27

THE D-DOMAIN OF FIBRIN - STRUCTURAL AND FUNCTIONAL STUDIES.

by

MAUD PURVES (M.Sc.Rand)

Thesis submitted for the degree of

Doctor of Philosophy (Medicine)

to the

UNIVERSITY OF CAPE TOWN

June 1987

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

Dedicated to

My mother.

My husband and children for their encouragement and support.

The memory of my friend Helen Nelson.

ACKNOWLEDGEMENTS

This study was carried in the Department of Chemical Pathology, University of Cape Town.

I was supported in part by a bursary from the South African Medical Research Council and by the funds allocated to L.R.Purves from the S.A.M.R.C., the University Staff Research Fund and the Nellie Atkinson Fund.

I wish to thank my supervisors Professor Langley Purves and Professor Mervyn Berman for their support and discussions.

This study has resulted in the following publications :-

Purves, L. R., Purves, M., Lindsey, G. G. & Linton, N. J. (1986). Specific cleavage of fibrin-derived D-dimer by a metalloproteinase isolated from venom of the puffadder (Bitis arietans). South Afr. J. Sci. 82, 30-33.

Purves, L.R., Purves, M. and Brandt, W. (1987) Cleavage of fibrin derived D-dimer into monomers by an endopeptidase from puffadder venom (Bitis arietans) acting at the cross-linked sites of the γ-chain. Sequence of carboxy-terminal cyanogen bromide γ-chain fragments. Biochemistry (scheduled for publication June, 20 1987)

The thesis was produced on a Sperry microcomputer using Wordstar

CONTENTS

Page

Abbreviations 11

SUMMARY 12

1.INTRODUCTION 17

1.1 Aspects of fibrinogen structure and function. 17

1.2 Amino acid sequence of the alpha, beta and gamma chains. 21

1.3 Homologies between chains and interspecies homologies. 23

1.4 Fibrinogen to fibrin conversion. 27

1.4.1 Polymerisation sites. 27

1.4.2 Transglutaminases. 31

1.4.3 Assignment of the fibrin γ-chain cross-link site. 33

1.4.4 Role of calcium in the clotting of fibrinogen and the structure of fibrin. 35

1.4.5 Binding of fibronectin and plasminogen to fibrin clot. 36

1.5 Fibrin(ogen)olysis 37

1.5.1 Plasmin degradation of fibrinogen 37

1.5.2 Plasmin degradation of fibrin. 38

1.5.3 Physiological interactions of fibrin(ogen) degradation products. 41

1.5.4 Regulation of fibrinogen synthesis. 43

1.5.5. Synthesis of fibrinogen. 45

1.6 Assay of fibrinogen/fibrin degradation products. 48

1.7 Snake venoms and fibrinolysis. 50

1.7.1 Proteinases from snake venoms. 50

1.7.2 Puffadder (Bitis arietans) venom proteases. 52

2 METHODS 55

2.1 Assay methods. 55

2.1.1 Polyacrylamide gel electrophoresis. 55

2.1.2 Protein concentration. 55

2.1.3 Chromozym assays 55

2.1.4 Fibrinogenolytic activity of puffadder venom protease. 56

2.1.5 Caseinolytic assay for puffadder venom proteases. 56

2.1.6 Monoclonal antibody radioisotope assay. 57

2.1.7 Dimertest agglutination assay. 60

2.1.8 Fluorimetry. 61

2.1.9 Preparation of plasmin. 61

2.1.10 Definition of PAV protease activity with f-D-dimer as substrate. 61

2.2 Preparation of fibrinogen and fibrin plasmin derivatives 62

2.2.1 Preparation of fibrinogen. 62

2.2.2 Preparation of fibrinogen, fibrin and plasmin derivatives. 62

2.2.3 Gel filtration of the pooled fractions from DEAE anion exchange chromatography. 63

2.2.4 Preparation of non-fluorescent D-dimer. 64

2.2.5 Preparation of D-monomer. 64

2.2.6 Preparation of D-PAV-monomer. 65

2.3 Purification of protease(s) from puffadder venom 66

2.3.1 Cation exchange chromatography. 66

2.3.2 Gel filtration on Ultrogel AcA 34. 66

2.3.3 Gel filtration on Sephadex G 100. 67

2.3.4 Purification by HPLC. 68

2.4 Sequencing of the carboxy-terminal cyanogen bromide fragment of the γ-chain of D-PAV-monomer. 68

2.4.1 Preparation of D-PAV-monomer. 68

2.4.2 Isolation of the γ- and β-chains from D-PAV-monomer 69

2.4.3 Cyanogen bromide digestion of the γ-chain of D-PAV-monomer. 69

2.4.4 Isolation of the carboxy-terminal peptide from the γ-chain of D-PAV-monomer digest. 70

2.4.5 Peptide hydrolysis and amino acid composition. 70

2.4.6 Manual gas-phase microsequencing. 70

2.4.7 High performance liquid chromatography. 72

3 RESULTS 74

3.1 Purification of fluorescent and non-fluorescent fibrin(ogen) plasmin derivatives 74

3.1.1 Fluorescent and non-fluorescent D-Dimer. 74

3.1.2 Fluorescent D-monomer. 74

3.1.3 Non-fluorescent D-dimer. 77

3.1.4 D-PAV-monomer. 77

3.2 Purification of puffadder venom protease 77

3.2.1 CM cellulose cation exchange chromatography. 77

3.2.2 Gel filtration on AcA 34 and Sephadex G 100. 82

3.2.3 HPLC of puffadder venom protease. 91

3.3 Characterisation of the puffadder venom protease. 91

3.3.1 Identity of the puffadder venom protease. 91

3.3.2 Comparison of PAV protease and plasmin activity using chromogenic substrates. 98

3.3.3 Caseinolytic activity of the PAV protease. 98

3.4 Definition of the PAV protease activity using D-dimer as substrate. 98

3.4.1 Timed digestion of the fluorescent D-dimer by the PAV protease. 98

3.4.2 Effect of zinc ions on PAV protease activity. 103

3.4.3 Effect of inhibitors on PAV protease activity. 103

3.4.4 Comparison of PAV protease with plasmin using D-dimer as substrate. 108

3.4.5 Cleavage of D-dimer by venom from different species of snakes. 108

3.4.6 Effect of PAV protease on D-dimer from rat fibrin. 111

3.5 Immunological approach to the study of the D domain. 111

3.5.1 Radioisotope assay for D-dimer. 114

3.5.2 Dimertest agglutination assay. 117

3.6 Sequencing of the carboxy-terminal cyanogen bromide fragment of the γ-chain of D-DAV. 117

4 DISCUSSION 130

5 REFERENCES 143

TABLES

I. Amino acid sequences at the amino-termini of fibrin α- and β-chains from various species. 26

II. Enzyme distribution in various snake venoms. 51

III. PAV protease, plasmin and Dimertest assay.... 117

IV. Amino acid composition of isolated peptide. 124

V. Gas-phase Edman degradation microsequencing. 126

VI. Crosslink configurations of the human di-γ-chain carboxy-terminal peptide....... 136

VII. Comparison of carboxy-terminal sequences at the crosslink sites of rat, bovine, human and lamprey γ_a-chain. 139

FIGURES

1. Diagram of vertebrate fibrinogen molecule. 19

2. Primary structure model of human fibrinogen. 22

3. Aminoacid sequence of the three chains of human fibrinogen. 24

4. Model for human fibrin polymerisation. 29

5. Schematic representation of fibrin polymerisation. 32

6. Diagram of fibrinogen digestion by plasmin. 39

7. Sequence of the carboxy-terminal end of the γ-chain 44

8. Anion exchange chromatography of plasmic digest 75

9. Gel filtration of pooled fractions containing f-D-dimer. 76

10. Anion exchange chromatography of plasmin digests of fibrin 78

11. Gel filtration of D-dimer on Ultrogel AcA 34 79

12. SDS-PAGE of materials used as substrates for PAV protease. 80

13. Fractionation of crude puffadder venom on CM cellulose. 81

14. Gel filtration of crude puffadder venom on Ultrogel... 83

15. Fractionation of pooled PAV fractions on DE-52 cellulose.. 85

16. Molecular sieving of crude PAV on Sepadex G 100. 87

17. Activity of pooled PAV fractions using Chromozyms... 89

18. Purification of pool 1 from Sephadex G 100 column... 90

19. Purification of PAV by means of HPLC. 92

20. Effect of PAV on the clotting time of fibrinogen. 94

21. Comparison of digestion of fibrinogen by PAV and plasmin. 96

22. Activity of the crude PAV with Chromozym substrates. 97

23. Optimum pH of PAV protease with casein as substrate. 99

24. Fluorescence spectra of dansylated proteins. 100

25. Timed digest of f-D-dimer by puffadder venom protease. 101

26. Changes in fluorescence during digestion of f-D-dimer.. 102

27. Decrease in absolute fluorescence of f-D-dimer... 104

28. Effect of various inhibitors, calcium and zinc ions.. 105

29. Effect of inhibitors on decrease in fluorescence... 107

30. Timed digest of f-D-dimer with purified PAV protease.. 109

31. Timed digestion of f-D-dimer by PAV.... 110

32. A two hour digestion of f-D-dimer with nine snake venoms. 112

33. Timed digest of rat D-dimer. 113

34. Standard curve for monoclonal antibody radio assay. 114

35. Radioisotope immunoassay with various antigens. 115

36. Radioisotope immunoassay with PAV added to the wells. 116

37. Isolation of reduced and alkylated β- and γ-chains..... 118

38. Separation of the cyanogen bromide peptides of the γ-chain119

39. HPLC separation of carboxy-terminal peptides of γ-chain.. 121

40. HPLC separation of pooled carboxy-terminal peptides... 122

Abbreviations

ATEE - acetyl tyrosine ethyl ester

BTEE - benzoyl tyrosine ethyl ester

Chromozym substrates (Boehringer)

PK - benzoyl-pro-phe-arg-p-nitranilide (pNA)

PL - tosyl-gly-pro-lys-PNA

TH - tosyl-gly-pro-arg-PNA

TRY - Cb3-val-gly-arg-pNA

EDTA - ethlene diamine tetra-acetic acid

f-D-dimer / monomer - Fluorescent D-domain from a plasmin digest of fibrinogen substituted with dansyl-cadaverine

βME - beta- mercaptoethanol

PAV - puffadder snake venom

PMSF - phenyl methyl sulphonyl fluoride

SDS - sodium dodecyl sulphate

SDS-PAGE - polyacrylamide gel electrophoresis with SDS

TAME - tosyl arginine methyl ester

Tris - tris(hydroxymethyl)-methylamine

SUMMARY

The D-domain of fibrin(ogen) was separated from the parent molecule by plasmin digestion in the presence of calcium and isolated by means of DEAE-anion exchange chromatography followed by gel-filtration in buffer containing 4 M urea. Fluorescent-D-dimer (f-D-dimer) was isolated from a plasmic digest of fibrin clotted in the presence of 2.45 mM dansyl cadaverine, a fluorescent lysine analogue. Fluorescent-D-monomer was a by-product of f-D-dimer purification, the yield being determined by the concentration of dansyl cadaverine. At 2.45 mM f-D-monomer was always present in the digest. The f-D-monomer is probably formed directly and not as a result of degradation of f-D-dimer. The molecule elutes in the fibrinogen-derived-D-monomer position on gel-filtration.

A protease was isolated and partially purified from venom of the puffadder (Bitis arietans). Puffadder venom protease is characterized by its ability to cleave D-dimer into symmetrical D-monomers, smaller than plasmin-derived D-monomers from fibrinogen. This characteristic was used to detect the puffadder venom protease activity with fluorescent-D-dimer being used as the substrate. Fractions obtained were assayed for D-dimer cleavage activity and the samples analyzed by means of SDS-PAGE on 4-20% gradient gels under reducing (βME) and non-reducing conditions. The fluorescent bands were located under U.V light and photographed prior to staining with Coomassie Blue.

Several methods for the purification of the protease were investigated. Cation exchange chromatography on CM-cellulose at pH 5.2 proved to be unsatisfactory. Gel filtration on Ultrogel

AcA 34 and Sephadex G 100 gave similar separations and fractions with D-dimer cleavage activity were located in a peak containing proteins of molecular weight less than 40,000 daltons. The fractions from this peak were pooled, dialysed and subjected to anion exchange chromatography on DEAE-cellulose. The active fractions were also fractionated by a second gel-filtration step on Ultrogel AcA 54. High performance liquid chromatography using a molecular sieving column revealed that the protein capable of cleaving fluorescent and non-fluorescent D-dimer had a molecular weight ± 25,000 daltons and was associated with smaller proteins. Crude puffadder venom was able to hydrolyse casein, the serine protease substrate Chromozym TH (Boehringer) as well as cleaving D-dimer. The purified protein was able to hydolyse casein and cleave D-dimer but was not active against Chromozyms PL, PK, TRY or TH, as well as TAME, BTEE and ATEE. The D-dimer cleavage activity of the crude and purified enzyme could be completely inhibited by EDTA. The purified enzyme was not inhibited by the usual serine protease inhibitors such as PMSF or protease inhibitors of bacterial origin. Addition of low concentrations of zinc potentiated D-dimer cleavage activity of the enzyme at least tenfold.

Collection of the venom into EDTA completely destroyed D-dimer cleavage activity. No further attempt was made to purify the enzyme for fear of removing the zinc moeity that was found to be essential for D-dimer cleavage activity.

The purified enzyme was utilised in the study of the cross-link site in the D-domain of fibrin. Cleavage of f-D-dimer by the

enzyme resulted in two symmetrical monomers - called D-PAV-monomer - that was smaller than plasmin D-monomer from fibrinogen. The earliest event in the action of PAV protease appears to be the cleavage of the di-gamma bond with the production of only one size of γ-species, either fluorescent or non-fluorescent, with separation of the dimer into monomers. This was suggestive of an 'isopeptidase' activity. The β-chain is hydrolysed at a slower rate and this is a progressive process yielding a smaller chain size (β-PAV). Early in the digestion of D-dimer, two species of D-monomers are seen, suggesting that cleavage of the di-gamma bond can precede and be independent of the progressive reduction in size of the β-chain (that determines the smaller D-monomer species). D-PAV-monomer was utilized in the study of the cross-link site of fibrin.

D-PAV-monomer was prepared by digestion with PAV protease of non-fluorescent D-dimer to which was added a small amount (1:100) of fluorescent D-monomer as marker to facilitate location of the γ-chain in the subsequent manipulations of the molecule. The D-PAV-monomer was isolated from the PAV protease digest by DEAE-anion exchange chromatography.

The D-PAV-monomer was reduced and alkylated and the γ-chains isolated by means of anion exchange chromatography on DEAE-cellulose CL 6B in buffer containing 8M urea.

The γ-chains were cleaved with cyanogen bromide and the peptides isolated by gel-filtration on Sephadex G50 in 10% acetic acid. The carboxy-terminal peptide was located by means of the fluorescence after evaporation of the acid solvent and the addition of ammonium bicarbonate and the peptide was finally

purified by means of high performance liquid chromatography. This peptide could be identified by means of the known composition of the post-methionine peptides and this was facilitated by the unique absence of serine. The peptide was sequenced by a manual gas-phase microsequencing technique and the results checked with automated gas-phase microsequencing. The sequence found revealed two amino-terminal residues and two sequences for 7 residues and thereafter a single sequence. Comparison with the expected linear sequence of the carboxy-terminal post-methionine peptide allowed the site of cleavage to be clearly identified as occuring before the alanine, 7 residues from the carboxy-terminal end, with a slightly heterogeneous loss of about 4 to 6 residues on the aminoterminal side of this alanine between the crosslinked glutamines (13 or 14) and the lysine, 6 residues from the carboxy-terminus.

The expected peptide sequence is :-

M $_{385}$K I I P F N R L T I G E G Q Q H H L G G A K Q A G D V$_{411}$

but the sequence found could be resolved by inspection as :-

$_{385}$K I I P F N R L T I G E G Q Q* H$_{400}$ (Q Q marked * *) - either Q

↕

$_{405}$A K* Q A G D V$_{411}$ - K

[where (↕) indicates a cross-link site]. There is therefore a (heterogeneous) loss of $_{401}$H L G G$_{406}$ and possibly also $_{399}$Q and $_{400}$H in subfractions of the peptide.

The loss of amino acids between the cross-link sites suggests that PAV protease cleaves D-dimer by means of hydrolysis of a peptide bond between the cross-link sites, and not by hydrolysis through the 'iso-peptide' bond, as was originally suggested.

The results were unexpected, in that contrary to current dogma, the major partner in the (γ-glutamyl)-(ϵ-lysine) reciprocal cross-link of fibrin would appear to be predominantly residue glutamine γ-399 with minor involvement of glutamine γ-398. The susceptibility of rat and bovine D-dimer to cleavage suggests that the consensus site for cleavage might lie on the carboxy-terminal side of the two glycines, 8 and 9 residues from the end and between the reciprocal crosslinks. The loss of about 4 to 6 residues from between the crosslinked residues may be due to carboxypeptidase activities of the protease or a contaminant.

1 INTRODUCTION

1.1 ASPECTS OF FIBRINOGEN STRUCTURE AND FUNCTION

Proteins in general are members of two classes - soluble and insoluble proteins eg. globular cytoplasmic and blood plasma proteins and insoluble membrane and fibrous proteins. There are few proteins that exist in both forms. Fibronectin is an example of a protein that has a major role as an integral membrane protein promoting cell anchorage, migration and differentiation (Yamada and Kennedy, 1979), and is also present in small amounts (3 mg %) as a soluble plasma protein. Whether the soluble form contributes to cell anchorage in vitro is still uncertain, although it does promote cell anchorage in tissue culture. Fibrinogen appears to be unique in that it is present in large amounts (200-400 mg%) in blood in a soluble form and is capable of increasing in times of increased demand and yet can readily be converted to an insoluble covalently stabilized fibrous form by activation of the zymogens of the coagulation cascade. An unique fibrous protein indeed since it carries within its structure the seeds of its own destruction in the form of plasmin susceptible sites and the anticipatory binding of the inactive plasmin zymogen - plasminogen (Varadi and Patthy, 1983; Lewis et al, 1984).

Fibrinogen is one of the most ancient multichain proteins, present in all vertebrates from the lamprey to man, and arose more than 450 million years ago. All the evidence points to the evolution of the three genes, coding for the fibrinogen chains from one ancestral gene (Doolittle et al, 1976; Kant et al, 1983; Kant et al, 1985). Fibrinogen similar to the vertebrate type

has not been in found in vertebrates or protochordates. Coagulable proteins are found in invertebrates (Fuller and Doolittle, 1971) but they are not related to fibrinogen and are not activated by thrombin. In common with most large proteins that have a well-defined function eg. haemoglobin, the fibrinogen molecule has several domains through which multiple functions of the molecule are mediated. In addition coagulation of the protein involves interactions between the domains. Fibrinogen is a dimeric molecule M.W. 340,000 comprising three pairs of non-identical chains $A\alpha_2$ $B\beta_2$ γ_2 (Figure 1). Electron microscopy has demonstrated details of the gross domainal structure of the molecule with electronmicrographs depicting the trinodular structure of the fibrinogen molecule that has stood the test of time. More recent work confirms the original model of Hall and Slayter (1959); Fowler et al, 1979; Ericson and Fowler, 1983; Slayter, 1983). The central E domain of the molecule is flanked by two larger distal D-domains consisting of the carboxy-terminal ends of the chains. The D-domains are connected to the E-domain by a rod-like system of interdomainal 'coiled-coils' formed by each of the three chains in alpha helical conformation wrapped around each other (Doolittle,1978). The set of proteins originally grouped by similar X-ray diffraction patterns, called the 'kmef' group (keratin- myosin- epidermin- fibrinogen), have in common regions of helical super coiling (Baily et al, 1943). The 'coiled coils' of fibrinogen begin in the E domain, relax centrally at the site of plasmic interdomainal cleavages, re-coil and terminate in the D-domains. The known amino acid sequences

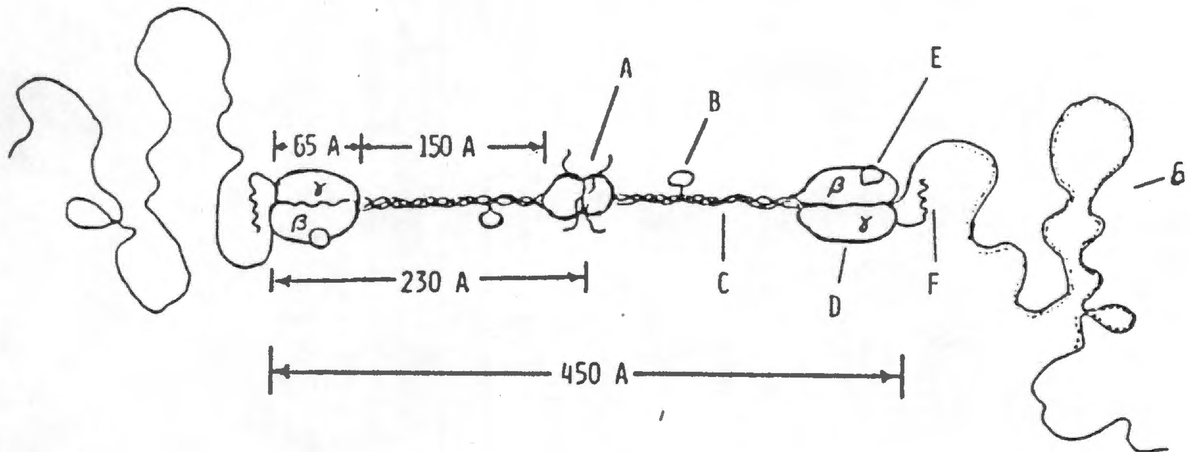


Figure 1. Diagram of vertebrate fibrinogen molecule.
(Doolittle,1983)

A = E domain with fibrinopeptides.
B = γ-chain carbohydrate cluster.
C = interdomainal connector made up of the three chains forming a coiled-coil.
D = D domain.
E = β-chain carbohydrate cluster.
F = carboxy-terminal end of the γ-chain containing the cross-linking site.
G = α-chain carboxy-terminal extension.

for the three chains are compatible with the formation of such helices (Doolittle et al, 1978; Plow et al, 1983). The carboxy-terminal ends of the alpha chains have long extensions that lie outside the D-domains. The difficulties in crystallizing fibrinogen are due to these alpha chain extensions lacking a defined conformation. The packing or disposition of this extension is still controversial. Ericson and Fowler, (1983) using electron microscopy have demonstrated the presence of an extra central nodule smaller than the E-nodule. This nodule is always missing in fragment X where the α-chain extensions have been cleaved by plasmin. Shrager et al, (1976) postulate that the carboxy-terminal ends of the α-chain are therefore folded back over the E-domain forming a protective 'umbrella' protecting it against proteolytic attack from enzymes other than thrombin. An alternative possibility is that the α chain extension may be flexible and lie free outside the confines of the D-domain (Doolittle, 1973). Cierniewski et al, (1984) have shown that the carboxy-terminal regions of the Aα-chains are surface oriented with systematic ordering of the tertiary structure. Using antibody probes they demonstrated several surface epitopes present in native fibrinogen, as well as several cryptic epitopes that are exposed after digestion of fibrinogen by plasmin.

The six peptide chains in fibrinogen are held together by a series of 29 inter and intra-chain disulphide bonds. (Henschen, 1978; Henschen et al, 1983). The two halves of the dimeric molecule are connected by 3 of 11 disulphide bridges in the central E domain of the molecule with 8 interchain disulphide bridges forming the so-called N-terminal disulphide knot - NDSK

(Blomback et al, 1968). In each of the two D-domains there are six-disulphide bridges connecting the chains, the remaining six disulphide bridges being intra-chain found at the carboxy-terminal ends of the chains (Figure 2). Hoeprich and Doolittle (1983) postulate that the two halves of the dimeric molecule are joined by the disulphide bonds in an anti-parallel orientation.

1.2 AMINO ACID SEQUENCE OF THE ALPHA, BETA AND GAMMA CHAINS.

In the mid 1970's, the most important advance in the elucidation of structure and function of fibrinogen was the complete determination of the amino acid sequence of the three chains of fibrinogen. Cyanogen bromide treatment of whole fibrinogen resulted in a large number of fragments from which the N-terminal disulphide knot was isolated and purified, and the amino acid sequence determined (Blomback et al, 1972; Blomback et al, 1973).

The three chains were isolated after reduction and alkylation of fibrin(ogen) and chromatography on CM cellulose with elution order of γ-β-α-chains (McDonagh et al, 1972). Chemical cleavage of the chains was achieved by digestion of methionyl bonds with cyanogen bromide resulting in peptides that were isolated and purified by gel filtration. These peptides were studied further using enzymes such as trypsin, plasmin and carboxypeptidases. By means of sequencing overlapping peptides using various techniques such as Edman degradation, enzyme digestion and total amino acid analysis, the entire sequence of all three chains was elucidated:- alpha -chain (Blomback et al,

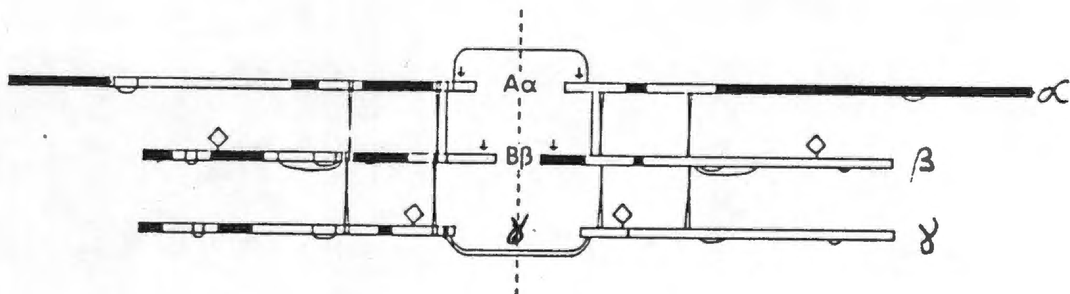


Figure 2. Primary structure model of human fibrinogen (Henschen,1983).
The chains are aligned according to homology, the N-terminal ends proximal and the C-terminal ends distal. The connecting lines represent disulphide bridges, the arrows thrombin cleavage sites and the diamonds carbohydrate side chains. On the left side of the model the disulphide knots formed by cyanogen bromide cleavage are white, on the right side of the model the plasmic fragments E and D are white.

1972; Takagi and Doolittle, 1975; Cottrell and Doolittle, 1976; Doolittle et al, 1977, a, b and c; Lottspeich and Henschen, 1978, 1979; Doolittle et al, 1979). The beta chain (Henschen and Lottspeich, 1977) and the gamma-chain (Blomback et al, 1973, Lottspeich and Henschen, 1977a and b) (Figure 3). Recent work by Wolfenstein-Todel and Mosesson, (1980) has confirmed the presence of a γ-chain variant in normal human plasma fibrinogen found by Francis et al, (1980). The γ'- (γb)-chain is larger than the γ- (γa)-chain having a carboxy-terminal extension of 16 amino acids accounting for their more negative charge and larger size. The carboxy-terminal amino acid in the γa-chains is valine while that of the γb-chain variant is leucine. The chain functions normally with regard to cross-linking and polymerization. This γ-chain variant is found only in plasma fibrinogen and not in platelet fibrinogen which has only the smaller form of γ-chains (Mosesson et al, 1984).

1.3 HOMOLOGIES BETWEEN CHAINS AND INTERSPECIES HOMOLOGIES

All vertebrates from the primitive lamprey to man have fibrinogen composed of three pairs of non-identical chains - $A\alpha_2\ B\beta_2\ \gamma_2$ - and all are converted to fibrin on removal of amino-terminal fibrinopeptides A and B by thrombin. The amino acid sequences of the three polypeptide chains are similar enough to suggest that they arose from a common ancestor (Doolittle, 1983). If the three chains are aligned with their cysteines in register, the similarity between the three chains becomes very clear. The most obvious differences between the chains are the

A α

1 A D S G E G D F L A E G G G V R G P R V V E R H Q S A C K D S D W P F C S D E D

41 W N Y K C P S G C R M K G L I D E V N Q D F T N R I N K L K N S L F E Y Q K N N

81 K D S H S L T T N I M E I L R G D F S S A N N R D N T Y N R V S E D L R S R I E

121 V L K R K V I E K V Q H I Q L L Q K N V R A Q L V D M K R L E V D I D I K I R S

161 C R G S C S R A L A R E V D L K D Y E D Q Q K Q L E Q V I A K D L L P R S D R Q

201 H L P L I K M K P V P D L V P G N F K S Q L Q K V P P E W K A L T D M P Q M R M

241 E L E R P G G N E I T R G G S T S Y G T G S E T E S P R N P S S A G S W N S G(G)

281 S G P G(G)T G N R N P G S S G T G G T A T W K P G S S G P G S T G S W N S G S S

321 G T G S T G N Q N P G S P R P G S T G T W N P G S S E R G S A G H W T S E S S V

361 S G S T G Q W H S E S G S F R P D S P G S G N A R P N N P D W G T F E E V S G N

401 V S P G T R R E Y H T E K L V T S K G D K E L R T G K E K V T S G S T T T T R R

441 S C S K T V T K T V I G P D G H K E V T K E V V T S E D G S D C P E A M D L G T

481 L S G I G T L D G F R H R H P D E A A F F D T A S T G K T F P G F F S P M L G E

521 F V S E T E S R G S E S G I F T N T K E S S S H H P G I A E F P S R G K S S S Y

561 S K Q F T S S T S Y N R G D S T F E S K S Y K M A D E A G S E A D H E G T H S T

601 K R G H A K S R P V

B β

1 Z G V N D N E E G F F S A R G H R P L D K K R E E A P S L R P A P P P I S G G G

41 Y R A R P A K A A A T Q K K V E R K A P D A G G C L H A D P D L G V L C P T G C

81 Q L Q E A L L Q Q E R P I R N S V D E L N N N V E A V Q(S,T)S S S S Q F Y M Y L

121 L K D L W Q K R Q K Q V K D N E N V V N E Y S S E L E K H Q L Y I D E T V N S N

161 I P T N L R V L R S I L E N L R S K I Q K L E S D V S A Q M E Y C R T P C T V S

201 C N I P V V S G K E C E E I I R K G G E T S E M Y L I Q P D S S V K P Y R V Y C

241 D M N T E N G G H T V I Q N R Q D G S V D F G R K W D P Y K Q G F G N V A T N T

281 D G K N Y C G L P G E Y W L G N D K I S Q L T R M G P T E L L I E M E D W K G D

321 K V K A H Y G G F T V Q N E A N K Y Q I S V N K Y R G T A G N A L M D G A S Q L

361 M G E N R T M T I H N G M F F S T Y D R D N D G W L T S D P R K Q C S K E D G G

401 G W W Y N R C H A A N P N G R Y Y W G G Q Y T W D M A K H G T D D G V V W M N W

441 K G S W Y S M R K M S M K I R P F F P Q Q

γ

1 Y V A T R D N C C I L D E R F G S Y C P T T C G I A D F L S T Y Q T K V D K D L

41 Q S L E D I L H Q V E N K T S E V K Q L I K A I Q L T Y N P D E S S K P N M I D

81 A A T L K S R K M L E E I M K Y E A S I L T H D S S I R Y L Q E I Y N S N N Q K

121 I V N L K E K V A Q L E A Q C Q E P C K D T V Q I H D I T G K D C Q D I A N K G

161 A K Q S G L Y F I K P L K A N Q Q F L V Y C E I D G S G N G W T V F Q K R L D G

201 S V D F K K N W I Q Y K E G F G H L S P T G T T E F W L G N E K I H L I S T Q S

241 A I P Y A L R V E L E D H N G R T S T A D Y A M F K V G P E A D K Y R L T Y A Y

281 F A G G D A G D A F D G F D F G D D P S D K F F T S H N G M Q F S T W D N D N D

321 K F E G N C A E Q D G(S)G W(W)N N K C H A G H L N G V Y Y Q G G T Y S K A S T P

361 N G Y D N G I I(W)A T(W)K T R W Y S M K K T T M K I I P F N R L T I G E G Q Q H

401 H L G G A K Q A G D V

Figure 3. Amino-acid sequence of the three chains of fibrinogen. (Henschen,1983).

lack of the fibrinopeptide region in the γ-chain and the long carboxy-terminal extension of the α-chain (Figure 2).

The homologies between the β- and γ-chains are greater than for either the β- or γ-chain with the α-chain, indicating that the β- and γ-chain divergence is a more recent event. The fibrinogen chains from different species are remarkably similar; rat and human chains are 82% identical, bovine and human β-chains 84% identical. The γ-chain of the lamprey resembles all other γ-chains in that it lacks the fibrinopeptide region and is also cross-linked by Factor XIII after polymerization. Lampreys diverged from other vertebrates about 450 million years ago, thus the three chains of fibrinogen evolved long before this time. In vertebrate fibrinogen there are regions that have changed rapidly and regions which have been tightly conserved. Evolutionary stability within a molecule often indicates a high degree of functional significance. The most divergent regions of the fibrinogen molecule are the fibrinopeptides A and B (Doolittle and Blomback, 1964). Their only functional role is to mask the potential polymerization sites in the α- and β-chains. By contrast the amino terminals of the α- and β-chains revealed after removal of the fibrinopeptides are highly conserved, this region being essential for fibrin-monomer polymerization (Table I). There is also a great deal of variability in the interdomainal coiled-coils where exact amino acid residues are not necessary for the α-helical coiling of the three chains around one another, as long as polar residues and non-polar residues occur in the correct register (Doolittle et al, 1978). Lamprey α- β- and γ-chains differ more from each other than the human chains. The

Table I: Amino acid sequences at the amino-termini of fibrin α- and β-chains from various species.

α-chain	
Human	Gly-Pro-Arg-Val-Val-Glu-Arg...
Bovine	Gly-Pro-Arg-Leu-Val-Glu-Lys...
Dog	Gly-Pro-Arg-Ile-Val-Glu-Arg...
Chicken	Gly-Pro-Arg-Ile-Leu-Glu-Asn...
Lamprey	Gly-Pro-Arg-Leu- ? -Glx-Glx...
β-chain	
Human	Gly-His-Arg-Pro-Leu-Asp-Lys...
Bovine	Gly-His-Arg-Pro-Tyr Asx-Lys...
Dog	Gly-His-Arg-Pro-Leu-Asp-Lys...
Chicken	Gly-His-Arg-Pro-Leu-Asp-Lys...
Lamprey	Gly-Val-Arg-Pro-Leu-Pro- ? ...

(Doolittle,1983)

lamprey α-chain contains large amounts of glycine, serine and threonine and unusually low amounts of lysine. Antibodies raised against mammalian fibrinogen (rat and humans) do not recognise lamprey fibrinogen (Doolittle et al, 1976).

There is a great deal of homology between the carboxy-terminal regions of the γ-chains of human and lamprey, the pattern being similar to that of human β and γ-chains (Strong et al., 1985). Conservation of the carboxy-terminal γ-chain sequence is not surprising in the light of the important functions of this region of the γ-chain. These are, reciprocal cross-linking by Factor X111 (Chen and Doolittle, 1971), calcium-binding (Purves and Lindsey, 1978), staphylococcal clumping (Strong et al, 1982), platelet aggregation (Hawiger et al, 1982), as well as this region being a primary contact site for polymerization (Olexa and Budzynski, 1981). At the cross-linking site of the γ-chains of lamprey the acceptor glutamine and donor lysine are only 4 residues apart, but cross-linking does occur, with formation of γ-dimers (Doolittle and Wooding, 1974; Murtaugh et al, 1974).

G E G Q Q H H L G G A K Q A G D V   Human.

G H G G Q Q Q S K G N S R G D N   Lamprey.

(Sequence from Strong et al, 1975).

1.4 FIBRINOGEN TO FIBRIN CONVERSION

1.4.1 Polymerization sites

Four steps occur in the formation of fibrin (Hermans and McDonagh, 1982).

1) Activation of fibrinogen by thrombin cleavage of

fibrinopeptide A from the amino-terminal end of the α-chain, followed by slower cleavage of fibrinopeptide B from the amino-terminal end of the β-chain resulting in the formation of fibrin-monomer.

2) End to end polymerization of monomeric fibrin to long strands two molecules in diameter called protofibrils.
3) Lateral association of protofibrils to form fibrin fibres.
4) Covalent cross-linking of fibrin by activated Factor XIII resulting in the formation of γ-chain dimers and α-chain cross-linked polymers.

Step 1 results in the exposure of polymerization sites in the central E domain of the fibrinogen molecule. Cleavage of fibrinopeptide A by thrombin exposes the gly-pro-arg site followed by the slower removal of fibrinopeptide B exposing the gly-his-arg site. Olexa and Budzynski, (1980) have defined four polymerization sites involved in fibrin formation. Two sites 'A' and 'B' unmasked in the E-domain by the action of thrombin, and 'a' located in the D-domain complementary to 'A'. A fourth polymerization site 'b' also located on the D-domain is formed by the alignment of the D-domain of two fibrin monomer molecules (Figure 4).

