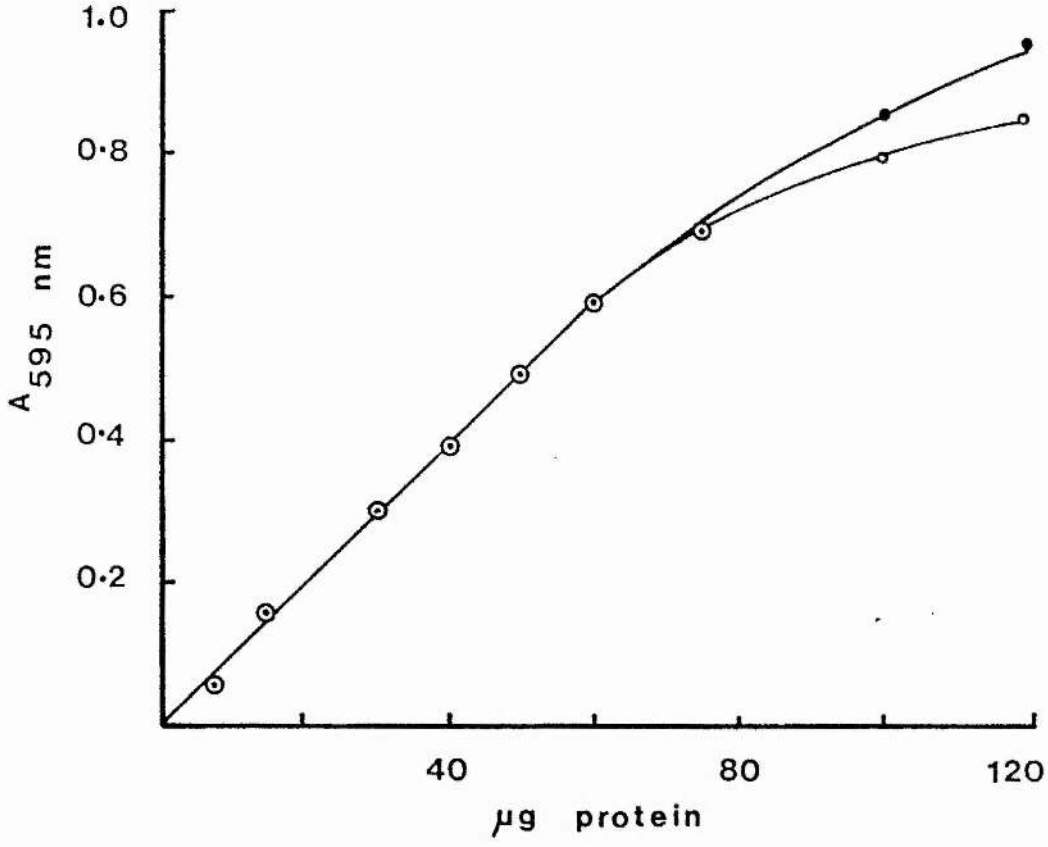


Fig. 2.1. STANDARD CURVE FOR PROTEIN ESTIMATION USING BSA AS STANDARD PROTEIN

●——● Bio-Rad kit reagents

o——o Home-made reagent

2.6. Fibrinogen/Fibrin Estimation

The method used was based on that of Dacie and Lewis (1975a).

2.6.1. Assay procedure

Fibrin clots were made as (3.2.4) and were washed in 0.85% (w/v) NaCl 3 times. 1.0 ml of 2.5 M NaOH was added to each clot and boiled for 15 min. to dissolve the clot. The solution was cooled, then 1.5 ml of Folin-Ciocalteau reagent were added. The mixture was incubated at room temperature for 20 min., for the colour to develop.

The absorbance was read at 650 nm. The blank was prepared as above by omitting the fibrin clot. At the same time, standard was prepared using 0.2 ml of 0.2 mg/ml tyrosine in 0.1 M HCl, in place of fibrin clot.

2.6.2. Calculation of the results

$$\frac{t}{s} \times vs \times cs \times \frac{1}{vt} \times 11.7 = \text{mg/ml concentration of fibrinogen.}$$

t = absorbance of the test solution - blank

s = absorbance of the standard solution - blank

vs = volume of the standard in ml

cs = concentration of the standard in mg/ml

vt = volume of the original sample which was used to make the fibrin clot

11.7 = conversion factor for liberated tyrosine to fibrinogen concentration.

Standard curve was constructed using different volume of the precipitate of 25% Ammonium sulphate

gel fractionation (25 AS) of plasminogen free bovine plasma/to make fibrin clot. The standard curve is used shown in Fig. 2.2.

2.7. Plasminogen Assay

2.7.1. Fibrinogen plate substrate

The method used was a modification of Noren et al. (1975). 0.1 g of agar was dissolved in 8 ml of 0.05 M Tris-HCl, pH 7.4 by boiling. The temperature of agar was brought to 56°C. 20 mg of human fibrinogen (Kabi Vitrum, London) was dissolved in 2 ml buffer, and mixed with the agar. The mixture was poured into a glass petri dish, and allowed to set at room temperature on a level surface. At the same time another plate was prepared as above except the fibrinogen was added to the agar at 70°C to inactivate the contaminating plasminogen. 5-25 µl of samples were placed towards the centre of each quarter of the plates which were then incubated at 37°C for 24 h.

2.7.2. α-Casein substrate

The method used was based on that of Alkjaersig et al. (1959).

2.7.2.1. Reagents preparation

1.5 g of α-casein (Hammarsten) was dissolved in 100 ml of 0.05 M phosphate/0.1 M NaCl buffer, pH 7.4 and the volume was brought to 250 ml with distilled water.

36 mg of tyrosine was dissolved in 900 ml of

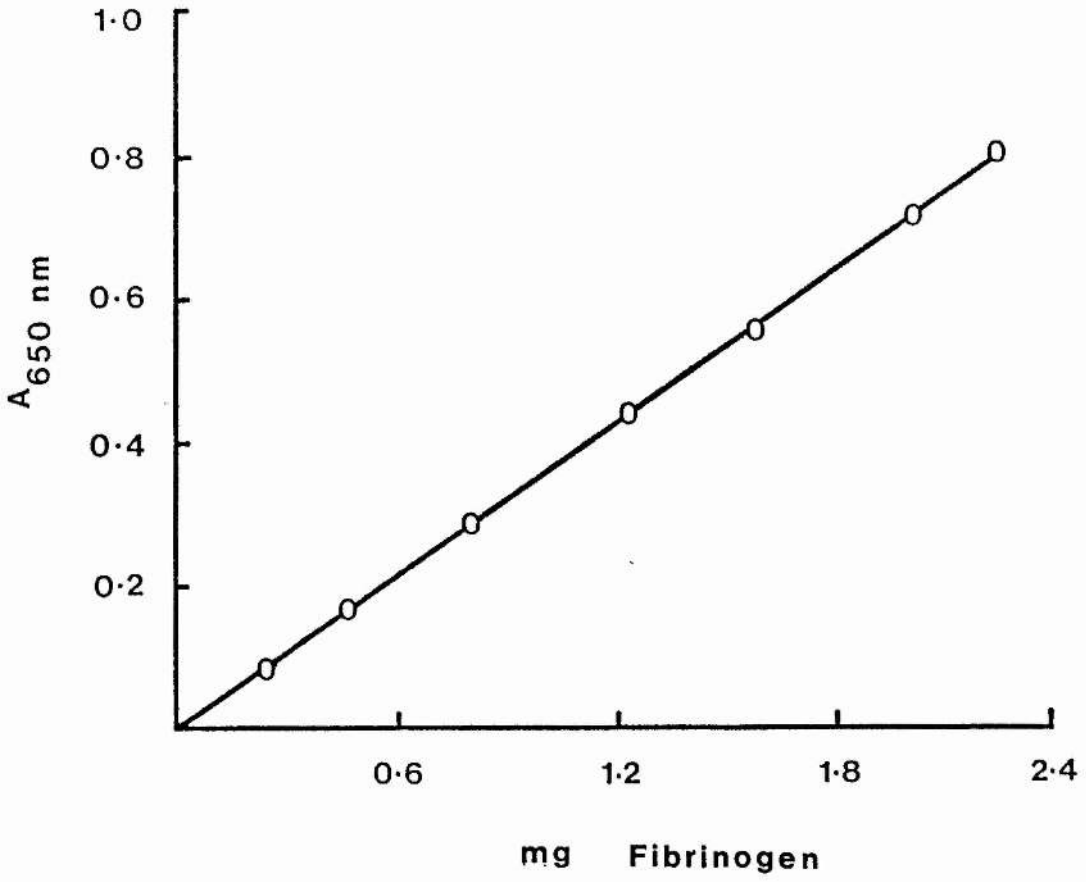


Fig. 2.2. STANDARD CURVE FOR FIBRIN CONTENT OF CLOT

Variation of absorbance at 650 nm. A graph of variation of absorbance at 650 nm versus the concentration of fibrinogen used to make fibrin clot.

of 0.1 M HCl/100 ml of isopropanol.

The tyrosine was used as a standard.

2.7.2.2. Assay method

0.5 ml of sample was mixed with 0.1 ml of 0.1 M barbitone/HCl buffer, pH 7.4. 0.1 ml of 2000 units streptokinase (Behring) was added to the sample, followed by the addition of 2.0 ml of α-casein. The mixture was incubated in a water bath at 37°C, and the time was noted. After 2 min. 1.0 ml of incubation mixture was transferred to a tube containing 1 ml of 10% (w/v) TCA. After 60 min. the subsampling was repeated. The two samples were centrifuged at 1500 g for 10 min. The precipitates were discarded and 2.5 ml of 0.5 M NaOH, 0.75 ml of 5% (w/v) TCA and 0.75 ml of Folin-Ciocalteu reagent were added to the supernatents. The mixtures were left at room temperature for 15 min. for the colour to develop. The absorbance was read at 650 nm. The two min. sample served as a blank for the 60 min. sample.

2.7.2.3. Plasminogen standard curve

Standard curve relating absorbance at 650 nm to concentration of tyrosine was constructed, Fig. 2.3.

Definition of units/ml plasminogen

Tyrosine standard containing 0.5 ml of stock solution 3.6 mg % (w/v) in assay system has been given an arbitrary value of 4 plasminogen units.

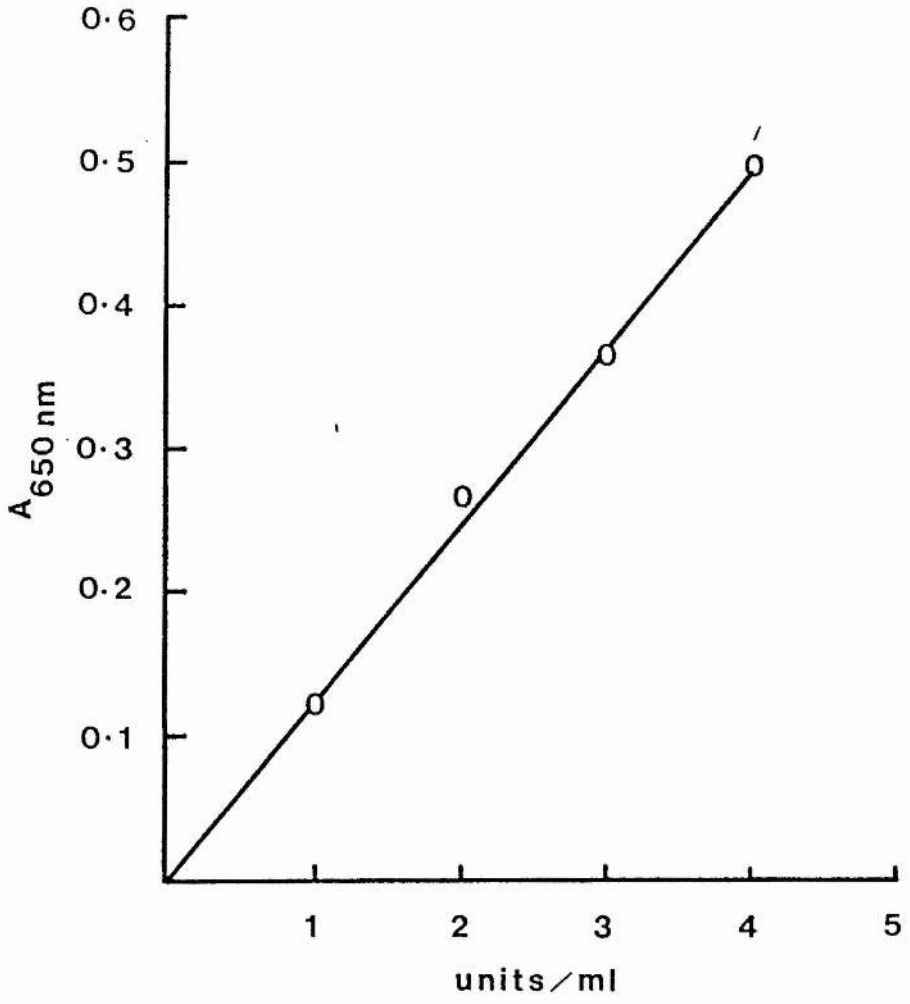


Fig. 2.3 STANDARD CURVE FOR PLASMINOGEN ESTIMATION

Variation of absorbance with concentration of tyrosine (units of plasminogen).

2.7.3. Synthetic substrate S-2251

The chromogenic substrate S-2251 (H-D-val-Leu-Lys-pNA) is a sensitive and specific substrate for plasmin and streptokinase-activated plasminogen. S-2251 is a soluble and stable substance and is also very sensitive to small amounts of plasmin ($< 10^{-11}$ moles) (Friberger et al., 1978).

2.7.3.1. Assay method

Substrate stock solution was prepared by dissolving 25 mg of S-2251 (Kabi Vitrum, London) in 15 ml distilled water. A Cecil spectrophotometer with cell carriage at 37°C was used. The spectrophotometer was set at 405 nm and zeroed with distilled water. Plasminogen buffer (0.05 M Tris-HCl, 0.012 M NaCl, pH 7.4 was equilibrated at 37°C. 100 µl of the sample and 100 µl of streptokinase (2000 units/ml) were mixed in 1 ml plastic cuvette and incubated at 37°C for 10 min. 700 µl of buffer and 100 µl of substrate were added to the mixture, and the absorbance was recorded until plateau formation.

2.7.4. Estimation of bovine plasminogen

Summaria et al. (1974) reported that bovine plasminogen (0.1 mg) could not be activated to plasmin even with as much as 100,000 units of streptokinase. However, addition of small amount of human plasminogen could trigger the activation of bovine plasminogen to plasmin. To estimate bovine plasminogen, a known concentration

of human plasminogen was added to the bovine plasminogen and the mixture was assayed as 2.7.3.1. To calculate the activity of bovine plasminogen in the mixture, the activity due to the human plasminogen added originally was subtracted from the total.

2.8. Fluorometric Measurement of Fibrin-Stabilizing Factor (F XIII).

Some amines inhibit the crosslinking of fibrin, by acting as a pseudo substrate and blocking the acceptor group of normal crosslinking.

$$H_2N.F.Co.Y + H_2N.F.Co.Y \xrightarrow[Ca^{++}]{FXIII} H_2N.F.CoF.CoY + HY$$

Fibrin Fibrin Ca Cross linked fibrin

$$H_2N.F.Co.Y + H_2NR \xrightarrow[Ca^{++}]{FXIII} H_2N.F.Co.NH.RN. + HY$$

Fibrin pseudosubstrate

Monodansyl cadaverine was used as the amine donor, and fibrinoligase was measured fluorometrically.

2.8.1. The assay

Method used was based on that of Lorand et al.(1969).

2.8.2. Reagents

(I) 0.2 M glutathione was prepared in 50% (v/v) aqueous glycerol and the pH was adjusted to 7.5, with 3 M NaOH. (II) 125 NIH units/ml thrombin (Sigma) was made in 25% (v/v) aqueous glycerol, and made 0.02 M with respect to $CaCl_2$, pH 7.5. (III) 2 mM monodansyl cadaverine was prepared in 0.05 M tris-HCl, pH 7.5, containing 3 mM $CaCl_2$. (IV) 0.4%

(w/v) casein was made in a mixture containing 10% (v/v) glycerol, 3 mM $CaCl_2$ and 0.05 M Tris-HCl, pH 7.5, then it was dialysed against the same buffer. The dialysed solution was centrifuged at 1500 g for 60 min. (V) The solution for measuring the protein bound amine was prepared using 8 M Urea, 0.5% (w/v) Na dodecyl sulphate in 0.05 M Tris-HCl, pH 8.0.

2.8.3. Assay procedure

0.2 ml of plasma and 0.05 M (v/v) aqueous glycerol were mixed, and brought rapidly to 56°C, and kept at that temperature for 2.5 min ("desensitization" of fibrinogen by heat). The mixture was cooled by immersing the sample in an ice bath. 0.05 ml of 0.2 M glutathione solution and 0.2 ml of thrombin were added to the mixture and after mixing the solution was left at room temperature for 20 min. 0.5 ml of 2 mM dansylcadaverine solution and 1.0 ml of 0.4% (w/v) casein were added. The mixture was incubated for a further 30 min.

The incorporation of amine was stopped by addition of 2 ml of 10% (w/v) TCA. The protein precipitate was washed with ethanol/ether (1:1 v/v) and dried. The dried precipitate was dissolved in 2 ml of 8 M Urea solution for protein bound amine measurement. Fluorescence intensity was measured at an emission wave length of 518 nm and excitation of 335 nm.

Calculation of units of F XIII per ml of plasma

$$\frac{2}{0.2} \times \frac{1}{3} \times \frac{1}{0.9} \quad \frac{iT - iB}{iR - iS}$$

$\frac{2}{0.2}$ = 10 dilution factor for sample in test system

$\frac{1}{3}$ = assay in 10 min incubation instead of 30 min.

$\frac{1}{0.9}$ = 10% correction for loss of Factor XIII during the heating of plasma.

iT = fluorescent intensity of test

iB = fluorescent intensity of blank

iR = 1 μ mol/liter monodansyl cadaverine in solvent system

iS = solvent system, fluorescent intensity.

Blank = same as the test without monodansyl cadaverine

Solvent system = 8 M Urea, 0.5% (w/v) SDS in 0.05 M Tris-HCl, pH 8.0.

2.9. Prothorombin Estimation

2.9.1. Preparation of brain thromboplastin

Method used was based on that of Dacie and Lewis (1975b). Fresh rabbit brain was used. The membrane and blood vessels were removed from the brain which was macerated in 5-10 times volumes of acetone. The acetone was changed at least 3 times, until the granular material was grease-free. The granular material was air dried and stored in an evacuated desiccator at 4°C. For use in the assay system, 5 ml of 0.85% (w/v) NaCl was added to 30g of the dry brain powder. The mixture was incubated at 37°C for 15-30 min. with occasional mixing. The coarse particles were left to sediment, or centrifuged at 100g for 5 min. The supernatent (thromboplastin) was used in the

assay of prothrombin.

2.9.2. Performance of the assay

The method used was based on that of Dacie and Lewis (1975). To a 75 mm x 12 mm tube containing 0.1 ml thromboplastin, 0.1 ml of the sample was added. The mixture was incubated at 37°C for 1-2 min. 0.1 M of prewarmed (37°C) 0.025 M $CaCl_2$ was added to the mixture, and a stopwatch started simultaneously, and the time taken for fibrin clot to form was noted.

2.10. Purification of fibrin stabilizing factor (F XIII) from bovine plasma

The method used was based on that of Lorand and Gotoh (1970).

6 l of bovine blood was mixed with 0.08 M potassium oxalate (ratio of 9:1), and centrifuged at 200 g for 2 h. The plasma was separated and recentrifuged to remove any remaining red blood cells. 2 l of plasma was mixed with 500 ml of saturated ammonium sulphate and stirred at 4°C for 1 h. The solution was centrifuged at 10,000 g for 30 min. at 4°C.

The precipitate was dissolved in 0.15 M KCl, and the pH adjusted to 5.4 with 1 M acetic acid. 312.5 ml of solution was mixed with 60 ml of saturated ammonium sulphate, stirred and centrifuged as before. The precipitate was dissolved in 0.05 M Tris-HCl/0.1 M NaCl, pH 7.0. To remove fibrinogen the solution was mixed with equal volume of 0.05 M Tris-HCl/0.1 M NaCl, pH 7.0.

The mixture was heated at 56° for 3 min. by rapid immersion of the solution in a water bath at 80°C. Denatured fibrinogen was removed with the help of glass rod, and the supernatent centrifuged at 2000 g for 15 min. to remove any residual fibrinogen. The supernatent (200 ml) was cooled to 4°C, mixed with 60 ml of saturated ammonium sulphate, and centrifuged as before. The precipitate was dissolved in 10 ml of 0.05 M Tris-HCl/.001 M EDTA, pH 7.5, and dialysed against the same buffer for 18 h at 4°C.

2.10.1 Ion-exchange chromatography

2.10.1.1 DEAE-cellulose 52 (Whatman).

The DEAE-cellulose resin was precycled and degassed. A column of 40 cm x 2.5 cm was then packed with the resin, and allowed to equilibrate by pumping 0.05 M Tris-HCl/0.001 M EDTA, pH 7.5 at a flow rate of 36 ml/h. The semi-purified sample of Factor XIII which has been dialysed against the above buffer, was applied. The column was eluted with equilibration buffer, until no protein was detected in the effluent. A linear gradient to a final concentration of 0.3 M NaCl, and with a total volume of 600 ml was then applied.

2.10.2 Gel filtration

2.10.2.1 Sephadex G 200

Sephadex G 200 (Pharmacia) was swollen for two days at room temperature in an excess of buffer (0.05 M Tris-HCl, 0.001 M EDTA, 0.1 M NaCl, pH 7.5 . The resin was degassed and packed into a column of

35 cm x 2.2 cm. The buffer was allowed to go through the column by gravity, at the rate of 6.8 ml/h. The sample was applied to the column and fractions were collected every 20 min.

2.10.3. F XIII purification results

2.10.3.1 DEAE cellulose

Three major peaks were obtained, Fig. 2.4. Samples under each peak were pooled separately and assayed for F XIII. The F XIII activity was measured using fibrin clot assay 3.2.5 (Fibrinogen Grade L Kabi Vitrum, London) which had been passed through lysine sepharose column and also shown to be lacking in F XIII, activity was used as a source of fibrin clot. Peak II and Peak III both showed activity, with the highest concentration in Peak II. SDS gel electrophoresis was performed on an aliquot of each pool, using 5%, 10% SDS gels and using reduced and non-reduced samples. F XIII was detected in both peaks. As the SDS gel revealed the presence of other proteins, a further purification step was necessary.

The pool from Peak II was concentrated by ammonium sulphate precipitation (35% saturation) as before. The precipitate was dissolved in 2.0 ml of 0.05 M Tris-HCl/0.001 M EDTA, 0.1 M NaCl, pH 7.5, and dialysed against the same buffer, before being subjected to gel filtration.

2.10.3.2 Sephadex G 200.

Elution pattern from Sephadex G 200 column showed two

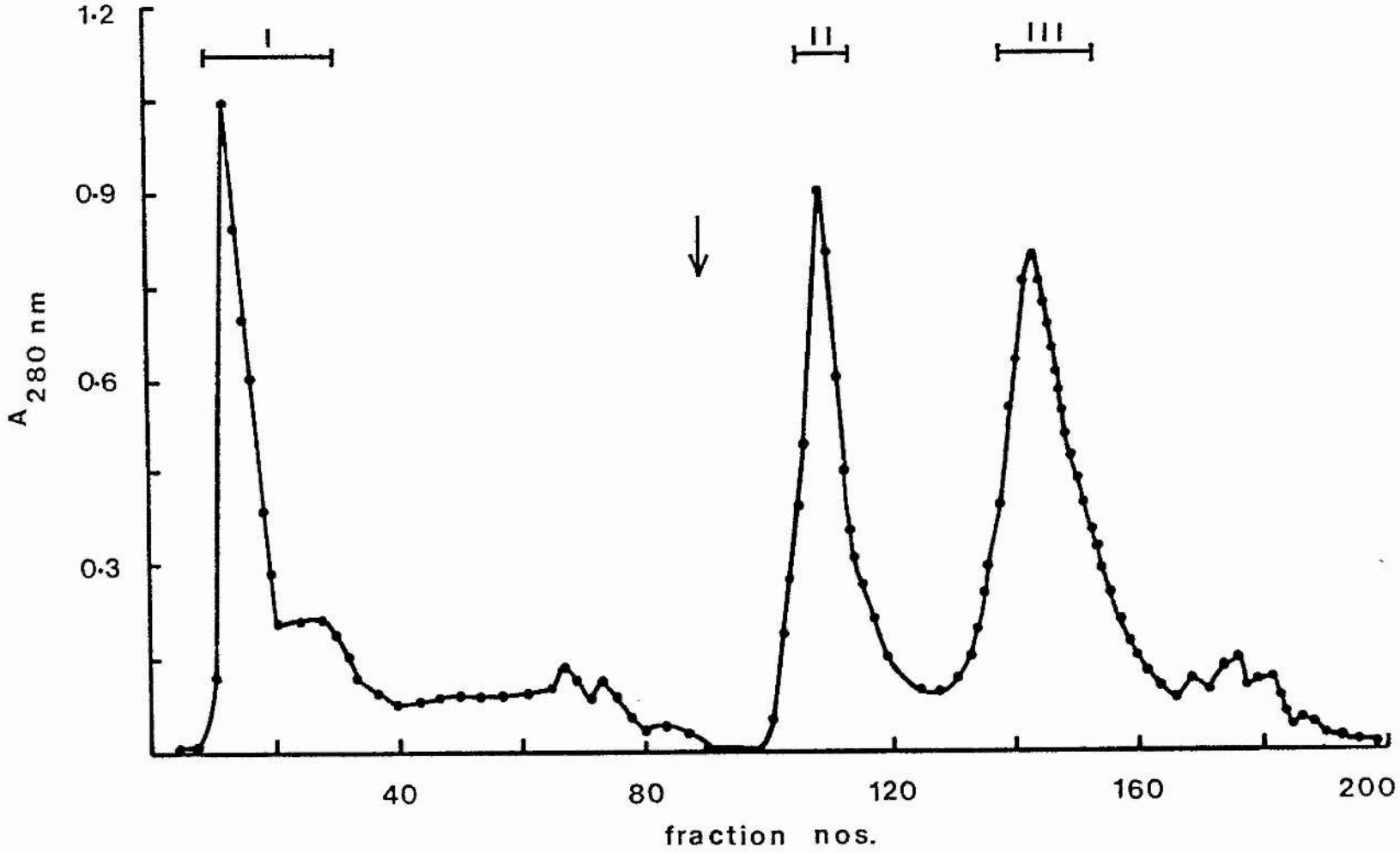
Fig. 2.4 DEAE CELLULOSE COLUMN CHROMATOGRAPHY OF BOVINE FACTOR XIII

absorbance at 280 nm

salt gradient applied(section 2.10.1.1.)

fractions pooled

Pool II and III had Factor XIII activity with highest concentration in Pool II.


1·2
0·9
0·6
0·3
A 280 nm
I
II
III
40
80
120
160
200
fraction nos.

peaks, Fig. 2.5. Fractions 20-30 were assayed for Factor XIII as 2.8.3 . All fractions showed activity with the highest levels in fractions 21-26. SDS gel electrophoresis was performed as 2.1.12 , and fractions 21, 22 and 23 showed no contamination. These fractions were pooled, concentrated as 2.10 , using ammonium sulphate, and dialysed against 0.05 M Tris-HCl/0.001 M EDTA/0.01 M NaCl, pH 7.5. Glycerol was added to a final concentration of 20% (v/v) and the sample was stored at -20°C.

2.11. Purification of Bovine Fibrinogen

The method used was based on that of Lawrie et al.(1979). Bovine blood was collected at slaughter, and allowed to run into a solution of 0.106 M Tri-sodium citrate 9:1 (v/v) blood to anticoagulant . The blood was centrifuged at 1200-1500 g for 1 h. The clear plasma was applied to a lysine-sepharose column (4.4.1.2) for preparation of plasminogen-free plasma.

2.11.1 Ammonium sulphate fractionation (AS)

Plasminogen-free plasma was brought to 25% saturation with ammonium sulphate, and after 1-2 h. the suspension was centrifuged at 1500 g for 20 min. The supernatent was discarded and the precipitate was washed with 23% saturated ammonium sulphate. The suspension was centrifuged as before, and the precipitate was suspended in 0.05 M Tris-HCl/0.05 M NaCl, pH 8.6 and dialysed against the same buffer for 24 h. with 2 changes of buffer.

The sample was applied to DEAE cellulose column.

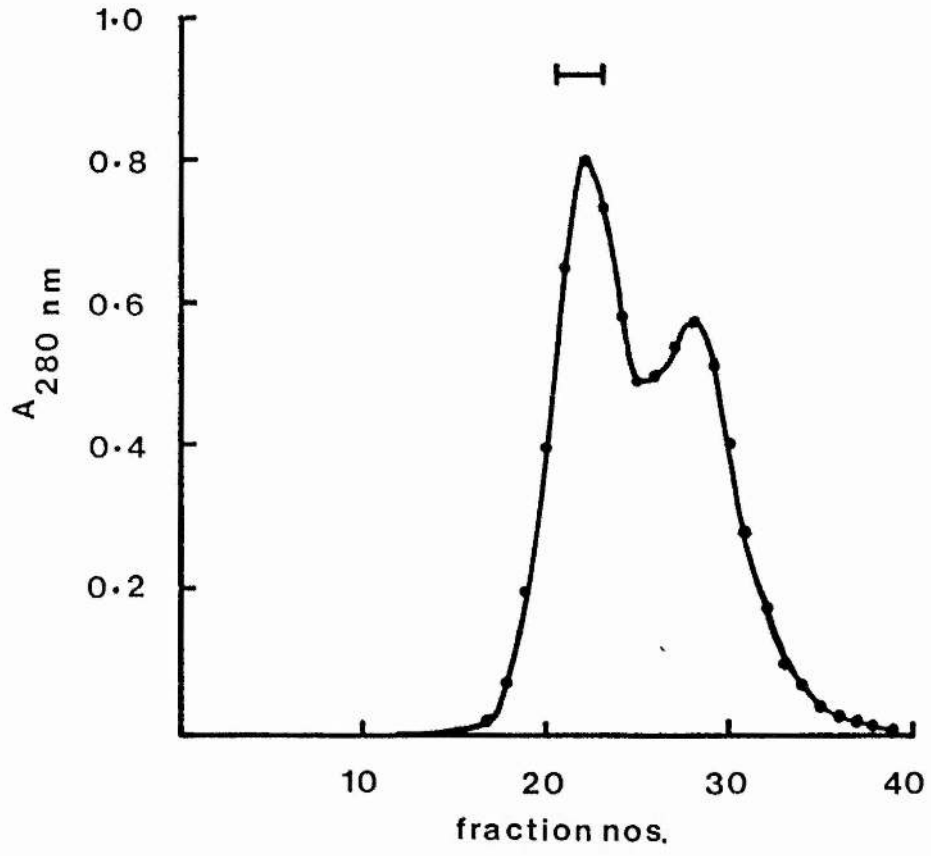


Fig. 2.5 SEPHADEX G 200 COLUMN CHROMATOGRAPHY OF BOVINE FACTOR XIII

●——● absorbance at 280 nm

⊢——⊣ fractions with activity

Sample applied was the pool from Peak II of DEAE cellulose, which has been concentrated and dissolved in the equilibrating buffer 0.05 M Tris-HCl/0.001 EDTA, 0.1 M NaCl, pH 7.5.

2.11.2. DEAE-cellulose 52 (Whatman)

The DEAE cellulose resin was precycled and degassed. A column 24 cm x 1.6 cm was packed with the resin and allowed to equilibrate with 0.05 M Tris-HCl/0.05 M NaCl, pH 8.6, at the flow rate of 50 ml/h. The sample which had been dialysed in the same buffer was applied to the column, and fractions were collected every 6 min. The starting buffer was pumped through until the effluent had an absorbance of less than 0.1 at 280 nm. Then 0.05 M Tris-HCl/0.1 M NaCl, pH 8.6 was applied and fractions were collected as before.

2.11.3. Results

Two peaks were obtained from DEAE cellulose column, Fig. 2.6. Both peaks were tested for fibrinogen, using thrombin clotting time. Most of the fibrinogen was in Peak II. SDS gel electrophoresis was performed on the fractions, using 5% gel. Fractions 29-33 showed one single band on non-reduced samples, and three bands on reduced sample, Fig. 2.7.

2.12. Purification of plasminogen

The method used was based on that of Deutsch and Mertz (1970) and Rickli and Cuendent (1971). To prepare human and bovine plasminogen, fresh frozen plasma was applied to the lysine sepharose column as 4.4.1.2. Once the plasminogen-free plasma was collected, the column was washed with 0.05 M Tris-HCl/0.3 M NaCl, pH 7.5 until the effluent had absorbance of less than 0.05. Plasminogen was eluted

Fig. 2.6 DEAE CELLULOSE COLUMN CHROMATOGRAPHY OF BOVINE FIBRINOGEN

absorbance at 280 nm

fractions with pure fibrinogen

Sample applied in equibibrating buffer 0.05 M Tris-HCl/0.05 M NaCl, pH 8.6, and fractions were eluted with 0.05 M Tris-HCl/0.1 M NaCl, pH 8.6.

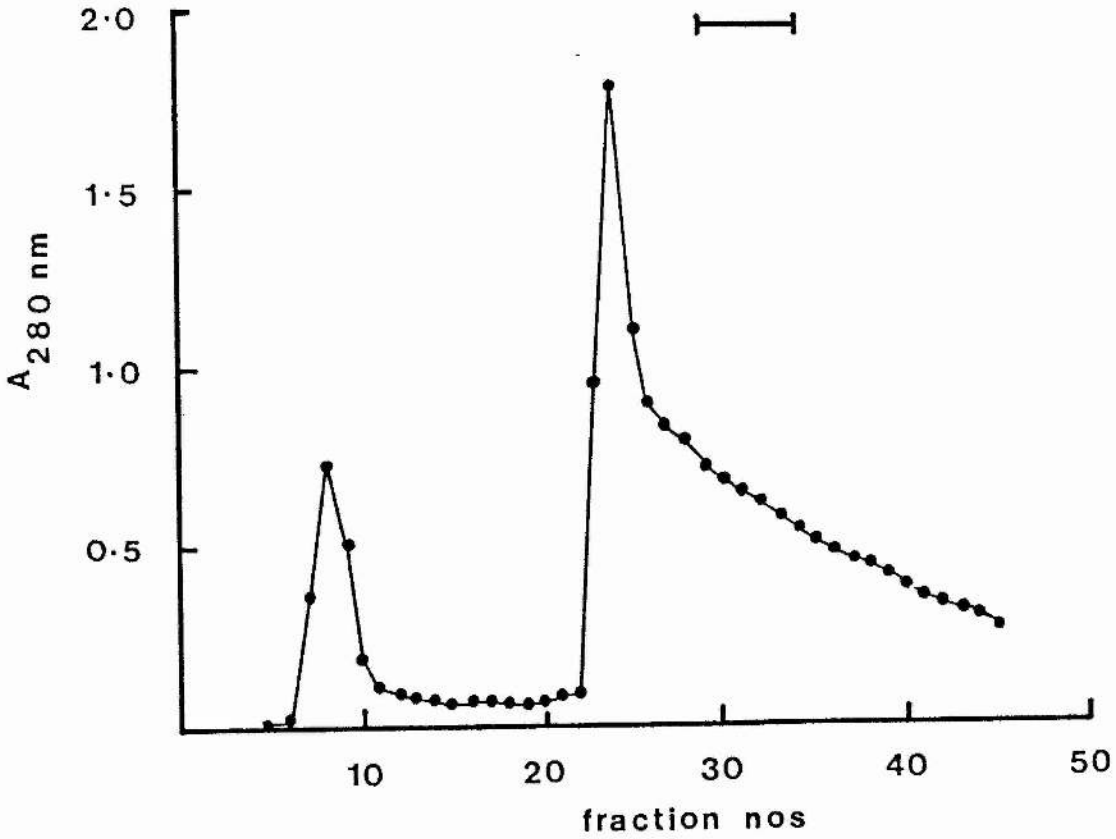
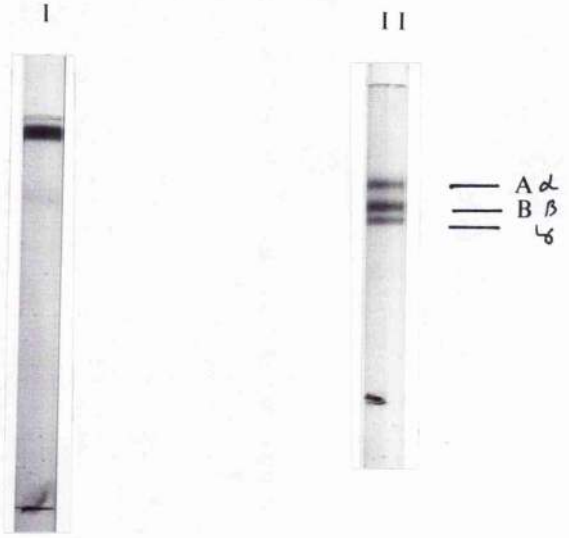

2·0
1·5
1·0
0·5
$A_{280\ nm}$
10
20
30
40
50
fraction nos

Fig. 2.7. SODIUM DODECYL SULPHATE (SDS) POLYACRYLAMIDE GEL ELECTROPHORESIS OF BOVINE FIBRINOGEN

I Non-reduced

II Reduced



from the column with 100 ml of 0.2 M ε-aminocaproic acid (EACA) and fractions with absorbance of more than 0.2 at 280 nm were collected. Then 100 ml of 0.05 M Tris-HCl/0.2 M EACA, 1 M NaCl, pH 7.5 was applied to clean the column.

2.13. Preparation of Fragments D and E from Fibrinogen

The method used was based on that of Lawrie et al. (1979). Fibrinogen was prepared as 2.11. The fibrinogen was dialysed against 0.05 M Tris-HCl, pH 7.5. overnight at 4°C. The flow chart of the purification , Fig. 2.9.

2.13.1. Fibrinogen digestion

To 0.5 M plasminogen, 50 µl of 5000 units/ml streptokinase was added and incubated at 37°C for 15 min. Then the activated plasminogen (plasmin) was added to bulk of fibrinogen at 37°C. After approximately 4 h, an aliquot of the mixture was subjected to electrophoresis to determine the extent of the digestion.

When the digestion was completed, the sample was applied to DEAE 52 cellulose column.

2.13.2. DEAE cellulose

A DEAE 52 cellulose column was prepared as 2.11.2 and was equilibrated with 0.05 M Tris-HCl/0.05 M NaCl, pH 8.6.

Degraded fibrinogen was applied to the column and fractions were collected until no protein was detected in the effluent. This peak contained fragment D

Fig. 2.8, To elute fragment E, a linear salt gradient was applied from 150 ml of 0.05 M Tris-HCl/1 mM $CaCl_2$, pH 7.5 to 150 ml of 0.05 M Tris-HCl/1 mM $CaCl_2$, 0.5 M NaCl, pH 7.5, (Fig. 2.8).

2.14. Purification of Pepsin and Gastricsin from Gastric Juice

Gastric juice was generously supplied by Ninewells Hospital (Dundee).

2.14.1. Preparation of sample

The method used was based on that of Tang (1970). 250 ml of gastric juice was filtered through glass wool, and dialysed at 4°C against distilled water for 48 h. with several changes of distilled water. The dialysed sample was freeze dried.

2.14.2. Amberlite IRC-50(H) column chromatography

The freeze dried gastric juice was dissolved in 12 ml of 0.2 M citrate buffer, pH 3.0 and centrifuged at 30,000 rpm for 2 h. The clear supernatent was applied to the equilibrated column as 4.2 . Three peaks were obtained, Peak I at pH 3.2, Peak II at pH 4.0 and Peak III at pH 4.4, (Fig. 2.10).

Samples under the Peak II and III showed proteolytic activity, using haemoglobin and fibrin clot as substrates. Samples under Peak II and III were pooled separately and dialysed at 4°C for 72 h. against 5-10 times changes of distilled water. After dialysis each pool was freeze dried separately.

Fig. 2.8. DEAE CELLULOSE COLUMN CHROMATOGRAPHY OF FIBRINOGEN FRAGMENTS D AND E

o——o Absorbance 280 nm

I Fractions with pure fragment D*

II Fractions with pure fragment E*

——→ Salt gradient was applied

* photograph of an SDS PAGE run shown

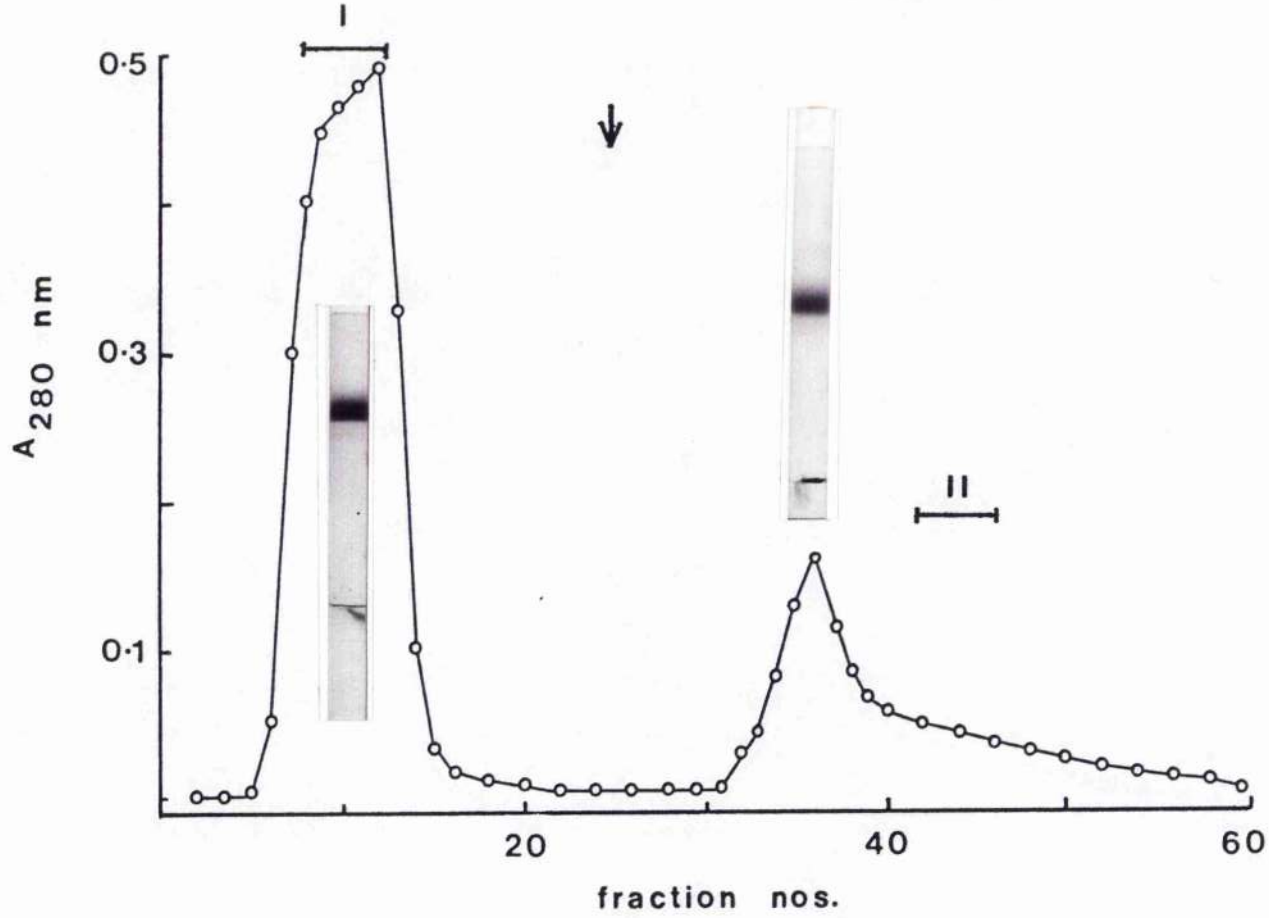

I
II
0·5
0·3
0·1
A_{280} nm
20
40
60
fraction nos.

Fig 2.9

SUMMARY OF PURIFICATION FLOW CHART FOR FIBRINOGEN, PLASMINOGEN, FRAGMENTS D AND E

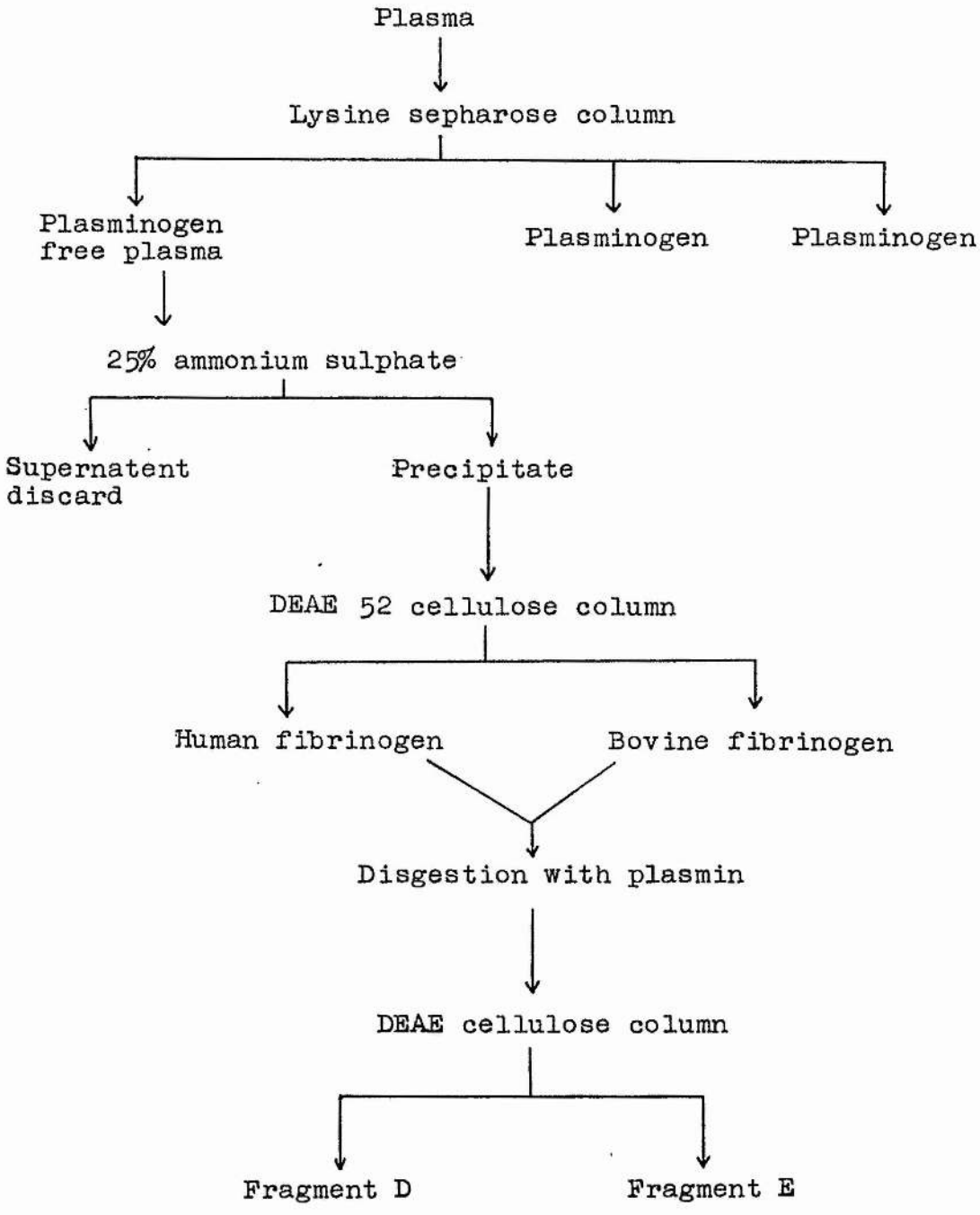


Fig. 2.10.

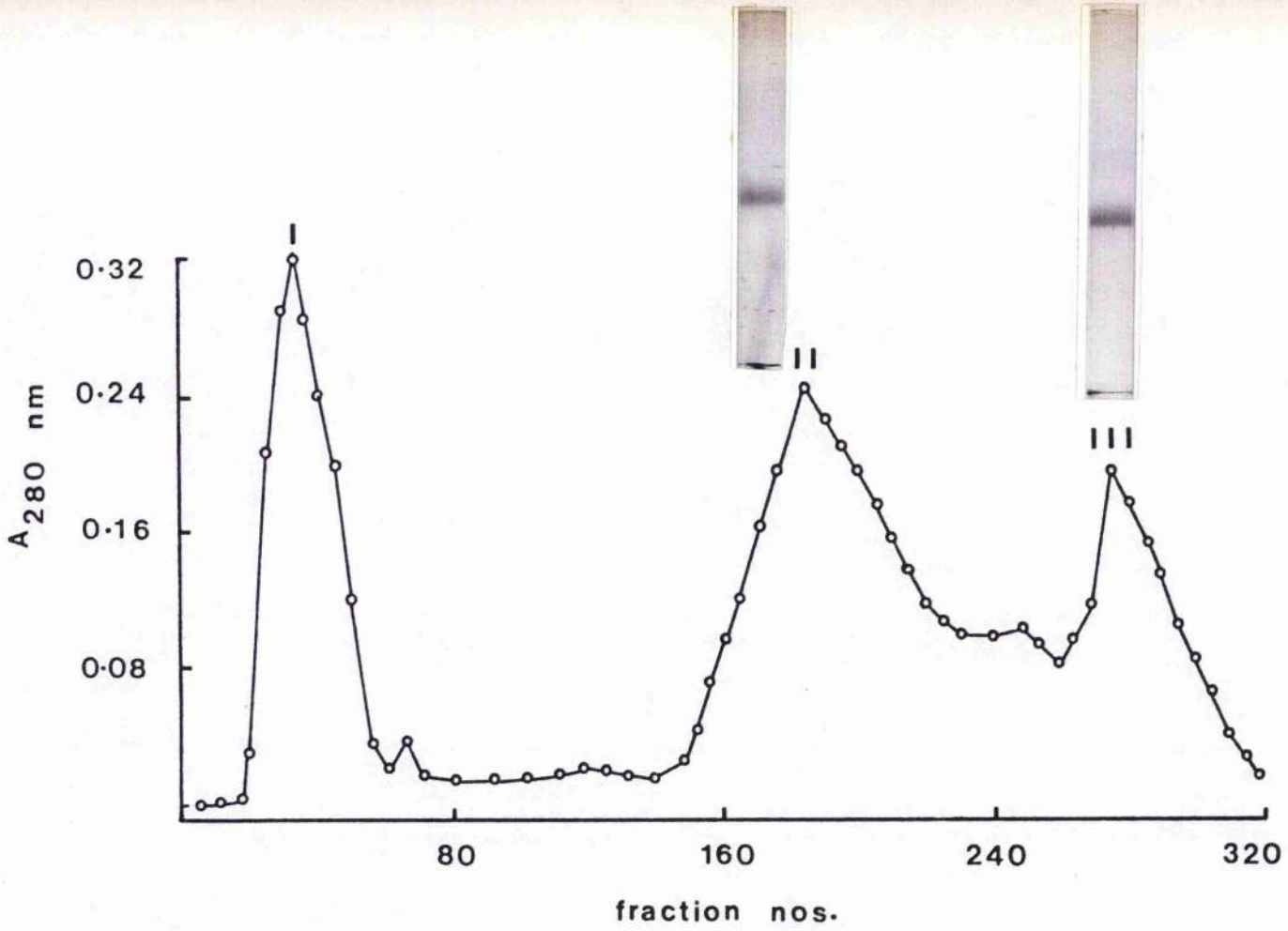
AMBERLITE IRC-50(H) COLUMN CHROMATOGRAPHY OF PEPSIN AND GASTRICSIN

o——o absorbance at 280 nm

II = pH 4.0 pepsin*

III = pH 4.4 gastricsin*

* photograph of an SDS PAGE run shown


I
II
III
0·32
0·24
0·16
0·08
A_{280} nm
80
160
240
320
fraction nos.

2.14.3. Identification of proleolytic enzymes of Peaks II and III of Amberlites column.

2.14.3.1 Molecular weight determination

A sample from Peaks II and III were electrophoresed on 5% and 10% gels (2.1) in reduced and non-reduced form.

2.14.3.2. Amino acid analysis

A sample of both peaks were subjected to complete hydrolysis in 6 M HCl. The amino acid composition of hydrolysates were determined using automated ion-exchange chromatography, in amino acid analyser. (The amino acid analysis was performed very kindly by Mr. Angus Grieve.)

2.14.3.3. Enzyme assay

Proteolytic activity of both peaks were measured using a) haemoglobin assay at pH 2.0 and 3.0; b) synthetic substrate, N-acetyl-L-phenylalanyl-L-diiodotyrosine (APDT); c) fibrin clot assay.

2.14.4. Results

Molecular weights of Peak II and Peak III were 39,000 and 34,000 respectively, values which are higher than those recorded in the literature for pepsin and gastricsin. However, commercial hog pepsin (Sigma, Victrum, London) with a stated molecular weight of 34,700 gave a molecular weight of 39,000 in this system. The amino acid composition of both peaks were similar to that of pepsin

and gastricsin respectively. Proteolytic activity of Peak II, using haemoglobin as substrate was highest at pH 2.0 and of Peak III at pH 3.0. Both were inhibited with pepstatin but, when fixed concentration of both enzymes were treated with different amounts of pepstatin, the results indicated that Peak II was x 100 more sensitive towards inhibition with pepstatin than Peak III.

Peak II hydrolysed synthetic substrate (APDT), but Peak III had no effect. By comparing the different parameters with that of Tang's(1970) work, it was clear that Peak II was pepsin and Peak III gastricsin.

CHAPTER 3

SUBSTRATE

3.1. General Introduction to Substrates

The fact that there are many different types of substrate in the literature, indicates the problem of finding a substrate which is best for a particular enzyme.

There are many criteria which one should consider, and it is not always possible to find a substrate with all the necessary parameters. Sometimes there is a need to use more than one type of substrate to overcome the inefficiency of the system. There are different types of substrates such as protein substrates and synthetic substrates which are used in the assay of proteolytic enzymes. Protein substrates include natural substrate proteins, "other proteins" and artificial substrate proteins (Bohley and Langner, 1982).

(i) Natural protein is the substrate which has been isolated from the same cell or tissue as the proteinase enzyme. The advantages of using natural substrates is that a direct comparison to the in vivo system is possible. However, it is difficult to create the physiological conditions in vitro, and unfortunately many experiments are set up in very low or high pHs. Although these pH values are optimal for the activity of the enzyme, they create an artificial condition, i.e. expose sensitive regions of the protein by conformational changes.

The main disadvantages of using protein substrates

is that they are unsuitable for kinetic studies of proteinases, for they present multiple sites of cleavage, and peptide products probably change constantly, creating a mixture of secondary substrates and inhibitors (Knight, 1977). Another problem with protein substrate is that, no matter how precisely the protein concentration has been measured, none of the protein estimations are perfect. Usually the extent of hydrolysis is determined after acid precipitation of protein with TCA and measuring the absorbance of the supernatent (which contains cleaved small peptides) at 280 nm. This, however, depends on the presence of aromatic amino acids (Reimerdes and Klostermeyer, 1976). Sometimes the substrate is attacked by the enzyme in a rather limited way, and the resulting fragments are only partially soluble in acid solution. In addition, it is possible that some of the proteolytic products do not contain aromatic amino acids.

