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BIOCHEMICAL STUDIES ON THE HEMOLYMPH TRYPSIN INHIBITORS OF THE TOBACCO
HORNWORM MANDUCA SEXTA

A Thesis Presented

By

Narayanaswamy Ramesh

Submitted to the Faculty of the
University of Massachusetts Medical School in partial
fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES

March 1986

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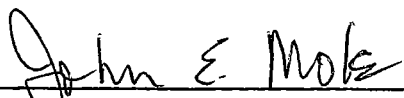
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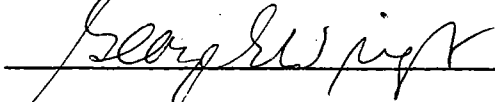
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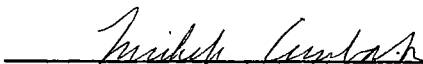
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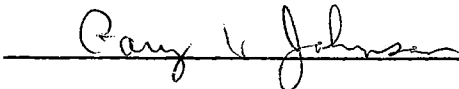
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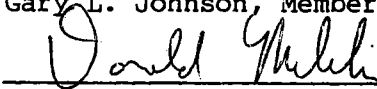
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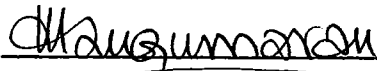
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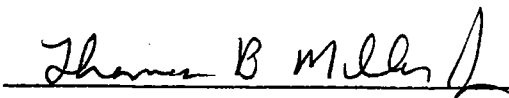
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Graduate Studies

March 1986

To my parents and brother
And to the memory of Professor Herbert Lipke.

ACKNOWLEDGEMENTS

I wish to express my gratitude to Doctors M. Sugumaran, J. E. Mole, and G. E. Wright for their guidance, and to the members of my graduate committee, Doctors D. Melchior, D. A. Laufer, M. Cimbala, and G. Johnson.

I also wish to thank my colleagues Vic, Brian, and Chad for their help.

Financial assistance from the University of Massachusetts at Boston, and the University of Massachusetts Medical School, Worcester are gratefully acknowledged.

Last, but not the least, I wish to thank my wife, Vijaya, for her patience, encouragement, and wise counsel.

ABSTRACT

Trypsin inhibitory activity from the hemolymph of the tobacco hornworm, Manduca sexta, was purified by affinity chromatography on immobilized trypsin and resolved into two fractions with molecular weights of 13700 (inhibitor A) and 8000 (inhibitor B) by Sephadex G-75 gel filtration. SDS-polyacrylamide gel electrophoresis under non-reducing conditions gave a molecular weight estimate of 15000 for inhibitor A and 8500 for inhibitor B. Electrophoresis of these inhibitors under reducing conditions on polyacrylamide gels gave molecular weight estimates of 8300 and 9100 for inhibitor A and inhibitor B, respectively, suggesting that inhibitor A is a dimer. Isoelectrofocusing on polyacrylamide gels focused inhibitor A as a single band with pI of 5.7, whereas inhibitor B was resolved into two components with pIs of 5.3 and 7.1. Both inhibitors A and B are stable at 100° C and at pH 1.0 for at least 30 minutes, but both are inactivated by dithiothreitol even at room temperature and non-denaturing conditions. Inhibitors A and B inhibit trypsin, chymotrypsin, plasmin, and thrombin but they do not inhibit elastase, papain, pepsin, subtilisin BPN' and thermolysin. In fact, subtilisin BPN' completely inactivated both inhibitors A and B. Inhibitor A and inhibitor B form stable complexes with trypsin. Stoichiometric studies showed that inhibitor A combines with trypsin and chymotrypsin in a 1:1 molar ratio. The inhibition constants (K_i) for trypsin and chymotrypsin inhibition by inhibitor A were estimated to be 1.45×10^{-8} M and 1.7×10^{-8} M, respectively. Inhibitor A in complex with chymotrypsin does not

inhibit trypsin (and vice versa) suggesting that inhibitor A has a common binding site for trypsin and chymotrypsin. The amino terminal amino acid sequences of inhibitors A and B revealed that both these inhibitors are homologous to the bovine pancreatic trypsin inhibitor (Kunitz).

