METABOLISM OF PLANT CELLS

Studies on papain inhibitors of Vigna catjang

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C O N T E N T S

CHAPTER I

INTRODUCTION

INTRODUCTION

Section I

Proteinase inhibitors are proteins which inhibit proteinases by forming complexes with them.

Kunitz and Northrop (1936) crystallised the basic trypsin inhibitor from bovine pancreas in 1936 and showed that it was a protein. Trypsin inhibitors have subsequently been purified from animal tissues, plants and microorganisms. Major advances have been made in the study of their structure, properties, kinetics and mechanisim of action. Studies on the inhibitors of other proteolytic enzymes have, however, been relatively few. Papain inhibitors have been shown to be present in animal tissues, plants and microorganisms, but the only ones to be purified are those from chicken egg white (Fossum and Whitaker, 1968; Sen and Whitaker, 1973) and from the healing skin of Arthus type inflammation in rabbits (Udaka and Hayashi, 1965). Low molecular weight polypetide inhibitors of papain have been obtained from microorganisms (Umezawa, 1972). None of these papain inhibitors is specific for papain except those from egg white (Fossum and Whitaker, 1968; Sen and Whitaker, 1973) and rabbit skin (Udaka and Hayashi, 1965). The occurrence of specific papain inhibitors in plants has not been reported.

The study of papain inhibitors of plants and especially of the legume, Vigna catjang, was undertaken in the present study. Papain inhibitors were found to occur in seeds as well

as in rapidly growing tissue cultures of all plants which were investigated, whereas inhibitors of other proteolytic enzymes such as trypsin, chymotrypsin and subtilisin were present only in some and not in all the callus cultures tested. Specific protein isoinhibitors, which act only on papain (and the related enzymes ficin and chymopapain), but not on other proteinases such as trypsin, chymotrypsin and subtilisin were demonstrated for the first time in a plant source (seeds of Vigna catjang) and were separated from each other and purified. The occurrence of papain inhibitors in plants and the purification, properties and kinetics of the isoinhibitors of papain from Vigna catjang form the subject of this thesis.

Section II

Historical

The isolation of a trypsin inhibitor from bovine pancreas by Kunitz and Northrop (1936) in 1936 was followed by the discovery of an antigrowth factor in soybean flour by Ham and Sandstedt (1944) and independently by Bowman (1944). This was followed by reports of the inhibition of animal growth by other members of the Leguminosae such as lima bean (Tauber, Kershaw and Wright, 1949), and lucerne (Ramirez and Mitchell, 1960). In 1947 Kunitz (Kunitz, 1947) crystallised the soybean trypsin inhibitor as well as the enzyme-inhibitor complex. The methods of isolation and assay of the inhibitor, the study of the kinetics of interaction of the proteinase and the inhibitor and the dissociation of the complex, which were introduced by Kunitz, are still of importance in the study of these inhibitors.

This pioneering work was followed by numerous reports on the isolation and purification of proteinase inhibitors from several sources such as animal organs, sera, extracellular fluids, plant tissues and microorganisms. Their kinetics and nutritional and pharmacological effects were studied. The stability of several of these inhibitors to heat and acid facilitated their purification and many of them were isolated in pure form and their amino acid composition determined. This was followed by the determination of the primary sequence of the bovine pancreatic trypsin inhibitor by Kassel,

Radicevic, Ansfield and Laskowski in 1965 and of a number of other trypsin inhibitors such as Kazal's inhibitor of bovine pancreas (Greene and Bartelt, 1969), porcine pancreas inhibitor (Tschesche and Wachter, 1970) and inhibitor of Ascaris lumbricoides (Fraefel and Acher, 1968).

The unravelling of the three dimensional structure of Kunitz's pancreatic trypsin inhibitor and the enzyme-inhibitor complex by X-ray crystallography by Huber, Kukla, Ruhlmann and Steigemann (1970) and Huber, Kukla, Ruhlmann, Epp and Formanek (1970) was a major achievement.

A landmark in this field is the synthesis of the bovine pancreatic trypsin inhibitor (Kunitz) by Noda, Terada, Mitsuyasu, Waki, Kato and Izumiya (1971) and by Yajima and Kiso (1974).

Rapid advances have been made in the study of the mechanism of action of trypsin inhibitors, the identification of the active site, and the reversible cleavage of a peptide bond, atleast in some of the inhibitors (Laskowski and Sealock, 1971).

The physiological function of many of the inhibitors, especially in plants is obscure. The detection of isoinhibitors in a number of seeds has made this problem more intriguing. Several theories have been put forward about their physiological role, but none of them has gained general acceptance. A few of the inhibitors have found clinical applications. Trasylol (bovine parotid and lung trypsin inhibitor) has been used in the treatment of shock (Back, 1966; 1968) and early

pancreatitis (Thompson, 1968). The microbial leupeptin has been used as an anti-inflammatory agent (Umezawa, 1972). Clinical trials have proved that pepstatin, a pepsin inhibitor is an effective antidote for stomach ulcers in pylorus ligated rats (Umezawa, 1972).

Scope of literature survey:

The wide occurrence of inhibitors of a large number of proteolytic enzymes has stimulated considerable interest in this field and the literature on this subject is vast (Reviews - Vogel, Trautschold and Werle, 1968; Kassel, 1970; Laskowski & Sealock, 1971; Liener and Kakade, 1969; Fritz and Tschesche, 1970). This survey will therefore be limited to general aspects of inhibitors of proteinases and will deal mainly with papain inhibitors. The term "proteinase inhibitor" or "inhibition" is used in this thesis to refer only to proteinaceous inhibitors of proteolytic enzymes.(Antigen-antibody interactions and non-protein inhibitors will not be considered in this survey).

Section III

Papain

The properties of the enzyme papain (E.C.No. 3.4.4.10) are outlined in this section.

Papain is one of the proteolytic enzymes obtained from the latex of the tree Carica papaya. The term "papain" was coined by Wurtz and Bouchut (1879) in 1879 to describe the proteolytic enzyme from the latex. Balls and Lineweaver (1939) were the first to isolate the enzyme from fresh papaya latex. In 1954 Kimmel and Smith (Kimmel and Smith, 1954) successfully crystallised papain from commercially available dried latex.

Papain activity is only a part of the total proteolytic activity of the latex. Shack (1967) showed the presence of four distinct fractions containing proteolytic activity by means of carboxymethyl-sephadex chromatography. All the four required a thiol reagent for activation. The soluble protein of the latex consisted of papain activity, chymopapain activity and two new enzymes. One of the new enzymes was termed peptidase A and was purified to homogeneity. The activity of peptidase A was 40% of that of papain when casein was used as a substrate. The optimum pH of peptidase A was between 8 and 10 with casein as substrate, while that of papain was between 7-8 with the same substrate. No other information is available on the fourth enzyme, except the fact that it is a thiol activated enzyme.

Heterogeneity of papain:

Papain itself consists of three types of proteins (Blumberg, Schechter and Berger, 1970; Brocklehurst and Kierstan, 1973). (1) Active papain in which the side chain of cysteine 25 (active centre) is free. (2) Inactivated papain which can be converted to active papain by thiol reagents such as cysteine, reduced glutathione etc. and cyanide. (3) Inactive papain which is non-activatable, probably due to the active -SH being converted irreversibly to the sulfonic form. Of these papain (1) and (2) consistute 40-60% of the total protein. Hence different batches of papain vary in their specific activities, depending on the amount of proteins (1) and (2).

Activators:

It is well known that papain is activated by thiol reagents such as cysteine, reduced glutathione and cyanide (Glazer and Smith, 1971; Arnon, 1970). Traces of heavy metals are removed by the addition of EDTA.

Inactivators:

Papain is inactivated in the presence of air. Oxidised papain is reactivatable by thiol (Sluyterman, 1967) agents. Metals such as Cd^{+}, Fe^{+2}, Cu^{+2}, Hg^{+2}, Pb^{+2} inactivate papain (Arnon, 1970). As mentioned above, these heavy metals can be removed and the enzyme reactivated by the addition of a chelating agent such as EDTA and a thiol activator simultaneously. p-chloromercuric benzoate forms a stable inactive complex with the active SH group of the enzyme. Iodoacetic

acid and iodoacetamide also cause irreversible inactivation.

Chloromethyl ketones of phenylalanine and lysine also bring about total loss of activity. They act on the SH group rather than the imidazole groups of histidine as in the case of trypsin and chymotrypsin (Whitaker and Perez-Villasenor, 1968).

Stability:

The crystalline enzyme shows high stability as a suspension in NaCl solution and can be stored at 4 °C for several months without appreciable loss of activity. In solution, the enzyme loses 1-2% of its activity per day, while the mercuric form can be stored for months without loss of activity. Compared to other enzymes, papain is remarkably temperature stable. Dried papain can be heated to 100 °C for three hours without loss in activity. Its stability in solution is pH dependent. It is stable in the neutral pH range, unstable in acid solution at elevated temperatures and is rapidly destroyed below pH 2 at 25 °C with spectral changes (Lineweaver and Schwimmer, 1941).

Papain is stable to organic solvents, such as 70% methanol, and 15% dimethylsulphoxide (Barel and Glazer, 1969). It retains its activity in 8 M urea with no observable conformational changes (Gundlach and Turba, 1965). However it is unstable in 6 M guanidine hydrochloride and 10% trichloroacetic acid (Sluyterman, 1967).

Optimum pH:

The optimum pH range for activity is from pH 6 to 8,

depending upon the substrate used.

Specificity:

Papain has a broad specificity towards proteins and small molecular weight substrates (Kimmel and Smith, 1957). Most peptide bonds are hydrolysed although the relative rates for different synthetic substrates vary. Peptide bonds formed by the carboxy groups of α-amino lysine and arginine were found to be the most susceptible bonds (Hill, 1965). Thus benzoyl-L-arginine amide and ethyl esters were found to be the most sensitive substrates. Papain also shows transamidase and transesterase activities (Mycek and Fruton, 1957; Yu-Kun and Chen-Lu, 1963; Glazer, 1966).

PHYSICAL PROPERTIES

Molecular weight:

Sedimentation diffusion studies and the approach to equilibrium technique showed the molecular weight to be 20,700 and 20,900 respectively. However recent studies indicate the molecular weight to be 23,000 (Drenth, Jansonius, Koekoek, Swen and Wolthers, 1968). Papain has an isoelectric point of 8.75. The absorbance of a 1 mg/ml solution of the enzyme for 1 cm lightpath at 280 nm is 2.5.

Amino acid composition:

Papain has 212 amino acid residues and is devoid of any carbohydrate moiety. (Drenth, Jansonius, Koekoek, Swen and Wolthers, 1968). All the common amino acids are present except methionine. Papain is rich in arginine and lysine. The total number of arginine and lysine residues is more than that

of glutamic acid and aspartic acid residues and hence the isoelectric point is at pH 8.75. The amino acid sequence of papain has been determined by a number of workers (Light, Frater, Kimmel and Smith, 1964; Mitchel, Chaiken and Smith, 1970). The N terminal amino acid sequence was found to be Ile-Pro-Glx., while the C terminal was established as -Lys-Asn COOH. Later X-ray structure studies on papain (Drenth, Jansonius, Koekoek, Swen and Wolthers, 1968) confirmed the amino acid sequence and structure of papain. Disulfide bonds were found to be present between Cys 22 and Cys 63, Cys 56 and Cys 95 and Cys 153 and Cys 200.

Three dimensional structure of papain:

The molecule is ellipsoidal in structure with the dimensions 50 x 37 x 37 Å. The hydrophillic moities are at the surface, while the hydrophobic groups are oriented inwards (Drenth, Jansonius, Koekoek, Swen and Wolthers, 1968).

Active centre:

Papain is binuclear. There are two hydrophobic cores, with a groove at the top. The reaction between the enzyme and the substrate occurs in this groove. Cysteine 25 is the free SH group at the active centre. As stated earlier, reaction of this sulfhydryl group by an alkylating reagent or a heavy metal or disulfide formation or oxidation to the sulfonic group renders the enzyme inactive. Reactivation of Cys 25 is essential for enzyme activity. An imidazole ring of histidine 159 is in close proximity and is postulated to be involved in catalytic action.

Section IV

Distribution of proteinase inhibitors

Proteinase inhibitors have been isolated from a variety of tissues. The frequent presence of isoinhibitors in the same tissue offers considerable difficulty in arriving at a suitable nomenclature. Steiner and Frattali (1969) proposed a nomenclature based on the active centre of the inhibitor e.g. the trypsin inhibitors can be classified as arginine or lysine inhibitors depending on the particular amino acid present at the active centre. The above suggestion would no doubt lead to a more systematic classification, but it is at present premature, since the reactive sites of a number of inhibitors have yet to be determined. In this thesis the inhibitors will be classified on the basis of enzyme activity which is inhibited e.g. trypsin inhibitors, chymotrypsin inhibitors, subtilisin and papain inhibitors. Most of the work till now nas been on the above inhibitors from various sources such as animal tissues, plants and microorganisms. The literature on the above inhibitors is extensive and has been surveyed in several reviews and books (Vogel, Trautschold and Werle, 1968; Kassel, 1970; Fritz and Tschesche, 1970; Laskowski and Sealock, 1971; Liener and Kakade, 1969; Weyer, 1968). Only the literature on papain inhibitors will be dealt with in detail in this section while only some aspects of the literature on other inhibitors will be reviewed.

PLANTS:

Trypsin inhibitors:

Seeds of the Leguminosae family such as soybean, (Kunitz, 1947; Birk, 1961; Rackis, Sasame, Mann, Anderson and Smith, 1962; Yamamoto and Ikenaka, 1967), and lima bean (Jones, Moore and Stein, 1963; Haynes and Feeney, 1967) are rich in trypsin inhibitors. Sohonie and Bhandarkar (1955) have shown trypsin inhibitors to be present in a wide wariety of seed tissues. Seeds of 20 species were screened for trypsin inhibitor activities and for inhibitors of chymotrypsin and subtilisin (Sumathi and Pattabiraman, 1976). Trypsin inhibitor activity was found to be widely distributed. The trypsin inhibitors are not restricted to the dicotyledons. Monocotyledonous plants such as wheat (Shyamala and Lyman, 1964), barley (Burger and Siegelmann, 1966) and corn (Hochstrasser, Muss and Werle, 1967) also contain trypsin inhibitors. They have been detected in tubers such as potato (Honavar and Sohonie, 1955), beet (Vogel et al., 1968) etc. Sumathi and Pattabiraman (1975) have shown the presence of trypsin inhibitors in a wide variety of tubers. Colocasia was found to have the highest anti-trypsin activity. Extensive physio-chemical work has been carried out on purified trypsin inhibitors.

Chymotrypsin inhibitors:

A chymotrypsin trypsin inhibitor from potato has been isolated in pure form and studied extensively (Ryan and Balls, 1962; Balls and Ryan, 1963; Ryan and Kassel, 1970; Melville and Ryan, 1972). It also inhibits subtilisin, pronase (partly),

kallikrein and trypsin. The trypsin inhibitors from other sources also inhibit chymotrypsin. A small dialyzable inhibitor having a molecular weight of 3000-4000 has been isolated from potato (Rancour and Ryan, 1968). It inhibits α-chymotrypsin but is not specific since it also inhibits an endogenous bradykinin inactivating carboxypeptidase of potato (Ryan, 1973). Recent investigations by Belitz, Kaiser and Santarius (1971) indicate the presence of as many as thirteen isoinhibitors in potato which inhibit trypsin and chymotrypsin.

Subtilisin inhibitors:

No specific subtilisin inhibitor of plants was known until recently. Three isoinhibitors specific for subtilisin were isolated from Vigna catjang (Vartak, 1975), and one from barley (Yoshikawa, Iwaśki, Fujii and Oogaki, 1976). The subtilisin inhibitors from Vigna catjang have molecular weights ranging from 10,000 to 11,700, whereas the one from barley has a molecular weight of 20,000. A non-specific inhibitor has been reported to occur in potatoes (Balls and Ryan, 1963) and in barley (Mikola and Suolinna, 1971).

Isoinhibitors:

A number of reports deal with the presence of isoinhibitors in plant tissues. Soybean, lima bean and potato have been found to contain a number of isoinhibitors of trypsin and chymotrypsin. A family of chromatographycally distinct polypeptide inhibitors of bromelain has recently been isolated (Reddy, Keim, Heinrikson and Kezdy, 1975). The isoinhibitors were found to have a molecular weight of about 5,600. They

contain 50 amino acids each, and five disulfide bridges. The inhibitor was also found to contain a carbohydrate moiety, possibly attached to a threonine residue. Isoinhibitors may arise by the action of proteolytic enzymes or deamidation or amidation during seed formation or during isolation and may therefore be artifacts (Reddy et al., 1975). However the occurrence of isoinhibitors even in tissue extracts prepared with trichloroacetic acid (Warsy, Norton and Stein, 1974), perchloric acid (Jones, Moore and Stein, 1963), diisopropyl-phosphofluoridate (Pubols, Barteit and Green, 1974) and other reagents which inactivate proteolytic enzymes suggests the probability of isoinhibitors occurring naturally in plants.

Localisation:

In soybean the trypsin inhibitor was found to be concentrated in the cotyledons (Rackis and Anderson, 1964). In the case of cereals such as barley, wheat and rye (Mikolo+Kirsi, 1972) etc. the endosperm was found to be rich in trypsin inhibitors. The embryo of wheat, barley and rye also showed inhibitor activity. In the case of tubers such as potato, the inhibitor was found to be concentrated in the cortex of the tubers (Ryan, Huisman+Vandenburgh, 1968).

Inhibitors of Animal Origin - Trypsin and chymotrypsin inhibitors:

The presence of proteinase inhibitors in animal tissues was first established as early as the end of the last century. These substances were termed as anti-enzymes. The inhibitors were found in exudates such as serum (Bundy and Mehl, 1959; Schwick, Heimburger and Haupt, 1966), pancreatic secretions

(Kazal, Spicer and Brahinsky, 1948), colostrum (Laskowski, Kassell and Hagerty, 1957) and urine (Astrup and Nissen, 1964). The pancreatic secretory inhibitor (also known as Kazal's inhibitor) was found in most of the animals tested. The bovine trypsin kallikrein inhibitor crystallised from pancreas by Kunitz and Northrop in 1936 was found to be distributed in tissues such as lung, parotid glands, lymph nodes, spleen and liver. Similar inhibitors are found in goat and pig but not in other mammals (Kassel, 1970). Proteinase inhibitors have been reported in nematodes (Peanasky and Ghaleb, 1970)and eggs of certain birds such as chicken, turkey, duck and quail (Rhodes, Bennett and Feeney, 1960). Most of the above inhibitors are mainly trypsin inhibitors. However most of them also inhibit chymotrypsin and kallikrein.

Subtilisin inhibitor:

The trypsin and chymotrypsin inhibitors of chicken egg white also inhibit subtilisin. (Liu, Means and Feeney, 1971). No specific subtilisin inhibitor has been reported to be present in animal tissues.

Carboxypeptidase inhibitor:

An endogenous carboxypeptidase inhibitor was isolated from goat pancreas by Dua, Bedi and Dixit (1975). The inhibitor was obtained in the dialysate by dialysis of the pancreatic extract at pH 5 against citrate buffer.

Inhibitors of microbes:

During the last decade proteinase inhibitors have been

shown to be present in microorganisms. The most widely studied proteolytic inhibitors of microbial origin are the ones from Streptomyces strains (Umezawa, 1972). Extensive work on these interesting polypeptides has been carried out by Umezawa et al. (1972). Leupeptin inhibits trypsin, papain and (very weakly) plasmin. Chymostatin inhibits chymotrypsin, but has only weak activity with papain and none with trypsin. Pepstatin inhibits pepsin and cathepsin D. They are very low molecular weight polypeptide inhibitors.

A specific subtilisin inhibitor from Streptomyces albogriseolus S-3253 has been purified. It has a molecular weight of 27,000 and does not act on trypsin and chymotrypsin. (Murao and Sato, 1972; Sato and Murao, 1973; 1974).

Saccharomyces cerevisiae:

Lenney, Maitle, Wiemken, Schellenberg and Meyer (1974) have shown the presence of three different proteinases and their corresponding inhibitors in Saccharomyces cerevisiae. The two proteinase inhibitors A and B have been now partially purified and characterized. The A and B inhibitors were found to be thermostable. The proteinase inhibitor A has a molecular weight of 22,000. It could be dissociated into two chains, each having a molecular weight of 11,000. Proteinases B and C were found to be serine proteinases, whereas proteinase A was shown to be an acid proteinase (Lenney, 1975). There are very few reports in the literature of different proteinases and specific inhibitors of these proteinases occurring in the same

tissue. This work on the yeast enzymes and inhibitors, represents an important advance in the characterization of proteinase inhibitors and further work may throw light on their metabolic role in yeast.

Proteinase inhibitors of Vigna:

Since this work deals with the papain inhibitors of Vigna catjang the occurrence of other proteinase inhibitors in this source will be briefly described. A protein inhibitor was purified from Vigna sinensis by Ventura and Filho (1966). The inhibitor was found to be more active against chymotrypsin, than trypsin. The molecular weight was found to be 17,000 by sedimentation equilibrium studies. However in a recent report by Ventura, Filho, Moreira, Aquino and Pinheiro (1971) the molecular weight was corrected to 10,000. The inhibitor contains 2% tryptophan but no methionine. Fractionation of the crude extract on sephadex revealed four different fractions having trypsin inhibiting activity and with molecular weights of 11,000 to 21,000 (Filho, 1973).

The trypsin inhibiting activity of the cotyledons was found to decrease during germination (to 5% of the value of the dry seeds). The disappearance of the activity was found to be controlled by the axial parts of the plant. A weak inhibition of an endogenous proteinase by the crude extract was reported. When this work was nearing completion Gennis and Cantor (1976) reported the purification of two new inhibitors of an endogenous proteinase from Vigna sinensis (trypsin and chymotrypsin inhibitors). They have also

studied the structure and formation of the trypsin and chymotrypsin inhibitor complexes.

Royer, Miege, Grange, Miege and Mascherpa (1974) have in a recent report demonstrated the presence of five trypsin inhibitors in the cotyledon extract of Vigna unguiculata (from Zaire), strain H.81. It was found that trypsin inhibitors have a partial activity towards an endogenous proteinase. The presence of an inhibitor-enzyme complex is postulated. Sohonie and Bhandarkar (1955) reported the presence of two trypsin inhibitors in Vigna catjang, but their purification was not carried out. Earlier work from this laboratory showed that extracts of Vigna catjang seeds contained a large number of proteins, which inhibit trypsin, chymotrypsin, subtilisin and papain. They were separated from each other and two trypsin inhibitors, three specific subtilisin and two papain inhibitors were purified to homogeneity. The properties and kinetics of some of them were studied in detail (Vartak, 1975). Their properties will be discussed in later sections. The characterization of such a large number of specific inhibitors of different proteinases from a single plant source is of considerable interest.

Papain inhibitors:

Proteinase inhibitors of papain have been detected in plants, microorganisms and animal tissues. The only inhibitors specific for papain, which have been purified, are those from two animal sources chicken egg white and from the healing skin

of Arthus type inflammation in rabbits. Papain inhibitors of Actinomycetes are small peptides and are non-specific in nature. Antipapain factors have been detected in a few plant tissues, but have not been purified and their specificity is not known. The papain inhibitors described below will be classified into non-specific inhibitors and those specific for papain and ficin, which are closely related -SH activated plant enzymes.

SPECIFIC PAPAIN INHIBITORS:

Egg white:

A protein inhibitor of ficin and papain was purified from chicken egg white by Fossum and Whitaker (1968) and by Sen and Whitaker (1973). It inhibited bromelain feebly. The purification and properties will be discussed later.

Healing skin of Arthus type inflammation in rabbits:

A papain inhibitor from the site of healing Arthus type inflammation of the skin in rabbits was purified by Hayashi and Udaka (1965). The properties and purification will be dealt with in a later section.

Plants:

No specific papain inhibitor has been isolated and purified, except for a potato inhibitor (unpublished work Huff, 1972) and the two inhibitors purified in this laboratory by Vartak (1975). However inhibitors from plant sources which inhibit other enzymes such as trypsin, chymotrypsin, subtilisin have been reported. They will be discussed below.

Non specific papain inhibitors:

Learmonth (1951; 1958) was the first to report the presence

of an antipapain factor in soybean flour extract, while studying its influence on bread doughs. He showed the factor to be concentrated almost entirely in the germ. The factor was also reported to be present in haricot beans and broad beans. However the results were not conclusive since the activities were low and the inhibition could have been due to non-protein factors.

Radoeva (1973) reported the presence of an antipapain factor in soybean meal and extract. The factor was found to be heat stable and was different from the thermolabile fractions which inhibited trypsin and chymotrypsin.

When the present work was nearing completion, isoinhibitors of bromelain from commercial stem bromelain were described by Perlstein and Kezdy (1973). However the isoinhibitors were non-specific and inhibited trypsin, chymotrypsin and papain. The molecular weights ranged from 5000 to 6000.

Broad bean:

Two isoinhibitors having a broad specificity for trypsin, chymotrypsin, papain, pronase and thrombin have been purified recently (Warsy, Norton and Stein, 1974). The broad bean trypsin inhibitor also inhibits papain and chymotrypsin (Sohonie, Huprikar and Joshi, 1959).

A papain inhibitor was reported to be present in wheat flour (Hites, Sandstedt and Schaumburg, 1951).

Animal source - Inhibitor from the mucous membrane of the respiratory passages:

An inhibitor was detected in the mucous membrane of the

respiratory passages and in their secretions in humans and other mammals by Haendle, Trautschold, Vogel and Werle (cited in Vogel *et al*. 1968). It was found to be active against plasmin, pronase and papain, though not as effective as against chymotrypsin.

Inhibitor from seminal vesicles and semen of mammals:

An organ specific and sex specific proteinase inhibitor in the vesicular glands and semen of all mammals examined so far has been reported by Haendle, Trautschold, Fritz and Werle (1965). Though essentially a trypsin inhibitor it also inhibits plasmin, papain and bacterial proteinase from *Streptomyces griseus*. Kallikrein and chymotrypsin are however not inhibited (cited from Vogel *et al*., 1968).

Serum:

A papain inhibitor has been detected in the sera of many species (Grob, 1949).

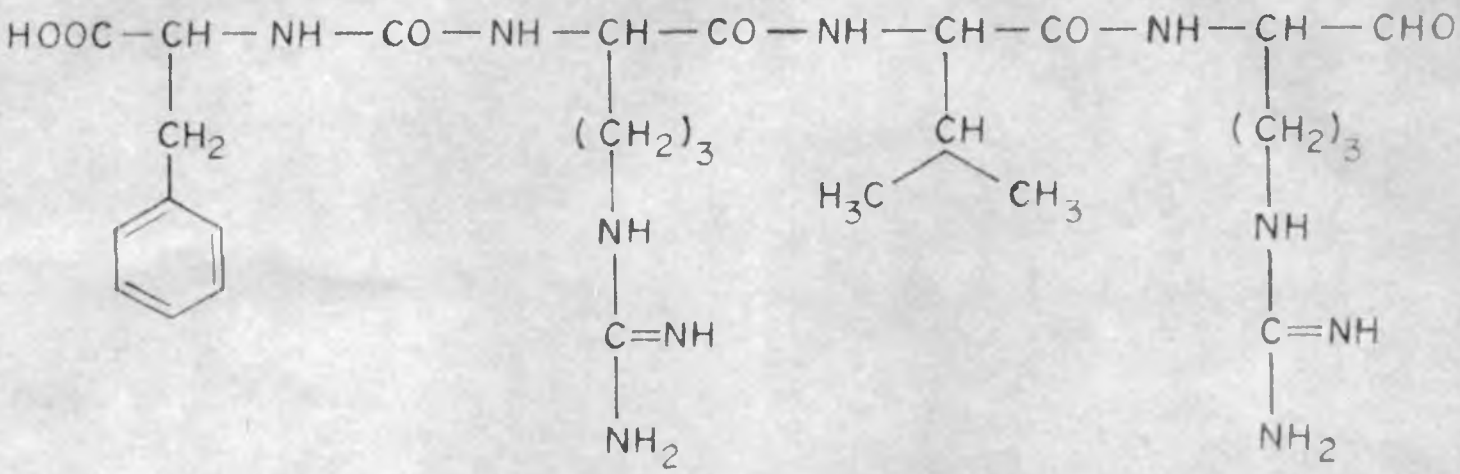
A trypsin inhibitor from the submandibular glands of dogs inhibits chymotrypsin and papain (Trautschold, 1965).

Skin:

An antipapain factor has been shown in rabbit skin (Martin and Axelrod, 1958).

Micro-organisms:

Antipain is a small peptide produced by a few species of *Streptomyces*. It was detected in the culture filtrates of *Streptomyces michiganensis*, *Streptomyces yokosukanensis* and *Streptomyces violascens*. The following structure was given by Umezawa.



STRUCTURE OF ANTIPAIN (UMEZAWA, 1972)

Both antipain and leupeptin have arginine residues. Both inhibit papain and trypsin, but antipain is a weaker inhibitor of plasmin than leupeptin.

Section V

Methods of estimation

Papain activity may be measured either by determining the rate of digestion of proteins or by following the rate of hydrolysis of small molecular weight synthetic substrates. Since in its native state papain shows very low activity, all assays are carried out in the presence of activators such as cysteine and EDTA. Inhibitor activity is measured by the decrease in papain activity caused by the inhibitor.

Determination of proteolytic activity:

This method consists of measuring the formation of small molecular weight trichloroacetic acid soluble digestion products of the protein. Casein is generally used as the substrate. Hemoglobin is used for acidic pH ranges at which casein is insoluble. Other proteins such as bovine serum albumin, which can be obtained in pure form, can also be used as substrates. The reaction is stopped by the addition of a specified concentration of trichloroacetic acid and the reaction mixture filtered after 30 minutes. The free amino acids and small peptides in the filtrate are measured either by determining the absorbance at 280 nm (due mainly to tyrosine and tryptophan and their peptides) (Kunitz, 1947) or by the well known method of Folin-Ciocalteau (Anson, 1938) or by the ninhydrin method (Moore and Stein, 1948).

Use of synthetic substrates:

The use of simple synthetic substrates for the assay of proteolytic enzymes was introduced by Bergmann (1942) in 1942.

Generally the esters or amides of amino acids are used. Papain hydrolyses peptides, amides and esters, especially bonds involving arginine lysine, leucine and glycine.

Assay of esterase activity:

The method is based on the assay of the free carboxyl group or the liberated alcohol. When BALE (benzoyl-L-arginine ethyl ester) is used as the substrate, the liberated carboxyl groups are titrated against a standard base (Smith and Parker, 1958; Blumberg, Schechter and Berger, 1970).

When BALE is hydrolysed to benzoyl arginine and ethyl alcohol there is an increase in absorbance which is maximum at 253 nm. This forms the basis for the spectrophotometric assay when BALE is used as the substrate. However the substrate also absorbs strongly at 253 nm; hence the titrimetric method is preferred. When CTNP (p-nitrophenyl carbobenzoxy tyrosine) is used as a substrate, the liberated p-nitrophenol is measured spectrophotometrically at 410 nm. The substrate has no absorption at 410 nm (Bender, Begue-canton, Blakely, Brubacher, Feder, Gunter, Kezdy, Killheffer, Marshall, Miller, Roeske and Stoops, 1966).

Assay of amidase activity:

The amide substrates are cleaved to give carboxyl groups and the corresponding amine. When benzoyl-L-arginine amide (BAA) is used as the substrate, the free carboxylic groups released are estimated by titration in the presence of ethanol (Smith and Parker, 1958). The free amine is estimated spectrophotometrically at 410 nm, when benzoyl-DL-arginine

p-nitroanilide is used as the substrate. The substrate does not show any absorption at 410 nm. (Arnon, R., 1965).

Units:

No generally accepted convention exists for expressing proteolytic inhibitor activity. Vogel has suggested that one international inhibitor unit (IU) may be defined as "the quantity of inhibitor which completely inhibits the activity of one international unit of proteolytic activity" i.e. which catalyses the conversion of one micromole of the substrate per minute under standard conditions (Vogel et al., 1968). This definition is applicable only to synthetic substrates. If a high molecular weight substrate such as casein or hemoglobin is used, the definition given by Kunitz is used (Kunitz, 1947).

Kunitz's inhibitor unit:

It is the quantity of inhibitor that causes a decrease in absorbance of 1.0 per minute at 280 nm for 1 cm light path, during the cleavage of a natural substrate, in the trichloro-acetic acid filtrate, when the reaction is carried out under specified condition of pH, temperature etc. (Kunitz, 1947).

Section VI

Purification

Conventional techniques for protein purification such as ammonium sulfate fractionation, precipitation with organic solvents, deproteinizing reagents, ion-exchange resin chromatography, exclusion chromatography etc. are generally used. However, because of the stability of these inhibitors to denaturing conditions such as high temperature and acidity, drastic conditions can be employed for the purification of these proteins. Kunitz used boiling trichloroacetic acid solution for the extraction of the pancreatic inhibitor and denaturation of most of the other proteins. This treatment was also used to isolate the inhibitor from the complex with the enzyme (Kunitz and Northrup, 1936). Similar procedures have been adopted by other workers to separate and purify the inhibitors. When the enzyme-inhibitor complex is sufficiently stable, the complex can be separated by exclusion chromatography and lowering the pH causes denaturation of the enzyme and facilitates the separation of the inhibitor. 6 M guanidine hydrochloride was used to denature trypsin and release the soybean inhibitor from the complex after exclusion chromatography (Sealock and Laskowski, 1969).

An attractive method for the purification of the inhibitor is affinity chromatography. The enzyme is bound to an insoluble support and the insolubilised enzyme is used for binding the inhibitor. A resin which is used for binding the inhibitor is the polyanionic trypsin resin introduced by Levin, Pecht, Goldstein and Katchalski (1964). They used the

maleic anhydride ethylene copolymer as the support and 1,6-diaminohexane as the crosslinking reagent. The resin was used to isolate the Kazal type of inhibitors from human (Fritz, Huller, Wiedemann and Werle, 1967) porcine, canine and bovine pancreas (Fritz, Schult, Neudecker and Werle, 1966) and from seminal vesicles of mice (Fritz, Huller et al., 1967). For inhibitors with isoelectric point below pH 5, which were not bound to the resin due to interference by free carboxylates on the resin, a modified resin in which the carboxylates of the resin were blocked by N-N dimethyl ethylene diamine was used (Fritz, Gebhardt, Fink, Schramm and Werle, 1969). The new resin was used to purify inhibitors from wheat, rye (Hochstrasser and Werle, 1969), potatoes (Hochstrasser, Werle, Siegelmann and Schwarz, 1969) and peanuts (Hochstrasser, Illchmann and Werle, 1969). Chymotrypsin-Sepharose and trypsin-Sepharose were used respectively for the purification of chicken ovoinhibitors (Fienstein, 1971). The complex is dissociated with 0.2 M KCl or by urea solution at pH 2. In some cases the method has the advantage that the resin-linked enzyme can be reused several times.

However, modification of the inhibitor on the insoluble enzyme resin may also take place. Hochstrasser, Muss and Werle (1967) and Hochstrasser, Illchman and Werle (1969) obtained a mixture of modified inhibitors and native inhibitor while purifying trypsin inhibitors from maize extract, wheat and rye germs.

PURIFICATION OF SPECIFIC PAPAIN INHIBITORS

Chicken egg white: (Fossum and Whitaker, 1968; Sen and Whitaker, (1973).

Purification of the egg white inhibitor was carried out by conventional purification techniques. The initial step consisted of the removal of ovomucin and some other proteins by adding sodium chloride. This was followed by 50% ammonium sulfate precipitation, Sephadex-G-75 chromatography and CM-cellulose chromatography, which yielded a fraction with a specific activity of 3.22 units/mg of protein (1100-fold purification). The CM-cellulose fractions were found to be unstable to lyophilisation in dilute solutions. The yield was about 7% of the initial inhibitor. The preparation was not homogeneous on disc-electrophoresis. Sen and Whitaker (1973) improved the purification procedure and obtained an electrophoretically pure material. Concentration of the material was carried out by ultrafiltration. A linear gradient pH elution on a CM-cellulose column was carried out, which gave a homoegeneous material. The final specific activity was 5.3 units/mg of protein where a unit was equivalent to the inhibition of 10 µg of ficin according to Kunitz's caseinolytic method of assay. The inhibitor was purified 2130-fold.

The purification of the inhibitor by affinity chromatography was attempted by Sen and Whitaker using ficin bound to CM-cellulose. But none of the methods used to dissociate the complex such as treatment with 9 M urea at pH 3, 20% glycerol or with tetrathionate were successful.

Purification of a papain inhibitor from the healing skin of Arthus type inflammation in rabbits:(Udaka and Hayashi, 1965)

The inhibitor was isolated from acetone pretreated skin. The acetone powder extract was fractionated with ammonium sulfate and then by chromatography on Sephadex G-50 and DEAE-cellulose. Concentration was carried out using 20% polyvinyl pyrrolidone. The specific activity of the purified material was 1700 units/mg of protein using the caseinolytic assay. The preparation was homogeneous on paper electrophoresis. The yield was about 3% of the initial activity. The inhibitor was purified 212 fold.

Section VII

General properties

Stability:

The most remarkable property of this class of proteins is their stability to the usual denaturing agents. Several inhibitors e.g. Kunitz's (Kunitz and Northrop, 1936) and Kazal's pancreatic inhibitors, (Laskowski and Wu, 1953), bovine and porcine colostrum inhibitors (Laskowski and Laskowski, 1951; Laskowski, Kassel and Hagerty, 1957) and the various bean inhibitors (Vogel et al., 1968) retain their activity even after exposure for several minutes to 2-3% trichloroacetic acid at elevated temperatures (95°C). Kunitz's soybean trypsin inhibitor (Kunitz, 1947) and serum inhibitors are exceptions (Laskowski and Sealock, 1971). They are heat and acid labile. Kazal's pancreatic inhibitor and Bowman Birk's inhibitor from soybean are unaffected by 90% ethanol. Many of the above inhibitors are stable to denaturation by urea at neutral pH and room temperature. With a few exceptions, these proteins are unique in their stability and possess a rigid structure. Laskowski suggests that the instability of serum inhibitors may be due to the lack of cystine residues. The cross linkage by numerous cystine residues contributes to the rigidity of the molecule. The labile nature of Kunitz's soybean trypsin inhibitor (Kunitz, 1947) may arise from the fact that it contains only 4 half-cystine residues in 148 amino acid residues. In contrast other stable inhibitors contain a higher percentage of cystine moieties. Most of them contain 9-15% half cystine. The lima bean inhibitor component 2 and

the Bowman-Birk inhibitor from soybean (Frattali, 1969; Jones, Moore and Stein, 1963) contain 14 half-cystine residues. Bromelain inhibitors were also found to have a high half-cystine residue content ranging from 8-9 in about 52 amino acid residues (Reddy et al., 1975). However unpublished work from this laboratory has shown that heat and acid stable subtilisin inhibitor from Vigna catjang has only one mole of half-cystine per mole of inhibitor and a low proline content. The stability of these inhibitors may be due to factors other than disulfide crosslinking (Vartak, 1975).

Most of the inhibitors are relatively stable to degradation by proteolytic enzymes. The polyvalent bovine proteinase inhibitors are resistant to pepsin digestion (Vogel et al., 1968). The Bowman-Birk inhibitor from soybean is also stable to pepsin action (Vogel, et al., 1968). On the contrary Kunitz's soybean trypsin inhibitor is readily attacked by pepsin (Kunitz, 1947). However as in the case of other denatured proteins, denatured inhibitors are readily acted upon by proteolytic enzymes. It has been suggested (Laskowski and Sealock, 1971) that the secondary structure is responsible for this stability.

Molecular weight:

The molecular weights of proteinase inhibitors usually range from 6000 to 60,000, most being around 20,000. The serum inhibitors are of high molecular weight. The plant inhibitors have usually molecular weights of 6,000 to 20,000. However the isoinhibitors active against carboxypeptidase from potato have molecular weights ranging from 3,000 to

4,000 (Rancour and Ryan, 1968). They are dialyzable polypeptides. Inhibitors of the Streptomyces species are of very low molecular weight (500-1200) (Umezawa, 1972).

Amino acid composition:

The amino acid composition of several inhibitors has been determined. The common occurrence of cystine residues in inhibitors has already been discussed earlier. Another feature common to the inhibitors is the presence of numerous prolyl moieties in the molecule, especially near the active centre. It has been suggested that prolyl residues (Laskowski and Sealock, 1971) also add to the rigidity of the molecule.

The similarity of the properties of several inhibitors suggests the presence of a similar conformational structure, which accounts for the stability of the molecule. Jirgensons Ikenaka and Gorguraki (1960) compared the optical rotatory dispersion spectra in the visible spectrum of four lima bean fractions, Kunitz's soybean inhibitor and chicken ovomucoid inhibitor. The results showed that the latter two are quite distinct from the former. The optical rotatory dispersion spectra (visible and ultraviolet) of the 1.9S inhibitor from soybean and the above inhibitors were compared by Ikeda, Hamaguchi, Yamamoto and Ikenaka (1968). The inhibitors were found to differ from each other, both quantitatively and qualitatively.

However the trypsin inhibitors have been found to contain similar reactive sites. The details of the reactive site will be discussed in a later section.

Carbohydrate content:

The serum trypsin inhibitors are known to contain varying amounts of carbohydrate groups 10-20%. The inhibitors from egg white, other than the papain inhibitor, also have carbohydrate moieties. However the only report of a plant proteinase inhibitor having a carbohydrate group is that of the bromelain inhibitor from pineapple stem (Reddy et al., 1975).

Inhibitor constant K_i (Association or Dissociation constant):

Kinetics:

The dissociation constant (K_i) is a quantitative expression to denote the affinity of an inhibitor for an enzyme. It is given by the equation $K_i = \frac{(E)\ (I)}{(EI)}$ where E, I and EI represent enzyme, inhibitor and enzyme-inhibitor complex respectively. The complex is generally found to be labile with decreasing pH, until dissociation is practically complete below pH 3 (Steiner, 1954). The dissociation constants of many of the inhibitors have been determined. Most of the complexes have a dissociation constant around 10^{-3} to 10^{-10}M, whereas enzyme substrate complexes generally have much higher dissociation constants.

Stoicheiometric ratio:

This designates the number of molecules of the enzyme combining with a molecule of the inhibitor. The molar combining ratio for trypsin and the lima bean inhibitor, was found to be 1:1 (Ryan, Clary and Tominatsu, 1965). The ratio was the same for the egg white ovomucoid inhibitors and trypsin. However it has been shown that in addition to the 1:1 complex 1 mole of

the above inhibitor can also combine with two moles of trypsin. The 1:1 complex (K_i = 5.8 x 10^{-9}M) was found to be more stable than the 1:2 complex (K_i = 2.5 x 10^{-6}M) (Vogel et al., 1968).

Trypsin derivatives such as polytyrosyl trypsin and poly DL alanyl trypsin also combine with the inhibitors. The combining ratio of SbI-poly-DL-alanyl trypsin was found to be 2:1 while with serum trypsin inhibitors it was shown to be 1:1. Thus the tyrosyl and alanyl residues offer no steric-hindrance to inhibition (Epstein,Anfinsen and Sela, 1962).

