PROTEINASE INHIBITORS

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H. G. VARTAK, M. Sc.

DIVISION OF BIOCHEMISTRY
NATIONAL CHEMICAL LABORATORY
POONA - 411 008 (India)
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LIST OF ABBREVIATIONS

BAEE	N-benzoyl-L-arginine ethyl ester
BAPA	Benzoyl arginine p-nitroanilide
BTEE	N-benzoyl-L-tyrosine ethyl ester
CM-cellulose	Carboxymethyl cellulose
DEAE-cellulose	Diethylaminoethyl cellulose
DTNB	5-5' dithio-bis-2-nitrobenzoic acid
EDTA	Ethylenediamine tetraacetate
g	gram (s)
x g	Acceleration due to gravity
GSH	Reduced glutathione
h	Hour(s)
mg	milligram(s)
min	Minute(s)
O.D.	Optical density (absorbance)
Δ O.D.	Change in optical density (absorbance)
SBTI	Soybean trypsin inhibitor (Kunitz)
SDS	Sodium dodecyl sulphate
STD	Standard
TEMED	Tetraethylmethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane

CHAPTEH I

INTRODUCTION

INTRODUCTION

Section 1

"The term 'protein proteinase inhibitor' refers to a protein which may associate reversibly with one or more proteinases to form complexes of distinct stoicheiometry in which all the catalytic functions of the proteinase are competitively inhibited" (Laskowski and Sealock, 1971).

Kunitz and Northrop (1936) were the first to isolate and crystallize a trypsin inhibitor from bovine pancreas in 1936. Subsequently reports regarding the isolation of proteolytic inhibitors from various sources have been published. In plants, especially in Leguminosae, trypsin inhibitors are present in abundance. However the occurrence of specific subtilisin and papain inhibitors has been reported only in a few cases. The occurrence of specific subtilisin inhibitors in a plant or an animal source has not hitherto been described. Moreover the simultaneous occurrence of all the above mentioned inhibitors in a single plant source has also not been reported.

The present work deals with the isolation, purification and partial characterization of subtilisin, papain and trypsin inhibitors from seeds of Vigna catjang.

Section II

Historical

Fields of investigation

Research on proteinase inhibitors has been carried out in several different fields, especially in three major fields. One is the isolation, purification and characterization of a large number of inhibitors from plant, animal and microbial sources. The second is the possible physiological role and pharmacological and nutritional significance of the inhibitors. The third is the attempt to understand enzyme-inhibitor interaction and specificity in molecular terms.

Occurrence of inhibitors

The first major achievement in inhibitor research was the crystallization of a trypsin inhibitor from bovine pancreas and the crystallization of its complex with trypsin by Kunitz and Northrop (1936). The first known plant trypsin inhibitor was that from soybean, which was discovered by Ham and Sandstedt (1944) and Bowman (1944) and later crystallized by Kunitz (1947). Another trypsin inhibitor was later found in lima beans as well as in many of the Leguminosae (Borchers and Ackerson, 1947). Subsequently trypsin inhibiting activity was found not only in other families of plants (Werle, Mier and Ringelmann, 1952) but also in microorganisms. Thus trypsin inhibitors were shown to be widely distributed in nature. Several of these inhibitors also inhibit, in addition to trypsin, other enzymes such as chymotrypsin, thrombin and plasmin.

Although inhibitors of trypsin are widespread in nature the 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 (Bessho and Kurosawa, 1966; Yoshikawa, Kiyohara and Ito, 1963), barley (Mikola and Suolinna, 1971) and avian ovomucoids (Rhodes, Bennett and Feeney, 1960), but these are not specific for subtilisin and also inhibit other proteolytic enzymes. A specific subtilisin inhibitor has been purified from the culture broth of Streptomyces albogriseolus (Murao and Sato, 1972 and Sato and Murao, 1973, 1974). A few papain inhibitors have been reported to be present in animal tissues, plants and microorganisms of which only the inhibitors from chicken egg white (Fossum and Whitacker, 1958; Sen and Whitaker, 1973) and Streptomyces (Umezawa, 1972) have been purified. The latter are small peptides and non-specific in nature. Rhodes, Marsh and Kelley (1963) have reported the purification of two different chymotrypsin inhibitors from Ascaris. Pepsin inhibitors were isolated and purified from the culture broths of Streptomyces strains (Umezawa, Aoyagi, Morishima, Matsuzaki, Hamada and Takeuchi (1970) and from Ascaris (Peanasky and Abu-Erreish, 1971).

Major contributions to the study of inhibitors

Kunitz was a pioneer not only in the isolation but also in the study of the properties and kinetics of proteinase inhibitors. His contributions include crystallization of the trypsin inhibitor, establishment of 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 low pH and reassociation at higher pH. Detailed kinetic studies have been subsequently made on the inhibitors from other sources.

Amino acid analysis, sequence determination and synthesis

Several other workers have studied the mechanism of action of different inhibitors. With the isolation of pure inhibitors and the

availability of increasingly sophisticated methods for amino acid analysis, the amino acid composition of several of the inhibitors was determined. This was followed by the determination of the amino acid sequence of bovine basic pancreatic inhibitor by Kassal, Radicevic, Ansfield and Laskowski in 1965. In 1966 Anderer and Hornle described the amino acid sequence of a kallikrein inhibitor from bovine lung and parotid gland. Since the sequence presented for these inhibitors turned out to be identical in every respect it was concluded that the inhibitors were identical even though they occurred in three different bovine organs.

Noda, Terada, Mitsuyasu, Waki, Kato and Izumiya (1971) synthesized the bovine pancreatic trypsin inhibitor (Kunitz) by carrying out a stepwise solid phase synthesis. Subsequently in 1974, Yajima and Kiso ~~and~~ synthesized the same inhibitor by fragment condensation on polymer support. As compared to the activity of the native inhibitor (Kunitz) the products obtained by Noda et al. and Yajima and Kiso had 30% and 82% activity respectively. X-ray crystallographic studies and the elucidation of the three dimensional structure of the Kunitz pancreatic trypsin inhibitor were carried out by Huber, Kukla, Ruhlmann and Steigemann (1971) and Huber, Kukla, Ruhlmann, Epp and Formanek (1970).

Temporary inhibition

Gorini and Audrain (1952, 1953) observed that a complex of trypsin and chicken ovomucoid on standing releases trypsin by slowly destroying the inhibitor. A similar observation was made by Laskowski and Wu (1953) in the case of a complex of trypsin and bovine secretory pancreatic inhibitor. This phenomenon was termed "temporary inhibition" and described in detail by Laskowski and Wu. Inactivation of the inhibitor on prolonged

incubation with trypsin was also shown recently in the case of a plant inhibitor - lima bean trypsin inhibitor (Sakura and Timasheff, 1974).

Modified inhibitor

On the basis of studies on the interaction between the Kunitz soybean trypsin inhibitor and trypsin (Lebowitz and Laskowski, 1962; Finkenstadt and Laskowski, 1965 and Ozawa and Laskowski, 1966) put forward an interesting new hypothesis for the interaction. The first step is the splitting of a single peptide bond Arg-Ile at the active site of the inhibitor to form a modified inhibitor. Complex formation then takes place between trypsin and the modified inhibitor through a covalent linkage. Whether this mechanism is valid for all trypsin inhibitors is controversial.

The above survey indicates the progress made in the study of the isolation, characterization and mode of action of the protease inhibitors. Their physiological role is however obscure.

Scope of the literature survey

A survey of the literature on proteinaceous proteinase inhibitors from animal, plant and microbial source is presented in this chapter. In the case of the inhibitors from microbial source a detailed description of a specific subtilisin inhibitor from Streptomyces albogriseolus is given. However other inhibitors from microbial sources, except for a brief mention of the inhibitors from actinomycetes and yeast, are not dealt with in the present report. Emphasis is laid on the isolation, purification and properties of the inhibitors from different sources, since they are related to the studies undertaken. The main subject of this thesis is the occurrence of a large number of inhibitors of trypsin, chymotrypsin, subtilisin and papain in seeds of Vigna catjang, the separation and purification of these inhibitors and the study of some of their properties. Detailed studies on the kinetics and mode of action and structural studies of such a large

number of inhibitors were not possible. The extensive literature on the kinetic and molecular basis of their action will only briefly be discussed. The term proteinase inhibitor is used to refer only to inhibitors which are polypeptides.

Section III

Inhibitors from different sources

a) Inhibitors from plant and animal tissues

Proteinase inhibitors are widely distributed in plants, animals and microorganisms. In plants they are particularly abundant in legumes. Several of the proteinase inhibitors have been isolated and purified. This section is restricted to a brief description of some of the well characterized proteinase inhibitors from plant and animal tissues.

Soybean: Fifteen years after the crystallization of a trypsin inhibitor from soybean (Kunitz, 1947), Birk (1961) found a second trypsin inhibitor ("acetone insoluble") which was also an inhibitor of bovine α-chymotrypsin. The Kunitz inhibitor inhibits trypsin, plasma kallikrein, plasmin and chymotrypsin B, contains tryptophan and is unstable to heat, acid, trichloroacetic acid and pepsin, whereas Birk's inhibitor has no tryptophan and is stable to heat, acid and pepsin (Birk, Bondi, Gestetner and Ishaya, 1963; Birk, Gertler and Khalef, 1967).

Four other trypsin inhibitors have subsequently been purified from soybean. These include SBTI A_1 (Rackis, Sasame, Mann, Anderson and Smith, 1962), 1.9 S inhibitor (Yamamoto and Ikenaka, 1967), F1 and F3 (Frattali and Steiner, 1968). SBTI A_1 has the lowest molecular weight (14,300). The 1.9 S inhibitor also inhibits chymotrypsin. It contains no glycine. F1 and F3 are weak inhibitors of trypsin. F3 contains no tyrosine.

Lima bean: Lima bean inhibitors have been separated into four components by Jones, Moore and Stein (1963). More recently Haynes and Feeney (1967) obtained six chromatographically distinct inhibitors of trypsin and chymotrypsin from lima beans. All the fractions studied 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 86 residues respectively) could each be derived from component 3 (93 residues) by proteolytic cleavage, probably by losing terminal peptides. Component 6 (89 residues) of Haynes and Feeney could similarly be a precursor of fraction 4 (84 residues).

Blood serum: There are several inhibitors of proteolytic enzymes in blood serum. Some are polyvalent (showing bread specificity) while others show narrow specificity. Several of these inhibit the blood-clotting enzymes plasmin and fibrinolysin as well as trypsin or α-chymotrypsin or both. At least four different inhibitors have been isolated and characterized from different fractions of human serum. The post albumin region contains a specific chymotrypsin inhibitor and a polyvalent inhibitor (Bundy and Mehl, 1959; Schwick, Heimburger and Haupt, 1966) for trypsin, chymotrypsin, thrombin and fibrinolysin is present in the α_1-globulin fraction. The α_2-globulin fraction also contains a polyvalent inhibitor (Shulman, 1955) for trypsin, chymotrypsin and (particularly) plasmin. A specific trypsin inhibitor is present between the bands α_1 and α_2 globulin fractions.

The proteinase inhibiting capacity of serum is mainly due to inhibitors 2 (90%) and 3 (9%). Inhibitor 2 is precipitated by trichloroacetic acid and is destroyed by chloroform and acetone while inhibitor 3 is soluble in trichloroacetic acid and is not affected by chloroform and acetone. All the four inhibitors are glycoproteins and possess high molecular weights ranging from 60,000 to 845,000. As stated earlier they are unstable to heat and acids.

Potato: Several inhibitors of different types have been isolated from potato. A chymotrypsin inhibitor ("Inhibitor 1") was purified and studied in detail (Ryan and Balls, 1962; Balls and Ryan, 1963; Ryan and Kassel, 1970; Melville and Ryan, 1972). One mole of inhibitor combines

with four moles of chymotrypsin. It inhibits α-chymotrypsin and chymotrypsin B as well as subtilisin, pronase (partly), human salivary and urinary kallikrein and trypsin. It is a weak inhibitor of trypsin. By using haemoglobin as the substrate 1 mg of the inhibitor was shown to inhibit 3.42 mg of chymotrypsin or 2.70 mg of subtilisin or 0.3 mg of trypsin. Moreover in the case of trypsin only the proteolytic and not the esterolytic activity is inhibited (Ryan, 1968). The inhibitor has a molecular weight of 38,000 and consists of four subunits.

Another inhibitor which inhibits carboxypeptidase B and α-chymotrypsin has been isolated from potato by Rancour and Ryan (1968). It is a dialyzable polypeptide having a molecular weight of 3,000–4,000. It also inhibits an endogenous bradykinin inactivating carboxypeptidase isolated from potato (Ryan, 1973). Three other isoinhibitors showing similar inhibition have also been detected in potato.

Two isoinhibitors specific for human plasma kallikrein have been isolated by Moriya et al. (1970) from this tuber. Recently Helitz, Kaiser and Santarius (1971) have shown the presence in potato of as many as thirteen isoinhibitors that inhibit trypsin and chymotrypsin.

Avian egg-white inhibitors: Chicken, duck, turkey and quail ovoinhibitors are inhibitors showing bread specificity. They inhibit trypsin, chymotrypsin, subtilisin and fungal proteinase from Aspergillus oryzae. These inhibitors have similar molecular weights (48,000±2,000) and similar combining ratios with trypsin and chymotrypsin. One mole of the inhibitor inhibits 2 moles of trypsin and two moles of chymotrypsin (Liu, Means and Feeney, 1971). Ovomucoids from eleven avian species have been studied by Rhodes, Bennett and Feeney (1960). They inhibit trypsin and chymotrypsin to varying degrees. Another inhibitor

from chicken egg white (Fossum and Whitaker, 1968; Sen and Whitaker,1973) inhibits papain and ficin. Papain and ficin compete for the same site in the inhibitor. This inhibitor inhibits cathepsin B1 and also forms a complex with the mercury form of cathepsin B1 (Keilova and Tomasek, 1974).

Ascaris: Three different types of specific inhibitors, one specific for trypsin, another for chymotrypsin and the third for pepsin (molecular weight 8,000) (Peanasky and Ghaleb, 1971) have been isolated and purified from Ascaris. The pepsin inhibitor also inhibits cathepsin E from rabbit bone marrow (Keilova and Tomasek, 1972).

Pancreatic inhibitors: The inhibitors from bovine pancreas are the best characterized inhibitors of proteolytic enzymes. Two inhibitors from bovine pancreas have been intensively studied(Kunitz 1938; Kassell 1966). The first inhibitor studied is called the Kunitz or basic inhibitor. This is a polyvalent inhibitor and inhibits trypsin, chymotrypsin, kallikrein and plasmin. It consists of a single polypeptide chain of only 58 amino acids and has a molecular weight of 6,300. The second inhibitor is present in pancreatic juice and is known as the Kazal pancreatic inhibitor (Kazal, 1948; Greene, Rigbi and Fackre, 1966). It is specific for trypsin. Recently Schneider and Laskowski (1974) have isolated three isoinhibiters from Kazal's crystalline inhibitor. Two forms of porcine pancreatic secretory trypsin inhibitors have been purified by Fritz, Huller, Wiedeman and Werle (1967) and by Burch, Cerwinsky and Grimman (1967). The molecular weight of inhibitor I is 6,040 and that of inhibitor II 5,400. Both inhibit trypsin. Five isoinhibitors of trypsin have been isolated and purified from human pancreas and pancreatic juice, with molecular weights of 6,242 and having 56 amino acid residues per mole. The inhibitors differ in their amide content (Pubols, Bartelt and Greene, 1974).

b) Inhibitors from Vigna and inhibitors of papain and subtilisin

In this subsection inhibitors studied from the genus Vigna and inhibitors of the proteinases, papain and subtilisin, obtained from various sources are described. The literature on these inhibitors is described separately since it is related to the subject of this thesis.

Inhibitors from black-eyed pea (Vigna sinensis)

Ventura and Filho (1966) have purified and partially characterised an inhibitor for α-chymotrypsin and trypsin from this seed. One mole of the inhibitor inhibits two moles of chymotrypsin and one mole of trypsin. Its molecular weight was earlier reported as 17,000, but in a recent report Ventura, Filho, Moreira, Aquino and Pinheiro (1971) have corrected it to 10,000. The discrepancy was explained to be due to the aggregation of the inhibitor molecules. The inhibitor contains 2 per cent tryptophan but no methionine and has an optical factor (Section IV) of 1.2. The purification steps involved extraction of the seeds with water, precipitation of impurities with trichloroacetic acid, ammonium sulphate precipitation and two chromatographies on DEAE-cellulose. The homogeneity of the purified inhibitor was verified by paper, starch gel and polyacrylamide electrophoresis and ultracentrifugation. A total of 150 amino acid residues was found per mole of inhibitor.

The crude seed extract showed on Sephadex G-75 chromatography (Filho, 1973) four different fractions having trypsin inhibiting activity, the molecular weights of which ranged between 11,000 and 21,000.

In a recent report Royer, Mdege, Grange, Miege and Mascherpa (1974) have demonstrated the presence of five trypsin inhibitors in the cotyledonary extract of Vigna unguiculata (from Zaire), Strain H 81. It was

also shown that these trypsin inhibitors partly inhibit the proteolytic activity of the extract. The presence of a complex of trypsin-like enzyme and trypsin inhibitor is postulated. Two types of proteinases were shown, one hydrolyzing BAPA and the other hydrolysing casein. Very little work has been done on the inhibitors from Vigna catjang. Sohonie and Bhandarkar (1955) have shown the presence of two trypsin inhibitors in these seeds, but no further purification of these inhibitors was carried out. Moreover, the presence of the inhibitors of other proteinases such as subtilisin, papain and chymotrypsin was also not shown in the seeds of this plant.

Papain inhibitors:

Although there are some reports regarding the occurrence of non-specific papain inhibitors, specific papain inhibitors have been only rarely reported. Broad bean trypsin inhibitor also inhibits chymotrypsin, papain (Sohonie, Huprikar and Joshi, 1959) and Aspergillus proteinase. Recently two non-specific proteinase isoinhibitors have been purified from broad beans, which inhibit trypsin, chymotrypsin, thrombin and papain (Waray, Norton and Stein, 1974). A trypsin inhibitor from the submandibular glands of dogs inhibits chymotrypsin and papain (Trautschold, 1965). A trypsin inhibitor from the seminal vesicles of guinea pigs (Trautschold, 1966) also inhibits plasmin, papain and bacterial proteinase from Streptomyces griseus. An antipapain factor has been shown in rabbit serum (Itoh, 1966) and skin (Martin and Axelrod, 1958). A papain inhibitor was reported to be present in wheat flour (Hites, Sandstedt and Schaumburg, 1951).

Recently several types of proteinase inhibitors have been obtained from some strains of Streptomyces (Umezawa, 1972). These are small peptides and are designated as leupeptins, pepstatin, antipain and chymostatin.

They find clinical application in different pathological conditions. Leupeptin inhibits trypsin, papain and kallikrein but not α-chymotrypsin. Pepstatin, a pentapeptide, inhibits pepsin. Antipain inhibits papain, trypsin and to a certain extent plasmin. Chymostatin inhibits chymotrypsin and cathepsin B.

The only known specific papain inhibitor which has been purified is from chicken egg white (Fossum and Whitaker, 1968). (Sen and Whitaker, 1973). In addition to papain it inhibits ficin. It does not inhibit trypsin, chymotrypsin and several bacterial proteinases. 1 mole of inhibitor inhibits 1 mole of papain or ficin. Papain and ficin apparently compete for the same site. The dissociation constant of the enzyme inhibitor complex is 1.5×10^{-8} M. At 100° in 30 min at pH 4 it loses only 10% activity whereas at pH 9 it loses 60% activity. Its molecular weight was found to be 12700 by gel filtration. It contains no carbohydrate. Huff (1972, unpublished) has crystallized a papain inhibitor from potato.

An inhibitor isolated from rabbit skin inhibits a dermatoproteinase also isolated from the skin. The inhibitor also inhibits papain (Matsuba, 1960). Aqueous extracts of raw soybeans were shown to inhibit the action of papain (Learmonth, 1951). The papain inhibitory factor was shown to be concentrated almost entirely in the germ (Learmonth, 1958) whereas the cotyledon was without inhibiting action. A similar distribution of papain inhibiting factors was shown to exist in other leguminous seeds.

Subtilisin inhibitors

A few non-specific inhibitors of subtilisin have been reported from avian egg white, potato and barley, but there is only one report of a specific subtilisin inhibitor which was shown to be present in a microbial source (Murao and Sato, 1972; Sato and Murao, 1973).

As mentioned earlier ovoinhibitors from duck, turkey, chicken and quail egg white inhibit trypsin, α-chymotrypsin, subtilisin and the alkaline proteinase from A.oryzae. Chymotrypsin and subtilisin compete for the same site (Liu et al. 1971). The polyvalent potato inhibitor (Type 1) inhibits chymotrypsin, trypsin, subtilisin, pronase (partly) and human salivary and urinary kallikrein (Balls and Ryan, 1963; Ryan and Kassell, 1970; Melville and Ryan, 1972). A nonspecific inhibitor from barley grains which has been purified about 30-fold (Mikola and Suolinna, 1971) inhibits alkaline proteinases of A.oryzae, B.subtilis, S.griseus and Alternaria tenuissima and chymotrypsin. Its molecular weight is about 25,000. By the casein hydrolysis method 1 mg of the inhibitor inhibits 0.36 mg of chymotrypsin or 0.29 mg of subtilisin. When the synthetic substrate (glutaryl-L-phenylalanine-nitroanilide) was used for chymotrypsin assay, 1 mg of the inhibitor inhibited 2 mg of chymotrypsin. It was resolved into 3-5 isoinhibitors of similar specific activity and properties. They differ only in their isoelectric points which range between 4.6 to 5.4. Very high proteinase inhibitor concentration for an animal tissue is found in the submandibular glands of dogs (Trautschold et al. 1963). Four inhibitors having broad specificity were isolated and purified from this source (Fritz et al. 1971). They inhibit trypsin, chymotrypsin, subtilisin, elastase, plasmin and A.oryzae proteinase. Their molecular weights vary between 12,750 and 12,878. The trypsin and chymotrypsin inhibiting sites are distinct while chymotrypsin and subtilisin reactive sites are identical.

All the inhibitors described so far in the section on "Subtilisin inhibitors" are not specific for subtilisin as stated earlier. A specific subtilisin inhibitor has been recently isolated and crystallized from the culture broth of Streptomyces albogriseolus (Murao and Sato, 1972; Sato and Murao, 1973, 1974). It was homogeneous on acrylamide gel and was

stable at 100° for 10 min at pH 4-6. 1 mg of the inhibitor combines with 1.64 mg of subtilisin. Its ultraviolet absorption spectrum shows a maximum at 280 nm and a minimum at 250 nm. Its optical factor is 1.22. It does not contain carbohydrate or phosphorus. The inhibitor was found to be highly specific towards microbial alkaline proteases. It inhibited subtilisin, BPN and alkaline proteases of B.subtilis'.,S04, B.subtilis var. amylosaccharitieus, Streptomyces sp. and Cephalosporium sp. It did not inhibit trypsin, α-chymotrypsin, thrombin, plasmin, papain, ficin, Pseudomonas aeruginosa neutral protease, acid proteases of Rhodotorula glutinis and Cladosporium sp. and pepsin.

The molecular weight of the inhibitor by sedimentation equilibrium, gel filtration and SDS gel electrophoresis was 23,000, 27,000 and 12,000 respectively, which suggests that the inhibitor exists as a dimer (Sato and Murao, 1974). The inhibitor contained a large amount of alanine (19 moles/mole) and valine (12 moles/mole), 4 moles of half cystine and no isoleucine or cysteine. The isoelectric point of the inhibitor was at pH 4.3. The amino acid sequence of this inhibitor was determined by Ikenaka, Odani, Sakai, Nabeshima, Sato and Murao (1974). It is postulated that the reactive site of the inhibitor is present in a disulfide loop and that the proline residues occur near the reactive site.

Studies regarding the complex formation of subtilisin and the inhibitor (Sato and Murao, 1974b) show that the complex is formed from one molecule of the inhibitor (two subunits of the inhibitor) and two molecules of subtilisin. Subtilisin which was inactivated by diisopropyl fluorophosphate or by carbobenzoxy-L-alanyl-glycyl-L-phenylalanine chloromethyl ketone, did not form a complex with the inhibitor. The isoelectric point of the complex was pH 5.5. Preliminary X-ray diffraction studies of

the crystals of the inhibitor and its complex with subtilisin were carried out by Satow, Mitsui, Iitaka, Murao and Sato (1973).

Recently Sato, Kimura and Murao (1975) immobilized this subtilisin inhibitor on sepharose. Surprisingly it was observed that although the original (non immobilized) inhibitor was specific for subtilisin, the immobilized inhibitor also inhibited α-chymotrypsin. The immobilized subtilisin inhibitor was used to purify crude subtilisin by affinity chromatography. 0.5 per cent SDS was used as a dissociating agent. A Dowex-2 column was simultaneously used to remove SDS from the dissociated enzyme. The immobilized inhibitor was also used for the purification of α-chymotrypsin.

More recently Inouye, Tonomura and Hiromi (1975) have shown that the subtilisin inhibitor has a wider inhibiting ability than originally reported by Sato and Murao (1973). Accordingly the inhibitor has been shown to feebly inhibit chymotrypsin and trypsin. The dissociation constants for the complexes of the inhibitor with subtilisin, chymotrypsin and trypsin were $<10^{-9}$M, 3×10^{-6}M and 1.1×10^{-4}M respectively.

Section IV

General properties of inhibitors

Different proteinases inhibited by inhibitors

The proteinases which are inhibited by the inhibitors are usually endopeptidases. However Rancour and Ryan (1968) have described inhibitors of carboxypeptidase B from potato. Peptidases which hydrolyse small peptides are not inhibited by the proteinase inhibitors which have hitherto been studied (Vogel et al. 1968). The proteinases which have been shown to be inhibited by the inhibitors obtained so far from different sources are trypsin, chymotrypsin, papain, ficin, plasmin, elastase, thrombin, pepsin, kallikreins, carboxypeptidase B, subtilisin, pronase, rennin, gastricin, bromelain, cathepsins, alkaline proteases of A.oryzae, S.griseus and Alternaria tenuissima, proteinase from Penicillium and different proteinases of bacteria as well as of seeds before and after germination.

Molecular weights

Proteinase inhibitors generally have molecular weights between 6,000-60,000. In a number of cases the molecular weights are less than 20,000. From potato Rancour and Ryan (1968) have obtained four isoinhibitors of carboxypeptidase B, having molecular weights as low as 3,000-4,000. These are dialysable polypeptides, and in addition to carboxypeptidases B also inhibit α-chymotrypsin. The recently discovered small peptides from culture broths of several strains of Streptomyces, which inhibit different proteinases, have still lower molecular weights ranging from 500 to 1,200. However these inhibitors are generally non-specific in nature.

Stability

Most of the inhibitors are remarkably stable towards denaturing conditions. Generally the low molecular weight inhibitors (molecular

weights less than 20,000) are highly stable to heat and acids. Several of the low molecular weight inhibitors are not precipitated by trichloroacetic acid. Several inhibitors such as those from the Leguminosae (Vogel *et al.* 1968), Kunitz (Kunitz and Northrop, 1936) and Kazal (Laskowski and Wu, 1953) pancreatic inhibitors, trypsin, chymotrypsin and pepsin inhibitors from *Ascaris* (Collier, 1941; Green, 1957) and inhibitors from colostrum of bovine and porcine origin (Laskowski and Laskowski, 1951; Laskowski *et al.* 1957) are stable to 2.5 per cent trichloroacetic acid at 95°. Several of these inhibitors are stable to 90 per cent ethanol and are not denatured by 8-9 M urea at neutral pH and room temperature.

Several of the inhibitors have high cystine content, 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 borohydride or dithiothreitol leads to a complete loss of inhibitory activity. Most of the inhibitors mentioned above contain 9 to 15% half-cystine. Bowman-Birk inhibitor from soybean and Lima bean inhibitor component '2' contain 14 half-cystines in the molecule (Frattali, 1969; Jones *et al.* 1963). Navy bean (*Phaseolus vulgaris*) trypsin inhibitor has 30 half-cystines per mole (Wagner and Riehm, 1967). Kunitz's soybean inhibitor (Kunitz, 1947) which contains only 4 half-cystines in its 198 residues (Wu and Scheraga, 1962; Laskowski, 1966) is unstable to heat, though it is stable to 9 M urea (Edelhoch and Steiner, 1963). Serum inhibitors (α_1-trypsin and α_1-chymotrypsin inhibitors) which do not contain cystine are unstable to heat and acids (Wu and Laskowski, 1960).

In addition to their stability to heat and acids many trypsin inhibitors are stable to the action of pepsin at pH 2-3 (Vogel *et al.*1968).

It is suggested (Laskowski and Sealock, 1971) that the secondary structure of the native inhibitors is responsible for thie stability, since previously denatured inhibitors are easily hydrolyzed by pepsin.

Proline, cystine, tryptophan and carbohydrate content

A number of inhibitors contain proline, while many do not contain tryptophan. Cysteine has not been shown to be present in the inhibitors studied so far. Ovomucoids and serum inhibitors are glycoproteins. A prolyl residue is always shown to be present near the reactive site of the trypsin inhibitors. Laskowski and Sealock (1971) suggest that "the presence of proline near the reactive site contributes to the required rigidity of the reactive site. Similarly, the presence of prolyl groups in the remainder of the inhibitor molecule is partly responsible for the rigidity and resistance to denaturation of the molecule".

Amino acid composition, amino acid sequence and X-ray studies

The amino acid composition of several inhibitors and the amino acid sequence of a few inhibitors have been determined. Some of the inhibitors whose amino acid sequence has been determined are bovine pancreatic trypsin inhibitor of Kunitz (Kassel et al. 1965; Anderer and Hernle, 1968), bovine pancreatic secretory trypsin inhibitor (Greene and Bartlet, 1969), cow colostrum inhibitor (Cechora et al. 1970), maize seed, peanut and ovine pancreatic trypsin inhibitors (Hochstrasser and Werle, 1970), secretory trypsin inhibitors from porcine pancreas (Tschesche and Wachter, 1970) and snake venom proteinase inhibitor (Takahashi, Iwanaga, Kitagawa, Nokama and Suzuki, 1974). X-ray investigations of different inhibitors and their complexes are being made by many workers. As stated earlier X-ray crystallographic studies and the elucidation of the three dimensional structure of the Kunitz pancreatic trypsin inhibitor were carried out by Huber et al. (1970, 1971).

Dissociation constant

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

Dissociation constants have been determined for several enzyme-inhibitor pairs at different pH values. Various physical and enzymic methods have been used for these determinations. The physical methods include examination of the complexes by analytical ultracentrifngation, free boundary electrophoresis and viscosity measurements (Sri Ram et al. 1954), gel filtration (Fritz et al. 1965), fluorescence quenching technique (Edelhoch and Steiner, 1965), preflavine displacement technique (Feinstein and Feeney, 1967) and the potentiometric technique developed by Laskowski Jr (Labowitz and Laskowski, Jr. 1962). The enzymic titration method uses the extent of deviation from stoicheiometric inhibition in the region of the equivalent point of a titration according to Green and Work (1953, 1957). Trypsin-pancreatic trypsin inhibitor complex has an unusually low dissociation constant (6×10^{-14}M at pH 8.0, Vincent and Lazdunski, 1972). Values for the dissociation constants of some of the proteinase-inhibitor complexes are given below: Trypsin-Kunitz's pancreatic inhibitor 3×10^{-5}M (at pH 3) and 6×10^{-14}M (at pH 8), trypsin-Kunitz's soybean inhibitor 6×10^{-3}M (pH 3.1) and 6×10^{-9}M (pH 7.8), subtilisin-penguin ovomucoid inhibitor 5×10^{-8}M (pH 8), ficin-chicken egg white inhibitor 1×10^{-9}M (pH 7), Ascaris chymotrypsin inhibitor 6.9×10^{-9}M (pH 7.5) (Peanasky and Erreish, 1971) and trypsin-snake venom inhibitor 7.6×10^{-10}M (Takahashi et al. 1974).

Separation of the inhibitor and enzyme from the complex

In the case of stable inhibitors the inhibitors can be easily isolated from the complex by precipitating the enzyme by boiling or by trichloroacetic acid. Gel filtration at lower pH is also used to separate inhibitor and enzyme provided the molecular weights of the inhibitor and enzyme differ significantly and the inhibitor is stable to acid. Although recovery of the inhibitor from the complex has been obtained in several cases, recovery of the enzyme portion has been reported only in a few cases. Hochstrasser et al. (1968) used thioethanol to inactivate the inhibitor and release the enzyme in active form. Herez (1973) has recently shown that SDS can be used selectively to inactivate blood α_1-trypsin inhibitor in its complex with trypsin. As mentioned earlier, recently Sato et al. (1975) immobilized subtilisin inhibitor on Sepharose and used it for the purification of subtilisin. The complex was cleaved with 0.5 per cent SDS at pH 7.5 and the SDS from the dissociated enzyme was rapidly removed by Dowex-2. 80 per cent recovery of subtilisin was obtained by this method. However after this treatment it was found that the inhibitor-sepharose had considerably lost its ability to combine with subtilisin. The same authors hve also used 6 M urea or 3 M guanidine hydrochloride, but the recovery of the enzyme by this method was less than 35 per cent.

Isoelectric point (p*I*)

The isoelectric points of the purified inhibitors are generally between pH 3.5 to 6. Some of the trypsin-chymotrypsin inhibitors (p*I* 5.1 to 9.2) showed higher values. The isoelectric point of basic bovine pancreatic trypsin-chymotrypsin inhibitor is pH 10.1. One of the human plasma trypsin inhibitors shows an isoelectric point as low as 2.8 whereas a kallikrein inhibitor showed a p*I* of 6.4. In the case of soybean trypsin

inhibitor (Kunitz) the isoelectric point (pH 5.0) of the complex lies between those of trypsin (pH 10.8) and the inhibitor (pH 4.5).

Optical factor

The optical factor (Kunitz, 1947) 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 the inhibitors tested range from 0.77 to 2.30. Some of the typical values for the optical factor are: potato chymotrypsin inhibitor 0.77, soybean trypsin inhibitor (Kunitz) 1.10, subtilisin inhibitor (from Streptomyces albogriseolus) 1.22 and soybean 1.9S trypsin-chymotrypsin inhibitor 2.3. A high optical factor indicates a low content of aromatic amino acids, especially tyrosine and tryptophan.

Section V

Different forms and types of inhibitors

Isoinhibitors

Many inhibitors which have been studied in detail have been found to exist in multiple form. Such inhibitors are called 'isoinhibitors' and are very similar in their specificities and in some of their properties. The origin and the role of isoinhibitors are not known. It is possible that in some cases at least the isoinhibitors may have been formed by the breakdown of the original inhibitor during extraction and purification and are artifacts. This may not however be the general case since isoinhibitors have been obtained from many different types of tissue by using many different isolation and purification techniques. In some instances the isolation of the inhibitors is made by washing the tissue with acetone or ethanol (Jones et al. 1963) and/or extracting the tissue with agents such as perchloric acid, diisoprpyl phosphofluoridate (Pubols, Bartelt and Greene, 1974), boiling or cold trchloroacetic acid (Warsy, Norton and Stein, 1974) which are likely to destroy proteolytic enzymes. Isoinhibitors are obtained even by using these procedures of extraction. Unequivocal evidence is however lacking which ca establish whether the isoinhibitors occur in the tissues or are artifact. A typical instance where the isoinhibitors could have been formed from thenative inhibitor is that of lima bean inhibitors which is described in Sectin III of this chapter.

Specificty and stoicheiometry

Th inhibitors from different sources vary in their specificity and stoicheiometry. Some inhibitors inhibit more than one molecule of the enzyme per molecle of the inhibitor. In the case of navy bean and mung bean trypsin inhibitors, one mole of inhibitor inhibits two moles of trypsin.

One mole of potato chymotrypsin inhibitor inhibits four moles of chymotrypsin.

Many inhibitors have broad specificity and inhibit different enzymes. Such inhibitors are designated as 'polyvalent' or 'broad spectrum' inhibitors. Typical examples of these are the following. Potato type I inhibitor inhibits trypsin, chymotrypsin, subtilisin, kallikrein and pronase. Dog submandibular gland inhibitor (Fritz et al. 1971) inhibits trypsin, chymotrypsin, subtilisin, elastase and plasmin. Barley grain inhibitor (Mikola and Suolinna, 1971) inhibits chymotrypsin, subtilisin and alkaline proteases of A.oryzae, S.griseus and Alternaria tenuissima. Snake venom proteinase inhibitors I and II inhibit bovine plasma kallikrein, trypsin, α-chymotrypsin and plasmin (Takahashi, Iwanoga and Suzuki, 1974).

In a number of cases where an inhibitor in addition to trypsin also inhibits chymotrypsin the inhibitory sites are usually distinct as in the case of lima bean inhibitor (Ryan and Clary, 1964), turkey and duck ovomucoid (Rhodes et al. 1960) inhibitor and Vigna sinensis inhibitor (Ventura and Filho, 1966). Rhodes et al. (1969) used the term "double headed" to describe such inhibitors. In such cases their complex with either enzyme is capable of still inhibiting the other. An exception is that of Kunitz's bovine pancreatic trypsin inhibitor. Wu and Laskowski (1955), Kraut and Bhargava (1967) and Ribgi (1971) found that in this case the 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. Some of the chymotrypsin inhibitors which also inhibit subtilisin show such behaviour. Another example of this type is a papain inhibitor from chicken egg white which also inhibits ficin.

In the case of the polyvalent potato inhibitor 1, which also inhibits trypsin, it is found that this inhibition can be observed only when casein

(protein substrate) is used in the assay system. However when synthetic ester substrates are used there is ano inhibition of trypsin. Thus only proteolysis is inhibited while esterolysis is not affected (Section III of this chapter). A different case where esterolysis is predominantly affected is that of the barley grain inhibitor. In this case five times more chymotrypsin is inhibitied by using synthetic substrate than by using casein as substrate (Section III of this chapter).

It is interesting to note that some trypsin inhibitors differentiate between bovine trypsin and human trypsin by selectively inhibiting one of them (Feeney et al. 1969). Trypsin inhibitors from the ovomucoids of chicken and turkey, chicken ovoinhibitor and Kazal's bovine secretory pancreatic inhibitor inhibit bovine trypsin but not human trypsin. Trypsin inhibitors from Lima bean, soybean (Birk) and Kunitz's 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 (1966) postulated that some trypsin inhibitors have a trypsin susceptible Arg-X bond in their reactive site while others have a Lys-X bond. This was confirmed by Liu et al. (1968) and Haynes et al. (1967). Thus the trypsin inhibitors can be classified as arginyl and lysyl inhibitors. Chicken ovomucoid trypsin inhibitor, chicken ovoinhibitor and Kazal's bovine pancreatic inhibitor are arginyl inhibitors while inhibitors from turkey ovomucoid, lima bean, soybean (Birk) and bovine pancreas (Kunitz) are lysyl inhibitors.

Section VI

Proteinase inhibitors and endogenous proteinases in plants

Inhibitors present in most of the plants inhibit corresponding enzymes from animal or microbial sources. However only a few cases are known where the plant inhibitors inhibit endogenous proteolytic enzymes of the plant from which they are derived (Ryan, 1973).

Ofelt *et al.* (1965) and Birk (1965), showed that the soybean inhibitors did not inhibit the endogenous proteinases although aqueous extracts of soybeans were shown to inhibit papain and the proteolytic enzymes of malt and wheat (Learmonth, 1951).

However Shain and Mayer (1965, 1968) reported that a trypsin inhibitor in lettuce seeds does inhibit an endogenous 'trypsin-like' endopeptidase. This enzyme has a pH optimum of 6.8 and a molecular weight of 3,000–4,000. This enzyme developed during germination of the seeds. As the proteolytic activity increased during germination, the inhibitor disappeared. This disappearance indicated that either the enzyme was complexed with the inhibitor, or that the enzyme which was present as a precursor became activated and then neutralized the inhibitor. The 'trypsin like' enzyme was purified 450 fold but was still not homogeneous.