Fragment D inhibits fibrin formation by binding to the protofibril via a portion of the same polymerization contacts that are used for attachment to another fibrin monomer (Hermans and McDonagh, 1982; Hantgan et al, 1983). The inhibitor action of fragment D is due to the blocking of both ends of the growing protofibrils, each by a molecule of fragment D (Knoll et al,1984).

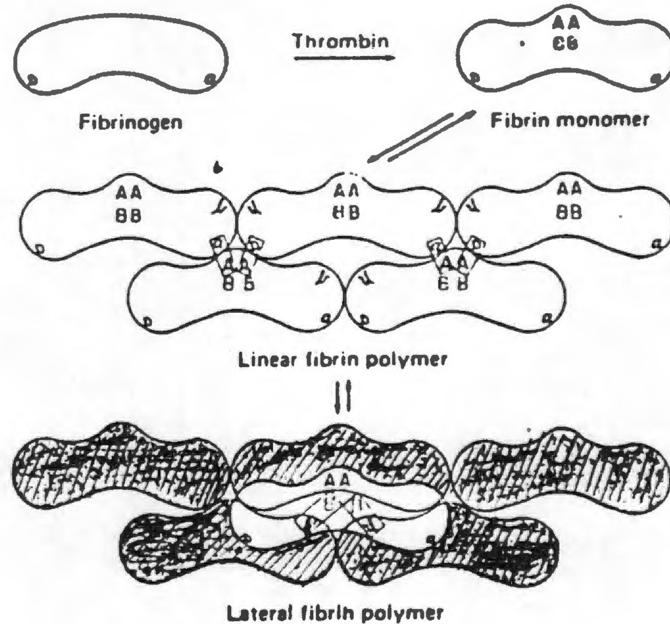


Figure 4. Model for human fibrin polymerization.
(Olexa and Budzynski,1983).
Thrombin cleavage of fibrinopeptides A and B exposes two sets of binding sites "AA" and "BB". The binding of "A" sites on the E-domain with complementary "a" sites on the D-domain results in linear polymerisation of molecules. This in turn gives rise to a bivalent "bb" site also in the D-domain complementary to the "B" site in the E-domain. These sites allow for lateral aggregation of the fibrin strands.

The work of Heene and Mathias (1973) showed that fibrinogen will bind to fibrin-monomer covalently linked to sepharose. It was subsequently demonstrated that fragment D also binds to immobilized fibrin monomer, specifically to the N terminal disulphide knot, supporting the theory of complementary binding sites on the D-domain, (Kudryk et al.,1974). The mode of alignment of the D-domains during polymerization is still not clear. Three kinds of contacts have been postulated, (Hermans and McDonagh, 1982) - these are:-

a) D E-staggered

b) D D-long

c) D D-lateral contacts

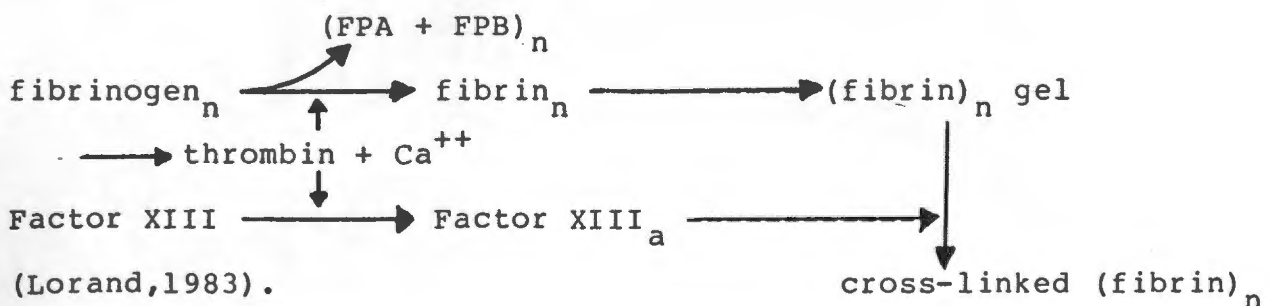
The D E-staggered contact represents the contact between the D and E domains. The site in the E domain is exposed after cleavage of the fibrinopeptides. It has been shown that fragment D binds to E, (Olexa and Budzynski, 1979) and to the amino-terminal disulphide knot (NDSK) of the E domain (Kudryk et al, 1974). Inhibition of fibrin assembly by a synthetic peptide gly-pro-arg, (Laudano and Doolittle, 1978), the sequence of the α-chain after cleavage of fibrinopeptide A, suggests that this part of the α-chain participates in the D-E staggered contact. The tetrapeptide gly-his-arg-pro, which is the sequence of the N-terminal of the β-chain after removal of fibrinopeptide B, binds to fragment D. This site is thought to strengthen the D E-staggered contact. (Laudano and Doolittle, 1981). The DD-long contact is formed between two D-domains that are adjacent to one another and results in an increase in length of the protofibrils. This contact site is strengthened by the cross-linking of the

γ-chains of the two D-domains. The D D-lateral contacts are thought to be responsible for the formation of fibres from protofibrils (Fowler et al, 1981) (Figure 5).

1.4.2 Transglutaminases

The transglutaminases are a group of enzymes that catalyze a calcium dependent acyl transfer in which the γ-carboxamide group of peptide bound glutamine residues are the acyl donors. Primary amino groups of a number of compounds including the ϵ-amino group of peptide bound lysine residues function as acyl acceptors. The transglutaminases are found in tissues such as liver and epidermis as well as circulating in zymogen form in plasma (Folk and Finlayson, 1977).

Following polymerization of fibrin-monomers, stabilization of the resulting clot occurs with the formation of ϵ-(γ-glutamyl) -lysine) cross-links between anti-parallel orientated γ-chains of the D-domains of fibrin. This cross-linking reaction is catalyzed by a transglutaminase: glutamyl peptide glutamylyl tranferase or Factor Xlll (EC.2.3.2.13). The zymogen form in plasma is converted to the active enzyme Factor Xllla by thrombin.



(Lorand,1983).

Thus the exposure of polymerization sites on fibrinogen is co-ordinated with the activation of the cross-linking enzyme.

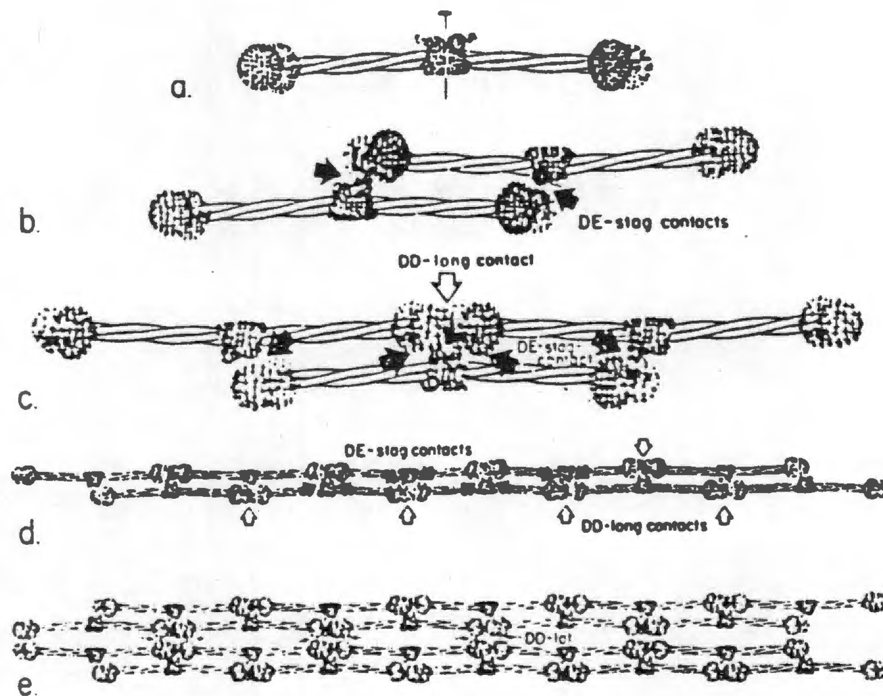


Figure 5. Schematic representation of fibrin polymerisation. (Hermans and McDonagh, 1982).

a) fibrinogen molecule with central E-domain and two terminal D-domains.
b) fibrin dimer showing D E-staggered contacts.
c) fibrin trimer with D E-staggered and DD-long contacts.
d) protofibril.
e) formation of fibres

Cross-linking of the γ-chains is rapid, not easily inhibited and precedes α-chain cross-linking. The γ-chain cross-links result in the formation of covalent γ-dimers. The α-chain cross-linking can incorporate 5 or 6 chains to form a complex α-chain polymer (McDonagh et al, 1971). The region of the γ-chain where cross-linking occurs is highly conserved in all vertebrate species, permitting hybrid cross-linking of the γ-chains of fibrin from different species eg. bovine-human (Chen and Doolittle, 1971). The finding of these hybrids also confirms the formation of intermolecular as opposed to intra-molecular cross-links.

It has recently been demonstrated that a peptide gly-pro-arg-pro that binds to the polymerization site in the D-domain of fibrin(ogen) (Laudano and Doolittle, 1981), modifies the glutamine cross-linking sites on both α and γ-chains of fibrinogen in such a way as to inhibit intermolecular cross-linking by transglutaminase (Achyuthan et al, 1986).

The formation of cross-links bestows a number of important qualities on the cross-linked protein: rigidity, strength, insolubility and stability. The last quality is a reflection of the resistance of the fibrin cross-link to proteolysis. Enzymes capable of separating the cross-linked D-domains of fibrin in the native state, at neutral pH and physiological concentrations of Ca^{++}, have not been reported.

1.4.3 Assignment of the fibrin cross-link site in the γ-chain.

The cross-link site lies in the carboxy-terminal end of the γ-chain. The acyl acceptor is lysine γ-406. One of two glutamine residues could be the putative acyl donor: glutamine

γ-398 or γ-399. Under natural conditions the carboxy-terminal peptide segments from adjacent γ-chains are aligned anti-parallel and are reciprocally cross-linked by two ε-(γ-glutamyl)-lysine isopeptide bonds, the lysine side chain of one segment being condensed with γ-glutamine side chain of the other and vice versa. The cross-link has been assigned to sites lysine γ-406 and glutamine γ-398 of reciprocal human γA-chains, seven residues apart (Chen and Doolittle, 1971; Sharp et al, 1972; Doolittle, 1973). The cross-link was assigned by locating [^{14}C]-glycine ethyl ester substituted onto fibrin by transglutaminase (Chen and Doolittle, 1970). The assumption made was that this occurred at the natural γ-chain cross-link site. Since the actual cross-link sites have not yet been demonstrated by direct sequencing of cross-linked γ-chains, it is possible that both glutamines at sites γ-398 and γ-399 could be the target for transglutaminase activity. The cross-link site has also been studied utilising a fluorescent lysine analogue - monodansyl-cadaverine - that is incorporated into the cross-link sites by transglutaminase. Digestion of this partially cross-linked fibrin by plasmin gives rise to a fluorescent D-dimer with an intact cross-link and dansyl-cadaverine substituted cross-link sites (Purves and Lindsey, 1978).

D-dimer can be separated into monomers when the molecule is rendered susceptible to plasmin cleavage by removal of calcium ions. Under these conditions two smaller D-monomers with shortened γ-chains, and a peptide containing the cross-link and the substituent fluorescent dansyl cadaverine, are produced (Haverkate and Timan, 1977; Purves and Lindsey, 1978).

1.4.4 Role of calcium in the clotting of fibrinogen and the structure of fibrin

Calcium ions are required for all stages of the clotting process and fibrinogen itself binds calcium (Kd 8.7 x 10^{-6}M). (Lindsey **et al**, 1978). Removal of Ca^{++} ions by chelation with EDTA renders fibrinogen more susceptible to thermal and alkali denaturation (Ly and Godal, 1973) and to further plasmin degradation beyond the natural terminal plasmin degradation products D-monomer, in the case of fibrinogen, and D-dimer in the case of fibrin (Haverkate and Timan, 1977; Purves and Lindsey, 1978) Since calcium ions are always present in physiological fluids **in vivo** consequences of the removal of Ca^{++} ions would appear to be irrelevant in plasmin-mediated fibrinolysis and related phenomena. It has been demonstrated that fibrinogens from man (Purves & Lindsey 1978), rat (Niewenhuizen **et al**, 1981) and cow (Marguerie, 1977) have three calcium binding sites. Two of these high affinity sites (Kd 10.6 x 10^{-6}M) have been shown to be situated in each of the two D-domains (Purves **et al**, 1978; Haverkate and Timan, 1977; Niewenhuizen, 1979).

Recently the calcium-binding site on the D-domain has been shown to be located in the peptide γ-311 to γ-336 using terbium fluorescence (Dang **et al**, 1985). The sequence of the γ-chain peptide from γ-315 to γ-329 is homologous to other calcium binding proteins notably calmodulin (Henschen, 1983) and parvalbumin (Kretzinger, 1973).

The third calcium-binding site (Kd 1.8 x 10^{-4} M) is situated in the central E domain of fibrinogen, specifically in the NDSK portion of the E domain involving residues Aα 17-19, Bβ 15-53

and/or γ-54-78 (Niewenhuizen et al, 1983).

1.4.5 Binding of fibronectin, and plasminogen to the fibrin clot

Fibrin clots are composed of fibrin, fibronectin (Mosher and Johnson, 1983) α-2 anti-plasmin (Tamaka and Aoki, 1981) and plasminogen (Patthy and Varadi, 1983). Fibronectin associates with the clot both by non-covalent binding and covalent cross-linking mediated by activated Factor X111, with the α-chain of fibrin. The α-chain of fibrin contributes the lysine donors while the fibronectin provides the glutamine acceptors for the formation of ε-(γ-glutamyl)-lysine cross-links. One of the functions of fibronectin association with the fibrin clot may be to form a surface for the attachment and spreading of cells during wound healing (Grinnell, et al, 1980). Plasminogen binds to both fibrinogen and fibrin but more strongly in the case of fibrin suggesting that the plasminogen binding sites are not fully fuctional in fibrinogen. (Lewis et al, 1984) have shown that one mole of fibrinogen will bind 4 moles of either lys- or glu-plasminogen with a progressive increase in the affinity of plasminogen for fibrinogen as the binding sites are occupied, suggesting co-operative binding behaviour. There are binding sites for plasminogen in both the D and E domains of fibrin. Proteolytic modification of fragment D_1 to D_{EDTA} causes a significant increase in a number of plasminogen binding sites suggesting that the carboxy-terminal end of the γ-chain may be involved in masking the plasminogen binding site in the D domain (Varadi and Patthy, 1983). Fibrin appears to have at least two types of plasminogen binding sites, one on intact fibrin clots

and another on partially plasmin degraded clots. All the early cleavages by plasmin during fibrinolysis are at lysyl bonds thus creating new plasminogen binding sites (Bok and Mangel, 1985).

1.5 FIBRIN(OGEN)OLYSIS

1.5.1 Plasmin degradation of fibrinogen

The mechanism for fibrinogen degradation by plasmin in vitro has been extensively studied (Marder et al, 1969; Pizzo et al, 1972, 1973a 1973b; Mosesson et al, 1973; Ferguson et al, 1975). However the in vivo degradation of fibrinogen is not as well understood. Both fibrinogen and fibrin have an affinity for the lysine binding sites of plasminogen. This plasminogen binding anticipates the eventual dissolution of the fibrin clot. Fibrinogen binds plasminogen less strongly than fibrin (Lucas et al., 1983). The bound plasminogen is activated by tissue plasminogen activators related to urokinase and converted to an active plasmin (Niewenhuizen et al, 1983; Lucas et al, 1983; Silverstein et al, 1985). As plasmin begins to digest the fibrin clot, more plasminogen binding sites are exposed. Plasmin hydrolyses proteins and peptides at the carboxyl side of arginyl and lysyl residues in both protein and ester substrates (Wainstein and Doolittle, 1972).

Plasmin digestion of fibrinogen proceeds through asymmetrical cleavage of the fibrinogen molecule to fragment X, Y and ultimately the terminal degradation fragments D and E (Lucas et al, 1983). First step in fibrinogen degradation is removal of peptides +/- 40,000 M.W. from the carboxy-terminal end of the αA-chain leaving an amino terminal remnant of αA attached

by the disulphide bonds to bβ and the γ-chains. This degradation is in fact difficult to prevent eg. in stored plasma. Plasmin then cleaves peptides 60,000 daltons from the amino-terminus of the βb-chains (Mosesson et al, 1972; Budzynski et al, 1974) followed by splitting of the molecule by cleavage through all three chains with assymetric products produced en route. These susceptible cleavage sites occur where the regularity of the coiled-coils is perturbed by proline residues imparting a structure that determines sensitivity to plasmin. A single cleavage gives rise to fragment Y with release of one fragment D. Another similar cleavage reduces Y to a second fragment D and frees the central region of the molecule, fragment E, containing the amino terminals of the six chains held together by disulphide bonds i.e. the NDSK or N-terminal disulphide knot (Figure 6). It has been found that fragment D and E of plasmin digested fibrinogen form non-covalent complexes (Plow et al, 1977). However this association does not occur in native fibrinogen or between individual fibrinogen molecules.

1.5.2 Plasmin degradation of fibrin.

Fibrin is degraded by plasmin in the same way as fibrinogen in an orderly sequence of proteolytic cleavages. The clearest model of fibrinolysis has been proposed by Francis and Marder, (1982, 1983). Their proposed model is summarized as follows:

1) Limited proteolysis, but all D and E domains still attached covalently to other parts of the clot matrix.
2) Some fragments are bound to the particulate matrix by non-covalent bonds.

Figure 6. Diagram of fibrinogen digestion by plasmin. (Lucas et al 1983).

The small vertical arrows represent plasmic cleavages; the dashed lines indicate the minimal number of disulphide bonds present in each fragment.

3) Release of soluble complexes into solution.

4) Degradation of soluble complexes to plasmin-resistant terminal forms D-dimer and fragment E, that are non-covalently associated (Olexa and Budzynski, 1979).

Under physiological concentrations of calcium plasmin is unable to cleave D-dimer (Haverkate and Timan, 1977; Purves et al, 1978). Francis and Marder (1982) by means of SDS-gel electrophoresis of fibrin digests, have characterised several soluble cross-linked fibrin degradation products: DD/E, DY/YD YY/DXD and XXD/DXY. Following their release into solution further plasmin degradation results in fragment D-dimer and fragment E. Due to the intact polymerization sites of fragment D-dimer and fragment E, they have a high affinity for each other and tend to associate non-covalently (Olexa and Budzynski, 1979).

Fragments D_1 and D-dimer have been shown to inhibit clot formation by blocking the polymerization sites on the E-domain of fibrin monomer (Hermans and McDonagh, 1982).

1.5.3 Physiological interactions of fibrin(ogen) degradation products

At least five species of fragment D can be formed in vitro, each differing in the extent of the carboxy-terminal degradation of the γ-chains (Purves and Lindsey, 1978). Physiological concentrations of Ca^{++} protects fragment D_1 from conversion to the other species (Haverkate and Timan, 1977; Purves et al, 1978). Fragment D_1 possesses the γ-chain cross-link site (Ferguson et al, 1975), a calcium binding site (Purves et al, 1978), a binding site for the platelet receptor for fibrinogen (Kloczewiak et al, 1982) and a staphylococcal binding site (Hawiger et al, 1982).

Clumping of staphylococci by fibrinogen is an example of a cell agglutination reaction which requires that the agglutinin i.e. fibrinogen, be bivalent in order to effect the binding of two cells together. This requirement is fulfilled as the staphylococcal binding site is in the D-domain, and there are two D-domains in the fibrinogen molecule. The breakdown of the bivalent fibrinogen molecule into two monovalent fragments D abolishes the clumping reaction but retains the ability to bind staphylococci (Hawiger et al, 1982). Fragment D stimulates fibrinogen synthesis (Kessler and Bell, 1980; Franks et al, 1981) and the proliferation of haemapoietic cells in vitro (Hatzfeld et al, 1982). Intravenous infusion of purified fragment D_1 induces hypoxemia and tachypnea and increases capillary permeability in rabbits (Luterman et al, 1977). The plasma level of fragment D is elevated in patients with the adult respiratory distress syndrome (Rinaldo and Rogers, 1982) and in

patients with disseminated intravascular coagulopathy (Bell, 1980). These syndromes all show severe endothelial cell abnormalities. Fragments D_1 and D_2 were found to disrupt endothelial cell monolayers in a dose responsive manner, indicating that fragment D directly disorganizes the architecture of the endothelial cell monolayer. Fragment D-dimer has the same effect. That the cross-link site is not necessary for this phenomenon is also shown by the effectiveness of D_2 which lacks the cross-link site. These results suggest that fragment D plays a role in the pathogenesis of syndromes with vascular endothelial damage (Dang et al, 1985). All these phenomena are lacking in fragments D_2-D_5. Fragment D has been shown to inhibit clotting in fibrinogen solutions (Kawaski, 1968), to prolong thrombin clotting times (Belitzer et al, 1975), to decrease the rigidity and alter the light scattering of fibrin gels (Williams, 1981). Fragment D_1 does not inhibit thrombin itself or the formation of fibres from protofibrils of sufficient length (Williams, 1981). Fragment D_1 does inhibit fibrin assembly (Williams, 1981) and fibrin polymerization (Hermans and McDonagh, 1982).

The region of the D-domain involved in staphylococcal clumping and platelet aggregation has been located in the carboxy-terminal region of the γ-chain by means of inhibition studies using a 27 residue cyanogen-bromide fragment of the carboxy-terminal end of the γ-chain. (Strong, 1982; Kloczewiak et al, 1982, 1984), have located the interaction of the D-domain of fibrinogen with platelets, in the last 15 residues from the carboxy-terminal end of the γ-chain. Varadi and Scheraga (1986)

using isolated fragment D_2 which lacks carboxy terminal γ-356-411, have shown that this fragment binds calcium, but does not inhibit polymerization. Thus the calcium binding site γ-311 to γ-333 does not overlap with the polymerization site in the D-domain. However the binding of the calcium may cause conformational changes that enhance reactivity of the adjacent polymerization site. Horwitz et al, (1984) have localized the fibrin γ-chain polymerization site within segment Thr-374 to Glu-396 of fibrinogen (Figure 7).

1.5.4 Regulation of fibrinogen synthesis

The main site of fibrinogen synthesis is the liver. Fibrinogen levels in plasma are closely correlated to physiological demands eg. in pregnancy, response to injury and bleeding and as an acute phase reaction in inflammatory states. Most work has shown that sharp reductions or elevations of plasma fibrinogen concentration do not influence the basal rate of fibrinogen synthesis (Alving et al, 1979). The most potent stimulator of fibrinogen synthesis in the intact animal was found to be fibrinogen fragment D_1. In contrast fibrin degradataion products, cross-linked or non-cross-linked, had no stimulatory effect on fibrinogen synthesis (Kessler and Bell, 1980; Franks et al, 1981). The reasons why degradation products from fibrinogen but not from fibrin, stimulate fibrinogen synthesis, remain unexplained. Ritchie et al (1982) using rat hepatocyte monolayer cultures, found that fragment D_1 stimulates fibrinogen synthesis through an indirect pathway involving cells of the reticuloendothelial system. When leucocytes were treated with

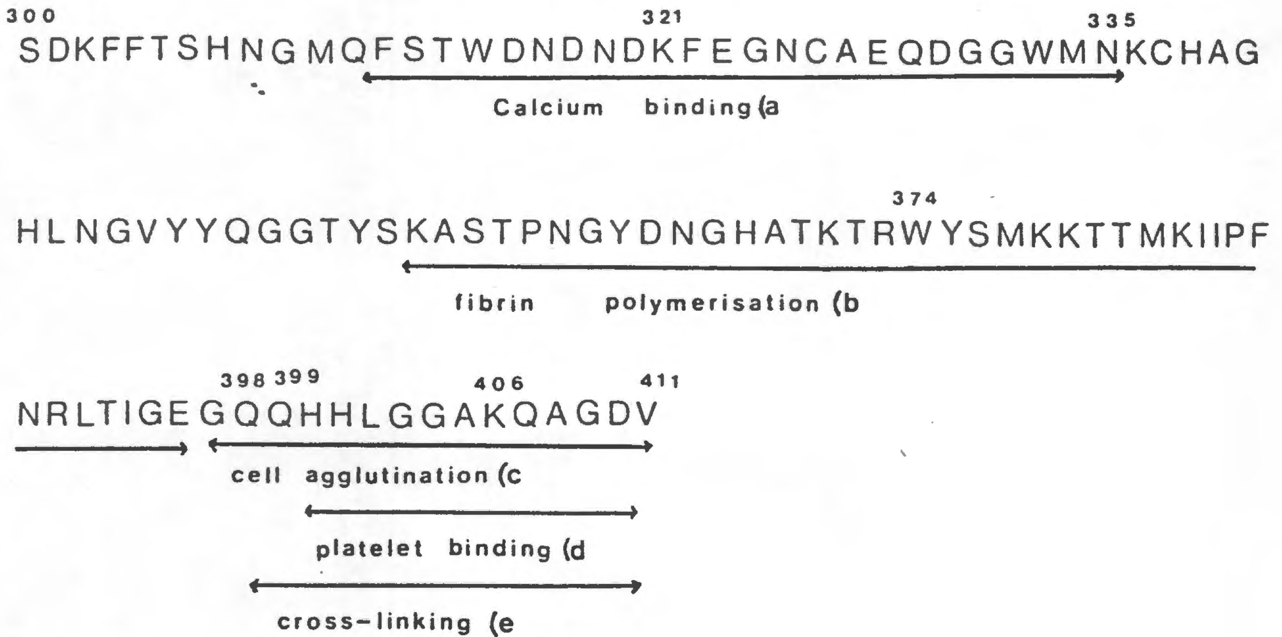


Figure 7. Sequence of the carboxy-terminal end of the γ-chain of human fibrinogen showing regions important for specific activities.

Information was compiled from the following references:

a) Dang, 1985
b) Olexa and Budzynski, 1980
c) Hawiger, 1982
d) Kloczewiak et al, 1984
e) Chen and Doolittle, 1971.

fragments D_1 and/or E they responded by producing an hepatocyte stimulating factor (HSF) that had a dramatic effect on fibrinogen synthesis. Addition of fibrinogen itself to the leucocytes did not stimulate production of HSF.

1.5.5 Synthesis of fibrinogen

Comparison of the amino acid sequences of the three chains of fibrinogen shows that the three chains are highly homologous and suggest that they arose from a common ancestral gene (Doolittle et al, 1979). Since all vertebrate fibrinogen including lamprey fibrinogen is made up of the three chains triplication of the gene must have occured more than 450 million years ago, making fibrinogen a very ancient multi-chain protein (Crabtree et al, 1985). Recent work has shown that each chain has an independent messenger-RNA indicating the presence of three genes (Nickerson and Fuller 1981; Crabtree and Kant 1981; Rixon et al, 1983; Kant et al, 1983). The work of Kant et al (1983) has confirmed the postulate that the genes for the three polypeptide chains have arisen by duplication and subsequent divergence of an ancestral gene. The α and β-genes arose first with subsequent splitting at a later stage of the beta and gamma genes.

Kant et al (1985) have shown that the genes encoding the α-, β- and γ-chain polypeptides of human fibrinogen occur as single copies closely linked in a 50 Kb region of chromosome 4. The α-gene lies between the γ- and β-genes. The α and γ-gene are oriented in tandem and transcribed towards the β-gene. The β-gene is transcribed from the opposite DNA strand toward the γ- and

α-genes. The structure of the γ-gene is closely related to the functional regions of the γ-chain. The human γ-chain gene consists of 10 exons coding for the 411 amino acid residues and a leader sequence of 26 amino-acids (Rixon et al, 1985). The positions of the nine introns divide the gene into 10 segments each roughly corresponding to separate functions in the γ-chain. Exon I codes for the signal peptide and Exon II for the cysteines forming the disulphide bridge linking the γ-chains together. Exon III codes for the cysteines of the first disulphide ring and a portion of the coiled-coil, and the carbohydrate attachment site at asparagine residue 52. Exon IV codes for more of the coiled-coil and exon V the rest of the coiled-coil and the second disulphide ring marking the end of the coiled-coil. The residues found in the D-domain are coded for by exons VI, VII and VIII. This region shows the highest degree of homology when compared to the β-chain are between γ-chains of different species. Exons IX and X code for the region containing the cross-linking amino acid residues as well as those residues involved in fibrin polymerization and platelet receptor recognition. The last intron contains sequences that code for the γ-chain extension and is generated by alternative processing and polyadenylation of the γ-chain transcript within the IXth intron resulting in read-through of the 9th intron/exon functional sequence (Rixon et al, 1985).

The mRNAs for the three genes are co-ordinately regulated at the transcription level (Fowlkes et al, 1984). Although there is only one gene for the γ-chain of fibrinogen, two γ-chains are synthesized γA and γB, differing only in their carboxy-terminal ends (Wolfenstein-Todel and Mosesson, 1984). Crabtree and Kant

(1982) propose an alternative pathway of splicing mRNA transcripts as a likely mechanism for the production of γB in rats.

Fornace et al (1984) have found that there is only one locus for the γ-gene and that γA and γB arise by alternate mRNA splicing near the 3' end of the gene. In the rat γB is produced by alternate splicing with identical polyadenylation sites (Crabtree and Kant, 1982). In humans γB is produced when the VIIIth intervening sequence remains unspliced and a polyadenylation signal within this intron is used. The new carboxy-terminus of the γB-chain is 16 amino acids longer than the γA-chain. The two γ-chains are produced at the same time in the liver, and their relative proportions remain the same even after subjecting the liver to a variety of stimuli known to induce fibrinogen messenger-RNA (Crabtree and Kant, 1982).

In vitro studies have indicated that the individual chains are assembled in the rough endoplasmic reticulum into the dimeric molecule (Kudryk et al, 1982). Fibrinogen assembly commences while nascent βB-chains are still attached to membrane-bound polysomes, followed by the nearly simultaneous incorporation of Aα and γ-chains (Alving et al, 1982) from the intra-cellular pool of these chains (Yu et al, 1983).

In humans and rodents fibrinogens of slightly different molecular weights are synthesised due to the existence of two γ-chains - γA and γB.

1.6 ASSAY OF FIBRINOGEN / FIBRIN DEGRADATION PRODUCTS

The immunological approach to the study of fibrinogen and its derivatives has assummed increasing importance. Current fibrinogen degradation product (FDP) assays are unreliable. The difficulties of accurate FDP assay are three-fold:

i) The specificity of the antibody in immunological assays

ii) The generation of fibrin degradation products during handling of the blood samples

iii) The loss of fibrin fragments generated in vitro and the development of soluble fibrin related to the processing of the sample in thrombin induced sera.

The conventional FDP assays give little real information about in vivo levels in patients (Gaffney and Perry, 1985). Any immunological method for detection of FDP's in vivo, must be able to distinguish between Y-D, $(Y\text{-}D)_{X\text{-}L}(D\text{-}Y)$ as well as X, Y, D, D-dimer and E.

The advent of monoclonal antibody techniques has facilitated the development of antibody probes with affinity for specific epitopes. In the review by Plow and Edgington (1982), roughly thirty-five epitope sets present in fibrinogen and its derivatives are described. They point out that large regions of the molecule remain unexplored. These include the central portion of the β-chain, the α-chain extension that lies outside the D-domain, the γ-chain that lies between the D and E domains that contains several plasmin cleavage sites, and the carboxy-terminal end between residues γ-265 and γ-411 which contains a number of physiologically important sites. There are two main types of epitope - simple epitopes where antibodies will bind

denatured polypeptide chains or limited peptide sequences contained within the epitope - and - complex epitopes that require a specific conformation of the polypeptide chains and often consist of non-sequential elements of the sequence of one or more peptide chains.

Several assay systems have been developed with a high degree of sensitivity for specific fibrinogen antigens at low concentrations in plasma. These include radio-immunoassays and enzyme linked immunoassays (ELISA), both using monoclonal antibodies. The epitopes used for the raising of monoclonal antibodies include whole fibrinogen which resulted in antibodies that bind D-monomer and fragment D obtained from non-crosslinked fibrin, but not D-dimer or plasmic cleavage products D_2 or D_3, thus indicating that the epitope lies in the carboxy-terminal end of the γ-chain (Wilner et al, 1982).

Monoclonal antibodies have been raised to the N-terminal end of the β-chain of fibrin that are able to distinguish between fibrinogen and fibrin and fragment E derived from these two molecules (Hui et al, 1983). Their method has been developed further resulting in the production of a species specific antibody, using a sequential epitope, the human post-thrombin amino-terminal sequence, peptide β(1-7) -

gly-his-arg-pro-leu-asp-lys-

that binds to human and canine fibrins but not bovine, ovine or porcine fibrins (Matsueda and Margolies, 1986).

Muller-Berghaus et al (1985) have developed a monoclonal antibody directed at the amino terminal end of the α-chain after

cleavage of fibrinopeptide A, exposing the sequence -

gly-pro-arg-val-val-glu-.

This antibody raised with the hexapeptide as antigen, is able to distinguish between fibrin and fibrinogen. Rylatt et al (1983) have developed a solid phase enzyme linked radio-immuno-assay with a sensitivity of 10 ng/ml, suitable for detection of cross-linked derivatives in serum and plasma samples in a clinical situation (Hafter et al,1985). One of the aims of the present study is the utilization of puffadder venom enzyme to expose further epitopes in the γ-chain of cross-linked D-dimer.

1.7 SNAKE VENOMS AND FIBRINOLYSIS

1.7.1 Proteinases from snake venoms

Snake venoms are a prolific source of enzymes many of which are useful in coagulation studies. At least 26 enzymes types have been described, 10 of which are found to be common to all snake venoms, and the other sixteen are scattered throughout the five families of poisonous snakes (Russel, 1980). These enzymes tend to be chacteristic of certain families or genera eg. elapid venoms are rich in acetyl cholinesterase whereas crotalid and viperid venoms lack this enzyme but are rich in endopeptidases (Table II).

Many of these enzymes interfere with blood coagulation (Dyr, 1983; Niewerowski, 1979; Ouyang, 1979) and have extensively studied due to their potential clinical importance. These enzymes affecting coagulation may be devided into two groups; those with thrombolytic activity eg. ancrod and batroxabin (Dyr, 1983; Kirby, 1979; Bajwa, 1982), and those with

Table II: Enzyme distribution in various snake venoms.

Snake venoms (crude)	Proteinase activity	Arginine ester hydrolase activity	Clotting activity
Crotalidae			
Agkistrodon halys blomhoffii	24.5	1.8	-
A. piscivorus piscivorus	35.3	4.6	±
A. contortrix contortrix	49.5	11.1	+
A. contortrix mokesan	46.5	10.2	+
A. acutus	25.5	2.9	++
Crotalus adamanteus	13.8	16.8	±
C. atrox	59.0	4.2	++
C. durissus terrificus	25.7	8.9	++
C. viridis viridis	27.5	9.1	+
C. basiliscus	77.0	4.0	±
Trimeresurus flavoviridis	22.5	1.5	±
T. okinavesis	19.0	2.2	-
T. macrosquamatus	26.8	34.1	±
T. gramineus	11.5	8.0	++
Bothrops atrox	17.8	3.8	++
Viperidae			
Vipera russelli	2.6	1.1	-
Echis carinatus	23.5	1.1	±
Bitis gavonica	11.2	2.7	±
bitis arieatans	10.0	1.0	-
Elapidae			
Naja naja atra	0.9	-	-
Naja nigricollis	0.5	-	-
Dendroaspis angusticeps	1.0	-	±

Protein activity expressed as ug of tyrosine equivalent of TCA-soluble product formed per minute per mg protein.
Arginine hydrolase activity expressed as umoles of a-toluene sulphonyl ethyl ester hydrolysed per minute per mg protein.
++ = fibrin fibres appear in 3 min., + = within 20 min., ± = within 2 hours.

Iwanaga et al,1976.

fibrin(ogen)olytic activity; (Moran and Geren, 1981; Bajwa and Pandya, 1984). The fibrinolytically active enzymes may be further grouped according to which chain of fibrinogen they preferentially attack, namely the α and β-fibrinogenases (EC 3.4.21.5) (Ouyang, Feng and Chen, 1977; Teng, Ouyang and Lin, 1985; Nikai et al, 1983). The α-chain of fibrinogen is very susceptible to proteolytic attack and many of the snake proteases disrupt it eg. ecarin from Echis carinatus, which digests the α-chain of fibrinogen rendering it incoaguable by preventing cross-linking of the α-chain (Kornalik, 1979). Venom from Naja nigricollis contains an enzyme that degrades only the α-chain of fibrinogen and the α-polymer from fibrin leaving the β- and γ-chains intact (Evans, 1981). Pandja and Budzynski (1984) have isolated four enzymes from Crotalus atrox, two of which were found to be alkaline serine proteases which only attack the β-chain of fibrinogen and two which are zinc ion dependent metalloproteinases.

1.7.2 Puffadder (Bitis arietans) venom proteases

The venom of the puffadder acts slowly by destroying tissue and haemolyzing the blood through the action of proteases unlike the fast acting fatal neurotoxic venom of the cobras. Van der Walt (1971) separated three major and two minor proteases by gradient elution from CM-cellulose. One of these proteases - protease A was further purified by Sephadex G 75 gel filtration. The enzyme MW 21,400 hydrolysed casein with a pH optimum of 10.