Quantitation of the trypsin inhibitory activity in the hemolymph of the larval and the pupal stages of Manduca sexta showed that the trypsin inhibitory activity decreased from larval to the pupal stage. Further, inhibitor A at the concentration tested caused approximately 50% reduction in the rate of proteolytic activation of prophenoloxidase in a hemocyte lysate preparation from Manduca sexta, suggesting that inhibitor A may be involved in the regulation of prophenoloxidase activation. However, inhibitor B was not effective even at three times the concentration of inhibitor A. Since activation of prophenoloxidase has been suggested to resemble the activation of alternative pathway of complement, the effect of inhibitors A and B and the hemolymph of Manduca sexta on human serum alternative pathway complement activity was evaluated. The results showed that, although inhibitors A and B do not affect human serum alternative complement pathway, other proteinaceous component(s) in Manduca sexta hemolymph interact(s) and cause(s) an inhibition of human serum alternative complement pathway when tested using rabbit erythrocyte hemolytic assay.

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LIST OF ABBREVIATIONS

BAPNA	Benzoyl-DL-arginine p-nitroanilide
BPTI	Bovine pancreatic trypsin inhibitor (Kunitz)
DFP	Diisopropylphosphofluoridate
DMSO	Dimethylsulfoxide
Dopa	3, 4-Dihydroxyphenylalanine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
EGTA	Ethylene-bis(oxyethylenitrilo)- tetraacetate
GPNA	Glutaryl-L-phenylalanyl p-nitroanilide
GVBE	Veronal buffer containing EDTA
GVBS	Veronal buffered saline containing gelatin
MUTMAC	4-Methylumbelliferyl N,N,N (p-tri- methylammonium) cinnamate.
NPGB	p-Nitrophenyl p'guanidobenzoate
SDS	Sodium dodecylsulfate
Tris	Tris (hydroxymethylamino) methane

LIST OF SYMBOLS

E_t	Total enzyme concentration
I_{50}	Inhibitor concentration required to produce 50% inhibition of an enzyme
RB serum	Serum depleted of complement factor B
RD serum	Serum depleted of complement factor D
RE	Rabbit erythrocytes
V_e	Elution volume from a gel filtration column
V_o	Void volume from a gel filtration column
v_i	Proteolytic activity in the presence of inhibitor
v_o	Proteolytic activity in the absence of inhibitor

ONE LETTER ABBREVIATIONS FOR AMINO ACIDS

Ala	A
Arg	R
Asn	N
Asp	D
Cys	C
Gln	Q
Glu	E
Gly	G
His	H
Ile	I
Leu	L
Lys	K
Met	M
Phe	F
Pro	P
Ser	S
Thr	T
Trp	W
Tyr	Y
Val	V

CHAPTER I

INTRODUCTION

A. Objectives and Significance

The class Insecta is the largest group of animals on earth and consists of a population which is larger than all other animal groups combined. Insects, as a group, have a large impact on mankind, both medically and agriculturally. Some insects such as the cotton boll weevil and the army worm cause considerable agricultural damage, while many other insects such as the mosquito and the tse-tse fly serve as vectors of dreaded diseases such as encephalitis and sleeping sickness. Thus, an understanding of the biochemistry of insects may provide insights into effective methods of insect control.

A well known pest of the tobacco crops in the United States is the larvae of the sphingid moth Manduca sexta. Until the large scale introduction of chemical pesticides three decades ago, these larvae (referred to as the hornworms) caused considerable economic damage especially to the flue cured and wrapper tobacco by foraging on the leaves. Although successful control methods for preventing crop damage by hornworm foraging have been developed, the tobacco hornworm has continued to be used as an experimental animal for studying the biochemistry and the physiology of insects. Two factors have contributed to the popularity of the tobacco hornworms as experimental animals: first, they can be grown on an artificial diet which allows controlled developmental studies; secondly, the effect of environmental factors such as the

photoperiod and temperature on growth and development, and the developmental control by insect hormones have been well described. Therefore, this insect is a useful model to correlate biochemical and physiological findings with developmental changes.

It is well recognized that proteolytic enzymes are involved in important regulatory functions in both prokaryotic and in eukaryotic organisms (1, 2, 3). Many of the physiologically significant processes in insects also require proteolytic enzymes. For example, metamorphosis in insects involves the catabolism of the tissues of one stage of development and the reformation of the tissues at the next stage. The catabolic steps during metamorphosis require proteolytic enzymes. In addition, the enzyme phenoloxidase, which plays a key role in insect immunity, is activated from its precursor by limited proteolysis, and the coagulation process in insects is also thought to be activated by limited proteolysis.

Although the importance of proteases in insect physiology is well known, the physiological regulation of insect proteases, particularly the role of endogenous protease inhibitors in the control of protease activity, are not well understood. In an effort to understand the biochemical function(s) of insect protease inhibitors, the hemolymph protease inhibitors from the tobacco hornworm, Manduca sexta, were studied. This thesis reports the isolation, and physicochemical characterization of two trypsin inhibitors present in the hemolymph of the tobacco hornworm as a prelude to elucidation of their possible physiological significance.

