ENZYME INHIBITORS

A THESIS
SUBMITTED TO THE
UNIVERSITY OF POONA
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
(IN BIOCHEMISTRY)

BY

ARVIND M. BODHE
M. Sc.

577.15.014(043)
BOD

DIVISION OF BIOCHEMICAL SCIENCES
NATIONAL CHEMICAL LABORATORY
PUNE 411008 (INDIA)

JANUARY 1988

COMPUTERISED

DEDICATED TO MY BELOVED FATHER

CONTENTS

Page No.

DECLARATION

ACKNOWLEDGEMENT

LIST OF ABBREVIATIONS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I : INTRODUCTION 1 - 56

Section I : Background 1

Section II : Historical 3

Section III : Inhibitors from different sources 7

Inhibitors from plants 7

Inhibitors from animals 16

Inhibitors from microbial sources 21

Section IV : General properties of the inhibitors 27

Section V : Different forms and type of inhibitors 38

Section VI : Proteinase inhibitors and endogenous proteinases in plants 39

Section VII : Mechanisms of action of proteinase inhibitors 42

Section VIII: Physiological role of proteinase inhibitors 47

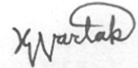
Section IX : Uses of proteinase inhibitors 53

Section X : Present work 55

		Page No.
CHAPTER II :	**MATERIALS AND METHODS**	57 - 81
	Materials	57
	Methods	59
CHAPTER III :	**EXPERIMENTAL**	82 - 118
Section I :	Development of simple separation methods	82
Section II :	Purification of proteinase inhibitors	97
Section III:	Occurrence of DTi	111
Section IV :	Isolation and purification of horse gram DTi	115
CHAPTER IV :	**PROPERTIES**	119 - 144
Section I :	Properties of the purification subsilisin and associated trypsin inhibitor	119
Section II :	Properties of the DTi	138
CHAPTER V :	**DISCUSSION**	145 - 155
CHAPTER VI :	**SUMMARY AND CONCLUSIONS**	156 - 158
BIBLIOGRAPHY		159 - 182

DECLARATION

Certified that the work incorporated in the thesis entitled: **"ENZYME INHIBITORS"** submitted by Shri Arvind M. Bodhe was carried out by him under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.



(H.G. VARTAK)
Supervisor

ACKNOWLEDGEMENT

I wish to express my deep sense of gratitude to my teacher and guide Dr. H.G. Vartak for his constant encouragement and valuable guidance throughout the course of this investigation. His unitiring zeal and patience at all times kept me going till the very end.

I am grateful to Dr. (Mrs.) V.V. Deshpande for her constructive suggestions and keen interest during the course of this work.

I take this opportunity to thank Dr. (Mrs.) M.V. Rele, Dr. G.D. Ghadge, Mr. H.S. Pawar for the kind help rendered by them. I gratefully acknowledge the technical assistance rendered by Mr. S.T. Dhume and Mr. M.N. Kamthe especially in carrying out the amino acid analysis. I appreciate the help extended by all the members of the Biochemical Sciences Division during this work. I wish to place on record my thanks to Mrs. Indira Mohandasan for flawless typing of this thesis.

I would also like to thank Professor John Barnabas, Head, Biochemical Sciences Division and Dr. C. SivaRaman, for their encouragement during the course of this work.

Finally, my thanks are also due to the Director, NCL, Poona for permitting me to submit this work in the form of a thesis for the degree of Doctor of Philosophy.

AmBodhe

ARVIND M. BODHE

ABBREVIATIONS

BAEE	:	N-Benzoyl-L-arginine ethyl ester
BAPNA	:	N-Benzoyl-DL-arginine-*p*-nitroanilide
Bis	:	N-N'-methylene-bis-acrylamide
CMC	:	Carboxymethyl cellulose
DABITC-PITC	:	Dimethylaminoazobenzene Isothiocyanate-phenylisothiocynate
DEAE	:	Diethylaminoethyl cellulose
DTi	:	Dialysable trypsin inhibitor
DTNB	:	5-5'-dithio-bis-2-nitrobenzoic acid
EDTA	:	Ethylene diamine tetraacetate
Mr	:	Molecular weight
MWCO	:	Molecular weight cut off
PAG	:	Polyacrylamide gel
PAGE	:	Polyacrylamide gel electrophoresis
pI	:	Isoelectric point
SDS	:	Sodium dodecyl sulphate
TCA	:	Trichloroacetic acid
TEMED	:	N,N,N',N'-Tetraethylmethylethylenediamine
TLCK	:	N-*p*-Toluenesulfonyl-L-lysine chloromethyl ketone
TNBS	:	2,4,6-Trinitrobenzenesulphonic acid
TPCK	:	N-*p*-Toluenesulfonyl-phenylalanine chloromethyl ketone
Tris	:	Tris(hydroxymethyl) aminomethane

LIST OF TABLES

Table No.		Page No.
I	A list of plant inhibitors from different sources inhibiting various proteolytic enzymes.	12
II	Principal proteinase inhibitors studied in human blood plasma	19
III	Dissociation constants of proteinase inhibitor complexes	32
IV	Optical factor of inhibitors	34
V	A typical assay of subtilisin inhibitor	63
VI	Purity and properties of pure proteinases	68
VII	Inhibition of proteinases by different Leguminous seed extracts	99
VIII	Purification of proteinase inhibitors from horse gram seeds	102
VIIIa	Major trypsin inhibitor	103
VIIIb	Elution of associated trypsin inhibitor	103
IX	Dialysable trypsin inhibitor from Leguminous seeds	113
X	Purification of DTi	117
XI	Stability of subtilisin and trypsin inhibitor	128

Table No		Page No.
XII	Effect of pH on the stability of subtilisin and trypsin inhibitor	129
XIII	Amino acid composition of subtilisin inhibitor	131
XIV	Amino acid composition of associated trypsin inhibitor	132
XV	Properties and kinetics of the proteinase inhibitors	158

LIST OF FIGURES

Fig. No.		Page No.
1	Elution unit	84
2	Elution unit assembled	85
3	Procedure for easy removal of high concentration PAG from glass tube	89
4	Electrophoresis pattern of fluorescent peptide markers	91
5.	Electrophoresis of cytochrome c, myoglobin, trypsin inhibitor, BSA and ferritin	95
6	Electrophoresis of histones	96
7	DEAE-cellulose column chromatography	105
8	Analytical PAGE pattern at different stages during purification of subtilisin inhibitor from horse gram seeds	106
9	Production of DTi	112
10	Analytical PAGE of DTi	118
11	PAGE of purified subtilisin inhibitor and associated trypsin inhibitor	120
12	Molecular weight of subtilisin and associated trypsin inhibitor by gel filtration	121
13	Molecular weight of subtilisin inhibitor by slope method	122

Fig. No.		Page No.
14	SDS PAGE of subtilisin inhibitor	123
15	Molecular weight of subtilisin and associated trypsin inhibitor by SDS method	124
16	Ultraviolet absorption spectrum of subtilisin inhibitor	125
17	Ultraviolet absorption spectrum of associated trypsin inhibitor	126
18	Dissociation constant of subtilisin inhibitor	130
19	SDS PAGE of DTi	139
20	Molecular weight of DTi by SDS method	140
21	Molecular weight of DTi by slope method	141
22	Ultraviolet absorption spectrum of DTi	142
23	Dissociation constant of DTi	143

CHAPTER I

INTRODUCTION

SECTION I

BACKGROUND

Protein inhibitors of proteinases are present in cells of almost all life forms and are thus ubiquitous in nature. Proteinase inhibitors inhibit proteinases by associating reversibly with one or more proteinases to form complexes with them [1]. Proteinase inhibitors are valuable reagents for controlling proteolytic enzymes. Their use in therapeutic applications, their potential toxic nature in foods and their possible physiological significance in life processes stimulated research in this class of proteins (2).

Proteinases have applications in food industry, leather industry, medicine, detergents and beer industry. Proteolysis is intimately involved in many fundamental processes such as hemostatis (3), inflammation (4), complement action (5) cell-cell interaction (6), gene depression (7) and hormone metabolism (8). Since majority of proteolytic enzymes are regulated by their inhibitors the role of inhibitors in controlling these processes is evident. Proteinase inhibitors are known to play an important role in dormancy of seeds, regulation of germination and differentiation. In medicine, they find applications as anti-inflammatory agent, for suppressing pain and blister formation, for inhibition of tumerogenesis, as contraceptives, in organ transplantation and liver disorders. They are also used in the treatment of pancreatitis, surgical haemorrhages, shocks and

burns (9-12). They can also be used in food processing and preservation. Their gross physiological function is to prevent unwanted proteolysis.

Natural proteinase inhibitors are often found as major components of the cytoplasm, secretions, and intercellular fluids of many organs and tissues. In plants they are usually concentrated in seeds and tubers, especially in those of the Leguminosae, Graminae and Solanaceae families. Trypsin and chymotyrpsin inhibitors occur widely and have been studied extensively (13). However, reports on the occurrence of specific inhibitors of other proteinases such as subtilisin, papain and pepsin as well as their simultaneous occurrence in a single source are rare. Similarly, only a few reports on the occurrence of low molecular weight/dialysable (less than 6000 daltons) protein inhibitors are available (14-19).

The present work deals with the isolation, purification and characterization of a specific subtilisin inhibitor and an associated trypsin inhibitor along with partial purification of a papain inhibitor and a low molecular weight trypsin inhibitor (molecular weight 4800), from the seeds of *Dolichos biflorus*/Horse gram/Hulga seeds. These seeds will be referred to as horse gram seeds throughout this work.

SECTION II

HISTORICAL

Kunitz and Northrop (20) were the first to crystallise a trypsin inhibitor and also its complex with trypsin from bovine pancreas. The first known plant trypsin inhibitor from soyabean was discovered by Ham and Sandstedt (21) and Bowman (22) and later crystallized by Kunitz (23). Subsequently trypsin inhibitors from several other legumes such as Lima bean, Soya bean, Gram bean, etc. were studied by Borcher and Ackerson (24). Later trypsin inhibitors were also found in other families of plants and in microorganisms (25), indicating a wide distribution of trypsin inhibitors in nature. Most of the trypsin inhibitors also inhibit chymotrypsin.

Occurrence of specific inhibitors of other proteolytic enzymes such as subtilisin, papain, chymotrypsin and pepsin has been reported only in a few cases. Inhibitors of subtilisin have been purified from potato (26, 27), barley (28) and avian ovomucoids (29). However, these inhibitors are not specific for subtilisin. They also inhibit other proteolytic enzymes such as trypsin, chymotrypsin, papain and ficin. Specific subtilisin inhibitors from the culture broth of *Streptomyces albogriseolus* has been purified by Murao and Sato (30) and Sato and Murao (31, 32). A few specific subtilisin inhibitors

were reported from *Vigna unguiculata* from this Laboratory (33), *Vicia faba* (34), black bean (35), barley (36) and Adjuki bean (37). Few papain inhibitors have also been reported in animals, plants and microorganisms such as inhibitors from chicken egg white (38, 39), *Streptomyces* sp. (40) and *Vigna unguiculata* (41). Peanasky *et al*. (42) and Rhodes *et al*. (43) have reported two different chymotrypsin inhibitors from Ascaris, which inhibit chymotrypsin, elastase and subtilisin at the same active site. Pepsin inhibitors from the culture broth of *Streptomyces* strain and from Ascaris were isolated and purified (44, 45).

Pioneering work on the purification and characterization of trypsin-chymotrypsin inhibitors was done by Kunitz (46). He established the proteinaceous nature of the inhibitor, the concept of enzyme-inhibitor complex formation in definite proportions, assay method for the inhibitor and dissociation of the complex at lower pH and reassociation at higher pH.

Several workers studied the mechanism of action of different proteinase inhibitors. An interesting hypothesis for the formation of an intermediate modified inhibitor before the formation of the enzyme-inhibitor complex was put forward by Laskowski (47), Finkenstadt (48) and Ozawa (49). An observation made in the case of bovine pancreatic secretory trypsin inhibitor where

the enzyme inhibitor complex is slowly broken by the complexed enzyme itself was termed as 'Temporary inhibition" by Laskowski and Wu (50). Amino acid composition of several purified inhibitors was determined. This was followed by the determination of amino acid sequence. Amino acid sequence of bovine basic pancreatic inhibitor was determined by Kassal et al. (51). Anderer and Hornle (52) determined the amino acid sequences of kallikrein inhibitors from bovine, lung and parotid gland. Since the sequence of these inhibitors turned out to be identical, it was concluded that the inhibitors are identical although they occur in three different bovine organs.

The bovine pancratic trypsin inhibitor was isolated in crystalline form and showed the typical 1:1 enzyme inhibitor stoichiometry (53). Noda et al. (54) synthesized the inhibitor by a stepwise solid phase synthesis. Yajima and Kiso (55) synthesized the same inhibitor by fragment condensation on polymer support. The product obtained by Noda et al. (54) and Yajima and Kiso (55) had 30% and 82% activity respectively. It contained 58 amino acid residues. X-ray crystallography studies and the three dimensional structure of the Kunitz pancreatic trypsin inhibitor was determined by Huber et al. (56, 57).

Low molecular weight inhibitors

Among the few reports on low molecular weight inhibitors, Umezawa (40,44) reported peptide inhibitors (molecular weight ranging from 3000-6000). Reports on low molecular weight proteinaceous proteinase inhibitors include inhibitors of carboxypeptidase A from potato (molecular weight 3000 - 4000) (14, 15) and trypsin and chymotrypsin inhibitors from different varieties of bean (molecular weight 8000 - 23000) (13, 14, 18, 19).

Scope of the literature survey

A survey of the literature on proteinaceous proteinase inhibitors from plant, animal and microbial sources is presented in this Chapter. Major emphasis is laid on the isolation, purification and properties of the inhibitors from plant sources, since they are related to the studies undertaken. The objectives of this thesis include (i) occurrence of a large number of inhibitors of trypsin, chymotrypsin (including low molecular weight/dialysable trypsin-chymotrypsin inhibitor), subtilisin and papain in the seeds of Horse gram; (ii) separation and purification of a specific subtilisin inhibitor and a low molecular weight/dialysable trypsin chymotrypsin inhibitor, and (iii) study of their properties.

The term proteinase inhibitor is used to refer only to inhibitors which are polypeptides.

SECTION III

INHIBITORS FROM DIFFERENT SOURCES

Literature on proteinaceous proteinase inhibitors from plant, animal and microbial sources is presented in this section.

Proteinase inhibitors occur in plants, animals, and microorganisms. In plants, particularly in legumes, they are abundant. Several inhibitors have been isolated and purified from plants.

Plants

<u>Inhibitors from beans</u>. Trypsin inhibitors from different varieties of bean are low molecular weight proteins ranging from 8000 to 23000 (13) and contain high cystine and low methionine. These inhibitors are thermostable type of inhibitors and the thermostability is due to high content of disulfide bridges (58).

(a) <u>Soyabean</u>: Inhibitors from soyabean have been described by Ham and Sandstadt (21) and by Bowman (22). The seeds contain about 2% trypsin inhibitor (59). They are present in the exterior parts of cotyledons in embryonic axles of seeds (60). Two trypsin inhibitors have been studied in detail from soyabean: (i) Kunitz type inhibitor which is built of a single peptide chain containing 181 amino acids. The active site Arg63-Ile64 can be hydrolyzed by trypsin (61). Kunitz inhibitor

inhibits trypsin, kallikrein, plasmin and chymotrypsin B; contains tryptophan and is unstable to heat, trichloroacetic acid and pepsin; (ii) Bowman-Birk inhibitor has no tryptophan and is stable to heat, acid and pepsin (62, 63) and contains 71 amino acid residues (64, 65). It has two different and independent active sites. For trypsin the active site is (Lys16 - Ser17) at the N-terminal and for chymotrypsin (Leu43 - Ser44) in the middle of the chain. This enables the formation of the complexes with trypsin and chymotrypsin at the same time. Both active sites have almost identical structure (66). Four other trypsin inhibitors have been purified from soyabean. These include: SBTI A_1 (67); 1.9S inhibitor (68); F_1 and F_3 (69). SBTI A_1 has the lowest molecular weight of 14300. The 1.9S inhibitor also inhibits chymotrypsin. It contains no glycine. F_1 and F_3 are weak inhibitors of trypsin, F_3 contains no tyrosine.

(b) Lima bean: The inhibitors from Lima bean have molecular weights of about 9000 and there are 4 - 9 inhibitors of this kind. They have separate binding places for trypsin (Lys-Ser) (70) and chymotrypsin depending on the molecular form (Leu-Ser or Phe-Ser) (67) They are resistant to the action of acids, bases, pepsin and papain (71, 72). These inhibitors have been separated into four components by Jones *et al*. (73).

Recently, Haynes and Feeney (74) obtained six chromatographically distinct inhibitors of trypsin and chymotrypsin from Lima beans. All fractions have been found to be devoid of tryptophan. from the amino acid analysis of the component inhibitors it appears that components 1, 2 and 4 (77, 76 and 80 residues, respectively) could have been derived from component 3 (93 residues) by proteolytic cleavage owing to the loss of terminal peptides. Component 6 (89 residues) of Haynes and Feeney (74) could similarly be a precursor of Fraction 4 (84 residues).

(c) Black eyed pea (*Vigna sinensis*) : Partial characterization and purification of an inhibitor for α-chymotrypsin and trypsin from this seed is carried out (75). Its molecular weight is shown to be 17000 but later Ventura *et al*. (76) corrected it to be 10000. This fallacy is explained by the aggregation of the inhibitor molecules. The inhibitor contains two percent tryptophan but no methionine and has an optical factor of 1.2. 150 amino acid residues are found per mole of the inhibitor. Gennis *et al*. (77) purified two inhibitors, both showing molecular weight of about 8000 but different pIs of 5.1 and 6.5. One of them possesses two independent binding places for trypsin and chymotrypsin while the other inhibitor is double headed since it binds two molecules of trypsin.

(d) *Vigna unguiculata*: Royer *et al*. (78) reported the presence of five trypsin inhibitors in the cotyledonary extract of *Vigna unguiculata* (from Zaire) strain H81. These trypsin inhibitors partly inhibit the proteolytic activity of the extract. Sohonie and Bhandarkar (79) showed the presence of two trypsin inhibitors in the seeds of *Vigna unguiculata*. Further purification of these inhibitors was not carried out. The presence of the inhibitors of other proteinases (like subtilisin, papain, chymotrypsin) is also shown in these seeds from this Laboratory (33).

(e) *Potato*: Potato and related plants are shown to be rich source of proteinase inhibitors (80, 81). These inhibitors are characterized by molecular heterogeneity (being polyvalent) since they inhibit proteinases of different structure at the binding and catalytic center (82). They inhibit trypsin, chymotrypsin, kallikreins, carboxypeptidases, papain and cell proteinases (Cathepsin A). On this basis they are distinguished into six different types of inhibitors (83) viz. Inhibitors of (1) chymotrypsin I; (2) Proteinase IIa; (3) Proteinase IIb; (4) Kallikrein; (5) Carboxypeptidase A and B and (6) Papain.

The individual types are distinguished on the basis of their different molecular weights, composition of amino acids and also stability during heating (84-86).

Potato I inhibitors are shown to be non-covalent tetramers of a single-chain inhibitor with only a single interchain disulfide bridge (85, 87), while potato II family inhibitors are low molecular weight chymotrypsin inhibitors (88). The inhibitors of chymotrypsin I has a molecular weight of 39,000 and contains four subunits, which dissociate in 6 M guanidine hydrochloride and 8 M urea in acid medium. It showed considerable thermostability and also did not lose the activity in the pH range of 2.0 to 10.0 (89, 90). Thermostable inhibitors of proteinase IIa and IIb (91 - 93) are isolated from the variety of Danshaku-Imo. Their molecular weights are around 10300. Inhibitor IIa inhibited both trypsin and chymotrypsin but IIb inhibited only chymotrypsin. From the same variety two inhibitors of kallikrein are also isolated. Their isoelectric points are 5.6 and 6.4. The inhibitor of carboxypeptidase A and B is purified from Russet-Burbank. It has a molecular weight of 3100 and does not inhibit trypsin and chymotrypsin (94). It occurs in two molecular forms (95). Papain inhibitor which also inhibits chymopapain is described. It is a glycoprotein of 80,000 molecular weight and possesses 8 active places for binding other enzymes. Four of them bind papain or chymopapain and the remaining four bind only chymopapain (96). Papain inhibitor from potato is crystallized by Huff (97).

A list of plant inhibitors from different sources inhibiting various proteolytic enzyme is given in Table 1(98).

TABLE I: A LIST OF PLANT INHIBITORS FROM DIFFERENT SOURCES INHIBITING VARIOUS PROTEOLYTIC ENZYMES(98)

Source	Type	Specificity	Comments
Soyabean	Kunitz	Trypsin	Has tryptophan:primary sequence of 181 amino acids known
	Bowman Birk	Trypsin-Chymotrypsin	Combines at separate non overlapping sites with trypsin and chymotrypsin:contains 7 disulfide bonds
Lima bean (*Phaseolus lunatus*)		Trypsin-chymotrypsin	There are at least 4 possibly 6 isoinhibitors: trypsin and chymotrypsin combine at separate independent, non overlapping sites
Garden bean (Greater Northern variety) (*Phaseolus vulgaris*)		Trypsin:chymotrypsin weakly inhibited by I and II. chymotrypsin strongly inhibited by IIIb	Isoinhibitor IIIb has independent binding sites for trypsin and chymotrypsin
Navy bean (*Phaseolus vulgaris*)		Trypsin-chymotrypsin	Possibly 4 isoinhibitors
Mung bean (*Phaseolus murcus*		Trypsin endopeptidase of mung bean	Another inhibitor of molecular weight 2000 was also reported
Pinto bean (*Phaseolus vulgaris* cultivar Pinto)		Trypsin-chymotrypsin	Two isoinhibitors present; probably different binding site for trypsin and chymotrypsin
Broad bean (*Vicia faba*)		Trypsin-chymotrypsin thrombin pronase papain(slightly)	

Table I Contd.

Source	Type	Specificity	Comments
Chik pea (*Cicer arietinum*)		Chymotrypsin, trypsin	Does not contain carbohydrate: independent binding sites for trypsin and chymotrypsin
			Probably two isoinhibitors with 4 more found as a result of proteolysis.
Black eyed pea (*Vigna sinensis*)	Chymotrypsin and trypsin inhibitor	Trypsin chymotrypsin	Independent binding sites for trypsin and chymotrypsin
	Trypsin inhibitor	Trypsin	Binds with two molecules trypsin simultaneously
Broad bean (*Vicia faba*)		Trypsin, chymotrypsin thrombin, pronase papain (slightly)	
Chik pea (*Cicer arietinum*)		Chymotrypsin, trypsin	Does not contain carbohydrate: independent binding sites for trypsin and chymotrypsin
			Probably two isoinhibitors with 4 more found as a result of proteolysis

...3/-

Table I Contd.

Source	Type	Specificity	Comments
Black-eyed pea (<u>Vigna sinensis</u>)	Chymotrypsin and trypsin inhibitor	Trypsin chymotrypsin	Independent binding sites for trypsin and chymotrypsin
	Trypsin inhibitor	Trypsin	Binds with two molecules trypsin simultaneously
Potato	Chymotrypsin inhibitor I	Chymotrypsin	Binds four moles of chymotrypsin
	Proteinase inhibitor IIa	Chymotrypsin nagarse, trypsin	
	Proteinase inhibitor IIb	Chymotrypsin nagarse	
	pKI-56 pKI-64	Kallikrein	
	Carboxypeptidase A and B inhibitor	Carboxypeptidase A and B	No activity on trypsin or chymotrypsin
	Papain inhibitor	Papain, chymopapain	Glycoprotein. There are eight binding sites, four of which can bind either papain or chymopapain, the remaining four bind only chymopapain.
Sweet potato (<u>Ipomoea batatas</u>)	Inhibitor II	Trypsin, plasmin kallikrein	Also inhibitor I present
	Inhibitor III	Trypsin, plasmin kallikrein	Both inhibitors II and III are arginine type

---4/-

Table I Contd.

Source	Type	Specificity	Comments
Tomato leaves	Inhibitor I Inhibitor II	Both inhibit trypsin and trypsin-like enzymes chymotrypsin and chymotrypsin like enzymes	Only inhibit proteinases from animals not plants
Corn		Trypsin	Chymotrypsin not inhibited
Sorghum grain		Trypsin	No other enzyme tested: the 6000 MW component may be tannins
Barley		Alkaline proteinases of *Aspergillus oryzae* *Streptomyces griseus* *Alternaria tenuissima* *Bacillus subtilis* chymotrypsin	Contains 4-5 compounds with inhibitory activity
Eggplant (*Solanum melongena* L.)		Trypsin	Arginine-type inhibitor
		Trypsin, chymotrypsin weakly	No carbohydrate
Pineapple stem	Acidic cysteine proteinase inhibitors	Papain and ficin	There are atleast seven isoinhibitors
Ground nuts		Trypsin chymotrypsin	Sites for trypsin and chymotrypsin separate but overlapping

Inhibitors from animals

(a) Avian egg white inhibitor: chicken, duck, turkey and quail ovinhibitors show broad specificity. , They inhibit fungal proteinases from *Aspergillus oryzae*, subtilisin, trypsin and chymotrypsin and have similar molecular weights (48000 ± 2000). They show similar combining ratios for trypsin and chymotrypsin; one mole of the inhibitor inhibiting 2 moles of trypsin or 2 moles of chymotrypsin. Liu *et al*. (99) and Rhodes *et al*. (29) studied ovomucoids from eleven avian species which inhibit trypsin and chymotrypsin to varying degree. Fossum and Whitaker (38) and Sen and Whitaker (39) described an inhibitor from chicken egg-white which forms a complex with the mercury form of cathepsin B1 (100).

(b) Ascaris: Three specific inhibitors of trypsin, chymotrypsin and pepsin (molecular weights about 8000) are isolated and purified from Ascaris by Peanasky and Ghaleb (42, 101). the pepsin inhibitor also inhibits cathepsin E from rabbit bone marrow.(102).

(c) Pancreatic inhibitor : The inhibitors from this class are well characterized and intensively studied (46, 103). The first inhibitor is called the Kunitz or basic inhibitor. It being a polyvalent inhibitor inhibits trypsin, chymotrypsin, plasmin and kallikrein. It contains 58 amino acids and a single polypeptide chain and shows a molecular weight of 63000. Its sequence

TH-555

and three dimensional structure are also determined. The physiological function of this inhibitor is not known. It is shown to be present in all bovine organs (104) such as liver, lung, parotid glands, spleen and is restricted to bovids and caprids (105). So far Kunitz type of inhibitors were reported from birds. However, snake venon has proved an unexpectedly rich source from which four different single domain Kunitz type inhibitors (toxins I, K, B and E) are isolated. Three of them (I, K and E) are sequenced from the venom of the black mamba (106-108). A more clearer example of this phenomenon is shown by Kunitz type inhibitor from red sea, turtle egg-white, chelonianin (109). This inhibitor inhibits trypsin and subtilisin independently. The first is closely homologous to Kunitz type inhibitor, while the second is not homologous to any of the inhibitor families described. Therefore it represents a new inhibitor family. The Kunitz type inhibitors are not confined to vertebrates.

The second inhibitor present in pancreatic juice is known as Kazal pancratic inhibitor (110, 111). It is found in the vertebrates only and is specific for trypsin. Schneider and Laskowski (112) isolated three isoinhibitors from Kazal's crystalline inhibitor. Two forms of porcine pancratic secretory trypsin inhibitors are purified (113, 114). The molecular weights of inhibitor I and inhibitor II are 6040 and 5400, respectively.

577.15.014(043)
BOD

Both inhibit trypsin. Five isoinhibitors of trypsin are purified from human pancreas with molecular weights of 6242, containing 56 amino acid residue per mole. The inhibitors differ in their amide content (115). Kazal type inhibitors are not limited to vertebrates. Crude extract from Leeches, Hirudo medicinalis contains numerous trypsin inhibitors called bdellins (116, 117). One of these, bdellin B-3 is partially sequenced. It is clearly shown to be a single domain Kazal inhibitor (118).

(d) Blood serum: Several inhibitors are located in blood plasma. Some show polyvalent nature (showing broad specificity) while others exhibit narrow specificity. They inhibit the blood clotting enzymes plasmin and fibrinolysin as well as trypsin and chymotrypsin. Principle proteinase inhibitors studied in human blood plasma (119) are listed in Table II.

Four different inhibitors are isolated and characterized from different fractions of serum. They are glycoproteins and unstable to heat and acids and have molecular weights ranging from 50,000 to 8,00000. A specific chymotrypsin inhibitor is located in post albumin region (120, 121). A polyvalent inhibitor for trypsin, chymotrypsin, thrombin and fibrinolysin is shown to be present in α_1-globulin fraction. The α_2-globin fraction contains a polyvalent inhibitor for trypsin and chymotrypsin and (particularly) plasmin (122). A specific trypsin

TABLE II : PRINCIPAL PROTEINASE INHIBITORS STUDIED IN HUMAN BLOOD PLASMA

	Concentration mg/100 ml	Molecular weight	No. of polypeptide chains	Heads[a]
1. α_1-Proteinase inhibitor	290	52,000	1	1
2. α_1-antichymotrypsin	49	69,000	1	1
3. Inter-α-trypsin inhibitor	50	160,000	1	2
4. α_2-antiplasmin	7	70,000	1	1
5. Antithrombin III	24	65,000	1	1
6. C_1-inactivator	24	70,000	1	1
7. α_2-macroglobulin	260	720,000	4	1-2 or more

[a] No. of enzyme molecules inhibited by one inhibitor molecule

inhibitor is present betwen α_1 and α_2 globulin fractions. α_2-Macroglobulin is shown to be a general endopeptidase trap (23). α_1-Proteinase inhibitor contains no interchain disulfide bridge (124) and inhibits serine proteinases. α_2-Antiplasmin a major plasma inhibitor (125-127) is shown to react slowly with several trypsin like enzymes but reacts exceptionally fast with plasmin. Antithrombin III is identified as a major thrombin directed inhibitor in plasma. Complete amino acid sequence of antithrombin III is reported (128), but its reactive site is not easily identified. Based on a small portion of the α_1-proteinase inhibitor sequence it is postulated that antithrombin III and α_1-proteinase inhibitor may be homologous.