The enzyme was inhibited by EDTA but not inhibited by DFP, mercuric compounds, or chloromethylketones. The protease did not

hydrolyse TAME or ATEE. It was found to have a broad specificity with three different protein substrates hydrolysed randomly yielding a large number of small peptides. Synthetic peptides such as met-phe-gly, gly-leu-tyr, gly-leu, gly-ile and poly-L-tyr were not attacked by protease A, suggesting that it requires a substrate exceeding a certain minimum number of amino acids (Van der Walt, 1970). He concluded that a hydrophilic region stretching over two residues as well as an hydrophobic residue in the substrate polypeptide chain promote the affinity of protease A for that region and are desirable for the hydrolysis of the peptide bond in that region. Iwanaga (1976) has stated that proteinases from a number of snake venoms (Table II) hydrolyse many kinds of substrates with a specificity that is directed toward bonds in which the amino groups are contributed by hydrophobic residues (Iwanaga, S., Oshima, G. T., Suzuki, T.). In all cases studied specificity is dictated by the identity of the residue contributing the amino group to the bond, and preference is for a hydrophobic residue in this position. There is also a minimal substrate size for hydrolysis.

Several workers have found that puffadder venom is the most potent of the snake venoms tested, preferentially destroying proteinase inhibitors in human plasma (Kress and Paroski, 1978; Kress, 1979). The enzyme or enzymes that are zinc ion dependent metalloproteinases destroy the serine protease inhibitors present in plasma thus rendering other enzymes eg. renin (Lawrence and Morris, 1981) and prekallikrein active (Morris et al, 1980). The effects of puffadder venom on fibrinogen and fibrin have not been studied.

An haemorrhagic principle was isolated from puffadder that had no caseinolytic or esterase activity and was not associated with the lethal properties of the venom (Mebs and Panholzer, 1982). Zinc dependent metalloproteinases with low molecular weight 21,000-24,000 daltons have been isolated from many snakes (Ouyang et al, 1979; Evans, 1981; Dyr et al, 1983; Pandya et al, 1983; Pandya and Budzynski, 1984). They have all been tested for activity against fibrinogen, but only venom from Naja nigricollis was tested for fibrinolytic activity. This enzyme cleaved the α-polymer but not the di-γ-chain of fibrin (Evans, 1981). The low molecular weight, zinc-dependent metalloproteinase from puffadder venom has the ability to cleave D-dimer, from cross-linked fibrin, into two apparently symmetrical monomers (Purves et al, 1986).

The aim of this study was to utilize the properties of this enzyme from puffadder venom to try and distinguish between any special conformational features of the D-domains from fibrinogen and fibrin. Since the enzyme cleaved D-dimer into monomers it was necessary to establish whether the enzyme is an isopeptidase capable of directly cleaving the ϵ-(γ-glutamyl)-lysine isopeptide bond.

2 METHODS

2.1 ASSAY METHODS.

2.1.1 Polyacrylamide gel electrophoresis (SDS-PAGE).

(a) SDS-PAGE - 1.5 x 150 x 150 mm slab polyacrylamide gels containing 1% sodium dodecyl sulphate (SDS) were run at 50 V for 16 hours in a Hoefer Model SE 600 electrophoresis apparatus. The acrylamide gradients used were 4-20%, 6-8% and 10-12%. Gels were stained with Coomassie Blue or silver stained if greater sensitivity was required.

(b) Acid-Urea PAGE.

Acid urea gels were run in the same Hoefer apparatus as the SDS gels. The tank buffer was 0.9 M acetic acid. The separating gel was 8% acrylamide containing 2.5M urea and 5% acetic acid. The sample solvent was 0.1 M HCl containing 8 M urea, 5% BME and 0.5% pyronin Y.

2.1.2 Protein Concentration.

Protein concentrations were determined using a method based on the Bradford dye-binding procedure (Bradford, M., 1976). The dye Coomassie Blue G 250 was obtained from Biorad laboratories. A standard curve was constructed using bovine serum albumin from 0 - 100 μg in 0.1 ml; 0.5 ml of 5X diluted dye reagent added to an 0.1 ml aliquot of 1/10 or 1/100 diluted sample. The blank was 0.1 ml of distilled water or the buffer diluent. Urea, Tris and EDTA do not interfere with the assay.

2.1.3 Chromozym assays (Lottenberg et al, 1981).

Activity of the plasmin used was ascertained using the Chromozym assay with Chromozym PL as substrate.

Substrate: Chromozym PL(Boehringer)-tosyl-glycyl-prolyl-lysine-4-nitroanilide acetate M.W. 634,7. The substrate (20 mg) was dissolved in 1 ml distilled water adjusted to pH 4.0 with HCl and stored frozen.

Assay buffer 0.01 M Hepes
0.01 M Tris/HCl pH 7.8
0.1 M NaCl
0.1% Polyethylene glycol 6000.

Assay 1 ml buffer
0.1 ml substrate
10 µl plasmin/glycerol

The reaction was followed in a spectrophotometer at wavelength 405 nm.

Assays were performed to ascertain the specificity of crude puffadder venom with four different Chromozym substrates.

2.1.4 Fibrinogenolytic activity of puffadder venom protease.

Fibrinogenase activity was measured by the method of Ouyang and Teng (1976). Fibrinogen solution, 5 mg (10 mg/ml) was incubated at 37°C with 50 µl of puffadder venom (1 mg/ml). At each time interval a 0.5 ml aliquot was withdrawn, thrombin and calcium added and the time noted for the clot to develop. Aliquots, 50 µl, were withdrawn at the same time intervals and analyzed on a 4-20% SDS-PAGE gel under reducing and non-reducing conditions and stained with Coomassie Blue.

2.1.5 Caseinolytic assay for puffadder venom proteases.

Substrate 1% casein was made by dissolving 100 mg casein with heating in 5 ml of 0.012 N NaOH and made up to 10 ml with 0.1 M

Tris/HCL buffer pH 9.0.

Assay 100 µl casein

50 µl puffadder venom enzyme

10 µl 10 mM Zn Cl_2

The mixture was incubated at 37°C for thirty minutes, after which 500 µl of 0.44 M trichloracetic acid was added. The solution was left at 4°C overnight and centrifuged. To 250 µl supernatant, 1.25 ml 0.4 M Na_2CO_3, and 250 µl Folin-Ciocalteau reagent, was added, allowed to stand for 10 minutes and the absorbance read at 660 nm.

2.1.6 Monoclonal antibody radio isotope assay.

The monoclonal antibodies for this work were a gift from Dr. P. G. Bundesen MAbCO. Inc., Australia.

"Soft" chloramine T iodination method (Walters, M. University of Queensland).

Reagents:
1) Mix equal volumes of 0.3 M Na_2HPO_4 and 0.3 M NaH_2Po_4 to give phosphate buffer pH 7.
2) 56 mg Chloramine T in 10 ml H_20 diluted 1 in 100 with 0.3 M Phosphate buffer. 10 µl of chloromine T solution per 990 µl H_20.
3) 38 mg Sodium metabisulphite in 10 ml H_20 diluted 1 in 100 with 0.3 M sodium phosphate buffer. 10 µl metabisulphite per 990 µl H_20.
4) Phosphate buffered saline (PBS)
5) 2% bovine serum albumin (BSA)
6) 1% potassium iodide in H_20.

Method

Mix: 25 µl 0.3 M sodium phosphate buffer

25 µl 200 µg/ml protein or antibody in sodium phosphate buffer.

10 µl $Na^{125}I$ (1 mCi)

25 µl chloramine T.

Leave for 30 seconds

Add 25 µl Na metabisulphite and

1 drop of KI

Dilute the reaction mixture with 500 µl PBS and two drops 2% BSA. Recover iodinated protein by gel filtration on a Sephadex G 25 PD-10 column equilibrated with 15 ml of PBS. Collect 10 0.5 ml fractions. Count 10 µl of each fraction. Pool the fractions with the highest counts, usually tubes 5, 6, and 7.

In a capture/tag assay, the antigen in question is reacted with two antibodies with specificity for different regions of the same molecule. Usually a capture antibody is attached onto a solid phase and after addition of antigen to allow binding to occur, the presence of bound antigen can be detected after washing, by the addition of the second labelled antibody, labelled either with horse-radish peroxidase or with ^{125}I.

Radioisotope assay for cross-linked fibrin derivatives.

Reagents:

1) DD - 3B6/22 Stored at 4°C. Diluted immediately before use.

2) PBS 8g NaCl, 0.2g KCl, 1.25g Na_2HPO_4, 0.2g $KH_2PO_4.H_2O$ in 1 litre, pH 7.3.

3) D-dimer 2 mg/ml in 50% glycerol. Store at -20°C.

4) (^{125}I)DD-4D2

Store at 4°C. Dilute for tagging immediately before use. Diluted solution 1-2 x 10^6 cpm/ml.

The wells of a 96 well U-bottomed polyvinyl-chloride microtitre plate (Dynatech Cat. No. 1. 220. 24) were coated by adding 50 µl of a 1/500 dilution (± 10 µg/ml) of capture antibody DD-3B6/22 in phosphate buffered saline for 1 hour at room temperature. Unbound antibody was removed by inversion and tapping the plate followed by washing three times with PBS containing 0.05% Tween. Pour buffer to completely cover plate, leave 1 minute, invert and flick. Antigen capture was achieved by adding 50 µl of 0 - 1 µg/ml D-dimer standard, or 1/5 dilution of plasma or serum or test solution in PBS/Tween for 1 hour at room temperature (Figure 9). Remove unbound capture antigen and wash three times as before. The bottom of each well is cut off into a suitable counting tube, and counted in a gamma counter.

2.1.7 Dimertest agglutination assay.

Agglutination test for the detection of cross-linked fibrin derivatives. Monoclonal antibody DD 3B6/22 coupled to latex beads obtained from MAbCO, P.O. Box 475, Springwood Queensland 4127, Australia.

Buffer salts and standard were reconstituted as directed.

Method

1) Shake beads before use.
   Using pipette supplied place 0.025 ml beads on a clean slide.
   Add 0.01 ml sample and mix.
2) Rock slide gently. Read test after 3 minutes.
3) Add 0.02 ml standard to 0.025 ml latex beads as a positive control.

Estimation	Sample dilution			
	neat	1/2	1/4	1/8
range ng/ml 1-200	-	-	-	-
201-500	+	-	-	-
501-1000	+	+	-	-
1001-2000	+	+	+	-
>2001	+	+	+	+

2.1.8 Fluorimetry

The fluorescence spectra of f-D-monomer and f-D-dimer (dansyl fluorophore) were scanned in an Aminco SPF500 spectrofluorimeter. The optimum excitation wavelength was 340 nm and the emission wavelength 520 nm.

Puffadder venom protease activity was measured using f-D-dimer as substrate by following the change in fluorescence.

Assay. 1 ml of f-D-dimer (1 mg/ml) in Tris/HCl buffer pH 8.0 containing 150 mM NaCl, 1mM $CaCl_2$ and 1 mM $ZnCl_2$.
100 µl of crude or purified puffadder venom (100 µg/ml).

2.1.9 Preparation of plasmin.

Plasminogen, 10 mg (Sigma) was dissolved in 2 ml of 0.1 M Tris/HCl pH 7.4, 150 mM NaCl buffer and 500 units of urokinase added. The solution was incubated at room temperature for 1 hour and an equal volume of glycerol added resulting in a plasmin solution containing 2.5 mg/ml. Activity of the plasmin was checked using Chromozym PL (Boehringer) as substrate. The plasmin/glycerol solution was stored at -20°C.

2.1.10. Definition of PAV protease activity with f-D-dimer as substrate.

All assays were carried out for two hours at 37°C.

Buffer. 0.1 M Tris/HCl pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM $ZnCl_2$

Assay. 50 µl f-D-dimer (1 mg/ml in buffer)
50 µl PAV protease (10 µg/ml)

Digestions were terminated by the addition of an equal volume (100 µl) of SDS sample buffer containing EDTA with and without

β-mercaptoethanol and analyzed on SDS-PAGE.

2.2 PREPARATION OF FIBRINOGEN, FIBRIN AND PLASMIN DERIVATIVES

2.2.1. Preparation of fibrinogen.

Fibrinogen was prepared by repeated ammonium sulphate precipitation of citrated human plasma. The final precipitate was dissolved in 5 mM trisodium citrate and dialysed against 50 mM Tris/HCl buffer pH 7.4 with 150 mM NaCl.

Rat and hagfish fibrinogen were prepared using this method. The fibrinogen/fibrin plasmin derivatives were prepared from fibrinogen obtained from Sigma.

2.2.2. Preparation of fluorescent D-dimer and fluorescent D-monomer (Purves and Lindsey, 1980).

Dansyl cadaverine (Sigma) is a lysine analogue that inhibits cross-linking of the γ-chains. At a concentration of 2.45 mM only some of the cross-links are inhibited resulting in fibrin with fluorescently labelled γ-chains (Purves and Lindsey, 1980). Some of the cross-links sites are completely inhibited resulting in f-D-monomer that appeared to be identical to non-fluorescent D-monomer from digestion of fibrinogen by plasmin. Thus f-D-monomer was always found in f-D-dimer preparations. Fibrinogen, 1 g (Sigma) was dissolved in 100 ml of 0.1 M Tris/HCl pH 7.4 buffer containing 150 mM NaCl. Dansyl cadaverine, 80 mg (2.45 mM) was dissolved in a small volume of 6 N HCl and the pH neutralized with 6 N NaOH taking care to keep the dansyl cadaverine in solution. The solution was made up to 5 ml with 0.1 ml Tris/HCl buffer pH 7.4 containing 100 mM $CaCl_2$ 100 U of thrombin and 2 mg Factor Xlll. This entire solution was added

to the dissolved fibrinogen and the mixture incubated at 37°C overnight. The clot was placed between sheets of filter paper and pressed flat. The clot was resuspended in 50 ml 0.1 M Tris/HCl buffer pH 7.4 containing 150 mM NaCl and 10 mM $CaCl_2$ and 1.0 ml (2.5 mg) plasmin added. The suspension was incubated at 37°C overnight, by which time the fibrin clots were totally digested. The solution containing fluorescent D-dimer and fragment E, was dialyzed against 5 mM Tris/HCl pH 8.6, 1 mM $CaCl_2$ filtered, and loaded onto a DEAE-52 cellulose (Whatman) column (20 x 2 cm) washed with starting buffer and eluted with a salt gradient 0-0.3 M NaCl. The fractions were analysed by PAGE-electrophoresis. Pools were made of the fractions containing f-D-dimer, and f-D-monomer. These were concentrated by ultrafiltration using Millipore submersible CX-10 units,prior to gel filtration of the fragments.

2.2.3. Gel filtration of the pooled concentrated fractions from DEAE anion exchange chromatography

The concentrated pools of fractions containing f-D-monomer and f-D-dimer were molecular sieved on a column of Ultrogel AcA 34 (100 x 2 cm) equlibrated with 0.1 M Tris/HCl buffer pH 7.4 containing 1 mM $CaCl_2$ and 4 M urea. Flow rate 30 ml/hour, fraction volume, 3ml. Aliquots of the fractions were analyzed by means of SDS-PAGE, and fluorescent fragments located with U.V. light prior to staining of the gel with Coomassie Blue. Fractions containing f-D-monomer and f-D-dimer were pooled, concentrated and stored at -20°C. Protein concentration was ascertained using the Biorad Coomassie Blue dye-binding method.

2.2.4 Preparation of non-fluorescent D-dimer.

Non-fluorescent fragment D-dimer was isolated from a plasmin digest of fibrin clotted in the absence of dansyl cadaverine, using the same methods as for the isolation of f-D-dimer.

2.2.5 Preparation of D-monomer (non-fluorescent).

Fibrinogen, 1 g (Sigma) was dissolved in 100 ml of 0.1 M Tris/HCl buffer pH 7.4 containing 150 mM Nacl, 500 μl of plasmin solution (2.5 mg/ml) was added and the mixture incubated at 37°C. After ten minutes incubation calcium was added to give a final concentration of 5 mM, and the solution incubated overnight at 37°C. The digestion was terminated by addition of Trasylol (1000 U/ml) and dialysed against 5 mM Tris/HCl buffer pH 8.6 containing 1 mM $CaCl_2$. After dialysis the solution was passed through an 0.45 μ Millipore filter. The solution was chromatographed on cellulose DE-52 (Whatman) column to separate D-monomer from fragment E (Nussenzweig et al, 1961). The fractions were collected on an LKB fraction collector flow rate 50 ml/hour, fraction volume 5.0 ml and the D-monomer located by SDS-PAGE electrophoresis. The fractions containing D-monomer were pooled, concentrated by ultrafiltration using a Millipore immersible CX-10 ultra-filtration unit and stored frozen. Fluorescent D-monomer was obtained as a by-product during preparation of fluorescent D-dimer due to incomplete cross-linking on addition of 2.45 mM dansyl-cadaverine, the yield being determined by the amount of dansyl-cadaverine present (Purves et al, 1980).

2.2.6. Preparation of D-PAV-monomer.

Fluorescent or non-fluorescent D-dimer was incubated at 37°C for 18 hours with purified puffadder venom protease (PAV) in 0.1 M Tris/HCl buffer pH 8.0 containing 150 mM NaCl, 1 mM $CaCl_2$ and 1mM $ZnCl_2$. Enzyme to substrate ratio was 10:1. Cleavage of the D-dimer to D-PAV-monomer was checked by means of SDS-PAGE The reaction was stopped by the addition of EDTA (5 mM final concentration).

2.3 PURIFICATION OF PROTEASE(S) FROM PUFFADDER VENOM.

Lyophilized puffadder venom was obtained from Sigma and from the Transvaal Snake Park, Halfway-House, RSA.

2.3.1 Cation exchange chromotography.

The proteases of crude puffadder venom were not retained on DEAE-cellulose (Van der Walt, 1970) so an attempt at purification was made using CM-cellulose equilibrated with 5 mM Na acetate pH 5.6 eluted with a gradient of 5 mM Na acetate to 0.35 M Na acetate pH 5.6. The fractions were checked for activity against f-D-dimer caseinolytic activity, and Chromozym activity.

2.3.2. Purification of puffadder venom protease by gel-filtration on Ultrogel AcA 34.

Crude puffadder venom, 250 mg, was dissolved in 1 ml saline, filtered and applied to a column of Ultrogel AcA 34 (LKB), (200 x 2 cm), equilibrated with 0.1 M Tris/HCl buffer pH 7.4 containing 1 mM $CaCl_2$ and 150 mM NaCl. The proteins were eluted with the same buffer at a flow rate of 50 ml/h (fractions 4 ml). Aliquots of the fractions were run on 4-20 % SDS-PAGE and stained with Coomassie Blue. Aliquots, 50 µl, of the same fractions were incubated with 50µl of f-D-dimer at 37° C for 2 hours. The reaction was terminated by the addition of 100µl of SDS-sample buffer, 20µl aliquots run on 4-20% SDS-PAGE and the gel photographed under U.V.light. Fractions 135-165 showing maximum D-dimer cleavage activity were pooled, dialysed against 5 mM Tris/HCl pH 8.0 and loaded onto a column (20 x 2 cm) of DE 52 cellulose equilibrated with the same buffer. The proteins were eluted with a gradient of 0 - 0.15 M NaCl in the same buffer,

flow rate 60 ml/hour, fraction volume 6.0 ml. 50 µl Aliquots of the fractions were run on 4-20% SDS-PAGE and stained with Coomassie Blue, Aliquots of the same fractions were incubated with f-D-dimer for 4 hours at 37°C. The reaction was stopped with an equal volume of SDS-sample buffer and aliquots run on a 6-10% SDS-PAGE and photographed under U.V. light prior to staining with Coomassie blue. Fractions 16-45 were pooled, concentrated by ultrafiltration and stored at 20°C.

2.3.3. Purification of puffadder venom protease(s) by gel filtration on Sephadex G 100.

Crude lyophilized venom, 100 mg was dissolved in saline to form a bright yellow milky suspension. The filtered venom contained 37 mg of protein and was subjected to gel-filtration on Sephadex G 100 (Pharmacia) equilibrated with 0.1 M Tris/HCl buffer pH 7.4 containing 150 mM NaCl and 1 mM $CaCl_2$. Enzyme activity of the fractions was monitored by the cleavage of f-D-dimer (seen on SDS-PAGE under UV light), proteolysis of casein and hydrolysis of Chromozym TH. The fractions with activity against f-D-dimer were pooled and concentrated by ultrafiltration using Millipore CX 10 immersible filtration units and subjected to a further gel-filtration step on an Ultrogel AcA 54 column (100 x 2 cm) equilibrated with the same buffer as the previous gel-filtration. The fractions were again tested for D-dimer activity, pooled, concentrated and stored at -20°C, after adding glycerol to 50% to prevent freezing. The enzymes were stable for many months.

2.3.4. Purification of PAV protease by means of HPLC.

Protein, 1 mg from the peak on Ultrogel AcA 54 gel filtration that showed maximum activity against D-dimer was subjected to high performance liquid chromatography. The column used was an LKB Ultrapak TSK G 4000 SW (7.5 x 300 mm). Buffer 20 mM NaH_2PO_4 pH 7.4. Flow rate was 1 ml/min and eluant monitored at 280 nm. 20 μl Aliquot of the fractions were tested for activity against f-D-dimer. Aliquots were run on SDS-PAGE.

2.4 SEQUENCING OF THE CARBOXY-TERMINAL CYANOGEN-BROMIDE FRAGMENT OF THE γ-CHAIN OF D-PAV-MONOMER.

2.4.1 Preparation of D-PAV-monomer.

Purified puffadder venom, 5 mg was added to a mixture of 500 mg non-fluorescent and 5 mg fluorescent D-dimer in a buffer : 0.1M Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$. The fluorescent D-dimer was added to facilitate location of the γ-chain. The digestion was carried out for 18 hours at 37°C and monitored by SDS-PAGE in 4-20% gels. The reaction was stopped by addition of 5 mM EDTA (final concentration).
The digest containing D-PAV-monomer was dialyzed against a 10 mM Tris, pH 8.6 buffer and applied to a DEAE cellulose (Whatman) column (20 x 2 cm) equilibrated with the same buffer and the protein eluted with a gradient of 0-0.3 M NaCl. Fluorescent fractions were located by SDS-PAGE. A pool was made of the major fluorescent peak.

2.4.2 Isolation of γ- and β-chains from D-PAV-monomer.

The D-PAV-monomer, 345 mg was made 6 M with respect to guanidinium hydrochloride and reduced and alkylated (Doolittle, 1977b). The solution was desalted on a Sephadex G25 column equilibrated with a 10 mM Tris, pH 7.5 buffer containing 8 M urea. The reduced and alkylated γ- and β-chains were separated on a DEAE-Sepharose CL 6B column (20 x 2 cm) equilibrated with a 10 mM Tris, pH 7.5 buffer containing 8 M urea, and eluted using a gradient of 0-0.3 M NaCl. Aliquots were analysed by acid/urea PAGE.The pools from the major peaks were exhaustively dialysed against distilled water and lyophilized. The yield of γ-chain was 100 mg.

2.4.3 Cyanogen bromide digestion of the γ-chain of D-PAV-monomer.

Lyophilized γ-chain, 30 mg was dissolved in 70% formic acid (5 mg/ml). Cyanogen bromide was added (10 mg/ml) and the reaction vial was kept under nitrogen for 18 hr at room temperature. The solution was thereafter diluted 10 x with distilled water and lyophilized. The dried peptides were dissolved in 10% acetic acid and molecular sieved on a Sephadex G 50 column (200 x 1 cm) using 10% acetic acid. The fluorescence in the fractions was detected after evaporation of the acid solvent by vacuum centrifugation and addition of 200 μl of 0.1% NH_4HCO_3. The fluorescent peak was pooled and lyophilized.

2.4.4 Isolation of the carboxy-terminal peptide from the γ-chain of D-PAV-monomer.

The lyophilized peptides were redissolved in 0.1% NH_4HCO_3 or 0.1% TFA and separated by high performance liquid chromatography (HPLC). In order to visualize the fluorescent peptide (added at 1:100 ratio) one HPLC separation used an alkaline ammonium bicarbonate buffer system with an acetonitrile gradient to elute from a RadialPak C8 column. It was anticipated that the fluorescent dansyl group could cause the peptide to be more hydrophobic and elute later. The relevant peaks were pooled and a portion of the pool run on an acid HPLC system.

2.4.5 Peptide hydrolysis and amino acid composition.

The Picotag (Waters) method was used to hydrolyse aliquots of the dried peptide (18 hours at 105°C under nitrogen with gas-phase 6 N HCl hydrolysis). The Waters system for PITC amino acid derivatisation and separation was used. The carboxy-terminal peptide could be readily identified by the characteristic signature of the absence of serine and an appropriate amino acid composition. The yield of carboxy-terminal peptide was calculated on the basis of the lysine content.

2.4.6 Manual gas-phase microsequencing by Edman degradation.

Reagents were all Pierce Sequanal grade. The method used was that of W.F.Brandt and G.Frank, Biochemistry Dept., UCT (not yet published). Oxygen scavengers and dithiothreitol were not added to reagents but these were always freshly opened or stored at -40°C under nitrogen.

Preparation of glass paper filter.

A disc of Whatmans GF/C paper, 0.8 cm diameter, was inserted into a 2 cm glass tube with a drawn-out end. The filter was washed with trifluoroacetic acid (TFA) 100 µl, followed by 4 additions of 100 µl butyl chloride. This was repeated twice and the glass filter dried **in vacuo** at 50°C for 15 minutes. Polybrene (Pierce), 10 µl of a 50 mg/ml solution in water was added to the center of the filter and then dried **in vacuo** for 15 minutes. The tube containing the filter was perched on glass tubing and placed together with a tube containing 0.5 mls TFA in a 50 ml Reaction bottle with a tap permitting evacuation and nitrogen replacement. The Picotag (Waters) vessels were convenient for this purpose. A blank cycle was performed by exposing the filter to TFA vapours at 50°C for 15 minutes. Thereafter the filter was washed with 3 x 200 µl additions of ethylacetate and then 2 x 200 µl additions of butyl chloride.

Addition of sample.

The filter was dried in vacuo and the peptide, 5 nmole in 10 µl 50% TFA in water, was added accurately to the center of the filter and then dried in vacuo.

Coupling reaction.

10 µl of 5% PITC in heptane was added to the centre of the filter. A tube containing 0.5 ml of 5% triethylamine was also added to the reaction vessel that was then flushed with nitrogen and kept at 50°C for 40 minutes. The filter was dried **in vacuo** and washed with additiions of 2 x 200 µl heptane followed by 2 x 200 µl ethylacetate and then dried in vacuo again (adequate drying was assessed by a pressure less that 50 millitorr).

Cleavage reaction.

A tube containing 0.5 ml TFA was added to the reaction vessel and flushed briefly with nitrogen. After exposure to TFA vapour at 50°C for 15 minutes the filter was dried briefly (to 200 millitorr) and extracted with 3 x 200 µl washes of butyl chloride collected into a 10 x 75 mm tube (previously washed with TFA). The filter was dried in vacuo and was then ready for the next cycle of the coupling reaction. The washings were dried in vacuo under a stream of nitrogen at 50°C.

Conversion reaction.

The dried thiazolinone was converted to the phenylthiohydantoin (PTH) derivative by the addition of 1 drop of 50% TFA in water, heated at 90°C for 10 minutes in the reaction vessel. The tube was then dried in vacuo and the contents analysed by HPLC.

Automated gas-phase microsequencing.

A method was used in which a spinning cup sequencer was converted into a vapour (gas)-phase sequencer (Brandt et al, 1984).

2.4.7 High performance liquid chromatography.

A Waters system was used throughout with wavelengths of 214 nm or 254 nm used for peptide detection together with fluorescence detection at alkaline pH. PTH-amino acids were detected at 254 nm. Injections were manual and temperature was maintained at 45°C. Flow rates were always 1 ml/minute. Peptides were separated by either:

a) 5 µ Bondapak C18 (Waters) cartridge (8 mm x 10 cm) in a radial compression system. The column was equilibrated with 0.1% NH_4HCO_3 and the sample eluted with a linear gradient up

to 25% acetonitrile over 40 minutes and then up to 40% over 10 minutes.

b) Ultrapore RPSC (Beckman) C3 column (4.6 x 75 mm) was equilibrated with 0.1% TFA in water containing 10% acetonitrile and the sample eluted with a linear gradient over 40 minutes up to 40% acetonitrile in 0.1% TFA.

Amino acid composition was performed using the complete Picotag (Waters) system with gas phase 6N HCl hydrolysis of samples and PITC derivatives and separation of the thiazolinone derivatives on the Picotag selected C18 column using commercial reagents.

PTH amino acids were separated by either:

a) Novapak C18 (Waters) column (3.9 mm x 15 cm). The column was equilibrated with 5% tetrahydrofuran (Merck) containing 30 ml 3M sodium acetate, pH 3.8 and 7 ml 3M sodium acetate, pH 4.6 per litre. The sample was eluted with linear gradients of acetonitrile; 5% over 2 minutes; 30% in 20 minutes; isocratic at 30% for 2 minutes; 70% over 5 minutes.

b) The same column was used with equilibration with Picotag Buffer A followed by elution of sample with a linear gradient over 20 minutes up to 30% acetonitrile in H_2O. Picotag Buffer A consists of 0.14 M sodium acetate containing 0.5 ml triethylamine per litre titrated to pH 6.40 with glacial acetic acid and 6% acetonitrile is added before use.

3. RESULTS

3.1 PURIFICATION OF FLUORESCENT AND NON-FLUORESCENT FIBRIN(OGEN) PLASMIN DERIVATIVES.

3.1.1 The purification of fluorescent and non-fluorescent D-dimer.

Fluorescent D-dimer was isolated from an over-night plasmin digest of dansyl cadaverine substituted fibrin. Aliquots of fractions collected after DEAE-cellulose anion exchange were analyzed on a 4-20% SDS-PAGE (Figure 8A). The gel was photographed under U.V. light and the fluorescent fragments located prior to staining with Coomassie Blue (Figure 8B). The fractions containing f-D-monomer and f-D-dimer, and those containing f-D-dimer and fragment E were pooled and concentrated. Due to non-covalent binding of fragment E to fragments D-monomer and D-dimer (Olexa and Budzynski, 1979) the buffers for molecular sieving contained 4M urea. D-dimer was separated from other fragments by molecular sieving on a column of Ultrogel AcA 34 (Figure 9A). Aliquots of fractions collected were run on a 4-20% SDS-PAGE and f-D-dimer located by fluorescence under U.V. light prior to staining with Coomassie Blue (Figure 9B).

3.1.2 Purification of fluorescent D-monomer

Fluorescent fragment D was a contaminant of fluorescent D-dimer preparation due to partial inhibition of cross-linking by dansyl-cadaverine. It appeared to be identical to plasmin D-monomer with respect to molecular weight and physical properties (Purves et al, 1980). Non-fluorescent D-monomer was prepared from an overnight plasmin digest of fibrinogen, the necessary calcium only being added after the first fifteen minutes of digestion to

A

2·0
1·0
O.D. 280
0·3M
0·15M
NaCl
40 50 60 70 80 90 100 110
Fraction no.

B

Figure 8. Anion exchange chromatography of a plasmic digest of dansyl-cadaverine labelled fibrin.

A. The dialysed, filtered digest was loaded onto a cellulose DE 52 column (2Ø x 2 cm) and eluted with a gradient of NaCl, Ø - Ø.3M. Flow rate 8Øml/h.

B. Aliquots of the fractions were run on a 4-2Ø% SDS-PAGE, and the wet gel photographed under U.V. light prior to staining with Coomassie Blue. Fractions containing f-D-monomer and f-D-dimer were pooled, concentrated and molecular sieved on Ultrogel AcA 34.

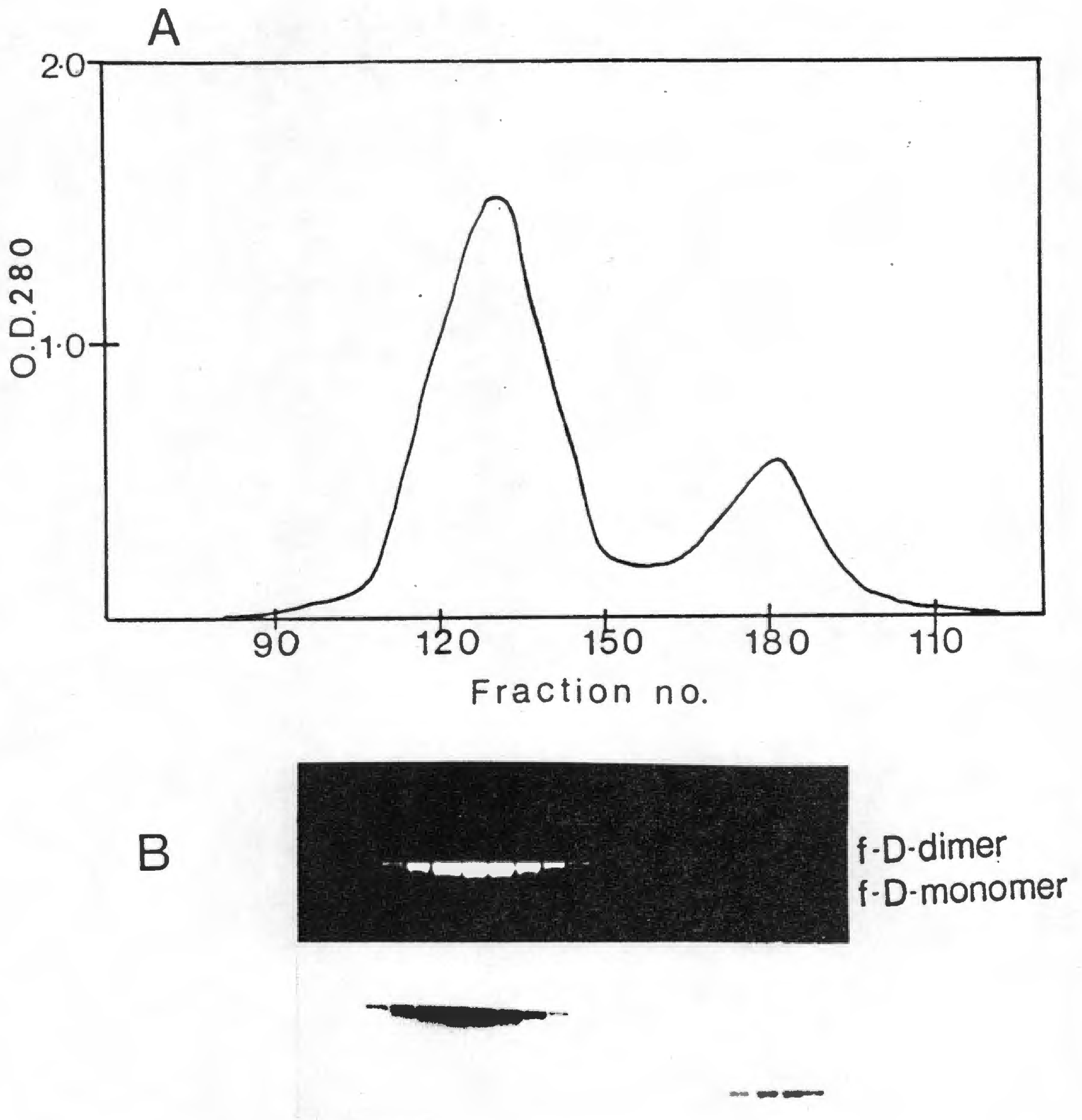


Figure 9. Gel filtration of pooled concentrated fractions containing f-D-dimer.

A. A column of Ultrogel AcA 34 (200 x 1cm) was equilibrated with 0.1 M Tris/HCl buffer, pH 7.5, containing 150 mM NaCl and 4 M urea.

B. Aliquots of the fractions were run on a 4-20% SDS-PAGE and photographed under U.V. light prior to Coomassie Blue staining.

avoid clotting of the fibrinogen. The D-monomer was isolated using the same method as for the D-dimer.

3.1.3 Purification of non-fluorescent D-dimer.

Non-fluorescent D-dimer was isolated from a plasmic digest of non-fluorescent fibrin in the same way as f-D-dimer (Figure 10).