B. The Role of Proteases in Insect Physiology

B.1. Proteases and insect development

The life cycle of holometabolous insects consists of three stages, the larva, the pupa and the adult. During the pupal stage the larval tissues are histolysed and the adult structures are developed from a group of cells set aside for this purpose from the larval stage (for example in Drosophila these cells are termed imaginal discs). The transformation of the larvae through pupae into the adult is under rigorous hormonal control. Juvenile hormone (JH) is responsible for the maintenance of larval structures while a steroid called ecdysone stimulates the metamorphosis of the larvae into pupae and adults.

The larval stage of insects is subdivided into several instars. During each successive instar the larvae grow bigger. At the end of each instar the outer layer of the insect integument has to be shed (molted) and a new larger layer synthesized in order to accommodate the increase in size of the larva. During the molting process the old cuticle is digested by a battery of enzymes which includes proteases and chitinases, and the digested material is reabsorbed while the undigested matter is shed as exuvia. Two proteases have been identified from the molting fluid of the silkworm Antheraea polyphemus (4). These enzymes have molecular weights of 30,000 daltons and 34,000 daltons and are inhibited by diisopropylphosphofluoridate (DFP), an irreversible inhibitor of serine proteases. Both proteases hydrolyse benzoyl-L-arginine ethyl ester and tosyl-L-arginine methyl ester and, therefore, have trypsin-like specificity. These proteases also are inhibited by soybean trypsin inhibitor; however, the effect of insect

protease inhibitors on these proteases have not been explored. A trypsin-like protease has also been reported in the molting fluid of Manduca sexta, and it has been suggested that this enzyme is responsible for the digestion of chitin associated proteins during molting. In addition, a metal chelator-sensitive protease has been identified in the molting fluid of Manduca sexta, but the nature of the metal ion has not been established (5). This metalloprotease may be involved in the digestion of cuticle associated proteins as well as the degradation of the cuticular chitinase activity (6).

During the pupal stage, larval tissues are histolysed by both lysosomal and non-lysosomal proteases (7). The insect hormone ecdysone stimulates histolysis of the larval muscles by stimulating the proliferation of the lysosomes (8). The involvement of lysosomal proteases is further supported by the finding that homogenates of Antheraea pernyi larval muscles undergoing histolysis contain a particulate fraction associated protease with pH optimum of about 4.0 (9). Ultrastructural and cytochemical studies on metamorphosing Lepidopterous larval muscles have shown that the mitochondria and the endoplasmic reticulum are degraded by the lysosomes (7).

More recent studies have suggested that non-lysosomal proteases may be necessary to initiate myofibrillar degradation in muscles undergoing histolysis. The main support for this contention derives from the finding that neither pepstatin (an inhibitor of the lysosomal protease cathepsin D) nor chloroquin (an inhibitor of intralysosomal proteolysis) inhibited the dissolution of the organized myofibrillar structure (10, 11). The origin of non-lysosomal protease(s) is (are)

uncertain, although, it is possible that the phagocytic hemocytes present in insect muscle undergoing histolysis may be the source.

The mechanisms by which the activities of the molting fluid proteases and the proteases involved in histolysis are regulated is poorly understood. Histolysis is known to be regulated by neural as well as hormonal factors. For example, an increase in the ecdysone level and the cessation of motor impulse to the muscles trigger muscle histolysis (7). However, the mechanisms that couple the hormonal and the neural stimuli to the action of proteases are not known. It is likely that at least part of the immediate regulation of protease activity involves protease inhibitors. To the author's knowledge no direct studies on the control of histolysis by endogenous insect protease inhibitors have been performed. The lack of information is probably attributable to two factors: first, histolysis is a complex phenomenon and the proteases involved have not been well characterized; second, protease inhibitors have been isolated and characterized for only a few insects, and often no information is available on the histolytic process of the insect from which the inhibitor was isolated.