"Other protein" substrates are proteins such as haemoglobin, casein, albumin, etc., which are widely used without being a natural substrate. The disadvantages of these substrates are similar to those of natural substrates.

Artificial substrates are proteins coupled to a matrix such as sepharose or agarose, or free soluble protein, which can be labelled with a chromogenic or radioactive group, and used in vivo or vitro. The advantages of matrix-coupled proteins lie in the possibility of determination of the splitting of peptide

bonds without the use of TCA.

Synthetic substrates have well defined chemical structures. Their advantages compared to the natural substrate are: they can have better solubility; and their enzymic cleavage can be easily followed by photometric or fluorometric methods (Claeson et al., 1978). Synthetic substrates can be highly specific and sensitive. They are useful for the investigation and characterisation of an enzyme, especially once the enzyme has been purified. However, the hydrolysis of these substrates is not necessarily an accurate reflection of an enzyme's activity against protein substrates (Lin et al., 1969).

The important points to consider, regardless of the type of substrate, are availability, sensitivity, specificity and lability. The substrate must be hydrolysed by the enzyme at a reasonable rate, and its products measured easily (Knight, 1977).

3.2. Fibrin Clot Substrate

3.2.1. Introduction

A blood clot is formed after a chain of enzymic reactions. The last stage is the conversion of the soluble plasma protein fibrinogen into a visible insoluble network of the fibrin clot (Ratnoff, 1977).

Fibrinogen has a dimeric structure, in which the two halves of the molecule are made up of three polypeptide chains (Aα, Bβ, and γ).

Fibrin monomers are formed by the proteolytic action of thrombin, which cleaves two pairs of small peptides, (A and B) from the amino terminus of [A]α and [B]β chains respectively. Once the fibrin monomers form, they link up spontaneously to form a fibrin polymer. The monomers are held together by weak forces such as electrostatic and hydrogen bonding. The clot formed is not very stable and would disperse in denaturing media such as 1% (w/v) monochloro-acetic acid (MCA) or 5 M urea (Doolittle, 1977).
A more stable fibrin clot is formed by the catalytic action of another plasma protein, Factor XIII (fibrin stabilizing factor). Factor XIII catalyses a transpeptidation reaction between the lysine of one chain (donor) and the glutamine residue of another chain (acceptor), leading to γ-glutamyl-ε-lysine bridges, cross-linking the two chains covalently (Lorand et al., 1968). Factor XIII cross-linked fibrin does not dissolve in 1% (w/v) MCA or 5 M urea

and is more resistant to enzymic dissolution than is a fibrin clot formed in the absence of Factor XIII (Lorand et al., 1981). Cross-linking of fibrin monomers takes place between γ-chains of adjacent molecules, within the first few min. of polymerization, while the α-chains take much longer to form polymers. The β-chains are not involved in this process. Although a fibrin clot formed in this way is very stable, it would dissolve eventually in physiological condition (in vivo) and in acid solution in vitro in the presence of plasma enzymes (Doolittle, 1981).

In addition to the major role of thrombin and F XIII in the formation of a stable clot, the environment (pH and ionic strength) in which the fibrin polymerization takes place, is also important (Crum, 1977). The pH of the media affects the kind of clot which is formed and the ionic strength of the medium affects the rate of polymerization. Steiner and Laki (1951), using the light scattering method, found that fibrin clot formed at high pH and ionic strength is a "fine clot" which has low opacity, relatively high rigidity, while at low pH or ionic strength, the clot is coarse with high opacity and less rigidity. At pH values below 5.1 or greater than 10.1, no gel is formed and gelation time as a function of pH showed that there is a minimum clotting time between pH 6.0 and 8.5. The gelation time is further dependent on the ionic strength.

3.2.2. Purified fibrinogen preparation

Human fibrinogen (Kabi Vitrum, London) was passed through lysine-sepharose 4B as 4.4.1.2 to remove any plasmin(ogen) contamination. Most commercial preparations are contaminated with plasmin(ogen) (Dacie and Lewis, 1975c). Bovine fibrinogen was purified as 2.11. Fibrin clots were prepared as 3.2.4 except $CaCl_2$ solution contained 25 units/ml thrombin.

3.2.3. Semi purified fibrinogen preparation

Bovine blood was treated as 2.11 up to the stage of ammonium sulphate precipitation. The precipitate was dissolved in and dialysed against 0.85% (w/v) NaCl at 4°C for 24 h. The NaCl was changed at least 3 times. The dialysed solution was stored at -20°C if it was not to be used immediately. To prepare fibrin clots, the dialysed fibrinogen was diluted with 0.85% (w/v) NaCl to give a final fibrinogen concentration of 2-3 mg/ml. Fig. 3.1.

3.2.4. Fibrin clot formation

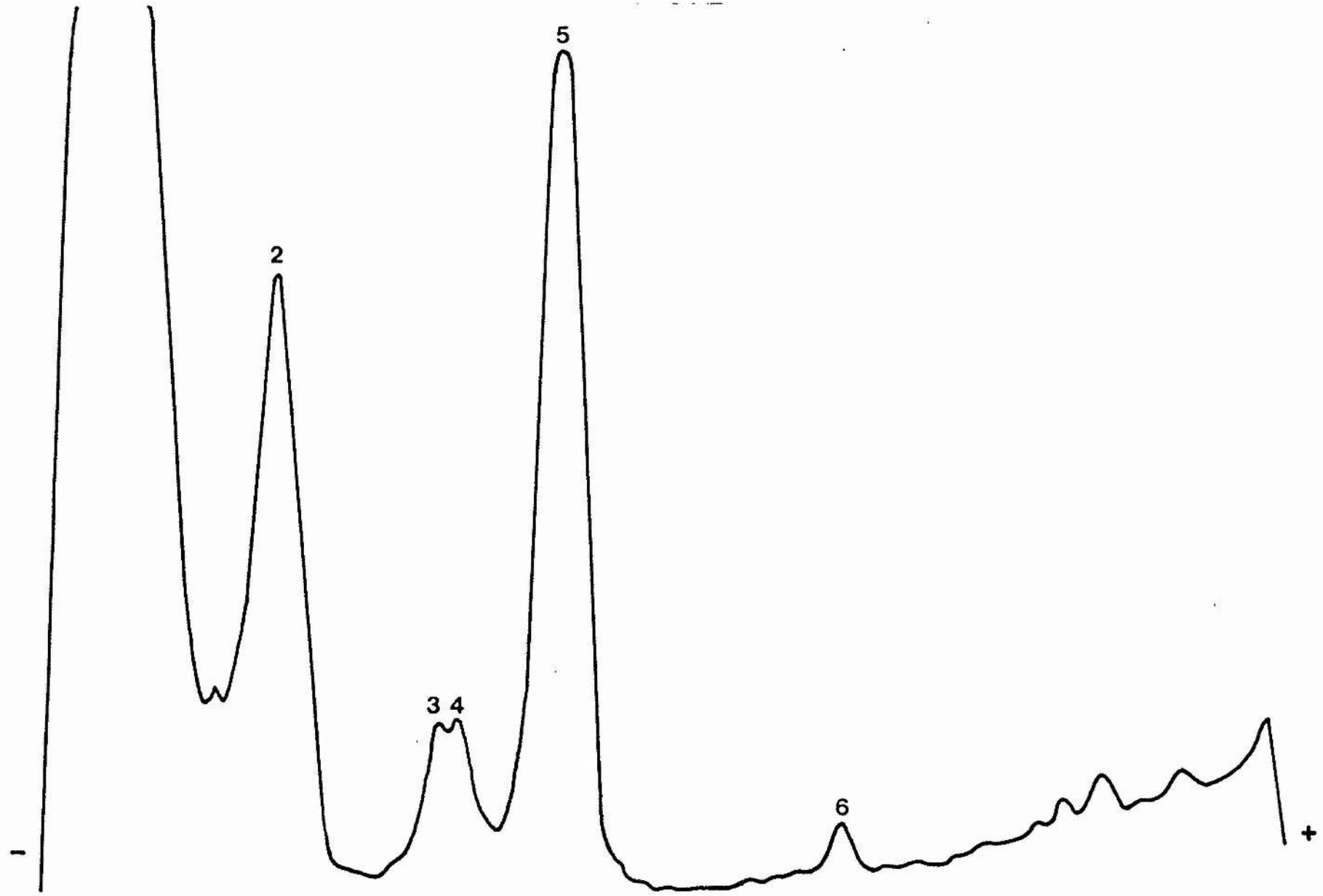
The method used was based on that of Ikemori et al., (1975).

0.6 ml of the sample was placed in a 75 mm x 12 mm glass tube, in 37°C water bath. Clotting was initiated by the addition of 0.2 ml of pre-warmed (37°C) 0.05 M $CaCl_2$, and the fibrin clot was collected by winding on a glass rod. It was then incubated at 37°C for 4-5 h in its own serum. The clot was removed from the glass rod and washed in 0.85% (w/v) NaCl for 18 h,

Fig. 3.1

DENSITOMETRIC SCAN OF AN ALIQUOT OF CRUDE FIBRINOGEN SEPARATED BY SDS PAGE

Peak I = fibrinogen


5
2
3 4
6
–
+

with at least 3 changes of NaCl. To remove the salt, the clot was washed and left in distilled water for about 3 h.

3.2.5. Fibrin clot assay

The method used was based on that of Ikemori et al., (1975).

2.6 M of 1% (w/v) monochloro-acetic acid (MCA) was added to the washed clot in a centrifuge tube. Test solution (0.05-0.3 ml) was added, and the mixture incubated at 37°C for 16 h. The solution was centrifuged at 200 g for 20 min. The supernatent was removed and discarded. The remaining clot was washed with saline 3 times, and the protein content of the remaining clot was assayed as 2.6.

3.2.6. Fibrin clot substrate result

Clots prepared from purified fibrin dissolved rapidly (within 5 min.) in 1% (w/v) monochloro-acetic acid (MCA), in the absence of a proteolytic enzyme, indicating that fibrin clots had not been cross-linked. As Ca^{++} and thrombin had been added in excess, this suggested a lack of Factor XIII. To overcome the Factor XIII deficiency, partially purified bovine fibrinogen was used. Factor XIII precipitates at approximately 17% saturation with ammonium sulphate (Lorand and Gotoh, 1970), and fibrinogen at about 25% saturation (Lawrie et al., 1979). Therefore a 25% ammonium sulphate precipitate has sufficient Factor XIII and fibrinogen for the purpose of preparing a fibrin clot substrate.

Fibrin clots formed from partially purified fibrinogen were stable in 1% (w/v) MCA, indicating that sufficient Factor XIII and prothrombin were present. The clots, however, were also resistant to lysis in MCA in the presence of plasma enzyme.

To investigate the cause, plasminogen which had been removed from the original plasma was added back to the crude fibrinogen in varying amounts, before clot formation. As the results (Table 3.1) show, the addition of plasminogen made the clot more susceptible to lysis by plasma enzyme in MCA. Theoretically, plasminogen should not have any effect at low pH, so the buffer (0.05 M Tris-HCl/0.02 M lysine/0.1 M NaCl, pH 9.0) against which the plasminogen had been dialysed was tested as well as plasminogen before dialysis (plasminogen in 0.1 M EACA from lysine-sepharose column). In addition the plasminogen solution was examined for the presence of plasmin. As Table 3.2 shows, the change in the susceptibility of the clot to lysis was not due to the action of the plasminogen as the clots become less resistant to lysis with the addition of buffer only. The next step was to investigate the reason for the change in the susceptibility of the clot by the buffer. The buffer was made without the addition of lysine (0.05 M Tris-HCl/0.15 M NaCl, pH 9.0). The results did not change significantly. Different amounts of buffer without lysine were added to the crude fibrinogen, and the pH of the solution was measured

Substrate	mg fibrin dissolved by 0.2 ml enzyme in 16 h.
Crude bovine fibrinogen /0.15 M NaCl 9:1 (v/v)	0.12
Crude bovine fibrinogen /plasminogen 9:1 (v/v)	1.92
Crude bovine fibrinogen /plasminogen 19:1	1.55

Table 3.1. Effect of the presence of plasminogen (in crude fibrinogen preparation) in the susceptibility of fibrin clot to lysis by plasma enzyme in acid media.

prior to clot formation.

As the results show (Table 3.2) the addition of buffer changes the pH of the solution. As mentioned before (3.2.1) the pH of the media has an effect on the type of clot formed. From the above experiments it was concluded that with the correct environment for fibrin clot formation it was possible to make a fibrin clot which was stable enough to resist lysis in 1% (w/v) monochloro acetic acid, but which would lyse readily in the presence of plasma enzyme. The method adapted for the preparation of substrate is described in detail in Section 3.2.4.

To have a standard substrate the above method was used to prepare fibrin clots. However, during the course of this work it was found that although the same procedure was followed each time, there was a wide variation in the susceptibility of clots formed from different batches of bovine plasma. As there is a wide range for the normal levels of coagulation factors, it is probable that different batches of plasma have different amounts of Factor II (prothrombin) and Factor XIII, both of which are directly involved in the type of clot formed (3.2.1). The concentrations of both factors were studied (3.2.7 and 3.2.8).

3.2.7. <u>The effect of prothrombin concentration on the susceptibility of the fibrin clot to lysis</u>

One stage prothrombin time assay was performed on 20 different bovine plasmas as 2.9.2. The clotting times

Substrate	pH	mg fibrin dissolved by 0.2 ml enzyme in 16 h.
Fibrinogen + NaCl v/v (9:1)	6.5	0.3
Fibrinogen + buffer v/v (9:1)	8.5	1.6
Fibrinogen + buffer v/v (19:1)	8.3	1.0

Table 3.2. Effect of the pH of crude fibrinogen on the susceptibility of fibrin clot lysis by plasma enzyme in acid media.

were within 16 ± 3 secs. suggesting no significant differences between the test plasmas. Crude fibrinogen samples were also tested. All the results were above 60 secs., indicating low levels of at least one of Factors II, V, VII or X. As the prothrombin time was above 60 secs., the sensitivity of the test is lost and comparison between the different batches of plasma is not valid.

However, the addition of excess thrombin to crude fibrinogen had no effect on the fibrin clot susceptibility to lysis, indicating that crude fibrinogen preparation had enough prothrombin.

3.2.8. Effect of F XIII on the susceptibility of fibrin clot to lysis

Clots prepared from pure fibrinogen dissolved in 1% (w/v) monochloroacetic acid within 5 min., but became resistant to lysis after the addition of purified F XIII. The concentration of Factor XIII in crude fibrinogen was measured fluorometrically as 2.8. The results were unsatisfactory. There was a wide variation (0-16 units) within the duplicate tests.

The inaccurate results were not due to the inefficiency of the assay system, as F XIII concentration in normal human and bovine plasma ranged from 18-20 units in accordance with the literature. The problem was due to a high concentration of fibrinogen, which precipitated at 56°C, and the precipitate interfered with the reading of the results in

the fluorimeter.

Because of the above technical problems, the presence of Factor XIII in crude fibrinogen preparation was measured indirectly.

The cross-linking of fibrin was measured by the observation of gel electrophoretic patterns of reduced fibrin clots, as 3.2.9.

3.2.9. Use of SDS gel electrophoresis in the assessment of fibrin clot stability

3.2.9.1. Method

The method used was based on that of Schwartz et al., (1971).

Fibrin clots were prepared as 3.2.4, from different batches of crude bovine fibrinogen. Fibrin clots were made in triplicate. One clot was used to estimate fibrin concentration and another was used in fibrin clot assay. The third fibrin clot was reduced as 2.1.11 and was electrophoresed as 2.1. on 5% gel. The gels were scanned and the formation of γ-γ dimer and disappearance of α and γ chains were measured by dividing the height of the appropriate peak by that of the β chain, which does not change during the course of polymerization (Table 3.3). Figs.3.2,3,4 and 5.

3.2.9.2. Results

Samples with γ chain present were very susceptible to lysis in 1% (w/v) monochloro acetic acid in the presence of plasma enzyme and also to eventual

Table 3.3. DENSITOMETRIC ANALYSIS OF THE RATIO OF α AND γ CHAIN TO β CHAIN OF BOVINE FIBRIN(OGEN)

	$\frac{\gamma}{\beta}$	$\frac{\gamma_2}{\beta}$	$\frac{\alpha}{\beta}$
Crude bovine fibrinogen	0.98	0	0.62
Fibrin made from bovine plasma	0	0.62	0
Fibrin made from crude fibrinogen (S_1)	0	0.35	0.14
Fibrin made from crude fibrinogen (S_2)	0.8	0.2	0.5
Fibrin made from crude fibrinogen (S_3)	0	0.5	0.48

S_1 - Fibrin clot which was resistant to lysis in acid medium

S_2 - Partially cross-linked fibrin clot which was not stable in acid medium

S_3 - Partially cross-linked fibrin clot used in assay system

Fig. 3.2

DENSITOMETRIC SCAN OF AN ALIQUOT OF PURE REDUCED BOVINE FIBRINOGEN

Peak 1 α chain

Peak 2 β chain

Peak 3 γ chain

-
1
2
3
+

Fig. 3.3

DENSITOMETRIC SCAN OF AN ALIQUOT OF REDUCED FIBRIN CLOT

(fibrin clot which was resistant to lysis in acid medium)

Peak 1 - α polymer

Peak 2 and 3 - α polymer (intermediate)

Peak 4 - γ-γ dimer

Peak 5 - β chain

Peak 6 - degradation of fibrin

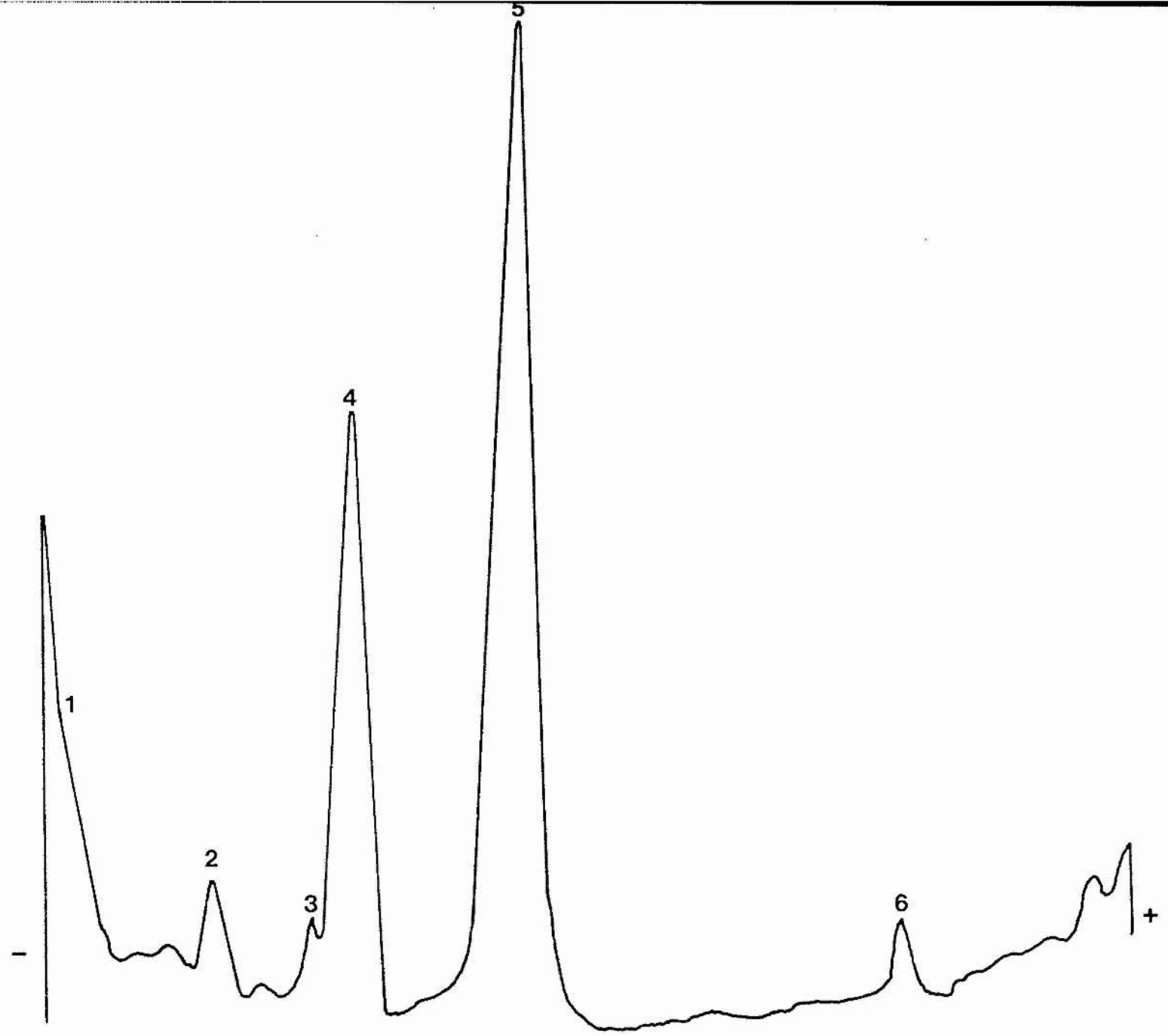

1
2
3
4
5
6
–
+

Fig. 3.4

DENSITOMETRIC SCAN OF AN ALIQUOT OF REDUCED FIBRIN CLOT

(partially cross-linked fibrin clot which was not stable in acid medium)

Peak 1 - γ-γ dimer

Peak 2 - α chain

Peak 3 - β chain

Peak 4 - γ chain

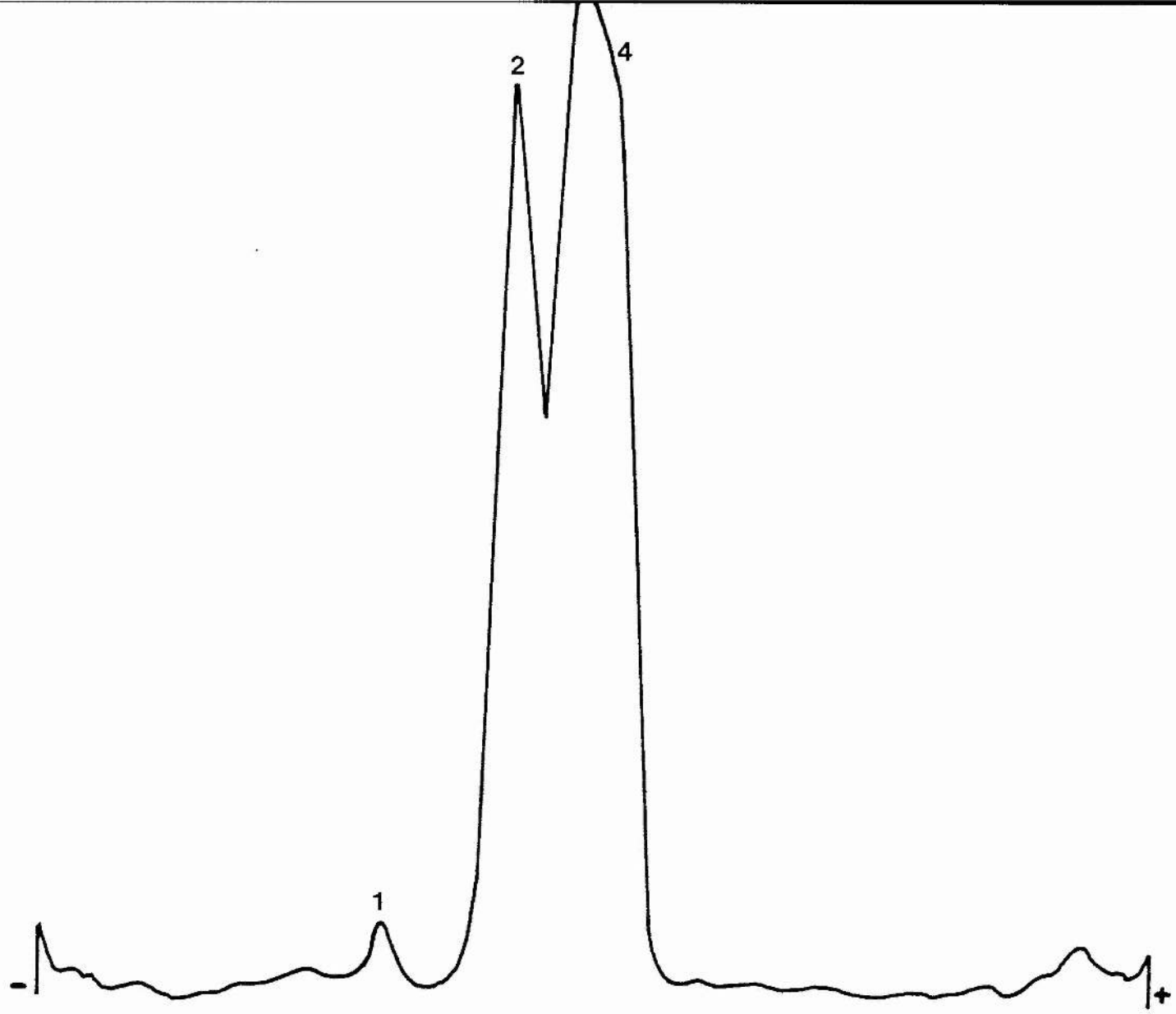

2
4
1
-
+

Fig. 3.5

DENSITOMETRIC SCAN OF AN ALIQUOT OF REDUCED FIBRIN CLOT

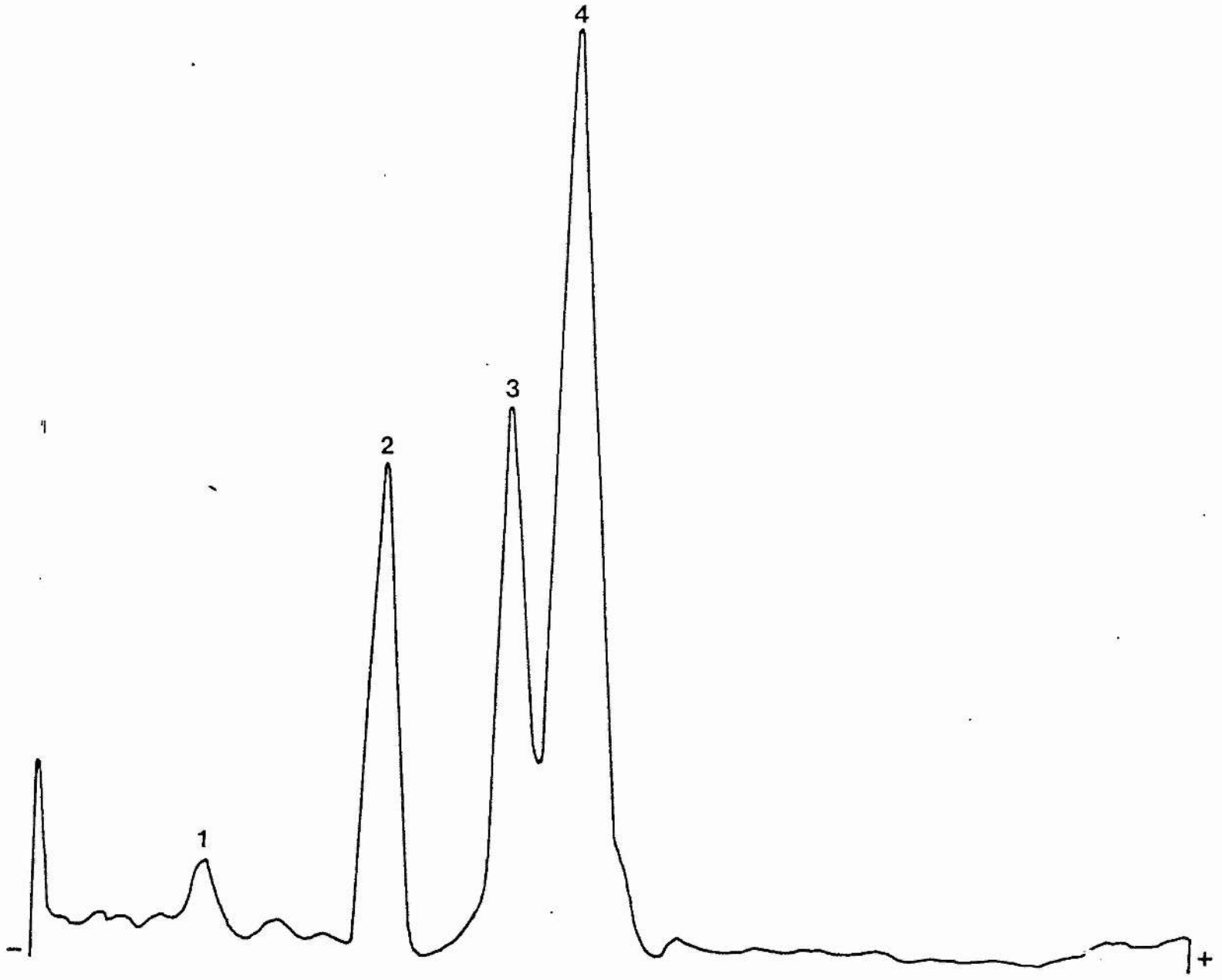
(partially cross-linked fibrin clot used in assay system)

Peak 1 - α polymer intermediate

Peak 2 - γ-γ dimer

Peak 3 - α chain

Peak 4 - β chain


4
3
2
1
–
+

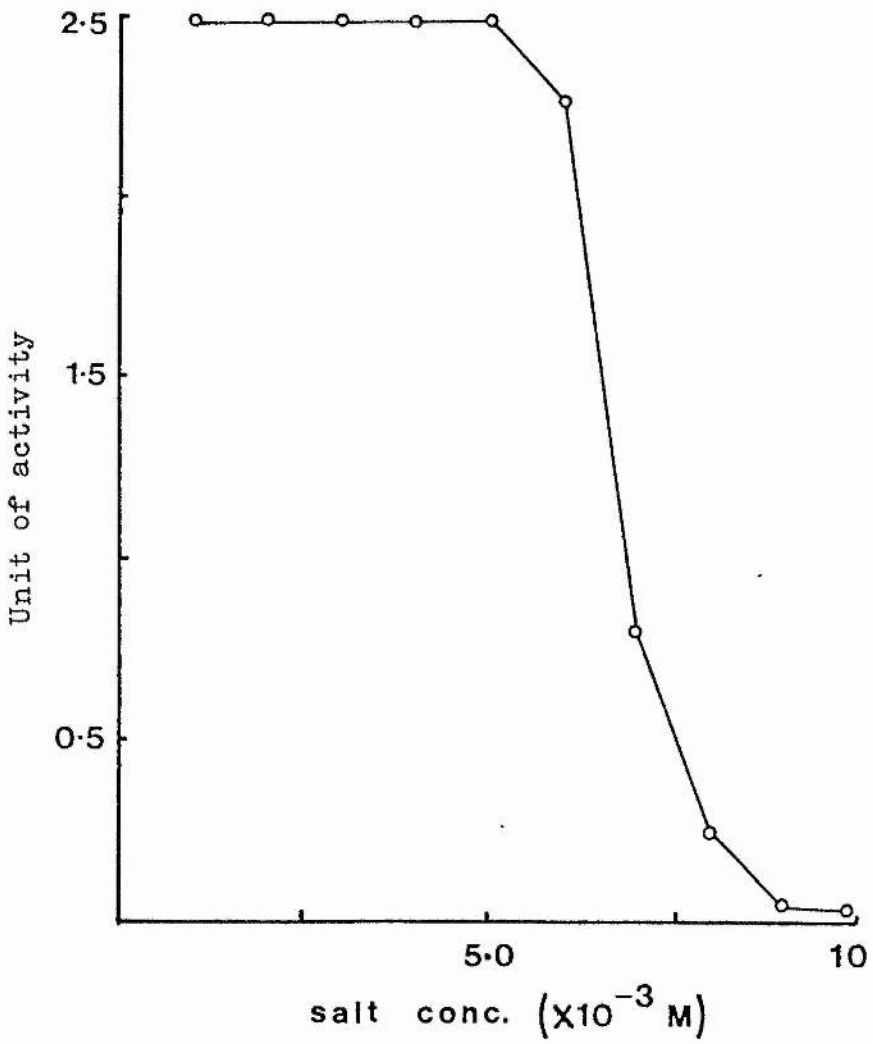
lysis in the absence of the plasma enzyme. Samples with no γ chain, and an ($\frac{\alpha}{\beta}$) ratio of less than 0.25 were very resistant to lysis in acid media. The results were in accordance with the work of Schwartz et al., (1973). They reported that prolongation of lysis times coincided with the appearance of extensively cross-linked α chains, and those clots with the longest lysis times had virtually all of their α chains cross-linked.

3.2.10. Effect of salt concentration on the susceptibility of fibrin clot to lysis by plasma enzyme in acid media

During the course of fibrin clot assays, it was noticed that fibrin clots in 1% (w/v) monochloroacetic acid become swollen, elongated, and translucent. On the addition of NaCl, the fibrin clots became shrunken and opaque. These physical changes had a great effect on the susceptibility of the clot to lysis in the presence of plasma enzyme. As the concentration of NaCl was increased ($\geqslant$0.05 M) the fibrin clot became completely resistant to lysis by the enzyme (Fig. 3.6). Presumably the swelling of the fibrin clot caused the exposure of the enzyme to the susceptible bonds . Bowes and Kenten (1950) studied the swelling of the collagen in hydrochloric acid and sodium hydroxide with the pH range of 0.5-14. The swelling of the collagen increased sharply at approximately pH 2.0, 11.5 and 13. In the presence of different concentrations of NaCl (0.05-0.5 M) in acid solution, the swelling of the

Fig. 3.6. EFFECT OF SALT CONCENTRATION ON THE SUSCEPTIBILITY OF FIBRIN CLOT TO LYSIS IN ACID MEDIA

A graph of enzyme unit of activity versus the final salt concentration in the assay system.


2·5
1·5
0·5
Unit of activity
5·0
10
salt conc. (X10⁻³ M)

collagen decreased progressively as the concentration of NaCl increased, and at a concentration of 0.5 M, the swelling was completely suppressed. Swelling of fibrous proteins in acid and alkaline solution depends on two factors. The first of these, the Donnan effect, is due to differences in the osmotic pressure between protein phase and the external solution due to protein salt formation. This is in turn a consequence of the concentration of diffusable ions between the protein and external phases. The other factor is the cohesive force within the protein, which restricts the swelling. The cohesive factors include the interweaving of the molecules, the inter molecular forces and bonds both covalent and non-covalent. Therefore the extent of swelling depends on the balance between the osmotic pressure and the cohesive forces (Bowes and Kenten, 1950).

Sodium chloride effects the swelling of different fibrous proteins differently. For example, NaCl completely suppresses the swelling of gelatin at alkaline pH, but not collagen. The reason for this is that with gelatin the uptake of water is much higher than with collagen, due to weaker cohesive forces, and the concentration of the diffusable ions in the gelatin phase is low. Therefore, a very small amount of NaCl is needed to reduce the difference in the concentration of ions in the external and internal phase.

Yasukouchi et al., (1979) measured the fibrino-

lytic action of plasmin in the presence of NaCl (0-1.5 M), using fibrin agar plate, and clot lysis time. In both methods the higher the concentration of NaCl the higher the enzyme activity. From their results, they concluded that the sodium chloride causes conformational changes in plasmin rather than in the fibrin clot.

Buckell and Truscott, (1959), reported that sodium chloride in low concentration 0.2-0.4% (w/v) shortens the clot lysis time, but in higher concentration 0.4-1% (w/v) prolongs the clot lysis time. Weiner (1959) diluted blood in phosphate buffer and sodium chloride (0-0.075 M). In both systems the higher the molarity of the salt the higher the fibrinolytic activity.

In this project it was observed that even a very low concentration of NaCl (final concentration 0.05 M) is needed to suppress the swelling of the fibrin clot, with a consequent inhibition of proteolysis.

3.2.11. Reproducibility of fibrin clot assay

Since the preparation of fibrin clot is rather time-consuming, a large number of fibrin clots were made, and kept in 0.9% (w/v) NaCl at 4°C. Fibrin clots were tested at different intervals for a period of 3 weeks. There was no change in the rate of fibrin clot lysis by plasma enzyme.

To determine the stability of fibrin clots in an acid environment, in the absence of plasma enzyme,

20 fibrin clots were made, and incubated in 1% (w/v) monochloro acetic acid for 24 h at 37°C. Fibrin concentration was measured in all the clots as 2.6, and results were statistically analysed.

Mean = 0.50

Standard deviation = ± 0.03

To study the reproducibility of the assay system, 15 fibrin clots were incubated in 1% (w/v) monochloro acetic acid in the presence of identical plasma enzyme concentrations at 37°C for 24 h. The amount of fibrin clot remaining was estimated as 3.2.5.

Result - Mean = 0.126

Standard deviation = 0.021

3.2.12. Precision

Coefficients of variation were determined by duplicate measurements of the same samples in the same assay and in different assays.

The coefficient of variation (CV) was determined using the formula $CV = \frac{d^2}{2m}$

where m = number of duplicate determination

and $d = \frac{\text{highest value of each duplicate}}{\text{lowest value of sample duplicate}} - 1 \times 100$

Abraham et al., (1971).

10 duplicate determinations were carried out with the same batch of substrate, but different enzyme concentrations. (Residual fibrin concentration ranged from 0.6-2.6 mg) The coefficient of variation was

8.0%.

In addition 30 duplicate determinations were carried out with different batches of substrate and different enzyme concentrations.(Residual fibrin concentration ranged from 0.4-3.0 mg) The coefficient of variation was 12%.

3.2.13. Comment

During the course of study the fibrin clot assay was used for the enzyme purification. Meanwhile the search for a better substrate continued, because of the general problem of using protein substrates and also because of particular problems directly associated with this substrate, such as inter-batch variation, and the tedious nature of the assay.

3.3. Azocasein Substrate

3.3.1. Introduction

Almost all known proteolytic enzymes hydrolyse casein, and it is often used as a substrate for the determination of proteolytic activity (Reimerdes and Klostermeyer, 1976). The extent of proteolysis is measured in the supernatant after acid precipitation. If the acid soluble fragments of the casein contain aromatic amino acids, it is possible to measure the amount of hydrolysis at 280 nm. However, there are disadvantages in using casein as a substrate:

a) casein is a cow's milk product, and there is a batch to batch variation according to the way in which it has been prepared and stored. A good sample of purified casein contains β-casein, α_{s1}-casein and κ-casein. There are other minor components such as γ-casein, R-, S-, TS-, and $\alpha_{s2\text{-}5}$-casein. Some samples are even contaminated with albumin and globulin. Acidic proteinases such as chymosin specifically hydrolyse the Phe23-Phe24 bond in α-casein and the Phe105-Met 106 in κ-casein, but only the latter fragment is acid soluble.

b) Acid precipitation can lead to another problem in that not all the acid soluble fragments contain aromatic amino acids, and other fragments are only partially soluble in acid solution. c) Casein is not soluble at acid pH and is not a very suitable substrate for acid proteinases.

To attempt to produce a more sensitive and

versatile substrate, Charney and Tomarelli (1947) used azocasein as a colourimetric method.

3.3.2. Principle of the assay

Diazotized sulphanilic acid is coupled to the side chains of histidine and tyrosine residues of casein in alkaline solution to form a chromoproteide. Hydrolysis of this azo protein with proteolytic enzymes results in the formation of coloured components which are soluble in TCA. The absorbance of the acid soluble peptides containing the chromophore is measured at 366 nm.

The increase in colour in TCA filtrate is thus the function of enzyme activity.

3.3.3. Preparation of azocasein

The method used was based on that of Barrett and Kirschke (1981) and Kirschke et al., (1982). 50 g of α-casein (Hammarsten) was dissolved in 1 L of distilled water by heating and adjusting the pH to approximately 9.0. The solution was centrifuged at 2000 g for 20 min. to remove undissolved casein. 50 g of sulphanilic acid was dissolved in distilled water containing 6 ml of 5 M NaOH and 32.2 g of $NaNO_2$. Then 18 ml of 5 M HCl was added to the sulphanilic acid solution to form the diazotized sulphanilic acid. The solution was stirred for 2 min., and 18 ml of 5 M NaOH added. This solution was then added to the casein, while stirring. On mixing the two solutions a dark purple colour was developed. The mixture was stirred for 1 h. The

azo-protein was precipitated out by acidifying the solution with 25% (v/v) acetic acid. The solution was centrifuged at 2000 g for 20 min.

The supernatant was discarded and the precipitate dissolved by dropwise addition of 1 M NaOH. The precipitation procedure was repeated twice for further purification. The precipitate was then suspended in ethanol and filtered. The washing with solvent was repeated 3 times or until the filtrate was colourless. To obtain a very pure substrate with low blank, acid precipitation and washing with ethanol was repeated once more. The precipitate was air dried at room temperature.

3.3.4. General assay method

1% (w/v) azocasein was freshly prepared in the appropriate buffer. In a plastic tube 100 µl of enzyme solution was mixed with 100 µl of azocasein solution in an ice bath. The tubes were incubated at 37°C for an appropriate time. The reaction was stopped by the addition of 200 µl of 10% (w/v) TCA. The tubes were kept at room temperature for 10 min. The absorbance of the supernatent read at 366 nm. The blank was prepared in the same way except that the enzyme solution was replaced by buffer.

In this project a modified method which has been used for cathepsin D assay (Kirschke et al., 1982) was used.

3.3.5. Assay method

2% (w/v) azocasein was made in 0.4 M sodium citrate

buffer, pH 3.5. Two volumes of buffer, one volume of enzyme solution and one volume of azocasein were mixed and incubated at 37°C for different time intervals. The reaction was stopped by addition of four volumes of 10% (w/v) TCA. After 10 min. the mixture was centrifuged at 200 g for 20 min. and the absorbance of the supernatant read at 366 nm.

3.3.6. Results

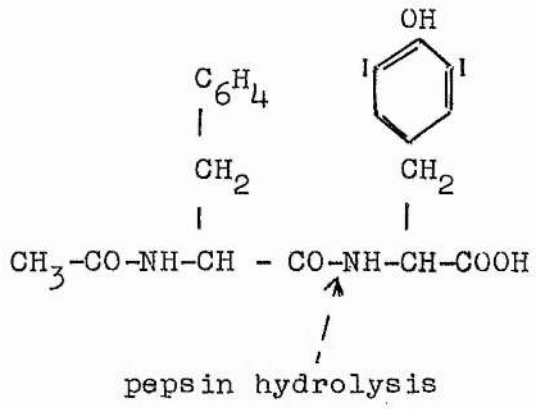
Different concentrations of plasma enzyme which showed proteolytic activity in the fibrin clot assay system were used.

Each concentration of enzyme was incubated with a fixed amount of azocasein substrate at 37°C for a period of 1-18 h. None of the concentrations showed any proteolytic activity. The experiment was repeated using the same buffer at different pH's. Plasma enzyme exhibited maximal activity towards the azocasein substrate at pH 3.1, Fig. 5.6.

3.4. Synthetic Substrate N-acetyl-L-phenylalanyl-diiodotyrosine (APDT)

3.4.1. Introduction

Fruton and Bergmann (1939) used different synthetic substrates to measure the specificity of pepsin. They concluded that the substrate to be hydrolysed by pepsin should contain aromatic amino acid residues tyrosine or phenylalanine, preferably combined with glutamic acid. They showed the best substrate was carbobenzoxy-l-glutamyl-l-tyrosine, but carbobenzoxy-l-glutamyl-l-diiodotyrosine was resistant to pepsin action. In contrast Baker (1951) found that the diiodotyrosine peptide was hydrolysed much more rapidly than the tyrosine peptide, at all pH values. She concluded that N-substituted dipeptides with two residues of the L forms of the aromatic amino acids, tyrosine, diiodotyrosine or phenylalanine will be hydrolysed by pepsin. Peptides containing D forms of these amino acids or amino acids with their phenolic group blocked are not hydrolysed by pepsin. Ryle (1970) used N-acetyl-l-phenylalanyl l-diiodotyrosine (APDT) to assay minor pepsins and pepsin. The activity of the enzyme was measured colorimetrically, using ninhydrin. The substrate APDT is hydrolysed by pepsin liberating diiodotyrosine, which gives a colour reaction with ninhydrin.



3.4.2. The APDT assay

The method used was based on that of Moore and Stein (1954) and Tang (1970).

3.4.3. Substrate preparation

2 mM APDT in 5 mM sodium hydroxide was prepared by dissolving 62.2 mg of APDT in 2.5 ml of 0.1 M NaOH and taking the volume to 50 ml with distilled water.

3.4.4. Ninhydrin preparation

Oxygen free nitrogen gas was bubbled through 750 ml of 2 methoxy ethanol (methyl cellosolve), to remove any peroxides which may have been formed during storage. The following procedure was used to test for the presence of peroxides. A small crystal (approximately 1-2 mg) of potassium iodide was added to 1-2 ml of methyl cellosolve. In the presence of peroxides the colour changed from straw to yellow. 250 ml of 4 M Na acetate buffer, pH 5.5, was prepared and oxygen free nitrogen bubbled through for about 30 min. 20 g of ninhydrin and 3 g of hydrindantin were added to the mixed solution of methyl cellosolve and Na acetate buffer. Nitrogen gas was bubbled through

and the solution stored in a dark bottle at 4°C.

3.4.5. Assay procedure

0.5 ml of the test sample, 0.25 ml of 0.208 M HCl and 0.25 ml of APDT substrate were mixed and the solution incubated at 37°C for 1 h. 1.0 ml of ninhydrin solution was added and the mixture boiled for 15 min. After cooling, 0.5 ml of 60% (v/v) ethanol was added and the solution thoroughly mixed (vortex mixer) for 30 secs.

The absorbance was read at 570 nm against a blank prepared the same way as the test except APDT was added after ninhydrin. All the tests were carried out in duplicate.

3.4.6. Results

Normal plasma gave very high blank, and it was almost impossible to record the absorbance. With samples from later stages of plasma enzyme purification, it was possible to obtain reasonable reading at 570 nm, but the tests and blanks were identical. Increasing the enzyme concentration and the incubation time did not improve the results, indicating that there is no detectable hydrolysis of the synthetic substrate APDT. To verify the assay, commercial hog pepsin (BDH) was used. Pepsin also showed very high blank.

To find out the cause of the high blank, individual reagents were tested, and tyrosine was used to prepare a standard curve for the ninhydrin reagent. Results were satisfactory and a linear relationship between tyrosine concentration and colour development

obtained. This suggests that the high blank value is due to pepsin and not to the reagents.

To verify this, a) pepsin was extensively dialysed to remove any peptides. b) pepsin was prepared fresh prior to the test. Two samples of pepsin which had been incubated at 25°C and 37°C respectively were also tested. Dialysis reduced the high reading of the blank, but not completely.

Freshly prepared pepsin had a lower blank, indicating some self digesting of pepsin had taken place at 25°C and 37°C. However, even with high blank it was possible to obtain a straight line with different concentrations of pepsin. From the above results it can be deduced that plasma enzyme does not hydrolyse synthetic substrate APDT. Therefore its action is similar to cathepsin D, and unlike pepsin (Chiang et al., 1966).

3.5. Haemoglobin (Hb) Substrate

3.5.1. Denatured haemoglobin

3.5.1.1. Principle

The proteinase digest denatured haemoglobin, and small peptides are released (Mycek, 1970). Undigested fragments are precipitated with TCA and the soluble peptides can be estimated spectrophotometrically at 280 nm.

3.5.1.2. Method

The method used was based on that of Aoyagi et al. (1971).

0.5% (w/v)bovine haemoglobin in 0.03 M HCl was prepared. 0.8 ml of 0.02 M KCl/HCl buffer, pH 2.0, was added to 1.0 ml of the haemoglobin solution. The mixture was incubated at 37°C for 3 min. Then 0.1 ml of enzyme was added, and the mixture incubated for further 25 min. The reaction was stopped by the addition of 2.0 ml of 1.7 M perchloric acid or 10% (w/v) TCA. The mixture was incubated at room temperature for 1 h before being centrifuged at 200 g for 30 min. The absorbance of the supernatant (acid soluble fraction) was read at 280 nm.

3.5.2. Haemoglobin-polyacrylamide gel (Hb-gel)

The haemoglobin assay can be adapted for the detection of proteinase activities in polyacrylamide gels either by incorporating denatured haemoglobin into the gel before polymerization, or by diffusing soluble substrate into the gel, after electrophoresis (Andary and Dabich, 1974).

3.5.2.1. Hb-gel electrophoresis (insoluble-Hb)

The method used was based on that of North and Harwood (1979).

3.5.2.2. Gel preparation

Separating gel

2.5% (w/v) bovine haemoglobin in 0.1 M Tris-HCl, pH 7.5, was prepared and incubated at 70°C for 5 min., followed by approximately 5 min. sonication (until the particles stay in suspension while the gel sets).

1% (w/v) ammonium persulphate was prepared in distilled water just before use. The gel solution was prepared to give a final concentration of 12% (w/v) acrylamide, 0.075% (w/v) bisacrylamide, 0.2% (v/v) denatured haemoglobin, and 0.05% ammonium persulphate. The reagents were mixed quickly, deareated, and TEMED added to a final concentration of 0.05% (v/v). The solution was poured into the gel holder, until the liquid level was about 1.5 cm. from the top of the tubes. Distilled water was layered on the top of the gels and they were left at room temperature for 30-60 min. to allow polymerization.

Stacking gel

The stock solution of riboflavin was prepared by adding 4 mg of riboflavin to 100 ml distilled water. The stacking gel solution was prepared to give a final concentration of 2.5% (w/v) acrylamide, 0.6% (w/v) bisacrylamide, and 1% (v/v) riboflavin. The

pH of the mixture was adjusted to 6.8 and it was degassed. 0.01% (v/v) TEMED was added and the solution was poured on the top of the separating gel (0.5 cm), replacing the water on the top of the gel. Distilled water was again layered on the top of the stacking gels. The gels were exposed to fluorescent light for 30-45 min. to allow photopolymerization to take place. Once the gels were set, water was replaced by chamber buffer, and the gels were stored at 4°C if not in use immediately.

3.5.2.3. Gel electrophoresis

The upper chamber (cathode) buffer consisted of 0.03 M sodium tetraborate, containing 0.002% (w/v) bromophenol blue, while the lower chamber (anode) buffer was 5 mM Tris-HCl/38.5 mM glycine, pH 8.5.

Samples in 20% sucrose were placed on the top of stacking gels, and were electrophoresed at 4°C at 1 mA/gel until the tracking dye entered the separating gel. Thereafter the current was raised to 2 mA/gel.

3.5.2.4. Assay for proteolytic activity

The proteinase bands were developed in 0.1 M glycine/HCl buffer, pH 2.5-2.8 for 6 h. The buffer was changed at least twice in the first 30 min. The proteolysis was stopped by placing the gels in the staining solution 0.1 M Tris-HCl buffer, pH 7.5 containing 0.1% (w/v) nigrosine for 5-6 h.

The gels were washed in destaining solution (0.1 M Tris-HCl buffer, pH 7.5) for at least 48 h before the clear bands were visible.

3.5.3. Hb-gel electrophoresis (soluble haemoglobin)

The method used was based on that of Andary and Dabich (1974).

3.5.3.1. Gel preparation

7.76 g acrylamide and 0.24 g bisacrylamide were dissolved in 100 ml of 25 mM citric acid buffer, pH 3.1, and stored at 4°C (stock solution). To prepare 8% gel, 7.5 ml of gel stock solution, 2.5 ml of 3% (w/v) ammonium persulphate and *0.1 ml of TEMED were mixed, degassed and poured in a) sets of tubes, b) sets of plates (12 cm x 12 cm). The top of the gels were covered with 50% (v/v) ethanol and left at room temperature to polymerize, then ethanol was replaced with the above buffer, and the gels were stored at 4°C in a humid chamber.

3.5.3.2. Gel electrophoresis

Samples were dialysed against chamber buffer (25 mM citric acid, pH 3.1). Dialysed samples were mixed with glycerol and bromophenol blue, before being applied to the gels. Gels were electrophoresed at 200 volts for about 5 h at room temperature.

3.5.3.3. Detection of proteolytic activity

The method used was based on that of Ryle (1980). Tube gels were immersed in 0.65% (w/v) bovine haemoglobin in 0.2 M citric acid. The gels from plates were covered with No. 3 Whatman paper which had been soaked in haemoglobin solution (0.3 ml haemoglobin/cm^2 paper).

* High percentage of ammonium persulphate and TEMED were used to facilitate gel formation at low pH.

The gels and Whatman paper were covered with cling film, and were incubated at 37°C for 30 min. Gels were fixed in 5% (v/v) acetic acid in 50% (v/v) ethanol (Samloff, 1969) overnight. Then they were stained in coomasie blue solution, and were destained in a solution of ethanol, acetic acid, and H_2O (20:5:75).

3.5.4. Haemoglobin-agar (Hb-agar)

The method used was based on that of Samloff (1969).

1.5 g I.D. agar (Oxoid), was added to 0.05 M barbitone buffer, pH 8.6, and dissolved by boiling. The molten agar was poured onto a glass plate (15 cm x 15 cm), and left at room temperature to set. Plates were stored at 4°C in a humid chamber. For use, slots of 1 cm x 0.1 cm were made on the gels and filled with the mixture of dialysed sample and bromophenol blue. Electrophoresis was carried out long enough for the marker dye to travel to the end of the gel($\sim$ 4 h). The electrophoresis was run at 112 volts/cm. Because of the rather high voltage, a tank with cooling system was used.

3.5.4.1. Detection of proteolytic activity

Plates were immersed in 0.65% (w/v) bovine haemoglobin in 0.06 M HCl, pH 1.6, for 20 min. at 37°C and then left in a humid chamber for a further 2 h. Plates were fixed, stained and destained as (3.5.3.3.).

3.5.5. Preparation of N,N-Dimethyl haemoglobin

The method used was based on that of Lin et al., (1969).

1.5 g of bovine haemoglobin was dissolved in 150 ml of 0.1 M borate buffer, pH 9.0. 5-10 drops of hydrogen peroxide were added to the solution, which was warmed gently to reduce its visible absorbance. 300 mg sodium borohydride was added followed by 5-10 drops of 2-octanol to stop the production of foam. 3.0 ml of formaldehyde was added in a period of 30 min. (100 µl formaldehyde at a time). 5 min. after the addition of all the formaldehyde, the pH of the solution was brought to pH 6.0 with 50% (v/v) acetic acid. Methylated haemoglobin was dialysed against distilled water. It was then freeze dried and stored at -20°C.

3.5.6. Haemoglobin-substrate results

3.5.6.1. Denatured haemoglobin

Samples from different stages of plasma enzyme purification were tested as (3.5.1.2). No proteolytic activity was detected. Hog pepsinogen was used as a control, and it digested Hb at concentration of 1 µg/ml. A set of experiments was performed to find the optimum condition for the plasma enzyme. Once the incubation time was extended to 18 h, some proteolytic activity was detected. At the end of 18 h incubation, a linear relationship was found between enzyme concentration and the absorbance of the supernatent, Fig. 5.2. The optimum activity was detected at pH 3.0 - 3.2. Although plasma enzyme hydrolysed denatured haemoglobin, the absorbance readings were very low.

High concentrations of enzyme were needed and the incubation time was long. These factors made the Hb-substrate unsuitable for routine use and for the detection of enzyme during the purification procedure.