3.1.4 Purification of D-PAV-monomer.

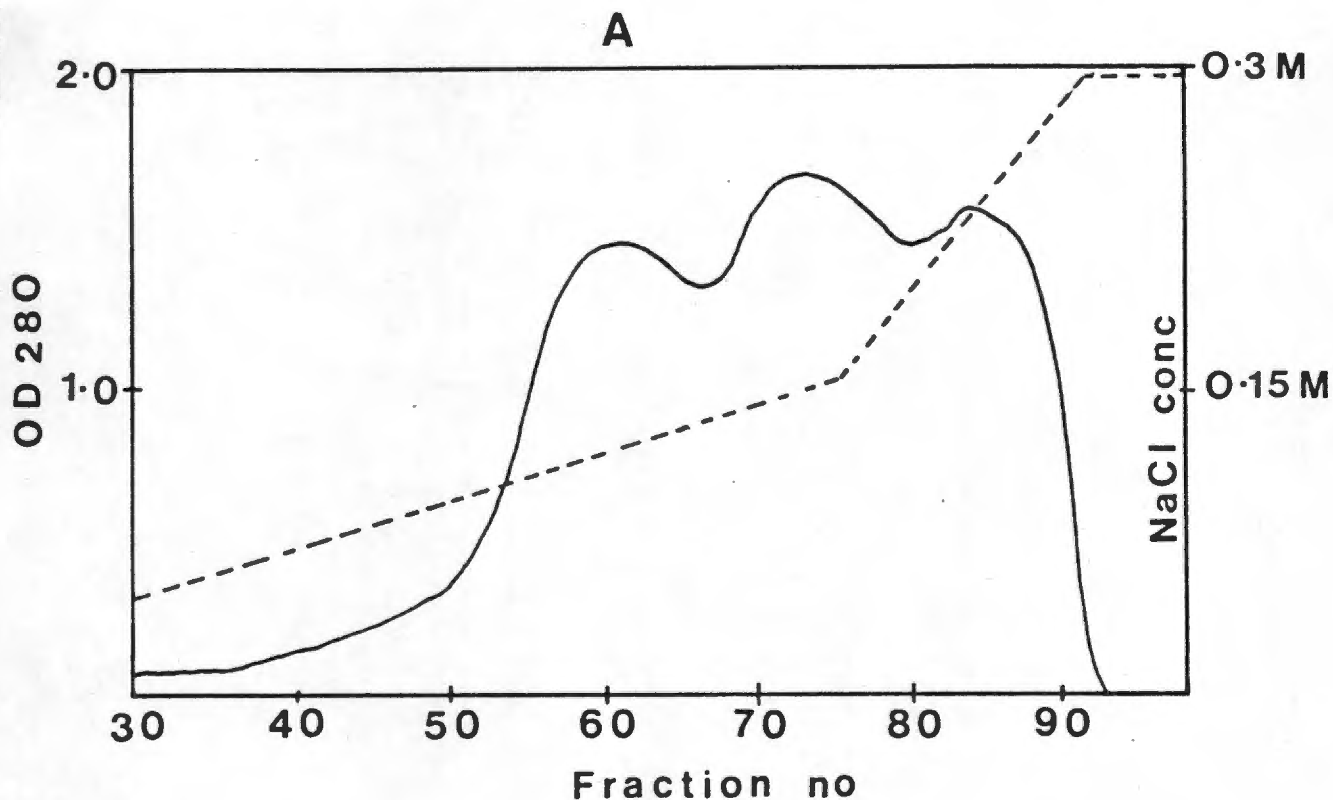
D-PAV-monomer was isolated as described in the methods from an overnight digest of fluorescent or non-fluorescent D-dimer using the enzyme isolated from puffadder venom and buffers containing 1 mM $ZnCl_2$. The D-PAV-monomer was isolated by DEAE-cellulose anion-exchange chromatography and/or molecular sieving on Ultrogel AcA 34. Purity and molecular weights of the isolated fragments used on experiments with PAV protease (Figure 12).

3.2 PURIFICATION OF PUFFADDER VENOM PROTEASE.

The enzyme used to study the D-domain of fibrin was puffadder venom protease I, first isolated and characterized by Van der Walt (1971).

3.2.1 CM cellulose cation exchange chromatography.

Crude venom (1g) was chromatographed on CM cellulose. The fractions were tested using Chromozym TH, and D-dimer as substrates. The D-dimer cleavage activity extended over a number of rather ill-defined peaks, and was clearly separated from the peak containing Chromozym TH activity (Figure 13). The enzyme was not purified further.



B

E

Figure 10. A. Anion exchange chromatography of a plasmic digest of fibrin.

The proteins were loaded onto a cellulose DE 52 column (20 x 2 cm) equilibrated with 5 mM Tris/HCl buffer pH 8.6, and eluted with an NaCl gradient 0-0.3 M.

B. Aliquots of the fractions were run on a 4-20% SDS-PAGE and stained with Coomassie Blue. The fractions containing D-dimer were pooled, comcentrated and molecular sieved on Ultrogel AcA 34.

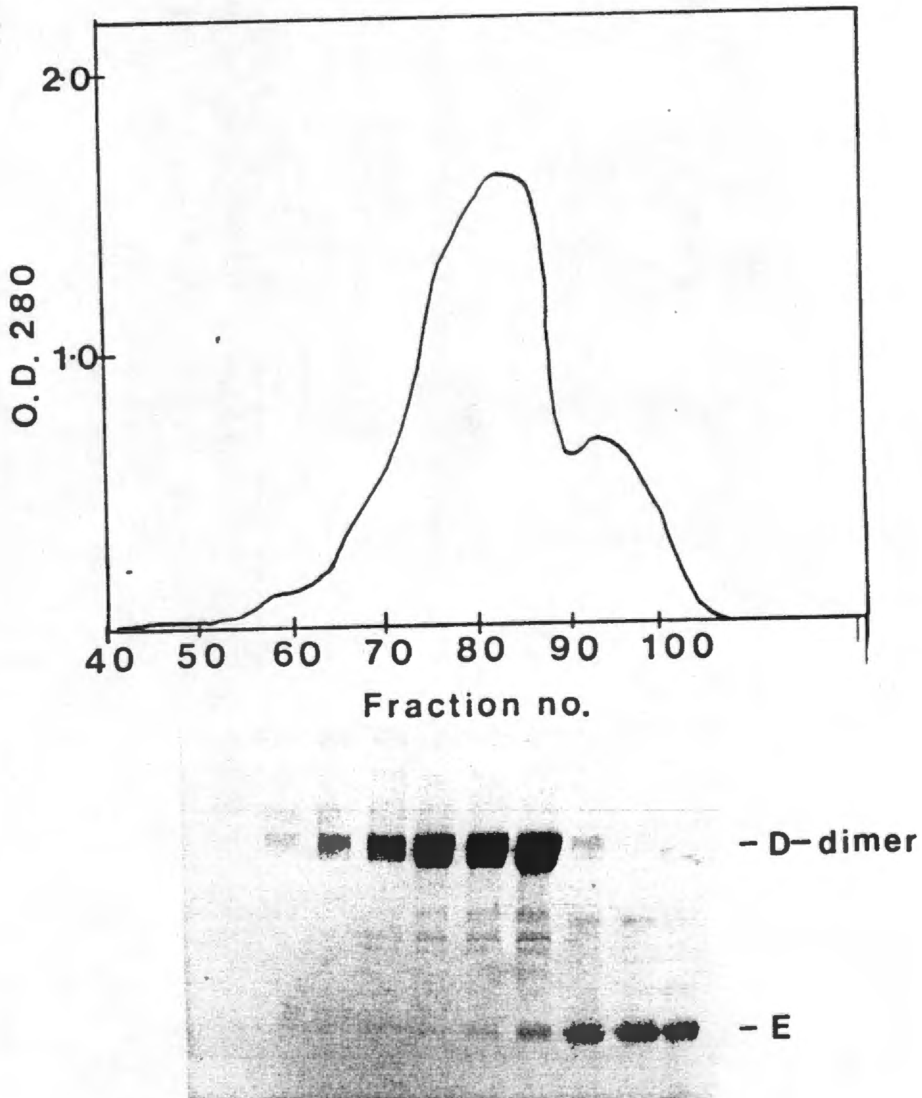


Figure 11. Gel-filtration of D-dimer on Ultrogel AcA 34. After concentration the fractions containing D-dimer were applied to a column of Ultrogel AcA 34 (100 x 1 cm) equilibrated with 0.1 M Tris/HCl buffer, pH 7.4, containing 150 mM NaCl.The proteins were eluted with the same buffer, flow rate 30 ml/hour.

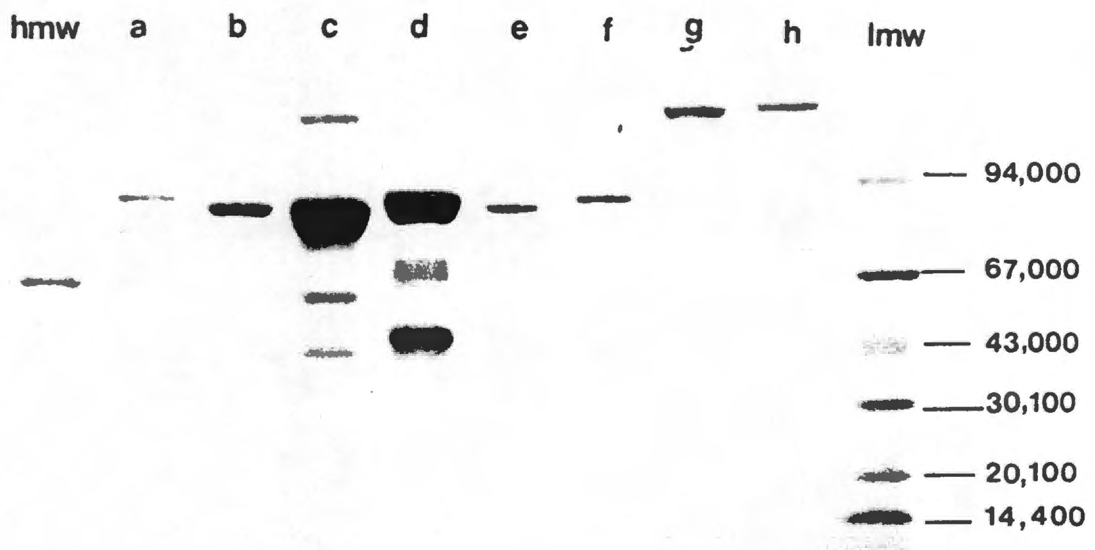


Figure 12. SDS-PAGE of materials used as substrates for puffadder venom protease.

a & d = non-fluorescent D-monomer (plasmin)
f = fluorescent D-monomer (plasmin)
b & c = non-fluorescent D-PAV-monomer
e = fluorescent D-PAV-monomer
g = non-fluorescent D-dimer
h = fluorescent D-dimer.

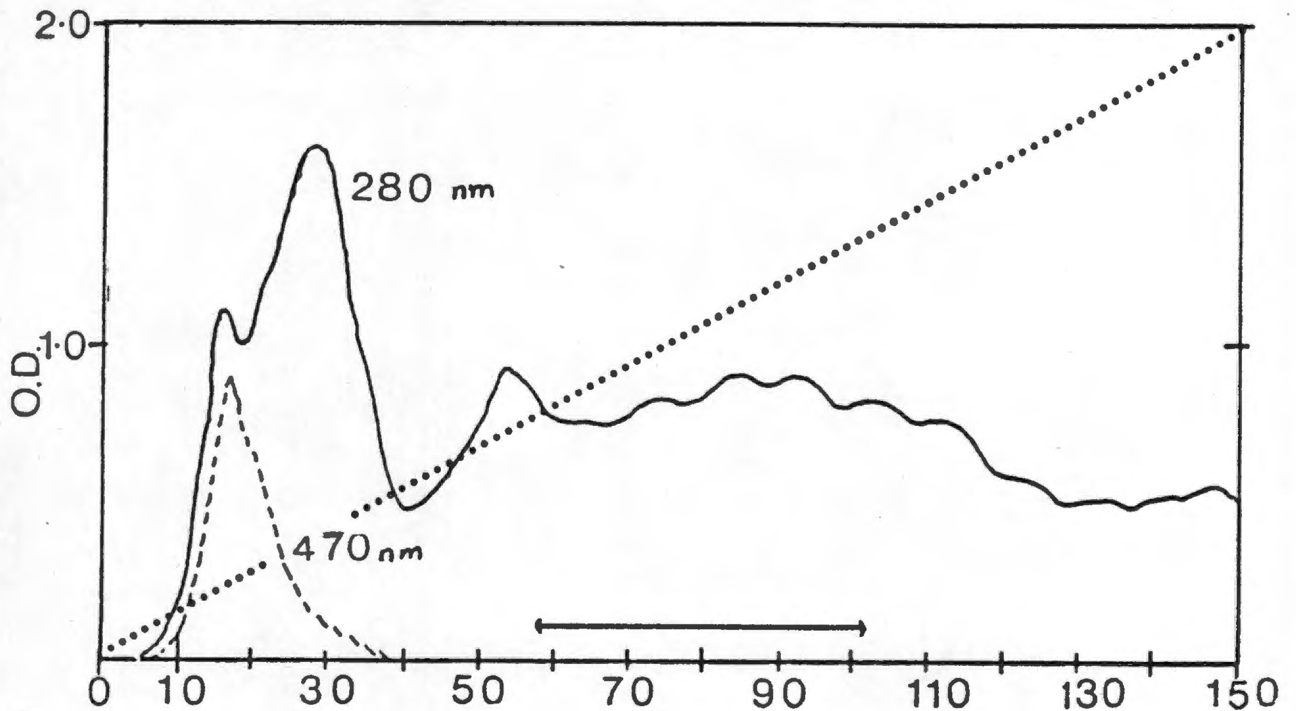


Figure 13. Fractionation of crude puffadder venom on CM cellulose.

Crude puffadder venom, 100 mg in 5 mM Na acetate buffer was applied to a column of CM cellulose (20 x 2 cm) equilibrated with the same buffer. The proteins were eluted with a linear gradient of 5 mM - 0.35 M Na acetate pH 5.6. (---) = fractions with Chromozym activity. The bar indicates the fractions with D-dimer cleavage activity.

3.2.2 Gel filtration of puffadder venom proteases.

a) Gel filtration on AcA 34:

Crude puffadder venom, 230 mg was dissolved in saline, filtered and applied to an AcA 34 column (2 metres x 2 cm) equilibrated with 0.1M Tris/HCl ph 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $ZnCl_2$ buffer. The venom separated into four peaks (Figure 14A). The fractions were assayed for caseinolytic and D-dimer cleavage activity. There were two main peaks of caseinolytic activity (Figure 14A).Aliquots of the fractions were assayed for D-dimer activity, the peak being from fraction 135 to 160 (Figure 14C). These fractions were pooled, dialysed and applied to a cellulose DE-52 column (20 x 2 cm) equilibrated with 5 mM Tris pH 8.0 and eluted with an NaCl gradient from 0 to 0.15M (Figure 15A). The starting material contained proteins with molecular weights ranging from ± 30,100 to ± 15,000. The material fractionated into fractions containing a protein of ± 25,000 (fractions 5-9) and ± 29,000 (fractions 33-41, Figure 15B) D-dimer cleavage activity was found in fractions 9-48 with the most active peak being fractions 5 to 15 which were pooled (Figure 15C).

b) Gel filtration on Sephadex G 100.

Puffadder venom separated into three main peaks with molecular sieving on Sephadex G 100 (Figure 16A). D-dimer cleavage activity occured in peak 3 (Figures 16A & B), maximum Chromozym TH activity in peak 2, and caseinolytic activity in peaks 1 and 3 (Figure 16A). There was no activity against TAME, BTEE or ATEE in any of the peaks.The three peaks were pooled and concentrated by ultrafiltration. The activity of each pool with Chromozym TH was determined. EDTA (final concentration 5 mM) had no effect on

Figure 14. Caption overleaf

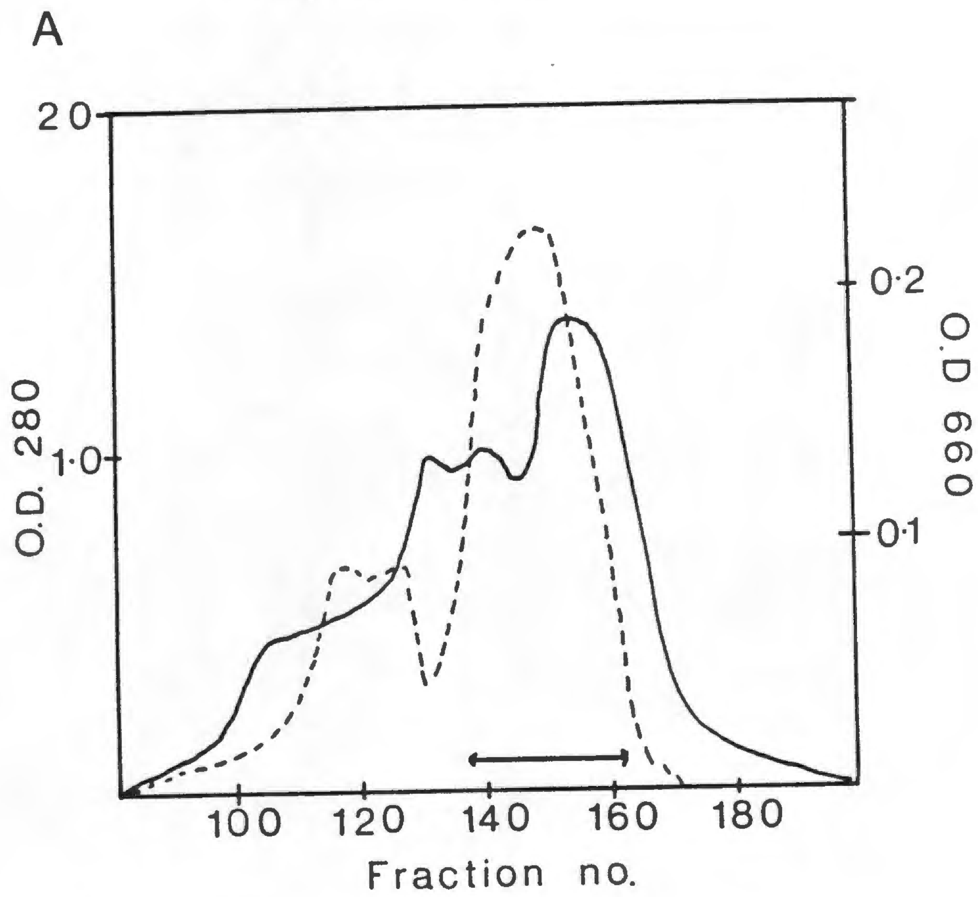


Figure 14. Gel filtration of crude puffadder venom on Ultrogel AcA 34.

A. Crude puffadder venom, 250 mg dissolved in saline was applied to a column of Ultrogel AcA 34 (200 x 1 cm) equilibrated with 0.1 M Tris/HCl buffer pH 7.4 containing 150 mM NaCl and 1 mM CaCl2. The proteins were eluted with the same buffer. Flow rate 30 ml/hour. Aliquots of the fractions were assayed for caseinolytic activity.
OD 280 (——)
caseinolytic activity (- - -)

B. Aliquots of fractions were run on 4-20% SDS-PAGE and stained with Coomassie Blue.

C. Aliquots of the fractions were incubated with f-D-dimer for two hours at 37oC, run on SDS-PAGE and the wet gels photographed under U.V. light prior to staining with Coomassie Blue.
Fractions indicated by the bar were pooled dialysed and fractionated further on cellulose DE 52.

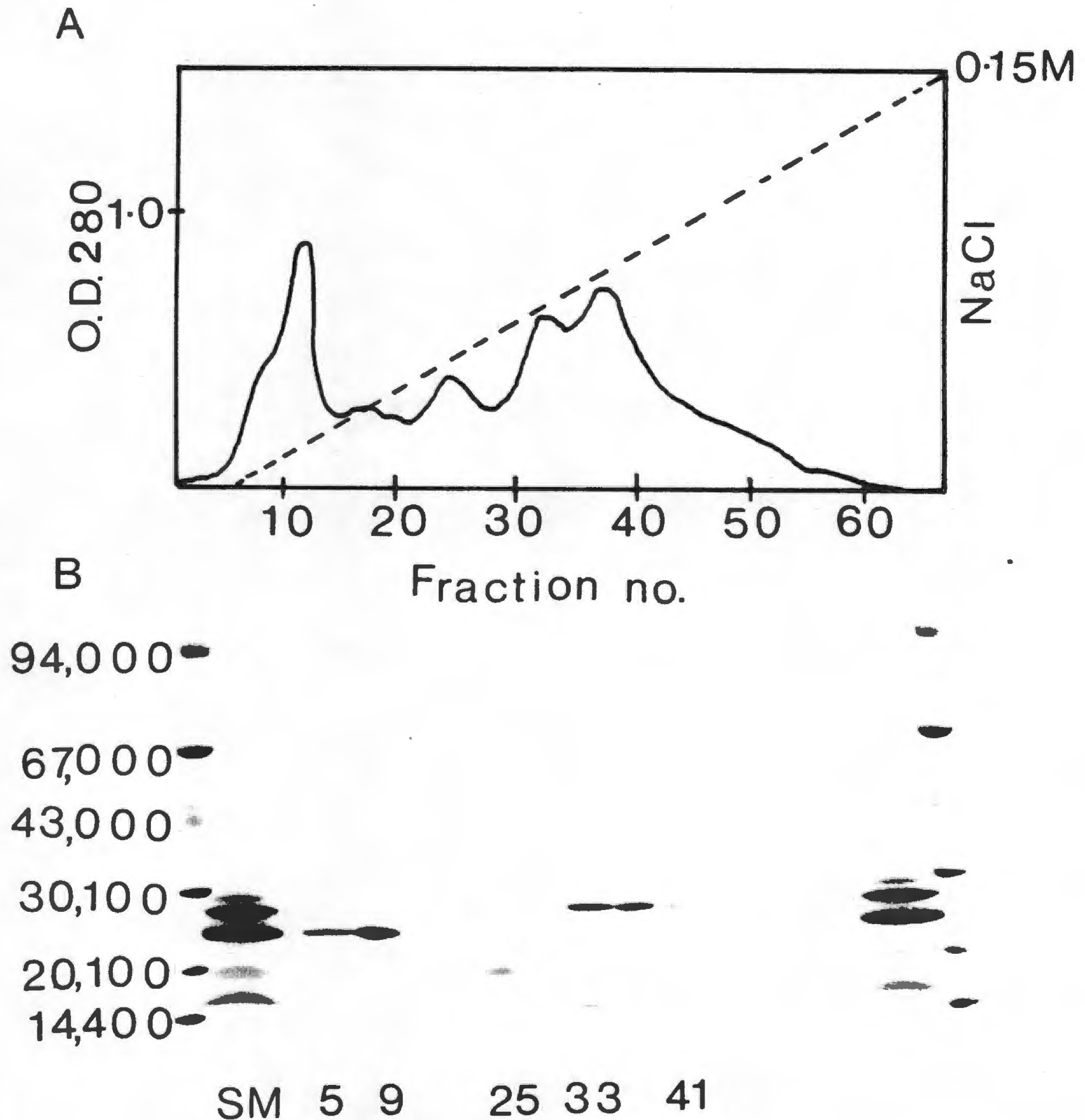


Figure 15. Fractionation of the dialysed pooled fractions from the AcA 34 gel filtration of crude puffadder venom.

A. The pooled dialysed fractions were applied to a column of cellulose DE 52 (20 x 2 cm) equilibrated with 5 mM Tris/HCl buffer pH 8.0 and eluted with a linear gradient of NaCl in buffer from 0 - 0.15 M.

B. Aliquots of the fractions were run on a 4-20% gradient SDS-PAGE and stained with Coomassie Blue.

C. continues overleaf

C

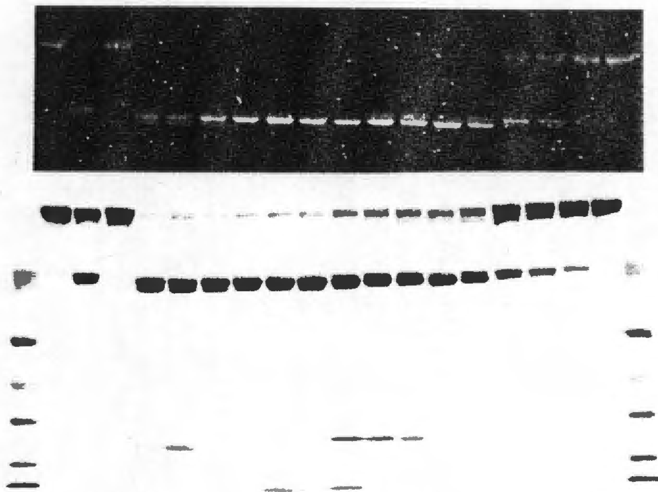


Figure 15 contd.

C. Aliquots of the fractions were assayed for f-D-dimer cleavage activity and run on a 4-20% gradient SDS-PAGE, photographed under U.V.light and stained with Coomassie Blue. Fractions 5-50 were all active against f-D-dimer with the strongest cleavage activity in fractions 5 - 15 associated with a protein of 25,000 daltons as compared to the protein standards.

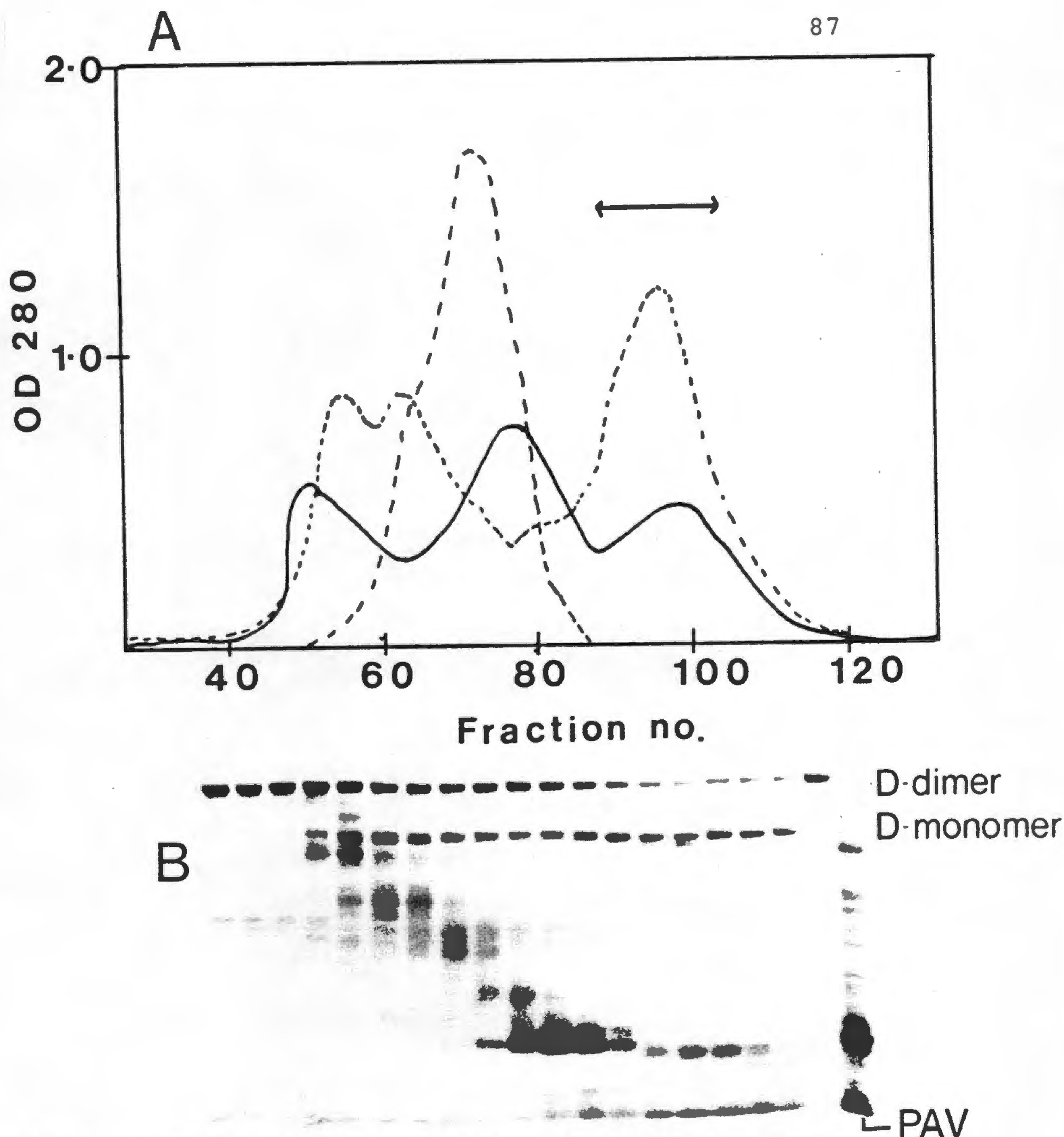


Figure 16. Molecular sieving of crude puffadder venom on Sephadex G 100.

A. The filtered venom containing 37 mg protein was applied to a column of Sepahadex G (100 x 2 cm) equilibrated with 0.1 M Tris/HCL buffer pH 7.4 containing 150 mM NaCl and 1 mM CaCl2.
The proteins were eluted with the same buffer. Flow rate 30 ml/h, fraction volume 3.0 ml.
——— = OD 280 nm;
.... = caseinolytic activity;
- - - - = activity with chromozym TH (t-gly-pro-arg-pNA)

B. SDS-PAGE of PAV fractions incubated with D-dimer for 4h at 37oC.

the Chromozym activity of pools 1, 2 and 3 (Figure 17 A). A timed digest of f-D-dimer with each of the three pools was performed. Aliquots were withdrawn at 2', 5',10', 15', 25' and 35 minutes and analyzed on a 4-20% SDS-PAGE. Most of the Chromozym activity was associated with pool 1,a small amount with pool 2 and no activity at all in pool 3. The enzymes in pool 1 were unable to cleave D-dimer despite having a high caseinolytic activity.
Pool 2 was weakly active against D-dimer. Pool 3 was very active against D-dimer giving rise to D-PAV-monomer after 10 minutes (Figure 17B). The concentrated pool 3 was applied to an Ultrogel AcA 54 column (100 x 2 cm) (Figure 18A). The main fraction contained a protein with maximal activity against D-dimer (Figure 18C).
The size of this protein was approximately 25,000 daltons, and several smaller components were separated on SDS-PAGE. The smaller proteins are probably associated in solution , since they emerge at the same hydrodynamic radius as the larger protein on molecular sieving.

Figure 17. Activity of pools 1, 2 and 3 with chromozym TH, and f-D-dimer as substrates.

A. Activity of pools 1,2 and 3 with chromozym TH as substrate.

- -○-○- pool 1
- -▽-▽- pool 2
- -●-●- crude PAV
- -▼-▼- pool 3

In all cases 100 mg of protein was used in the assay. The assay was performed as described in the methods. The arrows indicate the time at which EDTA was added (final concentration 5 mM) to the assay.

B. Pools 1,2 and 3 from Sephadex G100 gel-filtration were concentrated and a timed digest with f-D-dimer as substrate performed. A 50 µl aliquot was withdrawn at each time interval and added to an equal volume of SDS sample buffer and run on a 4-20% SDS-PAGE.

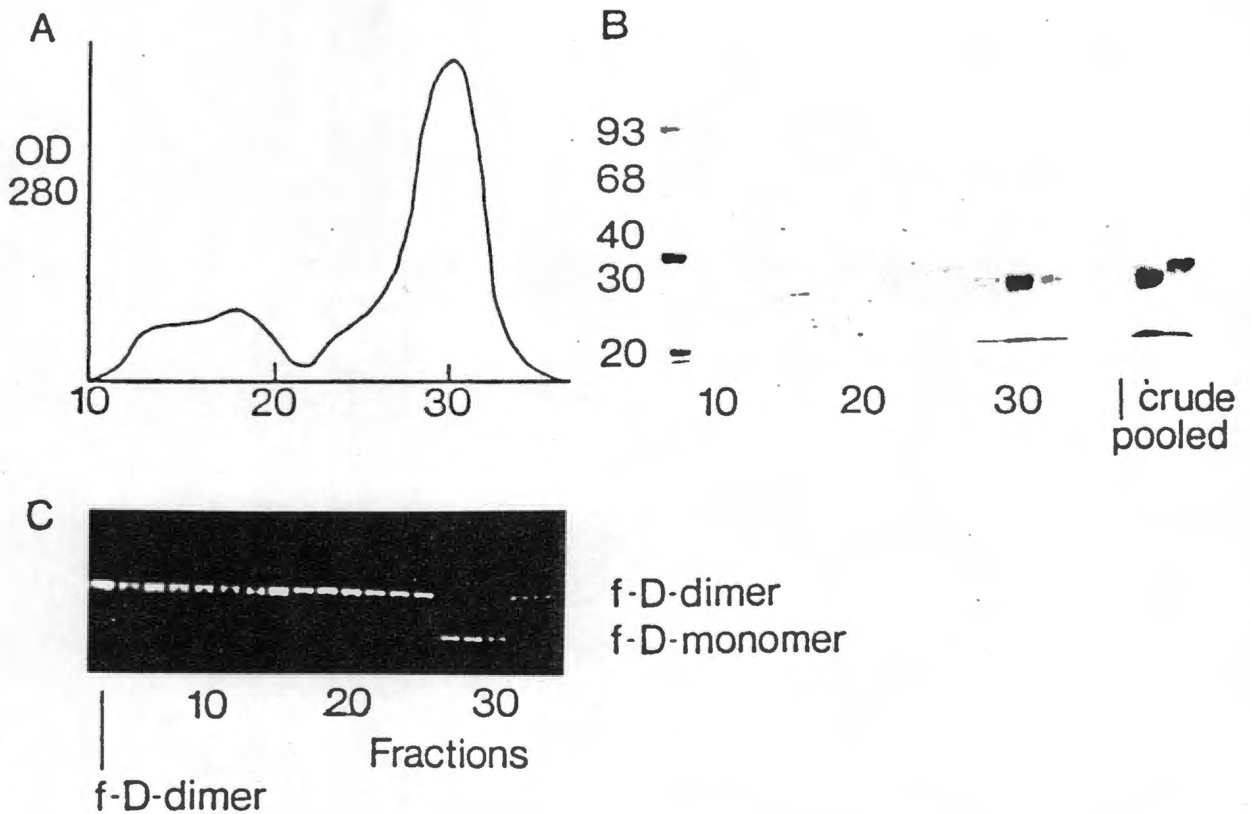


Figure 18. Purification of pool 3 from Sephadex G100 column, on Ultrogel AcA 54.

A. Molecular sieving of pool 3 on Ultrogel AcA 54 column (100 x 2 cm).

B. Aliquots of fractions run on 6 - 10% SDS-PAGE and stained with Coomassie Blue.

C. SDS-PAGE of fractions incubated with f-D-dimer at 37oC for 2 hours.

3.2.3 High performance liquid chromatography of puffadder venom protease.

An aliquot from the pooled and concentrated Peak 3 from the Sephadex G 100 gel filtration gave several peaks on HPLC (Figure 19A). Maximal D-dimer activity was found in peak 6 (fractions 15 and 16) (Figure 19B), associated with a protein of molecular weight $\pm$ 25,000 daltons (Figure 19C).

3.3 Characterization of puffadder venom protease.

3.3.1 Identity of the PAV protease.

Although this enzyme is probably the same as protease I isolated and characterized by Van der Walt (1971), its specific effects on fibrin and fibrinogen have not yet been studied. It inhibits clotting of fibrinogen (Figure 20). It is capable of lysing fibrin clots albeit at a much slower rate than plasmin. A comparison between digestion of fibrinogen by plasmin and purified puffadder venom protease shows that PAV enzyme rapidly degrades the α-chain, then the β-chain at a slower rate, and appears to leave the γ-chain intact even after lengthy incubation. Plasmin degrades all three chains (Figure 21A & B).

3.3.2 Comparison of PAV protease and plasmin activity using chromogenic substrates.

Various chromogenic serine protease substrates were tested with crude PAV, and compared to plasmin activity with Chromozym PL. PAV showed the greatest activity with Chromozym TH (Figure 22). The activity was unaffected by the addition of Zn^{++} or EDTA. The pools of the three peaks from Sephadex G 100 were each tested for activity with Chromozym TH. Only peak 2 was active (Figure 16).