B.2. The Proteolytic activation of insect prophenoloxidase

Phenoloxidases are copper containing monooxygenases, widely distributed among the eukaryotes, which are capable of oxygenating monophenols to diphenols and further oxidizing diphenols to quinones (Fig. 1). In insects, phenoloxidases appear to have important roles in sclerotization, wound healing, and in insect immunity. In sclerotization, the phenoloxidases generate catechols and quinone derivatives which crosslink insect cuticular proteins and harden (sclerotize) the

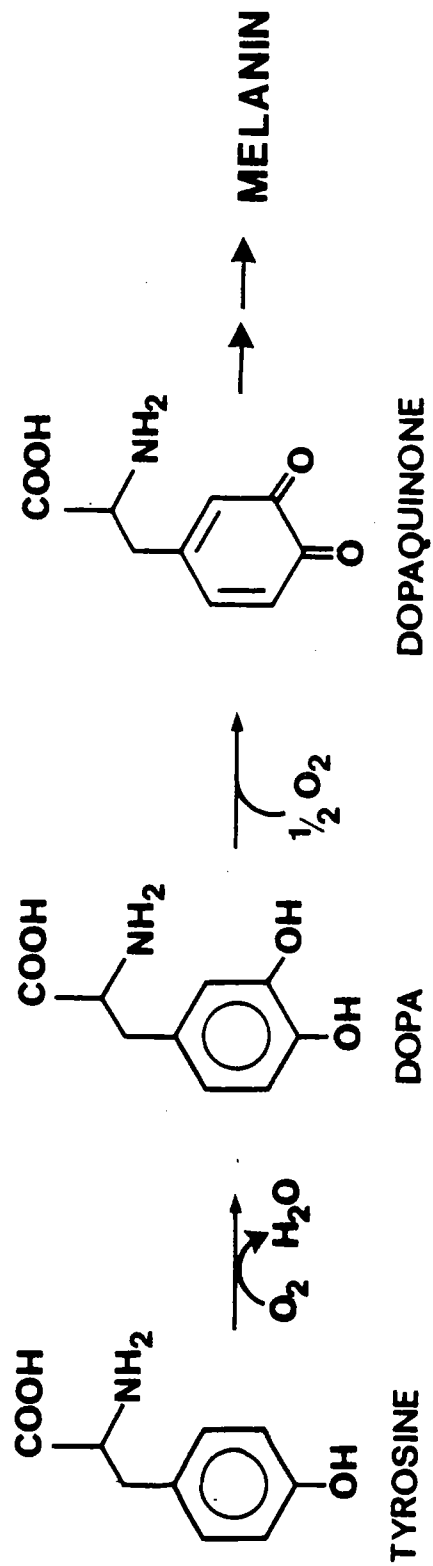


Fig. 1. Reactions catalyzed by phenoloxidase.

cuticle. Crosslinking of the cuticular proteins locally at the site of an injury aids wound healing. The quinones generated by the phenolox- idases have both bacteriocidal and fungistatic properties. In addition, the quinones form melanin pigments which when deposited on the surfaces of invading parasites isolate them from the insect tissues. These melanin depositions attract phagocytic hemocytes and thus promote insect immunity.

There are two classes of phenoloxidas in insects, the cuticular phenoloxidas and the hemolymph phenoloxidase(s). The cuticular phenoloxidas appear to be responsible for sclerotization reactions while the hemolymph enzyme is thought to play a vital role in insect defense mechanisms. The cuticular phenoloxidas in turn can be subclassified into two types. The first type is sensitive to inhibition by phenylthiourea and accepts both mono and ortho diphenol substrates. The second type accepts both ortho and para diphenol substrates and are less sensitive to inhibition by phenylthiourea. The phenylthiourea sensitive phenoloxidas are usually readily solubilized in aqueous buffers, in contrast to the phenylthiourea resistant type of phenoloxidas which are difficult to solubilize and often require limited proteolysis to dissociate them from the cuticle. The properties of the cuticular phenoloxidas and the chemistry of sclerotization have been reviewed (12, 13). The hemolymph phenoloxidase is soluble and is present in both the hemolymph and the hemocytes. Hemolymph phenoloxidase initially was thought to be enclosed within membrane-bound granules and released when needed (14). However, it is now

generally accepted that the hemolymph phenoloxidase is synthesized as an inactive proenzyme which is activated by limited proteolysis (15).

Prophenoloxidase has been purified from the hemolymph of the silkworm Bombyx mori (16), from the larvae of the blowfly Calliphora (17) and from the hemolymph of the tobacco hornworm Manduca sexta (18). A diisopropylphosphofluoridate-sensitive protease capable of activating prophenoloxidase has been purified to homogeneity from the cuticle of Bombyx mori (19) and partially purified from Manduca sexta cuticle (18). In vitro studies using purified preparations of Bombyx mori hemolymph prophenoloxidase and prophenoloxidase activating enzyme from cuticle have demonstrated that activation involves the cleavage of an activation peptide of molecular weight ca. 5000 daltons from a 40,000 dalton prophenoloxidase of Bombyx mori (20). The prophenoloxidase activating serine protease from Bombyx mori hydrolyzes benzoyl-L-arginine ethyl ester and tosyl-L-arginine methyl ester, and therefore possesses trypsin-like specificity. It is still not entirely clear how a protease present in the cuticle can activate prophenoloxidase present in the hemolymph (or hemocytes), although several modes may be proposed. The cuticular activator could be secreted directly into the hemolymph where it would activate the prophenoloxidase. The cuticular protease also could activate a hemolymph enzyme which in turn would cleave the prophenoloxidase. Alternatively the cuticular protease could act on the prophenoloxidase locally near the site of a wound. The latter mechanism could regulate the extent of prophenoloxidase activity by preventing disseminated prophenoloxidase activation in the hemolymph.