Three types of proteinase inhibitors are present in ungerminated barley extracts (Kirsi and Mikola, 1971). The firsttype inhibits endogenous proteinases of barley (Mikola and Enari, 1970), the second type inhibits microbial proteinases and the third inhibits trypsin. As in the case of lettuce, during germination the inhibitors of endogenous proteinases disappear. A small molecular weight, dialyzable inhibitor from barley was shown to inhibit an endogenous proteinase in barley kernels (Burger and Siegelman, 1966). The inhibitor was not isolated or identified.

In the case of black eyed pea (Vigna sinensis) the trypsin inhibitory activity of the extracts of cotyledons falls continuously during germination to a value which corresponds to 5 per cent of the value for the dry seeds. It was also shown that the disappearance of the trypsin inhibiting activity was controlled by the axial parts. It was shown by Filho et al. (1973, unpublished results) that the "crude inhibitor" fraction of Ventura and Filho (1966) showed a weak but finite inhibition of the endogenous proteases of the seeds. According to Filho this fraction could contain specific inhibitors of endogenous proteinases. Hoyer et al. (1974) have shown that the extract of Vigna unguiculata contained two types of proteinases, one hydrolyzing BAPA and the other hydrolyzing casein. It was also shown that the casein hydrolyzing proteinases were partly inhibited by the trypsin inhibitors from the same source.

An inhibitor for mammalian carboxypeptidase B and α-chymotrypsin was isolated from potatoes (Rancour and Ryan, 1968; Ryan, 1971). ~~Ryan (1973)~~ Ryan (1973) found that the same inhibitor strongly inhibits the bradykinin inactivating carboxypeptidase from potato. It is the smallest proteinase inhibitor isolated from a plant source (molecular weight 3,500). It was suggested that the inhibitor may have a function in regulating the activity of the enzyme in dormant potatoes.

Section VII

Mechanism of action of proteinase inhibitors

The interaction between proteinases and their inhibitors has been the subject of numerous studies. The mechanism of interaction can only briefly be described in the following pages, since it will not be possible here to survey the extensive work done in this field.

Kunitz and Northrop showed that the reaction between a proteolytic enzyme and its inhibitor involves the formation of a complex between the two:

$$E + I \rightleftharpoons EI$$

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

The inhibitors contain 'active sites' for the inhibition of proteinases which give them their specificity. Inhibitors specific for trypsin always have either a Lys-X or Arg-X sequence at the binding site, where X is isoleucine, alanine or leucine. Chymotrypsin specific inhibitors usually have leu-X sequence at their active centres, where X is serine (lima bean inhibitor). The reactive site of the inhibitor is situated in a disulfide loop.

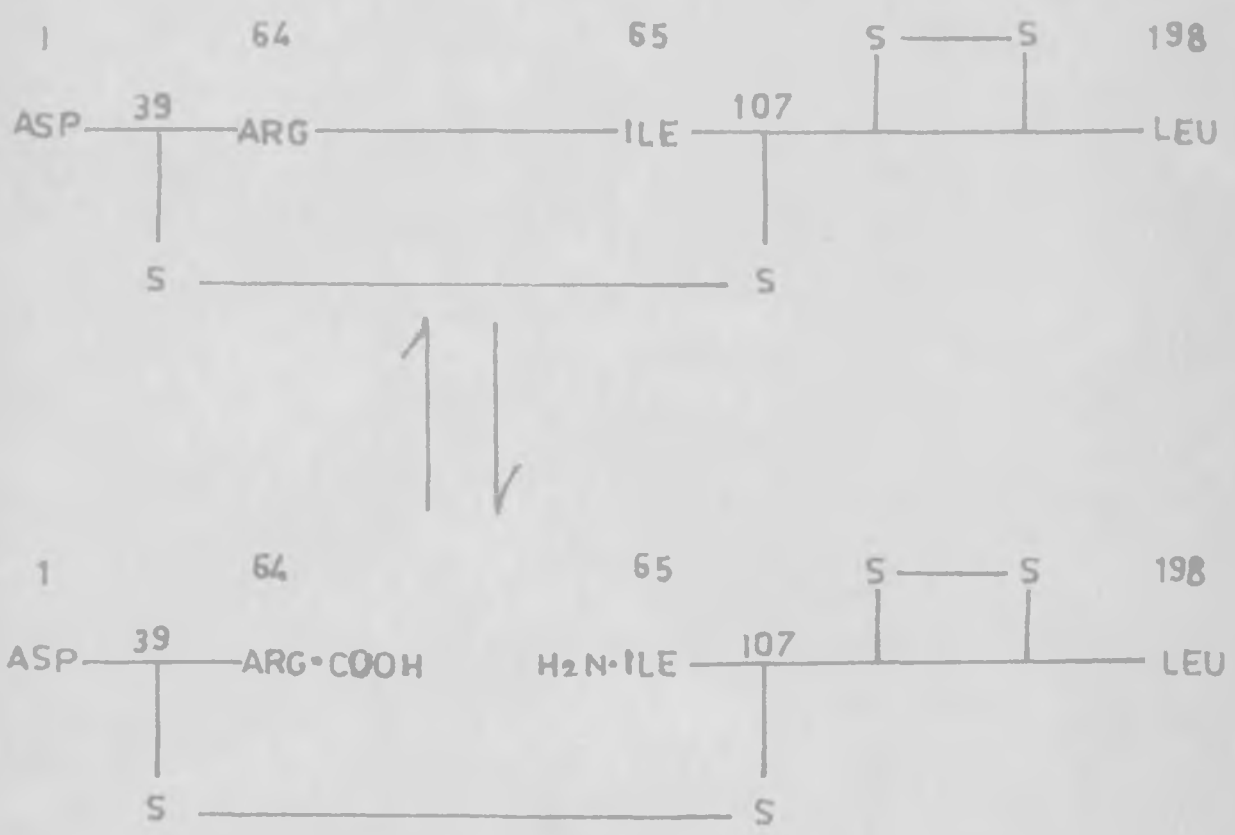
Laskowski Jr. postulated that the formation of a protease-inhibitor complex involves a cleavage of a peptide bond at the active site of the inhibitor (such as lys-X or arg-X in the case of a trypsin inhibitor) by the proteinase. The original (the 'native' or the 'virgin') inhibitor

thus gets 'modified' due to this bond cleavage. Subsequently the 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 (probably an ester bond between a (OH) group of trypsin and the newly formed COOH-terminal of the inhibitor). The sequence of events in the mechanism was given as:

$$E + I \underset{}{\overset{\text{fast}}{\rightleftharpoons}} EI \overset{\text{slow}}{\rightleftharpoons} E + I^*$$

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

Ozawa and Laskowski Jr.(1966) proposed the following as the reaction scheme in the case of soybean trypsin inhibitor (Kunitz).



In support of this the following evidence was presented. It was assumed that the native inhibitor consisted of one chain and the modified inhibitor due to bond cleavage would consist of two chains joined by an S-S bridge. By reducing the S-S bridges in the native and modified inhibitor and subsequently separating the corresponding fragments on Sephadex, Ozawa and Laskowski (1966) showed that the modified inhibitor had an additional chain (joined by S-S bridge). It was also shown that the newly formed C-terminal arginine residue could be cleaved by carboxypeptidase B whereby the inhibitory activity was lost. Alternately the additional α-NH_2 group in the modified inhibitor could be blocked by citraconic anhydride (Kowalski and Laskowski Jr. (1972) which also inactivated the modified inhibitor.

The results were similar with chicken ovomucoid. Many other investigators (Birk et al. 1967; Frattali and Steiner, 1969; Rigbi and Green,1968) have subsequently confirmed that there are bonds split in a variety of different inhibitors by the proteinases which they inhibit and that there are losses of activity when the new COOH-terminal amino acids are removed with carboxypeptidases. Kowalski and Laskowski Jr (1972) studied the action of citraconic anhydride on the mixtures of virgin and modified inhibitors of several inhibitors (having arg-X bond at the reactive sites) and showed that citraconic anhydride exclusively and quantitatively inactivates the modified inhibitor only.

Finkenstadt and Laskowski (1967) showed that the modification was reversible. The complex was prepared by using modified soybean trypsin inhibitor (Kunitz) and trypsin. After dissociating the complex the native inhibitor was obtained in considerable yield. Similar behaviour has also been shown in the case of the temporary inhibitors such as porcine or

bovine pancreatic secretory inhibitors (Schneider et al. 1973; Schneider and Laskowski, 1974). Formation of the modified inhibitor is favoured at lower pH values while at higher pH virgin inhibitor is favoured. Sealock and Laskowski Jr. (1969) and Laskowski Jr. (1970) have also shown that the arg-X bond in the peptide chain of the soybean trypsin inhibitor could be cleaved and then could be resynthesized by substituting the arginine moiety with lysine. It was shown that the resynthesized product also had inhibitory activity.

However, there is no general agreement that the above mechanism of inhibition is common to all proteolytic inhibitors. No tryptic cleavage could, for instance, be shown with Kunits and Northrop's bovine pancreatic trypsin inhibitor (Dlouha, Keil and Sorm, 1968). In the case of turkey and cassowary ovomucoids Feinstein et al. (1966) could still show inhibiting activity 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 the same treatment, when given to chicken ovomucoid and soybean trypsin inhibitor, did not cause loss of activity (Haynes et al. 1967). Uy and Feeney (1971) modified penguin and turkey ovomucoids with either α-chymotrypsin or subtilisin and the newly formed amino groups were substituted by dimethylation; however the inhibitory activity of the inhibitors against either enzyme was not abolished. Feinstein and Feeney (1966), Fossum and Whitaker (1968), Feeney (1971), Ryan (1973) and Sen and Whitaker (1973) have shown that inactive derivatives of the enzymes such as TLCK-trypsin, TPCK-chymotrypsin, anhydro-chymotrypsin, trypsin (histidine residue substituted), chymotrypsin (serine modified by dehydration), subtilisin (serine substituted by tosylation),

mercury-papain and alkylated ficin, also bind inhibitors to form complexes that could not involve an intermediate (modified inhibitor) as proposed by Laskowski Jr.

According to Feeney (1971), "the formation of an inhibitory complex does not require the primary structure of the inhibitor to be broken and the principal forces are the ones usually occurring in protein-protein interactions. These would involve the binding and fitting which occur between a biologically active protein and the substance with which it reacts. In the case of the enzyme-inhibitor complex, the selectivity of the binding site on the enzyme for a particular type of side chain on the inhibitor would be important. Catalysis and bond splitting, however, would be secondary and generally not essential". Further work is however needed to resolve this controversial issue.

Section VIII

Physiological role of proteinase inhibitors

Although nothing definite is known at present regarding the physiological function of the proteinase inhibitors, several hypotheses have been suggested. Proteinase inhibitors are considered as (a) regulatory agents in controlling endogenous proteinases (b) protective agents against animal, insect or microbial proteinases (c) storage proteins (especially in leguminous seeds and (d) since in a number of cases no direct evidence of these roles could be shown, they are even considered as biochemical peculiarities.

In pancreas the role of the secretory pancreatic trypsin inhibitor may be to prevent premature release of the pancreatic enzymes trypsin and chymotrypsin. It may also control the activation of a trypsin-like enzyme responsible for the conversion of proinsulin to insulin (Frank and Veros, 1968).

In blood the inhibitors may hve a role in the clotting of blood. It was shown that two inhibitors from blood serum inhibit some of the blood clotting enzymes. It was also shown that individuals genetically deficient in serum inhibitors are usually susceptible to lung and liver disorders such as pulmonary emphysema (Erickson, 1964) and liver cirrhosis (Sharp, Bridges, Kirvit and Friuer, 1969).

The pepsin resistant colostrum inhibitor which inhibits trypsin and chymotrypsin is regarded as useful in preventing the proteolysis of milk antibodies in the digestive system of the newborn (Laskowski and Laskowski, 1951; Laskowski *et al*. 1957).

Regarding the function of egg white inhibitors, it is suggested that the inhibitors may be acting as antimicrobial or antiviral substances during

the hatching of the egg (Feeney and Allison, 1969).

The secretory proteinase inhibitors from the submandibular gland of Canidae and Felidae inhibit a variety of proteinases which include microbial proteinases also. It is suggested (Werle, Trautscheld, Haendle and Fritz, 1968) that the inhibitors protect the mucosal membrane of the mouth and esophagus of the animal from the proteinases ingested with the food, especially stale flesh.

Inhibitors similar to bovine polyvalent inhibitor which are present only in ruminants, may be important with regard to the relationship between the complicated digestive system of the ruminants and the growth of bacteria and protozoa in the gastrointestinal tract (Werle et al. 1968). Astrup (1968) considers that the absence of certain respiratory diseases in cattle is due to the action of this inhibitor in the lungs.

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

Regarding plant inhibitors Mansfeld et al.(1959) suggested 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. (1968) suggested that the presence of a 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.

In some cases such as lettuce, barley and potatoes (Section VI of this chapter) inhibitors are known to inhibit endogenous proteolytic enzymes of the plant, but in most instances inhibition of plant proteinases has not been reported.

According to Ryan the most usefnl function of plant inhibitors is of plant protection. Proteinase from Tribolium a common larva that destroys stored grains, is inhibited by soybean extracts. Also the proteinases from Tenebrio a common pest that consumes stored grain are inhibited by lima bean and soybean inhibitors. The trypsin like and chymotrypsin like digestive proteinases of a number of insect genera are inhibited by plant proteinase inhibitors (Fox and Kassell, 1974). Green and Ryan (1972) noted that when the potato or tomato plants were wounded by potato beetles or their larvae it caused a rapid accumulation of a proteinase inhibitor throughout the aerial tissues 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 microorganism from the harmful effects of their own proteases (Umezawa, 1972). It is stated that after the hydrolysis of organic nitrogen compounds in the medium the free proteolytic enzymes outside the microbial cells become harmful to the cells.

The presence of three different proteinases and their corresponding inhibitors was shown in Saccharomyces cerevisiae (Lenney, Matile, Wiemken, Schellenberg and Meyer, 1974). The proteinases are localized in the vacuole while their inhibitors are present in the extravacuolar cytosol. It is proposed that 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.

Another explanation for the existence of a large amount of trypsin-chymotrypsin inhibitors in the seeds may be their role in the endozooic disposal of seeds (Mikola and Soulinna, 1969). It is suggested that seeds

consumed by animals are distributed as viable seeds due to the protective action of seed inhibitors against the alimentary canal proteinases.

Schnebli and Burger (1972) have studied the effect of five protease inhibitors on the growth of transformed mouse and hamster cells. It was observed that the inhibitors selectively inhibit the growth of transformed cells while they did not affect the growth of the non-transformed cells. It is suggested that the inhibitors block a protease like activity that is required for the unrestrained growth of transformed cells. Faber, Wertheimer and Golrick (1973) have demonstrated that 1-chloro-4-phenyl-3-tosylamido-2 butanone (a specific chymotrypsin inhibitor) inhibits the growth of HeLa cells. It is postulated that the inhibitor may be inhibiting a proteolytic cleavage mechanism that normally cleaves some high molecular weight proteins to lower molecular weight proteins. The possibility that proteolytic inhibitors have a role in regulating protease activity during cell division and protein synthesis remains to be investigated.

Zanerald, Polakoski and Williams (1973) have shown that acrosomal extracts of mammalian (boar, rabbit and stallion) epididymal spermatozoa possess high proteinase (acrosin) activity. This activity is absent in neutral extracts of ejaculated spermatozoa. This absence of activity was found to be due to the formation of a complex betwwen acrosin and acrosin-inhibitor. The complex could be dissociated at pH 3 and reassociated at pH 8. It is postulated that the inhibitor is added to acrosin during ejaculation and is removed from the spermatozoa during its stay in the female genital tract.

Section IX

Uses of proteinase inhibitors

The occurrence of certain pathological conditions such as inflammation, pancreatitis and various types of shock is due to the uncontrolled liberation of kinins by proteinases. Inhibition of these proteinases is essential in such cases and bovine inhibitor (Trasylol) due to its broad inhibition spectrum, low molecular weight and low toxicity is a valuable drug in the treatment of early pancreatitis (Thompson, 1968) and in the prevention and treatment of surgical hemorrhages (Amris, 1966; Matis and Morl, 1968). It has also reduced mortality in experimental shock caused by trauma, burn and anaphylaxis (Back, 1966, 1968). It has been claimed that bovine pancreatic inhibitor is useful in preventing skin transplant rejection in rats (Bertelli *et al.* 1963).

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 occurring in crude tissue extracts.

Several uses have been suggested for the recently discovered low molecular weight inhibitors isolated from the culture broths of *Streptomyces* strains (Aoyagi *et al.* 1969; Umezawa, 1972). These inhibitors have very low toxicity and are absorbed even when given orally. Orally given leupeptin shows 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 tumorigenesis in mouse skin initiated by 7,12-dimethylbenzanthracene and croton oil. Chymostatin, pepstatin and also leupeptin show inhibition of carrageenin edema formation. Pepstatin was shown to be very effective in suppressing ulcers in the stomach of pylorus-ligated rats.

Section X

Assay methods and definition of inhibitor units

The activity of inhibitors is measured in terms of the corresponding proteinase inhibited and thus all the methods used for the assay of proteinases are applicable to the determination of inhibitors.

Proteinases can be estimated by several methods which fall into two major groups:- 1) use of natural substrates 2) use of synthetic substrates.

1) Use of natural substrates

The methods using natural substrates are based on the determination of the rate of proteolysis and are applicable to several proteolytic enzymes. The substrates which have been used include casein, haemoglobin, globin, fibrin, gelatin and serum albumin.

A representative method of this type is the widely used spectrophotometric method of Kunitz (1947) in which casein is used as a substrate. In this method casein is digested with the proteolytic enzyme (without and with the addition of the inhibitor) for 20 min at 35°. The reaction is stopped by the addition of trichloroacetic acid and the undigested casein is removed by filtration or by centrifugation. The amount of split products in the solution is determined by measuring their absorbance at 280 nm or by the Folin-Ciocalteau reagent (Anson, 1938).

2) Use of synthetic substrates

Simple synthetic substrates for the assay of proteolytic enzymes were introduced by Bergman (1942). The peptide bonds susceptible to the action of different proteinases could be identified by the use of synthetic peptides.

Trypsin hydrolyses peptides, amides, and esters at bonds involving the carboxyl group of L-arginine or L-lysine while chymotrypsin hydrolyses them at bonds involving the carboxyl groups of aromatic L-amino acids. Subtilisin shows a wide specificity and attacks ester bonds whereas the amide bond is more resistant. Papain hydrolyses peptides, amides and esters especially at bonds

involving basic amine acids, leucine, or glycine.

Trypsin and chymotrypsin can hydrolyze the synthetic substrate BAEE (α-N-benzoyl L-arginine ethyl ester) and BTEE (N-benzoyl-L-tyrosine ethyl ester) respectively. During the hydrolysis of these compounds there is an increase in absorbance at 253 nm due to the formation of benzeyl arginine or benzoyl tyrosine. This increase in absorbance has been used as a measure of proteolytic activity of trypsin and chymotrypsin (Schwert and Takenaka,1955; Kassell et al. 1963). The inhibition of this increase in absorbance by the corresponding inhibitor is used as a measure of inhibitor activity.

Since subtilisin can hydrolyze BAEE and BTEE, and papain can hydrolyze BAEE, these substrates have also been used for the assay of these proteinases and their inhibitors. The chromogenic amide substrate BAPA is hydrolyzed by trypsin and papain and the increase in absorbance at 410 nm is used for their assay (Arnon, 1970). Care is however required in the use of these assays, since it has been noted earlier that an inhibitor may inhibit the caseinolytic activity of a proteinase but not its esterolytic activity (Section V).

Definition of unit

i) When a protein substrate is used in the assay system, one proteinase unit is defined as the activity that causes an increase of one optical density unit at 280 nm per min (for 1 cm light path) under the standard conditions. Proteinase-inhibiting activity is expressed as units of proteinase inhibited, and specific activity is expressed as units of proteinase inhibited per microgram of inhibitor (Kunitz, 1947).

ii) When a synthetic substrate is used, one unit of proteinase activity is usually defined as the amount of proteinase that causes the hydrolysis of 1 micromole of the substrate per min under standard conditions. One inhibitor unit is equal to the reduction of the proteinase activity by one unit.

Several methods using different natural and synthetic substrates for the assay of different proteolytic enzymes and their inhibitors are summarized by Vogel et al. (1968).

Section XI

Purification of proteinase inhibitors

The stability of inhibitors to heat, acid, solvents and deproteinizing reagents has been made use of for their purification. In general the usual methods of enzyme purification are also applied for the purification of inhibitors. They include salt fractionation, electrophoresis and chromtography using different adsorbents (bentonite,kaiselguhr or calcium phosphate gel), ion exchangers (DEAE-cellulose, SE-cellulose, cellulose phosphate and CM-cellulose) or Sephadex. Enzyme inhibitor complexes have also been used for purification.

An important advance in recent years is the immobilization of enzymes without loss of activity, for example by covalently linking trypsin to CM-cellulose (Mitz and Summaria, 1961) or to a copolymer of maleic anhydride and ethylene (Levin, Pecht, Goldstein and Katchalski, 1964). Subsequently enzymes were also linked covalently to Sephadex, agarose, Sepharose (Porath, Axen and Ernback, 1967) or polyacrylamide carriers (Fritz, Gebhardt, Meister and Schult, 1970). Fritz, Schult, Neudecker and Werle (1966) have purified inhibitors from the pancreas of hog, cattle and dog and from the seminal vesicles of mice, by using enzymes (trypsin, chymotrypsin and kallikrein) inselubilised on ethylene-maleic anhydride copolymer. Chymotrypsin-Sepharose and trypsin-Sepharose were used respectively for the purification of chicken ovoinhibitor (Fienstein,1971) and bovine lung or colostrum trypsin inhibitor (Kassell and Marciniszyn,1971; Cechova, 1974) by affinity chromatography.

According to the general procedure minimally pretreated extracts are treated with the immobilized enzyme at neutral pH, either on columns or batch-wise. The impurities not bound to the carrier are then removed by washing with a neutral salt solution. Elution is done by acid salt solutions. The enzyme-carrier may be repeatedly reused.

The formation of modified inhibitor must however be considered in this procedure, especially when dissociation is carried out at low pH. Hochstrasser,

resin for purifying trypsin inhibitors from maize extract and wheat and rye germs. In each case they obtained a mixture of the native inhibitor and a modified form.

Section XII

PRESENT WORK

In the work presented in this thesis preliminary evidence is presented to show the presence of a large number of proteinase inhibitors with different specificities in the seeds of Vigna catjang. Out of these inhibitors two trypsin inhibitors, two subtilisin inhibitors and two papain inhibitors were purified to homogeneity and their properties and kinetics were studied. A third subtilisin inhibitor was purified partially and its properties were studied.

The presence of such a large number of inhibitors with different specificities in a single source is of interest. The purification of subtilisin inhibitors and papain inhibitors from a plant source has been reported here for the first time.

The study of the properties and kinetics of the inhibitors include the stability of the inhibitors under different conditions, polyacrylamide gel electrophoresis, amino acid composition, isoelectric pH, association constant, mechanism of action, molecular weight determination, molar combining ratio and ultraviolet absorption spectrum.

Chapter II of this thesis deals with the materials and experimental methods used in these studies.

Chapter III deals with the isolation of inhibitors, their separation from each other and purification.

Chapter IV describes the properties and kinetics of purified inhibitors.

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

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

A bibliography of the literature references cited in this thesis is presented in the final section.

Section I

MATERIALS

Seeds: Vigna catjang seeds (local name "chavli") were obtained from the local market and stored at 4° till use. Only those lots in which the seeds were relatively big (average weight of each seed was 200 mg) were used.

According to recent nomenclature (Verdcourt, 1970) Vigna catjang and Vigna sinensis are both grouped under the same species having the name Vigna unguiculata. However they are further regarded as subspecies of V.unguiculata as follows:

V.catjang - V.unguiculata. Sub. sp. cylindrica

V.sinensis - V.unguiculata. Sub. sp. unguiculata

The two subspecies are mainly differentiated by pod size: The pod of Vigna catjang is 7.5-12.5 cm long whereas the pod of Vigna sinensis is 20-30 cm long.

In this thesis the old nomenclature "Vigna catjang" and "Vigna sinensis" will be retained.

Chemicals: All common chemicals used were of analytical grade. The following chemicals and enzymes were obtained from Sigma Chemical Company, U.S.A.: 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 special strain of B.subtilis, type VIII), ficin (from fig tree latex, 2 x crystallized), trypsin inhibitor (from soybean, type I-S), cytochrome c (from horse heart), myoglobin, albumin (from bovine serum crystallized), reduced glutathione, BAEE, BTEE and BAPA.

Cysteine hydrochloride and casein (Hammersten) were obtained from E.Merck, Germany. Haemoglobin was purchased from Serva Laboratories,Germany

and pronase (B grade) from Calbiochem, U.S.A. Papain (electrophoretically pure) was obtained from Biochemicals Unit, Delhi. Pepsin (porcine origin, crystallized) was from Armour and Company, U.S.A. Insulin (crystalline) was purchased from B.D.H. England.

The chemicals used for polyacrylamide gel electrophoresis (acrylamide, N, N'-methylene-bis-acrylamide, N,N,N',N'-ethylmethyl ethylenediamine (TEMED) and amido black 10B) were obtained from Eastman Kodak Company, U.S.A. Glycine was from Koch Light Laboratories, U.K.

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

Cellogel strips were purchased from Reeve Angel Scientific Ltd.,U.K. The strips were preserved in 30 per cent methanol, and washed and soaked in the required buffer before use.

Celluloses were washed according to Peterson and Sober (1956). The chromatographic columns were generally used with flow of liquid under gravity without the application of external pressure.

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Section II

METHODS

All the reagents and buffers were prepared in glass distilled water. Centrifugations were carried out at 0°, in the International Refrigerated Centrifuges (Models PR-1 and PR-2), Sorvall centrifuge (Model SS-1) and Spinco ultracentrifuge (Model L); and at room temperature, using a Sharples supercentrifuge (AS 12 clarifier) and Sharples supercentrifuge (laboratory model).

Spectrophotometric determinations were carried out with a Beckman DU spectrophotometer or a Unicam Spectrophotometer SP 500 model using cuvettes with 10 mm light path. Ultracentrifugal studies were carried out in a Beckman Model E Analytical Ultracentrifuge.

Methods of estimation

Phosphorus: Inorganic phosphate was determined by the method of Fiske and Subba Row (1925).

Protein: Protein estimations were carried out by the method of Warburg and Christian (1941). It was assumed that a 0.1 per cent solution of protein shows an optical density of 1 at 280 nm for 1 cm light path. A blank was also taken using a buffer of the same composition. A correction for ultraviolet absorbing impurities was made by subtracting the absorbance obtained at 340 nm.

Protein was also estimated by the method of Lowry *et al.* (1951). Crystalline bovine serum albumin was used as a standard. The concentration of serum albumin was calculated from ~~th~~ its extinction coefficient at 280 nm ($E^{1\,cm}_{1\%}$ - 6.6) according to Cohn Hughes and Weare (1947). Interfering substances such as glucose, phosphate, thioethanol and ammonium sulphate were removed by dialysis before estimating the protein.

The protein determinations during purification were made by measuring absorbance at 280 mm 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 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.

Ammonium sulphate precipitations: Ammonium sulphate concentrations at 0° were calculated according to Jagannathan, Kartar Singh and Damodaran (1956). The equation used for addition of solid ammonium sulphate was

$$X = \frac{50\ (S_2 - S_1)}{1 - 0.28 \times S_2}$$

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 the g of solid ammonium sulphate and Y is the number of ml of saturated ammonium sulphate solution to be added to a 100 ml of solution. S_1 and S_2 are the initial and final saturations of ammonium sulphate respectively at 0°.

Dialysis: Dialysis was carried out in cold at 3 to 5°. Small scale dialysis was done under stirring and by giving frequent changes (at least three changes) of the buffer. For one volume of the material to be dialyzed approximately 50 to 100 fold volume of the buffer was used for each change. 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 approximately 20 to 50 fold volume of the buffer was used each time.

Ultracentrifuge measurements: Spinco Model E ultracentrifuge was used for carrying out analytical runs. The instrument was equipped with a phase plate-Schlieren optical system and a rotor temperature indicator and control device. A 4° sector, 12 mm path length standard cell was used in sedimentation velocity runs. Sedimentation coefficients were calculated from plots of the logarithm of distance of sedimenting boundary from the axis of rotation versus time (Schachman, 1957). The sedimentation coefficients were standardized to water at 20° ($S_{20,w}$) after making density and viscosity corrections (Schachman, 1957).

Molecular weight determinations were made by the approach-to-sedimentation-equilibrium method of Archibald (1947) as described by Schachman (1957). A Beckman valve-type synthetic boundary cell of 4° sector and 12 mm light path was used. The phase plate was used at an angle of 80°. The speeds of centrifugation for linear extrapolation of the gradient curve were calculated according to LaBar (1966). Only readings at the air meniscus were taken. Photographic plates were measured on a Hilger L 50 two-way micrometer.

Polyacrylamide gel electrophoresis: Acrylamide gels were prepared according to the method of Davis (1964) with slight modifications. Riboflavin was used instead of ammonium persulphate. Cylindrical gels were 3-4 mm in diameter and 7% in acrylamide concentration. Tris-glycine buffer (pH 8.5) was used, the concentration of running buffer being 0.0025 M with Tris and 0.02 M with glycine. The electrophoresis was run at 0° with 3-5 milliamps current per gel for a period of 90-120 min. The protein was stained with 0.5% Amido Black 10 B solution. 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.

Preparative polyacrylamide gel electrophoresis: This was used for the purification of the individual inhibitors. A polyacrylamide gel column of 12 cm height and 5 cm diameter was prepared. Riboflavin was used for polymerization. Spacer gel of 0.5 cm height was layered on the separation gel. 500 ml of tris-glycine, pH 8.5 were used as a bath buffer in each ~~anod~~ compartment. 0.5 to 1 ml of 0.05% bromophenol blue was used as a marker in the upper (cathodic) compartment. 5 ml of the inhibitor solution (containing 50 to 80 mg protein) in 10% sucrose was loaded on the spacer gel. The electrophoresis was run at 5 milliamperes current at a voltage of 50 volts. After every 12 to 16 hours the bath buffer was replaced with fresh buffer. Electrophoresis was continued till the marker band moved about 10 cm towards the anode (about 60 to 65 hours).

The gel black was removed and cut horizontally into sections of 0.5 to 1 cm thickness. They were then separately extracted with water using a homogenizer and the extract was filtered. The extract containing the inhibitor activity was processed further. The approximate location of the particular inhibitor on the gel block was determined by a previous small scale polyacrylamide run.

Polyacrylamide extracts contain non-protein impurities, which interfere with the protein assay. These could be partly removed by dialysis. The nondialyzable impurities were removed by passing the extract through DEAE, which does not adsorb the impurities.

Isoelectric point (pℓ): Isoelectric points for different inhibitors were determined by using cellogel electrophoresis, at pH values ranging from 3.5 to 8.0. Acetate buffers for pH 3.5 to 6.0 and phosphate buffers for pH 6.0 to 8.0 were used for the runs. 10 to 20 μg (in 1 to 2 μl) of the

inhibitor dialyzed against the respective buffer (0.005 M) were used for each run. To 0.1 ml of the inhibitor (1% concentration) 0.01 ml of 0.05% of bromophenol blue was added before the run.

Cellogel strip size was, length - 5 cm (Bridge length), width - 5 cm. The ionic strength of the buffers was 0.005. The current used was 1 milliamp and the time of run was 15 min. After the run the strips were removed and stained for 1 min with 0.5% Amido black and then destained for 5 min in 7% acetic acid. The movement of the protein at various pH values was measured and the pH at which the protein does not show any movement was noted as the isoelectric point ($p\mathcal{I}$) of the particular inhibitor.

Dissociation constant: The dissociation constant of the enzyme (proteinase) - inhibitor complex was determined by the method of Green and Work (1953). According to the method an inhibition curve was obtained by adding increasing amounts of inhibitor to a fixed amount of enzyme near the equivalence point. The residual proteinase inhibitor activity in the presence of one equivalent of inhibitor was used to calculate 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 the ultraviolet absorption spectrum, the inhibitors were dialyzed against 0.001 M phosphate buffer pH 7.5 and the optical densities at various wavelengths in the ultraviolet region were recorded using the same buffer as blank.

The optical factor of the inhibitor was determined as the reciprocal of its absorbance at 280 nm, when the protein concentration was 1 mg/ml.

Molecular weight determination of inhibitors: Three different methods were used for the molecular weight determinations depending on the amount of pure inhibitor available. Since trypsin inhibitor 1 was obtained in large amounts, homogeneity and molecular weight determinations were carried out by ultracentrifugal studies. The molecular weight of the inhibitor was determined by the approach to sedimentation equilibrium method of Archibald.

For other inhibitors molecular weight determinations were carried out by SDS and/or gel filtration methods.

Molecular weight determination by SDS-polyacrylamide gel electrophoresis: The method used by Shapiro *et al* (1967) and Weber and Osborn (1969) was followed with slight changes. The principle on which the method is based is that the SDS minimizes the native charge differences of the proteins and that all proteins migrate as anions due to the formation of complexes with SDS. The migration rate is then proportional to the molecular weights.

To a 50 µg solution of inhibitor in 0.1 ml water was added 0.1 ml of a solution containing 2% SDS, 2% thioethanol and 0.2 M sodium phosphate buffer, pH 7.2. The mixture was incubated at 37° 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 0.01 M 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, 0.1 M sodium phosphate pH 7.2 and 0.0004% riboflavin. The bath buffer contained 0.1% SDS and 0.1 M sodium phosphate, pH 7.2. For each gel 0.03 ml 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. The current was 8 milliamps per gel. The anodic compartment was in the lower chamber. Electrophoresis was discontinued after about

4 h when the marker band moved about three fourths of the distance in the gel. Gels were fixed overnight in 0.5% amido black in 7% acetic acid.

Standard proteins, insulin (molecular weight of sub unit, approximately 2,800), cytochrome c (13,000), myoglobin (17,000), soy trypsin inhibitor (21,000) and egg ovalbumin (48,000), were also processed and run as above (molecular weights in parenthesis). 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 known polypeptide chain molecular weights a smooth curve was obtained. From this curve the molecular weight of the unknown protein was calculated.

Molecular weight by gel filtration: The molecular weights of inhibitors were also determined by Sephadex G-50 filtration (Andrews, 1965). Sephadex G-50 was suspended in water and was allowed to swell by heating in a boiling water bath for one hour. After cooling the material was packed in a column (1.5 x 100 cm) and was equilibrated by passing 500 ml of 0.05 M phosphate buffer, pH 7.5 through it. 1 ml of 0.05 M phosphate buffer, pH 7.5, containing a mixture of 0.5 mg soybean trypsin inhibitor, 1 mg myoglobin, 1 mg cytochrome c and 1,000 units of inhibitor was loaded on the column and eluted with the same buffer. The flow rate was 18 ml per hour and 3 ml fractions were collected. The fractions were assayed for the different proteins loaded. Cytochrome c and myoglobin were estimated by determining the absorption at 412 nm and 405 nm respectively. Trypsin, subtilisin and papain inhibitors were assayed by the casein digestion method of Kunitz (1947). Separate columns were run for different inhibitors. The molecular weight of the inhibitor was then determined by plotting the eluant volume vs logarithm of molecular weight from which the molecular weight was calculated.

Amino acid analysis: For amino acid analysis three 1 mg samples were dissolved in 2 ml of 6N HCl in test tubes and the tubes were evacuated and sealed. They were hydrolyzed at 110° for 24, 48 and 72 h respectively, after which excess acid was removed by vacuum evaporation at room temperature (30°). Evaporation was repeated three times, each time using 2 ml of water for dissolving the hydrolysate.

Hydrolytic destruction of proline, threonine and serine was corrected for by extrapolation to zero hydrolysis time from 24, 48 and 72 h hydrolysis.

Tyrosine and tryptophan were determined by the method of Goodwin and Morton (1946) by determining the absorbance of the protein at 294.4 nm, 257.15 nm and 280 nm.

Estimation of free sulfhydryl groups: Free -SH group assays were carried out according to Ellman (1958, 1959). 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 M^{-1} was used to calculate the thiol concentration. The accuracy of the method was checked with glutathione.

In a 1 ml cuvette with 10 mm light path, 0.1 ml of the inhibitor solution (containing 1 mg of the inhibitor), 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 the protein: For these determinations the method of Cavallini et al. (1966) was followed. According to this method sodium borohydride ($NaBH_4$) in 8 M urea was used as a reducing agent and DTNB was used as a thiol disulphide exchanger. The increase in absorbance at 412 nm was measured and the number of sulfhydryl

groups (N) formed was calculated using the following formula:

$$N = \frac{M_w \times A \times V}{12{,}000 \times m}$$

where M_w is the molecular weight of the protein, A is absorbance, V is the volume of the final solution and m is the weight in mg of the protein sample analyzed. The accuracy of the assay 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 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 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°, the mixture was incubated at the same temperature for 45 min. 0.5 ml of 1 M KH_2PO_4 in 0.2 M HCl and 2 ml of 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.

Inhibitor Assay

The Kunitz (1947) spectrophotometric method using casein was followed with slight modifications for the assay of proteinases and proteinase inhibitors. This method was also used for following the purification of the inhibitors. Different proteinases whose inhibition was studied by this method included trypsin, chymotrypsin, subtilisin, papain, ficin and bromelain.

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. The test tubes containing the reaction mixture were incubated for 20 min at 35°. After 20 min, 3 ml of 5% trichloroacetic acid were added and the contents of the tubes were mixed well. After standing for 20 min the solutions were filtered and the absorbance of the filtrates was measured at 280 nm. The 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 give 280 nm absorbing material and thus will interfere with the assay of proteinases and their inhibitors, if these blanks are not subtracted from the total absorbance at 280 nm obtained in the assay system after 20 min incubation.

Reagent blank (mainly due to casein) was taken by omitting enzyme and inhibitor from the reaction mixture. The optical density of this blank varied between 0.080 and 0.150.

Enzyme blank - For crude enzyme preparations the enzyme blank was taken as follows. The inhibitor was omitted from the reaction mixture. To

the reaction mixture enzyme was added after the addition of trichloroacetic acid. This blank reading (designated as zero min blank) when subtracted 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 enzyme blanks were not necessary for purer preparations of enzyme, since the amount of enzyme used had no detectable reading at 280 nm.

Inhibitor blank: As explained below, two types of blanks - zero min and 20 min incubated - were taken whenever the crude extract of seeds was used as a source of inhibitor. In the case of both types of blanks the enzyme was omitted from the reaction mixture. Zero min inhibitor blank was taken by adding the inhibitor to the reaction mixture after the addition of trichloroacetic acid. The other type of blank is necessary in some cases where the seed extract itself shows proteolytic activity. In such cases specific inactivation of the proteolytic activity (and not of the inhibitor) is desirable or alternately the above mentioned 20 min incubated blank can be taken. For this type of blank the enzyme was omitted and the crude inhibitor was added to the reaction mixture in the beginning only and the reaction was stopped as usual with trichloroacetic acid after 20 min incubation. The 280 nm value obtained by zero min blank when subtracted from the 20 min incubated blank gives the proteolytic activity of the crude inhibitor. For calculating the inhibition a correction for this proteolytic activity should be made.

In the present work the crude acid extract (0 - 0.70 ammonium sulphate precipitate, Fraction II, Chapter III) of Vigna catjang seeds did not show any detectable proteolytic activity at pH 7.5. Hence in this case no correction for this activity was necessary. However in the case of alkaline extract

of Vigna catjang seeds (extracted with 0.1 M phosphate buffer, pH 7.5) it was found that the extract possesses a strong proteolytic activity. Hence in this case it is necessary to take the 20 min incubated blank and correct for the proteolytic activity of the extract.

Care was also taken to see that the inhibition of the enzyme obtained is not apparent i.e., is not due to the hydrolysis of the enzyme by the proteolytic activity of the extract itself. This can be confirmed by pre-incubating the enzyme with the extract for different periods of time before adding the substrate and then testing the inhibition. In this case if the inhibition in the different preincubated samples is the same, it can be concluded that the inhibition is due to an inhibitor and not due to an endogenous protease activity acting on the protease being tested. This was routinely carried out for all the purified inhibitors and it was shown that the inhibitor was not a protease.