Specificity of inhibitors towards enzymes and substrates:

Most inhibitors combine with more than one enzyme. They generally react with closely related enzymes. Such inhibitors are designated as polyvalent or broad spectrum inhibitors. Most trypsin inhibitors act on chymotrypsin and vice versa. The potato inhibitor type I inhibits, trypsin, chymotrypsin, subtilisin, kallikrein and pronase. The inhibitor from barley inhibits chymotrypsin, subtilisin and an alkaline proteinase of A.oryzae, S. griseus, and Alternaria tenuissima (Mikola and Suolinna, 1971). The subtilisin inhibitors from Vigna catjang are specific for subtilisin. They do not bind trypsin and chymotrypsin (Vartak, 1975). It has been shown that when an inhibitor acts on two enzymes, such as trypsin and chymo-trypsin, the inhibitory sites are usually distinct. Examples are the lima bean inhibitor (Ryan and Clary, 1964), turkey and duck ovomucoid (Rhodes et al., 1960) and Vigna sinensis inhibitor (Ventura and Filho, 1966). These inhibitors were termed "double headed" (Rhodes et al., 1960). The Kunitz

bovine pancreatic trypsin inhibitor was found to be an exception. The same reactive site was involved in the inhibition of both trypsin and chymotrypsin (Wu and Laskowski, 1955; Kraut and Bhargava, 1967). Another example is the papain inhibitor from chicken egg white which binds both papain and ficin at the same reactive site of the inhibitor. The papain inhibitors from Vigna catjang were also shown to bind ficin and papain at the same reactive site of the inhibitor (Vartak, 1975).

Substrate specificity:

The polyvalent inhibitor from the potato (type I) was found to inhibit trypsin only when casein was used as the substrate. No inhibition was observed when a synthetic ester substrate was used. It is devoid of esterolytic inhibitory activity (Ryan, 1966). In another instance (barley grain inhibitor) amidase activity is inhibited predominatly. The inhibitor showed five times more anti-chymotrypsin activity with a synthetic substrate than with a protein (Mikola and Suolinna, 1971).

Another interesting observation was made by Feeney, Means and Bigler (1969). Some of the trypsin inhibitors differentiated between bovine trypsin and human trypsin, by selectively inhibiting one of them. Trypsin inhibitors from lima bean, soybean (Birk) and Kunitz's bovine pancreatic inhibitor inhibit both human and bovine trypsin, whereas the trypsin inhibitors from the ovomucoids of chicken and turkey inhibit bovine trypsin and not human trypsin.

Temporary inhibition:

Gorini and Audrain (1953) observed that tryptic activity reappeared upon prolonged incubation of the inhibitor-enzyme complex. A detailed study was made by Laskowski and Wu (1953) of this phenomenon which they termed "temporary inhibition". The chicken ovomucoid and possibly all the tested mammalian (Gorini and Audrain, 1953) pancreatic secretory inhibitors (Kazal) (Tschesche and Klein, 1968; Green, Rigbi, and Fackre, 1966) and several derivatives of Kunitz's pancreatic trypsin inhibitor (Kress, Wilson, and Laskowski, 1968) (where the cys 14-cys 38 disulfide bridge was selectively cleaved and cysteinyls modified) were found to be temporary inhibitors. The unmodified pancreatic Kunitz inhibitor is not a temporary inhibitor and was found to be resistant to proteolytic activity.

Further detailed study of the pancreatic trypsin inhibitor with respect to temporary inhibition was carried out by Tschesche, Klein and Reidel, (1970). They showed that the inactivation of the inhibitor proceeded via formation of small peptides. One of the early reactions they showed was the hydrolysis of the bond Arg^5-Glu^6 thus generating des-Thr-Ser-Pro-Glu-Arg inhibitor which is still active. By means of kinetic data they established that the cleavage of the reactive site peptide bond precedes the cleavage of the other arginine or lysine bonds. The exact number and sequence of other bonds broken have yet to be determined.

Tschesche *et al*. (1970) explain the physiological

significance of temporary inhibition on the basis that trypsinogen activated prematurely is inhibited. After secretion into the duodenal tract, however, the trypsin may be liberated and used for digestion of food proteins whereas the inhibitor is degraded and absorbed.

Cleavage of enzyme inhibitor complex:

Various methods have been used by workers to separate the inhibitor and the enzyme from the complex. The classical method of Kunitz (1936) using boiling trichloroacetic acid for the bovine polyvalent trypsin inhibitor is well known. The enzyme is denatured by boiling trichloroacetic acid thus setting the inhibitor free.

Another useful technique is the use of gel exclusion chromatography at low pH if the inhibitor is stable to acid. This method was used in the case of the trypsin-ovomucoid inhibitors (Schrode, cited from Laskowski and Sealock, 1971). 6 M guanidine hydrochloride at pH 7 followed by gel chromatography has been used to separate-soybean trypsin inhibitor from its complex (Sealock and Laskowski, 1969). Sulfoethyl sephadex has been used recently to separate the soybean trypsin inhibitor from its complex at pH 2.6 (Papaioannou and Liener, 1970).

However the above are examples of the inhibitor being obtained active and free from the complex. There are very few instances or reports of the active enzyme being released from the complex. Hochstrasser et al.(1968) have used thioethanol to inactivate the inhibitor and release the active enzyme. Sodium dodecyl sulfate has selectively been used

by Hercz (1973) to inactivate blood α_1, trypsin inhibitor from its complex with trypsin. Sato et al. (1975) immobilised a subtilisin inhibitor on Sepharose and used it for the purification of subtilisin. The complex was cleaved with 0.5% SDS at pH 7.5 and the SDS was removed effectively from the solution by use of Dowex-2. The recovery of subtilisin was 30%. However this treatment partially destroyed the ability of the bound inhibitor to combine with subtilisin again. The same authors have also used 6 M urea and 3 M guanidine HCl, but the recovery of the enzyme was only 35%.

The development of suitable methods for the recovery of the enzyme from the complex is of importance. It is possible that some inhibitors occur in combination with endogenous enzymes and the recovery of the enzymes free from inhibitors will facilitate the purification and study of their properties. The lability of the inhibitors to proteinase action at low pH made it possible to separate the inhibitors and proteinases of yeast as discussed earlier (Lenney et al., 1974).

PROPERTIES OF PAPAIN INHIBITORS

Not much work has been done on the properties of papain inhibitors, except for the specific papain inhibitors of chicken egg white (Fossum and Witaker, 1968; Sen and Whitaker, 1973) and rabbit skin, which will be discussed in this section.

Chicken egg white:

Molecular weight:

The molecular weight of the inhibitor determined by gel filtration was found to be 12,700. The ficin-inhibitor complex had a molecular weight of 24,600 and the mercuric papain-inhibitor complex 25,300. Since the molecular weight of papain is 21,000 the reason for the low values for the molecular weights of the complexes is not known.

Amino acid composition:

The amino acid composition of the papain-ficin inhibitor was found to be different from that of the ovomucoid and ovoinhibitor. A striking feature was the higher content of serine, glutamic acid, methionine, isoleucine, leucine and arginine in the papain inhibitor compared to the other egg protein inhibitors and the lower threonine, cystine and histidine content.

Carbohydrate:

The papain inhibitor contains no carbohydrate moiety. In this respect it differs markedly from the other chicken egg white inhibitors (trypsin, chymotrypsin etc.), which contain appreciable amounts of carbohydrate.

Stability:

At 35°C the inhibitor was stable for 30 min at pH 4.9. A slow loss of activity of a 5×10^{-5}M solution of inhibitor at pH 7 (0.2 M phosphate) at 30°C occurred. An unusual pattern of heat stability was observed. The first 10 min of heating resulted in a 40% loss in activity. Thereafter the

inhibitor was found to be stable to heating for a further period of 50 min. When the solution was reheated at 80°C a further loss of activity was observed in 5 min. Additional heating for 20 min more did not result in any further loss of activity. The inhibitor lost about 64% of its activity at 100°C within a period of 3 min but continued heating did not result in any further loss.

Effect of pH:

Combination of the inhibitor with ficin was found to be independent of pH, over the range 4-9.

Dissociation constant of ficin inhibitor complex:

The constant was found to be $1.47 \pm 0.68 \times 10^{-8}$M by equilibrium inhibitory studies. By kinetic studies involving the effect of substrate concentration it was found to be $1.32 \pm 0.43 \times 10^{-8}$M. The constant was found to be lower than that of the chicken ovoinhibitor complex (4×10^{-8}M). The dissociation rate constant for the complex was found to be $6.3 \times 10^{-2} sec^{-1}$ (t 1/2 = 11 secs.).

Mechanisim of complex formation:

According to Sen and Whitaker (1973) the forces involved in complex formation are not electrostatic, but probably hydrophobic. Hydrogen bonding may be involved to some extent. The above kinetic data indicate the formation of a strong complex. Secondly the dissociation rate constant was found to be in the order of (t 1/2 = 11 secs.) which is very slow.

As mentioned earlier Laskowski's theory required the participation of an active enzyme. If it is so, the -SH group

of the enzymes ficin and papain is essential. However in an earlier paper Fossum and Whitaker (1968) showed the combination of mercuric papain with the inhibitor. Later Sen and Whitaker have shown that carboxymethylated ficin (enzymatically inactive) combines with the inhibitor. Gel filtration and competitive binding studies indicated that the alkylated enzyme-inhibitor complex had a lower dissociation constant. The ratio of combination between the inhibitor and the enzyme was found to be 1:1 (for both native and alkylated ficin). Keilova and Tomasek (1974) observed that the mercury derivative of cathepsin B combined effectively with the chicken egg white papain inhibitor. The above observations contradict the assumption that participation of an active enzyme is an essential prerequisite for complex formation.

Inhibitor isolated from the healing skin of Arthus-type inflammation in rabbits: (Udaka and Hayashi, 1965)

Properties:

Appearance:

The inhibitor had a fibrous appearance when viewed under the microscope. The purified inhibitor was found to be homogeneous on ultracentrifugation and paper electrophoresis at pH 3.85 and 6.8. Its sedimentation co-efficient was 1.2S by the moving boundary technique. The ratio of absorbance at 276 nm/260 nm, was found to be 1.61 which indicates the absence of nucleic acids.

Stability:

The inhibitor was found to be stable at 80°C for 30 min. At 100°C it was stable for 5 min. At -5°C there was no loss

in activity in 7 days. The inhibitor was not precipitated by 3% trichloroacetic acid and was insoluble in water and soluble in salt solutions.

Specificity:

Besides papain it inhibited an SH dependent proteinase responsible for the cutaneous lesion of Arthus type inflammation. It had no effect on trypsin and chymotrypsin. It also inhibited an -SH proteinase released in the antigen antibody reaction in tissue culture, but had no effect on cellular proteinase released on irradiation.

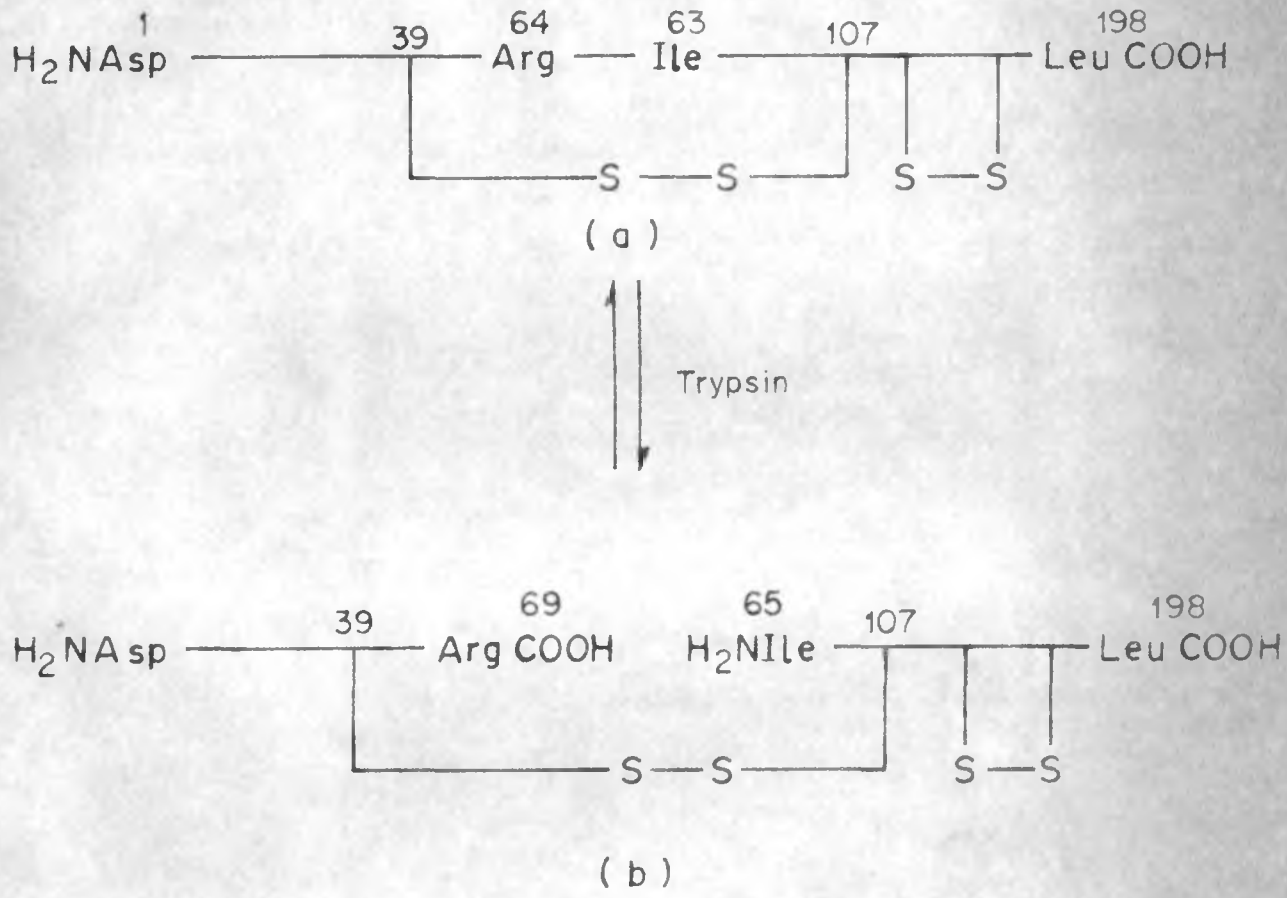
The properties of non-specific inhibitors will not be described as most of the property studies pertain to trypsin or other unrelated enzymes.

Section VIII

Active centre and mechanism of action

The elucidation of the mode of action and active centre of trypsin inhibitors is a major advance in this field. It has been suggested that the specific interaction between trypsin and trypsin inhibitors involves the cleavage of a peptide bond in the active centre of the inhibitor, (but see below for a discussion of this hypothesis). It has been demonstrated that the trypsin inhibitors have either an arginine or lysine residue at the active centre. The mechanism will be dealt with later. (See page 45).

Chemical modification of inhibitors by specific reagents has facilitated the classification of trypsin inhibitors into lysine or arginine inhibitors (Liu, Feinstein, Osuga, Haynes and Feeney, 1968). Since trypsin does not act on lysyl bonds where the ϵ-amino group of lysine is modified (acetylated, succinylated, maleylated, deaminated, or teated with trinitrobenzene sulfonic acid), modification of the lysine moiety at the active centre of the inhibitors would destroy its activity. As a result only the arginine inhibitors will be acted upon by trypsin. Similarly modification of the arginine moiety by reagents such as 1,2-cyclohexadione (Liu, et al., 1968), 2,3-butadione (Fritz, Fink, Gebhardt, Hochstrasser and Werle, 1969) will render the inhibitor inactive. Trypsin will then selectively act on lysyl containing inhibitors. Thus it is experimentally possible to classify the trypsin inhibitors into lysine or arginine inhibitors.



(a) ACTIVE CENTRE OF KUNITZ'S SOYBEAN TRYPSIN INHIBITOR

(a→b) CONVERSION OF VIRGIN TO MODIFIED SOYBEAN TRYPSIN INHIBITOR

OZAWA AND LASKOWSKI (1966)

The inhibitor with the bond intact is known as the virgin inhibitor and the one with the cleaved bond is referred to as the modified inhibitor. Haynes and Feeney (1968) observed that treatment of the modified inhibitor (Soybean trypsin inhibitor, Kunitz) with trinitrobenzene sulfonic acid, leads to a rapid loss of inhibitor activity. The virgin inhibitor was unaffected. It was therefore suggested that the newly formed NH_2 (i.e. the NH_2 from the active centre) should be free, if the modified inhibitor is to be active. Kowalski and Laskowski Jr. (1972) report that maleylation, citraconylation and carbamylation of the newly formed NH_2 terminal of the modified soybean trypsin inhibitor (Kunitz) or chicken ovomucoid leads to loss of inhibitor activity. Secondly if the modified inhibitor is to be active, then the peptide chains of the modified inhibitor must be held in a definite conformation relative to each other by the disulfide bridges.

Enzymatic replacement of residues at or near the active site:

The presence of certain common characteristics among the trypsin inhibitors suggest that the replacement of the arginine moiety at the reactive centre by a lysine molecule or *vice versa* should not alter the inhibitor activity. This has been studied in detail by Laskowski Jr. and Sealock (1969). Kunitz's soybean trypsin inhibitor was used for these studies. The virgin inhibitor was incubated with catalytic quantities of trypsin at pH 3.75 to get the modified inhibitor. The modified inhibitor was then treated with carboxypeptidase B at pH 7.6 to get the des-arginine inhibitor. The des-arginine inhibitor was devoid of inhibitor activity. In the

next step the des-arginine inhibitor was incubated with trypsin, carboxypeptidase and 0.1 M lysine at pH 6.7 for a few days to synthesize the arginine inhibitor which combines with trypsin. The synthesised complex designated as C_2, was then separated by sephadex chromatography and subjected to kinetic control dissociation in 6 M guanidine HCl to release the new lysine containing inhibitor. Studies on the (Lys 64) inhibitor have shown that the substitution of arginine by lysine does not alter the thermodynamic properties of the inhibitor.

Enough data are not available on the reactive site of other inhibitors. Chymotrypsin acts on the tryptophan -x or tyrosine -x or phenylalanine or leucine-x peptide bonds. When the chymotrypsin (Bowman Birk) inhibitor (Frattali and Steiner, 1969) was teated with catalytic quantities of chymotrypsin, a modified inhibitor was produced. When the modified inhibitor was treated with carboxypeptidase A, it lost its activity.

Krahn and Stevens (1970) determined the reactive site of the chymotrypsin lima bean component 3 inhibitor. It was found to be Ser-Thr-Leu-↓Ile-Pro. An interesting feature is the presence of 3 hydroxy amino acids, surrounding the active site leucine.

Leary and Laskowski (1973) recently reported a change from tryptic to chymotryptic specificity in the case of the soybean trypsin inhibitor (Kunitz). They report that the reactive site arginine or lysine can be replaced by tryptophan, tyrosine or leucine or phenylalanine thereby converting it into

a chymotrypsin inhibitor. The method is similar to the arginine → lysine conversion. The virgin Trp^{64} inhibitor inhibits chymotrypsin very effectively and trypsin is inhibited weakly if at all.

The reactive sites of other enzyme inhibitors such as those of papain and subtilisin are not known.

Mechanism of action of inhibitors:

The mechanisim of interaction between proteinases and their inhibitors will be disscussed only briefly since the work in this field is too extensive for a comprehensive review. Two theories have been put forward for the mechanisim of action of these inhibitors - the first being peptide bond splitting by the active enzyme at the active site of the inhibitor and the second being the absence of a requirement for an active enzyme.

Kunitz and Northrop showed that the reaction between a proteolytic enzyme and its inhibitor results in the formation of an inactive complex. The complex was stable at neutral pH and in acid solution dissociated into the individual components which retain their respective properties.

The inhibitors contain an active centre which combines with the enzyme. It is the configuration of the binding site of the inhibitor which probably gives its specificity. The trypsin inhibitors were found to contain an arginine or lysine residue at the active centre. The chymotrypsin inhibitor from lima bean (compenent III) was found to contain a leucine residue at the active centre[Krahn and Stevens (1970)].

Bond splitting mechanism:

Laskowski Jr. has postulated that cleavage of the peptide bond at the active centre by the proteinase first occurs, and that it is subsequently followed by the formation of the complex. The inhibitor with the bond intact is called the virgin inhibitor and the inhibitor with the bond cleaved is called the modified inhibitor. Ozawa and Laskowski (1966) proposed the following as the reaction scheme for the soybean trypsin inhibitor. (See Fig. on page 45).

Their theory is supported by the following. They showed that in the case of the soybean trypsin inhibitor and chicken ovomucoid the native inhibitor consists of a single chain whereas the modified inhibitor due to a bond cleavage consists of two chains held by s-s bridges. Reduction of the disulfide bridge leads to loss of activity of the modified inhibitor. It was also shown that the removal of the newly formed terminal NH_2 group by carboxypeptidase or modification of the NH_2 group leads to a loss of the modified inhibitor activity. Similar results in support of peptide cleavage were obtained by other workers (Birk et al., 1967; Frattali and Steiner, 1969; Rigbi and Green, 1968).

Finkenstadt and Laskowski (1967) showed that the modification was reversible. The complex was prepared by using modified soybean trypsin inhibitor and trypsin. The complex was dissociated under drastic conditions (very low pH and 6 M gauinidine HCl) when virgin inhibitor was obtained predominantly. They also showed that the complexes prepared from

virgin or modified inhibitor and trypsin were indistinguishable on disc gel electrophoresis. Therefore the following mechanism was proposed by them

$$T + I \rightleftharpoons L \rightleftharpoons C \rightleftharpoons L^* \rightleftharpoons T + I^*$$

where I and I* are native and modified inhibitors and C is the stable complex and L and L* are loose complexes of the Michaelis-Menten type involving non-covalent forces. The reactions on the left are faster than the reactions on the right. This results in the predominant release of virgin inhibitor from the complex obtained with trypsin and virgin or modified inhibitor. They postulate that an acyl intermediate is formed between the newly formed COOH of the inhibitor and serine of the active site of the enzyme. A tetrahedral intermediate is also proposed. Reports from this laboratory have shown that the subtilisin inhibitor, once modified cannot be converted back to the native inhibitor at pH 7.5. This observation is different from the observed reconversion of the modified to the native inhibitor in the case of trypsin inhibitors. However there is no general agreement that the above mechanism is true for all proteolytic inhibitors.

Absence of peptide bond hydrolysis has been reported by other workers. Dlouha, Keil and Sorm (1968) have shown that there is no cleavage of the peptide bond by trypsin at the active site in the case of Kunitz and Northrop's bovine pancreatic trypsin inhibitor. In the case of turkey and cassowary ovomucoids the modified inhibitor retained its activity even after treatment with carboxypeptidase

(Feinstein et al., 1966). Secondly substitution of the newly exposed amino group with trinitrobenzene sulfonic acid should lead to a loss of trypsin inhibitor activity. However this was not true in the case of all the inhibitors. Uy and Feeney (1970) modified penguin and turkey ovomucoid with either α-chymotrypsin or subtilisin. The newly exposed amino groups of the modified inhibitor were then substituted by dimethylation and tested for inhibitor activity. No loss of inhibitory activity was detected. It is possible that substitution of the H of the NH_2 by methyl groups does not always result in loss of activity.

The reactive site hydrolysis theory requires the participation of an active enzyme. However many authors have reported that inactive enzymes are also capable of combining with inhibitors. Foster and Ryan (1965), Feinstein and Feeney (1966), Fossum and Whitaker (1968), Feeney (1971), Ryan (1973), Sen and Whitaker (1973) have shown that inactive derivatives of the enzymes trypsin, chymotrypsin, subtilisin and papain such as TLCK-trypsin, TPCK-chymotrypsin, anhydrochymotrypsin, substituted subtilisin (serine substituted by tosylation), mercury papain and alkylated ficin combine with inhibitors to form complexes. However these results only indicate that non-covalent forces are involved in complex formation and do not rule out peptide bond splitting and covalent bond formation with active enzyme.

Modification of the lysine and arginine residues by acetylation, succinylation or by treatment with reagents

such as 2,3-butadione should render the inhibitors inactive since trypsin does not act upon lysine or arginine groups when the ϵ-amino group is modified. However, Kassel and Chow (1966) found that when the pancreatic trypsin inhibitor (Kunitz) was guanidinated and amidinated it still retained its inhibitor activity. Other inhibitors which show inhibitor activity on guanidination or amidation or treatment with reagents such as 2,3-butadione. 1,2-cyclohexadion are turkey, cassowary and duck ovomucoids. In the case of the lima bean inhibitor the modified (guanidinated) inhibitor was found to inhibit trypsin more rapidly than the untreated inhibitor (Haynes and Feeney, 1968).

According to Feeney the formation of the complex does not require the hydrolysis of a bond and the principal non-covalent forces in protein interaction are involved in complex formation. The specificity of an inhibitor is dependent on the side chain of the amino acids present at the binding site of the inhibitor. Catalyses and bond splitting are however secondary and not essential.

Each of the two theories (bond splitting and non splitting hypothesis) therefore seems to be applicable only to some inhibitors. Further work is needed with other inhibitors to clarify their mechanism of action. An insight into this interesting protein - protein combination may be possible when X-ray structures of a number of proteinase-inhibitor complexes are determined.

Section IX

Physiological role of proteinase inhibitors

The wide spread occurrence of proteinase inhibitors suggests an important role for these proteins. The role of inhibitors in animal tissues is better understood than in the case of plants. There are several theories regarding the role of plant inhibitors, which lack experimental support.

It has been suggested that the function of the pancreatic inhibitor is to inhibit prematurely activated trypsin. It is thought that trypsin is an activator of other zymogens present in the pancreas. Trasylol (polyvalent inhibitor) is used as an effective agent in pancreatitis (Vogel et al., 1968). It may also have a role in the control of a trypsin-like enzyme responsible for the conversion of proinsulin to insulin (Frank and Veros, 1968).

In blood the inhibitors may have a role in the clotting of blood. Some of the blood clotting enzymes are inhibited by two serum inhibitors. Individuals genetically definient in serum inhibitors were shown to be susceptible to lung and liver disorders such as pulmonary emphysema (Erickson, 1964) and liver cirrhosis (Sharp, Bridges, Kirvit and Friuer (1969). Chan and Rees (1975) have shown that the deficient state is associated with a lower content of sialic acid in the proteinase inhibitor. These patients were found to have a correspondingly lower sialic acid transferase activity. The two proteins i.e. the normal and the protein from deficient patients were found to differ in the content

of other carbohydrate groups also.

Alpha-1-antitypsin deficiency - liver disease:

Eriksson and Larrson (1975) have also recently reported that deficiency of serum alpha-1-antitrypsin is associated with some cases of chronic obstructive disease and of fatal cirrhosis in the prenatal or juvenile period. Hepatocellular carcinoma has also been associated with a partial deficiency of the above inhibitor. The liver damage was found to be related to the accumulation of the PAS-positive material which is immunologically similar to the α1-antitrypsin but is deficient in a terminal sialic acid moiety. The inability to add sialic acid was shown by Kuhlenschmidt, Yunis, Lammarino, Turco, Peters and Glew (1974) to be due to a deficiency of sialyl transferase. Therefore it is likely that patients deficient in the above two factors may be more prone to liver diseases, indicating that inhibitors may have an important regulatory role at the cellular level.

Role of a proteolytic inhibitor in protein synthesis:

Taber, Wertheimer and Golrick (1973) have shown that TPCK (1-chloro-4-phenyl-3 tosylamido-2 butanone) inhibits the cleavage of a large polypeptide chain synthesised in uninfected Hela cells. Earlier reports by Taber, Rekosh and Baltimore (1971); Summers and Maizel (1971) and Rekosh (1972) showed that the newly synthesised viral protein of the picorna-virus group was cleaved into three products while still on the ribosomes. These were further cleaved to form ten proteins. The above cleavage was found to be reproducibly inhibited by TPCK which is a chymotrypsin inhibitor.

The above reports indicate that some inhibitors may have a more basic role in cell division and that all of them do not merely have a protective role as postulated in the case of the pancreas.

The pepsin resistant colostrum inhibitor is considered to prevent proteolysis of the milk antibodies in the digestive system of the new born (Laskowski and Laskowki, 1951).

The physiological function of the polyvalent typsin inhibitor which occurs in cattle in large quantities and in several organs is obscure. It is probably concerned with some regulatory function of enzymes in these tissues. According to Astrup (1968) the absence of respiratory diseases in cattle is related to the presence of these inhibitors in lungs.

A proteinase inhibitor acting on a range of enzymes, including microbial proteinases, was found in the submandibular and sublingual glands of foxes and cats. It is suggested that its function is to protect the animal against proteinases of the bacteria taken with the food, especially in stale flesh (Vogel et al., 1968).

A protective role for the inhibitors of egg white against bacterial action has been suggested (Feeney and Allison, 1969).

The inhibitors from Ascaris lumbricoides are thought to protect the organisim against proteinases of the host during passage through the intestines (Vogel et al., 1968). In the case of Streptomyces, the inhibitors are produced at the end of the fermentation process possibly as a defense

mechanisim to stem auto-digestion (Umezawa, 1972).

Relation between tumour growth and proteinase inhibitor:

It is known that normal cells in tissue culture cease to divide when a monolayer is formed. The above phenomenon is termed as contact inhibition. Virus transformed cells or tumour cells do not show contact inhibition. Sefton and Rubin (1970) have shown that when normal mouse fibroblasts are treated with proteolytic enzymes, contact inhibition is lost and the cells show surface properties characteristic of transformed cells. Hence the role of endogenous proteolytic enzymes in contact inhibition is of interest. The effect of proteinase inhibitors on transformed cells was studied by Schnebli and Burger (1972). The effect of TAME, (tosyl arginine methyl ester) TPCK and TLCK, soybean trypsin inhibitor and ovomucoid II were tested on the growth of transformed mouse (Py3T3, SV3T3 and 3T12) and hamster (Py3ISK) cells. They found that there was a selective inhibition of the growth of transformed cells by the proteinase inhibitors. The restoration of contact inhibition by proteinase inhibitors indicates that a proteolytic enzyme is involved in the transformation of normal cells. Thus protein inhibitors may have a regulatory function and their role in tumour growth deserves further study.

The presence of three distinct and different proteinases and their corresponding inhibitors in Saccharomyces cerevisiae (Lenney, Matile, Wiemken, Schellenberg and Meyer (1974) has already been discussed in Section IV. The

proteinases are present in the vacuole while their inhibitors are localized in the extravacuolar cytosol. The role postulated for these inhibitors is that they protect the extravacuolar proteins from the accidental leakage of proteinases from the vacuoles. Secondly it is proposed that the proteinases are transferred after their synthesis on the cytoplasmic polysomes to the vacuoles as proteinase-inhibitor complexes and thus play a role in transportation of enzymes.

Uses:

Certain pathological conditions such as inflammation, pancreativ necrosis and shock are influenced by the premature or uncontrolled activity of proteolytic enzymes. Trasylol (bovine polyvalent inhibitor) has shown promising results in the treatment of early pancreatitis (Thompson, 1968). It has low toxicity and is a broad spectrum inhibitor. It has also been used in the treatment of surgical hemorrhages (Amris, 1966; Matis and Morl, 1968). The mortality rate is reduced in experimental shock caused by burn and trauma (Back, 1966).

The low molecular weight inhibitors from the culture broths of Streptomyces strains (Umezawa, 1972) have found clinical applications. They are of low toxicity and are absorbed when given orally. Leupeptin shows an anti-inflammatory effect and has been 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. Pepstatin was shown to be effective in suppressing stomach ulcers of pylorus-ligated rats.

Specific proteinase inhibitors can be used as a suitable analytical test to distinguish between different proteolytic enzymes present in tissue extracts.

Physiological role of plant proteinase inhibitors:

The role of proteinase inhibitors in plants has remained obscure. The following are some of the theories about their role.

Regulatory agents in controlling endogenous proteinases:

In 1965 Shain and Mayer (1965; 1968) reported that a proteinase inhibitor in lettuce seeds inhibits an endogenous proteinase. Recent investigations by the authors showed the enzyme to be an endopeptidase which has a trypsin like specificity. With the onset of germination the inhibitor disappeared. This suggests that the enzyme was in a cryptic form or that the enzyme was activated from its zymogen state by an activator and that the inhibitor is complexed. Ungerminated barley extracts were shown to contain an endogenous endopeptidase which exhibits a similar pattern of activity with the commencement of germination (Kirsi & Mikola, 1971; Mikola and Enari, 1970).

As stated in an earlier section, in Vigna sinensis the trypsin inhibitor activity of the cotyledon falls continuously during germination to a value which corresponds to 5% of the value for dry seeds. The axial parts were shown to control the disappearance of the inhibitor activity. It was shown by Filho et al,(1973) (unpublished results) that the crude "inhibitor" fraction of Ventura and Filho (1966) weakly

inhibits an endogenous proteinase. The extract of Vigna unguiculata was shown to contain two types of proteinases, one hydrolysing BAPNA and the other digesting casein. The proteinase acting on casein was shown to be inhibited weakly by trypsin inhibitor from the extract (Royer et al., 1974).

A carboxypeptidase B and ∝-chymotrypsin inhibitor from potatoes (Rancour and Ryan, 1968; Ryan, 1971) was found to inhibit the bradykinin inactivating carboxypeptidase from potato. It is thought that the inhibitor may have a regulatory function in dormant potatoes.

Plant protection:

It has been suggested that the proteinase inhibitors may have a protective function against invading microorganisms. They act on proteolytic enzymes produced by the invading bacteria and thus prevent cell wall hydrolysis.

Applebaum (1964) proposed that proteinase inhibitors of legumes may have an important role as a defensive mechanism against insects. In 1947, Mickel and Standish (1947) observed that larvae of certain pests showed abnormal growth when fed on soybean extracts. Lipke, Fraenkel and Leiner (1954) studied the effect of soybean inhibitors on the development of Tribolium confusum. They showed the inhibition of the digestive proteinases of the organisim by a specific inhibitor in soybeans. A tribolium inhibitor has also been reported from wheat (Applebaum & Konijn, 1966). Later reports showed the inhibition of Tenebrio molitor larval trypsin by lima bean inhibitor, ovomucoid and Bowman Birk inhibitor (Applebaum, Birk, Harpaz, and Bondi, 1964). The above results are very

similar to the inhibiting effect of soybeans and other legume seeds on the growth of animals.

Green and Ryan (1972) report that wounding of leaves of potato and tomato plants by adult Colorado beetle larvae induced a rapid accumulation of a proteinase inhibitor throughout the aereal parts of the plant. This was stimulated by mechanical damage. They report the isolation of a proteinase inhibitor inducing factor which stimulates the production of an inhibitor on wounding of the leaves. This phenomenon was found to be temperature and light dependent. The authors therefore suggest the production of an inhibitor as a response by the plant against invading insects. This theory may be of considerable importance in the control of pests, if it is proved to be correct, and requires further study.

Storage:

Yet another role has been suggested by various authors. The inhibitors may have a storage role, as the inhibitor content of many seeds is high e.g. they represent about 6% of the soybean protein (Rackis and Anderson, 1964) and upto 10% of the soluble protein of barley grains (Mikola and Kirsi, 1972).

Chymotrypsin inhibitor studies showed that the inhibitor behaved as a storage protein (potato). The apical cortex of the tuber was shown to contain the highest levels of the inhibitors. When the seed pieces of potato tubers are planted, inhibitor (chymotrypsin) entirely disappeared within a few days as the new plants emerged and grew. The aereal

parts of the plants contained appreciable amounts of the inhibitor. When new tubers were formed, the inhibitors accumulated in the tubers with a concomitant decrease in the aereal parts. Ambe and Sohonie (1956) showed the presence of trypsin inhibitors in all parts of double and field beans at all stages of growth. The inhibitors decreased in concentration in the cotyledons and increased in plants during germination.

The role suggested by Mikola and Soulinna (1969) is that the inhibitors may have a role in the endozooic disposal of seeds. The seeds remain viable even after having been consumed by animals. It is thought that the inhibitors protect the seeds against the alimentary canal proteinases.

Thus the role of inhibitors in plants is not very clear. They may have a multipurpose function or different inhibitors may have varied functions.

Section X

PRESENT WORK

This work presents evidence for the presence of a papain inhibitor$^{(S)}_{\wedge}$ in a wide variety of plant tissues tested. The purification of four specific isoinhibitors of papain from Vigna catjang is described. A comparative study of the properties and kinetics of isoinhibitors of papain has been carried out. The purification of specific papain inhibitors from a plant source has not been reported so far except for a brief earlier report from this laboratory. The study of the properties and kinetics of the inhibitors include amino acid composition, molecular weight determinations, molar inhibition ratio, UV spectrum, mechanism of action, stability of the inhibitors under different conditions etc.

Chapter II deals with the materials and experimental methods used for these studies.

Chapter III deals with the occurrence of papain inhibitors in different plant tissues.

Chapter IV deals with the separation and purification of the specific isoinhibitors of papain from Vigna catjang.

Chapter V deals with the properties and kinetics of the isoinhibitors of papain from Vigna catjang.

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

Chapter VII contains a summary and conclusions of this work.

Chapter VIII gives a bibliography of the literature references cited in this thesis.

C H A P T E R II

M A T E R I A L S A N D M E T H O D S

Section I

Materials:

The chemicals used were of analytical grade. Reduced glutathione was from Reanal, Hungary and p-chloromercuric benzoate was from Fluka, Switzerland. Cysteine hydrochloride and Hammarsten's casein were from E.Merck, Germany. Hemoglobin was purchased from Serva Laboratories, Germany.

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,) BAEE, BAPNA, albumin (from bovine serum crystallised), cytochrome c, (from horse heart), myoglobin (from horse heart), trypsin inhibitor (from soybean, type I-S), trypsin (from bovine pancreas - Type III), α-chymotrypsin (from bovine pancreas - Type II), subtilisin (subtilopeptidase-A, from special strain of Bacillus subtilis, Type VIII), ficin (from fig tree latex, 2 x crystallised), and chymopapain. The method of Peterson and Sober (1956) was followed for the washing of celluloses.

Papain was obtained from Biochemicals Unit, Delhi. Pepsin (porcine origin, crystallised) was obtained from Armour and Company, USA. Insulin (crystalline) was purchased from B.D.H., England.

Sephadex G-200, G-100, G-75 and G-50 were obtained from Pharmacia Fine Chemicals, Sweden. Sephadex was kept on a boiling water bath as specified by the manufacturers and cooled for the required time before use.

The chemicals used for polyacrylamide gel electrophoresis (acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethyl ethylenediamine (TEMED) and Amido Black 10B) were obtained from Eastman Kodak Company, USA. Glycine was from Koch Light Laboratories, U.K.

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

Phosphate buffers used were of potassium salts, unless otherwise mentioned.

Seeds:

The various seeds were purchased from the local market and stored at 4°C. In the case of Vigna catjang seeds of various sizes were available, and only those batches in which the seeds were relatively big (average weight of each seed was 200 mg) were used.

The recent nomenclature (Verdecourt, 1970) includes Vigna catjang and Vigna sinensis under the same species, Vigna unguiculata. However they are further regarded as subspecies of V.unguiculata and are differentiated mainly by their pod size.

V.catjang = V.unguiculata: sub.sp. cylindrica - pod length - 7.5 - 12.5 cms.

V.sinensis = V.unguiculata: sub.sp. unguiculata - pod length - 20 - 30 cms.

According to the new nomenclature the particular seed which has been used for this work belongs to the sub-species

cylindrica. However in this thesis the old nomenclature Vigna catjang and Vigna sinensis will be retained.

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Section II

Methods:

Centrifugations were carried out at 0°C in an International Centrifuge (Model PR-1 and PR-2), Sorvall (Model SS-1) and Spinco (Model 1). Radiometer TTT 60 was used for enzymic titrations. Amino acid analysis was carried out in a Beckman 120C analyser. Asborbance measurements were carried out in a Beckman Ultraviolet spectrophotometer using 1 ml cuvettes with 1 cm light path.

The pH of ammonium sulfate solutions was determined after diluting them four times with water.

Chromatographic columns were packed under gravity by hydrostatic pressure. No external pressure was applied during the operation of the column. All purification operations were carried out in the cold, unless otherwise stated. All solutions were prepared with glass distilled water unless otherwise stated.

Ammonium sulfate precipitation:

Precipitation with ammonium sulfate was carried out at 0°C under constant stirring. The precipitates were collected by centrifugation or gravity filtration after keeping the solutions for a period of a minimum of one hour. The concentrations of ammonium sulfate calculated at 0°C were according to Jagannathan, Kartar Singh and Damodaran (1956).

The equation used for the addition of solid ammonium sulfate was

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

and the equation for addition of saturated ammonium sulfate solution was

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

where X is grams of solid ammonium sulfate to be added for every 100 ml. Y is ml of saturated ammonium sulfate solution to be added for every 100 ml. S_1 is the initial concentration and S_2 is the required saturation of ammonium sulfate at 0°C.

The precipitates were suspended in a known volume of the appropriate buffer. The increase in volume was assumed to be due to ammonium sulfate at the saturation at which the precipitate was obtained and a correction was made for the ammonium sulfate concentration in the enzyme or inhibitor, if used directly. Otherwise the solutions were dialysed exhaustively against the buffer employed. Dialysis was carried out at 4°C to 5°C. One volume of the material was dialysed approximately against 50 to 100 volume of the buffer used. A minimum of three changes were given in the case of small scale experiments. The external buffer was kept stirred. However in the case of large scale experiments several changes of the buffer were given. In the case of ammonium sulfate precipitates, the dialysates were tested for sulfate ions by using $BaCl_2$-HNO_3 solution. The period of dialysis ranged from 20 to 30 h.

Estimation of protein:

A modification of the Warburg and Christian method (1941) was used for the estimation of protein. A correction for

nucleic acid and ultra-violet absorbing impurities was made by the following equation (Jagannathan, Kartar Singh and Damodaran, 1956).

$$\frac{4}{7} \times \left(2.3\,(OD_{280} - OD_{340}) - (OD_{260} - OD_{340})\right) = \text{mg of protein per ml.}$$

It was assumed that a 0.1 percent protein solution has an absorbance of 1.0 at 280 nm for a 1 cm light path. Inhibitor solutions were diluted, if necessary, with 0.05 M phosphate buffer, pH 7.5. A buffer of the same composition was used as blank.

Protein was also determined, especially of purified preparations according to the method of Lowry, Rosenbrough, Farr and Randall (1951), using bovine serum albumin as standard. The concentration of serum albumin was calculated from its extinction co-efficient at 280 nm ($E^{1\,cm}_{1\%}$ - 6.6) according to Cohn, Hughes and Weare (1947). Inhibitor solutions, dialysed and free from ammonium sulfate and phosphate and other interfering impurities, were used. In each case the dialysate was also assayed for protein as a control. The Warburg method was used routinely to determine protein concentrations during the purification procedure.

Phosphorus:

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

Papain:

The purity of papain was determined with synthetic

substrates, BAPNA (benzoyl-DL-arginine p-nitroanilide) according to the method of Arnon (Arnon, 1965) and BAEE (benzoyl-L-arginine ethyl ester) by the pH stat method of Blumberg, Schechter and Berger (1970).

In the present work the purity of papain was calculated on the assumption that the papain purified by Kimmel and Smith (1954) was a pure preparetion. However Blumberg et al. (1970) in a recent report have shown that the above papain can be further purified two-fold by affinity chromatography. The percent purity of papain used for the present work ranged from 55 to 58% for different batches of papain. This corresponded to a specific activity of 3000-3200 units/mg of protein when assayed by Kunitz's caseinolytic method. The purity of papain was also checked electrophoretically. It was arbitrarily assumed that impurities present in the enzyme do not combine with the inhibitor. The absorbance of a 0.1% solution of pure papain at 278 nm is 2.5 for a 1 cm light path.

Papain used for affinity chromatography (purification of the inhibitor) was purified in this laboratory according to the method of Kimmel and Smith (1954). Papain was stored under nitrogen. Bound papain was converted to the mercuric form by adding $HgCl_2$ in Tris/HCl, 0.01 M, pH 7.5. The suspension was kept overnight and excess $HgCl_2$ was washed off with the above buffer.

Preparation of S-carboxyamidomethyl papain:

Papain was bound on Sephadex G-200 according to the method of Axen and Ernback (1971). Bound papain was activated with 0.01 M EDTA and 0.01 M cysteine, pH 7.5. The bound

material was then washed several times with 0.1 M phosphate buffer, pH 7.5 to remove cysteine. The material was then treated with freshly prepared iodoacetamide (1×10^{-2}M) in the presence of 0.3 M KCl. The reaction was carried out at 30°C in the dark. Excess iodoacetamide was washed off and the material suspended in 0.1 M phosphate buffer, pH 7.5. The suspension was tested for residual proteolytic activity, using casein. The carboxyamidomethyl derivative had no enzyme activity and was used for binding studies.