A trypsin inhibitor from Submandibular glands of dogs inhibits chymotrypsin and papain (129). Trypsin inhibitor from guinea pig seminal vesicles (130) also inhibits plasmin, papain and bacterial proteinase from <u>Streptomyces griseus</u>. An antipapain factor is shown to be present in rabbit serum (131) and skin (132).

The only known specific papain inhibitor purified by Fossumand Whitaker (38) and Sen and Whitaker (39) is from chicken egg white. It inhibits papain and ficin but does not inhibit trypsin, chymotrypsin and several bacterial proteinases. One mole of inhibitor inhibits one mole of papain or ficin which apparently compete for the same site. The dissociation constant

of the enzyme inhibitor complex is 1.5 x 10^{-8}M. At 100°C, pH 4.0, it loses 10% activity while at pH 9.0 it loses 60% activity. Molecular weight of the inhibitor by gel filtration is 12,700 and it does not contain carbohydrate.

The inhibitor isolated from rabbit skin inhibits dermato proteinase isolated from the skin. This inhibitor inhibits papain also (133). Aqueous extract of raw soyabean is shown to inhibit the action of papain (134). The papain inhibitory factor is concentrated almost entirely in the germ (135), while cotyledon is without inhibitory action. A similar observation of papain inhibiting factor is made in other leguminous seeds. Seven isoinhibitors from pineapple *Annanas sativus* (136) inhibit ficin and papain and have two subunits.

Inhibitors from microbial sources : Several types of proteinase inhibitors have been reported from *Streptomyces* (40). These are identified as small peptides and designated as leupeptin, antipain, and chymostatin. They are shown to have clinical applications in different pathological conditions. Leupeptin shows inhibition of trypsin, papain and kallikrein but are of chymotrypsin. Pepstatin a pentapeptide inhibits pepsin. Antipain inhibits papain, trypsin and to a certain extent plasmin while chymostatin showed chymotrypsin and cathepsin B inhibition.

A proteinaceous subtilisin inhibitor (molecular weight around 23,000) is isolated and crystallized from culture broths of *Streptomyces albogriseolus* (30-32).

Subtilisin inhibitors from different sources

(a) *Plants*

Subtilisin inhibitor from barley is isolated and purified by Yoshikawa *et al*. (36). It has a molecular weight of 20,000. Its dissociation constant is 1.5 x 10^{-10} M. Enzyme inhibitor complex is in a molar ratio of 1:1. Both carboxy and amino terminals are alanine. Munday *et al*. (138) purified subtilisin inhibitors from barley. Its molecular weight, dissociation constant and isoelectric pH are 21,000, 4 x 10^{-8} M and 7.2, respectively. It is heat labile. A non-specific inhibitor from barley grain is purified by about 30-folds (28). It shows inhibition of alkaline proteinases of *A. oryzae*, *B. subtilis*, *S. griseus* and *Alternaria tenuissima* and chymotrypsin. Its molecular weight is 25,000. By casein hydrolysis method 1 mg of the inhibitor inhibits 0.36 mg of chymotrypsin or 0.29 mg of subtilisin. However, when synthetic substrate (glutarayl-L-phenyl-alanine nitroanilide) is used, 1 mg of the inhibitor inhibits 2 mg of chymotrypsin. It is resolved into 3-5 isoinhibitors of the same specific activity and properties. Their isoelectric points range from 4.6 to 5.4. Seidl *et al*. (35) purified subtilisin inhibitor from Black bean seeds. Its molecular weight is 10,000

by gel filtration and SDS-gel electrophoresis. Archer (137) purified subtilisin inhibitor from *Hevea brasiliensis* latex. Its molecular weight is 11,730 and has an iso-electric point of 4.15. Two subtilisin inhibitors are purified from *Vigna unguiculata* (33) in this Laboratory. Their molecular weights are 11,000 and 10,000 by gel filtration and 8100 and 8500 by SDS-gel electrophoresis. Their isoelectric points, dissociation constants and optical factors are shown to be 5.1, and 5.9, 9 x 10^{-10}M and 1.4 x 10^{-9} M, and 1.3 and 1.0 respectively. They do not contain cystine. The polyvalent potato inhibitor type I shows inhibition of trypsin, chymotrypsin, subtilisin and pronase (partly) and human salivary and urinary kallikrein (85, 142, 143, 163).

Hejgaard and Chavan (34) purified subtilisin inhibitor from broad bean (*Vicia faba*). Its molecular weight and isoelectric point are 10,000 and 4.8. The inhibitor is shown to be homologous with barley and leach inhibitor family. Cleavage studies show that the inhibitor site is at alanine-aspargine bond. A very low content of cysteine shows that Vicia and Vigna inhibitors are similar to 'potato inhibitor I' family (139). Yoshikawa *et al.* (37) purified subtilisin inhibitor from *Adzuki bean* (*Phaseolus angularis*). The molecular weight of the inhibitor by gel filtration is 12,300 and on the basis of amino acid composition it is 11,000. Its isoelectric

point (pI) is 3.7. Dissociation constant of the complex of the inhibitor with subtilisin is 1.6×10^{-9} M. It contains large amounts of glutamic acid and valine residues.

(b) Animals: A few non-specific subtilisin inhibitors have been reported from avian egg white. Ovo-inhibitors from duck, turkey, chicken and quail egg-white inhibit trypsin, α-chymotrypsin, subtilisin and the alkaline protease from *A. oryzae*. Chymotrypsin and subtilisin are shown to compete for the same site (99). Submandibular gland contains very high concentration of proteinase inhibitors (144). Four inhibitors having broad specificity are isolated and purified by Fritz *et al*. (145). They inhibit trypsin, chymotrypsin, subtilisin, elastase, plasmin and *A. oryzae* proteinase. Their molecular weights are around 13,000. The trypsin and chymotrypsin inhibiting sites are shown to be distinct while chymotrypsin and subtilisin reactive sites are identical. All the above inhibitors for subtilisin are non-specific.

(c) Microorganisms: A specific subtilisin inhibitor is isolated and crystallized from the culture broth of *Streptomyces albogriseolus* (30 - 32). It is homogenous in polyacrylamide gel and stable at 100°C for 10 minutes between pH 5.0 and 6.0. It shows maximum UV absorption at 280 nm and minimum at 250 nm. Its optical factor is found to be 1.22. It does not contain any carbohydrate

or phosphorus. The inhibitor is specific towards microbial alkaline proteinase. It inhibits subtilisin, BPN and alkaline proteinases of B. subtilis, SO_4, B. subtilis var. amylsacchariticus, Streptomyces sp. and Cephalosporium sp. Its molecular weight by sedimentation equilibrium, gel filtration and SDS gel electrophoresis is 23,000, 27,000 and 12,000, respectively which suggests that the inhibitor exists as a dimer (32). The inhibitor contains large amounts of alanine (19 moles/mole) and valine (12 moles/mole), 4 moles of half cystine and no isoleucine or cysteine.

Sato and Murao (32) showed that the complex of subtilisin and the inhibitor is formed from one molecule of the inhibitor (two subunits of the inhibitor) and two molecules of subtilisin. Isoelectric point of the complex is 5.5. Subtilisin inactivated by diisopropylfluorophosphate or carbobenzoxyl-L-alanyl-l-glycyl-L-phenylalanine-chloromethyl ketone does not form a complex with the inhibitor. X-Ray diffraction studies of the crystals of the inhibitor and its complex with subtilisin are carried out by Satow et al. (140). Inouye et al. (141) showed that this subtilisin inhibitor has a wider inhibiting ability than reported by Sato and Murao (31). The inhibitor feebly inhibits chymotrypsin and trypsin. Dissociation constants for the complexes of the inhibitor with subtilisin, chymotrypsin and trypsin are 1×10^{-9}M,

3×10^{-6} M and 1.1×10^{-4} M, respectively.

The low molecular weight peptides of Umezawa (40) although have a broad specificity do not inhibit subtilisin.

SECTION IV

GENERAL PROPERTIES OF THE INHIBITORS

Molecular weight

The molecular weights of proteinase inhibitors generally range between 6000 to 60,000. In a majority of cases the molecular weights are less than 20,000. Inhibitors from different variety of beans are shown to have low molecular weights (between 8000 to 23,000) (13). Rancour and Ryan (17) obtained four isoinhibitors of carboxypeptidase B from potato having low molecular weights of 3000 - 4000. These polypeptides are dialyzable and inhibit α-chymotrypsin besides carboxypeptidase B Small peptide inhibitors from culture broths of several strains of Streptomyces have been described (146). They inhibit different proteinases such as trypsin, chymotrypsin, papain and cathepsin B and posses very low molecular weights ranging from 500 to 1200. However, these inhibitors are shown to be generally non-specific in nature.

Stability

Most of the proteinase inhibitors are shown to be stable even at denaturing conditions. Generally all the low molecular weight inhibitors (molecular weight less than 20,000) are more stable to heat and acids. Many low molecular weight inhibitors are not precipitated by trichloroacetic acid. Several leguminasae inhibitors (147), Kunitz (20) and Kazal (50) pancreatic inhibitors,

trypsin, chymotrypsin and papain inhibitors from Ascaris (148, 149) and Clostrum inhibitors from bovine and porcine origin (150, 151) are stable to 2.5% trichloroacetic acid at 95°C and also stable to 90% ethanol and are not denatured by 8 - 9M urea at neutral pH and room temperature.

Several trypsin-chymotrypsin inhibitors contain high cystine which forms extensive cross-linking in the molecule. The stability of the inhibitors is attributed to this cross-linking. Reduction of the disulfide groups with dithiothreitol or borohydride shows complete loss of inhibitory activity. Most of these inhibitors have 9 - 15% half-cystine. Bowman-birk inhibitor from soyabean and Lima-bean inhibitor component '2' contain 14 half cystine in the molecule (73, 152). Navy bean (Phaseolus vulgaris) trypsin inhibitor contains 30 half cystines per mole (153), however, Kunitz soyabean inhibitor (23) has only 4 half cystines in its 198 residues (49, 154) and is unstable to heat but stable to 9 M urea (155). Serum inhibitors (α_1-trypsin and α_1-chymotrypsin inhibitors) do not contain cystine and are unstable to heat and acids (156).

In addition to stability towards heat and acids many trypsin inhibitors are stable to the action of pepsin at pH 2.0 - 3.0 (147). It is suggested (157) that the secondary structure of native inhibitors is

responsible for this stability, since previously denatured inhibitors can be easily hydrolysed by pepsin.

Amino acid composition, sequence and X-ray studies

Amino acid composition of several proteinase inhibitors has been determined. Many proteinase inhibitors contain proline but do not contain tryptophan. So far cysteine has not been shown to be present in a number of inhibitors. ovomucoids and serum inhibitors are glycoproteins. Generally prolyl residue is present near the reactive site of the trypsin inhibitor. Laskowski and Sealock (157) postulated that the presence of prolyl near the reactive site contributes to the required regidity of the reactive site. Similarly the presence of prolyl groups in the remainder of the inhibitor molecule is shown to be partly responsible for the resistance to denaturation of the molecule.

Sequences of a few proteinase inhibitors have also been determined. Sequences of bovine pancreatic trypsin inhibitor or Kunitz bovine pancreatic trypsin inhibitor of Kunitz (51, 52), bovine pancreatic secretory trypsin inhibitor (158), cow clostrum inhibitor (159), maize seed, peanut and ovine pancreatic trypsin inhibitor (160), secretory trypsin inhibitor from porcine pancreas (161), snake venom proteinase inhibitor (162), *Streptomyces* subtilisin inhibitor (163) and subtilisin inhibitor from barley (139) are determined. X-ray studies of

different inhibitors, and their complexes are also studied by different workers. Studies on X-ray crystallography and three dimensional structure of Kunitz pancreatic trypsin inhibitor are carried out by Huber *et al*. (56, 57).

Dissociation constant

The affinity of an inhibitor for a proteinase is inversely related to the dissociation constant and designated as the inhibitor constant. The dissociation of the trypsin-trypsin inhibitor complex is pH dependent. the complex is stable at neutral and slightly alkaline pH-values. On lowering the pH, the complex is more labile and at pH 2 it gets completely dissociated.

Dissociation constants have been reported for many enzyme inhibitor pairs at different pH values. Physical and enzymatic methods have been used for these determinations. The physical method includes analytical ultracentrifugation, free boundary electrophoresis, viscosity measurements (164), gel filtration (165), fluorescence quenching (166), proflavine displacement (167) and the potentiometric technique of Laskowski Jr. (47). Enzymatic titration method uses the extent of deviation from the stoichiometric inhibition in the region of the equivalent point of titration as described by Green and Work (149, 168). Pancreatic trypsin inhibitor complex shows an unusually low dissociation constant (6×10^{-14} at pH 8.0) (169). Dissociation constants

of some of the proteinase inhibitor complexes are summarized in Table III.

Separation of the inhibitor and enzyme from their complex

Active inhibitor can be easily isolated from the complex by precipitating the enzyme either by boiling or by trichloroacetic acid. Gel filtration at lower pH can also be used to separate inhibitor and enzyme provided the molecular weights of the inhibitor and enzyme differ significantly. Hochstrasser *et al.* (170) used thioethanol to inactivate the inter α_1 trypsin inhibitor and release the enzyme in active form. Horez (171) showed that SDS can be used to inactivate serum α_1-trypsin inhibitor from its complex with trypsin. Sato *et al.* (172) immobilised subtilisin inhibitor on sepharose and used it for the purification of subtilisin. The complex is cleaved with 0.5% SDS at pH 7.5 and SDS from the dissociated enzyme is removed by Dowex 2. 80% recovery of subtilisin is obtained by this method. However, when 6M urea or 3M guanidine-HCl is used, the enzyme recovery is less than 35%.

Isoelectric points

Isoelectric points of the inhibitors generally lie between pH 3.5 to 6.0. Some of the trypsin chymotrypsin inhibitors show higher values (pI 5.1 to 9.2). pI of the basic pancreatic trypsin-chymotrypsin inhibitor

TABLE III : DISSOCIATION CONSTANTS OF PROTEINASE INHIBITOR COMPLEXES

		pH	Dissociation constant
1.	Trypsin-Kunitz pancreatic	3.0	3 x 10^{-5}
		8.0	6 x 10^{-4}
2.	Trypsin-Kunitz soyabean	3.1	6 x 10^{-3}
		7.8	5 x 10^{-9}
3.	Subtilisin-penguin ovomucoid inhibitor	8.0	6 x 10^{-8}
4.	Ficin-chicken egg white inhibitor	7.0	1 x 10^{-9}
5.	Ascaris-chymotrypsin inhibitor (45)	7.5	5.9 x 10^{-9}
6.	Trypsin-snake venom inhibitor (162)		7.6 x 10^{-10}
7.	*Vigna unguiculata* subtilisin inhibitor 1(33)	7.5	9 x 10^{-10}
	Vigna unguiculata subtilisin inhibitor 2a(33)	7.5	1.4 x 10^{-9}
	Vigna unguiculata subtilisin inhibitor 2b(33)	7.5	1.54x 10^{-9}
8.	Barley subtilisin inhibitor		4 x 10^{-8}
9.	*Streptomyces albogriseolus* subtilisin inhibitor (non-specific) (21 - 23)		
	Subtilisin inhibitor	7.5	1 x 10^{-9}
	Chymotrypsin inhibitor	7.5	3 x 10^{-6}
	Trypsin inhibitor	7.5	1 x 10^{-4}

is shown to be 10.1 (173). Human plasma trypsin inhibitor showed low pI (2.8) (174) whereas kallikrein inhibitor showed a pI of 6.4 (174).

Optical factor

The optical factor (23) is defined as the reciprocal of the absorbance at 280 nm for a light path of 1 cm when the concentration of protein is 1 mg/ml. The optical factors of inhibitors range between 0.77 to 2.30.

High optical factor indicates a low content of aromatic amino acids especially tyrosine and tryptophan. Optical factors for some of the inhibitors are given in Table 4.

Specificity and stoichiometry of inhibitors

Before discussing on the specificity of proteinase inhibitors it is necessary to mention about the specificity of the proteinases which they inhibit.

Specificity of proteinases

Trypsin: Hydrolyses peptides, amides, esters, etc. at bonds involving the carboxyl group of L-arginine or L-lysine.

Chymotrypsin: Hydrolyses peptides, amides, esters, etc. especially at bonds involving the carboxyl groups of aromatic L-amino acids.

Subtilisin: Hydrolyses peptides, attacks ester bonds but the amide bond is more resistant. The enzyme

TABLE IV: OPTICAL FACTOR OF INHIBITORS

		Optical factor
1.	Potato-chymotrypsin inhibitor	0.77
2.	Soyabean trypsin inhibitor (Kunitz)	1.10
3.	Subtilisin inhibitors from *Streptomyces albogriseolus*	1.22
4.	Trypsin-chymotrypsin inhibitor (Soyabean 1.9 trypsin chymotrypsin inhibitor)	2.3

also catalyzes transesterification reactions between L-tyrosine ethyl ester and a number of aliphatic alcohols.

Papain: Hydrolyses peptides, amides, and esters especially at bonds involving basic amino acids, or leucine or glycine.

Pepsin: Hydrolyses peptides, including those with bonds adjacent to aromatic or dicarboxylic L-amino acid residues. Esters and amides are not hydrolysed, only the peptide bond is hydrolysed.

Specificity and stoichiometry of inhibitors

Inhibitors from different sources vary in their specificity and stoichiometry. Some inhibitors inhibit more than one molecule of the enzyme per molecule of the inhibitor. In the case of trypsin inhibitor from Navy bean and from many other beans (175), one mole of the inhibitor inhibits two moles of trypsin. One mole of the potato chymotrypsin inhibitor inhibits four moles of chymotrypsin (175). Most of the inhibitors having broad specificity inhibit different enzymes and are designated as polyvalent or broad spectrum inhibitors. Potato type I inhibitor inhibits trypsin, chymotrypsin, subtilisin, kallikrein and pronase. Dog submandibular gland inhibitor (116) shows the inhibition of trypsin, chymotrypsin, subtilisin, elastase and plasmin. Barley grain inhibitor (28) combines with chymotrypsin, subtilisin and alkaline proteases of *A. oryzae*, *S. griseus* and

Alternaria tenuissima. Snake venom inhibitors I and II inhibit bovine plasma kallikrein, trypsin, α-chymotrypsin and plasmin (162). When an inhibitor possesses two distinct sites for the inhibition of trypsin and chymotrypsin it is described as a double headed inhibitor (29). In such cases their complex with either enzyme is capable of stil inhibiting the other enzyme. Kunitz's bovine pancreatic trypsin inhibitor is found to be an exception. Wu and Laskowski (176), Kraut and Bhargava (177) and Ribgi (178) found that in this case same reactive site is involved in the inhibition of both the enzymes. In some cases there is a competition between two proteinases for the same reactive site of the inhibitor. Chymotrypsin inhibitor which inhibits subtilisin shows such behaviour (179). Papain inhibitor from chicken egg white also inhibits ficin at the same active site (38).

In the case of the polyvalent potato inhibitor I which also inhibits trypsin, it is found that this inhibition can be observed only when casein (Protein substrate) is used in the assay system. When synthetic substrates are used then there is no inhibition of trypsin. In contrast barley grain inhibitor is shown to be an example where esterolysis is predominantly affected. In this case five times more chymotrypsin is required for synthetic substrate than for casein as substrate (180).

Trypsin inhibitors are shown to differentiate between bovine trypsin and human trypsin by selectively inhibiting one of them (181). Trypsin inhibitors from ovomucoids of chicken and turkey, chicken ovoinhibitors and Kazal's bovine secretory pancreatic inhibitor (51) inhibits bovine trypsin but not human trypsin. Trypsin inhibitors from Lima bean, soyabean (Birk) and Kunitz bovine pancreatic inhibitor inhibit the enzymes from both the sources.

Trypsin inhibitors can be divided into two classes on the basis of the differences in their reactive sites. Ozawa and Laskowski (49) postulated that some trypsin inhibitors have a susceptible Arg-x bond in their reactive site while others have Lys-x bond. This is confirmed by Liu *et al*. (182) and Haynes *et al*. (74). Hence trypsin inhibitors, can be classified as arginyl or lysyl inhibitors. Chicken ovomucoid trypsin inhibitor, chicken ovoinhibitor and Kazal's bovine pancreatic inhibitors are shown as arginyl inhibitors. While inhibitors from turkey, ovomucoid, Lima bean, soyabean (Birk) and bovine pancreas (Kunitz) are found to be lysyl inhibitors (183).

SECTION V

DIFFERENT FORMS AND TYPES OF INHIBITORS

Isoinhibitors

Many inhibitors are found to exist in multiple forms. These inhibitors are called isoinhibitors. They are similar in their specificities and in some of their properties. The origin and role of isoinhibitors are not known. Isoinhibitors may have been formed by the breakdown of the original inhibitor during extraction and purification and may be artefacts. A typical example where isoinhibitors are formed from native inhibitor is that of Lima bean inhibitors as described in Section III.(184).

Isoinhibitors can be obtained from different types of tissues by using different isolation techniques. In some instances isolation of the inhibitor is made by washing the tissue with solvents like acetone or ethanol (73), or by extracting the tissue with perchloric acid, diisopropyl phosphofluoridate (115) or cold TCA (185) which can destroy proteolytic enzymes and prevent the formation of artefacts.

SECTION VI

PROTEINASE INHIBITORS AND ENDOGENOUS PROTEINASES IN PLANTS

Plant inhibitors inhibit corresponding enzymes from animal or microbial sources but in few cases they are also shown to inhibit endogenous proteolytic enzymes of the plant from which they are derived (80)

Ofelf *et al*. (186) and Birk (62) showed that purified soyabean trypsin inhibitor does not inhibit the endogenous proteinases but aqueous extracts of soyabean inhibit papain and the proteolytic enzymes of malt and wheat (134).

Shain and Mayor (187, 188) reported that trypsin inhibitor in lettuce seeds inhibits an endogenous 'trypsin like' endopeptidase. This enzyme shows pH optimum of 6.8 and molecular weight of 3000 - 4000. This enzyme is developed during germination of the seeds. When proteolytic activity increases during germination, inhibitor activity disappears. This indicates that either the enzyme is complexes with the inhibitor or is present as a precursor and during germination gets activated and neutralizes the inhibitor. This enzyme is purified 450 fold but still it is not homogeneous.

Three types of proteinase inhibitors are shown in ungerminated barley extract (189). The first type inhibits endogenous proteinases of barley (190). The

second inhibits microbial proteinases and the third type inhibits trypsin. A small molecular weight dialyzable inhibitor from barley is shown to inhibit endogenous proteinases in barley kernals (191). The inhibitor is not identified or isolated.

In black eyed pea (Vigna sinensis) the trypsin inhibitory activity of the extracts of cotyledons decreases continuously during germination. The disappearance of trypsin inhibiting activity is controlled by the axal parts. Filho (192) showed that the crude inhibitor fraction of Ventura and Filho (75) contains a weak but finite inhibitor of the endogenous proteinases of the seeds. Royer et al. (78) described two types of proteinases from Vigna unguiculata. One hydrolyzing BAPA and other hydrolyzing casein. The casein hydrolyzing proteinases are partly inhibited by the trypsin inhibitors from the same source.

Inhibitor for mammalian carboxypeptidase-B and α-chymotrypsin is isolated from potato (17, 193). The same inhibitor strongly inhibits the bradykinin inactivating carboxypeptidase from potato (80). It is found to be the smallest proteinase inhibitor from plant source (molecular weight 3500) and may have a function in regulating the activity of the enzymes in dormant potatoes.

Different proteinases inhibited by inhibitors

The proteinases inhibited by the inhibitors are usually endopeptidases. However, Rancour and Ryan (17) showed that the inhibitors from potato are of carboxypeptidase B type. Vogel *et al*. (147) reported a peptidase acting on small peptides which is not inhibited by proteinase inhibitors. Proteinases which have been shown to be inhibited by inhibitors are trypsin, chymotrypsin, papain, ficin, plasmin, elastase, thrombin, pepsin, kallikreins, chatepsins, carboxypeptidase B, subtilisin, pronase, rennin, gastricin, bromelein, alkaline proteinases of *A. oryzae*, *S. griseus* and *Alternaria tennissima*, proteases from Penicillium and different proteinases of bacteria as well as seeds before and after germination.

SECTION VII

MECHANISM OF ACTION OF PROTEINASE INHIBITORS

Kunitz and Northrop (20) demonstrated that the reaction between proteolytic enzyme and its inhibitor involves the formation of a complex.

E + I (unmodified) ⇌ EI = E + I (modified)

While working on the soyabean trypsin inhibitor he isolated a complex which showed all the properties expected for an addition compound of one mole of soyabean trypsin inhibitor and one mole of trypsin. The complex was stable at neutral pH, but at acidic pH it dissociated into component parts which retained their respective activities.

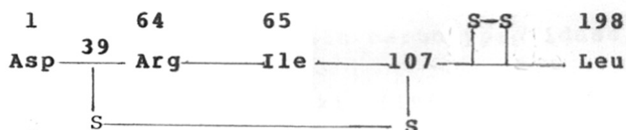
It was postulated that the inhibitors contain active sites for the inhibition of their proteinases which give them their specificity (48). Inhibitors specific for trypsin always have either Lys-x or Arg-x at their binding sites. Where x is Lysine, isoleucine or alanine, chymotrypsin specific inhibitors usually have Leu-x, sequence at their active centers, where x is serine (Lima bean inhibitor) and the reactive site of the inhibitor is situated in a disulfide loop.

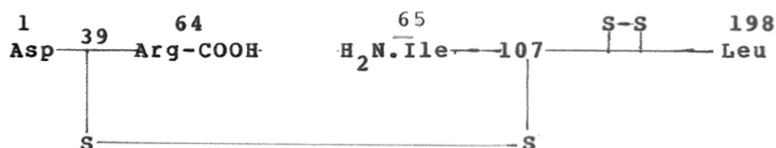
Laskowski Jr. (157) postulated the formation of a protease-inhibitor complex involving a cleavage of

a peptide bond at the active site of the inhibitor (such as Lys-x or Arg-x in the case of trypsin inhibitor) by the proteinase. The original (native) inhibitor gets modified due to this bond cleavage. Thus complex is formed by the formation of a covalent bond between a group at the active site of trypsin and the newly formed COOH-terminal of the modified inhibitor (an ester bond between OH of trypsin and newly formed COOH terminal of the inhibitor). The sequence of mechanism is given as :

where E is a proteinase, I is a native inhibitor and I* is the modified inhibitor.

Ozawa and Laskowski Jr. (49) suggested the following mechanism in the case of soyabean trypsin inhibitor (Kunitz).





Conversion of native soyabean inhibitor to modified inhibitor

It is shown that the native inhibitor consists of a single chain but the modified inhibitor contains two polypeptide chains connected by a S-S-bridge. By reducing S-S-bridge in the native and modified inhibitor and subsequent separating the fragments on Sephadex Ozawa and Laskowski (49) showed that the modified inhibitor has an additional chain (joined by S-S bridge). Newly formed C-terminal arginine residue can be cleaved by caroboxypeptidase B, where the inhibitory activity is lost. Kowalski and Laskowski Jr. (194) stated that additional $-NH_2$ group formed in the modified inhibitor can be blocked by citraconic anhydride which inactivates the modified inhibitor.

The results are similar with chicken ovomucoid. Many others (63, 152, 195) confirmed that in a variety of different inhibitors loss of activity is due to COOH-terminal amino acid removal with carboxypeptidase.

Finkenstadt and Laskowski (196) showed that the modification is reversible. The complex is prepared by using modified soyabean trypsin inhibitor (Kunitz) and trypsin and is dissociated under drastic conditions (very low pH and 6M guanidine HCl) yielding native inhibitor predominantly. Sealock and Laskowski Jr. (197) and

Laskowski Jr. (157) have shown that the Arg-x bond in the peptide chain of soyabean trypsin inhibitor can be cleaved and resynthesized by replacing arginine moiety with lysine and this resynthesized product also shows inhibitory activity.

However, there is no agreement that the above mechanism is common to all proteolytic inhibitors. No tryptic cleavage was shown in Kunitz and Northrop bovine pancreatic trypsin inhibitor (198). In turkey and cassowary ovomucoids, Feinstein et al. (199) could show inhibiting activity even after modification with trypsin and treatment with carboxypeptidase B.

Substitution of the newly exposed amino group with trinitrobenzene sulfonic acid of penguin and turkey ovomucoids, colostrum and Lima bean trypsin inhibitors led to loss of trypsin inhibitory activity. While in the case of chicken ovomucoid and soyabean trypsin inhibitor there was no loss of activity (Haynes et al. (74). Uy and Feeney (200) modified penguin and turkey ovomucoids with either α-chymotrypsin or subtilisin. The newly exposed amino group of the modified inhibitor was substituted by dimethylation and tested for inhibitor activity. No loss of inhibitory activity was detected. The reactive site hydrolysis theory requires the participation of an active enzyme. However, Feinstein and Feeney (167), Fossum and Whitaker (38), Feeney (201), Ryan (80) and

Sen and Whitaker (39) showed that the inactive derivatives of the enzymes like TLCK-trypsin, TPCK-chymotrypsin. anhydrochymotrypsin, trypsin (histidine residue substituted) chymotrypsin (serine modified by dehydration), subtilisin (serine substituted by tosylation) mercury papain and alkylated ficin, combined with inhibitors to form complexes which did not involve an intermediate modified inhibitor as proposed by Laskowski Jr.

Feeney (201) reported that the formation of the complex does not require the hydrolysis of a bond and only non-covalent forces are involved in complex formation. The specificity of an inhibitor is dependent on the side chain of the amino acid present at the binding site of an inhibitor. Catalysis and bond splitting are, however, secondary and are not essential.

Temporary inhibition

The complex of trypsin and chicken ovomucoid on standing releases trypsin by slowly destroying the inhibitor (202, 203). A similar observation is reported by Laskowski and Wu (50) in the case of a complex between trypsin and bovine secretory pancreatic inhibitor. This is termed as temporary inhibition and described in detail by Laskowski and Wu. Inactivation of the inhibitor on prolonged incubation with trypsin is shown by Sakura and Timasheff (204) in the case of a plant inhibitor viz. Lima bean trypsin inhibitor.