Figure 19. Caption overleaf

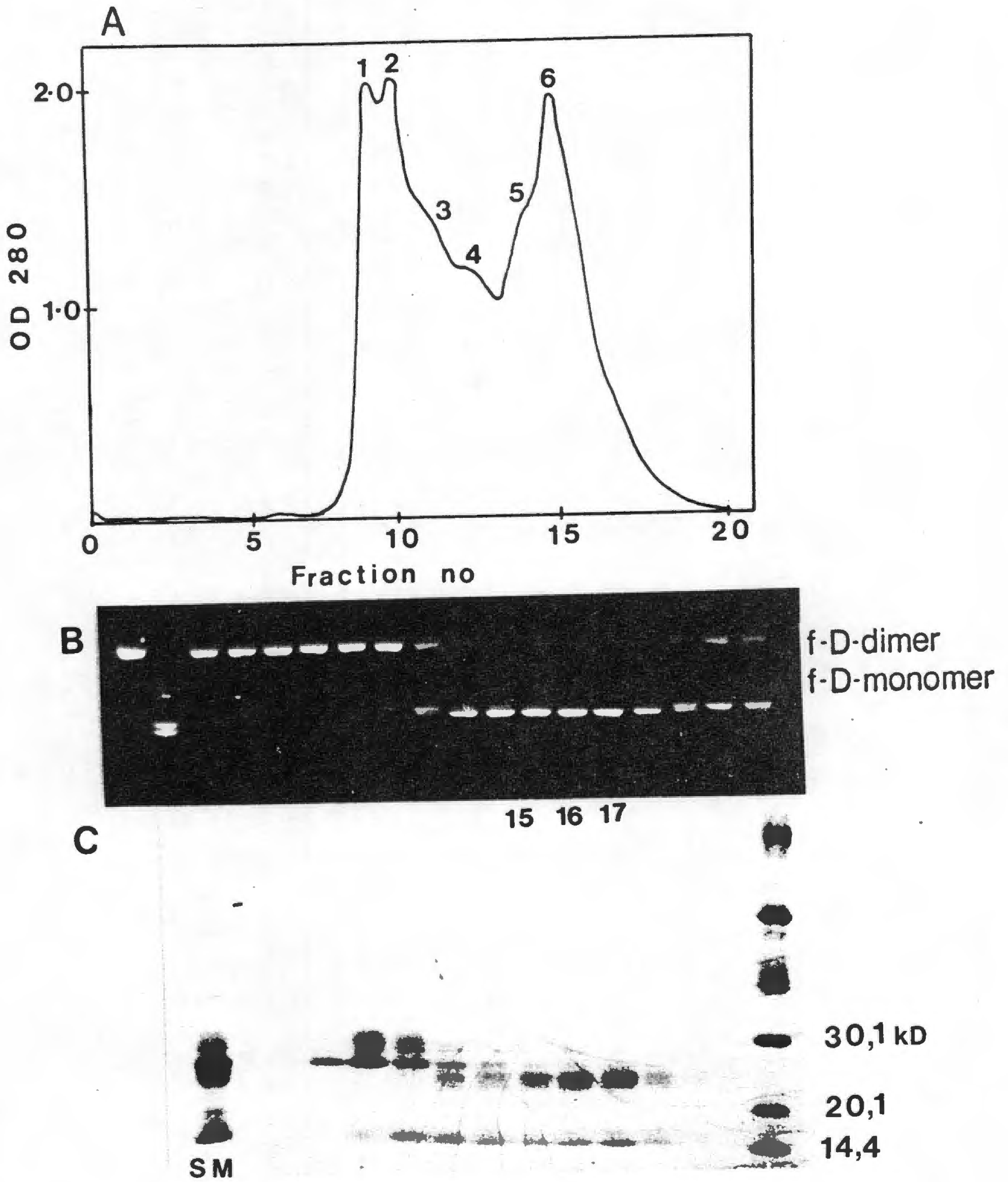


Figure 19. Purification of PAV by means of high performance liquid chromatography (HPLC).

A. 1 mg of protein from pool 3 of Sephadex G 100 gel filtration was subjected to HPLC. Column Ultrapak TSK G4000 SW (7.5 x 300 mm). Buffer 20 mM NaH_2PO_4. Flow rate 1 ml/min. Fraction volume 1 ml.

B. 20µl aliquots of the fractions were assayed for f-D-dimer cleavage activity and run on a 4-20% SDS-PAGE and photographed under U.V. light.

C. Aliquots of the fractions were run on a 6-8% SDS-PAGE and stained with Coomassie-Blue. The fractions with maximum f-D-dimer cleavage activity were associated with a protein of molecular weight 25,000 daltons as compared to the molecular weight standards.

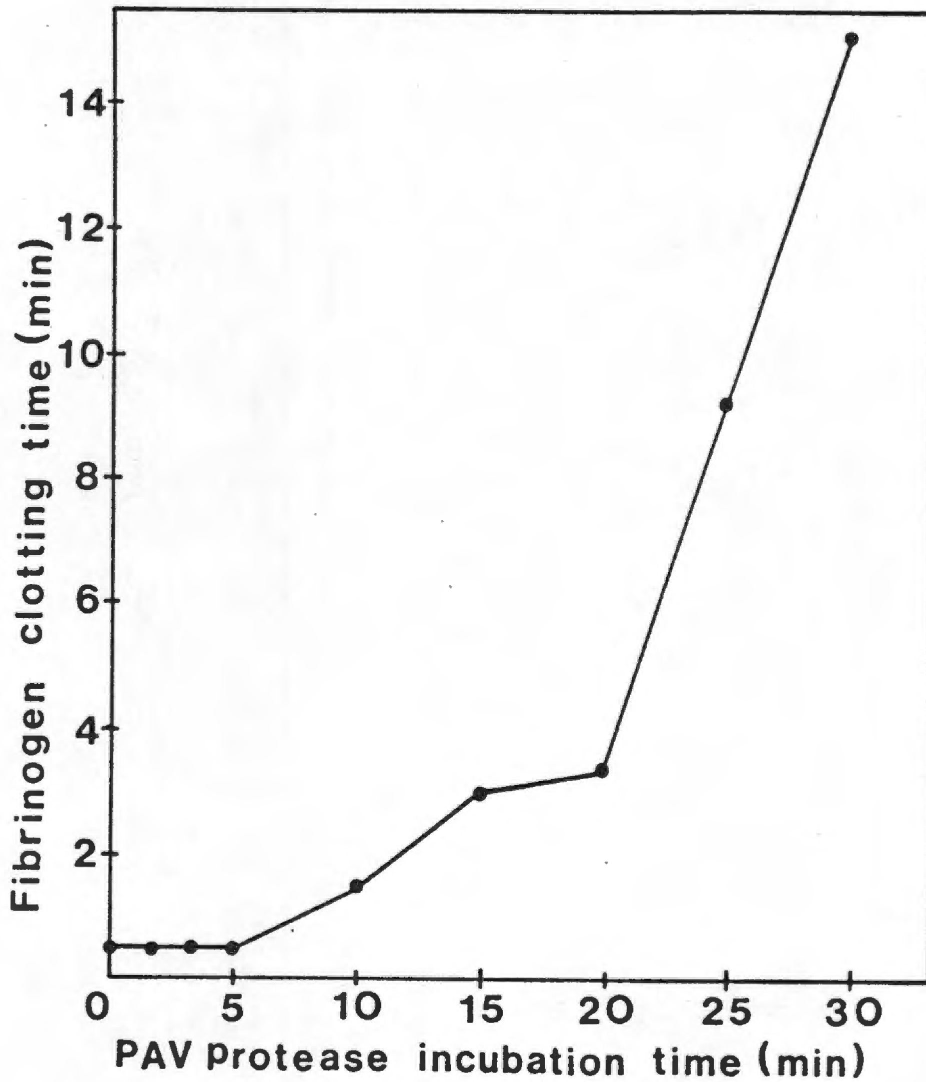


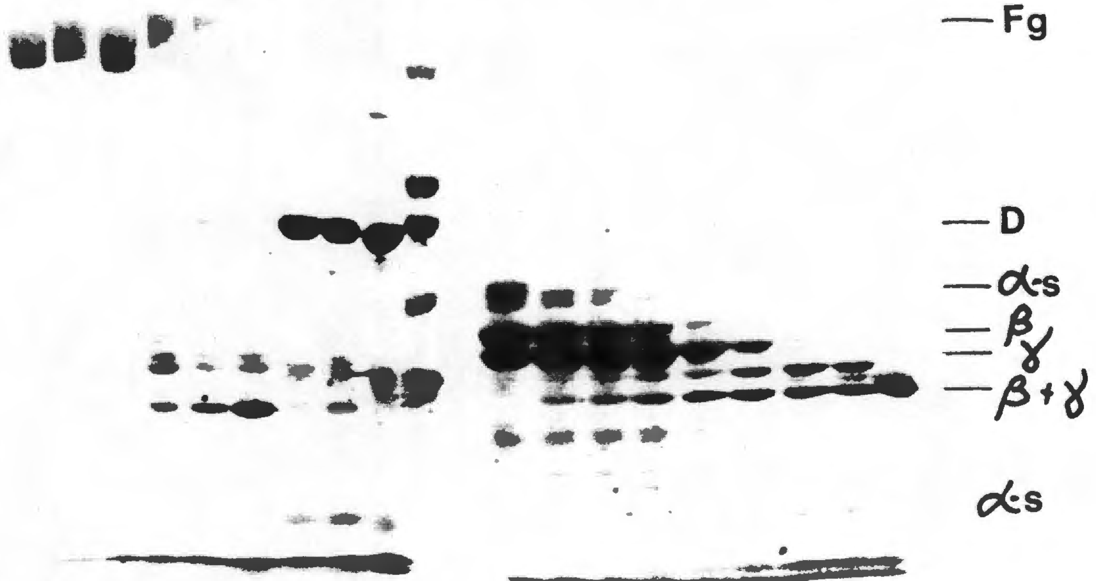
Figure 20. The effect of PAV protease on the clotting time of fibrinogen.

Fibrinogen was incubated for 30 minutes at 37°C with crude PAV protease. At 5 minute intervals aliquots were withdrawn, calcium and thrombin added and the time noted for a clot to develop. Purified PAV protease rendered fibrinogen incoaguable after 30 seconds.

Figure 21. Caption overleaf

A

0' 5' 10' 15' 30' 1, 2, 4, 24 h with plasmin



B

SDS STDs βME

0' 5' 10' 15' 30' 1 2 4 24h with PAV

Figure 21. Comparison of digestion of fibrinogen by plasmin and PAV protease.

A. Fibrinogen (10 mg/ml) was incubated with plasmin at 37oC in buffer containing 1 mM $CaCl_2$. Aliquots were withdrawn at timed intervals and run on 4-20% SDS-PAGE under non-reducing (SDS) and reducing (BME) conditions.

B. Fibrinogen incubated with PAV protease using same conditions as A.

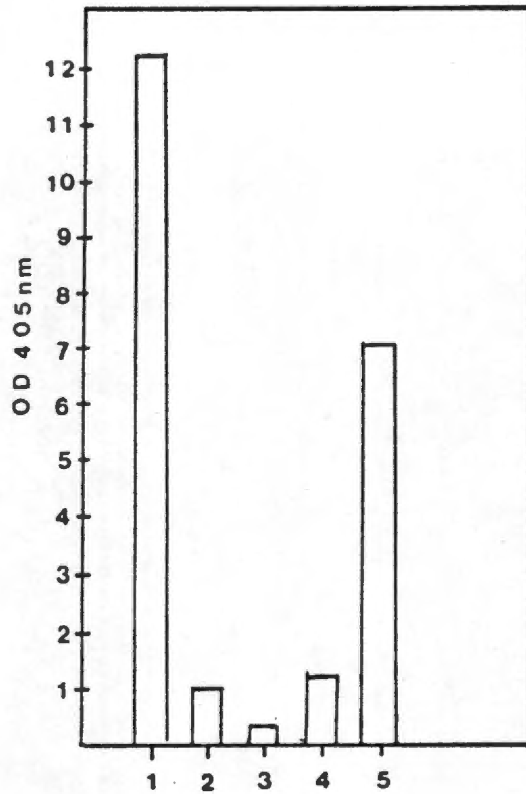


Figure 22. Activity of crude puffadder venom with Chromozym substrates.

1 = Chromozym PL + plasmin (10μg)
2 = Chromozym PL + PAV (100 μg)
3 = Chromozym TRY + "
4 = Chromozym PK + "
5 = Chromozym TH + "

3.3.3 Caseinolytic activity of PAV protease.

The pH optimum for caseinolytic activity of PAV protease was established using buffers with pH ranging from 7.0 to 10. The pH optimum for caseinolytic activity of PAV protease is about pH 9.0 (Figure 23).

3.4 DEFINITION OF PAV PROTEASE ACTIVITY USING D-DIMER FROM CROSS-LINKED FIBRIN AS SUBSTRATE.

Before using puffadder venom as a tool in the study of the D-domain of fibrin it was necessary to define parameters for its activity with respect to D-dimer. The fluorescence spectra of f-D-monomer, f-D-dimer and the fluorophore dansyl cadaverine were determined (Figure 24).

3.4.1 Timed digest of fluorescent D-dimer by PAV protease.

The assays for D-dimer activity were carried out at pH 8.0. Fluorescent D-dimer was incubated with PAV protease and aliquots for given times were run on a 4-20°C SDS-PAGE (Figure 25A & B). The D-dimer is cleaved to a monomer with the same molecular weight as D-monomer-plasmin. After two and three hours of digestion a second D-monomer (D-PAV-monomer) can be seen with a slightly smaller molecular weight (Figure 25A, lane 4). The activity of the PAV protease was also followed with a spectrofluorimeter. Both fluorescent D-dimer and fluorescent D-monomer showed a decrease in absolute fluorescence during PAV digestion (Figure 26A). Only fluorescent D-monomer species, with no fluorescent fragments, were produced on PAV digestion and the digestion was both time and concentration dependent as monitored

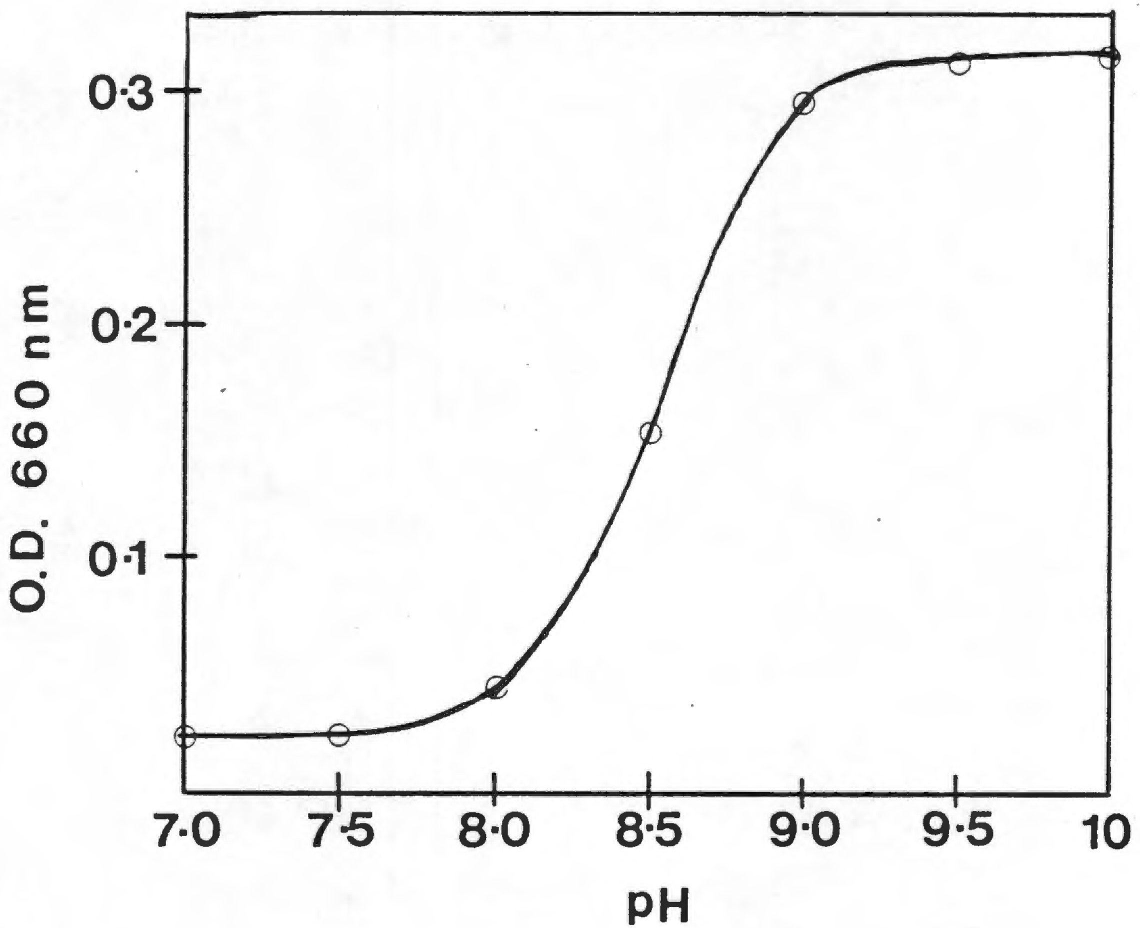


Figure 23. Optimum pH of PAV protease with casein as substrate.

Caseinolytic activity of PAV protease was determined in Tris/HCl buffer of different pHs.
The optimum activity was found to be at pH 9.

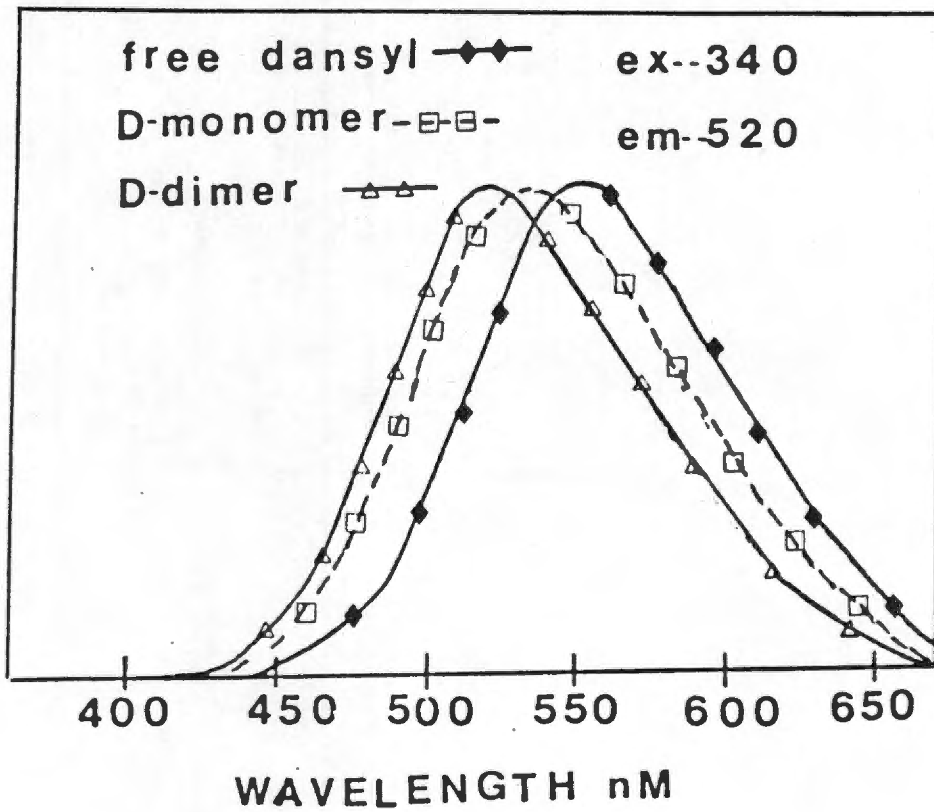


Figure 24. Fluorescence spectra of D-monomer, D-dimer and monodansyl-cadaverine.

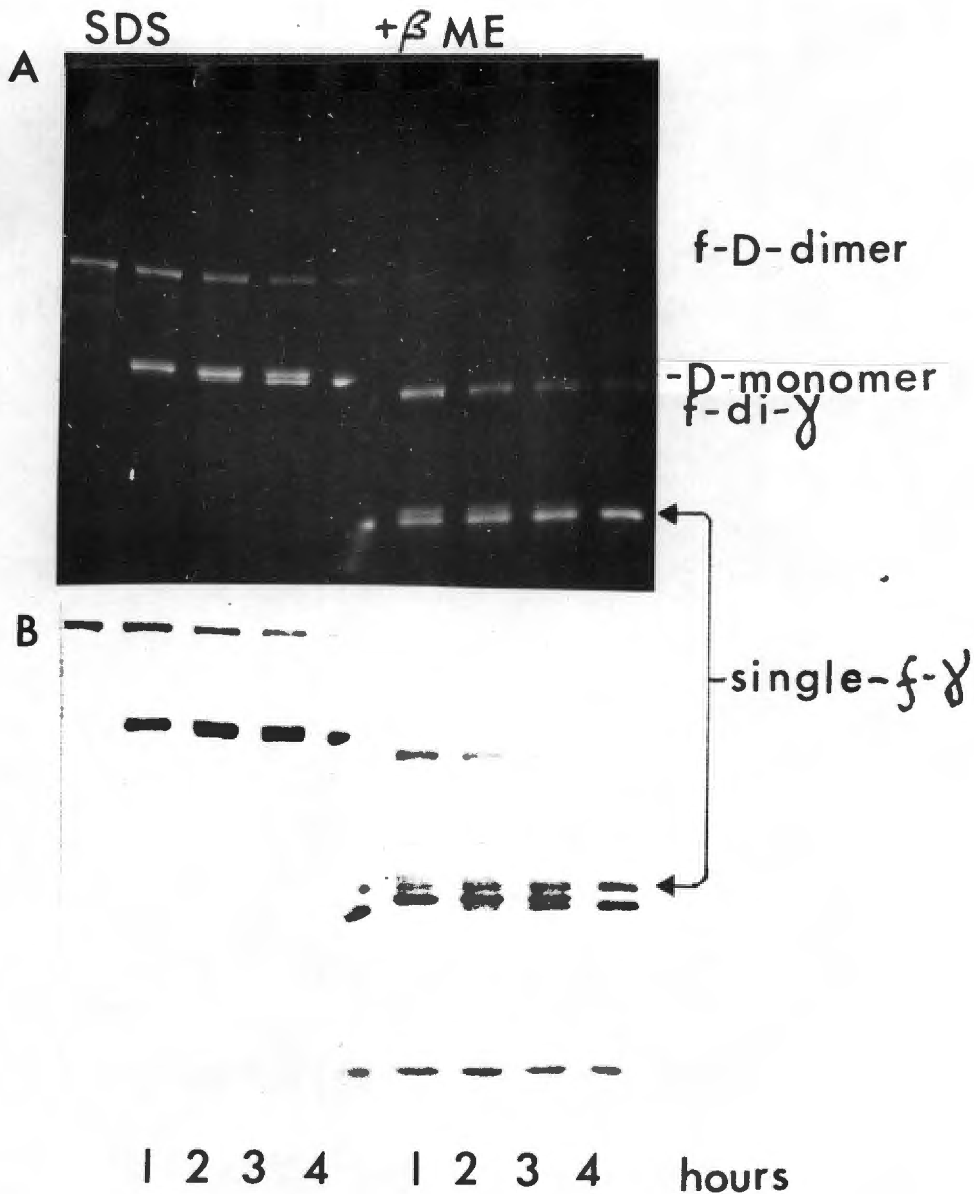


Figure 25. Timed digestion of fluorescent D-dimer by PAV protease showing the distinct single γ-chain.

f-D-dimer (1 mg/ml) was incubated with PAV protease (10 μg/ml) at 37oC. Aliquots were taken at 0, 1, 2, 3 and 4 hour intervals and analysed by SDS-PAGE under reducing (βME) and non-reducing conditions. The gel was photographed under U.V. light prior to staining with Coomassie blue.

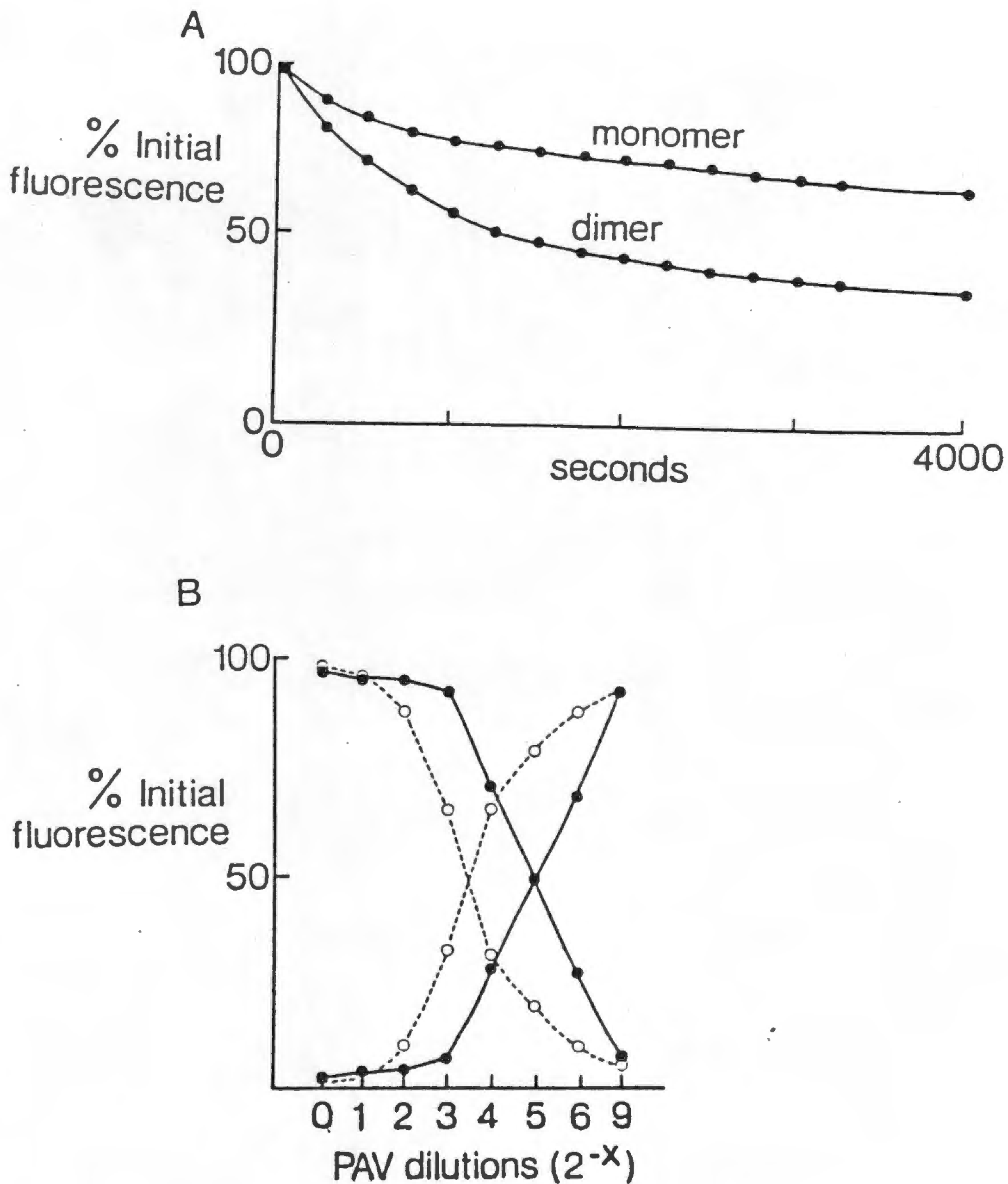


Figure 26. Changes in fluorescence during digestion of f-D-dimer with PAV protease. Excitation wavelength 340 nm, emission wavelength 520 nm.

A. Decrease in the initial fluorescence of f-D-dimer and f-D-monomer with time. Both species are converted to a form of f-D-monomer smaller than plasmin D-monomer.

B. Relative changes in amounts of f-D-dimer and f-D-monomer after 1h (——) and 2h (- - -) digestion with dilutions of PAV. The undiluted venom contains 0% f-D-dimer and has been completely converted to f-D-monomer for both durations.

by fluorescent scanning of PAGE gels of f-D-dimer that had been exposed for one or two hours to dilutions of PAV (Figure 26B).

3.4.2 Effect of zinc ions on PAV protease activity.

The change in fluorescence during conversion of f-D-dimer to f-D-monomer PAV protease was used to study the effects of added zinc on the enzyme. The effect of added zinc was to increase the loss of absolute fluorescence immediately but the same final value was attained whether the zinc was added at zero time or later (Figure 27). There was a 10 fold potentiation of enzyme activity when zinc was added. The correlation of changes in absolute fluorescence and D-dimer cleavage was confirmed by SDS-PAGE of aliquots taken during the digestion. These changes in fluorescence probably reflect changes in the hydrophobic environment of the dansyl group on the substituted cross-link site. This is possible since the cross-link sites must both lie between the large D-domains.

3.4.3 Effect of inhibitors on PAV protease activity.

A preliminary study of inhibitors revealed that only metal chelators e.g. EDTA, were effective (Figure 28). No other class of effective inhibitor e.g. mercury compounds, fluoride compounds, chloromethylketones, or bacterial inhibitors e.g. clostripain, pepstatin or leupeptin have been found (Figure 29). Calcium appears to play no role in the action of PAV or in determining the susceptibility to PAV digestion even when plasmin-susceptible D-dimer (Chelex-treated D-dimer, very low Ca^{++} concentration) was used. The presence or absence of the E-domain associated with the D-dimer did not alter susceptibility to PAV (Figure 28A).

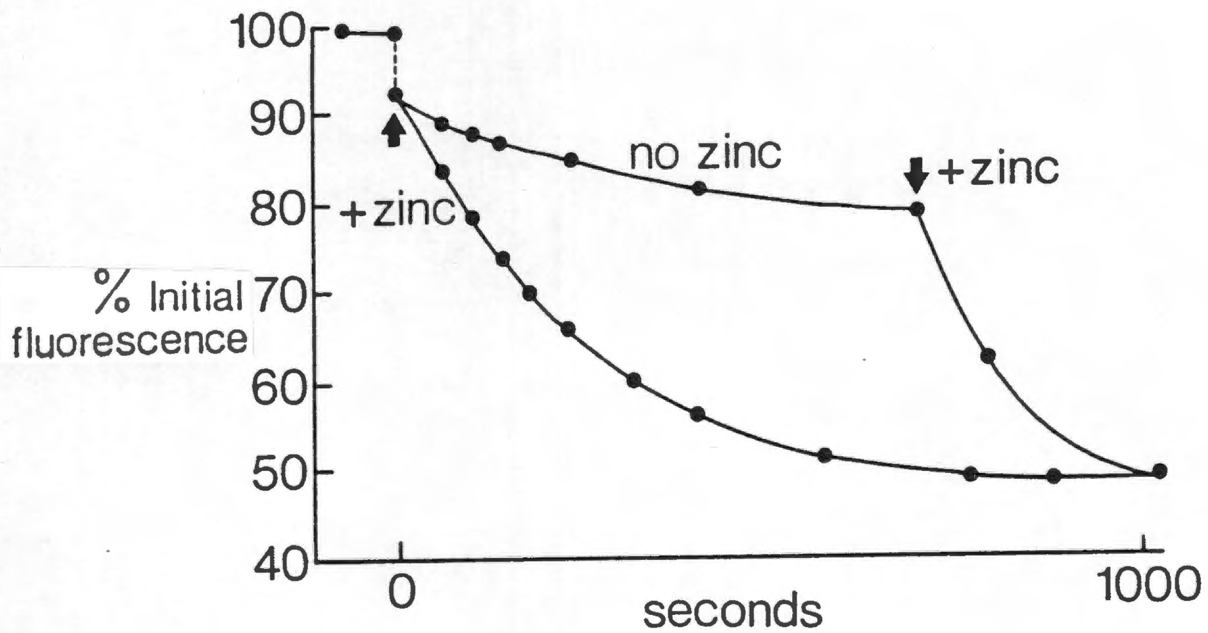


Figure 27. The decrease in absolute fluorescence of f-D-dimer on digestion with PAV is potentiated by the addition of Zn^{++} but the same total decrease is attained.

Figure 28. Caption overleaf

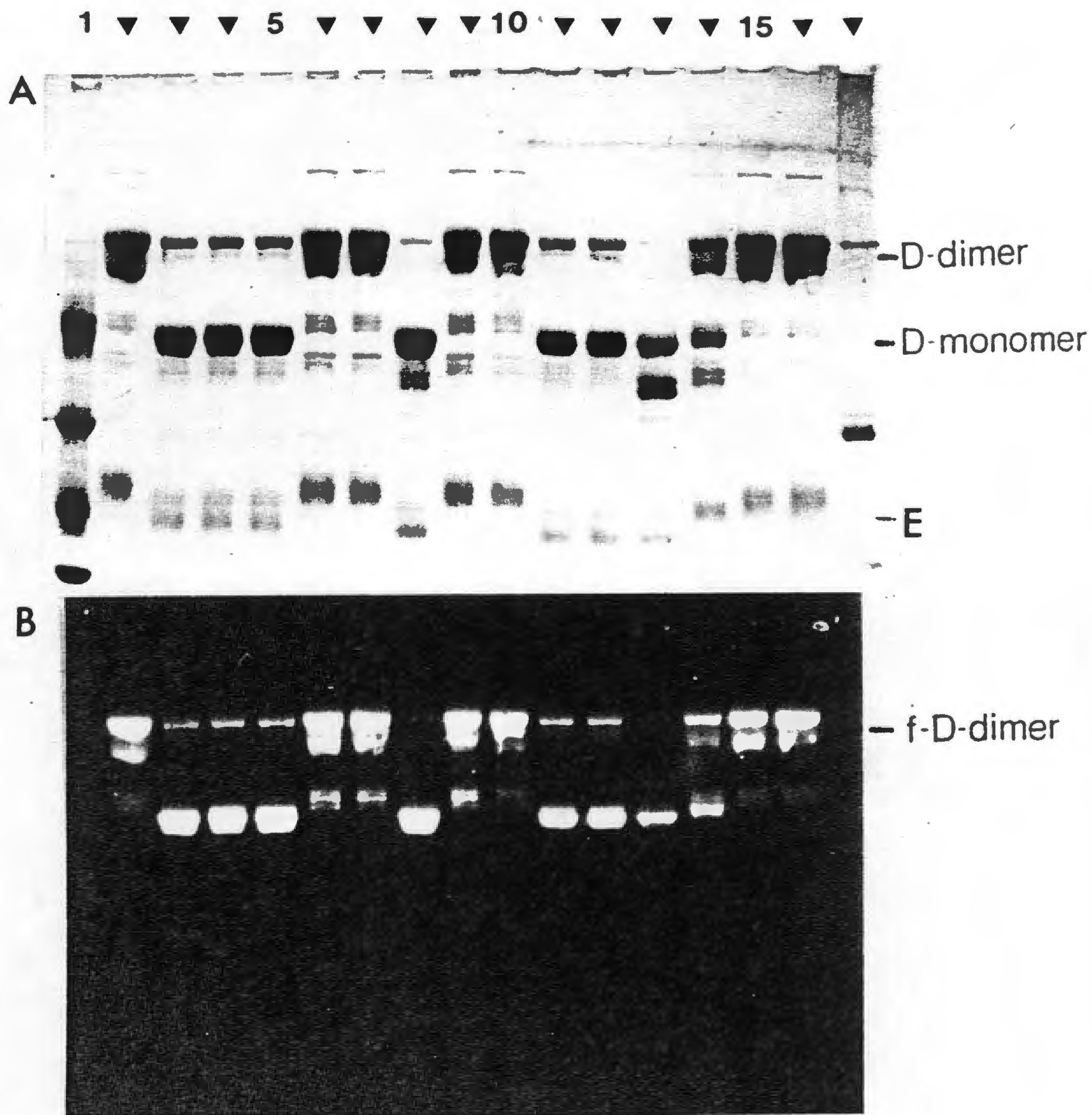


Figure 28. The effect of various inhibitors, calcium, and zinc ions on the cleavage of f-D-dimer by PAV.

A. Fluorescence picture of SDS-PAGE.

B. SDS-PAGE stained with Coomassie Blue.

Lanes	
1	LMW standards
2	f-D-dimer
3	" + PAV + hexane-diamine
4	" + PAV + PMSF
5	" + PAV
6	" + PAV + 1 mM EDTA
7	" + PAV + 3 mM EDTA
8	" + PAV + 1 mM EDTA + 1 mM $ZnCl_2$
9	" + PAV + 3 mM EDTA + 1 mM $ZnCl_2$
10	f-D-dimer Chelex treated
11	" + PAV + 0.1 mM $CaCl_2$
12	" + PAV + 1.0 mM $CaCl_2$
13	" + PAV + 0.1 mM $ZnCl_2$
14	" + PAV + 1.0 mM $ZnCl_2$
15	" + plasmin + 1.0 mM Ca^{++}
16	" + plasmin + 0.1 mM Ca^{++}
17	HMW standards

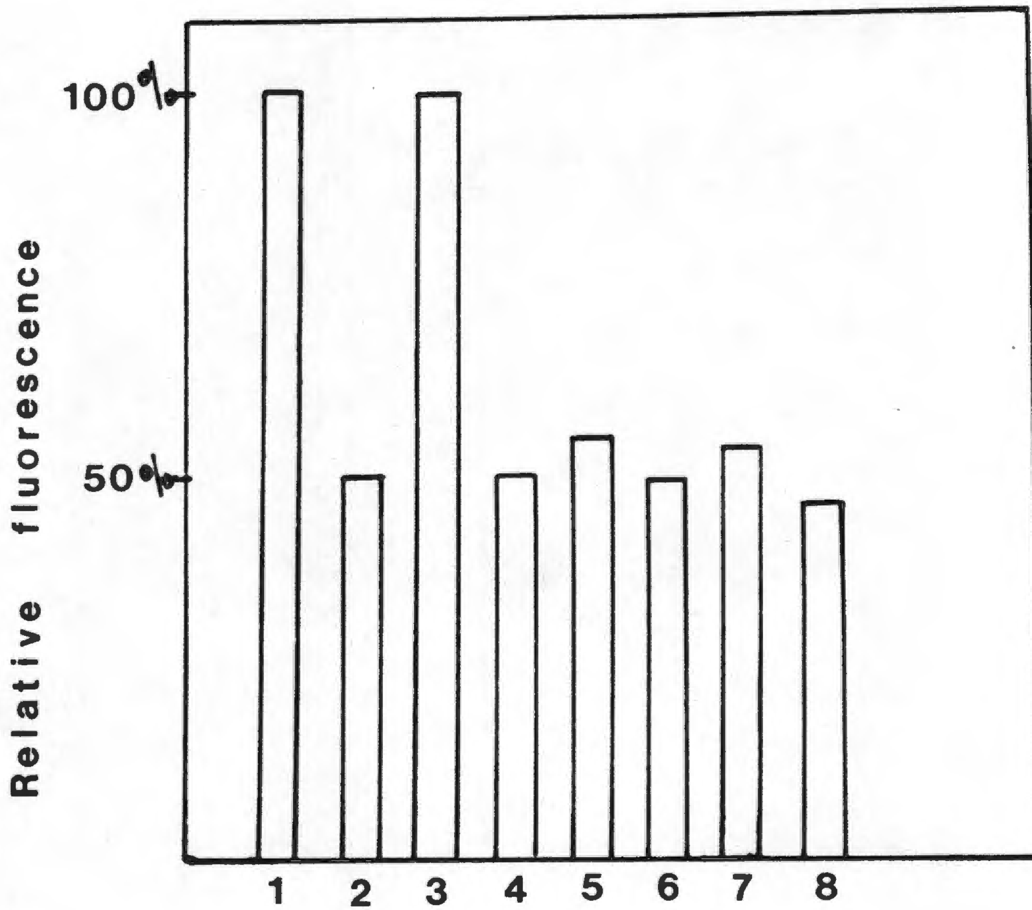


Figure 29. Effect of inhibitors on the decrease in fluorescence of f-D-dimer on digestion with PAV.