Papain purified in this laboratory as well as that purchased, were tested for chymopapain activity by lowering the pH to 2 and testing for proteinase activity (Papain is destroyed at pH 2 and below, while chymopapain is stable). There was no chymopapain activity in any of the samples of papain.

A stock solution of papain was prepared in 0.02 M reduced glutathione and 0.1 M phosphate (or Tris/HCl buffer), pH 7.5. Dilution of the stock solution was carried out with GSH buffer solution as and when required. Reduced GSH solutions were neutralised before use with 2 M $KHCO_3$. It was observed that the stock solution could be stored at -20°C for about two weeks under nitrogen without appreciable loss of activity.

Linearity of papain activity with concentration of enzyme and time:

As seen from Figs. 1 and 2 papain activity is proportional both to enzyme concentration and time. Linearity is observed when the increase in absorbance of the trichloroacetic acid filtrate is less than 0.550. The assay was carried out

according to the method of Kunitz.

Substrate:

A stock solution of casein was prepared by suspending 1 g of casein in 100 ml of 0.1 M potassium phosphate buffer, pH 7.5. The suspension was heated for 15 min. in a boiling water bath to dissolve the whole of the solid casein. The 1% casein solution was stored at -20°C and could be used for about 30 days.

Activation by cysteine hydrochloride:

Cysteine hydrochloride was neutralised with 2 M $KHCO_3$ immediately before use and then mixed with casein solution. The casein solution was 0.05 M with respect to cysteine.

Inhibitor solutions:

Inhibitor solutions dialysed against either phosphate or Tris/HCl buffer of appropriate concentration were used for assay. Suitable blanks were taken which are discussed below in detail.

Buffer:

0.1 M potassium phosphate buffer or Tris/hydrochloride buffer, pH 7.5 was used for the assay.

Kunitz's caseinolytic method: (Kunitz, 1947)

This method was used routinely for assaying papain inhibitor activity during purification of the inhibitor and for the study of some of its properties. The details of the method are as follows except when stated otherwise.

The assay system consisted of 10 mg of casein, 200 μmoles of potassium phosphate buffer, pH 7.5, 25 μmoles of cysteine, and the inhibitor solution and papain in a final volume of

2 ml. The final pH of the reaction mixture was 7.5 and the mixture was incubated at 35°C. The reaction was initiated by the addition of 1 ml of the substrate and the reaction mixture was incubated for 30 min. The reaction was stopped by adding 3 ml of 5% trichloroacetic acid. The tubes were kept for a minimum period of 30 min. and then filtered through Whatman No. 1 filter paper. The absorbance of the filtrate was determined at 280 nm. 3% trichloroacetic acid was used for dilution if the absorbance exceeded 0.700 (The concentration of trichloroacetic acid in the filtrate is 3%).

Blanks:

Trichloroacetic acid filtrates of casein, crude enzyme and inhibitor preparations contain 280 nm absorbing material, which will therefore interfere with the assay of proteolytic enzymes and inhibitors. In order to obtain the true readings, the readings of the above solutions at 280 nm will have to be subtracted, suitably to get the final value. The different blanks were run as follows.

Reagent Blank:

The reading obtained is mainly due to the substrate, casein or hemoglobin. The optical density for this blank for casein varied from 0.080-0.150. The reading was obtained by omitting the inhibitor and the enzyme from the reaction mixture.

Enzyme blank:

This blank was necessary for crude preparations of enzymes. It was obtained as follows. The enzyme was omitted

initially from the reaction mixture and was added to the reaction mixture after the addition of trichloroacetic acid. This blank is also referred to as the zero minute enzyme blank. This reading when deducted from the 280 nm reading of the trichloroacetic acid filtrate of the 30 min. incubated reaction mixture gave the true enzyme activity. Zero minute blanks were not necessary for pure preparations of enzyme, as the quantity of the enzyme used had no detectable reading at 280 nm.

Inhibitor blank:

Two blanks were taken when crude preparations were used. In both the cases the enzyme, whose inhibition was being studied, was omitted from the reaction mixture. One blank (zero minute inhibitor blank) was run by adding the inhibitor solution after the addition of trichloroacetic acid. This is used for correcting for ultraviolet absorbing impurities in the crude inhibitor. The second blank is necessary in the case of crude extracts containing proteolytic activity. In the case of the second blank the inhibitor solution was added to the reaction mixture in the beginning itself and incubated for the required time. The reaction was stopped as usual by the addition of trichloroacetic acid. The 280 nm reading obtained for the zero minute inhibitor blank when subtracted from the above blank (incubated blank) gives the proteolytic activity of the extract. This has to be taken into account while calculating the inhibition values.

The possibility that the inhibition is not a true inhibition, but is due to the hydrolysis of papain by

proteolytic enzymes in the crude extract was also taken into account. This possibility was ruled out by incubating the enzyme, namely papain, with crude extracts for different periods of time before adding the substrate and then testing the inhibition. In the above case if the inhibition is the same in all the samples which were preincubated for different times, inhibition is due to a papain inhibitor and not due to an endogenous proteinase acting on papain. This was carried out routinely for all samples of tissues tested and for the purified preparations of the inhibitors.

A typical assay for inhibitor activity using 0-0.90 ammonium sulfate precipitate (Fraction II, Section III, Chapter IV) is illustrated in Table 1. The table illustrates the different types of blanks taken and the corrections made for them in calculating the final inhibitor value.

Amount of enzyme and inhibitor in the reaction mixture:

The amount of enzyme taken in the reaction mixture was such that an optical density increase of about 0.4-0.5 was obtained. The papain inhibitor activity is proportional to inhibitor concentration when the decrease in papain activity was not more than 60% of the initial activity. The amount of inhibitor taken in the reaction mixture was adjusted such that the decrease in enzyme activity was between 0.09-0.250. Only those inhibitor values which did not exceed 60% inhibition of the enzyme activity were taken for calculating the inhibitor values, since the inhibition was found to be proportional to the inhibitor concentration in this range.

The sensitivity of the assay was increased by decreasing the enzyme concentration and increasing the period of incubation proportionally, when required.

EDTA was found to have no effect on papain activity in the case of the caseinolytic method and hence was deleted from the assay system. For every variation in experimental conditions, suitable controls were run to ensure that the compound did not interfere with enzyme activity.

Units:

A unit of papain activity gives an increase in absorbance at 280 nm of 0.001 per minute per ml of the reaction mixture under the experimental conditions. A unit of papain inhibitor activity is that which causes inhibition of one unit of papain. The same procedure was used for the assay of inhibitor activity when hemoglobin and serum albumin were used as substrates.

Assay of papain and inhibitor using BAPNA as substrate: (Arnon, 1965)

The reaction mixture consisted of 5 µmoles of BAPNA, 500 µmoles of dimethyl sulfoxide, 15 µmoles of EDTA pH 7.5, 30 µmoles of cysteine pH 7.5 and 500 µmoles of Tris/HCl buffer, pH 7.5. The total volume of the reaction mixture was 6 ml. 50-100 µg of papain and the required amount of inhibitor to give 50% inhibition were added. The various blanks as discussed earlier were taken. The mixture was incubated for 30 min at 25°C. The reaction was stopped by adding 1 ml of 3% acetic acid and the liberated p-nitroaniline was estimated at 410 nm, (Fig 3)

Units and specific activity:

The amount of substrate hydrolysed by papain can be

calculated from the molar extinction coefficient of p-nitroaniline at 410 nm ($E^{1\%}_{1\ cm}$ = 8800). One unit of BAPNA activity is the amount of papain which will hydrolyse one micromole of the substrate per minute under the experimental conditions. The specific activity is expressed as number of units per milligram protein. One unit of inhibitor activity is that amount which inhibits one unit of papain activity under the same experimental conditions.

Englund's assay of ficin by using the synthetic substrate BAPNA:

This was used to determine the specific activity of ficin. The method is similar to that of Erlanger, Kokowsky and Cohen (1961). A total volume of 0.8 ml contained 0.1 M sodium phosphate buffer, pH 7, ficin, 0.02 M mercaptoethanol and 0.001 M EDTA. 3 ml of the substrate were added to start the reaction. (BAPNA solution was prepared by dissolving 43.5 mg of BAPNA in 100 ml of 0.1 M phosphate buffer, pH 7 containing 0.001 M Versene and 1 ml of dimethyl sulfoxide). The tubes were incubated for 60 min at 37°C. The reaction was terminated by the addition of 0.2 ml of glacial acetic acid. The increase in optical density at 410 nm was measured. Suitable blanks were taken. One unit of activity is defined as the quantity of ficin required to catalyse the hydrolysis of BAPNA at the rate of 1.00 umole per minute (Englund, King, Craig and Walti, 1968), (Fig 5) The specific activity of pure ficin was calculated to be 9000 µnits/mg according to Kunitz's caseinolytic method of assay, (Fig 4)

BALE titrimetric assay:

Papain was also assayed by the pH stat method of Blumberg, Schechter and Berger (1970). The acid liberated

during the hydrolysis of BAEE by papain was titrated with 0.02 M NaOH. The reaction mixture volume was 15 ml and it contained 300 μmoles of BAEE, 30 μmoles of EDTA, 75 μmoles of cysteine pH 6, 150 μg of papain and 4,500 μmoles of KCl. The reaction was carried out at pH 6 at 21°C. The volume of NaOH added was recorded graphically and the activities were calculated from the initial rates of hydrolysis of BAEE. Papain purified according to Kimmel and Smith (1954) gives a k_{cat} of 13.0 sec^{-1}.

Specific activity of chymopapain was taken as 1920 units/mg of protein according to Kunitz's caseinolytic method of assay (Ebata and Yasunobu, 1962).

Polyacrylamide gel electrophoresis:

Polyacrylamide gels were prepared according to the method of Davis (1964) with slight modifications. Riboflavin was used instead of ammonium persulfate. The spacer gel was deleted. 9% gels were prepared in cylindrical tubes of 3-4 mm diameter. The pH of the gel was generally around pH 8.9 and the pH of the buffer (Tris-glycine) was 8.3. The concentration of the bath buffer was 0.02 M with respect to glycine and 0.0025 M with respect to Tris. The current applied was about 3-4 milliamps per tube and the run took usually about 120 min. Bromophenol Blue was used as the tracking dye. Protein was stained with 0.5% Amido Black 10B in 7% acetic acid. Destaining was carried out with 3% acetic acid. 0.5% Coomassie Blue in 20% trichloroacetic acid was also used occasionally to stain the protein bands. Destaining was carried out in 3% trichloroacetic acid.

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.

Washing of gels:

Certain non-protein impurities from the polyacrylamide gel inactivated the inhibitor. The yield of the inhibitor on elution was poor. Hence the gels were removed from the tubes and washed with glass distilled water first and then washed with the concentration of the buffer used to prepare the gel (0.25 M with respect to Tris). The gels were then shrunk in the cold and inserted into the tubes and allowed to swell at room temperature and stored in the cold (4°C). This washing was essential for preparative purposes and detection of the inhibitor on the gel.

The non-protein impurities also interferred in the protein assay. These could be partly removed by dialysis, and washing. However the non-dialyzable impurities could be removed only by passing the extract through DEAE-cellulose or CM-cellulose, depending on the nature of the inhibitor used. The non-protein impurities were in general not adsorbed and could mostly be removed by a thorough washing of the cellulose, by the buffer used for adsorption. Gels at low pH (4.4) were prepared according to the method of Reisfeld, Lewis and Williams with slight modifications (pamphlet from Ames Co., Indiana). Riboflavin was used instead of ammonium persulfate and 9% gels were used. It was observed that in the case of the acid gels, certain impurities from the gel impeded the mobility of the protein. Basic Fuchsin was used as the tracking

dye. The marker moved extremely slowly and 8 to 10 h were required for the marker to traverse 6 to 8 cm. However washing the gels with buffer of the same concentration as that used to prepare the gels (0.25 M with respect to acetic acid) enhanced the movement of the marker as well as the protein. Washing of the gels was essential for good separation on the preparative scale. The bath buffer used was 0.01 M potassium acetate buffer, pH 4.4. The current applied was 3 to 4 milliamp per tube and the runs usually took 180 min. 0.5% Amido Black 10B in 7% acetic acid was used to stain the protein bands. Destaining was carried out in 3% acetic acid.

Preparative gel electrophoresis:

Polyacrylamide gel columns of height 11 cm and diameter 1.8 cm were prepared as mentioned above depending on the pH at which the gels were to be run. Riboflavin was used for polymerisation. The gels were washed as mentioned above and shrunk in the cold. They were reinserted into the tubes and allowed to swell in the appropriate washing buffer in the cold. The above operation took about two weeks. 5% sucrose was first layered on the gel. 800 ml of Tris-glycine, pH 8.3 were used as the bath buffer in both the compartments. 800 ml of 0.01 M potassium acetate buffer were used in each of the compartments for runs at pH 4.4. 0.05% Bromophenol Blue was used as the marker in the upper compartment for runs at higher pH and Basic Fuchsin at lower pH. One ml of the inhibitor solution (containing 8 to 10 mg of protein) in 10% sucrose was loaded on 5% sucrose. Electrophoresis was run at 5 milliamp per tube. After every 12 to 16 h the buffer

was replaced with fresh buffer. Electrophoresis was continued till the marker reached the end of the tube. In the case of one of the inhibitors (run at pH 8.3), the marker was allowed to flow out and fresh marker was loaded in 5%sucrose and allowed to reach the end before the electrophoresis was stopped. This was essential as some impurities moved close to the inhibitor and a longer run gave better separation.

The gels were removed and cut horizontally into sections of 0.5 to 1 cm thickness. Each portion was extracted separately with 0.05 M potassium acetate buffer, pH 4.4. A homogeniser was used to extract the fractions which contained the inhibitor activity. The gels were reextracted several times at intervals of 24 h each with 0.05M potassium acetate buffer, pH 4.4. The time factor was important for total recovery, as diffusion from the acrylamide particles was very slow. It was also observed that better recoveries were obtained with extraction carried out at low pH than at pH 7.5. The extracts containing the inhibitors were processed separately. Non-protein impurities were removed by dialysis and the use of ion-exchange celluloses.

Iso-electric point (pI):

Cellogel electrophoresis was carried out to determine the isoelectric point of the inhibitors. 5 cm long and 5 cm wide cellogel strips were used. Acetate buffer for pH 3.5-6, phosphate buffer for pH 6-8 and NaOH-glycine buffers for pH 8-12 were used. 10 to 20 µg of the inhibitor (in 0.1 ml to 0.2 ml) and dialyeed against the appropriate buffer were used for each run. 0.01 ml of 0.05% Bromophenol Blue was

added to the inhibitor before the run. The buffer concentration was 0.005 M. The current used was one milliamp and the time of the run was 15 min. After the run the cellogel strip was stained for 1 min in 0.5% Amido Black and then destained for 5 min in 7% acetic acid. The movement of the protein at different pH values was measured and the pH at which there was no movement was noted as the isoelectric point of the inhibitor used.

Ultraviolet absorption spectrum:

The inhibitor was dialysed against 0.001 M phosphate buffer, pH 7.5 and the optical densities at various wavelengths in the U.V. region were recorded. The same buffer was used as the blank. The optical factor of the inhibitor was the reciprocal of its absorbance at 280 nm, when the protein concentration was 1 mg/ml for 1 cm light path.

Molecular weight by gel filtration:

The molecular weights of the inhibitors were determined by Sephadex G-50 filtration (Andrews, 1965). Sephadex G-50 was suspended in water and was allowed to swell in a boiling water bath for about an hour, cooled and deaerated. A column (100 cm x 1.5 cm) was then packed with gel and equilibrated by passing several column volumes of 0.05 M phosphate buffer, pH 7.5 through it. 0.5 ml of phosphate buffer containing a mixture of 2 mg each of soybean trypsin inhibitor (21,000), cytochome c (13,000), myoglobin (17,000) and blue dextran and 1000 units of the inhibitor was loaded on the column and eluted with the same buffer. The flow rate was 30 ml per hour and 3 ml fractions were collected. The fractions were

assayed for the different proteins loaded. Cytochrome c and myoglobin were detected by determining the absorption at 412 nm and 405 nm respectively. Trypsin and papain inhibitors were assayed by the caseinolytic method of Kunitz. Separate columns were run for the inhibitors. A graph was drawn by plotting the eluant volume against the logarithms of molecular weight from which the molecular weight was calculated.

Molecular weight determination by SDS-polyacrylamide gel electrophoresis:

The method of Shapiro, Vinuela and Maizel (1967) and Weber and Osborn (1975) was adopted.

Gels of length 10 cm and diameter 0.6 cm were prepared containing 15% acrylamide, 0.36% Bis, 0.1% SDS and 0.05% TEMED, 0.1 M sodium phosphate, pH 7.2 and 0.0004% riboflavin. The bath buffer used was 0.1 M sodium phosphate buffer, pH 7.2 containing 0.1% SDS.

One part of protein solution in 0.01 M sodium phosphate buffer, pH 7.2 was added to nine parts of 0.01 M sodium phosphate buffer, pH 7.2 containing 0.1% SDS and 1% 2-mercaptoethanol kept in a boiling water bath (98°C). The final protein concentration ranged from 0.05 to 1.0 mg/ml. The above mixture was incubated for 4 min and cooled to room temperature. The ratio of SDS to protein was always maintained at 3:1. 10 to 20 µg of the denatured protein was loaded on each gel. Dialysis was essential only if the ionic strength of the buffer was high or the material contained salts. Bromophenol Blue was used as the tracking dye. Electrophoresis was carried out by using 8 milliamp per tube.

The anodic compartment was the lower chamber. Electrophoresis was discontinued when the marker had reached three-fourths of the distance of the gel. The dye front was marked by inserting a thin nylon thread. Gels were fixed overnight in 2.5% Coomassie Blue prepared in 45% methanol and 9% glacial acetic acid. Destaining was carried out by using 5% methanol - 7.5% acetic acid mixture. Insulin (molecular weight of subunit approximately 2,800), cytochrome c (13,000), myoglobin (17,000), and soybean trypsin inhibitor (21,000) were used as standard proteins. They were processed in a similar way. Migration of Bromophenol Blue was used as the reference point for each gel. The electrophoretic mobilities (distance of protein migration/ distance of dye migration) were plotted against the logarithms of the known polypeptide chain molecular weights. A straight line was obtained from which the molecular weight of the unknown protein was calculated.

Amino acid analysis:

Three 0.2 mg samples were dissolved in 2 ml of 6N HCl in test tubes. The test tubes were evacuated and sealed. The samples were hydrolysed at 110°C for 24, 48 and 72 h respectively. The excess acid was removed by vacuum evaporation at room temperature (35°C). Evaporation was done thrice adding 2 ml of water each time for dissolving the residue.

Proline, threonine, serine and tyrosine are partially destroyed on hydrolysis. Hence a correction was applied by extrapolating to zero hydrolysis time from the data for 24, 48 and 72 h hydrolysis.

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

Estimation of free SH groups:

Free SH groups were assayed according to the method of Ellman (1958, 1959). When the protein is treated with 5-5' dithiobis-2-nitrobenzoic acid (DTNB), the increase in absorbance at 412 nm is proportional to the SH content. Molar extinction of 13,600 $M^{-1}cm^{2}$ was used to calculate the thiol concentration. The accurancy of the method was checked with reduced glutathione.

0.1 ml of the inhibitor solution (containing (200 µg), 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 taken in a 1 ml cuvette with 10 mm light path and the increase in absorbance at 412 nm was measured.

Dissociation constant:

The dissociation constant of the enzyme - proteinase inhibitor complex was determined by the method of Greene and Work (1953). An inhibition curve was obtained by adding increasing amounts of inhibitor to a fixed amount of enzyme near the equivalence point. Straight lines (dotted) are drawn from the base to find out the equivalence point and the amount of free enzyme. The residual enzyme 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 the enzyme and I is the inhibitor. However it has to be noted that the above

method for determination of the dissociation constant of the enzyme-inhibitor complexes is approximate and the values could differ by an order of ten. E°/K_i was calculated and correlated to the theoretical plot of fractional free enzyme as a function of molar ratios of inhibitor to enzyme $(I^{\circ})/(E^{\circ})$, for the indicated values of the ratio E°/K_i. The above correlation gives an idea as to how firm the binding is between the inhibitor and the enzyme (Beith, 1973).

Performic acid oxidation and cysteic acid estimation:

The total half-cystine content was determined as cysteic acid after oxidation with performic acid according to Moore (1963), followed by HCl hydrolysis and amino acid analysis. 0.2 - 0.4 mg of the inhibitor was used for cysteic acid estimation.

Total carbohydrate determination:

This was carried out by the orcinol-sulfuric acid method of Winzler modified by Francois, Marshall and Neuberger (1962).

Partial specific volume:

Partial specific volume of the inhibitors was determined from the data on amino acid analysis. The values for $\bar{v}$ for the amino acid residues were taken from Schachman (1957).

Determination of degree of hydrophobicity:

The degree of hydrophobicity of the papain inhibitor A_1 was calculated from its amino acid composition. Three different methods were followed.

Fisher's method (1964):

According to Fisher, the degree of hydrophobicity is

expressed in terms of polarity ratio p, which is defined by the equation given below:

$$p = V_e/V_i$$

where V_e and V_i are the volumes occupied by polar and non-polar residues respectively.

Bigelow's method: (1967)

This method gives the average hydrophobicity H Øave based on Tanford's free energies of transfer of amino acid side chains from an organic environment to an aqueous environment. H Øave is the total hydrophobicity divided by the total number of residues. Values for the H Ø cal/res for each amino acid were taken from Bigelow (1967).

Waugh's method:

According to the method of Waugh (1954) the degree of hydrophobicity is measured in terms of NPS, i.e. frequency of non-polar side chains. NPS is calculated by counting the tryptophan, isoleucine, tyrosine, phenylalanine, proline, leucine and valine residues and expressing the sum as a fraction of the total number of residues.

Ultracentrifuge measurements:

Spinco Model E ultracentrifuge was used for carrying out analytical runs. It was provided 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 co-efficients were calculated from plots of logarithm of

distance of sedimenting boundary from the axis of rotation versus time (Schachman, 1957). The sedimentation co-efficients were standardised to water at 20°C ($s_{20,w}$) after making density and viscosity corrections. A partial specific volume of 0.73 was used.

Molecular weight determinations were made by the approach to sedimentation equilibrium method of Archibald (1947) as described by Schachman (1957). A synthetic boundary cell was used. The plate phase was at an angle of 60°. The speed of centrifugation for linear extrapolation of the gradient curve was calculated according to LaBar (1966). Only readings at the air meniscus were taken. Photographs were taken at intervals of 15 min. Photographic plates were measured on a Hilger L 50 two-way micrometer.

Table 2: Values used for activity calculations of proteinases:

E.C.Number	Proteinases	Molecular weight	Substrate used.	Specific activity of pure proteinase
				K.Units/mg
3.4.4.10	Papain	21,000	Casein	5430
3.4.4.11	Chymopapain*	27,000	Casein	1920
3.4.4.12	Ficin	25,000	Casein	9300

*Ebata et al. (1962).

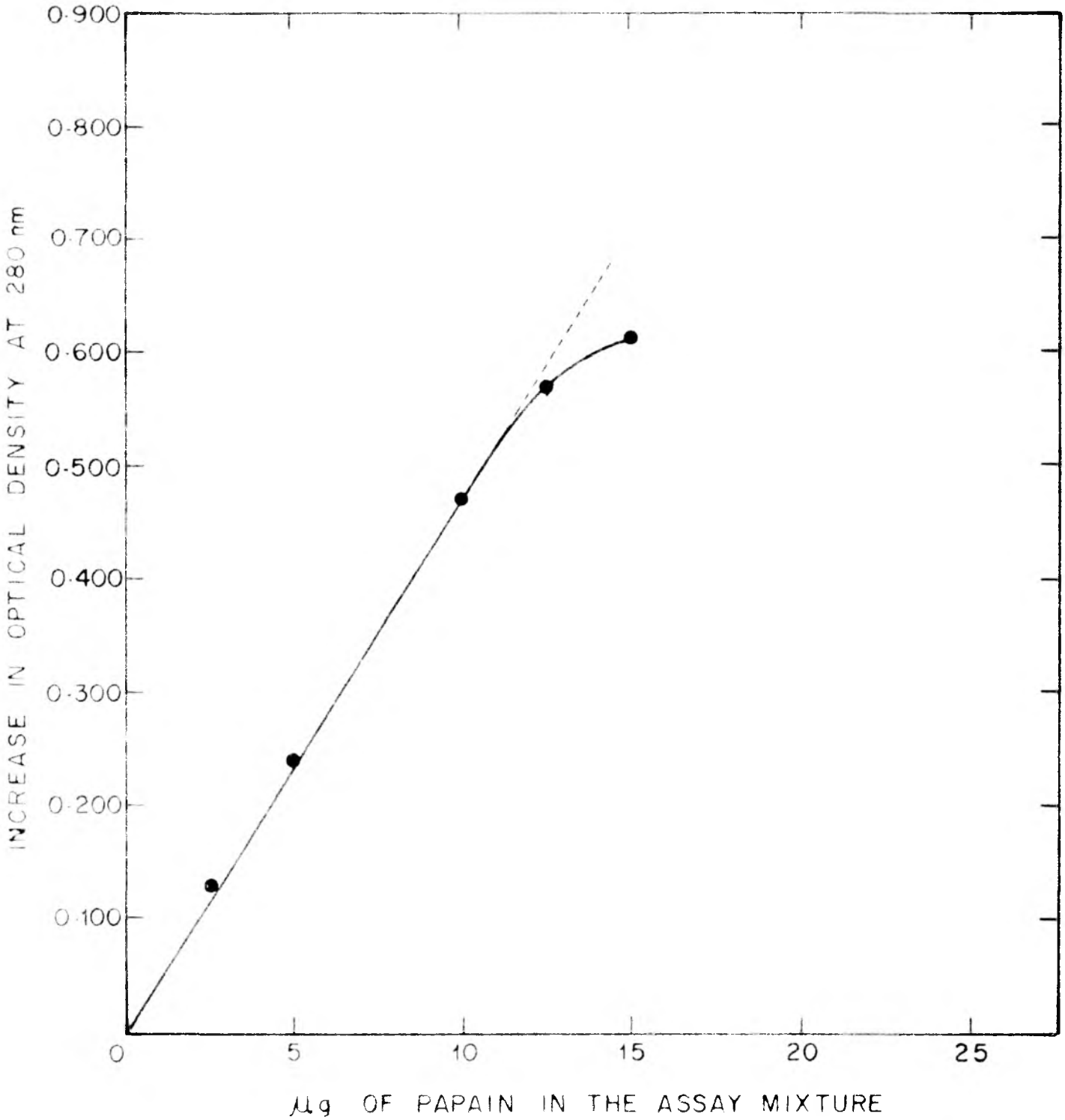


FIG. 1

PAPAIN ACTIVITY AS A FUNCTION OF ENZYME CONCENTRATION

Kunitz's caseinolytic assay method was used. Conditions are as described in Materials and Methods.

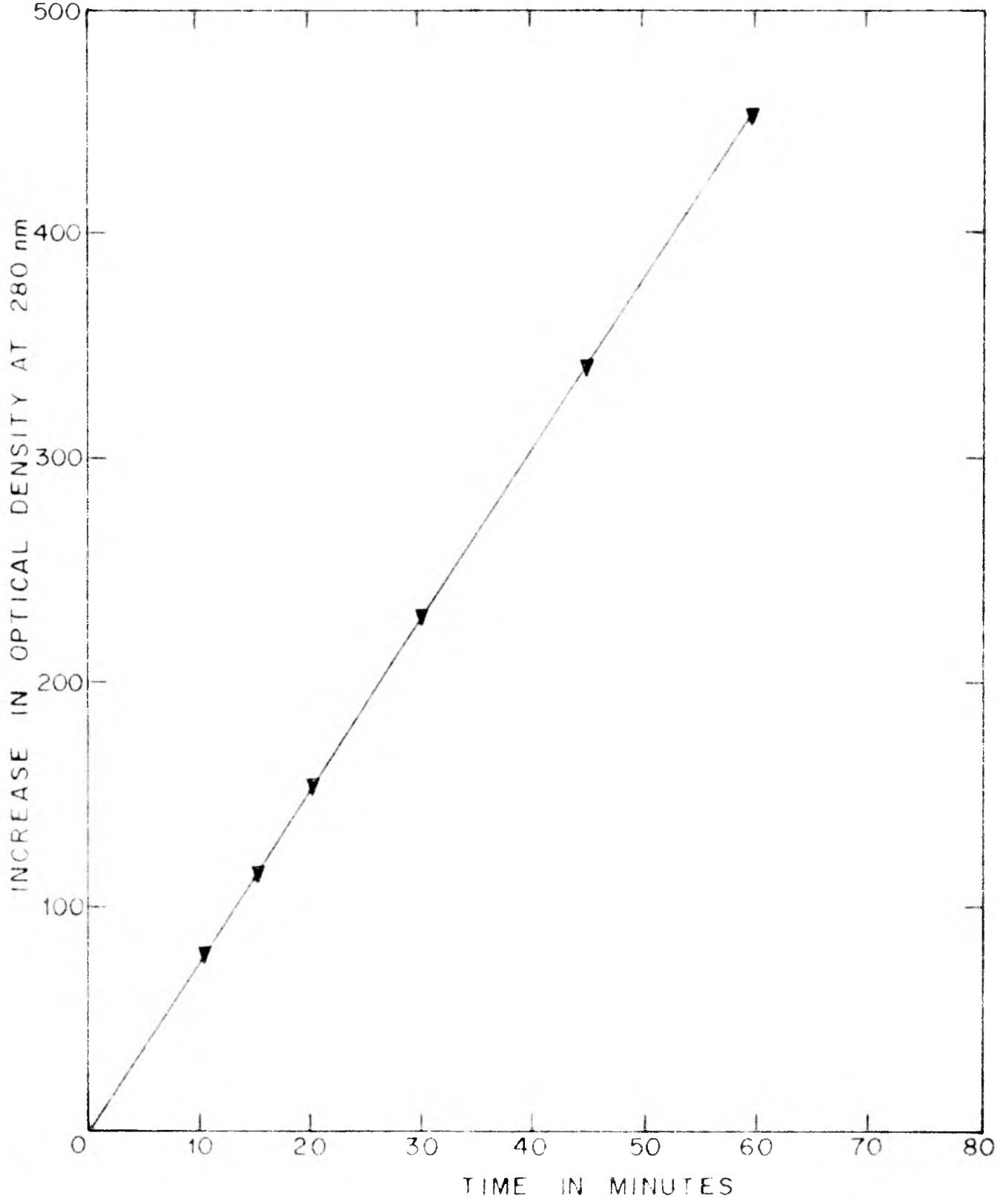


FIG. 2

PAPAIN ACTIVITY AS A FUNCTION OF TIME

Kunitz's caseinolytic assay method was used. 5 ug of papain were used. Other conditions are as described in Materials & Methods.

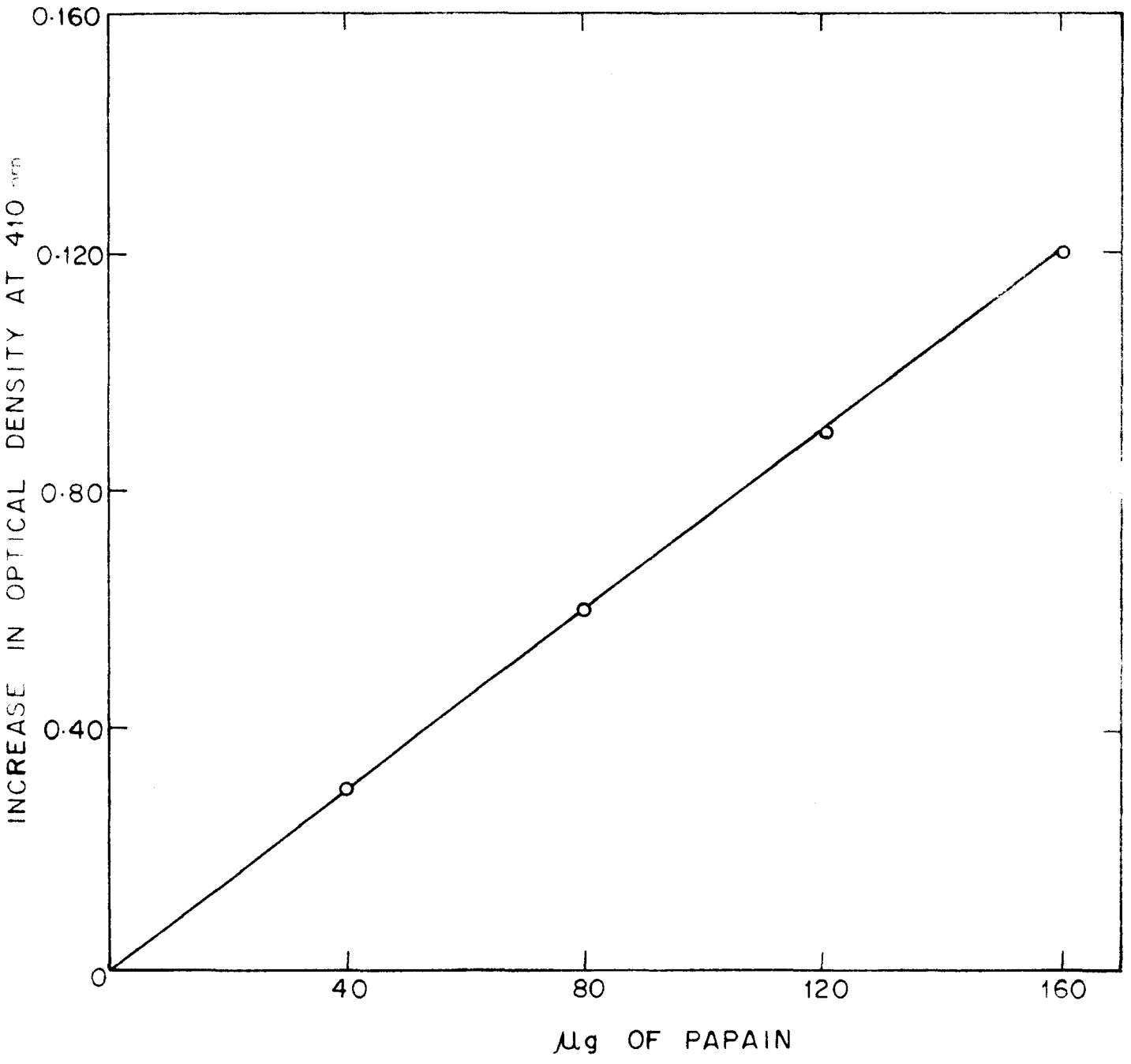


FIG. 3.

PAPAIN ASSAY USING SYNTHETIC SUBSTRATE (BAPNA)

Reaction was carried out at 20°C for 20 min. Other conditions are as described in Materials and Methods. Purity of papain used as compared with the calibration curve of Arnon is 56%.

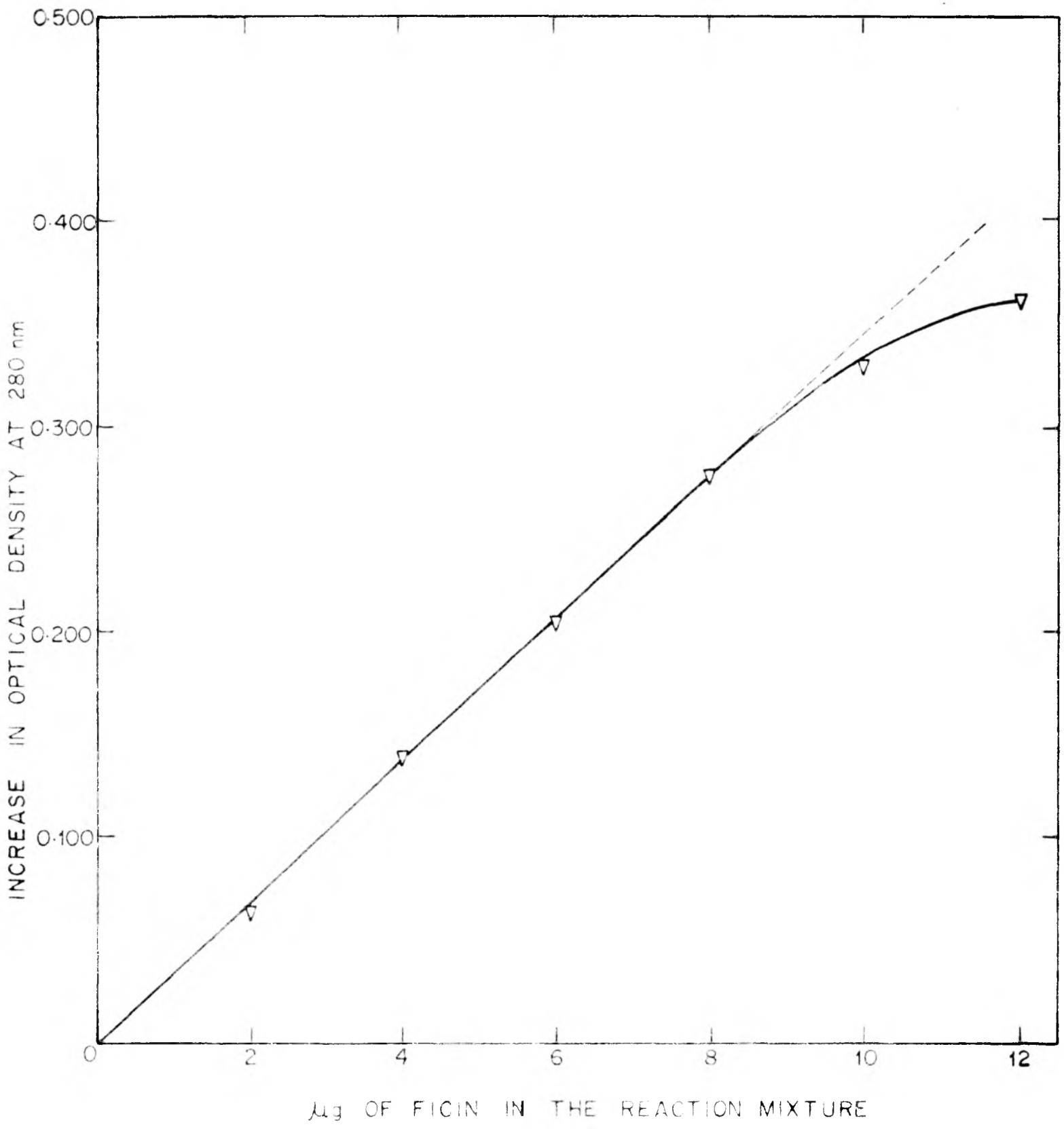


FIG. 4

CASEINOLYTIC ASSAY OF FICIN (KUNITZ'S PROCEDURE)

Kunitz's method of assay was used as described in Chapter II, Materials and Methods. Specific activity of ficin - 3300 units/mg. Specific activity of pure ficin - 9300 units/mg.

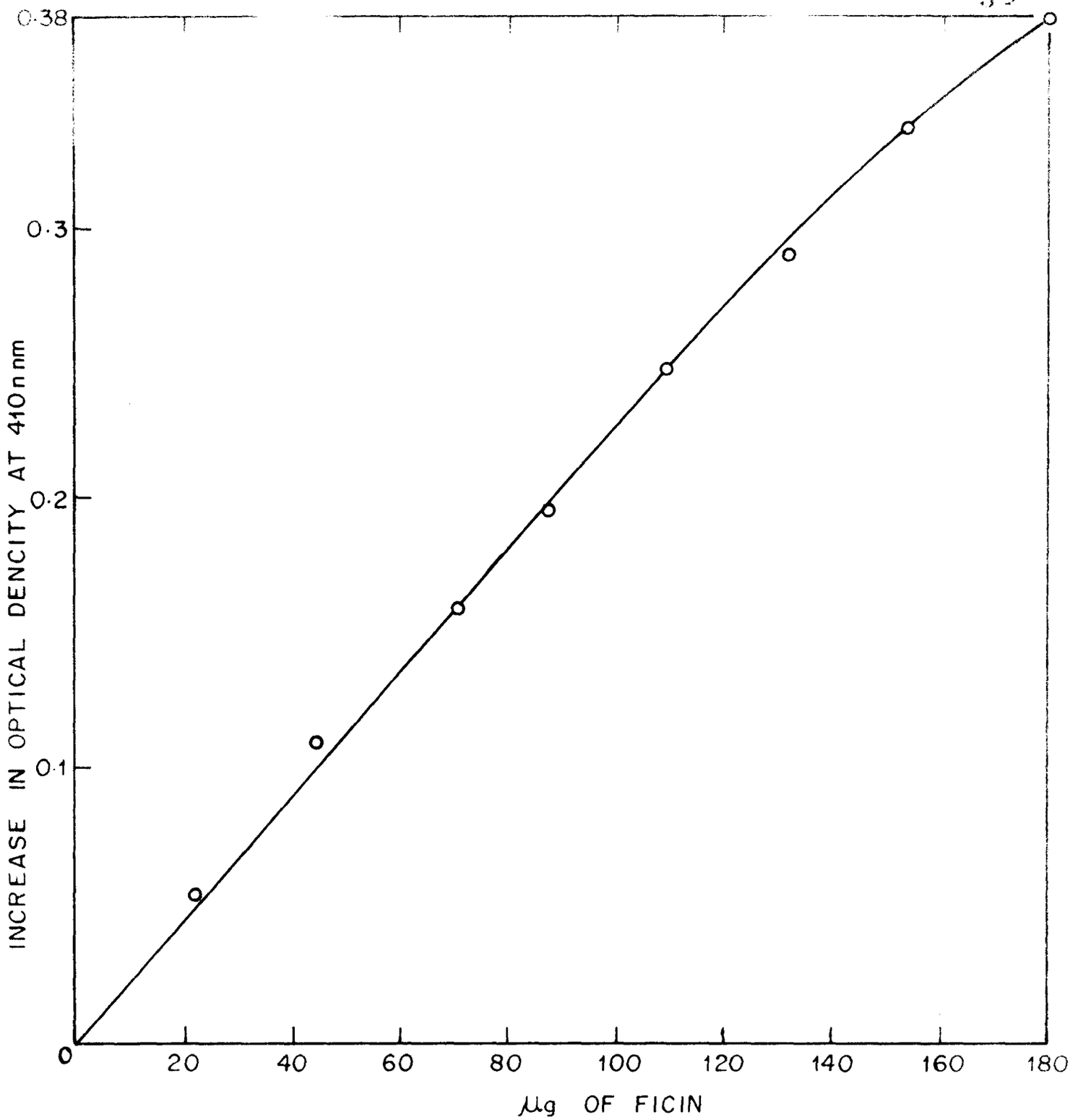


FIG. 5.

"ENGLUND'S" ASSAY OF FICIN BY USING SYNTHETIC SUBSTRATE (BAPNA)

SPECIFIC ACTIVITY OF FICIN USED—

0·5 MOLE BAPNA HYDROLYZED/MOLE

SPECIFIC ACTIVITY OF PURE FICIN—

C H A P T E R III

OCCURRENCE OF PAPAIN INHIBITORS IN PLANTS

Section I

Occurrence of papain inhibitors in plants

Introduction:

As described in Chapter I (Section IV) the occurrence of trypsin inhibitors in seeds, especially of Leguminosae, is well known. Papain inhibitor activity has been reported to occur in a few plant tissues, but no survey of its distribution has so far been carried out. In this Chapter the distribution of papain inhibitors in a wide variety of plant tissues is reported for the first time. The inhibitor was found to be present in seeds as well as actively growing cells. The inhibitor distribution in different parts of the plant and its variation during germination were also studied.