3.5.6.2. Insoluble haemoglobin-polyacrylamide gel

Different volumes (5-50 µl) of normal plasma were applied to the Hb-gels (3.5.2.1). Plasma volume of 30 µl showed a clear zone with the mobility (relative to marker dye) of 0.82-0.86. Hog pepsinogen was used as a control, and clear bands were detected with mobilities of 0.95-0.98, (Fig. 3.7). Some plasma samples showed up to four clear bands, with the mobilities of 0.82-0.95, with the prominent clear band always at 0.82-0.86. Samples from different stages of purification of plasma enzyme showed the same characeristic clear band. Samples which were treated with pepstatin showed no activity (Fig. 3.8). The plasma samples which did not give any proteolytic activity with fibrin clot assay also had no activity in this system.

Comment

The haemoglobin gel assay is extremely sensitive in detecting very small amounts of plasma enzyme. It has, however, some disadvantages. It is time consuming, it takes at least 48 h for the clear bands to become visible, it is difficult to carry out densitometric scanning or even photography. Clear zones are much more readily detected by eye. It is not possible to measure the proteolytic

Fig. 3.7 DENSITOMETRIC SCAN OF ALIQUOTS

FROM A Normal plasma

B 25-75% (AS) step

C Blue-sepharose step, Table 4.7

D DEAE-cellulose step, Table 4.7

trough I clear band on Hb polyacrylamide gel.

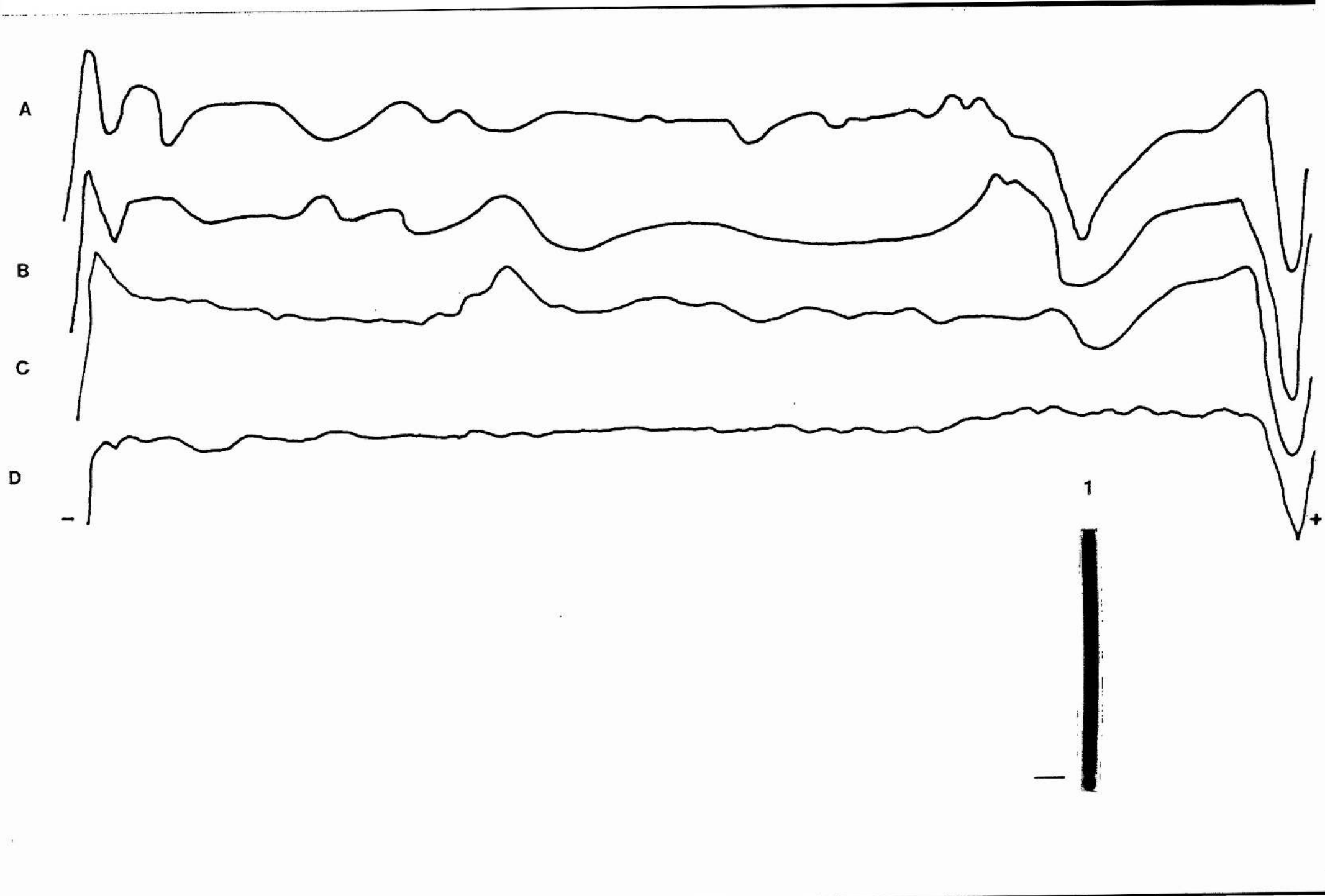

A
B
C
D
–
+
1

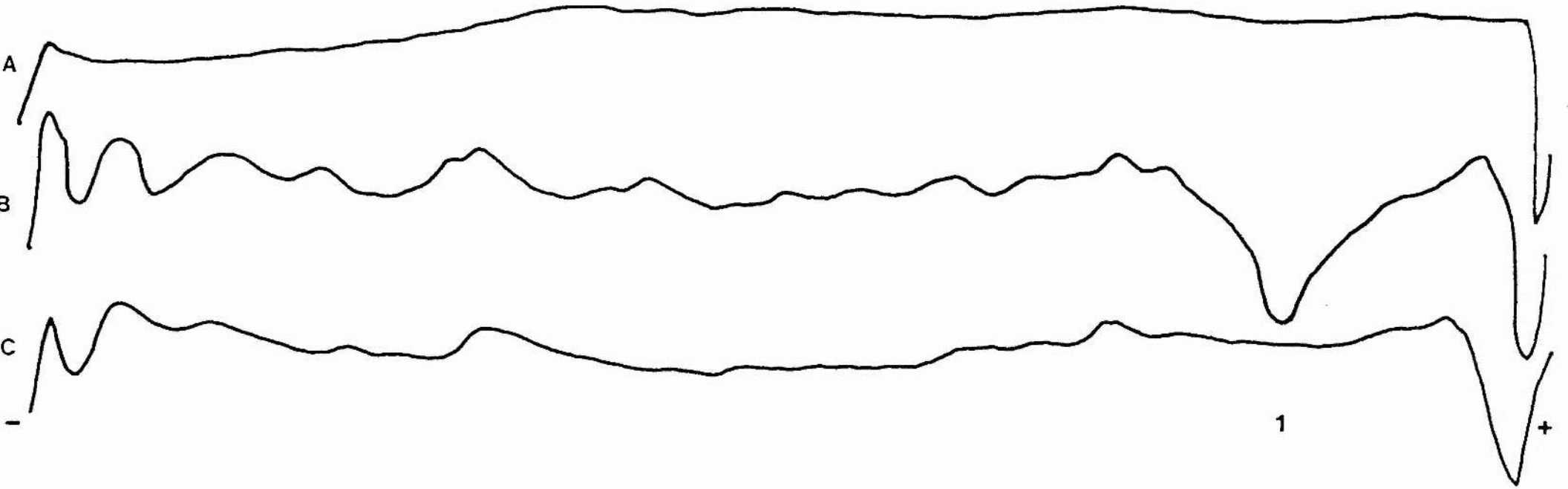
Fig. 3.8 DENSITOMETRIC SCAN OF Hb-GEL

A Control electrophoresis of the gel with no sample

B An aliquot of plasma added to the gel before electrophoresis

C Pepstatin was added to the plasma before the application of the sample to the gel

t.rough I clear band on Hb-polyacrylamide gel.


A
B
C
–
1
+

activity of the enzyme in the later stages of purification, as the gels are electrophoresed at alkaline pH and the enzyme, having been in acid pH, will be inactivated by the pH change. To investigate the possibility of improving the visibility of the zone against the dark background of the gel, and also shortening the destaining time, two different types of nigrosine were used.
a) water soluble; b) alcohol soluble. Different concentrations of each dye 0.01-0.1% (w/v) were used, and the incubation time of the gels in the staining solution was varied. The best results were achieved by using 0.1% (w/v) alcohol soluble nigrosine with 6 h incubation in the staining solution.

3.5.6.3. Hb-gel (soluble haemoglobin)

Normal human plasma, samples from different stages of purification and hog pepsinogen which had shown the same level of proteolytic activity with the fibrin clot substrate, were tested. No activity was detected with plasma enzyme. Although pepsinogen showed proteolytic activity, the sensitivity was 100x less than the insoluble haemoglobin-polyacrylamide gel.

Comment

The plates and tube gels were very heavily coloured, even with extensive destaining. The concentration of the staining solution was reduced, incubation time of the gels in haemoglobin was varied, as

was the pH of the chamber buffer. None of these procedures improved the method.

3.5.6.4. Haemoglobin-agar

This did not prove to be a very satisfactory method, the results were very similar to 3.5.7.3; even with varying the different parameters of the assay system no improvement was detected.

3.5.6.5. Methylated Hb

To overcome the dark background of the soluble Hb-gel (3.5.6.3), methylated haemoglobin was used. Clear bands were visible with pepsin and the sensitivity of the results was comparable with that of Hb gel (insoluble Hb). However, plasma enzyme did not show any proteolytic activity, which made the method inappropriate.

3.5.7. Summary

Haemoglobin was used in different ways in an attempt to find a sensitive method for the detection of plasma enzyme. None of the methods was entirely satisfactory.

Acid denatured haemoglobin was used as a substrate for the detection of proteolytic activity of the plasma enzyme. The method proved insensitive, as the amount of proteolysis was low, giving a very low absorbance reading at 280 nm. To improve the technique, insoluble Hb was incorporated into polyacrylamide gels, before polymerization. The sample was electrophoresed at alkaline pH, and the gels were then incubated in acid media. The enzyme activity was

detected by the development of a clear band. The method was sensitive to the enzyme's proteolytic activity, but the problem was that the gels were electrophoresed in alkaline pH, and once the enzyme had been in acid pH, it was not possible to measure its activity as a change of pH from acid to alkaline caused the loss of enzyme activity. To overcome this difficulty, the plasma enzyme was electrophoresed in acid pH, using polyacrylamide, agarose and agar. Then haemoglobin was diffused into the gels. The technique lost its sensitivity. When methylated haemoglobin was used no proteolytic activity was detected with plasma enzyme.

On using methylated haemoglobin a clear background was achieved but did not improve the sensitivity of the assay system.

3.6. General Discussion on Substrate

Laing (1975) and Taylor (1976) used dyed fibrin as the substrate in preliminary investigations of the plasma enzyme on the grounds of the similarity with the phenomenon which brought the enzyme to light. Their assay system had its limitations and was improved by Law (1978). However, doubts still remain as to the specificity of the system she developed, as reliable quantitative results were not easy to obtain.

In this project the fibrin clot assay was used throughout the purification and for much of the enzyme characterization. Quantitation with this assay is possible, but the protein nature of this substrate

makes a study of the kinetics of the enzyme impractical.

Although denatured haemoglobin is widely used as a substrate for acid proteinases it was not an effective substrate for this enzyme. Haemoglobin incorporated into polyacrylamide gel was an effective and sensitive means of comparing the plasma with other enzymes. This method is, however, time-consuming and purely qualitative.

Azo-casein was not very sensitive as a substrate for this enzyme. Its insolubility at acid pH makes it generally unsuitable. N-acetyl-L-phenylalanyl-L-diiodotrosine did not appear to be cleaved by the plasma enzyme to any detectable degree.

In spite of its technical drawbacks the fibrin clot assay remains the more suitable assay for this particular enzyme, although there is scope for further work with a range of synthetic substrates to clarify the kinetics and specificity of the plasma enzyme.

CHAPTER 4

PURIFICATION

4.1.1. Ammonium sulphate fractionation

The sample was brought to 25% and 75% saturation with solid ammonium sulphate.

The suspension was stirred gently at 4°C for 8h, then it was centrifuged at 10,000 g. for 1 h. The precipitate was dissolved in and dialysed against 0.9% (w/v) NaCl for 48 h, with at least 4 changes of NaCl. To prepare (25-75%) saturation another aliquot of the sample was first brought to 25% saturation with ammonium sulphate, and then the supernatant was brought to 75% saturation, and treated as above.

4.1.2. Aluminium hydroxide $Al(OH)_3$ gel fractionation

4.1.2.1. Gel preparation

The method used was based on that of Ikemori et al., (1975). 50 ml of 0.2 M aluminium sulphate was heated at 63°C. 5 ml of 58% (w/v) ammonium hydroxide was added to 45 ml of distilled water at 67°C.

The aluminium sulphate was mixed with ammonium hydroxide, and the temperature of the mixture was maintained at 62-63°C for 10 min. The gel was washed with distilled water several times, until no free sulphate was detected by testing with 1% (w/v) barium sulphate.

4.1.2.2. Fractionation

1 volume of aluminium hydroxide was mixed with

10 volumes of sample. The mixture was stirred at room temperature for 1 h. or at 37°C for 10 min.

The suspensions were centrifuged at 2500 g for 30 min. The supernatant was kept separate and the precipitate was washed with 0.9% (w/v) NaCl until no protein was detected in the wash.

The gel was then extracted twice with 0.05 M phosphate, pH 7.0, and the eluted material was collected.

4.1.3. Poly(ethylene glycol) 6000 fractionation

The method used was based on that of Barrett et al., (1979).

1 volume of sample was mixed with 0.28 volume of 25% (w/v) poly(ethylene glycol) 6000 (B.D.H.) in 0.05 M Tris-HCl, pH 7.0 final concentration of poly (ethylene glycol) 5.5% (w/v) .

The mixture was stirred at room temperature for 30 min. and centrifuged at 12,000 g for 30 min. The precipitate was dissolved in 0.1 volume of the above buffer, and kept separately. 0.72 volume of poly (ethylene glycol) 6000 was added to the supernatant final concentration of poly(ethylene glycol) 12.5% (w/v) .

The mixture was treated as before, and the precipitate was dissolved in 0.1 volume of 0.05 M Tris-HCl, pH 7.0.

The two precipitates and the supernatant were dialysed against 0.05 M Tris-HCl, pH 7.0.

4.2. Ion Exchange Chromatography

4.2.1. Sulphopropyl Sephadex (SP-Sephadex)

The SP-Sephadex (Pharmacia) resin was precycled and degassed. A column of 25 cm x 1.8 cm was packed with the resin and it was equilibrated with 0.05 M sodium acetate, pH 4.0. The flow rate was adjusted to 40 ml/h. The sample was dialysed against above buffer at 4°C for 48 h, and applied to the column. Fractions were collected every 10 min. The starting buffer was pumped through until no protein was detected in the effluent at 280 nm. Then 0.05 M sodium acetate, pH 5.0 was applied until again no protein was detected in the effluent, and the pH of the effluent was 5.0. Then 0.05 M sodium acetate, pH 6.0 was applied until the pH of the effluent reached 6.0.

4.2.2. CM-Cellulose 52 (Whatman)

CM-cellulose resin was precycled and degassed. A column of 25 cm x 1.8 cm was packed with the resin, and was equilibrated with 0.05 M sodium acetate buffer, pH 4.0. The flow rate was adjusted to 40 ml/h. The sample which had been dialysed against the above buffer was applied to the column. Fractions were collected every 10 min. The starting buffer was pumped through until no protein was detected in the effluent at 280 nm. Then 0.05 M sodium acetate, pH 5.0 was applied until again no protein was detected in the effluent and the pH was 5.0. Next 0.05 M sodium acetate, pH 6.0 was applied in the same way.

4.2.3. DEAE-Cellulose 52 (Whatman)

The DEAE-cellulose resin was precycled and degassed. A column of 26 cm x 2 cm was packed with the resin and it was equilibrated with 0.05 M Tris-HCl, pH 8.0. The sample was dialysed against the above buffer and applied to the column at the rate of 30 ml/h. Fractions were collected every 6 min., until no protein was detected in the effluent. Then 0.05 M Tris-HCl/ 0.05 M phosphoric acid, pH 3.8 was applied.

4.2.4 Amberlite IRC-50(H)

4.2.4.1. Generation of Amberlite IRC-50 resin

The method used was based on that of Hirs et al., (1953).

300 ml of distilled water was added to 100 g of resin, and stirred for 20 min. The suspension was left undisturbed for the resin to settle. The supernatent was removed and washing was repeated four times, until the supernatant was clear. The resin was filtered through a Buchner funnel and 200 ml acetone was added, stirred and left for 3 h. The resin was filtered, washed with 500 ml acetone, followed with 5 ml of distilled water to remove the acetone. Once the resin was cleaned, 200 ml distilled water was added followed by 95 ml of 40% (w/v) NaOH solution, which was added gradually within 30 min., and stirred for 3 h (pH 11).

The sodium form of resin was washed with distilled water until the pH of filtrate was 10. The resin was washed with 1 l of 2 M HCl over a 4h

period, followed by washing with the buffer to be used in the experiment.

4.2.4.2. Amberlite IRC-50 chromatography

A column of 38 cm x 2.5 cm was packed with the resin and equilibrated by pumping 0.2 M citrate buffer pH 3.0. Flow rate was adjusted to 30 ml/h. The sample was dialysed against the starting buffer or in the case of freeze dried sample, which had been dialysed against water, dissolved in the above buffer and applied to the equilibrated column. The column was eluted with the starting buffer (0.2 M citrate buffer, pH 3.0). Fractions were collected every 10 min. The elution was continued until the eluate had a constant absorbance of less than 0.1 at 280 nm. Then 0.2 M sodium citrate buffer pH 3.8 was applied until the absorbance of the effluent was less than 0.05 at 280 nm. Then 0.2 M sodium citrate buffer pH 4.2 was applied until the effluent pH reached 4.2, after which 0.2 M sodium citrate buffer pH 4.6 was applied.

4.3. Gel filtration

4.3.1. Sephadex-G200 (Pharmacia)

Sephadex-G200 resin was swollen in excess 0.02 M phosphate, pH 5.5 for 48 h at room temperature. The resin was degassed and packed in a 45 cm x 2 cm column.

The sample was dialysed against the above buffer, freeze dried, and then dissolved in the minimum amount

of distilled water before applying to the column. The column was eluted by gravity flow at the rate of 14 ml/h, and fractions were collected every 30 min.

4.4. Affinity Chromatography

4.4.1. Lysine-Sepharose 4B

4.4.1.1. Coupling lysine to Sepharose

The method used was based on that of Chibber et al., (1974).

40 g of Sepharose 4B was suspended in 100 ml of distilled water. 5% (w/v) cyanogen bromide was prepared in cold water (4°C) and added to the Sepharose. The mixture was stirred gently. The reaction was allowed to proceed for 10 min. Meanwhile the temperature of the reaction was kept at 18-20°C with the addition of ice, and the pH of the reaction was kept constant at 11.0 by the addition of 5 M sodium hydroxide. The reaction was stopped by washing the mixture in a Buchner funnel with cold (4°C) 0.1 M sodium biocarbonate buffer, pH 8.9. The activated sepharose was mixed with lysine (10.0 g in 25 ml distilled water pH 8.9), and stirred gently for 18 h. 10 g glycine in 50 ml distilled water was added to the mixture and stirred for a further 2-3 h.

The lysine-Sepharose was washed with 1.0 l of 1.0 M sodium chloride, 2.0 l of distilled water and it was stored at 4°C in 0.02% (w/v) sodium azide.

4.4.1.2. Lysine Sepharose column

A column of 18 cm x 1.8 cm was packed with lysine-Sepharose and allowed to equilibrate by pumping 0.05 M Tris-HCl buffer, pH 7.5 through at a flow rate of 36 ml/h. The sample was applied to the column and fractions were collected every 10 min. Starting buffer was pumped through until the eluate had an absorbance of less than 0.05 M at 280 nm. Then 0.05 M Tris-HCl/0.05 M NaCl, pH 7.5, was applied and fractions were collected as before. Next, 0.2 M ϵ-amino caproic acid (EACA) was applied, followed by 0.05 M Tris-HCl/0.2 M EACA/1.0 M NaCl.

4.4.2. Cibacron Blue-Sepharose column

The method used was based on that of Virca et al., (1978).

The blue Sepharose resin was very kindly provided by Dr. Wray (Biochemistry Department, St. Andrews University). A column of 25 cm x 2 cm was packed with the resin, and washed with 0.05 M Tris-HCl, pH 8.0. The sample was dialysed against the above buffer, and applied to the column. Fractions were eluted from the column by gravity flow at the rate of 8 ml/h. Fractions were collected every 20 min. until no protein was detected in the effluent at 280 nm. The column was washed with 0.15 M NaCl, 2 M NaCl, and fractions were collected as before.

4.4.3. Haemoglobin (Hb)-Sepharose

4.4.3.1. Gel preparation

The method used was based on that of Smith and

Turk (1974).

Sepharose 4B resin was activated as (4.4.1.1.). 6.0 mg bovine haemoglobin was added per 1 ml of wet Sepharose at pH 6.4.

The resin was stirred gently at 4°C for 24 h. The resin was washed with 1 l of 1 M NaCl and 2 l of distilled water, and stored in 0.02% (w/v) sodium azide at 4°C if not in use.

4.4.3.2. Haemoglobin-Sepharose column

Haemoglobin-Sepharose-4B resin was washed extensively with 0.05 M sodium acetate, 1 M NaCl, pH 3.5, and degassed. A column of 23 cm x 1 cm was packed with the resin and was equilibrated by pumping through the above buffer. The flow rate was adjusted to 18 ml/h. The sample was dialysed against the starting buffer and applied to the column. Fractions were collected every 10 min. After the application of the sample the column was washed with 0.1 M sodium acetate, 1 M NaCl, pH 5.0, until the effluent had an absorbance less than 0.05 at 280 nm. Then 0.1 M Tris-HCl/1 M NaCl, pH 8.6 was applied and fractions were collected until the pH of the eluate reached 8.6.

4.5. Results

4.5.1. Removal of plasminogen

Normal fresh frozen plasma was applied to lysine-Sepharose column as 4.4.1.2, and plasminogen free plasma, obtained from the column, was kept frozen at -20°C if not used immediately.

4.5.2. Ammonium sulphate (AS) fractionation (batch test)

Small aliquots of plasminogen free plasma were fractionated as 4.1.1. The precipitates and supernatents from 25%, 75%, and 25-75% were dialysed against 0.45% (w/v) NaCl and they were examined for enzyme activity, using fibrin clot assay results Table 4.1.

Table 4.1

Fractions	25% (AS) (a)	75% (AS) a	25-75% (AS) (a)	75% (AS) (b)	25-75% (AS) (b)
*% Activity	2	90	94	6	1.4

a = precipitate

b = supernatant

* % activity of the original sample recovered.

The results showed (Table 4.1) that the 75% and 25-75% (AS) precipitates had high activity. The 25-75% (AS) was used for further purification as it had the advantage over 75% (AS) in that fibrinogen had been removed at 25% (AS) precipitation.

4.5.3. Aluminium hydroxide gel fractionation (batch test)

A small aliquot of 25-75% (AS) was fractionated as 4.1.2.2. Two fractions were obtained: a) material whichdid not bind to the $Al(OH)_3$ gel and b) material eluted from the $Al(OH)_3$ gel. As the results show (Table 4.2) material which did not bind to the gel had the higher activity and was used for the further purification.

Table 4.2

Fraction	% Activity
Material did not bind to the $Al(OH)_3$ gel	87%
Eluate	2.0%

Comment The unbound material had to be dialysed to remove the high concentration of salt before it could be assayed.

4.6. Purification of plasma enzyme (first batch)

The flow chart of the purification method is summarized in Fig. 4.1. and results in Table 4.3.

100 ml of fresh frozen plasma was applied to lysine-Sepharose column as 4.4.1.2. 140 ml of plasminogen free plasma was obtained. To 100 ml of plasminogen free plasma, ammonium sulphate was added as 4.1.1.

90 ml of 25-75% (AS) fraction was subjected to $Al(OH)_3$ gel fractionation as 4.1.2.2. Specific activities of the above steps are shown in Table 4.3.

4.6.1. Ion exchange chromatography

SP-Sephadex

Unbound material from $Al(OH)_3$ gel (80 ml fraction) was dialysed against 0.05 M sodium acetate, pH 4.0 and applied to the colum (4.2.1). Material which did not bind to the column, Peak I, Fig. 4.2, had the greatest activity. Table 4.3.

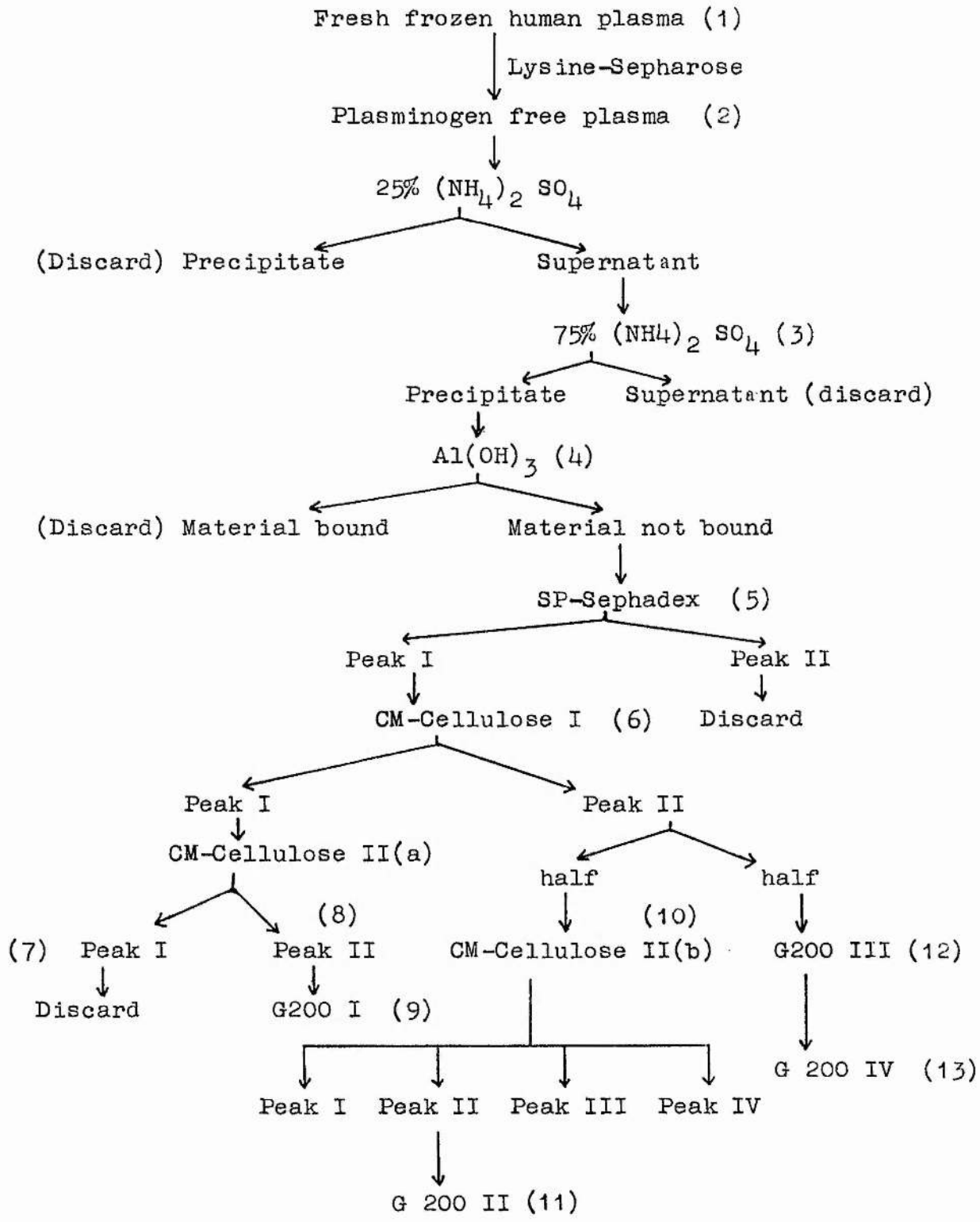


Fig. 4.1 Flow chart for the plasma enzyme purification (first batch).

Table 4.3 Purification of plasma enzyme (first batch)

Procedure	Volume (ml)	Protein conc. "mg/ml" A_{280}^{nm}	Total Protein (mg)	Enzyme Activity Units/ml)	Enzyme Activity Total Units	S.A. Units/ml	Yield %	Purification Factor (Fold)
1. Normal plasma	96	60	5760	9.7	931	0.16	100	1.0
2. Plasminogen free plasma	110	40	4400	7.8	858	0.19	92	1.2
3. $(NH_4)_2\ SO_4$	74	35	2590	9.9	732	0.28	79	1.75
4. $Al(OH)_3$	82	30	2460	10.1	825	0.34	89	2.1
5. SP-Sephadex (pH 4-6)	85	24	2040	7.9	672	0.33	72	2.1
6. CM-Cellulose I	78	3.9	304	3	234	0.77	25	4.8
7. CM-Cellulose II(a)	120	4.5	540	1.8	216	0.4	23	2.5
8. CM-Cellulose I	61	0.77	47	43	262	5.6	28	35
9. G200I	14	0.62	8.7	8.8	123	14.2	13	89
10.CM-Cellulose II(b)	84	0.45	38	0.2	16.8	0.44	1.8	2.8
11.G200II	15	0.26	3.9	0.58	8.7	2.2	0.9	13.7
12.G200III	36	1.3	47	1.2	43	0.92	4.6	5.7
13.G200IV	12	1.0	12	1	12	1	1.3	6.2

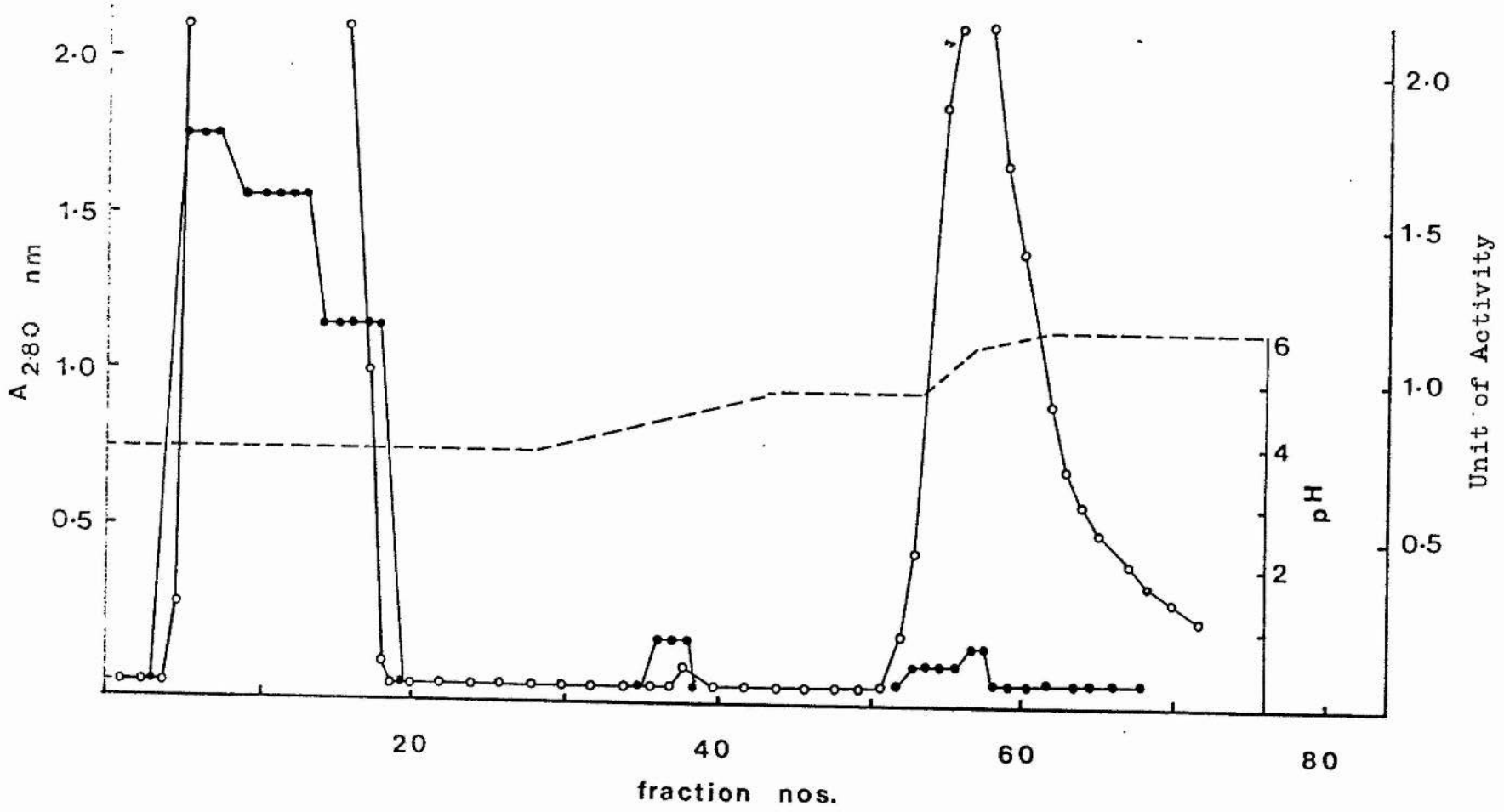
Fractions pool from Peak II of CM-Cellulose I was divided into two, and each half treated differently. For the purpose of this Table of purification results were multiplied by two where appropriate.

Fig. 4.2 SP-SEPHADEX COLUMN CHROMATOGRAPHY OF PLASMA ENZYME (Step 5, Table 4.3)

o——o Absorbance 280 nm

•——• mg fibrin clot dissolved by 0.2 ml enzyme in 16 h.

- - - - pH of eluate


2·0
1·5
1·0
0·5
A 280 nm
6
4
2
pH
2·0
1·5
1·0
0·5
Unit of Activity
20
40
60
80
fraction nos.

As the enzyme did not bind to the column, and very low yield was achieved, the fractions from Peak I were pooled and applied to a CM-cellulose column.

4.6.2. CM-cellulose I

Unbound material from SP-Sephadex column (85 ml of fractions) was applied to the column as 4.2.2. Two peaks were obtained. Fig. 4.3. Some material did not bind to the column (Peak I), and material eluted at about pH 5.0 (Peak II). Both peaks had activity. Obtaining two peaks with activity could be due to the existence of two distinct enzymes, one of which was retarded at pH 4.0, or to the fact that the Peak I was caused by over-loading the column. To solve the problem, fractions from Peak I were pooled and applied to another CM-cellulose column, under the same conditions.

4.6.3. CM-cellulose II(a)

78 ml of pool fractions from Peak I of CM-cellulose I was applied to the column as 4.2.2. Two peaks were obtained, Fig. 4.4. The first peak was small and with low activity, and Peak II which was eluted between pH 5-6 had the highest activity. Therefore the Peak I of CM-cellulose I (4.6.2) was due to over-loading.

4.6.4. Sephadex G200 (I)

Fractions of Peak II of CM-cellulose II(a) (4.6.3) were pooled, dialysed against 0.02 M phosphate, pH 6.8, and freeze dried. The sample was then dissolved in

Fig. 4.3 CM-CELLULOSE (I) COLUMN CHROMATOGRAPHY OF PLASMA ENZYME (Step 6, Table 4.3)

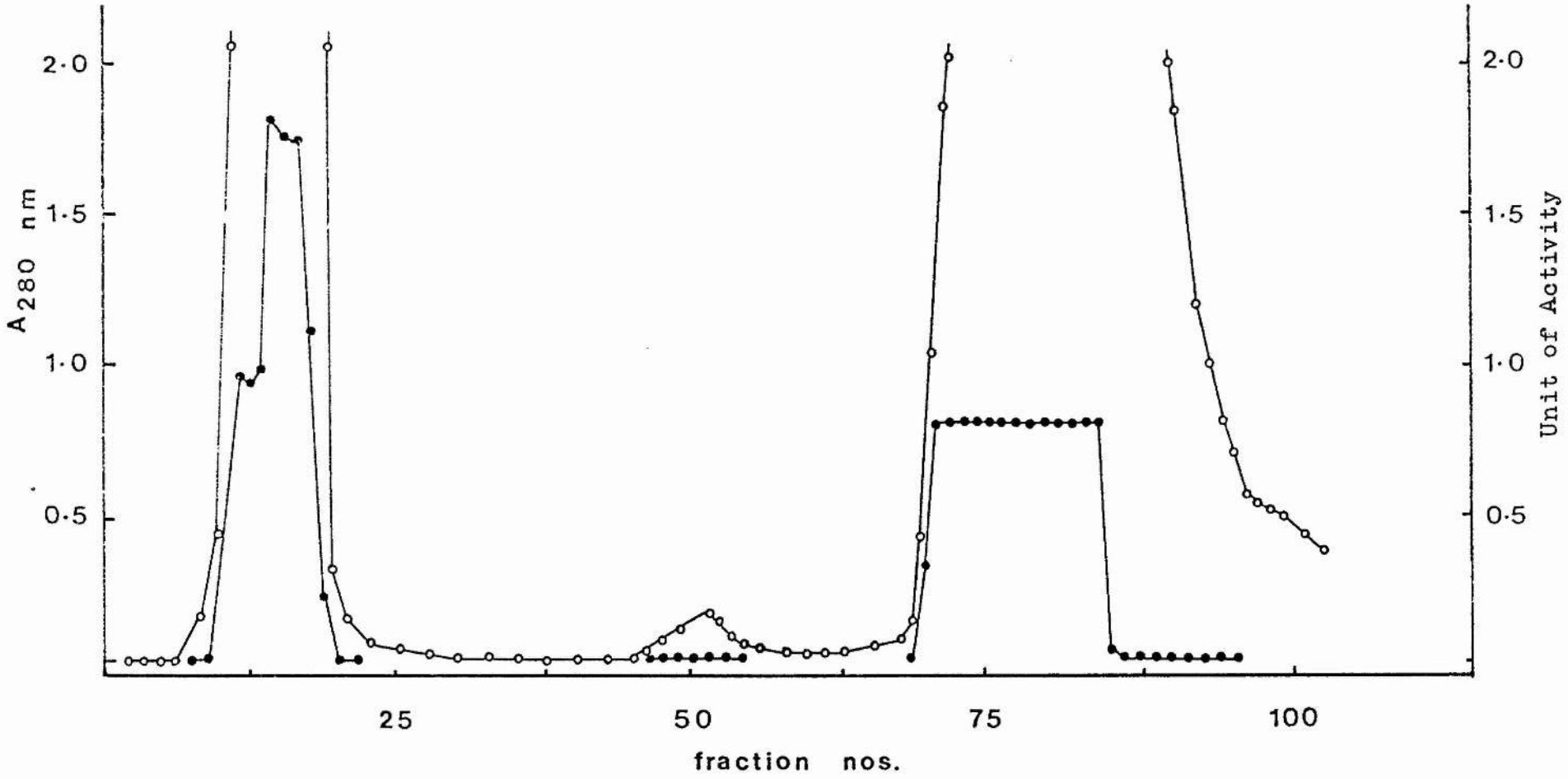
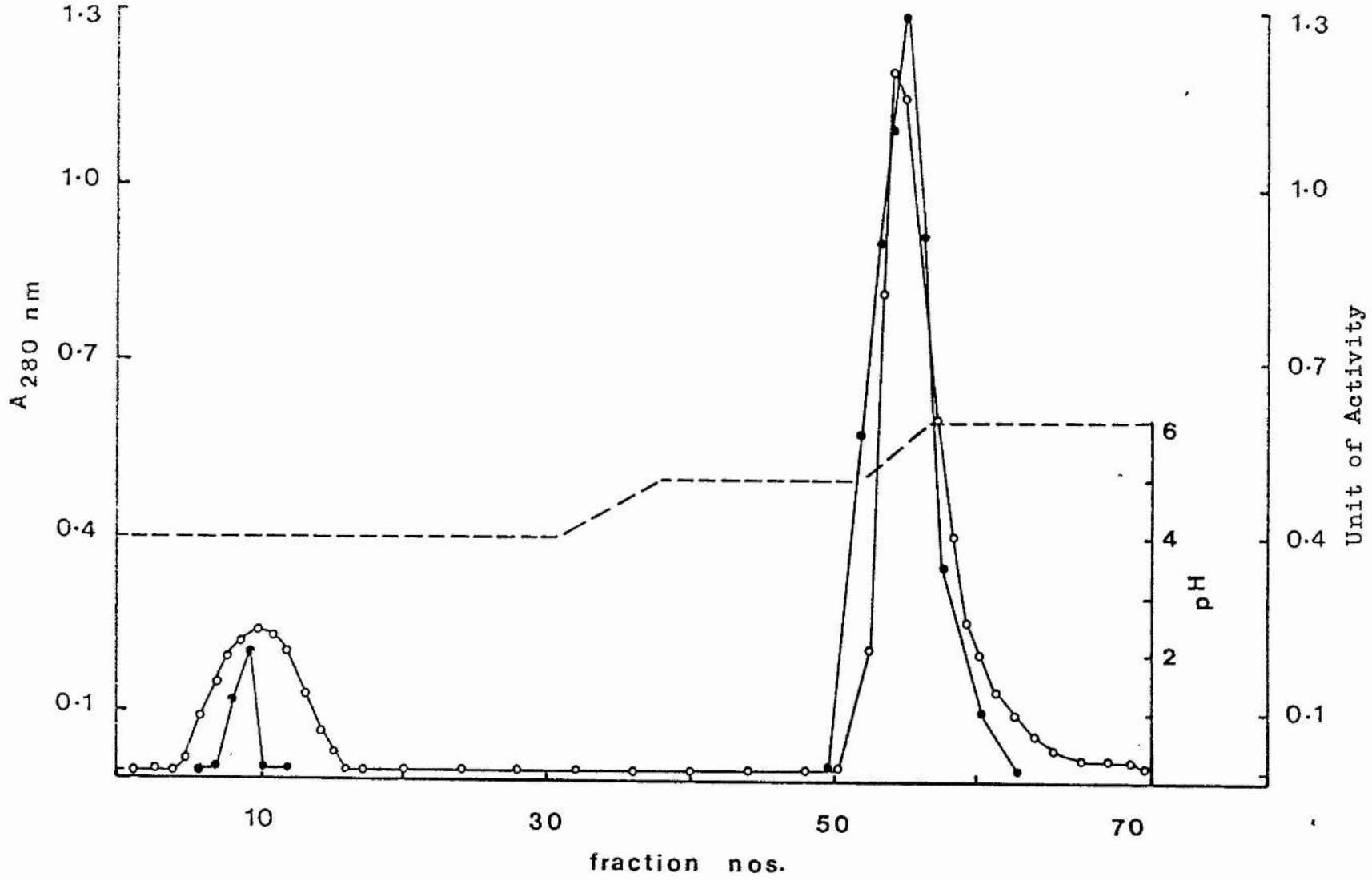

2·0
1·5
1·0
0·5
A_{280} nm
2·0
1·5
1·0
0·5
Unit of Activity
25
50
75
100
fraction nos.

Fig. 4.4 CM-CELLULOSE (IIa) COLUMN CHROMATOGRAPHY OF PLASMA ENZYME (Step 7, Table 4.3)

o——o Absorbance 280 nm

•——• mg fibrin clot dissolved by 0.2 ml enzyme in 16 h.

----- pH of eluate


1·3
1·0
0·7
0·4
0·1
A 280 nm
6
4
2
pH
1·3
1·0
0·7
0·4
0·1
Unit of Activity
10
30
50
70
fraction nos.

5.0 ml of distilled water and applied to the column as 4.3.1. Fractions 8 and 9 had activity, but the yield was poor (Table 4.3). SDS gel electrophoresis of an aliquot of each fraction showed four distinct protein bands with apparent molecular weights of 54,000, 92,000 and two bands with molecular weights of higher than 120,000, Fig. 4.6.

Immunoelectrophoresis of fractions 8 and 9 against antihuman antiserum showed one and three precipitation lines respectively. Both showed a precipitation line in a position similar to that expected for albumin. However, neither of the two fractions gave precipitation line with antialbumin antiserum. Both fractions showed a faint line with anti- α_2-macroglobulin antiserum.

4.6.5. CM-cellulose (IIb)

Fractions 67-90 from Peak II of CM-cellulose I, Fig. 4.3, were pooled and half of the sample (60 ml) was dialysed against 0.05 M sodium acetate, pH 4.0 and applied to the column as 4.2.2. The column was eluted with a stepwise salt gradient, Fig. 4.7. Four peaks were obtained. Fractions under each peak were pooled, desalted and freeze dried. The highest activity was detected in the sample from Peak II, Table 4.3. SDS gel electrophoresis on Peak II pools showed two major bands with molecular weights of 55,000 and 90,000 and one minor band with molecular weight of $>100,000$, Fig. 4.8.

Fig. 4.5 SEPHADEX G200(I) COLUMN CHROMATOGRAPHY OF PLASMA ENZYME (Step 9, Table 4.3)

o——o Absorbance at 280 nm

•——• mg fibrin clot dissolved by 0.2 ml enzyme in 16 h.

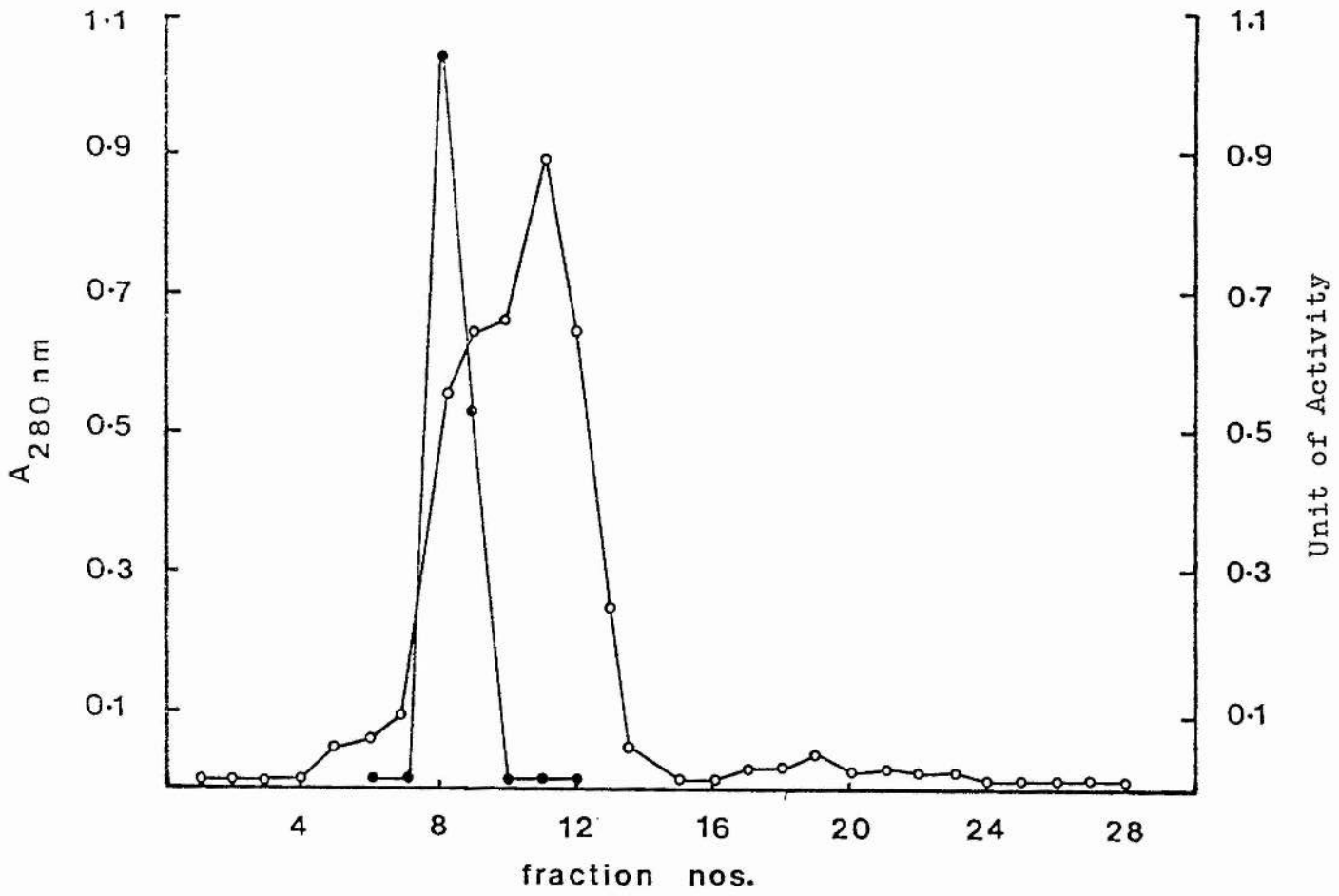

1·1
0·9
0·7
0·5
0·3
0·1
$A_{280\,nm}$
Unit of Activity
4
8
12
16
20
24
28
fraction nos.

Fig. 4.6 DENSITOMETRIC SCAN OF AN ALIQUOT FROM FRACTION NO. 8 OF SEPHADEX G200(I) COLUMN.

Estimated molecular weights are 92,000 Peak 3, 54,000 Peak 4.

1
1
2
3
4
+

Fig. 4.7 CM-CELLULOSE (IIb) COLUMN CHROMATOGRAPHY OF PLASMA ENZYME (Step 10, Table 4.3)

o——o Absorbance at 280 nm

•——• mg fibrin clot dissolved by 0.2 ml enzyme in 16 h.

I 0.05 M Na Acetate/0.1 M NaCl, pH 4.0 applied

II 0.05 M Na Acetate/0.2 M NaCl, pH 4.0 applied

III 0.05 M Na Acetate/0.3 M NaCl pH 4.0 applied

IV 0.05 M Na Acetate/0.4 M NaCl pH 4.0 applied

V 0.05 M Na Acetate/0.5 M NaCl, pH 4.0 applied

VI 0.05 M Na Acetate, 1.0 M NaCl, pH 4.0 applied

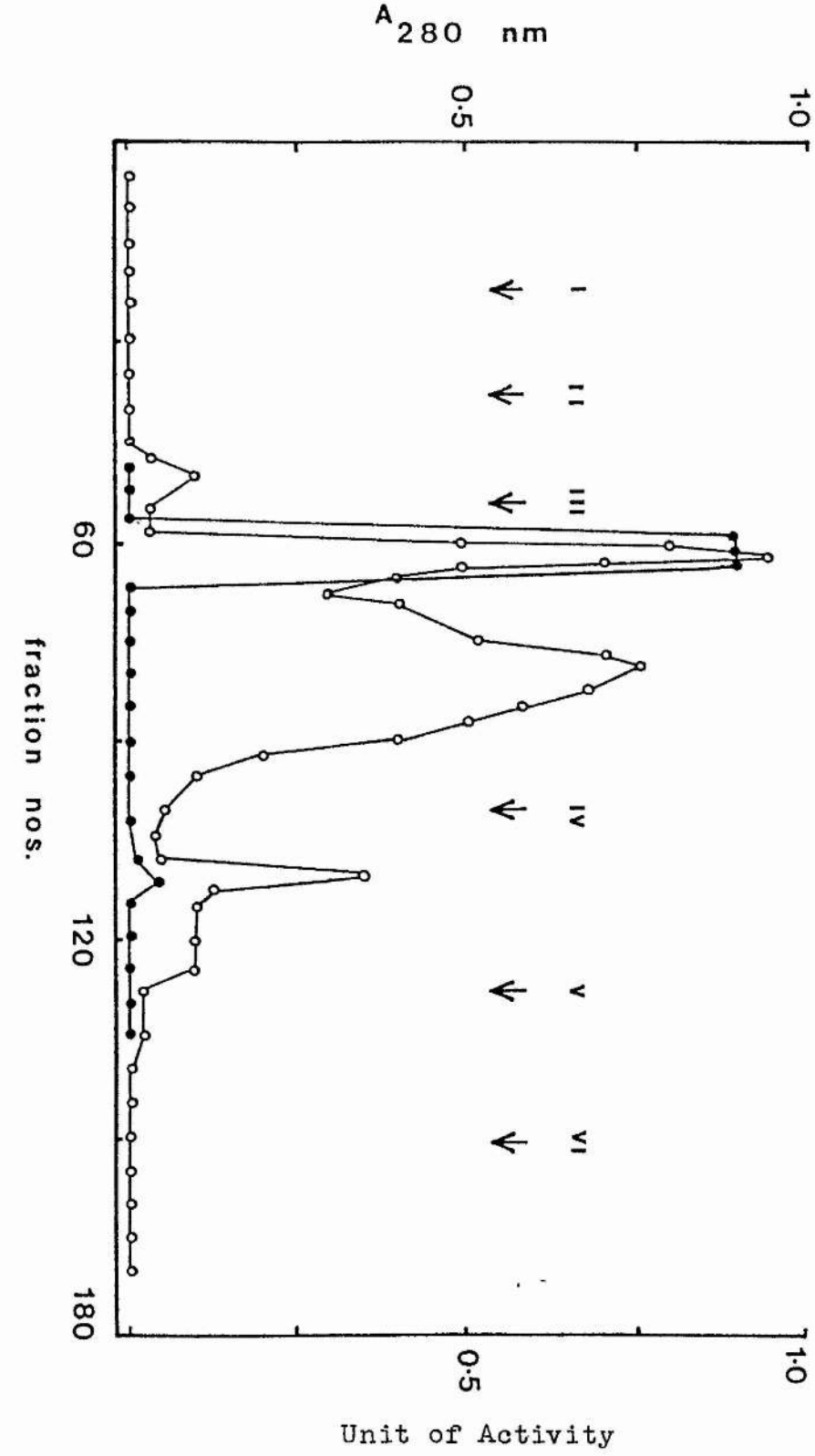
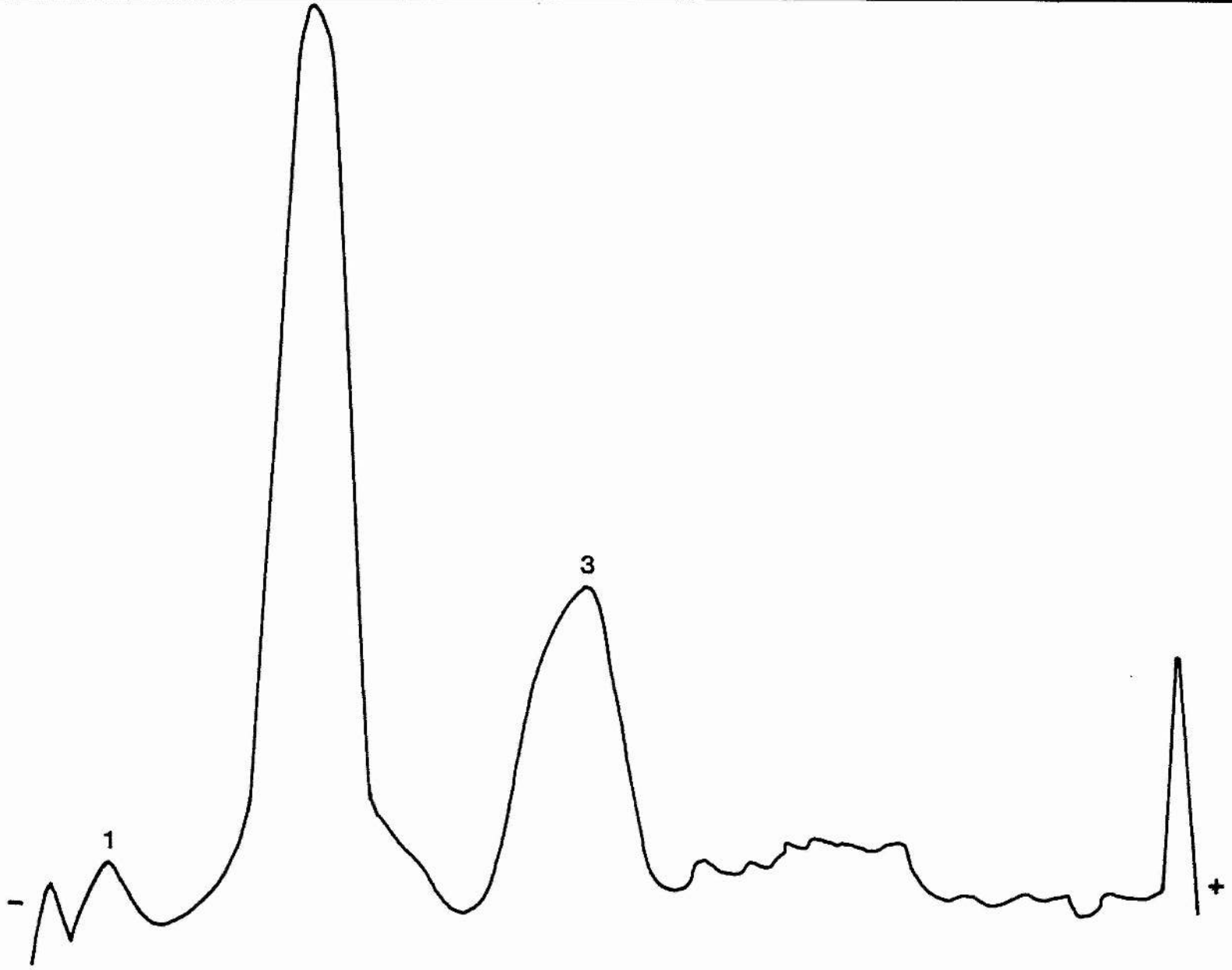

A_{280} nm
0·5
1·0
I
II
III
IV
V
VI
60
120
180
fraction nos.
0·5
1·0
Unit of Activity

Fig. 4.8 DENSITOMETRIC SCAN OF AN ALIQUOT FROM POOLS OF FRACTIONS OF PEAK II OF CM-CELLULOSE (IIb)

Estimated molecular weights are 100,000 Peak 1, 90,000 Peak 2, and 55,000 Peak 3


1
3
-
+

4.6.6. Sephadex G200(11)

Fractions from peak 11 of CM-cellulose 11(b) , 4.6.5 were pooled (42 ml) and dialysed aginst 0.05 M sodium acetate, pH 5.5, and freeze dried. The sample was dissolved in 5 ml distilled water, and applied to the Sephadex-G200 column as 4.3.1. Two peaks were obtained (Fig. 4.9). Fractions 16-18 had activity, Table 4.3.