SECTION VIII

PHYSIOLOGICAL ROLE OF PROTEINASE INHIBITORS

The physiological function of the inhibitors of proteolytic enzymes is not sufficiently explained though they constitute often considerable portion of plant proteins. Several hypotheses have been suggested by various workers. Proteinase inhibitors are essential for the protection of organism against tissue damage by active proteinases. Proteinase inhibitors have been implicated as a safeguard against proteolytic damage that might result from leaky or broken vacuoles and as a regulatory control element of proteinase activity (205). Proteinase inhibitors are considered as (a) regulating agent in controlling endogenous proteinases (b) protective agents against insect or microbial proteinases, (c) storage protein (especially in leguminous seeds) and (d) since in a number of cases no direct evidence of these roles could be shown, they are also considered as biochemical peculiarities.

It has been suggested that in pancreas the role of the secretory pancreatic trypsin inhibitor is to inhibit prematurely activated trypsin which is responsible for activating zymogens of other proteinases present in the pancreas. Trasylol (a polyvalent inhibitor) is used as an effective agent in pancreatitis (147). It may also control the activation of a trypsin like

enzyme responsible for the conversion of proinsulin to insulin (206).

In blood the inhibitors have a role in blood-clotting. Some of the blood clotting enzymes are inhibited by two serum inhibitors. Individuals genetically deficient in serum inhibitors are shown to be susceptible to lung and liver disorders such as pulmonary emphysema (207) and liver cirrhosis (208). The deficient state is associated with a lower content of sialic acid in the proteinase inhibitors (209).

The pepsin resistant colostrum inhibitor which also inhibits trypsin and chymotrypsin is regarded as useful in preventing the proteolysis of milk antibodies in the digestive system of the new born (150, 151).

Beumgartner _et al_. (210) have reported that the inhibitors of endopeptidase of mung bean (_Phaseolus murcus_) play little part in the regulation of the protein metabolism, but they have more significant influence on the protection of cytoplasm.

Erickson and Larson (211) have reported that deficiency of serum alpha-1-antitrypsin is associated with some cases of chronic obstructive disease and fatal cirrhosis in the prenatal or juvenile period. Hepatocellular carcinoma has also been associated with a partial deficiency of the above inhibitor. The damage was found

to be related to the accumulation of the PAS-positive material which is immunologically similar to the α_1-antitrypsin but is deficient in terminal sialic acid molecule. The inability to add sialic acid was shown by Kuhlenschmidt et al. (212). to be due to a deficiency of sialyl transferase. Hence pateints deficient in the above two factors may be more prone to liver disease, indicating that inhibitors may have an important regulatory role at the cellular level.

Taber, Wertheimer and Golrick (213) stated that TPCK (1-chloro-4-phenyl-3-tosylamido-2-butane), which is a specific chymotrypsin inhibitor, inhibits the cleavage of a large polypeptide chain synthesised in uninfected hela cells. This indicates that some inhibitors may have a role in cell division. Inhibitors similar to bovine polyvalent inhibitor, which are present in ruminants may be important with the relationship between the complicated digestive system of the ruminants and the growth of bacteria and protozoa in the gastrointestinal tract (214).

Astrop (215) reported that the absence of certain respiratory diseases in cattle is due to the presence of this inhibitor in lungs.

The presence of pepsin, trypsin and chymotrypsin inhibitor in Ascaris may protect it from its proteolysis in the digestive tract of the host.

Mansfel et al. (216) stated that the plant inhibitors, in general, play a role in maintaining the latent life form

by inhibition of autolysis.

Legumes exist in symbiosis with the Rhizobia present in the root nodules. Vogel *et al*. (147) suggested that the presence of high proportion of proteinase inhibitors in the roots of these plants may offer a protection to the plant against excessive invasion by these bacteria by inhibiting their proteinases.

Ryan (217) proposed that the most useful function of plant inhibitors is of plant protection. Proteinases from *Tribolium* a common larva that destroys stored grains, is inhibited by soyabean extracts. The proteinases from *Tenobrio*, a common paste that consumes stored grains are inhibited by Lima and soyabean inhibitors. The trypsin and chymotrypsin like digestive proteinases of a number of insect genera are inhibited by plant proteinase inhibitors (218). Green and Ryan showed that when the potato and tomato plants were wounded by potato beetles or their larvae, it caused a rapid accumulation of a proteinase inhibitor throughout the aerial tissue of the plants. This type of response may be effective in repelling insects or their larvae and pathogenic microorganisms.

The microbial proteinase inhibitors from some strains of *Streptomyces* are thought to be protecting the microorganisms from the harmful effects of their own proteases (40). It is proposed that after the hydrolysis of organic nitrogen compounds in the medium, the free proteolytic enzymes outside

cells may become harmful to the cells, if they are not inhibited.

Leeney *et al.* (219) showed three different proteinases and their inhibitors in *Saccharomyces cerevisiae*. The proteinases are localized in vacuole, while inhibitors are present in the extravacuolar cytosol.

The proteinases after their synthesis in the cytoplasmic polysomes are transferred to the vacuoles as enzyme inhibitor complexes. Also the inhibitors may be protecting the extra-vacuolar proteins from the accidental leakage of proteinases from the vacuoles.

Mikola and Suolinna (220) suggested that the inhibitors may have a role in the endozoic disposal of seeds. The seeds remain viable even after having been consumed by animals. The inhibitors may be protecting the seeds against the alimentary canal proteinases.

During storage of food products unfavourable biochemical transformations are often observed causing their spoilage. In some cases inhibitors of proteinases can be very beneficial. For instance potato inhibitors inhibit the activity of exogenic proteinases synthesized by microorganisms, probably protecting potato tubers against the attack of molds and bacteria during their storage for many months.

Inspite of various theories put forward the role of plant proteinase inhibitors is not very clear. They may have multipurpose function or different inhibitors may have varied function.

Kinship relationship of proteinases and their inhibitors

Amino acid sequence of Human α_1-proteinase inhibitor shows striking homology to that of α_1-antichymotrypsin and antithrombin III and strangely enough also to that of ovalbumin inhibitor which has no known relationship to proteolytic enzymes. This homology suggests that the plasma inhibitors, like the proteinases which they inhibit, had a common ancestor. In fact the evolutionary distance between α_1-protease inhibitor and antithrombin III - that is, 160 accepted point mutations per 100 residues - is the same as that which separates the respective enzymes elastase and thrombin. Similarly, the degree of sequence identity of α_1-protease inhibitors and α_1-antichymotrypsin is similar to that between the cognate enzymes trypsin and chymotrypsin (about 42 percent). This parallelism suggests that the speciali-zation of the inhibitors has occurred in response to and simultaneously with that of the corresponding pro-teinases (205).

SECTION IX

USES OF PROTEINASE INHIBITORS

There are a number of reports published about the use of trypsin inhibitors in the therapy of certain diseases. Proteinase inhibitors may have an application in controlling proteolytic enzymes in the processing of foods.

Inhibitors which are specific for their proteinases can be used as an analytical tool in distinguishing between proteolytic enzymes. By the use of pure inhibitors of different specificities it will be possible to distinguish between different proteinases occuring in crude tissue extracts.

There are adherents of the use of inhibitors of proteinases in the cardiac infarction. Indication regarding the use of inhibitors in this disease is the liberation of cathepsins from the necroticly changed cardiac muscle, and also the influence of inhibitors on pulmonary vessels, which is expressed by the decreased penetration of fluids to the pulmonary tissue and prevents the thickening of blood (222).

Proteinase inhibitors are used in the therapy of shocks of different aetiology, inflammation of pancreas and in the prevention of thrombosis. It is proposed to use inhibitors showing antikallikrein activity against

pain. In allergic states the inhibitors prevent unfavourable symptoms as well as stimulate the formation of blocking antibodies, which is important for desensitisation (222).

Several uses have been suggested for the low molecular weight inhibitors, isolated from culture broths of *Streptomyces* strains (40, 146). These inhibitors have very low toxicity and are absorbed even when given orally. Orally given leupeptins show anti-inflammatory effect. Use of leupeptin is suggested for the treatment of pancreatitis and for the inhibition of kinin formation.

In the case of burns leupeptin ointment suppresses pain and blister formation. Leupeptin inhibits tumarogenesis initiated by 7, 12-dimethylbenzanthracene and croton oil in mouse skin. Chymostatin, pepstatin and also leupeptin show inhibition of carrageenin edama formation. Pepstatin is shown to be very effective in suppressing ulcers in the stomach of pylorus-ligated rats.

Tests have been carried out concerning the use of the inhibitor of acrosin from human cervical muscus as the non-hormone contraceptive agent (223).

SECTION X

PRESENT WORK

The work described in this thesis deals with the finding of a large number of proteinase inhibitors with different specificities in the seeds of horse gram. Out of these inhibitors, a subtilisin inhibitor and a trypsin inhibitor are purified to homogeneity and characterized with respect to their properties and kinetics.

A unique observation made during the course of this work is regarding the presence of dialysable low molecular weight trypsin-chymotrypsin inhibitors in leguminose seeds. A dialysable trypsin inhibitor from horse gram is partially purified and characterized in this work.

The presence of such a large number of inhibitors with different specificities in a singlesource is of interest. In the course of this work various simple and efficient techniques have been developed and used for the purification and characterization of the inhibitors. These include (a) Electrophoretic elution of proteins from gels; (b) Method of electrophoresis in high concentration gels; (c) Use of fluorescent markers for locating the protein bands on gel and (d) Polymerization of acrylamide at acid pH using uranyl nitrate.

The properties and kinetics of the inhibitors include stability of the inhibitors under different

conditions, amino acid composition, N-terminal amino acid, chemical modification studies, isoelectric pH, dissociation constant, mechanism of action, molecular weight, UV-spectrum and molar combining ratio.

The work in this thesis is presented in six Chapters as follows:

Chapter I : deals with the historical account of proteinase inhibitors with more emphasis on inhibitors from plant source.

Chapter II: gives the information about the materials and experimental methods used in this work.

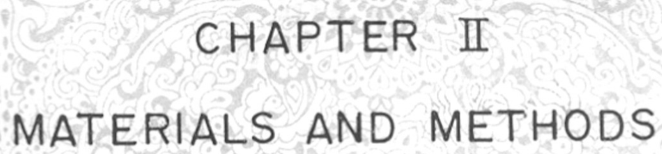
Chapter III: deals with the isolation of inhibitors of subtilisin and trypsin, their separation from each other and purification from the seeds of horse gram

Chapter IV: describes the properties and kinetics of purified inhibitors.

Chapter V: deals with the discussion on the results of these studies.

Chapter VI: contains a summary of the results and conclusion of this work.

A bibliography of the literature references cited in this thesis is presented at the end.



CHAPTER II

MATERIALS AND METHODS

Materials

Seeds

Various seeds used were purchased from the local market and stored at 4°C till use.

Nomenclature of Hulga seeds

The Hulga seeds are designated as *Macrotyloma uniflorum* or *Dolichos biflorus* Linn Horse gram.

It is a leguminous plant. A branched sub-erect or trailing annual, with small trifoliate leaves, bearing, when mature, narrow, flat, curved pods, 1½ - 2 inches long, tipped with a persistent style. The pods contain 5 - 6 flattened, ellipsoid seeds, 1/8 - 1/4th inch long. The seeds are brown, light red, grey, black or mottled (224).

The seeds used were light red in colour.

Chemicals

All chemicals used were of analytical grade. Chemicals and enzymes obtained from Sigma Chemical Company, U.S.A. were: DEAE-cellulose (0.9 meq per g, medium mesh), CM-cellulose (0.6 meq per g, medium mesh) trypsin (from bovine pancreas, type III), α-chymotrypsin (from bovine pancreas, type II), subtilisin (subtilopeptidase A) from a special strain of *B. subtilis* type III), cytochrome c and myoglobin (from horse heart), and albumin (from bovine serum, crystallised), BAEE, BTEE and BAPA, aprotinin.

Cysteine hydrochloride and casein (Hammersten) were obtained from E. Merck, Germany. Hemoglobin was obtained from Serva Laboratory, Germany, and pronase (B grade) from Calbiochem, U.S.A. Papain (electrophoretically pure) was procured from Biochemicals Unit, Delhi. Papain (porcine origin, crystallized) was from Armour and Company, U.S.A. Insulin (crystalline) was from B.D.H., England.

Polyacrylamide gel electrophoresis chemicals viz. acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethyl-ethylenediamine (TEMED), amido black 10B and Coomassie blue G-250 were obtained from Eastman Kodak Company, U.S.A. Glycine was from Koch Light Laboratory, U.K.

Sephadex (G-100, G-75, G-50) was obtained from Pharmacia Fine Chemicals, Sweden. The material was suspended in water and kept on boiling water bath for a specified time for swelling, cooled and deaerated before use.

Phosphate buffers used were of potassium salts unless otherwise mentioned.

Celluloses were washed according to Peterson and Sober (225). The chromatographic columns were used with flow of liquid under gravity without the application of external pressure.

Dialysis tubing of MWCO 10,000 was obtained from from Cole Parmer, USA.

Methods

All the reagents and buffers were prepared in glass distilled water. Centrifugations were carried out at 0°C in an International Centrifuge (Model PR-I and PR-II), Sorvall (Model SS-1) and Spinco (Model I), and at room temperature using a Sharples Supercentrifuge (A: 12 clarifier) and Sharples Supercentrifuge (Laboratory Model). Absorbance measurements were carried out in a Beckman Ultraviolet DU-spectrophotometer or Shimadzu Spectrophotometer using cuvettes with 10 mm light path. Radiometer TTT-60 was used for enzymic titrations. Amino acid analysis was carried out in a Beckman 120C analyser. Isoelectric pH was determined using LKB Isoelectric focusing column.

Inhibitor assay

The activity of the proteinase inhibitor was determined using two types of substrates for the proteinase viz.

(i) Protein substrates (casein, hemoglobin, albumin etc.) and

(ii) Synthetic substrates (BAEE, BAPNA).

1. Protein substrates

The Kunitz (23) spectrophotometric method using casein was generally used for the assay of different proteinases and proteinase inhibitors.

Assay system

2 ml of the reaction mixture contained proteinase or a mixture of proteinase and inhibitor, 200 micromoles

of potassium phosphate buffer, pH 7.5 and 10 mg of casein. Test tubes containing the reaction mixture were incubated for 20 min at 35°C. After 20 min, 3 ml of 5% trichloroacetic acid was added to precipitate the unreacted protein and arrest the reaction. After standing for 30 min, the mixture was filtered and the absorbance of the clear filtrate was measured at 280 nm. Readings were corrected for the values of blanks for casein (reagent blank), enzyme and inhibitor as described below.

Blanks - reagent blank, enzyme blank and inhibitor blank

Trichloroacetic acid filtrates of casein, crude enzyme preparations and crude inhibitor extracts show absorbance at 280 nm and thus interfere in the assay system. Hence these blanks are subtracted from the total absorbance obtained at 280 nm in the assay system, after 20 min incubation.

Reagent blank

The optical density obtained by omitting enzyme and inhibitor from the reaction mixture is the reagent blank and it ranged between 0.080 to 0.150 which was mainly due to casein.

Enzyme blank

For crude enzyme preparation the enzyme blank was obtained by omitting the inhibitor from the reaction mixture. The enzyme was added to the reaction mixture after the addition of trichloroacetic acid. This blank

reading (referred to as zero min blank) when substracted from the 280 nm reading of the trichloroacetic acid filtrate of the 20 min incubated reaction mixture (containing enzyme but no inhibitor) gives the true enzyme activity. Zero min blanks were not necessary for purer preparation of enzyme since the amount of enzyme used had no detectable reading at 280 nm.

Inhibitor blank

When crude preparations of the inhibitor were used two inhibitor blanks viz. zero min and 20 min incubated blanks were run. In both the cases, the enzyme, whose inhibition was being studied, was omitted from the reaction mixture. Zero min blank was obtained by adding the inhibitor to the reaction mixture after the addition of trichloroacetic acid. The other type of blank (20 min incubated blank) was essential in some cases where the seed extract itself showed proteolytic activity. In such cases specific inactivation of the proteolytic activity (and not of the inhibitor) is desirable.

In the present work the crude extract (0 - 0.9 ammonium sulphate precipitate) of Dolichos biflorus seeds did not show any detectable proteolytic activity at pH 7.5.

It was essential to see that the inhibition of the enzyme is not apparent, i.e. is not due to the hydrolysis of the enzyme by the proteolytic activity of the extract itself. This was confirmed by preincubating the enzyme

with the extract for different periods of time before adding the substrate and then testing the activity. This was routinely carried out for purified inhibitors and it was shown that the inhibitor had no proteinase activity.

A typical assay of a proteinase inhibitor (subtilisin inhibitor) from the acid extract of Dolichos biflorus seeds (0 - 0.9 ammonium sulphate precipitate) is described in Table V. The Tables show how the different blanks were taken and how the activity was calculated.

Amount of proteinase and inhibitor in the reaction mixture

The quantity of the proteinase in the reaction mixture was adjusted to give an optical density increase of about 0.3. In the range of 0.1 - 0.3 the proteinase activity (except trypsin) was proportional to the enzyme concentration. In the case of trypsin the enzyme concentration was referred from the standard curve for trypsin (46).

The amount of the inhibitor used in the assay was so adjusted that the decrease in the enzyme activity corresponded to an optical density between 0.05 and 0.150 and the inhibition of the enzyme was not more than 50%. Under these conditions the activity of the inhibitor was found to be proportional to the concentration of the inhibitor.

In some experiments, when the inhibitor concentration was very low, the sensitivity of the assay was increased

TABLE V: A TYPICAL ASSAY OF SUBTILISIN INHIBITOR

	Experiment No.					
	1 Reagent blank	2 Enzyme blank*	3	4 Zero min inhibitor blank	5 20 min incubated inhibitor** blank	6
Subtilopeptidase A (50 ug/ml of 0.1 M phosphate buffer, pH 7.5)		0.1 added after TCA	0.1			0.1
Inhibitor (Protein 1.9 mg/ml of 0.005 M phosphate buffer pH 7.5)				0.1 added after TCA	0.1	0.1
Phosphate buffer (0.1 M, pH 7.5)	1.0	0.9	0.9	0.9	0.9	0.8
Casein 1% solution (in 0.1 M phosphate buffer, pH 7.5)	1.0	1.0	1.0	1.0	1.0	1.0
280 nm reading (O.D. x 10^3)	110	110	495	130	130	425
- Reagent blank (-110)	0	0	385	20	20	315
- Inhibitor blank(-20)			385	0	0	295
Proteinase units/ml (reaction mixture) (-20)			19.25			14.95
Proteinase units/2 ml reaction mixture (x 2)			38.50			29.50
Inhibitor units/0.1 ml inhibitor						9
Inhibitor units/ml inhibitor (x 10)						90

Table V Contd.

Reaction mixture (volume 2 ml) was incubated at 35°C for 20 min and the reaction mixture was stopped by adding 3 ml of 5% trichloroacetic acid. The solutions were filtered and filtrates were read at 280 nm.

*Enzyme blank (experiment No. 2) was not necessary for this assay since pure preparation of enzyme was used.

**20 min incubated blank for inhibitor (Experiment No. 5) was also not needed since the crude inhibitor used (0 - 0.9 ammonium sulphate precipitate of the acid extract of seed) does not show any proteolytic activity at pH 7.5.

by using longer incubation times (upto 1 h). During incubation for 1 h the enzyme concentration was reduced to one third of the amount required for 20 min assay. Enzyme activity was proportional to the concentration and time of incubation.

Trypsin was also standardized with crystalline soyabean trypsin inhibitor (23), on the basis that 1 mg of the inhibitor inhibits 1:1 mg of pure trypsin. Trypsin, chymotrypsin and ficin were also standardised by using caseinolytic assay curves of Kunitz (46), Laskowski (226) and England *et al*. (227), respectively.

A unit of proteinase was defined as the activity which during the cleavage of substrate (casein) gave rise to an increase of 0.001 of absorbance at 280 nm per min, per ml of the reaction mixture under the assay conditions (46). An inhibitor unit was expressed in terms of units of proteinase inhibited and was defined as the quantity of inhibitor which caused a decrease of 0.001 in optical density at 280 nm, per min, per ml of the reaction mixture.

(ii) Synthetic substrates

With highly purified preparations of the inhibitor, inhibition of the proteinase was also determined by the use of synthetic substrates e.g. (BAEE for trypsin and subtilisin).

During the assay using synthetic substrates, proteinase blank of the inhibitor was not necessary since highly purified inhibitors were used. One unit of the inhibitor corresponded to a decrease in hydrolysis of 1 μmole of synthetic substrate per min. Specific activity was expressed as units per mg of protein.

Assay for trypsin inhibitor

The method of Schwert and Takenaka (228) was used with minor changes. The reaction mixture (Volume 1 ml) consisted of 2 to 3 μg of trypsin, 0.5 to 1.5 μg of purified inhibitor, 1 μmole of BAEE and 100 μmoles of phosphate buffer, pH 7.0. A control was also run with trypsin without the inhibitor. The reaction mixture was incubated at 26°C and the increase in optical density at 253 nm was monitored for 5 min. Enzyme activity was proportional to the increase in optical density till the difference in optical density was not more than 0.200.

Assay for chymotrypsin inhibitor (229)

Assay was carried out with BTEE as the substrate. The reaction was carried out at 30°C in a total volume of 1 ml containing 0.5 μmole of BTEE, 40 μmoles of Tris, HCl, pH 7.8, 40 μmoles of $CaCl_2$ pH 7.8, 0.3 - 1.0 μg of chymotrypsin and the inhibitor. Increase in absorbance at 256 nm was recorded for a period of about 5 min. A control for chymotrypsin without inhibitor was also run. The enzyme activity was proportional to the increase

in absorbance when the change in absorbance was less than 0.120 O.D.

Assay for subtilisin inhibitor

Titrimetric procedure using pH-stat (230) was used for the assay of subtilisin inhibitor. The hydrogen ions liberated during the hydrolysis of the ester substrate (BAEE) were titrated against 0.02 M NaOH at pH 8.0 at 37°C. 10 ml of the reaction mixture contained 500 μmoles of BAEE, 1000 μmoles of KCl and 100 μg of subtilopeptidase A with and without 30 μg of the subtilisin inhibitor. Pure subtilopeptidase A has a KC (Sec^{-1}) value of 16.1.

The purity (the amount of functional enzyme) of different proteinases (trypsin, chymotrypsin and subtilisin), used in the assay was ascertained by comparing with the standard curves obtained by various workers (Table VI) using synthetic substrates as well as casein for assay. Percent purity of different proteinases used in this work was found to be: trypsin, 52; chymotrypsin 90 and subtilisin, 53.

Quantities of the proteinases mentioned in the present work represent the amount of enzyme only. Values of Kunitz units by caseinolytic assay corresponding to 1 mg of pure proteinase are given in Table VI.

TABLE VI : PURITY AND PROPERTIES OF PURE PROTEINASES

Proteinase	Molecular weight (Mr)	Optical factor	Substrate used	$M^{-1}cm^{-1}$	Specific activity of pure proteinase Units/mg	Reference
Trypsin	23,300	0.67	BAEE	808 (253 nm)	70	(228)
			Casein		6000	
Chymotrypsin	25,300	0.50	BTEE	964 (256 nm)	48	(229)
			Casein		5000	
Subtilisin	27,600	1.04	BAEE		35	(230)
			Casein		83009	
Papain	21,000	0.4	BAPA	8800 (410 nm)		(231)
			BAEE			
			Casein		5500	(232)

Protein

Protein estimations were carried out according to the method of Warburg and Christian (233). It was assumed that 0.1 percent solution of protein shows an optical density of 1.0 at 280 nm for 1 cm light path. A blank was taken using a buffer of the same composition.

Protein was also determined by the method of Lowry *et al*. (234), using cyrstalline bovine serum albumin as a standard. Serum albumin concentration was determined from its extinction coefficient at 280 nm ($E_{1\%}^{1\ cm} = 6.6$) according to Cohn *et al*. (235). Interfering substances such as phosphates, ammonium sulphate and glucose were removed by dialysis before estimating the protein. The protein determinations during purifications were made by measuring absorbance at 280 nm assuming an absorbance of 1 to be equivalent to a protein concentration of 1 mg per ml. For all other studies the protein determinations were made by Lowry's method using crystalline bovine albumin as standard. The specific activities were usually presented in terms of protein determined by the spectrophotometric method but the values for the final purified inhibitors were given in terms of the Folin's method since the optical factor was not 1 in all cases.

Phosphorus

Inorganic phosphate was determined by the method of Fiske and Subba Row (236).

Ammonium sulphate precipitation

Precipitation with ammonium sulphate was carried out at 0°C under constant stirring. The precipitates were collected by centrifugation after keeping the solutions for a period of minimum one hour. The concentrations of ammonium sulphate calculated at 0°C were according to Jagannathan et al. (237). The equation used for solid ammonium sulphate was

$$X = \frac{50\ (S_2 - s_2)}{1 - 0.28 \times S_2}$$

and the equation for the addition of saturated ammonium sulphate solution was

$$Y = \frac{100\ (S_2 - S_1)}{1 - S_2}$$

where X is grams of solid ammonium sulphate to be added for every 100 ml and Y is ml of saturated ammonium sulphate solution to be added for every 100 ml. S_1 and S_2 are the initial and final saturations of ammonium sulphate, respectively at 0°C.

Dialysis

Dialysis was carried out in cold at 3 to 5°C against 100 volumes of buffer under stirring for a period of 24 h and by giving frequent changes (atleast three changes) of the buffer. For large scale dialysis, especially of the ammonium sulphate precipitates obtained during the purification of the inhibitors, several changes of

the buffer were given till the dialysates were free from sulphate ions as tested by $BaCl_2$ - HNO_3. For this dialysis 20 to 50 fold volume of the buffer was used each time.

Polyacrylamide gel electrophoresis

Acrylamide gels were prepared as described by Davis (238) with slight modification. Riboflavine or ammonium per sulphate was used for polymerisation. Cylindrical gels were of 4 to 5 mm diameter and 7.5% in acrylamide concentration. Tris-glycine buffer (pH 8.3) was used in the electrode compartments. The concentration of this buffer was 0.0025 M with respect to Tris and 0.02 M with respect to glycine. Electrophoresis was run at 0°C with 3 - 5 milliamps current per gel for a period of 90 - 120 min and the protein was stained with Coomassie blue G-250 solution prepared according to the procedure of Blakesley and Boezi (239). Relative mobility of the protein concerned was calculated as the ratio of the distance travelled by the protein to the distance travelled by the marker (bromophenol blue).

Preparative polyacrylamide gel electrophoresis

Preparative polyacrylamide slab gels were used for the purification of the subtilisin inhibitor. This was according to the method of Ghadge *et al*. (240) developed in this Laboratory. The apparatus consisted of two vertical chambers, which formed the electrode compartments, adjacent to each other and were separated by a common partition

wall with a 0.8 cm gap at its base. One cm thick gel was cast at the base of the chamber which sealed the gap (0.8 cm) kept below the partition wall and made the two chambers, separate and leakproof. This basal gel (contact gel) also helped in keeping electrical contact between the chambers. Gel cassettes were placed above the contact gel. 7.5% acrylamide gel was polymerized in cassettes, bath buffer was filled in the two chambers and electrophoresis was carried out. Thus in this device one can make the gel as well as run the electrophoresis in the same apparatus.

Electrophoresis at alkaline pH

Solutions were prepared according to Davis (238). Both upper and lower electrode buffers contained 0.005 M Tris and 0.04 M glycine, pH 8.3. The gel composition was 0.38 M Tris and 0.06 M HCl, pH 8.9. 7.5% acrylamide 0.18% Bis, 0.03% TEMED and 0.07% ammonium persulfate.

Composition of contact gel

Contact gel was made in the bath buffer. Its composition was 0.005 M Tris and 0.04 M glycine, pH 8.3, 7.5% acrylamide, 0.18% Bis, 0.03% TEMED and 0.07% ammonium persulfate. Contact gel can be reused several times by preserving the gel in cold under a layer of the buffer.

Operating procedure

Two slab gels (16.5 x 11.0 x 0.9 cm) were prepared. About 50 mg of protein with tracking dye (bromophenol blue) was loaded on each gel. About 1.5 lit Tris-glycine

buffer, pH 8.3 was filled in both the electrode chambers. A voltage of 100 V and current of 30 mA were applied and electrophoresis was carried out for about 30 h until the tracking dye reached to 1 - 2 cm above the bottom of the gel. The gels were removed and the inhibitor was eluted using an electrophoretic elution apparatus developed during this work (described in Section III).

Isoelectric point

Isoelectric point of the inhibitor was determined according to the method described by Vesterberg and Svendson (241). The isoelectric focusing column (LKB Producter AB, Broma, Sweden) of 110 ml capacity was used. Density gradient was made with sucrose using an automatic gradient mixer. Dense solution, light solution and electrode solution were prepared as given in LKB instruction manual. The purified inhibitor was dialyzed against 0.001 M K-PO_4 buffer, pH 7.5 to reduce salt concentration. Ampholine Carrier Ampholytes and inhibitor solution were mixed in light and dense solutions in equal amounts before generation of the gradient. Ampholine Carrier Ampholytes (1% w/v) in the pH range of 3.5 - 10 was used for the run. Electrodes were placed so that anode was at the bottom of the column. Electro-focusing was carried out at 4°C for 48 h. After completion of electrofocusing the column was emptied at a rate of 30 - 40 ml/h and fractions of approximately 1 ml were collected. Fractions were processed for the determination of pH, activity and protein.

Dissociation constant

The dissociation constant of the enzyme (proteinase) inhibitor complex was calculated by the method of Green and Work and Bieth (242, 243). An inhibition curve was obtained by adding increasing amounts of inhibitor to a fixed amount of enzyme near the equivalence point. The residual enzyme activity in the presence of one equivalent of inhibitor was used to determine an approximate dissociation constant for the equilibrium, $EI \rightleftharpoons E + I$, where E is enzyme (proteinase) and I is inhibitor.