Nature of the inhibitor:

It is well-known that a number of metals inhibit enzyme activity. Hence it is important for the investigator, working on "protein inhibitors", to rule out the possibility of metal inhibition of enzymes. In the case of proteolytic inhibitors it is equally important to distinguish between "true inhibition" of the enzyme and apparent inhibition due to the hydrolysis of the enzyme under consideration, by proteolytic enzymes present in crude extracts. Hence the following experiments were carried out to determine the nature of inhibition of papain, before proceeding to purify the inhibitors.

Experiment 1:

The crude extracts were precipitated with ammonium sulfate at 90% saturation. The precipitated material in each

case was dialysed exhaustively and tested for inhibitor activity. The above experiment makes it less likely that inhibition of papain is due to small molecules.

Experiment II:

The precipitated inhibitor samples were dialysed against 0.01M EDTA, pH 7.5 and tested for activity. In addition, the inhibitor activity was also tested in the presence of EDTA in the reaction mixture itself. The experiments rule out inhibition of the enzyme by heavy metals chelated by EDTA.

Experiment III:

Crude extracts often contain proteolytic enzymes. These may destroy papain and cause apparent inhibition. To eliminate this possibility, papain was incubated with the crude extract for different intervals of time. The inhibitor activity was then determined. Proteolytic hydrolysis of papain, will be time dependent.

Experiment IV:

In another set of experiments papain and the crude extract were incubated at different temperatures (0°C, 4°C, 15°C and 35°C) for a known period and the inhibitor activities of the preincubated samples were determined. It was found to be the same in all the samples. Interaction between enzymes and protein inhibitors is very rapid and virtually independent of temperature. If the decrease in papain activity was due to proteolytic enzymes present in the crude extract, inhibition would progressively increase with increase in temperature.

Experiment V:

Polyacrylamide gel electrophoresis of a partially purified sample was carried out in some cases. The location of the inhibitor was determined after elution. The above experiment also suggested that the inhibitor was a protein.

EXPERIMENTAL:

A papain inhibitor was shown to be present in all the plant tissues tested so far (Table 3 & 4).

25 g of the seeds were washed several times with tap water, distilled water and glass distilled water to remove traces of preservatives which may have been added. All operations were carried out at 0°C unless otherwise stated. The seeds were soaked in a definite quantity of glass distilled water at room temperature (35°C) for 30 min. The water was drained off and the seeds were homogenised with 25 ml of 0.1 M potassium phosphate buffer, pH 7.5, (5 ml/g dry seeds) for 2 min in a Waring blendor. The extract was allowed to stand for 30 min. with occasional stirring and then passed through muslin cloth to remove the coarse particles. The homogenate was centrifuged at 4000 x g for 20 min. The resultant supernatant liquid was dialysed against one litre of 0.01M phosphate buffer, pH 7.5 over a period of 18 h at 4°C with two changes. The dialysed sample was estimated for papain inhibitor activity.

A portion of the supernatant liquid was precipitated with ammonium sulfate at 90% saturation and filtered under gravity after keeping for two hours. The precipitate was dissolved in a small volume of the same buffer and dialysed

exhaustively under the same conditions as mentioned above with several changes of buffer.

The extracts in all cases were treated with EDTA (0.01 M) before assaying the inhibitor activity. A portion was also dialysed against EDTA in the buffer and the inhibitor activity was determined after dialysis.

Vigna catjang seeds were germinated for four days in the dark in a tray containing a layer of moist cotton at 30°C. The whole seedlings were harvested and homogenised with 0.1 M potassium phosphate buffer, pH 7.5 as mentioned earlier. The extract was dialysed and an aliquot was precipitated with ammonium sulfate and dialysed exhaustively as described above. An aliquot of the dialysed extract was also heated in a boiling water bath for 7 min. at 98°C and cooled immediately in an ice bath. The heated dialysed extract, the dialysed extract and the ammonium sulfate precipitated fraction were assayed for papain inhibitor activity.

The inhibitor content was also determined at different stages of germination. Vigna catjang seeds were grown as mentioned above and harvested at 1 day intervals. The extract was prepared as mentioned earlier and precipitated with ammonium sulfate and dialysed exhaustively before testing the inhibitor activity. (EDTA treatment was also given).

In another set of experiments 4-day old seedlings were separated into leaves, stem and roots. Each portion was extracted and processed separately as above. The inhibitor content of the individual parts of the plant was determined. The inhibitor content after heating the extracts was also

measured.

Tissue cultures:

The inhibitor content of several callus cultures from plants which are maintained in this laboratory, was determined.

Callus cultures (about 3-4 weeks old) were collected and lyophilised. The dry weights were determined. The tissues were first powdered and extracted with 0.1 M phosphate buffer, pH 7.5 (15 ml to 25 ml buffer/gm of dry tissue) after grinding in a glass mortar. The homogenate was allowed to stand for 30 min. sieved through a muslin cloth and fuged at 4000 x g for 20 min. The supernatant liquid was dialysed against 0.01 M phosphate buffer, pH 7.5. Extracts were tested for papain inhibitor activity with and without EDTA.

A few of the cultures were treated with ammonium sulfate to 90% saturation, and dialysed exhaustively before determining the inhibitor content.

The inhibitor content of all the tissues was determined after heating the extracts at 98°C for 7 min.

RESULTS AND DISCUSSION

Table 3 shows the papain inhibitor content of the seeds tested with or without ammonium sulfate precipitation. Table 4 shows the papain inhibitor content of the callus cultures tested before and after heating. As seen from Tables 3 & 4 papain inhibitor activity occurs in all the plant tissues tested.

Seeds:

Some of the dicots were found to be rich sources of the

Table 3: Papain inhibitor activity of plant seeds

Name	Dialysed extract	Precipitated with ammonium sulfate and dialysed°
	Papain inhibitor activity* Units/g dry seeds	
1. Phaseolus vulgaris	220	224
2. Arachis hypogaea	640	488
3. Glycine max	690	432
4. Dolichos biflorus	450	326
5. Cyamopsis psoralioides	480	600
6. Vigna catjang	600	580
7. Vigna catjang (germinated, fresh extract)	175	180
8. Triticum vulgare	112	47
9. Sorghum vulgare	120	87
10. Zea mays	220	260
11. Oryza sativa (polished seeds)	16	1.8
12. Oryza sativa (bran)	156	-
13. Brassica oleracea var. capitata	117	126

* Papain inhibitor activity represents total inhibitor activity.

° Mean inhibitor activity of three batches of seeds.

Table 4: Papain inhibitor activity of plant tissue cultures

Name	Papain inhibitor activity				Proteinase activity
	Fresh Extract		Heated at 98°C		
	Units/g dry tissue	Units/mg protein	Units/g dry tissue	Units/mg protein	Units/mg protein
1. Vigna catjang	34	3	94	10	0
2. Malus sp.- Pyrus malus	71	4	154	8	0
3. Daucus carota	0	0	234	11	3
4. Capsicum sp.	225	8	225	8	-
5. Digitalis purpurea	0	0	170	5	-
6. Papaver somniferum	40	1	44	1	-
7. Nicotiana tabacum (normal)	98	3	140	4	0
8. Nicotiana tabacum (crowngall)	330	6	448	9	0
9. Parthenocissus tricuspidata (normal)	300	29	780	84	0
10. Parthenocissus tricuspidata (crowngall)	92	11	154	18	0.5
11. Helianthus tuberosus (crowngall)	0	0	188	13	9
12. Saccharum officinarum	98	6	358	23	18
13. Lilium sp.	40	1	77	3	10

contd..

Name	Papain inhibitor activity				Proteinase activity
	Fresh Extract		Heated at 98 °C		
	Units/g dry tissue	Units/mg protein	Units/g dry tissue	Units/mg protein	Units/mg protein
14. Oryza sativa	103	4	720	28	-
15. Sorghum vulgare	129	4	1370	40	-
16. Tritioum vulgare (callus culture)	260	6	326	7	-
17. Triticum vulgare (root culture)	23	3	67	7	-
18. Zea mays (callus culture)	0	0	145	3	0
19. Zea mays (root culture)	375	9	1315	35	0
20. Zea mays (root culture medium) (1 ml)	5	35	9	60	0

inhibitor. Arachis hypogaea, Glycine max and Vigna catjang were found to be equally rich in this particular inhibitor. As compared to the dicots, the monocots have a lower content of the inhibitor. In the case of rice the inhibitor was found to be present almost entirely in the bran. Polished rice contains only 10% of the inhibitor as compared to an equal weight of rice bran.

Heating the extracts at 98°C for 7 min. had no marked effect on the inhibitor activity of seeds. Treatment with EDTA or incubating papain with crude extracts of the seeds at different temperatures for different periods did not result in any change in inhibitor activity. This indicates that metals and proteinases are not responsible for the inhibition since the dialysed ammonium sulfate precipitates retain the activity. The presence of a protein inhibitor of papain in all the seeds tested so far is indicated.

Tissue cultures:

In the case of callus cultures also, the inhibitor activity was unaffected by treatment with EDTA or by incubation for varying periods with papain, before and after addition of the substrate. Hence it is improbable that the net decrease in activity of papain is caused by proteolytic enzymes which hydrolyse papain or by heavy metals.

As seen from Table 4 the inhibitor activity increased on heating in the case of the callus cultures. Since all the inhibitors are non-dialysable and the activity increased on heating it is likely that the inhibition is due to a

Table 5: Papain inhibitor activity of dialysed ammonium sulfate precipitate of tissue extracts

	Papain inhibitor activity of fresh extract (unheated)		Papain inhibitor activity heated at 98°C	
	Units/g dry tissue	Units/mg of protein	Units/g dry tissue	Units/mg protein
1. Malus sp. pyrus malus	175	15	234	22
2. Oryza sativa vk 41	317	23	450	39
3. Saccharum officinarum	712	25	2049	80
4. Brassica oleracea var. capitata	0	0	2643	11
5. Zea mays (callus culture)	0	0	145	4
6. Zea mays (root culture)	370	9	1470	33

protein and not due to heavy metals. The ammonium sulfate precipitated extracts (Table 5) also showed increase in activity on heating. Some of the extracts showed inhibitor activity only after heating. The extracts of all tissue cultures except Capsicum sp showed an increase in activity after heating. (Removal of phenolics from dark coloured extracts such as those from Capsicum (unpublished work), which showed slight inhibitor activity only after treatment with polyclar, is probably necessary for accurate assay.)

It is of interest that the liquid medium in which maize roots were grown also showed papain inhibitor activity, suggesting that the inhibitor is secreted into the medium.

Parthenocissus tricuspidata (normal and crowngall), Nicotiana tabacum (normal and crowngall), Vigna catjang and Saccharum officinarum were tested for trypsin and subtilisin inhibitor activities (private communication from Vartak). Subtilisin inhibitor activity was present in all the cultures except Saccharum officinarum, but it decreased on heating at 98°C for 7 min. whereas trypsin inhibitor was absent in all the cultures tested except Saccharum officinarum. These results show that the papain inhibitor is different from subtilisin and trypsin inhibitors.

Tumour tissues:

The normal and tumour tissues of Nicotiana tabacum and Parthenocissus tricuspidata were screened to find out if there was a definite marked variation between normal and tumour tissues. In all the cases the inhibitor activity increased

on heating, but no uniform difference was observed between the normal and tumour tissues. The inhibitor activity of Nicotiana tabacum (normal) was higher than that of the crowngall tissue. The reverse was true of the Parthenocissus tricuspidata normal and tumour tissues. However it will be shown in a subsequent Chapter that Vigna catjang seeds contain a large number of isoinhibitors of papain. Further work is therefore required to study not merely the total inhibitor content but also the isoinhibitor pattern in order to determine whether there is a difference between normal and tumour tissues.

Several of the extracts of tissue cultures contained proteinase activity at pH 7.5, which was completely destroyed on heating at 98°C for seven min. The inhibitor is, however, stable to heating. The increase in papain inhibitor activity on heating suggests that a heat labile proteinase (or a related protein capable of binding the inhibitor specifically) and inhibitor are both present in varying amounts in the callus cultures and that the observed activity is the difference between the activities of inhibitor and enzyme. Heating the extract destroys the enzyme and results in an apparent increase of the heat stable inhibitor. Preliminary work indicates the presence of more than one proteinase possibly of inhibitor in plant tissues. Further work is needed to isolate the specific proteinase free from the inhibitor.

Differentiating tissues:

Another interesting observation was the high content

of inhibitor in differentiating tissues such as Saccharum officinarum, Brassica oleracea and Zea mays. The above tissues differentiate readily to form roots or whole plantlets. Other tissues, which contain lower inhibitor activities, showed no differentiation. In the case of Zea mays it was observed that the root culture has a higher content of inhibitor when compared with the callus and a part of the inhibitor is excreted into the medium. However in the case of Triticum vulgare, the undifferentiated callus was found to contain higher inhibitor activity than the callus exhibiting differentiation into roots. It is possible that a part of the inhibitor is excreted into the medium but unfortunately sufficient material was not available to study the inhibitor content of medium and tissue in greater detail. Further work is required to find out whether papain inhibitors have a role in differentiation of tissues.

Seedlings:

Vigna catjang seedlings (Table 6) showed an increase in inhibitor activity on boiling whereas in the seeds the activity remained constant on heating. The increase in inhibitor activity on heating indicated the presence of an enzyme or protein binding the inhibitor, which was destroyed on heating, thereby releasing the inhibitor. Hence it was decided to study the inhibitor pattern with and without heating the extracts obtained at different stages of germination. The inhibitor activity decreased on the second day. However there was no increase in inhibitor activity on

Table 6: Variation of papain inhibitor activity of Vigna catjang seeds during germination. Total papain inhibitor activity per 100 g of Vigna catjang seeds.

	Fresh extract (unheated)	Heated at 98°C	Proteinase activity in fresh extract (unheated)
	* Units x 10^3	Units x 10^3	Units x 10^3
Ungerminated seeds	48	48	27
Ist day of germination	47	41	22
IInd day of germination	27	27	6
IIIrd day of germination	4	11	6
IVth day of germination	9	31	5

The extracts were precipitated with ammonium sulfate and dialysed exhaustively before papain inhibitor activity was determined.

* Units x 10^3 represents units multiplied by 1000.

heating. On the third day the inhibitor activity was markedly reduced and was only 8% of the initial activity, but it increased three-fold on heating. There was an increase in the inhibitor activity on the fourth day, but after heating there was a striking increase in activity and the activity of the heated extract was nearly 65% of that of the seeds.

The above changes in inhibitor pattern indicate destruction of inhibitor activity on the second day and the synthesis of a new enzyme after the second day of germination which complexes one or more of the isoinhibitors of the seeds. The increase in activity of the germinated seedlings on the fourth day probably indicates synthesis of a papain inhibitor. However more rigorous experiments and rapid methods for the separation of isoinhibitors are necessary to study these interesting variations observed during germination.

Localization in plantlets:

The distribution (Table 7) of the inhibitor in different parts of the seedling was studied. The experiment was done with the seedlings on the 4th day of germination. Unheated extracts of the root showed no inhibitor activity, but activity appeared on heating. On the other hands extracts of leaves showed activity which did not increase on heating. The unheated extracts of the stem, cotyledons and leaf had nearly the same activity, but the heated cotyledon extract showed a higher activity than the boiled stem extract. The stem extract showed a 50% increase in activity whereas the cotyledon extract showed a 250% increase in activity on

Table 7. [illegible]

seedling

Parts of the germinated seedling	Papain inhibitor activity. Ammonium sulfate precipitated dialysed fresh extract (unheated)	Papain inhibitor activity. Ammonium sulfate precipitated dialysed extract. Heated at 98°C.	Ammonium sulfate precipitated dialysed extract-Proteinase activity.
	Units x 10^3/100 g dry seeds		
Roots	0	1	0.1
Stem	2.2	3.3	0.2
Cotyledons	2.6	6.5	4.8
Leaves	3	3	0.2

Germinated seedlings - 4 days old were used for this experiment.

heating. The cotyledon extract also showed much higher proteolytic activity. More detailed experiments are required to characterise the differences in the inhibitors from different parts of the plant.

Conclusion:

A papain inhibitor was found to be present in all the seeds and plant tissues examined so far. The activity was found to be present in actively dividing cells such as callus and root cultures and germinating seeds of Vigna catjang. In contrast the trypsin and subtilisin inhibitors were detected in some but not all of the callus cultures tested. The increase in inhibitor activity on heating extracts of actively growing tissues showed the presence of a latent inhibitor. The inhibitor was probably present as a complex with an enzyme in the unheated extracts and was released on destroying the labile enzyme by heating. The wide-spread occurrence of a papain inhibitor in all plant tissues which were tested and especially in actively growing plant cells suggests that it may have an important role in the metabolism of plant cells.

C H A P T E R IV

P U R I F I C A T I O N

Section I

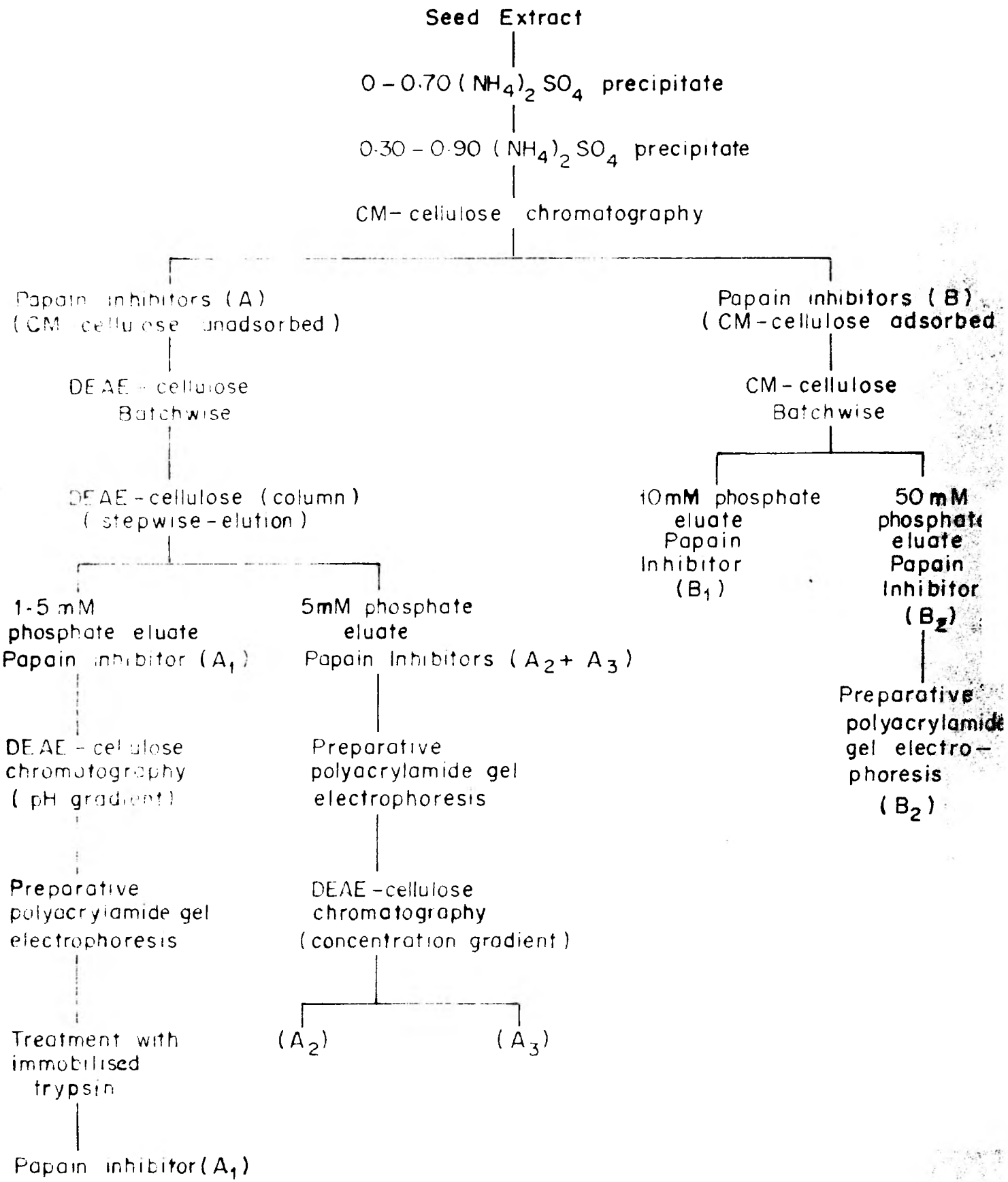
Purification of papain inhibitors

As indicated in Chapter III, seeds of Glycine max, Arachis hypogea etc. were found to be rich in papain inhibitors. Purification of papain inhibitors was undertaken from Vigna catjang, since these seeds were available in plenty throughout the year and since the purification of other proteinase inhibitors from these seeds was in progress in this laboratory. Earlier work in this laboratory indicated the presence of a large number of isoinhibitors of trypsin, subtilisin and papain in V.catjang. Two trypsin inhibitors, two subtilisin and two papain inhibitors were purified to homogeneity (Vartak, 1975). The purification of the other isoinhibitors of papain was carried out in the present work. Purification of papain inhibitors from both alkaline extract as well as acid extract of Vigna catjang was carried out. The alkaline extract contained six times more papain inhibitor activity than the acid extract. Both conventional techniques of protein purification as well as affinity chromatography were used to purify the inhibitor C from the alkaline extract. However the inhibitor was unstable on purification and even after 600-700 fold purification, was not homogeneous. The acid extract of the seeds contained five isoinhibitors of papain, three of which were adsorbed on DEAE-cellulose (A_1, A_2 and A_3), and two were adsorbed on CM-cellulose (B_1 and B_2). They were separated from each other and purified. Isoinhibitor A_1 was purified to homogeneity and studied in

detail. Inhibitors A_2 and A_3 were purified by Vartak (1975). The isoinhibitors B_1 and B_2 were partially purified and some of their properties studied. Isoinhibitor B_2 was found to be homogeneous by ultracentrifugation gel electrophoresis and SDS-gel electrophoresis, but contained impurities, possibly non-protein in nature. Table 8 indicates a brief outline of the purification procedure for the isoinhibitors A_1, B_1, B_2 of papain from the acid extract. The separation and purification of isoinhibitors A_1, B_1, B_2 and C are described in this chapter.

Chromatography on CM-cellulose of the fraction precipitating between 0.30 and 0.90 ammonium sulfate saturation gave two groups of inhibitors. The unadsorbed portion when treated with DEAE-cellulose further separated into two fractions, papain inhibitors A_1 and A_2 (which was further resolved into two isoinhibitors A_2 and A_3 on rechromatography on DEAE-celluloses). The two inhibitors differed in their elution properties. Papain inhibitor A_1 was weakly adsorbed on DEAE-cellulose and was eluted by 1.5 mM phosphate buffer, pH 7.5 while papain inhibitor A_2 and A_3 were eluted by 3 mM and 5 mM phosphate buffer, pH 7.5 respectively. Papain inhibitor A_1 was further purified by rechromatography on a DEAE-cellulose column, preparative polyacrylamide gel electrophoresis and treatment with immobilised trypsin to remove traces of trypsin inhibitor. The above procedure gave a homogeneous preparation. The inhibitors A_2 and A_3 were separated on a DEAE-cellulose column after preparative polyacrylamide gel electrophoresis (Vartak, 1975).

TABLE 8



ISOINHIBITORS OF PAPAIN FROM VIGNA CATJANG

The more basic inhibitors (i.e. those unadsorbed on DEAE-cellulose) were further separated into B_1 and B_2 on rechromatography on CM-cellulose. Papain inhibitor B_1 was eluted by 10 mM phosphate buffer, pH 7.5 and papain inhibitor B_2 by 50 mM phosphate buffer, pH 7.5.

Purification procedure for papain inhibitor A_1:

Small scale extractions were carried out using a Waring blendor. However this was found to be time-consuming and laborious for large scale extractions. Hence a power driven meat-mincer was used instead of a blendor and centrifugations were carried out in a Sharples centrifuge. An 18 kg batch of seeds was processed, the initial extraction and precipitation at 0-0.70 ammonium sulfate saturation being carried out with three batches of 6 kg each.

Step I - Extraction:

6 kg of Vigna catjang seeds of fairly uniform size were washed with distilled water, glass distilled water and soaked in glass distilled water for 30 min at room temperature. Washing of the seeds was essential to remove traces of insecticides often added as a preservative. All subsequent operations unless otherwise stated were carried out at 0°C. The seeds were ground to a fine meal by passing them twice through a meat mincer. The ground material was extracted with a chilled solution, containing 30 l of 0.01 potassium acetate buffer, pH 5.7, 450 g of KCl and 6 l of 0.6 M HCl. The final pH of the extract was 3.5. The extract was allowed to stand for 30 min with occasional stirring. The

extract was then passed through muslin cloth to remove coarse particles. The filtrate was neutralised to pH 6 by the addition of 1200 ml of 2 M $KHCO_3$ and the liquid was centrifuged in a Sharples centrifuge (15,000 x g). The clear supernatant liquid was used for the next step. (Fraction I).

Step II: Ammonium sulfate precipitation:

0-0.70 precipitation:

13,485 g of ammonium sulfate were added with constant stirring to the extract (31 l) till it dissolved completely. The liquid was then allowed to stand for a minimum period of 1 h for the precipitation to be complete and then centrifuged at 18,000 x g. The supernatant liquid was discarded and the precipitate was stored at -20°C. Two additional batches of 6 kg each were similarly processed and the ammonium sulfate precipitates pooled. (Fraction II).

Step III: Ammonium sulfate fractionation:

0.30 - 0.90 saturation: The precipitate obtained from the 18 kg batch (Fraction II) was dissolved in 7700 ml of 0.025 M potassium acetate buffer, pH 5.7 (final volume 8400 ml, 0.058 with respect to ammonium sulfate saturation, volume of the precipitate 700 ml). The ammonium sulfate saturation of the liquid was raised from 0.058 to 0.30 by adding 1100 g of ammonium sulfate (13.92 g/100 ml). The extract was allowed to stand for 1 h. The precipitate obtained was removed by centrifugation at 18,000 x g and the supernatant liquid was collected (volume 7900 ml). 3160 g of ammonium sulfate were added to the supernatant liquid to increase the ammonium sulfate saturation to 0.90. The solution was allowed

to stand for 60 min and was centrifuged at 18,000 x g. The precipitate was collected and dissolved in about 800 ml of 0.05 M potassium phosphate buffer, pH 7.5 and dialysed against six changes of 5 l of 0.01 M phosphate buffer, pH 7.5 over a period of 50 h. The supernatant liquid was collected (Fraction III) after centrifugation to remove a precipitate.

Step IV (a): Chromatography on CM-cellulose:

10 ml of 1 M H_3PO_4 were added to 2800 ml of the extract (Fraction III) to adjust the pH to 6.0. To this solution were then added 7200 ml of a suspension of 560 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 about 60 min and then filtered through a Buchner funnel. The cellulose was washed twice with 4800 ml of 0.005M phosphate buffer, pH 6.0 and filtered. The filtrates were pooled and the pH was raised to 7.5 with solid $KHCO_3$ (Total volume 15,320 ml). The above liquid containing unadsorbed material was precipitated with 10,056 g of ammonium sulfate at 0.95 saturation. The mixture was kept overnight and filtered. The precipitate was suspended in 500 ml of 0.05 M phosphate buffer, pH 7.5 and dialysed exhaustively against 0.03 M phosphate buffer, pH 7.5 to remove ammonium sulfate. After centrifugation the supernatant liquid (volume 2140 ml) was collected. (Fraction IV a).

Step IV (b): CM-cellulose chromatography:

CM-cellulose was eluted with 11,000 ml of 0.1 M phosphate buffer, pH 7.5 and then with 4000 ml of the same buffer (volume 15,800 ml). The above eluate was precipitated with 10,000 g of

ammonium sulfate (0.95 saturation). The eluate was allowed to stand for 60 min and filtered. The precipitate was dissolved in a 200 ml of 0.05 M phosphate buffer, pH 7.5 and dialysed exhaustively against 0.0005 M phosphate buffer, pH 6. The volume after dialysis was 500 ml. (Fraction IV b).

Step V: Chromatography on DEAE-cellulose:

Negative adsorption was carried out to remove the trypsin inhibitor and other impurities. 400 g of DEAE-cellulose suspended in 10 litres of 0.03 M phosphate buffer, pH 7.5 (equilibrated with 0.03 M phosphate buffer, pH 7.5) were added to 2140 ml of CM-cellulose supernatant liquid (Fraction IV a). The mixture was stirred for about 60 min and filtered through a Buchner. The DEAE-cellulose was washed twice with 6 l to 0.03 M phosphate buffer, pH 7.5 and filtered. The filtrates were pooled (volume 15 l) and precipitated with 9,750 g of ammonium sulfate at 0.95 saturation. The liquid was allowed to stand for 60 min and then filtered. The precipitate was dissolved in 0.01 M phosphate buffer, pH 7.5, (1000 ml), and dialysed against buffer of the above concentration. The dialysed material was concentrated by lyophilization, redialysed and reconcentrated to a volume of 320 ml. The material was stored in 0.01 M phosphate buffer, pH 7.5 at -20°C and was dialysed against 0.0005 M phosphate buffer, pH 7.5 before use. (Fraction V).

Step VI: Rechromatography on DEAE-cellulose:

300 g of DEAE-cellulose were washed and equilibrated with 0.0005 M phosphate buffer, pH 7.5. A column (8 x 40 cm) was packed with this DEAE-cellulose. The column was washed with

several volumes of the above buffer. Four such columns were run. 80 ml of the concentrated inhibitor solution (Fraction V) containing 96 x 10^3 units of papain inhibitor and 2.65 g of protein were loaded on the column. The column was washed with two column volumes of 0.0005 M phosphate buffer, pH 7.5. The inhibitor was then eluted by successive addition of 400 ml each of 0.0015 M and 0.005 M phosphate buffer, pH 7.5. 400 ml fractions were collected. The papain inhibitor content and protein of each fraction were then determined. Two isoinhibitors of papain, one weakly adsorbed and eluted at 0.0015 M phosphate buffer, pH 7.5 and the other eluted at 0.005 M phosphate buffer, pH 7.5, were obtained. The specific activity did not increase much, but this step was essential to separate the two isoinhibitors. The inhibitor eluted at 0.0015 M phosphate buffer, pH 7.5 was processed further. (The inhibitor eluted at 0.005 M potassium phosphate buffer, pH 7.5 was purified further by Vartak). Fractions having a specific activity ranging from 80 to 100 units/mg were pooled and concentrated by lyophilisation, dialysed against water and reconcentrated by lyophilisation to a volume of 250 ml (Fraction VI).

Step VII: Rechromatography on DEAE cellulose: (pH gradient)

Preliminary work done on a small scale showed that this isoinhibitor was strongly adsorbed on DEAE-cellulose at pH 9. (0.02 M Tris). The inhibitor was not eluted even at a concentration of 0.1 M Tris, pH 9.4-9.5. However the inhibitor was eluted at a pH of 8 or less. A considerable

amount of impurities could be removed by the above procedure and hence a pH gradient was used.

100 g of DEAE-cellulose were washed and equilibrated with 0.02 M Tris base. A column (4 x 40 cm) was packed with above DEAE-cellulose and washed with about two column volumes of 0.02 M Tris. The inhibitor solution (250 ml) (Fraction VI) containing 143 x 10^3 papain inhibitor units and 700 mg of protein was loaded on the column. The inhibitor solution was made 0.02 M with respect to Tris by the addition of 5 ml of 1 M Tris to 250 ml of the inhibitor solution. The column was first washed with 2000 ml of 0.02 M Tris, pH 9 and then with 2000 ml of 0.1 M Tris. The washings were collected in fractions of 100 ml each. The inhibitor was then eluted with a linear gradient formed from 2000 ml of 0.1 M Tris and 2000 ml 0.1 M Tris/HCl, pH 7.2 buffer. 40 fractions of 100 ml each were collected. The flow rate was 2 ml per min. The pH, protein and inhibitor activity of the individual fractions were determined and fractions having a purity of 1000 units/mg of protein and above were pooled. The pooled solution was concentrated, dialysed and lyophilised to a volume of about 8 ml (Fraction VII).

An electrophoresis run at this stage showed the presence of four to five impurities. The impurities were removed by preparative polyacrylamide gel electrophoresis as described below.

Step VIII: Purification by preparative polyacrylamide gel electrophoresis:

Glass columns (2.5 x 12 cm) containing 9% gels at pH 8.9

were used. The gels were washed by the procedure described in Chapter II prior to use. Tris-glycine buffer, pH 8.3 and 0.002 M with respect to glycine was used as the bath buffer. Several such polyacrylamide columns were run. 1 ml of the inhibitor solution (equivalent to 8 mg of protein and 8000 units of inhibitor) in 10% sucrose and containing 0.1 ml of 0.05% Bromophenol Blue was loaded at the cathodic end of the column, previously layered with 5% sucrose. Two gel columns were run at a time. A current of 5 mamps was applied and the run was carried out at 4°C for 30 to 40 h. The marker was allowed to flow out into the anodic compartment. Initial experiments on a small scale showed that reloading the marker and taking a second run gave better resolution. Hence the marker in 10% sucrose was loaded at the cathodic end of the column and it was allowed to reach the anodic end. Electrophoresis was then discontinued and the gel cut into eight equal portions. Each fraction was crushed and eluted with 0.1 M acetic acid. The crushed gel fractions were kept overnight and the individual fractions were estimated for inhibitor activity. Eight such columns were run. Individual fractions containing inhibitor activity were checked for homogeneity on polyacrylamide gels. Fractions which showed a homogeneous preparation were pooled. The pooled fraction was homogenised in a blendor to disperse the gel particles. The crushed material was extracted daily with 0.05 M acetate buffer, pH 4.4 over a period of ten days. The pooled extracts were concentrated, dialysed against water and

reconcentrated to a volume of 4 ml (Fraction VIII). The inhibitor was then tested for its homogeneity by gel electrophoresis. The pooled fractions showed a single band. However it was found that even though the inhibitor was apparently homogeneous it had trypsin and chymotrypsin inhibitor activity. A small amount of the above fraction was then treated with immobilised trypsin to determine whether the papain inhibitor itself had antitrypsin and antichymotrypsin activities. This experiment showed that the trypsin inhibitor activity could be removed by the bound trypsin leaving behind papain inhibitor, which was specific only for papain, ficin and chymopapain. The antichymotrypsin activity was also removed simultaneously with the trypsin inhibitor. The trypsin inhibitor was recovered from the complex by heating the complex in a boiling water bath. Thus a trypsin inhibitor was also obtained in homogeneous form. No further studies were carried out on the trypsin inhibitor since it was present in very small quantities and was not related to the object of this thesis.

Step IX: Treatment with immobilised trypsin:

The fraction obtained from polyacrylamide gel electrophoresis contained about 8000 units of trypsin inhibitor and 20,000 units of papain inhibitor. Bovine pancreatic trypsin was bound to Sephadex G-200 by the method of Axen and Ernback (1971). A suspension of immobilised trypsin in 0.1 M phosphate buffer, pH 7.5 (30 ml) and containing 10,000 units of trypsin was added to the inhibitor solution (Fraction VIII). The suspension was stirred continuously for 60 min. at 0°C. The suspension was then filtered through a Whatman No. 1 filter

paper. The residue was washed three times with 20 ml lots of 0.1 M phosphate buffer, pH 7.5. The filtrates were pooled, dialysed against water and then concentrated by lyophilisation. The preparation was tested for trypsin and chymotrypsin inhibitor activities and for homogeneity by gel electrophoresis. The above treatment successfuly removed the trypsin inhibitor impurity and a homogeneous papain inhibitor was obtained. This was used for the study of its properties (Fraction IX).

Removal of acrylamide impurities:

Polyacrylamide extracts contain non-protein impurities which absorb at 280 nm and also interfere in the estimation of protein by the method of Folin. An attempt was made to use DEAE-cellulose to remove the non-dialysable impurities. However at high pH 9 the impurities were adsorbed along with the inhibitor. It was necessary to do the adsorption at high pH, as adsorption of the inhibitor at pH 7.5 was weak. The impurities were partly eluted along with the inhibitor at pH 7.2. Hence true protein and specific activity of the inhibitor could not be determined by using routine methods.

Protein was determined by amino acid analysis, by taking a known dry weight of the sample. Specific activity of the A_1 inhibitor was found to be 4000 units/mg of protein. The above sample was homogeneous on gel electrophoresis and SDS-gel electrophoresis. The results of the purification of the inhibitor are shown in Tables 9 & 10. Its properties are described in a later chapter.

Yields of the inhibitor:

It is difficult to calculate the yield of the individual inhibitors due to the presence of a very large number of isoinhibitors. The amounts present in the crude extract of each isoinhibitor are not known and hence initial specific activity and the degree of purification cannot be calculated accurately. However the recoveries on DEAE-cellulose and on preparative polyacrylamide gel electrophoresis were poor and the reason for this is not clear since the inhibitors are stable over a wide pH and temperature range. The poor recovery at Step III is due to defective dialysis tubing which resulted in loss of material.

Table 9: Separation of inhibitor groups A and B

No.	Fraction	Volume	Activity	Total Activity	Protein	Total protein	Apparent specific activity
		ml	Units/ml	Units x 10^3	mg/ml	mg	Units/mg
1.	Seed Extract	-	-	-	-	-	-
2.	0-0.70 $(NH_4)_2SO_4$ saturation precipitate	700	3200	2240	170	118,000	19
3.	0.30-0.90 $(NH_4)_2SO_4$ saturation precipitate	2800	500	1400	18.5	52,000	27
4.	CM-cellulose* chromatography						
	a) CM-cellulose supernatant precipitated with $(NH_4)_2SO_4$ (A_1, A_2 & A_3)	2140	327	700	14.8	31,000	22
	b) CM-cellulose 0.1 M phosphate buffer, pH 7.5 eluate $(NH_4)_2SO_4$ precoipitate (B_1 and B_2).	500	636	318	9.2	4,600	69

*Chromatography on CM-cellulose yields two groups of iso-inhibitors A and B.

Table 10: Purification of isoinhibitor A_1

No.	Fraction	Volume ml	Activity Units/ml	Total activity Units x 10^3	Protein mg/ml	Total protein mg	Specific activity Units/mg
1.	CM-cellulose supernatant 0-0.90 $(NH_4)_2SO_4$ saturation precipitate (A_1, A_2 & A_3)	2140	327	700	14.8	31,000	22
2.	DEAE-cellulose 0.03 M phosphate eluate, pH 7.5 (A_1, A_2 & A_3)	320	1200	385	33	10,600	36
3.	DEAE-cellulose*						
	a) 0.0015 M phosphate eluate, pH 7.5 (A_1)	250	570	143	2.8	700	203
	b) DEAE-cellulase 0.005M** phosphate eluate, pH 7.5 (A_2 + A_3)	300	167	50	1.6	480	104
4.	DEAE-cellulose rechromatography, pH-gradient A_1	8	8000	64	8	64	1000
5.	Preparative polyacrylamide° gel electrophoresis A_1	4	6250	25	-	-	-
6.	Treatment with immobilised° trypsin A_1	10	2100	21	-	-	-
7.	DEAE-cellulose Rechromatography A_1	2	3600	7.2	0.9	1.8	4000***

* Chromatography on DEAE-cellulose yields inhibitors (A_1) and (A_2 & A_3)

° Protein could not be determined owing to acrylamide impurities.

*** Protein was determined by amino acid analysis.

** Two papain isoinhibitors A_1 & A_2 were obtained in homogeneous form by Vartak (1975). Their properties are given in a later Chapter.

Section II

Purification procedure for papain inhibitors B_1 and B_2

preliminary work

Occurrence of isoinhibitors in the seeds:

It is of importance to determine whether all the inhibitors occur in the seeds or whether they are artifacts due to the action of a proteolytic enzyme or other enzymes on one or more of the inhibitors. An extract was prepared by boiling powdered Vigna catjang seeds in KCl-HCl mixture for 2 min at 98°C. The material was processed in a similar way upto the CM-cellulose stage. The extract was further fractionated on CM-cellulose which gave two groups of inhibitors, one of which was adsorbed and the other not adsorbed on CM-cellulose. This suggests that the main groups of inhibitors are probably present originally in the seeds and are not artifacts obtained during the isolation procedure. Further work is required to determine whether the isoinhibitors in each main group are also present in the seeds or whether they are obtained during the purification procedure.

This section deals with the purification of the CM-cellulose adsorbable papain inhibitors.

A preliminary experiment was carried out to determine whether the CM-cellulose adsorbable inhibitor (B) could be further purified on DEAE-cellulose.

DEAE-cellulose treatment of CM-cellulose eluate:

2 ml of CM-cellulose eluate equivalent to 32 mg of protein and 800 units were added to 400 mg of DEAE-cellulose

equilibrated with 0.0005 M phosphate buffer, pH 7.5. The mixture was kept for 60 min and then filtered through a Buchner funnel and washed with the above buffer twice. The DEAE-cellulose was eluted twice with 3 ml of 0.1 M phosphate buffer, pH 7.5. The filtrates and eluates were tested for protein and inhibitor activity. All the inhibitor activity and protein were in the unadsorbed fraction and none in the eluate. The inhibitor was therefore sufficiently basic not to be adsorbed on DEAE-cellulose under these conditions. The above experiment also shows that the above inhibitor is different from the inhibitors A_1, A_2 and A_3. As DEAE-cellulose chromatography gave no purification it was decided to use CM-cellulose for further purification.

A small batchwise experiment on CM-cellulose was initially carried out.

CM-cellulose chromatography (Batchwise):

10 g of CM-cellulose were washed and equilibrated with 0.001 M phosphate buffer, pH 6. A column (20 x 3 cm) was packed with the cellulose under gravity and washed with two column volumes of 0.001 M phosphate buffer, pH 6. 2 ml of the inhibitor solution containing 63 mg of protein and 4000 units in 0.001 M phosphate buffer, pH 6 were loaded on the column. The column was washed with 200 ml of 0.001 M phosphate buffer, pH 6. The inhibitor was eluted with buffers of different phosphate concentrations. The elution was first carried out with 200 ml of 0.01 M phosphate, pH 7.5. 25 ml fractions were collected, the flow rate being 5 ml per 5 min.

The concentration of the buffer was then increased to 0.03 M and 8 fractions of 25 ml each were collected. The column was then successively eluted with 200 ml of the following concentrations 0.05 M, 0.075 M and 0.1 M phosphate buffer, pH 7.5. Individual fractions were tested for inhibitor activity and protein. 52% of the inhibitor activity was obtained in the 0.05 M phosphate eluate and about 30% in the 0.01 M phosphate fraction and about 15% remained unadsorbed. There was very little activity in the other fractions. The unadsorbed fraction was not further examined to determine whether it is a separate inhibitor or not. The above experiment showed that this fraction contains at least two inhibitors with different elution characteristics. On the basis of this experiment, a gradient elution was carried out to confirm the presence of two isoinhibitors.

Rechromatography on CM-cellulose:

Gradient elution:

A column of the following dimensions was used (28 x 1.2 cm), the effective length being 25 cm. The column was filled with 4 g of washed CM-cellulose equilibrated with 0.001 M, pH 6, phosphate buffer. 10 ml of the inhibitor solution equivalent to 143 mg of inhibitor protein and 10^4 units in 0.001 M phosphate buffer, pH 6 were loaded on the column. The column was washed with 200 ml of 0.001 M phosphate, pH 6.0. A gradient mixer containing 200 ml of 0.001 M phosphate buffer, pH 7.5 and 200 ml of 0.1 M phosphate buffer, pH 7.5 was connected to the column. The concentration of the buffer was increased slowly and the flow rate of the column was adjusted to 5 ml

per 5 min. Eighty 5 ml fractions were collected. The inhibitor activity and protein of each fraction were determined. Maximum purity of 500 units/mg was obtained, the starting purity being 69 units/mg of protein. The above fraction showed three bands on polyacrylamide gel electrophoresis. A further gradient rechromatography of the above 0.05 M fraction on CM-cellulose column gave a purity of 1000 units/mg protein. However this preparation was also found to be inhomogeneous on acrylamide and showed a major band and two faint bands. Hence polyacrylamide gel electrophoresis was carried out to determine whether a pure preparation could be obtained directly.