4.6.7. Sephadex G200(111)

Half of the pool of peak 11 of CM-cellulose 1 (Fig. 4.3) was dialysed aginst 0.05 M sodium acetate, pH 5.5 and freeze dried. The sample was dissolved in 5 ml distilled water and applied to the Sephadex column as 4.3.1. One peak was obtained (Fig.4.10). Fractions with activity were pooled and an aliquot was subjected to SDS gel electrophoresis, which showed at least seven protein bands. Therefore the sample was applied to another Sephadex G200 column.

4.6.8. Sephadex G200(1V)

Fractions pool of Sephadex G200(111) (4.6.7.) was freeze dried, dissolved in 2 ml of distilled water, and applied to another Sephadex G200 column as 4.3.1 (Fig 4.11)

Specific activity:-Table 4.3. SDS gel electrophoresis of an aliquot from fraction No. 12 G200 :- Fig.4.12.

4.7. Plasma Enzyme Purification (second batch)

A flow chart of purification steps is given in Fig.4.13.

The purification steps were similar to the first batch, but with the following minor changes.
In the first batch material did not bind to the SP-Sephadex column at pH 4.0 (Fig.4.2), therefore a larger column

Fig. 4.9 SEPHADEX G200(II) COLUMN CHROMATOGRAPHY OF PLASMA ENZYME (Step 11, Table 4.3)

o——o Absorbance at 280 nm

•——• mg fibrin clot dissolved by 0.2 ml enzyme in 16 h

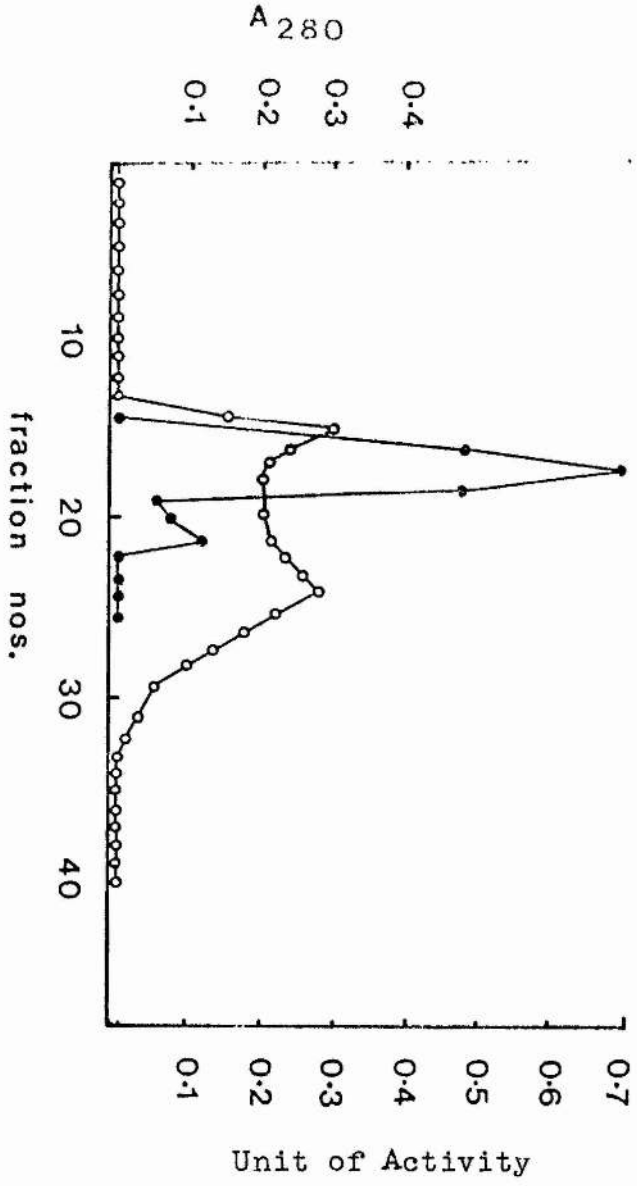

A 280
0·1
0·2
0·3
0·4
10
20
30
40
fraction nos.
0·1
0·2
0·3
0·4
0·5
0·6
0·7
Unit of Activity

Fig. 4.10 SEPHADEX-G200(III) COLUMN CHROMATOGRAPHY OF PLASMA ENZYME (Step 12, Table 4.3)

o——o Absorbance at 280 nm

•——• mg fibrin clot dissolved by 0.2 ml enzyme in 16 h.

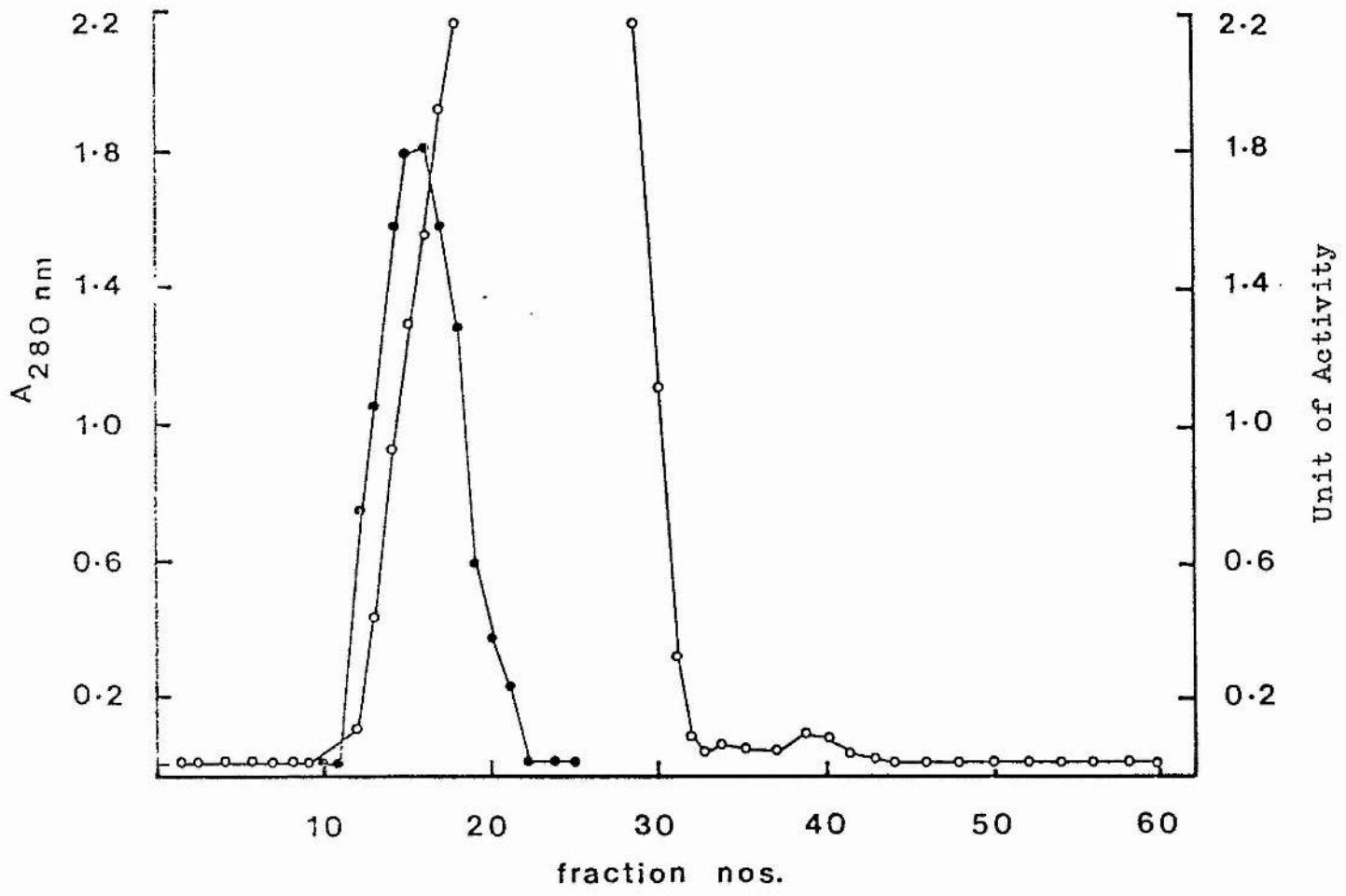
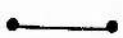

2·2
1·8
1·4
1·0
0·6
0·2
$A_{280\ nm}$
Unit of Activity
10
20
30
40
50
60
fraction nos.

Fig. 4.11 SEPHADEX G200(IV) COLUMN CHROMATOGRAPHY OF PLASMA ENZYME (Step 13, Table 4.3)

Absorbance at 280 nm



mg fibrin clot dissolved by 0.2 ml enzyme in 16 h.

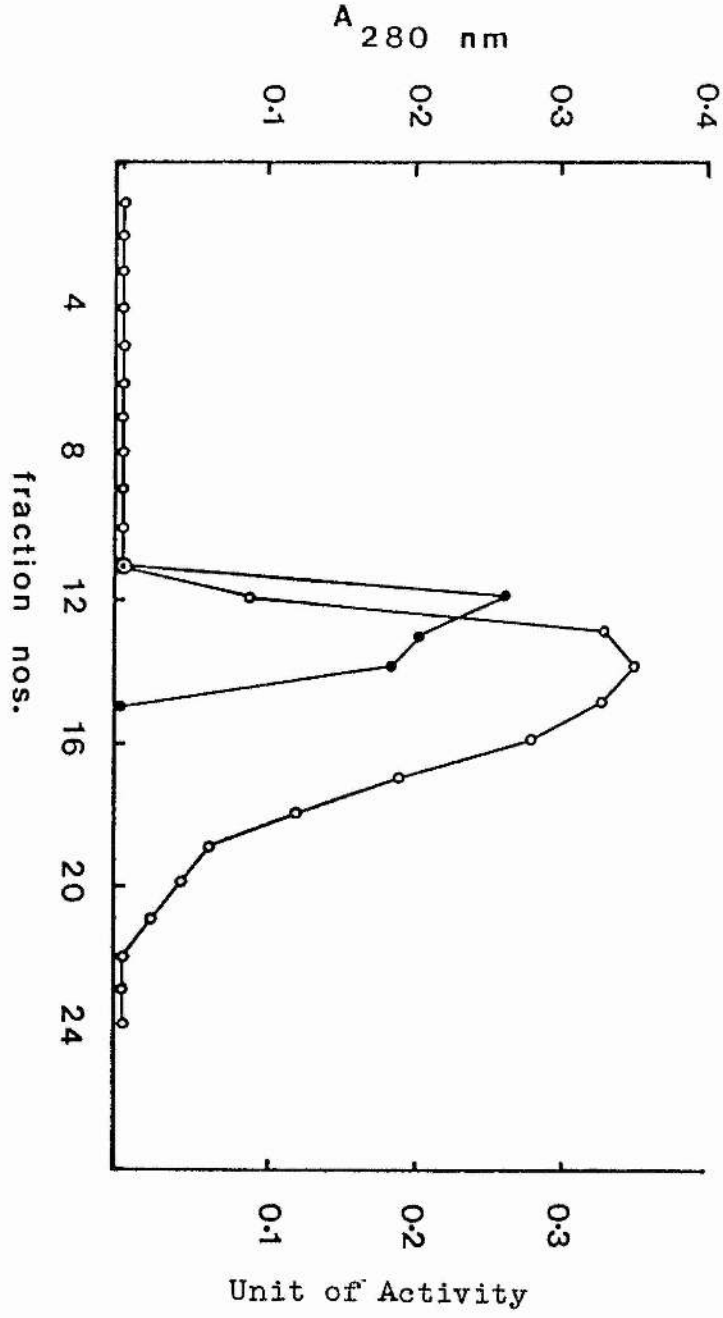
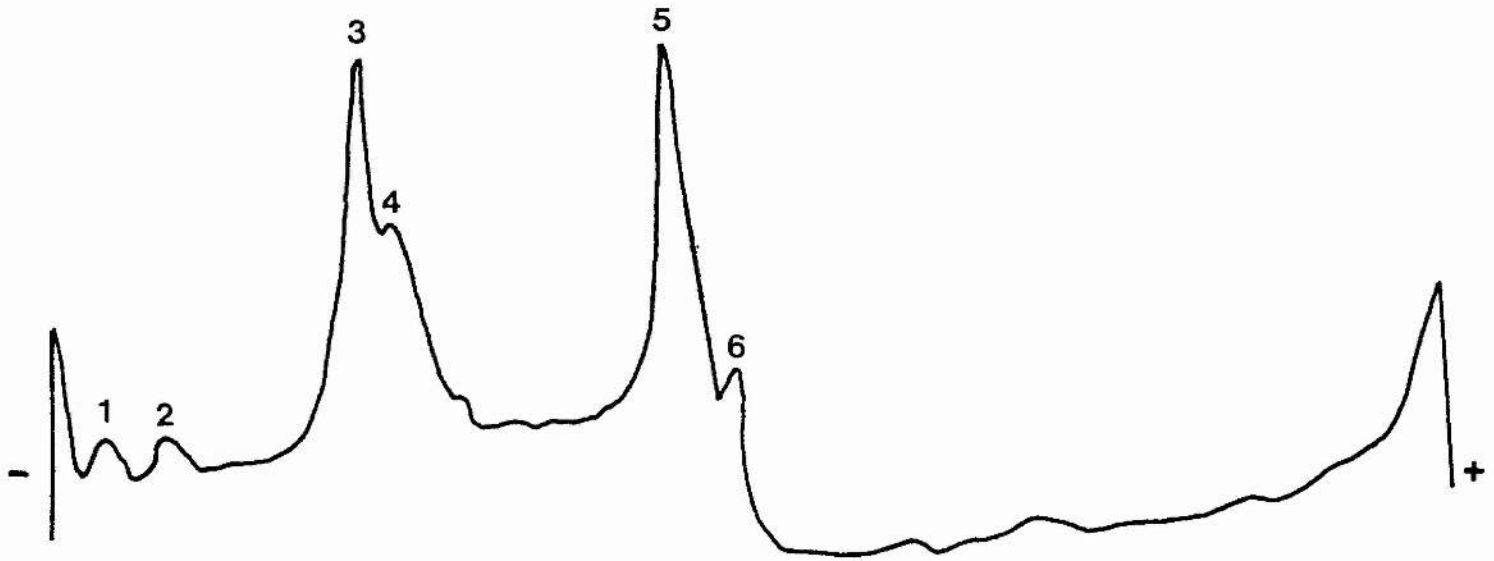

A 280 nm
0·1
0·2
0·3
0·4
4
8
12
16
20
24
fraction nos.
0·1
0·2
0·3
Unit of Activity

Fig. 4.12 DENSITOMETRIC SCAN OF AN ALIQUOT FROM FRACTION NO. 12 G200(IV) COLUMN

Estimated molecular weights are

Peak 3	90,000
Peak 5	52,000


3
5
4
6
1
2
-
+

(40 cm x 1.8 cm) was used to reduce the possibility of over-loading. With the new column, two peaks were obtained, Fig. 4.14. Most of the activity was in Peak I which was eluted at pH 4.0 without being bound to the column. The second peak with a lower activity was eluted between pH 5.3-5.8, Fig. 4.14, with the highest activity at pH 5.5. The fractions with activity from Peak I were pooled and applied to a CM-cellulose column (40 cm x 1.8 cm), (larger than previous batch). This time most of the material was bound to the column and was eluted at pH 5-6 (Fig. 4.15). However, a small amount of the material did not bind to the column (Peak I, Fig. 4.15). The fractions under this peak were pooled and applied to another CM-cellulose column. All the material was bound to the column, and fractions eluted at pH 5.0 were active (Fig. 4.16), with a better specific activity than in the last batch. However, the yield was rather poor, Table 4.4. Fractions from the second CM-cellulose were pooled, freeze dried and applied to a Sephadex G200 column. Fractions collected from the Sephadex G200 column, (Fig. 4.17) had low activity, even after concentration by freeze drying or pressure filtration (Table 4.4).

Comment

Although a larger column of SP-Sephadex was used in this batch of purification, still most of the material did not bind to the column. Either the column was still over-loaded or SP-Sephadex is not a suitable resin for the purification of plasma enzyme.

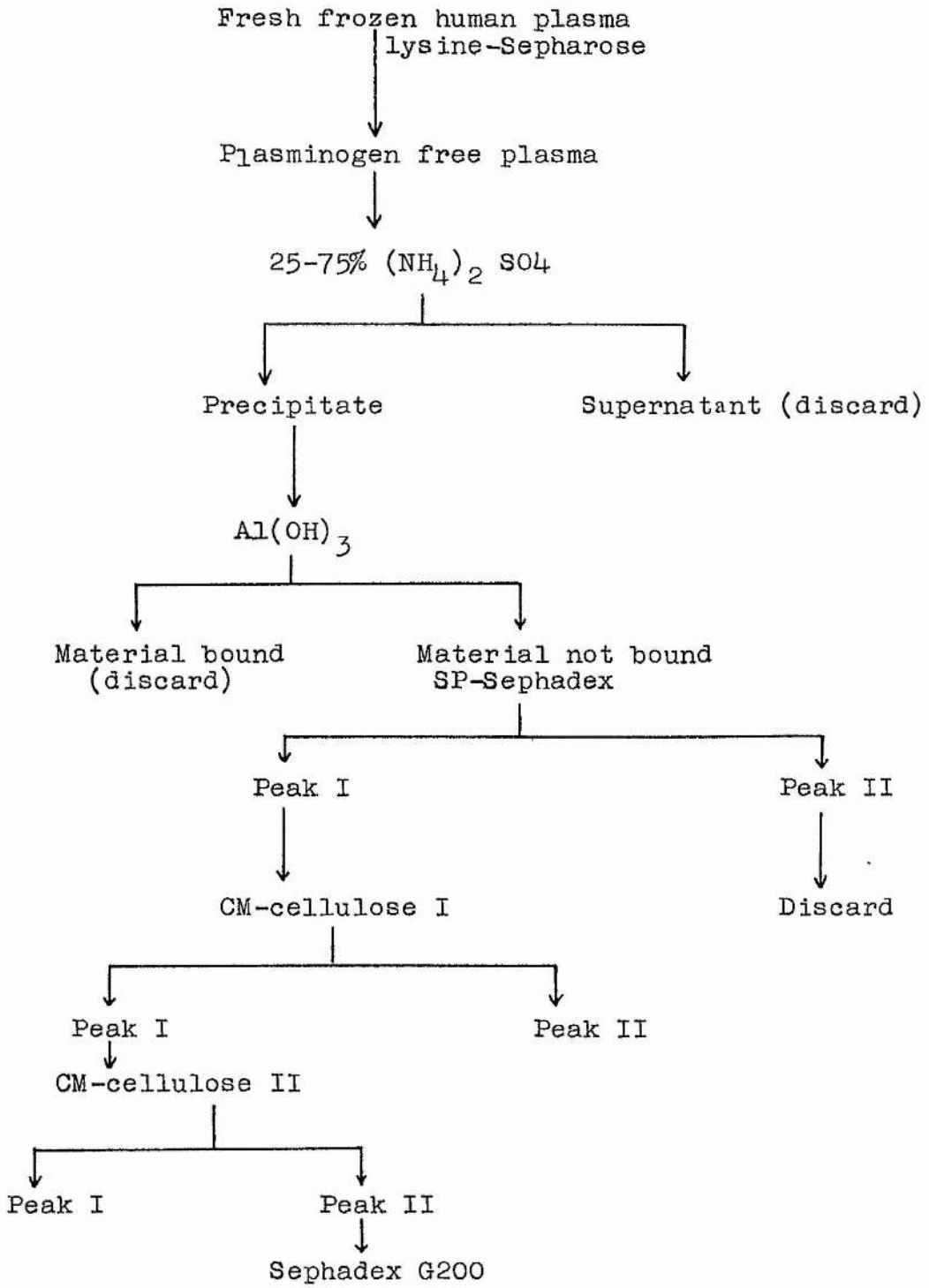


Fig. 4.13 FLOW CHART FOR THE PLASMA ENZYME PURIFICATION (Second batch).

Table 4.4 Purification of plasma enzyme (second batch)

Procedure	Volume (ml)	Protein conc. "mg/ml" $A_{280\ nm}$	Total Protein (mg)	Enzyme Activity (Units/ml)	Enzyme Activity Total Units	S.A Units/ml	Yield %	Purification Factor (Fold)
1. Normal plasma	96	63	6048	10.2	972	0.16	100	1.0
2. Plasminogen free plasma	110	50	5500	10.0	1100	0.21	113	1.3
3. $(NH_4)_2\ SO_4$	90	35	3150	11	910	0.3	101	1.87
4. $Al(OH)_3$	123	22.6	2780	7.5	922	0.33	95	2.1
5. SP-Sephadex	140	13.65	1911	5.0	700	0.37	72	2.3
6. CM-Cellulose I	40	11.8	472	10.5	420	0.89	43	5.6
7. CM-Cellulose II	40	0.1	4	5	200	50	20	312
8. G200	8	0.05	0.4	0.55	4.4	11	4.5	69

Fig. 4.14 SP-SEPHADEX COLUMN CHROMATOGRAPHY OF PLASMA ENZYME

o——o Absorbance at 280 nm

•——• mg fibrin clot dissolved by 0.2 ml enzyme in 16 h.

- - - - pH of eluate

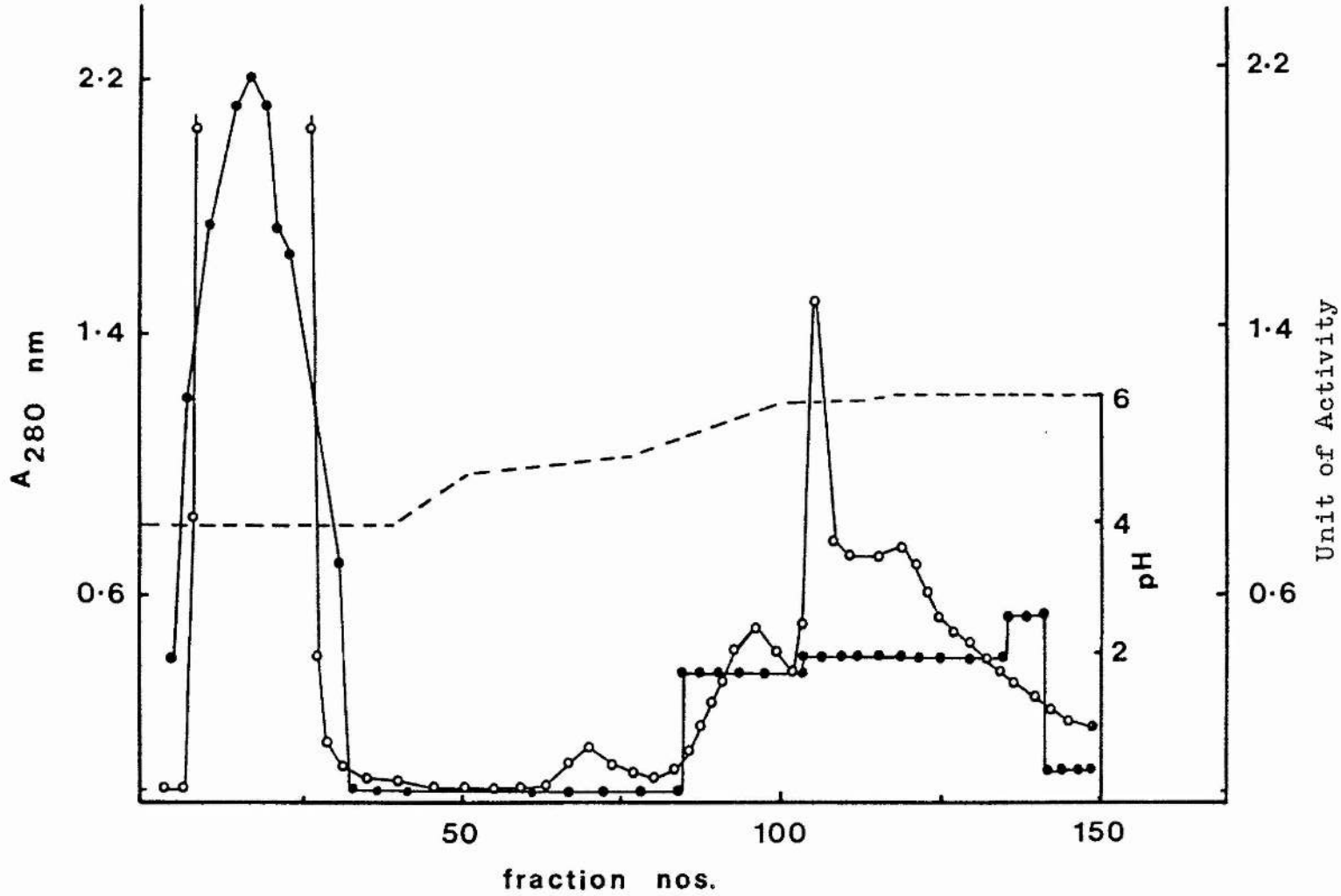

2·2
1·4
0·6
A_{280} nm
6
4
2
pH
2·2
1·4
0·6
Unit of Activity
50
100
150
fraction nos.

Fig. 4.15 CM-CELLULOSE (I) COLUMN CHROMATOGRAPHY OF PLASMA ENZYME

o——o Absorbance at 280 nm

•——• mg fibrin clot dissolved by 0.2 ml enzyme in 16 h.

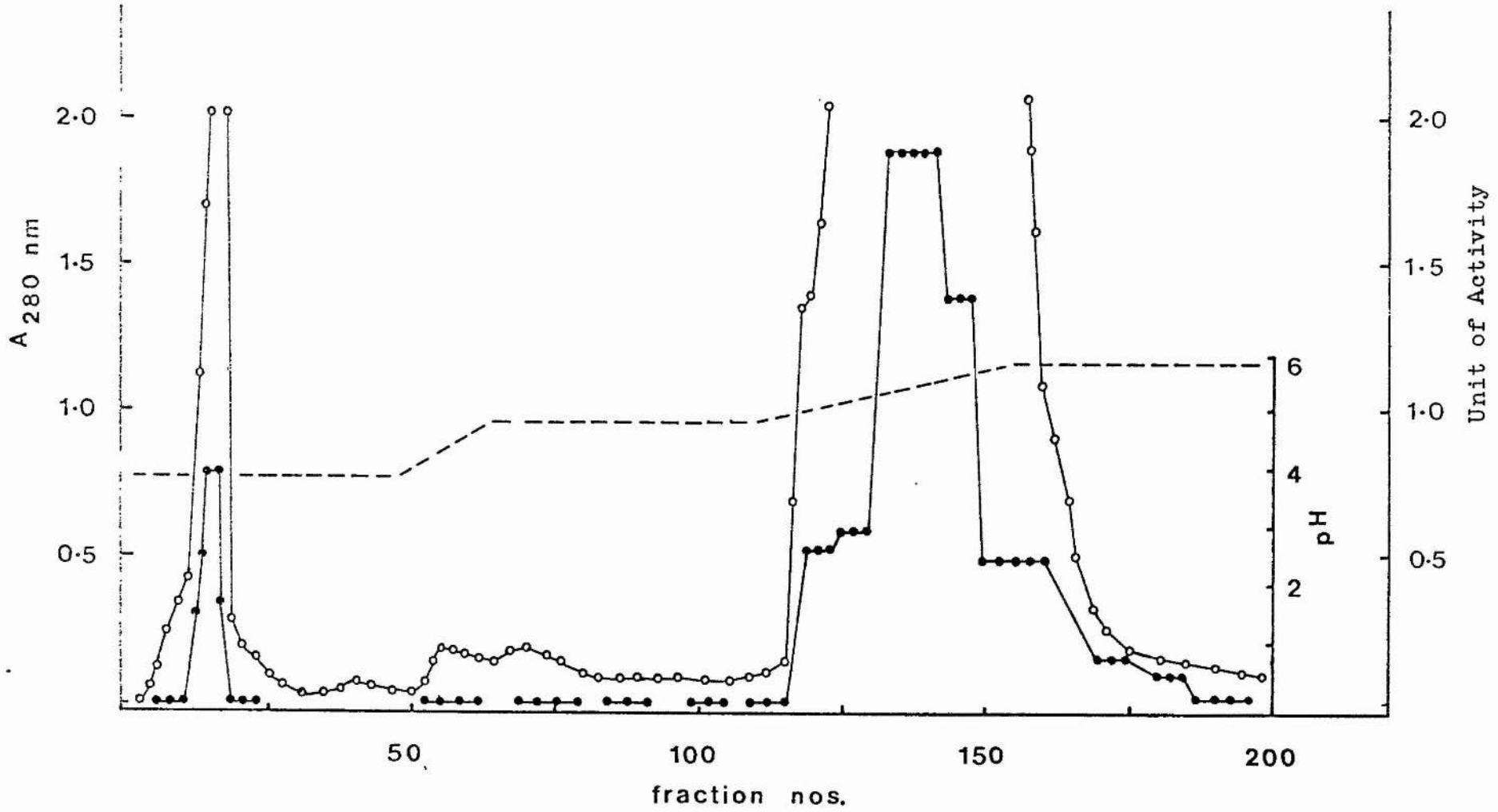

2·0
1·5
1·0
0·5
A 280 nm
6
4
2
pH
2·0
1·5
1·0
0·5
Unit of Activity
50
100
150
200
fraction nos.

Fig. 4.16 CM-CELLULOSE (II) COLUMN CHROMATOGRAPHY OF PLASMA ENZYME

o——o Absorbance at 280 nm

•——• mg fibrin clot dissolved by 0.2 ml enzyme in 16 h.

I 0.05 M Na citrate buffer, pH 5.0 applied

II 0.05 M Na citrate buffer, pH 6.0 applied

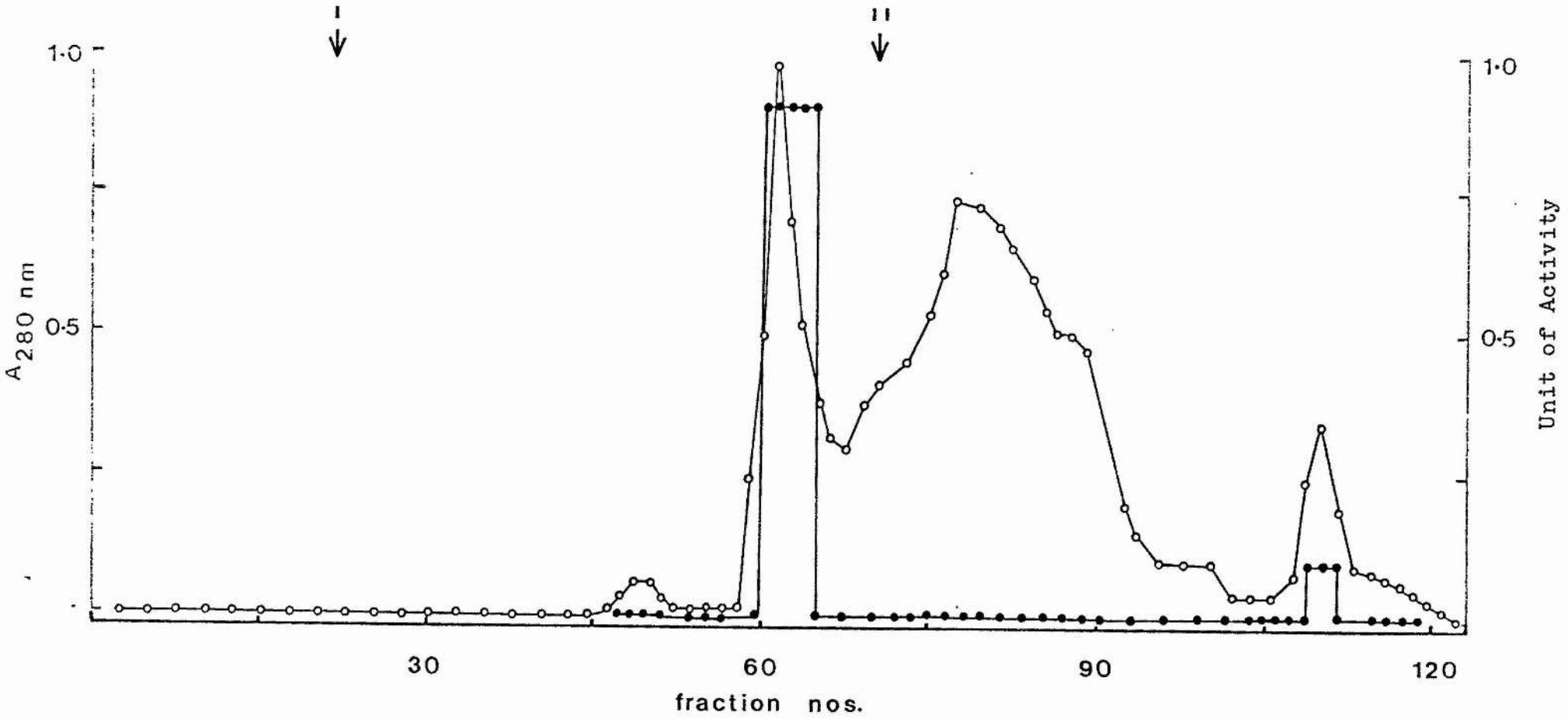
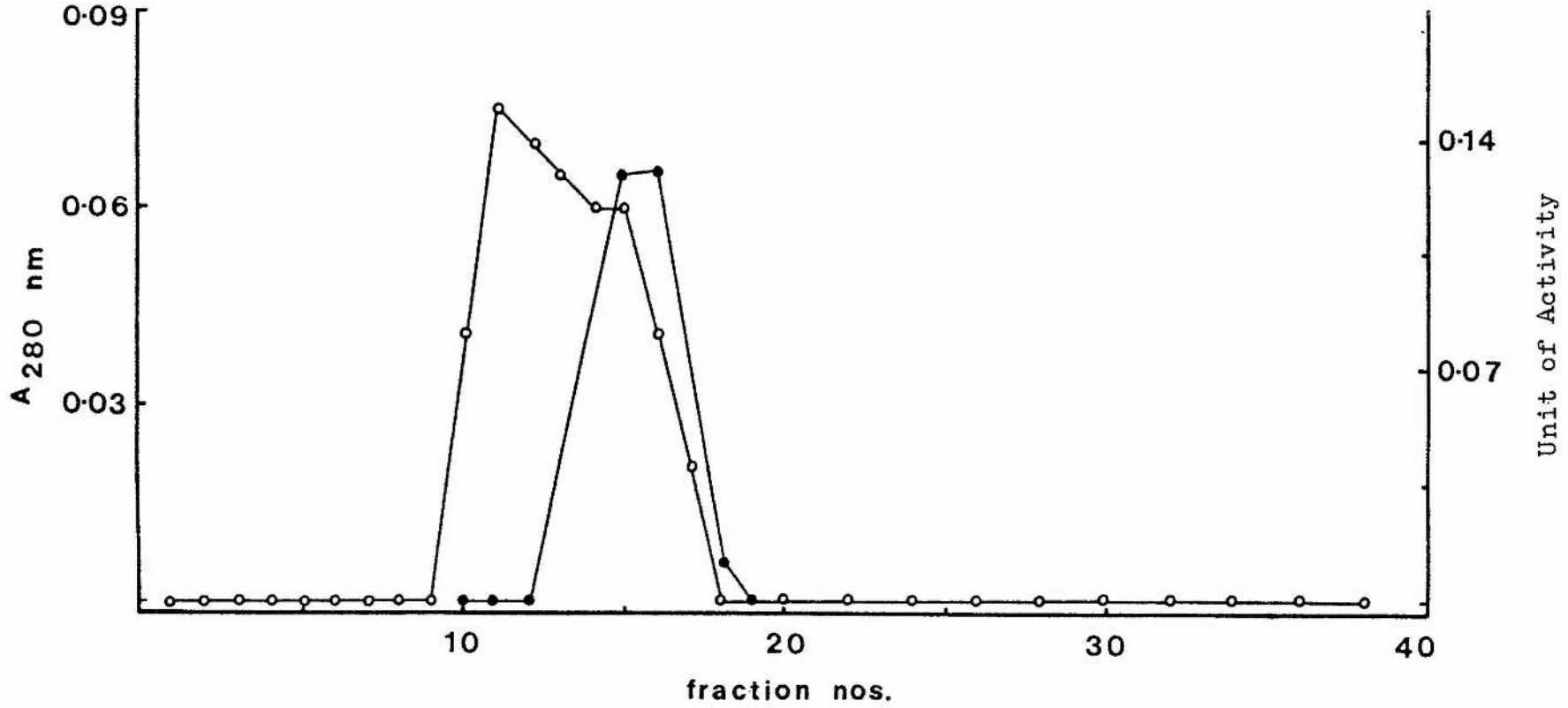

I
II
1·0
0·5
$A_{280\ nm}$
1·0
0·5
Unit of Activity
30
60
90
120
fraction nos.

Fig. 4.17 SEPHADEX-G200 COLUMN CHROMATOGRAPHY OF PLASMA ENZYME

o——o Absorbance at 280 nm

•——• mg fibrin clot dissolved by 0.2 ml enzyme in 16 h.


0·09
0·06
0·03
A 280 nm
0·14
0·07
Unit of Activity
10
20
30
40
fraction nos.

From these two batches of purification as well as other attempts in small batch purifications, it seems that the enzyme activity is lost at the stage of gel filtration. This suggests that the enzyme is more stable in the presence of other proteins.

Table 4.5

POLY(ETHYLENE GLYCOL) 6000 FRACTIONATION

Procedure	Volume (ml)	Enzyme activity (units/ml)	Total enzyme activity
Plasminogen free plasma	15	23	345
PEG 1st precipitate (5.5%)	2	2.85	5.7
PEG 2nd precipitate (5 - 12.5%)	2	4.5	9.0
PEG supernatant of 12.5% precipitate	50	7.0	350

Fig.4.18 FLOW CHART FOR THE PLASMA ENZYME (third batch)

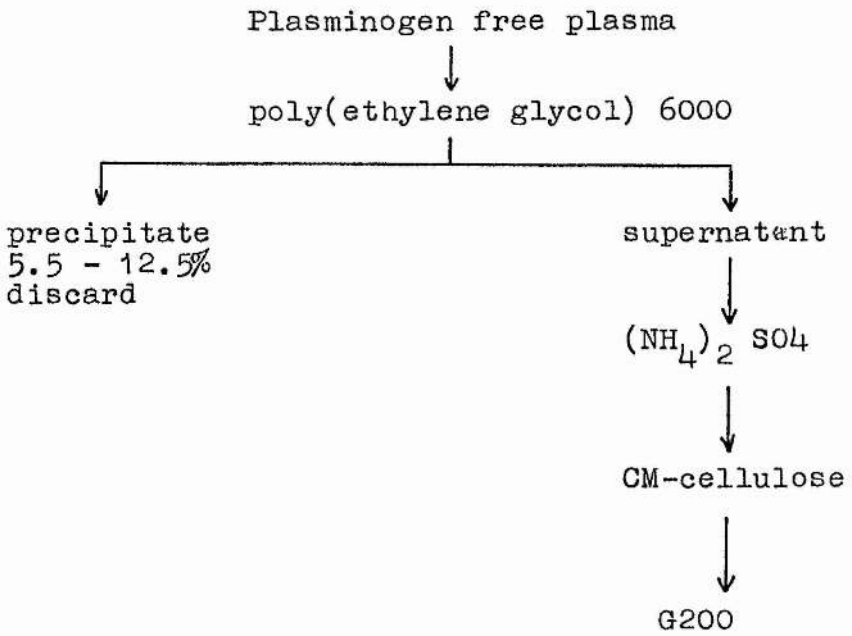
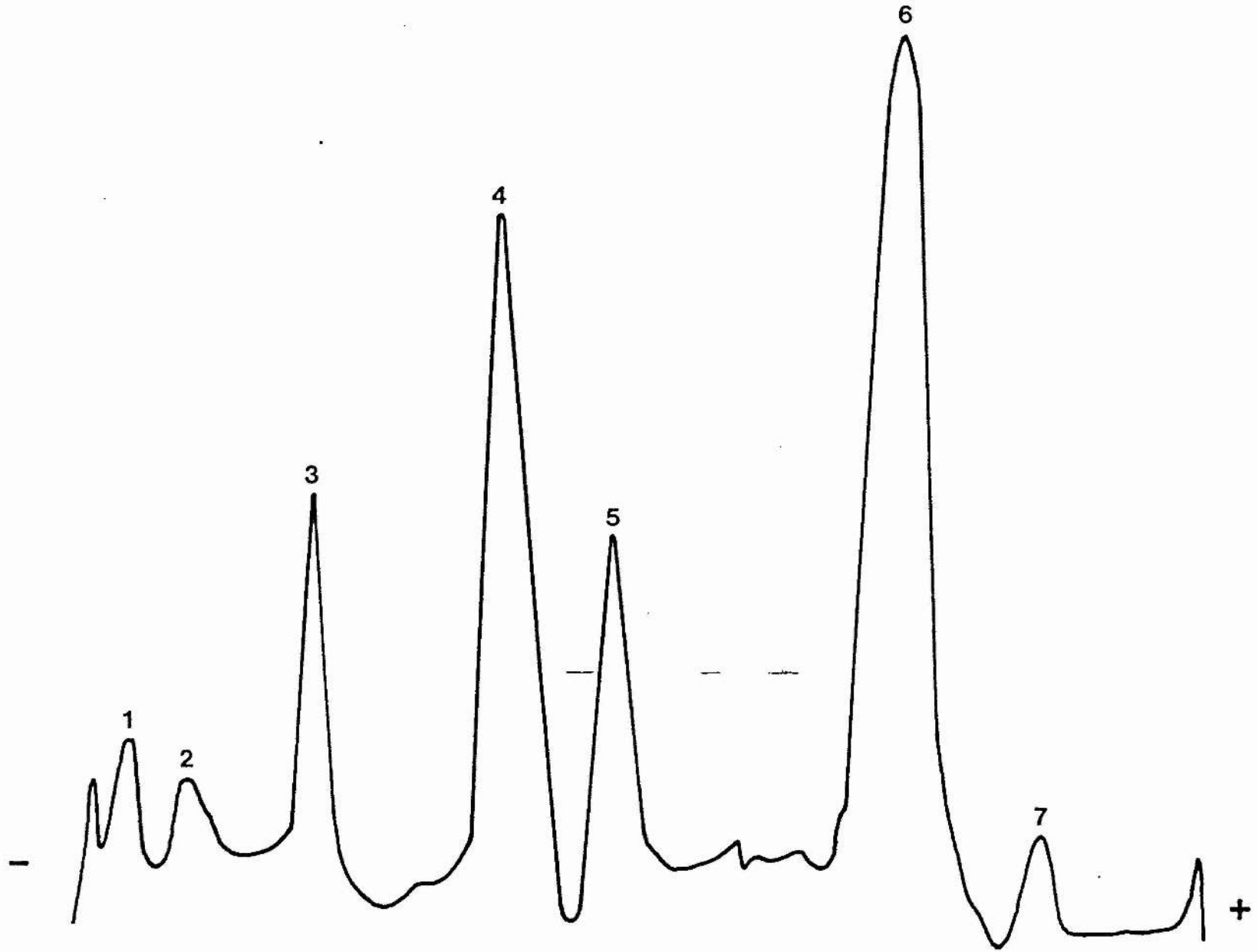


Fig. 4.19 DENSITOMETRIC SCAN OF AN ALIQUOT FROM THE SUPERNATENT AT 12.5% (w/v) POLY(ETHYLENE) GLYCOL 6000

Estimated molecular weights are:

Peak 3 approximately 90,000

Peak 4 approximately 53,000


1
2
3
4
5
6
7
–
+

4.8. Plasma Enzyme Purification (third batch)

4.8.1. Poly(ethylene glycol) 6000 (PEG)

In the last two batches of plasma enzyme purification, a very high molecular weight band was present which might be α_2-macroglobulin (α_2 M). A poly(ethylene glycol) precipitation has been used by Barrett et al., (1979), for α_2 M precipitation. This might prove a similar initial procedure in the purification of this enzyme.. Purification flow chart Fig. 4.18.

4.8.1.1. Procedure

15 ml plasminogen free plasma was added to poly (ethylene glycol) as 4.1.3, and the precipitate at 5.5% (w/v) PEG plus the supernatent and precipitate following adjustment of the 5.5% (w/v) PEG to 12.5% (w/v) PEG, were assayed for proteolytic activity, using the fibrin clot assay. More than 95% of the activity remained in the supernatent at 12.5% (w/v) PEG (Table 4.5). However, α_2 M is present in the precipitate at this concentration of PEG (Barrett et al., 1979).

SDS gel electrophoresis was performed on an aliquot from the active fraction, and as Fig. 4.19 shows, there are many contaminating protein bands.

For further purification, it was necessary to remove PEG. Because of its high molecular weight and structure, it was not possible to remove it by dialysis, gel filtration or pressure filtration.

Ammonium sulphate was used to precipitate proteins. With 70% saturation it was possible to separate proteins, but not as a precipitate. After centrifugation, the proteins "floated" on the top of the solution. However, there was not a very clearcut boundary between the two phases even after prolonged (4-5 h) centrifugation at 1200 g. The "floating" material was dissolved and dialysed against 0.05 M sodium acetate, pH 4.0. The "liquid" phase was also dialysed against the same buffer. Both fractions were assayed for proteolytic activity, using fibrin clot assay. The "floating" fraction had fibrinolytic activity, Table 4.6. This fraction was applied to CM-cellulose column.

4.8.2. CM-cellulose

30 ml of 70% AS, (floating material) 4.8. which had been dialysed aginst 0.05 M sodium acetate, pH 4.0, was applied to a CM-cellulose column which had been equilibrated with the above buffer. The material bound to the column and was eluted between pH 5.3 and 5.9 with the highest activity at pH5.5, Fig. 4.20. SDS gel electrophoresis of an aliquot from fraction 66 of CM-cellulose column, Fig. 4.21.

Fractions with activity were pooled and freeze-dried. The sample was dissolved in 2 ml of distilled water and applied to a Sephadex G200 column, Fig.22. No activity was recovered.

Table 4.6 PURIFICATION OF PLASMA ENZYME (third batch)

Procedure	Volume (ml)	Protein conc. mg/ml A_{280}	Total protein mg	Enzyme activity units/ml	Enzyme activity total units	S.A. units/ml	Yield %	Purification factor (fold)
Plasminogen-free plasma	7.5	50	375	23	172	0.46	100	1
12% (w/v) PEG	25	3.2	80	7.0	175	2.18	102	4.7
Ammonium sulphate 70%	30	1.2	36	3.9	117	3.25	68	7.1
CM-cellulose	38	0.6	22.8	3	22.8	5	13	10.9
Sephadex-G 200	40	0.08	3.2					

Fig. 4.20 CM-CELLULOSE COLUMN CHROMATOGRAPHY OF PLASMA ENZYME

o——o absorbance 280 nm

•——• mg fibrin clot dissolved in 0.2 ml enzyme in 16 h.

- - - - - pH of eluate

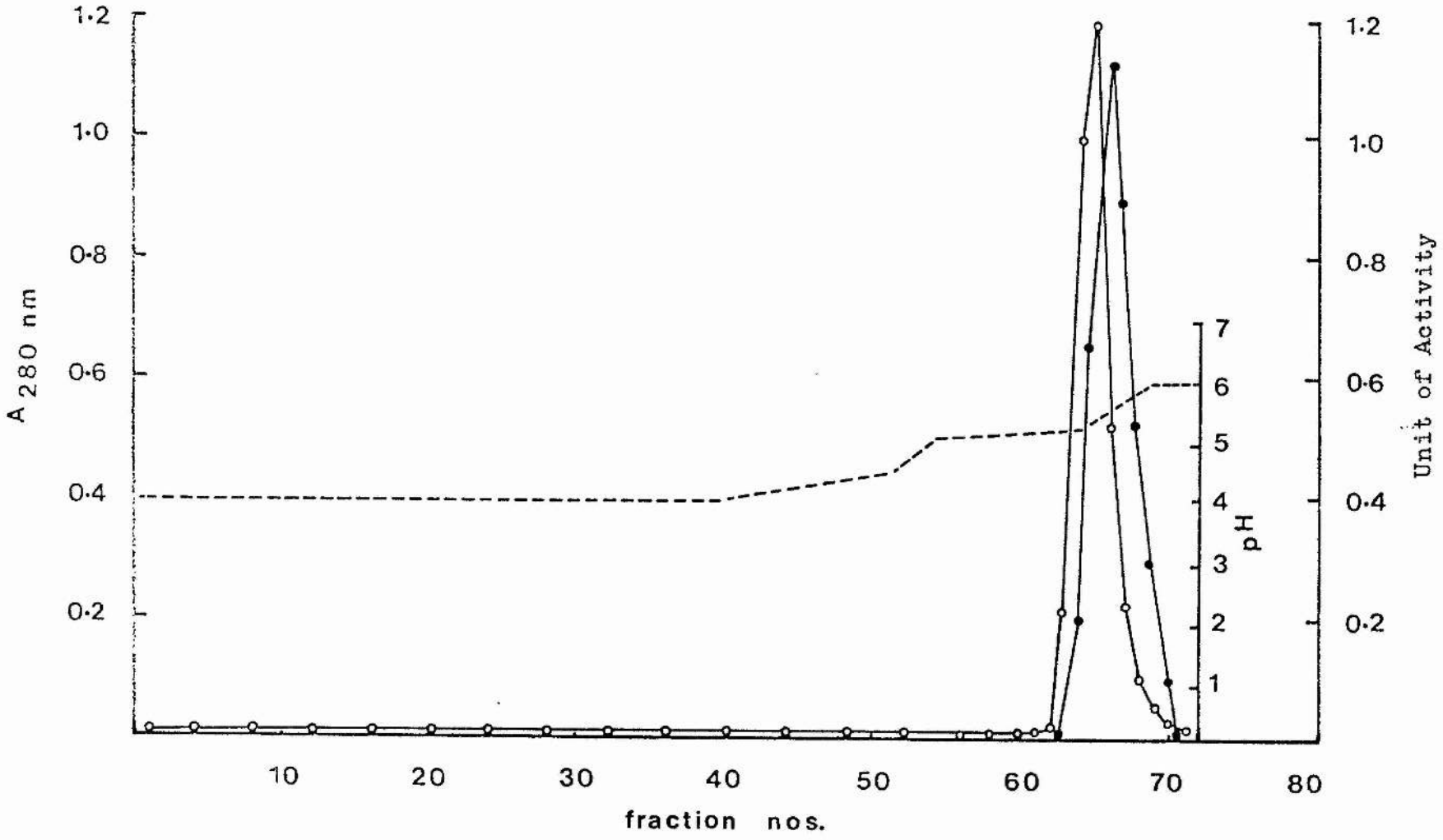

1·2
1·0
0·8
0·6
0·4
0·2
A 280 nm
7
6
5
4
3
2
1
pH
Unit of Activity
10
20
30
40
50
60
70
80
fraction nos.