A typical assay of a proteinase inhibitor (subtilisin inhibitor) from the acid extract of Vigna catjang seeds (0 - 0.70 ammonium sulphate precipitate, Fraction II, Chapter III) is illustrated in Table 1. The table shows how the different blanks were taken and how the activity was calculated. Also to illustrate how the blank due to the proteolytic activity of the alkali extract (seeds extracted with 0.1 M phosphate buffer, pH 7.5) is eliminated for calculating the inhibitor activity an example is given in Table 2.

-SH requiring enzymes: In the case of -SH requiring enzymes such as papain, ficin and bromelain, the assay system (reaction mixture) in addition to the usual constituents also contained 40 micromoles of neutral cysteine hydrochloride.(The stock solutions of these enzymes were also made in 0.02 M neutral reduced glutathione). Moreover the enzyme was activated by pre-incubating it for 10 min at 0° with 0.01 M neutral cysteine hydrochloride

before adding it to the reaction mixture.

Amount of enzyme and inhibitor in the reaction mixture: The amount of enzyme 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 for trypsin) was proportional to the enzyme concentration. In the case of trypsin the enzyme concentrations were read from the standard curve (Kunitz, 1948).

The amount of the inhibitor taken was also adjusted so that when a mixture of enzyme and inhibitor was used in the reaction mixture, the decrease in the enzyme activity (the fall in the optical density rise) was between 0.05 and 0.150. Only those inhibitor values which did not exceed 50% inhibition of the enzyme were taken for calculation since the inhibition was found to be proportional to the inhibitor concentration in this range.

In some experiments when the inhibitor concentration was very low the sensitivity of the assay was increased by using longer incubation times (upto 1 h). During this incubation time, the amount of enzyme had to be reduced to one-third the amount required for the usual 20 min assay. Enzyme activity was proportional to the concentration and time of incubation between these ranges.

The purity of the proteolytic enzyme was also established by using assay methods wherein synthetic substrates were used. (These assay methods are described at the end of this section). Trypsin was also standardized with crystalline soybean trypsin inhibitor (Kunitz, 1947) on the basis that 1 mg of the inhibitor inhibits 1.1 mg of pure trypsin. Trypsin, chymotrypsin and ficin were also standardized by using the caseinolytic assay curves of Kunitz (1948), Laskowski (1955) and Englund et al.(1968) respectively.

A unit of proteinase is defined as the activity which gives rise under the conditions described to an increase of 0.001 of absorbance at 280 nm per min per ml of reaction mixture (Kunitz, 1948). An inhibitor unit is expressed in terms of units of proteinase inhibited. The inhibitor unit is defined as the quantity of inhibitor which causes a decrease in extinction of 0.001 per min at 280 nm during the cleavage of casein.

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

Typical methods using synthetic substrates are described below. During these assays no protease blank for inhibitors was necessary since highly purified inhibitors were used. One unit of the inhibitor was equal to the decrease in hydrolysis of 1 μmole of synthetic substrate per min. Specific activity was expressed as units per mg of protein.

Trypsin inhibitor: assays were carried out according to Schwert and Takenaka, 1955 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. A control was also run with trypsin without the inhibitor. The temperature was 26°. The increase in optical density at 253 nm was noted for 5 min. Enzyme activity was proportional to the increase in optical density when Δ O.D. was not more than 0.200.

The values used for calculation of activity of pure proteinases (trypsin, chymotrypsin, subtilisin and papain) using synthetic substrates are summarized in the table given at the end of this section (Table 3).

Chymotrypsin inhibitor assays (Walsh and Wilcox, 1970) were carried out with BTEE as the substrate. The reaction was carried out at 30°, 1 ml of the reaction mixture contained 0.5 μmole of BTEE, 40 μmoles of tris-HCl pH 7.8 and 40 μmoles of $CaCl_2$ pH 7.8. To this a mixture of 0.3-1.0 μg of chymotrypsin and the inhibitor was added and the absorbance difference at 256 nm was recorded for a period of about 5 min. A control using chymotrypsin without the inhibitor was run. The enzyme activity was proportional to the increase of absorbance when the change in absorbance was less than 0.120.

Subtilisin inhibition was determined by the titrimetric procedure using a pH-stat (Glazer, 1967). The hydrogen ions liberated during the hydrolysis of the ester substrate-BAEE were titrated against 0.02 M NaOH, pH 8, at 37°. 10 ml of the reaction mixture contained 500 μmoles of BAEE, 1000 μmoles of KCl and 100 μg of subtilopeptidase A, without and with approximately 30 μg of the subtilisin inhibitor. Pure subtilopeptidase A has a ko (sec^{-1}) value of 15.1.

Papain inhibitor: BAPA was used for the assay of papain and its inhibitor (Arnon, 1965). The reaction mixture (volume 6 ml) contained 5 μmoles of BAPA, 500 μmoles of dimethylsulfoxide, 15 μmoles of EDTA pH 7.5, 30 μmoles of cysteine pH 7.5 and 500 μmoles of Tris buffer pH 7.5. To this a mixture of 50 to 100 μg of papain and the inhibitor was added, and the reaction was carried out for 20 min at 25°. A control was run without the inhibitor. The reaction was stopped by adding 1 ml of 30% acetic acid and the liberated p-nitroaniline was estimated at 410 nm.

Papain assay was also carried out by the pH-stat method of Blumberg, Schechter and Berger (1970). The acid liberated during the hydrolysis of BAEE was titrated with 0.02 M NaOH. The reaction was carried out at pH 6.0

at 25°. 15 ml of the reaction mixture contained 300 µmoles of BAEE, 30 µmoles of EDTA, 75 µmoles of cysteine, 4,500 µmoles of KCl and 150 µg of papain. The activities were calculated from the initial rates of BAEE hydrolysis. Papain purified according to Kimmel and Smith (1954) gives a kcat of 13.0 sec^{-1}.

In the present report the purity of papain used was calculated on the assumption that the papain purified by the method of Kimmel and Smith (1954) represented a pure preparation (Arnon, 1965). However in a recent report Blumberg et al. (1970) have shown that this pure papain can be further purified two-fold by affinity chromatography.

As mentioned earlier the purity (amount of functional enzyme) of different proteinases was established by the assay methods based on the use of synthetic substrate as well as by comparing the standard caseinolytic assay curves by different workers. Per cent purities of different proteinases used in this work were found to be as follows. Trypsin 52, chymotrypsin 90, subtilisin 83, papain 58 and ficin 39. In the present work it is arbitrarily assumed that the impurities present in the enzymes do not combine with the inhibitor.

Quantities of the proteinases mentioned in the present work represent the amount of pure enzyme only. Values of caseinolytic assay units corresponding to 1 mg of pure proteinase are given below (Table 3).

TABLE 1

0 - 0.70 ammonium sulphate precipitate (fraction II, Chapter III) obtained from seed acid extract was dialyzed against 0.05 M phosphate buffer, pH 7.5. The precipitate formed during dialysis was centrifuged and the supernatant liquid was diluted 10-fold with 0.05 M phosphate buffer, pH 7.5 and used for the assay of subtilisin inhibitor (protein 1.9 mg/ml).

Experiment No.	1 Reagent blank	2 Enzyme blank *	3	4 Zero min. inhibitor blank	5 20 min incubated inhibitor blank **	6
			ml			
Subtilopeptidase A (50 µg/ ml of 0.1 M phosphate buffer, pH 7.5)		(0.1) added after trichloro-acetic acid	0.1			0.1
Inhibitor (protein,1.9 mg/ml of 0.05 M phosphate buffer, pH 7.5)				(0.15) added after trichloro-acetic acid	0.15	0.15
Phosphate buffer (0.1 M, pH 7.5)	1.0	0.9	0.9	0.85	0.85	0.75
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 ($O.D. \times 10^3$)	90	90	395	125	125	280
-Reagent blank(-90)	0	0	305	35	35	190
-Inhibitor blank (-35)			305	0	0	155
Proteinase units/ml reaction mixture (÷ 20)			15.3			7.8
Proteinase units/2 ml reaction mixture(X 2)			30.6			15.6
Inhibitor units/ 0.15 ml inhibitor						15.0
Inhibitor units/ml inhibitor						101

Legend to Table 1

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

* Enzyme blank (Expt. no.2) was not necessary to this assay since pure preparation of enzyme was used.

** 20 min incubated blank for inhibitor (Expt. no. 5) was also not needed since the crude inhibitor used (0 - 0.70 ammonium sulphate precipitate of the acid extract of seed) does not show any proteolytic activity at pH 7.5.

TABLE 2

30 g of Vigna catjang seeds were extracted with 150 ml of 0.1 M phosphate buffer pH 7.5. The extract (protein 50 mg/ml) was used for the 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
			ml			
Subtilopeptidase A (50 µg/ml of 0.1 M phosphate buffer, pH 7.5)		(0.1) added after trichloro-acetic acid	0.1			0.1
Inhibitor (protein, 50 mg/ml of 0.1 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 mm reading ($O.D. \times 10^3$)	85	85	380	225	340	465
-Reagent blank (-85)	0	0	295	140	255	380
-Zero min inhibitor blank (-140)			295	0	115	240
-20 min incubated inhibitor blank (-115)			295		0	125
Proteinase units/ml reaction mixture (÷ 20)			14.8			6.3
Proteinase units/2 ml reaction mixture (X 2)			29.6			12.6
Inhibitor units/0.1 ml inhibitor						17.0
Inhibitor units/ml inhibitor						170

Legend to Table 2

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

* Enzyme blank (Expt. no. 2) was not necessary in this assay since pure preparation of enzyme was used.

** Since the alkaline (pH 7.5) extract itself showed proteolytic activity this blank (20 min incubated inhibitor blank, expt. no. 5) was essential.

TABLE 3

VALUES USED FOR ACTIVITY CALCULATIONS OF PROTEINASES

Proteinase	Mol.wt.	Optical factor	Substrate used	$\triangle\epsilon$ Substrate product	Specific activity of pure proteinase	Reference	Fig.No.
				$M^{-1}cm^{-1}$	units/mg		
Trypsin	23,300	0.67	BAEE	808 (253 nm)	70	Schwert and Takenaka (1955)	
			Casein		6000		
Chymotrypsin	25,300	0.50	BTEE	964 (256 nm)	48	Walsh and Wilcox (1970)	
			Casein		5100		
Subtilisin (Subtilo-peptidase A)	27,600	1.04	BAEE		35	Glazer (1967)	
			Casein		8300		
Papain	21,000	0.4	BAPA	8800 (410 nm)		Arnon (1965)	
			BAEE			Blumberg et al. (1970)	
			Casein		5430		
Ficin			BAPA			Englund et al. (1968)	
			Casein		9300		

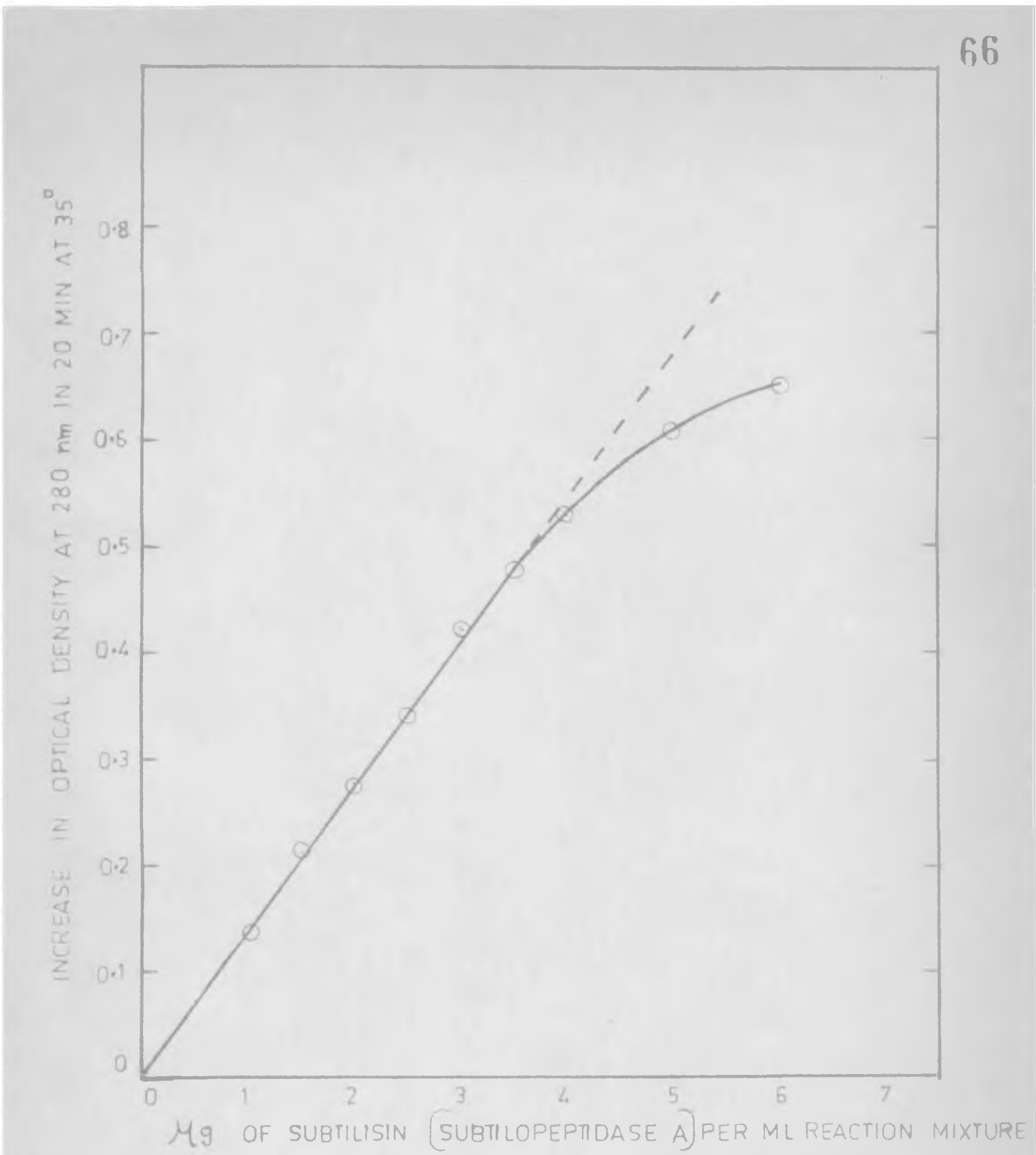


FIG-1 CASEINOLYTIC ASSAY OF SUBTILISIN (KUNITZ'S ASSAY PROCEDURE)

SPECIFIC ACTIVITY OF SUBTILISIN USED – 6875 UNITS/MG, (PURITY 83 %

SPECIFIC ACTIVITY OF PURE SUBTILISIN – 8280 UNITS/MG

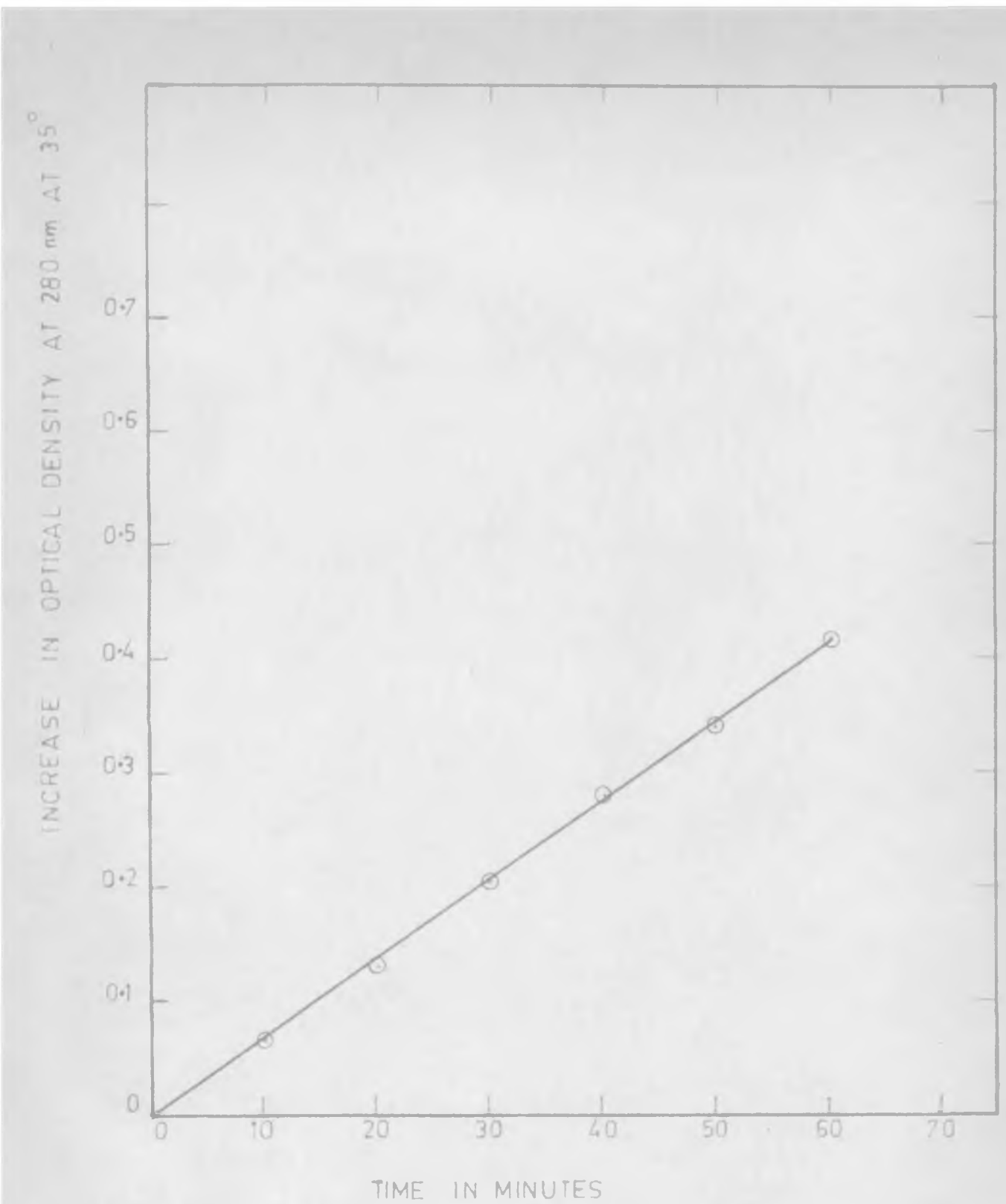


FIG-2 EFFECT OF TIME OF REACTION ON THE ACTIVITY OF SUBTILISIN (KUNITZ'S CASEINOLYTIC ASSAY)
1 μg (PER ML OF REACTION MIXTURE) OF SUBTILISIN OF 83% PURITY WAS USED (SPECIFIC ACTIVITY 6875 UNITS/MG)

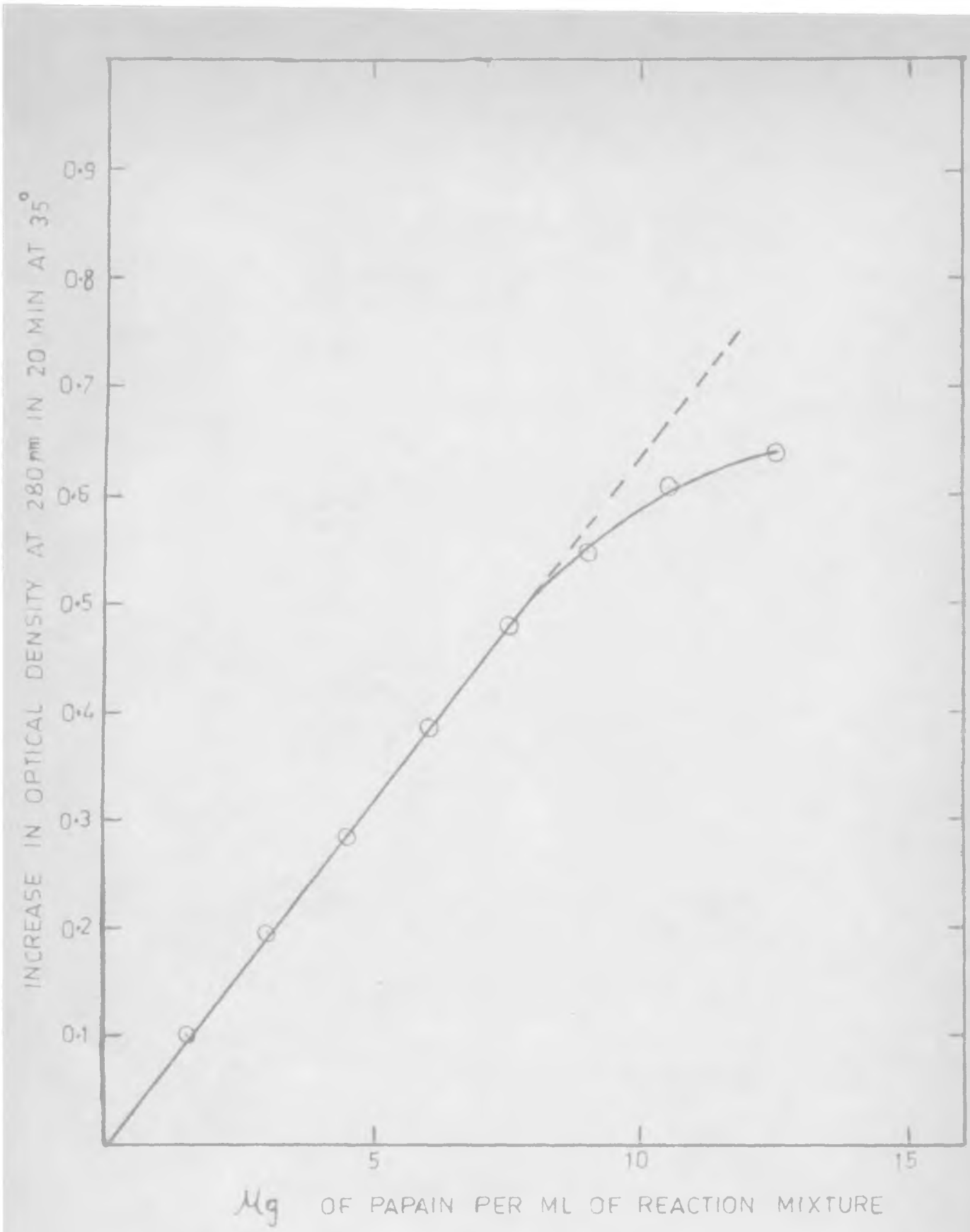


FIG-3 CASEINOLYTIC ASSAY OF PAPAIN (KUNITZ'S ASSAY PROCEDURE)

SPECIFIC ACTIVITY OF PAPAIN USED—3200 UNITS/MG, PURITY 58%

(SPECIFIC ACTIVITY OF PURE PAPAIN—5500 UNITS/MG)

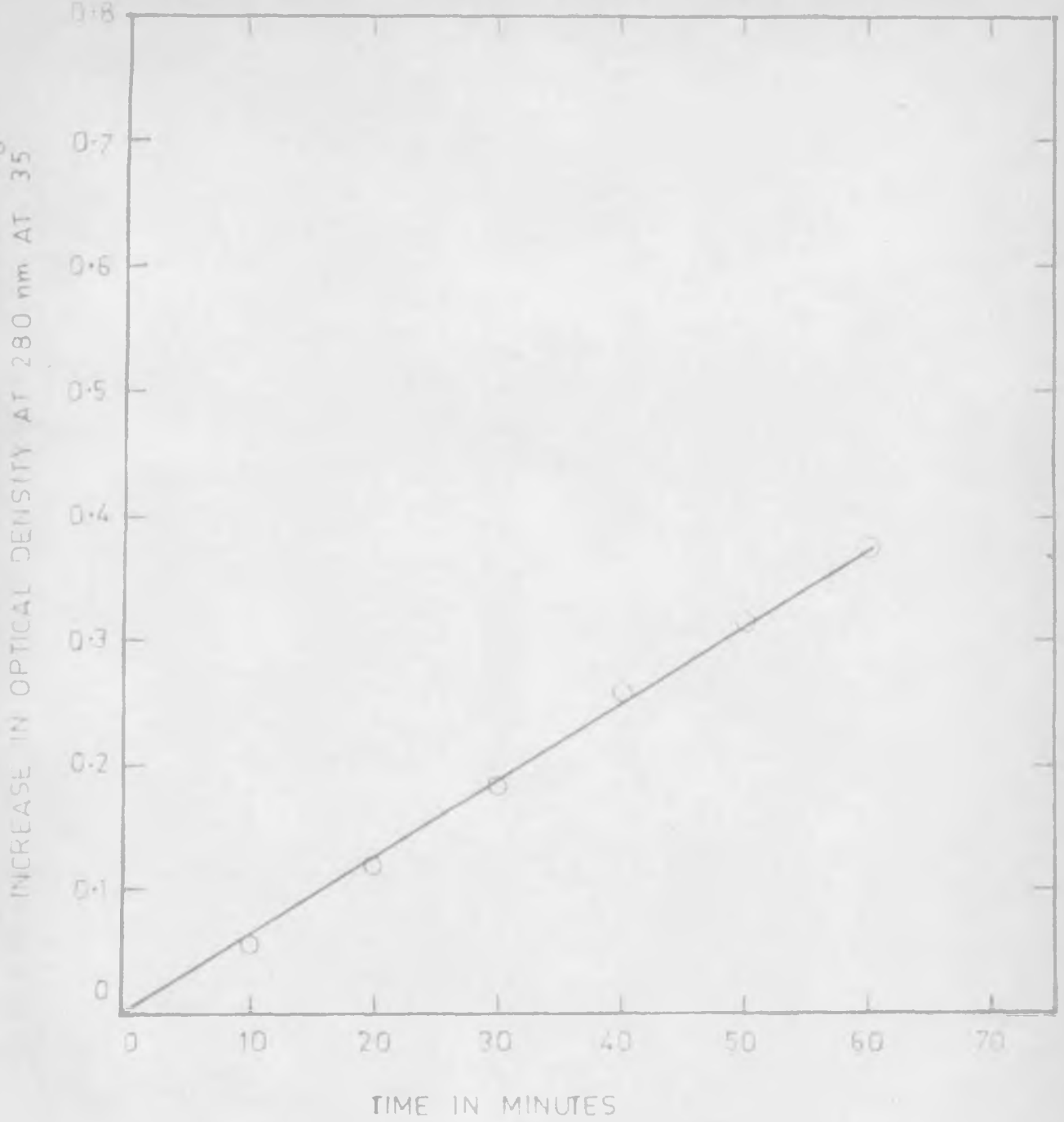


FIG-4 EFFECT OF THE TIME OF REACTION ON PAPAIN ACTIVITY (KUNITZ'S CASEINOLYTIC ASSAY)

2 μg OF PAPAIN (SPECIFIC ACTIVITY 3200, PURITY 58%) WAS USED

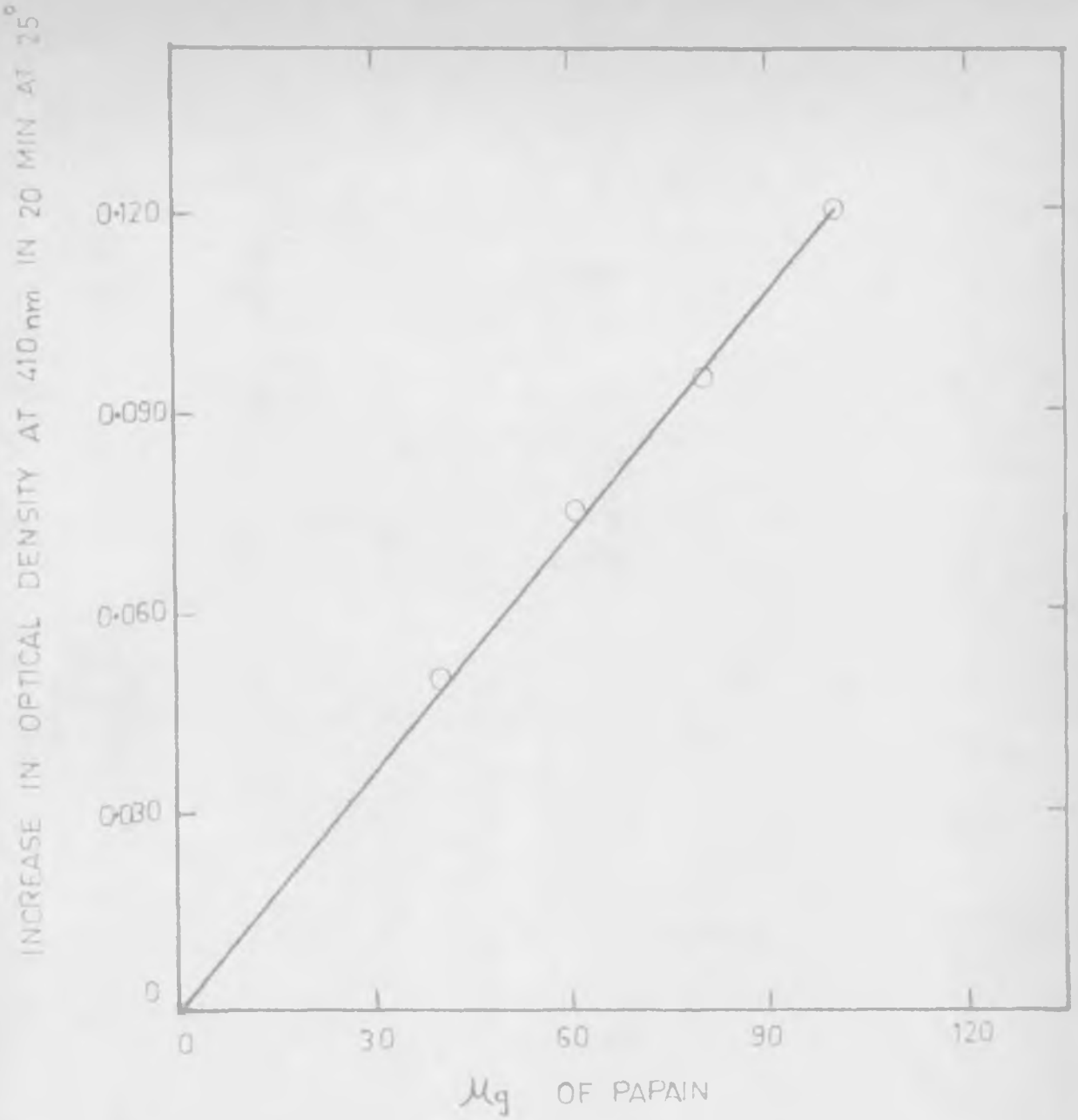


FIG-5 ARNON'S STANDARD CALIBRATION CURVE FOR THE ASSAY OF PURE PAPAIN (TWICE CRYSTALLIZED) BY USING THE SYNTHETIC SUBSTRATE BAPA.

100 μg OF PURE PAPAIN GIVES AN OPTICAL DENSITY RISE OF 0·121 UNDER THE ASSAY CONDITIONS.

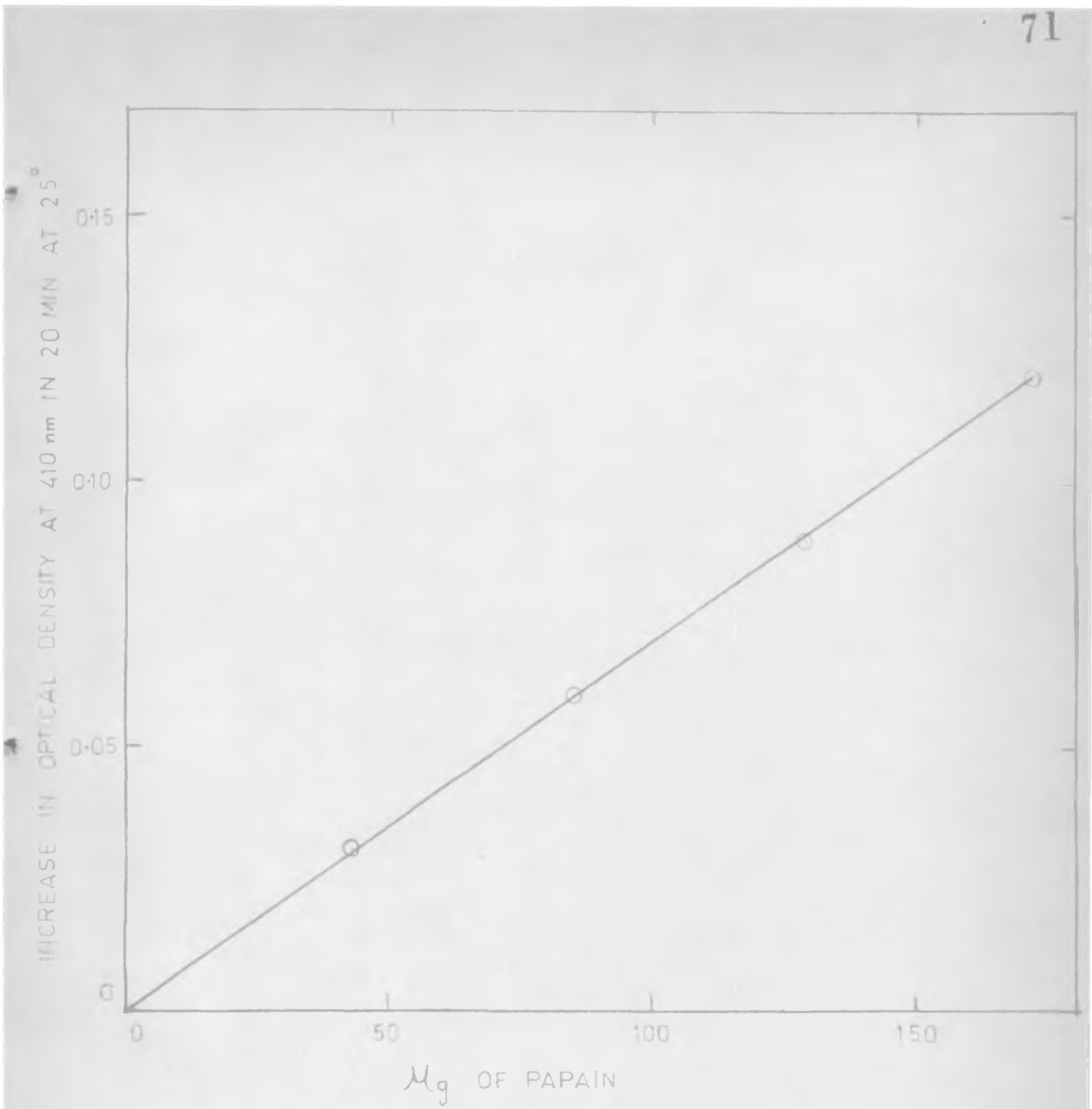


FIG-6 ASSAY OF PAPAIN BY USING SYNTHETIC SUBSTRATE (BAPA)

PURITY OF PAPAIN USED (COMPARED WITH THE CALIBRATION CURVE OF ARNON) IS 58%

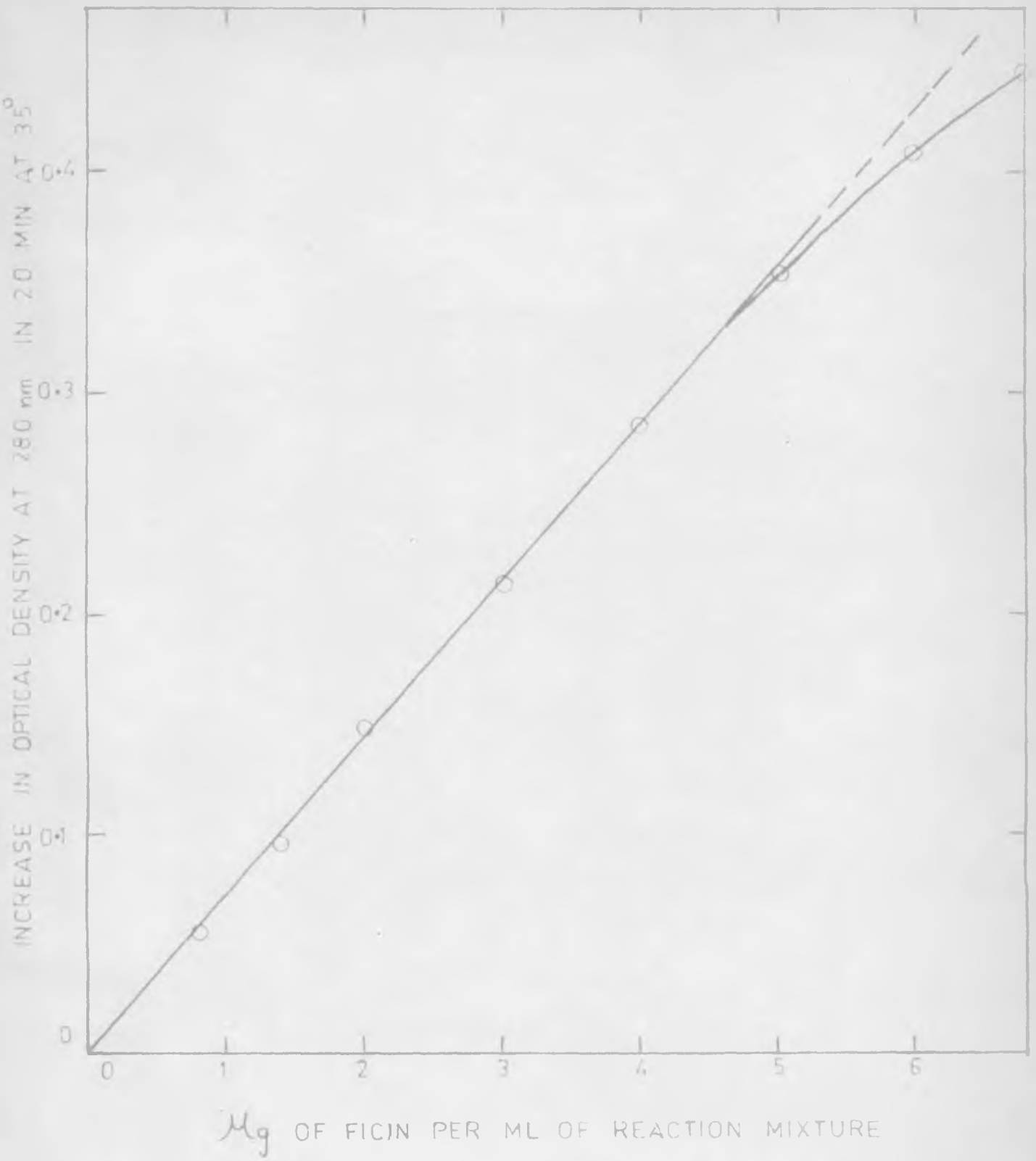


FIG-7 CASEINOLYTIC ASSAY OF FICIN (KUNITZ'S ASSAY PROCEDURE)

SPECIFIC ACTIVITY OF FICIN USED—3650 UNITS/MG, PURITY 39%

SPECIFIC ACTIVITY OF PURE FICIN—9300 UNITS/MG

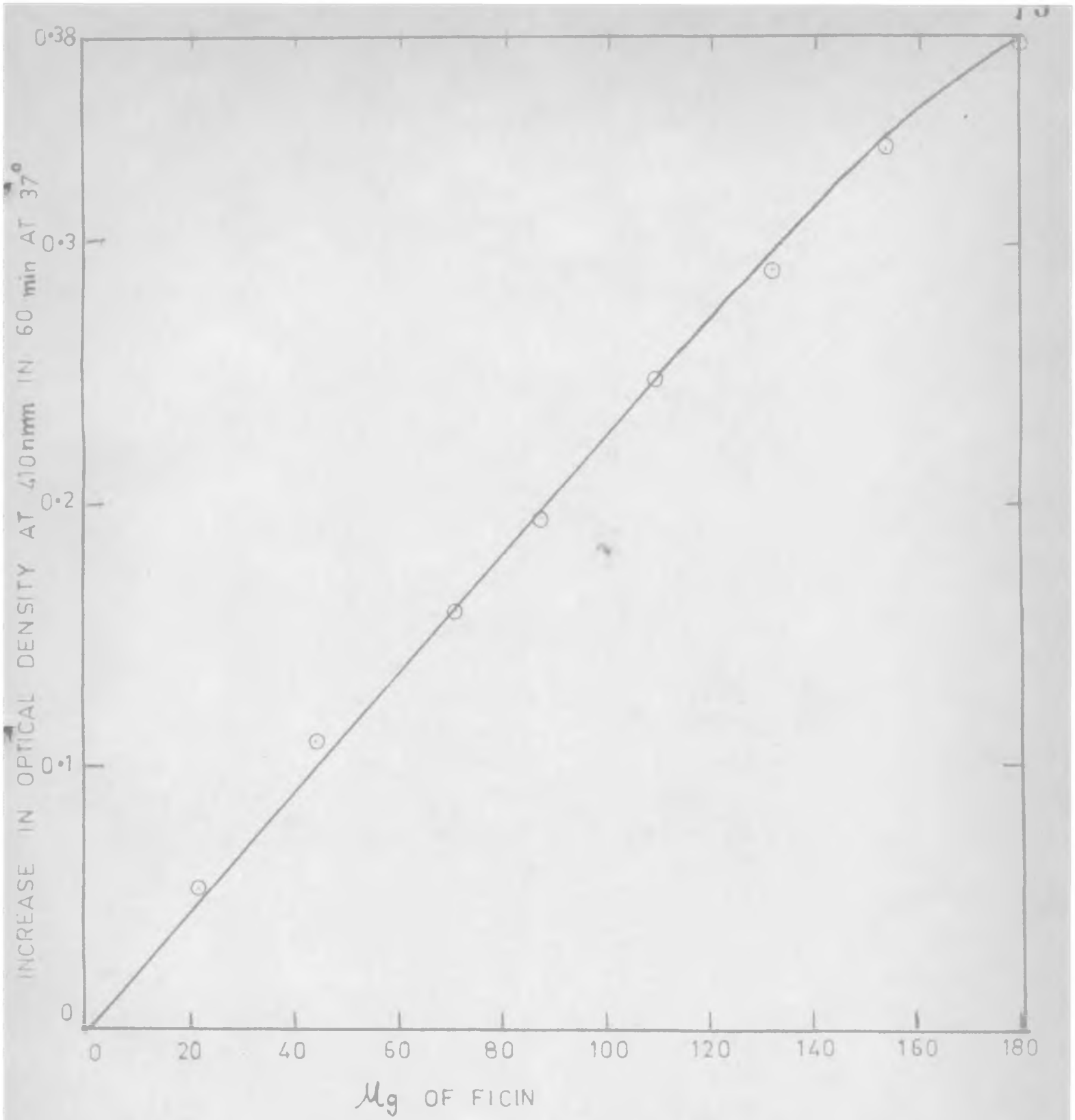


FIG-8 "ENGLUND'S" ASSAY OF FICIN BY USING SYNTHETIC SUBSTRATE (BAPA)

SPECIFIC ACTIVITY OF FICIN USED — 0·5 MOLE BAPA HYDROLYZED/MOLE FICIN

SPECIFIC ACTIVITY OF PURE FICIN — 1·6 MOLES BAPA HYDROLYZED/MOLE FICIN

CHAPTER III

EXPERIMENTAL AND RESULTS

Section I

Preliminary work

Preliminary work on proteinase inhibitors showed that acid extracts of several legumes inhibit not only trypsin and chymotrypsin but also subtilisin and papain.