Polyacrylamide electrophoresis:

Electrophoresis on a small scale was carried out. Gels were washed according to the procedure described in Chapter II. At pH 8.3 (9% gels) there was hardly any movement of the protein (A cathodic run was carried out). 0.002 M Tris-glycine buffer was used as the bath buffer and Brilliant Cresyl Blue as the marker. 0.1 ml of the inhibitor solution (equivalent 30 units of inhibitor) containing 0.01 ml of 0.05% Brilliant Cresyl Blue was loaded at the anodic end of the polyacrylamide column. The current was 3 to 4 mamp. The movement of the bands was extremely slow and two bands of equal intensity were obtained. One of the two bands moved about 1 cm from the loading end, while the other showed no movement. Hence electrophoresis was carried out at low pH (4.4) using the method of Reisfield (1962) with slight modifications.

Polyacrylamide gel electrophoresis at pH 4.4:

9% washed gels were used for the run. 0.01 M potassium acetate buffer, pH 4.4 was used as the bath buffer. 0.1 ml of the solution containing 30 units and 0.7 mg protein was

loaded at the anodic end of the column (0.6 x 10 cm). The solution was in 10% sucrose and contained 0.003 ml of Basic Fuchsin as the tracking dye. The marker was allowed to reach about three fourths of the column length when electrophoresis was stopped. The gel was stained with 0.05% Amido Black and kept for 60 min and destained with 3% acetic acid. Four to five bands were seen. One of the bands moved with the marker and a band of equal intensity was close to it. Another gel was run simultaneously for detection of the inhibitor. This gel was sliced into eight equal fractions. Each fraction was eluted separately with water and allowed to stand for 24 h after crushing the gel. The inhibitor activity of each fraction was determined. The inhibitor activity was found to coincide with the component moving with the marker. A control was run to correct for inhibition of papain by the dye or by acrylamide extract. The purified inhibitor was found to have the same mobility as the marker. An important point to note here is that in unwashed gels, the movement of the marker as well as proteins was impeded by impurities from the gel and virtually no separation or movement of the protein bands was observed in unwashed gels. Hence washing was essential for rapid and effective resolution. On the basis of these preliminary studies the following procedure was used for the separation and purification of these isoinhibitors.

Final purification procedure:

A batchwise CM-cellulose adsorption and elution to separate the isoinhibitors and the purification of one of

them by preparative gel electrophoresis were the two steps adopted. Gradient chromatography on CM-cellulose was not adopted since it failed to give a homogeneous preparation. Moreover the fraction obtained by this procedure was unstable on storage. CM-cellulose adsorption was also done batchwise, since column runs took too much time. Preparative polyacrylamide electrophoresis was used as the final purification step since the impurities could not be removed by gradient chromatography on CM-cellulose. Secondly the inhibitor had a mobility similar to that of the tracking dye, which facilitated the purification of the inhibitor by preparative gel electrophoresis. The inhibitor obtained by this procedure was very stable on storage.

The procedure used for extraction of seeds (step I) and precipitation of the extract with ammonium sulfate at 0.70 saturation (step II), subsequent ammonium sulfate fractionation (0.3 - 0.90) (step III) and CM-cellulose chromatography (step IV b) were the same as for the purification of the DEAE-cellulose adsorbable inhibitor.

Step V: Rechromatography on CM-cellulose:

Inhibitors B_1 and B_2 were separated at this step. 200 g of CM-cellulose were washed and equilibrated with 0.0005 M potassium phosphate buffer, pH 6. To this 500 ml of the dialysed inhibitor solution (from step IV b) were added and kept for 60 min with constant stirring. The suspension was then squeezed through muslin cloth and the filtrate centrifuged at 4000 x g for 30 min to remove finer

particles of CM-cellulose. It was then washed with 2500 ml of 0.001 M potassium phosphate buffer, pH 6 and eluted with 2500 ml of 0.01 M phosphate buffer, pH 7.5. The cellulose was washed twice with 2500 ml of 0.01 M phosphate buffer, pH 7.5. This fraction (0.01 M eluate) contained the B_1 inhibitor. It was precipitated with ammonium sulfate at 0.90 saturation and stored at -20°C. No further purification of this inhibitor was undertaken as it was found to be unstable on purification. A few of the properties were studied using this fraction after dialysis. Finally it was eluted with 2500 ml of 0.1 M phosphate buffer, pH 7.5 and washed twice with 0.1 M phosphate buffer, pH 7.5. Each fraction was assayed separately for papain inhibitor activity. The 0.1 M fractions were pooled and concentrated by lyophilisation to a smaller volume and dialysed against water and reconcentrated to a volume of 10 ml. (Fraction V).

Step VI: Preparative polyacrylamide gel electrophoresis:

Polyacrylamide gel columns (2.5 x 12 cm) were used. pH 4.4 gels were prepared and washed by the procedure described in Chapter II. 0.01 M potassium acetate buffer, at pH 4.4 was used as the bath buffer. Two gel columns were used each time and ten such experiments were carried out to collect sufficient material. 0.5 ml of the inhibitor solution (equivalent to 3750 units and 38 mg) in 10% sucrose and containing 0.04 ml of 0.05% Basic Fuchsin were loaded on each gel (earlier layered with 5% sucrose) at the anodic end of the column. A current of 5 mamp per tube was applied

in the beginning (14 h) and then increased to 10 mamp per tube. Each run took about 30 h. The marker was allowed to flow up to 2 cm from the cathodic end, when electrophoresis was discontinued. The gels were removed and each gel cut into eight equal portions. Each section of the gel was crushed separately into fine particles and eluted overnight with water. Each fraction was then tested for inhibitor activity the next day. The active fractions were tested for homogeneity by polyacrylamide gel electrophoresis. Those fractions which showed a single band were pooled, homogenised in a blendor and extracted daily with 0.05 M potassium acetate buffer, pH 4.4, over a period of ten days. The filtrates were collected, pooled, concentrated dialysed against water and lyophilised to a volume of 8 ml (Fraction VI).

Step VII: CM-cellulose chromatography:

This step was essential to remove some of the non-dialysable acrylamide impurities. 25 g of CM-cellulose, was equilibrated with 0.001 M phosphate buffer, pH 6. 8 ml of the inhibitor solution in 0.001 M phosphate buffer, pH 6 containing 25 x 10^3 units were added and stirred for 60 min. The slurry was then filtered through a Buchner funnel and washed several times to remove adhering acrylamide impurities. The cellulose was then eluted with 100 ml of 0.1 M phosphate buffer, pH 7.5 thrice. The eluates were then pooled, dialysed and concentrated by lyophilisation. The above homogeneous preparation was used to study the properties of the inhibitor.

The preparation obtained by polyacrylamide gel

electrophoresis was found to be homogeneous on SDS-gel electrophoresis and ultracentrifugation. However the specific activity of the preparation was only 100 units/mg. Since a specific activity of 1000 units/mg was obtained on gradient elution from a CM-cellulose column, when fraction V was loaded it is likely that the preparation contained impurities which had the same mobility as the inhibitor or inactive inhibitor or non protein impurities which interfered with the Folin and spectrophotometric protein assays. Work on fractionation with CM-cellulose showed that the inhibitor lost activity on concentration by lyophilisation. Hence it is possible that this fall in activity was due to inactivation of the preparation on concentration. Attempts to determine its true purity were made by using immobilised papain. Only the active material was bound by papain. Elution of the inhibitor was carried out by heating the complex at 80°C at pH 2. 40% of the activity was recovered. A specific activity of 1000 units/mg was calculated from the difference in the initial "protein" content and the "protein" remaining unadsorbed by immobilised papain. However further work is required to determine the reason for the low specific activity of this apparently homogeneous fraction.

Yields of the inhibitor:

As stated earlier the true yield and increase in specific activity on purification could not be calculated due to the presence of a large number of isoinhibitors. Rechromatography on CM-cellulose (step 4) gave two inhibitors

which were present in nearly equal quantities. The final specific activity could not be accurately determined due to the presence of non-protein impurities. The losses on CM-cellulose chromatography as in the case of DEAE-cellulose chromatography could not be explained. Inhibitor B_1 was not purified further due to its lability on purification, but the properties of the partially purified material were studied. The separation of B_1 and B_2 and purification of B_2 are shown in the table given below: (Table 11).

Table 11: Purification of papain inhibitors B_1 and B_2

No.	Fraction	Volume ml	Activity Units/ml	Total activity Units x 10^3	Protein mg/ml	Total protein mg	Apparent specific activity Units/mg
1.	CM-cellulose 0.1 M eluate phosphate buffer, pH 7.5, 0-0.90 $(NH_4)_2SO_4$ saturation (B_1 + B_2)	500	636	318	9.2	4,600	69
2.	CM-cellulose rechromatography* 0.1 M phosphate eluate, after dialysis and lyophilisation Isoinhibitor B_2	10	7500	75	75	750	100
	0.01 M phosphate eluate Isoinhibitor B_1	2000	30	60	1.5	3,000	20
3.	Preparative polyacrylamide(B_2)	8	3125	25	-	-	-
4.	CM-cellulose rechromato-graphy	2	3500	7	35	70	100**
5.	Immobilised papain	2	3500	7	3.5	7	1000***

* Separated into two inhibitors B_1 and B_2.

** Protein determined on dry weight basis.

*** Specific activity was found to be 1000 units/mg by using immobilised papain.

Section III

Purification of papain inhibitor C from alkaline extract of cholai seeds:

Preliminary experiments indicated that extraction at pH 7.5 with 0.1 M potassium phosphate buffer, pH 7.5 gave nearly six times the quantity of papain inhibitor activity as compared to that of the acid extract. Hence purification of the inhibitor from the alkaline extract was initially attempted.

Preliminary work:

100 g of seeds were washed with distilled water and glass distilled water and soaked for 30 min at room temperature. The soaked seeds were blendorized for 2 min with 500 ml of 0.1 M phosphate buffer, pH 7.5. The extract was allowed to stand for 30 min and then passed through muslin cloth to remove the coarse particles. The filtrate was centrifuged at 4000 x g for 20 min. The clear supernatant liquid was used for purification (volume 485 ml) (Fraction I).

Ammonium sulfate precipitation:

485 ml of the extract were precipitated at 0.9 saturation by the addition of 300 g of ammonium sulfate under constant stirring. The precipitated extract was filtered through a Whatman No. 1 filter paper overnight. The precipitate was scooped out and dissolved in 60 ml of 0.05 M Tris/HCl buffer, pH 7.5. The above liquid was dialysed against several volumes of 0.01 M Tris/HCl buffer, pH 7.5 over a period of 30 h. The volume after dialysis

was 236 ml (Fraction II).

Acid precipitation:

100 ml of the above fraction were mixed with 100 ml of glass distilled water and precipitated with 0.9 ml of 2 M acetic acid at pH 5. The precipitate obtained was centrifuged and both the precipitate and the supernatant liquid were tested for inhibitor and proteinase activity. The supernatant liquid contained all of the inhibitor activity, while the proteniase was precipitated out. However the supernatant liquid did contain traces of proteinase activity. The specific activity increased from 2 to 15 units/mg of protein (Fraction III).

Ammonium sulfate precipitation:

178 ml of the acid precipitated supernatant liquid were precipitated with 135 g of ammonium sulfate at 0.9 saturation. The precipitate was collected by centrifugation and suspended in a known volume of 0.05 M phosphate buffer, pH 7.5 and dialysed against 0.0005 M phosphate buffer, pH 7.5 (Fraction IV).

DEAE-cellulose chromatography:

Earlier batchwise experiments with Fraction II (dialysed 0.90 extract) showed that the inhibitor was adsorbed on DEAE-cellulose and eluted at 0.01 M phosphate buffer, pH 7.5. Hence a column with the following dimensions 1.5 x 35 cm was packed with washed DEAE-cellulose equilibrated with 0.0005 M phosphate buffer, pH 7.5. The column was washed with the same buffer and the inhibitor solution (7500 units and 250 mg) was loaded on the column.

The column was washed with several volumes of 0.0005 M phosphate buffer, pH 7.5 and then a gradient elution between 0.0005 M phosphate buffer, pH 7.5 and 0.05 M phosphate buffer, pH 7.5 was used to elute the inhibitor. Substantial purification was obtained. The purity was raised from 30 to 500 units/mg of protein in some of the fractions. However 75% of the inhibitor activity was lost within 24 h. As the supernatant liquid contained traces of proteinase activity, the sudden loss of inhibitor activity was possibly due to the action of the proteinase. Hence various methods were tried to destroy the proteinase before proceeding with further purification (Fraction V).

Removal of proteinase activity:

The following compounds were used to see whether they had any effect on the proteinase activity. The effect of these reagents on the inhibitor activity was tested before testing them on proteinase activity. (1) DFP (2) $HgCl_2$ (3) p^{CMB}. DFP at even a concentration of 10^{-3}M had no action on the proteinase, while both $HgCl_2$ and p^{CMB} at a concentration of 0.005 M destroyed only 30% of the proteinase activity. None of the reagents had any effect on the inhibitor.

Heating experiments:

Heating the extract at pH 7.5 (Fraction II and Fraction I) and at 90°C did not destroy the proteinase activity. However heating the acid supernatant (Fraction III) at pH 5 at 80°C destroyed it. The inhibitor activity was not lost on heating at pH 7.5 at 90°C. The supernatant liquid after precipitation with acid was found to be unstable at pH 5, when not

heated. Hence heating at pH 5 at 80°C was adopted for destroying the proteinase.

Streptomycin treatment of the alkali extract:

As several non-protein impurities interfered in the purification of the inhibitor and also to avoid dialysis after ammonium sulfate precipitation of the extract (step 2), which was extremely laborious, the extract was precipitated with streptomycin sulfate (1% streptomycin base). The extract was prepared in 0.01 M Tris-HCl buffer pH 7.5 instead of phosphate. When streptomycin treated extract was precipitated with acetic acid to pH 5, 75% of the inhibitor activity was lost on dialysis in 24 h. The dialysate contained no inhibitor activity. However if the extract was adjusted to pH 5 and neutralised immediately there was no fall in activity. This instability could not be explained. As this method was not successful it was not included in the final purification procedure. On the basis of the above results the following procedure was adopted for the purification of inhibitor C from the alkaline extract. The yield of the inhibitor was not good. A large number of experiments (direct extraction of seeds at 80°C at pH 5, fractionation with solvents etc.) which were tried were ineffective in increasing the yield and hence the following procedure was used without further modifications.

Final purification procedure:

Step I: Extraction:

Two kg seeds were washed with distilled water and then

with glass distilled water, soaked in glass distilled water for 30 min at room temperature and then passed through an electrically operated meat mincer twice. The fine meal obtained was suspended in 10,000 ml of previously chilled 0.1 M potassium phosphate buffer, pH 7.5. The extract was allowed to stand for 30 min at 4°C and then passed through muslin cloth to remove coarse particles. The filtrate was then centrifuged in a Sharples centrifuge at 15,000 x g. The precipitate was discarded and the supernatant liquid was processed further (volume 9,000 ml) (Fraction I).

Step II: Ammonium sulfate precipitation:

5,400 g of ammonium sulfate were added over a period of 60 min to 9000 ml of the extract to give 0.90 saturation. The material was allowed to stand for 2 h and then filtered through Whatman No. 1 filter paper overnight. The next morning the precipitate was suspended in 0.05 M Tris/HCl buffer, pH 7.5 (700 cc). The volume was one litre and the ammonium sulfate saturation 0.4. The precipitate was homogenised and filled in cellulosic casing tubings. The suspension was dialysed against 85 l of 0.01 M Tris/HCl buffer, pH 7.5. Two changes of 85 l each were given. The material was dialysed over a period of 48 h. The volume after dialysis was 4060 ml. (Fraction II).

Step III: Acid precipitation:

4000 ml were used for acid precipitation. The dialysed extract was mixed with an equal volume of glass distilled water (4000 ml) and precipitated with 40 ml of 2 M acetic

acid at pH 5. (If the dilution with water was not carried out there was loss of inhibitor). All operations were carried out in the cold and as rapidly as possible in this step and the next. The precipitate was removed by centrifugation in a Sharples centrifuge at 15,000 x g (Volume 6,700 ml) (Fraction III).

Step IV: Heating at pH 5 at 80°C.

6000 ml were filled in dialysis tubings and the bags were dropped into a water bath containing about 80 litres of 0.01 M sodium acetate buffer, pH 5 at about 80°C. The material was heated for 3 min at 80°C. The tubings were then plunged into ice chilled glass distilled water. Cellulosic casing was used to heat the material since the required temperature is attained rapidly. This was necessary to avoid the action of the proteinase during slow heating (volume 5,900 ml). (Fraction IV).

Step V: Ammonium sulfate precipitation at pH 5:

5900 ml were precipitated at 0.95 saturation by the addition of 4000 g of ammonium sulfate. The extract was precipitated without removal of denatured proteins. This was necessary since filtration of the ammonium sulfate precipitate was otherwise difficult. If the heat denatured protein was removed first, the volume was too large for high speed centrifugation. Hence precipitation was carried out in the presence of denatured protein. The ammonium sulfate precipitated suspension was filtered through Whatman filter paper No. 1 overnight. The precipitate was suspended in 250 ml of 0.05 M Tris/HCl, pH 7.5 and the

whole suspension was dialysed against 0.001 M Tris/HCl buffer, pH 7.5. The denatured protein was removed by centrifugation at 4000 x g. Removal of the precipitate by filtration and subsequent dialysis gave poor recovery. (Fraction V).

Step VI: DEAE-cellulose chromatography (Batchwise):

800 ml of a suspension of DEAE-cellulose (40 mg/ml) washed according to the method of Peterson and Sober were added to 480 ml of the inhibitor solution (Fraction V) in 0.001 M Tris/HCl buffer, pH 7.5. The volume was made to 2500 ml with glass distilled water. The suspension was stirred for 60 min and filtered through a Buchner funnel. The cellulose was washed twice with 1500 ml of glass distilled water. It was then eluted with 950 ml of 0.01 M phosphate buffer, pH 7.5. The eluates were pooled, concentrated by lyophilisation, dialysed against water and reconcentrated. (Fraction VI). 30% of the inhibitor activity remained unadsorbed. This unadsorbed fraction was not further studied.

Step VII: Refractionation on a DEAE-cellulose column:

A column of the following dimensions was used (1.3 x 29 cm). 25 g of washed DEAE-cellulose were packed in a column. 760 mg of protein equivalent to 163 x 10^3 units in 0.0005 M phosphate buffer, pH 7.5 were loaded on the column. The column was washed with several volumes of 0.0005 M phosphate buffer, pH 7.5. A gradient was applied between 0.0005 M and 0.02 M phosphate buffer, pH 7.5. The

flow rate was 14 ml per 16 min. 200 such fractions were collected. The individual fractions were tested for protein as well as inhibitor activity. Fractions having a purity of 1000 units/mg of protein and above were pooled, lyophilised, dialysed and reconcentrated to a volume of 8 ml (Fraction VII).

Step VIII: Sephadex G-100 fractionation:

A column of the following dimensions was used (100 x 4 cm). The void volume of the column as determined by using blue dextran was 380 ml. Sephadex G-100 was prepared as mentioned in Chapter II and packed after deaeration (70 g). The column was washed with 0.05 M phosphate buffer, pH 7.5. 2 ml of the inhibitor solution equivalent to 5 mg protein and 10^4 units in 0.05 M phosphate buffer, pH 7.5 were loaded on the column. The flow rate was 10 ml per 15 min. 120 fractions (each 10 ml) were collected. Individual fractions were tested for protein and inhibitor activity. Four such columns were run. The maximum specific activity obtained by the above column was 5000 units/mg of protein. The recovery of activity was about 70%. Fractions having a specific activity of 5000 units/mg of protein were pooled, lyophilised and dialysed and reconcentrated. (Table 12)

A polyacrylamide gel electrophoresis run at this stage showed four bands. (Two major and two minor bands).

Preliminary experiments on polyacrylamide gel electrophoresis:

Unwashed gels when used gave no recoverable inhibitor activity. Two experiments were run simultaneously. One gel was used for detecting the bands and the second to determine the inhibitor activity after elution. Electrophoresis was

Table 12: Purification of papain inhibitor C from alkaline extract.

No.	Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity
		ml	Units/ml	Units x 10^3	mg/ml	mg	Units/mg
1.	Seed extract	10,000	140	1400	50	500,000	2.8
2.	0-0.90 $(NH_4)_2SO_4$ saturation precipitate	4,000	300	1200	40	162,000	7.5
3.	Acid supernatant pH 5.	6,700	58	389	3.8	25,460	15
4.	Acid supernatant heated at pH 5 and pH 7.5 at 80°C.	6,000	45	270	1.5	9,000	30
5.	0-0.95 $(NH_4)_2SO_4$ saturation precipitate	480	500	240	6.4	3,072	78
6.	DEAE-cellulose (Batchwise) 0.01 M phosphate buffer, pH 7.5 eluate.	1,900	86	163	0.4	760	215
7.	DEAE-cellulose column concentration gradient.	8	5000	40	2.5	20	2000
8.	Sephadex G-100	4	4000	16	0.8	3.2	5000

2 kg seeds were used.

run at pH 8.3. 500 units were loaded on each gel. One gel was stained with Amido Schwarz and destained with 3% acetic acid. The other gel was cut into 8 equal portions and each portion was eluted with 0.1 M acetic acid and kept overnight for elution at 20°C. Four protein bands were observed out of which two bands were active. One of the bands corresponded to 40% and the other to 60% of the inhibitor activity. The stability of these fractions was tested after 10 days. The inhibitor having a relatively lower mobility lost all of its activity while the other one remained active. Further work is necessary to determine the reason for this unexpected loss of activity of one of the fractions. Preliminary experiments indicated that the purity of a preparation of specific activity 5000 units/mg of protein was about 80% pure on the basis of the intensity of staining of the different bands.

Purification of papain inhibitor C by affinity chromatography:

Purification of papain inhibitor C by using papain, immobilised on Sephadex G-200 was tried. Papain was immobilised according to the method described in Chapter II by coupling to Sephadex G-200 activated by cyanogen bromide.

The extract was prepared as mentioned in step I (purification from alkaline extract Section III). 10 ml of bound papain (equivalent to 12×10^3 units) were activated by the addition of 0.2 ml of 0.5 M neutralised cysteine HCl and then added to 90 ml of the extract (11×10^3 units) containing 0.01 M cysteine. The suspension

was stirred for 60 min and then centrifuged at 4000 x g for 30 min. The bound material was washed several times with 0.02 M phosphate buffer, pH 7.5 containing 0.005 M EDTA. The bound inhibitor was then suspended in 30 ml of 0.1 M phosphate buffer, pH 7.5 and the pH was lowered to 1 by the addition of 5.75 ml of 3 M HCl. The suspension was kept for 20 min at 0°C and then centrifuged. The residue was washed twice with 20 ml and 10 ml as described above. The eluates were pooled, neutralised with 2 M $KHCO_3$ and dialysed against 0.02 M phosphate buffer, pH 7.5. The unadsorbed supernatant liquid as well as the eluate were tested for activity. Protein in the eluate was determined by Folin's method. About 50-60% of the inhibitor activity could be recovered. The eluate had a high specific activity ranging from 3300 to 4000 units/mg of protein in different batches. The stability of the inhibitor was checked after storage at -20°C or at 0°C for about ten days. However the material was found to be unstable and about 75% of the activity was lost in ten days. Elution at pH 2 resulted only in 10 to 15% recovery of the inhibitor. Even though a thorough washing was given after binding the inhibitor, traces of proteolytic activity acting on the inhibitor were possibly present. Heating the material at pH 5 before elution also did not stabilise the inhibitor. It was thought that the excess papain (i.e. unbound papain) may be acting on the inhibitor when bound. Hence bound mercury papain was used. Hg bound papain was prepared as described in Chapter II. Adsorption and elution were done as

described above except that cysteine and EDTA were eliminated from the mixture and 0.1 M Tris/HCl was used instead of phosphate. All the inhibitor in the seed extract was bound to the Hg bound papain. However the recovery on elution was about 35%. The solutions were dialysed against EDTA prior to estimation. EDTA was also added in the assay system to remove traces of mercury. However the inhibitor obtained from the Hg bound immobilised system also lost activity on storage at -20°C. Binding to S-alkyl papain attached to Sephadex G-200 was also tried and though binding of inhibitor was quantitative its recovery was very poor.

Since the material was found to be unstable, purification by this procedure was not continued though relatively good recovery and very high purification were obtained in a single step. The reason for the instability of the inhibitor after treatment with immobilised papain and mercury papain is not known. In separate experiments it was shown that the papain inhibitor purified from alkaline seed extract by the procedure described earlier was quite stable after addition of acid to pH 1, followed by neutralisation. The instability was therefore not due to the acid treatment. Neither could it be due to residual papain activity, since papain was totally destroyed at pH 1 and since Hg-papain gave the same result as papain. The results of the purification of the above inhibitor by affinity chromatography are tabulated in Table 13.

Table 13: Purification of papain inhibitor C by affinity chromatography

Fraction	Total Inhibitor activity	Total protein	Recovery	Specific activity
	Units x 10^3	mg	%	Units/mg
Original Extract	11	180	-	6.1
Supernatant liquid after binding	0	-	-	-
Eluate	6	1.7	55	3350*

*Protein was determined by Folin's method.

C H A P T E R V

P R O P E R T I E S

Section I

Homogeneity and specificity

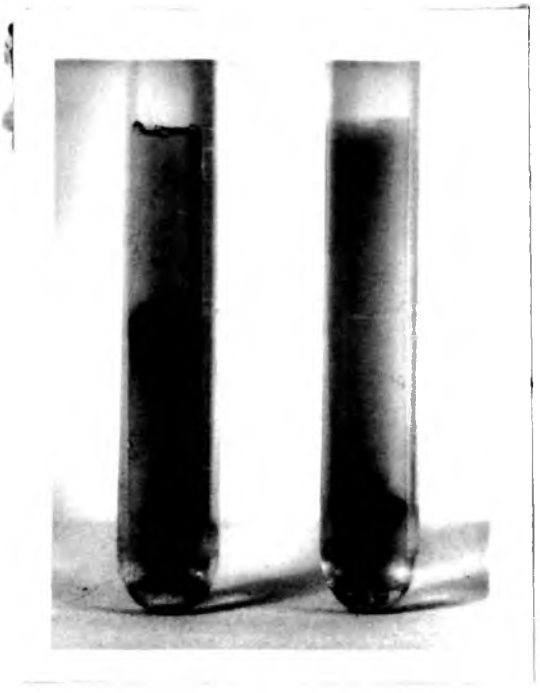
General:

The seeds of Vigna catjang contain six isoinhibitors of papain. They have been separated and characterized. Papain inhibitors A_1 A_2 and A_3, B_1 and B_2 were isolated from acid extracts of the seeds, while inhibitor C was isolated from the alkaline extract. Papain inhibitor A_1 was purified to homogeneity and had a specific activity of 4000 units/mg of protein. Inhibitors A_2 and A_3 were homogeneous and had a specific activity of 6250 and 5000 units/mg of protein respectively (Vartak, 1975). Papain inhibitor C was purified to a specific activity of 5000 units/mg of protein. Inhibitors B_1 and B_2 were separated and B_2 was purified to a specific activity of about 1000 units/mg of protein.

Homogeneity:

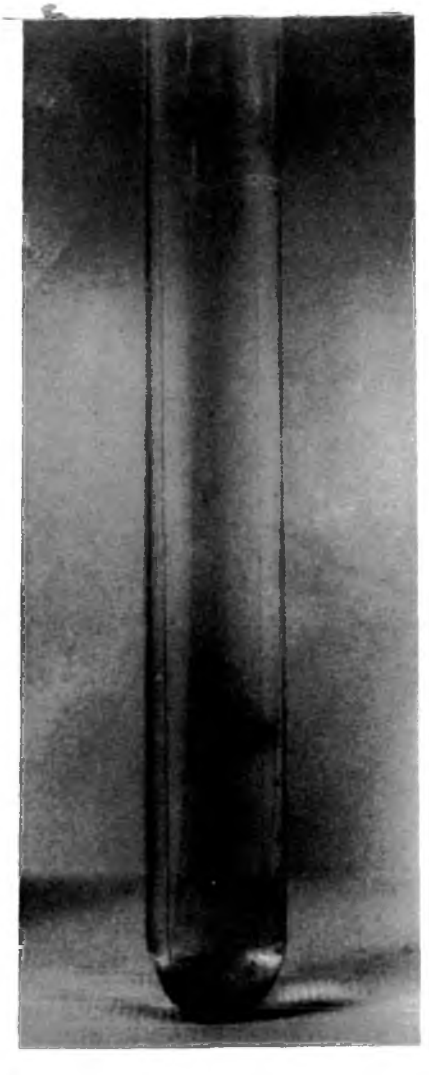
Papain inhibitors A_1 A_2 and A_3 were homogeneous on polyacrylamide gel electrophoresis and SDS-gel electrophoresis. Electrophoresis was carried out using 9.3% gels. Inhibitor A_1 was homogeneous at pH 9.9 and 8.3 (anodic run was carried our). 20 to 30 µg of inhibitor were used for each electrophoretic run (photograph 1). As stated earlier inhibitor C even at a specific activity of 5000 units/mg of protein showed four bands, of which two bands had inhibitor activity. One of these inhibitors was found to be unstable on storage at -25°C. It is not known whether two inhibitors are

Photograph No. 1: Disc gel electrophoresis of papain inhibitor A_1



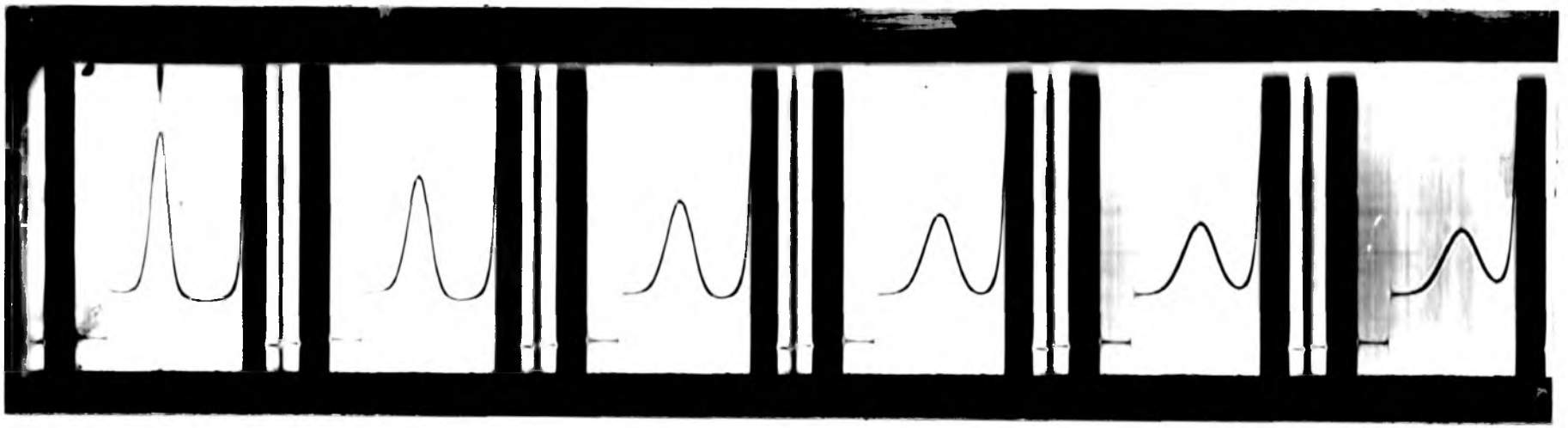
50 ug of inhibitor protein were used. Electrophoresis was carried out at pH 8.3 and pH 9.9. (a) pH 8.3 and (b) pH 9.9. Tris-glycine system at pH 8.3 and 0.1 M Tris base system at pH 9.9 were used. Electrophoresis was carried out in 9.3% gels. Migration was towards the anode. Other conditions are as described in Materials and Methods.

Photograph No. 2: Disc gel electrophoresis of papain inhibitor B_2



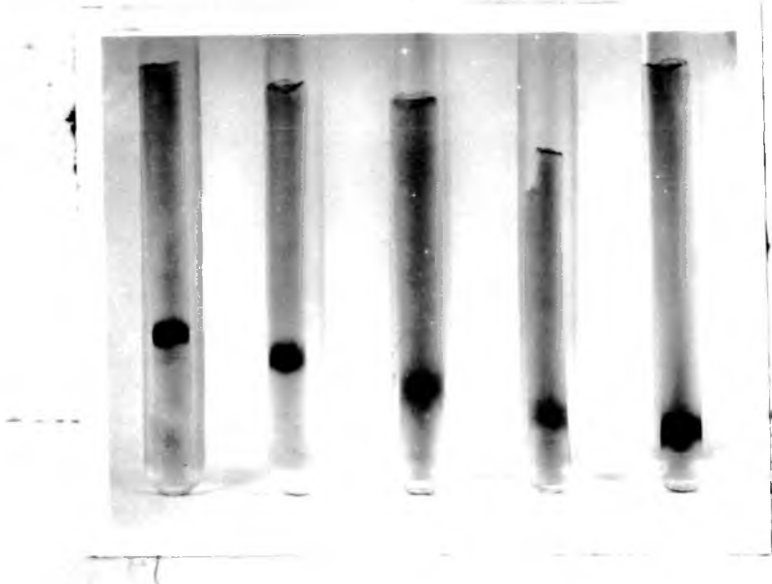
25 ug of inhibitor protein were used. Electrophoresis was carried out in 9.3% gels at pH 4.4 at 3-4 mA/gel. Migration was towards the cathode. Other conditions are as described in Materials and Methods.

Photograph No. 3: Ultracentrifuge profiles of papain inhibitor B_2



Protein concentration, 10 mg/ml in 0.1 M phosphate buffer, pH 7.5; Speed, 59,780 rpm; temperature 23°C; phase plate 60°; cell, synthetic boundary cell. Other conditions are as described in Materials and Methods.

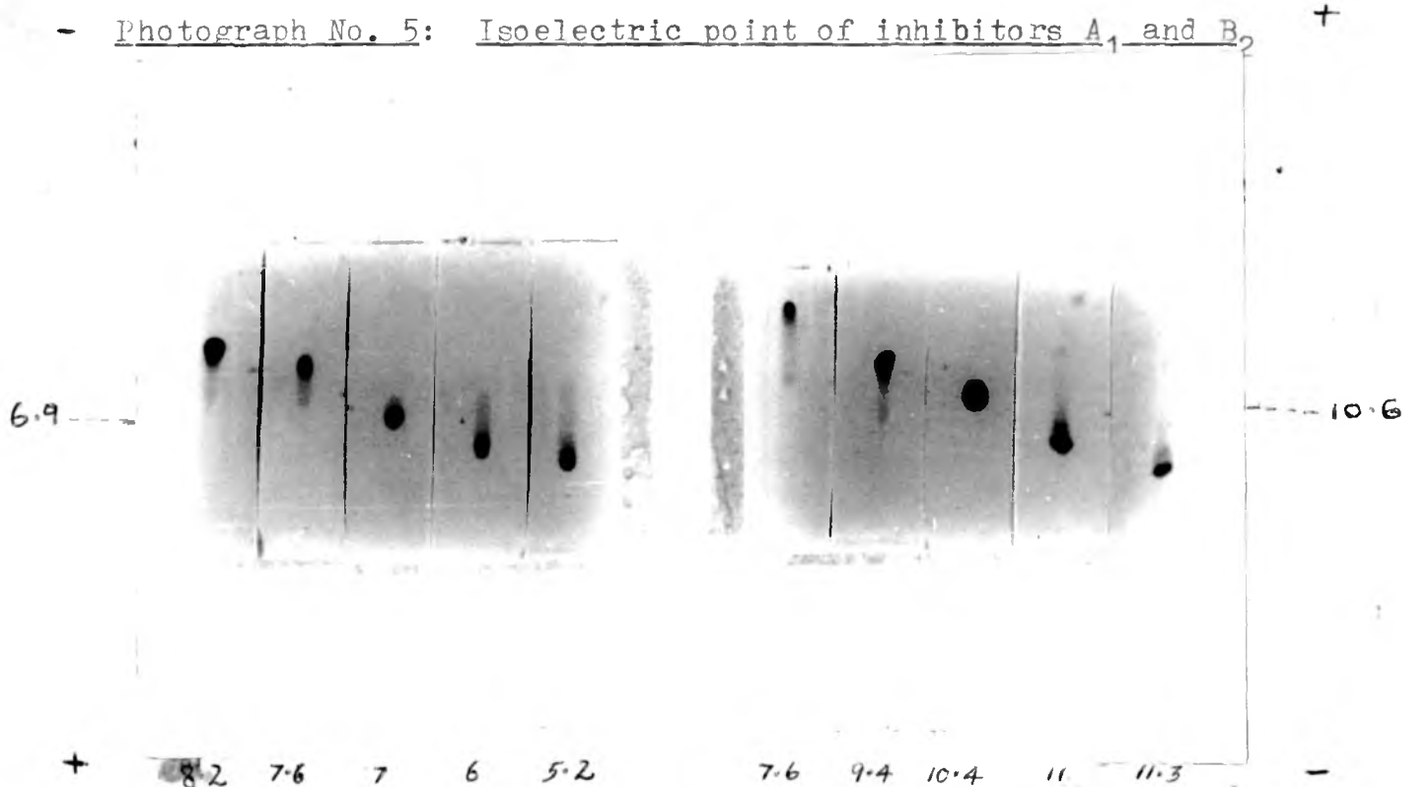
Photograph No. 4: SDS-gel electrophoresis of papain inhibitors A_1 and B_2



Standard proteins used as markers were myoglobin - 10 ug, cytochrome c - 10 ug and insuline 10 ug. 10-15 ug of each inhibitors were used. Electrophoresis was run at pH 7.2, phosphate buffer containing 1% SDS. Staining of the gels was with Coomassie Blue. Other conditions are as described in Materials and Methods.

Position of tracking dye.

Photograph No. 5: Isoelectric point of inhibitors A_1 and B_2



10 ug of concentrated inhibitor were used. Cellogel strips were used for electrophoresis. Buffer concentration employed in each case was 0.005 M. Staining was by Amido Schwarz and destaining by acetic acid. Other conditions are as described in Materials and Methods.

present in C or whether one of the bands contains the inhibitor in association with a proteinase, which causes loss of activity of the inhibitor.

Inhibitor B_2 moved towards the cathode at pH 4.4, along with the marker, as a single component (photograph 2). The inhibitor was found to be homogeneous at pH 7 and 8.5 (cathodic run). It moved as a single component in 15% gels at pH 4.4 and at pH 7. It was also found to be homogeneous by SDS-gel electrophoresis and ultracentrifugation (photograph 4 & 3). The inhibitor sedimented as a single symmetrical peak at 59,780 rev per min in the ultracentrifuge. The profiles obtained after 15, 30, 45, 60, 75 and 90 min of ultracentrifugation showed only a single component. (These results are discussed further in the Section on "Molecular weight"). As stated earlier the specific activity of this inhibitor was only 100 units/mg of protein whereas chromatography on CM-cellulose gave a specific activity of 1000 units/mg of protein suggesting that the preparation is not homogeneous. Further work is required to determine the reason for the low specific activity of this apparently homogeneous preparation.

Specificity of isoinhibitors of papain:

All the papain inhibitors also inhibit ficin and chymopapain. They do not inhibit any of the serine proteinases such as trypsin, chymotrypsin and subtilisin. They show no inhibition of pepsin and fruit bromelain.

A comparative study of the inhibition of papain, chymopapain and ficin by the different isoinhibitors with

different substrates was carried out. The results of these experiments are summarised in the table given below (Table 14). The figures in parenthesis indicate the molar inhibition ratio of inhibitor to enzyme. Molar inhibition ratio is the ratio of the number of molecules of enzyme inhibited by a molecule of the inhibitor. ($\frac{\text{mole of enzyme}}{\text{mole of inhibitor}}$). Molar inhibition ratios were calculated using the specific activities of pure enzymes (Table 2), and inhibitors. In the case of inhibitor C a maximum purity of 5000 units/mg of protein was assumed for calculations. (Since this inhibitor is not homogeneous, the actual molar inhibition ratio for the pure inhibitor will be higher). The molecular weights of enzymes and inhibitors used for calculations are summarised in tables 2 and 22. Striking variations in the activities of the inhibitors with different enzymes, different protein substrates and synthetic substrates were observed. The results are discussed below separately with regard to enzymes and substrates.

Casein:

As seen from table 14, A_1 shows high inhibition of papain and chymopapain, but inhibition of ficin is only one sixth of that with the other enzymes. Inhibitors A_2 and A_3 show high inhibition of papain and ficin, the inhibition being nearly the same. Since A_1 shows only feeble inhibition of ficin, it is different from A_2 and A_3. Inhibitor C shows high inhibition with papain when compared to A_1 but low activity towards ficin and chymopapain. Hence it is different from A_1, A_2 and A_3.

A_1, A_2, A_3 and C have molar ratios of 0.2 to 1.0 except for A_1. The ratio for A_1 with ficin is only 0.13.

B_1 and B_2 inhibitors show very low molar inhibition ratios. For B_1 0.003 is maximum, while for B_2 it is about 0.1 - 0.2. B_2 has a higher ratio with ficin than with papain and chymopapain.

Hemoglobin:

The molar inhibition ratio with hemoglobin as substrate is only a fifth of that with casein for A_1 with papain. A_1 shows no inhibition of ficin and chymopapain with hemoglobin. Inhibitor C shows lower activity with papain and hemoglobin, about a fourth of that with casein, none with chymopapain, but suprisingly with ficin it shows higher inhibition with hemoglobin than with casein. B_1 and B_2 show marked differences in their relative activities with casein and hemoglobin. B_1 shows more inhibitory activity on hemoglobin than on casein with papain, ficin and chymopapain. For B_2 it is the reverse with papain, the inhibition is nearly the same with ficin and hemoglobin when compared with casein and B_2 shows no inhibition of chymopapain when hemoglobin is used as the substrate.

Serum albumin:

None of the inhibitors showed any inhibition with papain, ficin and chymopapain with serum albumin as substrate.

Esterase activity:

Inhibition of esterase activity was tested only with papain. BAEE was used as the substrate. A_1 shows only slightly higher molar inhibition ratio with papain, for

inhibition of esterase activity, than inhibition of caseinolytic activity. But C shows only half the ratio with the synthetic substrate as it does with caseinolytic activity. Slightly higher inhibition of esterase activity than caseinolytic activity is observed for the inhibitors B_1 and B_2.

Amidase activity:

BAPNA was used as the substrate for amidase activity. A_1 shows slightly less inhibition than with casein for papain which is the reverse of that for esterase activity. C shows slightly more inhibitory activity with BAPNA than with casein (which is the reverse of that observed for A_1), but A_2 and A_3 show twice the inhibition with the synthetic substrate than with casein for papain. Inhibition of amidase activity of ficin by A_1 is strikingly greater than with casein, nearly ten times more. B_1 shows only a third of the inhibition with BAPNA (amidase) as it does with BAEE (esterase), B_2 shows the reverse, the activity with BAEE is twice that with BAPNA. With C inhibition of amidase activity with papain was similar to that with casein, and with ficin also the results are similar. B_1 and B_2 again showed disimilar results with papain and ficin. B_1 showed twice the inhibition of caseinolytic activity than of amidase activity with papain, but with ficin the reverse was the case. With B_2 there was slightly more inhibition of amidase activity than of caseinolytic with papain and ficin. The above differences in specificity show that the

six isoinhibitors are different.

The low molar inhibition ratio obtained in the case of B_1 and B_2 inhibitor is probably due to the fact that papain may not be similar to the endogenous enzyme present in the plant which is inhibited by it. Isolation of the native enzymes of Vigna catjang which are inhibited by the papain isoinhibitors and determination of the binding constants with those enzymes will be necessary to determine the effectiveness of these inhibitors under physiological conditions with the enzymes occurring in the same plant.