Fig. 4.21 DENSITOMETRIC SCAN OF AN ALIQUOT FROM FRACTION 66 OF CM-CELLULOSE COLUMN

Estimated molecular weights are:

Peak 2 approximately 91,000

Peak 3 approximately 54,000

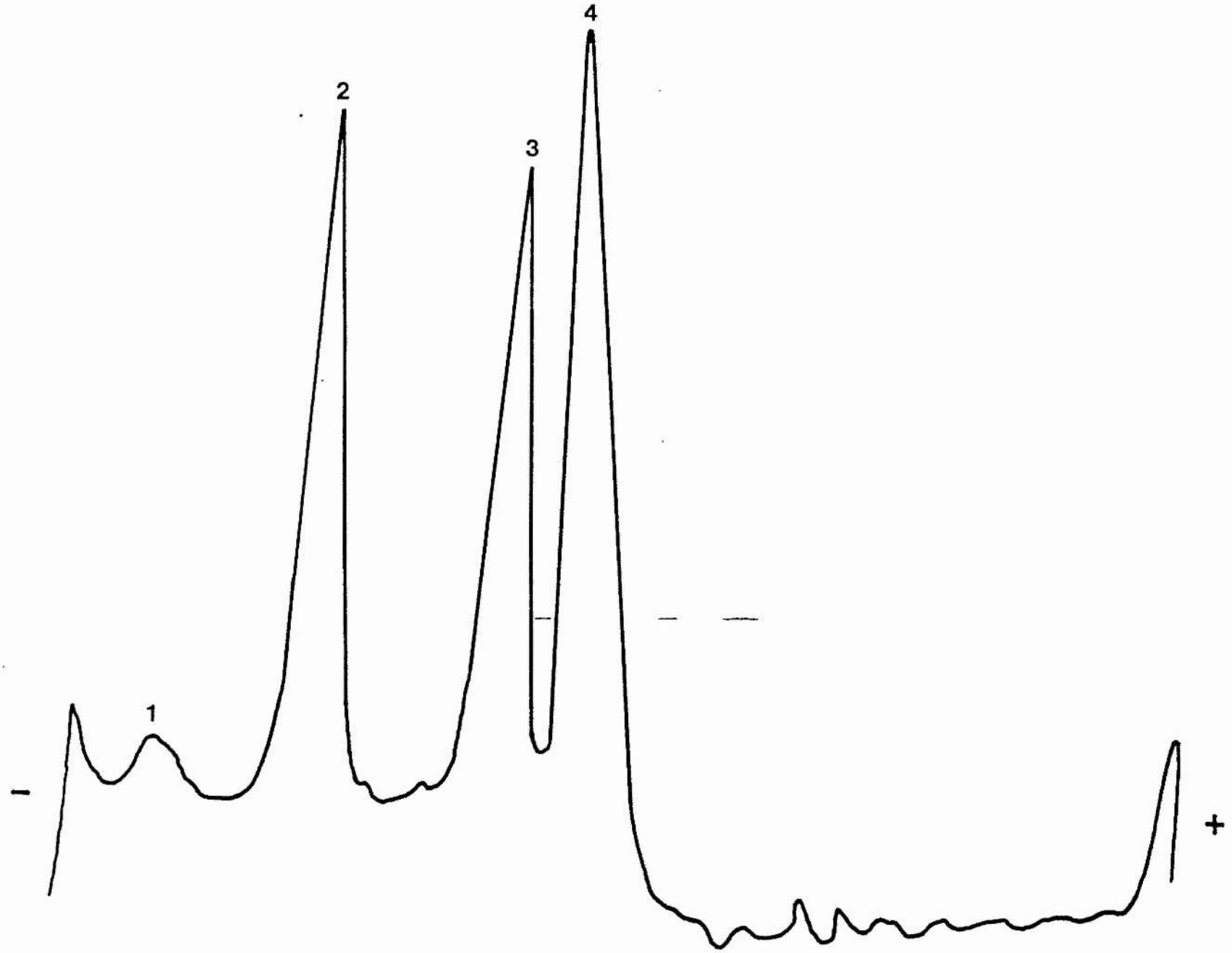
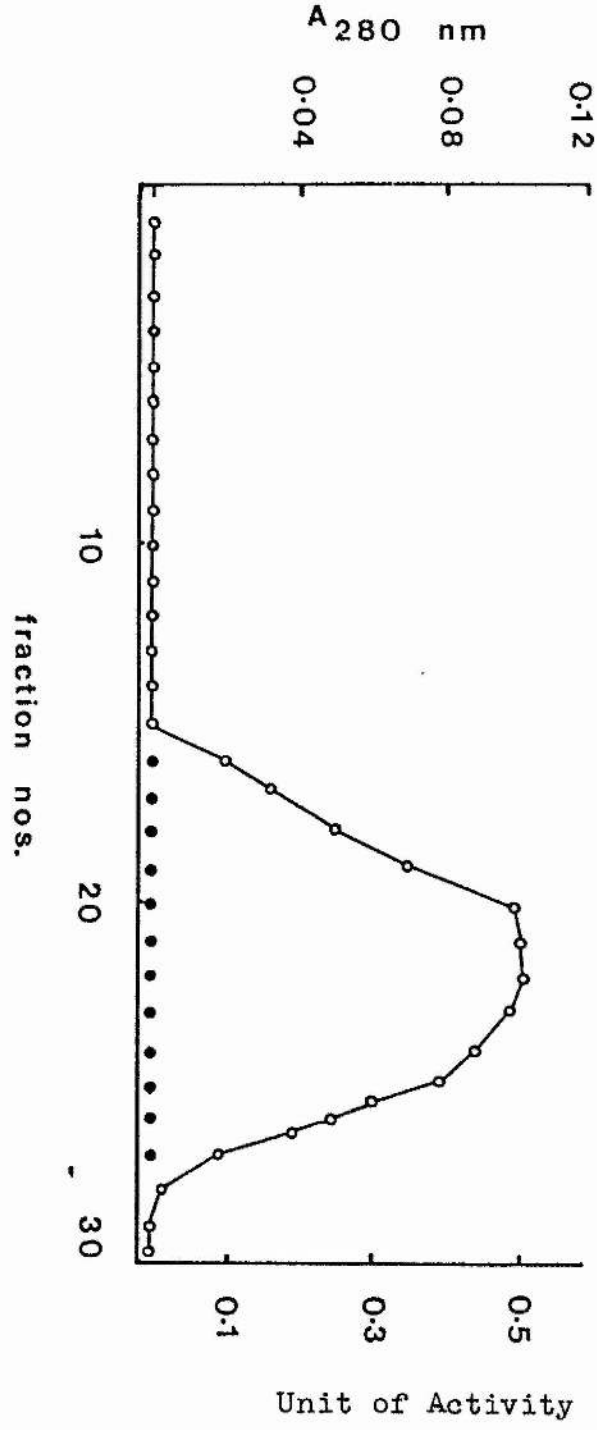

1
2
3
4
−
+

Fig. 4.22 SEPHADEX G 200 COLUMN CHROMATOGRAPHY OF PLASMA ENZYME

o——o absorbance at 280 nm

•——• mg fibrin clot dissolved by 0.2 ml enzyme in 16 h.


A_{280} nm
0·04
0·08
0·12
10
20
30
fraction nos.
0·1
0·3
0·5
Unit of Activity

4.9. Blue-Sepharose Column Chromatography

In the previous batch purification, an attempt was made to remove α_2 M. Although α_2 M was removed by poly (ethylene glycol) PEG a problem arose in the removal of the PEG. Blue-sepharose was used instead to remove α_2 M (Virca et al., 1978). The 25-75% precipitate of ammonium sulphate (AS) fraction of normal plasma was used as starting material. 11 ml of 25-75% (AS) fraction was dialysed against 0.05 M Tris-HCl buffer, pH 8.0, and applied to the column as 4.4.2. As the results show, Fig. 4.24, fractions that did not bind to the column contained proteolytic activity. The specific activities of the above steps are given in Table 4.7. and the SDS gel electrophoresis pattern of the pool of fractions with activity is shown in Fig. 4.25.

Immunoelectrophoresis of an aliquot from Peak I pool (containing activity) against anti-α_2 macroglobulin antiserum showed no precipitation line, Fig. 4.26. As the Blue-Sepharose column proved to be a useful step in purification, plasma was purified under the same conditions up to the Blue-Sepharose fractionation step. The fractions with activity from Blue-Sepharose were used as starting material for further purifications.

A flow chart of the further purification steps tried are given in Fig. 4.23.

Fig. 4.23 FLOW CHART OF PLASMA ENZYME PURIFICATION

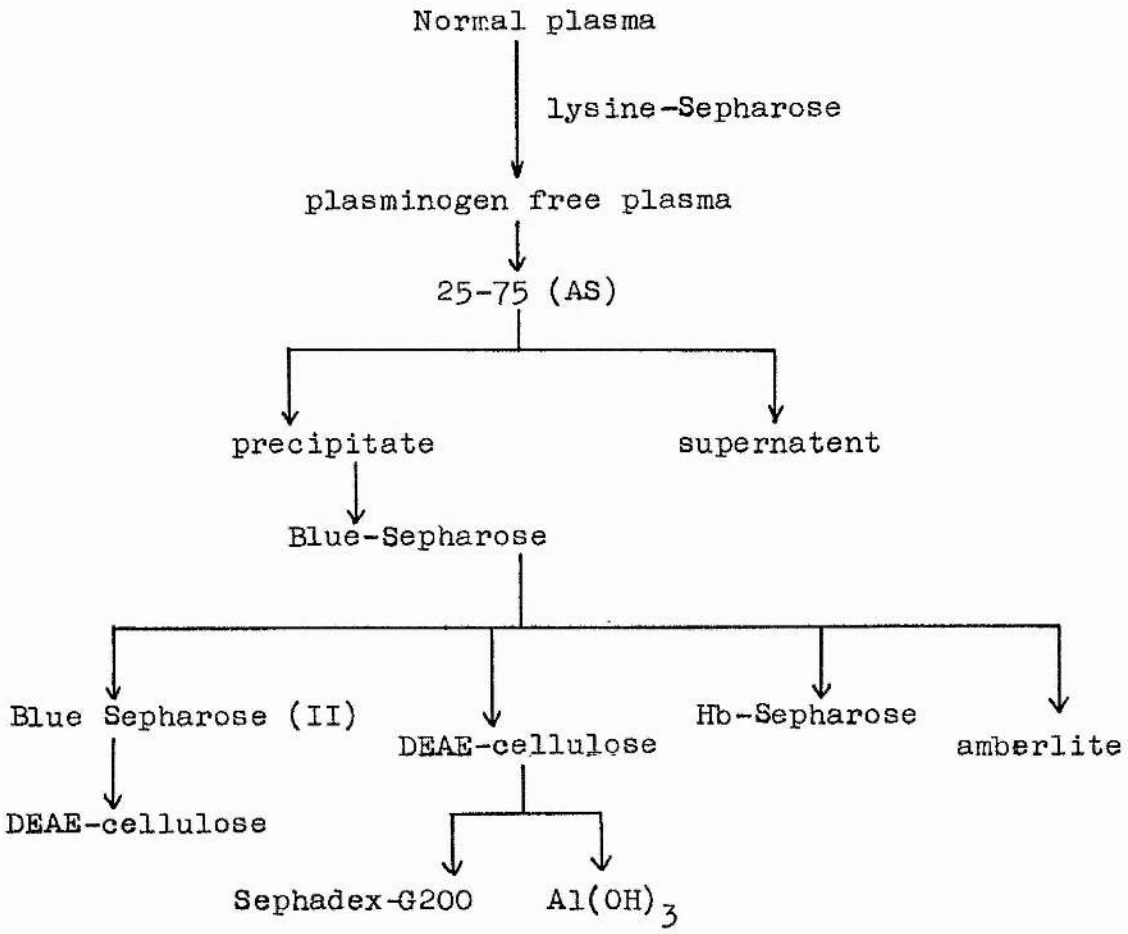


Fig. 4.24 BLUE-SEPHAROSE COLUMN CHROMATOGRAPHY OF PLASMA ENZYME

o——o absorbance at 280 nm

•——• mg fibrin clot dissolved by 0.2 ml enzyme in 16 h.

——→ I 0.1 M NaCl applied

——→ II 2 M NaCl applied

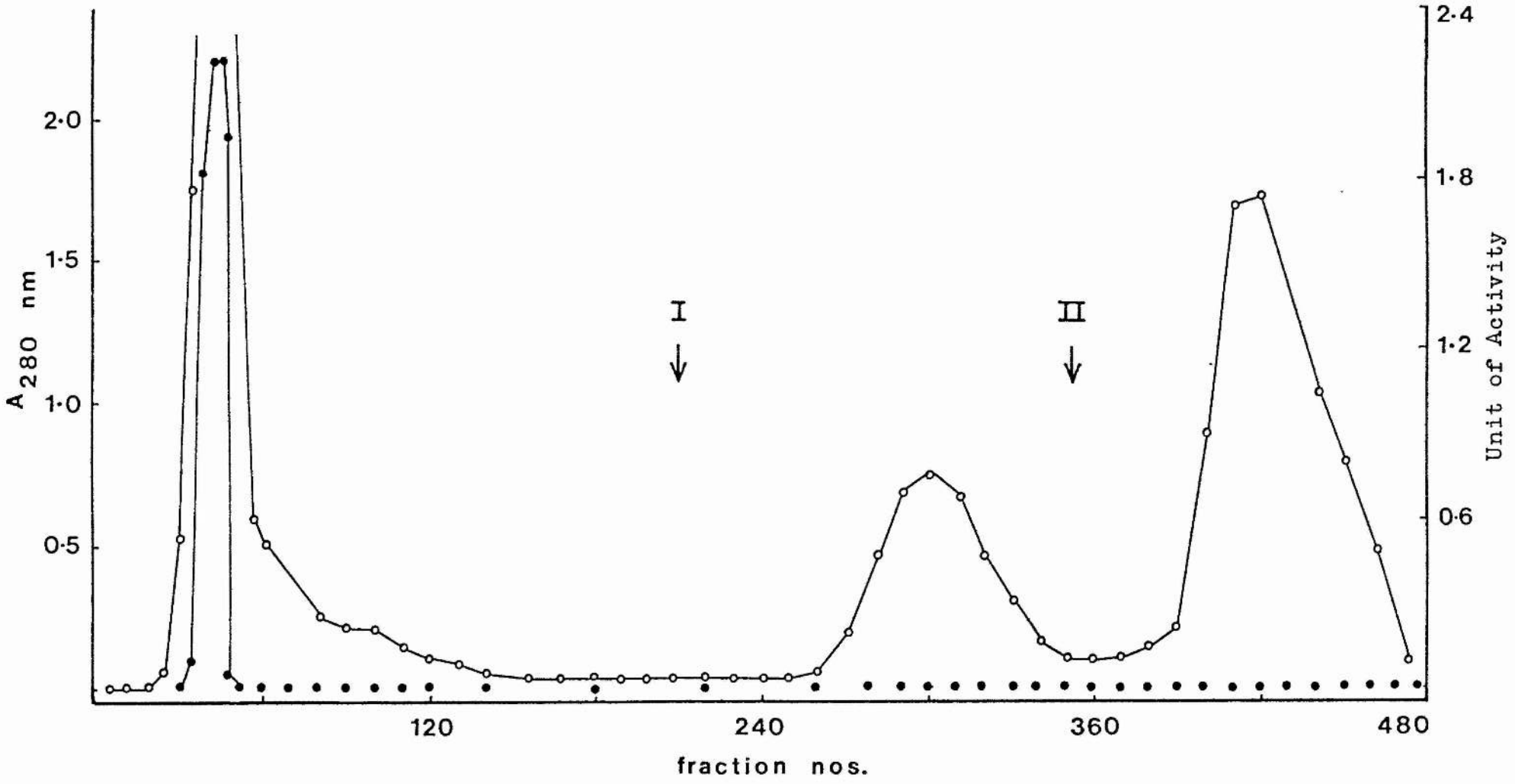

2·0
1·5
1·0
0·5
A280 nm
I
II
2·4
1·8
1·2
0·6
Unit of Activity
120
240
360
480
fraction nos.

Fig. 4.25 DENSITOMETRIC SCAN OF AN ALIQUOT OF THE POOL FROM BLUE SEPHAROSE

Estimated molecular weight of peaks 3 and 5 are approximately 90,000 and 54,000

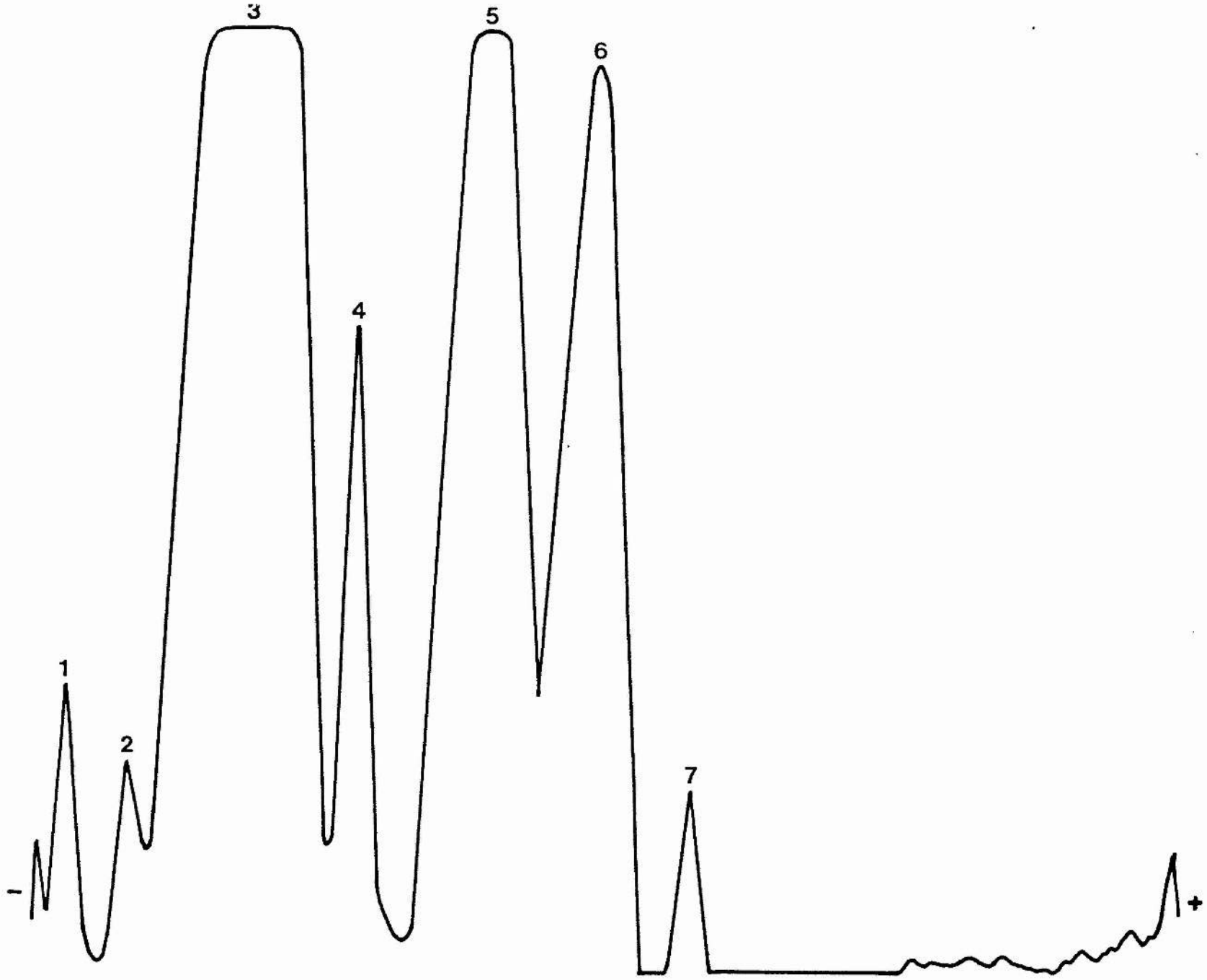

1
2
3
4
5
6
7
–
+

Fig. 4.26 IMMUNODIFFUSION PATTERN OF ALIQUOTS OF THE POOL FROM BLUE-SEPHAROSE AGAINST ANTI α_2-M ANTISERUM

Anti α_2-M antiserum in centre well

Well 1 Normal plasma

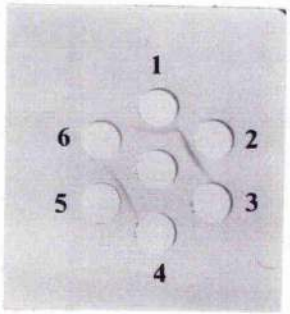
Well 2 25-75% (AS) step Table 4.1

Well 3 Blue-Sepharose step Table 4.7

Well 4 DEAE-cellulose step Table 4.7

Well 5 Pure α_2-M

Well 6 DEAE-cellulose step Table 4.7



4.10. Plasma Enzyme Purification (fourth batch)

Plasma enzyme was purified up to the stage of Blue-Sepharose.

The specific activities are given in Table 4.7, and a summary of the purification is given in Fig. 4.27.

4.10.1. DEAE-cellulose

The fractions with activity from Blue-Sepharose column were pooled (12 ml) and applied to a DEAE-cellulose column, which had been equilibrated with 0.05 M Tris-HCl, pH 8.0.

The sample was applied to the column, and eluted with a different buffer, (0.05 M Tris/0.05 M phosphoric acid, pH 3.8) as 4.2.3.

As Fig. 4.27a. shows, three peaks were obtained, fractions under each peak were pooled and concentrated by Amicon ultrafiltration. The pool from Peak III (pH approximately 4.0) contained the activity, Table 4.7. Immuno electrophoresis of an aliquot of the sample with activity against bovine antiserum showed no precipitation line. However, when human antiserum was used a precipitation line in the position of albumin was detected, Fig. 4.28. SDS gel. Fig. 4.29.

4.10.2. Sephadex G 200

The pool of Peak III of DEAE-cellulose column was concentrated (freeze-drying or ultrafiltration) to 2 ml volume and the sample applied to sephadex G 200 column. Fractions were collected, but no activity was detected even after concentration.

Fig. 4.27 FLOW CHART OF PLASMA ENZYME PURIFICATION

(fourth batch)

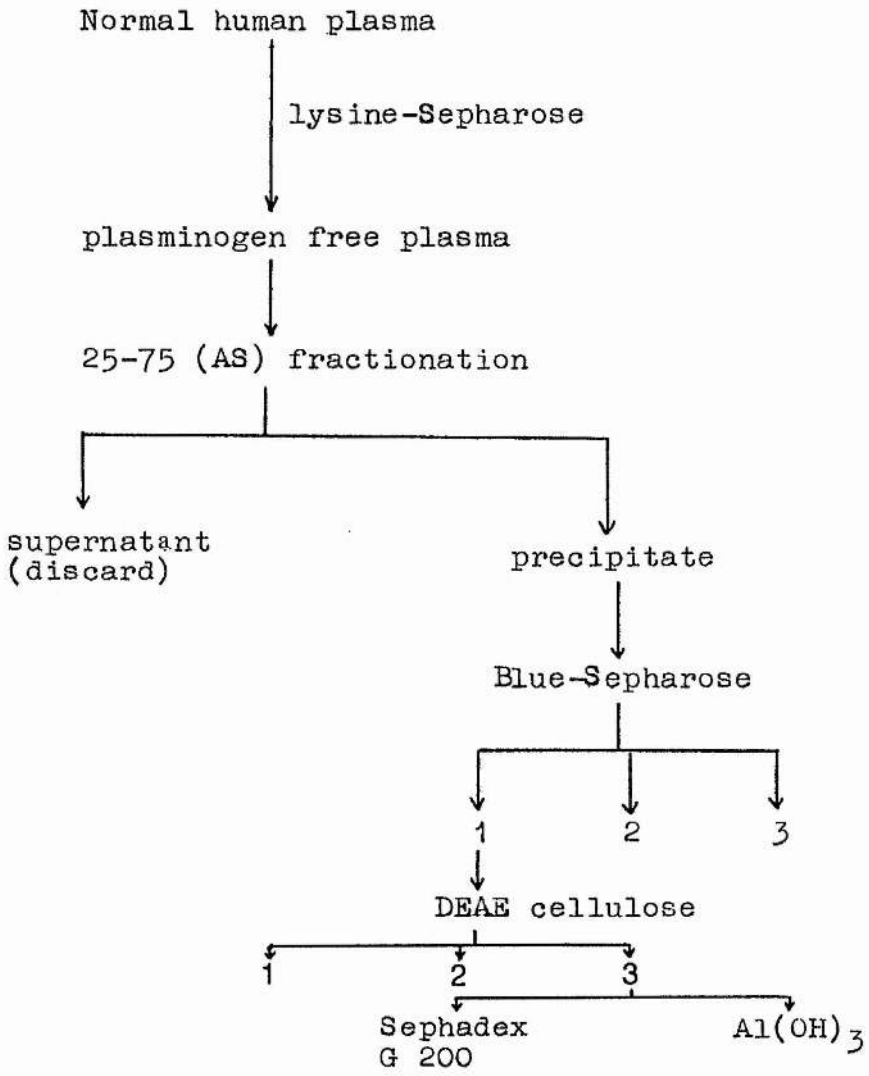


Table 4.7

PURIFICATION OF PLASMA ENZYME (fourth batch)

Procedure	Volume (ml)	Protein mg/ml A_{280}	Total protein	Units enzyme/ ml	Total enzyme	Specific activity	% yield	Fold purification
Normal plasma	12	62	744	10	120	0.16	100	1.0
Plasminogen free plasma	14	46	644	8.9	124	0.19	103	1.2
25-75 (AS) fraction	11	33	363	8.6	94.6	0.26	79	1.6
Blue Sepharose	12	3.9	46.8	8.9	106.8	2.3	89	14
DEAE-cellulose	4	0.7	2.8	14	56	20	47	125
Sephadex-G200	2							
$Al(OH)_3$								

Fig. 4.27a DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF PLASMA ENZYME

o——o absorbance at 280 nm

•——• mg fibrin clot dissolved by 0.2 ml enzyme in 16 h.

——→ 0.05 M Tris, 0.05 M phosphoric acid, pH 3.8, applied

I Peak I

II Peak II

III Peak III

– – – – – pH of eluate

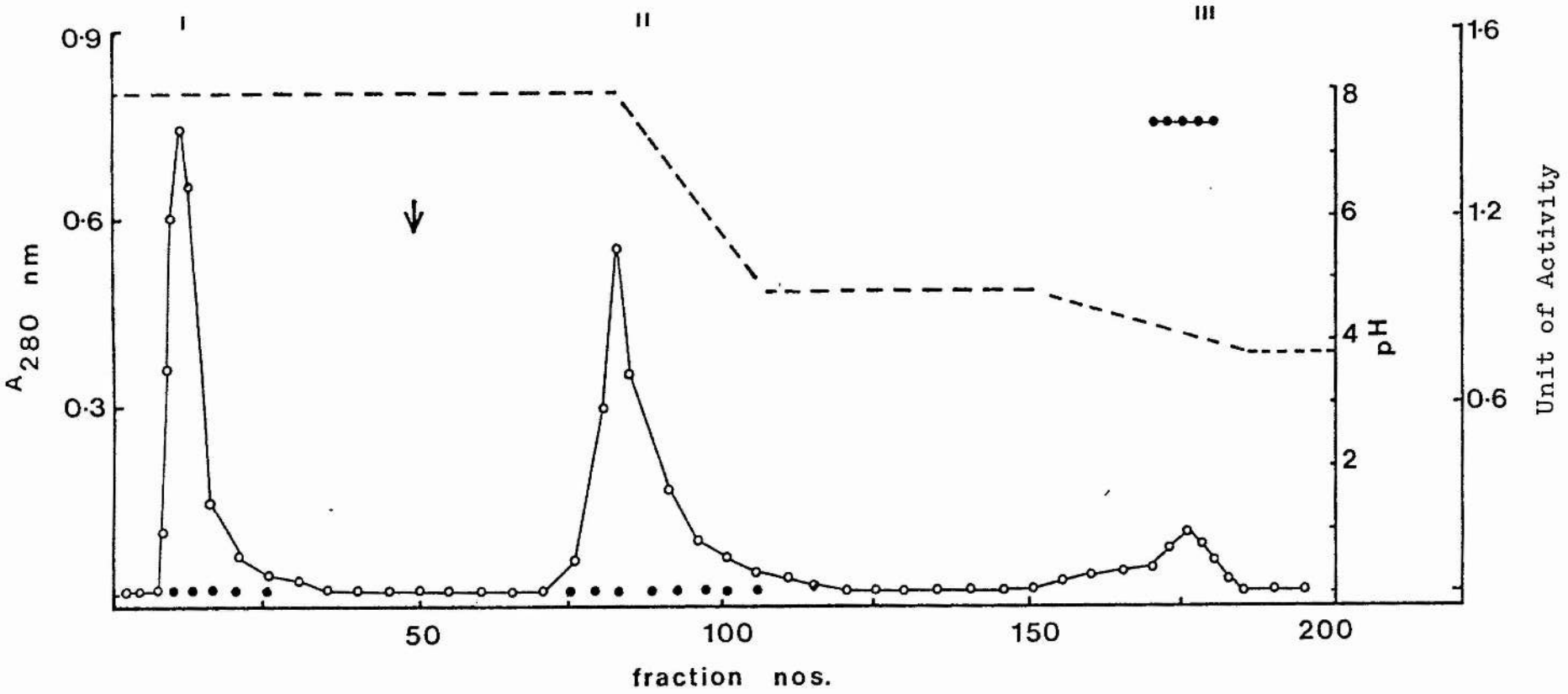

I
II
III
0·9
0·6
0·3
A_{280} nm
8
6
4
2
pH
1·6
1·2
0·6
Unit of Activity
50
100
150
200
fraction nos.

Fig. 4.28 IMMUNO ELECTROPHORESIS ON ALIQUOTS OF THE MATERIAL ELUTED FROM DEAE CELLULOSE

a) Anti-human antiserum in the trough

b) Anti-albumin antiserum in the trough

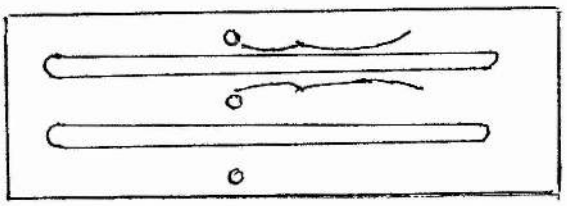


Fig. 4.29 DENSITOMETRIC SCAN OF AN ALIQUOT FROM POOL OF PEAK III FRACTIONS OF DEAE-CELLULOSE COLUMN. ESTIMATED MOLECULAR WEIGHTS ARE APPROXIMATELY 90,000 and 52,000

1
1
2
+

4.10.3. $Al(OH)_3$ gel fractionation

As the activity of semi-purified plasma enzyme from DEAE-cellulose column was lost following further purification on Sephadex G 200, another sample of plasma was purified up to the stage of the DEAE-cellulose column, and the fractions with activity from DEAE-column were pooled, concentrated and subjected to $Al(OH)_3$ gel fractionation (4.1.2). No activity was detected in the material adsorbed to the gel nor did the unbound material have any activity.

4.11. Plasma Enzyme Purification (fifth batch)

In the previous batch purification, plasma enzyme activity was lost after the $Al(OH)_3$ fractionation (4.10.2). It was decided to change the order of the DEAE-cellulose column and the $Al(OH)_3$ steps.

The flow chart of purification steps is given in Fig. 4.30. and the specific activity in Table 4.8.

A sample from Blue-Sepharose column was subjected to the $Al(OH)_3$ gel. Material which did not bind to the gel had activity. SDS gel electrophoresis of an aliquot from unbound material to $Al(OH)_3$ showed two major bands with molecular weights of 90,000 and 53,000 and one minor band, Fig. 4.31.

The sample with activity was dialysed aginst 0.05 M tris-HCl buffer, pH 8.0, and applied to DEAE-cellulose column which had been equilibrated with the same buffer. Samples were collected until no protein was detected in the eluate. Then 0.5 M NaOH was applied to the column, Fig. 4.32.

Table 4.8 PURIFICATION OF PLASMA ENZYME (fifth batch)

Procedure	Volume ml	Protein mg/ml A_{280}	Total protein	Units enzyme/ ml	Total enzyme	Specific activity	% yield	Fold purification
25 - 75 (AS) fraction	11	24	264	9	99	0.375	100	1.0
Blue Sepharose	11.5	3.5	40.2	8.1	93	2.3	94	6.1
$Al(OH)_3$	12	2.5	32	6	72	2.4	73	6.4
DEAE-cellulose	3	0.4	1.2	4.5	13.5	11.25	14	30

Fig. 4.30 FLOW CHART OF PLASMA ENZYME (fifth batch)

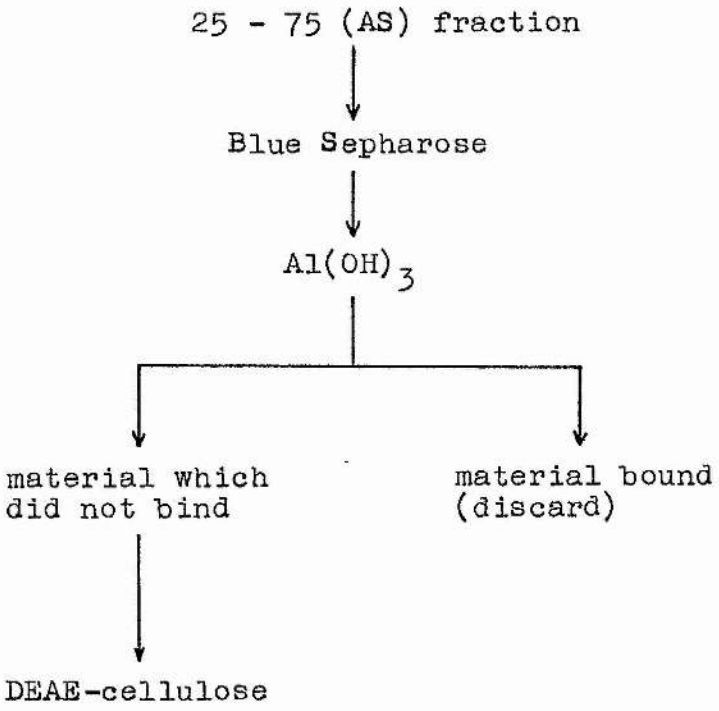


Fig. 4.31 DENSITOMETRIC SCAN OF AN ALIQUOT FROM UNBOUND MATERIAL TO $Al(OH)_3$

Estimated molecular weights are

Peak 1	approximately 90,000
Peak 3	approximately 53,000

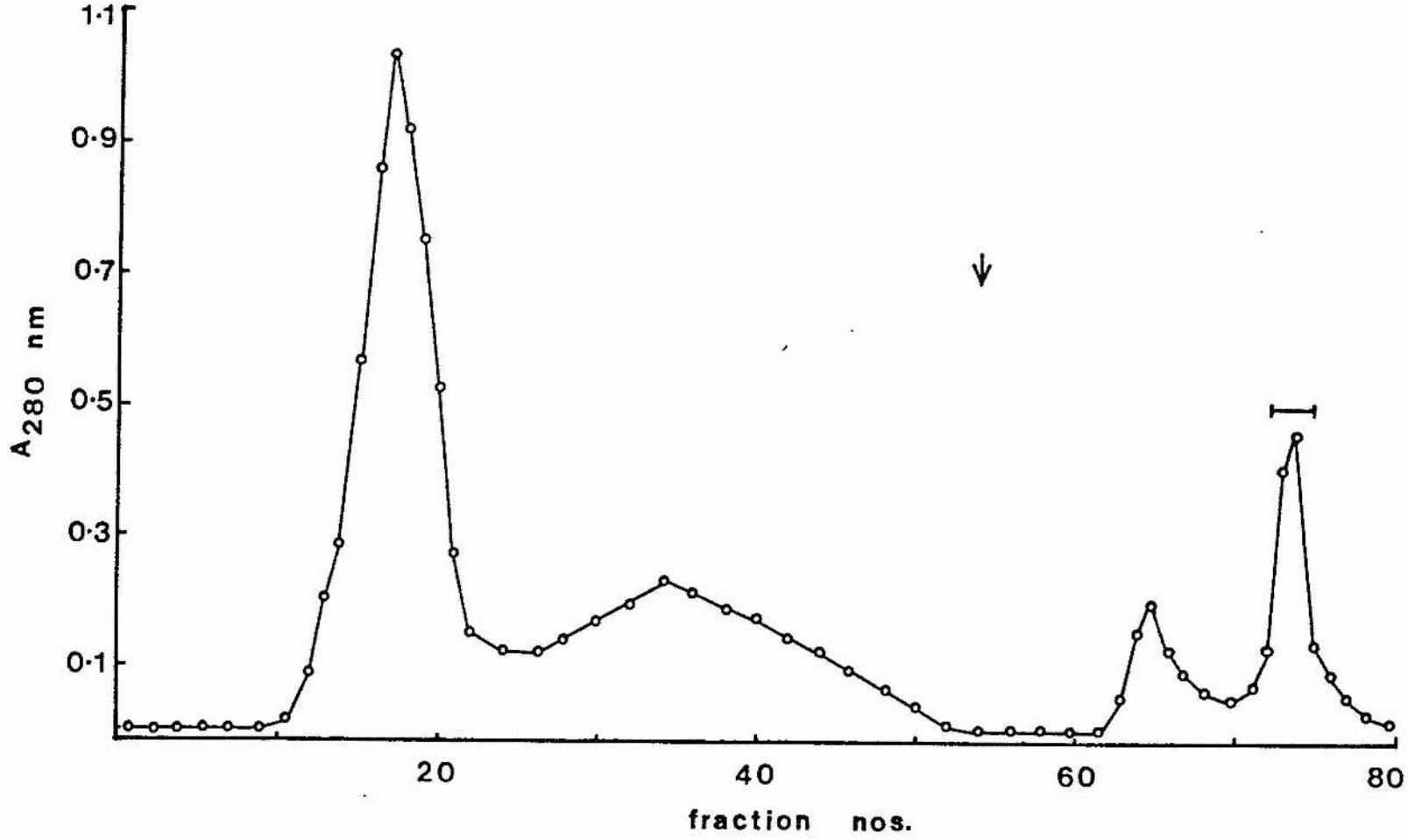
-
1
2
3
+

Fig. 4.32 DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF PLASMA ENZYME

o——o absorbance at 280 nm

⊢——⊣ pool of fractions with proteolytic activity

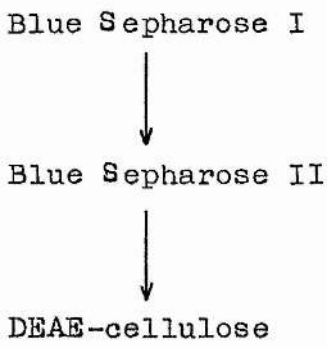
——→ 0.5 M NaOH applied


1·1
0·9
0·7
0·5
0·3
0·1
A_{280} nm
20
40
60
80
fraction nos.

4.12. Plasma Enzyme purification (sixth batch)

The purification of the fifth batch was not any improvement on the fourth batch (4.10). It was decided to use Blue-Sepharose column chromatography twice before the application of the sample to DEAE-cellulose column. A flow chart of purification steps is given in Fig. 4.33 and the specific activities in Table 4.9.

Fig. 4.33 FLOW CHART OF PLASMA ENZYME (sixth batch)



Elution pattern from Blue-Sepharose column II. Fig. 4.34.

SDS gel electrophoresis of an aliquot from fractions pool of Blue-Sepharose column II. Fig. 4.35.

Table 4.9 PURIFICATION OF PLASMA ENZYME (sixth batch)

Procedure	Volume ml	Protein mg/ml A_{280}	Total protein	Units enzyme/ ml	Total enzyme	Specific activity	% yield	Fold purification
Blue Sepharose I	10	3.7	37	13	130	3.5	100	1
Blue Sepharose II	10	2	20	8	80	4.0	62	1.2
DEAE-cellulose	3.5	0.28	0.98	5	17.5	17.9	5	4.9

Fig. 4.34 BLUE-SEPHAROSE II COLUMN CHROMATOGRAPHY OF PLASMA ENZYME

o——o Absorbance at 280 nm

•——• mg fibrin clot dissolved by 0.2 ml enzyme in 16 h.

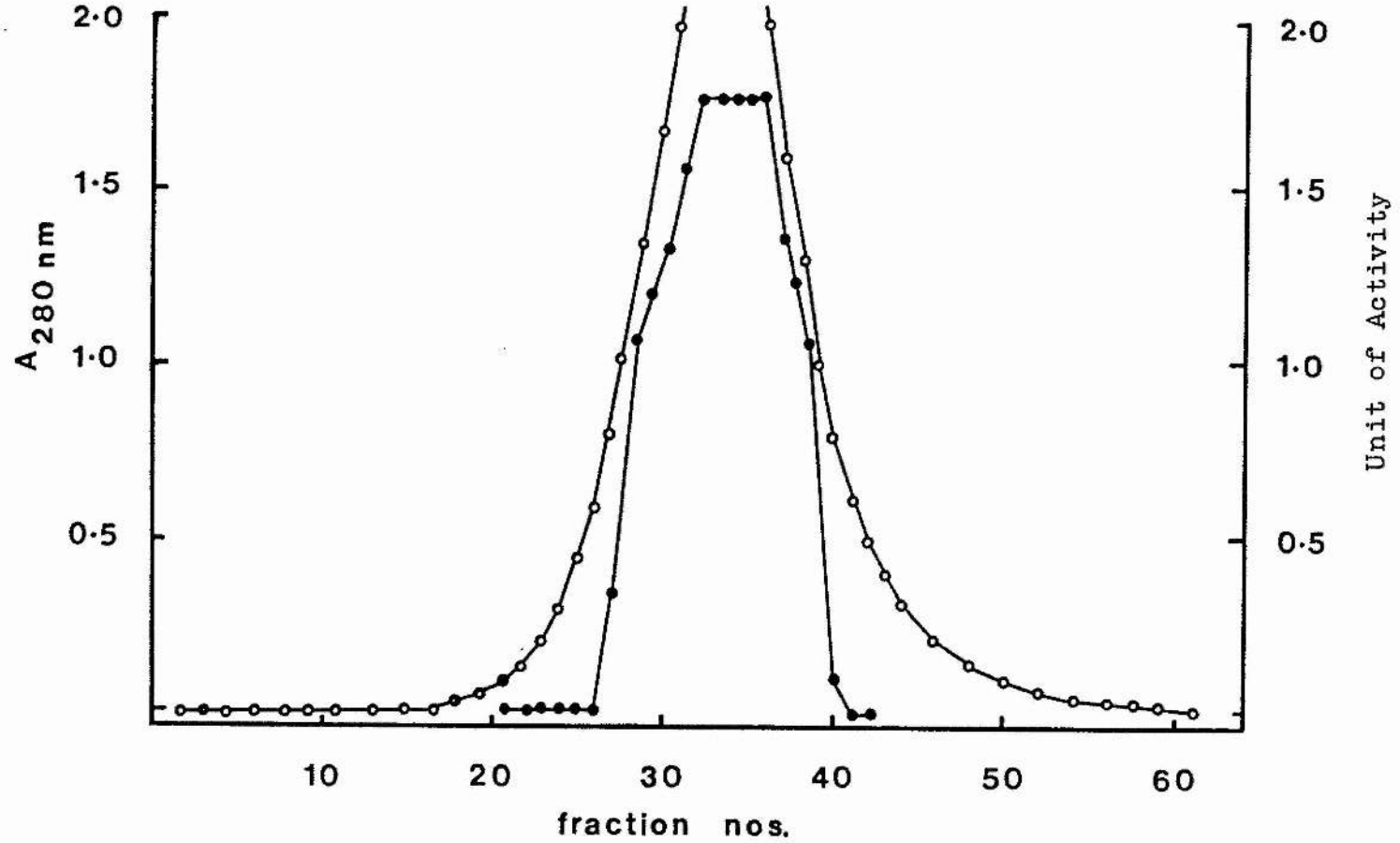
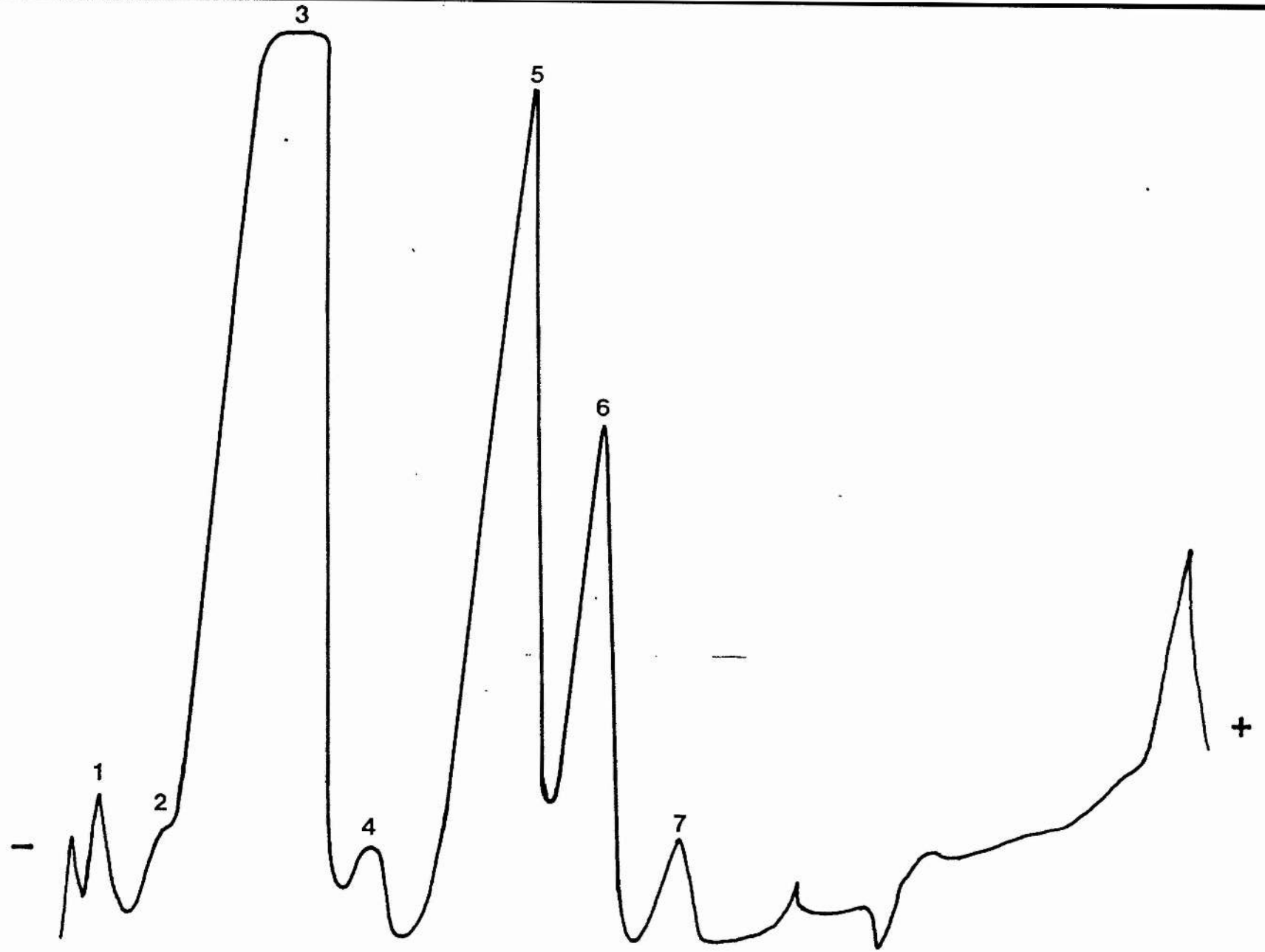

2·0
1·5
1·0
0·5
$A_{280\ nm}$
2·0
1·5
1·0
0·5
Unit of Activity
10
20
30
40
50
60
fraction nos.

Fig. 4.35 DENSITOMETRIC SCAN OF AN ALIQUOT FROM FRACTIONS POOL OF BLUE-SEPHAROSE II

Estimated molecular weight of Peaks 3 and 5 are approximately 90,000 and 54,000.


3
5
6
1
2
4
7
−
+

4.13. Plasma Enzyme Purification (seventh batch)

In the last three purification batches of plasma enzyme, the SDS gel electrophoresis patterns at the penultimate stage showed two protein bands with molecular weights of approximately 54,000 and 92,000, but on further purification the enzyme activity was lost. It was decided to use a different ion exchange resin.

Amberlite resin CG-50 (H) was used as it is a useful step in the purification of pepsin and gastricsin from gastric juice (2.14.2).

4.13.1 Amberlite resin CG-50 (H)

A sample from Blue-Sepharose column was used as starting material.

8 ml of sample from Blue-Sepharose with activity was dialysed against 0.2 M sodium citrate, pH 3.0, and applied to the Amberlite column as 4.2.4.2. The plasma enzyme was eluted between pH 5.4 - 5.6, Fig. 4.37 As Table 4.10 shows, the percentage recovery of plasma enzyme was good, but SDS gel electrophoresis showed contamination with other proteins, Fig. 4.38. Amberlite column step was abandoned as it took a very long time for the pH to change from 3 to 6. In addition, fractions had to be pooled, desalted and concentrated before assay.

Fig. 4.36 FLOW CHART OF PLASMA ENZYME(batch seven)

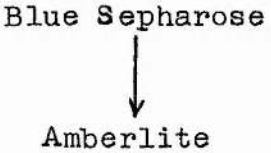


Fig. 4.37 AMBERLITE CG-50 (H) COLUMN CHROMATOGRAPHY OF PLASMA ENZYME

o——o absorbance at 280 nm

|——| pool of fractions with proteolytic activity

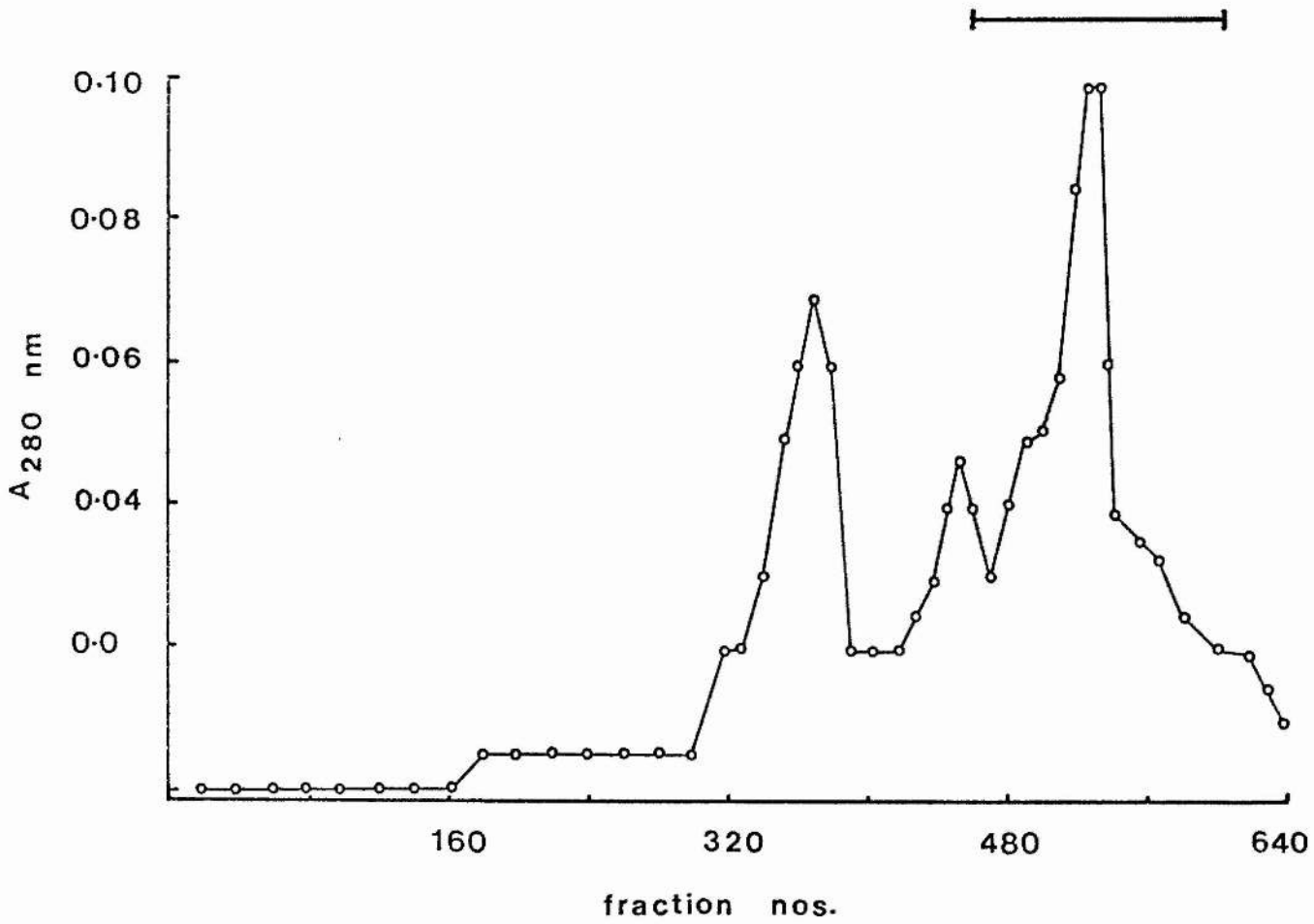

0·10
0·08
0·06
0·04
0·0
A280 nm
160
320
480
640
fraction nos.

Table 4.10 PURIFICATION OF PLASMA ENZYME (seventh batch)

Procedure	Volume ml	Protein mg/ml 280 nm	Total Protein	Units enzyme/ ml	Total enzyme	Specific activity	% yield	Fold purification
Blue-Sepharose column	8	3.5	28	11	88	3.1	100	1
Amberlite column	10	1.5	15	7.5	75	5	85	1.6

Fig. 4.38 DENSITORMETRIC SCAN OF AN ALIQUOT OF THE POOL FRACTIONS FROM AMBERLITE SEPARATED BY SDS-GEL ELECTROPHORESIS

Estimated molecular weight of Peaks 3 and 4 are approximately 91,000 and 53,000.

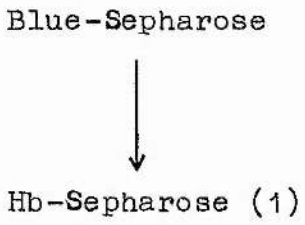
1
1
2
3
4
+

4.14 Plasma Enzyme Purification (eighth batch)

Hb-Sepharose was used, as it proved successful in the purification of acid proteinases such as renin and cathepsin D, (Smith and Turk (1974) and Chou et al., (1978)).

The flow chart of purification is given in Fig. 4.39, and specific activities in Table 4.11.