Several microbial polysaccharides including yeast zymosan, bacterial endotoxins and laminarin (a fungal polysaccharide) have been shown to cause activation of prophenoloxidase in the hemolymph of Bombyx mori (21) and in the hemocytes of Galleria mellonella (22). Purified Bombyx mori prophenoloxidase is not activated by laminarin confirming that an additional factor is necessary to couple the microbial polysaccharide stimulation to the activation of the prophenoloxidase. The exact sequence of reactions which couple microbial polysaccharide stimulation to prophenoloxidase activation in insects is not clear, although similar studies on the activation of prophenoloxidase in hemocyte lysates of the crayfish Astacus astacus demonstrated that at least one serine protease is involved (23).

Once formed, the enzyme phenoloxidase catalyses the formation of melanin pigments, and the melanization reaction has been demonstrated to induce host defense responses such as phagocytosis (24). The addition of p-nitrophenyl p'-guanidinobenzoate (an inhibitor of trypsin-like proteases) to Galleria mellonella hemocyte culture completely blocks both prophenoloxidase activation and phagocytosis, suggesting that phagocytosis and prophenoloxidase activation both require a trypsin-like protease activity (22). Histological studies have defined the relationship between melanin formation and phagocytosis: prophenoloxidase present in the granulocytes (a type of hemocyte) is secreted onto a foreign surface (e.g. bacterial cells) when the granulocytes come in contact with them and is subsequently activated. The activated phenoloxidase catalyses the formation of melanin pigments, and these in turn stimulate phagocytosis by phagocytic hemocytes (22). The possible

sequence of events which culminates in the phagocytosis of foreign material in the hemolymph is presented in Fig. 2.

The activation of the prophenoloxidase system in insects (and in arthropods in general) is analogous to the initial events in the activation of the alternative pathway of complement (21, 25) in mammals. Microbial polysaccharides are known to stimulate both systems, and in the process proteolytic enzymes are activated. However, it is not known if insects have a complement-like system.

Although the importance of phenoloxidase in insect physiology is well recognized, very little is known concerning the control of the proteases involved in the activation of prophenoloxidase. In particular the role of hemolymph protease inhibitors in the regulation of prophenoloxidase activation has not been examined in insects. One of the aims of this research was to determine whether the hemolymph trypsin inhibitors of Manduca sexta had any role in the regulation of prophenoloxidase activation in this insect.

B.3. The activation of the insect coagulation system

The hemolymph of insects serves a similar function as the vertebrate blood, although, it differs from it in not having erythrocytes. Cells present in the hemolymph are termed hemocytes and are comprised of a complex group of cells derived from the mesoderm. All hemocytes are nucleated cells which resemble vertebrate leukocytes. A clear classification of insect hemocytes is difficult because of their pleiomorphism. The seven major hemocyte types described by Arnold (26) and Crossley (27) are listed in Table 1 along with their putative function(s).