Seeds of four leguminous plants, Vigna catjang (chavli bean), Vicia faba (double bean), Phaseolus vulgaris (french bean) and Phaseolus lunatus (lima bean) were extracted as follows:

50 g of seeds were washed with distilled water to remove any preservatives added to the seeds. The seeds were then soaked in distilled water for 30 min at room temperature. All subsequent operations were carried out at 0-5° unless otherwise stated. The soaked seeds were homogenized for 2 min in a Waring blendor with 250 ml of 0.01 M K-acetate buffer, pH 5.7. To the homogenate were added 50 ml of 0.6 M HCl and 3.75 g of solid KCl and the extract was kept for 30 min with occasional stirring. It was then filtered through muslin, neutralized to pH 5.7 with 2M $KHCO_3$ and centrifuged. From the clear supernatant liquid (vol. 240 ml) the crude mixture of inhibitors was precipitated at 0.70 saturation with solid ammonium sulphate (43.5 g/100 ml). The solution was centrifuged and the precipitate was dialyzed against 0.05 M K-phosphate buffer, pH 7.5. The solution after dialysis was recentrifuged and the clear supernatant liquid (volume 75 ml) was tested for inhibitors (Table 4).

TABLE 4

Inhibition of trypsin, subtilisin and papain by different leguminous seed extracts

	Vigna catjang	Vicia faba	Phaseolus vulgaris	Phaseolus lunatus
	units/g seeds			
Trypsin inhibitor	9,285	17,850	11,220	59,160
Subtilisin inhibitor	214	153	112	510
Papain inhibitor	54	102	85	122
	mg/g seeds			
Protein	6.6	12.8	5.6	11.0

In a separate experiment an extract of Vigna catjang seeds was prepared as before and inhibition of other proteinases by the extract was determined. It was found that 1 g of seeds contained also 2,500 units of chymotrypsin inhibitor and 85 units of pronase inhibitor.

The isolation of the individual inhibitors was necessary to establish whether they are separate inhibitors specific for each enzyme or whether the inhibitors are non-specific. Work on the purification of the inhibitors was undertaken with chavli beans (Vigna catjang) since these seeds were available locally in abundance and throughout the year.

Ion exchange chromatography of the extract of Vigna catjang seeds using CM-cellulose and DEAE-cellulose

An extract of Vigna catjang seeds was prepared using 50 g of seeds and the precipitate at 0.70 ammonium sulphate saturation ("0-0.70 precipitate") was obtained as stated earlier. The 0-0.70 precipitate was dissolved in a small volume of water and dialyzed against 0.01 M phosphate buffer, pH 6.0. The solution after dialysis was centrifuged and the supernatant liquid (volume 9 ml, protein 170 mg) was used for adsorption on CM-cellulose.

To 9 ml of the solution was added 20 ml of a suspension of 1.5 g of CM-cellulose which was equilibrated with 0.01 M phosphate buffer pH 6.0. The mixture after stirring for 30 min was filtered through a Buchner. The CM-cellulose was washed twice each time using 12 ml of 0.01 M phosphate buffer, pH 6.0 and filtered. The pooled filtrates were neutralized to pH 7.5 with solid $KHCO_3$ and dialyzed against 0.001 M phosphate buffer, pH 7.5. This dialyzed solution (volume 30 ml, protein 54 mg) was used for DEAE-cellulose chromatography. A preliminary study of adsorption of the crude inhibitors on CM-cellulose showed that the activity which inhibits trypsin, chymotrypsin and subtilisin mostly remained unadsorbed, whereas papain inhibitory activity was

partly adsorbed and partly not adsorbed.

A small scale experiment was carried out and the CM-cellulose supernatant liquid (unadsorbed portion of crude inhibitors) was chromatographed on DEAE-cellulose column (Fig.9, Table 5). The crude inhibitor mixture corresponding to 10 mg protein (5.8 ml) was applied to a DEAE-cellulose column (1.5 x 40 cm) which was equilibrated and developed with 0.001 M phosphate buffer, pH 7.5. A linear gradient formed from 300 ml each of starting buffer (0.001 M phosphate buffer, pH 7.5) and 0.15 M phosphate buffer, pH 7.5 was used for elution. The flow rate was 60 ml/h and each effluent fraction was 5 ml per tube.

The elution pattern in the figure 9 and table 5 shows the presence of several fractions inhibiting one or more proteinases such as trypsin, chymotrypsin, subtilisin and papain.

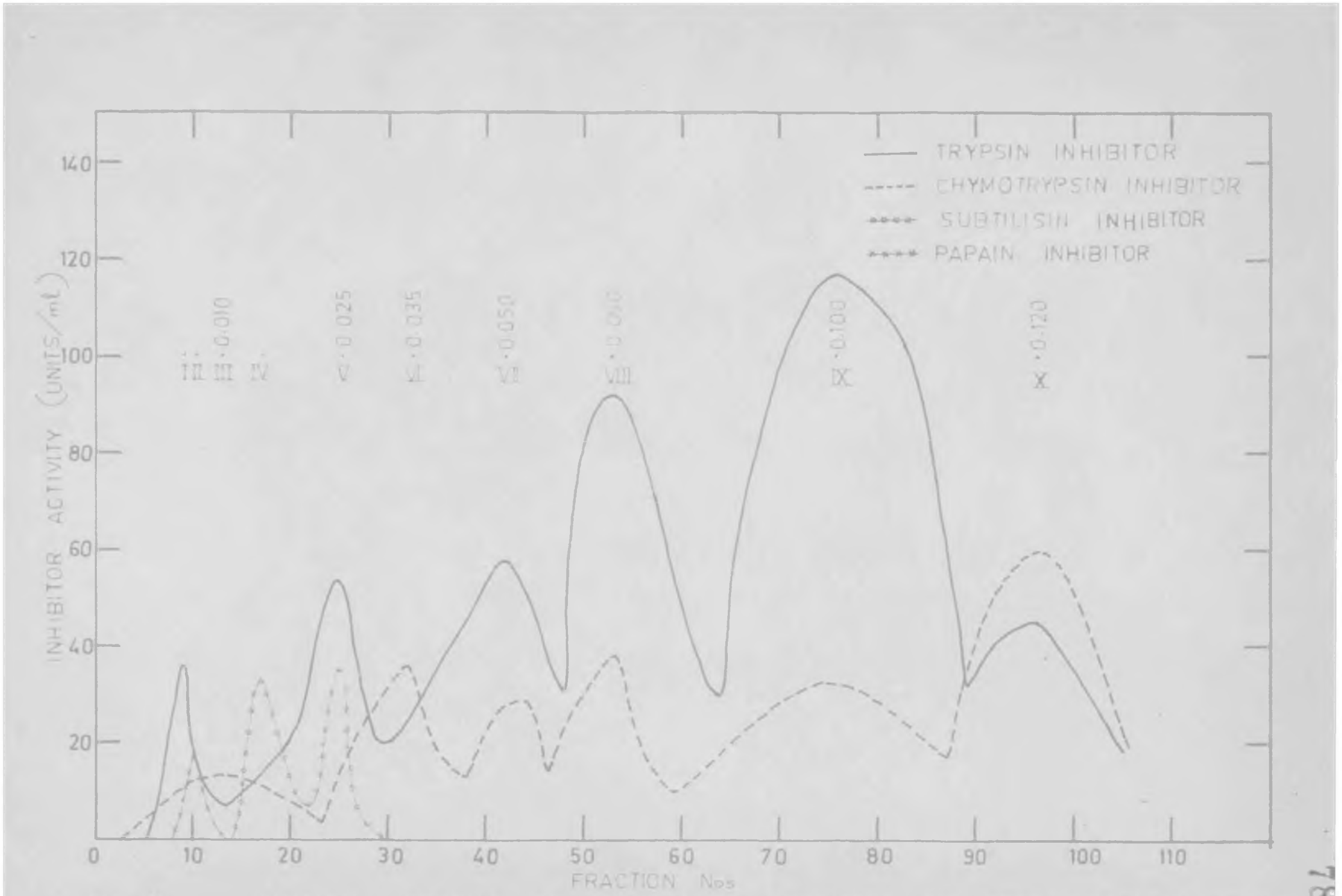


FIG - 9 ELUTION PROFILE OF THE CRUDE INHIBITORS ON DEAE - CELLULOSE COLUMN

TABLE 5

DEAE-cellulose chromatography of crude inhibitors

Peak No.	Fraction No.	Phosphate concentration required for elution	Trypsin inhibitor	Chymo-trypsin inhibitor	Subtilisin inhibitor	Papain inhibitor
		M			units/ml	
I	9	0.003	35	11	0	7
II	10	0.005	15	12	0	16
III	13	0.010	7	13	0	0
IV	17	0.015	12	10	33	0
V	25	0.025	54	3	35	0
VI	32	0.035	24	36	0	0
VII	42	0.050	58	28	0	0
VIII	53	0.060	92	42	0	0
IX	75	0.100	116	32	0	0
X	96	0.120	45	60	0	0

Another experiment was performed to see whether the inhibition of different proteinases was due to some metal in the extract of Vigna catjang seeds. For this the extract was first treated as follows with EDTA and then tested for inhibitors. The extract (0-0.70 precipitate) was dialyzed against 0.05 M K-phosphate buffer, pH 7.5, containing 0.001 M EDTA, pH 7.5. During the assay of the inhibitors the assay mixture also contained 0.01 M EDTA, pH 7.5. The table 6 shows that the inhibition of different proteinases, obtained with and without EDTA treatment was almost the same indicating that most probably a metal is not involved in the inhibition of the proteinases.

TABLE 6

Inhibition of trypsin, chymotrypsin, subtilisin and papain by Vigna catjang extract with and without EDTA-treatment

	Control experiment (without EDTA treatment)	With EDTA treatment*
	units/g seeds	
Trypsin inhibitor	9,285	9,325
Chymotrypsin inhibitor	2,500	2,455
Subtilisin inhibitor	214	220
Papain inhibitor	54	51

*The extract (0-0.70 precipitate) was dialyzed against 0.05 M K-phosphate buffer containing 0.001 M EDTA, pH 7.5. The assay mixture also contained 0.01 M EDTA, pH 7.5.

After these preliminary findings a systematic purification of the inhibitors of trypsin, subtilisin and papain was undertaken.

Section II

Purification of trypsin inhibitor 1

One of the trypsin inhibitors which was present in largest quantity (peak IX, Fig.9) was purified to homogeneity. For the purification of this trypsin inhibitor, a purification procedure different from that for subtilisin inhibitor and papain inhibitor was used, since it was found to be more convenient.

Large scale extraction procedure: Although the extraction procedure described in Section I of this chapter was suitable for small scale experiments, it was found to be laborious and time-consuming for large scale operations. Hence it was necessary to develop a modified procedure for extraction. In this procedure the blender was replaced by an electrical meat mincer and the centrifugations were carried out in a Sharples centrifuge.

Step I- Extraction

Three kg of chavli seeds were washed and soaked in distilled water for 30 min at room temperature. All further operations were carried out at 0° to 4°. The seeds were minced in a meat mincer. The minced material was extracted with cold buffer containing 15 litres of 0.01 M potassium acetate buffer pH 5.7, 225 g KCl and 3 litres of 0.6 M HCl, stirred for 30 min and then passed through muslin. The filtrate, after neutralization to pH 6.0 with 600 ml of 2 M $KHCO_3$, was centrifuged in a Sharples supercentrifuge (Fraction I).

Step II - Ammonium sulphate precipitation

0 - 0.70 precipitation: To the supernatant liquid (volume 15,500 ml- Fraction I) 6,740 g of ammonium sulphate were added with constant, gentle stirring till it dissolved completely. The solution was allowed to stand for at least an hour and then centrifuged in a Sharples centrifuge. The

supernatant liquid was discarded and the precipitate was dissolved in 250 ml of 0.1 M K-acetate buffer, pH 5.7 (Final volume 310 ml, 0.14 saturation - Fraction II).

Step III - Ammonium sulphate fractionation

0.20 - 0.50 saturation: The saturation of the solution (Fraction II) was increased from 0.14 to 0.20 by adding 16.2 g ammonium sulphate and the solution was centrifuged for 30 min. To the supernatant liquid (300 ml, 0.20 saturation), 53 g of ammonium sulphate (17.5 g/100 ml) were added and the solution centrifuged. The 0.20-0.50 precipitate was dissolved in 90 ml of 0.01 M K-phosphate buffer, pH 7.5 and dialyzed against the same buffer. The precipitate formed during dialysis was centrifuged off and the supernatant liquid (180 ml) was used for further purification (Fraction III).

Step IV - Chromatography on DEAE-cellulose: Three columns were operated simultaneously. Each column contained 30 g DEAE-cellulose (equilibrated with 0.01 M K-phosphate buffer, pH 7.5), filled under a slight pressure of three pounds per sq in (column diameter 6 cm, height 12.5 cm). 60 ml of the dialyzed liquid were loaded on each column and the column was washed first with 300 ml of 0.01 M phosphate buffer, pH 7.5, then with 1000 ml of 0.06 M phosphate buffer, pH 7.5, and finally eluted with 0.1 M phosphate buffer, pH 7.5. 100 ml fractions of the eluate were collected.

Fractions of the eluate showing a specific activity higher than 10,000 inhibitor units per mg were pooled, concentrated in a lyophilizer and precipitated at 0 - 0.90 saturation with ammonium sulphate. The precipitate obtained after centrifugation was dissolved in 0.1 M phosphate buffer, pH 7.5 and reprecipitated at 0.50 saturation with ammonium sulphate. The precipitate obtained on centrifugation was then dialyzed against 0.01 M phosphate buffer, pH 7.5 and concentrated to a small volume by lyophilization (Fraction IV).

Step V - Gel filtration using Sephadex G-100

It was then passed through a column (1.8 x 100 cm) of Sephadex G-100 using 250 ml of 0.05 M phosphate buffer, pH 7.5 as the eluant (flow rate 12 ml per h). ~~Fractions of 8 ml per h).~~ Fractions of 3 ml each were collected. Colouring matter and other impurities were separated in this step and the fractions showing trypsin inhibitor activity, having a specific activity of 18,000 units per mg were pooled, dialyzed against water and lyophilized to give a white powder. The purified trypsin inhibitor showed a single band on polyacrylamide gel electrophoresis and was homogeneous in the ultracentrifuge. The results of a typical fractionation are given in Table 7.

TABLE 7

Purification of trypsin inhibitor 1

No.	Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity
		ml	units/ml	units$\times 10^3$	mg/ml	mg	units/mg
I	Neutralized extract	15500	3480	53940	10.1	156550	345
II	0-0.70 saturation	310	169150	52437	78	24180	2170
III	0.20-0.50 saturation	180	146390	26350	40	7200	3660
IV	DEAE-cellulose chromatography	30	228850	6866	12	360	19070
V	Sephadex G-100 gel filtration	15	358200	5375	19.8*	297	18090

(3 kg seeds were used)

*Protein estimation by Folin's method

The yield of this final product was 10% and the specific activity was increased from 345 to 18090 units/mg giving a 52 fold purification.

An alternative purification procedure was also used after Step III. The fraction obtained after Step III was dialyzed against water and concentrated to a small volume by lyophilization. Further purification was achieved by preparative gel electrophoresis.

Preparative polyacrylamide gel electrophoresis

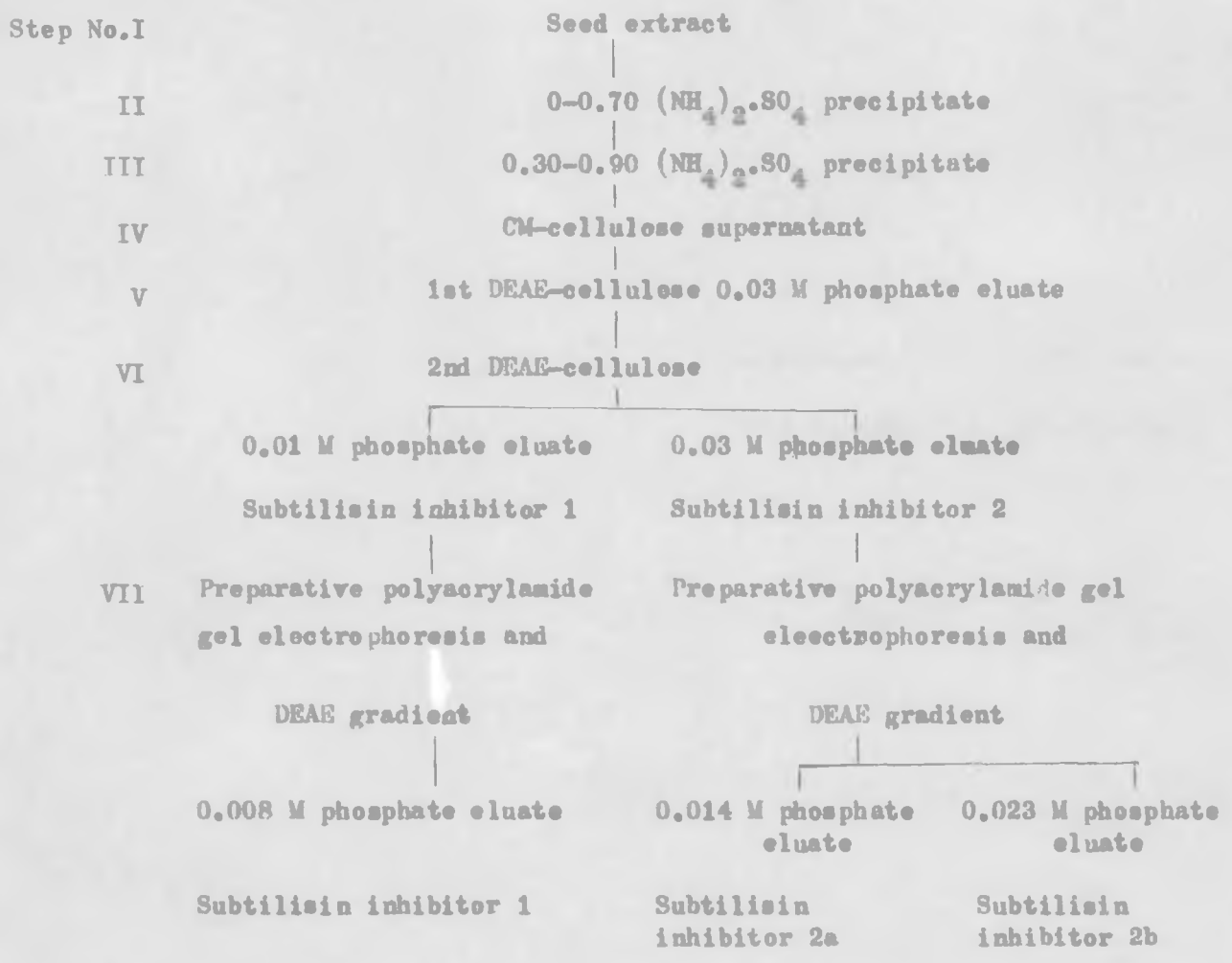
5 ml of the solution containing 50 mg protein were loaded on the cathodic end of a polyacrylamide gel column (5 x 12 cm). A current of 5 to 10 milliamperes was applied and the electrophoresis was run in the cold for 48 to 60 h. The movement of the marker (bromophenol blue) band which moved towards the anode was followed and when it was 1 cm before the anodic end, the electrophoresis was discontinued. It was observed that the inhibitor band very closely followed the marker band. Hence a 1.5 cm band just behind the marker was cut out from the gel and eluted with water. The eluate was filtered to remove insoluble polyacrylamide. The soluble impurities from polyacrylamide were removed by dialysis and by passing the eluate through a DEAE-cellulose column (1.6 x 50 cm). The impurities are not retained by the column and could be easily removed by washing the column with 0.01 M phosphate buffer, pH 7.5. After washing the column the inhibitor was eluted with 0.1 M phosphate buffer, pH 7.5. This preparation also gave a homogeneous material as tested by gel electrophoresis. Its specific activity was 17950 units per mg.

On the basis of the work presented in this chapter preliminary evidence shows the presence of six trypsin isoinhibitors. Out of these, two trypsin inhibitors were purified to homogeneity. (The purification of the second trypsin inhibitor is given in the next section on "the purification of subtilisin inhibitors").

Section III

Purification of the inhibitors of subtilisin

Before describing the detailed purification procedure a brief outline of the purification procedure for different subtilisin inhibitors is shown below:



The procedure used for the extraction of seeds (Step I) and for precipitation of the 0–0.7 ammonium sulphate fraction (Step II) was the same as described for the purification of trypsin inhibitor in Section II of this chapter.

Step III – Ammonium sulphate fractionation

0.30–0.90 saturation: The 0–0.70 precipitate obtained from 9 kg seeds was dissolved in 3850 ml of 0.025 M K-acetate buffer, pH 5.7 (final volume 4000 ml, 0.045 saturation). The ammonium sulphate saturation of the solution was raised from 0.045 to 0.30 by adding 557 g of ammonium sulphate (13.92 g/100 ml). It was allowed to stand for 30 min and centrifuged for 45 min at 3500 x g. The precipitate was discarded and to the supernatant liquid (volume 3800 ml, 0.30 saturation) 1520 g of ammonium sulphate (40 g/100 ml) were added to raise the saturation to 0.90. The solution was filtered overnight and the precipitate obtained was dissolved in 400 ml of 0.05 M K-phosphate buffer, pH 7.5 and dialyzed against four changes of 4 litres each of 0.01 M phosphate buffer, pH 7.5. After centrifugation the clear supernatant liquid was collected (volume 1370 ml, Fraction III).

Step IV - Chromatography on CM-cellulose

To 1350 ml of the solution (Fraction III) 12.6 ml of 1M H_3PO_4 were added to adjust the pH to 6.0. To this solution were then added 3400 ml of a suspension of 280 g of CM-cellulose (equilibrated with 0.005 M phosphate buffer, pH 6.0) in 0.005 M phosphate buffer, pH 6.0. The mixture was stirred for 45 min and then filtered through a Büchner. The CM-cellulose was washed twice, each time using 2400 ml of 0.01 M, pH 6.0 phosphate buffer and filtered. The filtrates were pooled and the pH was raised to 7.0 with solid $KHCO_3$.

To the combined filtrate were added 5500 g ammonium sulphate (0.95 saturation). The mixture was kept overnight and then filtered. The precipitate was dissolved in 210 ml of water and dialyzed against 0.03 M phosphate buffer, pH 7.5. After centrifugation the supernatant liquid (volume 725 ml, Fraction IV) was collected.

Step V - Chromatography on DEAE-cellulose

To 680 ml of Fraction IV were added 180 g of DEAE-cellulose (equilibrated with 0.03 M phosphate buffer, pH 7.5) suspended in 5000 ml of 0.03 M phosphate buffer, pH 7.5. The mixture was stirred for 45 min and then filtered through a Büchner funnel. The DEAE-cellulose was washed with 5000 ml of 0.03 M phosphate buffer, pH 7.5 and filtered. The filtrates were pooled (10,000 ml) and 5100 g of ammonium sulphate were added (0.95 saturation). The solution was filtered and the precipitate dissolved and dialyzed against water and lyophilized to a volume of 100 ml (Fraction V).

Step VI - Rechromatography on DEAE-cellulose

300 g of DEAE-cellulose were washed and equilibrated with 0.001 M K-phosphate buffer, pH 7.5. A (8 x 40 cm) column was prepared and washed with the same buffer. Two such columns were run. On each column 50 ml of the inhibitor solution (Fraction V) were loaded and eluted by stepwise addition of 4000 ml each of 0.001 M, 0.005 M, 0.010 M and 0.030 M phosphate buffer, pH 7.5. 400 ml fractions were collected and estimated for protein and for the activity of subtilisin inhibitor.

It was observed that the subtilisin inhibitor activity was resolved into two isoinhibitors one eluting at 0.01 M phosphate concentration (subtilisin inhibitor 1) and another elutable at 0.03 M phosphate concentration (subtilisin inhibitor 2). Active fractions with specific activities higher than 4500 units/mg (for subtilisin inhibitor 1) and 800 units/mg (for

subtilisin inhibitor 2) were pooled separately and concentrated from a volume of about 1.2 litre to a volume of approximately 300 ml by lyophilization. The inhibitors were then precipitated individually with ammonium sulphate at 0.95 saturation by adding 65 g ammonium sulphate for every 100 ml solution. The precipitates were dissolved in water, dialyzed against water and lyophilized to a small volume (5 ml), (Subtilisin inhibitor 1- Fraction VIa, Subtilisin inhibitor 2 - Fraction VIb).

Step VII- Purification by preparative polyacrylamide gel electrophoresis

Even after Step VI the inhibitors were not homogeneous but showed several bands (5-6) on polyacrylamide gel. They were purified further by using preparative polyacrylamide gel electrophoresis.

Purification by preparative polyacrylamide gel electrophoresis was carried out by the procedure described in Chapter II. The inhibitor was applied on the gel at the cathodic end. The electrophoresis was discontinued when the marker band moved approximately 10 cm towards the anodic end of the column.

Subtilisin inhibitors 1 and 2 purified by the above method were subjected to gradient DEAE-cellulose chromatography to remove impurities dissolved from the gel as well as other impurities in the inhibitors. 14 g of DEAE-cellulose were washed and equilibrated with 0.001 M phosphate buffer, pH 7.5. A (1.6 x 50 cm) column was prepared and washed with water. The inhibitor solution was loaded on the column and the column was washed with 300 ml water. After washing with water, a linear gradient formed from water and phosphate buffer, pH 7.5 at the concentrations given below, was used for elution of the inhibitor. The flow rate was 55 ml per h and fractions containing 4.6 ml effluent per tube were collected. Linear gradient columns used for subtilisin inhibitor 1 and 2 are described below.

Subtilisin inhibitor 1: A linear gradient formed from 250 ml each of water and 0.02 M phosphate buffer, pH 7.5 was used for elution. This inhibitor eluted as a single peak at a phosphate concentration of 0.008 M. It was however found that the fraction was not homogeneous on polyacrylamide gel, but showed two bands. Both the bands showed inhibitory activity towards subtilisin. 1 mg of the inhibitor inhibits 13910 units of subtilisin. The inhibitor was not fractionated further.

Subtilisin inhibitor 2: A linear gradient formed from 250 ml each of water and 0.045 M phosphate buffer, pH 7.5 was used for elution. It was observed that this inhibitor was further resolved into two isoinhibitors. The first inhibitor fraction appeared in the eluate at a phosphate concentration of 0.014 M and the second inhibitor fraction eluted at 0.023 M phosphate concentration. The first inhibitor fraction (subtilisin inhibitor 2a) was homogeneous and showed a single band on polyacrylamide gel. 1 mg of the inhibitor inhibits 16060 units of subtilisin. The second inhibitor fraction (subtilisin inhibitor 2b) was also homogeneous on polyacrylamide gel; however this fraction in addition to the inhibition of subtilisin also showed inhibition of trypsin. 1 mg of this inhibitor inhibited 3020 units of subtilisin and 2400 units of trypsin.

Table 8

Purification of subtilisin inhibitors

No.	Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity
		ml	units/ml	units$\times 10^3$	mg/ml	mg	units/mg
1	Seed extract	46000	53	2438	8.9	409400	6
2	0–0.70 $(NH_4)_2SO_4$ saturation	900	2522	2270	140	126000	18
3	0.30–0.90 $(NH_4)_2SO_4$ sat.	1370	1575	2160	21	28770	75
4	CMC supernatant 0–0.95$(NH_4)_2SO_4$ ppt.	680	2400	1635	16	10880	150
5	DEAE 0.03 M phosphate eluate 0–0.95$(NH_4)_2SO_4$ ppt.	100	11500	1150	39	3900	290
6	DEAE						
	0.01 M phosphate eluate Subtilisin inhib. 1	5	54000	270	11.4	57	4750
	0.03 M phosphate eluate Sub.inhib. 2	5	56000	280	51	255	1100
7	Preparative polyacrylamide and DEAE gradient						
	0.008 M phosphate eluate Subtilisin inhib.1	3	40330	121	2.9*	8.7	13910
	0.014 M phosphate eluate Subtilisin inhib.2a	3	16300	53	1.1*	3.3	16060
	0.023 M phosphate eluate Subtilisin inhib.2b	3	29300	88	9.7	29	3020

9 kg seeds were used

*Protein estimation by Folin's method

Further purification of subtilisin inhibitor 2b:
(Separation of the mixture of subtilisin inhibitor 2b and trypsin inhibitor 2 from Fraction 7)

Subtilisin inhibitor 2b which also showed trypsin inhibition, was not only homogeneous on polyacrylamide gel but even after SDS treatment the inhibitor moved as a single band. It was extremely difficult to separate this subtilisin inhibitor from the trypsin inhibiting impurity by conventional methods. However as described below, it was possible to obtain the subtilisin inhibitor free from trypsin inhibitor, by preparing a trypsin inhibitor-trypsin complex and separating the complex from the subtilisin inhibitor by gel filtration using Sephadex G-50. By this method one more trypsin inhibitor (trypsin inhibitor 2 which is different from the trypsin inhibitor purified in Section II) was also obtained by cleaving the trypsin inhibiter-trypsin complex which was recovered from the Sephadex.

Fraction 7 (0.023 M phosphate eluate) containing a mixture of subtilisin inhibitor 2b and trypsin inhibitor 2 was dialyzed against water and concentrated to a small volume (2 ml). (Total protein 29 mg, subtilisin inhibitor 88,000 units, trypsin inhibitor 69,600 units). To 1 ml of the concentrated solution were added 0.05 ml of 1 M phosphate buffer, pH 7.5 and 14.5 mg of trypsin (purity 40%, containing 34,800 tryptic units) and the mixture was loaded on a Sephadex G-50 column (1.5 x 100 cm) which was equilibrated and eluted with 0.05 M phosphate buffer, pH 7.5 (3 ml fractions were collected, flow rate was 10 min per fraction). The trypsin inhibitor-trypsin complex appeared in the void volume and the subtilisin inhibitor eluted at a later stage. The subtilisin inhibitor fractions (specific activity of 7,150 units/mg) were pooled, dialyzed against water and concentrated to a small volume (1.5 ml). The fractions containing trypsin-trypsin inhibitor complex were located by measuring absorbance at 280 nm and were pooled and concentrated

to a small volume (3 ml). To the concentrate an equal volume of 10% trichloroacetic acid was added. The precipitate obtained was centrifuged and the supernatant liquid containing the free trypsin inhibitor was dialyzed against water and concentrated to a small volume (1.5 ml, specific activity 3,300). The results obtained are summarized in Table 9.

Table 9

Separation of the mixture of subtilisin inhibitor 2a and trypsin inhibitor 2 from Fraction 7

No.	Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity
		ml	units/ml	units$\times 10^3$	mg/ml	mg	units/mg
7	Preparative polyacrylamide and DEAE gradient 0.023 M phosphate eluate						
	Sub.I 2a and Try. I 2	3	29300 23200	88 69.6	0.7	29	3020 2400
8	Trypsin addition and separation on Sephadex G-50						
	Sub.I 2b	3	15020	45	2.1*	6.3*	7150
	Try.I 2	3	9570	28.7	2.9*	8.7*	3300

*Protein estimation by Folin's method

9 kg seeds were used

The work presented in this chapter shows the presence of three isoinhibitors of subtilisin. Out of these one subtilisin inhibitor (subtilisin inhibitor 1) has been purified partially and the other two (subtilisin inhibitors 2a and 2b) were purified to homogeneity.

Purification by the use of affinity chromatography

A preliminary experiment was performed for the purification of subtilisin inhibitor from the 0.30–0.90 ammonium sulphate precipitate (Fraction III, Step III) by using Sephadex bound subtilisin.

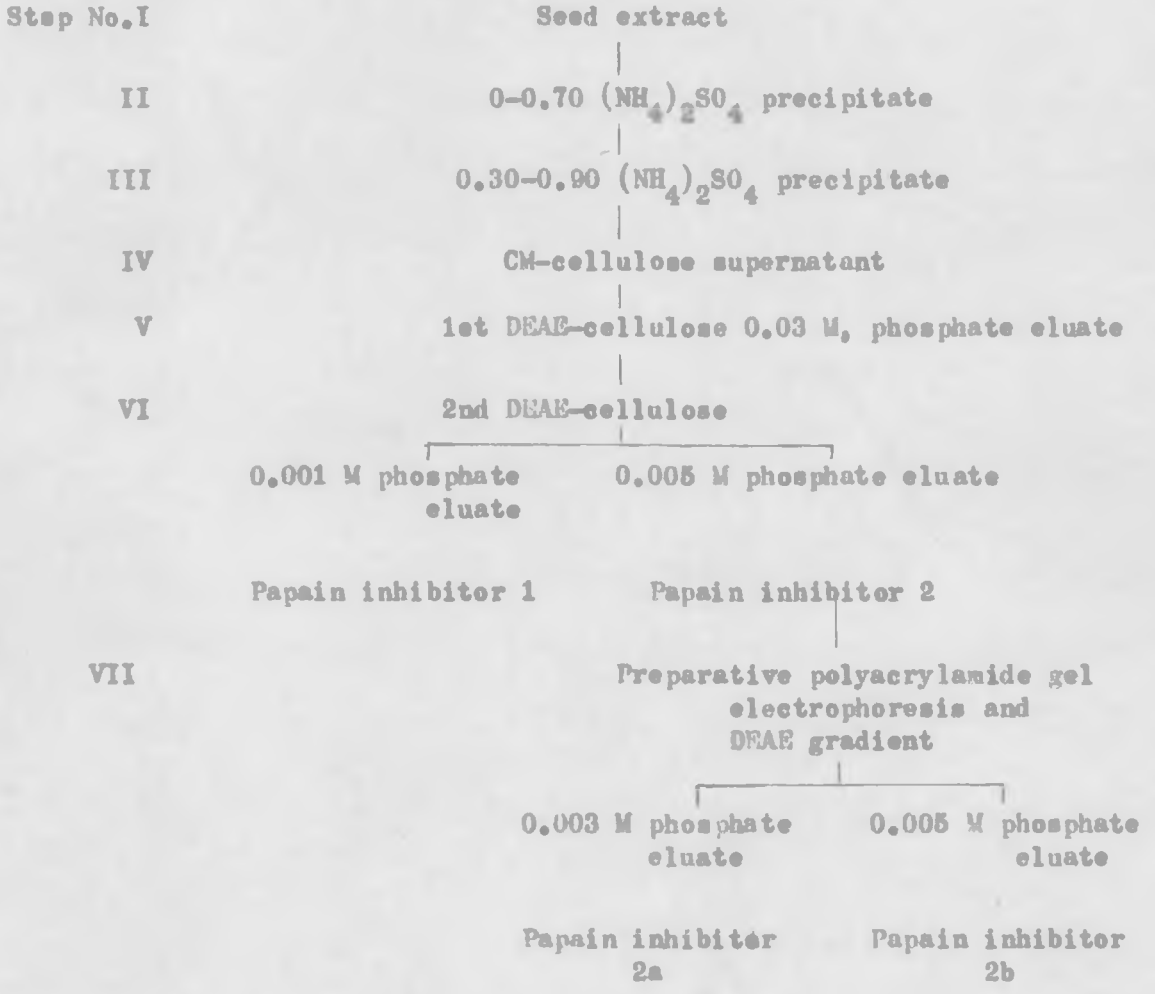
Sephadex-G 200 was activated with 4 cyanogen bromide and coupled with subtilisin by using the procedure of Axen and Ernback (1971). To a 10 ml Sephadex suspension containing 0.5 mg (4200 units) of immobilized subtilisin in 0.1 M phosphate buffer, pH 7.5 were added 1.5 ml of Fraction III containing 2360 units of subtilisin inhibitor (protein 31.5 mg, specific activity 75 units/mg). After stirring for 15 min the Sephadex-subtilisin-subtilisin inhibitor complex was washed with 0.1 M $KHCO_3$ and then the bound inhibitor was released by treatment with 2.5% trichloroacetic acid. 0.8 mg protein containing 2100 units of subtilisin inhibitor with a specific activity of 3600 units/mg was obtained. This gave approximately a 50-fold purification of the inhibitor in one step.

However this procedure of purification was not generally used since there is a possibility of obtaining artifacts (modified inhibitors) by this method.

Section IV

Purification of Papain Inhibitors

An outline of the purification procedure is given below, before describing the detailed procedure.



During the purification of papain inhibitors the procedures used for the extraction of seeds (Step I), 0-0.70 precipitation of the extract with ammonium sulphate (Step II), 0.30-0.90 fractionation with ammonium sulphate (Step III), CM-cellulose chromatography (Step IV) and chromatography on DEAE-cellulose (Step V) were the same as mentioned in the purification of subtilisin inhibitors in Section III of this chapter.