Fig. 4.39 FLOW CHART OF PLASMA ENZYME (batch eight)



4.14.1. Hb-Sepharose 4B (1)

Fractions from the Blue-Sepharose column with activity were pooled and used as starting material. 5 ml of the sample was dialysed against 0.05 M sodium acetate 1 M NaCl, pH 3.5, and applied to the Hb-sepharose column as 4.4.3.2. After the column was washed with 0.1 M sodium acetate/1M NaCl, pH 5.0, the proteins were eluted with 0.1 M Tris-HCl 1 M NaCl buffer, pH 8.6. Three peaks were obtained, Fig. 4.40 . Fractions under each peak were tested for proteolytic activity using

Table 4.11 PURIFICATION OF PLASMA ENZYME (eighth batch)

Procedure	Volume	Protein mg/ml 280 nm	Total protein	Units enzyme/ ml	Total enzyme	Specific activity	% yield	Fold purification
Blue-Sepharose	6	3.8	22.8	10	60	2.6	100	1
Hb-Sepharose	11.5	0.8	9.2	4.0	46	57.5	77	22

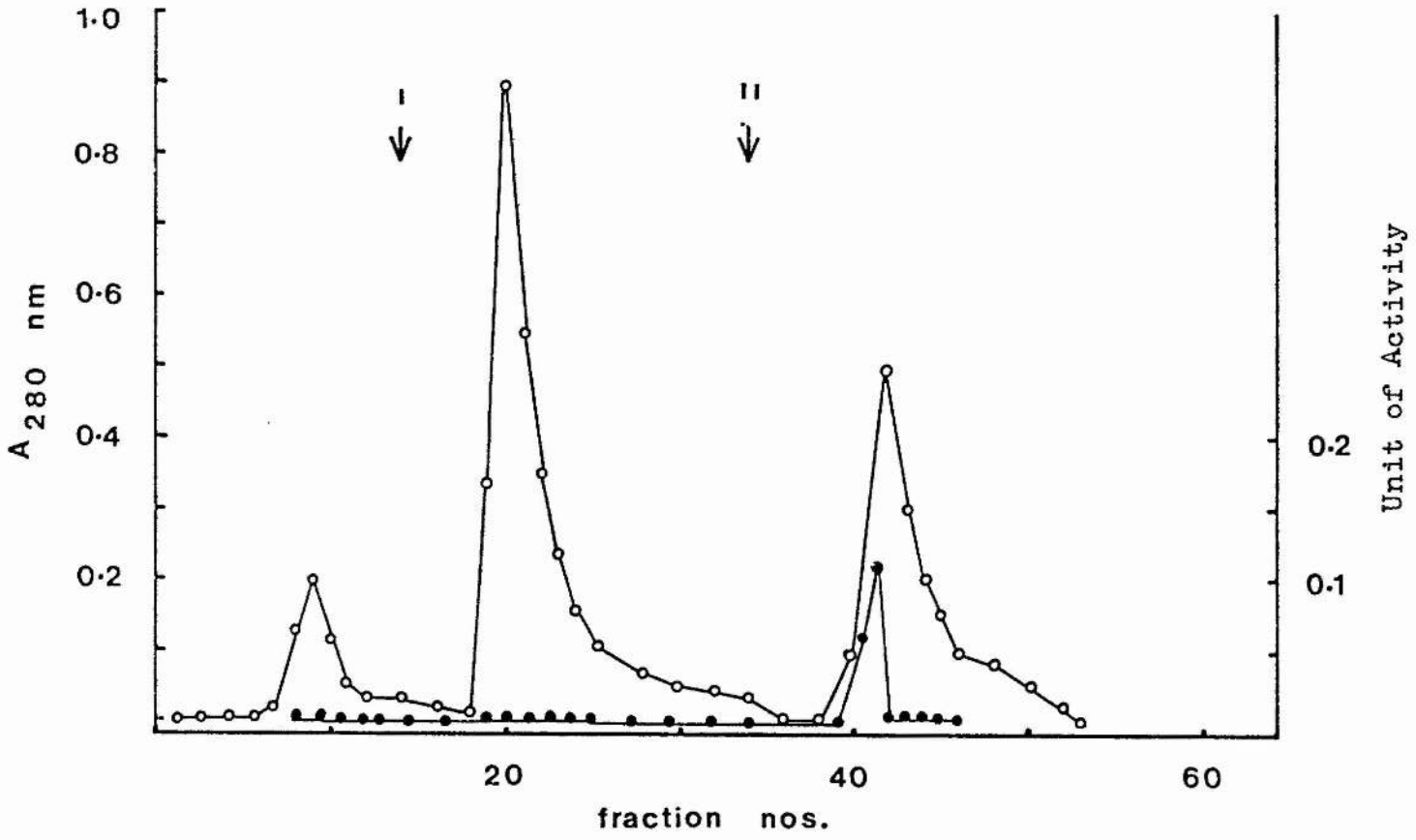
Fig. 4.40 Hb-SEPHAROSE 4B (I) COLUMN CHROMATOGRAPHY OF PLASMA ENZYME

absorbance at 280 nm

mg fibrin clot dissolved by 0.2 ml enzyme in 16 h

I 0.1 M sodium acetate/1 M NaCl, pH 5.0 applied

II 0.1 M Tris-HCl/1 M NaCl, pH 8.6 applied


1·0
0·8
0·6
0·4
0·2
A 280 nm
I
II
0.2
0.1
Unit of Activity
20
40
60
fraction nos.

Hb-assay. Also after dialysis against 0.05 M sodium citrate, pH 3.5, (to remove salt) they were assayed using the fibrin clot assay.

Fractions from Peak I and II did not show any activity in either assay system, nor did they have any activity after being concentrated.

Fractions No. 40 and 41 of Peak III had very low activity. The low activity could be due to the fact that the fractions with activity had a pH in the vicinity of 6.5, but the pH changed very rapidly (within three fractions) to 8.5. Some of the activity might have been eluted at the higher pH and the activity destroyed by the alkaline environment.

4.14.2. Haemoglobin-Sepharose 4B (II)

The method used was based on that of Chou et al., (1978).

Fractions from the Blue-Sepharose column with activity were used as starting material.

5 ml of the sample was dialysed against 0.05 M sodium acetate/1 M NaCl, pH 3.5, and applied to the Hb-sepharose column which had been equilibrated with the same buffer as 4.4.3.2. The proteins were eluted with 0.1 M Tris-HCl/M NaCl, pH 6.5.

Plasma enzyme bound to the column at pH 3.5 and was eluted between pH 5.9 - 6.5 with the highest activity at pH 6.3, Fig. 4.41.

Hb-sepharose column had a major disadvantage, as the SDS gel electrophoresis shows, Fig. 4.42.

There are many contaminating protein bands and also, some of the haemoglobin had been eluted from the column.

Fig. 4.41 Hb-SEPHAROSE 4B (II) COLUMN CHROMATOGRAPHY OF PLASMA ENZYME

o——o absorbance at 280 nm

•——• mg fibrin clot dissolved by 0.2 ml enzyme in 16 h

——→ 0.1 M Tris-HCl/1 M NaCl, pH 6.5 applied

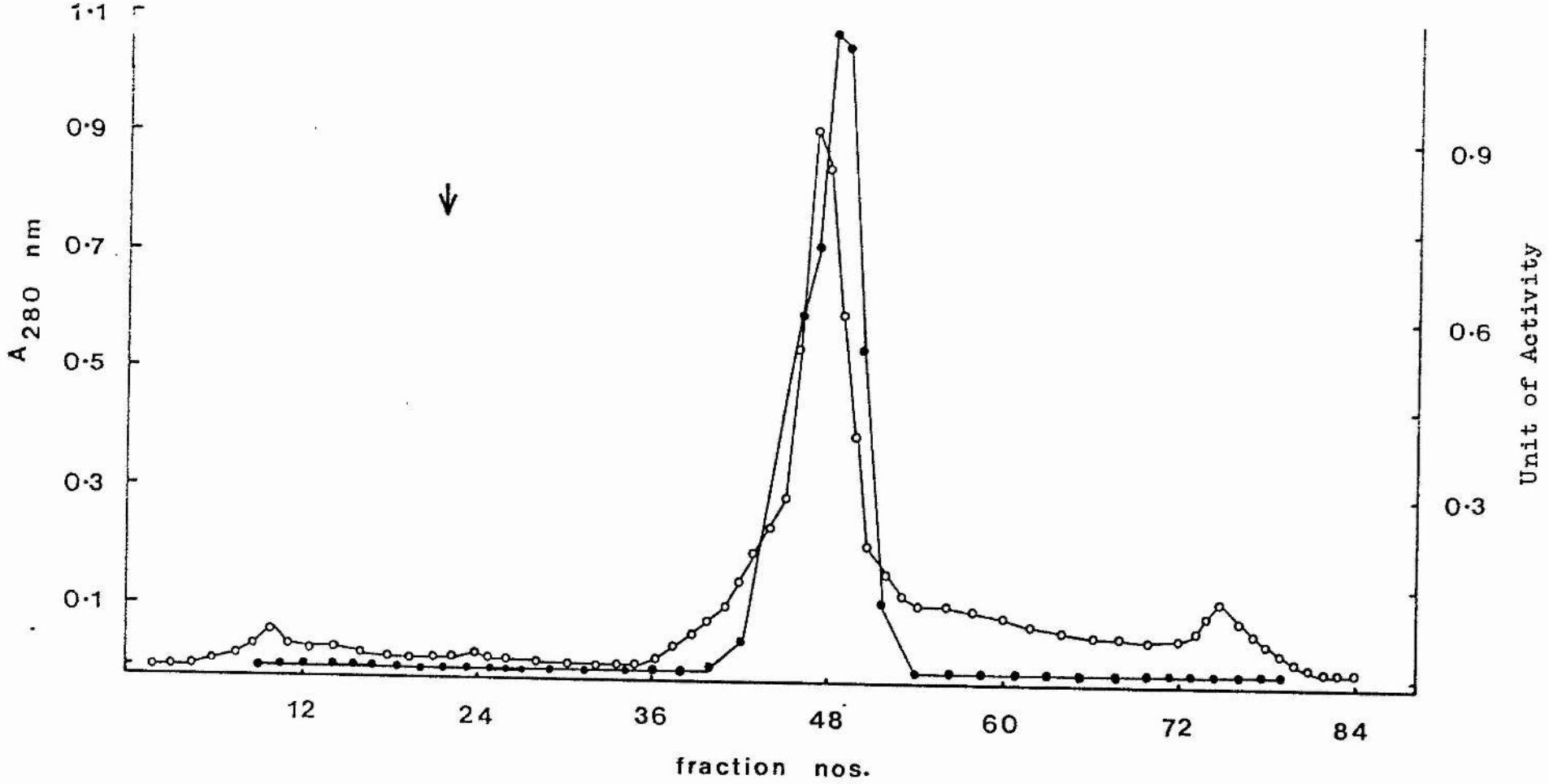
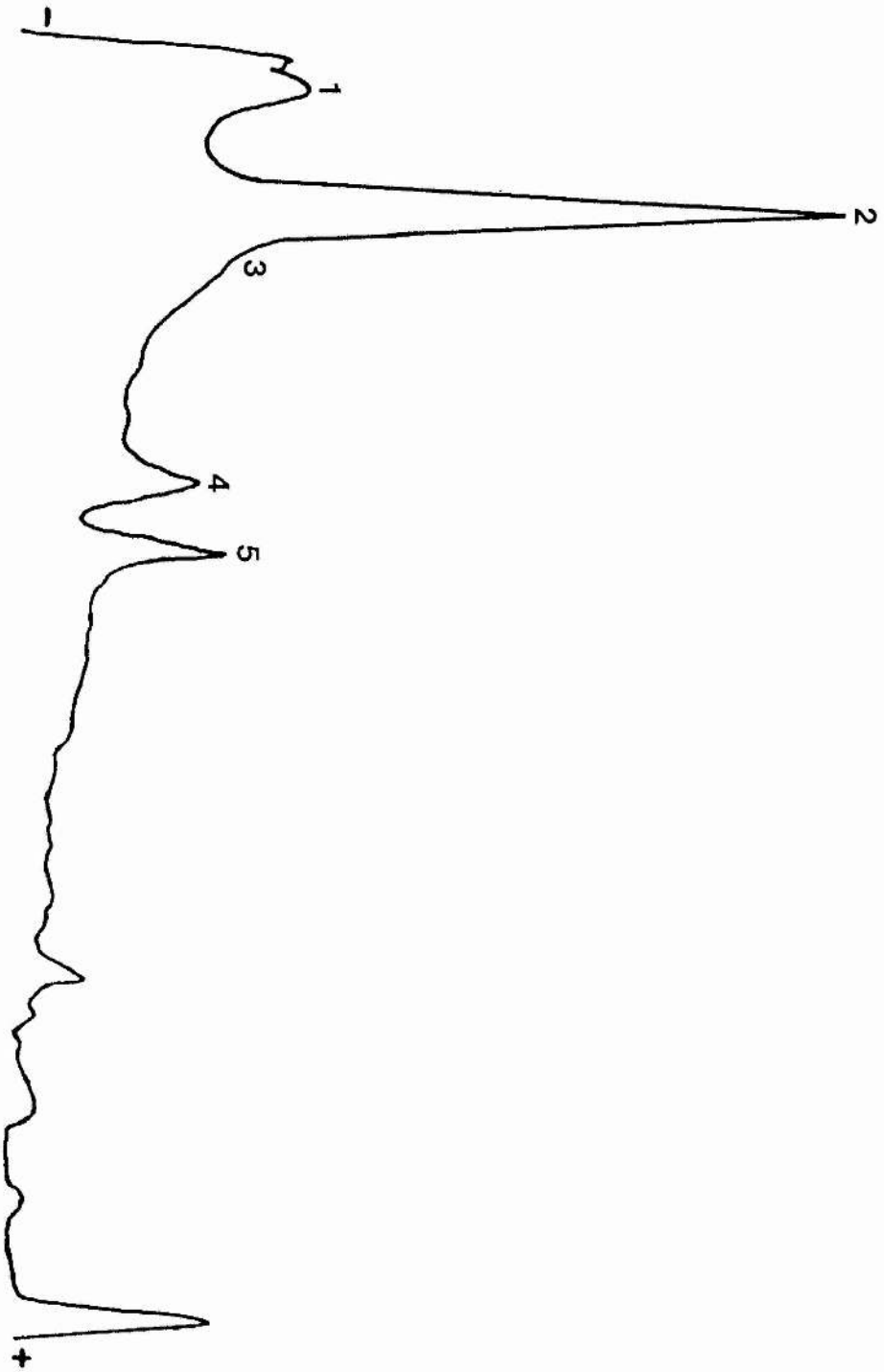

1·1
0·9
0·7
0·5
0·3
0·1
A280 nm
0·9
0·6
0·3
Unit of Activity
12
24
36
48
60
72
84
fraction nos.

Fig. 4.42 DENSITOMETRIC SCAN OF AN ALIQUOT FROM POOL OF FRACTIONS FROM Hb-SEPHAROSE 4B (II)

Estimated molecular weights are

Peak 3 approximately 90,000

Peak 5 approximately 55,000


1
1
2
3
4
5
+

4.15. Conclusion

Different methods were used to purify plasma enzyme in the final stage of each purification, before the loss of enzyme activity. There were always two protein bands on SDS gel electrophoresis with molecular weights of approximately 90,000 and 54,000.

Law (1978) attempted the purification of the same enzyme, and also reported the molecular weights of approximately 91,000 and 54,000 with the highest activity associated with the protein with the molecular weight of 54,000. Law discovered that her preparations were contaminated with albumin and was not able to separate the enzyme from albumin.

During conformational investigation on bovine plasma albumin (BPA) Wilson and Foster (1971) noted the presence of a proteolytic enzyme which cleaved BPA in the F form and hydrolysed several bonds in acid expanded BPA. Partial purification revealed a preparation with bands of molecular weights of 46,700, 14,300 and a high molecular weight component on SDS gel electrophoresis.

After crystalization of BPA the proteolytic enzyme was still present but in smaller quantity, and the protein with molecular weight of 46,000 was still present. They concluded that the enzyme was not trypsin, chymotrypsin or pepsin, as it did not hydrolyse the native form of BPA, which is relatively easily attacked by the above proteolytic enzymes.

The plasma enzyme electrophoretic pattern was

similar to that of Low (1978) and Wilson and Foster (1971), and the immunoelectrophoresis results showed a precipitin line with human antiserum in the position of albumin.

However, anti-albumin antiserum gave no precipitin line in the later purification stages, Fig. 4.28.

The concentrated pool fractions from DEAE-cellulose column, fourth batch of purification (4.10) was used for characterization of plasma enzyme. As it had high specificity, it was stable on storage and only two bands were present on SDS gel electrophoresis, Fig. 4.29.

4.16. Further Work

4.16.1. Purification of plasma enzyme using radioiodinated derivative of pepstatin

As the carboxy group of pepstatin is not involved in its effectiveness as an inhibitor, it can be modified or coupled to different reagents. Matthews et al., (1981), synthesised N-pepstatinyl-S-bimanyl-2-amino-ethane thiol, a fluorescent probe for the subcellular location of cathepsin D. Pourmotabbed et al., (1981), coupled pepstatin to dextran by the cyanogen bromide activation procedure, and the pepstatin-dextran conjugate retained the high water solubility of dextran and the inhibitory effect of pepstatin. The fact that this compound is water soluble makes it extremely useful for in vivo investigation of acid proteinases.

Workman and Burkitt, (1979), prepared radioiodinated pepstatin, by coupling L-tyrosine methyl ester. Then pepstatin-L-tyrosine methyl ester was iodinated with ^{125}T and chloromine-T radioiodinated pepstatin reacted with pepsin similar to unmodified pepstatin.

As pepstatin inhibited plasma enzyme, it was decided to use radiolabelled pepstatin to locate the plasma enzyme on gel-chromatography or SDS gel electrophoresis, also to determine the dissociation constants for the binding of pepstatin to plasma enzyme.

4.16.2. Preparation of pepstatin L-tyrosine methyl ester

The method used was based on that of Workman and

Burkitt, (1979).

Pepstatin, tyrosine methylester and dicyclohexylcarbodiimide were added to 8 ml of dichloromethane in molar ratio of 1.4.4, (30 mg, 34.3 mg, 36.25 mg). The mixture was stirred at room temperature for 96 h. The solvent was removed by evaporation, and the residue was dissolved in 2 ml of methanol, and applied to L-H_{20} column (1 Cm x 110 Cm). The samples were eluted by gravity flow using methanol. Fractions were collected until the absorbance at 280 nm was less than 0.05. Three peaks were obtained. Fractions from the first peak containing pepstatin-L-tyrosine methyl ester were pooled. The solvent evaporated and redissolved in 1.5 ml of chloroform, methanol, ammonia 75:24:1, and applied to silica gel (silicar CC-4) column (1 Cm x 25 Cm), to separate tyrosine methyl ester from unreacted pepstatin. Methanol was used as solvent fractions containing pepstatin methyl ester were pooled, and the solvent evaporated to dryness, ready for iodination.

The iodination of pepstatin-L-tyrosine methyl ester with ^{125}I and chloromin-T was not carried out for personal reasons.

CHAPTER 5

CHARACTERIZATION

5.1. Enzyme Kinetics

In Chapter 3 (substrate) it was concluded that none of the substrates used were ideal. Although the fibrin clot assay proved to be the most suitable for plasma enzyme, it had its problems. The fact that it is a protein substrate made kinetic studies rather difficult. Kinetic studies became even more complicated as the enzyme was not 100% pure. However, an attempt was made to find out the limitations of the system and the best possible conditions which may help to establish a certain order in the plasma enzyme characterization.

5.1.1. Effect of substrate concentration

Fibrin clots were prepared as 3.2.4 using a series of different concentrations of crude fibrinogen (1-6 mg/ml). Plasma enzyme was added to the fibrin clots as 3.2.5.

A graph of units of activity versus substrate concentration was plotted, Fig. 5.1. As the graph shows, a straight line was obtained up to 6 mg/ml fibrinogen, which suggests that the enzyme activity is proportional to the substrate concentration, i.e. the region of first order kinetics $[S] < 0.01$ Km, (well below Km).

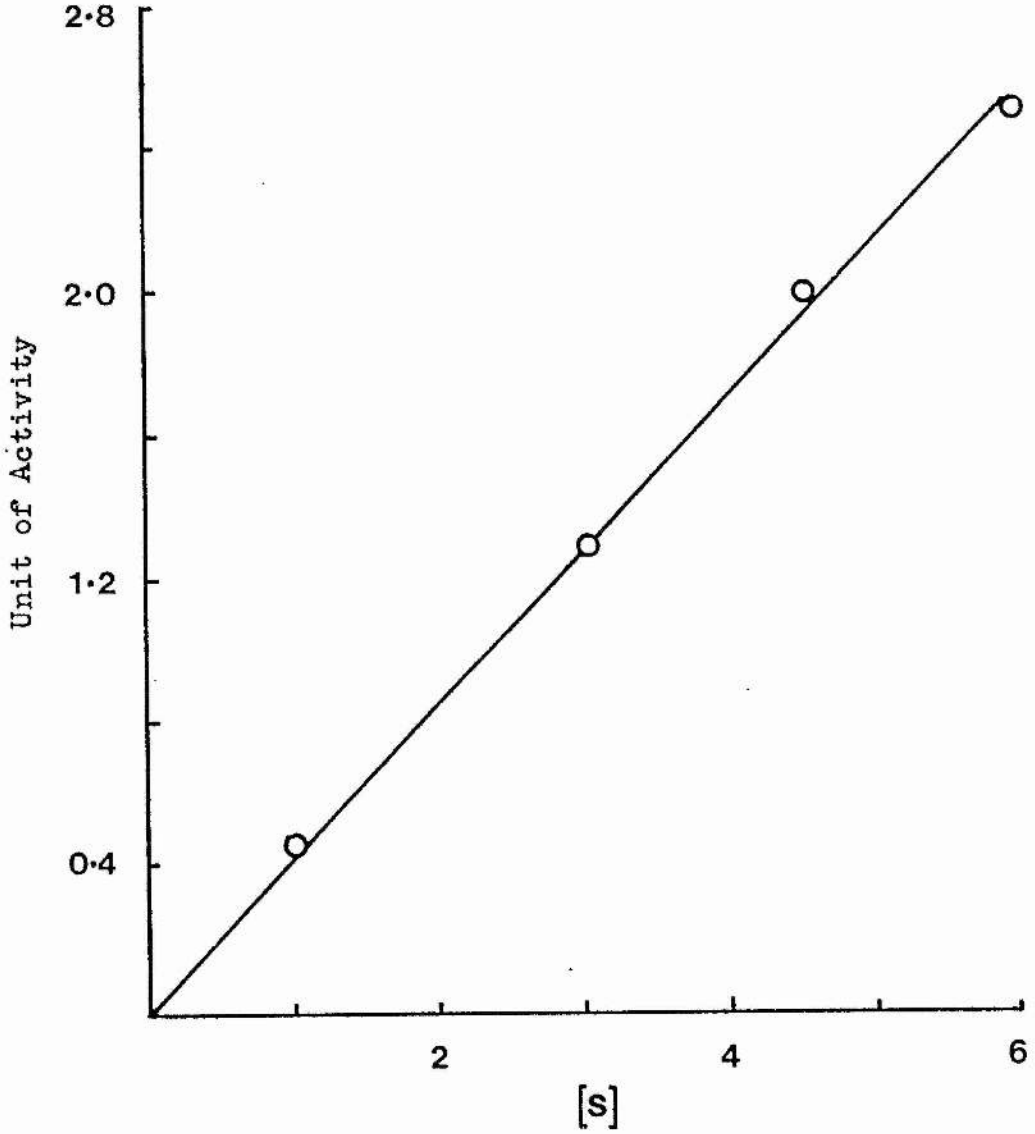
Although this is not an ideal range to work with, in all the assays performed with a given batch of substrate the concentration of fibrin was kept constant.

The need for a very high concentration of

Fig. 5.1 THE EFFECT OF SUBSTRATE CONCENTRATION. A GRAPH OF UNITS OF ACTIVITY VERSUS CONCENTRATION OF FIBRIN

S - mg/ml fibrinogen

enzyme - DEAE cellulose step Table 4.7


2·8
2·0
1·2
0·4
Unit of Activity
2
4
6
[S]

substrate could be a reflection of the restricted specificity, i.e. enzyme cleaves only at a limited number of sites in fibrin.

5.1.2. Effect of enzyme concentration

To study the effect of enzyme concentration, a range of plasma enzyme volumes was chosen and all made up to the total volume of 0.8 ml (highest volume of plasma enzyme). Assays with fibrin clots were performed in duplicate. A graph was plotted of activity versus plasma enzyme concentration, Fig. 5.2 A and B.

As results show, Fig. 5.2A, a linear relationship between the enzyme activity and concentration was achieved up to the point in which $\frac{1}{2}$ to $\frac{2}{3}$ of the fibrin clot had been dissolved, which could be due to the exhaustion of the substrate. When crude enzyme (plasma) was used, an inhibitory effect was obtained, Fig. 5.2 B, at the point where the graph levels off in Fig. 5.2 A. This could be due to the presence of fibrinogen in the plasma, which competes with the fibrin. Once the fibrinogen was removed, the graph of the enzyme activity versus enzyme concentration became similar to the other graphs from different stages of purification.

5.1.3. Effect of period of hydrolysis

Fibrin clots were incubated in 1% (w/v) monochloro acetic acid in the presence of the plasma enzyme at 37°C for various time intervals. The amount of fibrin clot being hydrolysed was estimated at each

Fig. 5.2(A) THE EFFECT OF ENZYME CONCENTRATION

A graph of units of activity versus a range of plasma enzyme dilution

substrate - fibrin clot

enzyme - DEAE-cellulose step Table 4.7

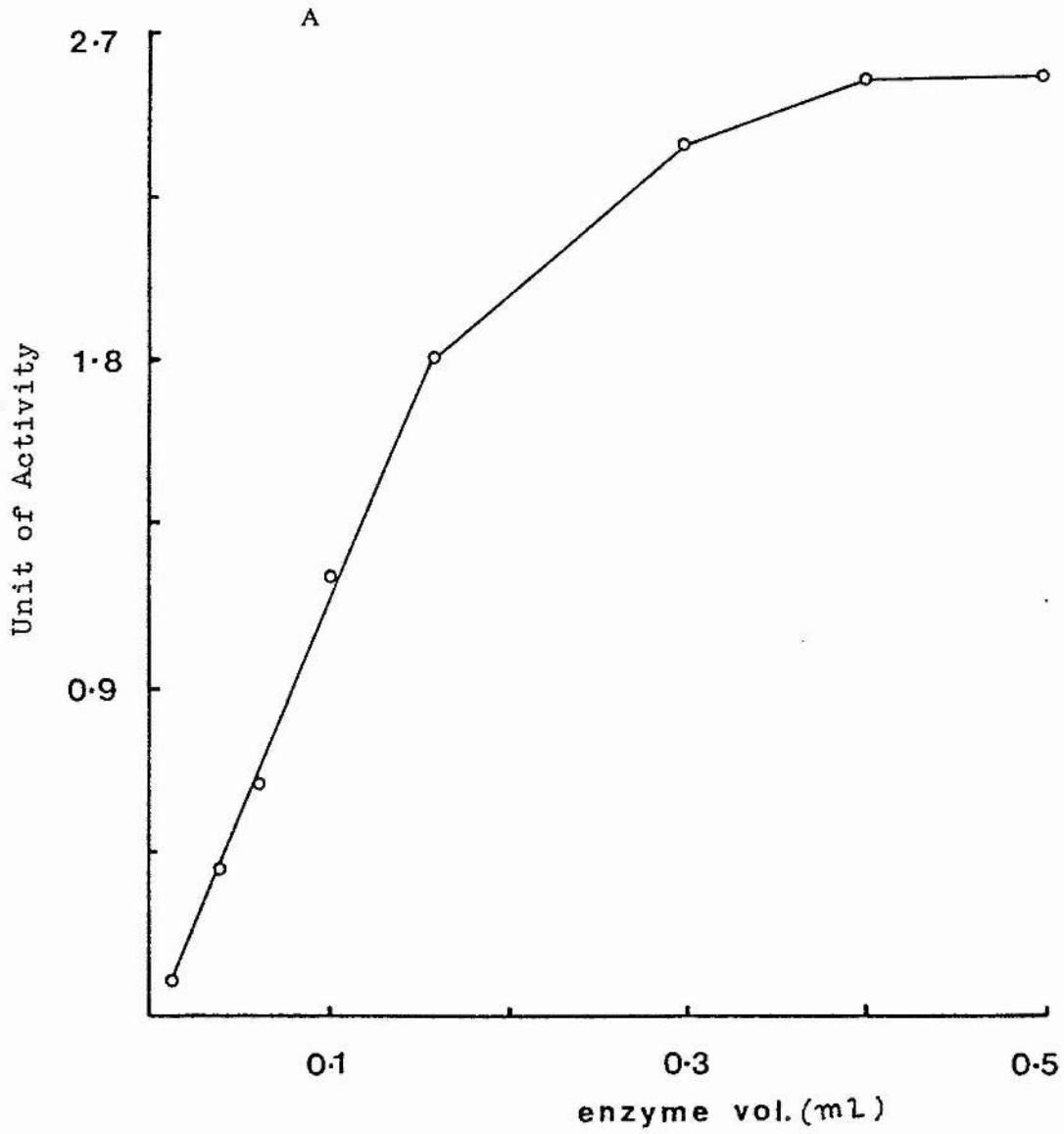
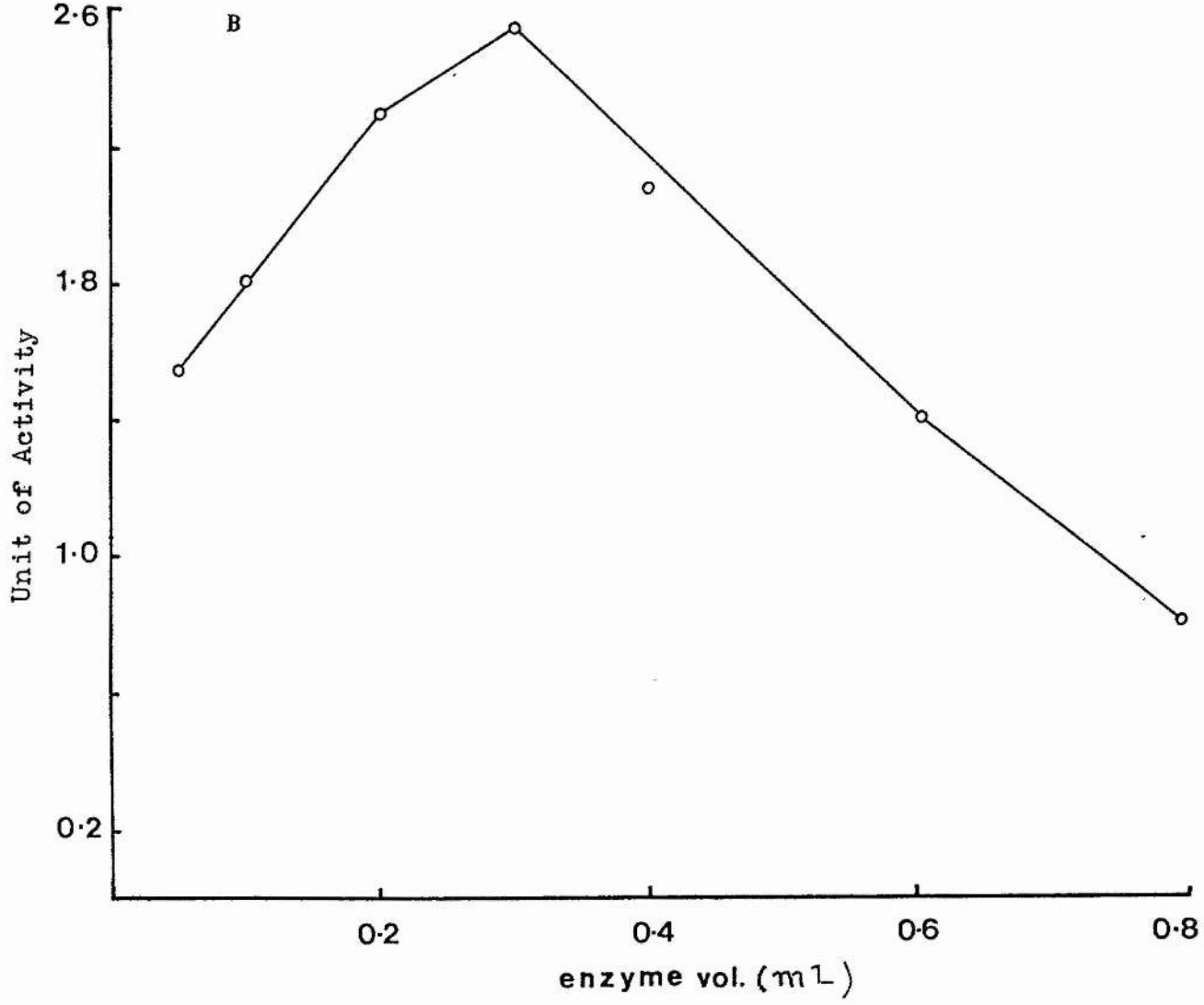

A
2·7
1·8
0·9
Unit of Activity
0·1
0·3
0·5
enzyme vol. (ml)

Fig. 5.2 (B) THE EFFECT OF ENZYME CONCENTRATION

A graph of units of activity versus a range of plasma enzyme dilution

substrate - fibrin clot

enzyme - plasminogen free plasma


B
2·6
1·8
1·0
0·2
Unit of Activity
0·2
0·4
0·6
0·8
enzyme vol. (ml)

time interval. Tests were carried out in triplicate. A graph of enzyme proteolytic activity versus time was constructed, Fig. 5.3. As the results show, after a short lag period a linear relationship between time and enzyme activity was found to be maintained up to about 16 h.

5.1.4. The effect of temperature on the proteolytic activity of plasma enzyme

Plasma enzyme was added to fibrin clots in acid media in different tubes and each one was incubated at different temperatures for 16 h. At the end of the incubation time the extent of enzyme proteolytic activity was measured and plotted against different temperatures. As results show (Fig. 5.5) the plasma enzyme was not stable at a high temperature, and all its activity was lost at 60°C.

5.1.5. Effect of temperature on enzyme stability

Plasma enzyme at 3 different stages of purification was dialysed against 0.05 M sodium citrate, pH 3.5, and kept at different temperatures for 2 and 4 h. The residual activity of these samples were estimated using fibrin clot assay as 3.2.5.

Results

Incubation at 4°C and 22°C had no effect on the enzyme proteolytic activity. At 37°C a high percentage of enzyme activity was lost in the case of the most purified plasma enzyme, Table 5.1.

Fig. 5.3 EFFECT OF PERIOD OF HYDROLYSIS

A graph of plasma enzyme units of activity versus time intervals (h)

substrate - fibrin clot

enzyme - DEAE cellulose step Table 4.7

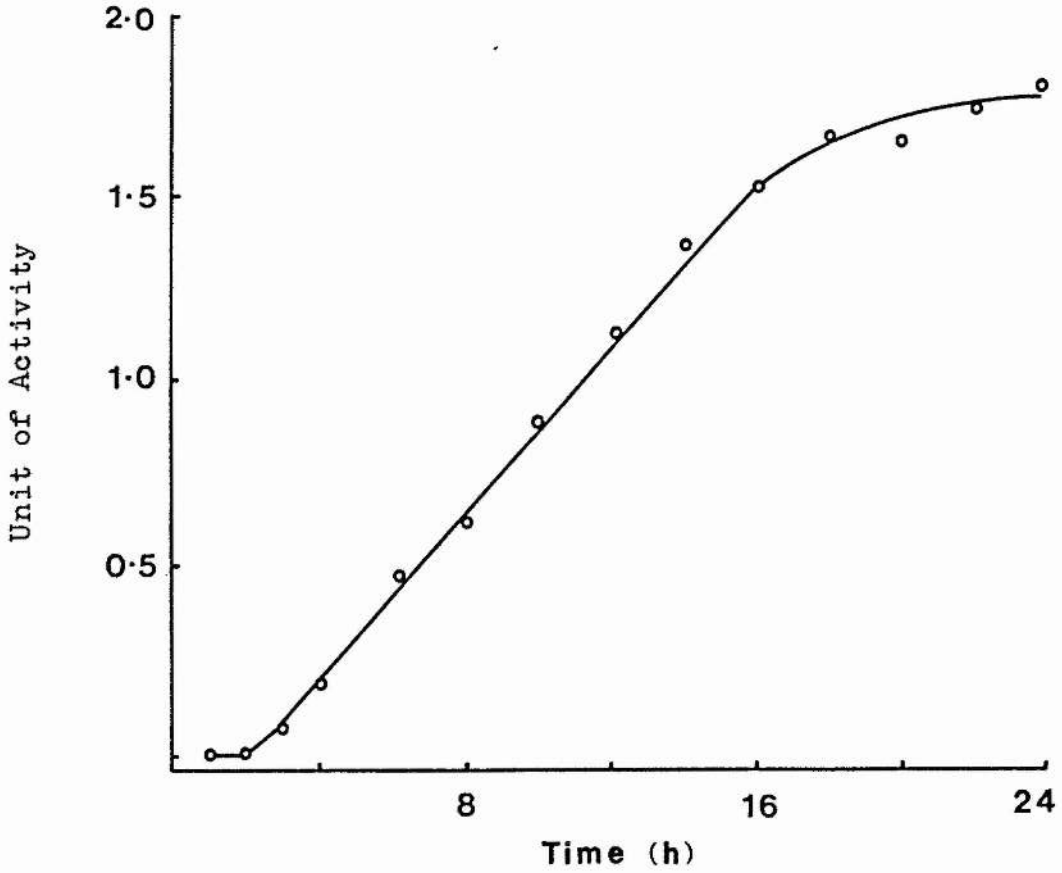

2·0
1·5
1·0
0·5
Unit of Activity
8
16
24
Time (h)

Fig. 5.5 THE EFFECT OF TEMPERATURE ON THE PROTEOLYTIC ACTIVITY OF PLASMA ENZYME

A graph of units of activity versus a range of temperature

substrate - fibrin clot

enzyme - DEAE-cellulose step Table 4.7

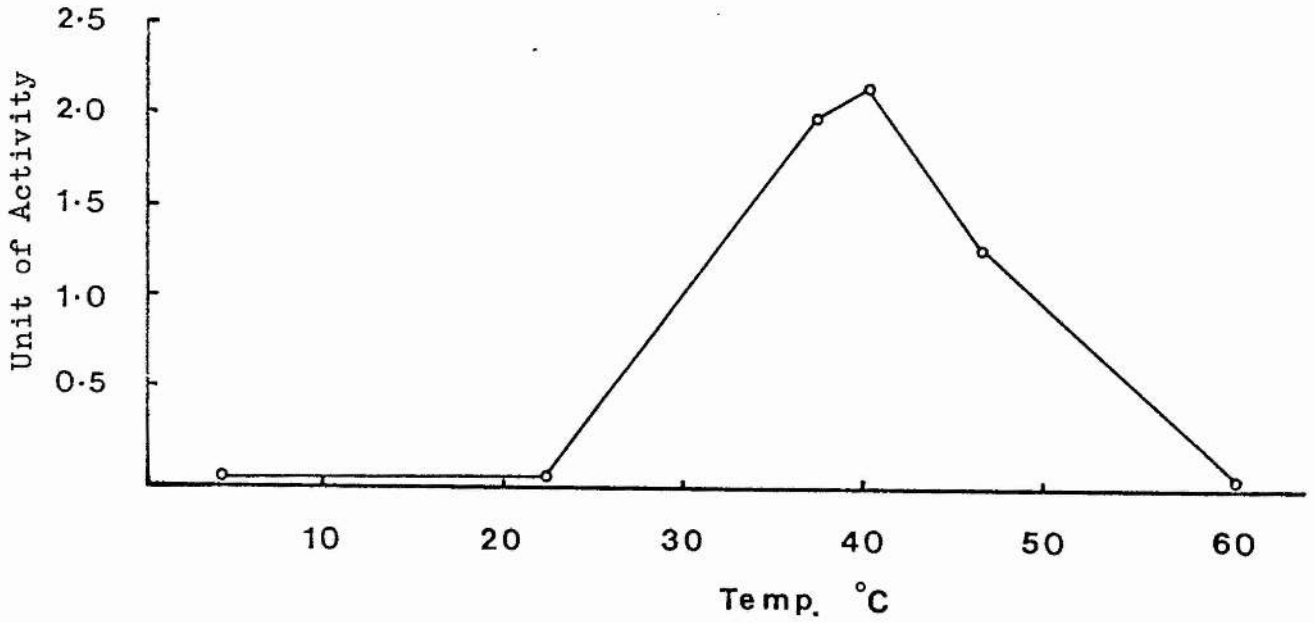

Unit of Activity
2·5
2·0
1·5
1·0
0·5
10
20
30
40
50
60
Temp. °C

Table 5.1 EFFECT OF TEMPERATURE ON ENZYME STABILITY

Plasma enzyme	(%) losses of enzyme activity 2 h 37°C	4 h 37°C
25-75% (AS)	0	0
Blue-sepharose step	5	21
DEAE-cellulose step Table 4.7	57	62

5.1.6. Stability of enzyme in the presence of substrate

Plasma enzyme at 3 different stages of purification was dialysed against 0.05 M sodium citrate, pH 3.5. Fibrin clots were added to each solution and were incubated at 37°C for 2 and 4 h. At the end of the incubation period a sample of plasma enzyme was removed and added to new fibrin clot in 1% (w/v) monochloro acetic acid, and proteolytic activity in each case was determined. As results show, Table 5.2, most of the enzyme activity is lost, which could be due to the adsorption of the enzyme to the fibrin clot.

5.2 The Effect of pH on the Proteolytic Activity of Plasma Enzyme

Azocasein, denatured bovine haemoglobin and fibrin clots were used as substrate, and proteolytic activity of enzyme was measured at range of pH using all 3 substrates.

As the results show, Fig. 5.6 A, B and C, the pH optimum for proteolytic activity of plasma enzyme was approximately 3.0.

Table 5.2 STABILITY OF ENZYME IN THE PRESENCE OF SUBSTRATE

Plasma enzyme	% losses of enzyme activity	
	2 h (37°C)	4 h (37°C)
25-75% (AS)	0%	30%
Blue-Sepharose step	48%	67%
DEAE-cellulose step	100%	100%

Fig. 5.6 (A) PROTEOLYTIC ACTIVITY OF PLASMA ENZYME DEPENDING ON pH

Substrate - azocasein

I Normal plasma

II 25-75% (AS)

III Blue-Sepharose step Table 4.7

IV DEAE-cellulose step Table 4.7

Buffer- 0.4M sodium citrate/HCl

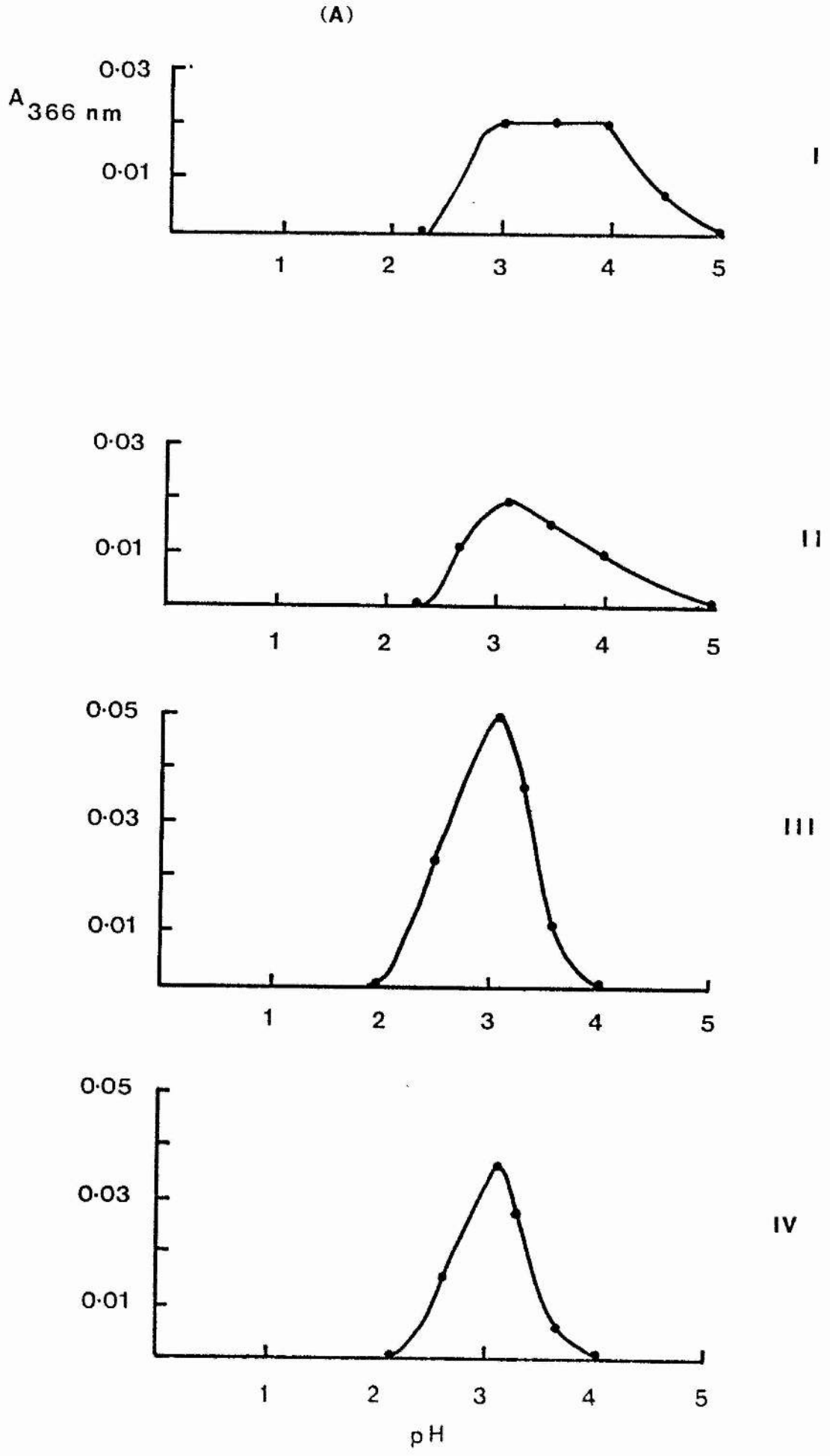

(A)
0·03
A 366 nm
0·01
I
1
2
3
4
5
0·03
0·01
II
1
2
3
4
5
0·05
0·03
0·01
III
1
2
3
4
5
0·05
0·03
0·01
IV
1
2
3
4
5
pH

Fig. 5.6 (B) PROTEOLYTIC ACTIVITY OF PLASMA ENZYME DEPENDING ON pH

Substrate - denatured bovine-haemoglobin

enzyme - Blue Sepharose step Table 4.7

Buffer- 0.1M sodium citrate/HCl

(B)

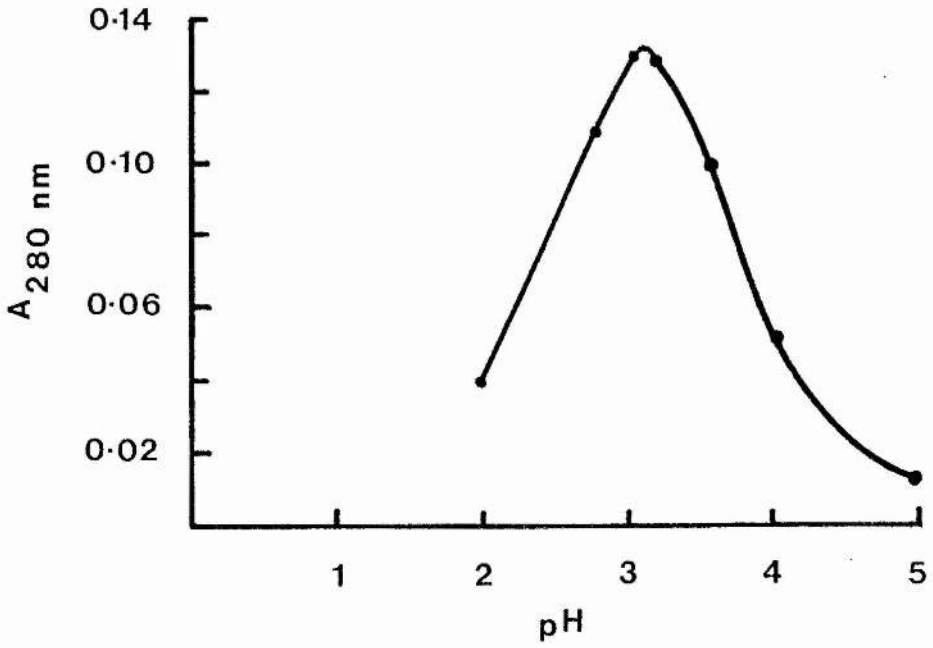


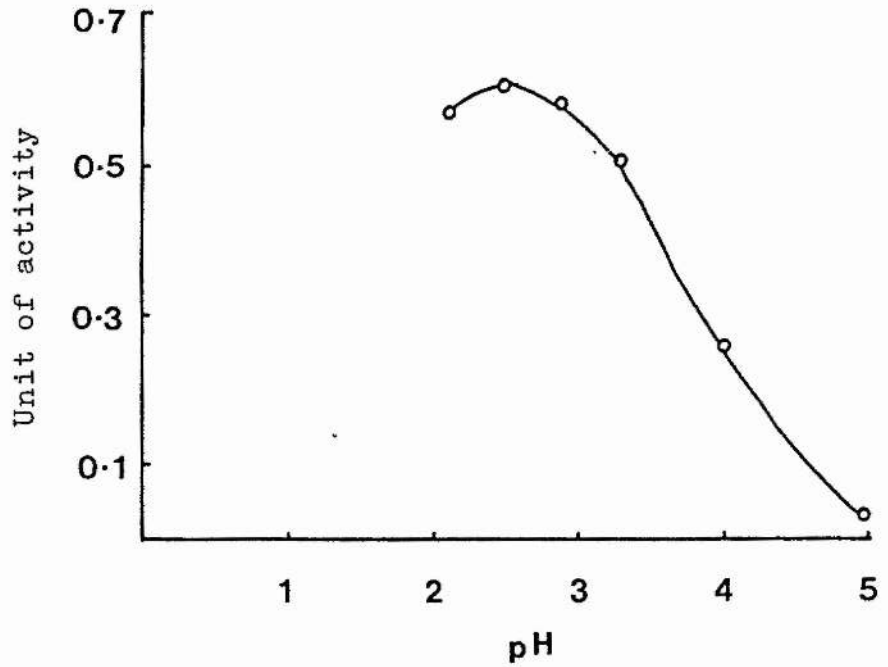
Fig. 5.6 (C) PROTEOLYTIC ACTIVITY OF PLASMA ENZYME DEPENDING ON pH

Substrate - fibrin clot

enzyme - Blue-Sepharose step
Table 4.7.

Buffer- 1% MCA(w/v)/NaOH

(C)



5.3. Inhibitors

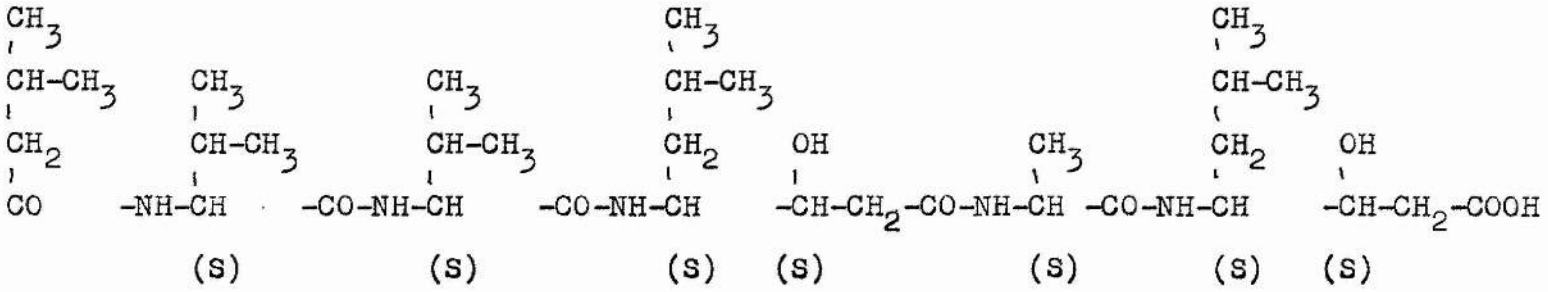
The endopeptidases are the only group of enzymes for which classification and identification on the basis of substrate specificity seems impossible,(Barrett, 1977).

Hartley (1960) classified the endopeptidases on the bases of catalytic mechanism. He divided them into four distinct groups according to the amino acid or ion which is involved in the catalytic process, serine, aspartate, metallo and cysteine proteinases.

Inhibitors give a clear evidence as to the type, the physical and chemical architecture of the active site and the kinetic mechanism of the reaction. They also help in the identification of an individual proteinase and in the investigation of its biological functions (Barrett, 1977).

A large number of proteinase inhibitors are present in many tissues of animals and plants, as well as micro organisms. Their major physiological role is to prevent unwanted proteolysis and in plants act as a defence by inhibiting the insect proteinases to prevent insect infestation (Laskowski and Kato, 1980). Proteinase inhibitors can be either reversible or irreversible.

For complete inhibition with reversible inhibitor large molar excesses may be needed, and if the binding is not very tight the inhibition may be reversed by gel-chromatography or dialysis. Irreversible inhibitors can completely inactivate the enzyme by an equimolar or slight excess in concentration. There are two groups



Pepstatin-isovaleryl-L-valyl-L-valyl- (3S, 4S)-4-amino-3-hydroxy-6-methyl heptanoyl-L-alanyl- (3S, 4S)-4-amino-3-hydroxy-6-methyl heptanoic acid.

Umezawa (1976).

of proteinase inhibitors, one group of inhibitors are those with class specific reactive sites, which inhibit only proteinases belonging to one of the four classes of proteinases. The other group contains α_2-macroglobulin (or strictly, the family of α-macroglobulins), which bind and inhibit the majority of proteinases from all four catalytic classes (Kirschke et al., 1982).

For the classification of the plasma enzyme at least one inhibitor from each class of proteinase inhibitor, as well as α_2M was used.

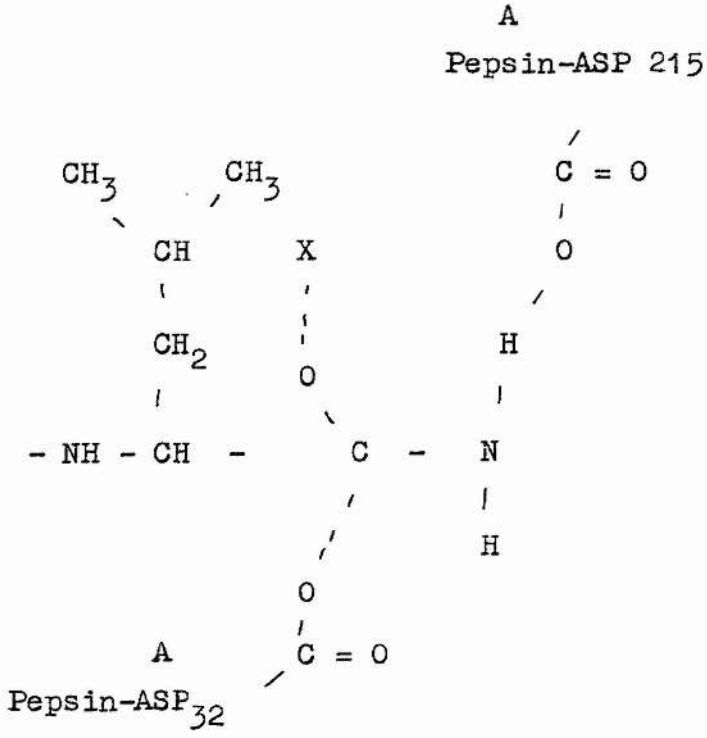
5.3.1. Inactivation of plasma enzyme with pepstatin

Pepstatin is a low molecular weight, strong inhibitor of acid proteinases, with a Ki value of approximately 1×10^{-10}M, and generally a chemical structure of isovaleryl-L valyl-L-valyl-stayl-alanyl-statine Marciniszyn et al., (1977). Pepstatin was found in the culture filtrate of various species of actinomycetes (Umezawa et al., 1970). Production of individual pepstatins depends on the fermentation conditions. In casein medium the main pepstatin contains n-caproyl group and in peptone medium, pepstatin contains an isovaleryl group. There are other pepstatin producing strains of streptomyces, but most work has been done with the pepstatin containing an isovaleryl group (Umezawa, 1976).