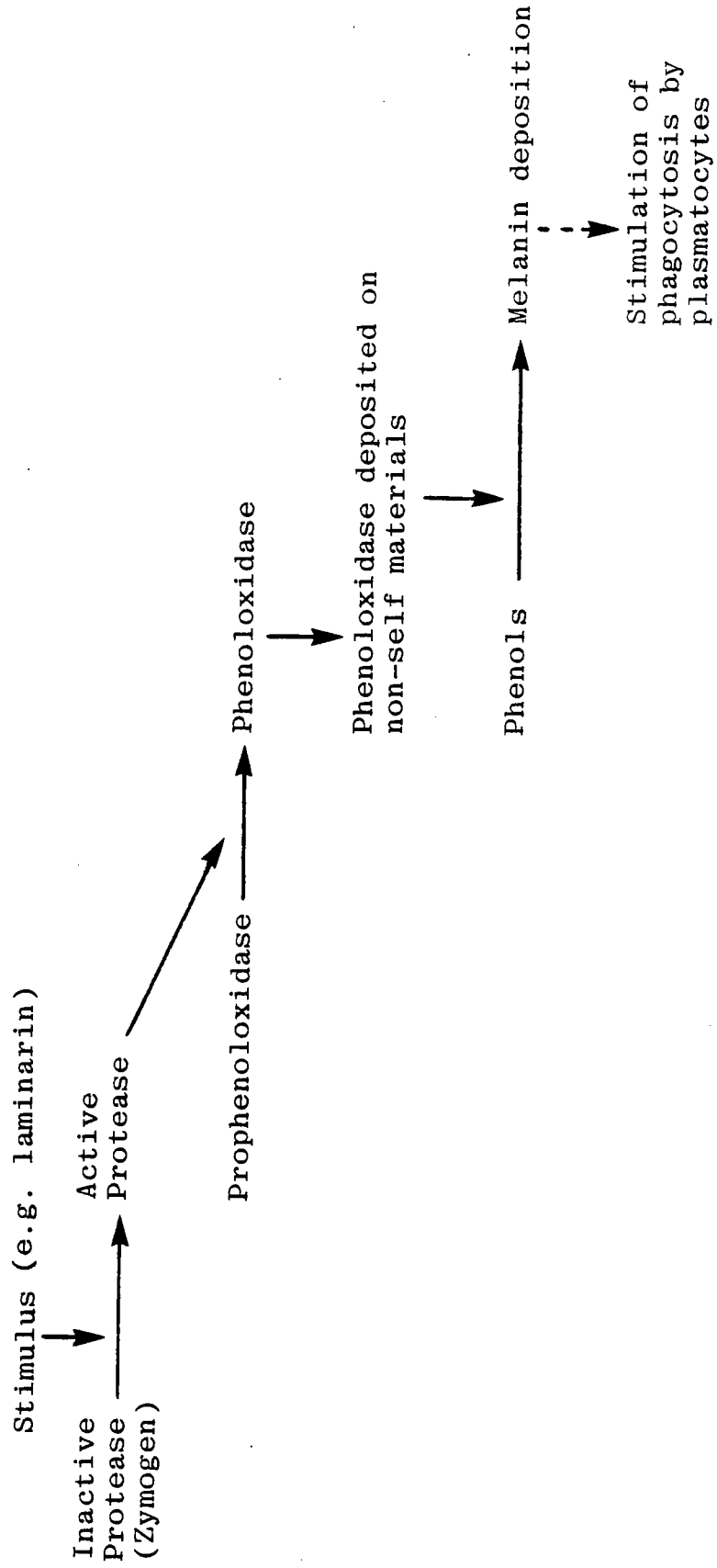


Fig. 2. Hypothetical scheme relating phenoloxidase activation and phagocytosis.

Table 1.

List of hemocyte types in insect hemolymph

<u>Hemocyte type</u>	<u>Suggested function(s)</u>
a) Prohemocyte	Ellipsoidal cells which are the germinal stem cells that differentiate into other cells.
b) Plasmatocytes	Pleiomorphic cells, has lysosomal apparatus, mainly phagocytic* in function.
c) Granulocytes	Disk shaped cells with many periodic-Schiff positive granules. Involved in phenol metabolism* has phenoloxidase activity.
d) Adipohemocytes	Prominent fat globules; lipid and other intermediary metabolism.
e) Cystocytes (Thrombocytoids)	Fragile cells that lyse and cause granular precipitate in the surrounding plasma. Also called <u>coagulocytes</u> , functions in hemolymph* coagulation.
f) Spherulocytes	Function similar to granulocytes.
g) Oenocytoids	Unstable cells which discharge their contents rapidly. Cytoplasm has crystal-like inclusions. Function not clear, may have function similar to granulocytes.

* Indicated function is carried out by more than one cell type.

Many soft bodied invertebrates such as the molluscs prevent hemorrhaging by simply closing the wound by muscular contraction. By contrast, most arthropods, both in the larval and the adult stage possess a rigid exoskeleton and hence cannot close the wound by simple muscular contraction. Arthropods, therefore, must possess an effective hemostatic mechanism which will prevent the loss of a large quantity of hemolymph after being wounded. The clotting of the hemolymph observed in many arthropod species, including *Xiphisura* (e.g., horse shoe crab) (28), Crustacea (e.g., crabs and shrimps) (29) and Insecta (e.g., locusts, roaches) (30, 31), serves as an effective hemostatic mechanism.