Ultraviolet absorption spectrum

For the determination of ultraviolet absorption spectrum, the inhibitor was dialyzed against 0.001 M $K\text{-}PO_4$ buffer, pH 7.5 and the optical densities at various wavelengths in the ultraviolet region (190 - 400 nm) was recorded in the UV spectrum graph using the same buffer as blank.

Optical factor of the inhibitor was determined as the reciprocal of its absorbance at 280 nm, when the protein concentration was 1 mg/ml. Thus higher optical factor shows a lower content of aromatic amino acids and vice versa.

Molecular weight determination of inhibitors

Three different methods were used for the determination of molecular weight of pure inhibitors.

(1) SDS-polyacrylamide gel electrophoresis

The method of Shapiro *et al.* (244) and Weber and Osborn (245) was used with slight changes. SDS minimizes the native charge differences of the proteins and thus all the proteins migrate as anions due to the formation of complexes with SDS. The migration rate is then proportional to the molecular weights.

To 50 µg of inhibitor in 0.1 ml water, was added 0.1 ml of a solution containing 2% thioethanol and 0.2M sodium phosphate buffer, pH 7.2. The mixture was incubated at 37°C for 3 h. The denatured and reduced solution of the inhibitor was then dialyzed overnight at room temperature against 100 ml of a solution containing 0.1% SDS, 0.1% thioethanol and 0.01M sodium phosphate buffer, pH 7.2.

Gels (length 10 cm and diameter 0.6 cm) were made containing 10% acrylamide, 0.13% Bis, 0.1% SDS, 0.05% TEMED and 0.1M sodium phosphate buffer, pH 7.2 and 0.0005% riboflavin. The bath buffer contained 0.1% SDS and 0.1M sodium phosphate buffer, pH 7.2. For each gel 0.03 ml of tracking dye (0.05% bromophenol blue), 0.2 ml 20% sucrose and 0.2 ml of the above treated inhibitor solution were mixed and loaded. Current used for each gel was 8 mA and 200 V. The anodic compartment was in the lower chamber. Electrophoresis was discontinued after about 4 h when marker moved about three fourth

the distance of the gel. Gels were fixed in 0.5% amido black in 7% acetic acid for 24 h, and destained with 7% acetic acid. Cytochrome C (13000), myoglobin (17000) and egg albumin (43000) were used as standard proteins.

The migration of bromophenol blue was used as a reference point within each gel. When the electrophoretic mobilities (distance of protein migration/distance of dye migration) were plotted against the logarithm of the molecular weights of known proteins a smooth curve was obtained and from this curve the molecular weight of the unknown protein was calculated.

(2) Polyacrylamide gel electrophoresis (slope method)

The molecular weight of native proteins using gel electrophoresis was determined according to the method described by Hedrick and Smith (246). Separation gels with various concentrations of acrylamide (7.5% to 20%) were prepared according to Davis (238) except that acrylamide to bis ratio of 30:1 was maintained constant in all the gels. Samples (50 µl) in 20% sucrose and 0.05% bromophenol blue were layered on the top of the gels. Electrophoresis was carried out at 3 mA/tube. for 3 h at 4°C. Under these conditions proteins migrate into the gel as a function of their size, charge and the acrylamide concentration of the gel. At the end of the run dye front was marked by inserting nylon thread. The gels were stained with Coomassie blue G-250 and

the ratio (Relative mobility (R_m) of the distance of the migration of the protein to that of bromophenol blue for various gel concentrations was determined. Plots of log molecular weight versus gel concentration gives straight line with slopes which are linearly related to the molecular weight of the proteins. The molecular weight of the inhibitor was determined from the slope on the concentration curve.

(3) Gel filtration

Molecular weight determination of the inhibitors by Sephadex G-50 filtration was carried out as described by Andrews (247). Sephadex G-50 was suspended in water and kept for swelling in boiling water bath for one hour. After cooling, the material was packed in a Pharmacia column (100 x 1.5 cm) and was equilibrated by passing 500 ml of 0.05 M phosphate buffer. 1 ml of 0.05 M phosphate buffer, pH 7.5 containing a mixture of 0.5 mg of soyabean trypsin inhibitor, 1 mg myoglobin, 1 mg cytochrome C, and 1000 units of the inhibitor was loaded on the column and eluted with the same buffer. Flow rate was 18 ml per hour and 2 ml fractions were collected. The fractions were assayed for the different proteins. Cytochrome C and myoglobin were estimated at 550 nm and 415 nm respectively (248, 249). Trypsin and subtilisin inhibitors were assayed by the casein digestion method of Kunitz (23). The molecular weight of the inhibitor was then determined by plotting the elution volume versus

logarithm of molecular weight of the standard proteins.

Amino acid analysis

Amino acid composition of the inhibitor was determined by hydrolyzing it according to Spackman (250). Three samples (1 mg each) were dissolved in 2 ml of 6 N HCl in test tubes. After evacuating and sealing the tubes the samples were hydrolyzed for 24, 48, and 72 h at 110°C. Excess of acid was removed by vacuum evaporation at room temperature (30°C). Evacuation was repeated three times, each time using 2 ml of water for dissolving the hydrolysate. Proline, threonine and serine are partially destroyed on acid hydrolysis. Hence correction was applied by extrapolating the results to zero hydrolysis time.

Determination of tyrosine and tryptophan

Protein shows selective absorption in ultraviolet region and absorption maximum varies with pH. Majority of the amino acids do not show any absorption in the range of 250 - 320 nm. It is known that phenylalanine tyrosine and tryptophan show absorption in the ultraviolet region. In 0.1 M NaOH, absorption of tyrosine and tryptophan is more and that of phenylalanine is negligible. Under these conditions protein solutions may be treated as a two component system for spectrophotometric analysis. The intensity of absorption at the point where the curves for tyrosine and tryptophan intersect is a direct measure

of the total molar solute concentration and will be the same however the proportions are varied. At any other wave length the intensity of absorption will vary with the relative proportions of the components. Using 0.1 N NaOH as a solvent the two absorption curves intersect at 294.4 nm (ϵ = 2375) and 275.15 nm (ϵ = 2738). By determining the absorption of the protein in 0.1 N NaOH at the above two wavelengths and at any other wavelength (e.g. at 280 nm), it is possible to determine relative proportions of tyrosine and tryptophan in the protein.

Thus if X = total mol/l in solution

Y = g mole of tyrosine

X-Y = g mole of tryptophan

At any wavelength other than the point of intersection, let ϵ tyrosine be A, ϵ tryptophan be B and the observed intensity of absorption for a 10 mm cell be E, then

$$E = YA + (x - y) B \text{ or}$$

$$Y = \frac{\epsilon - XB}{A - B}$$

$$X = \frac{\text{absorption value at an intersection}}{\epsilon \text{ at an intersection}}$$

Determination of free sulfhydryl groups

Free -SH groups were assayed according to the method of Ellman (252). When the protein was treated with 5-5'-dithiobis-2-nitrobenzoic acid (DTNB) the increase

in absorbance at 412 nm was proportional to the sulfhydryl content. Molar extinction of 13,600 was used to calculate the thiol concentration using reduced glutathione as a standard.

In a 1 ml cuvette with 10 mm light path, 0.1 ml of the inhibitor solution (containing 1 mg of the inhi-itor) 0.9 ml of 10 M urea (in 0.05 M phosphate buffer, pH 7.5) and 0.02 ml of 0.01 M DTNB were added and the increase in absorbance at 412 nm was measured.

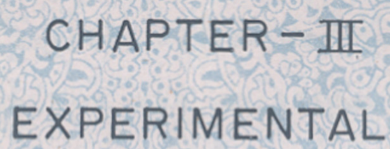
Determination of total sulfhydryl and disulfide groups in protein

Determination of thiol (-SH) and disulfide (S-S) groups were carried out by the method described by Cavallini *et al*. (253). In this method sodium boro-hydride ($NaBH_4$) in 8 M urea was used as a reducing agent, and DTNB was used as a thiol disulfide exchanger. Increase in absorbance at 412 nm was measured and the number of sulfhydryl group (N) formed was calculated using the following formula:

$$N = \frac{Mw \times A \times V}{12000 \times M}$$

where Mw is molecular weight of the protein sample, A is absorbance, V is volume of the final solution and M is weight in mg of the protein sample analyzed. Accuracy of the method was checked with bovine serum albumin as a standard.

Reduction and colour development of the sample and the standard were carried out in test tubes (18 x 110 mm) with marks at 3 ml and 6 ml. The following reagents were added to the test tubes in the order shown: 1.44 g of solid urea; 0.1 ml of 0.1 M EDTA; pH 7.5, 1 mg of the purified inhibitor in 0.1 ml of water and 1 ml of 2.5% $NaBH_4$ (prepared just before use) and water to make up to 3 ml. After dissolving the urea by shaking at 38°C, the mixture was incubated at the same temperature for 45 min. 0.5 ml of 1 M KH_2PO_4 in 0.2 M and 2 ml acetone were added, one after the other at an interval of 5 min to destroy the excess borohydride. Nitrogen was bubbled for 5 min through it and 0.5 ml of 0.01 M DTNB was added to the mixture and nitrogen was again passed for 1-2 min. The solution was kept for 15 min and the absorbance at 412 nm was measured.



CHAPTER – III

EXPERIMENTAL

SECTION I

DEVELOPMENT OF SIMPLE SEPARATION METHODS

During the course of this work following simple, efficient and useful techniques/devices were developed:

1. Electrophoretic elution of proteins from gel.
2. Method for removal of high concentration gels.
3. Use of fluorescent markers for the location of protein bands.
4. Polymerization of acrylamide at acid pH using uranyl nitrate.

These methods were successfully employed in the present work for the purification and characterization of the inhibitors.

1. Electrophoretic elution of proteins from gel.

A simple and efficient electrophoretic method for the elution of proteins from the gel was developed (254). Proteins were eluted vertically through the thickness of the gel by keeping the gel in a horizontal position on polyurethane foam from which the eluted proteins can be easily recovered by squeezing. This method was used, for the elution of the inhibitor from polyacrylamide gel during its purification.

Elution equipment

The design principle: Gel from which protein is to be eluted is placed horizontally on a recovery cell assembly containing foam strips. Vertical downwarm electrophoretic elution of the proteins is carried out

which get collected in the foam. The escape of proteins from the foam is prevented by wrapping the recovery cells with semipermeable membrane. Since the elution is carried out through the thickness of the gel, the distance travelled by the protein is very short. This results in rapid elution of proteins.

The mode of construction

Figures 1 and 2 give a general idea regarding the design and the method of elution. The main part of the elution unit, the recovery cell assembly (Fig. Id) consists of six to seven recovery cells with frames made from perspex strips of 1 to 1.5 mm thickness into which foam pieces are inserted. Each recovery cell frame with inner dimension 75 x 8 x 15 mm is open at the top as well as at the bottom. For the experiment each frame is wrapped in cellulose casing (with rubber bands or by tying knots at both the ends of the cell) and only the top portion of the cell is kept open. Cellulose casing (molecular weight cut off of 2000) was used in preference to dialysis membrane for holding low molecular weight proteins. The inner top edges of the walls, along the length of the recovery cells are tapered to minimize the wall thickness between the two cells. A slightly loose fitting foam is inserted in the recovery cell. The buffer holding capacity of the foam in each cell was between 8 to 10 ml. The height

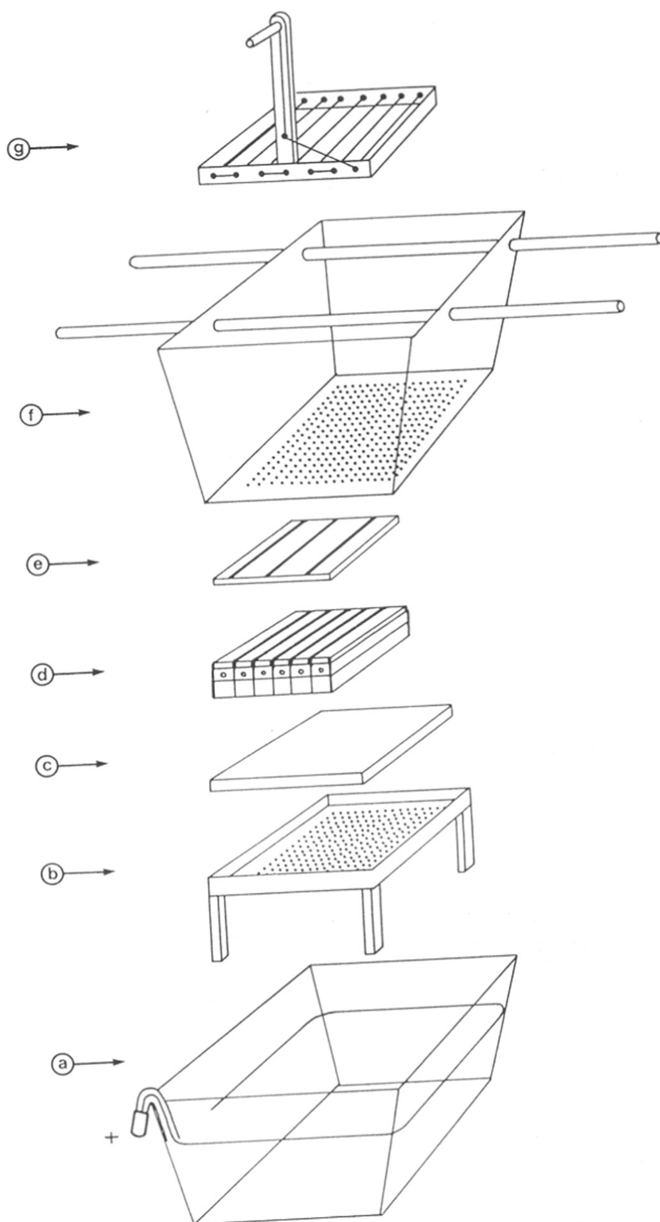


FIG. 1. Elution unit: (a) lower buffer chamber (12.5 × 11 × 6 cm) with platinum electrode, (b) perforated Perspex platform (8 × 8 × 0.5 cm; height of the legs, 4.5 cm), (c) contact Foam (8 × 8 × 0.5 cm), (d) recovery cell assembly (7.5 × 0.8 × 1.5 cm), (e) gel to be eluted (7.5 × 7.5 × 0.3 cm) showing positions of protein bands, (f) upper buffer chamber (12.5 × 11 × 8 cm) with perforated base in which two rods are inserted through its brim for keeping the chamber on a stand, and (g) upper platinum electrode (8 × 8 cm).

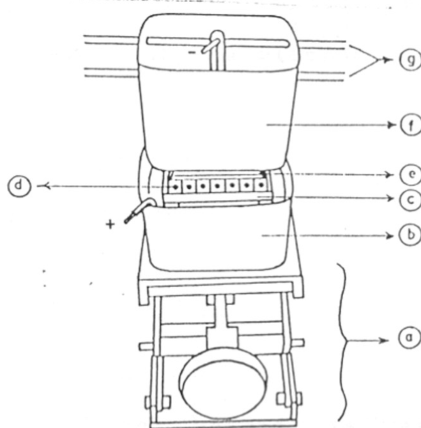


FIG. 2. Elution unit assembled: (a) laboratory jack, (b) lower buffer chamber (12.5 × 11 × 6 cm), (c) perforated Perspex platform with contact foam (8 × 8 × 0.5 cm; height of the legs, 4.5 cm), (d) recovery cell assembly (7.5 × 0.8 × 1.5 cm), (e) gel to be eluted (7.5 × 7.5 × 0.3 cm), (f) upper buffer chamber (12.5 × 11 × 8 cm), and (g) supporting rods.

of the foam strip is kept 2 mm more than the cells to make a proper contact with the gel to be eluted. Dimensions of the lower buffer chamber (Fig. 1a) are 12.5 x 11 x 6 cm. Platinum wire is wound around its inner surface to make the lower electrode. The perforated perspex platform (8 x 8 cm) (Fig. 1b) is supported by four legs of 4.5 cm height. A 0.5 cm collar of the perspex strip is made around the perforated plate so that the contact of the 'contact-foam' with the lower buffer is attained only from the base of the plate. The total height of the platform is 5.5 cm. The contact foam (8 x 8 x 0.5 cm) (Fig. 1c) is placed on the perforated platform and embedded in the lower buffer. The recovery cell assembly rests directly on the contact foam. The upper electrode chamber (12.5 x 11 x 8 cm) (Fig. 1f) is similar to the lower chamber (Fig. 1a). However in this case the central portion (8 x 8 cm area) of the bottom of the chamber is perforated. During the run the bottom of the chamber is covered with a dialysis membrane (27 x 23 cm) and tied on the chamber with rubber bands. The chamber is made leakproof by covering its inner bottom with a 1 cm thick layer of 7% polyacrylamide, (0.005 M Tris and 0.04 M glycine, pH 8.3 0.18% Bis, 0.03% TEMED and 4 ug/ml riboflavin).

The construction of the holder of the upper platinum electrode is also different from that of the lower electrode (Fig. 1a). It is a frame (8 x 8 cm) made from perspex

strips and the platinum wire is wound in rows parallel to each other. The electrode rests on the bottom of the upper chamber.

Operating procedure

The lower buffer chamber (Fig. 1a) with the platform and the contact foam (Fig. 1b and 1c) is filled with the bath buffer untill it reaches the platform collar and covers half of it. The foam in each recovery cell is also soaked in the appropriate buffer (Tris-HCl). The recovery cells (Fig. 1d) are assembled together with a rubber band and placed on the contact foam. The gel to be eluted (Fig. 1a) is then placed horizontally on the recovery cell assembly in such a way that bands to be eluted lie parallel to the length of the recovery cells. The upper electrode filled with bath buffer chamber (Fig. 1f) is kept on a stand with the help of two rods (Fig. 2g) and brought in contact with the gel to be eluted by raising the lower buffer chamber with a laboratory jack (Fig. 2). The upper chamber with the sealing gel and the recovery cells with the wrapped cellulose casing can be reused, if they are preserved in their respective buffers.

Buffer solution for elution under alkaline condition

Both upper and lower chambers contained 0.005 M Tris and 0.04 M glycine, pH 8.3. The foam strips in the recovery cells contained 0.05 M Tris and 0.013 M HCl, pH 8.1.

All the electrophoretic experiments were performed at 5 - 10°C in cold room.

2. <u>Method for removal of high concentration gels; (255)</u>

A simple technique was developed for the easy removal of high concentration gel rods. Usually it is very difficult to remove high concentration acrylamide gels (more than 10%) from the glass tubes without breaking the glass tube or gel. The use of mylar sheet while casting the gel was found to be convenient for the easy and effective removal of high concentration gels.

<u>Procedure</u>

Polyacrylamide gels (15 - 30% concentration) were made in glass tubes (10 x 0.7 cm) using the following procedure. A mylar sheet or a polyester sheet (12 x 2.5 x 0.005 cm) was folded in the form of a tube and inserted into the glass tube leaving an extra 1.5 cm portion of the sheet above the tube. Acrylamide of high concentration (15 - 30%) was polymerised by pouring it in the assembly by the conventional method of Davis. Electrophoresis of 50 µg of subtilisin inhibitor in each gel tube was carried out as usual.

After the electrophoretic run the gel was removed by pulling out the extra portion (1.5 cm) of mylar sheet. The gel wrapped inside the mylar roll comes out very easily. Fig. 3 gives a general outline of the procedure.

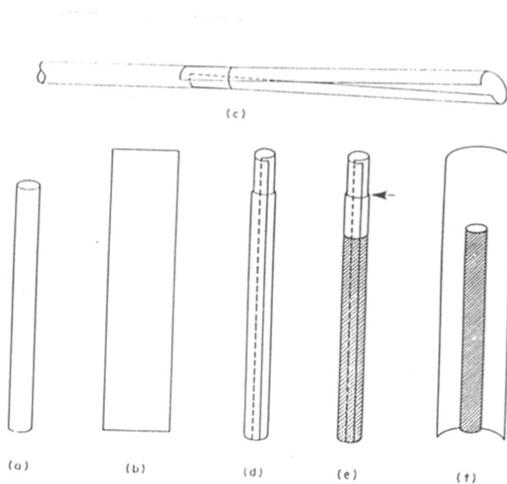


FIG. 3. Procedure for easy removal of high concentration PAG from glass tube: (a) glass tube (10 × 0.7 cm i.d.); (b) Mylar sheet (11.5 × 2.5 cm, thickness 0.05 mm); (c) rolled Mylar sheet being pushed in the glass tube; (d) Mylar sheet placed in the form of a tube inside the glass tube; (e) the assembly (d) filled with high-concentration PAG (arrow indicates the junction between the glass tube and Mylar sheet); (f) pulled-out Mylar sheet (along with the gel) exposing intact and unharmed gel after electrophoresis.

3. Use of fluorescent markers for the location of protein bands (256)

Fluorescamine treated casein hydrolysate when subjected to electrophoresis showed fifteen different bands under UV. These fluorescent bands were used as markers for the location and subsequent purification of the subtilisin inhibitor.

Experimental procedure

Preparation of markers:Peptic digestion of casein: To a 20 ml of 1% casein solution (pH 1.8) 8 mg of pepsin (3000 units/mg) were added and incubated at 35°C for 2 h after which pepsin was destroyed by boiling the contents for 10 min in a boiling water bath. The contents were then centrifuged and after adjusting the pH to 7.5 with 0.1 M NaOH the supernatant was dialysed against equal volume of 0.001 M phosphate buffer, pH 7.5. Alternately the peptic digest was passed through Amicon UM 10 membrane. To 0.04 ml of this dialysate or Amicon filtrate corresponding to 200 µg casein 0.05 ml (50 µg) of fluorescamine was added and allowed to remain at room temperature for 15 min. By this time excess fluorescamine is completely destroyed. The above fluorescent peptide markers were loaded on 7.5% acrylamide gel tubes. After completion of electrophoresis fluorescent bands were visualised under UV (390 nm) (Fig. 4).

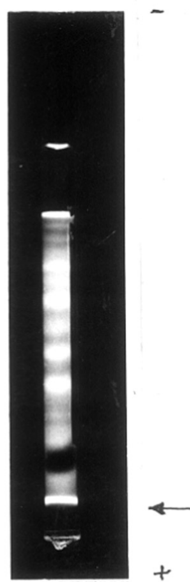


Fig. 4: Electrophoretic pattern of fluorescent peptide markers

Position of the dye marker (indicated by arrow) corresponds to the last fluorescent band.

Location and purification of subtilisin inhibitor

In a control experiment 0.4 ml (2 mg) of the above peptide markers were treated with 0.5 ml (500 μg) of fluorescamine and incubated for 15 min at room temperature. After 15 min incubation, crude extract containing subtilisin inhibitor (1 ml, 250 units) was added to the fluorescent markers and the mixture was loaded on analytical polyacrylamide gel tube (10 x 2 cm). After completion of electrophoretic run, the gel was visualised under UV and was cut at the marker bands into different pieces and each piece was extracted with 50 mM PO_4 buffer and estimated for subtilisin inhibitor activity. It was observed that the position of subtilisin inhibitor on the gel corresponded to the gel portion between 3rd and 4th marker band. Once the position of subtilisin inhibitor on the gel with respect to the marker bands was ascertained, it was possible to cut the active bands on a preparative gel, directly, between 3rd and 4th marker band after visualising the markers under UV. The active band could be further eluted by the electro-elution technique developed in this work.

4. Polymerization of acrylamide at acid pH using uranyl nitrate(266).

A new photopolymerizing agent, uranyl nitrate, is used for the polymerization of acrylamide gels at low pH. The amount of uranyl nitrate (0.2 mg/ml) required for the polymerization of gels at pH 3.0 is considerably less than that of persulfate (7 mg/ml). Use of this reagent obviates the need for the removal of excess of persulfate by preelectrophoresis. The electrophoretic separation of basic proteins in uranium-polymerized gels showed faster movement and better resolution of proteins and proved the gels to be versatile, uniform, and reproducible. Electrophoresis of trypsin in these gels does not affect the enzymatic activity. The catalyst can also be used for the polymerization of gels containing 3 M urea.

Experimental procedure

Preparation of uranium-polymerized (U-polymerized) gels(267): The gels were prepared according to Maurer (267) except that uranyl nitrate (0.2 mg/ml) was used as a polymerizing agent instead of persulfate. Polymerization was carried out under sunlight or UV light and was complete in 30 min.

Electrophoresis of a mixture of proteins containing 50 ug of cytochrome C, myoglobin, trypsin inhibitor, serum albumin, and ferritin was carried out simultaneously in acrylamide gels polymerized using persulfate or uranyl

nitrate (Fig. 5a, 5b). The separation of protein was significantly better in U-polymerized gels than in persulfate-polymerized gels. Also the movement of protein was faster in U-polymerized gels. It also shows the separation of proteins in U-polymerized gel containing 3 M urea. The results of this run show that uranyl nitrate can also be used as polymerization agent for the acrylamide gels containing urea. Figure 6 demonstrates the use of the new catalyst for the separation of histones. Here again the separation is better in U-polymerized gels than in persulfate polymerized gels.

Electrophoresis of trypsin was carried out in U-polymerized gels and elution of the protein from the gel resulted in 95% recovery of the trypsin activity in the eluates. These results show that uranium does not seem to interfere with the biological activity of the protein.

In the present work PAGE of the inhibitors at pH 2.9 was carried out using U-polymerized gels.

Fig. 5: Electrophoresis of cytochrome c, myoglobin, trypsin inhibitor, serum albumin, and ferritin
(a) persulfate-polymerized gel;
(b) U-polymerized gel, and
(c) U-polymerized gel containing urea

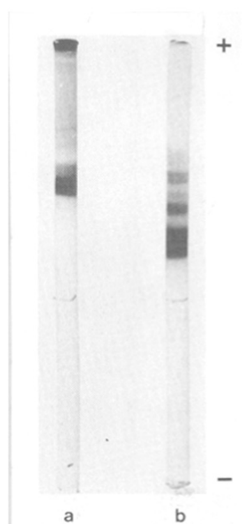


Fig. 6: Electrophoresis of histones

(a) persulfate-polymerized gel, and
(b) U-polymerized gel

SECTION II

PURIFICATION OF PROTEINASE INHIBITORS

Section IIA: Occurrence of inhibitors inhibiting different proteinases.

Preliminary work on proteinase inhibitors showed that extracts of different leguminous seeds inhibit trypsin, chymotrypsin, papain and subtilisin.

The occurrence of inhibitors of various proteinases was investigated in the seeds of nine leguminous plants, viz. cow pea/cholai (*Vigna catjang*), winged bean (*Phosphocarpus tetragonolobus*), soyabean (*Glycine max*), horse gram/hulga (*Dolichos biflorus*), masur bean (*Lens esculentum*), gram bean (*Cicer arientinum*), mung bean (*Phaseolus aerus*), lima bean (*Phaseolus lunatus*) and wal beans (*Dolichos lablab*).

Extraction of seeds

100 g of seeds were washed with distilled water to remove the traces of preservatives on the seeds and then soaked in distilled water for 30 min at 30°C. All other operations were carried out at 0 - 4°C, unless otherwise stated. Seeds were blendorized for 2 min in 500 ml of 0.12 M HCl containing 7.5 g of KCl and the slurry was allowed to stand for 30 min with occassional stirring. It was squeezed through muslin cloth, neutralized with 2M $KHCO_3$ to pH 6.0 and centrifuged at 10,000 g.

The clear supernatant liquid (460 ml) was precipitated with solid ammonium sulphate at 0.9 saturation (60.1 g/100 ml). The precipitate was dissolved in 30 ml of 0.01 M potassium phosphate buffer, pH 7.5 and dialyzed against 3-4 changes of one litre each of 0.01 M phosphate buffer, pH 7.5. The solution after dialysis was centrifuged and the clear supernatant liquid (Vol. 75 ml) was estimated for the inhibitor activity against various porteinases. The activities are summarized in Table VII.

Isolation of the inhibitors was necessary to establish whether they are separate inhibitors specific for each enzyme or whether they are non-specific (polyvalent). The seeds of *Dolichos biflorus* (horse gram) showed the presence of appreciable amount of inhibitors, and were easily available in the local market. Hence work on the subtilisin and trypsin inhibitors from these seeds was undertaken.

TABLE VII : INHIBITION OF PROTEINASES BY DIFFERENT LEGUMINOUS SEED EXTRACTS

Inhibitor	Inhibitor activity (u/g seeds)								
	Cholai seeds	Winged bean	Soya-bean	Horse gram seed	Masur seeds	Gram seed	Mung seed	Lima bean	Wal bean
Trypsin	14400	25920	48000	18720	5465	23184	13500	ND	ND
Chymotrypsin	5760	64800	5760	14040	3643	7617	90	1386	ND
Papain	211	216	166	125	167	33	360	370	ND
Subtilisin	480	1296	640	624	355	183	775	739	300

ND = Not determined

The seed extracts were precipitated to 0.9 saturation with ammonium sulphate and the precipitate was dissolved, dialysed and estimated for inhibitor activity.

Section IIB: Separation and purification of subtilisin and associated trypsin inhibitor from the seeds of horse gram

Step I: Extraction

Three kg of horse gram seeds were washed, dried at 30°C and ground in a wiely mill. The seed flour was extracted for 1 h with ice cold 18 l of 0.12 M HCl containing 225 g KCl. The slurry was squeezed through muslin cloth. The filtrate (15.5 l) was neutralized with solid $KHCO_3$ (185 g) to pH 7.5 and kept for 1 h. The neutralized filtrate was centrifuged in the Sharples super centrifuge at 10,000 g (supernatant liquid Vol.13.5 l).

Step II: Ammonium sulphate precipitation (0-0.9 saturation)

The supernatant liquid from Step I was precipitated to 0.9 saturation with 8500 g of ammonium sulphate with constant and gentle stirring till it dissolved completely. The solution was allowed to stand for atleast 1 h and centrifuged in a Sharples centrifuge laboratory model at 20,000 g. The supernatant liquid was discarded and the precipitate was dissolved in 825 ml of 0.001 M potassium phosphate buffer, pH 7.5 and dialysed against 3 to 4 changes of the same buffer and centrifuged (2810 ml).