Inhibition of an endogenous proteinase from the seedlings of Vigna catjang:

Seeds of Vigna catjang were germinated and harvested and the extract was prepared as described in Chapter III. It was precipitated with ammonium sulfate at 0.90 saturation. The precipitate was dialysed exhaustively against 0.01 M phosphate buffer, pH 7.5. The proteinase activity of the extract was determined. The inhibition of the proteinase by the various fractions of the acid extract of the seeds was determined. The results of the experiment are tabulated in the table given below (Table 15). Inhibition of the proteinase by inhibitor A_1 was low compared to that of the other fractions, but since A_1 was an apparently homogeneous preparation, the observed activity could possibly be due to A_1 itself, though the presence of traces of impurities could not be ruled out. The DEAE-cellulose adsorbable fraction, containing inhibitors

Table 15: Inhibition of endogenous proteinase activity from the seedlings of Vigna catjang

Fraction	Papain inhibitor activity	Germinated proteinase inhibitor activity	Ratio of papain inhibitor activity/Germinated proteinase inhibitor activity
	A/ml Units	A/ml Units	A/ml Units
0.30-0.90 ammonium sulfate precipitate (A_1 A_2 A_3, B_1 & B_2)	350	38	9
DEAE-cellulose, 0.005 M fraction after Sephadex G-100 chromatography (A_2 & A_3)	228	60	3.8
DEAE-cellulose 0.0015 M eluate A_1.	200	2	100
CM-cellulose 0.05M eluate	200	0	0

Phosphate buffer, pH 7.5 was used for elutions.

A_2 and A_3 showed a higher inhibition of the proteinase. However pure preparations of the inhibitors were not available when this experiment was carried out. It is possible that the low inhibition observed with inhibitor A_1 could also be due to the presence of several proteinases in the extract (which was confirmed by experiments not described here) of which only one was inhibited by A_1. The enzyme inhibited by A_1 could be present in very small quantities compared to the other enzymes. No inhibition was observed with inhibitor B_2. The above results are preliminary and the instability of the proteinases in the extract of germinating seeds made it difficult to undertake separation of the proteinases and testing the individual enzymes with the different purified papain isoinhibitors.

Competitive binding of papain and ficin by the inhibitors:

Experiments were carried out to find out whether the binding sites of the inhibitor are common for ficin and papain. The details of the experiments for the inhibitors A_1 and B_2 are given in Tables 16 and 17. The inhibitors after being saturated with papain could not inhibit ficin any further and vice versa. This shows that the binding site for ficin and papain is probably the same on the inhibitors and that the enzymes compete for the active site of the inhibitor. Similar results were obtained for the inhibitors A_2 and A_3 (Vartak, 1975). These results are in accordance with the ones obtained for the chicken egg white by Fossum and Whitaker (1968) and Sen and

Table 16: Competitive binding of ficin and papain by inhibitor A_1 from Vigna catjang

Reaction mixture		1	2	3	4	5
Papain	Units	19	-	19	-	19
Papain inhibitor A_1	Units	-	12	12	-	12
Ficin	Units	-	-	-	26	26
Proteinase activity of the reaction mixture (caseinolytic activity)	Units	19	0	7	26	33

Table 17: Competitive binding of ficin and papain by inhibitor B_2 from Vigna catjang

Reaction mixture	No.	1	2	3	4	5
Papain	Units	32	-	32	-	32
Papain inhibitor B_2	Units	-	10	10	-	10
Ficin	Units	-	-	-	30	30
Proteinase activity of the reaction mixture (caseinolytic activity).	Units	32	0	22	30	52

Whitaker (1973), who found that ficin and papain compete for the same active site of the inhibitor.

Section II

Stability of the inhibitors under varying conditions

All the inhibitors except B_1 were fairly stable to heating at 80°C at pH values ranging from pH 3 to 10. However the B_2 inhibitor as compared to the inhibitors A_1 A_2 and A_3 was less stable near pH 10. B_2 lost about 45% of the activity at this pH. However the B_1 inhibitor is more heat labile and lost 50% of its activity at all the pHs, when heated for 5 min. This clearly shows that B_1 is different from B_2. A general trend observed is that most of the inhibitors are comparatively more stable at low values of pH than at alkaline pHs. The results are summarised in Table 18.

Stability to ethanol:

The inhibitors again with the exception of B_1, were found to be stable in 75% ethanol at 30°C when incubated for 60 min. B_1 lost about 30% activity under these conditions (Table 19).

Stability to trichloroacetic acid:

A 0.02% solution of the inhibitor when heated in 3% trichloroacetic acid at 30°C for 15 min lost considerable activity. Inhibitor A_1 lost 65% of its activity and inhibitors B_1 and B_2 83% and 72% respectively. However inhibitor C retained most of its activity under these conditions. These results suggest that while the inhibitors are stable to treatment at low pH, they are less stable in the presence of a protein precipitant under similar conditions (Table 20).

Table 18: Stability of papain isoinhibitors at different values of pH

Inhibitors	Control (unheated) Units	pH 2.8 Units	pH 7.6 Units	pH 9.9 Units	pH 10.2 Units
A_1	243 (100%)	236 (97%)	224 (92%)	216 (89%)	-
A_2	16.5 (100%)	15 (90%)	12.5(75%)	-	11.5 (69%)
A_3	14 (100%)	12 (86%)	9.5(68%)	-	9.0 (64%)
C	100 (100%)	90 (90%)	85 (85%)	80 (80%)	-
B_1	120 (100%)	66 (55%)	60 (50%)	60 (50%)	-
B_2	256 (100%)	256 (100%)	230 (90%)	140 (54%)	-

0.02% solution of the inhibitor, in 0.1 M phosphate buffer, at different pH values, was heated at 85°C for 15 min. Inhibitor B_1 was heated for 5 min at 85°C. Figures in parentheses show percent activity remaining after heating.

Table 19: Stability of papain isoinhibitors in 75% ethanol at 30°C.

Experiment	A_1 Units	A_2 Units	A_3 Units	C Units	B_1 Units	B_2 Units
Control (at 30°C for 60 min,- Ethanol)	200	-	-	100	60	200
Incubated in 75% Ethanol for 60 min at 30°C.	180	-	-	100	35	200
% Activity remaining	90	-	-	100	68	100

A 0.02% solution of the inhibitor was used.

Table 20: Trichloroacetic acid stability of papain isoinhibitors at 80°C

Experiment	A_1 Units	A_2 Units	A_3 Units	C Units	B_1 Units	B_2 Units
Control (unheated, -trichloroacetic acid)	240	-	-	100	60	243
Heated in 3% trichloroacetic acid at 80°C for 15 min.	80	-	-	86	10	40
% Activity remaining	44	-	-	86	17	22

A 0.02% solution of the inhibitor was used.

Stability of the inhibitors to urea and borohydride:

A 0.02% solution of each inhibitor was incubated with 8 M urea or 0.1 M $NaBH_4$ or both at 35°C for 30 min. The incubated mixture was then dialysed exhaustively to remove the additives and assayed for inhibitor activity. About 15 and 25% inhibitor activity was lost in the case of A_1 and B_2 when incubated with $NaBH_4$. On the other hand C lost about 45% of its activity in the presence of urea but was stable to borohydride. The losses in the presence of both urea and $NaBH_4$ were not significantly higher than with urea or borohydride alone. It was noteworthy that B_1 which showed the least stability to heating and ethanol showed no loss of activity in the presence of urea and borohydride. The absence of loss in activity in the case of inhibitor A_1 is explained by the fact that no disulfide bridges are present. In the case of inhibitor B_2 which has two cystine bridges, it is possible that the disulfide bridges are not near the active site or are not required for activity. It is also possible that rapid reoxidation of the reduced bridges occurs. Further work is needed to confirm this (Table 21).

Action of pepsin on papain inhibitors:

When 0.02% solutions of the inhibitors (A_1 and B_2) were incubated with 30 ug of pepsin, at 35°C for 30 min, complete loss of inhibitor activity was observed in both the cases. The inhibitors are therefore highly susceptible to the action of pepsin.

Table 21: Urea and $NaBH_4$ stability studies of isoinhibitors of papain

Experiment	A_1 Units	C Units	B_1 Units	B_2 Units
Urea 8 M				
Control	240	420	-	245
Inhibitor + urea	180	220	-	180
% Activity remaining	75	53	-	74
0.1 M $NaBH_4$				
Control	240	420	-	245
Inhibitor + $NaBH_4$	200	420	-	200
% Activity remaining	85	100	-	84
8 M Urea + 0.1 M $NaBH_4$				
Control	250	1000	300	1000
Inhibitor + Urea + $NaBH_4$	185	640	330	750
% Activity remaining	75	64	100	75

0.02% solution of the inhibitor was used. The samples were dialysed exhaustively against 0.01 M phosphate buffer, pH 7.5 before estimation of inhibitor activity. The samples were incubated at 35°C for 30 min with the additives.

Section III

Physio-chemical properties

Molecular weights of the inhibitors:

The molecular weights of the different isoinhibitors of papain were determined by several methods, such as gel filtration on Sephadex G-50, SDS-gel electrophoresis and in the case of inhibitor B_2 by ultracentrifugation.

Gel filtration on Sephadex G-50:

The inhibitors were chromatographed on Sephadex G-50 column with marker proteins of known molecular weight [(Ref - Chapter II). Soybean trypsin inhibitor (21,000), myoglobin (17,000) and cytochrome c (13,000)]. A plot of eluant volume versus log molecular weight according to the procedure of Andrews (1964) gave a straight line and the molecular weights of the inhibitors, A_1 and B_2 were found to be 7760 and 11,430 respectively (Fig. 7 ' 8). The molecular weights of the other two inhibitors B_1 and C were also determined by the above procedure and were found to be 10,480 and 11,560 respectively. Partially purified samples of B_1 and C were used for these studies (Fig. 9 & 10).

SDS-gel electrophoresis:

The molecular weights of the purified preparations of the two inhibitors A_1 and B_2 were also determined by their migration in SDS-gel electrophoresis, according to the procedure described in Chapter II (photograph 4). Standard proteins used were myglobin (17,000), cytochrome c (13,000) and insulin (2,500). A plot of log molecular weight of

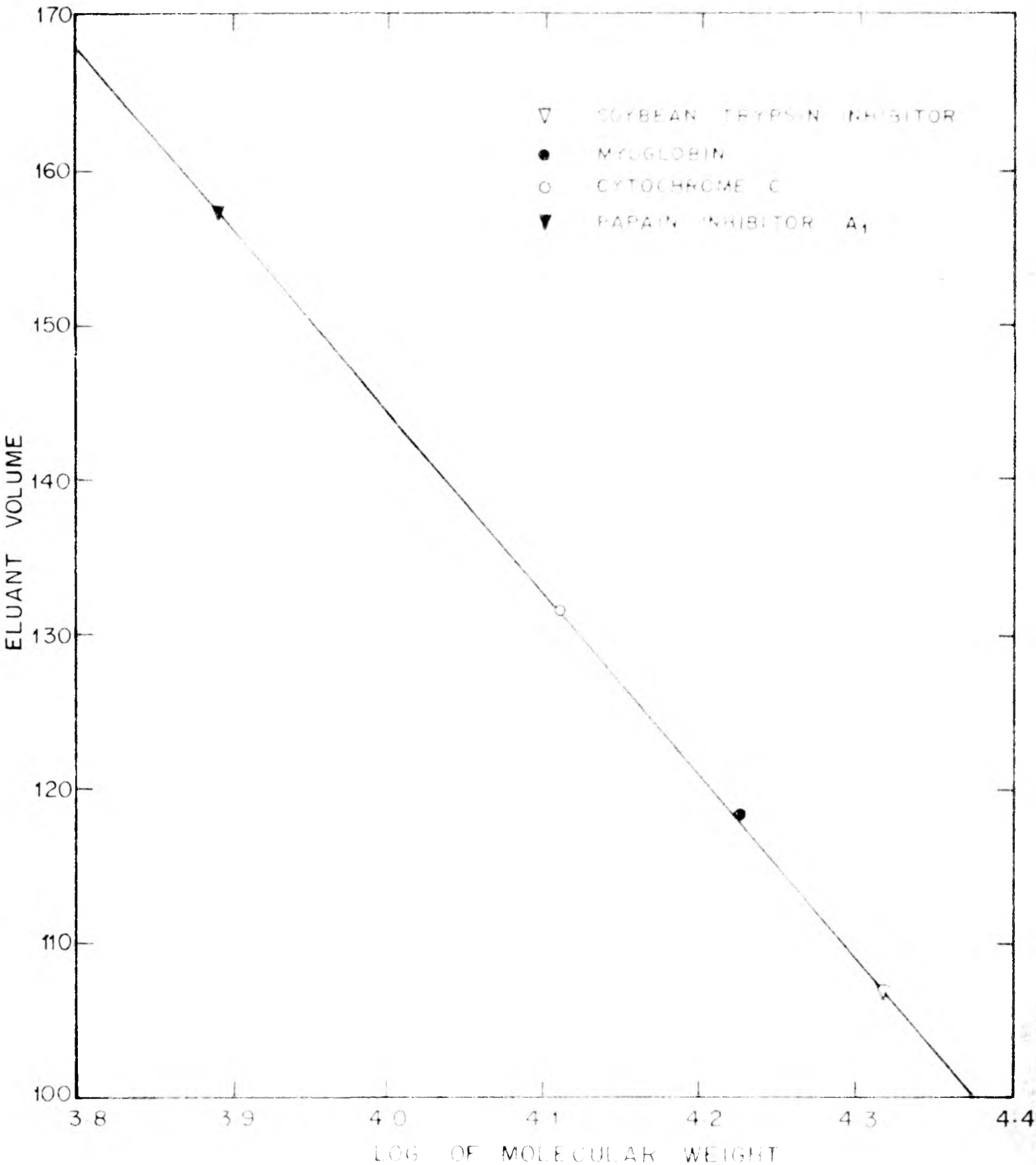


FIG 7

MOLECULAR WEIGHT OF PAPAIN INHIBITOR A_1

BY GEL FILTRATION

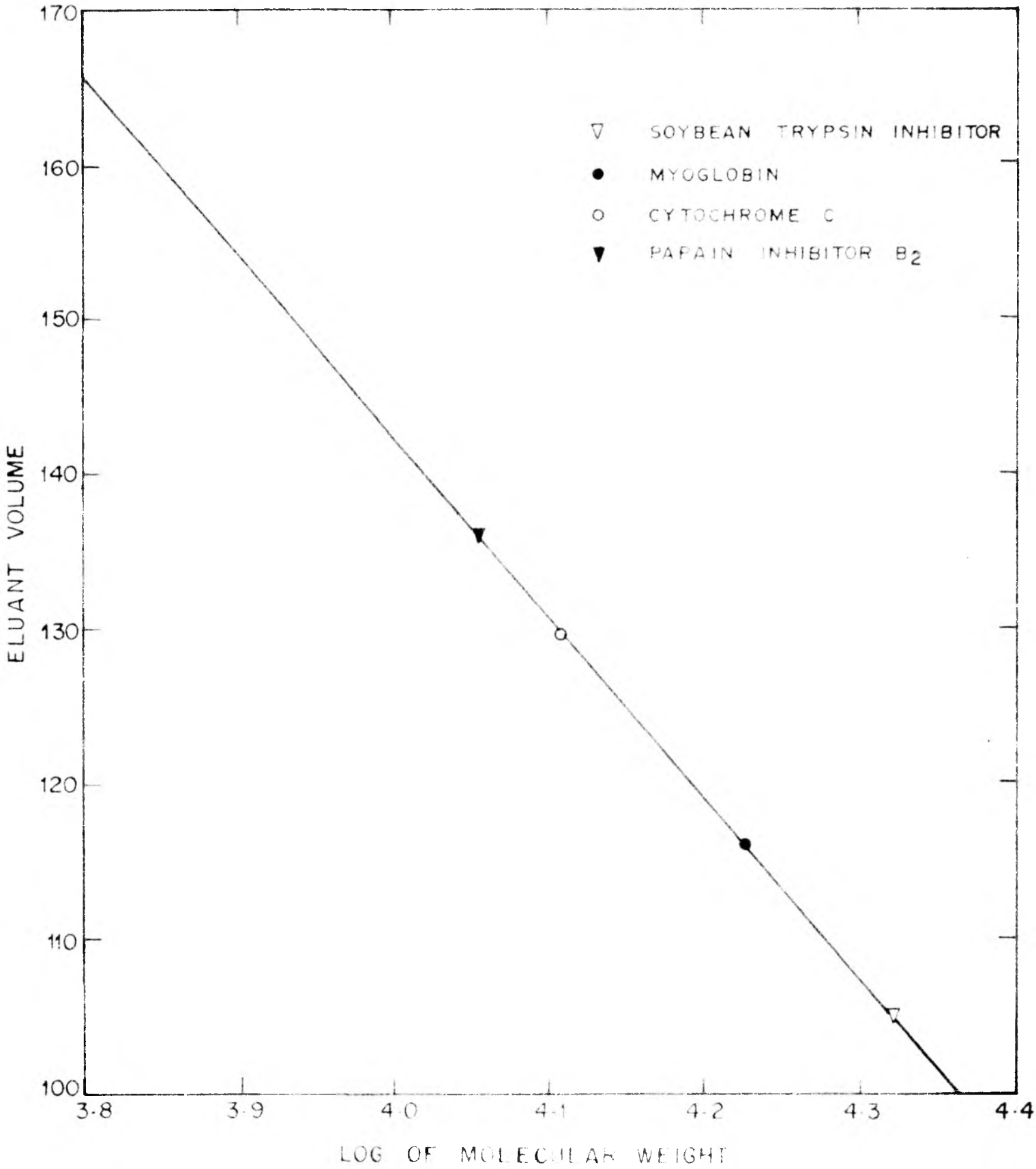


FIG. 8.

MOLECULAR WEIGHT OF PAPAIN INHIBITOR B_2 BY GEL FILTRATION

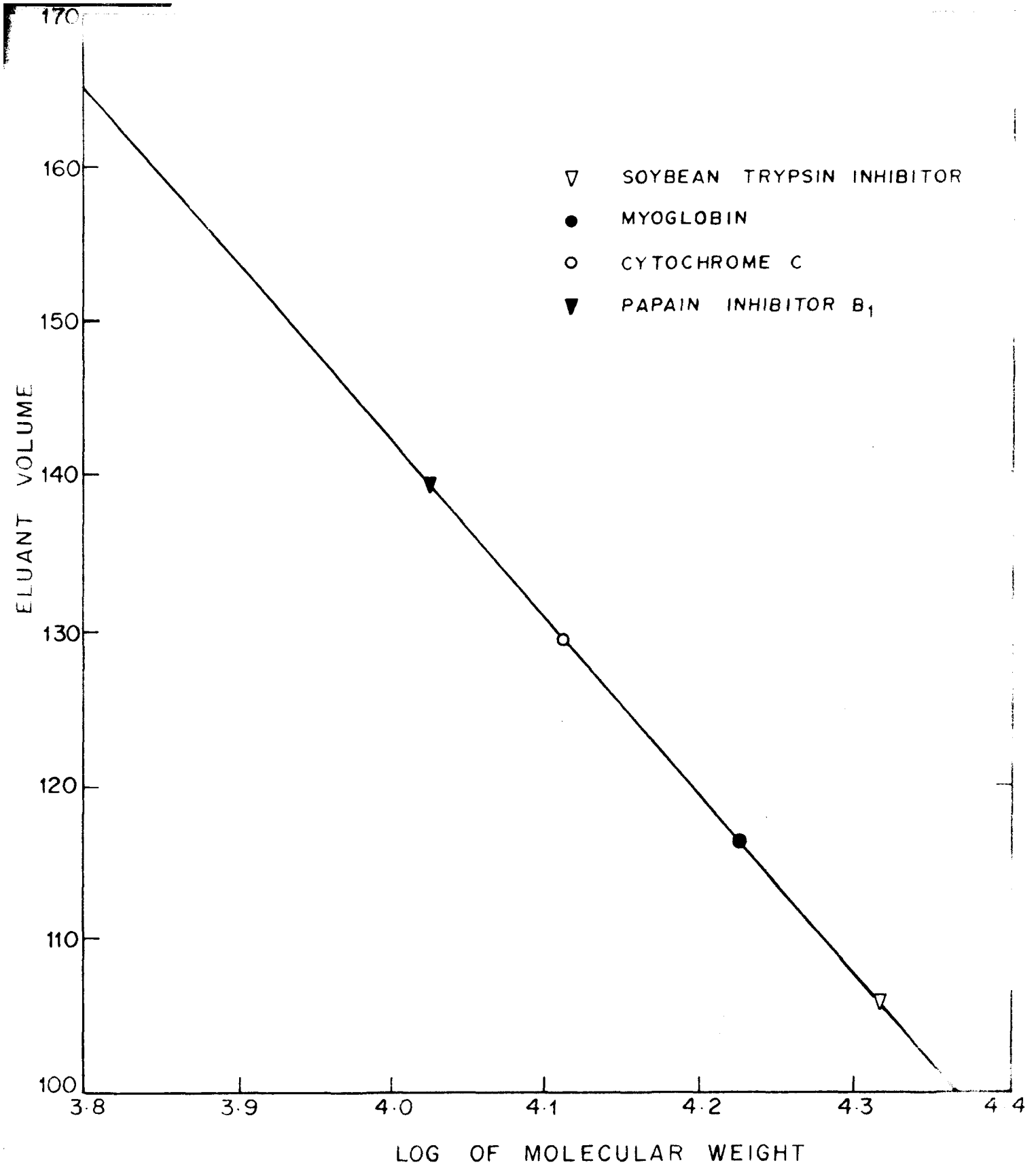


FIG. 9.

MOLECULAR WEIGHT OF PAPAIN INHIBITOR B_1 BY GEL FILTRATION

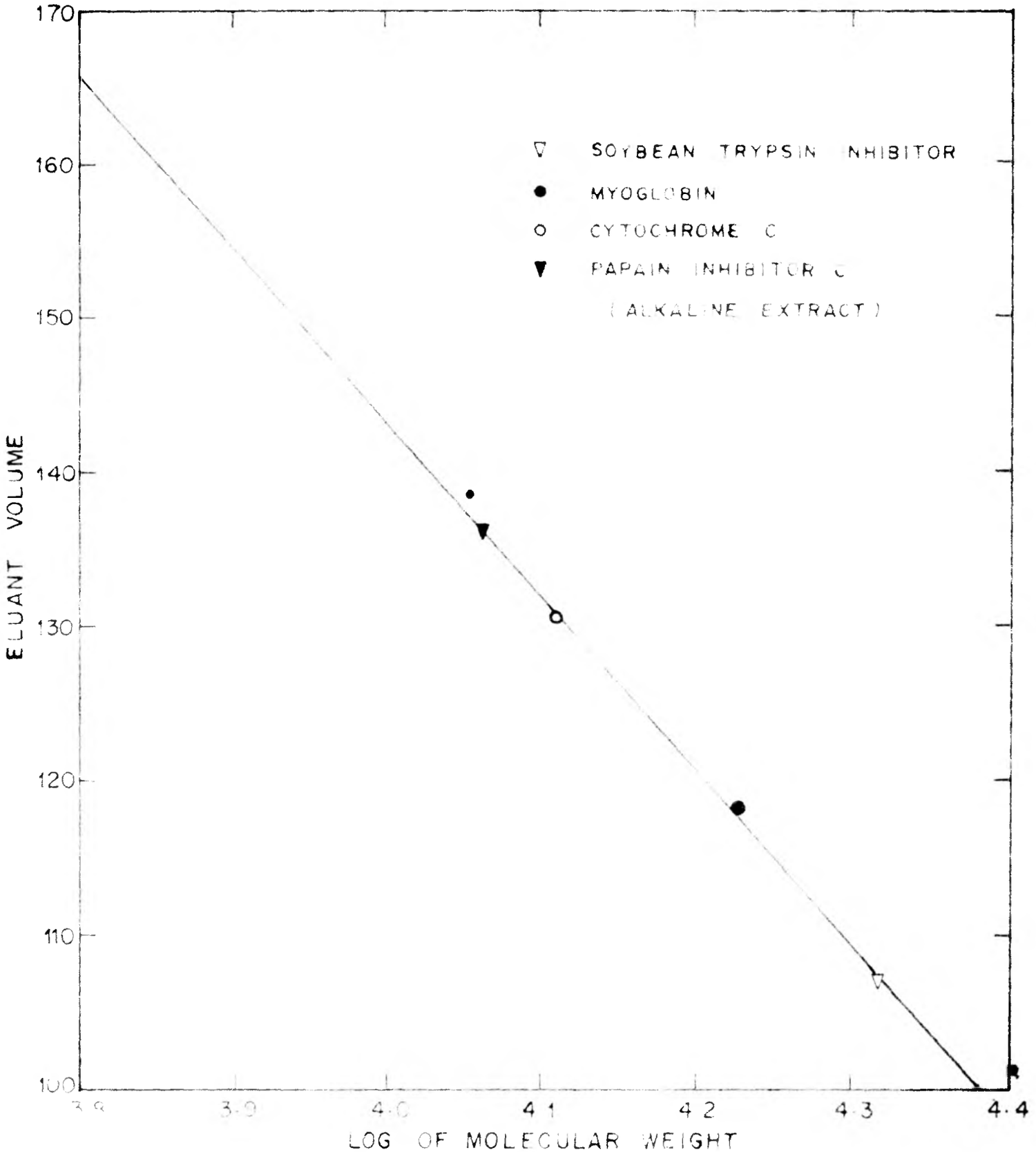


FIG. 10

MOLECULAR WEIGHT OF PAPAIN INHIBITOR C
BY GEL FILTRATION

marker proteins against relative mobilities (Rm) yielded a straight line. The molecular weights of the two inhibitors were found to be 7670 and 5500 respectively for A_1 and B_2 (Fig. 11). The value obtained for the B_2 inhibitor was found to be exactly half of that obtained by ultracentrifugation and gel filtration, within the limits of experimental error. The inhibitor appears to exist as a dimer and the monomers observed on SDS-gel electrophoresis are either identical or are non-identical, but with the same mobility.

Molecular weight determination by ultracentrifugation:

The molecular weight of the B_2 inhibitor was determined by the approach to equilibrium method of Archibald (1947). A synthetic boundary cell was used. A value of 0.73 ml/g was used for partial specific volume. A 1% solution of the inhibitor containing 4.5 mg in 0.1 M phosphate buffer, pH 7.5, was used for the run. Other experimental conditions were a calculated value of 16,200 revolutions per min, temperature 23°C and the movement of the boundary recorded by Schlieren photographs at 15 min intervals. The sedimentation constant was found to be 1.4 S. A value of 9855 for the molecular weight was obtained. This value is about 10% less than that obtained by gel filtration and is approximately double the value obtained by SDS-gel electrophoresis. Unfortunately enough A_1 inhibitor was not available for ultracentrifuge studies. The data on molecular weights of the isoinhibitors of papain by different methods are summarised in Table 22. The papain inhibitor from rabbit skin was found to have a sedimentation constant of 1.2 S.

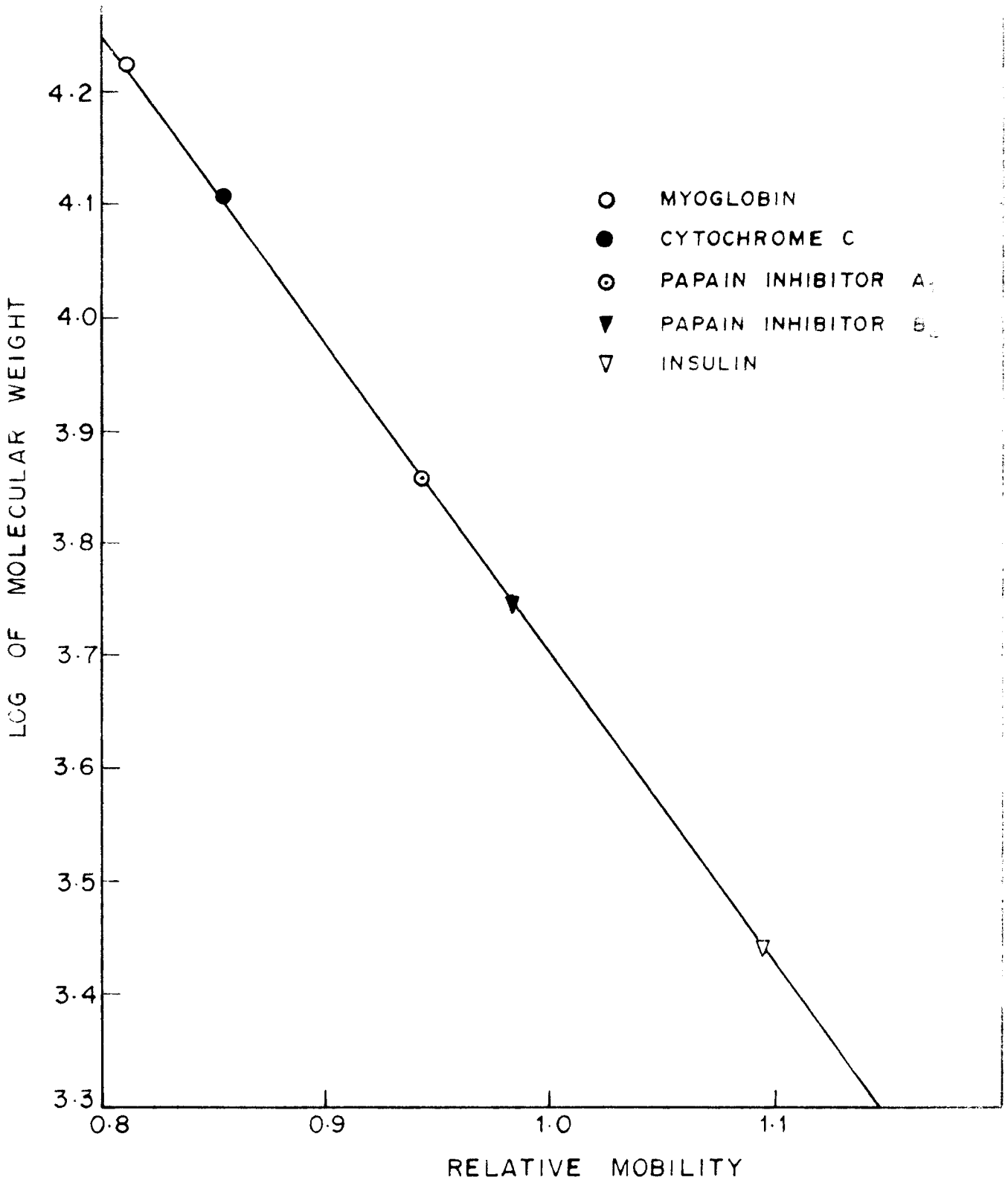


FIG. 11

MOLECULAR WEIGHT OF PAPAIN INHIBITORS A_1 AND B_2 BY SDS METHOD

Legend for Figure Numbers - 7, 8, 9 and 10:

A sample in 0.05 M phosphate buffer, pH 7.5 was applied to a Sephadex G-50 column. Markers used were soybean trypsin inhibitor, myoglobin and cytochrome c. Other experimental details are as described in Materials and Methods.

Legend for Figure Number - 11:

9% gels were used in SDS buffer system, pH 7.2. Conditions are as described in Materials and Methods. Markers used were myoglobin, cytochrome c and insulin treated with thioethanol and SDS.

Table 22: Molecular weights of papain inhibitors from Vigna catjang

Method	A_1	A_2^*	A_3^*	C	B_1	B_2
Gel filtration on Sephadex G-50	7760	11,430	12,220	11,560	10,480	11,480
SDS-gel electrophoresis	7670	9,700	9,700	-	-	5,500
Amino acid analysis	8560	-	-	-	-	5,900
Ultracentrifuge (Approach to equilibrium)	-	-	-	-	-	9,860

*from Vartak (1975).

Isoelectric point:

The isoelectric point of papain inhibitors A_1 and B_2 were at pH 6.9 and 10.6 respectively (photograph 4). The isoelectric point of the papain inhibitor from rabbit skin was at pH 6.6.

Absorption spectrum:

The ultraviolet spectrum of papain inhibitor B_2 is shown in Fig. 6. The figure represents the characteristic absorption spectrum of a typical protein. No other peaks were detected, indicating the absence of nucleotides. The presence of other ultraviolet absorbing material which also interferes with the protein estimation by the Folin's method cannot however, be ruled out in view of the unusually low specific activity of 100 units/mg of protein for this preparation, whereas a less pure preparation obtained by CM-cellulose chromatography as discussed earlier had a specific activity of 1000 units/mg of protein. Papain inhibitor B_2 shows a maximum absorption at 275 nm and a minimum at 250 nm and the 280/260 ratio was 1.43 as compared to a ratio of 1.80 for most proteins. The optical factor i.e. the reciprocal of the absorbance at 280 nm for a 1 mg/ml solution for B_2 was 1.1.

Amino acid composition:

The results of the amino acid analysis for the two inhibitors A_1 and B_2 are shown in tables 23 and 24. However the data on amino acid analysis of the B_2 inhibitor has to be interpreted with caution. Hydrolysis of the inhibitor was carried out for 24 h and 48 h as described in

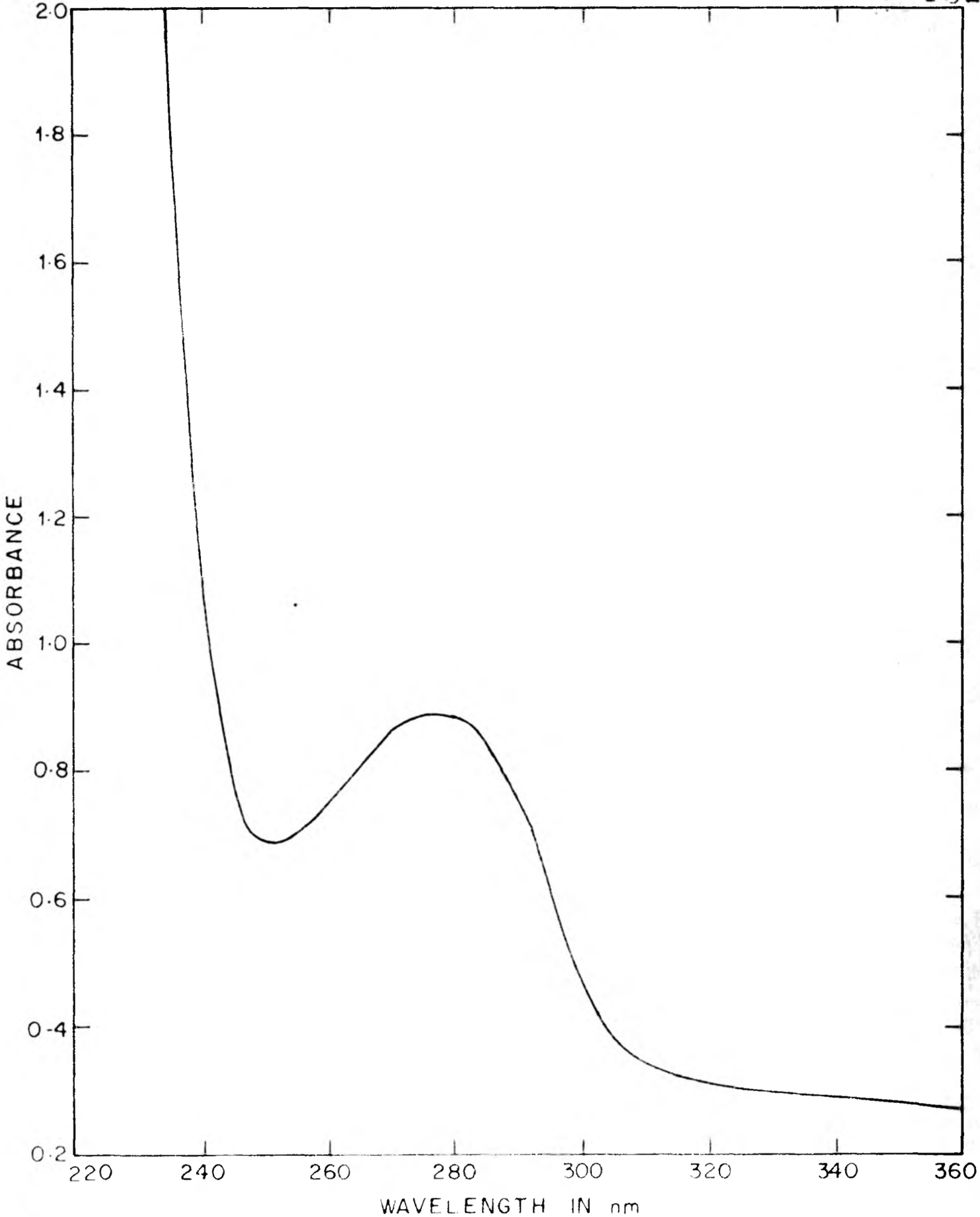


FIG. 11 a

ULTRAVIOLET ABSORPTION SPECTRUM OF PAPAIN INHIBITOR B_2

Chapter II and the values for the amino acids were then extrapolated to 0 h. A distinct difference is observed in the amino acid composition of the two inhibitors. Papain inhibitor A_1 has a total of 61 residues and the calculated value of 8560 for the molecular weight of the inhibitor is in agreement with that of 7760 and 7674 as determined by gel filtration and SDS-gel electrophoresis respectively. A mole of the inhibitor contained one mole of half-cystine and two moles of methionine. A_1 is very similar to the subtilisin inhibitor from Vigna catjang which also contains only one mole of half-cystine (Vartak, 1975). The inhibitor was found to be rich in histidine, glycine, alanine aspartic acid and threonine. Tryptophan could not be determined as enough material was not available. (Table 23)

The B_2 inhibitor had a total number of 40 amino acid residues, which corresponds to a minimum molecular weight of 5900 for the protein on the basis of amino acid analysis. The inhibitor was found to contain one mole of carbohydrate per mole of protein (calculated as hexose). Hence the true molecular weight should be greater than 5900. The molecular weight by gel filtration was 11,480 and by SDS-gel electrophoresis it was 5500. The inhibitor contains 4 half-cystine residues and one mole of methionine. The inhibitor has a predominance of aspargine, glutamine, lysine and arginine. 5 moles of aspartic acid and 3 moles of gluatamic acid per mole of inhibitor are present. A noteworthy observation is the absence of histidine, while A_1 is rich in histidine. Amino acid analysis showed a very low value of tyrosine (Table 24).

Table 23: Amino acid analysis of papain inhibitor A_1 from Vigna catiang

Amino acid	µmole/0.0931 mg sample	Residues/mole calculated	Assumed nearest integer
Aspartic acid	0.0704	5.870	6
Threonine	0.0677	5.650	6
Serine	0.0549	4.585	5
Glutamic acid	0.0502	4.194	4
Proline	0.0241	2.013	2
Glycine	0.0837	6.984	7
Alanine	0.0769	6.418	6
Half-cystine°	0.00766	0.6384	1
Valine	0.0260	2.173	2
Methionine°	0.01694	1.767	2
Isoleucine	0.0146	1.217	1
Leucine	0.0208	1.738	2
Tyrosine	0.0197	1.648	2
Phenylalanine	0.0066	0.5487	1
Lysine	0.0635	5.303	5
Histidine	0.0914	7.626	8
Arginine	0.0153	1.276	1
Tryptophan	-	-	-
Asparagine	0	0	0
Glutamine	0	0	0

Values for amino acids are the values extrapolated to 0 h. Only 0.3439 mole of ammonia was obtained and hence very little or no glutamine and asparagine were present.

°Half-cystine and methionine were determined by performic acid analysis.

Table 24: Amino acid analysis of papain inhibitor B_2 from *Vigna catjang*

Amino acid	µmole/0.950 mg sample	Residue/mole calculated		Assumed nearest integer
Aspartic acid*	0.8511	4.937	(5)	2
Threonine	0.4737	2.748		3
Serine	0.4522	2.872		3
Glutamic acid*	0.4357	2.527	(3)	1
Proline	0.4143	2.403		2
Glycine	0.7238	4.199		4
Alanine	0.5549	3.218		3
Half-cystine°	0.6367	3.692		4
Valine	0.3147	1.825		2
Methionine°	0.0879	0.5090		1
Isoleucine	0.3626	2.003		2
Leucine	0.4142	2.402		2
Tyrosine	0.0431	0.250	(0)	1
Phenylalanine	0.0974	0.565		1
Lysine	0.3077	1.784		2
Histidine	0.0652	0.378		0
Arginine	0.2929	1.698		2
Tryptophan	-	-		0
Asparagine	-	-		3
Glutamine	-	-		2

Values for the amino acids are the values extrapolated to 0 h. Tryptophan and tyrosine were determined by the spectrophotometric method of Goodwin and Morton (See Chapter II).

* Number of residues of aspartic acid and glutamic acid per mole of the inhibitor is 5 and 3 moles respectively. Asparagine and glutamine were obtained by alloting the number of NH_3 residues according to the percentage ratio of aspartic and glutamic acid present in one mole of the inhibitor. 5 moles of ammonia were liberated and were allotted in the ratio Asp3, Glu-2.

° Half-cystine and methionine were determined after performic acid oxidation.

However the spectrophotometric method of Goodwin and Morton (1946) showed one residue of tyrosine and no tryptophan. Partial destruction of tyrosine on acid hydrolysis of glycoproteins has been reported (Lugg, 1938). Hence it is possible that the low values of tyrosine could be due to the presence of a sugar moiety.

Partial specific volume:

The partial specific volume was calculated for inhibitor A_1 according to the method of Cohn and Edsall (1943) from the weight percentages of the individual amino acid residues and their respective specific volumes. The partial specific volume was calculated to be 0.75 ml/g from the amino acid composition of the inhibitor and the apparent specific volumes of the amino acids. Table 25 gives the calculation of partial specific volume for the A_1 inhibitor.

Determination of SH groups:

Treatment of 0.2 mg of the inhibitor B_2 with 18% urea, followed by spectrophotometric titration with DTNB yielded no colour, suggesting the absence of SH groups in the inhibitor. This is in agreement with amino acid analysis as an odd number of half-cystine residues was not present and the four half-cystine residues could account for two disulfide linkages. Unfortunately sufficient material was not available for the determination of the SH groups of the inhibitor A_1.

Determination of s-s groups:

These were determined in B_2 and A_1 inhibitors by performic acid oxidation followed by hydrolysis and amino acid analysis for half-cystine. As stated earlier A_1 was

Table 25: Partial specific volume of papain inhibitor A_1 from Vigna catjang

Amino acid Residue	No. of residues/ mole of Inhibitor	Wi Amino acid residue per 100 g of protein. (weight percent)	$\bar{v}i$ Specific volume of residue	$\bar{v}i$ Wi $\bar{v}i$ x Weight percent
Aspartic acid	6	9.674	0.59	5.804
Threonine	6	7.816	0.70	5.471
Serine	5	5.610	0.63	3.534
Glutamic acid	4	6.655	0.66	4.391
Proline	2	2.502	0.76	1.902
Glycine	7	5.148	0.64	3.296
Alanine	6	5.496	0.74	4.067
Half-cystine	1	2.864	0.63	1.748
Valine	2	2.556	0.86	2.198
Methionine	2	3.845	0.75	2.884
Isoleucine	1	1.457	0.90	1.311
Leucine	2	2.915	0.90	2.623
Tyrosine	2	4.206	0.71	6.863
Phenylalanine	1	1.897	0.77	1.461
Lysine	5	8.279	0.82	6.784
Histidine	8	14.150	0.67	9.454
*Asparagine	0	0	0.60	0
Glutamine	0	0	0.67	0
*Arginine	1	2.013	0.70	1.409
	61	87.083		65.201

Partial specific volume of the inhibitor was calculated as follows (Ref. Chapter III)

$$\sum Wi = 87.083 \qquad \sum Wi\ \bar{v}i = 65.201$$

$$V_p = \sum Wi\ \bar{v}i / \sum Wi$$

$$= 65.201/87.083 = 0.75\ ml/g$$

Values for $\bar{v}$ for the amino acid residues were taken from Schachman (1957).

found to contain one mole of half-cystine per mole of the inhibitor. B_2 was found to contain 4 residues of half-cystine per mole of the inhibitor corresponding to 2 disulfide bridges.