The further purification procedure used (for papain inhibitors) such as rechromatography on DEAE-cellulose (Step VI) and purification by preparative polyacrylamide gel electrophoresis (Step VII) were also broadly similar to those used for subtilisin inhibiters. In the following pages only the necessary changes involved in the purification of papain inhibitors during these steps are described.

Step VI - Rechromatography on DEAE-cellulose

Two DEAE-cellulose columns (as described in Step VI, Section III) each containing 300 g of DEAE-cellulose were run simultaneously.

On each column 50 ml of the inhibitor solution (Step V) containing 1610 units of papain inhibitor and 3.9 mg of protein per ml, with a specific activity of 41 units per mg protein, were loaded and eluted by stepwise addition of 4000 ml each of 0.001 M and 0.005 M phosphate buffer, pH 7.5. 400 ml fractions were collected and estimated for protein and inhibitor activity. It was observed that the papain inhibitor activity was resolved into two isoinhibitors, one of them was weakly adsorbed on DEAE-cellulose and eluted at 0.001 M phosphate concentration (papain inhibitor 1), while the other inhibitor was eluted at 0.005 M phosphate concentration (papain inhibitor 2).

Active fractions with specific activities higher than 500 units/mg for papain inhibitor 1 (volume 800 ml) and higher than 1200 units/mg for

papain inhibitor 2 (volume 800 ml), were pooled separately and concentrated by lyophilization to a volume of approximately 100 ml. The inhibitors were then precipitated individually with ammonium sulphate at 0.95 saturation and the precipitates dialyzed against water and concentrated by lyophilization to a small volume (5 ml).

Step VII. Purification by preparative polyacrylamide gel electrophoresis

Papain inhibitor 1: No further attempt was made to purify this inhibitor.

Papain inhibitor 2: This inhibitor was further purified by preparative polyacrylamide gel electrophoresis and subsequent gradient DEAE-cellulose chromatography.

For the gradient DEAE-cellulose chromatography a linear gradient formed from 250 ml each of water and 0.01 M phosphate buffer, pH 7.5 was used for elution. It was observed that the papain inhibitor was further resolved into two isoinhibitors. The first inhibitor fraction appeared in the eluate at a phosphate concentration of 0.003 M (papain inhibitor 2a) and the second inhibitor fraction eluted at 0.005 M phosphate (papain inhibitor 2b). Both the fractions were homogeneous on polyacrylamide gel. Their specific activities were 6250 and 5000 units per mg respectively.

The CM-cellulose adsorbed material contained papain inhibitor 3, 120×10^3 units of this inhibitor (protein 5 g, specific activity 23) could be eluted with 5500 ml of 0.1 M phosphate buffer, pH 7.5. This inhibitor was not studied further.

TABLE 10

Purification of papain inhibitors

No.	Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity
		ml	units/ml	unitsx10^3	mg/ml	mg	units/mg
1	Seed extract	46000	20	920	8.9	409400	2.2
2	0-0.70 $(NH_4)_2SO_4$ saturation	900	944	850	140	126000	6.7
3	0.30-0.90 $(NH_4)_2SO_4$ saturation	1370	591	810	21	28770	28
4	CMC supernatant 0-0.95 $(NH_4)_2SO_4$ precipitate	680	370	252	16	10880	23
5	DEAE- 0.03 M phosphate eluate 0-0.95 $(NH_4)_2SO_4$ precipitate	100	1610	161	39	3900	41
6	DEAE rechromatography						
	0.001 M phosphate eluate Pap inhib. 1	5	3600	18	7	35	510
	0.005 M phosphate eluate Pap inhib. 2	5	16200	81	9.6	48	1680
7	Preparative polyacrylamide and DEAE gradient of Pap. inhibitor 2						
	0.003 M phosphate eluate Pap.inhib. 2a	3	1000	3	0.16*	0.48	6250
	0.005 M phosphate eluate Pap.inhib. 2b	3	900	2.7	0.18*	0.54	5000

9 kg seed were used. *Protein estimation by Folin's method

Evidence for the presence of four isoinhibitors of papain was obtained and out of these, two inhibitors have been purified to homogeneity.

Further unpublished work on papain inhibitors in this laboratory showed two isoinhibitors in the fraction adsorbed by CM-cellulose and a different papain inhibitor in the alkaline extract of the seeds of Vigna catjang.

On the basis of the work presented in this chapter preliminary evidence for the presence of six trypsin isoinhibitors, three subtilisin isoinhibitors and four papain isoinhibitors has been obtained. Out of these inhibitors two trypsin inhibitors, two subtilisin inhibitors and two papain inhibitors have been purified to homogeneity. In addition the remaining third subtilisin inhibitor has been partially purified.

CHAPTER IV

PROPERTIES AND KINETICS

The properties and kinetics of the inhibitors of trypsin, subtilisin and papain, which are presented in the following sections, were studied with inhibitors of maximum purity. The inhibitors were dialysed against 0.001 M phosphate buffer, pH 7.5, before use.

Section 1

Properties and kinetics of trypsin inhibitors 1 and 2

Trypsin inhibitor 1

General - During the purification of the inhibitor a pale brown colour persisted which was completely removed during Sephadex gel filtration. A 1% solution of the final purified inhibitor was water clear and colourless.

Polyacrylamide gel electrophoresis - Polyacrylamide gel electrophoresis was carried out according to the procedure described in Chapter II. Electrophoresis was run using 7% and 12% gel concentrations at pH 8.5. The electrophoretic pattern of the purified inhibitor on a 7% gel is shown in Fig.10. A single sharp band was present when a gel concentration of either 7% or 12% was used.

Molecular weight and sedimentation constant - Molecular weight and sedimentation constant determinations were carried out by the ultracentrifugal method as described in Section II of Chapter II. 1% trypsin inhibitor solution in 0.1 M phosphate buffer, pH 7.5, was used. Velocity runs were carried out at 59,780 rev per min. A calculated speed of 16,200 rev per min was used for the molecular weight determination. The temperature was 20°. The movement of the boundary was recorded by Schlieren photographs at 16 min intervals. The value of 0.69 cm^3/g was used for the partial specific volume ($\bar{v}$) of the protein. Synthetic boundary ultracentrifugal patterns obtained are shown in Fig.11.

Fig.10

Polyacrylamide gel electrophoresis of purified trypsin inhibitor 1

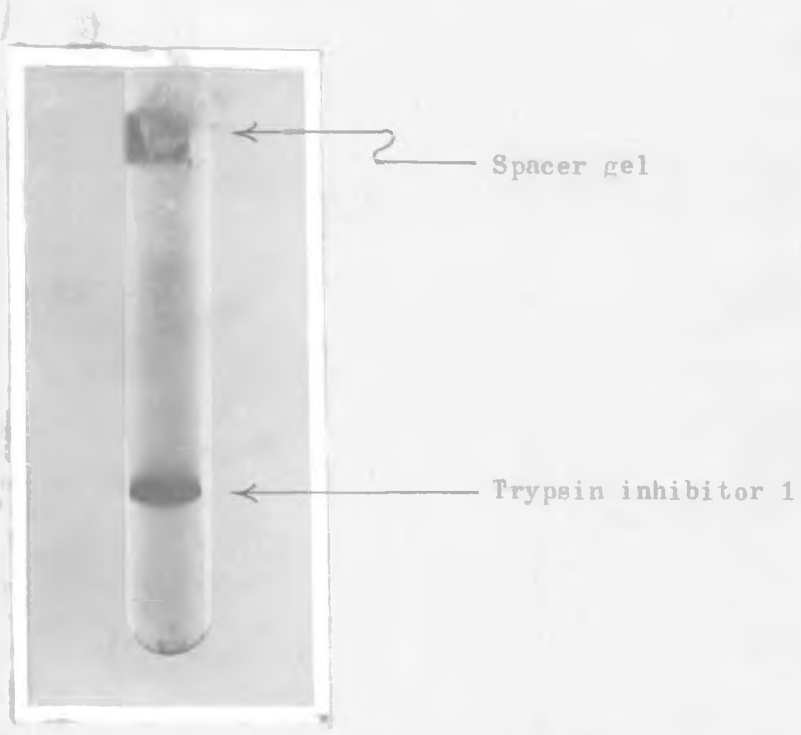
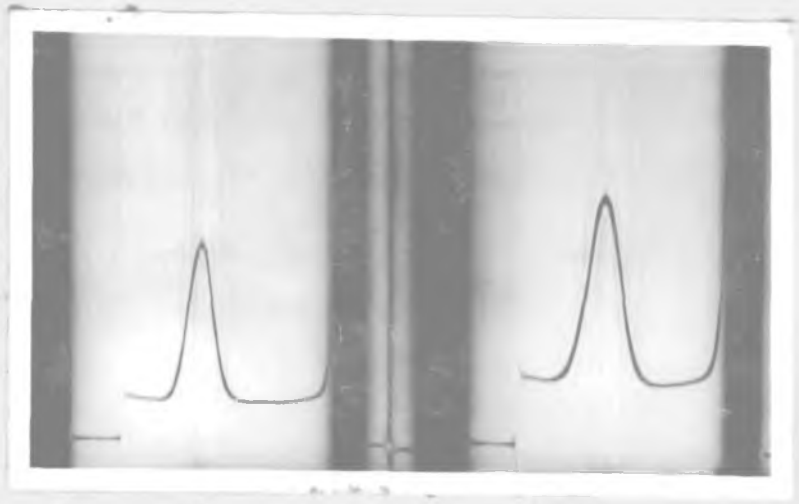
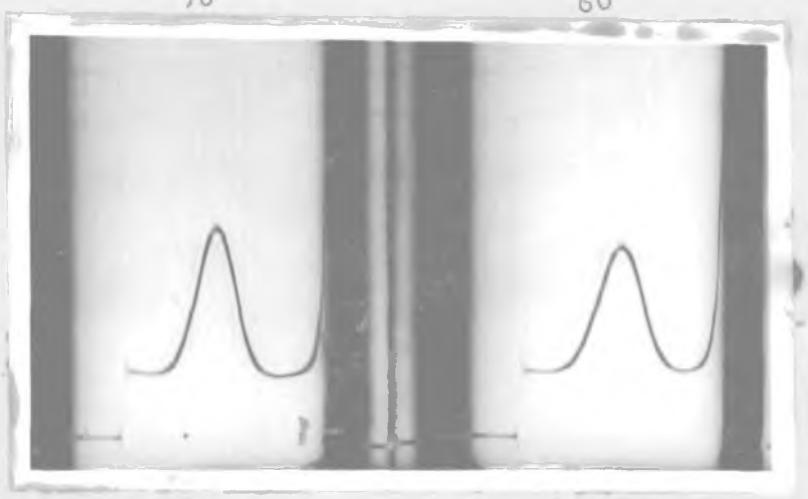


Fig.11

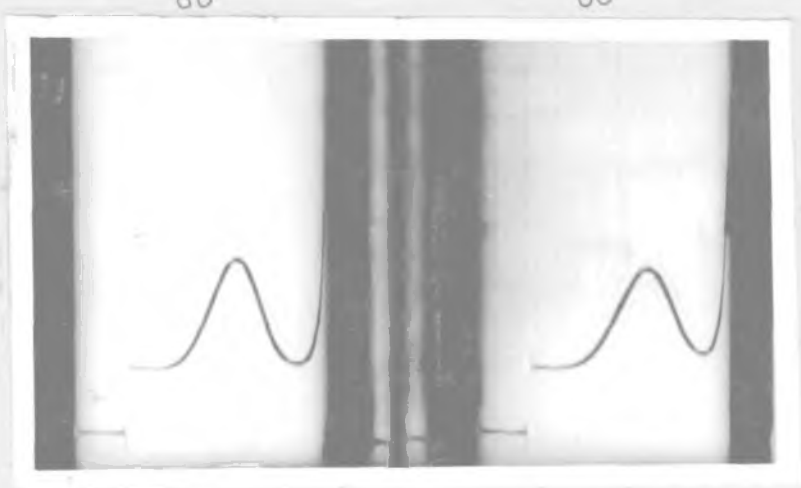
Synthetic boundary ultracentrifugal pattern of trypsin inhibitor



Time (min) 0 16
Phase plate 70° 60°



Time (min) 32 48
Phase plate 60° 60°



Time (min) 64 80
Phase plate 60° 60°

The sedimentation constant was calculated was calculated to be $S_w^{20} = 1.5 \times 10^{-13}$. The molecular weight was 14,500 daltons. The inhibitor showed only a single peak in the ultracentrifuge.

Ultraviolet absorption spectrum - Fig. 12 shows the ultraviolet absorption spectrum of a solution of 0.88 mg/ml of the purified inhibitor in 0.001 M phosphate buffer, pH 7.5. The inhibitor shows a maximum at 275 nm and a minimum at 252 nm. The absorption of the solution at 280 nm was 0.440 which is much less than that for many proteins. This low value can be explained taking into account the relatively low content of UV absorbing amino acids in the inhibitor. The ratio of absorption at 280:260 nm is 1.02. The optical factor for the inhibitor is 2.0

Dissociation constant - The dissociation constant of the inhibitor, which was determined by the method of Green and Work (1953), was found to be 8.4×10^{-10} moles/litre at pH 7.5 (Fig.13).

Stability of the inhibitor to heat, pH and trichloroacetic acid

Heat stability - A 0.03% solution of the inhibitor in 0.1 M phosphate buffer, pH 7.5 was heated at 90° for 15 min and 60 min and 7% and 19% inactivation were observed respectively.

pH stability - A 0.03% solution of trypsin inhibitor in 0.1 M phosphate buffers of different pH values was heated at 90° for 15 min. The destruction of the inhibitor at the different pH values is shown in Table 11. It will be seen that the inhibitor is stable upto pH 7.5 and that the activity rapidly falls above pH 9 at 90°.

Trichloroacetic acid stability - A 0.03% solution of the inhibitor in 2.5% trichloroacetic acid (pH 3.2) was heated at 90° for 15 min. A 6% destruction of the activity was observed.

Isoelectric point - The isoelectric point of the inhibitor was found to be at pH 5.0 (Fig.21).

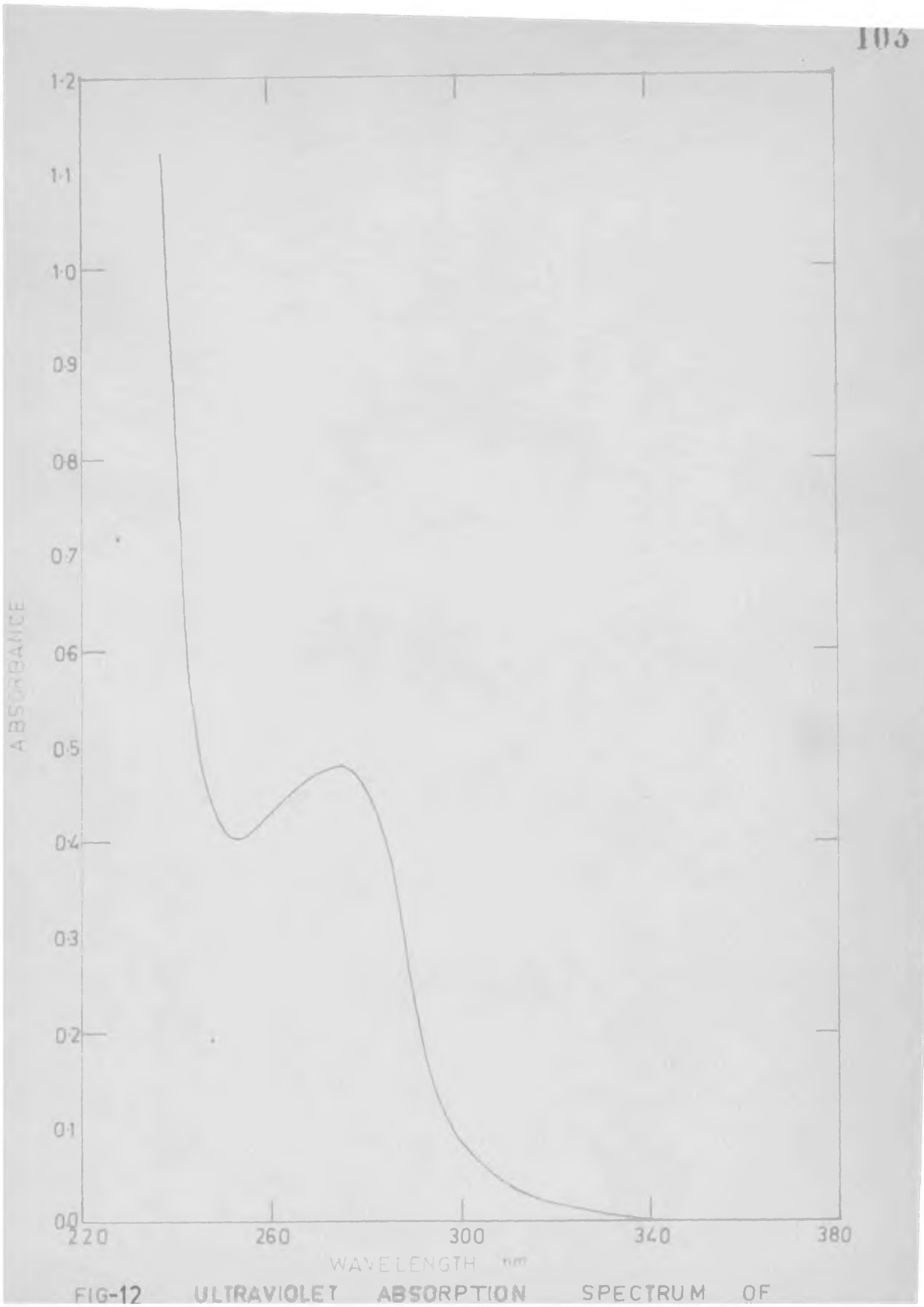


FIG-12 ULTRAVIOLET ABSORPTION SPECTRUM OF

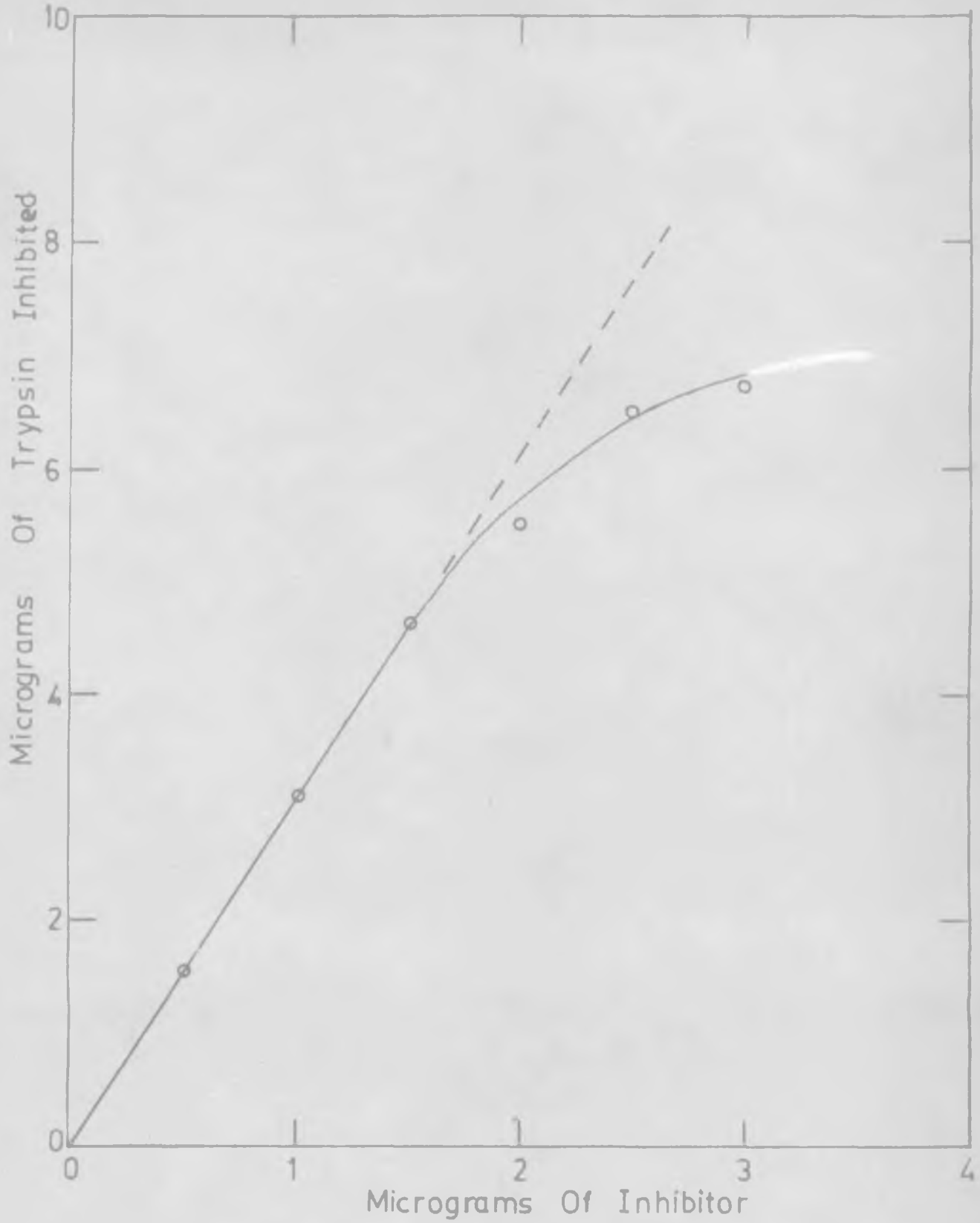


FIG - 12 A INHIBITION OF TRYPSIN WITH TRYPSIN INHIBITOR (1)

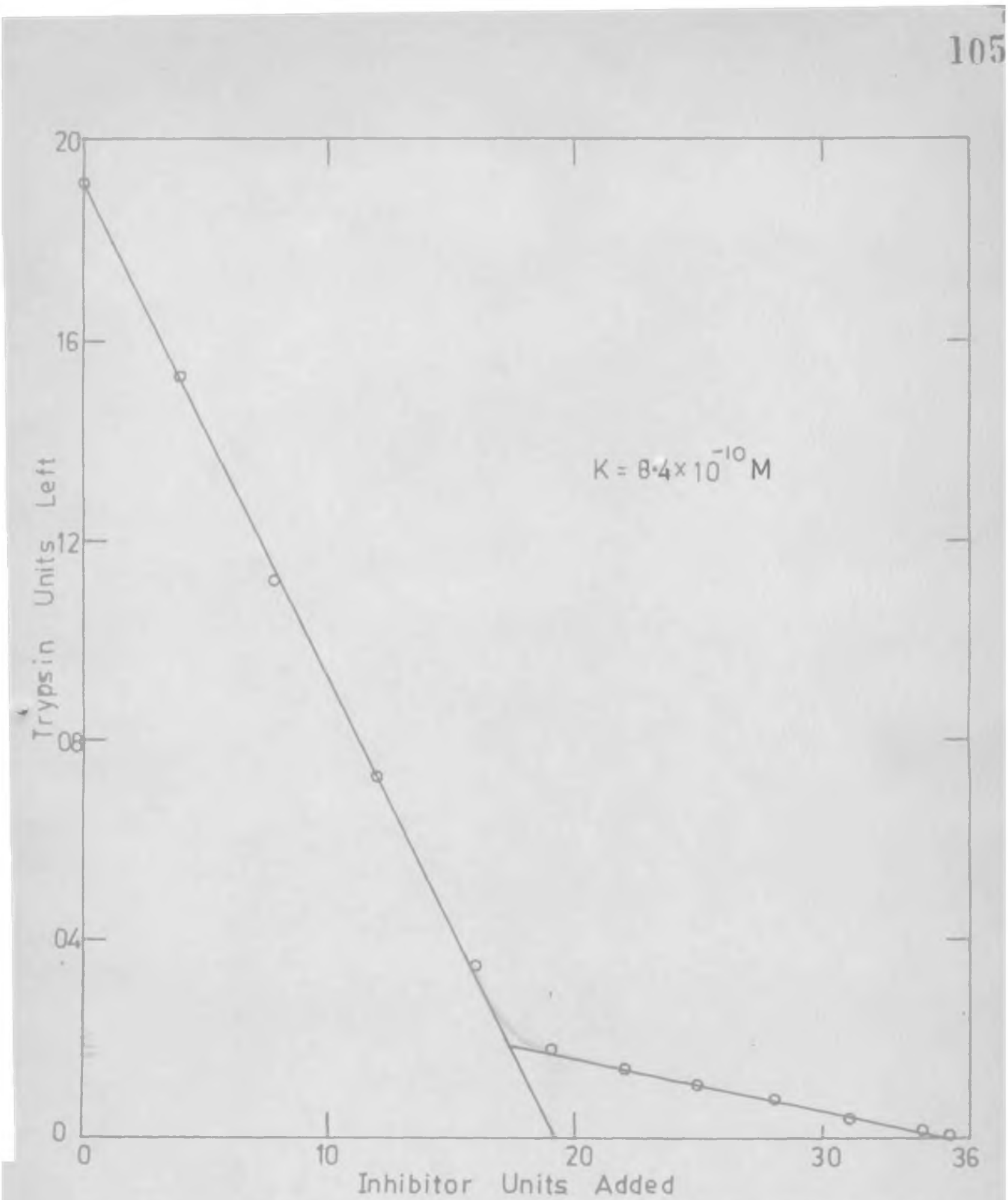


FIG-13 INHIBITION CURVE, SHOWING THE DISSOCIATION AT pH 7·5 OF TRYPSIN INHIBITOR (1)-TRYPSIN COMPLEX NEAR THE EQUIVALENCE POINT

Table 11

pH stability of trypsin inhibiter 1

pH	Trypsin inhibitor units left after heating	% activity left
Control	31.5	100
3.3	31.0	98.4
4.5	30.00	95.2
7.5	29.0	92.0
9.2	19.5	61.6
10.0	1.5	4.0

A 0.03% solution of the inhibitor, in 0.1 M phosphate buffer was heated at 90° for 15 min at different pH values

Inhibition of trypsin, chymotrypsin and other proteinases

The inhibition of trypsin by the inhibitor is shown in Fig.12A. The inhibitor-enzyme combining ratio was determined from the slope of the linear portion of the curve obtained at low inhibitor concentrations. The inhibitor also inhibits chymotrypsin. The molar combining ratios for trypsin and chymotrypsin with the inhibitor were different and were 1:2 and 2:1 respectively. Data regarding the inhibition of trypsin and chymotrypsin by the inhibitor are presented in Table 12. The molar combining ratio for trypsin was the same by the caseinolytic or BAEE assay. Other proteinases such as subtilopeptidase A and papain are not inhibited by the inhibitor. Thus 5 µg of subtilopeptidase A or papain were not inhibited even by 22 µg of the inhibitor.

Table 12

Trypsin and chymotrypsin inhibition by trypsin inhibitor 1

Enzyme	Assay	Amount of inhibitor	Amount of enzyme inhibited	mg of enzyme inhibited by 1 mg of inhibitor	Molar combining ratio (Inhibitor: Enzyme)
		µg	µg		
Trypsin	Caseinolytic	1.00	3.15	3.15	1:2
	BAEE	0.25	0.76	3.04	
Chymotrypsin	Caseinolytic	4.4	3.6	0.82	1:0.49 (2:1)

Inhibition of chymotrypsin by the inhibitor-trypsin complex was studied. To 25 µg of the inhibitor were added 78 µg of trypsin. This inhibitor-enzyme complex, which had no residnal inhibitory activity against trypsin, still inhibited 12 µg of chymotrypsin. These results indicate that the inhibitor is a "double headed inhibitor" since the trypsin and chymotrypsin inhibiting sites are separate.

Recovery of the inhibitor from its complex with trypsin

This could be achieved by three different methods as follows. The trypsin-trypsin inhibitor complex was prepared by dissolving 6 mg of trypsin and 2 mg of inhibitor (36000 units) in 3 ml of water.

a) To 0.5 ml of the complex (6000 units) was added 0.5 ml of 0.1 M phosphate buffer, pH 7.5 and the solution was heated for 15 min at 90°. Trypsin is inactivated and precipitates out. The supernatant liquid contained 90% (5400 units) of the inhibitor taken initially.

b) To 0.5 ml of the complex was added 0.5 ml of 5% trichloroacetic acid. The solution was kept for 30 min at 30°. Trypsin was precipitated and the supernatant liquid gave 99% (5950 units) recovery of the initial inhibitor.

c) To 0.5 ml of the complex was added 0.05 ml of 0.5 M phosphate, pH 2.5 and the solution was loaded on a Sephadex G-75 column (1.5 x 100 cm) which was equilibrated and eluted with 0.05 M phosphate, pH 2.5. The flow rate was 15 min per fraction. 3 ml fractions were collected. Trypsin (3500)units) appeared in the earlier fractions and inhibitor (4500 units) was obtained in the later fractions. The intermediate fractions contained a mixture of trypsin and the inhibitor.

Amino acid analysis - The data on amino acid analysis are given in Table 13.

Table 13

Amino acid analysis of trypsin inhibitor 1 from Vigna catjang

Amino acid	μmole/0.25 mg sample	Residues/mole calculated	Assumed nearest integer
Cysteic acid	0.0046	0.28	-
Aspartic acid	0.2713	16.28	16
Threonine	0.0801	4.81	5
Serine	0.2406	14.44	14
Glutamic acid	0.1707	10.24	10
Proline	0.1179	7.07	7
Glycine	0.0745	4.47	4
Alanine	0.0927	5.58	6
Half Cystine	0.2599	15.60	16
Valine	0.0269	1.76	2
Methionine	0.0199	1.19	1
Isoleucine	0.0792	4.75	5
Leucine	0.0442	2.65	3
Tyrosine	0.0274	1.64	2
Phenylalanine	0.0387	2.32	2
Lysine	0.1236	7.41	7
Histidine	0.0878	5.26	5
Arginine	0.0928	5.56	6
Tryptophan		0.70	1

These calculations were made on the basis of a molecular weight of 15000. A total of 112 residues was found. The calculated value of 14667 for the molecular weight of the inhibitor is in good agreement with that of 14500 as determined by the Archibald method.

Determination of tyrosine and tryptophan - Tyrosine and tryptophan were assayed by Goodwin and Morton's method. From the data (Table 14) it was calculated that the inhibitor contains 2.2 moles of tyrosine and 0.7 mole of tryptophan per mole of inhibitor. This value of tyrosine is in agreement with the value obtained by amino acid analysis.

Table 14

U.V. absorption of trypsin inhibitor 1 in alkali

(Absorbance of 1 mg of inhibitor/ml of 0.1 M NaOH)

Wavelength nm	Absorbance O.D.
257	1.200
280	0.495
294.4	0.460
305	0.285

Determination of free -SH groups: Free -SH group assays were carried out according to Ellmann (1958, 1959), (Chapter II). With 1 mg of the inhibitor no appreciable colour was developed (412 nm) with DTNB indicating the absence of -SH groups (cysteine) in the molecule.

Determination of total sulfhydryl plus disulfide groups: For these determinations the method of Cavallini et al. (1966) was followed. 1 mg of the inhibitor was used for the assay. The change in optical density after DTNB addition was 0.210. This gave a value of 15.8 -SH groups per mole of inhibitor. Since no free sulfhydryl group could be detected in the molecule it can be concluded that the inhibitor contains 16 half cystine residues per mole.

Trypsin Inhibitor 2

Tyrosine and tryptophan determination: Tyrosine and tryptophan contents of the inhibitor were determined by the method of Goodwin and Morton (1946). One mole of the inhibitor was found to contain 1.12 mole of tyrosine and 0.48 mole of tryptophan. This low value of aromatic amino acids is consistent with the high optical factor of the inhibitor.

Table 15

Absorbance of Trypsin inhibitor 2 in 0.1 M NaOH

Wavelength nm	Absorbance O.D.
257.15	0.320
280	0.130
294.4	0.115
305	0.080

360 µg of the inhibitor per ml of 0.1 M NaOH was used for the study

Homogeneity and molecular weight: The molecular weight of the inhibitor was determined by SDS gel electrophoresis and also by gel filtration using Sephadex G-50. The molecular weight by the SDS method was 13500 and by gel filtration 11700 (Fig. 14 and 15).

During the homogeneity determination by gel electrophoresis it was found that the relative mobilities of both the inhibitors (trypsin inhibitor 2 and subtilisin inhibitor 2b were the same (0.75). Hence the gel electrophoresis of a mixture of these two inhibitors showed a single band migrating towards the anode.

Isoelectric point: The isoelectric point (pI) of the inhibitor was at pH 5.9.

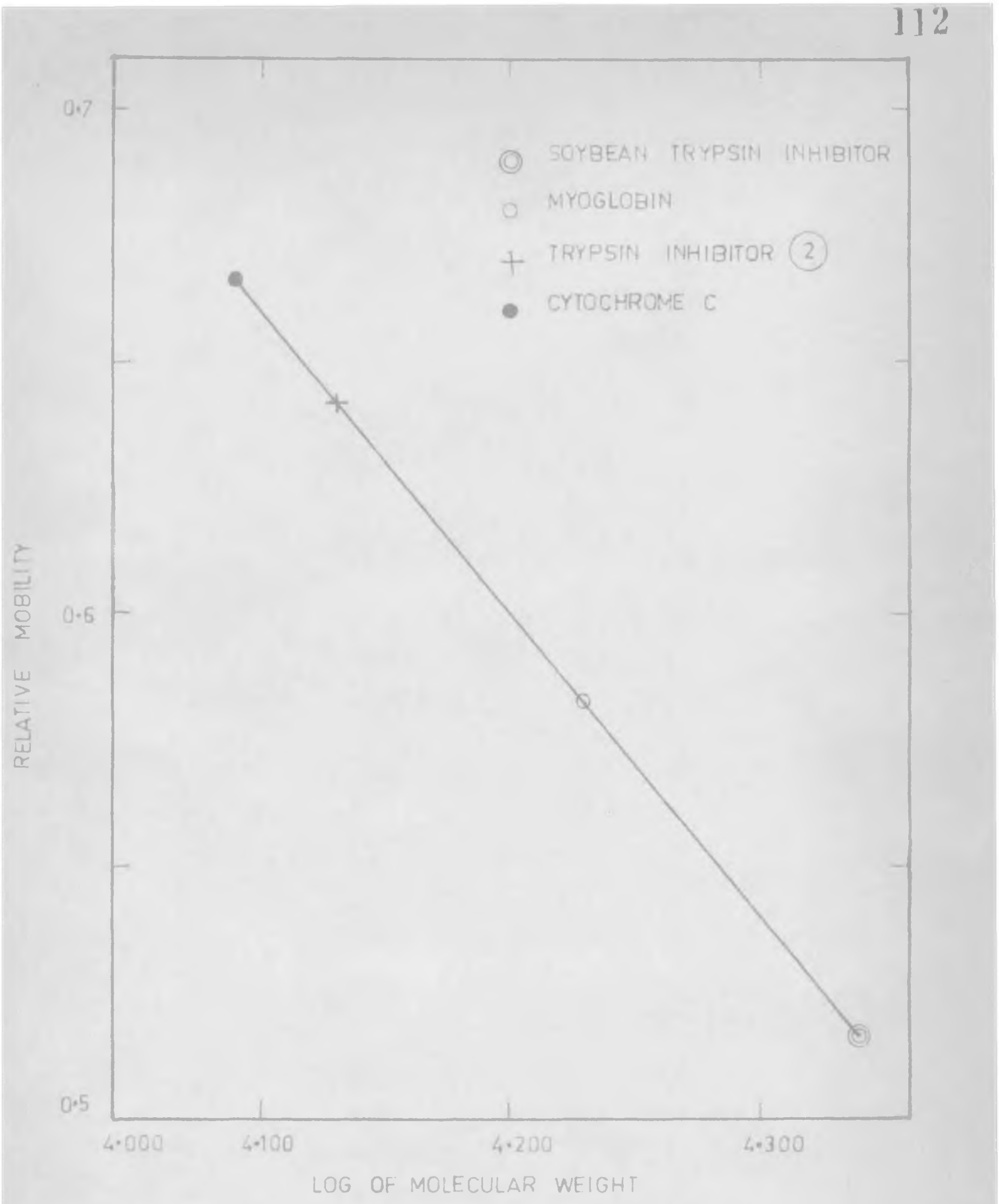
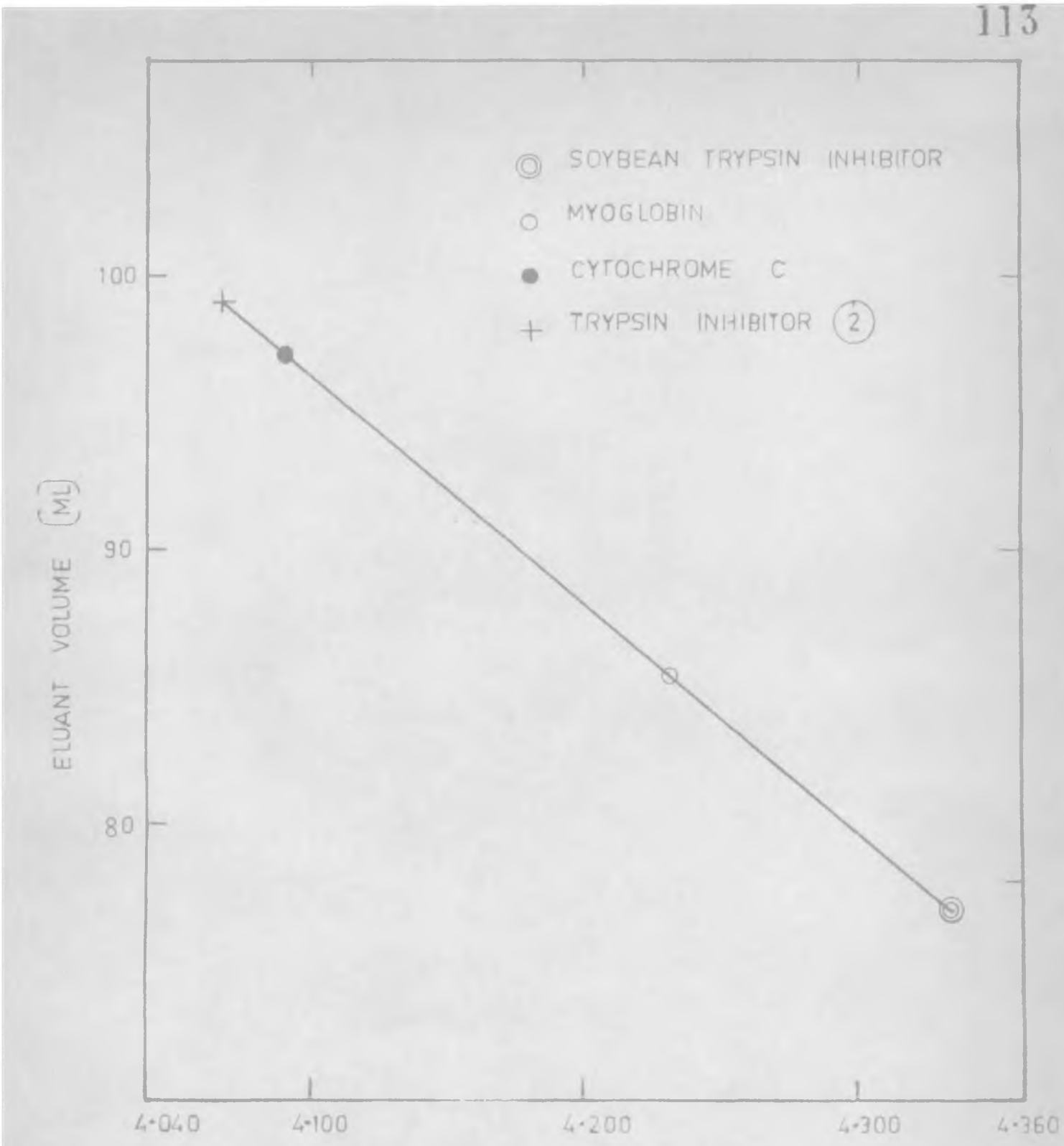


FIG-14 MOLECULAR WEIGHT OF TRYPSIN INHIBITOR (2) BY SDS METHOD



FIG·15 MOLECULAR WEIGHT OF TRYPSIN INHIBITOR (2)

BY GEL FILTRATION

Ultraviolet absorption spectrum: Fig.16 illustrates the ultraviolet spectrum of a solution of 0.46 mg/ml of trypsin inhibitor 2. It shows a maximum at 264 nm and a minimum at 254 nm. The 280:260 nm ratio is 0.77. The optical factor of the inhibitor is 3.6.