Step III: Precipitation of protein impurities at pH 5.0

Phosphate buffer concentration of the supernatant liquid from Step II was adjusted to 0.01 M by adding 8 ml of 1 M phosphate buffer, pH 7.5. 16 ml of 1 M

HCl was added to the solution to bring down the pH to 5.0, kept for 30 min and the precipitate obtained was removed by centrifugation at 10,000 g for 30 min. Supernatant liquid was readjusted to pH 7.5 with solid $KHCo_3$ (2770 ml).

Step IV: DEAE-Cellulose (PO_4 form) chromatography batchwise

280 g of DEAE-cellulose was equilibrated with 0.01 M potassium phosphate buffer, pH 7.5 and used for batchwise experiment. The solution from Step III (2770 ml) was adsorbed on the DEAE-cellulose. The slurry was kept for 1 h and then filtered on Buchner funnel. The cake was washed and filtered twice each time using 1200 ml of 0.01 M phosphate buffer, pH 7.5. The filtrates were pooled (4200 ml) and precipitated with ammonium sulphate (0.9 saturation). The precipitate was dissolved in 0.001 M phosphate buffer, pH 7.5 and dialysed against the same buffer. The dialyzed solution (960 ml) was used in Step V for the purification of subtilisin and closely associated trypsin inhibitor.

The DEAE-cellulose residue was eluted with 2,000 ml of 0.1 M phosphate buffer, pH 7.5 and further washed twice with 1000 ml buffer each time. This eluate contained the bulk of the trypsin inhibitor which was strongly adsorbed on DEAE-cellulose. This was referred as major trypsin inhibitor and was not processed further (Table VIIIa)

TABLE VIII : PURIFICATION OF PROTEINASE INHIBITORS FROM HORSE GRAM SEEDS

Step No.	Step	Volume	Protein	Subtilisin inhibitor		Trypsin inhibitor		Papain inhibitor	
				Activity	Specific activity	Activity	Specific activity	Activity	Specific activity
		ml	mg	U x 10^{-3}	U/mg	U x 10^{-3}	U/mg	U x 10^{-3}	U/mg
I.	Flour extract (from 3 kg seeds)	13,500	131,250	3,160	24	78,975	602	484	3.7
II.	Ammonium sulphate precipitation (0.9 saturation)	2,810	80,140	3,090	38	60,750	758	468	5.8
III.	pH 5.0 supernatant	2,770	37,620	2,946	78	57,834	1537	281	7.4
IV*	DEAE-cellulose (PO_4 form) chromatography (Batch-wise) 0.01 M filtrate	960	1,920	1,950	1,015	1,770	923	202	105
V.	DEAE-cellulose (OH form) column chromatography (0.035 M eluate)	38	310	1,230	3,960	1,100	3,550	-	-
VI.	Polyacrylamide gel electrophoresis	120	ND	928	ND	810	ND	-	-
VII	Affinity chromatography	830	ND	798	ND	0**	-	-	-
VIII	DEAE-cellulose (OH form) chromatography	50	26	590	22,500	-	-	-	-

ND = Not determined

TABLE VIIIa : *MAJOR TRYPSIN INHIBITOR

Step	Volume ml	Protein mg	Activity $U \times 10^{-3}$	Specific Activity (U/mg)
DEAE-cellulose (PO_4 form) from Step IV (Table VIII) 0.1 M eluate	3100	20800	45000	2160

TABLE VIIIb : **ELUTION OF ASSOCIATED TRYPSIN INHIBITOR

Step	Volume ml	Protein mg	Activity $U \times 10^{-3}$	Specific Activity (U/mg)
Affinity chromatography from Step VII (Table VIII)	500	45	640	14200

Step V: DEAE-cellulose (OH form) column chromatography

220 g of DEAE-cellulose was filled in a column (40 x 8 cm) and the supernatant liquid of Step IV (960 ml) was applied to the column. The column was washed with 2 litres of 0.001 M phosphate buffer and then gradient elution was carried out using a gradient formed from one liter each of 0.001 M and 0.1 M phosphate buffer, pH 7.5. Fractions (50 ml each) were collected at the rate of 300 ml/h. Eluted fractions were assayed for protein and papain, subtilisin and trypsin inhibitor activity.

It was observed that all the papain inhibitor was eluted at 0.001 M phosphate buffer, pH 7.5 and subtilisin and associated trypsin inhibitor eluated together at around 0.035 M phosphate concentration (230 ml) (Fig. 7). Active fractions were concentrated by lyophilization and dialyzed against 0.001 M phosphate buffer, pH 7.5 (38 ml).

Further work was restricted to the separation and purification of subtilisin and trypsin inhibitor from 0.035 M phosphate eluate. The purification of papain inhibitor was not persued further.

Step VI: Preparative polyacrylamide gel electrophoresis

The preparation from Step V was not homogeneous and showed the presence of 5-6 protein bands as revealed by analytical gel electrophoresis (Fig. 8). Hence it

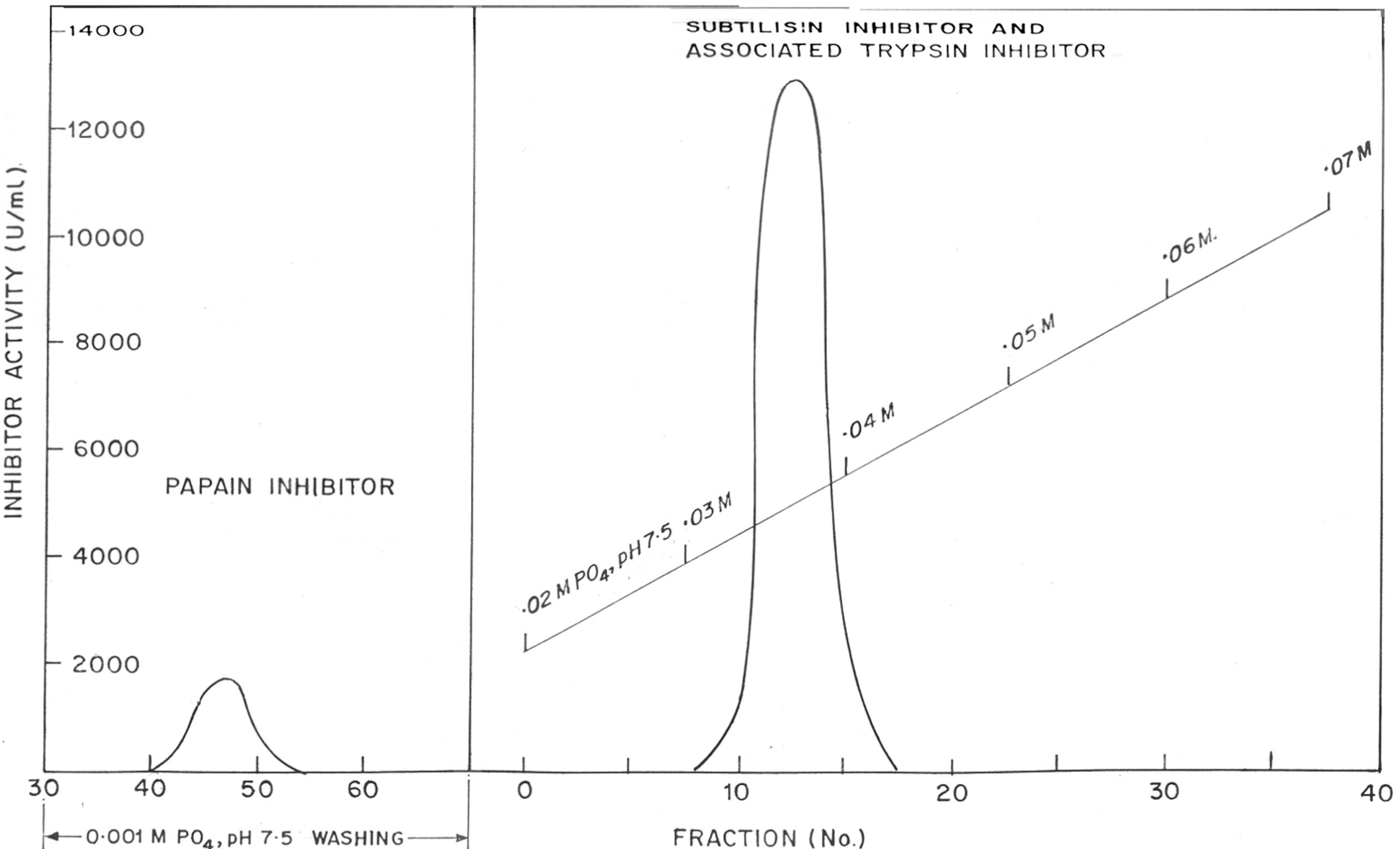


FIG. 7. DEAE CELLULOSE COLUMN CHROMATOGRAPHY

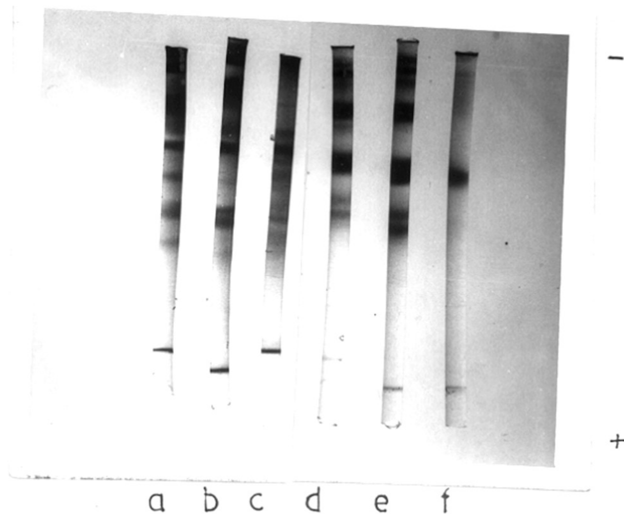


Fig. 8: Analytical PAGE pattern of different stages during purification of subtilisin inhibitor from horse gram seeds

(a) crude extract
(b) ammonium sulphate precipitate
(c) pH 5.0 supernatant
(d) DEAE ($-PO_4$ form) chromatography batchwise
(e) DEAE (-OH form) column chromatography
(f) electrophoretic elution after preparative PAGE

was subjected to further purification by preparative PAGE.

Preparative PAGE under alkaline conditions was carried out using the 'in situ' slab gel apparatus as described in Materials and Methods. At a time two polyacrylamide gel slabs (7%) were prepared in the apparatus. 5 ml of the solution from Step V containing 40 mg of protein was loaded on each slab. Buffer compartments contained Tris-glycine buffer, pH 8.3. Bromophenol blue was used as a marker. Cathode was on the top. Electrophoresis was carried out for 30 - 35 h using 10 - 15 mA and 100 V. Electrophoresis was discontinued when the marker band reached 1 cm above the bottom of the gel.

Gel slabs were removed and electrophoretic elution was carried out according to the method developed in this work (described in Section I.1) of Chapter III.

Before electrophoretic elution the inhibitor was located on the gel by using fluorescent markers developed during this work (Section I.3) of Chapter III. The portion of the gel containing the inhibitor protein was placed horizontally above the elution cell, such that the protein bands were parallel to the elution cell. Upper and lower chamber contained Tris-glycine buffer, pH 8.3 and elution cell contained Tris-HCl pH 8.1. The elution was carried out at 80 mA (100 V)

for 2 h. The inhibitor protein eluted in the sponge was recovered by squeezing and the eluate was assayed for subtilisin and trypsin inhibitor activity. Four such electrophoretic runs were carried out. The recovery of subtilisin inhibitor in this step was 75-80% (Table VIII). At this stage it was not possible to determine the protein due to the presence of acrylamide impurities which were subsequently removed.

Although the inhibitor protein from this step appeared homogenous by PAGE it was observed that the trypsin inhibitor activity was not separated from subtilisin inhibitor activity.

This may be due to two reasons (i) either the same protein may possess both subtilisin and trypsin inhibitor activities (polyvalent inhibitor) or (ii) the two inhibitors may have very similar physico-chemical properties.

Attempts were made to separate these two activities by affinity chromatography using immobilized trypsin.

Step VII: Affinity chromatography

Sephadex G-200 was activated with cyanogen bromide and coupled with subtilisin and trypsin according to the method described by Axen and Ernback (257).

To 210 ml CNBr-activated Sephadex G-200 (50 g) suspension containing 8,80,000 units) of immobilised trypsin in 0.05 M phosphate buffer, pH 7.5 was added 120 ml of the inhibitor solution from Step VI (Subtilisin inhibitor 9,28,000 units and trypsin inhibitor (8,10,000 units).

After 15 minutes Sephadex trypsin-trypsin inhibitor complex was filtered through buchner funnel and washed twice with 400 ml of buffer each time. The filtrate and washings were pooled and assayed both for subtilisin and trypsin inhibitor activity. It was observed that although there was almost quantitative recovery of subtilisin inhibitor in the filtrate, no trypsin inhibitor activity could be detected. This suggests that the two inhibitor activities although closely associated, reside on two separate proteins. This is also supported by the observed increase in the specific activity of both the inhibitors (Table VIII).

Step VIII: DEAE-Cellulose Chromatography

The soluble acrylamide impurities from the above preparation were removed by a batchwise adsorption of the inhibitor on DEAE-cellulose (OH form) and elution by 0.04 M phosphate buffer, pH 7.5.

Separation of associated trypsin inhibitor from trypsin-trypsin inhibitor complex

Sephadex G-200—trypsin-trypsin inhibitor complex was suspended in 200 ml of 0.05 M phosphate buffer pH 7.5 and heated on a water-bath at 90°C for 10 min.

On heating the trypsin-trypsin inhibitor complex, trypsin is denatured and the inhibitor is released which was recovered by filtering the suspension on Buchner

funnel and washing the residue twice with 200 ml of buffer each time. The filtrate was assayed for trypsin inhibitor activity, specific activity of the purified trypsin inhibitor was 14,200 (Table VIIIb).

Section IIC: Occurrence of dialysable trypsin inhibitor (DTi)

When boiled acid extract of various leguminous seeds was dialysed, the dialysate showed trypsin and chymotrypsi inhibitor activity but did not inhibit other proteolytic enzymes such as papain and subtilisin.

Preliminary experiments were carried out with acid extract of horse gram. The extract was heated at 90°C for 10 min, and centrifuged. The supernatant liquid when dialysed (MWCO = 10,000) against equal volume was distilled water at 6°C, the dialysate showed inhibition of trypsin and chymotrypsin. The dialysis was continued for 8 days and the dialysate was estimated periodically for the inhibitor activity. It was observed that the amount of inhibitor in the dialysate increased with time (Fig. 9). When the experiment was continued further for another week, the retentate still secreted the inhibitor, in increasing amount, in the dialysate.

In order to verify whether the occurrence of DTI in leguminous seeds is a general phenomenon, similar experiments were carried out with the heated acid extracts of the seeds of cowpea, soyabean, lima bean, double bean and french bean. It was observed that all the extracts exhibited considerable amount of dialysable trypsin inhibitor activity (Table IX).

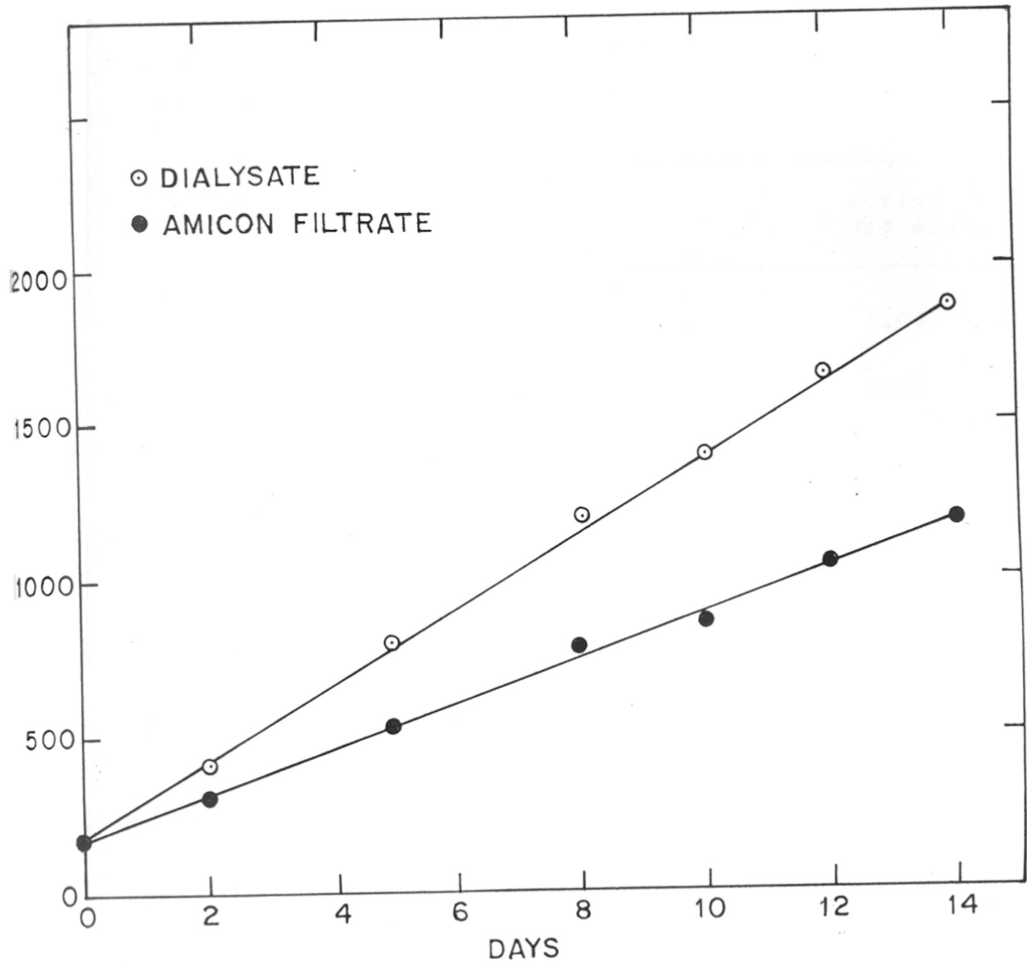


FIG. 9. PRODUCTION OF DTi

THE EXTRACTS WERE DIALYSED AGAINST EQUAL VOLUME OF DISTILLED WATER AT 6 °C. THE DIALYSATES WERE PERIODICALLY ESTIMATED FOR TRYPSIN INHIBITOR ACTIVITY. SIMULTANEOUSLY THE EXTRACT WAS KEPT AT 6°C AND SAMPLES WERE PERIODICALLY REMOVED, ULTRAFILTERED THROUGH UM 10 MEMBRANE AND THE FILTRATES WERE ASSAYED FOR TRYPSIN INHIBITOR ACTIVITY.

TABLE IX : DIALYSABLE TRYPSIN INHIBITOR FROM LEGUMINOUS SEEDS

	Name of the seed	Activity U/g seeds
1.	Horse gram	1300
2.	Cow pea	1200
3.	Soyabean	1250
4.	Lima bean	1350
5.	Double bean	1450
6.	French bean	1450

Heated seed extracts were dialysed at 6°C for 8 days and dialysates were estimated for trypsin inhibitor activity.

The seeds of horse gram were selected for further studies on dialysable trypsin inhibitor since the work on subtilisin and trypsin inhibitor was carried out from the same seeds.

Origin of DTi

When the heated acid extract of horse gram seeds was ultrafiltered through UM 10 Amicon membrane, trypsin inhibitor activity was found in the filtrates which increased continuously with time (Fig. 9).

This observation suggests that the low molecular weight trypsin inhibitor might be fforming from high molecular weight trypsin inhibitor.

It was thought that the DTi could have originated by proteolytic cleavage of the high molecular weight trypsin inhibitor originally present in the seed extract. To verify this the proteinase activity of the boiled acid extract was determined. However, it was found that the extract had no detectable acid or alkaline proteinase activity.

Section IID: Isolation and purification of horse gram DTi

Step I : Extraction: 100 g seeds were washed and soaked in distilled water for 1 h. Seeds were blendorised with 0.01 M K-acetate buffer, pH 5.7 (500 ml), 0.6 M HCl (100 ml) and KCl (7.5 g) for 3 min. Extract was kept for about 1 h and squeezed through muslin cloth and centrifuged at 4000 rpm for 30 min. The supernatant liquid (Vol. 520 ml, pH 2.4) was boiled on water bath at 90°C for 10 min. 100 ml of the boiled extract was dialyzed through a dialysis tubing with a MWCO of 10,000, against equal volume of distilled water at 6°C over a period of 8 days. Dialysate was assayed for trypsin inhibitor activity (90 ml).

Step II : Ammonium sulphate precipitation: The dialysate was precipitated with ammonium sulphate to 0.9 saturation (60 g/100 ml) kept at 0°C for 16 h and centrifuged at 14,000 x g for 30 min. The precipitate was dissolved in 20 ml of 0.01 M PO_4 buffer, pH 7.5 and dialysed against the same buffer using cellulose casing (MWCO 2,000).

Step III : Reprecipitation with ammonium sulphate: The insoluble impurities from the dialysate were removed by centrifugation and the clear supernatant liquid (15 ml) was saturated to 0.9 saturation with ammonium sulphate and the precipitate formed was dissolved in

0.01 M phosphate buffer, pH 7.5 and preserved at -20°C (8 ml) (Table X). This preparation in a forward run on PAGE at pH 8.3, gave a major protein band moving just behind the marker, along with a few very faint protein bands while no protein band was observed when a reverse PAGE was carried out at pH 8.3 (Fig. 10). The trypsin inhibitor activity was located in the major protein band as estimated by cutting and eluting the corresponding gel portion. No attempt was made to purify the inhibitor further.

TABLE X : PURIFICATION OF DTi

Step No.	Step	Volume (ml)	Total units (U)	Total protein (mg)	Specific activity (U/mg)
I.	Dialysate	90	24,000	68	350
II.	Ammonium sulphate precipitation	15	22,500	9	2,500
III.	Reprecipi-tation with ammonium sulphate	8.0	20,000	6.6	3,030

Extract corresponding to 20 g of horse gram seeds was used.

Fig. 10: Analytical PAGE of DTi

DTi Step II protein loaded 50 µg 7.5% PAGE, Tris glycine buffer, pH 8.3.

CHAPTER – IV

PROPERTIES

SECTION I

PROPERTIES OF THE PURIFIED SUBTILISIN AND ASSOCIATED TRYPSIN INHIBITOR

Homogeneity

Subtilisin inhibitor (Step VIII) and trypsin inhibitor (Step VII) showed single bands on 7.5% polyacrylamide gel electrophoresis at pH 8.3 and pH 2.9 (Fig. 11a, 11b).

Molecular weight (Mr)

Molecular weight of the subtilisin inhibitor by gel filtration using Sephadex G-75 (Fig. 12), slope method (Fig. 13a, 13b), SDS gel electrophoresis (Fig. 14) and by amino acid analysis was 7500, 7300, 7100 and 9450, respectively. The molecular weight of trypsin inhibitor by SDS gel electrophoresis (Fig. 14, 15), gel filtration (Fig. 12) and by amino acid analysis was 8500, 8650 and 8200, respectively.

Isoelectric point

The isoelectric point of subtilisin and associated trypsin inhibitor was 7.66 and 7.70, respectively.

UV absorption spectrum

The UV absorption spectra of subtilisin inhibitor and associated trypsin inhibitor are shown in Fig. 16, 17. Both showed the characteristic absorption spectrum of proteins. No other peaks were detected

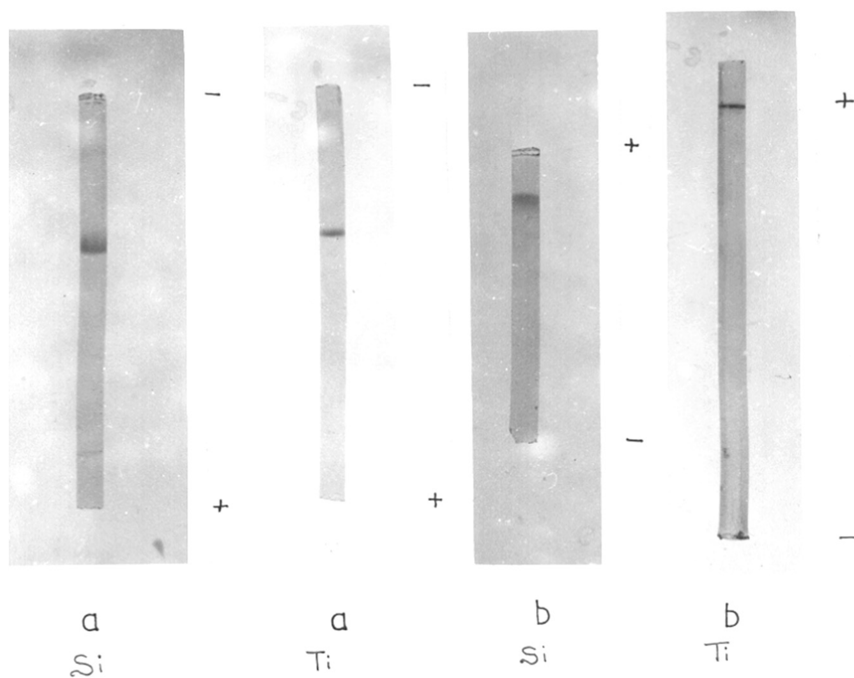


Fig. 11: PAGE of purified subtilisin inhibitor and associated trypsin inhibitor

7.5% PAG protein loaded 50 μg

Subtilisin inhibitor (Step VIII) and trypsin inhibitor (Step VII)

(a) pH 8.3 (b) pH 2.9

Si = Subtilisin inhibitor
Ti = Trypsin inhibitor

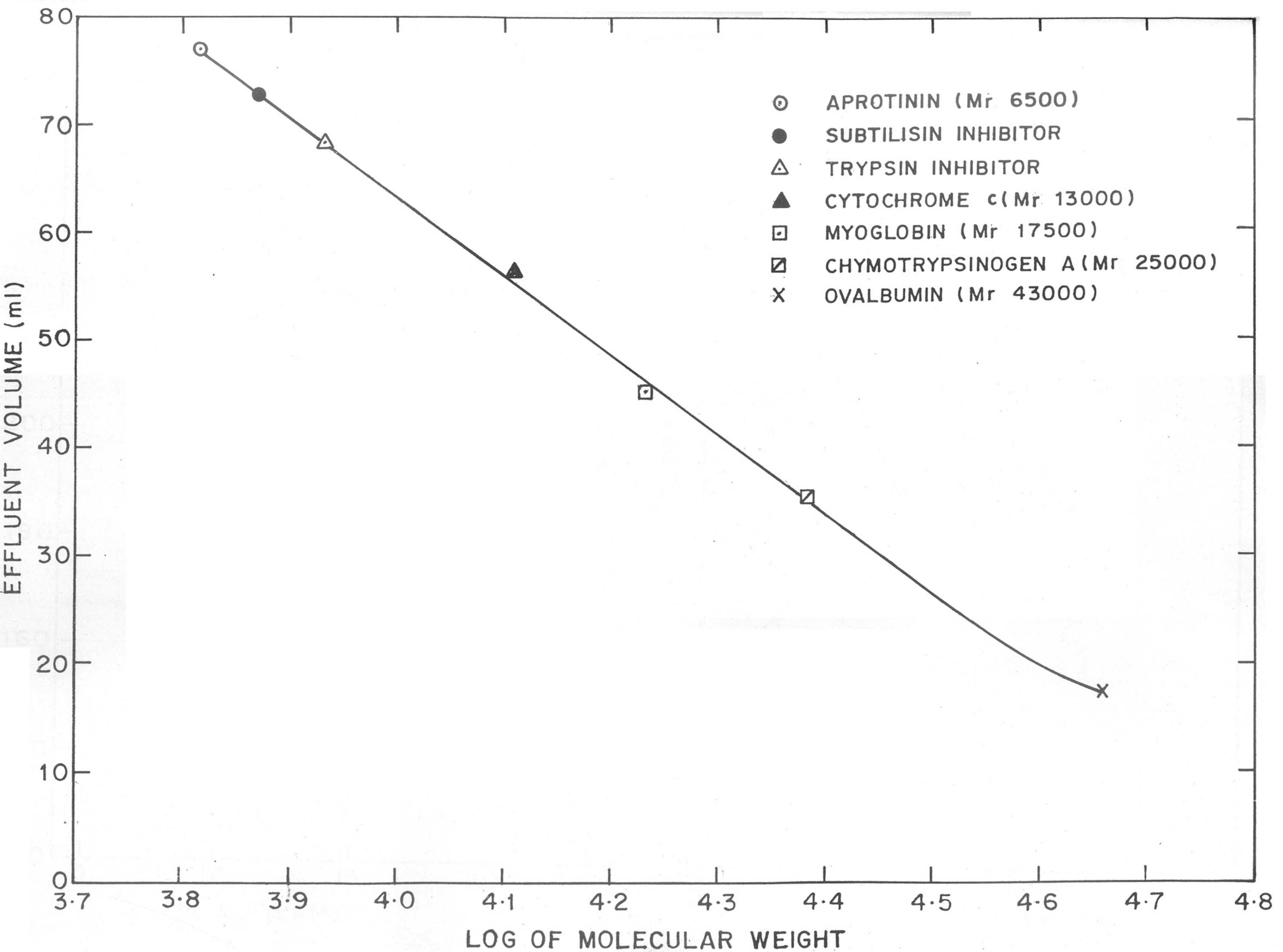


FIG. 12. MOLECULAR WEIGHT OF SUBTILISIN AND ASSOCIATED TRYPSIN INHIBITOR BY GEL FILTRATION

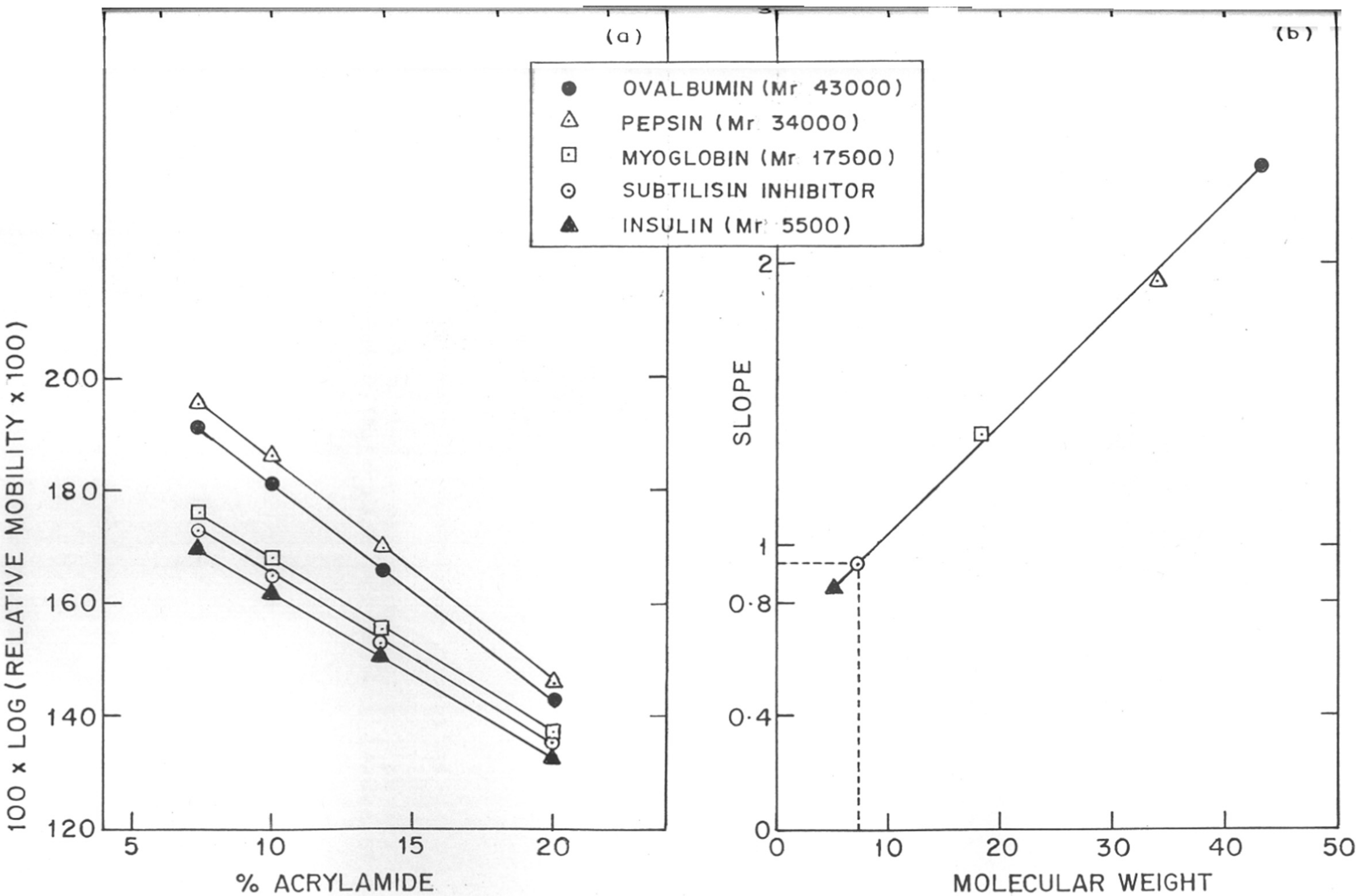


FIG. 13. MOLECULAR WEIGHT OF SUBTILISIN INHIBITOR BY SLOPE METHOD
(a) PLOT OF RELATIVE MOBILITY vs. ACRYLAMIDE CONCENTRATION
(b) SLOPE OF a vs MOLECULAR WEIGHT