Estimation of tyrosine and tryptophan:

Tyrosine and tryptophan were determined by the method of Goodwin and Morton (1946) for inhibitor B_2. 0.66 mg of the inhibitor on dry weight basis was taken in 1 ml of 0.1 M NaOH and the absorbance of the inhibitor at 294.4 nm, 280 nm and 305 nm was recorded. Calculations based on the data show that the inhibitor contains 0.4 mole of tryptophan and 0.9 mole of tyrosine per mole of the inhibitor. The absence of tyrosine by amino acid analysis after acid hydrolysis has been described earlier. In view of the possible presence of non-protein ultraviolet absorbing material in the preparation those results need independent confirmation (Table 26).

Total carbohydrate:

The B_2 inhibitor was found to contain 0.61 mole of sugar per mole of the inhibitor. Total carbohydrate was determined by the Orcinol-sulfuric acid method (Francois et al., 1962). 1.41 mg of the inhibitor on dry weight basis was used for analysis and was found to contain 0.028 mg of hexose. Glucose was used as the standard. This is equivalent to 0.61 mole of hexose per mole of inhibitor for a molecular weight of 5500.

It was also observed that a part of the inhibitor present in the CM-cellulose eluates containing (B_1 and B_2)

Table 26: Determination of tyrosine and tryptophan by Goodwin and Morton's method. UV absorbance of papain inhibitor B_2 in 0.1 M NaOH.

Wavelength nm	Absorbance OD
257	0.514
280.5	0.424
294.4	0.372
305	0.148

0.660 mg of the inhibitor per ml of 0.1 M NaOH was used for the assay.

was bound by Con A Sepharose and a lectin from Nata beans. CM-cellulose eluate (Fraction Step IV b) was treated with Con A Sepharose and the lectin from Nata beans separately in the presence of Mg^{++}, Mn^{++} and Ca^{++}. The ratio of protein to lectin was 1:0.5 in the former and 1:1 in the latter. The mixture was incubated for 30 min at 35°C. The filtrates were dialysed and estimated for inhibitor activity. It will be seen that part of the inhibitor activity is precipitated out by the lectin. The results of elution of inhibitor from the precipitate with 0.5 M methyl D-glucose (for Con A Sepharose) and 0.5 M glucose for (Nata bean) are shown in the Table. Dissociation of the lectin bound inhibitor was 8-15% (Table 27). These results further confirm the presence of a glycoprotein inhibitor in the CM-cellulose adsorbable fraction. It was, however, observed that the addition of Con A or the lectin from Nata beans to the reaction mixture had no effect on inhibitor activity of B_2 and B_1.

Degree of hydrophobicity:

The degree of hydrophobicity of papain inhibitor A_1 in terms of average hydrophobicity HØ, polarity index p, (Fisher, 1964) and the frequency of non-polar side chains (Waugh, 1954) was computed from the amino acid composition of the inhibitor, as shown in table 28. The above results indicate the molecule to be fibrous in nature. All the three parameters are in agreement with each other. Globular proteins generally have an average hydrophobicity of 1000-1100 cal. The average value for NPS for globular

Table 27: Combination of B_1 and B_2 inhibitors with lectins

Lectin used	Ratio of protein to lectin	Original activity	Filtrate activity	Fluate activity	% Activity bound	Bound activity eluted
		Units	Units	Units	%	%
Con A Sepharose	1:0.5	600	405	35	37	17
Nata beans	1:0.5	400	230		42	
	1:1	400	220	15	45	8

Table 28: Calculation of NPS, p and HØave for papain inhibitor A_1 from Vigna catjang

Amino acid	Number of residues	Volume ($Å^3$)	HØ (cal)
Non polar			
Tryptophan	-	-	-
Isoleucine	1	102.0	2950
Phenylalanine	1	113.9	2650
Proline	2	147.2	5200
Leucine	2	204.0	4800
Valine	2	170.2	3400
Methionine	2	195.4	2600
Alanine	6	315.6	4500
Glycine	7	254.1	
Half-cystine	1	68.3	1000
		1570.7	
Polar			
Tyrosine	2	232.4	5700
Lysine	5	525.5	7500
Arginine	1	109.1	0750
Threonine	6	427.5	2700
Serine	5	274.5	
Histidine	8	735.2	
Aspartic acid	6	410.4	
Glutamic acid	4	338.8	
	61	3053.1	43750

The amino acids are classified as polar and non-polar according to Fisher's definition.

NPS is calculated according to Waugh's method by adding the total number of residues of trp, ile, tyr, phe, pro, leu and val and expressing the sum as a fraction of the total number of residues:

NPS = 10/61 = 0.1640

'p' is the ratio of polar volume to non-polar volume

= 3053.1/1570.7 = 1.945

HØave is the total hydrophobicity divided by the number of residues

= 43,750/61 = 717.3

The above results do not take into consideration tryptophan as sufficient material was not available to determine it.

Table 29: Degree of hydrophobicities for some known fibrous proteins for comparison

* Protein		HØave	p	N.P.S.
Tropomyosin	Pinna nobilis	870	1.79	0.23
	Rabbit skeletal	870	1.94	0.20
	Squid	770	2.14	0.18
L-meromyosin		920	1.72	0.25
H-Meromyosin		1060	1.26	0.30
Collagens -	Soluble α-fraction from			
	Calf skin	940	0.89	0.29
	Spongin B	810	1.15	0.24
	Sturgeon swim bladder	1090	0.92	0.25
	Bovine ear	1120	0.26	0.40
Papain inhibitor A_1 from Vigna catjang		717	1.94	0.164

*Data taken from Bigelow (1967).

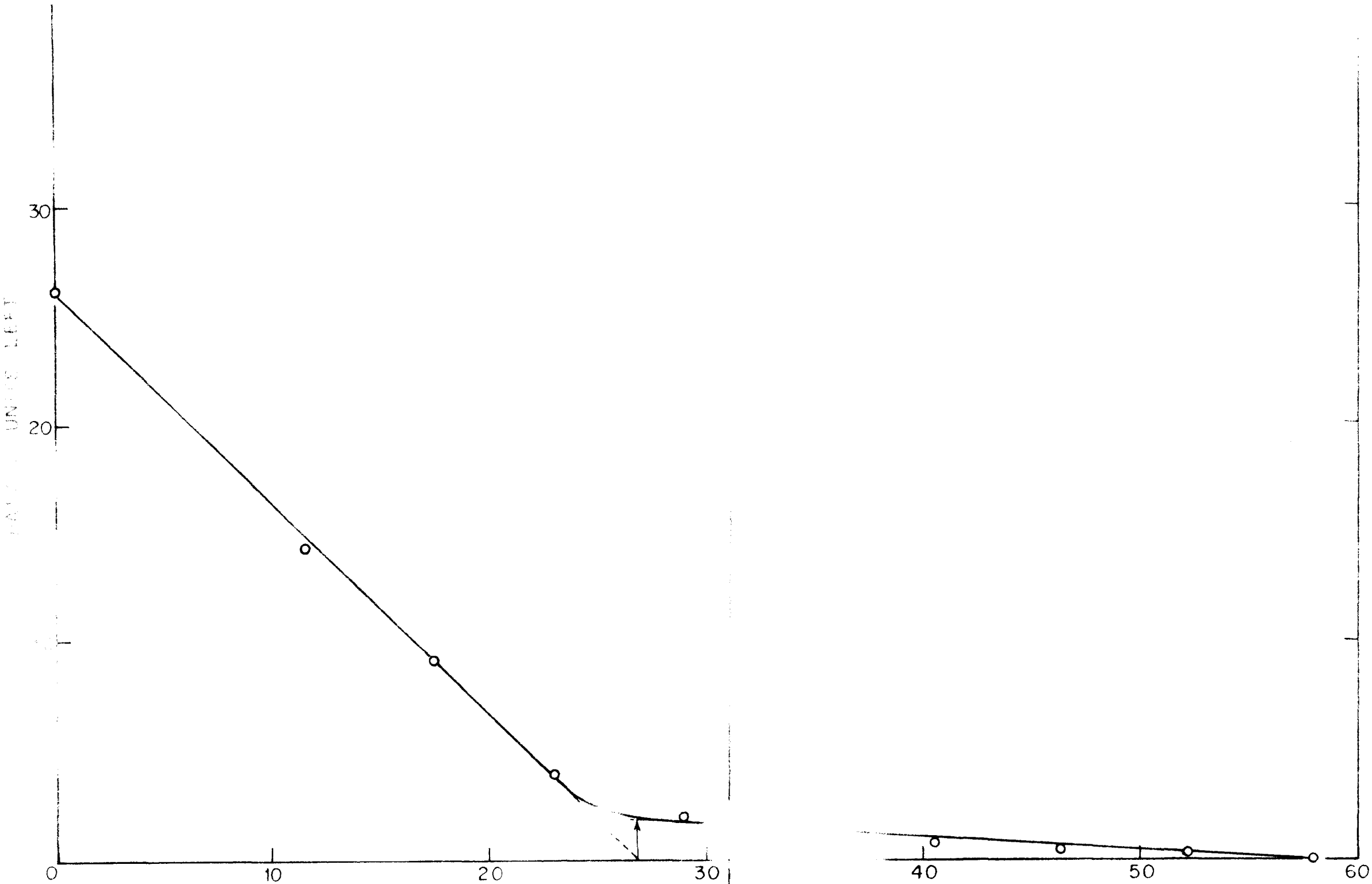
proteins is 0.3. A polarity ratio of about 1 indicates that the molecule is globular. Table 29 compares the values of the three parameters with some known soluble fibrous proteins.

Section IV

Dissociation constants of the inhibitor enzyme complex and dissociation of papain inhibitor complex:

Dissociation constants:

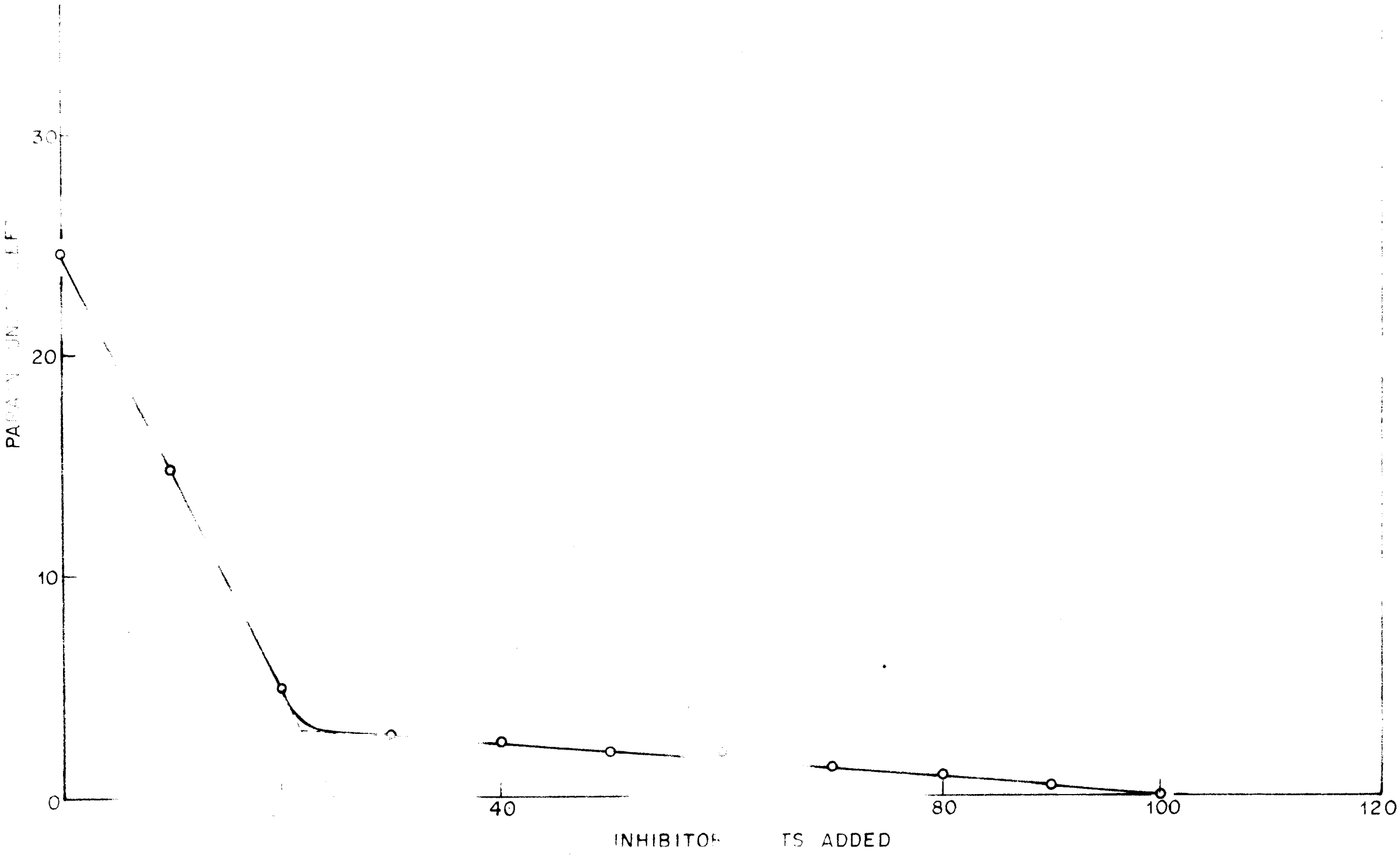
Dissociation constants for the two inhibitor complexes A_1 and B_2 were determined, according to the method of Green and Work (1953) using casein as the substrate. The reaction between inhibitor and papain was complete within 5 min (Incubation of inhibitor and papain for longer periods did not increase the inhibitor activity. Shorter periods of incubation were not tested). The dissociation constants at pH 7.5 were found to be 2.34×10^{-9}M and 4.3×10^{-8}M respectively for the complexes of A_1 and B_2 with papain. (Fig. 12 and 13). The dissociation constant reported for the chicken egg white complex with ficin was $1.47 \pm 0.68 \times 10^{-8}$M using p-nitrophenyl benzyloxycarbonyl glycinate as the substrate. As seen from the graphs, the dissociation curve is a rectangular hyperbola with a break near the equivalence point. This is observed in the case of complexes having a very low dissociation constant and dissociation is detected only after the enzyme is nearly saturated with the inhibitor i.e. near the equivalence point. It should be emphasised however that this method is only approximate though it has been widely used for the study of proteinase inhibitors due to its simplicity and the requirement of only small amounts of inhibitor.



FIG

INHIBITION CURVE SHOWING THE DISSOCIATION AT [illegible] OF PAPAIN INHIBITOR A_1-PAPAIN COMPLEX NEAR THE EQUIVALENCE POINT $K_i = 2 \times 10^{-9}$ M

Conditions are as described in Materials and Methods.



FIG

INHIBITION CURVE SHOWING THE DISSOCIATION AT pH 7·5 OF PAPAIN INHIBITOR B_2–PAPAIN COMPLEX NEAR EQUIVALENCE POINT $K_i = 4.3 \times 10^{-8}$ M

Conditions described in Materials and Methods.

Complex formation and dissociation of the papain-inhibitor complex:

The following experiments were carried out with soluble papain. The inhibitor was heated with an equivlent amount of papain to form a stoicheimmetric complex. Dissociation of the enzyme-inhibitor complex was attempted by several methods. The results are tabulated below (Table 30). From the data it will be seen that for both the inhibitors A_1 and B_2, heating the complex at pH 7.5 did not result in any dissociation of the complex. In the case of inhibitor A_1 no dissociation occurred at pH 2 at 30°C. However only partial dissociation of the complex was obtained on heating it at pH 2. When the pH was lowered to 1, the inhibitor was quantitatively released. In the case of inhibitor B_2, inhibitor activity was completely restored when the complex was heated at 95°C at pH 2. Partial dissociation was obtained when the incubation was carried out at pH 2 for 20 min at 30°C. The above results show that the A_1 inhibitor-enzyme complex is stabler at pH 2 than the B_2 inhibitor-enzyme complex. A peculiar observation was that if the B_2 inhibitor-papain complex was directly adjusted to pH 2, incubated for 20 min and then neutralised, 20% inhibitor activity was recovered, but if the complex was first heated at pH 7.5, 95°C and then kept at pH 2 for 20 min and then neutralised no activity was recovered. (Expts. 3 & 4 with B_2 in Table 30). This observation is surprising since separate experiments showed that boiled papain does not

Table 30: Dissociation of papain inhibitor complex

Treatment	Inhibitor A_1			Inhibitor B_2		
	Original activity	Activity after treatment	Recovery of activity	Original activity	Activity after treat-ment	Recovery of activity
	Units	Units	%	Units	Units	%
1. Inhibitor activity before complexing at pH 7.5	120	-	100	100	-	100
2. Heat the complex at pH 7.5 at 95°C for 5 min.	"	0	0	"	0	0
3. Heat the complex at pH 7.5 at 95°C for 5 min kept at pH 2 at 30°C for 20 min and neutralised to pH 7.5.	"	0	"	"	0	0
4. Complex inoubated at pH 2 for 20 min at 30°C and neutralised to pH 7.5.	"	0	0	"	28	28
5. Heat the complex at pH 2 at 95°C for 5 min cooled and neutralised.	"	48	40	"	100	100
6. Complex incubated at pH 1 for 30 min at 30°C and neutralised to pH 7.5.	"	118	98	-	-	-

Caseinolytic method of Kunitz was used for assaying inhibitor activity as described in Chapter II.

combine with the inhibitor.

Earlier experiments with immobilised papain and inhibitor C as described in Chapter IV showed that dissociation occurs only at pH 1. Even at this pH only 50% activity is released. In the case of inhibitor B_2 dissociation of the inhibitor bound to immobilised papain occurs only when the pH is lowered to pH 1. Only 10% dissociation is obtained at pH 2. This is in contrast to the results obtained with soluble papain (Refer table). Difficulty in obtaining dissociation of ficin-papain inhibitor complex was reported by Sen and Whitaker (1973) with the chicken egg white inhibitor. They failed to obtain dissociation with 20% glycerol, 9M urea at pH 3, or tetrathionate. Binding of the inhibitors by inactive papain derivatives will be disscussed under "mechanisum of inhibitors".

An interesting observation worth mentioning here, is that unlike papain-inhibitor complexes which are difficult to dissociate at pH 7.5, at 95°C the complex of inhibitor and enzyme (or other protein) from tissue culture and in germinated seedlings dissociates on heating as is apparent from the increase in inhibitor activity after heating the extracts of plant tissue cultures or germinated seedlings. This shows that the enzyme or other protein in the above cases is quite different from papain.

Section V

Mechanism of action of papain inhibitor

Finkenstadt and Laskowski (1965) have shown that when some of the trypsin and chymotrypsin inhibitors were incubated with catalytic quantities of the corresponding proteinase, modification of the inhibitor occurred by the cleavage of a peptide bond at the reactive site of the inhibitor. It was suggested that this step was essential for complex formation between the inhibitor and the proteinase. So far no work on the modification of a papain inhibitor during reaction with the enzyme has been reported. It was of interest to see whether modification of a papain inhibitor occurs and whether it is a prerequisite for complex formation. The inhibitor used for these studies was the B_2 inhibitor. The inhibitor was incubated with catalytic amounts of papain at acid pH 3.5. (0.3 ml equivalent to 300 units of papain inhibitor (300 µg) and 15 units of papain (2.8 µg)). The pH of the mixture was lowered to 3.5 by the addition of 4.5 µmoles of GSH, ~~HCl~~ which also served the purpose of activating papain. A control experiment wherein papain was omitted from the reaction mixture was also run simultaneously. The reaction mixtures were incubated for 18 h at 30°C, neutralised with 4.5 µmoles of Tris base and assayed for the inhibitor. The above incubated mixtures will be designated as follows: 'Reaction mixture N' where there is no papain and "Reaction mixture M" wherein the inhibitor is incubated with catalytic amounts of papain. For the assay of the inhibitor 0.1 ml of each reaction

mixture was diluted to 1 ml after neutralisation and aliquots were taken for assay. The inhibitor was incubated for 15 and 30 min with papain and then casein solution was added to start the reaction. The results showed that in both cases, "Reaction mixture N" and 'Reaction mixture M' there was no change in inhibitor activity. If modification had occurred and the modified inhibitor is less active than the native inhibitor, "Reaction mixture M" should show less inhibitor activity, and on incubation should gradually regain its inhibitor activity if the modification is reversible at higher pH. The above results indicate that no modification of the inhibitor had occurred similar to that of the trypsin inhibitor. However it is possible that the modified inhibitor may have the same activity as the native inhibitor. Further work is needed to determine whether a peptide bond in the inhibitor is cleaved by papain.

Temporary inhibition:

Temporary inhibition is one of the mechanisims by which the activity of a proteolytic enzyme can be blocked temporarily. This phenomenon has been observed in the case of the pancreatic secretory trypsin inhibitors. Trypsin activity reappears after prolonged incubation of the complex.

Papain inhibitors were tested for temporary inhibition. Inhibitors A_1 and B_2 were incubated with equivalent amounts of papain at 30°C for 24 h to determine whether the inhibition is temporary or not. 20 units of each inhibitor were taken separately and incubated with 20 units of

activated papain for 24 h. The complex was then reactivated with cysteine before estimation for release of papain activity if any. No papain activity was detected in either case, thereby showing that the inhibitors were not degraded with resultant release of papain from the complex. Longer periods of incubation of the complex were not tried.

Since no modification of the inhibitor was obtained it was of interest to determine whether enzymically inactive derivatives of papain will combine with the inhibitor. The mercury derivative as well as S-carboxy amido methylated papain were used. They were used as immobilized systems. Inhibitors B_2 and C were used for the above experiments. The results are shown in Table 31. It will be seen that both the inhibitors were bound by the mercury derivative as well as by the S-alkyl derivative of papain quantitatively. The binding of the inhibitors was as strong as the binding by active papain. There was no release of inhibitor activity after boiling the complex, formed by active papain or inactive derivatives at neutral pH. Release of the inhibitor from the complex with both active papain and inactive papain occurred to the same extent at pH 1 in the case of inhibitor C. Partial release of B_2 was also obtained at pH 1 from s-carboxy amido methyl papain. These results are in agreement with the observation of Fossum and Whitaker (1968) and Sen and Whitaker (1973). The binding and dissociation of the two inhibitors with papain derivatives were the same as with enzymatically active papain. Sen and Whitaker (1973) have suggested that protein interaction

Table 31: Binding of inhibitors by inactive derivatives of papain

Experiment	Inhibitor B_2				Inhibitor O			
	Original activity	Activity unbound	Activity bound	Activity eluted	Original activity	Activity unbound	Activity bound	Activity eluted
	Units	Units	Units	Units	Units	Units	Units	Units
Bound papain	584	0	584	230 (pH 1, 95°C for 5 min)	11,000	0	11,000	6,000 (pH 1, 30 min Phosphate 0.1 M/HCl mixture)
Bound Hg-papain	292	0	292	-	480	0	480	240 (pH 1, Tris/HCl mixture 0°C).
Bound carboxy amido methylated papain	810	22	788	96 (pH 2, 30°C for 60 min)				

Conditions of elution are given in parenthesis.

could be due to hydrophobic binding. Since boiled papain does not combine with the inhibitor it is likely that retention of the configuration of the active enzyme is essential and that binding of the protein molecules could be due to a number of forces such as hydrophobic interaction, hydrogen bonding and electrostatic forces. While these results show that peptide splitting in the papain inhibitor is not necessary for inhibitor binding, the mode of action of papain inhibitor remains obscure.

C H A P T E R VI

D I S C U S S I O N

DISCUSSION

This work presents evidence for the widespread occurrence of papain inhibitors in plant tissues and the presence of atleast six isoinhibitors of papain in a single source, *Vigna catjang*. The isoinhibitors have been separated from each other and one of them A_1, as described in Chapter IV, has been obtained in pure form and two (A_2 and A_3) were purified to homogeneity by Vartak (1975). Three other isoinhibitors, B_1, B_2 and C were partially purified, inhibitor C being nearly 80% pure. The purification of papain inhibitor C from the alkaline extract was not continued further since it was found to be unstable after purification. All the isoinhibitors are specific for papain and related enzymes (ficin and chymopapain) and have no action on trypsin, chymotrypsin, subtilisin and bromelain. There has so far been no report on the purification of specific inhibitors of papain from a plant source. The present work establishes for the first time the occurrence of specific isoinhibitors of papain in a plant material.

Occurrence:

The occurrence of a papain inhibitor, unlike inhibitors of other enzymes such as trypsin, chymotrypsin, subtilisin, in every one of a wide variety of tissues examined so far (Chapter III) is of considerable importance and interest with regard to the role of these inhibitors in plant metabolism. As shown in Chapter III, the papain inhibitor activity present in callus cultures and seeds was due to

protein inhibitors and not to heavy metals or proteinases. Purified preparations of the inhibitors from Vigna catjang were nondialysable and were active in the presence of EDTA, which makes it improbable that the inhibition was due to a metal. The inhibitors from Vigna catjang showed the typical behaviour of a protein, such as precipitation with ammonium sulfate, adsorption on and elution from DEAE and CM-celluloses, gel filtration on Sephadex, movement on polyacrylamide gel electrophoresis and on cellogel strips, staining with Amino Schwartz and Coomassie Blue and loss of activity with pepsin. Varying the period of incubation with papain showed no change in inhibitor activity, indicating that inhibition is not due to a proteolytic enzyme.

Pepain inhibitor activity was found to increase on boiling in the case of callus cultures. The increase in papain inhibitor activity on heating suggests that a heat labile proteinase (or a related protein capable of binding the inhibitor specifically) is present as a complex with the inhibitor. Increase in inhibitor activity was also observed when extracts of 3 and 4 days' old seedlings of Vigna catjang were heated.

The variation of inhibitor activity on germination as described in Chapter III suggests that after an initial fall in inhibitor activity on the 3rd day of germination, denovo synthesis of papain inhibitor occurs again on the 4th day, though this activity is observed only after boiling the extract. In this connection it should be noted that the enzyme or protein from callus cultures and

germinating tissues of Vigna catjang, which combines with the inhibitor is different from papain, since heating the former at 98°C at neutral pH releases inhibitor activity whereas heating the complex of inhibitor with papain shows no release of inhibitor activity. Another interesting observation was the high content of inhibitor in differentiating tissues (Chapter III). Tissues which do not readily differentiate were found to have a comparatively lower papain inhibitor content than those which differentiate. All the above observations suggest that papain inhibitors may have a basic role in cell metabolism or differentiation. Unfortunately no suitable method exists for selective destruction of the inhibitor and releasing the active enzyme if any, in callus tissue, which combines with the papain inhibitor. Acid treatment, heating etc. destroy the enzyme and release the inhibitor. Suitable methods for recovery of both enzyme and inhibitor would be invaluable for isolating the proteolytic enzymes occurring endogenously which correspond to the inhibitors and studying their metabolic role.

It is possible that there are several proteolytic enzymes in Vigna catjang, which are selectively inhibited by different isoinhibitors. Their separation and characterization would normally be difficult, but the purified isoinhibitors can be used for distinguishing between different papain-like proteolytic enzymes which may occur in Vigna catjang and callus tissues. As already shown in Chapter V, the DEAE-cellulose fraction containing the isoinhibitors

A_1, A_2 and A_3 shows appreciable inhibition of the endogenous proteinase from the seedlings of Vigna catjang. However, purified preparations of A_1 show very low inhibition. There is no inhibition by B_2. Further work is required for characterization of the inhibitors and enzymes.

Crude extracts of Vigna catjang contain inhibitors of trypsin, chymotrypsin, subtilisin and papain. The trypsin inhibitor content was found to be quite high as compared to papain inhibitor activity. The different inhibitors were found to be present in the amounts shown in Table 32. (The results are for acid extracts of the seeds. For alkaline extracts the papain inhibitor activity will be six times higher). It will be seen that papain inhibitor activity is very low compared to trypsin inhibitor activity.

Isoinhibitors:

There is no conclusive evidence to show whether or not the isoinhibitors are artifacts. However as described earlier (Chapter IV), extraction of papain inhibitors from ground seeds with boiling acid indicated the presence of atleast two distinct groups of isoinhibitors - those which are adsorbed and eluted from DEAE-cellulose and those which are comparatively basic and are adsorbed only on CM-cellulose. These observations make it improbable that the two distinct groups of isoinhibitors arise due to the action of proteolytic enzymes present in the extract. The relative amounts of the two groups of inhibitors also did not vary significantly in preparations from different batches of seeds.

Table 32: Inhibition of trypsin, subtilisin and papain by different leguminous seed extracts

	Vigna catjang	Vicia faba	Phaseolus vulgaris	Phaseolus lunatus
		Units/g seeds		
Trypsin inhibitor	9285	17,850	11,220	59,160
Subtilisin inhibitor	214	153	112	510
Papain inhibitor	54	102	85	122
		mg/g seeds		
Protein	6.6	12.6	5.6	11

Vartak (1975).

However further work is required to determine whether some of the isoinhibitors are artifacts formed during the purification steps or whether they are initially present in the seeds. It also needs to be determined whether the relative proportion of the isoinhibitors A_1, A_2 and A_3 vary on storage due to the action of proteolytic enzymes. In different batches of seeds processed for the purification of the inhibitors there was a considerable variation in the proportion of isoinhibitors A_1, A_2 and A_3. A_1 was present in large amounts compared to A_2 and A_3 in the batches of seeds used for this work. Earlier batches showed a larger proportion of A_2 and A_3. Whether this variation, in the proportion of the isoinhibitors A_1, A_2 and A_3 is due to genetic variation of seeds, or whether they have arisen due to the action of enzymes on storage remains to be seen. However the properties of the isoinhibitors separated and purified are sufficiently different for them to be designated as distinct isoinhibitors. The main groups of isoinhibitors A and B differ markedly in their properties. The differences in their properties will be discussed in later sections.

As seen in Chapter III all the parts of the plant were found to contain a papain inhibitor. A similar pattern of distribution of the trypsin inhibitor was observed by Ambe and Sohonie (1956) in broad beans. The presence of isoinhibitors in various seeds has been discussed in Chapter I (Section III). It is possible that these isoinhibitors, present in different parts of the plant, or seed, may have different functions in the metabolism of the tissues concerned.

The presence of papain inhibitors (unlike other inhibitors) in all plant tissues, which were examined, suggests that they may have a basic metabolic role in regulating tissue differentiation, cell division or other processes and not mere protective or storage function as in the case of other inhibitors, such as trypsin inhibitors, which are not universally present in plants.

Purification:

Extraction at acid pH eliminated a considerable amount of impurities, including non-protein material such as nucleic acids, calcium phytate etc. In general, conventional techniques of protein purification such as ammonium sulfate fractionation, CM-cellulose and DEAE-cellulose chromatography were used to obtain the inhibitors in a homogeneous form. Preparative polyacrylamide gel electrophoresis was used as the final step in the purification procedure. This was necessary since fractionation with ammonium sulfate, and column chromatography on CM- or DEAE-celluloses for the respective inhibitors gave preparations which were not completely homogeneous. Four to five bands were obtained even after repeated chromatography on ion-exchangers. The impurities had probably similar elution profiles and hence could not be separated by the above techniques. Hence further purification on polyacrylamide gel electrophoresis for both the inhibitors was carried out.

The A_1 inhibitor after preparative polyacrylamide gel electrophoresis gave a single band when tested by

polyacrylamide gel electrophoresis. This homogeneous preparation when tested for trypsin inhibitor activity showed feeble inhibition of trypsin. Since it is improbable that only this inhibitor could be non-specific, the preparation was tested with immobilised trypsin. The trypsin inhibitor activity was bound by trypsin. The trypsin inhibitor was obviously not removed by conventional techniques. The use of immobilised trypsin was the only technique which proved successful in removing the trypsin inhibitor.

The observation that highly purified, apparently homogeneous papain inhibitor A_1 had trypsin inhibitor activity raises an interesting point. (A similar observation was made by Vartak (1975), who obtained a subtilisin inhibitor which was homogeneous by several methods but contained trypsin inhibitor activity. The latter could be removed by immobilised trypsin leaving only subtilisin inhibitor). Earlier reports in the literature regarding the specificity of proteinase inhibitors in different tissues should be viewed with caution. The presence of more than one inhibitor activity is not necessarily an indication of lack of specificity for the proteinase even with a purified, apparently homogeneous inhibitor. The method described here (i.e. treatment with immobilised trypsin etc.) will be a good test to determine specificity, when more than one activity is present.

In the case of the B_2 inhibitor, preparative polyacrylamide gel electrophoresis gave a homogeneous preparation,

as indicated by other techniques such as ultracentrifugation and SDS-gel electrophoresis. However the specific activity of the preparation was only 100 units/mg of protein. Treatment of the inhibitor with immobilised papain, followed by elution with acid gave a preparation with a specific activity of 1000 units/mg of protein. This suggests that impurities which are difficult to separate by ultracentrifugation and electrophoresis are present in the preparation which are possibly non-protein in nature, or denatured inhibitor.

Purification of papain inhibitor C from the alkaline extract was attempted since the extract contained several times more papain inhibitor activity than the acid extract. Initially purification by using immobilised papain was successful, since a 1000 fold purification was obtained in a single step. The recovery of the inhibitor from the complex was fairly good and was about 50 to 60%. However the preparation was found to be unstable on storage at -20°C. The inhibitor preparation purified by conventional techniques was also found to be unstable to storage at -20°C. The reason for this instability is not known, since the inhibitor is markedly stable to heat and other denaturating conditions. The inhibitor was purified to 5000 units/mg of protein. The major loss in activity observed during acid fractionation could be due to the action of a proteolytic enzyme acting on an inhibitor which is rapidly destroyed at pH 5. Similar results were obtained by Lenney *et al.* (1974) in the case of *Saccharomyces cerevisiae*. This may be of physiological importance since

it could be a mechanism to regulate the action of the inhibitor *in vivo*.

Specific activities:

The final specific activities of the papain inhibitors were 4000, 1000 and 5000 units/mg of protein for inhibitors A_1, B_2 and C respectively. In comparison the papain inhibitor from chicken egg white and rabbit skin had a final specific activity of 5340 units and 1700 units/mg of protein respectively.

Yield of papain inhibition on purification:

Considerable loss of activity occurred during purification. The loss of inhibitor activity was mainly in the preparative gel electrophoresis step and chromatography on CM-cellulose and DEAE-cellulose. Yields of the inhibitor on polyacrylamide gel electrophoresis were improved considerably from practically nil recovery to 50% by the use of washed gels and repeated elution over a longer period of time. Losses on chromatography on DEAE-cellulose and CM-cellulose could not be readily explained. Washing the ion exchangers with EDTA and alcohol was of little use in improving the yield of the inhibitor. The yield of papain inhibitor C, inspite of heavy losses during acid precipitation, was better than that of the inhibitors from the acid extract.

Degree of purification:

Though it is difficult to estimate the true degree of purification when several isoinhibitors are present in the extract, an approximate estimate is made to determine the

degree of purification. The apparent purification of the isoinhibitors. A_1, B_1 and B_2 with respect to the crude acid extract was approximately 2000, 12 and 500-fold respectively. Inhibitor C from the alkaline extract was purified 2000-fold. If it is arbitrarily assumed that A_1, A_2, A_3, B_1 and B_2 are present in equal amounts initially in the acid extract (the activities of the inhibitors adsorbed and not adsorbed on CM-cellulose were nearly the same and the activities of B_1 and B_2 were nearly the same; the relative ratios of A_1 and A_3 were variable for different lots of seeds, but it is arbitrarily assumed for simplifying the calculations that they are present in equal amounts) the actual purification of these inhibitors A_1, B_1 and B_2 is atleast 10,000, 60 and 2,500-fold respectively. The acid extract of the seeds contains only 4.5 percent of the protein in the seeds and about one-sixth of the protein in the alkaline extract. Hence the actual purification of the papain inhibitors with respect to the extractable protein of the seeds is atleast 60,000, 360 and 15,000-fold for A_1, B_1 and B_2 and more than 2000 fold for C. (since the number of isoinhibitors in the crude alkaline extract is not known). It will be seen that these inhibitors constitute a very small portion of the seed proteins. This is in contrast to the trypsin inhibitors which may constitute nearly 1-5% of the proteins of some seeds. The chicken egg white papain inhibitor and the rabbit skin papain inhibitor were purified 2100 fold and 210 fold respectively. The difficulty involved in the isolation in pure form of the isoinhibitors and their separation from each other will be

apparent from the large number of papain isoinhibitors in the seeds and the minute quantities which are present.

The presence of several isoinhibitors of papain, and the very small amounts present in the seeds necessiate improved methods for their separation and characterization. The need for separation from proteolytic enzymes to stabilise the inhibitors and also from other proteinase inhibitors such as trypsin inhibitors from which they are difficult to separate suggest that improved methods of affinity chromatography require investigation. Rapid and sensitive methods for separation by gel electrophoresis or similar techniques and staining methods for identifying the isoinhibitor bands would be invaluable for determining which of the inhibitors are present originally in the seeds and whether any are formed during the isolation procedures. The differences in papain isoinhibitor pattern of different parts of the plant such as stem, leaves etc. as well as of cytoplasm, lysosomes etc. and during growth, differentiation and tumour formation would be of interest and require rapid methods of separation and sensitive methods of detection. Attempts to characterise some of the proteinase inhibitors directly in crude extracts after polyacrylamide gel electrophoresis were unsuccessful due to their presence in very small amounts. Initial concentration by affinity chromatography appears to be essential for their further separation. As indicated earlier these inhibitors will also be of value for the study of the endogenous proteinases which they inhibit.

PROPERTIES

Homogeneity of inhibitors: The homogeneity of the inhibitors A_1, B_2 and C has been discussed in detail in Chapter V.

Molecular weight:

The papain inhibitors can be classed in the category of small molecular weight inhibitors. The molecular weight of 7760 obtained for A_1 by gel filtration on Sephadex G-50 is in agreement with the value obtained by SDS-gel electrophoresis, 7670. The other DEAE-cellulose adsorbable papain isoinhibitors from the acid extract, A_2 and A_3, have been shown to have molecular weights of 11,430 and 12,200 respectively by gel filtration on Sephadex G-50. The values obtained for these inhibitors by SDS gel electrophoresis are 9700 and 9700 respectively (Vartak, 1975). The above data show that inhibitor A_1 is different from A_2 and A_3. The molecular weight of inhibitor C was found to be 11,560 by gel filtration.

In the case of inhibitors B_1 and B_2 the molecular weights determined by gel filtration on Sephadex G-50 corresponded to 10,480 and 11,480 respectively. However the molecular weight determined by SDS-gel electrophoresis for B_2 was 5500 which is half of that obtained by gel filtration. The value obtained by ultracentrifugation was 9,800. The existence of the inhibitor as a dimer is likely. It is similar to the subtilisin inhibitor from Streptomyces albogriseolus the monomers of which were found to have identical molecular weights. (Sato and Murao, 1974).

Other examples of oligomers of inhibitors are the Bowman Birk inhibitors from soybean (Harry and Steiner, 1969) lima bean inhibitor (Haynes and Feeney, 1967) and the inhibitor from potatoes (Melville and Ryan, 1970). These inhibitor aggregates were found to dissociate and bind trypsin in their monomeric form. However the potato inhibitor (Melville and Ryan, 1970) which exists as a tetramer was found to bind four molecules of chymotrypsin in its tetrameric form. The specific papain inhibitor from chicken egg white (Fossum and Whitaker, 1968) was shown to have a molecular weight of 12,700 by gel filtration. On this basis the molecular weight of the papain inhibitor - papain complex (assuming a mole to mole combination) should have been 34,000. However the molecular weight obtained by gel filtration was only 25,000. No explanation was given for this discrepancy. It is likely that this inhibitor also exists as a dimer, which however combines in its monomeric form with the enzyme.

Dissociation constants:

Dissociation constants were determined for both the inhibitors A_1 and B_2 using casein as the substrate by the method of Green and Work (1953). The B_2 inhibitor had a dissociation constant of 4.3×10^{-8}M. The A_1 inhibitor had a lower dissociation constant, 2.34×10^{-9}M. The value reported with p-nitrophenyl benzyloxycarbonyl glycinate for the complex of ficin with the chicken egg white inhibitor is $1.47 \pm 0.68 \times 10^{-8}$M. The inhibitors generally have a dissociation constant in the range 10^{-7} to 10^{-9}M.

However there are a few exceptions e.g. the trypsin-pancreatic trypsin inhibitor has an unusually low dissociation constant 6 x 10^{-14}M at pH 8 (Vincent and Lazdunski, 1972). Bieth (1974) has shown that the binding behaviour of an enzyme inhibitor system depends on the ratio $(E^\circ)/K_i$ and not only on K_i. [(E°) is the total enzyme concentration and K_i is the dissociation constant]. He has shown that if the $(E^\circ)/K_i$ values are high (about 100), the dissociation curves will show abrupt breaks at the equivalence point. This is illustrated in Fig. 14. The calculated values of $(E^\circ)/K_i$ for inhibitors A_1 and B_2 are 98 and 50. These high values show that the method of determining the dissociation constants used is valid for these inhibitors.