The biochemistry of coagulation in insects has not received as much attention as the biochemistry of the mammalian coagulation system. The clottable protein, coagulogen, has been purified from the hemolymph of the roach *Leucophaea maderae* (32), and has been shown to be a lipoprotein. Coagulogen of locusts has also been shown to be a lipoprotein (33). The clotting of roach hemolymph is strongly inhibited by EDTA, and both Ca^{+2} and Zn^{+2} can reverse this inhibition suggesting that divalent cations are needed for the clotting process (31). In addition to divalent metal ions, a hemocyte derived factor appears to be essential for coagulation (34). The nature of this factor in insects is not clear, but in crustaceans this factor has been shown to be a protease (29). Although the details of the activation reactions has not been described for insects, a cascade of proteolytic activities is likely to be involved in the activations since (a) larval bee hemolymph was shown to contain activities functionally equivalent to all the human coagulation factors, except Factor VII activity (35), and (b) the

hemolymph coagulation of another arthropod, the horse shoe crab, has been shown to be activated by a cascade of proteases (31). Mammalian blood protease inhibitors have been shown to be important in the control of the blood coagulation process, and it is likely that protease inhibitors in insect hemolymph also play an important role in the control of the clotting process.

C. Protease Inhibitors - An Overview

Proteolytic enzymes serve as key agents of biological regulation in all living organisms (36, 37). Hence, it is not surprising that protease inhibitors have been isolated from such diverse organisms as bacteria (38) and man (39). The protease inhibitors of mammalian and plant origin have been studied extensively, and their biochemistry has been summarized in several reviews (40, 41, 42).

The amino acid sequences of many protease inhibitors have been elucidated. Based on their primary structures, Laskowski and Kato have proposed a system to classify the serine protease inhibitors into eight major families (41). Although this system fails to include the protease inhibitors whose primary structures are not known, it has been useful for comparing properties of inhibitors of diverse origin. The most extensively studied families are the bovine pancreatic trypsin inhibitor (BPTI, Kunitz) family, the pancreatic secretory trypsin inhibitor (PSTI, Kazal) family and the Bowman-Birk family of inhibitors. The BPTI (Kunitz) family of inhibitors have a wide distribution and are found in both vertebrates and invertebrates. The PSTI (Kazal) type of inhibitors have been shown to be present in large amounts in pancreatic secretions

of mammals, and the Bowman-Birk family of inhibitors are widely distributed among the plant kingdom.

In contrast to the mammalian and plant protease inhibitors, much less is known about the biochemistry and the regulatory functions of insect protease inhibitors. Both trypsin and chymotrypsin inhibitors have been described in the larval homogenates of Drosophila (43), and in the alimentary tract of the tse-tse fly, Glossina morsitans (44), and the roach, Leucophaea maderae (45). Chymotrypsin specific inhibitors have been isolated from whole body homogenates of Drosophila (46), and from the mosquito Culex pipiens (47).

Trypsin and chymotrypsin inhibitory activities have been reported in the hemolymph of the silkworms, Antheraea pernyi and Philosamia cynthia ricini, but, these have not been purified (48). The hemolymph of the commercial silk worm Bombyx mori contains high molecular weight (>40,000 daltons) and low molecular weight (<20,000 daltons) inhibitors which inhibit trypsin and/or chymotrypsin (49, 50). To date, 7000 dalton and 43,000 dalton chymotrypsin-specific inhibitors and a 42,000 dalton trypsin inhibitor have been isolated from the hemolymph of Bombyx mori. A list of insect protease inhibitors which have been purified to homogeneity is given in Table 2.

C.1. The significance of protease inhibitors in biological processes

Viewed in the broadest sense, the physiological function of protease inhibitors would be to eliminate unwanted proteolysis. With few exceptions, the function of specific protease inhibitors have not been determined. The underlying reason for this lack of clarity of

Table 2.

List of some insect protease inhibitors purified to homogeneity

Inhibitor source	Protease inhibited	Molecular weight	Suggested function(s)	Reference
<u>Drosophila</u> larvae	Chymotrypsin	12,000	Thought to be involved in the regulation of catheptic activity.	48
<u>Bombyx mori</u> larvae	Chymotrypsin	7,000	Not known	51
<u>Bombyx mori</u> larvae	Chymotrypsin	43,000	Not known	52
<u>Bombyx mori</u> larvae	Trypsin	42,000	Not known	52

function is that for the most part protease inhibitors were purified and characterized using readily available proteases such as bovine trypsin or chymotrypsin, and consequently, the physiological target enzymes of these inhibitors are unknown. A trypsin inhibitor whose function has been well established is pancreatic secretory trypsin inhibitor (PSTI, Kazal). This inhibitor prevents the premature activation of trypsinogen prior to secretion, and thus protects the pancreas from the deleterious effects of trypsin (41). Likewise, the human serum α_1 -proteinase inhibitor has been shown to protect the lungs against leukocyte elastase (39).