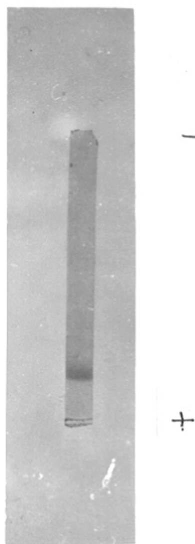


Fig. 14: SDS-PAGE of subtilisin inhibitor

0.1 M sodium phosphate buffer, pH 7.2
SDS 0.1%, 8 mA/tube; protein loaded 50 µg.

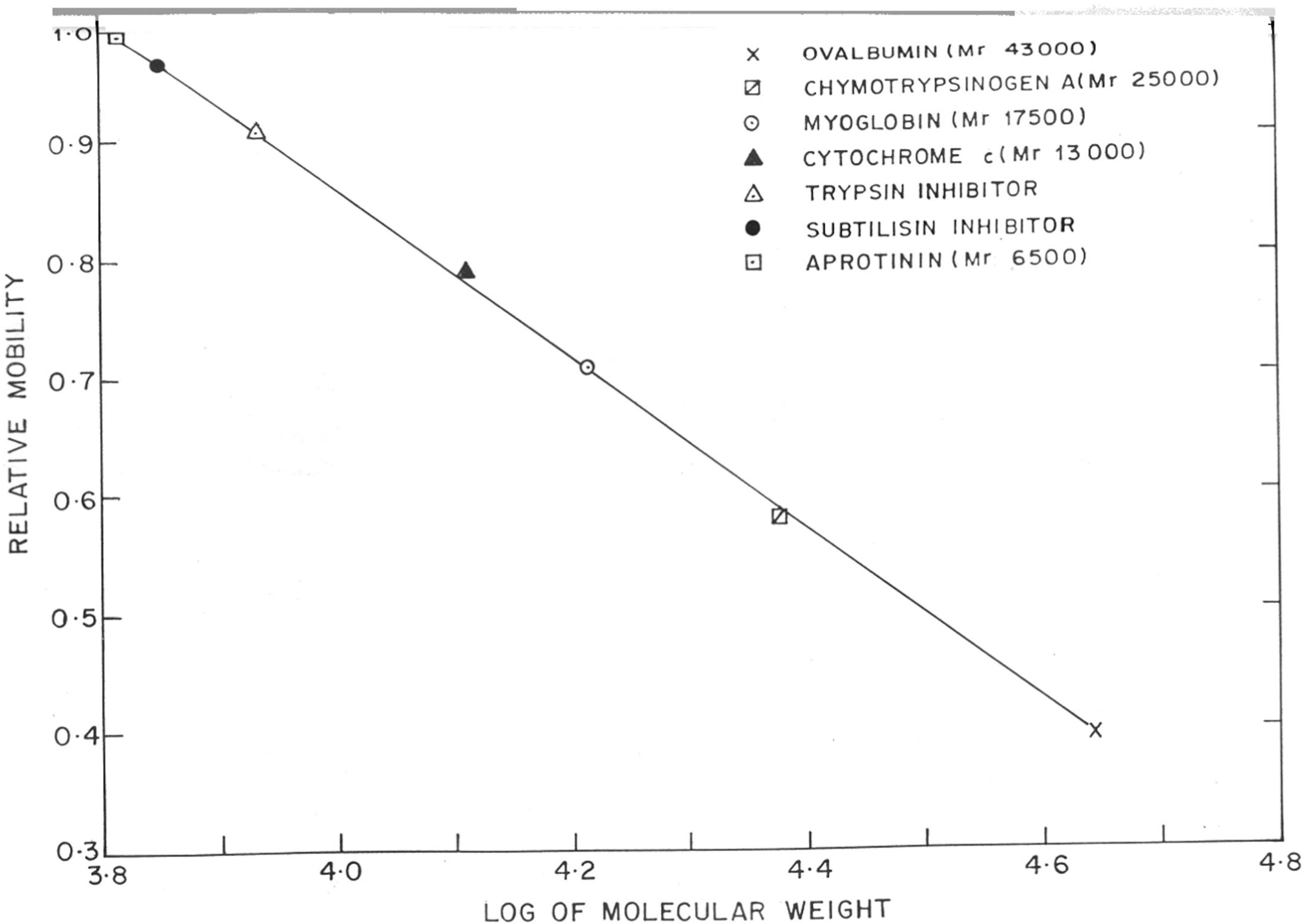


FIG. 15. MOLECULAR WEIGHT OF SUBTILISIN AND ASSOCIATED TRYISIN INHIBITOR BY SDS-PAGE

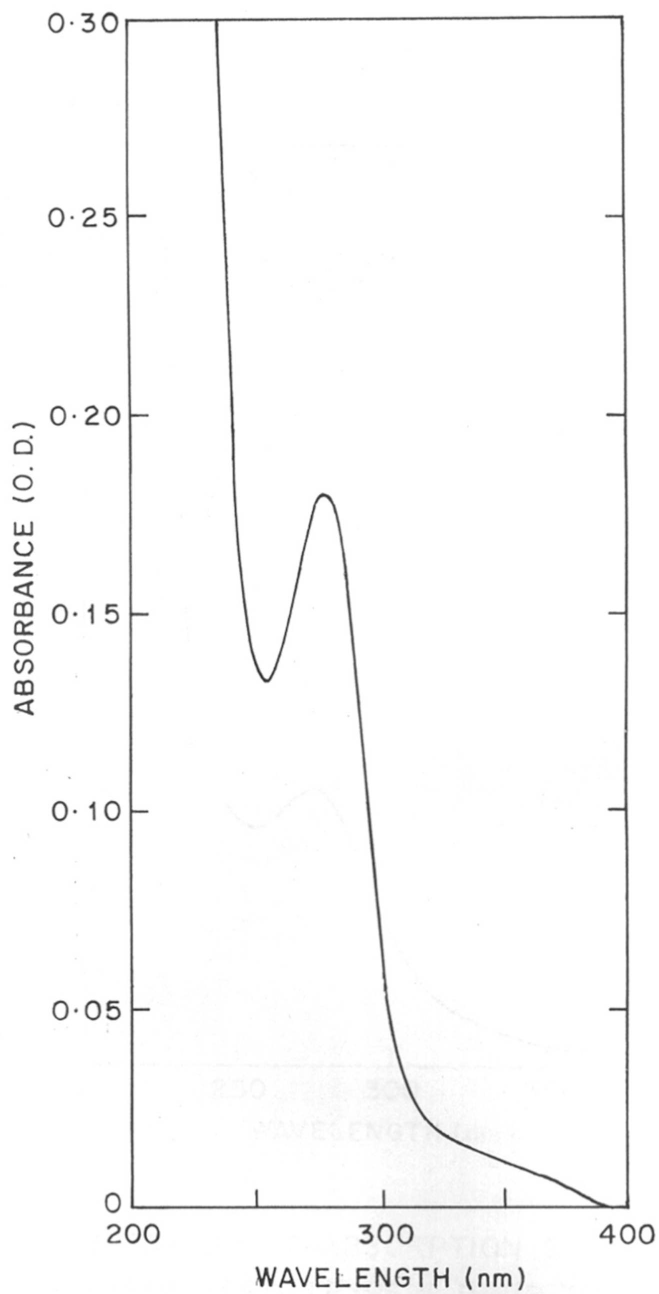


FIG. 16. ULTRAVIOLET ABSORPTION SPECTRUM OF SUBTILISIN INHIBITOR (0·170 mg/ml)

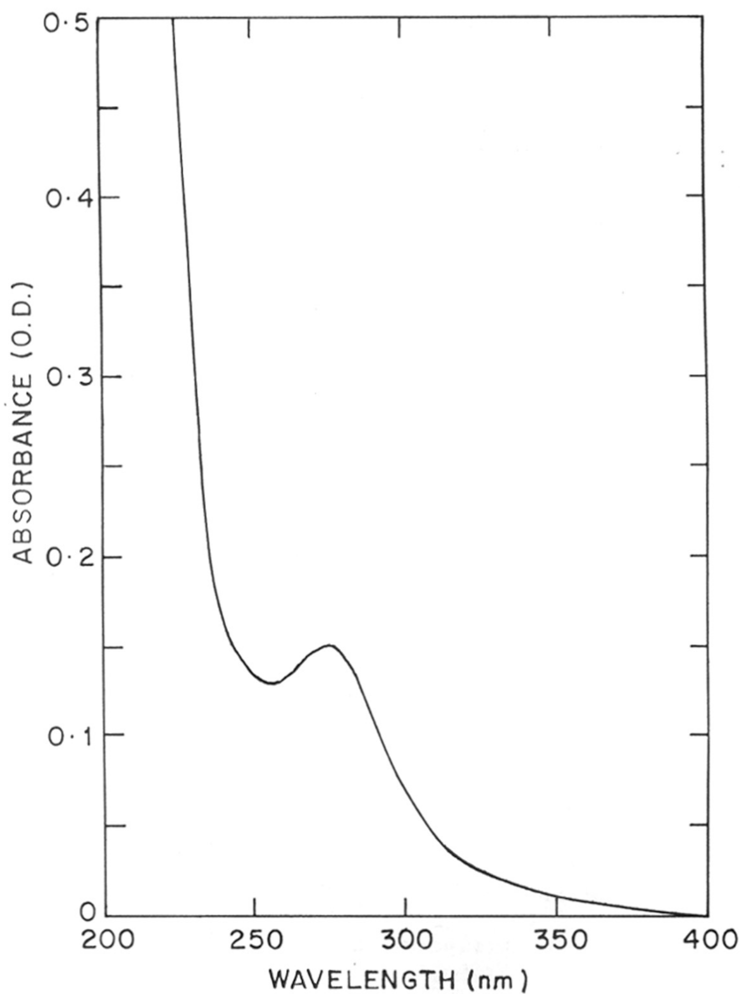


FIG. 17. ULTRAVIOLET ABSORPTION SPECTRUM OF ASSOCIATED TRYPSIN INHIBITOR (0·130 mg/ml)

indicating the absence of nucleotides or other UV absorbing material in the inhibitor. The subtilisin and trypsin inhibitor showed maximum absorbance at 275 nm and minimum at 254. The ratio of absorption at 280 to that of 260 nm for subtilisin and trypsin inhibitor was 1.30 and 1.11, respectively.

Optical factors

Optical factor of the subtilisin inhibitor and associated trypsin inhibitor was 0.88 and 0.93, respectively.

Stability

The subtilisin and associated trypsin inhibitors were stable to heat, trichloroacetic acid and ethanol in the pH range of 2.5 to 10.5 (Table XI, and Table XII).

Dissociation constant

The reaction of subtilisin inhibitor with subtilisin was complete within 5 min and on the basis of 1:1 complex formation dissociation constant at pH 7.5 was 2.69 x 10^{-10} mole/liter (Fig. 18).

Amino acid analysis

The amino acid composition of subtilisin and associated trypsin inhibitors is given in Table XIII, XIV.

TABLE XI: STABILITY OF SUBTILISIN AND TRYPSIN INHIBITOR

Treatment	Subtilisin inhibitor activity remaining (%)	Trypsin inhibitor activity remaining (%)
1. Heating at 90°C, 15 min.	94	95
2. Precipitation with TCA (2.5%)	86	88
3. Precipitation with ethanol (three volumes)	95	94

Inhibitor (0.02%) in 0.1 M phosphate buffer, pH 7.5 was used for the above experiment. The activity of the inhibitor was estimated by Kunitz caseinolytic assay

TABLE XII : EFFECT OF pH ON THE STABILITY OF SUBTILISIN AND TRYPSIN INHIBITOR

Sample	pH	Subtilisin inhibitor activity (%)	Trypsin inhibitor activity (%)
Control (unheated)	7.5	100	100
	2.5	78	76
	4.4	82	84
	7.5	98	98
	8.9	98	96
	10.5	77	75

0.02% solution of the inhibitor in 0.1 M phosphate buffer was heated at different pH at 80°C for 15 min.

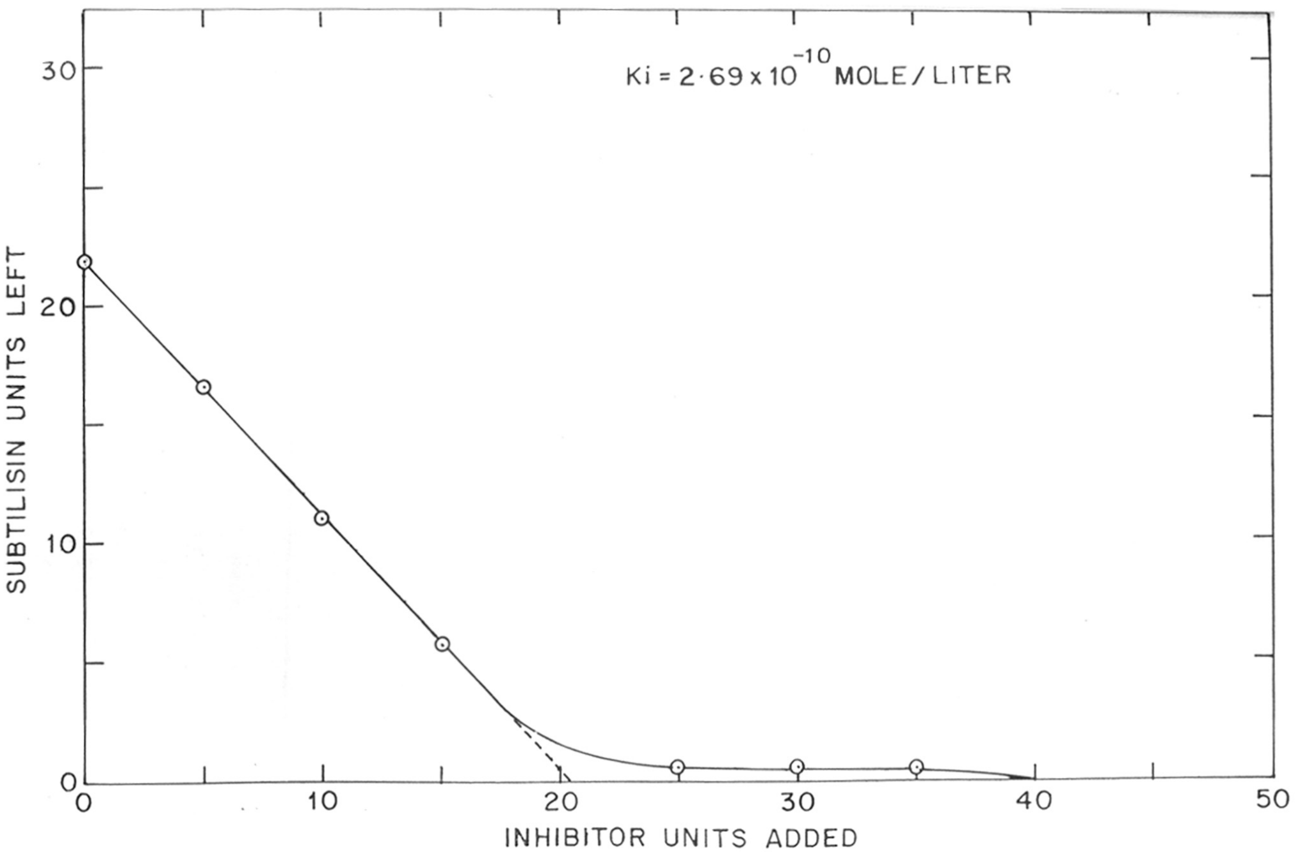


FIG. 18. DISSOCIATION CONSTANT OF SUBTILISIN INHIBITOR INHIBITION CURVE SHOWING THE DISSOCIATION AT pH 7·5 OF SUBTILISIN INHIBITOR–SUBTILISIN COMPLEX NEAR THE EQUIVALENCE POINT.

TABLE XIII: AMINO ACID COMPOSITION OF SUBTILISIN INHIBITOR

Amino acid	µmole/ 100 mg residue	Residues/ mole calculated	Assumed nearest integer
Aspartic acid	0.0786	5.90	6
Threonine	0.0898	6.75	7
Serine	0.0602	4.51	5
Glutamic acid	0.1472	11.07	11
Proline	0.0721	5.40	5
Glycine	0.0725	5.40	5
Alanine	0.0609	4.50	5
Half-cystine	-	-	-
Valine	0.1127	8.45	8
Methionine	0.0186	1.40	1
Isoleucine	0.0424	3.50	4
Leucine	0.0350	2.60	3
Tyrosine*	0.0240	1.80	2
Phenylalanine	0.0137	1.02	1
Glycine	0.0674	5.0	5
Histidine	-	-	-
Arginine	0.0560	4.20	4
Tryptophan*	-	-	1
Cysteic acid* *	-	-	-

The above calculations were made assuming the molecular weight of 7500 for the subtilisin inhibitor. A total of 73 residues of subtilisin inhibitor were obtained and on this basis the value for the minimum molecular weight of the inhibitor is 9450.

* Tyrosine and tryptophan were determined by Godwin and Morton's method

** The data on performic acid oxidized sample of subtilisin inhibitor showed the existence of one cysteine residue in subtilisin inhibitor.

TABLE XIV: AMINO ACID COMPOSITION OF ASSOCIATED TRYPSIN INHIBITOR

Amino acid	umole/ 100 mg residue	Residues/ mole calculated	Assumed nearest integer
Aspartic acid	0.0656	5.9	6
Threonine	0.0276	2.4	2
Serine	0.0801	7.2	7
Glutamic acid	0.0395	3.95	4
Proline	0.0577	5.1	5
Glycine	0.0216	1.9	2
Alanine	0.0386	3.4	3
Half-cystine	0.0871	7.8	8
Valine	0.0357	3.2	3
Methionine	0.0163	1.0	1
Isoleucine	0.0269	2.4	2
Leucine	0.0175	1.57	2
Tyrosine*	0.0029	1.0	1
Phenylalanine	0.0163	1.46	1
Lysine	0.0297	2.6	3
Histidine	0.0121	1.08	1
Arginine	0.0056	0.504	1
Tryptophan*	-	-	2
Cysteic acid**	0.0774	6.93	7

The above calculations were made assuming the molecular weight of 8500 for the trypsin inhibitor. A total of 64 residues of trypsin inhibitor were obtained and on this basis the value for the minimum molecular weight of the inhibitor is 8200.

* Tyrosine and tryptophan were determined by Godwin and Morton's method (251).

** The data on performic acid oxidized sample of trypsin inhibitor showed the existence of 7 cysteine residue in trypsin inhibitor.

Estimation of tyrosine and tryptophan

The absorbance of a solution of 0.520 mg of the inhibitor in 1 ml of 0.1 M NaOH was 0.770. From this data the tyrosine and tryptophan content of the molecule was calculated. It was observed that one mole of the inhibitor contained 2.4 moles of tyrosine and 1.4 moles of tryptophan. The value of tyrosine (2.4 moles) obtained by this method (251) was in fair agreement with that obtained by the amino acid analysis.

Estimation of cysteine content (-SH group)

0.200 mg of the inhibitor was used for this test. Reduction of inhibitor with sodium borohydride in the presence of EDTA and urea, and titration with DTNB at 412 nm showed the presence of 0.75 mole of SH per mole of inhibitor.

Specificity

The inhibitor inhibits subtilisin when assayed by the caseinolytic assay as well as by the BAEE assay. The inhibitor is specific for subtilisin and does not inhibit trypsin, chymotrypsin, papain and ficin. There is no amylase activity in the inhibitor. Even the amount as high as 36 µg of subtilisin inhibitor shows no detectable inhibition towards these proteinases. It inhibits subtilopeptidase A and B. The molar combining ratios of subtilisin inhibitor with subtilisin and

of trypsin inhibitor with trypsin are 0.84 and 0.90 respectively using casein as substrate. However the molar combining ratio of subtilisin inhibitor with subtilisin was 1.85 using synthetic substrate BAEE. It was also observed that alkaline protease from Conidiobolus species inhibited by the subtilisin inhibitor.

Modification studies using subtilisin inhibitor

Some of the trypsin and chymotrypsin inhibitors, on incubation with catalytic amounts of corresponding proteinases are known to get modified due to a bond cleavage at the reactive site of the inhibitor (48). It was suggested that this step is an essential step for complex formation between the inhibitor and the proteinase.

These modification studies have been reported only with the inhibitors of trypsin and chymotrypsin. Moreover no modification experiments were performed on an inhibitor which is devoid of cystine which is believed to give stability to the modified inhibitor. Hence it was interesting to determine whether subtilisin inhibitor gets modified and if it does whether it is possible to reconvert it from the the modified form into its native form. Based on the studies of Ozawa and Laskowski (49) on soyabean trypsin inhibitor (Kunitz) the following experiments were carried out with subtilisin inhibitor from Dolichos biflorus/horse gram.

Subtilisin inhibitor was incubated with catalytic amounts of subtilisin at acid pH in the presence of calcium ions. The reaction mixture (M) contained 200 µg (3600 units) of subtilisin inhibitor, 40 moles of calcium chloride and 10 µg (120 units) of subtilisin in a total volume of 1 ml. The reaction mixture was adjusted to 3.7 with 20 µl of 0.1 M HCl. A control experiment (N) wherein subtilisin was omitted from the reaction mixture was also run simultaneously. The reaction mixture was incubated for 18 h at 30°C, neutralized with 20 µl of 1 M Tris base and used for polyacrylamide gel electrophoresis and for the assay of the inhibitor.

It was observed that M and N had identical mobilities. M and N with and without pre-incubation with subtilisin for 15 min showed similar activities suggesting that the subtilisin inhibitor does not get modified.

<u>Active site modification of subtilisin inhibitor</u>

Effect of various active site group specific reagents on the subtilisin inhibitor was studied.

<u>Arginine</u>

Phenylglyoxal reacts with the guanidino group of arginine residue under mild conditions (258). The reaction also occurs with ϵ-amino group of lysine residues when proteins are treated with high concentrations

of phenylglyoxal for a long period. The arginine residue can also be modified at neutral pH with 2-3 butanedione (259).

The reaction was carried out in dark to avoid possible photochemical effects. Phenylglyoxal (final concentration 10 mM) was treated with 15 µg of subtilisin inhibitor in a total volume of 1 ml of 0.1 M PO_4 buffer, pH 8.2 and incubated at 30°C for 1 h. There was no inhibition of subtilisin inhibitor. Hence arginine may not be involved in the active site of the inhibitor.

2-3-butanedione (final concentration 2.5 mM) was incubated with 11 µg of subtilisin inhibitor in 1 ml at pH 8.0 for 1 h at room temperature. The lack of inhibition by this reagent again indicates that arginine is not essential for the active site of the inhibitor.

Lysine

0.1 ml of TNBS (10 mg/ml) was treated with 15 µg of subtilisin inhibitor in a total volume of 3 ml containing 0.7 M $NaHCO_3$ and kept for 1 h at 30°C. 30% destruction of the inhibitor indicated that lysine may be at the active site of the inhibitor.

Cysteine

15 µg of subtilisin inhibitor was incubated in a total volume of 1 ml with 10 µmoles each of N-methyl-maleimide, iodoacetamide and reduced glutathione at 30°C for 1 h. 50, 85 and 78% inhibition of the inhibitor

was obtained respectively suggesting the involvement of cysteine in the active site of the inhibitor.

Tyrosine

15 μg of subtilisin inhibitor was treated with N-acetyl imidazole (10 mM) in 1 ml of 0.1 M phosphate buffer, pH 7.5 and incubated for 1 h at 30°C. Inhibitor activity was lost by 50% indicating that tyrosine may be essential for the inhibitor activity.

N-terminal analysis

60 nMole of subtilisin inhibitor was used for the N-terminal analysis by DABITC-PITC method (260). N-terminal of subtilisin inhibitor was found to be alanine.

SECTION II

PROPERTIES OF THE DIALYSABLE TRYPSIN INHIBITOR (DTi)

Molecular weight (Mr)

Molecular weight of the inhibitor by SDS-gel electrophoresis and by slope method was 4800 and 4,900 respectively (Fig. 19 - 21). Gel filtration could not be used for molecular weight determination of DTi due to the formation of aggregates of the inhibitors giving different molecular weights.

UV absorption spectrum

The UV absorption spectrum of DTi is shown in Fig. 22. No other peaks were observed indicating the absence of nucleotides or other UV absorbing materials in the inhibitor. The inhibitor showed maximum and minimum absorption at 275 nm and 252 nm, respectively. The ratio of absorption at 280 nm to that of 260 nm for the inhibitor was 1.08.

Optical factor

Optical factor for the dialysable trypsin inhibitor is 1.

Isoelectric point

The isoelectric point of the inhibitor is 4.10.