1 = f-D-dimer
2 = " + PAV + Zn^{++}
3 = " + PAV + Zn^{++} + 5 mM EDTA
4 = " + PAV + DMSO
5 = " " + chymostatin
6 = " " + leupeptin
7 = " " + pepstatin
8 = " " + PMSF

Final concentration of inhibitors 1 mM.

3.4.4 Comparison of PAV protease with plasmin using D-dimer as substrate.

Plasmin is unable to cleave D-dimer in the presence of physiological concentrations of calcium (Figure 28, lane 15 &16). If calcium is chelated plasmin degrades D-dimer to several species of D-monomer from D_1 to D_5 (Purves et al,1978). The smaller D species are due to progressive shortening of the γ-chain from the carboxy-terminal end. The final product is two smaller D-monomers and a cross-linked peptide which remains associated with the D-monomers (Figure 30).

The earliest event in the action of PAV protease appears to be the cleavage of the di-γ-bond with the production of only one size of γ-species, either fluorescent or non-fluorescent (Figure 25) and separation of the dimer into monomers (Figure 31A). The β-chain is hydrolysed at a slower rate and this is a progressive process yielding a smaller chain size (β-PAV). Early in the digestion two species of f-D-monomer were seen (Figure 31A, lane 4), suggesting that cleavage of the di-γ-bond can precede and be independent of the progressive reduction in size of the β-chain that determines the smaller D-monomer species.

3.4.5 Cleavage of D-dimer by venom from different species of snakes.

Venom from seven members of the Viperidae and one Elapid snake were tested for D-dimer cleavage activity. Three out of the seven showed some activity, suggesting that the venom from Trimeresurus gramineus, T.okinavensis and Bothrops atrox may contain an enzyme similar to protease I from Bitis arietans. The other venoms tested; Cerastus cerastus (horned viper),

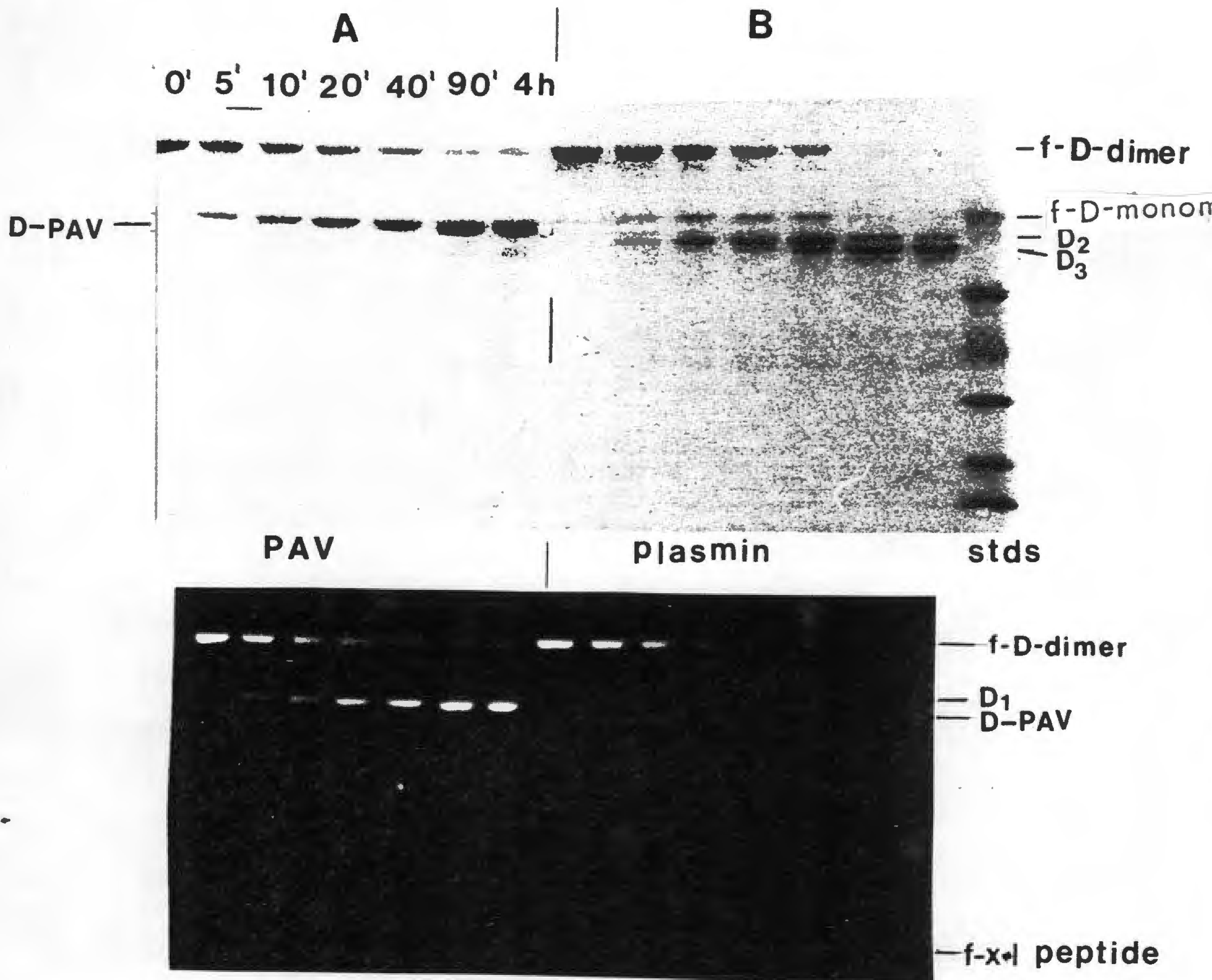


Figure 30. Timed digest of f-D-dimer with purified PAV protease compared to digestion of f-D-dimer by plasmin in the absence of calcium ions.

A. f-D-dimer (1mg/ml) was incubated with purified PAV (10 μg/ml) at 37oC in buffer containing Ca^{++} and Zn^{++}. At the times shown aliquots were withdrawn and added to SDS sample buffer and run on a 4-20% SDS-PAGE. The gel was photographed wet under U.V. light prior to staining with Coomassie Blue.

B. f-D-dimer was digested with plasmin under the same conditions as A except that the Ca was chelated with EDTA. This resulted in the excision of a cross-linked fluorescent peptide from f-D-dimer giving rise to a fluorescent D-monomer and some smaller non-fluorescent D-monomers.

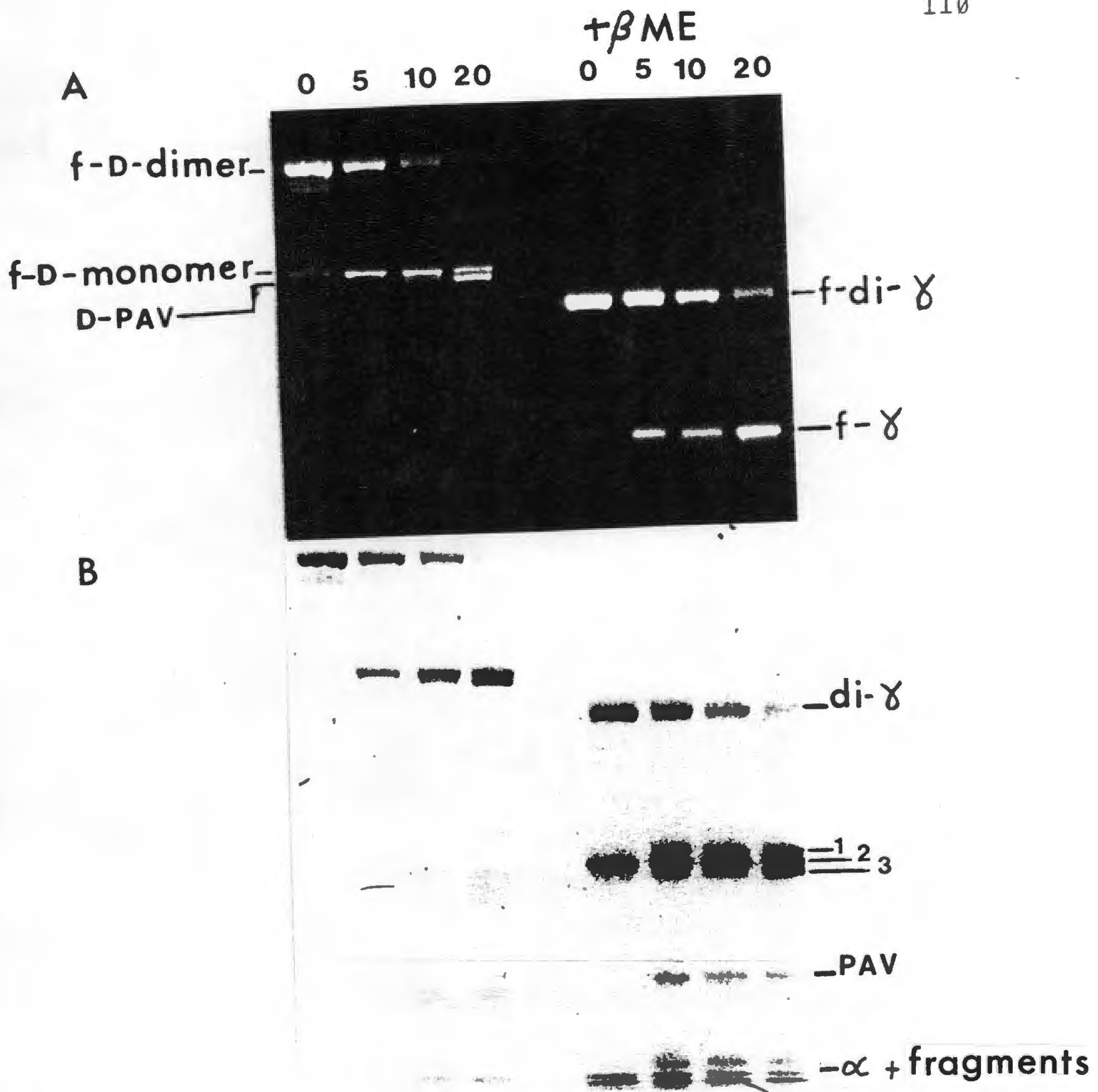


Figure 31. Timed digestion of f-D-dimer by PAV.

f-D-dimer was incubated with PAV protease for 20 minutes at 37oC.Aliquots were withdrawn at the times shown and run on a 4-20% SDS-PAGE. The wet gel was -

(A) photographed under U.V. light

(B) prior to staining with Coomassie blue.

After 10 minutes of digestion two D-monomer bands can be seen, both these bands are fluorescent. On reduction three bands can be seen that were identified as -

1) f-γ-chain,
2) plasmin-β-chain and
3) PAV-β-chain in decreasing order of size.

Only a single species of f-γ is produced with no fluorescent fragments but a plasmin-D-monomer is the first product with a smaller D-PAV-monomer later (note double band in lane 4 in SDS run).

Dendroaspis polylepis (black mamba), Crotalus adamanteus (diamond -back rattlesnake), Echis carinatus (saw-toothed viper) and Causus rhombeatus (night adder) showed no D-dimer cleavage activity (Figure 32).

3.4.6. Effect of PAV protease on D-dimer derived from rat fibrin. Fibrinogen was prepared from rat plasma as described in the methods. It was clotted in the presence of thrombin and calcium and the clot digested with plasmin.The resulting D-dimer was not purified further, but PAV protease added directly to the plasmin digest. Aliquots were removed at various time intervals up to 2 hours and analyzed on a 4-20% SDS-PAGE with non-reducing (SDS) and reducing (BME) conditions and stained with Coomassie Blue. After 30 minutes there is an increase in D-monomer (Figure 33A) and the two B-chains can be seen (Figure 33B).

3.5 IMMUNOLOGICAL APPROACH TO THE STUDY OF THE D-DOMAIN OF FIBRIN.

The advent of monoclonal antibody techniques has opened up new possibilities for the identification of fibrin/fibrinogen degradation products. The main avenue of attack has been the exposure of normally sequestered epitopes in an effort to distinguish the different antigens present in plasma in normal and disease states.

In this work a start has been made in the development of a monoclonal antibody to the site exposed by action of puffadder venom on D-dimer. The reactivity of D-PAV-monomer was tested against existing monoclonal antibodies developed by Rylatt.

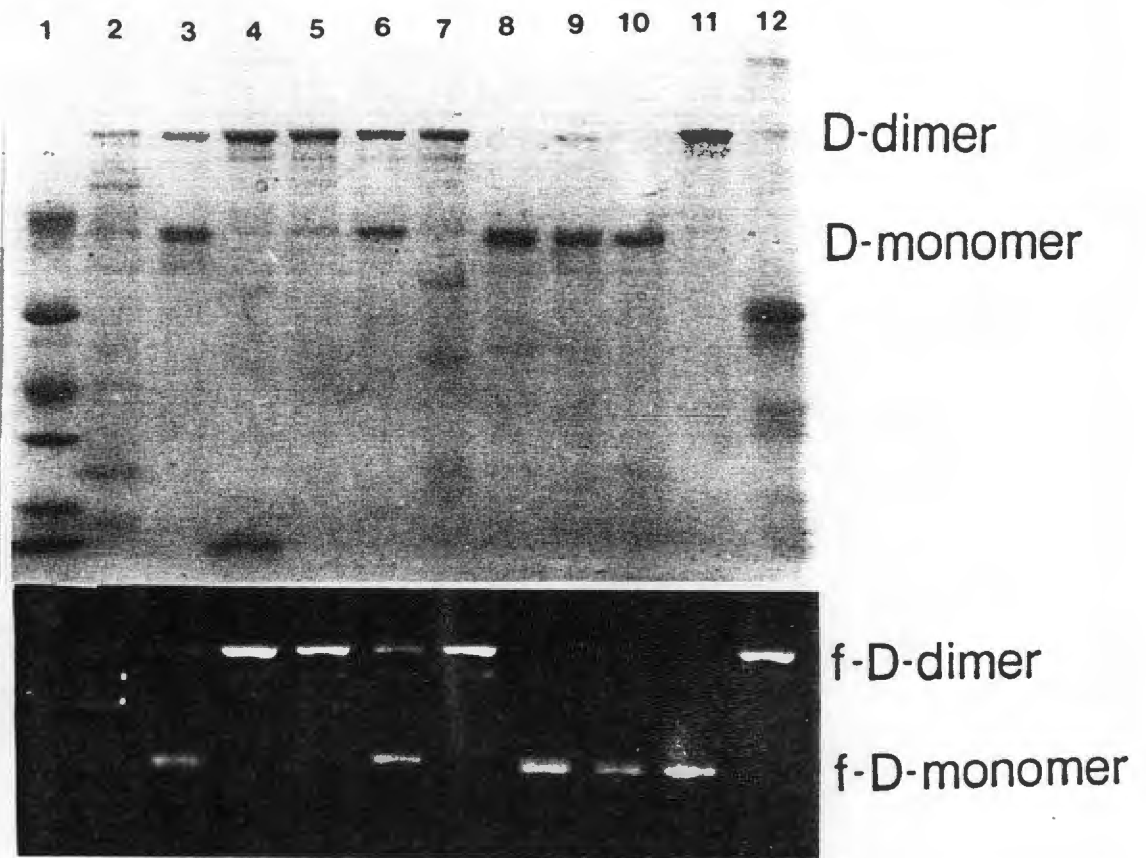


Figure 32. A two hour digest of f-D-dimer with nine different snake venoms.

Lane 1 = LMW Stds.
" 2 = Cerastus cerastus
" 3 = Trimeresurus gramineus
" 4 = Crotalus adamanteus
" 5 = Dendroaspis polylepis
" 6 = Echis carinatus
" 7 = Causus rhombeatus
" 8 = Bothrops atrox
" 9 = Trimeresurus okinavensis
" 10 = Bitis arietans (puffadder)
" 11 = f-D-dimer
" 12 = HMW Stds.

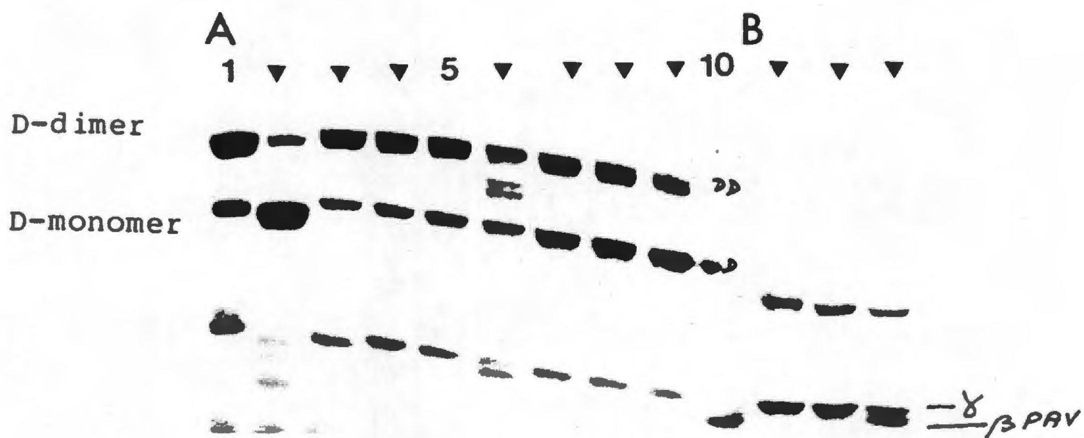


Figure 33. Timed digest of rat D-dimer by PAV protease.

Rat D-dimer was incubated with PAV protease for 2 hours at 37oC. Aliquots were withdrawn at the times shown and analyzed by SDS-PAGE with

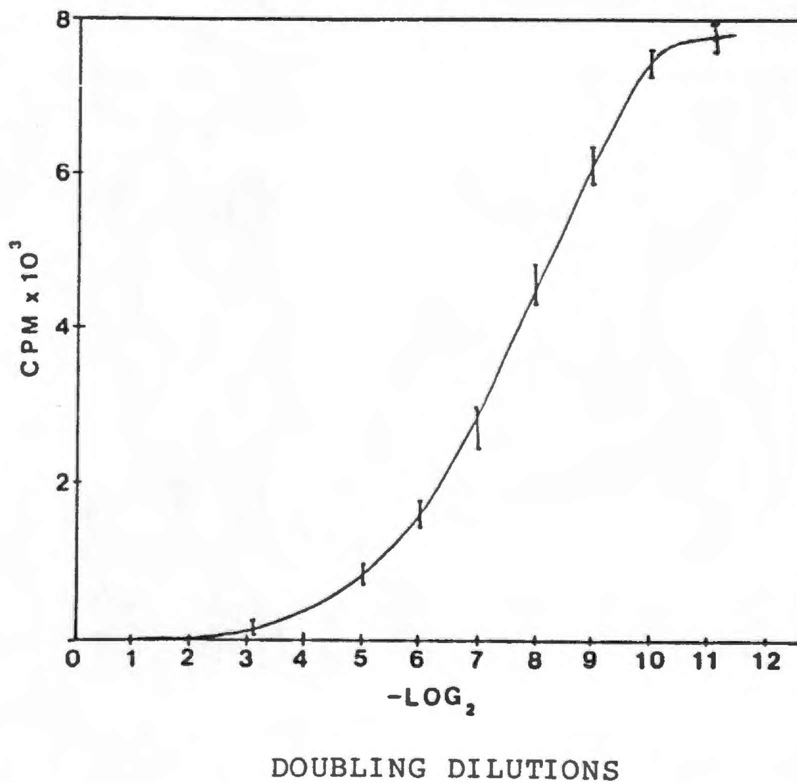
A. non-reducing conditions
1 - human D-dimer
2 - " + PAV 2 hours
3 - rat D- dimer
4 - rat D-dimer + PAV 2 mins
5 - " 5
6 - " 10
7 - " 20
8 - " 30
9 - " 60
10 - " 120

B. reducing conditions.
11 - " 0 mins + βME added
12 - " 5 "
13 - " 30 "

3.5.1 Radioisotope assay for D-dimer.

A standard curve was performed (Figure 34). All assays were performed in duplicate. D-PAV-monomer showed very little reaction with the monoclonal antibody directed at D-dimer (Figure 35).

A second assay was performed in which the wells were coated with D-dimer and PAV protease added to wells and left overnight, thus converting the bound D-dimer to D-PAV-monomer, which again showed very little reaction to the second antibody. D-PAV-monomer showed very little reaction with the monoclonal antibody directed at D-dimer (Figure 36).



DOUBLING DILUTIONS

Figure 34 . Standard curve for monoclonal antibody radioisotope assay.

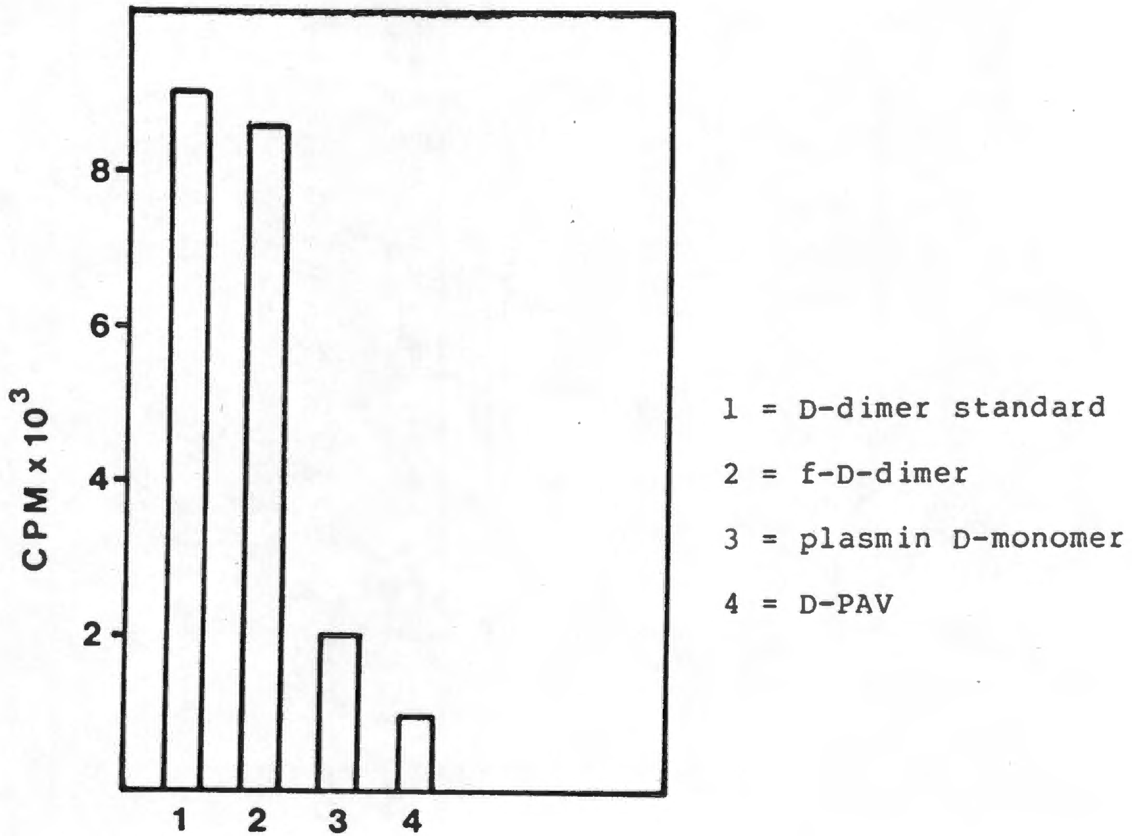


Figure 35. Radioisotope immunoassay with various antigens.

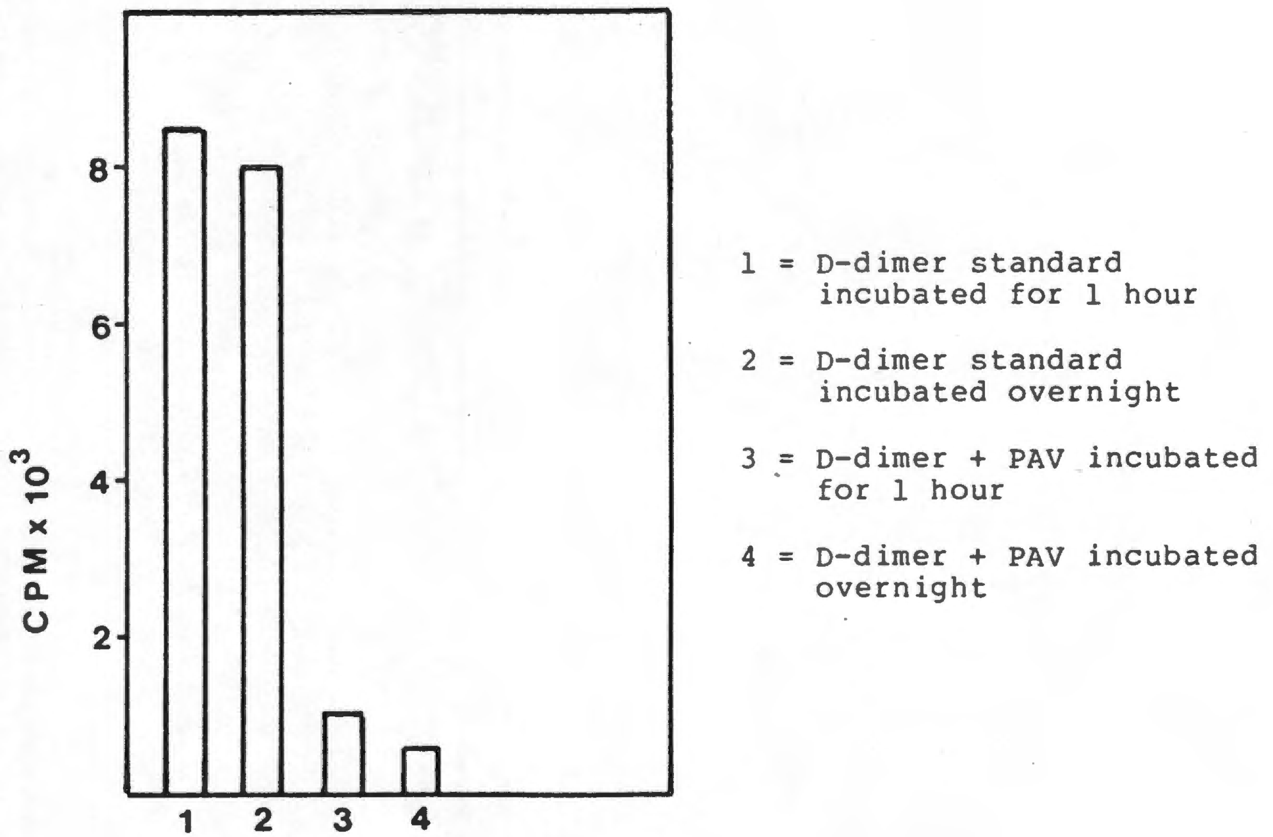


Figure 36. Radioisotope immunoassay with PAV added to the wells.

3.5.2 Dimertest agglutination assay.

f-D-dimer digestion was carried out at 37°C with plasmin and EDTA, and PAV protease. Aliquots were withdrawn at time intervals from 0 to 4 hours and run on SDS-PAGE (Figure 30). Another 20 µl aliquot was diluted 1/10 (± 500 ng) and tested with the Dimertest agglutination assay (Table III).

Table III: Reactivity of PAV protease and plasmin digested D-dimer with the Dimertest monoclonal antibody to D-dimer.

Time	0'	5'	10'	20'	90'	3h	18h
PAV	+	+	+	+	-	-	-
plasmin	+	+	+	+	+	+	+

3.6 Sequencing of the carboxy-terminal cyanogen-bromide fragment of the γ-chain of D-PAV-monomer.

The D-PAV-monomer from the DEAE-cellulose column was collected by pooling the fluorescent samples (due to the initial addition of 1% fluorescent D-dimer). After reduction and alkylation the β and γ-chains were clearly separated by anion exchange chromatography as shown by acetic acid/urea PAGE (Figure 37). After cyanogen bromide cleavage of the purified γ-chain the peptides were separated by molecular sieving. The lyophilised fractions, made alkaline with ammonium bicarbonate, showed that the fluorescence was located at one end of the β peak (Figure 38). This distribution conformed with the expectation of a carboxy-terminal peptide, 2864 daltons, with the other peptides having sizes: 19284, 4875, 4854, 3891, 3047, 1214, 615, 589 and 188 daltons.

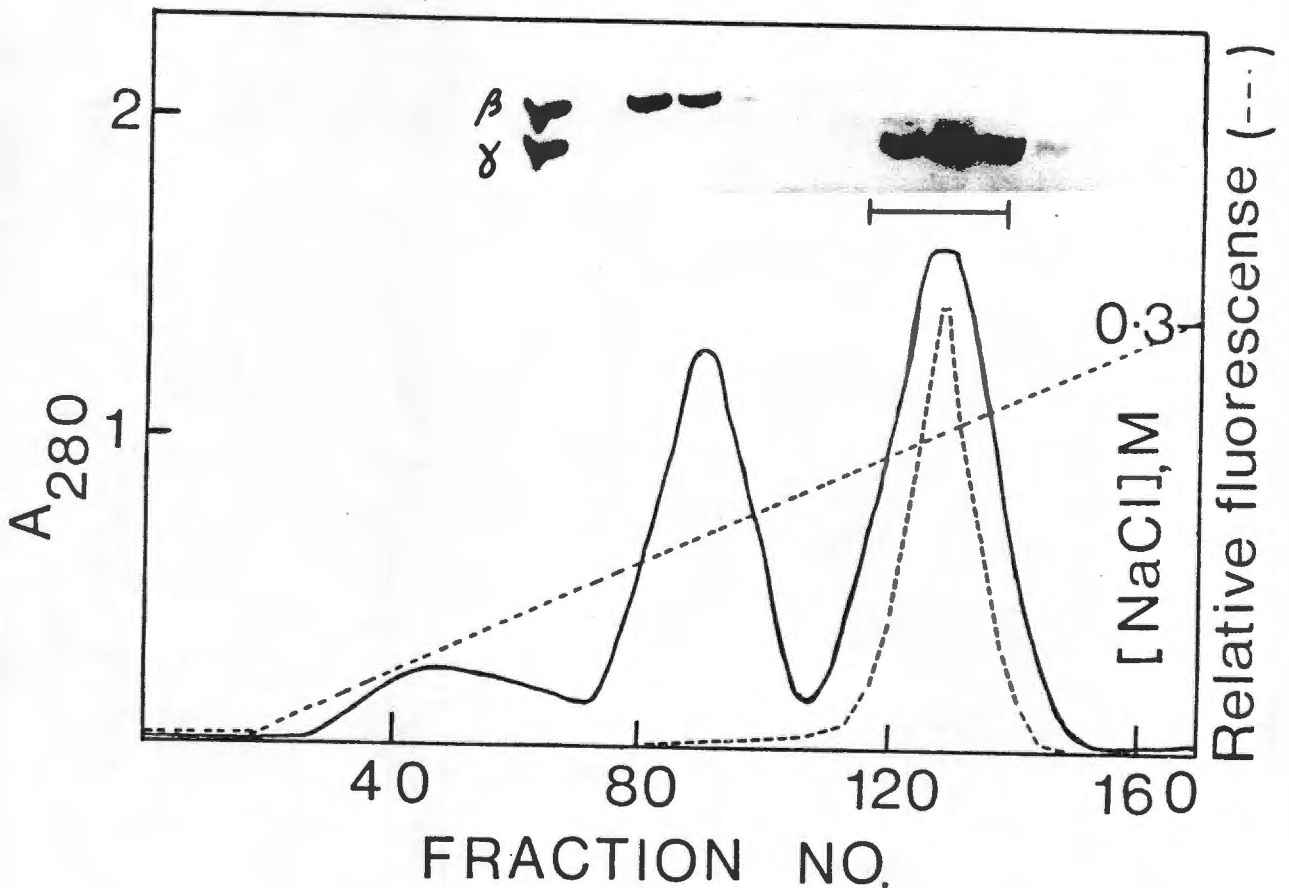


Figure 37. Isolation of reduced and alkylated β- and γ-chains (345 mg), on a DEAE-Sepharose CL 6B column (2 x 20 cm) equilibrated with 10 mM Tris/HCl buffer pH 7.5 containing 8M urea.

Flow rate 34 ml/hour. Fraction volume 2.25 ml. Absorbance 280 nm (——). Fluorescence in arbitrary units (- - -), excitation 340 nm, emission 520 nm (from the addition of 1% dansyl cadaverine substituted D-dimer to the starting material in the preparation of D-PAV-monomer). γ-Chains were pooled as indicated.
Inset. PAGE of indicated fractions in an acetic acid buffer containing 2.5 M urea.

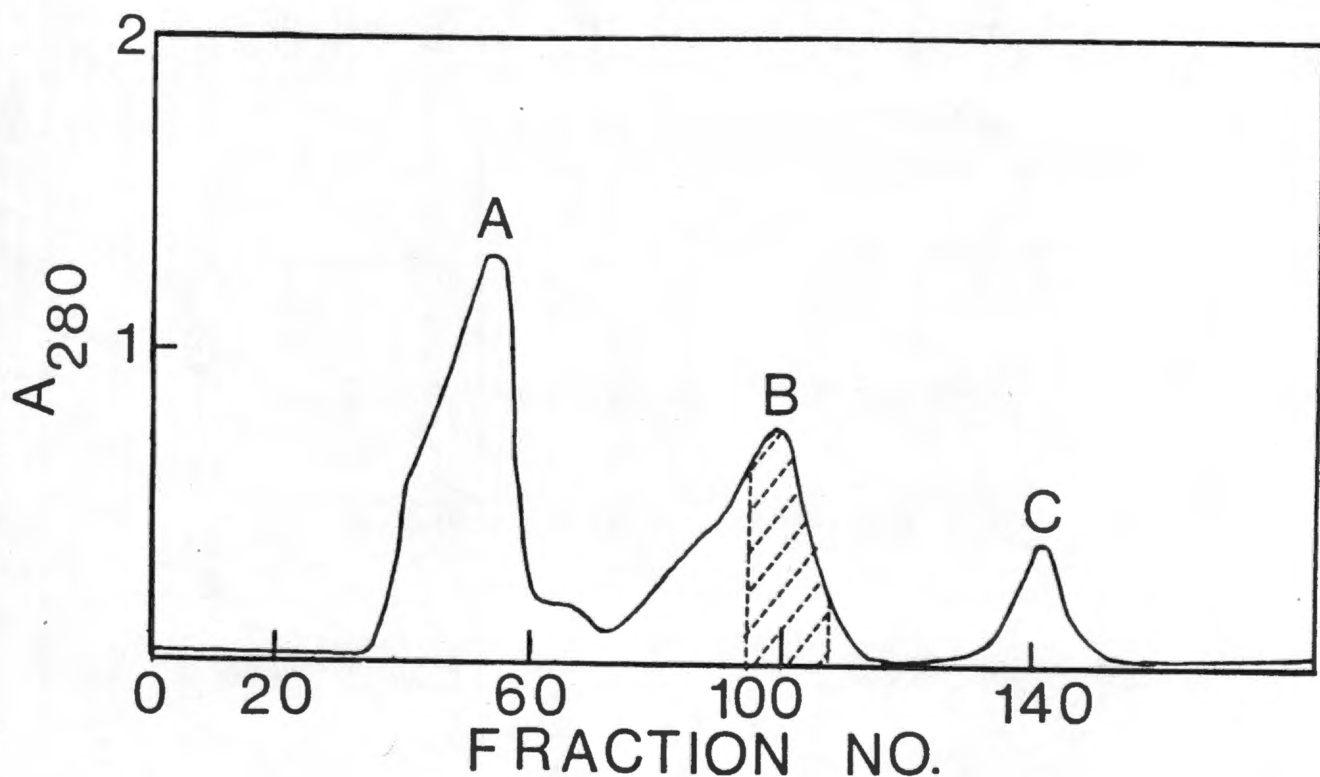


Figure 38. Separation of the cyanogen-bromide peptides of the γ-chain of D-PAV-monomer (30 mg) on a Sephadex G 50 column (1 x 200 cm) in 10% acetic acid.