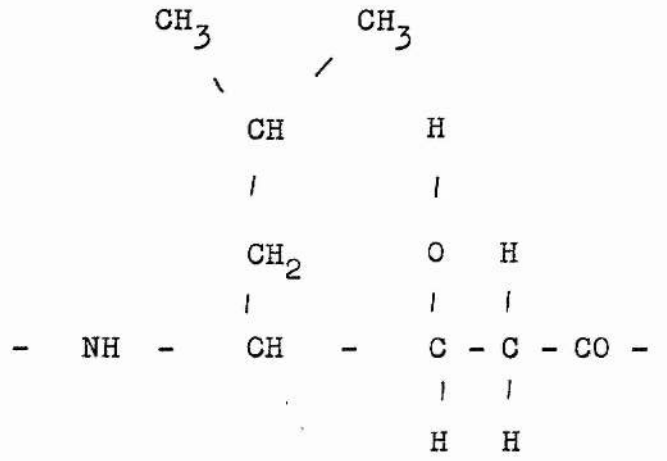
Kunimoto et al., (1974) suggested that the modification of the carboxy group of pepstatin has no effect in its inhibitory activity, but acetylation of its hydroxyl groups drastically reduces its inhibition of acid proteinases. Synthetic analogues have been

prepared and used to determine the mechanistically important groups on the molecule by Rich et al., (1980). In one compound the C-terminal β-hydroxy propionic acid group was deleted. There was no difference in its activity in comparison with pepstatin, supporting the work of Kunimoto et al., (1974). When the hydroxyl groups of the statine residues in position 3 and 5 were deleted the new compound was at least 2000 fold less effective than pepstatin in its inhibition of pepsin. Carrying this work further, they found that removal of a single hydroxyl group in the fifth residue of pepstatin had no effect, but the third residue of pepstatin is essential for its inhibitory activity. They also reported that the length of the acyl group on the amino group of statine is important too. As the chain length was extended from an acetyl group to an iso valeryl-val group, the dissocation constant decreased 10,000 fold.

Pepstatin is not an irreversible inactivator, but binds strongly to the enzyme. Marciniszyn et al., (1977) suggested that since pepstatin inhibits all the acid proteinases, and not the other groups of proteinases, there must be a common transition state which is a result of a common catalytic mechanism for acid proteinases.



Proposed transition state of peptic catalysis



A statyl residue

5.3.1.1. Assay method

Pepstatin was dissolved first in methanol (Agoyagi et al., 1971), and diluted with 1% (w/v) monochloroacetic acid. Different amounts of pepstatin (0-100 ng/assay tube) were added to fibrin clot in acid medium in the presence of plasma enzyme. The proteolytic activity of plasma enzyme was measured as 3.2.5. As Fig. 5.7 shows, 250 ng of pepstatin completely inhibited the proteolytic activity of 0.2 ml of plasma enzyme.

5.3.2. Inhibition of plasma enzyme with p-bromophenacyl bromide

p-bromophenacyl bromide is a specific inactivator of pepsin, causing 75-80% loss of activity with haemoglobin (Hb) as substrate, and causing complete inactivation with carbobenzoxy-L-glutamyl-L phenylalanine as substrate (Erganger et al., 1967). The β-carboxyl group of a residue of aspartic acid is esterified during the reversible inactivation of pepsin by p-bromophenacyl bromide (Gross and Morell, 1966).

5.3.2.1. Method

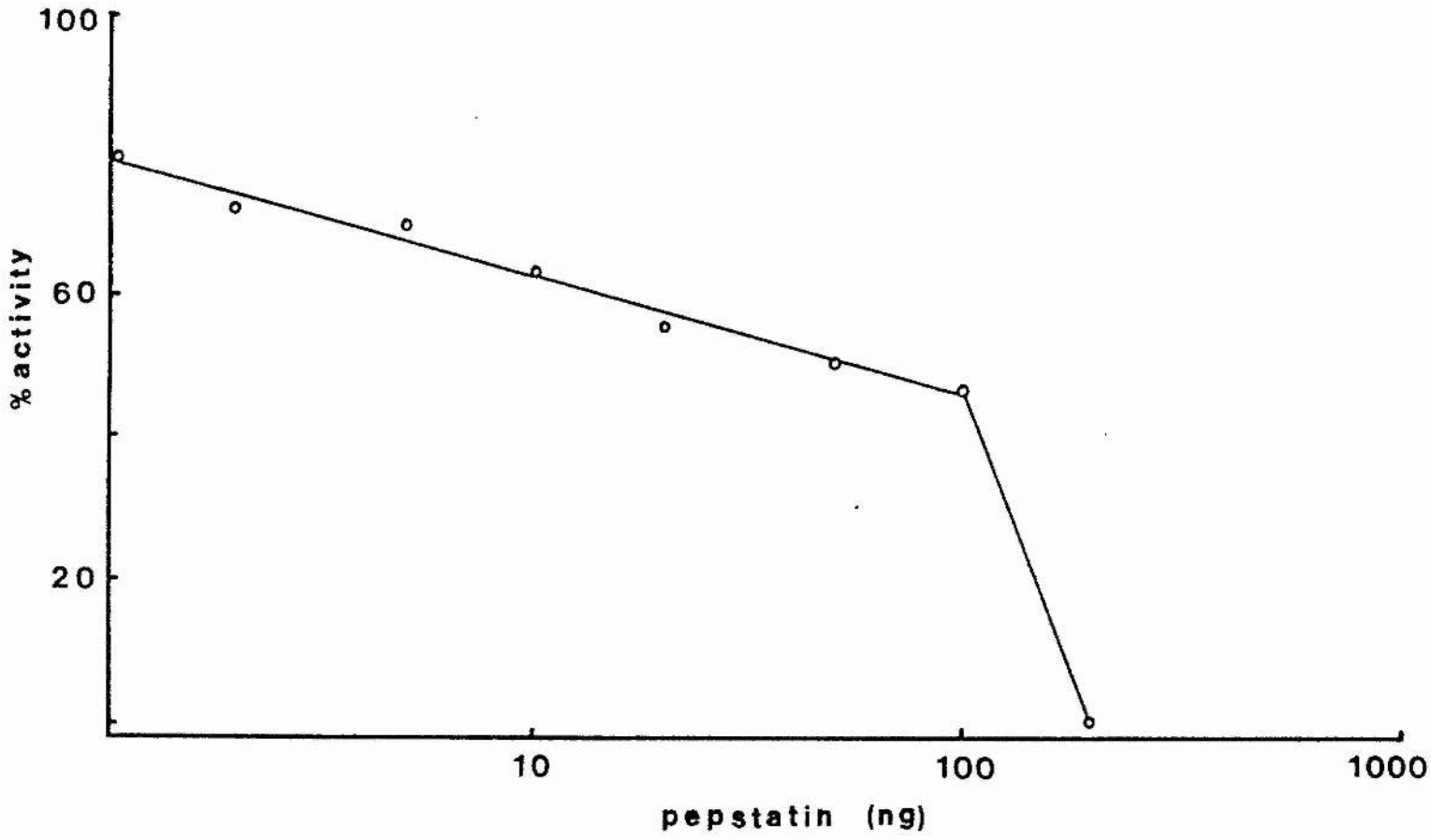
The method used was based on that of Erlanger et al., (1965, 1967). The sample was dialysed against 0.0033 M HCl, and different concentrations of the sample were mixed with 0.1 ml of 5.7×10^{-5} M p-bromophenacyl bromide. The mixtures were stirred either for 2 h at 25°C, and allowed to stand for a

Fig. 5.7 INHIBITION OF PROTEOLYTIC ACTIVITY OF PLASMA ENZYME BY PEPSTATIN

Graph of % activity versus range of pepstatin concentration

substrate - fibrin clot

enzyme - DEAE cellulose step Table 4.7.


100
60
20
% activity
10
100
1000
pepstatin (ng)

further 20 h, or the mixtures were incubated at 37°C for 3 h. Then aliquots of the samples were assayed, using denatured haemoglobin as substrate. As a control, 10 mg hog pepsin in 3.2 ml of 0.0033 M HCl (2.75×10^{-5} M) were mixed with 0.1 ml of p-bromophenacyl bromide and the mixture was treated as above.

5.3.2.2. Result

Semi-purified plasma enzyme was treated with p-bromophenacyl bromide as 5.3.2.1. The enzyme activity was tested against Hb substrate. No inhibition was detected. However, pepsin activity was 70-80% reduced. To determine whether the plasma enzyme is insensitive to the inhibitor or the condition which was ideal for pepsin was not optimal for the enzyme, a series of experiments was set up, varying parameters such as pH, incubation time, enzyme, and substrate concentration. Still no inhibition occurred in any of the experiments. The above experiments were repeated using fibrin clot as a substrate.

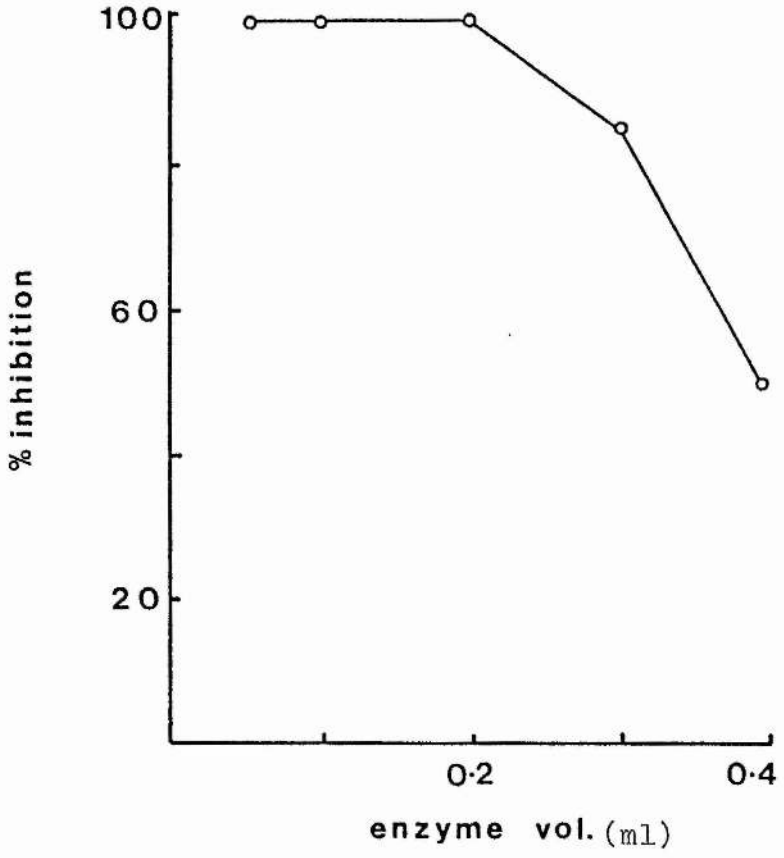
50% inhibition was obtained when the plasma enzyme was incubated with the inhibitor at 37°C for 3 h. However, when the concentration of inhibitor was increased by 10-fold, under the same conditions up to 100% inhibition was achieved, Fig. 5.8.

Fig. 5.8. INHIBITION OF PROTEOLYTIC ACTIVITY OF PLASMA ENZYME BY p-BROMOPHENACYL BROMIDE

Graph of % inhibition versus range of enzyme volume.

substrate - fibrin clot

enzyme - DEAE-cellulose step Table 4.7.


100
60
20
% inhibition
0·2
0·4
enzyme vol. (ml)

5.3.3. Inhibition of plasma enzyme with α_2-macroglobulin (α_2 M)

α_2 M is a plasma glycoprotein of high molecular weight. It is capable of binding many proteinases, mostly serine proteinases (Ogston and Bennett, 1977). Now it is believed that α_2 M will bind to enzymes of the other three classes of proteinases (Barrett and Starkey, 1973). For example, thiol proteinase cathepsin B, and a carboxyl proteinase cathepsin D. α_2 M does not bind to the inactive form of proteinases or other enzymes.

As Barrett and Starkey (1973) reported that α_2 M binds to cathepsin D, it was used to find out whether it would bind to plasma enzyme. α_2 M was prepared (Virca et al., method, 1978) and was mixed with plasma enzyme, and the mixture was assayed for proteolytic activity using fibrin clot assay (3.2.5.). There was no change in the enzyme activity. The results could be interpreted that either the α_2 M did not bind to the enzyme or it was dissociated from enzyme at low pH.

5.3.4. Inhibition of plasma enzyme with ε-amino caproic acid (ε-amino hexanoate) (EACA)

EACA is a synthetic fibrinolytic inhibitor. It differs from lysine only in the lack of an α-amino group. It is a competitive inhibitor of plasminogen activation at concentrations of 10^{-4} M and higher.

At concentration above 5×10^{-2} M EACA is also a non-competitive inhibitor of plasmin and other

proteolytic enzymes, including pepsin and trypsin (McNicol and Douglas, 1976). Woessner (1971), reported that EACA at 0.1 M inhibits the digestion of protein polysaccharide, light chain (PPOL) by cathepsin D but does not inhibit its action on Hb-substrate.

Because of the inhibitory effect of EACA on pepsin and cathepsin D, it was decided to find its effect on plasma enzyme.

Different concentrations of EACA (0.0 - 0.1 M) were mixed with plasma enzyme and the proteolytic activity of the enzyme was measured using fibrin clot assay (3.2.5).

No change in enzyme activity was detected up to 0.02 M EACA, but at about 0.04 M, the enzyme proteolytic activity was completely abolished.

The inhibitory effect of EACA (at high concentration) on plasma enzyme could be due to physical interference in the enzyme substrate interaction rather than to specific blocking of an active centre (Woessner, 1971). Or the inhibitory effect of EACA on plasma enzyme could be due to the fact that the EACA in high concentration changed the pH and salt concentration of the test solution. To overcome the problem of pH and salt concentration, aprotinin was used instead of EACA.

5.3.5. Inhibition of plasma enzyme with aprotinin (trasylol)

Aprotinin is a polypeptide of molecular weight of 6,500 from bovine lung. It is a competitive

inhibitor of plasminogen activation and a non-competitive inhibitor of plasmin. Aprotinin also inhibits kallikrein, trypsin, chymotrypsin and thromboplastin formation. Because of its anti-fibrinolytic and anti-coagulant activities, and its low toxicity, it could have possible therapeutic use for the defibrination syndromes of unascertained aetiology (McNicol and Douglas, 1975).

Since there was no conclusive result with EACA (5.3.5), aprotinin was used. Different concentrations (50-500 μ/ml) of aprotinin were mixed with plasma enzyme at pH 8.0 and the proteolytic activity of enzyme was measured using fibrin clot assay. There was no change in plasma enzyme activity.

5.3.6. Inhibition of plasma enzyme with phenyl methane sulphonyl fluoride (Pms-F)

Pms-F is a serine proteinase inhibitor. The inhibition is caused by sulphonylation of an active-site serine hydroxyl group (Means and Feeney, 1971).

Although Pms-F is primarily an inhibitor of serine proteinases, it will also inhibit thiol proteinases, but the inhibition, unlike that of serine proteinases, is reversible by dithiothreitol (Barrett, 1977).

Method

Stock solution

Stock solution of 100 mM Pms-F was prepared in isopropyl alcohol and kept in a dark bottle at 4°C. Pms-F was mixed with plasma enzyme with the

final concentration of 1.0 mM. The mixture of samples and the inhibitor were incubated at 4°C, 22°C and 37°C for 48 h. Controls were set up, by adding isopropyl alcohol to the enzyme without the Pms-F. At the end of the incubation period the samples were tested for enzyme activity using the fibrin clot assay, 3.2.5.

No significant changes in enzyme activity were detected.

5.3.7. Inhibition of enzyme with N-ethylmaleimide (NEM)

NEM is used for sulphydryl group modification. It has been used for the determination of the number of sulphydryl groups present in many different proteins. It also reacts with amino groups. The reaction is pH dependent. For example, at pH 7.0 its reaction with simple thiols is approximately 1000-fold greater than that of corresponding simple amino groups, (Means and Feeney, 1971).

Method

A stock solution of 100 mM NEM was prepared in 0.05 M Tris-HCl, pH 8.0.

The NEM was mixed with plasma enzyme, with the final concentration of 10 mM. The mixture was added to the fibrin clot in acid media, and the proteolytic activity of the enzyme was measured as under the above experimental conditions. NEM had no effect on the enzyme proteolytic activity.

5.3.8. Inhibition of plasma enzyme with ethylenediamine tetraacetic acid (EDTA).

Metallo proteinases are usually inhibited by EDTA. There are also some serine proteinases which are inhibited by chelating agents (Barrett, 1977).

Method

A stock solution of 200 mM EDTA in 0.05 M Tris-HCl, pH 8.0 was prepared. EDTA was mixed with plasma enzyme at a final concentration of 5 mM.

The proteolytic activity of the enzyme was measured using fibrin clot assay. No change was detected in the proteolytic activity of the plasma enzyme.

5.4. Effect of chemical modification of amino groups of fibrin clot on its susceptibility to lysis by plasma enzyme in acid media

Amino groups of proteins have been modified by different reagents. Riordan and Vallee (1972) used acetic anhydride for acetylation of amino groups, while Wang et al., (1978) used dinitrofluorobenzene (DNFB) or 2,4,6-trinitrobenzenesulfonic acid (TNBS) and potassium cyanate was used by Chu et al., (1969). In this project potassium cyanate was used to modify amino groups of fibrin clot. Cyanate reacts with α, ε-amino groups of protein as well as SH group, to form carbamyl derivatives. In the presence of excess cyanate, protein can be completely carbamylated.

5.4.1. Amino group modification

Method

The method used was based on that of Chu et al., (1969).

Fibrin clots were prepared as 3.2.4. Ten clots were added to 20 ml of 0.5 M Tris-HCl, pH 8.0, in 40°C water bath. 10 mg potassium cyanate was added every hour, for a period of 4 h. The incubating mixtures were kept at 40°C for a further 2 h. The pH of the solution was kept constant, using 0.1 M NaOH. Controls were prepared at the same time, by incubating clots in Tris buffer without the addition of cyanate. At the end of 6 h, clots were washed in distilled water for 18 h.

5.4.2. Results of effect of amino groups modification

Amino groups of fibrin clots were modified as 5.4.1. The effect of modification was determined by fibrin clot assay in the presence of plasma enzyme. As Table 5.3 shows, modified clots were more resistant to lysis in the presence of plasma enzyme.

Table 5.3 EFFECT OF AMINO GROUP MODIFICATION OF FIBRIN CLOT ON ITS SUSCEPTIBILITY TO LYSIS IN ACID MEDIA

Substrate	% lysis of fibrin clot in monochloroacetic acid control (Blue-Sepharose step Table 4.7)		
	No enzyme 18 & 48 h	Plasma enzyme 16 h	48 h
Modified fibrin clot	0	14.5	40
Unmodified fibrin clot in buffer	0	68	90
Unmodified fibrin clot in H_2O	0	63	86

As the above results indicate, clots were not completely resistant to lysis. Different experiments were set up to obtain an optimal condition for making fibrin clot 100% resistant to lysis. Different concentration of potassium cyanate was used, and the modification was carried out as 5.4.1. By increasing the concentration of potassium cyanate the modified clots become completely resistant to lysis, Table 5.4.

To determine the extent of modification of lysine groups, which caused the clot to become completely insoluble, the modified clots were treated with trinitro benzene sulphonic acid (TNBS).

Table 5.4

EFFECT OF CYANATE CONCENTRATION ON THE % OF AMINO GROUPS MODIFICATION OF FIBRIN CLOT AND ITS STABILITY IN ACID MEDIA IN THE PRESENCE OF THE PLASMA ENZYME

Cyanate concentration (a)	% Lysine modification (b)	% Lysis in monochloro acetic acid	
		Plasma enzyme	
		24 h	48 h
0	0	46	58
10	20%	10	26
25	38%	0	10
50	50%	0	0

(a) mg KCNO added at each hourly interval

(b) estimation as 5.5.1.

5.5. Determination of the Extent of Amino Group Blockage by Potassium Cyanate using 2,4,6-Trinitro Benzene Sulphonic acid (TNBS).

The method used was based on that of Habeeb (1966) and Kakade and Liener (1969)

TNBS reacts with free amino groups of amino acids to form trinitro phenyl (TNP) derivatives. Sulphite in TNBS is displaced by an attacking nucleophile NH_2. TNBS does not react with tyrosine or histidine side chains.

5.5.1. Method

Fibrin clots were modified, using potassium cyanate, as 5.4.1. For each fibrin clot 2 ml of distilled water, 1 ml of 4% (w/v) $NaHCO_3$, pH 8.5, and 1 ml of 0.25% (w/v) TNBS were used. $NaHCO_3$ and TNBS solutions were prepared fresh, just before use. The solutions containing fibrin clots were incubated at 40°C for 2 h.

Fibrin clots were hydrolysed by adding each clot to 3 ml of 12 M HCl, and autoclaving at 120°C, 15-17 lb/inch pressure for 1 h (Wang et al., 1978).

The hydrolysates were left at room temperature to cool. 5.0 ml of distilled water was added to each vial and were extracted twice with 10 ml ethyl ether to remove TNP-N terminal amino acids, excess TNBS, peptides and picric acid, which had been produced during the reaction.

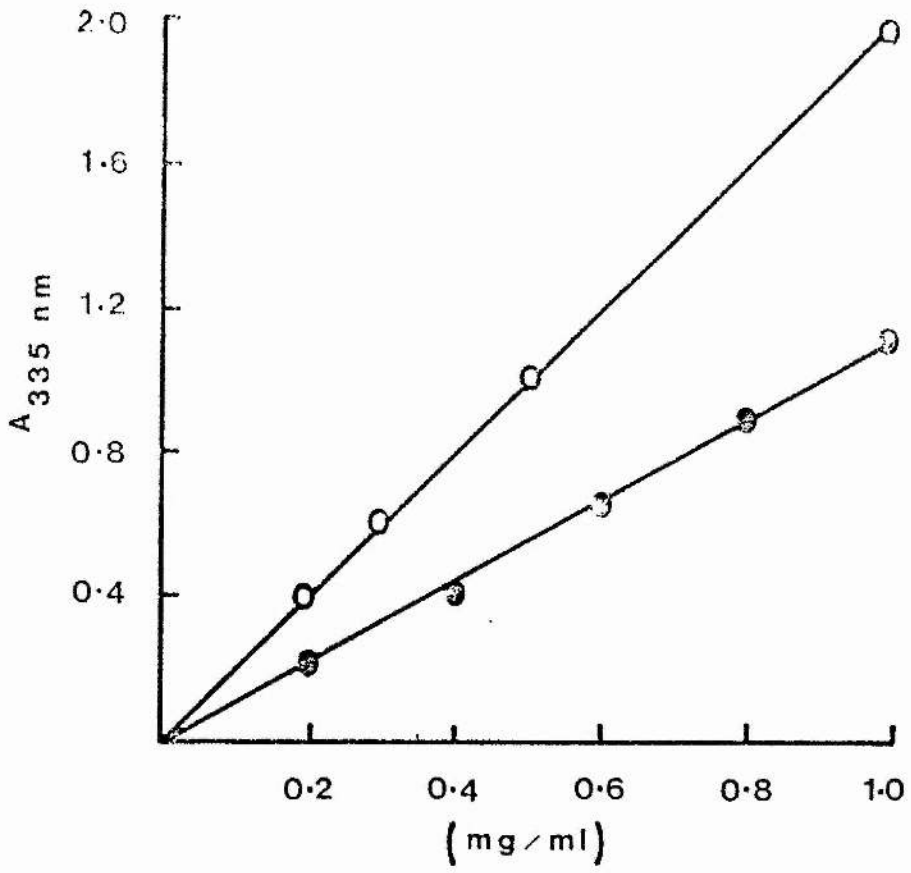
The excess ether was removed by placing the aqueous solution in boiling water for about 5 min.

The absorbance of the aqueous solution was measured at 335 nm, against a blank which had been treated as the test except that protein was replaced by distilled water. The amount of free amino groups is usually calculated from standard curve which has been constructed by using varying concentration of ε-TNP-L-lysine, and has been treated under the same condition of test. If this standard is not available, the value of 1.46×10^4 M^{-1} cm^{-1} for molar absorption of ε-TNP-lysine can be used for the calculation of lysine in protein. If the sample has been hydrolysed, value of 1.56×10^4 M^{-1} Cm^{-1} is used. To determine the sensitivity of the method bovine serum albumin (BSA) and lysozyme(muramidase) were used as standards. They were treated under the same conditions of the test. The number of amino groups per protein molecule was worked out. Standards were used with and without hydrolysis, Fig. 5.9.

As the results show, Table 5.4, 50% modification of lysine groups in fibrin clot was sufficient to make it insoluble in acid media in the presence of plasma enzyme.

Fig. 5.9 REACTION OF BOVINE SERUM ALBUMIN AND LYSOZYME WITH TNBS

A graph of variation of absorbance with concentration of BSA (O - O) and lysozyme (●–●).


2·0
1·6
1·2
0·8
0·4
$A_{335\ nm}$
0·2
0·4
0·6
0·8
1·0
(mg / ml)

5.6. Effect of Carboxyl Group Modification of Fibrin Clot on its Susceptibility to Lysis by Plasma Enzyme in Acid Media

5.6.1. Method

The method used was based on that of Hoare and Koshland, 1967.

Fibrin clots were made as 3.2.4. Each fibrin clot contained approximately 2.5 mg protein. 15 clots were added to 40 ml solution of 1.3 M glycine ethyl ester, 7.5 M urea, pH 4.75. The solution was equilibrated in 25°C water bath for 30 min. 13.5 ml of 0.4 M carbodiimide in 7.5 M urea was added, to initiate the reaction. The pH of the mixture was maintained constant at pH 4.75 by the addition of 0.5 M HCl, using radiometer pH stat assembly. Acid addition had ceased by 3 h at which point the clots were removed, and washed in 0.001 M HCl for 48 h. The test was also carried out using dansyl cadaverine (7 mg in 10 ml of 0.2 M HCl containing 7.5 M urea pH 4.7) instead of carbodiimide.

For both methods controls were prepared as follow: I) clots kept in distilled water at 25°C; II) clots were incubated at 25°C in 7.5 M urea.

5.6.2. Result

Effect of carboxyl group modification of fibrin clot on its susceptibility to lysis in acid media

Fibrin clots were modified as 5.6.1. The modified clots and controls both became resistant to lysis,

Table 5.5, which suggests that some chemical changes have taken place in urea solution as well as carboxyl group modification. Stark et al., (1960), reported that the chemical changes of ribonuclease in urea solution was due to the presence of cyanate. Also as previously (5.4) described, fibrin clots were modified with cyanate, the urea was acid washed as 5.6.3. The results did not change, i.e. at least cyanate at that concentration was not responsible. It is known that urea causes some physical disruption of protein, which could result in conformational changes. Because of the action of urea on fibrin clots interpretation of the results was not possible.

5.6.3. Preparation of cyanate free urea

The method used was based on that of Stark et al., (1960). Cyanate is known to react with amino groups, and a stable product is formed. The reaction takes place between unprotonated amino groups and the electrically neutral cyanic acid.

Method

To remove the cyanate from urea, the pH of 8 M urea was brought to pH 2.0 by using concentrated HCl. The urea was kept at room temperature for 1 h before use.

Cyanate is unstable at low pH and it breaks down to CO_2 and ammonium ion. The urea at low pH contain less than 0.001 M cyanate.

Table 5.5

EFFECT OF CARBOXYL GROUP MODIFICATION OF FIBRIN CLOT ON ITS SUSCEPTIBILITY TO LYSIS BY PLASMA ENZYME IN MONOCHLORO ACETIC ACID

	% lysis
Fibrin clot in dis H_2O	56
Fibrin clot in urea	0
Fibrin clot in urea/dansylcadaverine	0
Fibrin clot in urea/carbodiimide	0

5.7. SDS Polyacrylamide Gel Electrophoretic Patterns of Fibrin Degradation by Plasma Enzyme at Various Stages of Proteolysis

Fibrin clots were incubated in 1% (w/v) monochloro acetic acid in the presence of plasma enzyme at 37°C. Clots were removed at different time intervals. The clots were washed in 0.9% (w/v) NaCl, and reduced as 2.1.11 before being subjected to gel electrophoresis. Controls were set up by incubating fibrin clots in 1% (w/v) monochloro acetic solution, without the enzyme. Also, to apply the same amount of protein to gels, duplicates were set up and protein concentrations were measured simultaneously each time a clot was removed for application to SDS gel.

As the results show, Fig. 5.10, α-chain was the most susceptible to lysis by plasma enzyme, followed by γ-γ dimer, and the least amount of lysis was from β chain.

Comment

The results are rather interesting, since it may help to explain the reason why fibrin clot is a better substrate for plasma enzyme than casein, Chapter III. Jolles et al., (1978) comparing the structure of cow and sheep κ-casein with γ-chain of fibrinogen, found that 80% of whole protein of κ-casein is homologous to γ-chain. All homologies were limited to the N-terminal half of the κ-chain. Although there is a great similarity between the two, under the same condition chymosin splits only peptide bonds

Fig. 5.10 DENSITOMETRIC SCAN OF FIBRIN DEGRADATION BY PLASMA ENZYME AT VARIOUS STAGES OF PROTEOLYSIS

5.10. I Fibrin clot in acid media in the presence of plasma enzyme

Time = zero

Peak 1 α- polymer (intermediate)

Peak 2 γ-γ dimer

Peak 3 α-chain

Peak 4 β-chain

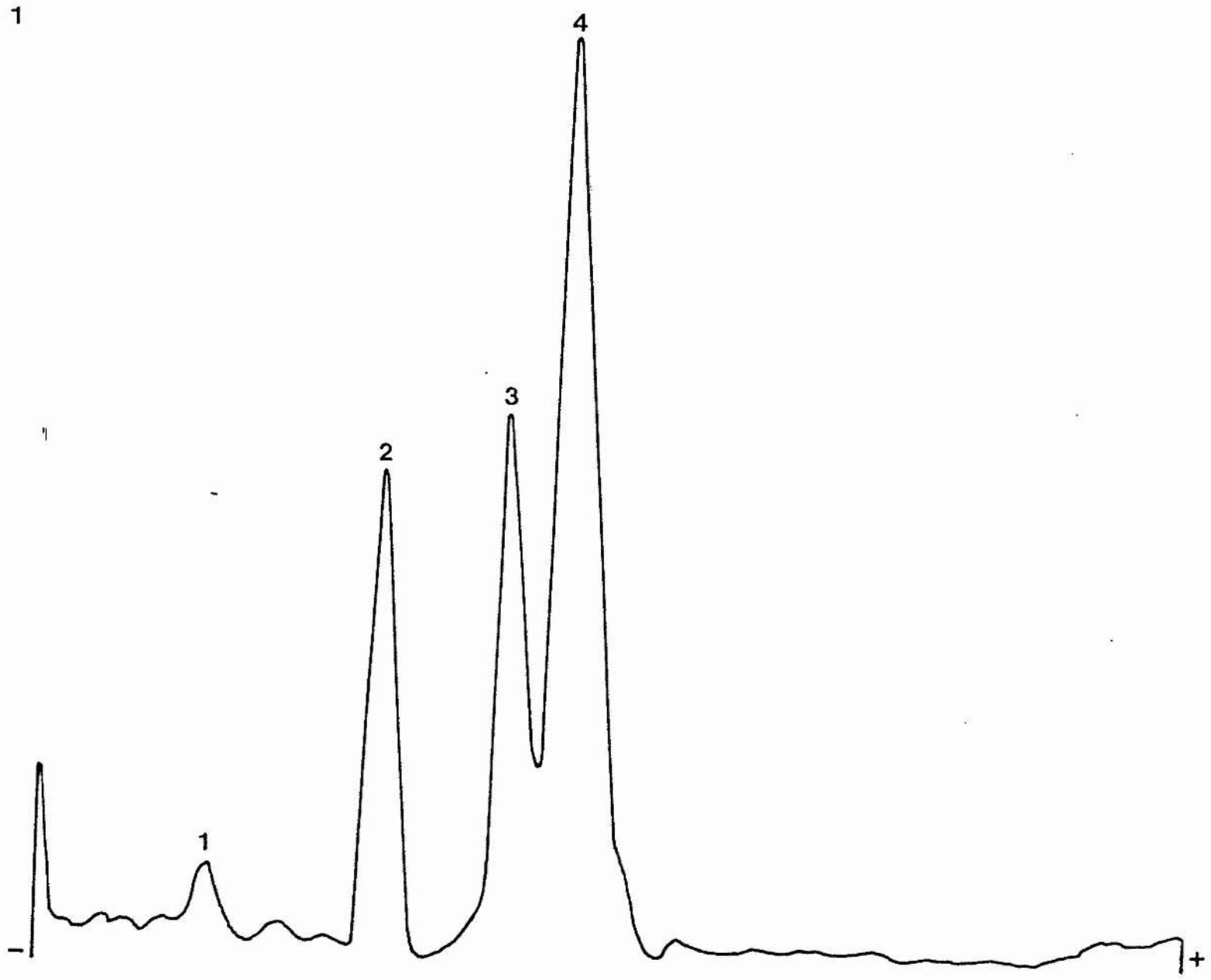

1
4
2
3
1
−
+

Fig. 5.10. II Densitometric scan of fibrin degradation by plasma enzyme

Fibrin clot in acid media in the presence of plasma enzyme

after 3 h

Peak 1 γ- γ dimer

Peak 2 α-chain

Peak 3 β-chain

II

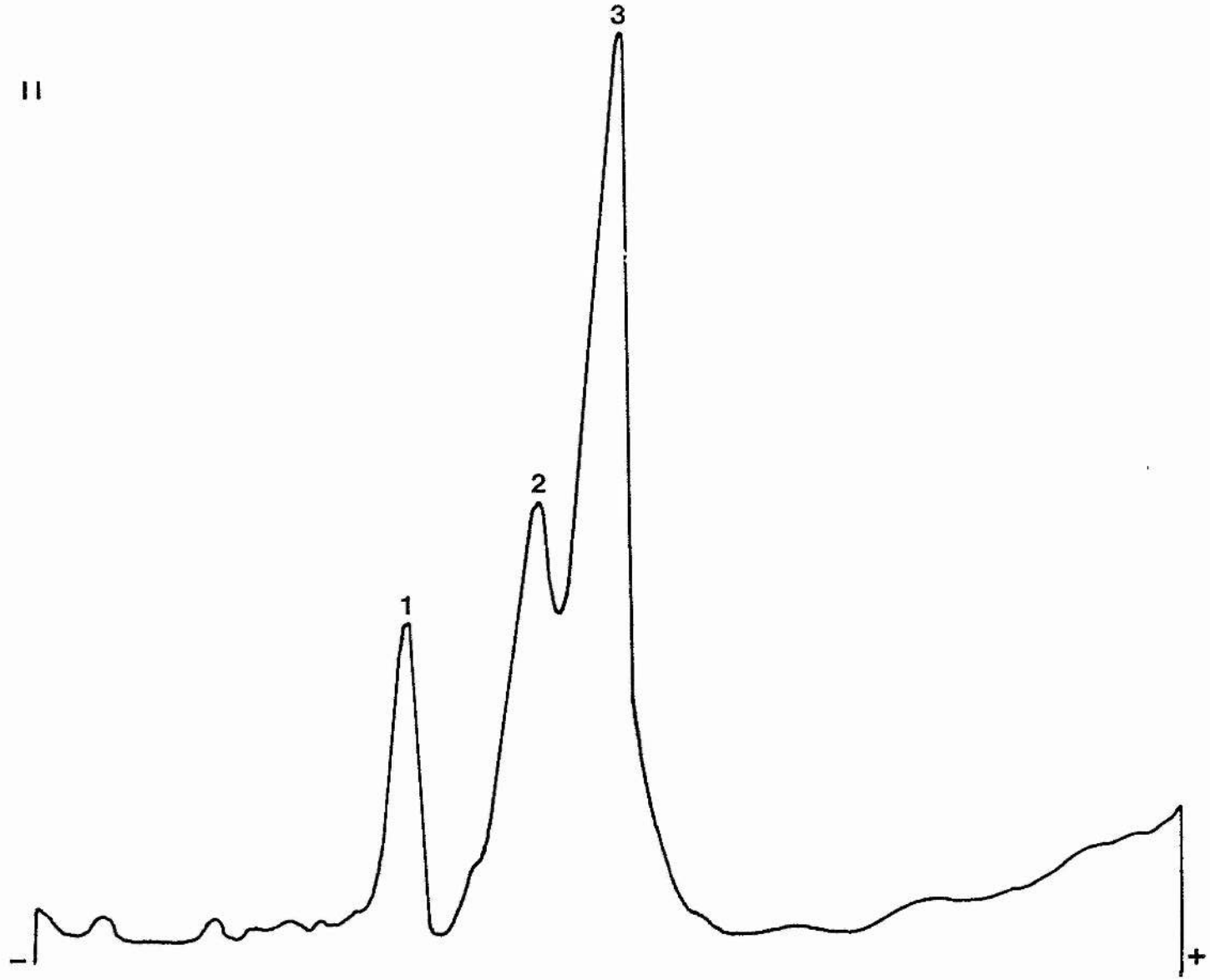


Fig. 5.10. III <u>Densitometric scan of fibrin degradation by plasma enzyme</u>

Fibrin clot in acid media in the presence of plasma enzyme

after 9 h

Peak 1 γ- γ dimer

Peak 2 α-chain

Peak 3 β-chain

Peak 4 γ-chain

III

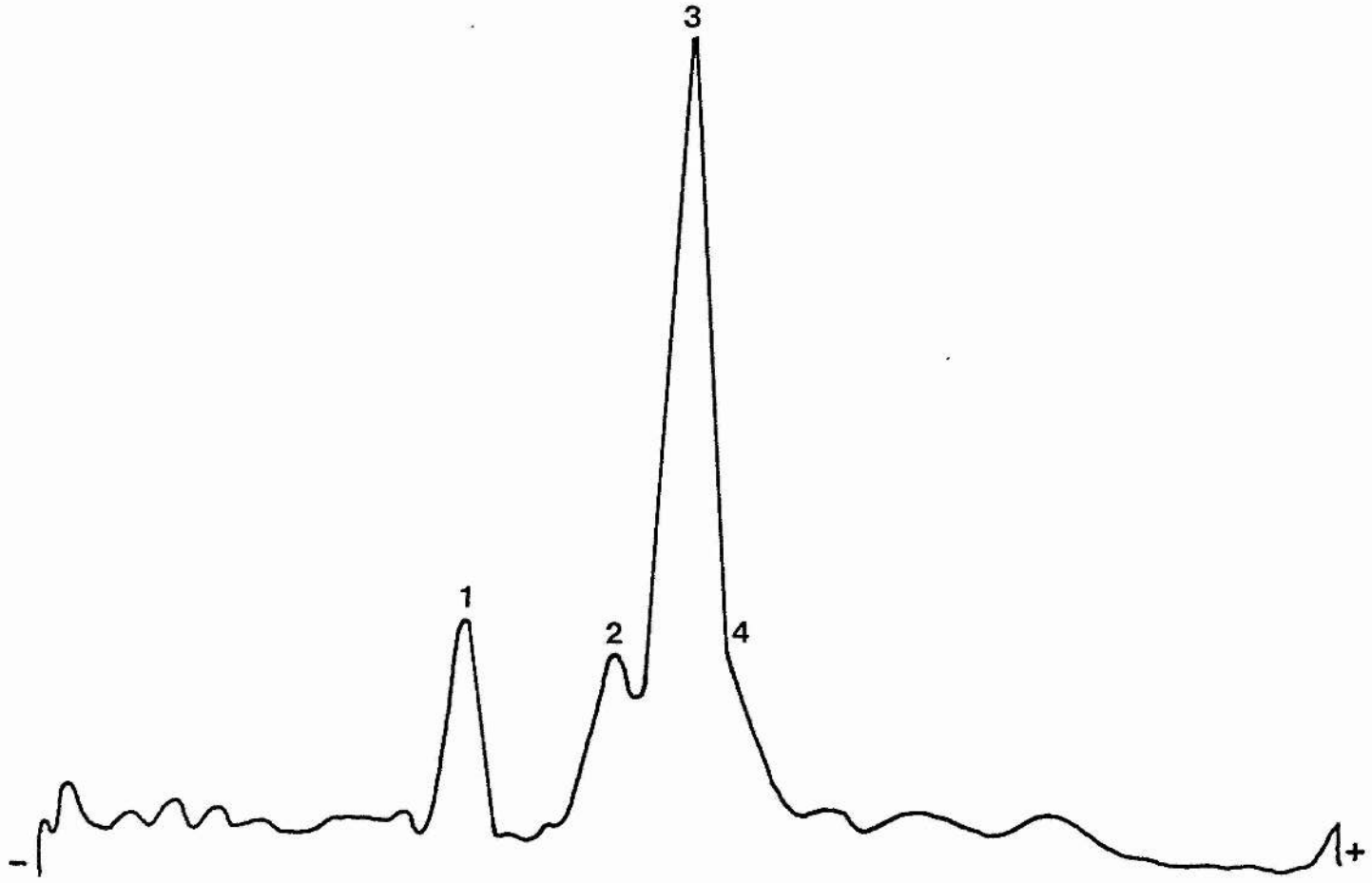
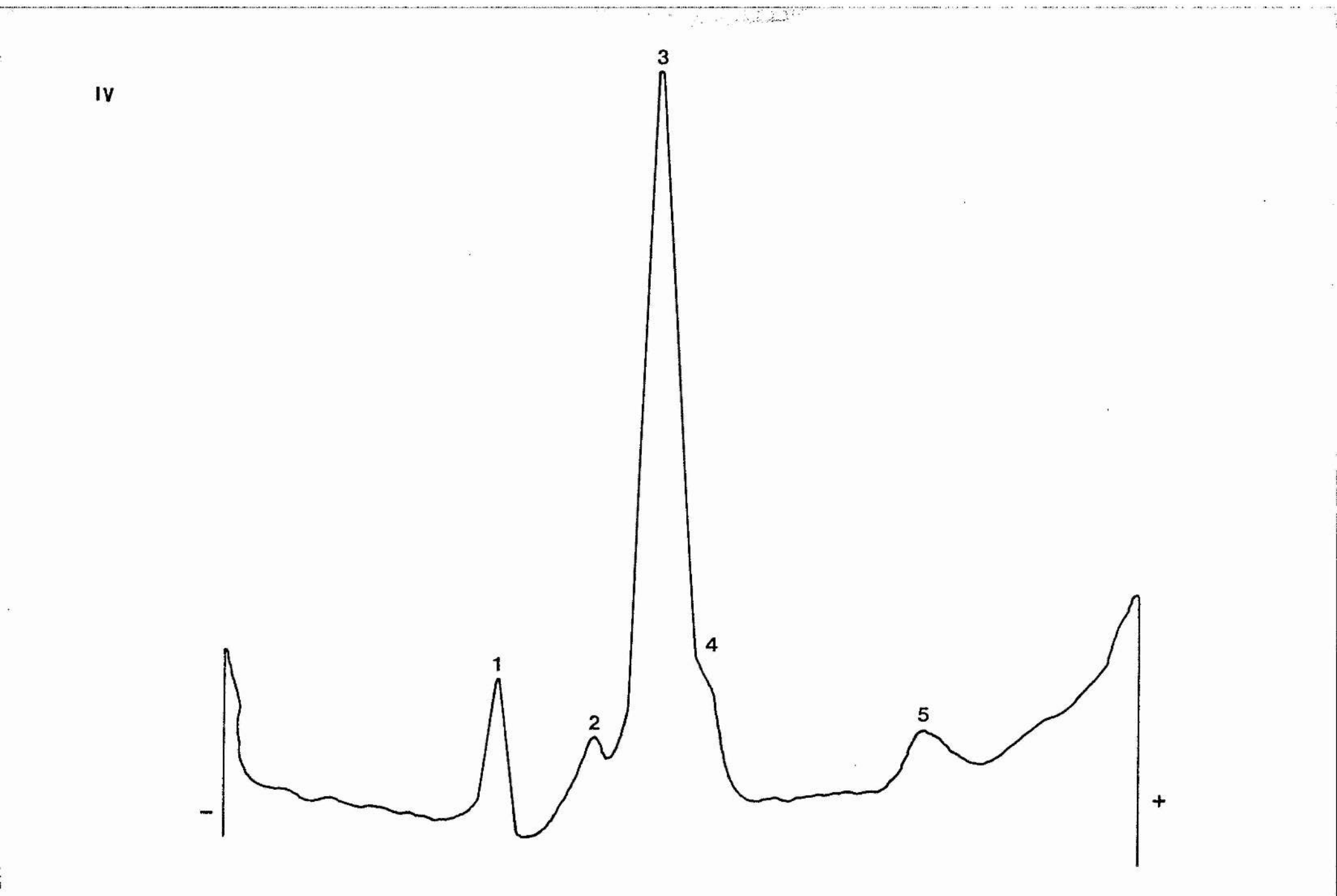


Fig. 5.10 IV <u>Densitometric scan of fibrin degradation by plasma enzyme</u>

Fibrin clot in acid media in the presence of plasma enzyme.

after 16 h

Peak 1	γ - γ dimer
Peak 2	α -chain
Peak 3	β -chain
Peak 4	γ -chain
Peak 5	fibrin degradation


IV
3
1
2
4
5
−
+

105-106 of κ-casein but rapidly splits several peptide bonds of the γ-chains.

The same statement as above could thus be true for plasma enzyme, i.e. like chymosin it can split more peptide bonds of α-chains of fibrin than casein. Plasma enzyme not only hydrolyses the γ-chains but also hydrolyses the α-chains at a faster rate.

5.8. Comparison of Plasma Enzyme with Other Proteinases

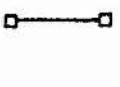
In all the experiments in which plasma enzyme was compared with pepsin, a fixed concentration of plasma enzyme and pepsin which showed the same amount of proteolytic activity in the fibrin clot assay were chosen. Usually 5-10 ng pepsin hydrolysed the same amount of fibrin clot as 0.2 M of plasma enzyme. Also, as the plasma enzyme was not completely pure, to overcome the difficulty of the interpretation of the results, pepsin was mixed with inactivated semi-purified enzyme before being subjected to the test system.

5.8.1. Comparison of plasma enzyme with human pepsin using different substrates

Fibrin clot, denatured haemoglobin, Azo`-casein and synthetic substrate (N-acetyl phenylanalyl-L-diiodotyrosine) were used. A fixed concentration of plasma enzyme which gave the same amount of activity with fibrin clot was used, as mentioned before, 3.2.5. Pepsin digests synthetic substrate, while the plasma enzyme had no effect.

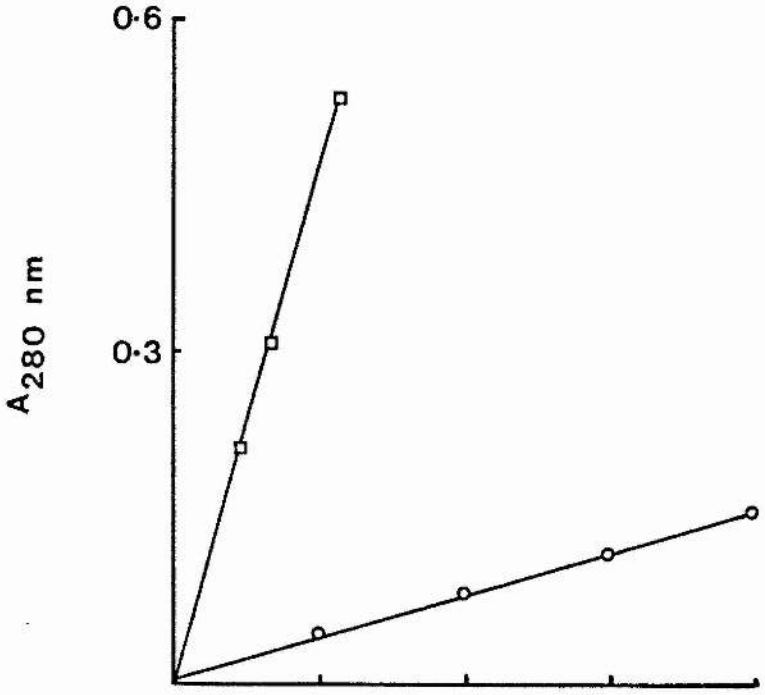
Pepsin had greater activity on azo-casein and denatured haemoglobin is at least 20 times more susceptible to pepsin than plasma enzyme, Fig. 5.11.

Fig 5.11 COMPARISON OF ACTIVITIES AGAINST DENATURED HAEMOGLOBIN AND FIBRIN CLOT SUBSTRATE



pepsin

plasma enzyme (DEAE-cellulose step, Table 4.7).



Enzyme "units"

(calculated from fibrin assay)

5.8.2. Effect of temperature on plasma enzyme and pepsin proteolytic activity

Plasma enzyme and pepsin were added to fibrin clots in acid media in different tubes and were incubated at different temperatures for 16 h. Then the proteolytic activity of the enzymes was measured.

As results show, Fig. 5.12, plasma enzyme is not stable at 45°C, while pepsin does not lose its proteolytic activity at that temperature.

5.8.3. Comparison of pH activity profile of plasma enzyme and pepsin

Proteolytic activity of plasma enzyme and pepsin were measured at different pH values using azo-casein as substrate.

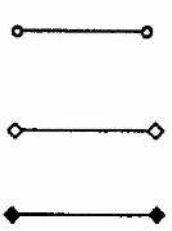
As results show, Fig. 5.13, there is a slight difference between the pH optima of two enzymes pH 3.2 and 2.6 for plasma enzyme and pepsin respectively.

5.8.4. Comparison of effect of salt concentration on plasma enzyme and pepsin proteolytic activity on fibrin clot

Proteolytic activity of plasma enzyme was compared with pepsin, using fibrin clot assay in the presence of different concentrations of NaCl.

As results show, Fig. 5.14, in contrast to plasma enzyme activity which was completely suppressed at salt concentration of 5 mM, the proteolytic activity of pepsin was increased as the salt concentration of the salt increased up to 7 mM. At higher salt

Fig. 5.12 EFFECT OF TEMPERATURE ON PLASMA ENZYME AND PEPSIN PROTEOLYTIC ACTIVITY - SUBSTRATE - FIBRIN CLOT



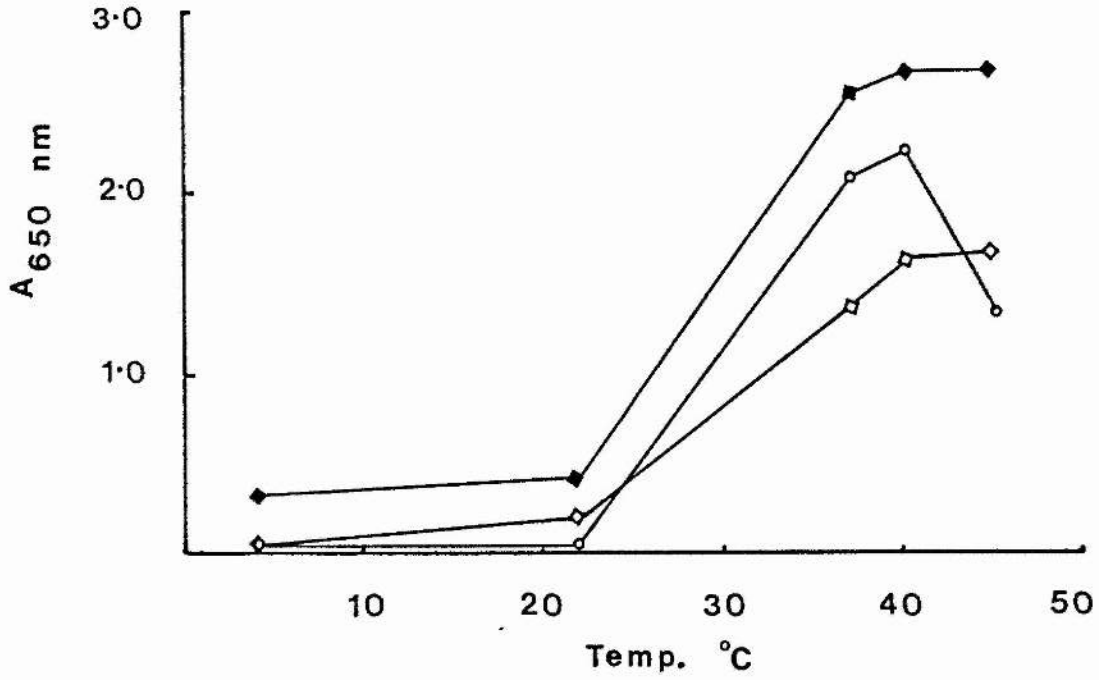

3·0
2·0
1·0
A_{650} nm
10
20
30
40
50
Temp. °C

Fig. 5.13 pH ACTIVITY PROFILES OF PLASMA ENZYME (●—●) AND PEPSIN (O—O) SUBSTRATE - AZOCASEIN

Graph of proteolytic activity versus a range of pH.

The pH optimum of the plasma enzyme and pepsin were approximately 3.2 and 2.6 respectively.

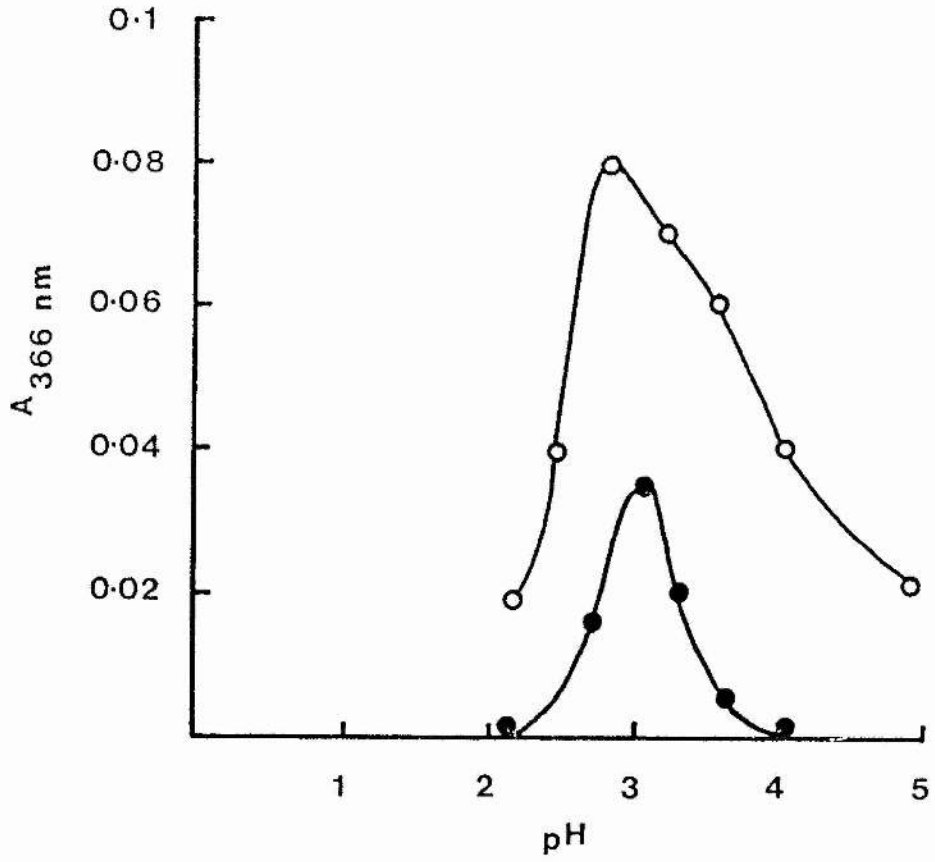
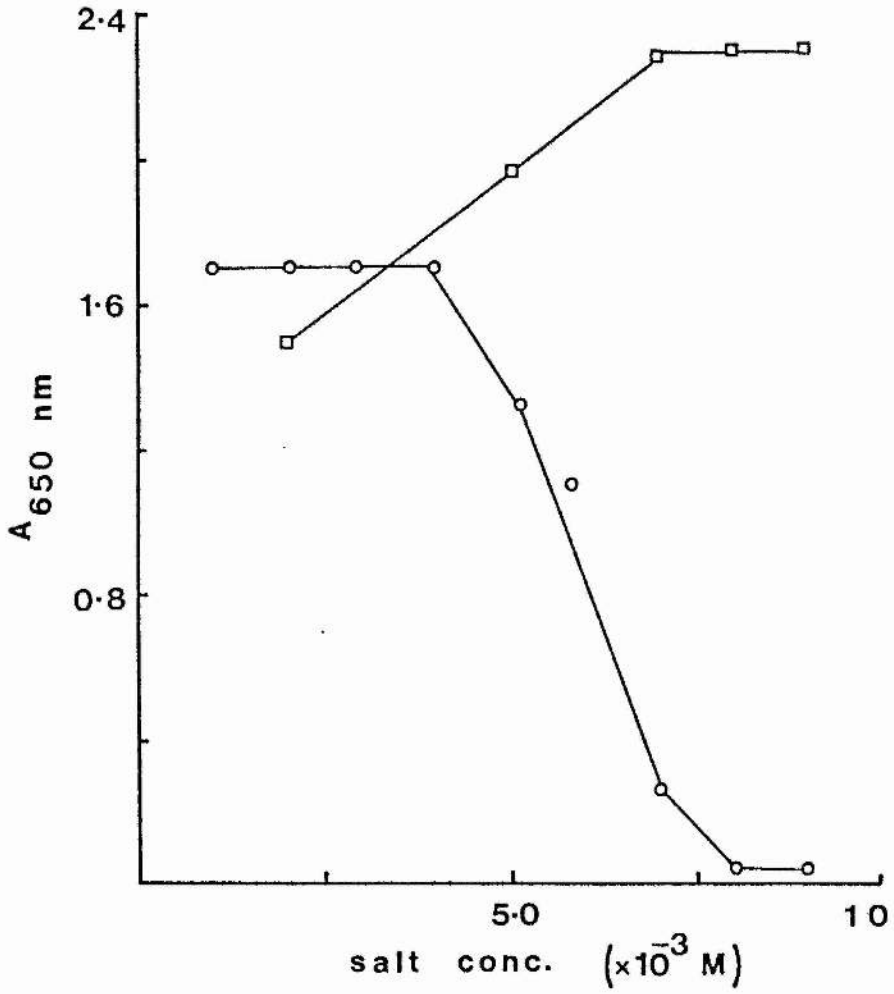

0·1
0·08
0·06
0·04
0·02
$A_{366\ nm}$
1
2
3
4
5
pH

Fig. 5.14 EFFECT OF SALT CONCENTRATION ON PLASMA ENZYME AND PEPSIN USING FIBRIN CLOT AS SUBSTRATE

A graph of salt concentration versus enzymes Proteolytic activity. (NaCl)

o——o plasma enzyme (DEAE-cellulose step Table 4.7)

◇——◇ pepsin



(NaCl)

concentration (7-10 mM) no further increase in the proteolytic activity of pepsin was detected.