Synthetic substrate

The DTi inhibits both the proteolytic (using casein

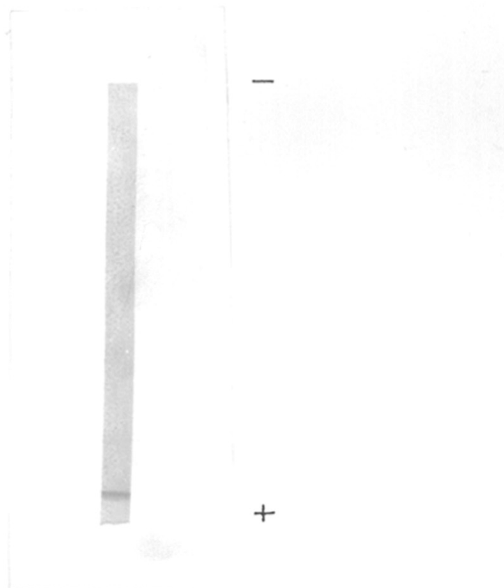


Fig. 19: SDS-PAGE of DTi

0.1 M sodium phosphate buffer, pH 7,2
SDS 0.1%, 8 mA/tube; protein loaded 50 µg.

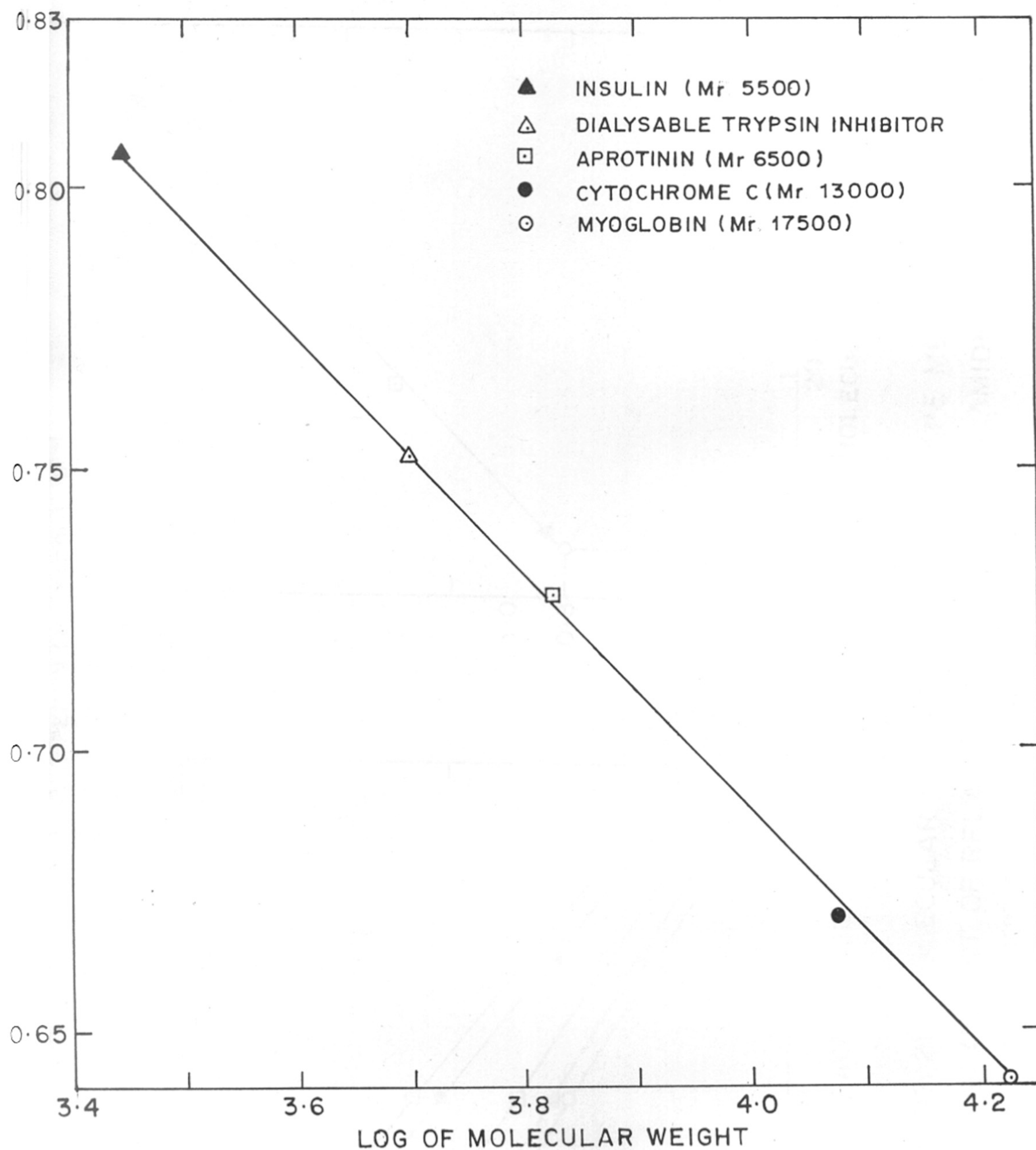


FIG. 20. MOLECULAR WEIGHT OF DIALYSABLE TRYPSIN INHIBITOR BY SDS-PAGE

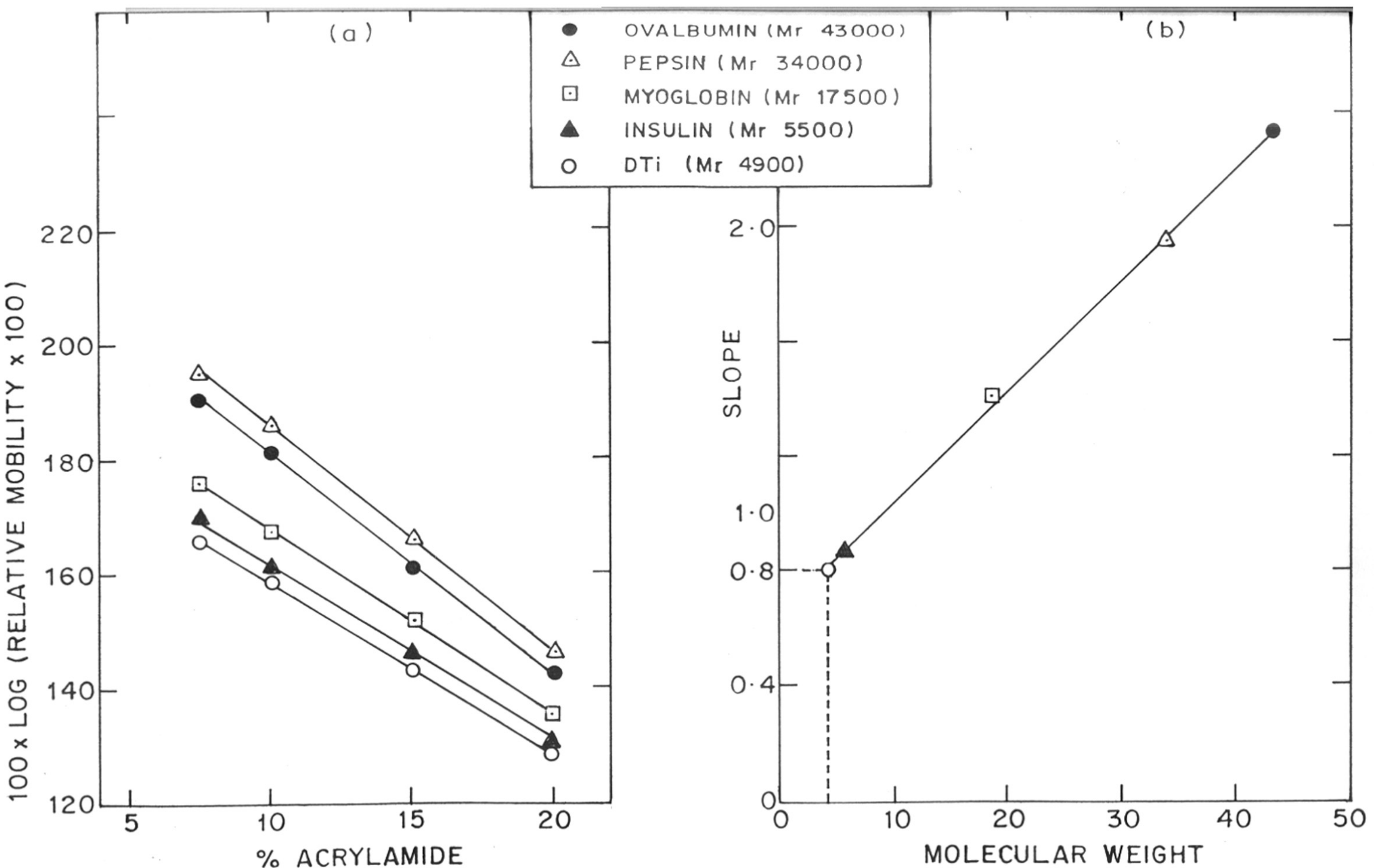


FIG. 21. MOLECULAR WEIGHT OF DTi BY SLOPE METHOD

(a) PLOT OF RELATIVE MOBILITY vs. ACRYLAMIDE CONCENTRATION

(b) SLOPE OF a vs. MOLECULAR WEIGHT

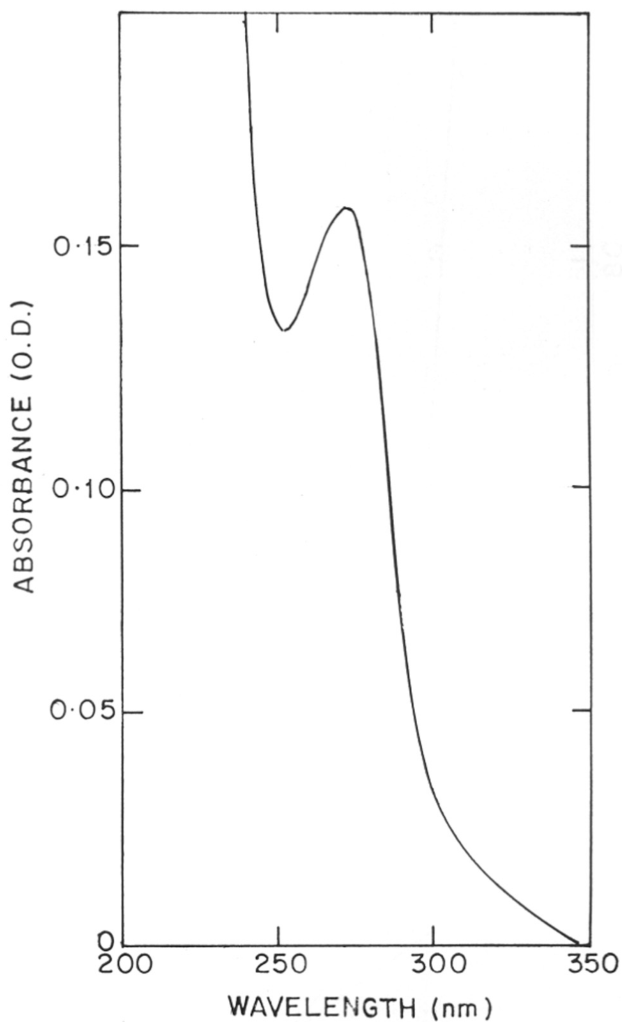


FIG. 22. ULTRAVIOLET ABSORPTION SPECTRUM OF DIALYSABLE TRYPSIN INHIBITOR (0·140 mg/ml)

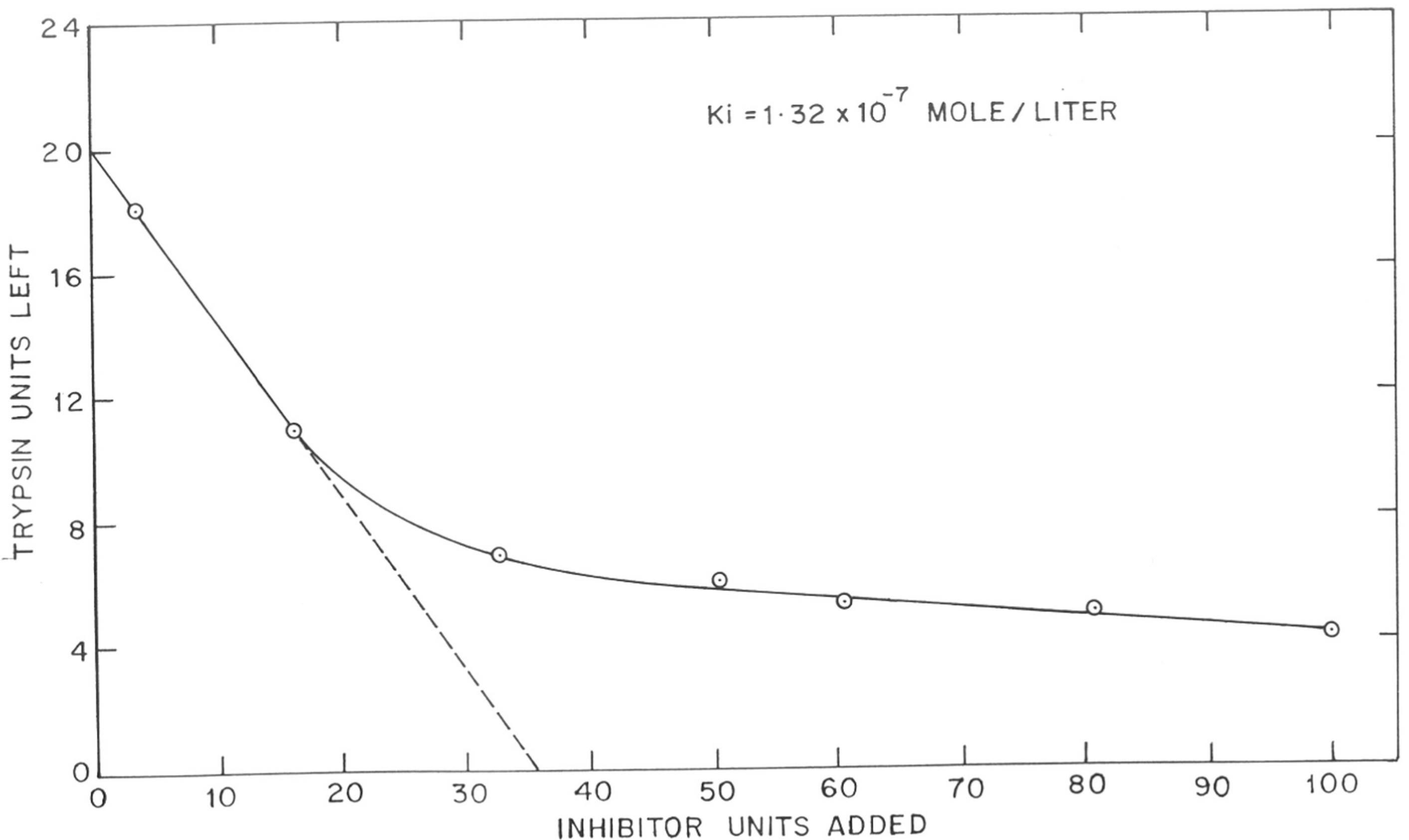


FIG. 23. DISSOCIATION CONSTANT OF DTi (INHIBITION CURVE SHOWING THE DISSOCIATION AT pH 7·5 OF DIALYSABLE TRYPSIN INHIBITOR–TRYPSIN COMPLEX NEAR THE EQUIVALENCE POINT.)

as substrate) as well as amidase activity (using BAPANA as substrate) of trypsin.

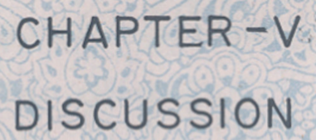
Dissociation constant

The dissociation constant of DTi-trypsin at pH 7.5 was 1.32×10^{-7} mol/liter (Fig. 23).

Active site modification of dialysable trypsin inhibitor

Lysine

The chemical modification for lysine using TNBS was carried out as described for subtilisin inhibitor. 80% destruction of inhibitor on treatment with TNBS indicated involvement of lysine at the active site of inhibitor. 100% destruction of the major inhibitor (0.1 M DEAE eluate, Table VIIIa) by TNBS suggests that lysine is also present in the active site of this inhibitor suggesting that the DTi might have arisen from the major trypsin inhibitor.



CHAPTER - V

DISCUSSION

The work presented in this thesis provides an evidence for the occurrence of the inhibitors of trypsin, chymotrypsin, subtilisin and papain in the seeds of horse gram. Out of these, subtilisin inhibitor and an associated trypsin inhibitor have been purified to homogeneity and characterized.

Preliminary work showed that the seed extracts of different legumes such as cow pea, double bean, winged bean, masur bean, mung bean, wal bean and horse gram inhibit different proteinases. Moreover, widespread occurrence of dialysable trypsin inhibitor in legumes has been observed which has not been so far reported.

The properties of the purified inhibitors of trypsin, and subtilisin show that they are specific and different from each other. Trypsin inhibitors also inhibit chymotrypsin and are considered to be specific since they do not inhibit subtilisin, papain and ficin. Similarly subtilisin inhibitors are regarded as specific, since they do not inhibit trypsin, cymotrypsin, papain and ficin.

Purification

Extraction of the seeds at acid pH (2.5) and subsequent precipitation of the filtrate at pH 5.0 eliminated considerable amount of impurities. The conventional methods of purification were applied for further purification of inhibitors. The major steps involved in the purification were (i) fractionation with ammonium sulphate, (ii) chromatography on DEAE-cellulose ($-PO_4$ and -OH forms),

(iii) polyacrylamide gel electrophoresis and its electro elution by a newly developed method and (iv) affinity chromatography using immobilized trypsin.

An elaborate purification procedure involving several steps had to be employed for the purification of subtilisin inhibitor due to its close association with various protein impurities in the crude extract. A negative adsorption of subtilisin inhibitor on DEAE-cellulose (-PO_4 form) helped to remove a major proportion of trypsin inhibitor (about 97%) with a concomittant reduction in the protein load (95%). Papain inhibitor was separated from subtilisin by its (subtilisin inhibitor) specific adsorption on DEAE-cellulose (-OH form) at a very low ionic strength.

Inspite of the ion exchange chromatography under two different conditions the subtilisin inhibitor preparation was not homogeneous and showed 5 to 6 protein bands on analytical gel electrophoresis. Hence preparative PAGE was carried out for further purification. Although the PAGE eluted protein showed single protein band on gel electrophoresis at two different pHs, it still possessed both, subtilisin and trypsin inhibitor activity. In order to ascertain whether a single protein exhibits both the activities or whether they are two distinct proteins. The preparation was treated with immobilized trypsin for selective removal of trypsin inhibitor activity. The affinity chromatography resulted in the complete separation of subtilisin and trypsin inhibitors. Thus the residual trypsin inhibitor

activity in this preparation could not be separated by conventional methods but could be removed only by immobilized trypsin. A similar observation was made with subtilisin and papain inhibitors from Vigna unguiculata (33, 41). Physical methods for determining homogeneity appear to be of limited value with these inhibitors. Our results suggest that reports in the literature regarding apparently pure but non specific proteinase inhibitors (polyvalent inhibitors) require re-examination by affinity chromatography. The subtilisin inhibitor from double bean, cow pea and Adzuki beans were also purified employing tedious purification procedure (33, 34, 37).

An elaborate purification procedure was also described by Svendsen (34) and Yoshikawa (37) from double bean and Adzuki beans. It would have been ideal to use immobilized subtilisin for the purification of subtilisin inhibitor. However, this procedure was not adopted due to the possibility of getting modified inhibitor.

The purification of DTi was comparatively simpler and involved only a few steps viz. extraction, dialysis and repeated ammonium sulphate precipitation.

Properties

Proteinaceous nature of the proteinase inhibitors

The purified inhibitors show characteristic protein absorption spectra with no indication of any other

ultraviolet absorbing material. The inhibitors show their activity in the presence of EDTA. Even after dialysing against EDTA the inhibitors retain their inhibiting activity indicating that the inhibition is not due to a metal (unless the metal is very tightly bound with the protein). Also the specificity of the different proteinase inhibitors rules out the possibility of a metal being an inhibitor of the different proteolytic enzymes. The inhibitors show the usual behaviour of a typical protein such as (i) precipitation with ammonium sulphate; (ii) adsorption and elution from DEAE-cellulose, (iii) gel filtration on Sephadex; (iv) staining with Coomassie Blue G-250 on PAGE; (v) amino acid content and (vi) destruction of lysine by TNBS with concomittant loss of the inhibitor activity. Complex formation with the corresponding proteinases in stoichiometric proportion and cleavage of the complex by either heating or acid treatment also indicates the proteinaceous nature of the inhibitors.

Homogeneity

All the purified inhibitors were homogeneous on PAGE at pH 8.3 and 2.9. After treatment with SDS-thioethanol only a single band was obtained on SDS PAGE. Homogeneity of subtilisin and the associated trypsin inhibitor was also confirmed by gel filtration on Sephadex G-75 where the inhibitor eluated as a single peak.

Molecular weight (Mr)

The subtilisin and trypsin inhibitors from horse gram fall in the category of small molecular weight inhibitors. The molecular weight of the purified inhibitors are in the range of 5000 to 8000 daltons. Subtilisins and papain inhibitors from cow pea, another legume, were also shown to have low molecular weights ranging from 8000 to 12000 daltons (33). By comparison of the molecular weights of native and SDS-treated samples of subtilisin associated trypsin and dialysable trypsin inhibitors, it appears that there are no subunits in the inhibitors. Specific subtilisin inhibitors from culture broth of *Streptomyces albogriseous* sp. (32) has molecular weight 27,000; 23,000 and 12,000 by gel filtration, sedimentation equilibrium and by SDS-PAGE respectively. In this case it was shown that the inhibitor exists as a dimer which is converted into monomer during SDS-PAGE. Subtilisin inhibitors from barley have high molecular weights of about 20,000 (36).

Based on its specific activity of 3000 and a molecular weight of 5000 the molar combining ratio of trypsin with DTi is calculated to be 0.1:1 which is much lower than that of high molecular weight trypsin inhibitor of legumes (usually 1:1).

A plausible explanation for this observed discrepancy may be explained as (1) Although the DTi appears as a single band on PAGE it is likely that it may be having major protein impurity with similar charge and molecular weight. (2) The lower specific activity of DTi might have arisen due to its formation from high molecular weight inhibitor, by cleavage, resulting in decreased efficiency of the inhibitor.

To clarify this point more detailed work is necessary viz. the use of immobilized trypsin to see whether DTi is associated with major protein impurity of identical charge and molecular weight. The separation of DTi from the complex with trypsin will give the true specific activity of the inhibitor.

Ultraviolet absorption spectrum

The ultraviolet absorption spectrum of the purified inhibitors showed characteristic protein absorption spectra with no indication of any other ultraviolet absorbing impurities. Subtilisin, associated trypsin and dialysable trypsin inhibitors show 280:260 ratio of 1.31, 1.11 and 1.08 and optical factors of 0.88, 0.93 and 1.0, respectively whereas the optical factors of the corresponding cow pea (33) inhibitors were in the range of 0.8 to 1.7, indicating varying proportions of aromatic amino acids.

Dissociation constant

Dissociation constants for the complex of subtilisin-subtilisin inhibitor and trypsin-dialysable trypsin inhibitor was 2.69×10^{-10} M and 1.32×10^{-7}, respectively at pH 7.5. Some of the values for the dissociation constants given by other workers for the non-specific subtilisin inhibitors are: potato inhibitor IIa 1.1×10^{-7}, potato inhibitors IIb 2.2×10^{-8} and penguin ovomucoid 1×10^{-9}. The DTi has a considerably higher dissociation constant. This may be because of the fact that it may have been produced by the cleavage of the major inhibitor and hence may be less efficient as compared to the original inhibitor.

Amino acid composition

Subtilisin inhibitor and associated trypsin inhibitor

The subtilisin inhibitor from horse gram contains 1 mole of cysteine per mole of inhibitor and no cystine. The subtilisin inhibitor from double bean (34) Adzuki bean (37) and cow pea (33) are also devoid of cystine. In contrast the subtilisin inhibitor from french bean (261) contains 3.4 moles of cystine. Untill now only the serum inhibitors are known to be devoid of cystine. The subtilisin inhibitor from S. albogriseolus has 4 moles of half cystine per mole of the inhibitor. It contains large amounts of alanine (19 moles/mole) and valine (13 moles/mole) and no isoleucine, while subtilisin inhibitors

from cow pea (33) has large amounts of glutamic acid (12 moles/mole), 7 moles/mole each of valine and alanine and 2 moles of isoleucine per mole of inhibitor. Subtilisin inhibitor from horse gram contains high amounts of glutamic acid (11 moles/mole) valine (8 moles/mole) and does not contain histidine.

Associated trypsin inhibitor has 4 cystine residues.

Isoelectric point

The isoelectric points of the subtilisin and associated trypsin inhibitors are 7.66 and 7.70 while that of dialysable trypsin inhibitor is 4.10. The subtilisin inhibitors 1, 2a and 2b from Indian cowpea (33) showed isoelectric points at pH 5.1, 5.9 and 5.9, respectively whereas trypsin inhibitors 1 and 2 from the same source showed isoelectric points of 5.0 and 5.9.

N-terminal

N-terminal residue of subtilisin inhibitor from horse gram is alanine while subtilisin inhibitor from barley (36) has alanine at both amino and carboxy terminal.

Modification studies using subtilisin inhibitor

Earlier work on modification studies was carried out by Ozawa and Laskowski on trypsin and chymotrypsin inhibitor which are known to possess disulfide linkages. The modification studies with subtilisin inhibitors are of interest since they contain no disulfide residues.

In the present report it is shown that treatment of native subtilisin inhibitor with catalytic amount of subtilisin was not able to form modified inhibitor. The relative mobility of the native and the treated subtilisin inhibitor was the same on PAGE. Hence it is concluded that the subtilisin inhibitor does not get modified probably due to the absence of disulfide linkage.

It was postulated (157) that the native and modified inhibitors will remain active only if the two peptide chains at the reactive site are strongly held together and that the reduction of the disulfide linkage would destroy the modified inhibitor. It may be concluded that this disulfide hypothesis cannot be applied to subtilisin inhibitor which has no cystine in the molecule.

Stability

The low molecular weight inhibitors are generally stable to heat, trichloroacetic acid, low pH and ethanol which is also observed for the trypsin, subtilisin and dialysable trypsin inhibitors from horse gram.

The subtilisin inhibitor from *Streptomyces albogriseolus* (30) is stable from pH 3.0 to 10.0 at 37°C for 25 h and on boiling for 10 min between pH 5.0 and 6.0.

Specificity

When casein is used as a substrate the subtilisin inhibitor from horse gram specifically inhibits subtilisin

in the stoichiometric ratio of 1:1 and does not inhibit trypsin/chymotrypsin and papain. A similar observation was made for the subtilisin inhibitor from cow pea purified by Vartak *et al*. (33). However, when a synthetic substrate (BAEE) was used, the horse gram subtilisin inhibitor showed a molar combining ratio of 1:2 whereas that from cow pea showed a ratio of 1:1 (33). The subtilisin inhibitor from *S. albogriseolus* inhibits chymotrypsin besides subtilisin.

Associated trypsin inhibitor and DTi inhibit trypsin and chymotrypsin but do not inhibit subtilisin and papain. 1 mole of dialysable trypsin inhibitor inhibits 0.1 mole of trypsin and 0.05 mole of chymotrypsin.

Both major trypsin inhibitor and DTi show the same trypsin:chymotrypsin inhibitor ratio of 2 (2/1 and 0.1/0.05 respectively) supporting the view that the origin of DTi may be in the major trypsin inhibitor. Low molar combining ratios have also been reported for the subtilisin inhibitor from barley (28) and for the papain inhibitors from cow pea (41). This may be due to the fact that the actual substrate or enzyme is different *in vivo*. Another explanation for the lower combining ratio of DTi from horse gram may be the decrease in its specific activity as it arises by the cleavage of usual high molecular weight trypsin inhibitor which normally has a higher combining ratio of 1:1 (20, 33, 151, 262, 263).

Isoinhibitors

The reported subtilisin and papain inhibitors occur as isoinhibitors. However, the subtilisin inhibitor from horse gram has no isoinhibitors.

Possible physiological role of DTi

Green and Ryan (264) noted that when the potato or tomato plants were wounded by potato beetles of their larvae it caused a rapid accumulation of a proteinase inhibitor throughout the aerial tissue of the plants probably as a preventive measure against the trypsin-like enzymes from the insects. Formation of low molecular weight inhibitors such as DTi from high molecular weight inhibitor from horse gram may facilitate its rapid distribution throughout the tissue for the effective protection against insects.

Green and Ryan have also reported the isolation of a proteinase inhibitors inducing factor which stimulates the production of an inhibitor by mechanical damage. This phenomenon was found to depend on temperature and light. The DTi might have been produced from the original high molecular weight trypsin inhibitor by an acid stable factor and requires further elaborate investigation.

Although the molar combining ratio of trypsin with DTi is 0.1:1, on the weight basis 1 mg of trypsin and 0.5 mg of chymotrypsin will be inhibited by 2 mg of DTi. which is quite significant and useful physiologically.

CHAPTER-VI

SUMMARY AND CONCLUSION

Acid extract of horse gram seed (at pH 3.0 - 3.5) contains a large number of proteinase inhibitors which inhibit trypsin, chymotrypsin, subtilisin and papain. From these inhibitors a specific subtilisin inhibitor and an associated trypsin inhibitor were purified to homogeneity and their properties and kinetics were studied. A specific papain inhibitor was also purified partially. A widespread occurrence of a low molecular weight/dialysable trypsin inhibitor in the acid extract of different legumes is demonstrated for the first time. A dialysable trypsin inhibitor from the seed of horse gram was isolated, purified and characterized.

The inhibitors were purified by ammonium sulphate precipitation, DEAE-cellulose chromatography, preparative PAGE and affinity chromatography. Purification of dialysable trypsin inhibitor involved extraction of the seeds, dialysis and repeated ammonium sulphate precipitation of the dialysate.

Following new separation methods were developed and used during the purification of the inhibitors.

1. Electrophoretic elution of proteins from gel.
2. Method for removal of high concentration gels.
3. Use of fluorescent markers for the location of protein bands.
4. Polymerization of acrylamide at acid pH using uranyl nitrate.

The subtilisin inhibitor, associated trypsin inhibitor and the dialysable trypsin inhibitor were stable to heat, acid and ethanol. Subtilisin inhibitor and associated trypsin inhibitor were purified 930and 23 folds respectively and were homogeneous on native PAGE at pH 2.9 and 8.3 as well as on SDS-PAGE. DTi was purified 8.7folds.

The subtilisin inhibitor is specific for subtilisin and does not inhibit trypsin, chymotrypsin and papain. The associated trypsin inhibitor and dialysable trypsin inhibitor inhibit both trypsin and chymotrypsin.

Subtilisin inhibitor could not be modified on treatment with catalytic amount of subtilisin. Similarly there was no modification in the case of dialysable trypsin inhibitor.

N-terminal amino acid of subtilisin inhibitor is alanine.

Data regarding the other properties and kinetics of the different inhibitors are summarized in the following Table (Table XV).

TABLE XV : PROPERTIES AND KINETICS OF THE PROTEINASE INHIBITORS

	Subtilisin inhibitor	Associated trypsin inhibitor	Dialysable trypsin inhibitor
Specific activity units/mg	22,500	14,200	3,000
Optical factor	0.88	0.93	1
Absorption spectrum			
Maximum (nm)	275	275	275
Minimum (nm)	254	254	252
280:260 nm ratio	1.30	1.11	1.08
Molecular weight			
by gel filtration	7,500	8,650	ND
SDS PAGE	7,100	8,500	4,800
Slope method	7,300	ND	4,900
Amino acid analysis	9,450	8,200	
Isoelectric pH (pI)	7.66	7.70	4.1
Dissociation constant Ki at pH 7.5 (M)	2.69×10^{-10}	ND	1.32×10^{-7}
Tyrosine content per mole of inhibitor	2	1	
Tryptophan content per mole of inhibitor	1	2	
N-terminal	Alanine	ND	ND
Molar combining ratio	1:0.84	1:0.90	1:0.1

ND = Not determined

BIBLIOGRAPHY

1. Laskowski, M. Jr. and Sealock, R.W. (1971) The Enzymes Vol. III, p. 378. (Boyer, P.D., Ed.)

2. Ryan, C.A. (1981) Biochemistry of Plants, Vol. 6, Chapter 9, p. 351. (Academic Press, New York).

3. Neurath, H. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D.B., and Shaw, E., eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, p. 51.