Flow rate 10 ml/hour. Fraction volume 1.0 ml. The hatched area indicates the fluorescent fractions that were pooled (see methods).

The dansyl-cadaverine substituted peptide was clearly separated from the main unsubstituted peptide(s) (Figure 39) in HPLC under alkaline conditions using a RadialPak (Waters) C8 column. The precise molecular constitution of the fluorescent peptide has not been studied further yet except to note that two main species appeared to be present as is the case with the unsubstituted peptides. The amino acid composition of the HPLC fractions showed that peaks A and B (Figure 39) both had the hallmarks of the carboxy-terminal peptide viz. no serine; equimolar valine, phenylananine, proline and threonine and more isoleucine than leucine (Table IV). There were minor differences in amino acid composition between peaks A and B. HPLC of individual peaks in an acid system again yielded two peaks. Peaks A & B were therefore pooled and run on an acid HPLC system and this yielded two overlapping peaks (Figure 40, peaks D, E) with similar amino acid composition and sequences.

The amino acid composition (Table IVA) showed that certain amino acids were at lower levels than expected - notably leucine, glycine, histidine, glutamine and possibly isoleucine. These amino acids, with the exception of isoleucine, occur as a group and could have been removed by exopeptidase activity after a chain cleavage (Table IVB). The deficient isoleucine could be due to incomplete hydrolysis of the two adjacent isoleucine residues in positions 2 and 3 of the peptide. Subsequently, the expected three isoleucine residues appeared in the sequence (Table V). Since the sequence of the 27 residue carboxy-terminal peptide of the γ-chain is known (Figure 7), identities were not confirmed by back-hydrolysis but several residues were

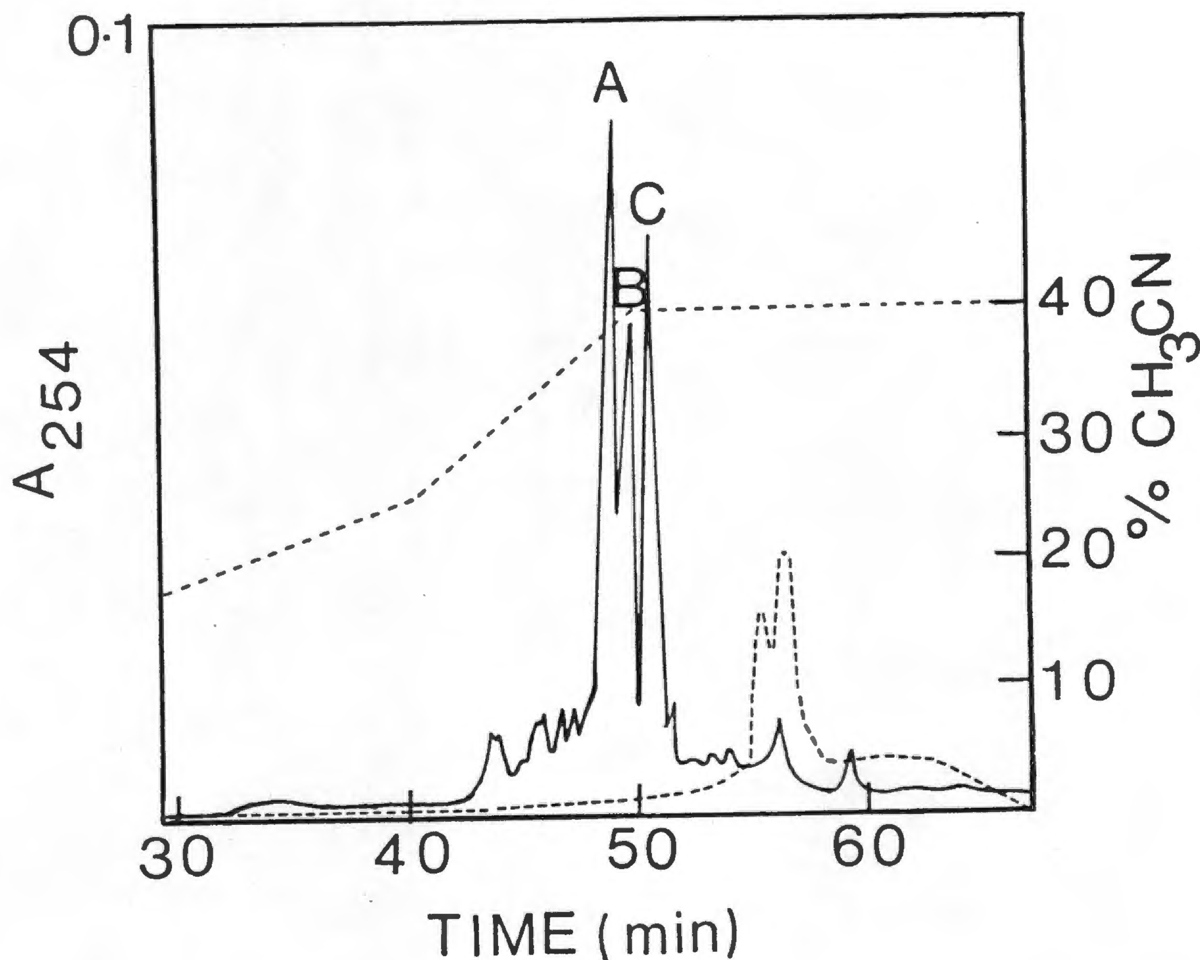


Figure 39. HPLC separation of the carboxy-terminal peptides of the γ-chain of D-PAV-monomer from the pooled fractions (Figure 31) using a RadialPak C8 (Waters) column.

The mobile phase was 0.1% NH_4HCO_3 containing 5% acetonitrile and the sample was eluted with an acetonitrile gradient up to 40%. Flow rate 1ml/minute. Absorption 254nm (——) and fluorescence in arbitrary units (- - -) (Waters detector). Peaks A and B were both shown to be carboxy-terminal peptides on the basis of amino acid composition (see results).

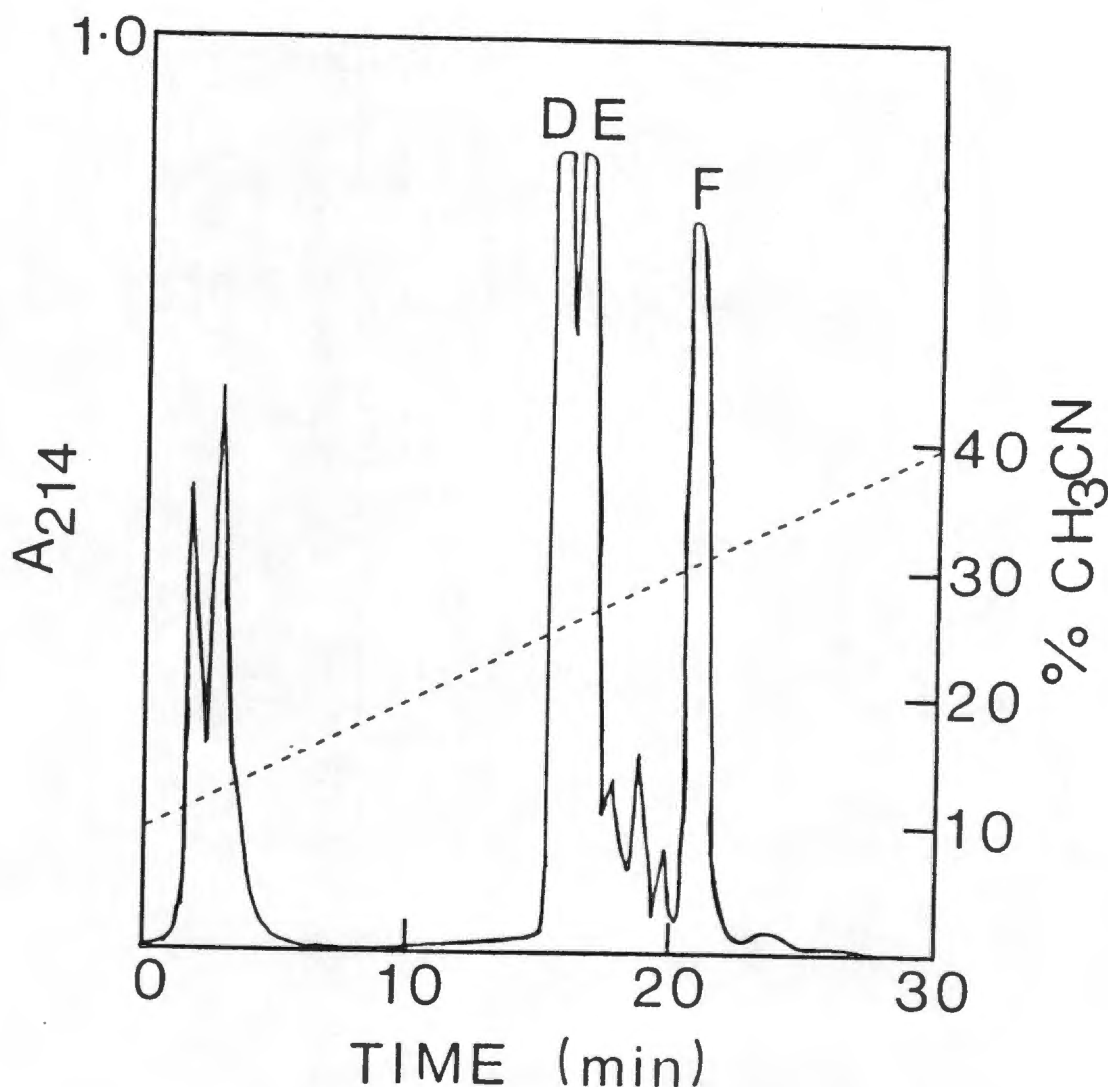


Figure 40. HPLC separation of pooled carboxy-terminal peptides (peaks A and B, Figure 32), on an Ultrapore RPSC (Beckman) C3 column.

The mobile phase was 0.1% TFA containing 10% acetonitrile and the peptides were eluted with a linear acetonitrile gradient over 30 minutes from 10% to 40%. Peaks D and E were both shown to be carboxy-terminal peptides on the basis of amino acid composition. (Two peaks were also seen when either peak A or B were applied separately - see discussion for comments on heterogeneity).

checked on a different PTH analysis system. Arginine (residue 7) was not identifiable as a single peak and threonine was present only as very hydrophobic dehydrothreonine with carry-over for 2 cycles. At residue 14, glutamine was found as well as a new peak (Table V, DP) eluting very early. A smaller amount of glutamine, possibly carried over, was found at residue 15 together with the early peak in larger amounts. The exact constitution of this early peak could not be ascertained but the synthetic dipeptide γ-L-glutamyl-ε-L-lysine, when subjected to the gas phase procedure, eluted in a similar position albeit with a few unidentified minor peaks. The di-PTH derivative of the dipeptide made by a solution method, however, yielded material eluting near to lysine but distinct from it and on hydrolysis in 6N HCl (gas phase) and re-derivatisation with PITC and conversion to the PTH derivative yielded glutamic acid and lysine. The differences have not been investigated further. Histidine was present in substoichiometric amounts (Table IV) suggesting a degree of heterogeneity. The manual sequence showed histidine to be present at residue 16 and possibly also at position 17. However, carry-over could not be excluded as the histidine was the terminal residue at either position 16 or 17. The carboxy-terminal amino acid of the γ-chain, i.e. valine, was seen in good yield despite its terminal position but occurred at cycle 7. The first cycle and the third to seventh cycles showed the presence of two residues. The second cycle contained only a single residue. The eight to thirteenth cycle showed a clearcut single residue and were all easily identified. Glutamine and a putative dipeptide (DP) were present at cycles 14 and 15 but with

Table IV :

A. Amino acid composition of isolated peptide (Peak A, Figure 39)

Amino acids	Corrected *[a] and normalised [b]	[c]	Probable number	Expected number	Difference
N + D	2.07	2.96	2	2	.
Q + E	2.25	3.21	2,3	4	-1,-2
S	0	0	0	0	.
G	1.90	2.71	2,3	5	-2,-3
H	0.34	0.48	0,1	2	-1,-2
R	1.05	1.40	1	1	.
T	0.86	1.22	1	1	.
A	1.76	2.5	2	2	.
P	1	1.40	1	1	.
V	0.70	1	1	1	.
I	1.60	2.28	2,3	3	-1
L	0.73	1.04	1	2	-1
F	1.05	1.50	1	1	.
K	1.70	2.42	2	2	.

B. Carboxy-terminal cyanogen bromide peptide of the human fibrinogen γ-chain.

[d] Sequence	K I I P F N R L T I G E G Q* Q* H H L G G A K* Q A G D V
[e] Losses	(i) (q h) H L G G

Table IV Caption.

[a] Corrected for recovery of hydrolysed amino acid standards.

[b,c] Normalised to 1 residue - [b]P, [c]V.

The values of probable and expected are given as residues per mole.

The known sequence [d] is aligned against the missing residues

[e] - (q h) represents probable extra losses in a subfraction of the peptide.

(i) the ratio of I to L was always 2:1 - 3:1 expected.

Partial hydrolysis of II could be responsible for low yield.

* Crosslink sites - at Q and K.

Table V : Gas-phase Edman degradation microsequencing.

	MANUAL				AUTOMATED	
Cycle	Residue 1	Carry over	Residue 2	Carry over	Residue 1	Residue 2
1	K 8.0	0	A 4.0	0	K 9.6	A 12.0
2	I 7.0	?	-		I	
3	I 6.0	2.0	Q 2.0	0.2	I 13.0	Q 6.1+E 4.0
4	P 5.0	0	A 5.0	0	P 9.3	A 11.0
5	F 4.0	1.0	G 4.0	1.0	F 11.0	G 10.6
6	N+D 8.0	1.5	(D)		N+D 3.4	(D)
7	[a] (R) ?	0	V 4.0	0.2	[e] R 1.6	[f] V 2.9
8	L 5.5	1.0			L 7.7	
9	[b] T 4.0	2.0 (0.2)			[g] T 3.6	
10	I 2.2	0.2			I 6.3	
11	G 2.0	?			G 6.5	
12	E 2.0	?			E 6.0	
13	G 1.0	?			G 5.2	
14	[c] Q 1.2	?	[d] DP 3.0	?	Q 0.1	(E 2.0)
15	Q 0.2	0.1	DP 4.0	?	[h] Q 1.0	(E 1.0)
16	H 1.0	?(0.05)	DP 3.0	0.2	[i] -	
17	(H 0.05)					
18	-					

Table V Caption.

The values in manual sequencing are relative peak areas. The repetitive yield was about 85%. Values in automated sequencing are in nmoles. Carry over refers to residues found in the subsequent cycle where it can be identified as such - (?) indicates where this cannot be done for technical reasons of peak separation or a repeated residue.

[a] R expected but 3 smaller peaks found instead.

[b] T only present as dehydrothreonine and carried over for 3 cycles.

[c] Q only a single major peak present.

[d] DP indicates presence of peak eluting at 1.5 minutes and not present in prior cycles.

[e] R identified as a small peak.

[f] V low yield of this carboxy-terminal residue.

[g] T identified as a triplet - the extra two peaks being dehydoalanine adducts with the dithiothreitol.

h Q identifiable above carry-over.

[i] no residue seen.

The early eluting DP peak could not be separated in the automated method.

more glutamine predominant at cycle 14 and the putative dipeptide predominant at cycle 15.

Automated sequencing of the carboxy-terminal peptide gave essentially similar results. Threonine was seen as a characteristic triplet and arginine and valine gave low yields at cycle 7. At cycle 14, glutamic acid and small amounts of glutamine were found. Glutamine and possibly carried-over glutamic were seen at cycle 15. The system did not permit the visualisation of very early eluting material. Histidine was not seen at all. The glutamine expected at cycle 3 was found partly as glutamic acid and this degradation could be expected to increase with each cycle perhaps accounting for glutamic acid instead of glutamine as the main component at cycle 14. (The glutamic acid is derived from the chemical deamidation of glutamine and is not the result of isopeptide bond hydrolysis although partial enzymatic deamidation or transamidation e.g. by transglutaminase cannot be excluded).

Using the known sequence of the human fibrinogen γ-chain the sequencing data could be resolved into a dipeptide cross-linked at lysine 406 and either glutamine 398 or 399 (residues 6 and 13 or 14 from the carboxy-terminal end of the γ-chain) with the loss of residues 401-404 that occur between the cross-link sites of both antiparallel cross-linked γ-chains. This effectively cleaves the D-dimer into two monomers and the cross-linked di-γ-chain into two symmetrical fragments consisting of a cross-linked dipeptide with the loss of 4 amino acids.

The sequence is therefore equivalent to the original fibrinogen sequence of -

$_{385}$ K I I P F N R L T I G E G (Q*Q*)H$_{400}$
X
$_{405}$ A (K$_*$) Q A G D V$_{411}$

[where (* X *) indicates a cross-link site]. There is heterogeneous loss of $_{401}$ H L G G$_{406}$ and possibly also $_{399}$Q and $_{400}$H in subfractions of the peptide.

A degree of heterogeneity seems to exist in two respects viz. -

1.) The cross-linked glutamine would appear to be either residue 14 or 15 with 15 predominating.

2.) The loss of carboxy-terminal amino acids may include both histidines and possibly even a leucine (residues 16, 17 and 18 of the peptide. There may also be deamidation of some of the glutamines near the cross-link site.

However, the amino-terminal residue of the secondary sequence is unequivocally alanine with the following lysine not appearing in the second cycle and presumably still cross-linked to the glutamines at either sites 14 or 15.

The recovery of carboxy-terminal peptide, quantitated by the lysine content of Peaks A and B, (Figure 3), accounted for 60% of the expected yield from the D-dimer used as starting material. Considering that only central components of peaks, e.g. as indicated by fluorescence, were collected the slightly heterogeneous carboxy-terminal peptides probably represented the major component and the existence of other variants was unlikely.

4. DISCUSSION

Fibrinogen is a molecule so perfectly adapted to its function that it has remained unchanged for 450 million years. The domainal nature of the molecule is essential both for its primary function, that of clot formation, and for the subsequent dissolution of the clot due to lysis at plasmin-vulnerable regions between the D and E domains. Fibrinolysis, in vivo, is achieved by plasminolysis at defined sites, releasing the E and D domains, but not affecting any D-domains cross-linked by transglutaminase. In the case of D-dimer removal of calcium exposes more sites for plasmin cleavage, putatively γ-356/357 and/or γ-g373/374 resulting in the excision of a cross-linked peptide of molecular weight ± 10,000 daltons, and therefore separation into monomers (Lindsey et al, 1978).

Southan et al (1985) report that conversion of Fg-D-Ca to Fg-D-EDTA occurs by progressive cleavage of three peptides γ-303-356, 357-373 and 373-405. The last two peptides form part of the polymerization site of the D-domain, as well as the site that reacts with platelets and staphylococci. These D-monomers have lost the ability to inhibit polymerization and are unable to cause platelet aggregation or staphylococcal clumping. The requirement for calcium removal probably indicates that cleavage of the carboxy-terminal end of the γ-chain by plasmin is physiologically irrelevant. Enzymes capable of separating the D-domains of fibrin in the natural state at neutral pH have not been reported.

There are three aspects to the work reported here:

i.) the isolation and characterization of an enzyme from puffadder (Bitis arietans) venom that is able to separate the cross-linked D-domain of D-dimer derived from fibrin, and

ii.) the nature of the cleavage affected by this enzyme i.e. whether there is direct cleavage of the ϵ-(γ-glutamyl)-lysine isopeptide bond or whether cleavage is due to endopeptidase activity of the enzyme, resulting in two D-monomer molecules.

iii.) the immunological reactivity of the crosslink region with antibodies.

Preliminary work on the action of puffadder on D-dimer was done using crude puffadder venom in which it was noted that cleavage of fluorescent-D-dimer to fluorescent-D-monomer was time and concentration dependent. The action of the puffadder venom on the dansyl-cadaverine labelled D-dimer results in the formation of apparently symmetrical monomers with only one species of γ-chain either fluorescent or non-fluorescent. Asymmetrical cleavage products with γ-chains having a size difference of 10 or more residues can be readily detected on SDS-PAGE gels with flat gradients (Purves and Lindsey, 1978). The enzyme from puffadder venom cleaved non-fluorescent D-dimer in exactly the same manner as the fluorescently labelled D-dimer, again giving rise to apparently symmetrical products. Early on in the work it was noted that after prolonged incubation of substrate and enzyme or using high concentrations of enzyme a double band appeared on SDS-PAGE gels, one equivalent to normal D-monomer and one with a slightly smaller molecular weight that

was called D-PAV-monomer. Using SDS-PAGE gels with reducing conditions smaller molecular weight of D-PAV-monomer was found to be due to cleavage of the β-chain, the γ-chain remaining intact. There was no indication of the asymmetrical products that might be produced if there was endopeptidase activity at the carboxy terminal end of the γ-chain.

Cleavage of the di-γ bond appears to precede and be independent of the progressive reduction in size of the β-chain. Thus the D-dimer cleavage into monomers appears to occur before shortening of the β-chain and is also not dependent on the calcium stabilized conformation producing resistance to continued plasmin cleavage (Purves & Lindsey 1978; Haverkate & Timan 1977). The purified enzyme gave exactly the same results with fluorescent and non-fluorescent-D-dimer as substrate. The caseinolytic activity found in peak 1 of the Sephadex G100 gel-filtration of PAV protease was associated with esterase activity and not with D-dimer cleavage activity. The D-dimer cleavage activity found in peak three was associated with the second and major peak of caseinolytic activity. The effect of the enzyme on fibrinogen was very rapid digestion of the α-chain, slower digestion of the β-chain with the γ-chain left intact. The effect of added zinc on cleavage of f-D-dimer by PAV protease was monitored by change in absolute fluorescence using a fluorimeter. The effect of added zinc was to increase the loss of absolute fluorescence immediately but the same final value was reached whether the zinc was added at zero time or later. The potentation was of the order of ten-fold.

These changes in fluorescence probably reflect changes in the hydrophobic environment of the dansyl group on the one blocked cross-link site. This is likely because both cross-link sites must lie between the large D-domains. Only metal chelators e.g. EDTA were effective in inhibiting PAV protease. Mercury compounds, methyl ketones and inhibitors of bacterial origon had no effect on PAV protease ability to cleave D-dimer. EDTA had no effect on the esterase activity of crude PAV enzyme, but totally inhibited cleavage of D-dimer. An equimolar amount of Zn and EDTA added to PAV enzyme did not inhibit the protease. However, PAV venom collected in the presence of EDTA showed no D-dimer activity. Dialysis of the EDTA-PAV venom against buffers containing zinc ions did not restore D-dimer cleavage activity. This may be due to auto digestion of the venom in the absence of calcium ions reported by Van der Walt (1971). The enzyme has a zinc moiety that once removed is unable to be restored. Many snake venom enzymes have been shown to be zinc dependent cationic metallo-proteinases with a molecular weight of between 20,000 and 30,000 daltons (Evans, 1981; Dyr et al, 1983; Pandya & Budzynski, 1984). Possibly several of them will be the same as or similar to the enzyme in puffadder venom. Three of the other snake venoms tested showed a similar activity with respect to D-dimer as puffadder venom. The enzyme does seem to be confined to snakes from Viperidae and Crotalidae and not found in Elapid snakes such as Dendroaspis polylepis (boomslang), or Echinis carinatus.

Several other proteases tested degraded the D-dimer molecule but showed no D-dimer cleavage into monomers. Clostripain,

leucine amino peptidase and cathepsin D were unable to degrade D-dimer at all.

The active fractions isolated from AcA 54 gel filtration were applied to an HPLC system and further fractionated into several peaks, one of which showed D-dimer cleavage activity. Further purification was not attempted, as it was found that the zinc moeity of the enzyme may be removed rendering the enzyme inactive. Although three bands were observed on SDS-PAGE gels, the smaller proteins are probably associated in solution since they emerge at the same hydrodynamic radius as the larger proteins on molecular sieving.

The HPLC results show D-dimer cleavage activity associated with a protein of $\pm$25,000 daltons on SDS-PAGE gels as compared to molecular weight standards. In conclusion the protease has a definite requirement for zinc ions, is active at pH's ranging from 7 to 9 with an optimum at about pH 8.0. It is unaffected by inhibitors such as DFP, the mercury compounds, bacterial derived inhibitors, or methylketones (Van der Walt, 1970).
It has no affects on substrates such as TAME, ATEE, BTEE or the Chromozym substrates. It is unable to cleave small peptides such as met-phe-gly, gly-leu-tyr, gly-leu, gly-ile and poly L-tyr, but randomly hydrolyzed three different protein substrates eg. casein into a large number of smaller peptides suggesting that a certain minimum number of amino acids are required (Van der Walt, 1970).

PAV protease degrades fibrinogen rendering it incoaguable within a short time, probably due to the rapid degradation of the a-chain. It does not degrade the γ-chain unlike plasmin which

attacks all three chains of fibrinogen.

The purified PAV protease was utilized to study the cross-link sites of the γ-chains of the D-domain of fibrin. Since the cleavage of D-dimer yielded apparently symmetrical monomers without γ-chain shortening or loss of substituted lysine analogues e.g. dansyl cadaverine, the simplest explanation would have been an isopeptide bond cleavage especially if, as has been assumed, one of the cross-link sites is always blocked by any substituent (Table VI).

The results provide the explanation for the ability of puffadder venom protease to produce cleavage of D-dimer into monomers. The presence of the cross-links between the v-chains of D-dimer provides a potential new conformation for selective γ-chain cleavage. The cleavage occurs between the cross-link sites i.e. between the lysine and glutamine acids 6 and 14 (or 13) residues from the carboxy-terminal end of the γ^{A}-chain (Table VI). Symmetrical products would be produced if <u>two</u> cross-links were present, irrespective of whether the cross-link site is at either glutamine residue (Table VI, E & F). However, the presence of only <u>one</u> cross-link due to a substitution should lead to assymmetrical products (Table VI, D) with differences as much as 18 residues. If both chains are nevertheless cleaved, a smaller difference of 7 residues between substituted and doubly cross-linked chains would be found (Table VI C).

Table VI : Crosslink configurations of the human di-γ-chain carboxy-terminal CNBr peptide and the mode of puffadder venom cleavage

```
A. Accepted configuration                        - two cross-links

    :---------------14-------6-----c
                    X        X
              c-----6-------14---------------:

B. Substitution by e.g. dansyl cadaverine (*)    - one cross-link

    :---------------14-------6-----c
                    X
              c-----6-----13-14--------------:
                          (*) *

C. Cleavage at the cross-link site by PAV     - assymetric fragments
                                              - 7 residue difference
    :---------------14--c         n-6-----c
                    X
   (γ-4)      c-----6-n      c--14---------------: f(γ-11)
                                 *

D. Cleavage at a single cross-link site     - assymetric fragments
                                            - 18 residue difference
    :---------------14-------6-----c
                    X
   (γ+7)      c-----6-n      c--14---------------: f-(γ-11)
                                 *

E. Cross-link not inhibited by a substitution on adjacent residues
                       *
    :--------------14-13-----6-----c
                    X        X
              c-----6-----13-14-----------------:
                           *

F. Alternate cross-link site
                    *
    :--------------14-13-----6-----c
                       X     X
                 c-----6-----13-14--------------:
                                 *

G.  Symmetrical cleavage products if model E or F appertains e.g.
                    *
    :--------------14-13-c       n-6-----c
                       X           X
                 c-----6-n       c-13-14---------------:
                                      *
```

Table VI Caption.

The diagram represents the cross-linked carboxy-terminal end of the γ-chain fragments after cyanogen bromide cleavage.

(:) - residue after cyanogen bromide cleavage

(n) - amino-terminal residue

(c) - carboxy-terminal residue

(f) - fluorescent substituent if dansyl-cadaverine used as lysine analogue for transglutaminase activity.

(X) - site of isopeptide cross-link.

(*) - substituent

(γ-4,γ-11,γ+7) - peptides differing from the γ-chain residue number.

The natural (unsubstituted) cross-linked γ-chains described in this report, appear to have both posssible cross-links present. The recovery of the cross-linked dipeptide was at least 60% of expected which implies that the predominant species was analysed.

The original assignment of the cross-link site was at glutamine 14 from the carboxy-terminal end based on [^{14}C]-glycine

ethyl ester substitution by transglutaminase and the lesser amount of substitution at glutamine 13 was attributed to the small size of the non-natural substrate. Model building also suggested that the glutamine, 14 residues from the carboxy-terminal end was advantageously positioned on the a-helix being in the same sector as the lysine, 6 residues from the carboxy-terminal end (Doolittle et al, 1972). However, labelling with a substituent is not the same as cross-linking.

Our data suggest that there is cross-linking at either glutamine with perhaps the glutamine at position 13 from the carboxy-terminal end (residue γ399) being predominant. This leaves open the possibility that <u>both</u> substitution and cross-linking could occur on the same molecule (Fig. VI, E + F). This would simplify explanations for the symmetry of substituted products after puffadder venom protease cleavage (Table VI, G). These questions will have to be resolved by direct sequencing of the substituted chains cleaved by puffadder venom protease.

Alanine (residue 419) was consistently found at the newly generated amino-terminal end but the carboxy-terminal end was heterogeneous to a certain extent (e.g. sub-stoichiometric amounts of histidine). This is an adequate explanation for the failure of previous attempts to determine the carboxy-terminal sequence by direct methods, e.g. carboxypeptidase-Y and tritium labelling (data not shown). The proximity of the cross-link site to the heterogeneous carboxy-terminal residues is another perturbing factor.

Table VII : Comparison of carboxy-terminal sequences at the cross-link sites of [a]rat, [b]bovine, [c]human and [d]lamprey γ_A-chain.

						*	+	+			+	+		*					
Rat	S	I	G	D	G	Q	Q	H	H	M	G	G	S	K	Q	V	G	D	M
Bovine	A	I	G	Q	G	Q	Q	H	Q	L	G	G	A	K	Q	A	G	D	V
Human	T	I	G	Q	G	Q	Q	H	H	L	G	G	A	K	Q	A	G	D	V
Lamprey	L	S	G	H	G	G	Q	Q	Q	S	K	G ↑	N	S	-	R	G	D	N
						14 *	13	12	11	10	9	8	7	6 *	5	4	3	2	1

* Indicates cross-link sites. The numbering begins at the carboxy-terminal end of the γ-chain.

(↑) Indicates the cleavage site in the human γ-chain.

(+) Possible consensus for puffadder venom protease specificity.

[a] (Hamandberg et al, 1985)

[b] (Chen & Doolittle, 1971)

[c] (Henschen et al, 1983)

[d] (Strong et al, 1985)

Since bovine and rat D-dimers are also susceptible to PAV protease there is an indication of a possible consensus (Table VII) of either di-glycyl or histidyl-glutamyl residues and the presence of the cross-link. D-dimer from hag fish, a close relative of the lamprey, also appears to be cleaved by puffadder venom protease (data not shown). However nothing is known about the cross-link site in hag fish. If similar to the lamprey, there will be fewer residues between the cross-links. There are only three residues between the cross-links of lamprey, and there could possibly be only one or two depending which glutamine is utilized for cross-linking. Susceptibility of the lamprey γ-chain cross-link to puffadder venom protease is still to be tested.

The possibility that the site-specific endopeptidase is contaminated by an exopeptidase that generates the heterogeneous ends cannot be excluded at present (Purves et al, 1986).

Since the presence of the cross-links provide the specificity necessary for γ-chain cleavage by the puffadder venom protease this suggests that the fibrinogen conformation is sufficiently altered so that new epitopes might be created and raises the possibility that monoclonal antibodies could be produced with specificity for cross-linked fibrin using puffadder venom-cleaved peptides as antigen.

The assays for fibrin(ogen) degradation products (FDPs) will only become more reliable as more specific monoclonal antibodies are developed. Synthetic peptides with the same sequence as the amino-terminal ends of the α- and β-chains of fibrin, have been the most frequently used antigens for raising monoclonal antibodies. Little attention has so far been given to the γ-chain except for

the raising of a monoclonal antibody that recognises cross-linked D-dimer (Rylatt et al, 1983) and attempts to exploit the enhanced reaction of anti-D-dimer antibodies (8-fold more than with D-monomer) (Purves et al, 1980). The carboxy-terminus of the γ-chain has not yet been a source of antigenic material for antibodies that could recognise the cross-link epitope and distinguish unequivocally between fibrinogen and fibrin degradation products in plasma and serum. PAV protease may offer a way of exposing useful epitopes in the carboxy-terminal end of the γ-chain. The cross-linked peptide from D-dimer can be isolated from a calcium free plasmin digest of D-dimer (Lindsey et al, 1978). This peptide could be used as a possible antigen for monoclonal antibodies. PAV protease cleaves D-dimer between the reciprocal cross-link sites, thus each D-PAV-monomer must retain one cross-link site. The carboxy-terminal peptide from the γ-chain of D-PAV-monomer or a synthetic peptide based on its structure, is another possible antigen for a monoclonal antibody for this region of cross-linked FDPs. In the preliminary work presented here D-PAV-monomer did not react with the monoclonal antibody to D-dimer, either in the radioimmunoassay or in the Dimertest agglutination assay. D-monomer from plasmin digested fibrinogen also gave a negative result with the radioimmunoassay and the Dimertest agglutination assay. A calcium free plasmin digest of D-dimer gave positive agglutination with Dimertest beads even after 18 hours digestion. This could be due to the association of the excised cross-linked peptide with the D-monomers (Purves et al, 1980).

REFERENCES

ACHYUTHAN, K. E., DOBSON, J. V. & GREENBERG, C. S. (1986).

Gly-Pro-Arg-Pro modifies the glutamine residues in the a- and γ-chains of fibrinogen, inhibition of transglutaminase cross-linking.

Biochim. Biophys. Acta 872, 261-268.

ALVING, B. M., EVATT, B. L., LEVIN, J., BELL, W. R., RAMSEY, R. B. & LEVIN, F. C. (1979).

Platelet and fibrinogen production: Relative sensitivities to endotoxin.

J. Lab. Clin. Med. 93, 437-448.

ALVING, B., CHUNG, S. I., MURANO, G., TANG, D. B.& FINLAYSON,J.S. (1982).

Rabbit it fibrinogen: time course of constituent chain production in vivo.

Arch. Biochem. Biophys. 217, 1-9

BAILEY, ASTBURY,W.T.,& RUDALL,K.M. (1943).

BAJWA, S. S., KIRAKOSSIAN, H., REDDY, K. W. N. & MARKLAND, F. (1982).

Thrombinlike and fibrinolytic enzymes in the venoms from the gaboon viper (Bitis gabonica).

Toxicon 20, 427-432.

BELITSER, V. A., VARETSKA, T.V., & TOLSTYKH, V. M. (1975).

Enhanced clotting activity of fragments D formed during plasmin hydrolysis of fibrinogen in the presence of calcium chloride.

Thrombosis Res.7, 797-806.

BELL, W. R. (1980).

Disseminated intravascular coagulation.

Johns Hopkins Med. J. 146, 289-299.

BELL, W .R., KESSLER, C. M., & TOWNSEND, R. R. (1983).

Stimulation of fibrinogen biosynthesis by fibrinogen fragments D and E.

British J. Haematol. 53, 599-610.

BLOMBACK, M., BLOMBACK, B., MAMMEN, E. F. & PRASAD, A. S. (1968).

Fibrinogen Detroit-a molecular defect in the N-terminal disulphide knot of human fibrinogen?

Nature 218, 134-137.

BLOMBACK, M., & BLOMBACK, B. (1972).

The molecular structure of fibrinogen.

Ann. N.Y. Acad. Sci. 202, 77.

BLOMBACK B., HESSEL B., IWANAGA S., REUTERBY J. & BLOMBACK M.J. (1972).

Primary structure of human fibrinogen and fibrin.

J. Biol. Chem. 247, 1496-1512.

BLOMBACK B., GRONDAHL, N. J., HESSEL B., IWANAGA S. & WALLEN P. (1973).

Primary structure of human fibrinogen and fibrin.

J. Biol. Chem. 248, 5806-5820.

BOK, R. A. & MANGEL, W. F. (1985).

Quantitive characterization of the binding of plasminogen to intact fibrin clots, lysine-sepharose, and fibrin cleaved by plasmin.

Biochemistry 24, 3279-3286.

BRADFORD, M. M. (1976).
A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.
Anal. Biochem. 72, 248-254.

BRANDT, W. F., ALK, H., CHAUHAN, M. & von HOLT, C. (1984)
A simple modification converts the spinning cup protein sequencer into a vapour phase sequencer.
FEBS Lett. 174, 228-232.

BRITTON, D. W., LAWRIE, J. S. & KEMP, G. D. (1982).
The influence of calcium ions on the conformation of fibrinogen fragment D: The use of chemical cross-linking agents.
Thrombosis Res. 27, 167-173.