Inhibition of trypsin and other proteinases: The inhibitor inhibits trypsin and chymotrypsin. Data regarding this inhibition are summarized in Table 16. The molar combining ratio for inhibitor and trypsin is the same by the caseinolytic or BAEE assay. 120 µg of the inhibitor do not show any inhibition of 5 µg of subtilisin or papain. The molar combining ratio of trypsin with the inhibitor was 4:1 whereas for chymotrypsin it was only 15:1.

Table 16

Inhibition of trypsin and chymotrypsin by trypsin inhibitor 2

Enzyme	Assay	Amount of inhibitor	Amount of enzyme inhibited	mg of enzyme inhibited by 1 mg of inhibitor	Molar combining ratio (Inhibitor: enzyme)
		µg	µg		
Trypsin	Caseinolytic	5.0	2.75	0.55	1:0.28
	BAEE	1.5	0.85	0.57	
Chymotrypsin	Caseinolytic	40	5.6	0.14	1:0.07

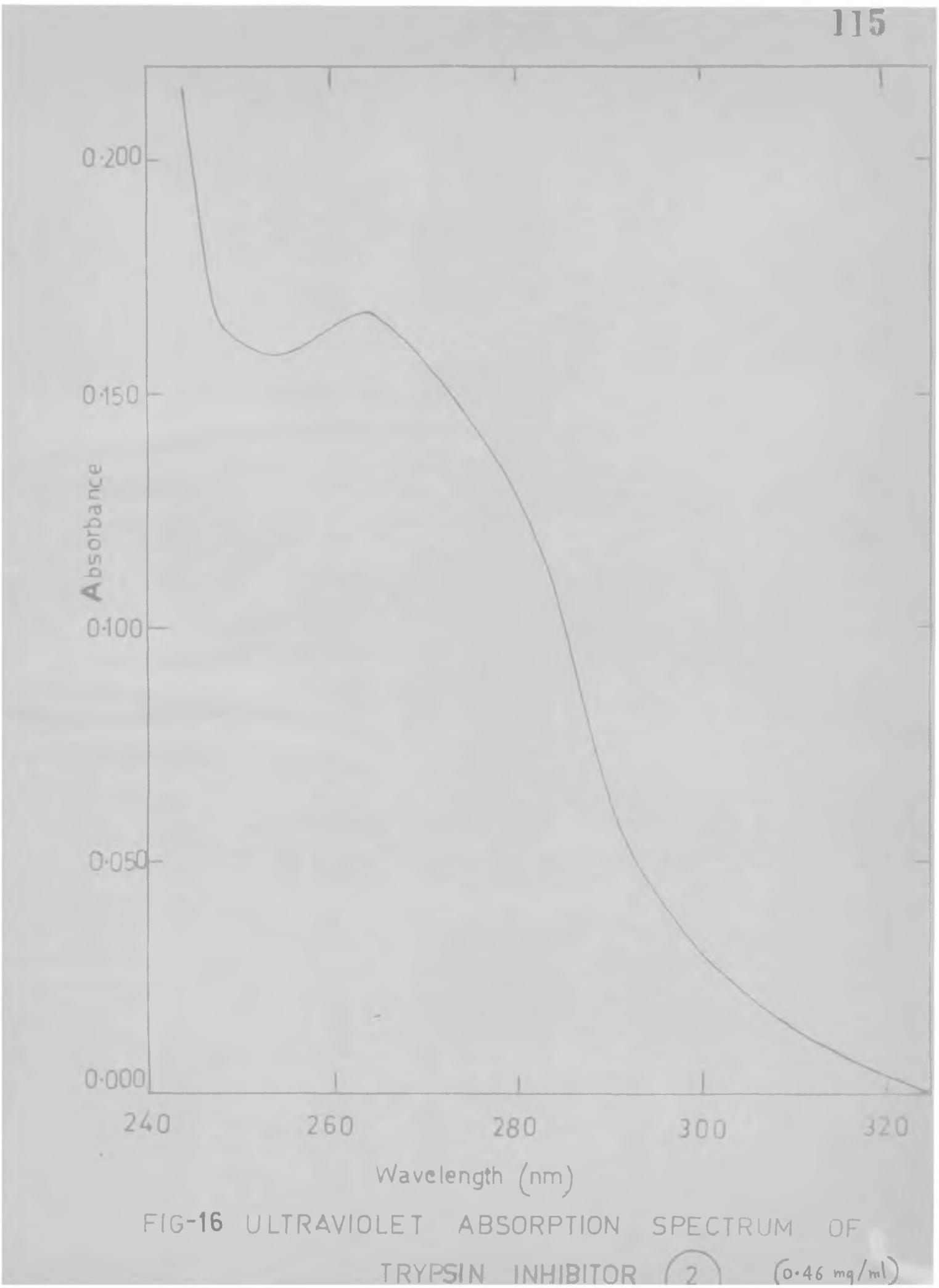


FIG-16 ULTRAVIOLET ABSORPTION SPECTRUM OF TRYPSIN INHIBITOR (2) (0·46 mg/ml)

Section II

Properties and kinetics of Subtilisin Inhibitors 1, 2a and 2b

General

Subtilisin inhibitors 1, 2a and 2b could be adsorbed on DEAE-cellulose and eluted at pH 7.5 with 0.008 M, 0.014 M and 0.022 M phosphate buffer respectively. Inhibitors 2a and 2b showed a greenish blue colour upto the preparative polyacrylamide gel electrophoresis stage. The colour was completely removed during the gradient DEAE-cellulose chromatography. Solutions of all the subtilisin inhibitors were water clear and colourless.

Homogeneity of purified inhibitors

Although subtilisin inhibitor 1 was eluted as a single peak at a phosphate concentration of 0.008 M, it was found that the fraction was not homogeneous on polyacrylamide gel at pH 8.5, but showed two bands (Fig.17). 80 µg of the inhibitor were loaded on the gel and electrophoresis was run at pH 8.5. After the run, 0.5 cm portions of the gel were cut and eluted with 0.01 M phosphate buffer, pH 7.5. The eluates were then assayed for subtilisin inhibition. It was found that the subtilisin inhibiting activity was associated only with those portions of the gel where the two bands were located, thus showing that both the bands had inhibitory activity towards subtilisin. However although the inhibitor showed two bands, after SDS treatment the inhibitor moved as a single band on gel electrophoresis (Fig.18).

Subtilisin inhibitors 2a and 2b were homogeneous on polyacrylamide gel electrophoresis (at pH 8.5, fig. 17) as well as on SDS gel electrophoresis (Fig.18). When the gel electrophoresis was carried out at pH 7 , all the three subtilisin inhibitors were found to be homogeneous and moved as single bands. Subtilisin inhibitor 1 may actually consist of two inhibitors.

Fig.17

HOMOGENEITY

Polyacrylamide gel electrophoresis of subtilisin inhibitors

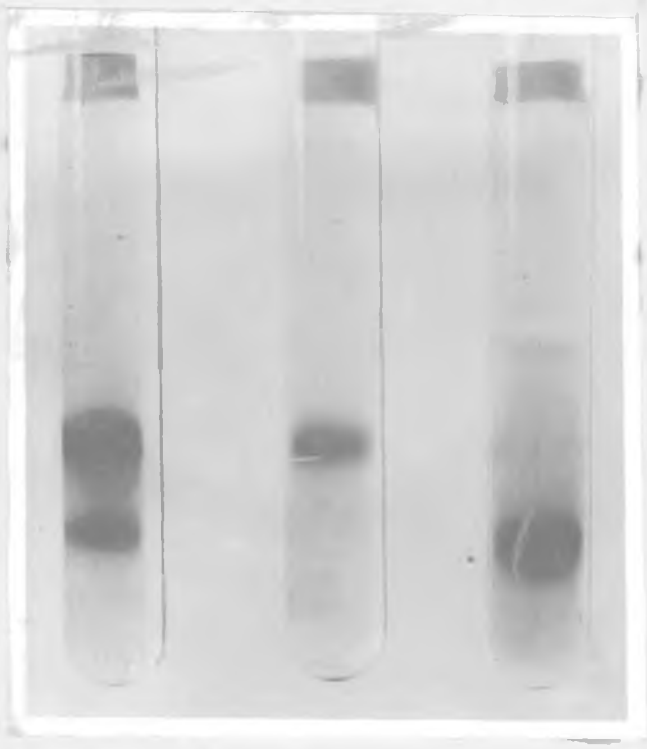
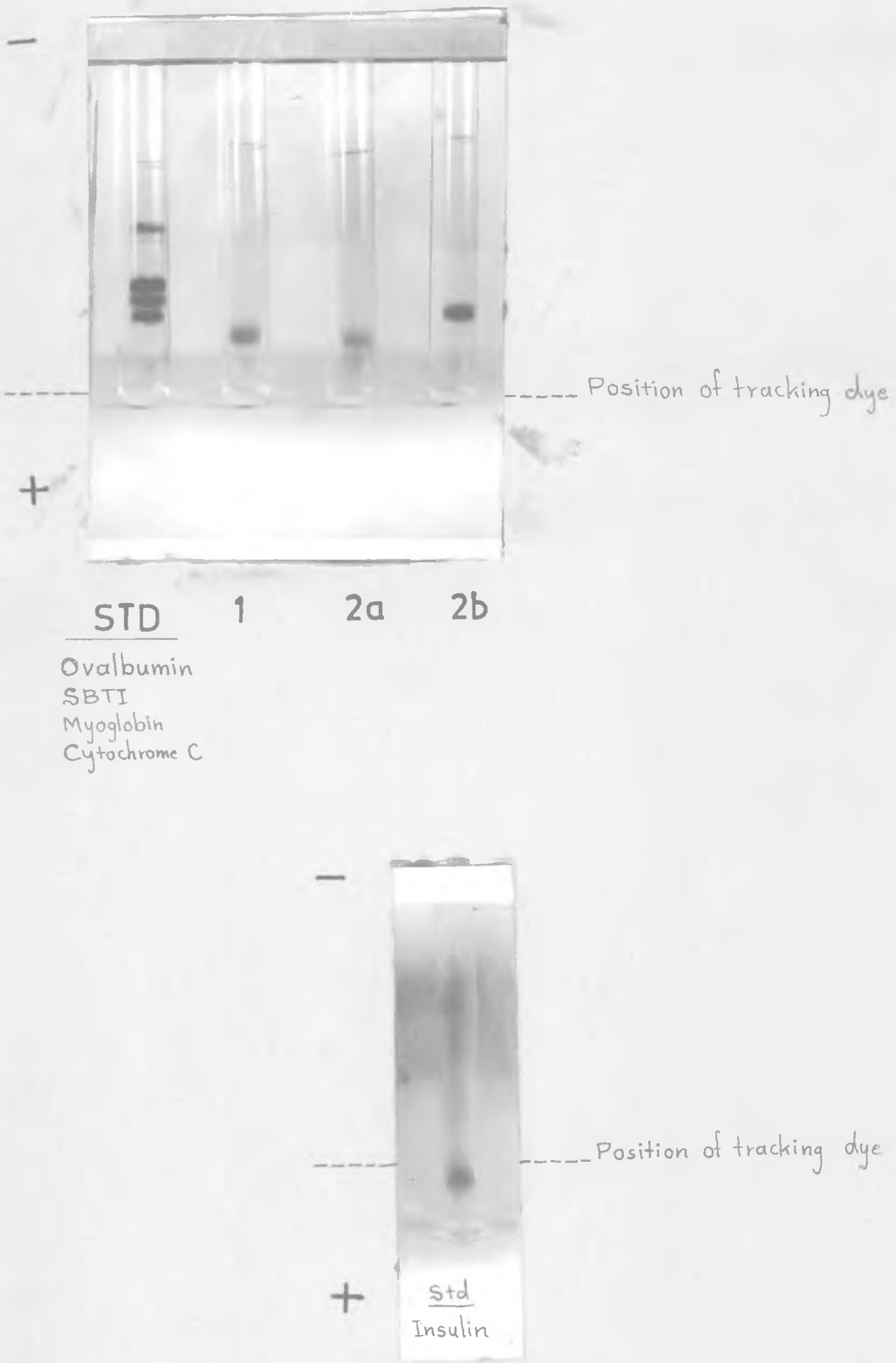


Fig.18

MOLECULAR WEIGHT BY SDS METHOD

(Gel electrophoresis of subtilisin inhibitors after SDS treatment)



Hence the observed properties may be a composite of both and hence one must await further purification for determining the properties of the individual inhibitor. It was not determined whether the two bands arise from two separate proteins ~~or whether the two bands arise from two separate proteins~~ or whether they are artifacts obtained from a single protein due to aggregation, combination with other ligands etc.

Molecular weight

The molecular weights of the inhibitors were determined by the SDS method and by gel filtration. The molecular weights of subtilisin inhibitors 1, 2a and 2b obtained by the SDS method were 8130, 8510 and 13500 respectively (Fig.19). The molecular weights by gel filtration were 11070, 10000 and 11700 respectively (Fig.20).

Isoelectric point

The isoelectric points of subtilisin inhibitors 1, 2a and 2b were found to be at pH 5.9, 5.9 and 5.1 respectively (Fig.21).

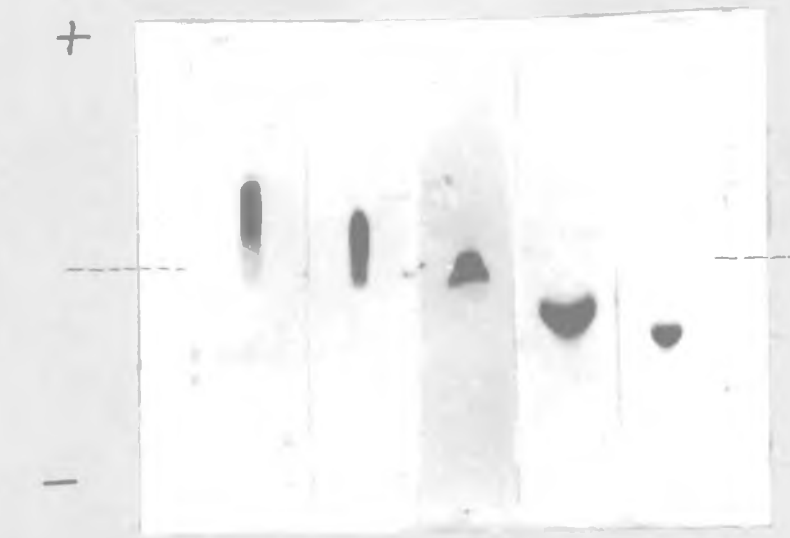
Ultraviolet absorption spectrum

The ultraviolet absorption spectra of subtilisin inhibiters 1, 2a and 2b are shown in Fig. 22, 23 and 24. The figures represent the characteristic absorption spectra of typical proteins. No other peaks were detected indicating the absence of nucleotides or other ultraviolet absorbing material in the inhibitors. Subtilisin inhibiters 1, 2a and 2b show maximum absorbance at 277, 275 and 276 nm and minimum at 250, 250 and 248 nm respectively. The ratios of absorption at 280:260 nm were found to be 1.7, 1.5 and 1.4 respectively.

~~Stab~~ The optical factors for the subtilisin inhibitors 1, 2a and 2b were 1.3, 1.0 and 1.2 respectively.

Fig. 21

Isoelectric point of inhibitors



Trypsin inhibitor 1

PI = 5·0

Subtilisin inhibitor 2b

PI = 5·1

Subtilisin inhibitor 1

PI = 5·9

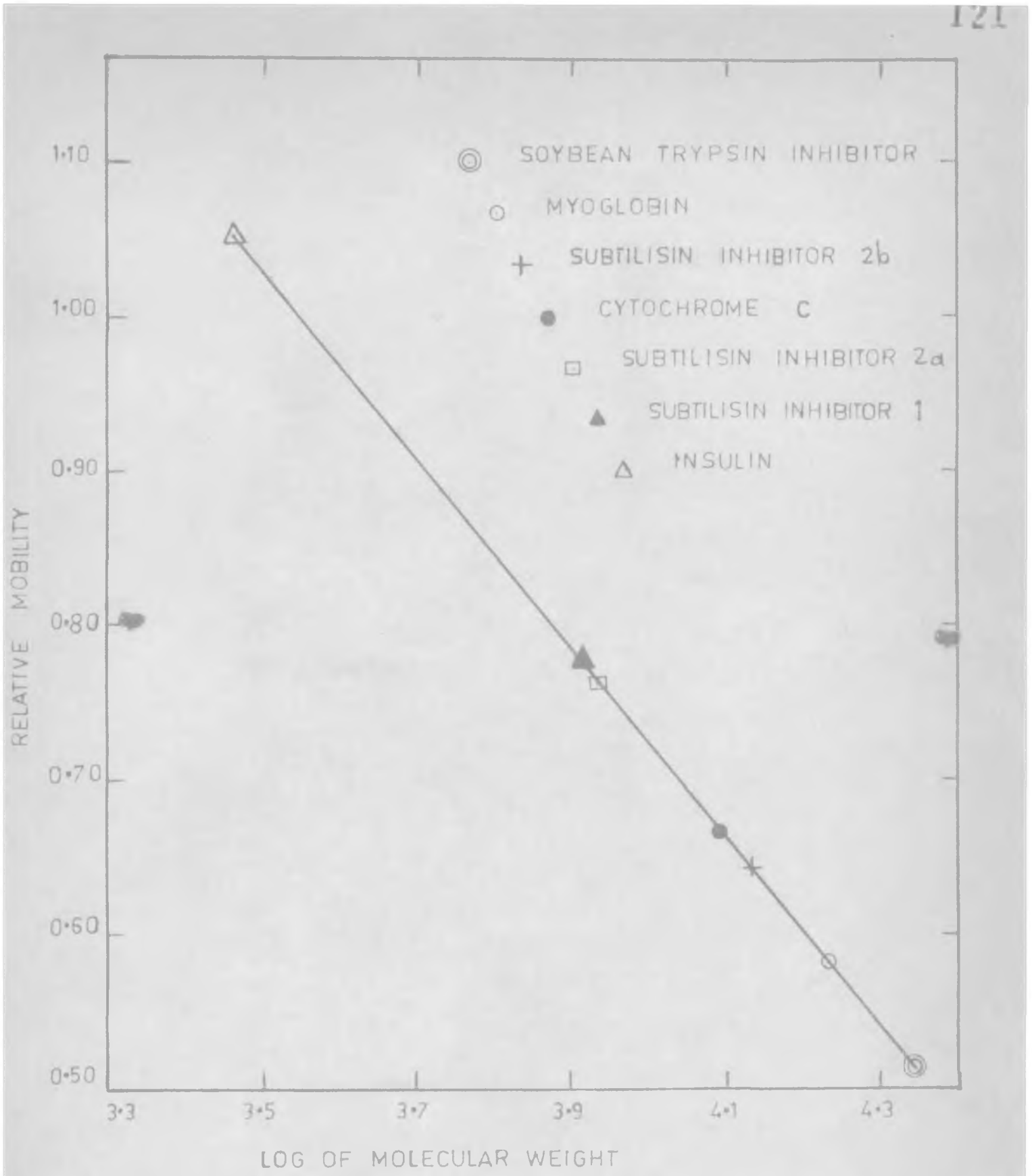


FIG-19 MOLECULAR WEIGHT OF SUBTILISIN INHIBITORS

BY SDS METHOD

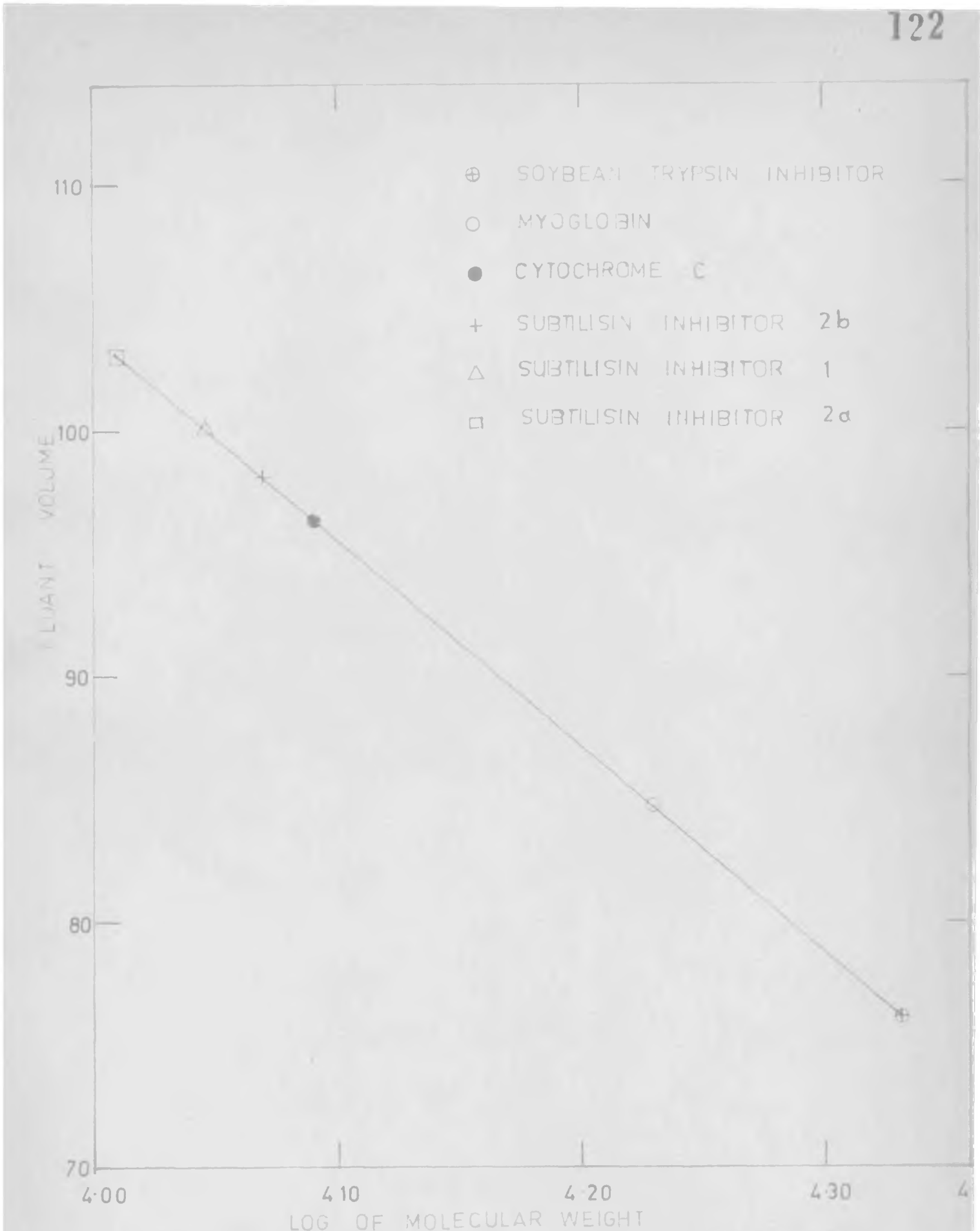


FIG -20 MOLECULAR WEIGHTS OF SUBTILISIN INHIBITORS BY GEL FILTRATION

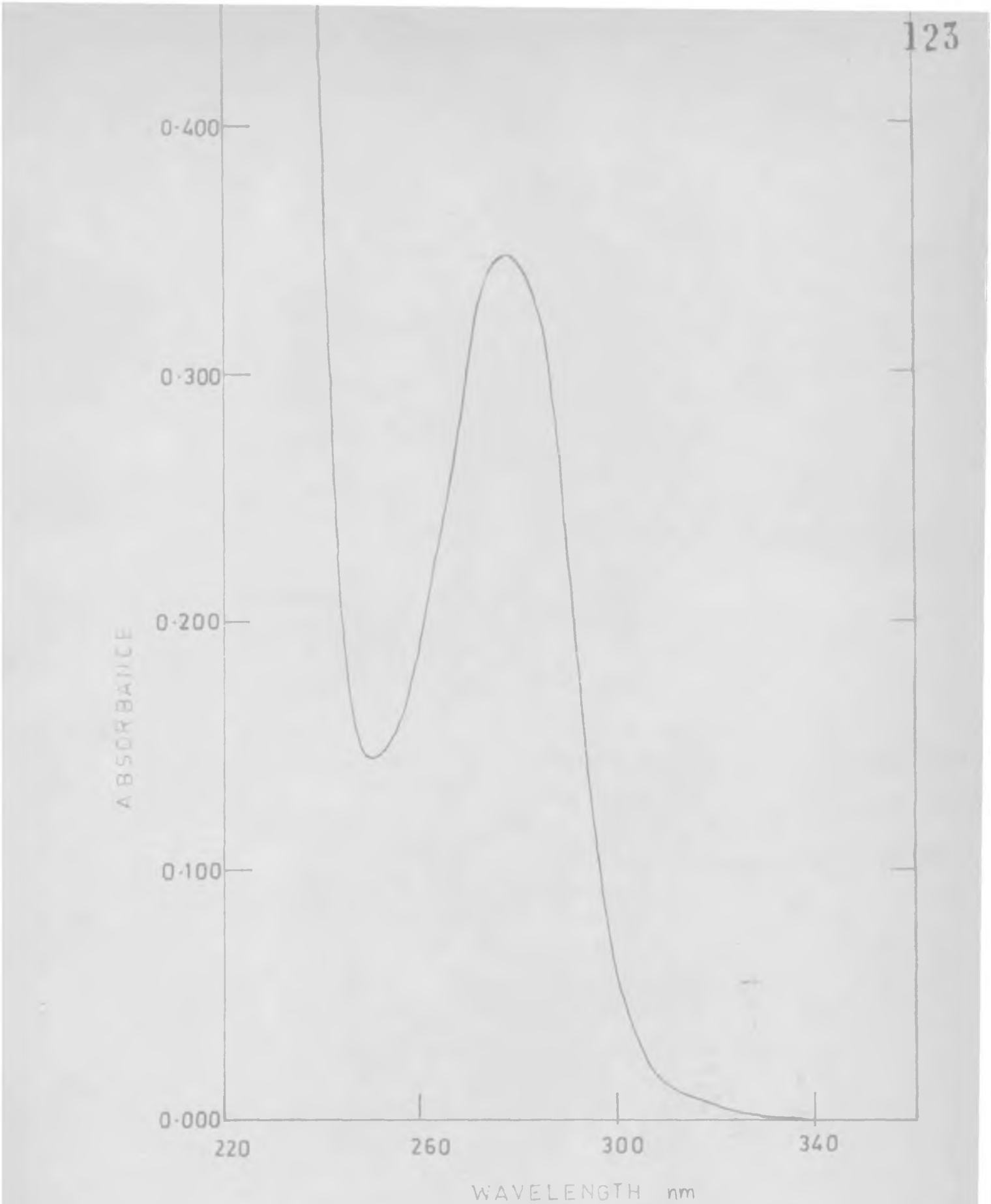


FIG - 22 ULTRAVIOLET ABSORPTION SPECTRUM OF SUBTILISIN INHIBITOR (1) (0·44 mg/ml)

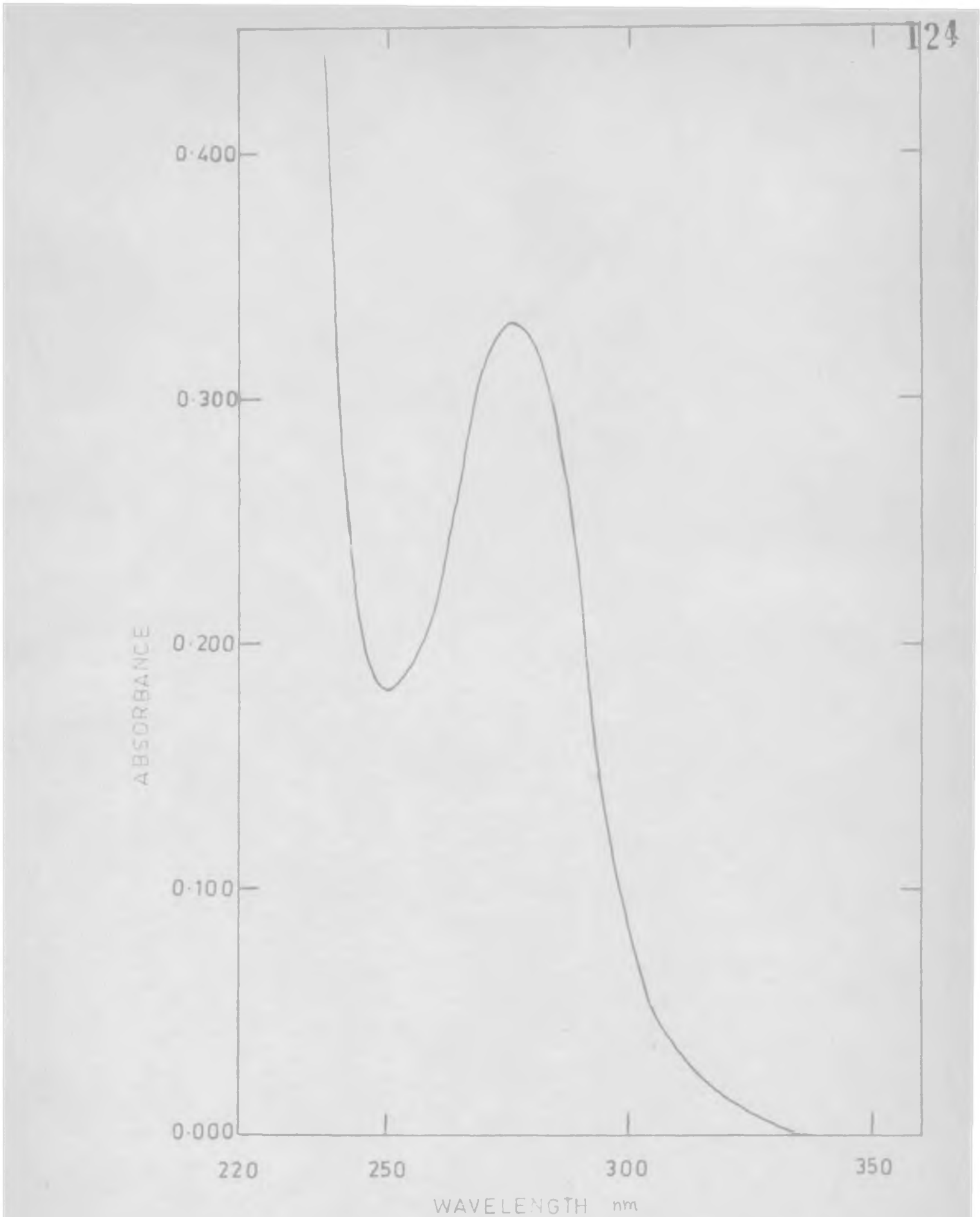


FIG-23 ULTRAVIOLET ABSORPTION SPECTRUM OF SUBTILISIN INHIBITOR (2α) (0·32 mg/ml)

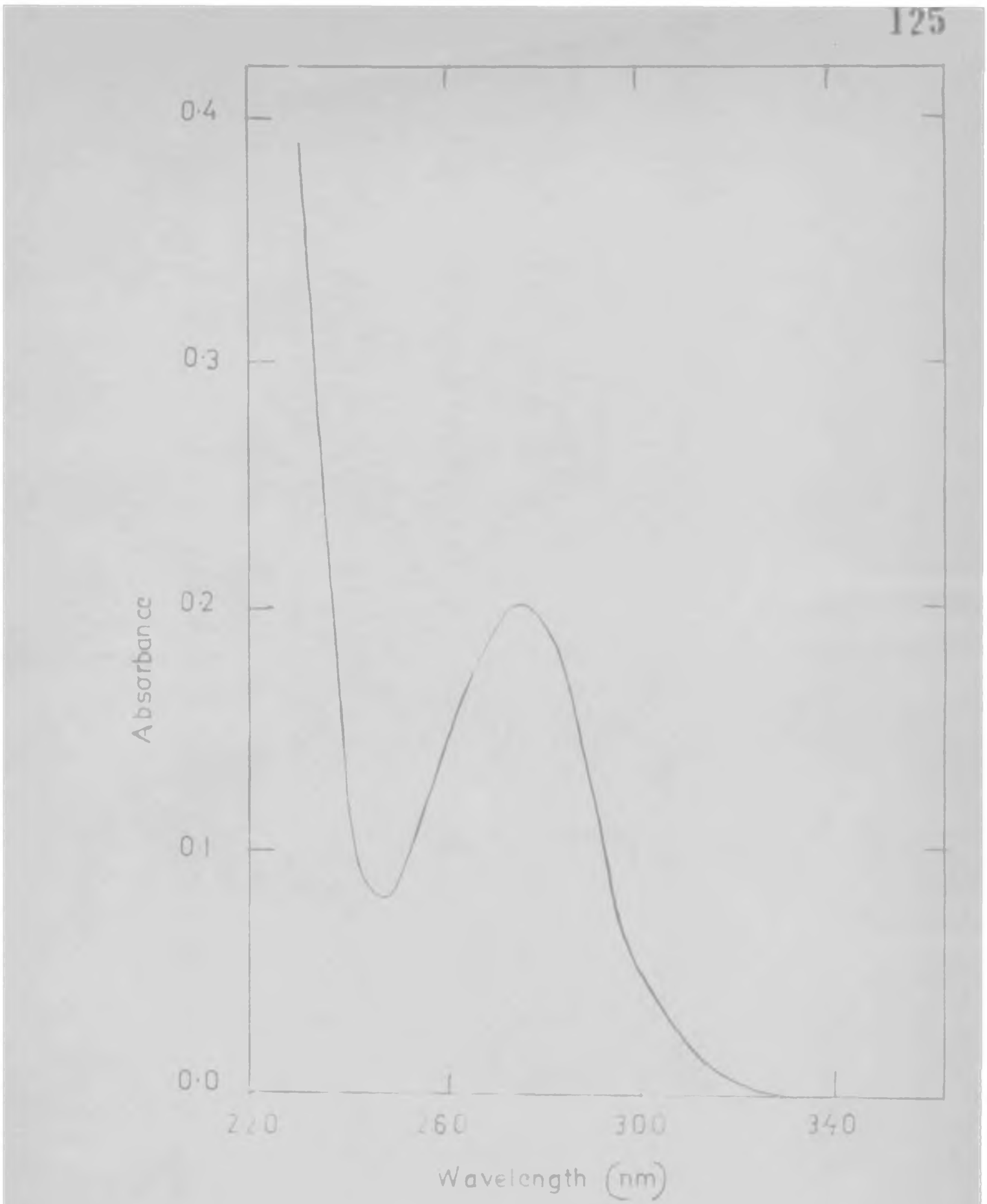


FIG-24 ULTRAVIOLET ABSORPTION SPECTRUM OF SUBTILISIN INHIBITOR (2b)
(0.27 mg/ml)

Stability

Data regarding the stability of the subtilisin inhibitors (1, 2a and 2b) towards heat, trichloroacetic acid, ethanol and different pH values are summarized in the following tables (17, 18). A 0.02% solution of the inhibitor was used for these experiments. The inhibitor assay was carried out by the caseinolytic assay of Kunitz (Chapter II).

All the three subtilisin inhibitors were found to be fairly stable to heat at pH values between pH 3 to pH 10, trichloroacetic acid treatment (2.5% concentration of trichloroacetic acid) and ethanol treatment (75% ethanol concentration).

Table 17

Stability of subtilisin inhibitors to heat, trichloroacetic acid and ethanol

Inhibitor	Inhibitor units left		% inhibitor activity left
	Control (unheated)	After heating at 90° for 15 min*	
1	18.5	15.5	84
2a	17.0	12.5	74
2b	18.5	13.5	73
	Control (untreated)	After trichloroacetic acid treatment**	
1	23.0	19.5	85
2a	21.5	21.0	93
2b	25.5	21.5	84
	Control (untreated)	After ethanol treatment***	
1	21.0	21.0	100
2a	20.5	20.5	100
2b	24.0	20.0	83

* 0.02% solution of the inhibitor in 0.1 M phosphate buffer was heated at 90° for 15 min.

** 0.02% solution of the inhibitor in 2.5% trichloroacetic acid was kept at 30° for 45 min.

*** 0.02% solution of the inhibitor was treated with 3 volumes of ethanol at 30° for 45 min.

Table 18

Stability of subtilisin inhibitors at different pH values

Inhibitor	pH	Inhibitor units left after heating at 80° for 15 min		% inhibitor activity left
1	2.8	21.0		93
	4.3	20.0		89
	7.6	19.5		87
	8.8	19.5		87
	10.2	19.0		84
	7.6	22.5	Control (unheated)	100
2a	2.8	20.0		89
	4.3	19.0		84
	7.6	17.0		76
	8.8	16.0		71
	10.2	15.5		69
	7.6	22.5	Control (unheated)	100
2b	2.8	15.0		72
	4.3	15.0		72
	7.6	15.5		74
	8.8	16.0		76
	10.2	17.5		83
	7.6	21.0	Control (unheated)	100

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

Specificity of inhibition

All the three inhibitors specifically inhibit the proteolytic enzyme subtilisin when assayed by the caseinolytic assay as well as the BAEE assay. The inhibitors are considered to be specific for subtilisin since they do not inhibit trypsin, chymotrypsin, papain and ficin. The inhibitors inhibit subtilopeptidase A, B. subtilisin proteinase, nagarse and one of the proteolytic enzymes from pronase. Data regarding the inhibition of subtilopeptidase A by the inhibitors and their molar combining ratio is given in Table 19. It was observed that the inhibition obtained by the inhibitor by using either the caseinolytic assay or the synthetic substrate assay was the same. Molar combining ratio of subtilisin inhibitor 1 and 2a with the enzyme was 1:1 and 1:1 respectively. However the ratio of subtilisin inhibitor 2b with the enzyme was 2:1

Caseinolytic assay was used to determine whether the inhibitors inhibit trypsin, chymotrypsin and papain. 5 µg of each proteinase was used for this study. Even by using 36 µg of subtilisin inhibitor 1, 32 µg of inhibitor 2a and 35 µg of inhibitor 2b, no detectable inhibition of these proteinases was observed.

Table 19

Inhibition of subtilisin (subtilopeptidase A) by subtilisin inhibitor 1, 2a and 2b

Inhibitor	Assay	Amount of inhibitor	Amount of enzyme inhibited	mg of enzymes inhibited by 1 mg of inhibitor	Molar combining ratio (Inhibitor:enzyme)
		µg	µg		
1	Caseinolytic	1.8	3.4	1.9	1:0.78(1;1)
	BAEE	13.5	29.6	2.1	
2a	Caseinolytic	1.5	3.5	2.2	1:0.85(1:1)
	BAEE	11.7	27.0	2.3	
2b	Caseinolytic	3.5	3.4	0.95	1:0.41(2:1)
	BAEE	28.0	30.2	1.1	

Dissociation constant

The reaction of subtilisin inhibitor 1, subtilisin inhibitor 2a or subtilisin inhibitor 2b with subtilisin was complete in 5 min and on the basis of a 1:1, 1:1 and 2:1 complex formation dissociation constants at pH 7.5 of 9.0×10^{-10}, 1.4×10^{-9} and 1.54×10^{-9} mole/liter were found for the respective complexes (Fig.25, 26, 27)

Amino acid analysis

Subtilisin inhibitor 2a was used for amino acid analysis. The data on amino acid analysis are given in Table 20.

Table 20

Amino acid analysis of subtilisin inhibitor 2a from Vigna catjang

Amino acid	μmole/0.200 mg sample	Residues/mole calculated	Assumed nearest integer
Aspartic acid	0.1535	7.68	8
Threonine	0.1301	6.51	7
Serine	0.0973	4.89	5
Glutamic acid	0.2416	12.08	12
Proline	0.0845	4.23	4
Glycine	0.0942	4.71	5
Alanine	0.1422	7.11	7
Half cystine	-	-	-
Valine	0.1310	6.55	7
Methionine	0.0485	2.41	2
Isoleucine	0.0467	2.34	2
Leucine	0.0573	2.87	3
Tyrosine	0.0308	1.54	2
Phenylalanine	0.0212	1.06	1
Lysine	0.0982	4.91	5
Histidine	0.0203	1.02	1
Arginine	0.0566	2.63	3
Tryptophan		1.28	1

The above calculations were made on the basis of a molecular weight of 10000 for the inhibitor. A total of 75 residues were obtained and on this basis the value for the minimum molecular weight of the inhibitor is 9833.