Amino acid composition:

The A_1 inhibitor contained only one mole of half-cystine per mole of the inhibitor. Unfortunately sufficient material was not available to determine SH groups. It is not certain whether it contains an SH group or whether two monomers are linked by a disulfide bond. The latter is unlikely since there was no difference in the molecular weight determined by gel filtration and SDS-electrophoresis in the presence of a reducing agent. On the other hand there is no report of any proteinase inhibitor containing an SH group. Further work is needed to determine whether this inhibitor contains an SH or S-S bond. The ficin-papain inhibitor from chicken egg white (Sen and Whitaker, 1973) contains 2.64 and 1.61 moles of half-cystine and methionine

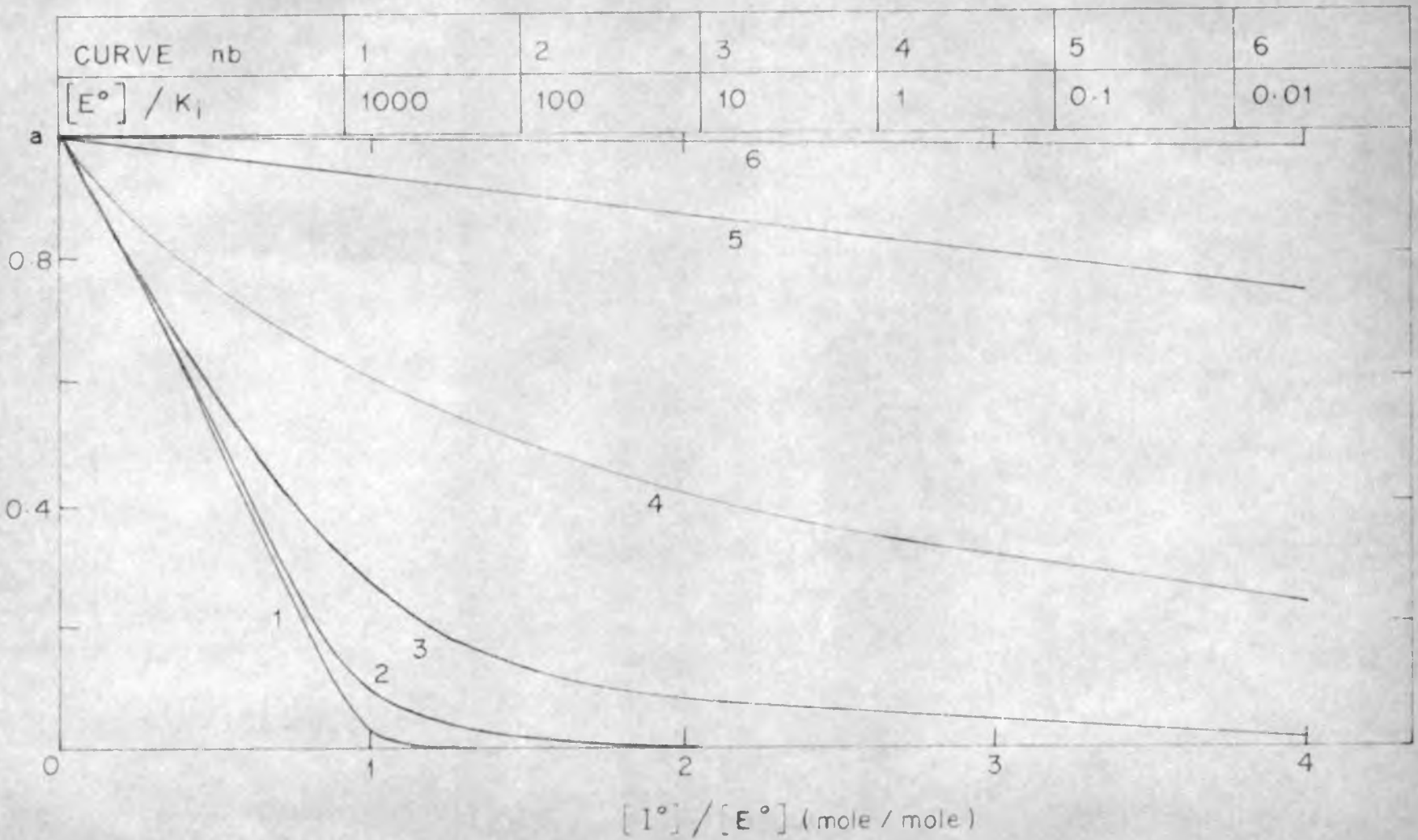


FIG. 14. THEORETICAL PLOTS OF FRACTIONAL FREE ENZYME (a) AS A FUNCTION OF MOLAR RATIOS OF INHIBITOR TO ENZYME ([I°] / [E°])

Bieth (1973)

Legend for Figure number 14:

Theoretical plots of fractional free enzyme (a) as a function of molar ratios of inhibitor to enzyme $(I^\circ)/(E^\circ)$ for the different values of the ratio $(E^\circ)/K_i$, where E° and I° are the total concentration of enzyme and inhibitor respectively and K_i is the dissociation constant of the inhibitor-enzyme complex.

respectively per 10,000 g of the inhibitor. The subtilisin inhibitor purified from Vigna catjang (Vartak, 1975) also contains one mole of half-cystine per mole of inhibitor.

The B_2 inhibitor contained 4 residues of half-cystine per mole of the inhibitor. Cysteine was absent as determined by DTNB titration. Both A_1 and B_2 have a very low content of aromatic amino acids and they are not unusually rich in proline.

Laskowski has postulated that the stability of most of the trypsin inhibitors is due to the high crosslinking by disulfide bridges and the presence of a high content of proline. However the papain inhibitors are very stable at acid pH and fairly stable at neutral pH at 80°C for 15 min. They are stable to other reagents such as ethanol and urea. The reason for their high stability, which is similar to that of the trypsin inhibitors, in spite of very low or no disulfide linkages and low proline content is not clear.

Carbohydrate:

B_2 was found to contain 0.61 mole of sugar per mole of the inhibitor. Preliminary experiments indicated that a part of the inhibitory activity present in the CM-cellulose eluate containing B_1 and B_2 combined with lectins. Other experiments showed that lectin does not have any effect on inhibitor activity. This suggests that the carbohydrate region is not required for activity. Owing to the difficulty of elution of the inhibitor activity from the complex with lectin, this work was not further continued. The use of immobilised lectin for isolation of this inhibitor deserves

further study. The serum inhibitors, and ovomucoids contain as much as 25% carbohydrate (Vogel, 1968). The plant bromelain inhibitor also contains carbohydrate (Reddy et al., 1975).

Stability:

All the papain inhibitors except B_1 were markedly stable to denaturing conditions such as heat, pH and ethanol and only B_1 lost 50% of its activity under similar conditions. They were also relatively stable to urea and borohydride, B_1 showing maximum stability. However all the inhibitors lost considerable activity on heating in 3% trichloroacetic acid at 80°C. In general the stability was higher at lower pHs. The B_2 inhibitor was found to be comparatively less stable at pH 10 on heating at 80°C.

The ficin-papain inhibitor from chicken egg white [Sen and Whitaker (1973); Fossum and Whitaker (1968)] was found to be comparatively unstable to heat. At 35°C it was found to be stable for 30 min in the pH range 4-9. However when a 5×10^{-5}M solution was heated at pH 7 at 80°C it lost 40% of its activity within 10 min.

Enzyme and substrate specificity of the papain isoinhibitors:

All the papain inhibitors inhibit ficin and chymopapain. They do not inhibit chymotrypsin, trypsin, subtilisin, and fruit bromelain. However care is needed in determining the specificity and activity of inhibitors, since it varies with the same enzyme, depending on which protein is used as the substrate, whether a large protein or a small synthetic substrate is used or whether esterolytic, amidase, or peptide bond hydrolytic activity is being determined. There have

so far been no studies of the inhibition of several closely related enzymes by isoinhibitors using a wide range of large and small molecular weight substrates. The data in table 14 are therefore of special interest in showing the complexity of these interactions. Different isoinhibitors can be distinguished from each other by such studies. The complete lack of inhibitory activity with serum albumin, the striking variation in the relative activities with casein and hemoglobin with different isoinhibitors and different enzymes, the ten fold higher inhibition by A_1 of amidase activity of ficin compared to caseinolytic activity, and the marked differences in inhibition of caseinolytic activities by the different isoinhibitors are among the noteworthy observations. Inhibitor activity should therefore be determined with different substrates and several related enzymes before any conclusions can be drawn, regarding their inhibitory activity or molar inhibition ratios.

The molar inhibition ratios, (number of moles of enzyme inhibited by 1 mole of inhibitor) especially for the B group of inhibitors is low. However this may be due to the fact that the actual substrate or enzyme is different in vivo. Some of the molar inhibition ratios for the inhibitors are as follows: 1:0,26 for the subtilisin inhibitor from barley (Mikola and Suolinna, 1971), and 1:0.35 for the same inhibitor for chymotrypsin inhibition. A ratio of 1:0.37 was obtained for the inhibition of subtilisin when hemoglobin was used as the substrate (Ryan, 1966). In the above case the inhibition of trypsin was seen only when TAME or

hemoglobin was used as the substrate and not with casein.

Ficin and papain were found to compete for the same active site of the inhibitor in the case of inhibitors A_1 and B_2.

Dissociation of enzyme-inhibitor complexes:

The inhibitors were found to bind firmly with papain and the complexes were difficult to dissociate. Papain-inhibitor complexes did not dissociate on heating at 98°C at neutral pH. As discussed earlier, these results are in accordance with the results obtained by Sen and Whitaker (1973). Quantitative recovery of the inhibitor was obtained only by treatment of the complex at pH 1 at room temperature (A_1), or by heating at pH 2 at 98°C. However the results were different when immobilised enzyme systems were used. Only partial dissociation of the complex was obtained at pH 1 in the case of inhibitors B_2 and C bound by immobilised papain or inactive derivatives. These results with papain inhibitor complexes are in contrast to other inhibitor-enzyme complexes, such as trypsin and subtilisin. The above complexes dissociate readily on heating or on lowering the pH to 3.

The results with callus tissues and germinated seedlings, which show increase in papain inhibitor activity on heating, suggest the presence of corresponding endogenous proteinases which interact with the inhibitors. These complexes are different from papain inhibitor complexes as they dissociate on heating at neutral pH. The characterization of these proteinases and study of their localization in different

organs and cell organelles and variation in activity during growth and germination may be expected to throw light on the physiological role of these isoinhibitors in the regulation of proteinase activities *in vivo*.

Mechanism of action:

It is evident that no modification of the inhibitor is required for complex formation. The combination of the inhibitor with inactive derivatives of papain such as Hg-papain and S-amido carboxymethylated papain indicate that an active enzyme is not a prerequisite for binding. There was also no inactivation of the inhibitor by incubation with papain as in the case of "temporary inhibition" of trypsin inhibitors. Interaction between inhibitor and enzyme appears to be dependent in these cases on specific protein - protein interaction due to charged groups, hydrophobic groups by van der Waals forces and hydrogen bonding and not due to covalent bonds between enzyme and inhibitor. But these studies do not preclude covalent linkages in the case of active enzyme and papain inhibitor. The results with inactive derivatives of papain are similar to the results obtained by Fossum and Whitaker (1968), Sen and Whitaker (1973), for the chicken egg white inhibitor.

Identity of papain isoinhibitors:

The isoinhibitors can be said to be different from each other for the following reasons:

They differ markedly in their activities towards different substrates and different enzymes as described

earlier. The two classes of inhibitors A and B differ qualitatively in their adsorption on DEAE-cellulose and CM-cellulose, the former class of inhibitors being adsorbed only by DEAE-cellulose and the latter only by CM-cellulose. A_1, A_2 and A_3 inhibitors differ in their elution profiles, A_1 being eluted by 15mM phosphate buffer, pH 7.5, A_2 and A_3 being eluted by 3 and 5 mMolar respectively. Inhibitors B_1 and B_2 are eluted at 10 and 50 mMolar of phosphate buffer, pH 7.5 respectively from CM-cellulose. Inhibitor C, purified from alkaline extracts of the seed is adsorbed by DEAE-cellulose and eluted with 10 mM phosphate buffer, pH 7.5. The elution profiles indicate that they are different from each other.

The inhibitors also vary in their relative mobilities on polyacrylamide gel electrophoresis. Inhibitors B_1 and B_2 move towards the cathode at pH 8.5 while inhibitors A_1, A_2, A_3 and C move towards the anode at the same pH. Inhibitors A_1, A_2 and A_3 have the following relative mobilities: 0.48, 0.56 and 0.68 at pH 8.5. Inhibitor B_2 has a relative mobility of 1 compared to the marker dye at pH 4.4.

The isoinhibitors also differ to some extent in their stability towards various denaturing agents and other conditions as stated earlier. These properties show that the isoinhibitors are quite different from each other. As mentioned earlier the existence of the two classes of inhibitors A and B in the seed has been established. However it remains to be shown that some of the different

isoinhibitors of each group are not formed from each other during the course of purification due to the action of hydrolytic enzymes.

CHAPTER VII

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Papain inhibitors were shown to be widely distributed in plant tissues. They were found to be present in all seeds (both monocots and dicots) and actively growing callus cultures which were tested. The existence of a cryptic inhibitor in all the callus cultures and in germinated seedlings of Vigna catjang was suggested, since the inhibitor activity was found to increase markedly on heating. Evidence was presented to show that the papain inhibitor activity was due to a protein and not due to heavy metals or proteinases.

The acid extracts of Vigna catjang seeds were found to contain five isoinhibitors of papain (A_1, A_2, A_3 and B_1 and B_2). The isoinhibitors were separated from each other and one of them, A_1, was purified to homogeneity. B_1 and B_2 were partially purified. The alkaline extract of Vigna catjang was found to contain appreciable quantities of papain inhibitor activity. The inhibitor C was purified to 80% homogeneity. All the inhibitors were specific for papain and the related enzymes ficin and chymopapain. The properties and kinetics of the inhibitors were studied in detail.

The inhibitors were purified by using several techniques such as fractionation with ammonium sulfate, acid precipitation, column chromatography on DEAE-cellulose and CM-cellulose, Sephadex gel filtration and affinity chromatography. The final specific activities of the inhibitors A_1, B_2 and C were 4000, 1000 and 5000 units/mg of protein

respectively, which represent 60,000, 15,000 and 2,000 fold purification relative to the extractable protein of the seeds, assuming that the isoinhibitors are present initially in equal amounts.

The specificity of the inhibitors with respect to different enzymes and substrates was studied in detail. They do not inhibit trypsin, chymotrypsin subtilisin, bromelain and pepsin. They inhibit papain, ficin and chymopapain. Striking differences were seen in their specificities with different substrates and enzymes. All the inhibitors blocked the amidase and esterase activity of papain. They generally showed higher inhibition with casein than with hemoglobin and no activity with serum albumin. The molar inhibition ratio of the isoinhibitors with different enzymes in the presence of different substrates show significant differences.

The inhibition of papain and ficin by A_1 or B_2 was not additive, suggesting that the same active site of the inhibitor is involved in the inhibition of both enzymes.

A crude mixture of inhibitors A_1, A_2 and A_3 was found to inhibit an endogenous proteinase from the germinated seedlings of Vigna catjang.

The amino acid composition of the two isoinhibitors A_1 and B_2 was determined. The A_1 inhibitor was found to contain one mole of half-cystine per mole of the inhibitor. It had a high content of histidine. The total number of amino acid residues was 61. The B_2 inhibitor had a total

number of 40 amino acid residues. It contained 4 half-cystine residues per mole of inhibitor. Both the inhibitors had a low content of aromatic amino acids. There were only two proline residues per mole of each of the inhibitors. The partial specific volume of A_1 was calculated to be 0.75 ml/mg. Inhibitor B_2 was found to contain 0.61 mole of sugar per mole of inhibitor.

The molecular weight of the inhibitors range from 5500 to 12000 as determined by several methods. An interesting observation was that the B_2 inhibitor existed as a dimer. Its molecular weight was 5500 as found by SDS-gel electrophoresis, which is half of that obtained by Sephadex G-50 chromatography.

Incubation of the inhibitor B_2 with papain showed no progressive loss of inhibitor activity with time. Enzymically inactive derivatives of papain (e.g. Hg-papain and S-alkyl derivative of papain) were found to bind the inhibitor, which suggests that an active enzyme for splitting of a peptide bond in the inhibitor or formation of a covalent bond between enzyme and inhibitor are not required for binding of the inhibitor as postulated for some trypsin inhibitors.

The dissociation constant for the inhibitors A_1 and B_2 were found to be 2.3×10^{-9}M and 4.3×10^{-8}M respectively.

The inhibitors bind firmly with papain and the complexes were difficult to dissociate. Heating the complex was ineffective in dissociating the complex at neutral pH. Quantitative recovery of the inhibitor was obtained only by

treatment of the complex at pH 1 at room temperature (A_1) or by heating at pH 2 (B_2) at 98°C. However this was true only for complexes in solution. Only partial dissociation of the complex was obtained at pH 1 in the case of the inhibitors B_2 and C bound by immobilised papain or inactive papain derivatives.

The inhibitors were found to be stable to heat at different pHs. The inhibitors were also stable in 75% ethanol at 80°C, sodium borohydride and urea. However the inhibitors lost considerable activity when heated in 3% trichloroacetic acid at 80°C. The inhibitors A_1 and B_2 were digested by pepsin.

Data regarding the other properties of the different inhibitors are summarised in the Tables given below (Table 33, 34 and 35).

The differences in stability to heat, pHs etc. and the differences in molecular weights, specificity to different enzymes and different substrates establish that the six isoinhibitors of papain from the seeds of Vigna catjang are different from each other.

In conclusion papain inhibitors, unlike others proteinase inhibitors such as trypsin inhibitors, are present in all plant tissues which were tested. Vigna catjang seeds contain several isoinhibitors of papain. They are protein in nature and specific for the enzymes papain, ficin and chymopapain and are different from the trypsin and subtilisin inhibitors of the seeds.

Table 33: Properties and kinetics of papain inhibitors of Vigna catiang

	A_1	A_2	A_3	B_1	B_2	C
DEAE-cellulose chromatography. Phosphate concentration required for elution (mM)	1.5	3	5	-	-	10
CM-cellulose chromatography. Phosphate concentration required for elution (mM).	-	-	-	10	50	-
Specific activity (units/mg of protein. Homogeneity on polyacrylamide gel electrophoresis at pH 8.5 (no. of bands)).	4000 Single band	6250 Single band	5000 Single band	-	1000 Single band	5000 four bands
Relative mobility at pH 8.5	0.48	0.56	0.68	-	-	-
Relative mobility at pH 10.	0.86	-	-	-	-	-
Relative mobility at pH 4.4.	-	-	-	-	1.0	-
Homogeneity by SDS gel electrophoresis.	Single band	Single band	Single band	-	Single band	-
Homogeneity by ultracentrifuge.	-	-	-	-	Single peak	-

Table 34: Properties and kinetics of papain inhibitors of Vigna catiang

	A_1	A_2	A_3	B_1	B_2	C
Optical factor (Absorbance^{-1} at 280 nm of 0.1% solution)	-	0.8	0.9	-	1.08	-
Absorption spectrum Maximum (nm)	-	275	276	-	275	-
Minimum (nm)	-	250	252	-	250	-
280/260	-	1.14	1.14	-	1.4	-
Molecular weight						
by gel filtration	7760	11,430	12,220	11,480	10,480	11,560
by ultracentrifuge	-	-	-	-	9,855	-
by SDS gel electrophoresis	7670	9,700	9,700	-	5,500	-
by amino acid analysis	8560	-	-	-	-	5,960
$s_{20,w}$	-	-	-	-	1.4S	-
Isoelectric point	6.9	-	-	-	10.6	-
Dissociation constant (M)	2.3×10^{-9}	-	-	-	4.3×10^{-8}	-

Table 35: Properties and kinetics of papain inhibitors of Vigna catjang

	A_1	A_2	A_3	B_1	B_2	C
Moles of tyrosine per mole of inhibitor	2	-	-	-	1	-
Moles of tryptophan per mole of inhibitor	-	-	-	-	0-1	-
Moles of half-cystine per mole of inhibitor	1	-	-	-	4	-
Moles of carbo-hydrate per mole of inhibitor	-	-	-	-	1	-

Properties of A_2 and A_3 from Vartak (1975).

C H A P T E R VIII

B I B L I O G R A P H Y

BIBLIOGRAPHY

Ambe, K.S. and Sohonie, K. (1956) — Experientia, 12, 302.

Ambe, K.S. and Sohonie, K. (1956) — J. Sci. Ind. Res. 15C, 136.

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

Andrews, P. (1965) — Biochemical Journal, 96, 595.

Anson, M.L. (1938) — J. Gen. Physiol. 22, 79.

Applebaum, S.W. (1964) — J. Insect Physiol. 10, 783.

Applebaum, S.W. and Konijn, A.M. (1966) — J. Insect Physiol. 12, 665.

Applebaum, S.W., Birk, Y., Harpaz, I. and Bondi, A. (1964) — Comp. Biochem. Physiol. 11, 85.

Archibald, W.J. (1947) — J. Phy. Colloid. Chem. 51, 1204.

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

Arnon, R. (1970) — Methods in Enzymology, Vol. 19, p. 226.

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

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

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

Back, N. (1966) — Fed. Proc. 25, 77.

Balls, A.K. and Lineweaver, H. (1939) — J. Biol. Chem. 130, 669.

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

Barel, A.O. and Glazer, A.N. (1969) — J. Biol. Chem. 244, 268.

Belitz, H.D., Kaiser, K.P. and Santarius, K. (1971) — Biochem. Biophys. Res. Commun. 42, 420.

Bender, M.L., Begue-Canton, M.L., Blakely, R.L., Brubacher, L.J., Feder, J., Gunter, C.R., Kezdy, F.J., Killhefler, J.V., Marshall, T.H., Miller, C.G., Roeske, R.W. and Stoops, J.K. (1966).	J. Amer. Chem. Soc. 88, 5890.
Bergmann, M. (1942)	Advances in Enzymology, 2, 49.
Bieth, J. (1973)	Proteinase Inhibitors, Proceedings of the 2nd International Conference. p. 462, Edited by Fritz, H., Tschesche, H., Green, L.J. and Truscheit, E. Springer-Verlag Edition, New York.
Bigelow, C.C. (1967)	J. Theor. Biol. 16, 187.
Birk, Y. (1961)	Biochem. Biophys. Acta, 54, 378.
Birk, Y., Gretler, A. and Khalef, S. (1967)	Biochim. Biophys. Acta, 147, 402.
Blumberg, S., Schechter, I. and Berger, A. (1970)	Eur. J. Biochem. 15, 97.
Bowman, D.E. (1944)	Proc. Soc. Exptl. Biol. Med. 57, 139.
Bowman, D.E. (1946)	Proc. Soc. Exptl. Biol. Med. 63, 547.
Brocklehurst, K. and Kierstan, M.P.J. (1973)	Nature, 242, 167.
Bundy, H.F. and Mehl, J.W. (1959)	J. Biol. Chem. 234, 1124.
Burger, W.C., Siegelmann, H.W. (1966)	Physiol. Plant, 19, 1089.
Cavallini, D., Graziani, M.T. and Dupre, S. (1966)	Nature, 212, 294.
Chan, S.K. and Rees, D.C. (1975)	Nature, 250, 240.
Cohn, E.J. and Edsall, J.T. (1943)	"Protein, Aminoacids and peptides" Reinhold Publishing Corp., New York.
Cohn, E.J., Hughes, W.L. Jr. and Weare, J.H.(1947)	J. Am. Chem. Soc. 69, 1753.

Davis, B. (1964) — Ann. N.Y. Acad. Sci. 121, 404.

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

Drenth, J., Jansonius, J.N., Koekoek, R., Swen, H.M. and Wolthers, B.G. (1968) — Nature, 218, 929.

Dua, R.D., Bedi, G.S. and Dixit, A. (1975) — Indian Journal of Biochem. Biophys. 12, 86.

Ebata, M. and Yasunobu, K.T. (1962) — J. Biol. Chem. 237, 1086.

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

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

Epstein, C.J., Anfinsen, C.B. and Sela, M. (1962) — J. Biol. Chem. 237, 3458.

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

Erikson and Larrson (1975) — Nutritional Reviews, 33, 189.

Erlanger, B.F., Kokowsky, N. and Cohen, W. (1961) — Arch. Biochem. Biophys. 95, 271.

Feeney, R.E. and Allison, R.G. (1969) — Evolutionary Biochemistry of proteins. Homologous and Analogous proteins from Avian Egg whites, Blood Sera, Milk and other substances, p. 51, Wiley-Interscience, New York.

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

Feeney, R.F. (1971) — Proc. Int. Res. Conf. Proteinase Inhibitors, 1st 1970, ed. 162.

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

Feinstein, G. (1971) — Proc. Int. Res. Conf. Proteinase Inhibitors Ist ed. 1970, p. 38.

Filho, X.J. (1973) — Physiol. Plant, 28, 149.

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

Fisher, H.F. (1964) — Proc. Natl. Acad. Sci. (USA), 51, 1285.

Fiske, C.H. and Subba Row, Y. (1925) — J. Biol. Chem. 66, 375.

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

Foster, R.J. and Ryan, C.A. (1965) — Fed. Proc. 24, 473.

Fraefel, W. and Acher, R. (1968) — Biochim. Biophys. Acta, 154, 615.

Fraenkel, G.S. and Leiner, I.E. (1954) — Agril. Food Chem. 2, 410.

Francois, C., Marshall, R.D. and Neuberger, A. (1962) — Biochem. J. 83, 335.

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

Frattali, V. (1969) — J. Biol. Chem. 244, 274.

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

Fritz, H., Schult, H., Neudecker, M. and Werle, E. (1966) — Angew. Chem. Internat. Ed. 5, 735.

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

Fritz, H., Gebhardt, M., Fink, E., Schramm, W., and Werle, E. (1969) — Hoppe-Seylers. Z. Physiol. Chem. 350, 129.

Fritz, H., Fink, E., Gebhardt, M., Hochstrasser, K. and Werle, E. (1969) — Hoppe-Seylers Z. Physiol. Chem. 350, 933.

Fritz, H. and Tschesche, H. (1970) — Proc. Int. Res. Conf. Proteinase Inhibitors (Munich) 1st Edition. Walter-de-Gruyter publications, New York.

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

Glazer, A.N. (1966) — J. Biol. Chem. 241, 3811.

Glazer, A.N. and Smith, E.L. (1971)	The Enzymes, Vol. 3, 3rd Ed. Edited Boyer, P.D. p. 511, Academic Press, N.Y.
Goodwin, T.W. and Morton, R.A. (1946)	Biochem. J. 40, 628.
Gorini, L. and Audrain, L. (1953)	Biochim. Biophys. Acta, 10, 570.
Green, N.M. and Work, E. (1953)	Biochem. J. 54, 347.
Greene, L.J., Rigbi, M. and Fackre, D.S. (1966)	J. Biol. Chem. 241, 5610.
Greene, L.J. and Bartlet, D.C. (1969)	J. Biol. Chem. 244, 2646.
Greene, T.R. and Ryan, C.A. (1972)	Plant Physiol. 51, 19.
Grob, D. (1949)	J. Gen. Physicl. 33, 103.
Gundlach, G. and Turba,F. (1965)	Biochem. J. 342, 303.
Haendle, H.I., Trautschold, I., Vogel, R., and Werle, E. (1965)	Cited from Vogel, R., Trautschold, I., Werle, E. (1968) "Natural Proteinase Inhibitors", Academic Press, New York, p. 104, 107.
Ham, W.E. and Sandstedt, R.M. (1944)	J. Biol. Chem. 154, 505.
Harry, J.B. and Steiner, R.F. (1969)	Biochemistry, 8, 5060.
Haynes, R. and Feeney, R.E. (1967)	J. Biol. Chem. 242, 5378.
Haynes, R., Osuga, D.T. and Feeney, R.E. (1967)	Biochemistry, 6, 541.
Haynes, R. and Feeney, R.E. (1968)	Biochim. Biophys. Acta, 159, 209.
Hercz, C. (1973)	Cand. J. Biochem. 51, 1447.
Hill, R.L. (1965)	Adv. Prot. Chem. 20, 37.
Hites, B.D., Sandstedt, R.M. and Schaumberg, L. (1951)	Ceral. Chem. 28, 1.

Hixson, H.F. and Laskowski, M. Jr.(1970)	J. Biol. Chem. 245, 2027.
Hochstrasser, K., Muss, M. and Werle, E. (1967)	Hoppe-Seylers Z. Physiol. Chem. 348, 1337.
Hochstrasser, K., Schwartz, C., Illchmann, K. and Werle, E. (1968)	Hoppe-Seylers Z. Physiol. Chem. 349, 1449.
Hochstrasser, K. and Werle, E. (1969)	Hoppe-Seylers Z. Physiol. Chem. 350, 249.
Hochstrasser, K., Illchmann, K. and Werle, E. (1969)	Hoppe-Seylers Z. Physiol. Chem. 350, 655.
Hochstrasser, K., Werle, E., Siegelmann, R. and Schwarz, S. (1969)	Hoppe-Seylers Z. Physiol. Chem. 350, 897.
Hochstrasser, K., Illchmann, K. and Werle, E. (1969)	Hoppe-Seylers Z. Physiol. Chem. 350, 929.
Honavar, M. and Sohonie, K. (1955)	J. Univ. Bombay, 24B, 64.
Huber, R., Kukla, D., Ruhlmann, A., Eppo, O. and Formanek, H. (1970)	Naturwissenshaften, 57, 389.
Huber, R., Kukla, D., Ruhlmann, A. and Steigemann, W. (1970)	Proc. Int. Res. Conf. on Proteinase Inhibitors. Munich. p. 56, Walter de Gruyter publications, New York.
Huff, J.E. (1972)	Personal Communication to Sen, L.C. and Whitaker, J.R. (1973). Arch. Biochem. Biophys. 158, 623.
Ikeda, K., Hamaguchi, K., Yamamoto, M. and Ikenaka, T. (1968)	J. Biol. Chem. (Tokyo), 63, 521.
Jagannathan, V., Kartar Singh and Damodaran, M. (1956)	Biochem. J. 63, 94.
Jirgensons, B., Ikenaka, T. and Gorguraki, V. (1960)	Makromol. Chem. 39, 149.
Jones, G., Moore, S. and Stein, W.H. (1953)	Biochemistry, 2, 66.

Kassel, B., Radicevic, M., Ansfield, M.J. and Laskowski, M.J. Fr.(1965)	Biochim. Biophys. Res. Commun. 18, 255.
Kassel, B. and Chow, R.B. (1966)	Biochemistry, 5, 3449.
Kassel, B. (1970)	Methods in Enzymology Vol. 19, page 840. Edited by Perlmann, G.E. and Lorand, L. Academic Press, New York.
Kazal, L.A., Spicer, D.S. and Brashinsky, R.A. (1948)	J. Am. Chem. Soc. 70, 3034.
Keilova, H. and Tomasek (1974)	Biochem. Biophys. Acta, 334, 179.
Kimmel, J.R. and Smith, E.L. (1954)	J. Biol. Chem. 207, 515.
Kimmel, J.R. and Smith, E.L. (1957)	Adv. Enzymol. 19, 267.
Kirsi, M. and Mikola, J. (1971)	Planta, 96, 281.
Kowalski, D. and Laskowski, M. Jr. (1972)	Cited from Laskowski, M. Jr. and Sealock, R.W. p. 420 - Enzymes Vol. III (Ed. Boyer, P.D.) Third Ed., Academic Press.
Krahn, J. and Stevens, F.C. (1970)	Biochemistry 9, 2646.
Kraut, H. and Bhargava, N. (1967)	Z. Physiol. Chem. 348, 1500.
Kress, L.F., Wilson, K.A. and Laskowski, M. Sr. (1968)	J. Biol. Chem. 243, 1758.
Kuhlenschmidt, M.S., Yunis, E.J., Lammarino, R.M., Turco, S.J., Peters, S.P. and Glew, R.H. (1974)	Lab. Invest. 31, 413.
Kunitz, M. and Northrop, J. H. (1936)	J. Gen. Physiol. 19, 991.
Kunitz, M. (1947)	J. Gen. Physiol. 30, 291.
LaBar, F.F. (1966)	Biochemistry 5, 2368.

Laskowski, M. Jr. and Laskowski, M. Sr.(1951)	J. Biol. Chem. 190, 563.
Laskowski, M. Sr. and Wu, F.C. (1953)	J. Biol. Chem. 204, 797.
Laskowski, M. Sr., Kassel, B. and Hagerty, G. (1957)	Biochem. Biophys. Acta, 24, 300.
Laskowski, M. Jr. and Sealock, R.W. (1969)	Biochemistry 8, 3703.
Laskowski, M. Jr. and Sealock, R.W. (1971)	"The Enzymes", vol. III, p. 376,(Edited by Boyer, P.D.) 3rd Ed., Academic Press, N.Y.
Laskowski, M. Jr. and Sealock, R.W. (1971)	"The Enzymes" Vol. III, p. 385, (Edited by Boyer, P.D.) 3rd Ed., Academic Press.
Laskowski, M. Jr. and Sealock, R.W. (1971)	"The Enzymes", Vol. III, p.378, (Edited by Boyer, P.D.), 3rd Ed., Academic Press.
Learmonth, E.M. (1951)	J. Sci. Food Agri. 2, 447.
Learmonth, E.M. (1958)	J. Sci. Food Agri. 9, 269.
Leary, T.R. and Laskowski, M. Jr. (1973)	Fed. Proc. Abs. 32, Ab. No. 1363.
Lenney, J.F., Matile, Ph., Wiemken. A., Schellenberg M. and Meyer, J. (1974)	Biochem. Biophys. Res. Commun. 60, 1378.
Lenney, J.F. (1975)	J. Bacteriology, 122, 1265.
Levin, Y., Pecht. M., Goldstein, L. and Katchalski, E. (1964)	Biochemistry 3, 1905.
Liener, I.E. and Kakade, M.L. (1969)	"Toxic constituents of plant food stuffs" Liener I.E. Edition, Academic Press, New York, p. 7.
Light A., Frater, R., Kimmel, J.R. and Smith, E.L. (1964)	Proc. Natl. Acad. Sci. US. 52, 1276.
Lineweaver, H. and Schwimmer, S. (1941)	Enzymologia, 10, 81.
Lipke, H., Fraenkel, G.S.& Leiner, I.E. (1954)	J. Agr. Food, Chem. 2, 410.

Liu, W.H., Feinstein,G., Osuga, D.T. and Haynes, R. and Feeney, R.E. (1968)	Biochemistry 7, 2886.
Liu, W., Means, G.E. and Feeney, R.E. (1971)	Biochem. Biophys. Acta, 229, 176.
Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.L. (1951)	J. Biol. Chem. 193, 265.
Lugg, J.W.H. (1938)	Biochemical J. 32, 775.
Martin, C.J. and Axelrod, A.E. (1958)	Biochim. Biophys. Acta, 27, 52.
Matis, P. and Morl, F.K. (1968)	Ann. N.Y. Acad. Sci. 146, 715.
Melville, J.C. and Ryan, C.A. (1970)	Arch. Biochem. Biophy. 138, 700.
Melville, J.C. and Ryan, C.A. (1972)	J. Biol. Chem. 247, 3445.
Mickel, C.E. and Standish, J. (1947)	Univ. Minn. Agr. Exp. Sta. Tech. Bull 173. Cited from Ann. Rev. plant Physiol. (1973) 24, 186.
Mikola, J. and Enari, T.M. (1970)	J. Inst. Brew. 76, 182.
Mikola, J. and Suolinna, E.M. (1971)	Arch. Biochem. Biophys. 144, 566.
Mikola, J., Kirsi, M. (1972)	Acta. Chem. Scand. 26, 787.
Mitchel, R.E.J., Chaiken, I.M. and Smith, E.L. (1970)	J. Biol. Chem. 245, 3485.
Moore, S. and Stein, W.H. (1948)	J. Biol. Chem. 176, 367.
Moore, S. (1963)	J. Biol. Chem. 238, 235.
Moore, S. and Stein, W.H. (1963)	Methods in Enzymology, Vol. 6, p. 819.
Murao, S. and Sato, S. (1972)	Agril. Biol. Chem. 36, 160.

Mycek, M.J. and Fruton, J. S. (1957) — J. Biol. Chem. 226, 165.

Neikamp, C.W., Hixson, H.F., Jr. and Laskowski, M.Jr. (1969) — Biochemistry 8, 16.

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

Ota, S., Moore, S. and Stein, W.H. (1964) — Biochemistry, 3, 180.

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

Papaioannou, S.E. and Liener, I.E. (1970) — J. Biol. Chem. 245, 4931.

Peanasky, R.J. and Ghaleb, M.A. (1970) — Proc. Int. Res. Conf. Proteinase Inhibitors 1st Ed. 1970 p. 282.

Perlstein, S.H. and Kezdy, F.J. (1973) — Ab. No. 5022 7f in chemical Abstracts 79 (9):110.

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

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

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

Rackis, J.J. and Anderson, R.L. (1964) — Biochem. Biophys. Res. Commun. 15, 230.

Radoeva, A. (1973) — Abst. No. 566470 in Chemical Abstracts 78 (9).

Ramirez, J.S., and Mitchell, H.L. (1960) — J. Agri. Food. Chem. 8, 393.

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

Reddy, N.M., Keim, P.S. Heinrikson, R.L. and Kezdy, F.J. (1975) — J. Biol. Chem. 250, 1741.

Rekosh, D. (1972) — J. Virol. 9, 479.

Reisfeld, R.A. (1962) — Nature, 195, 281. Reisfeld, Lewis and Williams Phamphlet from Ames Company, Division Miks Lab. Inc. Ellcharl, Indiana, U.S.A.

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

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

Royer, A., Miege, M.N., Grange, A., Miege, J. and Mascherpa, J.M. (1974) — Planta (Berl) 119, 1.

Rubin, H. (1970) — Science 167, 1271.

Ryan, C.A. and Balls, B.A. (1962) — Proc. Natl. Acad. Sci. US. 48, 1839.

Ryan, C.A. and Clary, J.J. (1964) — Arch. Biochem. Biophys. 108, 169.

Ryan, C.A., Clary, J.J., Tomimatsu, Y. (1965) — Arch. Biochem. Biophys. 110, 175.

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

Ryan, C.A., Huisman, O.C. and Vandenburgh, R.W. (1968) — Plant Physiol. 45, 484.

Ryan, C.A. and Kassel, B. (1970) — Methods in Enzymology, Vol. 19, 883.

Ryan, C.A. (1971) — Biochem. Biophys. Res. Commun. 44, 1265.

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

Ryan, C.A., Means, G.E., and Feeney, R.E. (1974) — Accounts of Chemical Research, 7, 315.

Sato, S. and Murao, S. (1973) — Agri. Biol. Chem. 37, 1067.

Sato, S. and Murao, S.(1974) — Agri. Biol. Chem. 38, 587.

Sato, S., Kimura, T. and Murao, S. (1975) — Agril. Biol. Chem. 39, 415.

Schachman, H.K. (1957) — Methods in Enzymology, Vol. 4, p. 32. Ed. Colowick, S.P. and Kaplan, O.N., Academic Press, New York.

Schack, P. (1967)	C.R.Travel Lab., Carlsberg, 36, 67.
Schnebli, H.P. and Burger, M.N. (1972)	Proc. Natl. Acad. Sci. (US) 69, 3825.
Schwick, H.G., Heimburger, N. and Haupt, H. (1966)	Z. ges. Inn. Med. 21, 193.
Schrode, J.	Cited from Laskowski, Jr. and Sealock, R.W. (1971). The Enzymes, 3, p.389 -(Ed. Boyer, P.D.) 3rd Edition, Academic Press, N.Y.
Sealock, R.W. and Laskowski, M. Jr. (1969)	Biochemistry 8, 3703.
Sefton, B.M. and Rubin, H. (1970)	Nature, 227, 843.
Sen, L.C. and Whitaker, J.R. (1973)	Arch. Biochem. and Biophys. 158, 623.
Shain, Y. and Mayer, A.M. (1965)	Physiol. Plant 18, 853.
Shain, Y. and Mayer, A.M. (1968)	Phytochemistry, 7, 1491.
Shapiro, A.L., Vinuela, E. and Maizel, J.V. (1967)	Biochem. Biophys. Res. Commun. 28, 815.
Sharp, H.L., Bridges, R.A., Krivit, W. and Friuer, E.F. (1969)	J. Lab. Clin. Med. 73, 934.
Shyamala, G. and Lyman, R.L. (1964)	Cand. J. Biochem. 42, 1825.
Sluyterman, L.A.E. (1967)	Biochim. Biophys. Acta. 139, 418.
Sluyterman, L.A.E. (1967)	Biochim. Biophys. Acta. 139, 430.
Smith, E.L. and Parker, M.J. (1958)	J. Biol. Chem. 233, 1387.
Sohonie, K. and Bhandarkar, A.P. (1955)	J. Sci. Ind. Res. 14C, 100.
Sohonie, K., Huprikar, S.V. and Joshi, M.R. (1959)	J. Sci. Ind. Res. 18C, 95.
Steiner, R.F. (1954)	Arch. Biochem. Biophys. 49, 71.

Steiner, R.F. and Fratalli, V. (1969) — J. Agri. Food and Chem. 17, 513.

Sumathi, S. and Pattabiraman, T.N. (1975) — Indian J. of Biochem. & Biophys. 12, 383.

Sumathi, S. and Pattabiraman, T.N. (1976) — Indian J. of Biochem. Biophys. 13, 52.

Summers, D.F. and Maizel, J.V. (1971) — Procd. Natl. Acad. Sci. (USA) 68, 2852.

Taber, R., Rekosh, D. and Baltimore, D. (1971) — J. Virol. 8, 395.

Taber, R., Wertheimer, R. and Golrick, J. (1973) — J. Mol. Biol. 80, 367.

Tauber, H., Kershaw, B.B. and Wright, R.D. (1949) — J. Biol. Chem. 179, 1155.

Thompson, A.G. (1968) — Ann. N.Y. Acad. Sci. 146, 540.

Trautschold, I. (1965) — Cited from Vogel, R., Trautschold, I. and Werle, E. (1968) "Natural Proteinase Inhibitors", Academic Press, New York, p. 31, 32 (801 Ref.)

Tschesche, H. and Klein, H. (1968) — Z. Physiol. Chem. 349, 1645.

Tschesche, H., Klein, H. and Reidel, G. (1970) — Proc. Int. Res. Conf. Proteinase Inhibitors, Ist Ed. p. 299.

Tschesche, H., and Wachter, E. (1970) — Hoppe-Seyler's Z. Physiol. Chem. 351, 1449.

Udaka, K. and Hayashi, H. (1965) — Biochim. Biophys. Acta. 97, 251.

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

Umezawa, H. (1972) — "Enzyme Inhibitors of Microbial Origin" p. 15-20, University park press, London.

Uy, R. and Feeney, R.E. (1970) — Proc. Int. Res. Conf. Proteinase Inhibitors Ist Ed. (1970), 164.

Vartak, H.G. (1975) — "Proteinase inhibitors". A thesis submitted to the University of Poona, India.

Ventura, M.M. and Filho, J. (1966)	Acad. Brasilevia de Ciencias Anais, 38, 553.
Ventura, M.M., Filho, X.J., Moreira, R.A., Aquino, A.M. and Pinheiro, P.A. (1971)	An. Acad. Bras. Cienc. 43, 233.
Verdecourt (1970)	Kew. Bulletin 24, 543.
Vincent, J.P. and Lazdunski, M. (1972)	Biochemistry, 11, 2967.
Vogel, R., Trautschold, I. and Werle, E. (1968)	"Natural Proteinase Inhibitors", Academic Press, New York.
Warburg, O. and Christian, W. (1941)	Biochem. Z. 310, 384.
Warsy, A.S., Norton, G. and Stein, M. (1974)	Phytochemistry 13, 2481.
Waugh, D.F. (1954)	Adv. Protein Chem., 9, 326. Ed. M.L. Anson and Edsall, J.T. Academic Press, New York.
Weber, K. and Osborn, M. (1969)	J. Biol. Chem. 244, 4406.
Weber, K. and Osborn, M. (1975)	"Proteins" 1, 4th Ed. Neurath, H. and Hill, R.L. Academic Press, N.Y.
Werle, E., Trautschold, I., Haendle, H. and Fritz (1968)	Cited from Vogel (1968).
Weyer, E.M. (1968)	Am. N.Y. Acad. Sc. 46, 361.
Whitaker, J.R. and Perez-villasenor, J. (1968)	Arch. Biochim. Biophys. 124, 70.
Wu, F.C. and Laskowski, M. Sr. (1955)	J. Biol. Chem. 213, 609.
Wurtz, A. and Bouchet, E. (1879)	Compt. Rend. 89, 425.
Yajima, H. and Kiso, Y.(1974)	Chem. Pharm. Bull. 22, 1087.
Yamamoto, M. and Ikenaka (1967)	J. Biol. Chem. (Tokyo) 62, 141.
Yoshikawa, M., Iwasaki, T. and Fujii, M. and Oogaki, M. (1976)	J. Biol. Chem. (Tokyo) 79,765.
Yu-Kun and Chen-Lu (1963)	Sci. Sinica (Peking), 12, 201.

LIST OF ABBREVIATIONS

BAEE	-	N-benzoyl-L-arginine ethyl ester.
BAPNA	-	N-Benzoyl-DL-arginine-p-nitroanilide.
Bis	-	N,N'-methylene-bis-acrylamide
CM-cellulose	-	Carboxymethyl cellulose
DEAE-cellulose	-	Diethylaminoethyl cellulose
DFP	-	Di-isopropyl fluorophosphate.
DTNB	-	5-5' dithio-bis-2-nitrobenzoic acid.
EDTA	-	Ethylenediamine tetracetate.
GSH	-	Reduced glutathione.
$NaBH_4$	-	Sodium borohydride.
OD	-	Optical density (absorbance)
ΔOD	-	Change in optical density (absorbance)
p^{CMB}	-	p-chloromercuribenzoate.
r.p.m.	-	Revolutions per minute
S	-	Svedberg unit (10^{-13}sec) of sedimentation coefficient.
-SH	-	Sulfhydryl group.
SBTI	-	Soybean trypsin inhibitor (Kunitz)
SDS	-	Sodium dodecyl sulfate.
TEMED	-	Tetraethylmethylethylenediamine
Tris	-	Tris (hydroxymethyl) aminomethane