BUDZYNSKI, A. Z., MARDER, V. J., & SHAINOFF, J. R. (1974).
Structure of plasmic degradation products of human fibrinogen.
J. Biol. Chem. 249, 2294-2302.

CHEN R. & DOOLITTLE, R. F. (1970).
Isolation, characterization, and location of a donor-acceptor unit from cross-linked fibrin.
Proc. Nat. Acad. Sci. 66, 472-479.

CHEN R. & DOOLITTLE, R. F. (1971)
γ-γ Cross-linking sites in human and bovine fibrin.
Biochemistry 10, 4486-4491.

CIERNIEWSKI, C. S., PLOW, E. F. EDGINGTON,T. S. (1984).
Conformation of the carboxy-terminal region of the Aa chain of fibrinogen as elucidated by immunochemical analysis.
Eur. J. Biochem. 141, 489-496.

COTTRELL, B. A. & DOOLITTLE, R. F. (1976).

The amino acid sequence of a 27-residue peptide released from the a-chain carboxy-terminus during the plasmic digestion of human fibrinogen.

Biochem. Biophys. Res. Comm. 3, 754-761.

CRABTREE, G. R. & KANT, J. A. (1981).

Molecular cloning of cDNA for the a, b & γ-chains of rat fibrinogen.

J. Biol. Chem. 256, 9718-9723.

CRABTREE, G. R. & KANT, J. A. (1982).

Organization of the rat γ-fibrinogen gene, alternative mRNA splice patterns produce the γA & γB (γ^1) chains of fibrinogen.

Cell 31, 159-166.

CRABTREE, G. R., COMEAU, C. M., FOWLKES, D. M., FORNACE Jr, A. J., MALLEY, J. D. & KANT, J. A. (1985).

Evolution and structure of the fibrinogen genes.

J. Mol. Biol. 185, 1-19.

DANG, C. V., BELL, W. R., KAISER, D. & WONG, A. (1985).

Disorganization of cultured vascular endothelial cell monolayers by fibrinogen fragment D.

Science 227, 1487-1490.

DANG, C. V., EBERT, R. F. & BELL, W. R. (1985). Localization of a fibrinogen calcium binding site between γ-subunit positions 311 and 336 by terbium fluorescence.

J. Biol. Chem. 260, 9713-9719.

DONOVAN, J. W. & MIHALYI, E. (1985).
Clotting of fibrinogen. 1. Scanning calorimetric study of the effect of calcium.
Biochemistry 24, 3443-3448.

DOOLITTLE, R. F. & BLOMBACK, B. (1964).
Amino acid sequence studies on fibrinopeptides from various mammals, evolutionary implications.
Nature 202, 147-152.

DOOLITTLE, R. F., CHEN, R. & LAU, F. (1971).
Hybrid fibrin, proof of the intermolecular nature of γ-γ-crosslinking units.
Biochem. Biophys. Res. Comm. 44, 94-100.

DOOLITTLE, R. F. (1973).
Structural aspects of the fibrinogen-fibrin conversion.
Avances in Prot. Chem. 27, 1-109.

DOOLITTLE R. F. & WOODING G. L. (1974).
The subunit structure of lamprey fibrinogen and fibrin.
Biochim. Biophys. Acta 271, 277-282.

DOOLITTLE, R. F., COTTRELL, B. A. & RILEY, M. (1976).
Amino acid composition of the subunit chains of lamprey fibrinogen, evolutionary significance of some structural anomalies.
Biochim. Biophys. Acta, 453, 439-452.

DOOLITTLE, R. F., CASSMAN, K. G., COTTRELL, B. A., FRIEZNER, S. J., HUCKO, J. T. & TAKAGI, T. (1977a).
Amino acid sequence studies on the a-chain of human fibrinogen. Characterization of 11 cyanogen bromide fragments.
Biochemistry 16, 1703-1709.

DOOLITTLE, R. F., CASSMAN, K. G., COTTRELL, B. A., FRIEZNER, S.J. & TAKAGI, T. (1977b).

Amino acid sequence studies on the a-chain of human fibrinogen. Covalent structure of the a-chain portion of fragment D.

Biochemistry **16**, 1710-1714.

DOOLITTLE, R. F., CASSMAN, K. G., COTTRELL, B. A. & FRIEZNER, S. J. (1977c).

Amino acid sequence studies on the a-chain of human fibrinogen. Isolation and characterization of two linked a-chain cyanogen bromide fragments from fully cross-linked fibrin.

Biochemistry, **16**, 1715-1719.

DOOLITTLE, R. F., GOLDBAUM, D. M. & DOOLITTLE, L. R. (1978).

Designation of sequences involved in the 'coiled-coil' interdomainal connections in fibrinogen: Construction of an atomic scale model.

J. Mol. Biol. **120**, 311-325.

DOOLITTLE, R. F., COTTRELL, B. A., STRONG, D. & WATT, K. W. K. (1978).

Preliminary report on the amino acid sequence of the a-chain of human fibrinogen.

Thrombosis Res. **14**, 787-792.

DOOLITTLE, R. F., WATT, K. W. K., COTTRELL, B. A., STRONG, D. D. & RILEY, M. (1979).

The amino acid sequence of the a-chain of human fibrinogen.

Nature **280**, 464-468.

DOOLITTLE, R. F. & LAUDANO A. P. (1980).

Synthetic peptide probes and the location of fibrin polymerization sites.

Protides of Biol. Fluids 28, 311-316.

DOOLITTLE, R. F. (1983).

The structure and evolution of vertebrate fibrinogen.

Ann. N.Y. Acad. Sci. 408, 13-27.

DYR, J. E., BLOMBAK, B. & KORNLIK, F. (1983).

The fibrinogenolytic and procoagulant activity of Southern Copperhead venom enzymes.

Thrombosis Res. 30, 185-194.

ERICKSON, H.P. & FOWLER, W. E. (1983).

Electron microscopy of fibrinogen, its plasmic fragments and small polymers.

Ann. N.Y. Acad. Sci. 408, 146-163.

EVANS, H. J. (1981).

Cleavage of the Aa-chain of fibrinogen and the a-polymer of fibrin by the venom of spitting cobra (Naja nigricollis).

Biochim. Biophys. Acta 660, 219-226.

FERGUSON, E. W., FRETTO, L. J. & McKEE, P. A. (1975).

A re-examination of the cleavage of fibrinogen & fibrin by plasmin.

J. Biol. Chem. 250, 7210-7218.

FOLK, J. E. & FINLAYSON, J. S. (1977).

The ϵ-(γ-glutamyl)lysine crosslink and the catalytic role of transglutaminases.

Adv.Prot. Chem. 31, 1-133.

FORNACE Jr., A. J., CUMMINGS, D. E., COMEAU, C. M., KANT, J. A. & CRABTREE, G. R. (1984).
Structure of the human γ-fibrinogen gene.
J. Biol. Chem. 259, 12826-12830

FOWLER, W. E. & ERICKSON, H. P. (1979).
Trinodular structure of fibrinogen.
J. Mol. Biol. 134, 241-249.

FOWLER, W. E., HANTGAN, R. R., HERMANS, J. & ERICKSON, H. P. (1981).
Structure of the fibrin protofibril.
Proc. Natl. Acad. Sci. 78, 4872-4876.

FOWLKES, D. M., MULLIS, N. T., COMEAU, C. M. & CRABTREE, G. R. (1984).
Potential basis for regulation of the coordinately expressed fibrinogen genes: Homology in the 5' flanking regions.
Proc. Natl. Acad. Sci. 81, 2313-2316.

FRANCIS, C. W.., MARDER, V. J., & MARTIN, S. E. (1979).
Detection of circulating cross-linked fibrin derivatives by a heat extraction-SDS gradient gel electophoretic technique.
Blood, 54, 1282-1295.

FRANCIS, C. W., MARDER, V. J. & MARTIN,S. E. (1980)
Demonstration of a large molecular weight variant of the gamma chain of normal human plasma fibrinogen.
J. Biol. Chem. 255, 5599-5604.

FRANCIS, C. W. & MARDER, V. J. (1982).
A molecular model of plasmin degradation of crosslinked fibrin.
Seminars in Thrombosis & Hemostasis 8, 25-35.

FRANCIS, C. W., & MARDER, V. J. (1983).

Plasmin degradation of cross-linked fibrin.

Ann. N. Y. Acad. Sci. 408, 397-406.

FRANCIS, C. W., KEELE, E. M. & MARDER, V. J. (1984).

Purification of three γ-chains with different molecular weights from normal human plasma fibrinogen.

Biochim. Biophys. Acta 797, 328-335.

FRANKS, J. J., FRITH, L., PURVES, L. R., FRANKS, W. T. & FRANKS, J. H. (1981).

Effects of fibrinogenolytic products D and E on fibrinogen and albumin synthesis.

J. Clin. Invest. 67, 575-580.

FULLER, G. M. & DOOLITTLE, R. F. (1971).

Studies of invertebrate fibrinogen. Purification and characterization of fibrinogen from the spiny lobster.

Biochemistry 10, 1305-1311.

GAFFNEY, P. J. (1977).

Fibrin(ogen) interactions with plasmin.

Haemostasis 6, 2-25.

GAFFNEY, P. J. & PERRY, M. J. (1985).

Unreliability of current serum fibrin degradation product (FDP) assays.

Thromb. Haemost. 53, 301-302.

GRAEFF,H. & HAFTER, R. (1982).

Detection and relevance of cross-linked fibrin derivatives in blood.

Seminars in Thrombosis and Haemostasis, 8, 57-68.

GREENBERG, C. S. & SHUMAN, M. A. (1982).
The zymogen forms of blood coagulation Factor X111 bind specifically to fibrinogen.
J. Biol. Chem. 257, 6069-6101.

GRINNELL,F. FELD, M. & MINTER, D. (1980).
Fibroblast adhesion to fibrinogen and fibrin substrata: requirement for cold insoluble globulin (plasma fibronectin).
Cell, 19, 517-525.

HAFTER, R., SCHROCK, R., VON HUGO, R. & GRAEFF, H. (1985).
Measurement of cross-linked fibrin derivatives in plasma and ascitic fluid with monoclonal antibodies against D-dimer using ElA and latex test.
Scand. J. Clin. Lab. Invest. 45, suppl.178, 137-144.

HALL, C. & SLAYTER, H. (1959).
The fibrinogen molecule: its size, shape and made of polymerization.
J. Biophys. Biochem. Cytol. 5, 11-16.

HANTGAN, R., MCDONAGH, J. & HERMANS, J. (1983).
Fibrin assembly.
Ann. N.Y. Acad. Sci. 408, 344-366.

HATZFELD, J. A., HATZFELD, A. & MAIGNE, J. (1982).
Fibrinogen and its fragments stimulate proliferation of human hemopoietic cell in vitro.
Proc. Natl. Acad. Sci. 79, 6280-6285.

HAVERKATE, F. & TIMAN, G. (1977).
Protective effect of calcium in the plasmin degradation of fibrinogen and fibrin fragments.
Thrombosis Res. 10, 803-812.

HAVERKATE, F., TIMAN, G. & NIEWENHUIZEN, W. (1979).
Anticlotting properties of fragments D from human fibrinogen and fibrin.
Eur. J. Clin. Invest. 9, 253-255.

HAWIGER, J., TIMMONS, S., STRONG, D. D., COTTRELL, B. A., RILEY, M. & DOOLITTLE, R. F. (1982).
Identification of a region of human fibrinogen interacting with staphylococcal clumping factor.
Biochemistry 21, 1407-1413.

HEENE, D. L. & MATTHIAS, F. R. (1973).
Adsorption of fibrinogen derivatives on insolubilized fibrinogen and fibrin monomer.
Thrombosis. Res. 2, 137-154.

HENNER G. M.D. & REIMAR H. (1982).
Detection and relevance of crosslinked fibrin derivatives in blood.
Seminars in Thrombosis & Hemostasis 8, 57-68.

HENSCHEN, A. & EDMAN, P. (1972).
Large scale preparation of S-carboxymethylated chains of human fibrin and fibrinogen and the occurrence of γ-chain variants.
Biochim. Biophys. Acta 263, 351-367.

HENSCHEN, A. & LOTTSPEICH F. (1977).
Preliminary note on the completion of the β-chain sequence.
Hoppe-Seyler Z. Physiol. Chem. 358, 1643-1646.

HENSCHEN, A., LOTTSPEICH, F. & HESSEL, B. (1978).
Amino acid sequence of human fibrin.
Hoppe-Seyler Z. Physiol. Chem. 359, 1607-1610.

HENSCHEN, A. (1978).
Disulphide bridges in the middle part of human fibrinogen.
Hoppe-Seyler Z. Physiol. Chem. 359, 1757-1770.

HENSCHEN, A., LOTTSPEICH, F., KEHL, M. & SAULTAN, C. (1983).
Covalent structure of fibrinogen.
Ann. N.Y. Acad. Sci. 408, 28-43.

HERMAN, J. & McDONAGH, J. (1982).
Fibrin, structure and interactions.
Seminars in Thrombosis & Hemostasis 8, 11-24.

HOEPRICH, P. D. & DOOLITTLE, R. F. (1983).
Dimeric half-molecules of human fibrinogen are joined through disulfide bonds in an antiparallel orientation.
Biochemistry 22, 2049-2055.

HOMANDBERG, G. A., EVANS, D. B., KANE, C. M. & MOSESSON, M. W. (1985).
Amino acid sequences of the carboxyl-terminal regions of rat plasma fibrinogen γA & γ^1 chains.
Thrombosis Res. 39, 263-269.

HORWITZ, B. H., VARADI, A. & SCHERAGA, H. A. (1984).
Localization of a fibrin γ-chain polymerization site within segment Thr-375 to Glu-396 of human fibrinogen.
Proc. Natl. Acad. Sci. 81, 5980-5984.

HUI, K. Y., HABER, E.G. & MATSUEDA, G. R. (1983).
Monoclonal antibodies to a synthetic fibrin-like peptide bind to human fibrin but not fibrinogen.
Science 222, 1129-1132.

IWANAGA, S., OSHIMA, G. & SUZUKI, T. (1976).
Endopeptidases of snake venoms.
Methods in Enzymology XLV, 459-468.
Colowick & Kaplan Eds.

KANT, J. A., LORD, S. T. & CRABTREE, G. R. (1983).
Partial mRNA sequences for human Aa, Bβ & γ-fibrinogen chain, evolutionary and functional implications.
Proc. Natl. Acad. Sci. 80, 3953-3957.

KANT, J. A., FORNACE, A. J., SAXE, D., SIMONS, O., McBRIDE, W. & CRABTREE, G. R. (1985).
Evolution and organization of the fibrinogen locus on chromosome 4: Gene duplication accompanied by transposition and inversion.
Proc. Natl. Acad. Sci. 82, 2344-2348.

KESSLER, C. M. & BELL, W. R. (1980).
Stimulation of fibrinogen synthesis: a possible functional role of fibrinogen degradation products.
Blood 55, 40-47.

KIRBY, E. P., NIEWIEROWSKI, S., STOCKER, K., KETTNER, C., SHAW, E. & BRUDZYSKI, T. M. (1980).
Thrombocytin, a serine protease from Bottrops atrox vemom.
1. Purification and characterization of the enzyme.
Biochemistry 18, 3564-3570.

KLOCZEWIAK, M., TIMMONS, S. & HAWIGER, J. (1982).
Localization of a site interacting with human platelet receptor on carboxy-terminal segment of human fibrinogen γ-chain.
Biochem. Biophys. Res. Comm. 107, 181-187.

KLOCZEWIAK, M., TIMMONS, S., LUKAS, T. J. & HAWIGER, J. (1984).
Platelet receptor recognition site on human fibrinogen. Synthesis and structure-function relationship of peptides corresponding to the carboxy-terminal segment of the γ-chain.
Biochemistry 23, 1767-1774.

KNOLL, D., HANTGAN, R., WILLIAMS, J., MCDONAGH, J. & HERMANS, J. (1984).
Characterization of soluble polymerized fibrin formed in the presence of excess fibrinogen fragment D.
Biochemistry 23, 3708-3715.

KRETSINGER, R. H. & NOCKOLDS, C. E. (1973).
Carp muscle calcium binding protein.
J. Biol. Chem. 248, 3313-3326.

KUDRYK, B. J. (1973).
Fragment D to E binding?
J. Biol. Chem. 248, 3313-3326.

KUDRYK, B. J., COLLEN, D., WOODS, K. R. & BLOMBACK, B. (1974).
Evidence for localization of polymerization sites in fibrinogen.
J. Biol. Chem. 249, 3322-3325.

KUDRYK, B. J., OKADA, M., REDMAN, C. M. & BLOMBACK, B. (1982)
Biosynthesis of dog fibrinogen. Characterisation of nascent fibrinogen in the rough endoplasmic reticulum.
Eur. J. Biochem. 125, 673-682.

LAUDANO, A. P. & DOOLITTLE, R. F. (1978).
Synthetic peptide derivatives that bind to fibrinogen and prevent the polymerization of fibrin monomers.
Proc. Natl. Acad. Sci. 75, 3085-3089.

LAUDANO, A. P. & DOOLITTLE, R. F. (1980).
Studies on synthetic peptides that bind to fibrinogen and prevent fibrin polymerisation. Structural requirements, number of binding sites, and species differences.
Biochemistry 19, 1013-1019.

LAUDANO, A. P., COTTRELL, B. A., & DOOLITTLE, R. F. (1983).
Synthetic peptides modelled on fibrin polymerization sites.
Ann. N.Y. Acad. Sci. 408, 315-329.

LAWRENCE, C. H. & MORRIS, B. J. (1981).
Mechanism of activation of inactive renin human plasma by puffadder venom.
Biochim. Biophys. Acta 657, 13-25.

LEWIS, M. S., CARMASSI, F. & CHUNG, S. I. (1984).
Cooperative association of plasminogen with fibrinogen.
Biochemistry 23, 3874-3879.

LINDSEY, G.G., BROWN, G. & PURVES, L. R. (1978a).
Calcium binding to human fibrinogen - Localization of two calcium specific sites.
Thrombosis Res. 13, 345-350.

LINDSEY, G. G., BROWN, G. & PURVES, L. R. (1978b).
Isolation of the cross-link peptide of fibrin.
Thrombosis Res. 12, 467-471.

LOTTENBERG, R., CHRISTENSEN, V., JACKSON, C. M. & COLEMAN, P.L. (1981)
Assay of coagulation using peptide chromogenic and fluorogenic substrates.
Methods in Enzymology 80, 341-361.
Colowick and Kaplan Eds.

LORAND, L. (1983).

New approaches to old problems in the clotting of fibrinogen.

Ann. N.Y.Acad. Sci. 408, 226-232.

LOTTSPEICH, F. & HENSCHEN, A. (1977a).

Amino acid sequence of human fibrin.

Hoppe-Seyler Z. Physiol. Chem. 358, 703-707.

LOTTSPEICH, F. & HENSCHEN, A. (1977b).

Preliminary note on three internal peptides obtained by cyanogen bromide cleavage of the β-chain.

Hoppe-Seyler Z. Physiol. Chem. 358, 1639-1642.

LOTTSPEICH, F. & HENSCHEN, A. (1977c).

Amino acid sequence of human fibrin.

Max-Plank-Institute Biochem. 358, 935-937.

LOTTSPEICH, F. & HENSCHEN, A. (1978a).

Amino acid sequence of human fibrin.

Hoppe-Seyler Z. Physiol. Chem. 359, 1451-1455.

LOTTSPEICH, F. & HENSCHEN, A. (1978b).

Amino acid sequence of human fibrin.

Hoppe-Seyler Z. Physiol. Chem. 359, 1611-1616.

LUCAS, M. A., FRETTO, L. J. & MCKEE, P. A. (1983).

The relationship of fibrinogen structure to plasminogen activation and plasmin activity during fibrinolysis.

Ann. N.Y. Acad. Sci. 408, 71-91.

LUCAS, M. A., STRAIGHT,D. L. FRETTO, L. J. & McKEE, P. A. (1983).

The effects of fibrinogen and its cleavage products on the kinetics of plasminogen activation by urokinase and subsequent plasmin activity.

J. Biol. Chem. 258, 12171-12177.

LUTERMAN, A., MANWARING, D. & CURREN, P. W. (1977)
The role of fibrinogen degradation products in the pathogenesis of the respiratory distress syndrome.
Surgery 87, 703-709.

LY, B. & GODAL, A. C. (1973).
Denaturation of fibrinogen: the protective effect of calcium.
Haemostasis 1, 204-209.

MCDONAGH, R. P., McDONAGH, J. & BLOMBACK, B. (1972).
Isolation and characterization of the S-carboxymethyl derivatives of crosslinked & noncrosslinked human fibrin.
Proc. Natl. Acad. Sci. 69, 3648-3652.

MCDONAGH, R. P., MCDONAGH, J., BLOMBACK, M. & BLOMBACK, B. (1971).
Cross-linking of human fibrin, evidence for intermolecular cross-linking involving a-chains.
FEBS Lett. 14, 33-36.

MARDER, V. J., SHULMAN, N. R. & CARROLL, W. R. (1969).
High molecular weight derivatives of human fibrinogen produced by plasmin.
J. Biol. Chem. 244, 2111-2119.

MARGUERIE, G., CHAGNIEL, G. & SUSCILLON, M. (1977).
The binding of calcium to bovine fibrinogen.
Biochim. Biophys. Acta. 490, 94-103.

MATSUEDA, G. R. & MARGOLIES, M. N. (1986).
Structural basis for the species selectivity of a fibrin specific monoclonal antibody.
Biochemistry 25, 1451-1455.

MATSUO, O., RIJKEN, D. C. & COLLEN, D. (1981).
Thrombolysis by human tissue plasminogen activator and urinokase in rabbits with experimental pulmonary embolus.
Nature 291, 590-591.

MEBS, D. & PANHOLZER, F. (1981).
Isolation of a hemorrhagic principle from Bitis arietans (puffadder) snake venom.
Toxicon 20, 509-512.

MORRIS, B. J., LAWRENCE, C. H. & CATANZARO, D. F. (1980).
Activation of plasma prekallikrein and inactive renin by puffadder venom.
Clin. Exp. Pharm. & Physiol. 7, 563-567.

MOSESSON, M. W., FINLAYSON, J. S., UMFLEET, R. A. & GALANAKIS, D. (1972).
Human fibrinogen heterogeneities.
J. Biol. Chem. 247, 5210-5219.

MOSESSON, M. W., HOMANDBERG, G. A. & AMRANI, D. L. (1984).
Human platelet fibrinogen gamma chain structure.
Blood 63, 990-995.

MOSHER, D. F. & JOHNSON, R. B. (1983).
Specificity of fibronectin-fibrin cross-linking.
Ann. N.Y. Acad. Sci. 408, 583-593.

MULLER-BERGHAUS, G., SCHEEFERS-BORCHEL, U., FUGHE, P., EBERLE, R. & HEIMBURGER, N. (1985)
Detection of fibrin in plasma by a monoclonal antibody against the amino-terminus of the alpha-chain of fibrin.
Scand. J. clin. Lab. Invest. 45, Suppl. 178, 145-151.

MURTAUGH, P. A., HALVER, J. E., LEWIS, M. S. & GLADNER, J. A. (1974).

Cross-linking reactions of lamprey fibrinogen and fibrin.

Biochim. Biophys. Acta 359, 415-420.

NICKERSON, J. M. & FULLER, G. M. (1981).

Modification of fibrinogen chains during synthesis: Glycosylation of Bβ and γ chains.

Biochemistry 20 2818-2821.

NIEWENHUIZEN, W., VERMOND, A., NOOIJEN, W. J. & HAVERKATE, F. (1979).

Calcium binding properties of human fibrin(ogen) and degradation products.

FEBS Lett. 98, 257-259.

NIEWENHUIZEN, W., VAN RUIJVEN-VERMEER, I. A. M., NOOIJEN, W. J., VERMOND, A. & HAVERKATE, F. (1981).

Recalculation of calcium binding properties of human and rat fibrinogen and fibrin(ogen) degradation products.

Thrombosis Res. 22, 653-657.

NIEUWENHUIZEN, W., VERMOND, A., VOSKUILEN M., TRAAS, D. W. & VERHEIJEN, J. H. (1983).

Identification of a site in fibrin(ogen) which is involved in the acceleration of plasminogen activation by tissue-type plasminogen activator.

Biochim. Biophys. Acta 748, 86-92.

NIEWENHUIZEN, W., VERMOND, A. & HERMANS, J. (1983).

Evidence for the localization of a calcium-binding site in the amino-terminal disulphide knot of fibrin(ogen).

Thrombosis Res. 31, 81-86.

NIEWIAROWSKI, S., KIRBY, E. P., BRUDZYNSKI, T. M. & STOCKER, K. (1979).

Thrombocytin or serine protease from Bothrops atrox, Interaction with platelets and plasma clotting factors. Biochemistry 18, 3570-3577.

NIKAI, T., KITO, R., MORI, N., SUGIHARA, H. & TU, A. T. (1983).

Isolation and characterization of fibrinogenase from Western Diamondback rattlesnake venom and its comparison to the thrombin -like enzyme, crotalase.

Comp. Biochem. Physiol. 76B, 679-686

NUSSENZWEIG, V., SELIGMAN, M., PELMONT, J. & GRABAR, P. (1961).

Le produits de degradation du fibrinogene humain par lar plasmine.

Ann. Inst. Pasteur. 100, 327-389.

OKUDE, M. & IWANAGA, S. (1971).

Carboxyl-terminal residues of mammalian fibrinogen and fibrin.

Biochim. Biophys. Acta 251, 185-196.

OKUDE, M. & IWANAGA, S. (1973).

Microheterogeneity of cross-linked γ-dimers isolated from bovine stabilized fibrin.

Biochem. Biophys. Res. Comm. 54, 53-61.

OLEXA, S. A. & BUDZYNSKI, A. Z. (1979).

Binding phenomena of isolated unique plasmic degradation products of human cross-linked fibrin.

J. Biol. Chem. 254, 4925-4932.

OLEXA, S. A. & BUDZYNSKI, A. Z. (1979).
Primary soluble plasmic degradation product of human crosslinked fibrin. Isolation and stoichiometry of the (DD)E complex.
Biochemistry 18, 991-995.

OLEXA, S. A. & BUDZYNSKI, A. Z. (1980).
Evidence for four different polymerization sites involved in human fibrin.
Proc. Natl. Acad. Sci. 77, 1374-1378.

OLEXA, S. A. & BUDZYNSKI, A. Z. (1981).
Localization of a fibrin polymerization site.
J. Biol. Chem. 256, 3544-3549.

OUYANG, C. & TENG, C. M. (1976).
Fibrinogenolytic enzymes of Trimeresurus macrosquamatus venom.
Biochim. Biophys. Acta 420, 298-308.

OUYANG, C. & HUANG, T. (1979).
a- And β-fibrinogenases from Trimeresurus gramineus snake venom.
Biochim. Biophys. Acta 571, 270-283.

OUYANG, C., TENG, C. & CHEN, Y. (1978).
Properties of fibrinogen degradation products produced by a- and β-fibrinogenases of Trimeresurus macrosquamatus snake venom.
Toxicon 17, 121-126.

PANDYA B. V., RUBIN, R. N., OLEXA, S. A. & BUDZYNSKI, A. Z. (1983).
Unique degradation of human fibrinogen by proteases from western diamondback rattlesnake (Crotalus atrox) venom.
Toxicon 21, 515-526.

PANDYA, B. V. & BUDZYNSKI, A. Z. (1984).
Anticoagulant proteases from western diamondback rattlesnake (Crotalus atrox) venom.
Biochemistry 23, 460-470.

PHILIPS, D. R. & BAUGHAN, A. K. (1983).
Fibrinogen binding to human platelet plasma membranes.
J. Biol. Chem. 258, 10240-10246.

PIZZO, S. V., SCHWARTZ, M. L. HILL, R. L. & MCKEE, P. (1972a).
The effect of plasmin on the subunit structure of human fibrinogen.
J. Biol. Chem. 247, 636-645.

PIZZO, S. V., SCHWARTZ, M. L., HILL, R. L. & MCKEE, P. A. (1972b).
The effect of plasmin on the subunit structure of human fibrin.
J. Biol. Chem. 248, 4574-4583.

PIZZO, S. A., SCHWARTZ, M. L., HILL, R. L. & MCKEE, P. H. (1973).
The subunit structure of fragment D from fibrinogen and cross-linked fibrin.
J. Biol. Chem. 248, 4584-4590.

PLOW, E. F., CIERNIEWSKI, C. S. & EDGINGTON, T. S. (1977).
The D:E complex as a discrete plasmic cleavage product of fibrinogen.
Thrombosis Res. 10, 175-181.

PLOW, E. & EDGINGTON, T. S. (1982).
Surface markers of fibrinogen and its physiological derivatives revealed by antibody probes.
Seminars in Thromb. Haemostasis 8, 36-56.

PLOW, E. & MARGUERIE, G. (1982).
Inhibition of fibrinogen binding to human platelets by the tetrapeptide glycyl-L-prolyl-L-arginyl-L-proline.
Proc. Natl. Acad. Sci. 79, 3711-3715.

PLOW, E. F., EDGINGTON, T. S. & CIERNIEWSKI, C. S. (1983).
Immunochemical analysis of the conformation of fibrinogen.
Ann. N.Y. Acad. Sci. 408, 44-57.

PURVES, L. R., LINDSEY, G. G., BROWN, G. & FRANKS, J. (1978).
Stabilization of the plasmin digestion products of fibrinogen and fibrin by calcium ions.
Thrombosis Res. 12, 473-484.

PURVES L. R. & LINDSEY, G. G. (1978).
Role of calcium in the structure and interactions of fibrinogen.
South Afr. J. Sci. 74, 202-209.

PURVES, L. R., LINDSEY, G. G. & FRANKS, J. J. (1980).
Sites of D-domain interaction in fibrin-derived D dimer.
Biochemistry 19, 4051-4058.

PURVES, L. R., PURVES, M., LINDSEY, G. G. & LINTON, N. J. (1986).
Specific cleavage of fibrin-derived D-dimer by a metalloproteinase isolated from venom of the puffadder (Bitis arietans).
South Afr. J. Sci. 82, 30-33.

RINALDO, J. E. & ROGERS. R. M. (1982).
Adult respiratory distress syndrome: Changing concepts of lung injury and repair.
New Eng. J. Med. 306, 900-909.

RITCHIE, D. G., LEVY, B. A., ADAMS, M. A. & FULLER, G. M. (1982).
Regulation of fibrinogen synthesis by plasmin derived fragments of fibrinogen and fibrin, an indirect feedback pathway.
Proc. Natl. Acad. Sci. USA. 79, 1530-1534.

RIXON, M. W., CHAN, W. Y., DAVIE, E. W. & CHUNG, D. W. (1983).
Characterization of a cDNA coding for the a-chain of human fibrinogen.
Biochemistry 22, 3237-3244.

RUSSEL, F. E. (1980).
Snake venom poisoning.
J. B. Lippincot Co.

RYLATT, D. B., BLAKE, A. S., COTTIS, L. E., MASSINGHAM, D. A., FLETCHER, W. A., MASCI, P. P., WHITAKER, A. N., ELMS, M., BUNCE, I., WEBBER, A. J., WYATT, D. & BUNDESEN, P. G. (1983).
An immunoassay for human D-dimer using monoclonal antibodies.
Thrombosis Res. 31, 767-778.

SCHRAGER, R. I., MIHALYI, E. & TOWNE, D. W. (1976).

Proteolytic fragmentation of fibrinogen. Kinetic modelling of the digestion of human and bovine fibrinogen by plasmin or trypsin.

Biochemistry 15, 5382-5386.

SELMAYR, E., MAHN, I. & MULLER-BERGHAUS, G. (1985).

Crosslinking of soluble fibrin and fibrinogen.

Thrombosis Res. 39, 467-474.

SILVERSTEIN, R. L., NACHMAN, R. L., LEUING, L. K. L. & HARPEL, P.C. (1985).

Activation of immobilized plasminogen by tissue activator.

J. Biol. Chem. 260, 10346-10352.

SHARP, J. J., CASSMAN, K. G. & DOOLITTLE, R. F.(1972).

Amino acid sequence of the carboxy-terminal cyanogen bromide fragment from bovine and human fibrinogen γ-chains.

FEBS Lett. 25, 334-337.

SLAYTER, H. S. (1983).

Electron microscopic studies of fibrinogen structure.

Ann. N.Y. Acad. Sci. 408, 131-145.

SOUTHAN, C., THOMPSON, E., PANICO, M., ETIENNE, T., MORRIS, H. R. & LANE, D. A. (1985).

Characterization of peptides cleaved by plasmin from the C-terminal polymerization domain of human fibrinogen.

J. Biol. Chem. 260, 13095-13101.

STRONG, D. D., LAUDANO, A. P., HAWIGER, J. & DOOLITTLE, R. F. (1982).
Isolation, characterization, and synthesis of peptides from human fibrinogen that block the staphylococcal clumping reaction and construction of a synthetic clumping particle.
Biochemistry 27, 1414-1420.

STRONG, D. D., MOORE, M., COTTRELL, B. A., BOHONUS, V. L., PONTES, M., EVANS, B., RILEY. M. & DOOLITTLE, R. F. (1984).
Lamprey fibrinogen γ-chain, cloning, cDNA sequencing, and general characterization.
Biochemistry 24, 92-101.

TAKAGI, T. & DOOLITTLE, R. F. (1975a).
Amino acid sequence of the carboxy-terminal cyanogen bromide peptide of the human fibrinogen β-chain, homology with the corresponding γ-chain peptide and presence in fragment D.
Biochim. Biophys. Acta 386, 617-622.

TAKAGI, T. & DOOLITTLE, R. F. (1975b).
Amino acid sequence studies on plasmin-derived fragments of human fibrinogen, amino-terminal sequences of intermediate and terminal fragments.
Biochemistry 14, 940-946.

TAKAGI, T. & DOOLITTLE, R. F. (1975c).
The amino acid sequences of those portions of human fibrinogen fragment E which are not included in the amino-terminal disulfide knot.
Thrombosis Res. 7, 813-818.

TAKAGI, T. & DOOLITTLE, R. F. (1975d).
Amino acid sequence studies on the a-chain of human fibrinogen. Location of four plasmin attack points and a covalent cross-linking site.
Biochemstry 14, 5149-5156.

TAMAKI, T. & AOKI, N. (1981).
Crosslinking of alpha-2 plasmin inhibitor and fibronectin to fibrin by the fibrin-stabilising factor.
Biochim. Biophys. Acta 661, 280-286.

TUSZYNSKI, G. P., KORNECKI, E., CIERNIEWSKI, C., KNIGHT, L. C., KOSHY, A., SRIVASTAVA, S., NIEWIAROWSKI, S. & WALSH, P. N. (1984).
Association of fibrin with the platelet cytoskeleton.
J. Biol. Chem. 259, 5247-5254.

VAN DER WALT, S. J. & JOUBERT, F. J. (1971.
Studies on puffadder (bitis arietans) venom-1. Purification and properties of protease A.
Toxicon 9, 153-161.

VAN DER WALT, S. J. (1972).
Studies on puffadder (bitis arietans) venom, IV. Association of protease A.
Hoppe-Seyler Z. Physiol. Chem. 353, 1217-1227.

VARADI, A. & PATTHY, L. (1983).
Location of plasminogen-binding sites in human fibrin(ogen).
Biochemistry 22, 2240-2446.

VARADI, A. & SCHERAGA, H. A. (1986).
Location of segments essential for polymerization and for calcium binding in the γ-chain of human fibrinogen.
Biochemistry 25, 519-528.

WEINSTEIN, M. J. & DOOLITTLE, R. F. (1972).
Differential specificities of thrombin, plasmin and trypsin with regard to synthetic and natural substrates and inhibitors.
Biochem. Biophys. Acta 258, 577-590.

WATT, K. W. K., TAKAGI, T. & DOOLITTLE, R. F. (1978).
Amino acid sequence of the β-chain of human fibrinogen, homology with γ-chain.
Proc. Natl. Acad. Sci. 75, 1731-1735.

WILLIAMS, J. E., HANTGAN, R. R., HERMANS, J. & McDONAGH, J. (1981).
Characterization of the inhibition of fibrin assembly by fibrinogen fragment D.
Biochem. J. 197, 661-668.

WILNER, G. D., MUDD, M. S., HSIEH, K. & THOMAS, D. W. (1982).
Monoclonal antibodies to fibrinogen, modulation of determinants expressed in fibrinogen by γ-chain cross-linking.
Biochemistry 21, 2687-2692.

WOLFENSTEIN-TODEL, C. & MOSESSON, M. W. (1980).
Human plasma fibrinogen heterogeneity, evidence for an extended carboxyl-terminal sequence in a normal γ-chain variant (γ^1).
Proc. Natl. Acad. Sci. 77, 5069-5073.

YAMADA, K. M. & KENNEDY, D. W. (1979).
Fibroblast cellular and plasma fibronectins are similar but not identical.
J. Cell. Biol. 80, 492-498.

YU, S., SHER, B., KUDRYK, B. & REDMAN, C. M. (1983).
Intracellular assembly of human fibrinogen.
J. Biol. Chem. 258, 13407-13410.

---

8 FEB 1988