4. Goldstein, I.M., (1974) in Mediators of inflammation (Weissman, G. ed.) Plenum Press, New York, p. 51.

5. Vogt, W. (1974) Pharmacol. Rev. 26, 125.

6. Schnebli, H.P. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D.B. and Shaw, E. eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, p. 785.

7. Meyn, M.S., Rossman, T., and Troll, W. (1977) Proc. Nat. Acad. Sci. USA 74, 1152.

8. Steiner, D.F., Kammlar, W., Tager, H.S., Rubenstein, A.H., Lernmark, A., and Zuhlke, H. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D.B., and Shaw, E., eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, p. 531.

9. Thompson, A.G. (1968) Ann. N.Y. Acad. Sci. 146, 50.

10. Amris, C.J. (1966) Scand. J. Haemat 3, 19.

11. Matis, P. and Merl, P.K. (1968) Ann. N.Y. Acad. Sci. 146, 715.

12. Back, N. (1966) Fed. Proc. 25, 77

13. Liener, I.E. and Kakade, M.L. (1969) Toxic Constituents of plant foodstuffs (Liener, I.E., ed.) p. 8, Academic Press, New York.

14. Hass, G.M., Hermodson, M.A., Ryan, C.A. and Gentry, L. (1982) Biochemistry 21, 752.

15. Hass, G.M. and Ryan, C.A. (1981) Methods in Enzymology Vol. 80, p. 778.

16. Takiuchi, I., Sci. Y., Takagi, H., and Sagawa, Y. (1984) Experientia 40, 846.

17. Rancour, J.M. and Ryan, C.A. (1968) Arch. Biochem. Biophys. 125, 380.

18. Kobayashi, S. and Watanabe, T. (1982) Igakuto Seibutsugaku 105, 227.

19. Baumgartner, B. and Chrispeels, M.J. (1976) Plant Physiol. 58, 1.

20. Kunitz, M. and Northrop, J.H. (1936) J. Gen. Physiol. 19, 991.

21. Ham, W.E. and Sandsteadt, R.M. (1944) J. Biol. Chem. 154, 505.

22. Bowman, D.E. (1944) Proc. Soc. Exptl. Biol. Med. 57, 139.

23. Kunitz, M. (1947) J. Gen. Physiol. 30, 291.

24. Borchers, R. and Ackerson, C.W. (1947) Arch. Biochem. Biophys. 13, 291

25. Werle, E., Maier, L. and Ringelmann, (1952) Naturwissenschaften 39, 328.

26. Besscho, H. and Kurosawa, S. (1966) Eiyo. To. Shokuryo 18, 365.

27. Yoshikawa, M., Kiyohara, T. and Ito, K. (1963) Hyogo Noka Digaku Kenkya Hokoku Nogei-Kagaku Hen. 6, 35.

28. Mikola, J. and Suolinna, E.M. (1971) Arch. Biochem. Biophys. 144, 566

29. Rhodes, M.B., Bennett, N. and Feeney, R.E. (1960) J. Biol. Chem. 235, 1686.

30. Murao, S. and Sato, S. (1972) Agr. Biol. Chem. 36, 160.

31. Sato, S. and Murao, S. (1973) Agr. Biol. Chem. 37, 1067.

32. Sato, S. and Murao, S. (1974) Agr. Biol. Chem. 38, 587.

33. Vartak, H.G., Bodhe, A.M., Paranjpe, S.V. and Jagannathan, V. (1980) Arch. Biochem. Biophys. 204, 129.

34. Svendsen, I.B., Hejgaard, J., and Chavan, J.K. (1984) Carlsberg Res. Commun. 49, 493.

35. Seidl, D.S., Abreu, H. and Jaffe, W.G. (1978) FEBS Lett. 92, 245.

36. Yoshikawa, M., Iwasaki, T., Fujii, M. and OOgaki, M. (1976) J. Biochem. 79, 765.

37. Yoshikawa, M., Tokota, K. and Hiraki, K. (1985) Agri. Biol. Chem. 42, 367.

38. Fossum, K. and Whitaker, J.R. (1968) Arch. Biochem. Biophys. 125, 367.

39. Sen, L.C. and Whitaker, J.R. (1973) Arch. Biochem. Biophys. 158, 623.

40. Umezawa, H. (1972) Enzyme inhibitors of microbial origin, p. 15.

41. Rele, M.V., Vartak, H.G. and Jagannathan, V. (1980) Arch. Biochem. Biophys. 204, 117.

42. Peanasky, R.J. and Ghaleb, M.A. (1971) Proc. Int. Res. Conf. Proteinase Inhibitors, Ist. 1970 ed. p. 281.

43. Rhodes, M.B., Marsh, C.L. and Kelley, G.W., Jr. (1963) Exptl. Parasitol. 13, 266.

44. Umezawa, H., Aoyagi, T., Morishima, H., Matsuzaki, M., Hamada, M. and Takeuchi, T. (1970) J. Antibiotics 23, 259.

45. Abu-Erreish, G.M. and Peanasky, R.J. (1974) J. Biol. Chem. 249, 1566.

46. Kunitz, M. (1948) J. Gen. Physiol. 32, 241.

47. Lebowitz, J. and Laskowski, M. Jr. (1962) Biochemistry 1, 1044.

48. Finkenstadt, W.R. and Laskowski, M. Jr. (1965) J. Biol. Chem. 240, 962.

49. Ozawa, K. and Laskowski, M. Jr. (1966) J. Biol. Chem. 241, 3955.

50. Laskowski, M. Sr. and Wu, F.C. (1953) J. Biol. Chem. 204, 797.

51. Kassell, B. and Laskowski, M. Sr. (1965) Biochem. Biophys. Res. Commun. 20, 463.

52. Anderer, F.A. and Hornle, S. (1966) J. Biol. Chem. 241, 1588.

53. Kunitz, M. (1947) J. Gen. Physiol. 30, 311.

54. Noda, K., Terada, S., Mutsuyasu, N., Waki, M., Kato, T. and Izumiya, N. (1971) Naturwiss 58, 147.

55. Yajima, H. and Kiso, Y. (1974) Chem. Pharm. Bull. 22, 1087.

56. Huber, R., Kukla, D., Ruhlmann, A., Eppo, O. and Formanek, H. (1970) Naturwissenshaften 57, 389.

57. Huber, R., Kulkla, D., Ruhlmann, A. and Steigemann (1971) Proc. on Proteinase Inhibitor Munich, p.56.

58. Bhatty, R.S. (1979) J. Inst. Can. Sci. Technol. Aliment. 12, 3.

59. Wolf, W.J. and Cowan, C.J. (1971) Soybeans as a food source: The Chemical Rubber Co. Cleveland Ohio 1971.

60. Polanowski, A. (1976) Acta. Univ. Vrasislaviensis 371, 3.

61. Koide, T.S., Tsunasawa and Ikenaka, T. (1973) Eur. J. Biochem. 32, 408.

62. Birk, Y., Bondi, A., Clestetner, B. and Ishaaya, T. (1963) Nature 197, 1089.

63. Birk, Y., Gretler, A. and Khalef, S. (1967) Biochem. Biophys. Acta. 147, 402.

64. Odani, S. and Ikenaka, T. (1972) J. Biochem. 71, 839.

65. Powers, J. and Whitaker, R.J. (1977) J. Food Chem. 1, 239.

66. Ikenaka, T., Odani, S., Sakai, M., Nabeshima, Y. Sato, S. and Murao, S. (1974) J. Biochem. 76, 1191.

67. Rackis, J.J., Sasame, H.A., Mann, R.K., Anderson, R.L. and Smith, A.K. (1962) Arch. Biochem. Biophys. 98, 471.

68. Yamamoto, M. and Ikenkaka, T. (1967) J. Biochem. 62, 141.

69. Frattali, V. and Steiner, R.F. (1968) Biochemistry 7, 821.

70. Krahn, J. and Stevens, J. (1970) Biochemistry 9, 2646.

71. Stevens, F.C., Wuerze, S. and Krahn (1974) Proc. Int. Res. Conf. 2nd Proteinase inhibitors.

72. Stevens, F.C. (1971) Proc. Int. Res. Conf. Ist Proteinase inhibitors

73. Jones, G., Moore, S. and Stein, W.H. (1963) Biochemistry 2, 66.

74. Haynes, E. and Feeney, R.E. (1967) J. Biol. Chem. 242, 5378.

75. Ventura, M.M. and Filho, J.X. (1966) An. Acad. Bras. Cienc. 38, 553.

76. Ventura, M.M., Filho, J.X., Moreira, R.A., Aquino, A.M. and Pihheiro, P.A. (1971) An. Acad. Bras. Cienc. 43, 233.

77. Gennis, L.S. and Cantor, C.R. (1976) J. Biol. Chem. 251, 734.

78. Royer, A., Miege, M.N., Grange, A., Miego, J. and Maschorpa, J.M. (1974) Planta (Berl.) 119, 1.

79. Sohonie, K. and Bhandarkar, A.P. (1955) JSIR, 14c, 100.

80. Ryan, C.A. (1973) Ann. Rev. Plant Physiol. 24, 173.

81. Richardson, M. (1977) Phytochemistry 16, 159.

82. Worowski, K. and Roszkowska, W. (1979) Postepy Biochemii 25, 229.

83. Whitaker, J.R. (1981) Impact of Toxicology on food processing

84. Hojima, Y., Moriwaki, C. and Moriya, H. (1973) J. Biochem. (Tokyo) 73, 933.

85. Melville, J.C. and Ryan, C.A. (1972) J. Biol. Chem. 247, 3445.

86. Ryan, C.A. (1968) Plant Physiology 43, 1880.

87. Ryan, C.A. (1966) Biochemistry 5, 1592.

88. Hass, G.M., Eddy, J., Gentry, L., Derr, J. and Ryan, C.A. (1978) Fed. Proc. 37, 1337.

89. Richardson, M. and Cossins, L. (1974) FEBS Lett. 45, 13.

90. Richardson, M. and Cossins, L. (1975) FEBS Lett. 52, 161.

91. Iwasaki, P., Iguchi, J., Kiyoharu, T. and Yoshikawa, M. (1974) J. Biochem. (Tokyo) 75, 1387.

92. Iwasaki, T., Wada, J., Kiyohara, T., Toshikawa, M. (1975) J. Biochem. (Tokyo) 78, 1267.

93. Iwasaki, P., Kiyohara, T., Yoshikawa, M. (1974) J. Biochem. (Tokyo) 75, 843.

94. Ryan, C.A., Hass, G.N. and Kuhn, R.W. (1974) J. Biol. Chem. 249, 5495.

95. Hass, G.M., Nau, H., Biemann, K., Grahn, D.T. and Erickson, L.H. (1975) Biochem. 14, 1334.

96. Rodis, P. (1974) Nature and function of cubical protein crystals in Solanum tuberosum, Ph.D. Thesis, Purdue University, Lafayette.

97. Huff, J.E. (1972) Personal Communication to Sen L.C. and Whitaker, J.R. (1973) Arch. Biochem. Biophys. 158, 823.

98. Mossar, G., Skupin, J. and Romanowska, B. (1984) Nahrung 28, 93.

99. Liu, W., Means, G.E. and Feeney, R.S. (1971) Biochem. Biophys. Acta. 229, 176.

100. Keilova, H. and Tomasek, V. (1974) Biochem. Biophys. Acta. 334, 179.

101. Peanasky, R.J., Bentz, Y., Paulson, B., Graham, D.L. and Babin, D.R. (1984) Arch. Biochem. Biophys. 232, 127.

102. Keilova, H. and Tomasek, V. (1972) Biochem. Biophys. Acta. 284, 461.

103. Kassell, B. and Laskowski, M. Sr. (1966) Acta. Biochem. Pllon, 13, 287.

104. Fritz, H., Kruck, J., Russe, I., Liebich, H.G. (1979) Hoppe-Seylers Z. Physiol. Chem. 360, 437.

105. Werle, E. (1964) Z. Physiol. Chem. 338, 228.

106. Joubert, F.J., Strydom, D.J. (1978) J. Biochem. 87, 191.

107. Strydom, D.J. (1973) Nature New Biol. 243, 88.

108. Strydom, D.J. (1977) Biochem. Biophys. Acta. 494, 361.

109. Kato, I., Tominaga, N. (1979) Fed. Proc. 38(3), 832 Abstr.

110. Kazel, L.A., Spicer, D.S. and Brahinsky, R.A. (1948) J. Ame. Chem. Soc. 70, 3034.

111. Greene, L., Righi, J.M. and Fackre, D.S. (1966) J. Biol. Chem. 241, 5610.

112. Schneider, S.L. and Laskowski, M. Sr. (1974) J. Biol. Chem. 249, 2009.

113. Fritz, H., Huller, I., Wiedemann, M. and Werle, E. (1967) Hoppe-Seylers Z. Physiol. Chem. 348, 405.

114. Burch, P.J., Cerwinsky, E.W. and Grinnan, E.L. (1967) Abstr. Am. Chem. Soc. Meeting, C-22 (Methods in Enzymology 19, 906, 1970).

115. Pubols, M.H., Bartelt, D.C. and Greene, L.J. (1974) J. Biol. Chem. 249, 2235.

116. Fritz, H., Gebhardt, M., Meister, R., Fink, E. (1971) Proc. Ist Int. Res. Conf. Proteinase Inhibitors Berlin, p. 271.

117. Fritz, H., Oppitz, K.H., Gebhardt, M., Oppitz, I., Werle, E., Marx, R. (1969) Hoppe-Seylers Z. Physiol. Chem. 350, 91.

118. Krejci, K., Fritz, H. (1976) FEBS Lett. 64, 152.

119. Collen, D., Wiman, B. and Verstraete, M. (1979) The Physiological Inhibitors of Blood Coagulation and Fibrinolysis, pp 295.

120. Bundy, H.F. and Mehl, J.W. (1959) J. Biol. Chem. 234, 1124.

121. Schwinck, H.G., Heimburger, N. and Haupt, H. (1966) Z. Ges. Inn. Med. 21, 193.

122. Shulmann, N.R. (1955) J. Biol. Chem. 213, 655.

123. Harpel, P.C. (1976) Methods in Enzymology Vol. 45B, p. 639.

124. Crawford, I.P. (1973) Arch. Biochem. biophys. 156, 215.

125. Moroi, M., Aoki, M. (1976) J. Biol. Chem. 251, 5956.

126. Collen, D. (1976) Eur. J. Biochem. 69, 209.

127. Mullertz, S., Cheemensen, I. (1976) Biochem. J. 159, 545.

128. Petersen, T.E. (1979) The Physiological Inhibitors of Blood Coagulation and Fibronolysis, p. 43.

129. Trautschold, I. (1965) Habilitations Schrift der Med. Fakultat Muchen Vogel _et al_. (1968) NPI 80/p.31

130. Trautschold, I., Fritz, H., Haendle, H. and Werle,E. (1966) Paper read at Conference on Chemistry Pharmacology and Clinical Applications of Proteinase Inhibitors, Academy of Sciences, New York.

131. Ito, A., Tokawa, K. and Shimuzu, B. (1972) Biochem. Biophys. Res. Commun. _49_, 343.

132. Martin, C.I. and Axelrod, A.E. (1958) Biochem. Biophys. Acta. _27_, 52.

133. Matusba, K. (1960) Mie. Med. J. _10_, 21.

134. Learmonth, E.M. (1951) J. Sci. Food Agric. _2_, 447.

135. Learmonth, E.M. (1958) J. Sci. Food Agric. _9_, 269.

136. Heinrikson, R.L. and Kezdy, F.I. (1976) Methods. Enzymol. _45B_, 740.

137. Archer, B.L. (1985) Phytochemistry _22_, 633.

138. Mundy, J., Svendsen, I.B. and Hejgaard, J. (1983) Carlsberg Res. Commun. _48_, 81.

139. Svendsen, I., Boisen, S. and Hejgaard, J. (1982) Carlsberg, Res. Commun. _47_, 45.

140. Satow, Y., Mitsui, Y., Iitaka, Y., Murao, S. and Sato, S. (1973) J. Mol. Biol. _75_, 745.

141. Inouye, K., Tonomura, B. and Hiromi, K. (1975) Agr. Biol. Chem. 39, 1159.

142. Balls, A.K. and Ryan, C.A. (1963) J. Biol. Chem. 238, 2976.

143. Ryan, C.A. and Kassell, B. (1970) Methods in Enzymol. 19, 883.

144. Trautschold, I., Werle, E., Haendle, H. and Sebening, H. (1963) Z. Physiol. Chem. 332, 328.

145. Fritz, H., Jaumann, E., Meister, R., Pasquay, P. Hochstrasser, K. and Finke, E. (1971) Proc. Int. Res. Conf. Proteinase Inhibitors Ist 1970 Ed., p. 257.

146. Aoyagi, T., Miyata, S., Nanbo, M., Kojima, F., Matsuzaki, M., Ishizuka, M., Takeuchi, T. and Umezawa, H. (1976) J. Antibiotics 22, 558.

147. Vogel, R., Trautschold, Il. and Werle, E. (1968) Natural Proteinase Inhibitors Academic Press, New York, p. 42.

148. Collier, H.B. (1941) Can. J. Res. B-19, 91.

149. Green, N.M. (1957) Biochem. J. 66, 407.

150. Laskowski, M. Jr. and Laskowski, M. Sr. (1951) J. Biol. Chem. 190, 563.

151. Laskowski, M. Sr., Kassell, B. and Hagerty, G. (1957) Biochem. Biophys. Acta. 24, 300.

152. Fratalli, V., and Steiner, R.F. (1969) Biochem. Biophys. Res. Commun. 34, 480.

153. Wagner, L.P. and Richm, J.P. (1967) Arch. Biochem. Biophys. 121, 672.

154. Wu, Y.V., and Scheraga, H.A. (1962) Biochemistry 1, 698.

155. Edelhoch, H. and Steiner, R.F. (1963) J. Biol. Chem. 238, 931.

156. Wu, F.C. and Laskowski, M. Sr. (1980) J. Biol. Chem. 235, 1680.

157. Laskowski, M. Jr. and Sealock, R.W. (1971) The Enzymes Vol. III, 378.(Boyer, P.D., Ed.).

158. Green, L.J. and Bartlet (1969) J. Biol. Chem. 244, 2646.

159. Cechora, D., Jonakova, V. and Sorm, F. (1971) Proc. on 'Proteinase Inhibitors' Ist Conference, p. 105.

160. Hochstrasser, K. and Werle, E. (1971) Proc. Int. Res. Conf. 'Proteinase Inhibitors' Ist 1970 ed,p.169.

161. Tschesche, H. and Wachter, E. (1970) Eur. J. Biochem. 16, 187.

162. Takahashi, H., Iwahaga, S., Kitagawa, T., Hokama,Y. and Suzuki, T. (1974) J. Biochem. 76, 721.

163. Ikenaka, T., Odani, S. and Koide, T. (1974) Proteinase Inhibitors 2nd Conference (Fritz and Tschesche Eds).

164. Sri Ram, J. Terminiello, L., Bier, M. and Nord, F.F. (1954) Arch. Biochem. Biophys. 52, 451.

165. Fritz, H., Trautschold, I. and Werle, E. (1965) Hoppe Seylers Physiol. Chem. 342, 253.

166. Edelhoch, H. and Steiner, R.F. (1965) J. Biol. Chem. 240, 2877.

167. Feinstein, G. and Feeney, R.E. (1967) Biochemistry 6, 749.

168. Green, N.M. and Work, E. (1953) Biochem. J. 54,347.

169. Vincent, J.P. and Lazdunski, M. (1972) Biochem. 11, 2967.

170. Hochstrasser, K., Schwartz, S., Illchmann, K. and Werle, E. (1968) Z. Physiol. Chem. 349, 1449.

171. Horez, C. (1973) Can. J. Biochem. 51, 1447.

172. Sato, S., Kimura, T. and Murao, S. (1975) Agric. Biol. Chem. 39, 415.

173. Astrup, T. and Nissen, U. (1964) Nature 203, 255.

174. Colman, R.W. and A. Bagdasarian (1976) Meth. Enz. Vol. 45B, p. 303.

175. Birk, Y. (1976) Methods in Enzymology, Vol. 45B,728.

176. Wu, F.C. and Laskowski, M. Sr., (1955) J. Biol. Chem. 213, 609.

177. Kraut, H. and Bhargava, N. (1967) Z. Physiol. Chem. 348, 1500.

178. Rigbi, M. (1971) Proc. Int. Res. Conf. Proteinase inhibitors. Ist 1970 Ed., p. 74.

179. Tschesche, H.J., Schorp, G. and Dietl, (1974) Proteinase Inhibitors 2nd Conference., p. 362.

180. Birk, Y. (1976) Methods in Enzymol. Vol. 45B, p.723.

181. Feeney, R.E., Means, G.E. and Bigler, J.C. (1969) J. Biol. Chem. 244, 1957.

182. Liu, W.H., Feinstein, G., Osuga, D.T., Haynes, R. and Feeney, R.E. (1968) Biochemistry 7, 2886.

183. Birk, Y. (1976) Methods in Enzymol. Vol. 45B, p.700.

184. Birk, Y. (1976) Methods in Enzymol. Vol. 45B, p.707.

185. Warsy, A.S., Norton, G. and Stein, M. (1974) Phytochemistry. 13, 2481.

186. Ofelt, C.W., Smith, A.K., Miles, J.M. (1955) Cereal Chem. 32, 53.

187. Shain, Y. and Mayer, A.M. (1965) Physiol. Plant. 18, 853.

188. Shain, Y. and Mayer, A.M. (1968) Phytochemistry 7, 1491.

189. Kirsi, M. and Mikola, J. (1971) Planta 96, 281.

190. Mikola, J. and Enari, T.M. (1970) J. Inst. Brew. 76, 182.

191. Burger, W.C. and Siegelman, W. (1966) Physiol. Plant. 19, 1089.

192. Filho, X.J. (1973) Physiol. Planta 28, 149.

193. Ryan, C.A. (1971) Biochem. Biophys. Res. Chem. 44, 1265.

194. Kowalski, D. and Laskowski, M. Jr. (1972) The Enzymes Vol. III (Boyer, P.D., Ed.) p. 420.

195. Rigbi, M. and Greene, R.J. (1968) J. Biol. Chem. 243, 5457.

196. Finkenstadt, W.R. and Laskowski, M. Jr. (1967) J. Biol. Chem. 242, 771.

197. Sealock, R.W. and Laskowski, M. Jr. (1969) Biochemistry 8, 3708.

198. Dlouha, V., Keil, B. and Sorm, F. (1968) Biochem. Biophys. Res. Commun. 31, 66.

199. Feinstein, G. and Feeney, R.E. (1966) J. Biol. Chem. 241, 5183.

200. UY, R., and Feeney, R.E. (1970) Proc. Int. Res. Conf. Proteinase Inhibitors Ist 1970 ed., p. 164.

201. Feeney, R.E. (1971) Proc. Int. Res. Conf. Proteinase Inhibitor Ist 1970 ed., p. 162.

202. Gorini, L. and Audrain, L. (1952) Biochem. Biophys. Acta. 8, 702.

203. Gorini, L. and Audrain, L. (1953) Biochem. Biophys. Acta 10, 570.

204. Sakura, J.D. and Timasheff, S.N. (1973) Arch. Biochem. Biophys. 159, 123.

205. Neurath, H. (1984) Science 224, 350.

206. Frank, B.H. and Veros, A.J. (1968) Fed. Proc. 27,392.

207. Erickson, S. (1964) Acta Med. Scand. 175, 197.

208. Sharp, H.L., Bridges, R.A., Krivit, W. and Friuer, E.F. (1969) J. Lab. Clin. Med. 73, 934.

209. Chan, S.K. and Rees, D.C. (1975) Nature 250, 240.

210. Beumgartner, B. and Chrispeels, M.J. (1976) Plant Physiol. 58, 1.

211. Eriksson, and Larsson, (1975) Nutritional Reviews 33, 189.

212. Kuhlenschmidt, M.S., Yunis, E.J., Lammarino, R.M., Turco, S.J., Peters, S.P. and Glew, R.H. (1974) Lab. Invest. 31, 413.

213. Taber, R., Wertheimer, R. and Golrich, J. (1973) J. Mol. Biol. 80, 367.

214. Werle, E., Trautschold, I., Haendle, H. and Fritz, (1968) Natural Proteinase Inhibitors, Vogel. 1968.

215. Astrup, T. (1968) Ann. N.Y. Acad. Sci. 146, 601.

216. Mansfel, V-, Ziegelhaffer, A., Horakova, Z. and Haldoree (1959) Naturwissenschaften 46, 172.

217. Ryan, C.A. (1973) Ann. Rev. Plant. Physiol. 24, 186.

218. Giebel, W., Zwilling, R., Pffeiderer, G. (1971) Comp. Biochem. Physiol. 38B, 197.

219. Lenny, J.E., Metile, Ph., Wieken, A., Schellenbery, M. and Meyer, J. (1974) Biochem. Biophys. Res. Commun. 60, 1378.

220. Mikola, J. and Suolinna, E.M. (1969) Eur. J. Biochem. 9, 155.

221. Green, G.M., and Ryan, C.A. (1972) Science 175,776.

222. Zbytniewski, Z. and Kanclerz, A. (1977) Post Hig. Med. Dosw. 31, 393.

223. Zaneveld, L.J.D., Schumacher, G.F.B., Tauber, P.F. and Propping, D. (1974) Proc. Int. Res. Conf. IInd on Proteinase Inhibitors, New York

224. The wealth of India, Raw material (1952), Council of Scientific and Industrial Research, Vol. III D - E, 101.

225. Peterson, E.A. and Sober, H.A. (1956) J. Am. Chem. Soc. 78, 751.

226. Laskowski, M. Sr. (1955) Methods in Enzymol. 2, 20.

227. Englund, P.T., King, T.P., Craig, L.C. and Walti,A. (1968) Biochemistry 7, 163.

228. Schwert, G.W. and Takenaka, Y. (1965) Biochim. Biophys. Acta 16, 570.

229. Walsh, K.A. and Wilcox, P.E. (1970) Methods in Enzymol. 19, 31.

230. Glazer, A.N. (1967) J. Biol. Chem. 242, 433.

231. Arnon, R. (1965) Immunochemistry 2, 107.

232. Blumberg, S., Schechter, I. and Berger, A. (1970) Eur. J. Biochem. 15, 97.

233. Warburg, O. and Christian, W. (1941) Biochem. Z. 310, 384.

234. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.L. (1951) J. Biol. Chem. 193, 265.

235. Cohn, E.J., Hughes, W.L. Jr., and Weare, J.H. (1947) J. Am. Chem. 69, 1753.

236. Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375.

237. Jagannathan, V., Kartar Singh and Damodaran, M. (1956) Biochem. J. 63, 94.

238. Davis, H. (1964) Ann. N.Y. Acad. Sci. 121, 404.

239. Blakesley, R.W. and Boezi, J.A. (1977) Anal. Biochem. 82, 580.

240. Ghadge, G.D., Bodhe, A.M., Dhume, S.T., Rele, M.V. and Vartak, H.G. (1984) J. Bioscience 6, 135.

241. Vesterberg, O. and Svenson, H. (1966) Acta. Chemica Scand. 20, 820.

242. Green, N.M. and Work, E. (1953) Biochem. J. 54, 347.

243. Bieth, J. (1974) Proteinase inhibitors Bayer Symposium V, p. 463.

244. Shapiro, A.L., Vinuela, H. and Maizel, J.V, Jr. (1967) Biochem. Biophys. Res. Commun. 28, 815.

245. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406.

246. Hedrick, A.L. and Smith, A.J. (1968) Arch. Biochem. Biophys. 126, 155.

247. Andrews, P. (1965) Biochem. J. 96, 595.

248. Tsou, C.L. (1951) Biochem. J. 49, 362.

249. Otto, M. and Snejdarkova, M. (1981) Anal. Biochem. 111, 111.

250. Spackman, D.H., Stein, W.H. and Moor, S. (1958) Anal. Chem. 30, 1190.

251. Godwin, T.W. and Morton, R.A. (1946) Biochem. J. 40, 628.

252. Ellman, G.L. (1958) Arch. Biochem. Biophys. 74, 443.

253. Cavallini, D., Graziani, M.T. and Dupre, S. (1966) Nature 212, 294.

254. Bodhe, A.M., Deshpande, V.V., Lakshmi Kantham, B.C. and Vartak, H.G. (1982) Anal. Biochem. 123, 133.

255. Ghadge, G.D., Bodhe, A.M., Modak, S.R. and Vartak, H.G. (1983) Anal. Biochem. 128, 468.

256. Bodhe, A.M., Vartak, H.G., Rele, M.V. and Dhamankar, V.S. (1987) Anal. Biochem. 164, 39.

257. Axen, R. and Ernback, S. (1971) Eur. J. Biochem. 18, 351.

258. Takahashi, K. (1966) J. Biol. Chem. 243, 6171

259. Grossberg, A.L. & Pressman, D. (1968) Biochemistry 7, 272.

260. Chang, J.Y. (1983) Methods in Enzymol. 91, Academic Press, New York, p. 455.

261. Seidl, D.S., Abreu, H. and Jaffe, W.G. (1982) Int. J. Peptide Res. 19, 153.

262. Birk, Y. (1976) Methods in Enzymol. 45B, 716.

263. Tauber, H., Kershaw, B.B. and Bartulowich, J.J. (1976) Aot. Biochem. Biophys. 115, 536.

264. Green, T.R. and Ryan, C.A. (1972) Science 175, 776.

265. Mehta, S.L. and Simlot, M.M. (1982) J. Bioscience 4, 295.

266. Deshpande, V.V., Bodhe, A.M., Pawar, H.S. and Vartak, H.G. (1986) Anal. Biochem. 153, 227.

267. Maurer, H.R. (1971) Disc gel electrophoresis and related techniques of PAGE. Berlin, Walter de Gruyter, New York.