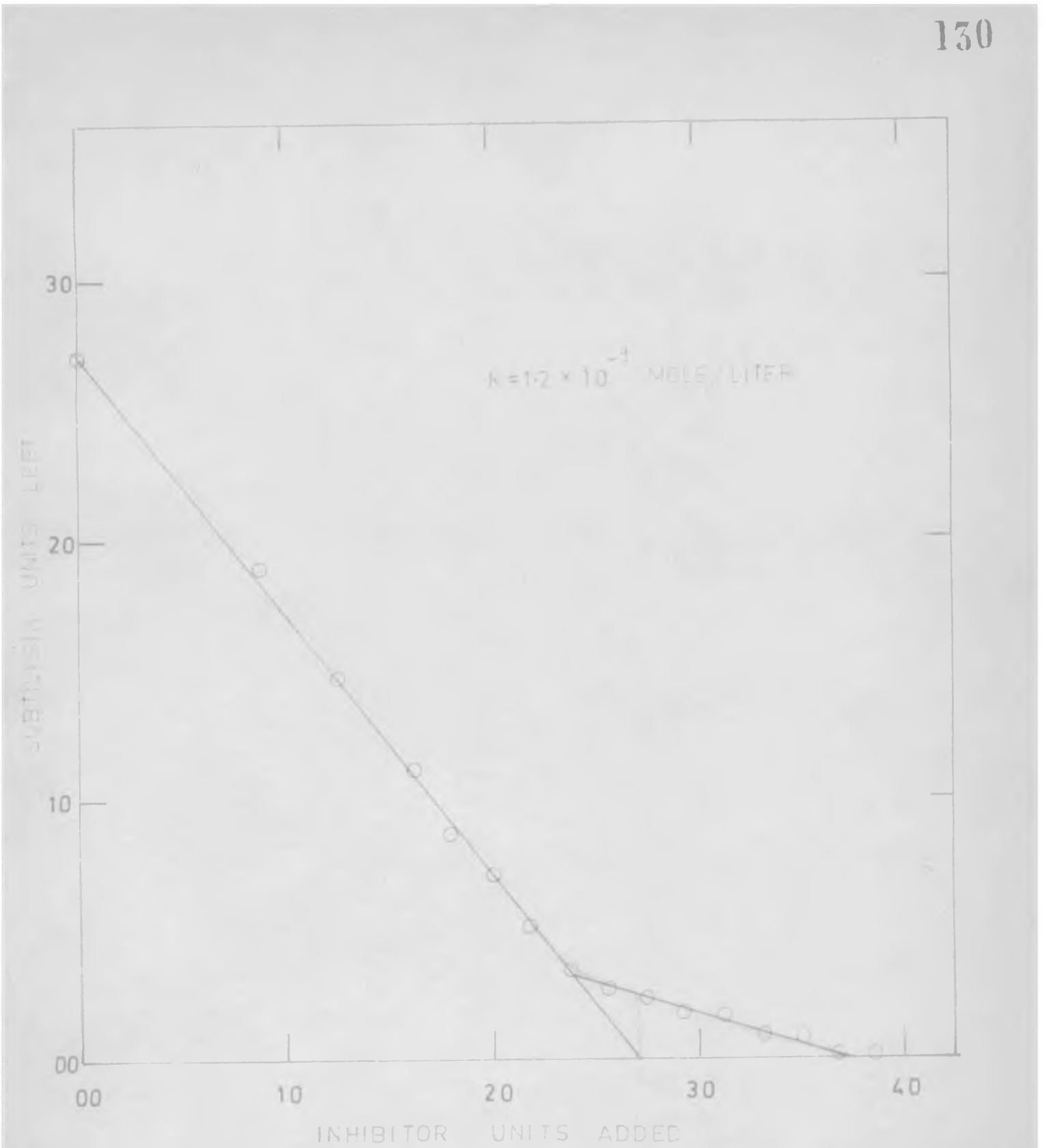


FIG-25 INHIBITION CURVE SHOWING THE DISSOCIATION AT pH 7·5 OF SUBTILISIN INHIBITOR (1) – SUBTILISIN COMPLEX NEAR THE EQUIVALENCE POINT

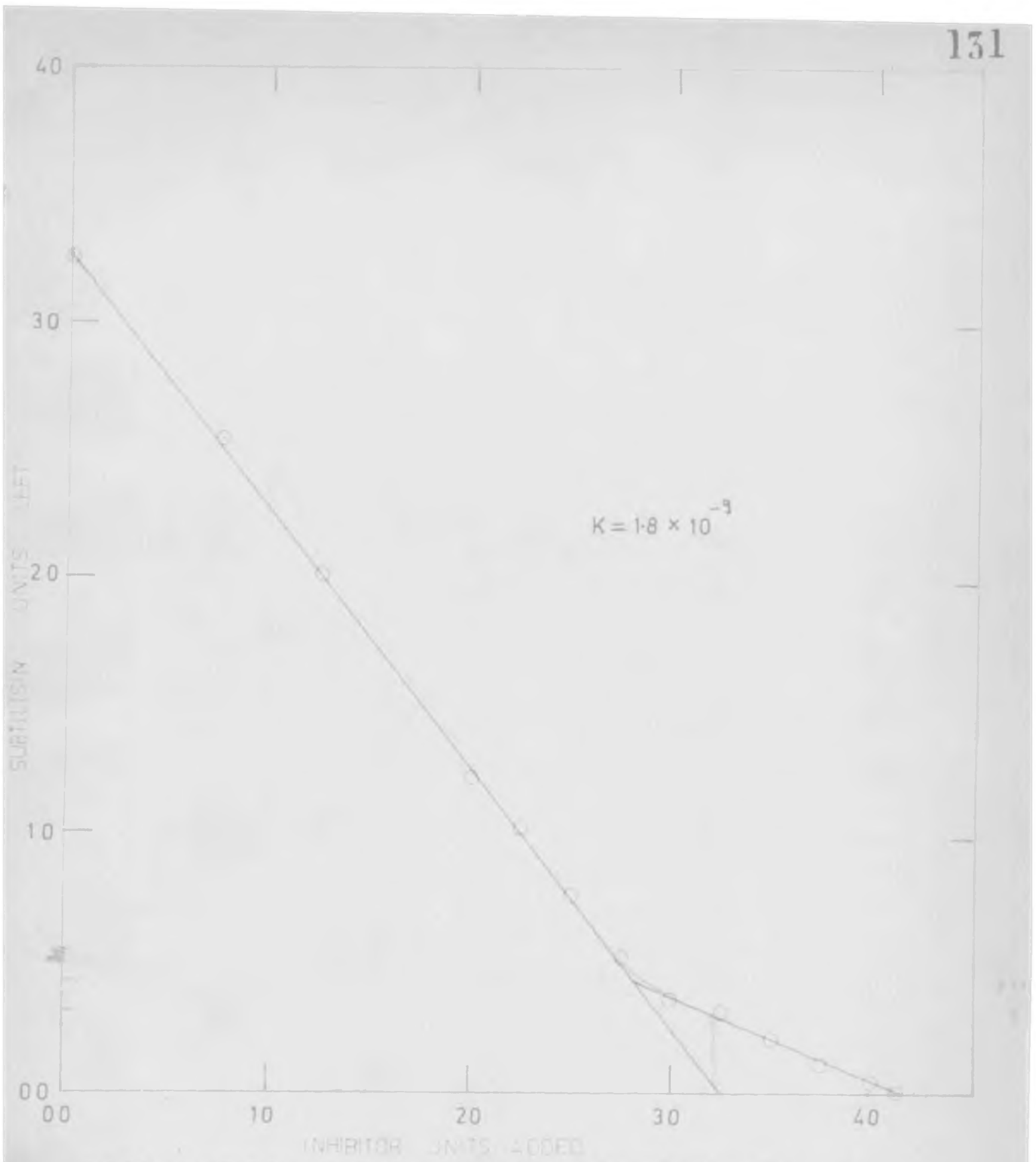


FIG-26 INHIBITION CURVE, SHOWING THE DISSOCIATION AT pH 7·5 OF SUBTILISIN INHIBITOR (2a) – SUBTILISIN COMPLEX NEAR THE EQUIVALENCE POINT

Estimation of tyrosine and tryptophan (Goodwin and Morton's method)

The absorbance of a solution of 0.200 mg of the inhibitor in 1 ml of 0.1 M NaOH was measured at 294.4 nm (O.D. 0.160) and 280 nm (O.D. 0.200). From these data the tyrosine and tryptophan content of the molecule was calculated. It was found that one mole of the inhibitor contains 2.09 moles of tyrosine and 1.28 mole of tryptophan. The value of tyrosine (2.09 moles) obtained by this method is in fair agreement with that obtained by the amino acid analysis (1.54 moles).

Estimation of cysteine content (free -SH group)
(Elmman's method)

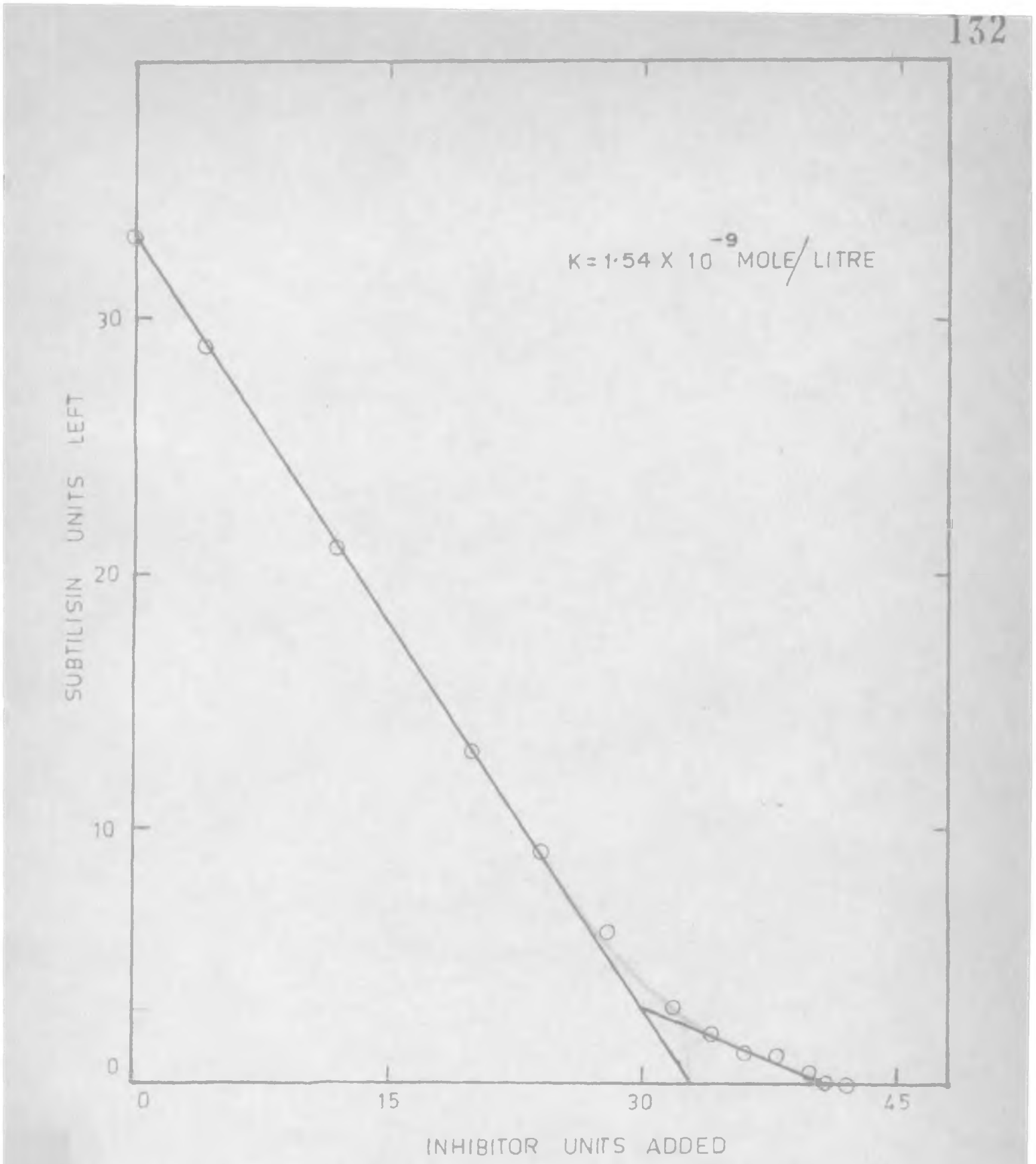
With 0.200 mg of the inhibitor no colour was developed with DTNB at 412 nm, indicating the absence of -SH groups in the molecule.

Estimation of total sulfhydryl plus disulfide groups
(Method of Cavallini et al.)

0.200 mg of the inhibitor was used for this test. Even after reducing the inhibitor with sodium borohydride in the presence of urea and EDTA, no colour was developed with DTNB at 412 nm indicating the absence of any disulfide bond or cystine in the inhibitor. This is an unusual finding and in this respect the plant subtilisin inhibitor is comparable with the serum trypsin inhibitors which are also devoid of cystine.

Modification studies using subtilisin inhibitor 2a

In the case of some of the trypsin and chymotrypsin inhibitors when they were incubated with catalytic amounts of the corresponding proteinases it was shown (Finkenstadt and Laskowski,1965) that the inhibitors were modified due to a bond cleavage at the reactive site. It was also suggested that this step is an essential step for complex formation between the inhibitor and the proteinase.



IG-27 INHIBITION CURVE SHOWING THE DISSOCIATION AT pH 7·5 OF SUBTILISIN INHIBITOR 2b — SUBTILISIN COMPLEX NEAR THE EQUIVALANCE POINT.

Estimation of tyrosine and tryptophan (Goodwin and Morton's method)

The absorbance of a solution of 0.200 mg of the inhibitor in 1 ml of 0.1 M NaOH was measured at 294.4 nm (O.D. 0.160) and 280 nm (O.D. 0.200). From these data the tyrosine and tryptophan content of the molecule was calculated. It was found that one mole of the inhibitor contains 2.09 moles of tyrosine and 1.28 mole of tryptophan. The value of tyrosine (2.09 moles) obtained by this method is in fair agreement with that obtained by the amino acid analysis (1.54 moles).

Estimation of cysteine content (free -SH group)
(Ellman's method)

With 0.200 mg of the inhibitor no colour was developed with DTNB at 412 nm, indicating the absence of -SH groups in the molecule.

Estimation of total sulfhydryl plus disulfide groups
(Method of Cavallini et al.)

0.200 mg of the inhibitor was used for this test. Even after reducing the inhibitor with sodium borohydride in the presence of urea and EDTA, no colour was developed with DTNB at 412 nm indicating the absence of any disulfide bond or cystine in the inhibitor. This is an unusual finding and in this respect the plant subtilisin inhibitor is comparable with the serum trypsin inhibitors which are also devoid of cystine.

Modification studies using subtilisin inhibitor 2a

In the case of some of the trypsin and chymotrypsin inhibitors when they were incubated with catalytic amounts of the corresponding proteinases it was shown (Finkenstadt and Laskowski,1965) that the inhibitors were modified due to a bond cleavage at the reactive site. It was also suggested that this step is an essential step for complex formation between the inhibitor and the proteinase.

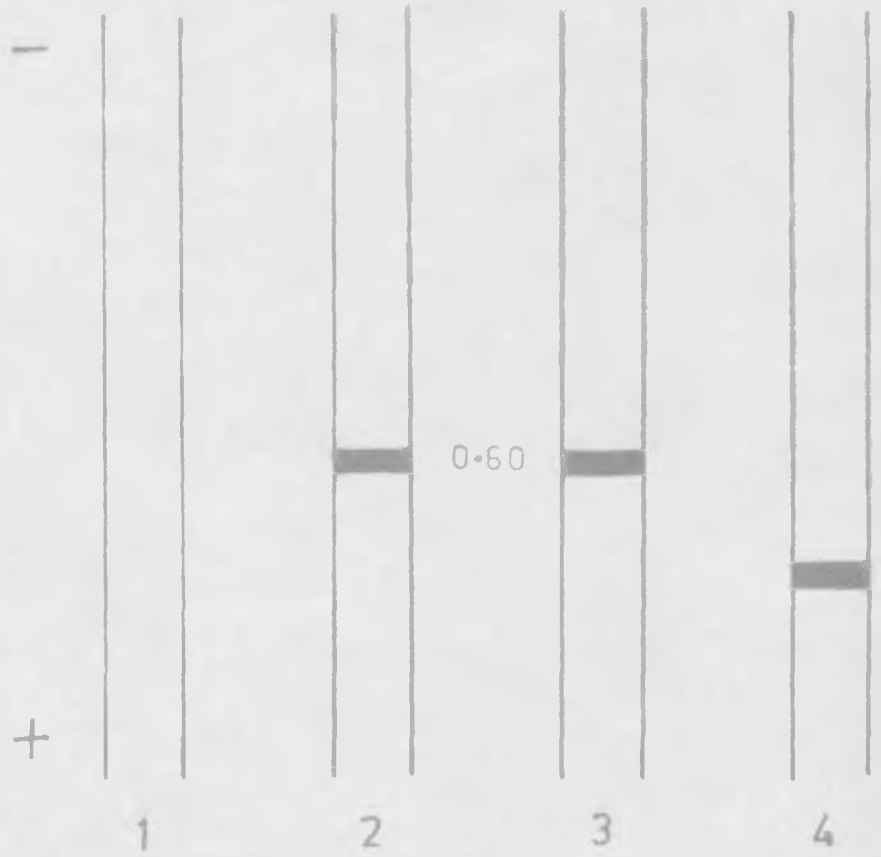
However such modification studies have been carried out only with the inhibitors of trypsin and chymotrypsin. Moreover no modification experiments were performed on an inhibitor which is devoid of cystine, which is believed to give stability to the modified inhibitor. Hence it was of interest to determine whether the inhibitor of subtilisin also gets modified and whether it is possible to reconvert the modified inhibitor to its native form.

The following experiments based on the studies of Ozawa and Laskowski (1966) on soybean trypsin inhibitor (Kunitz) were carried out using subtilisin inhibitor 2a.

Subtilisin inhibitor 2a was incubated with small amounts of subtilisin at acid pH (3.7) in the presence of calcium ions. 1 ml of the reaction mixture contained 200 µg (3200 units) of subtilisin inhibitor, 40 µmoles of calcium chloride and 10 µg (80 units) of subtilisin. The pH of the reaction mixture was adjusted to 3.7 with 20 µ liters of 0.1 M HCl. A control experiment wherein subtilisin was omitted from the reaction mixture was also run simultaneously. The reaction mixtures were then incubated for 18 h at 30°, neutralized with 20 µ litres of 1 M tris base and used for polyacrylamide gel electrophoresis and for the assay of the inhibitor.

The above incubated and neutralized reaction mixtures will be designated as follows and the terms will be used throughout this section. "Reaction mixture M" (wherein subtilisin inhibitor is incubated with small amounts of subtilisin) and "Reaction mixture N" (control experiment wherein the inhibitor is incubated without any subtilisin).

Longer gels (length 15 cm) were used for the polyacrylamide gel electrophoresis and 0.2 ml of the reaction mixture was loaded on the gel. It was observed (Fig.28) that the inhibitor in "reaction mixture M" was almost completely converted into a new form which had a different mobility. The relative mobility of the inhibitor in the control experiment ("reaction mixture N") was 0.60 while that of the new form ("reaction mixture M") was 0.75. Thus the new form migrates faster than the native inhibitor towards the anode.



1 PROTEINASE (SUBTILISIN) *

2 ORIGINAL SUBTILISIN INHIBITOR 2a

3 "REACTION MIXTURE N" (CONTROL EXPERIMENT)

4 ""REACTION MIXTURE M" (INHIBITOR INCUBATED WITH SUBTILISIN)

* SUBTILISIN MOVES TOWARDS CATHODE, HENCE NO BAND WAS OBSERVED IN 1

FIG-28 MODIFICATION STUDIES OF SUBTILISIN INHIBITOR (2a)

The above reaction mixtures were also assayed for their inhibitor content (Table 21). The details of the experiment carried out regarding this study are presented in the following table (Table 21). For the assay of the inhibitor in the "reaction mixture N" and in the "reaction mixture M" 5 µl of the reaction mixture and the enzyme-subtilisin (4.3 µg, 34 units) were allowed to react for different periods of time (from 5 min to 45 min) at 30° and then the casein solution was added to start the reaction. The results show that in the case of the native inhibitor (control experiment, "reaction mixture N") pre-incubation of the inhibitor with the enzyme for more than five min did not show increased inhibition of the enzyme and the inhibitor inhibited a total of 14.5 units of subtilisin. In the case of the modified inhibitor ("reaction mixture M") when the inhibitor and the enzyme were preincubated for 5 min only 1.8 units of the enzyme were inhibited. However the inhibition increased as the preincubation time was increased upto 30 min. After 15 min preincubation the inhibitor inhibited 7.3 units of subtilisin and only after 30 min preincubation the inhibitor showed full inhibitory activity and inhibited 14.5 units of subtilisin. This shows that although the native and the modified inhibitor inhibit subtilisin to the same extent the native inhibitor forms the complex much faster (within 5 min) than its modified inhibitor (30 min).

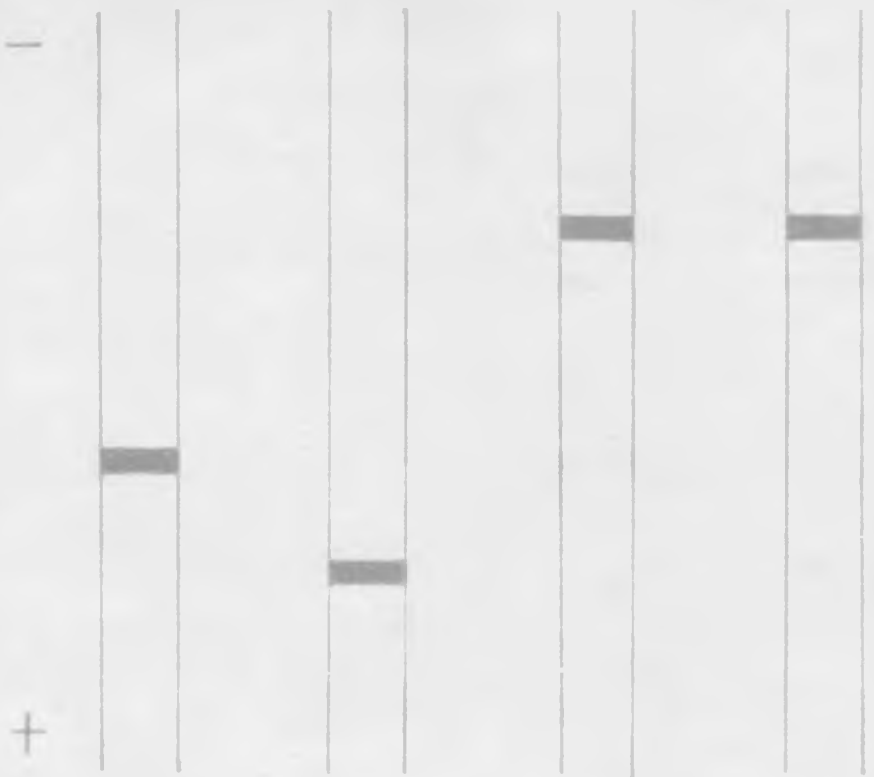
Table 21

Assay of subtilisin inhibitor in the reaction mixtures

Incubation time (min)	5	15	30	45
	Inhibition obtained - units			
"Reaction mixture N" (control experiment, native inhibitor)	15.0	14.6	15.0	14.5
"Reaction mixture M" (new form, modified inhibitor)	1.8	7.3	14.5	14.4

Inhibitors were assayed by using caseinolytic assay.
For the assay, the reaction mixture (5 µl) and the enzyme-subtilisin (4.3 µg, 34 units) were first incubated for 5 min to 45 min at 30° and then the casein solution was added to start the reaction.

Experiments were carried out to see whether the complexes formed by the native and the modified inhibitor are similar or different. To form the complexes both the inhibitors were saturated with subtilisin and then used for gel electrophoresis. To 0.25 ml of each reaction mixture ("reaction mixture N" - native inhibitor and "reaction mixture M" - modified inhibitor") were added 100 µg (800 units) of subtilisin in 0.05 ml of phosphate buffer, pH 7.5. The solutions were then incubated for 1.5 h at 30° and loaded on polyacrylamide gel. It was found (Fig.29) that the relative mobilities of both the complexes were the same (0.3) indicating that both the inhibitors may be forming similar complexes (~~Fig.~~) or that the complexes, though different, have the same mobility.



1 "REACTION MIXTURE N" (NATIVE INHIBITOR)

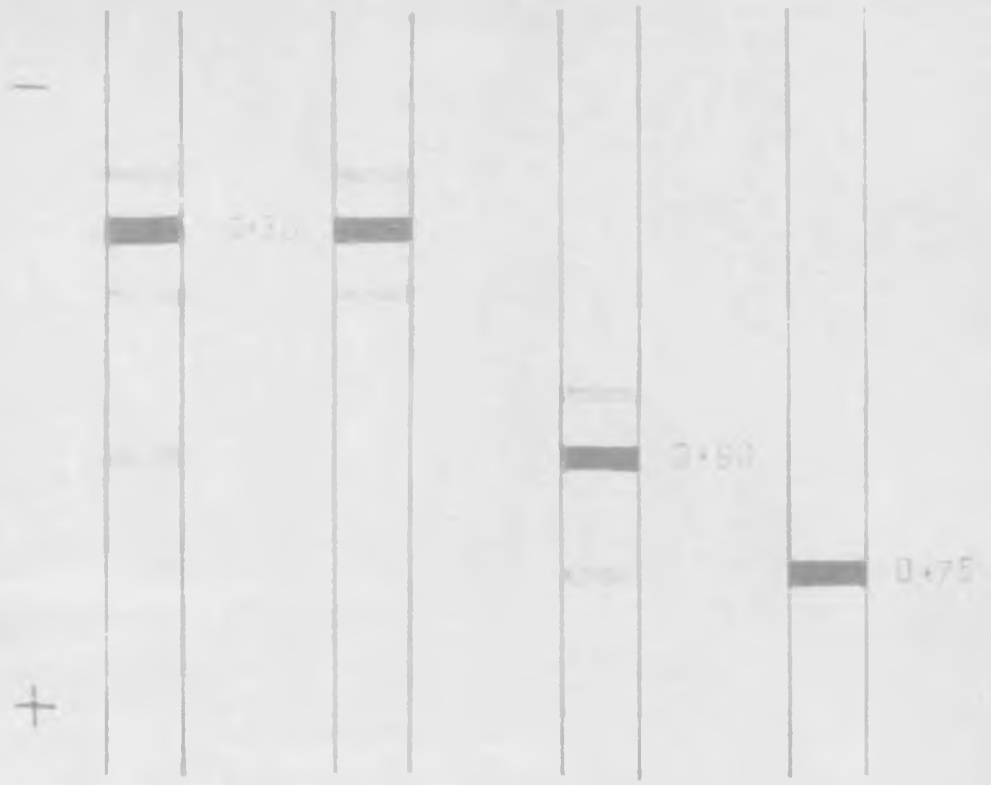
2 "REACTION MIXTURE M" (MODIFIED INHIBITOR)

3 REACTION MIXTURE N SATURATED WITH SUBTILISIN NATIVE INHIBITOR COMPLEX)

4 REACTION MIXTURE M SATURATED WITH SUBTILISIN MODIFIED INHIBITOR COMPLEX)

FIG-29 MODIFICATION STUDIES OF SUBTILISIN INHIBITOR 2a

Attempts were also made to recover the native inhibitor from the modified inhibitor. For this study first an enzyme-inhibitor complex was formed by saturating the inhibitor with subtilisin at pH 7.5 and then the complex was cleaved by heating. The released inhibitor was then tested on polyacrylamide gel. To 0.25 ml of each reaction mixture ("reaction mixture N" - native inhibitor and "reaction mixture M" - modified inhibitor) were added 100 µg (800 units) of subtilisin in 0.25 ml of 0.1 M phosphate buffer, pH 7.5. The solutions were incubated for 1.5 h at 30° and then heated at 95° for 5 min, cooled and centrifuged to remove the denatured subtilisin. The supernatant liquid was loaded on polyacrylamide gel. It was observed (Fig.30) that when the complex of native inhibitor-enzyme was heated, mostly the native inhibitor was obtained giving a trace of modified inhibitor. However when the complex of modified inhibitor-enzyme was heated only the modified inhibitor was obtained back. Thus by this method it was not possible to reconvert the modified inhibitor into the native form.



1 NATIVE INHIBITOR COMPLEX WITH SUBTILISIN

2 MODIFIED INHIBITOR COMPLEX WITH SUBTILISIN

3 NATIVE INHIBITOR COMPLEX WITH SUBTILISIN, HEATED

4 MODIFIED INHIBITOR COMPLEX WITH SUBTILISIN, HEATED

FIG-30 MODIFICATION STUDIES OF SUBTILISIN INHIBITOR (2a)

More work is needed regarding these modification experiments. It will include the effect of agents such as carboxypeptidase B and citraconic anhydride on the modified inhibitor, effect of pH on modification, reconversion of modified inhibitor to native inhibitor under different conditions and the identification of the newly exposed NH_2-terminal and COOH-terminal amino acids, if any, at the active centre of the inhibitor. It was unfortunately not possible to carry out these detailed studies since sufficient inhibitor was not available.

It may be pointed out that although the relative mobilities on polyacrylamide gel electrophoresis of subtilisin inhibitor 2a and subtilisin inhibitor 2b are different (relative mobilities of subtilisin inhibitor 2a and 2b are 0.60 and 0.75 respectively) the relative mobility of subtilisin inhibitor 2a after modification is the same (0.75) as that of subtilisin inhibitor 2b. This observation may indicate the possibility of subtilisin inhibitor 2b being a modified form of subtilisin inhibitor 2a. To clarify this point subtilisin inhibitor 2b was also assayed by preincubating it with subtilisin at different periods of time (from 5 min to 30 min, Table 22).

Table 22

Assay of subtilisin inhibitor 2b

Incubation time (min)	5	15	30
Inhibition obtained (units)	10.6	10.4	10.5

Inhibitor assay was carried out by using caseinolytic assay. For the assay, the inhibitor (1.5 µg, 10.5 units) and subtilisin (4 µg, 32 units) were first incubated for 5 min to 30 min at 30° and the reaction was then started by adding the casein solution.

The results (Table 22) show that the reaction between subtilisin inhibitor 2b and subtilisin is complete within 5 min, while the reaction between modified subtilisin inhibitor 2a and subtilisin is complete only after 30 min incubation (Table 21). This shows that modified inhibitor 2a and subtilisin inhibitor 2b are different. Moreover SDS gel electrophoresis data show that the molecular weight of subtilisin inhibitor 2b (13500) is higher than that of subtilisin inhibitor 2a (8510), (Fig.18).

Section III

Properties and kinetics of papain inhibitors 2a and 2b

General

Papain inhibitors 2a and 2b could be adsorbed on DEAE-cellulose and eluted at pH 7.6 with 0.003 M and 0.005 M phosphate buffer respectively. Aqueous solutions of the purified preparations of both the inhibitors were water clear and colourless.

Homogeneity

Polyacrylamide gel electrophoresis with and without SDS treatment was carried out according to the procedure described in Chapter II. Electrophoresis was run using 7% gel. Both the inhibitors were homogeneous on polyacrylamide gel at pH 8.5. After SDS and thioethanol treatment also, both the inhibitors moved as single bands on gel electrophoresis (Fig.31).

Molecular weight

The molecular weight of both the papain inhibitor 2a and 2b was found to be approximately 9700 by the SDS method (Fig. 32 and 33) and 11,400 and 12,200 respectively by the gel filtration method (Fig.34).

Ultraviolet absorption spectrum

Figs. 35 and 36 illustrate the ultraviolet absorption spectra of papain inhibitors 2a and 2b. They show maxima at 275 and 276 nm and minima at 250 and 252 nm respectively. The 280:260 nm ratio was found to be 1.14 for both the inhibitors. The optical factors of inhibitors 2a and 2b were 0.82 and 0.92 respectively.

Isoelectric point

The isoelectric point of papain inhibitors 2a and 2b were at pH 6.5 and 6.7 respectively.

Fig. 31. HOMOGENEITY

Polyacrylamide gel electrophoresis of papain inhibitors

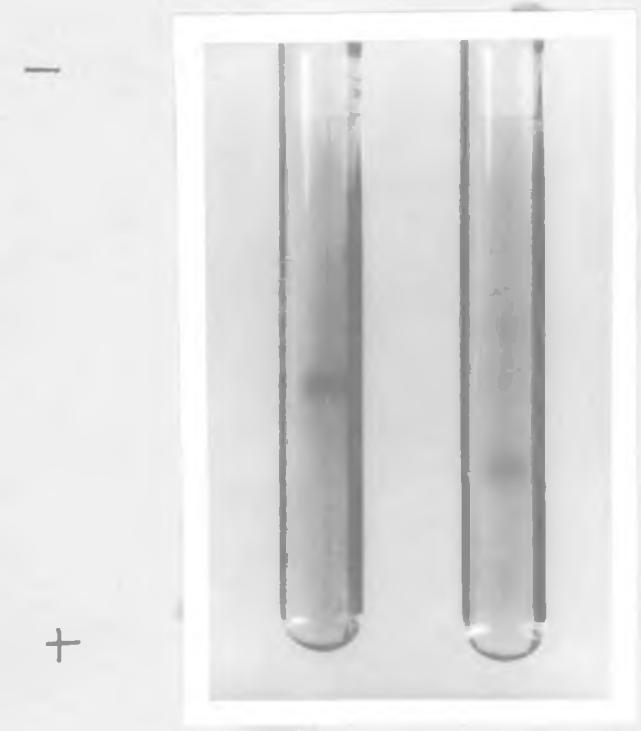
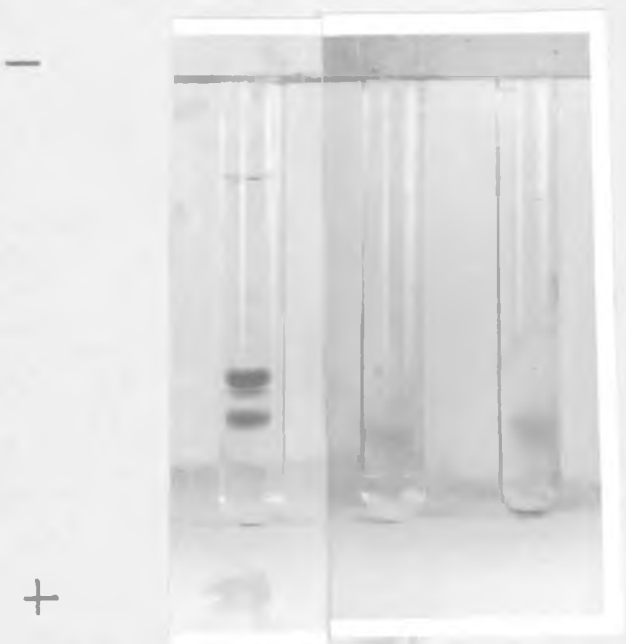