5.8.5. Effect of inhibitor, p-bromophenacyl-bromide on plasma enzyme and pepsin

Proteolytic activity of plasma enzyme was compared with pepsin, using fibrin clot assay in the presence of inhibitor as 5.3.2.1.

As results show, Table 5.6, a higher concentration of inhibitor is needed to inhibit plasma enzyme to the same extent as pepsin inhibition.

Table 5.6. EFFECT OF INHIBITOR, p-BROMO PHENACYL-BROMIDE ON PLASMA ENZYME AND PEPSIN

	% Inhibition	
Inhibitor concentration	5.7×10^{-5} M	5.7×10^{-4} M
Plasma enzyme Blue-Sepharose step-Table 4.7	50%	100%
Pepsin	100%	100%

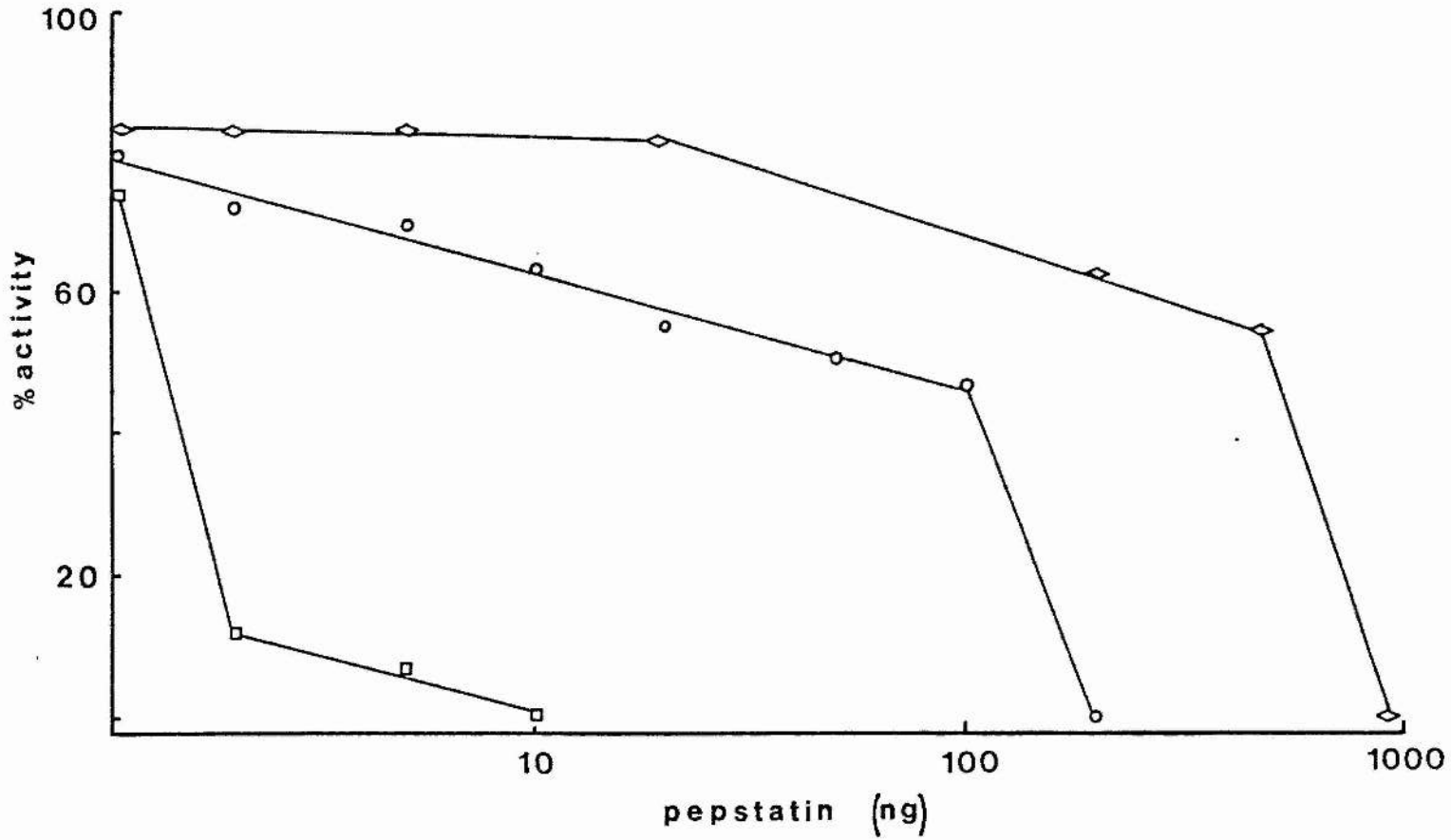
5.8.6. Effect of inhibitor pepstatin on plasma enzyme, pepsin and gastricsin

Proteolytic activity of plasma enzyme (DEAE-cellulose step, Table 4.7) was compared with pepsin and gastricsin using fibrin clot assay, in the presence of different concentrations of pepstatin.

As the results show, Fig. 5.15, pepstatin is 100-fold more effective on inhibiting pepsin than gastricsin, while only 20 times more effective in inhibiting plasma enzyme.

Fig. 5.15 EFFECT OF INHIBITOR PEPSTATIN ON PLASMA ENZYME o—o , PEPSIN □—□ AND GASTRICSIN ◇—◇

Fibrin clot was used as substrate


100
60
20
% activity
10
100
1000
pepstatin (ng)

5.8.7. Comparison of the effect of amino group modification of fibrin clot on its susceptibility to lysis in acid media in the presence of plasma enzyme and pepsin

Proteolytic activity of plasma enzyme (Blue-Sepharose step, Table 4.7) and pepsin were estimated using modified fibrin clot, 5.4.1.

Table 5.7

Substrate	% lysis in monochloroacetic acid			
	plasma enzyme		pepsin	
	24 h	48 h	24 h	48 h
Modified fibrin clot	14.5	40	39	100
Unmodified fibrin clot	68	90	70	100

As above results show, Table 5.7, modified clots are more resistant to plasma enzyme than to pepsin. Even after increasing the concentration of potassium cyanate (modifying reagent), Table 5.8, the modified clots were not completely resistant to pepsin in contrast to plasma enzyme. That was not surprising as Powers et. al., (1977) analysed the nature of substrate binding site of pepsin, by studying all the papers published during 1967-1972

Table 5.8.

Cyanate concentration (a)	% lysine modification (b)	% lysis in MCA			
		Plasma enzyme (24 h)	Plasma enzyme (48 h)	Pepsin (24 h)	Pepsin (48 h)
0	0	46	58	46	65
10	20	10	26	26	60
25	38	0	10	16	57
50	50	0	0	12	30

(a) mg KCNO added at each hourly addition

(b) estimated as 5.5.

in Biochemistry, J. Biol. Chem. and Biochem J. where information had been given regarding the cleavage of proteins and peptides by pepsin. The examination of data showed that the majority of the peptide bonds with high cleavage probabilities have aromatic or hydrophobic amino acids. They found some examples of positively (Met-Lys, Trp-Lys, His or Lys) or negatively charged (Asp or Glu) amino acids in bondswhich have been split by pepsin.

However, when fibrin clots were modified with TNBS under optimal conditions, they became completely resistant to lysis in the presence of pepsin. Even with a very high concentration of pepsin (0.1 M g/ml) in pH ranges of 2-5 at 37°C for 72 h, no lysis took place. This can be due to the fact that the actual nature and the site of cleavage is not the only factor, but other amino acid residues in either side of the bond can play an important role, e.g. conformational changes, or due to their charges.

5.8.8. Comparison of immunocross reactivity of fibrin degradation products by plasma enzyme and pepsin with fibrinogen fragments D and E

Human plasma was used to prepare fibrin clot. Plasma enzyme and pepsin were added to fibrin clots in 1% (w/v) monochloro acetic acid in 37°C. Once fibrin clots were completely degraded (visually), samples were removed and tested against rabbit anti-sera to human fibrinogen as 2.4. At the same time fibrinogen fragments D and E were tested. As the results show, fibrin degradation by plasma enzyme and pepsin are not distinguishable immunologically. The fragments produced showed no identity with fragment D and partial identity with fragment E. Fig. 5.16.

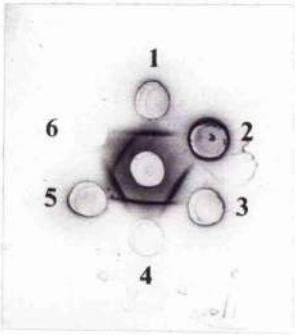
5.8.9. Comparison of SDS gel electrophoretic pattern of proteolytic derivatives of fibrin clot by plasma enzyme and pepsin

Fibrin clots were incubated in 1% (w/v) monochloro acetic acid at 37°C in the presence of plasma enzyme, human pepsin and hog pepsin for different time intervals. At each interval, the supernatents were removed, mixed with equal amounts of urea/SDS as 2.1.11, and boiled for 5 min, prior to the application to the gel. As the results show, Fig. 5.17, the two enzymes do not react exactly the same. However, there are similarities between the degradation products of both enzymes. Pepsin breaks more bounds as an extra fragment with molecular weight of approximately 82,000 was detected, Table 5.9.

FIG. 5.16 COMPARISON OF IMMUNOCROSS REACTIVITY PATTERNS OF FIBRIN DEGRADATION PRODUCTS (FDP) BY PLASMA ENZYME (a) AND PEPSIN (b) WITH FIBRINOGEN FRAGMENT D AND E USING ANTISERA TO WHOLE HUMAN FIBRINOGEN

A

1 FDP (b)
2 -
3 FDP (b)
4 Fragment D
5 FDP (a)
6 Fragment E

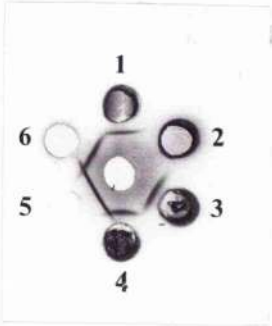


B

1 FDP (b)
2 Fibrinogen
3 Fragment D
4 Fragment E
5 FDP (a)

C

1 FDP (b)
2 -
3 FDP (a)
4 FDP (a)
5 Fragment D
6 FDP (a)



Anti fibrinogen antiserum in the centre wells

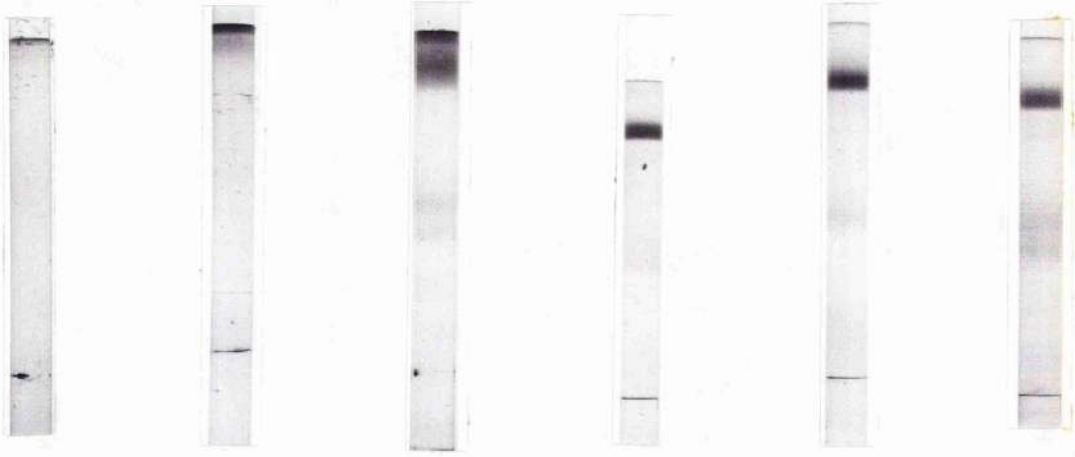
Fig. 5.17 COMPARISON OF SDS GEL ELECTROPHORESIS PATTERNS OF PROTEOLYTIC DERIVATIVES OF FIBRIN CLOT BY PLASMA ENZYME AND PEPSIN AT DIFFERENT TIME INTERVALS.

a) Plasma enzyme (DEAE-Cellulose step Table 4.7)

b) Human pepsin

Time sequence: 4,10,22,51,86 and 140.

(a)



(b)

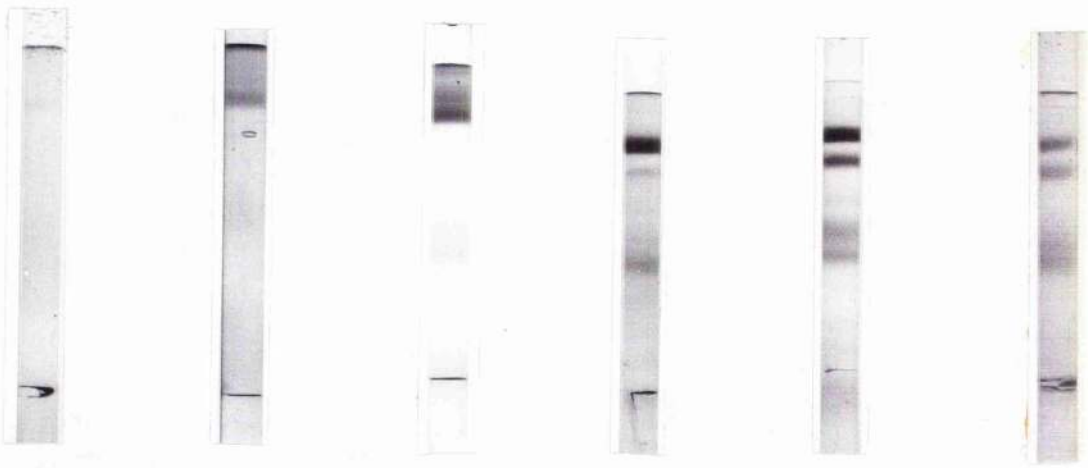


Table 5.9 MOLECULAR WEIGHT OF FIBRIN DEGRADATION PRODUCTS BY PLASMA ENZYME AND PEPSIN

Enzyme	Approximate molecular weight (10^3) of fibrin degradation products						
Plasma enzyme	150	150	-	58	48	36	18
Human pepsin	150	150	82	58	48	36	18
Hog pepsin	150	150	82	58	48	36	18

5.8.10. Comparison of the action of plasmin and plasma enzyme on fibrinogen/fibrin

At least three types of fibrin can be formed in vitro and probably in vivo as well (Gaffney, 1978), non cross-linked fibrin, partly cross-linked (γ-γ) chain and totally cross-linked (α^{P}-γ-γ). The order in which the chains are split by plasmin differs in each case, Table 5.10.

The proteolytic cleavage pattern of a fibrin clot by plasma enzyme is different to the action of plasmin on fibrin clot, Table 5.10.

5.8.11. Comparison of plasma enzyme with human pepsin and gastricsin: mobility on polyacrylamide gradient gel electrophoresis

Plasma enzyme, human pepsin, gastricsin, and a mixture of these enzymes were applied to gel gradient as 2.2.1.

Table 5.10 COMPARISON OF PROTEOLYTIC ACTION OF PLASMA ENZYME AND PLASMIN ON FIBRIN CLOTS

Substrate	Enzyme	Order of polypeptide chain susceptibility	
Fibrinogen	Plasmin	A $\alpha \longrightarrow$ B $\beta \longrightarrow \gamma$	(a)
Non cross linked fibrin	Plasmin	$\alpha \longrightarrow \beta \longrightarrow \gamma$	(a)
Partly cross linked fibrin	Plasmin	$\alpha \longrightarrow \beta \longrightarrow \gamma$	(a)
Totally cross linked fibrin	Plasmin	β or $\gamma \longrightarrow \alpha$	(a)
Partly cross linked fibrin	Plasma enzyme (DEAE-cellulose step Table 4.7)	$\alpha \longrightarrow \gamma \longrightarrow \beta$	

(a) P.J. Gaffney (1978)

As the results show, Fig. 5.18, plasma enzyme travels slower than pepsin and gastricsin, while there was no difference in the mobility of gastricsin and pepsin.

5.8.12. Comparison of the mobility of plasma enzyme and pepsin on insoluble haemoglobin-polyacrylamide gel

Plasma enzyme (blue-sepharose step Table 4.7) and hog pepsinogen were applied to the haemoglobin gel as 3.5.3.3. As Fig. 5.19 shows, plasma enzyme showed clear band with the mobility of 0.84, while the pepsinogen band had a mobility of 0.95.

Different concentrations of pepsinogen (0.01 - 100 ng/ml were prepared and 20 ul (2 pg-2 ng) were applied to the gels. All showed clear bands with the mobility of 0.95 - 0.98. When the concentration of pepsinogen was in ng/ml range, more than one clear band was detected, in accordance with the results of Samloff (1969).

The lower concentration of pepsinogen (pg/ml range) gave one distinct clear band at 0.95 in contrast to plasma enzyme where the clear band corresponded to a mobility of 0.84.

Electrophoresis was carried out following the addition of pepsinogen to normal plasma. The mixture showed (Fig. 5.19) clear bands in both positions, indicating that the mobility of pepsinogen is not affected by the presence of the plasma proteins.

Fig. 5.18 POLYACRYLAMIDE GRADIENT GEL ELECTROPHORESIS PATTERNS OF PLASMA ENZYME, HUMAN PEPSIN AND GASTRICSIN

1. Human pepsin
2. Blue Sepharose step, Table 4.7
3. Gastricsin
4. Pepsin
5. Blue Sepharose step, Table 4.7
6. Gastricsin
7. DEAE (incomplete)step, Table 4.7
8. Pepsin + Blue Sepharose
9. Gastricsin + Blue Sepharose

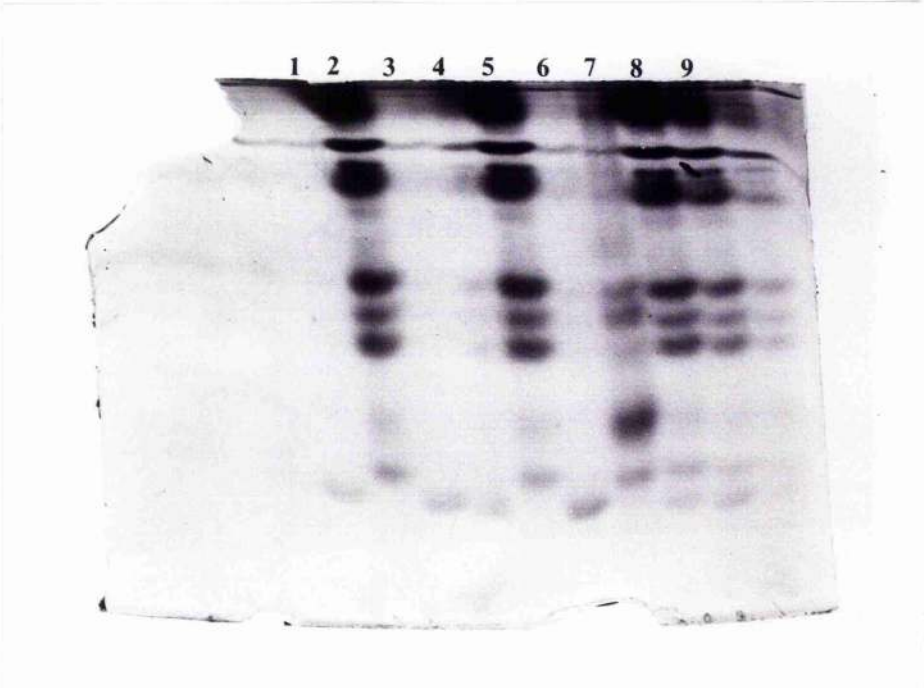


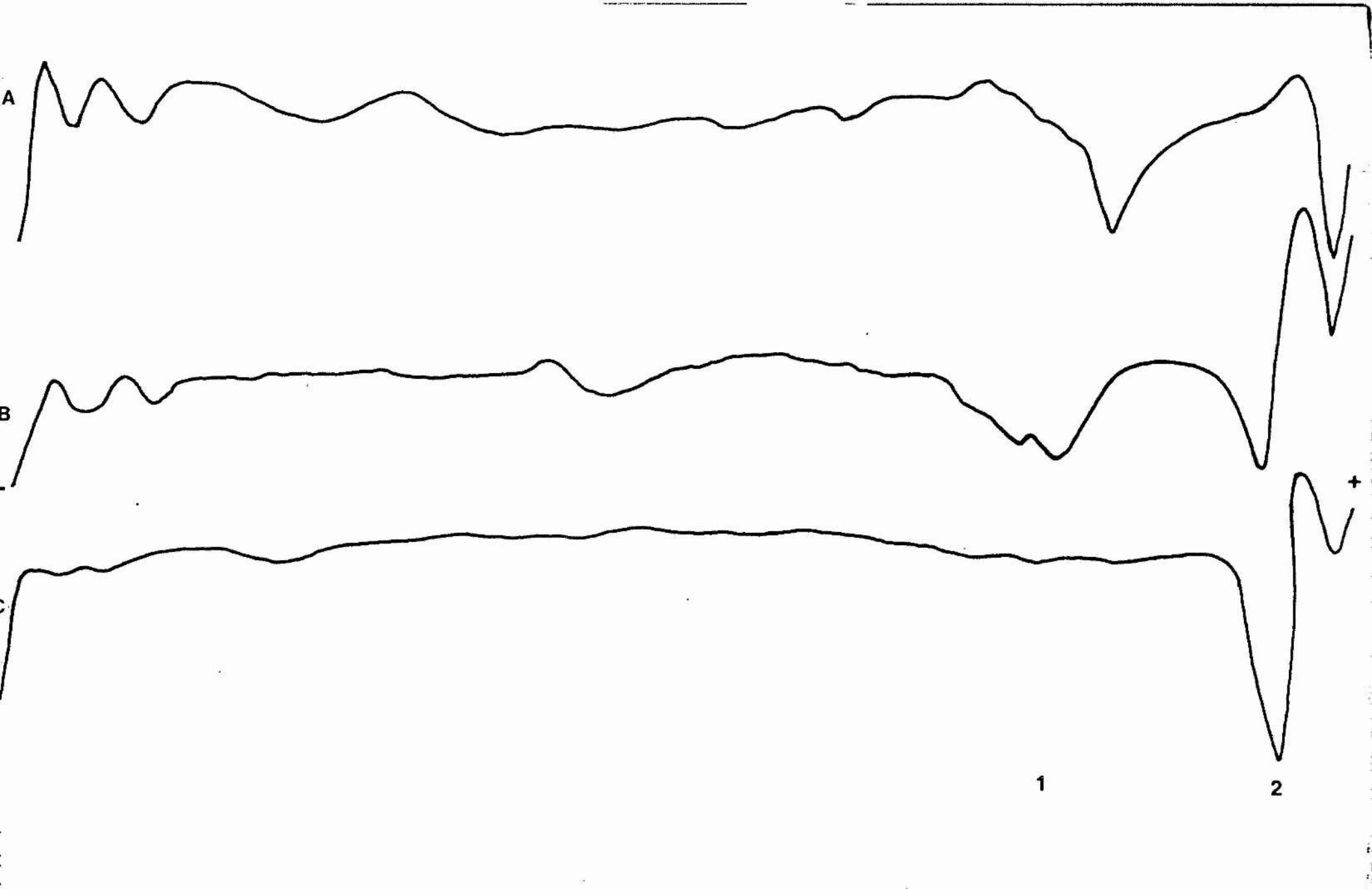
Fig. 5.19 DENSITOMETRIC SCAN OF PLASMA ENZYME AND PEPSIN ON HAEMOGLOBIN-POLYACRYLAMIDE GEL

The clear zones are shown by trough

A Plasma enzyme

B Plasma enzyme + pepsin

C Pepsin


A
B
C
1
2
+

5.8.13. Comparison of plasma enzyme pattern with other acid proteinases: elution from Hb-Sepharose column

Smith and Turk (1974) used Hb-Sepharose 4B to purify cathepsin D from bovine spleen and thymus. Cathepsin D was eluted at about pH 6.2. Chou et al., (1978), also used a similar technique to separate human renin and pseudo renin (cathepsin D). They reported that renin did not bind to the column at pH 3.5, but pseudo renin did, and was eluted at about pH 6.2.

To study the elution patterns of plasma enzyme and pepsin, two Hb-Sepharose 4B columns were set up under the same conditions as 4.4.3.2.

Plasma enzyme (Blue-Sepharose step, Table 4.7) was applied to one column and a mixture of plasma enzyme and hog pepsin to the other. As results show, Fig. 5.20, both enzymes were bound to the column at pH 3.5 in contrast to renin and were eluted at a similar position to cathepsin D (pH 5.9 - 6.5).

Fig. 5.20 Hb-SEPHAROSE COLUMN CHROMATOGRAPHY OF PLASMA ENZYME AND PEPSIN

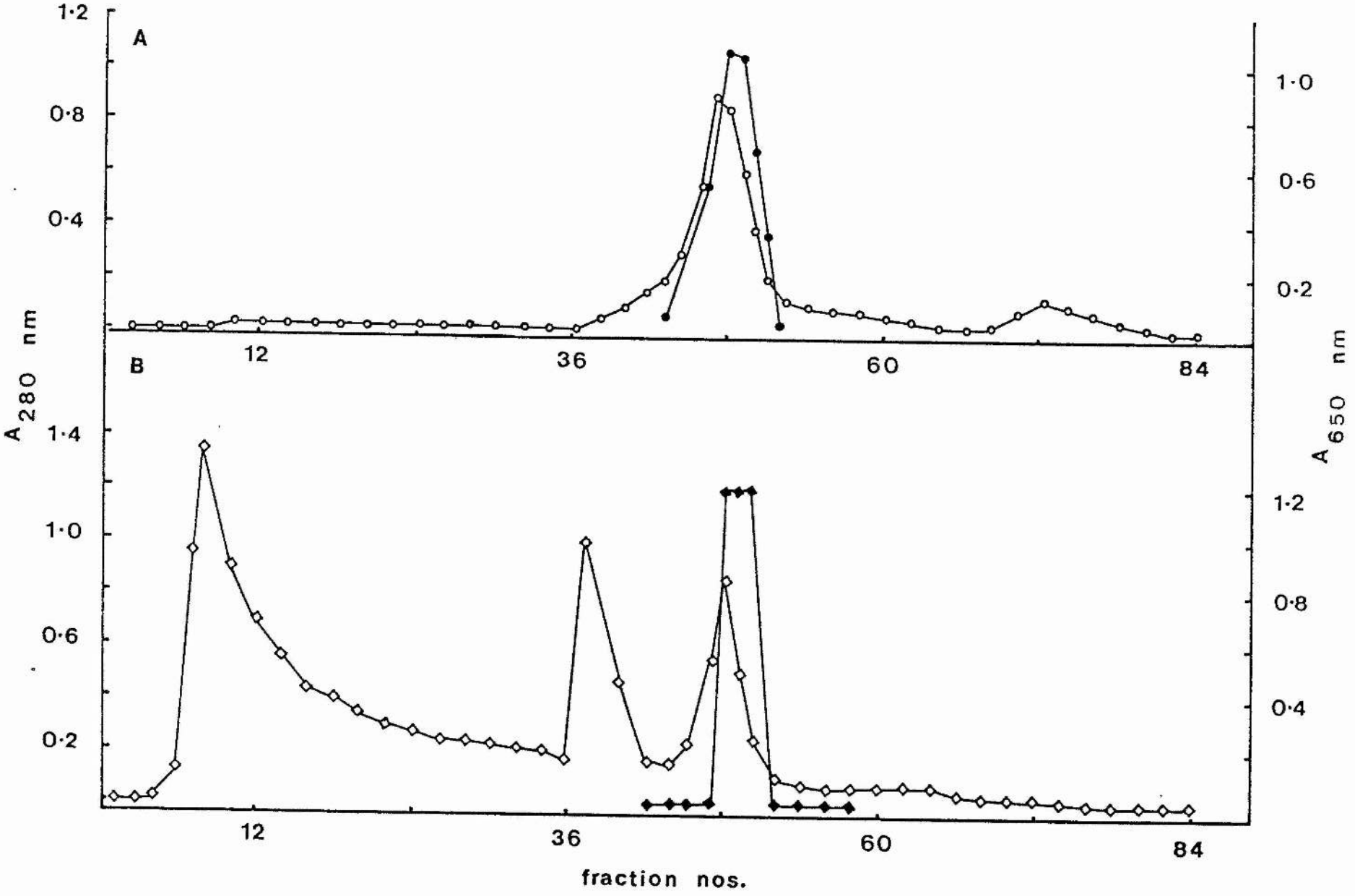
Graph A o—o absorbance at 280 nm plasma enzyme

•—• plasma enzyme proteolytic activity

Graph B ◇—◇ absorbance at 280 nm pepsin

◆—◆ pepsin proteolytic activity

Fibrin clot was used as substrate


A
B
1·2
0·8
0·4
1·0
0·6
0·2
12
36
60
84
A_{280} nm
A_{650} nm
1·4
1·0
0·6
0·2
1·2
0·8
0·4
12
36
60
84
fraction nos.

5.8.14. Comparison between plasma enzyme and pepsin elution pattern from Amberlite column

An amberlite column was set up under the same condition as was used for human pepsin and gastricsin purification (2.14.2). A sample of plasma enzyme (Blue-Sepharose fraction) was dialysed against 0.2 M sodium citrate, pH 3.0, and was applied to the column. The plasma enzyme was eluted between pH 5.4 - 5.6, Fig. 5.21, in contrast to pepsin and gastricsin which were eluted at pH 4.2 and 4.4 respectively.

Fig. 5.21 AMBERLITE COLUMN CHROMATOGRAPHY OF PLASMA ENZYME, PEPSIN AND GASTRICSIN

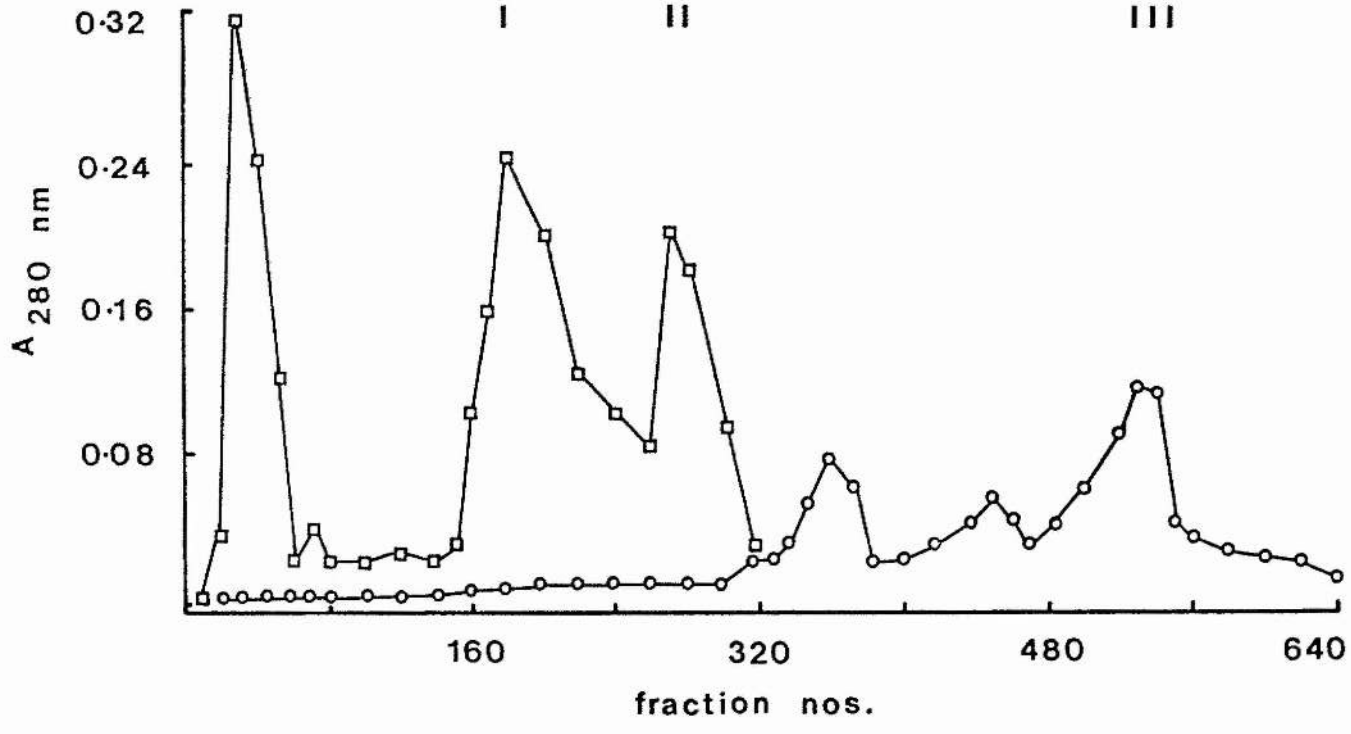
absorbance at 280 nm plasma enzyme

absorbance at 280 nm pepsin and gastricsin

I fractions with activity, pepsin pH 4.2

II fractions with activity, gastricsin, pH 4.4

III fractions with activity, plasma enzyme, pH 5.5


0·32
0·24
0·16
0·08
A 280 nm
I
II
III
160
320
480
640
fraction nos.

CHAPTER 6

GENERAL DISCUSSION

There is some evidence for the presence of acid proteinases in human plasma. Also, different acid proteinases have been discovered in various cellular components of blood as well as tissues.

Samloff et al., (1975), used a radio immuno assay (developed by Samloff and Leibman, 1974), to measure specifically Group I pepsinogens in serum, as they believed that usual proteolytic assay method measures Group I pepsinogen, Group II pepsinogen plus other acid proteinases.

Rotter et al., (1979) also reported the presence of two immuno chemicaly distinct groups of pepsinogen (I and II) in serum.

Vete et al., (1969) compared different substrates for measurement of pepsin and pepsinogen in blood and urine. Serum from normal and patients with various diseases hydrolysed haemoglobin and casein with highest activity at pH about 3-4. Commercial crystalline pepsin, however, digested these two substrates optimally at pH 2.0. Therefore they suggested that it is rather difficult to accept that the proteolytic activity of serum in pH range of 3-4 is totally due to pepsin and pepsinogen.

They introduced a new substrate, serum protein (the autologus serum was treated at pH 2.0, then adjusted to 8.0, ensuring the destruction of pepsin. Blood hydrolysed its own (autologous) serum protein optimally at pH 2.0. Also, crystaline pepsin digested the serum protein at pH 2.0. They concluded that serum protein is a better substrate for

blood pepsin and pepsinogen determination.

Mirsky et al., (1952) reported that the plasma of patients with duodenal ulcers has a higher pepsinogen content than normal. There is also a higher rate of pepsinogen excretion in the urine. Conversely the plasma pepsinogen levels are very low in patients with pernicious anemia and there is a negligible amount of excretion in urine. They assayed the proteolytic activity of the plasma at pH 1.5, using haemoglobin as substrate, and concluded that the major proportion of the total activity at this pH is due to pepsinogen. However, a small portion of the activity is due to enzymes other than pepsin.

Ruenwongsa and Chulavatnatol (1975) isolated an acid proteinase from human seminal plasma, which they originally thought was pepsin because its catalytic properties were similar to hog pepsin. It hydrolysed denatured haemoglobin at the same rate of hog pepsin, but was slightly less inhibited by p-bromophen-acyl bromide and 1,2- epoxy-3-(p-nitro-phenoxy) propane than was hog pepsin. When N-acetyl-L-phenylalanyl-L-diiodotyrosine was used as a substrate the acidic proteinase was 100-fold less active than hog pepsin, which might suggest a different active site from that of hog pepsin.

Ruenwongsa and Chulavatnatol (1977) compared the amino acid composition of the active proteinase and the proenzyme in seminal plasma, with bovine pepsin and pepsinogen, and found some similarity and differences. During the conversion of pepsinogen to pepsin 40 residues which carry most of the basic amino acids were released, while in acid proteinase of seminal

plasma 69 residues which contain about 30% of basic amino acids were released.

Morris and Lumberg (1972) reported the presence of an acid stable factor in amniotic fluid which has similar properties to pepsin. It activates renin at pH 3.3-3.6, is stable at pH 1.5, but loses its activity at pH 7.5.

Nackman and Ferris (1968), reported that intracellular granules of platelets contain proteases capable of the hydrolysis of undenatured human fibrinogen (pH optima 6-8), denatured haemoglobin (pH 6.5-7.8) and casein (pH 4.8 and 8.4).

They concluded that the proteolytic activity of platelet enzyme(s) was not due to plasmin as trypsin inhibitor, pancreatic trypsin inhibitor or EACA had no effect on the enzyme activity. In addition, the enzyme(s) were completely inactivated by heating to 60°C for 15 min. and proteolytic products were different from those of plasmin. At least one of the enzymes was regarded as cathepsin A, as it hydrolysed haemoglobin at pH 3.5, lost its activity on moderate heating, and did not require cystine for its activation. Nor was it inhibited by iodoacetamide, and it specifically hydrolysed the synthetic substrate N-CBZ-α-L-glutamyl-L-tyrosine at pH 5.0.

Reichelt et al., (1974) purified a proteolytic enzyme with molecular weight of 55,000 from human erythrocytes. This enzyme hydrolysed denatured haemoglobin with the highest activity at pH 2.8. The enzyme had no effect on denatured albumin, nor on the synthetic substrates specific for cathepsin A, B and C. The effects of EDTA and metal ions

on the enzyme activity were insignificant, and dithiothreitol in high concentration caused the inhibition of the enzyme activity, suggesting the presence of an essential disulphide group. However, they classified the enzyme as a cathepsin D, in that its origin was not lysosomal but mainly membrane bound.

Levy et al., (1974), and Levy and Chou (1974) studied the proteinases in Plasmodium falciparum and the red cell ghosts of its host aotus monkey, also Plasmodium knowlesi and its host rhesus monkey, and found that each species of parasite had a protease which degraded haemoglobinoptimally at pH 3.6. Another proteinase from Plasmodium berghei degraded haemoglobin at pH 2.5, to 3.0. They also found proteases in ghost cells of the hosts which were distinct from the parasites. The proteases in hosts and parasites were rather unusual in being susceptible to pepstatin, chymostatin, anti pain and leupeptin, with pepstatin being the most potent inhibitor. From their results they suggested that the major acid proteinases of both the hosts and parasites were like cathepsin D, because of their pH optimum and sensitivity to pepstatin.

Yago and Owers (1975) reported the presence of cathepsin D type proteinases in the rat thoracic duct lymphocytes and lymphoid tissues, with molecular weight of approximately 95,000 and 45,000. Both enzymes differed from cathepsin D by being more sensitive to inhibition by pepstatin, and they were not inhibited by antiserum which inhibited the rat liver cathepsin D. The reason for believing the enzymes were cathepsins was that the pH curves, using denatured haemoglobin were similar to that of cathepsin D. Haemoglobin was the

best substrate. The high molecular weight enzyme (95,000) was not cathepsin E as it hydrolysed bovine serum albumin more efficiently than human serum albumin.

Woessner, (1973), purified an enzyme with proteolytic activity from elastic cartilage of young rabbit ears and hyaline cartilage from the legs of chick embryos. Woessner identified the enzyme as cathepsin D. Cathepsin D rapidly degraded protein-polysaccharide light chain. The pH optimum for this process is about 4.0. The protein-polysaccharide complex digestion was almost completely inhibited (97%) by pepstatin, and also inhibited by EACA at high concentration. Woessner also suggested that apart from cathepsin D there are other proteases in cartilage which act at very low pH and neutral pH.

Sapolsky et al., (1974), reported that human articular cartilage contained two or more distinct proteases. One of the enzymes was cathepsin D which digests haemoglobin at pH 3.2 and proteoglycan at pH 5.0, while the other was a neutral protease. Cathepsin D has no activity at neutral pH and therefore its role in cartilage matrix breakdown must either be via intracellular digestion in the lysosomal system or occur in localised regions of low pH.

Barrett and McDonald, (1980b), reported the presence of cathepsin E, a proteolytic enzyme in bone marrow, polymorpho-nuclear leucocytes, macrophages and human platelets. Cathepsin E has a molecular weight of 100,000, and digests albumin and haemoglobin with highest activity at pH 2.5 in contrast to cathepsin D which has pH optimum of 3.5.

For many years the proteinases of tissues and leucocytes

which were active at mild acid pH (approximately 3.5) were called cathepsins until 1960, when Press et al. isolated a proteolytic enzyme from bovine spleen which had pH optimum of 3.0 on denatured haemoglobin and 4.2 with denatured albumin. The enzyme was heat labile and lost its activity below pH 2.5. The enzyme was present at least in 10 different forms, but with the same properties. They called the enzyme cathepsin D. Although different molecular weights (40,000 - 60,000) have been given for cathepsin D in the literature, Barrett and McDonald, (1980), reported that the generally accepted molecular weight of cathepsin D is close to 42,000 and also defined cathepsin D as proteolytic enzyme present in all mammals and a very wide range of other animals. It is located in lysosomes and red cell membranes. Its usual and the most sensitive substrate is haemoglobin and it has a pH optimum of 2.8-5.0 depending on the substrate.

From the above statements one can cast doubts as to whether all the protolytic enzymes classified in the literature as cathepsin D were really cathepsin D. As Barrett (1977b), mentions, there are similarities between carboxyl proteinases in their pH dependence, inhibitors, substrate specificity which all suggests that they show a common catalytic mechanism. For example, since the discovery that renin is a carboxyl proteinase, much attention has been focussed on its similarities to cathepsin D. Leckie, (1981) reported that although renin and cathepsin D have molecular weights of approximately 42,000, isoelectric points of approximately 4.5-5.5 with multiple forms, and are present in lysosomes, comparisons of the amino acid sequence of renin of mouse

submaxillary glands with cathepsin D have shown the two are distinct proteinases.

Inagami et al., (1974), suggested that renin may be an acid proteinase. It was not inhibited by inhibitors of serine, thiol or metalo-peptidases, but was inhibited by pepstatin. In addition renin from the mouse sub maxillary gland and renin from hog kidney were inactivated by diazo-acetyl-D, L-norleucine methyl ester, in the presence of cupric ions.

McKnown and Gregerman, (1974), also suggested that renin is related to acid proteinases. Human renin activity was inhibited by diazo acyl compounds such as diazo acetyl glycine ethyl ester.

Hsueh, et al., (1980), reported that normal plasma contains inactive renin which becomes activated by acidification or treatment with pepsin or trypsin.

Inagami et al., (1977), reported that renin isolated from kidney is in one or two molecular weight forms, big renin (molecular weight of 61,000) and big-big renin (molecular weight of 140,000). There is an inverse relationship between the molecular weight and activity. Tang suggested that the big renin is the precursor of the active enzyme, i.e. big-big renin $\longrightarrow$ big renin $\longrightarrow$ renin.

Ondetti and Cushman, (1982), reported that renin occurs in several forms with different molecular weight, and with different degrees of activity, from active form (molecular weight of 40,000) to partially or inactive form. They also reported that renin is a highly specific proteinase, which cleaves a Leu-Leu sequence in its only natural substrate the

plasma α-globulin, angiotensinogen to produce the decapeptide angiotensin I, which is converted by other enzymes to the vasoconstrictor octa peptide angiotensin II.

CONCLUSION

A proteolytic enzyme which showed the ability to dissolve fibrin clots in acid environment, and to a lesser extent denatured haemoglobin, azo-casein but not synthetic substrate : N-acetyl-phenylalanyl-L-diiodotyrosine, was almost purified from normal human plasma. Enzymological studies using different group-specific inactivators of proteinases indicated that plasma enzyme belongs to the class of acidic proteinases. Plasma enzyme was inhibited by pepstatin and p-bromophenacyl-bromide. It was stable at $-20^{\circ}C$ for a long period but heat labile. The enzyme activity was influenced by the ionic strength of the assay medium when fibrin clot assay was used. SDS gel electrophoresis of concentrated DEAE-cellulose fractions (Table 4.7) showed a major band of 52,000-54,000 and a minor band of approximately 90,000. These apparent molecular weights did not change following reduction of disulphide bonds.

In 1979, Law, in attempting to purify the same plasma enzyme, suggested that the enzyme differed from pepsin as during the course of purification, using $Al(OH)_3$ gel, the activity isolated was in a different fraction to the one which Ikemori et al., (1975), followed and called the enzyme pepsin.

In this project Law's work was confirmed, and on further work more dissimilarities become apparent.

I The digestion products of fibrin clot by two enzymes differed, 5.8.10.

II They had different mobility on gradient gels, 5.8.12.

III They had different mobility on haemoglobin gels, 5.8.13.

IV They were eluted at different pH from amberlite column, 5.8.14.

V Fibrin clot was the best substrate, while denatured haemoglobin was more sensitive to the pepsin proteolytic activity, 5.8.1.

Plasma enzyme differs from renin in that it bound to the Hb-sepharose column at pH 3.5 and was eluted at approximately pH 6.0, while renin does not bind to the Hb-sepharose at pH 3.5 (Chou et al., 1978). The pH optimum of plasma enzyme was 2.4-3.5 using three different substrates while the pH optimum for renin is approximately 6.5.

Plasma enzyme hydrolyses haemoglobin and bovine fibrin while haemoglobin is a potent inhibitor of renin ($ki=2 \times 10^{-6}$) and bovine fibrinogen is a weak inhibitor of renin ($ki=1 \times 10^{-4}$ M) (Workman et al., 1974).

Plasma enzyme also differs from cathepsin D as it has a molecular weight of approximately 54,000, while cathepsin D molecular weight is approximately 42,000.

Fibrin clot is a more sensitive substrate than haemoglobin for plasma enzyme, while the opposite is true for cathepsin D and cathepsin E.

Lebez et al., (1971), examined the hydrolytic activity of cathepsin D and cathepsin E towards different protein substrates.

The absorbance of hydrolysed haemoglobin was taken as 100% hydrolytic activity, and the hydrolysis of other proteins were expressed as percentage of haemoglobin hydrolysis.

Substrate	Cathepsin D	Cathepsin E
Haemoglobin	100	100
Fibrinogen	5 - 10	19
Fibrin	5 - 10	9

Comparison of hydrolytic activities of cathepsin D and cathepsin E from beef spleen.

Lebez et al., 1971

The pH optimum of plasma enzyme being about 2.4-3.5, makes it rather difficult to relate its role in physiological conditions, where the blood pH is about 7.35-7.4 and even in a very severe acidosis the fall of pH is to about 7.0 (Copley et al., 1980). However, most biological cells and surfaces, such as the endothelial wall are negatively charged and pH at these areas will be lower than bulk pH. Usually charges occur in patches, or clusters, and in these micro-regions, the pH could be 2, 3 or more pH units lower than in bulk (Copley et al., 1980). At pH below 6.5, the rigidity of fibrinogen surface layers is markedly increased and conformational changes in fibrinogen could trigger gel formation which appears indistinguishable from fibrin. Therefore there is a possibility of non-enzymic (no thrombin) aggregation of fibrinogen on the endothelium in micro-circulation and initiation of thrombus formation (Copley, 1980). Thrombus formation at these areas of low pH could happen in one of two ways. Where there is cellular damage, resulting in low pH, the flow properties of the blood in the proximity of damaged cells or tissue will be affected,

causing rigidity of red cells, and consequent red cell clumping, gelation of fibrinogen and thrombus formation. Thrombi could occur without an injury to the vascular wall as high wall shear forces of different blood vessels could cause conformational changes of fibrinogen at certain sites in the circulation. These sites may be where there is a lowering of the pH (Copley et al., 1980).

As there is a possibility of thrombus formation at localized areas where the pH is low, one can hypothesise that a plasma enzyme which is inactive at physiological pH could become active at low pH, Fig. 6.1. This is a similar picture to simultaneous fibrin clot formation and activation of plasminogen to plasmin, Fig. 6.2.

Another hypothesis is that the plasma enzyme is activated by other proteinases once in the region of acid media or when fibrin clot is formed in acid region similar to the work of Leckie, (1981). On reviewing inactive renin in human plasma he reported that endogenous serine proteinases which are involved in coagulation and fibrinolysis activate renin. Leckie found out that plasma defficient in pre-kallikrein could not activate in active renin, also that plasmin activates renin, but plasma def icient in plasminogen has normal acid activation. Therefore Leckie suggested as kallikrein and plasmin are rapidly inactivated by inhibitors in circulating blood, the only possible way of activating inactive renin is in restricted sites, e.g. within fibrin clot or close to vessel walls.

Derk et al., (1979) reported that activated Factor XII not only initiated the clotting, fibrinolysis, conversion of pre-kallidrein to kallikrein and kinin system, but is also

Fig. 6.1. FIBRINOLYSIS BY PLASMA ENZYME (HYPOTHESIS)

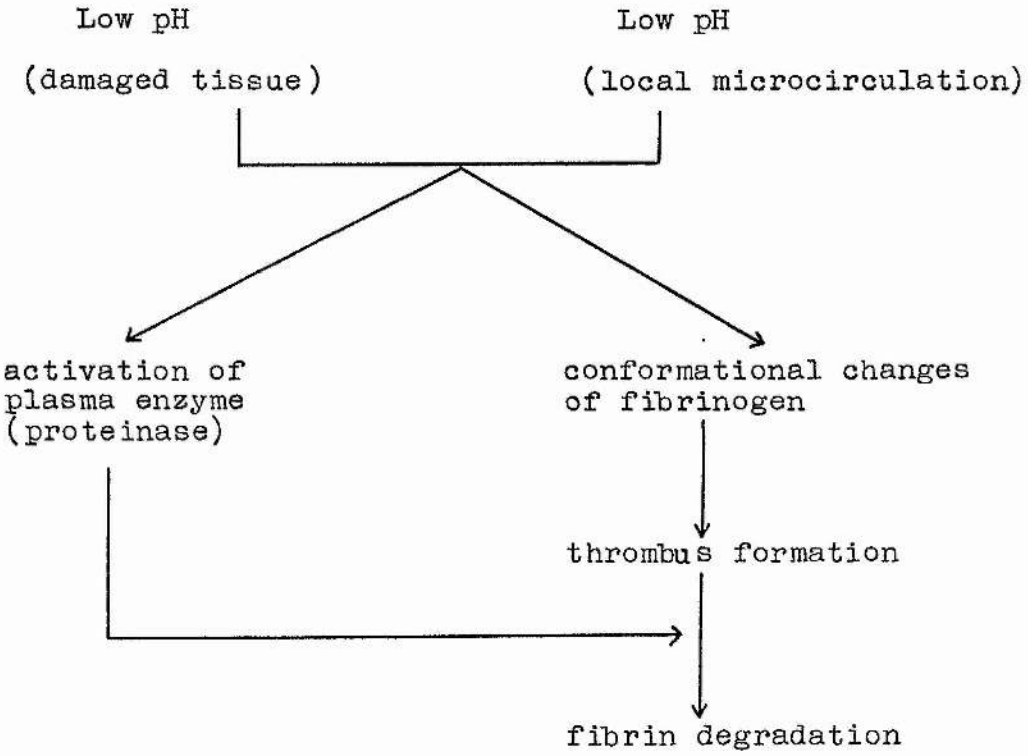
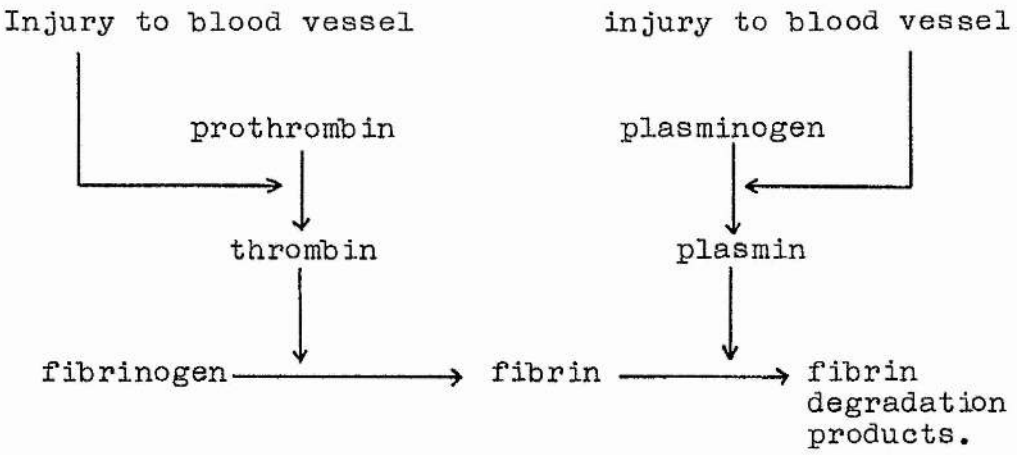


Fig. 6.2. SIMILARITY IN BROAD OUTLINE BETWEEN FIBRINOLYSIS AND THE PLASMA COAGULATION SEQUENCE



C.N. Chesterman, 1978.

involved in renin-angiotensin system. Sealy et al., (1978), also reported that urinary kallikrein is a very potent activator of inactive renin.

Considering most of the reports through the literature, plasmin can no longer be thought of as the only enzyme concerned in the physiological degradation of fibrin(ogen). Although it may be the most important enzyme involved in the degradation of fibrin/fibrinogen, the possible action of other proteinases including 'acid proteinases', should not be ignored.

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