Fig.32. MOLECULAR WEIGHT BY SDS METHOD

Gel electrophoresis of papain inhibitors after SDS treatment



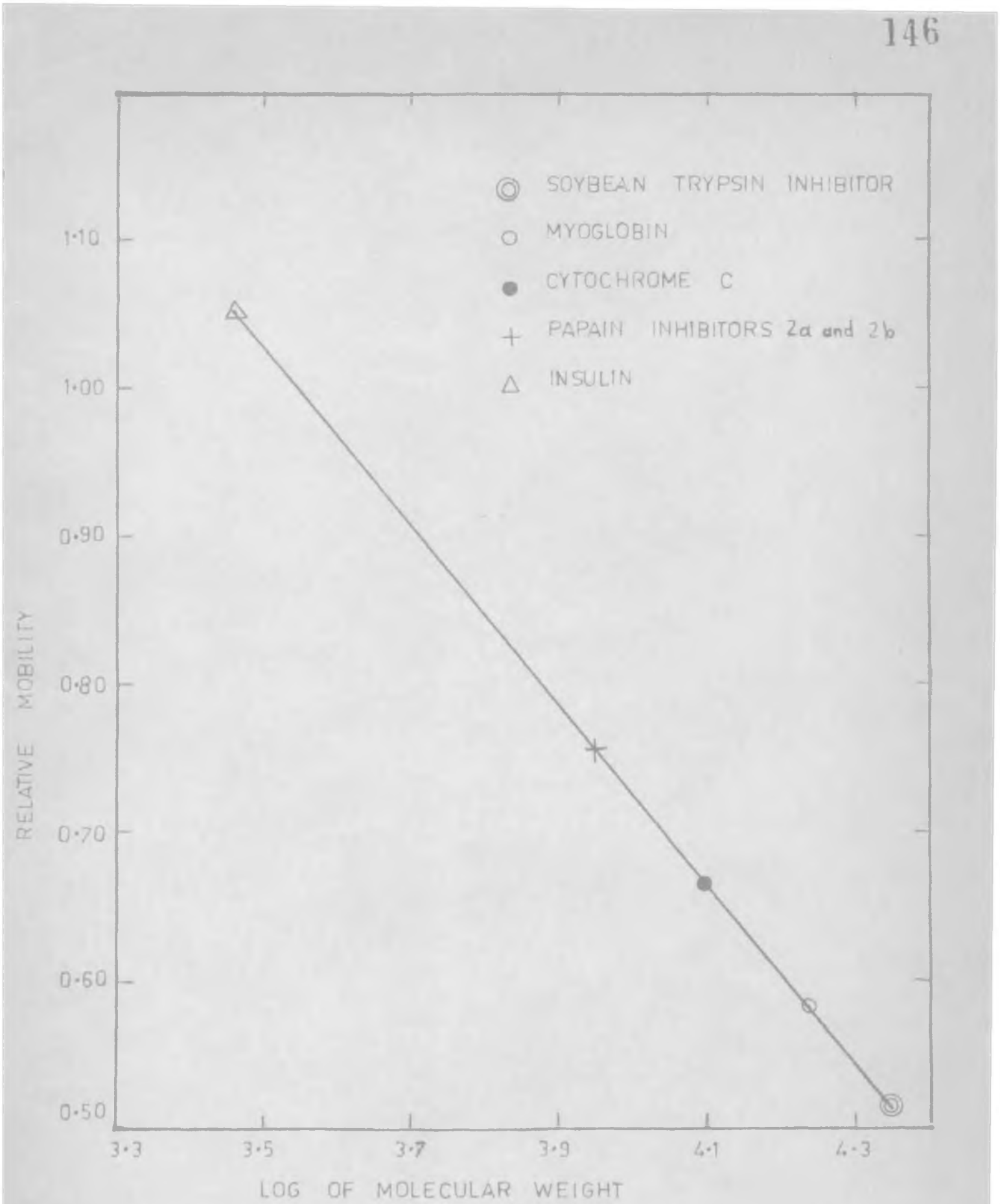


FIG-33 MOLECULAR WEIGHT OF PAPAIN INHIBITORS

BY SDS METHOD

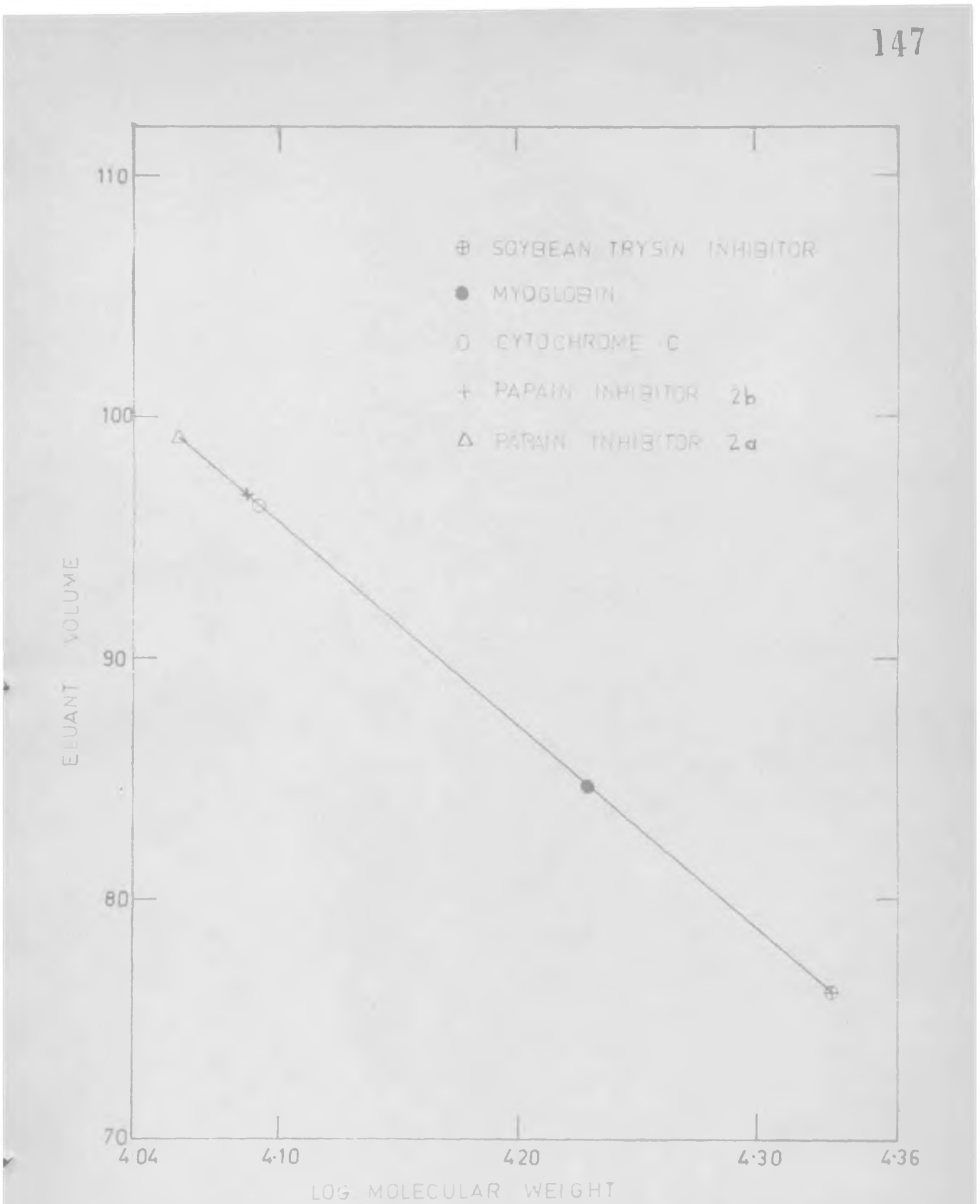


FIG-34 MOLECULAR WEIGHTS OF PAPAIN INHIBITORS BY GEL FILTRATION

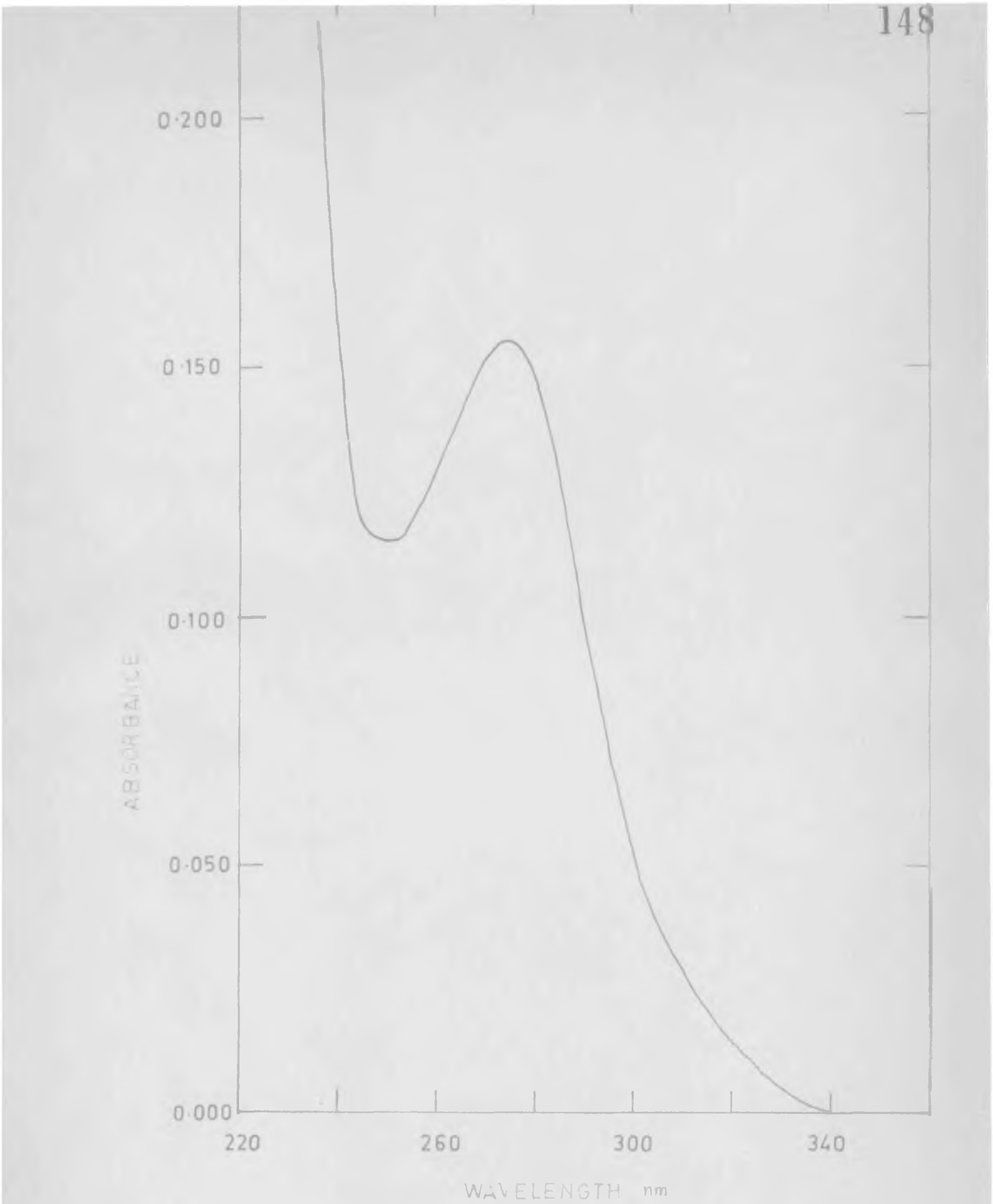


FIG- 35 ULTRAVIOLET ABSORPTION SPECTRUM OF PAPAIN INHIBITOR (2a) (120 Mg/ml)

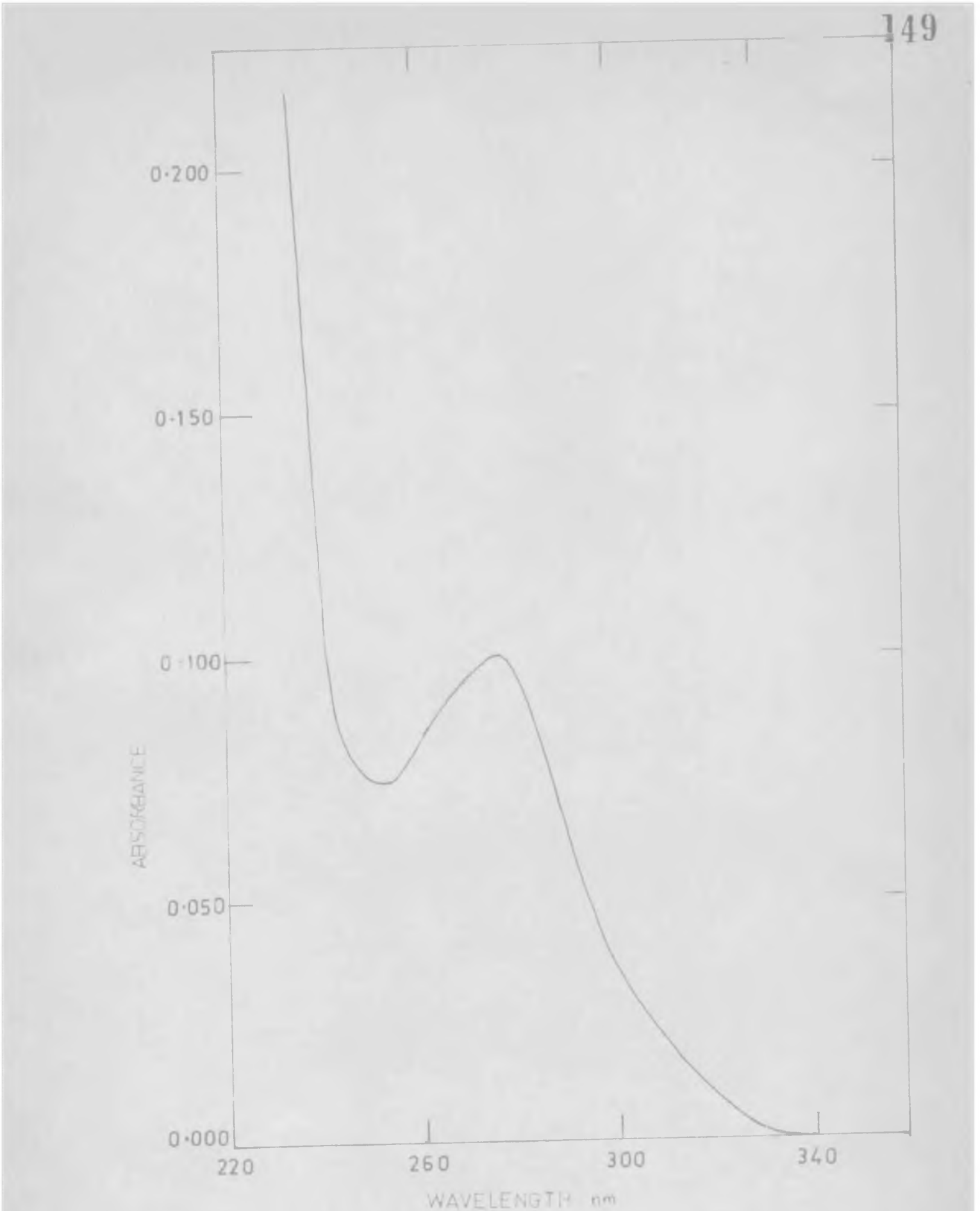


FIG 36 ULTRAVIOLET ABSORPTION SPECTRUM OF PAPAIN INHIBITOR (2b) (83 µg/ml)

Stability of papain inhibitors 2a and 2b towards trichloroacetic acid, ethanol, heat and pH

The inhibitors were completely stable when a 0.02% solution of the inhibitor in 2.5% trichloroacetic acid or in 75% ethanol was kept for 45 min at 30°.

Data about pH stability of the inhibitors are given in the following Table 23. Both the inhibitors were fairly stable between pH 2.8 to 10.2, when heated at 85° for 15 min. The inhibitors were more stable at lower pH values than at higher pH values.

Table 23

pH stability of papain inhibitors 2a and 2b

Inhibitor	pH	Inhibitor units left after heating at 85° for 15 min		% inhibitor activity left
2a	2.8	15.0		90
	7.6	12.5		75
	10.2	11.5		69
	7.6	16.5	Control (unheated)	100
2b	2.8	12.0		86
	7.6	9.5		68
	10.2	9.0		64
	7.6	14.0	Control (unheated)	100

0.02% solution of the inhibitor, in 0.1 M phosphate buffer, at different pH values, was heated at 85° for 15 min.

Inhibition of different proteinases

Both the inhibitors inhibit papain and ficin. The two inhibitors did not inhibit trypsin, chymotrypsin, subtilisin, and bromelain when 16 µg

of inhibitor 2a and 18.5 µg of inhibitor 2b were tested with 5 µg of each of the proteinases by using caseinolytic assay. Data regarding the inhibition of papain and ficin by caseinolytic as well as BAPA assays are summarized in Table 24. An interesting observation was that, by using BAPA assay the inhibition was approximately twice that obtained by the caseinolytic assay. This finding is in contrast with the observations made in the case of the inhibitors of trypsin and subtilisin (Sections I and II of this chapter). In these cases the inhibition obtained was the same by using either the synthetic substrate (BAPA) assay or the caseinolytic assay.

Complex formation between papain and papain inhibitor and its cleavage to release the inhibitor

To 1 ml of papain solution (95 µg protein, 280 units of proteinase) in 0.01 M phosphate buffer pH 7.5 and 0.01 M neutral cysteine was added 0.25 ml of CMC supernatant (Fraction IV, 4 mg protein, 92.5 units of papain inhibitor). After 5 min incubation at 30° the mixture (complex) was acidified to pH 2.5 with HCl, heated for 3 min at 90°, cooled, neutralized to pH 7.5 with $KHCO_3$ and tested for the inhibitor activity. Almost all the papain inhibitor activity (90 units) was recovered after heating. This shows that the papain gets inactivated and releases the inhibitor from the complex.

Table 24

Inhibition of papain and ficin by papain inhibitors 2a and 2b

Inhibitor	Enzyme	Assay	Amount of Inhibitor	Amount of enzyme inhibited	mg of enzyme inhibited by 1 mg of inhibitor	Molar combining ratio inhibitor: enzyme
			µg	µg		
2a	Papain	Caseinolytic	3.2	3.7	1.14	1:0.55
		BAPA	5.3	11.7	2.20	1:1.05(1:1)
	Ficin	Caseinolytic	3.2	4.1	1.28	
2b	Papain	Caseinolytic	3.7	3.4	0.92	1:0.44
		BAPA	6.0	10.8	1.80	1:0.86(1:1)
	Ficin	Caseinolytic	3.7	3.8	1.03	

Experiments were carried out to find out whether the inhibitory sites of the inhibitor for papain and ficin are the same or separate. Details regarding this study using papain inhibitor 2a are tabulated in Table 25. Similar observations were also made in the case of inhibitor 2b.

Both the inhibitors after being saturated with papain could not further inhibit ficin and vice versa. This shows that the papain and ficin inhibiting sites are not separate (as in the case of the trypsin-chymotrypsin inhibitor 1) but are probably the same, and that the enzymes compete for the same active site of the inhibitor.

Table 25

Experiment to establish whether the inhibiting sites in the inhibitor, for papain and ficin are same or distinct

(Reaction mixture no.)		1	2	3	4	5
Papain	units	42		42		42
Papain inhibitor 2a	units		28	28		28
Ficin	units				35	35
Proteinase activity of the reaction mixture (caseinolytic activity)	units	42	0	14	35	51

(Reaction mixture no.)		6	7	8	9	10
Ficin	units	35		35		35
Papain inhibitor 2a	units		28	28		28
Papain	units				42	42
Proteinase activity of the reaction mixture (caseinolytic activity)	units	35	0	7	42	50

DISCUSSION

This report presents evidence for the presence of a large number of proteinase inhibitors with different specificities in the seeds of Vigna catjang. Out of these, two trypsin inhibitors, two subtilisin inhibitors and two papain inhibitors have been purified to homogeneity. There has so far been no report on the purification of subtilisin inhibitors and papain inhibitors from a plant source.

The presence of such a large number of inhibitors with different specificities in a single source is of interest. The preliminary work done in the beginning of the present work also shows that the seed extracts of different legumes such as Vigna catjang, Vicia faba, Phaseolus vulgaris and Phaseolus lunatus inhibit the activities of trypsin, chymotrypsin, subtilisin and papain. This indicates the possibility of the wide spread occurrence of these inhibitors at least in legumes.

The properties of the inhibitors of trypsin, subtilisin and papain, which have been obtained in pure form, show that they are specific and different from each other. Trypsin inhibitors also inhibit chymotrypsin. They are considered to be specific since they do not inhibit subtilisin, papain and ficin. Subtilisin inhibitors are regarded as specific since they do not inhibit trypsin, chymotrypsin, papain and ficin. Papain inhibitors also inhibit ficin and they are specific in the sense that they do not inhibit trypsin, chymotrypsin, subtilisin and bromelain.

However there is no evidence regarding the isoinhibitors being artifacts or not. Further work is needed to determine whether they occur as such initially or are formed due to the action of endogenous proteases during the isolation of the inhibitors or during seed formation.

Purification

Extraction of the seeds at acidic pH eliminated a considerable amount of impurities. In general the usual methods of enzyme purification were applied for the purification of the inhibitors from the acid extract. The inhibitors were purified by conventional methods such as fractionation with ammonium sulphate, column chromatography on CM-cellulose, DEAE-cellulose and Sephadex (G-50 and G-100) and preparative polyacrylamide gel electrophoresis.

It may also be possible to purify the inhibitors by using affinity chromatography. A preliminary experiment wherein subtilisin inhibitor from the 0.30-0.90 ammonium sulphate precipitate was purified by using Sephadex bound subtilisin gave about a 50-fold purification in one step. However this procedure was not adopted due to the possibility of getting modified inhibitor.

Even after fractionation with ammonium sulphate and column chromatography on CM-cellulose and DEAE-cellulose, the inhibitors obtained were not homogeneous but showed 5 to 6 bands on polyacrylamide. Hence they were further purified by using preparative polyacrylamide gel electrophoresis. Polyacrylamide extracts contain non-protein impurities which interfere with the protein assay. These could be only partly removed by dialysis. The nondialyzable impurities were removed by passing the extract through DEAE-cellulose where the impurities were eliminated from the column as unadsorbed material. At this stage subtilisin inhibitor 2b which was homogeneous on polyacrylamide gel electrophoresis (with or without SDS-thioethanol treatment) also inhibited trypsin. It was not possible to separate this subtilisin inhibitor from the trypsin inhibitor by conventional methods. However it was possible to separate the subtilisin inhibitor from the trypsin inhibitor by preparing a trypsin inhibitor-trypsin complex and separating the complex

from the subtilisin inhibitor by gel filtration using Sephadex G-50. By this method one more trypsin inhibitor (trypsin inhibitor 2) was also obtained after cleaving the trypsin inhibitor-trypsin complex.

Yields of papain inhibitors were poor. The recovery loss was mainly in the preparative gel electrophoresis step. Whether this is due to poor extraction or due to some other reason is not clear and needs further study.

While purifying papain inhibitors during the CM-cellulose step, it was observed that part of the papain inhibiting activity was adsorbed on CM-cellulose. This fraction has been shown in this Laboratory (private communication from Miss K. S. Meenakshi) to contain two papain inhibitors.

One trypsin inhibitor (trypsin inhibitor 1) and one subtilisin inhibitor (subtilisin inhibitor 2a) were studied in greater detail while the other inhibitors were partially characterized. Trypsin inhibitor 1 was purified about 50-fold and subtilisin inhibitor 1, 2a and papain inhibitor 2a and 2b were purified about 2500-fold. Subtilisin inhibitor 2b was purified 1200-fold. The seeds contain several times more trypsin inhibiting activity than subtilisin or papain inhibiting activity. The acid extracts of the seeds contain more subtilisin than papain inhibiting activity. But other unpublished work from this laboratory shows that alkaline extracts of the seeds contain several times more papain inhibiting activity than the acid extracts. The relative amounts of subtilisin and papain inhibiting activity are not known precisely since the optimum conditions for quantitative extraction of the inhibitors have not been determined.

Properties

Proteinaceous nature of the proteinase inhibitors.

The purified inhibitors show characteristic protein absorption spectra with no indication of any other ultraviolet absorbing materials.

The inhibitors show their activity in the presence of EDTA. Even after dialyzing against EDTA the inhibitors retain their inhibitory activity indicating that the inhibition is not due to a metal (unless the metal is very tightly bound on the protein). Also the specificity of the different proteinase inhibitors lessens the chances of a metal being the inhibitor of the different proteolytic enzymes. The inhibitors show the usual behaviour of a protein, such as precipitation with ammonium sulphate, adsorption and elution on DEAE-cellulose or CM-cellulose, gel filtration on Sephadex, non dialyzability, movement on acrylamide gel and cellogel strip and staining with amido schwartz. Complex formation with the corresponding proteinase in stoicheiometric proportion and the 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 polyacrylamide gel electrophoresis at pH 6.8. After treatment with SDS-thioethanol also only a single band was obtained on electrophoresis. Homogeneity was also confirmed by gel filtration using Sephadex G-50, where each inhibitor eluted as a single peak. At pH 8.5 also all other inhibitors (except subtilisin inhibitor 1) were homogeneous on acrylamide gel. Subtilisin inhibitor 1 showed two bands and both the bands were active towards subtilisin. However on SDS-thioethanol treatment they moved as one band and also on Sephadex G-50 both eluted as one peak.

Homogeneity on acrylamide at pH 8.5 is expected since the inhibitors were purified by preparative acrylamide gel electrophoresis at this pH (pH 8.5). However the homogeneity obtained on pH 6.8 gel electrophoresis, SDS-thioethanol gel electrophoresis, cellogel strip electrophoresis

and gel filtration suggests that the inhibitors are homogeneous. However subtilisin inhibitor 1 consists of two inhibitors. Hence the observed properties are a composite of both and, therefore, one must await further purification for determining the properties of each of them.

Molecular weights

These inhibitors fall in the category of small molecular weight inhibitors. The molecular weights of the purified inhibitors ranged between 8000 to 15000 daltons.

It will be seen that there is some variation (about 25%) between the molecular weights determined by gel filtration and by SDS gel electrophoresis. No definite explanation can be given for these observations. It has been suggested (Sato and Murao, 1974) that molecular weight estimations by Sephadex-gel filtration are made on the assumption that the protein molecule is spherical and that the values obtained by this method may give erroneous results with non-spherical molecules. Moreover in the present work a sufficient number of low molecular weight reference proteins in the required range were not available for more accurate determinations. In the case of the specific subtilisin inhibitor obtained from the culture broths of Streptomyces albogriseolus (Sato and Murao, 1974) it was found that the molecular weights obtained by gel filtration, sedimentation equilibrium and by SDS gel electrophoresis were 27000, 23000 and 12000 respectively. In this case it was shown that the inhibitor exists as a dimer which is converted to the monomer during SDS gel electrophoresis. By comparing the molecular weights of trypsin, subtilisin and papain inhibitors determined by gel filtration and SDS gel electrophoresis it appears that there are no subunits in these inhibitors. However in order to confirm these observations the isolation of larger quantities of these inhibitors and the determination of their molecular weights e.g. by

ultracentrifugation with and without 8 M urea is desirable.

In the case of a specific papain inhibitor from chicken egg white it was shown (Fossum and Whitaker, 1968) that the molecular weight of the inhibitor by gel filtration was 12700. On this basis the molecular weight of the complex (assuming 1 mole:1 mole combination) should have been approximately 34000. However the molecular weight of the complex was found to be about 25000 (by gel filtration). No explanation was given for this discrepancy. It is possible that in this case also the inhibitor exists as a dimer which forms the complex in its monomeric form.

Ultraviolet absorption spectrum

The ultraviolet absorption spectra of the inhibitors showed characteristic protein absorption spectra with no indication of any other ultraviolet absorbing impurities. Except trypsin inhibitor 2 all the other inhibitors show maxima and minima in the range of 275 to 276 nm and and 248 to 252 nm respectively. Trypsin inhibitor 2 shows a maximum at 264 nm and a minimum at 254 nm. This inhibitor has an unusually low ratio of absorption at 280:250 nm (0.77). Moreover the inhibitor has a very high optical factor of 3.6 (reciprocal of absorbance at 280 nm of 1 mg/ml of inhibitor). This may be attributed to the fact that the inhibitor contains no tryptophan and has only one mole of tyrosine per mole of inhibitor. Optical factors of trypsin inhibitor 1, subtilisin inhibitor 1, 2a, 2b and papain inhibitor 2a and 2b were 2, 1.3, 1.0, 1.2, 0.8 and 0.9 respectively.

Dissociation constant

Dissociation constants were determined for trypsin-trypsin inhibitor 1, subtilisin-subtilisin inhibitor 1, subtilisin-subtilisin inhibitor 2a and subtilisin-subtilisin inhibitor 2b complexes by the method of Gree and Work and were found to be 8.4×10^{-10} M, 9.0×10^{-10} M, 1.4×10^{-9} M and 1.54×10^{-9} M respectively

at pH 7.5. Some of the values for the dissociation constants given by other workers for the non-specific subtilisin inhibitors are (Ki for subtilisin and inhibitor complex), potato inhibitor IIa 1.1×10^{-7}, potato inhibitor IIb 2.2×10^{-8} and penguin ovomucoid 1×10^{-9}.

Amino acid composition

Trypsin inhibitor 1: From the amino acid analysis a total of 112 residues were calculated and the calculated value of 14667 for the molecular weight of the inhibitor is in good agreement with that of 14500 as determined by the Archibald method. Cysteine is absent. It contains 16 half-cystine residues (14% per mole) which is comparable with the half-cystine content of several legume inhibitors which varies between 7% to 18% (Lima bean isoinhibitors - 16% to 17%, groundnut isoinhibitors - 7% to 18%, mung bean - 11%, kidney bean 14% and navy bean 14%). The inhibitor has low tyrosine (2 moles/mole inhibitor) and low tryptophan (1 mole/mole inhibitor) content.

Subtilisin inhibitor: The amino acid composition of subtilisin inhibitor 2a was determined and surprisingly the inhibitor was found to contain no cystine or cysteine. Subtilisin inhibitors 1 and 2b were also found to be devoid of cystine and cysteine. Until now only the serum inhibitors (α_1 trypsin and chymotrypsin inhibitors) were known to be devoid of cystine. The subtilisin inhibitor isolated from Streptomyces albogriseolus has 4 moles of half cystine per mole of inhibitor. This inhibitor has large amounts of alanine (19 moles/mole, 16%) and valine (13 moles/mole, 13%) and no isoleucine while subtilisin inhibitor 2a from Vigna catjang has large amounts of glutamic acid (12 moles/mole, 16%), 7 moles/mole (13%) each of valine and alanine and 2 moles of isoleucine per mole.

Modification studies using subtilisin inhibitor 2a: As stated earlier modification studies on proteinase inhibitors have been carried out hitherto only with the inhibitors of trypsin and chymotrypsin. Thus results with the subtilisin inhibitor are of interest especially since they contain no disulfide loops.

In the present report it was shown that the native subtilisin inhibitor 2a is converted to a new form by incubating it with catalytic amounts of subtilisin. On polyacrylamide gel at pH 8.5 the relative mobilities of the native inhibitor and the new form were 0.60 and 0.75 respectively, suggesting either a more positive charge on the modified inhibitor or fragmentation of the native inhibitor to a lower molecular weight structure. The new form also showed subtilisin inhibition and showed full activity towards subtilisin, similar to the native inhibitor. However the native inhibitor formed a complex with subtilisin much faster than the new form. This result is similar to the observations made by Finkenstadt and Laskowski (1965) on native and modified trypsin inhibitors.

The native inhibitor after incubating with catalytic amounts of subtilisin at pH 3.7 is almost completely converted into the new form (modified inhibitor). The complexes (with subtilisin) formed from the native and the modified inhibitors were found to have the same mobility on polyacrylamide gel (relative mobility 0.30). Another interesting observation is that although the conversion of the native inhibitor to the modified inhibitor was possible, the reverse process of converting the modified inhibitor to its native form was not possible. However studies over a wider range of conditions (pH etc.) are required since the equilibrium under the conditions used in the present studies may favour the modified form.

It was postulated (Laskowski and Sealock, 1971) 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 is clear that this disulfide loop hypothesis cannot be applied to subtilisin inhibitor 2a which has no cystine in the molecule. However it may be noted that although the modified subtilisin inhibitor is active, the conversion of the modified inhibitor to its native form has not been shown to take place at pH 7.5. It was unfortunately not possible to obtain enough material for more detailed studies on the mechanism and kinetics of the interaction of subtilisin and its inhibitor.

Stability

The inhibitors of trypsin, subtilisin and papain were markedly stable to denaturing agents such as heat, trichloroacetic acid, low pH and ethanol. In general the stability increases as the pH is lowered. Although subtilisin and papain inhibitors were comparatively stable at pH 10, trypsin inhibitor 1 was found to be unstable at this pH at elevated temperatures.

The subtilisin inhibitor from Streptomyces albogriseolus (Murao and Sato, 1972) is stable at pH 3 to10 at 37° for 25 h and on boiling for 10 min at pH 5 to 6. The papain inhibitor from chicken egg white (Fossum and Whitaker, 1968) is stable to boiling for 30 min at pH 4,while it loses 60% of its activity at pH 0 by the same treatment. Serum inhibitors are unstable to heat especially at acid pH. This instability of serum inhibitors is attributed to the abeence of cystine in the molecule. In this respect subtilisin inhibitors from Vigna catjang which are devoid of cystine are stable to heat treatment. The reason for this stability is

not known. Subtilisin inhibitor 2a does not also have an unusually high content of proline residues.

Specificity

Trypsin inhibitors 1 and 2 inhibit trypsin and chymotrypsin. They do not inhibit subtilisin or papain. 1 mole of trypsin inhibitor 1 inhibits 2 moles of trypsin and 0.5 mole of chymotrypsin, while the inhibitor of trypsin and chymotrypsin (Ventura and Filho, 1966), which was isolated from Vigna sinensis, inhibits 1 mole of trypsin and 2 moles of chymotrypsin per mole of the inhibitor. However in the case of both these inhibitors the inhibitory sites for trypsin and chymotrypsin are separate.

Trypsin inhibitor 1 and trypsin inhibitor 2 both inhibit trypsin and chymotrypsin and the inhibition ratio towards these proteinases (mg of trypsin inhibited: mg of chymotrypsin inhibitied per mg of the inhibitor) for both the inhibitors is the same (4). However trypsin inhibitor 2 is a weaker inhibitor of trypsin and chymotrypsin as compared to trypsin inhibitor 1. 1 mole of trypsin inhibitor 1 inhibits 2 moles of trypsin and 0.5 mole of chymotrypsin, while 1 mole of trypsin inhibitor 2 inhibits only 0.28 mole of trypsin and 0.07 mole of chymotrypsin. The inhibitors differ in their molecular weights, mobility on polyacrylamide gel electrophoresis, tyrosine and tryptophan content and optical factor.

Subtilisin inhibitors from Vigna catjang specifically inhibit subtilisin and do not inhibit trypsin, chymotrypsin and papain. Subtilisin inhibitors 1 and 2a inhibit subtilisin in a stoicheiometric ratio of 1:1 as observed in the case of the S.albogriseolus inhibitor. However in the case of subtilisin inhibitor 2b, two moles of the inhibitor inhibit one mole of subtilisin.

On polyacrylamide gel electrophoresis the relative mobilities of the two bands of subtilisin inhibitor 1 are 0.60 and 0.75 respectively. The relative mobility of subtilisin inhibitor 2a is 0.60 and that of subtilisin inhibitor 2b is 0.75. From these data one may conclude that subtilisin inhibitor 1 may be a mixture of subtilisin inhibitor 2a and 2b. However some differences in the properties of these inhibitors may be pointed out. During the purification of these inhibitors the elution pattern of each on DEAE-cellulose column chromatography is different. Subtilisin inhibitors 1, 2a and 2b are eluted at 0.008 M, 0.014 M and 0.023 M phosphate respectively. On SDS-gel electrophoresis subtilisin inhibitor 1 moves as a single band with a relative mobility of 0.80 while the relative mobility of subtilisin inhibitor 2b on SDS-gel electrophoresis is 0.70. This shows that although the relative mobility of the two inhibitors on plain polyacrylamide gel electrophoresis is the same (0.75) the inhibitors are not identical but have different molecular weights. Subtilisin inhibitor 1 has higher optical factor, lower molecular weight (SDS method) and lower dissociation constant than those of the other subtilisin inhibitors (2a or 2b). This would not have been possible if subtilisin inhibitor 1 had arisen from a mixture of subtilisin inhibitors 2a and 2b. Thus although more work is needed for a conclusive proof in this matter it is possible that subtilisin inhibitor 1 is separate from subtilisin inhibitor 2a or 2b.

That the subtilisin inhibitor 2a is different from subtilisin inhibitor 2b and not an artifact is evident from the data on SDS-gel electrophoresis, where the inhibitors have different mobilities and hence different molecular weights (moledular weight of 2a-8700 and 2b-13000). Also the molar combining ratio of subtilisin inhibitor 2a with subtilisin is 1:1 while that of subtilisin inhibitor 2b with subtilisin is 2:1.

However it is possible that all the isoinhibitors might have been formed from a parent subtilisin inhibitor due to proteolysis in the seed itself or during the isolation procedure.

Papain inhibitors 2a and 2b inhibit papain and ficin and do not inhibit trypsin, chymotrypsin and subtilisin. The inhibitory sites for papain and ficin are probably the same and not distinct as in the case of the trypsin-chymotrypsin inhibitor. In these respects the inhibitors resemble the papain inhibitor from chicken egg white.

Except the differences in their mobilities on polyacrylamide gel electrophoresis and in their elution pattern on DEAE-cellulose chromatography no other significant difference in the properties of papain inhibitor 2a and 2b is observed.

Further work carried out in this laboratory by Miss M. K. Salivati involves the purification of four additional papain inhibitors from Vigna catjang seeds (inhibitor 1, 3a, 3b and x). Papain inhibitor 1 is weakly adsorbed on DEAE-cellulose and elutes at 0.001 M phosphate concentration (Chapter III, Section IV). Papain inhibitors 3a and 3b are obtained from the inhibitor which is adsorbed on CM-cellulose (Chapter III, Section IV), while papain inhibitor x is recovered from the alkaline extract of Vigna catjang seeds.

The inhibition of trypsin or chymotrypsin by trypsin inhibitors and the inhibition of subtilisin by the subtilisin inhibitors was the same by using either the caseinolytic or the synthetic substrate assay procedure. However in the case of the papain inhibitors 2a and 2b it was observed that the inhibition of papain by using the synthetic substrate BAPA is nearly double that obtained by using casein as substrate. Further work is needed to explain this observation. In the case of chicken egg white papain inhibitor, the extent of the inhibition was the same with the synthetic substrate, p-nitrophenyl benzyloxycarbonyl glycinate and casein.

SUMMARY AND CONCLUSIONS

An extract of Vigna catjang seeds (at pH 3.0 to 3.5) was shown by chromatography on DEAE-cellulose to contain a large number of substances which inhibit trypsin, chymotrypsin, subtilisin and papain. From these inhibitors two trypsin-chymotrypsin inhibitors, two subtilisin inhibitors and two papain inhibitors were purified to homogeneity and their properties and kinetics were studied. The specific subtilisin and papain inhibitors (as well as their isoinhibitors) were obtained in purified form for the first time.

The inhibitors were purified by using a variety of methods such as fractionation with ammonium sulphate, column chromatography on CM-cellulose, DEAE-cellulose and Sephadex (G-100 and G-50), preparative polyacrylamide gel electrophoresis and affinity chromatography.

Trypsin inhibitors 1 and 2, subtilisin inhibitors 1, 2a and 2b and papain inhibitors 2a and 2b were purified 50, 10, 2300, 2650, 1200, 2840 and 2270-fold respectively.

Specificity and molar combining ratios: Trypsin inhibitor 1 and 2 inhibit both trypsin and chymotrypsin, and the inhibitory sites for the two proteinases are separate. They do not inhibit subtilisin, papain or ficin. The subtilisin inhibitors specifically inhibit subtilisin and not papain, trypsin or chymotrypsin. Papain inhibitors 2a and 2b inhibit only papain and ficin, the inhibitory sites for the two enzymes being the same. They do not inhibit bromelain, trypsin, chymotrypsin or subtilisin.

The molar combining ratios of trypsin inhibitors 1 and 2 with trypsin are 1:2 and 1:0.28 respectively and with chymotrypsin are 1:0.5 and 1:0.07 respectively. Molar combining ratios of subtilisin inhibitors 1, 2a and 2b

with subtilisin are 1:1, 1:1 and 2:1 respectively.

The combination ratios of trypsin inhibitors with trypsin and of subtilisin inhibitors with subtilisin are the same by using either the caseinolytic assay or the synthetic substrate (BAEE) assay. However in the cases of papain inhibitors 2a and 2b the molar combining ratios with papain are 2:1 respectively by the caseinolytic assay and 1:1 by the synthetic substrate (BAPA) assay.

The amino acid composition of trypsin inhibitor 1 and subtilisin inhibitor 2a was determined. Trypsin inhibitor 1 contains 112 amino acid residues giving a molecular weight of 14667. It has low tyrosine (2 moles/mole inhibitor) and low tryptophan (1 mole/mole inhibitor) content. Subtilisin inhibitor 2a contains 75 amino acid residues. The molecular weight calculated on this basis was 8223. An unusual finding is that all three subtilisin inhibitors do not contain cystine.

Modification studies were carried out using subtilisin inhibitor 2a. It was shown that the inhibitor could be modified to a new form (as tested by polyacrylamide gel electrophoresis) which also showed inhibition of subtilisin. However it was found that the reconversion of the modified inhibitor to the original native inhibitor at pH 7.5 was not possible.

Data regarding the other properties and kinetics of the different inhibitors are summarized in the following tables (Tables 26 and 27).

In conclusion it is shown that acid extracts of a number of leguminous seeds inhibit different proteinases such as trypsin, subtilisin and papain. Several isoinhibitors of the inhibitors of trypsin, subtilisin and papain were present in the extracts of Vigna catjang. The inhibitors of these enzymes which were isolated and purified from these seeds were proteinaceous in nature and were specific towards the corresponding proteinases.

Table 26

Properties and kinetics of the proteinase inhibitors

	Trypsin inhibitors		Subtilisin inhibitors			Papain inhibitors	
	1	2	1	2a	2b	2a	2b
DEAE-cellulose chromatography, phosphate concentration required for elution (M)	0.1	0.023	0.008	0.014	0.023	0.003	0.005
Specific activity (units/mg)	18090	3300	13910	16060	7150	6250	5000
Optical factor A_{280}^{-1} (Absorbance at 280 nm of 0.1% solution)	2.0	3.6	1.3	1.0	1.2	0.8	0.9
Absorption spectrum maximum (nm)	275	264	277	275	276	275	276
minimum (nm)	252	254	250	250	248	250	252
280:260 nm ratio	1.02	0.77	1.7	1.5	1.4	1.14	1.14
Homogeneity on polyacrylamide at pH 6.8	S i n g l e b a n d						
Homogeneity on polyacrylamide at pH 8.5 (number of bands)	one	one	two	one	one	one	one
Relative mobility at pH 8.5	0.95	0.75	0.60 0.75	0.60	0.75	0.56	0.68
Homogeneity by SDS-gel electrophoresis	S i n g l e b a n d						
Homogeneity by ultracentrifugation	Single peak						

Table 27

Properties and kinetics of the proteinase inhibitors

	Trypsin inhibitors		Subtilisin inhibitors			Papain inhibitors	
	1	2	1	2a	2b	2a	2b
Molecular weight							
by gel filtration		11700	11070	10000	11700	11430	12200
by SDS gel electrophoresis		13500	8130	8510	13500	9700	9700
by ultracentrifugation	14500						
by amino acid analysis	14667			8223			
$S_{20,w}$	1.5 S						
Isoelectric pH (pI)	5.0	5.9	5.1	5.9	5.9	6.5	6.7
Dissociation constant K at pH 7.5 (M)	$8.4x10^{-10}$			$9.0x10^{-10}$	$1.4x10^{-9}$	$1.54x10^{-9}$	
Tyrosine content per mole of inhibitor	2.20	1.12		2.09			
Tryptophan content " " "	0.70	0.48		1.28			
-SH groups " " "	0		0	0	0		
-S-S groups " " "	15.8		0	0	0		

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