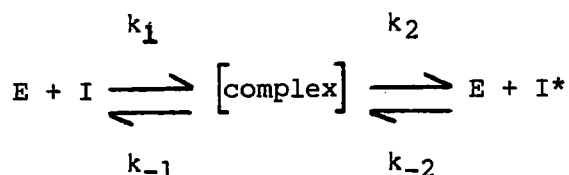
Interestingly, some plants use protease inhibitors as an effective defense against insect infestation. In response to injury by insects, potato leaves accumulate large amounts of chymotrypsin and carboxypeptidase inhibitors (51) which presumably inhibit the insect digestive proteases and prevent insects from feeding.

Three possible biological roles have been proposed for the protease inhibitors in insect hemolymph. First, these inhibitors may inhibit proteases that leak into the hemolymph from other tissues, especially the alimentary tract. Secondly, they may serve to regulate the activities of proteases normally present in the hemolymph, such as the proteases involved in activation of prophenoloxidase and the coagulation systems. The presence of large amounts of protease inhibitors in the hemolymph of Bombyx mori, Antheraea pernyi, and Philosamia cynthia ricini suggests an important role for these protease inhibitors in the control of proteolytic activation. Thirdly, the hemolymph protease inhibitors could protect against the proteases of invading parasites.

For example, the hemolymph of the silkworm, Bombyx mori has been shown to inhibit proteases derived from the fungus, Aspergillus mellus (48).

C.2. A model for the interaction of serine protease with serine protease inhibitors: the case of bovine pancreatic trypsin inhibitor and bovine trypsin

Polypeptide protease inhibitors form tight complexes with their respective proteases. The general mechanism for the interaction of serine proteases with their inhibitors may be depicted as follows:



Where E represents the serine protease, I is the virgin inhibitor and I* is the modified inhibitor (inhibitor whose reactive site peptide bond is hydrolysed - see below). The [complex] represents a collection of several intermediate steps which leads to the formation of a relatively stable protease-inhibitor species. Protease inhibitors display the characteristics of excellent substrates for the association step (k_1), but in contrast to hydrolysable substrates, are inhibitors by virtue of their slow dissociation ($k_2 + k_{-1}$) constants (52).

All polypeptide inhibitors of serine proteases contain at least one amino acid which is specifically recognized by its complementary protease, and a few inhibitors possess amino acid residues which are recognized by several proteases possessing different specificities. These latter inhibitors are known as multiheaded inhibitors (53). The amino acid which denotes the specificity of the inhibitor is termed the P₁ residue (Fig. 3) (40). For endopeptidase inhibitors the amino acid

residue on the carboxy-terminal side of P_1 is designated as the P_1' residue. The peptide bond linking the P_1 and the P_1' residues is designated the reactive site peptide bond (Fig. 3). For example, in BPTI the peptide bond between Lys 15 and Ala 16 has been shown to be the reactive site peptide bond (Fig. 3).

The physico-chemical and kinetic aspects of the interactions of bovine pancreatic trypsin inhibitor (Kunitz) (BPTI) and soybean trypsin inhibitor (Kunitz) (SBTI) with bovine trypsin have been studied in depth (52, 55, 56, 57). Moreover, the crystalline structures of the BPTI-bovine trypsin and the SBTI-porcine trypsin complexes have been elucidated, and we now have a clear understanding of the mechanism of action of these serine protease inhibitors (58, 59). The Manduca sexta hemolymph trypsin inhibitors reported here have strong sequence homology to BPTI. Therefore, BPTI-bovine trypsin interaction was chosen as a model for studying the interaction of insect hemolymph inhibitors with trypsin. In order to describe the nature of the complex formed between BPTI and bovine trypsin a brief description of the mechanism of trypsin catalysis is presented below.

The three amino acid residues intimately involved in the catalytic mechanism of trypsin are Ser 195*, His 57 and Asp 102. The sequence of events in trypsin catalysis is presented in Fig. 4. Polypeptide substrates bind to trypsin such that the trypsin labile peptide bond of the substrate is precisely oriented for nucleophilic attack by Ser 195 of trypsin. The favored mechanism for serine protease catalysis

*Note: The chymotrypsinogen enumeration has been followed throughout for describing the primary structure of trypsin and chymotrypsin.

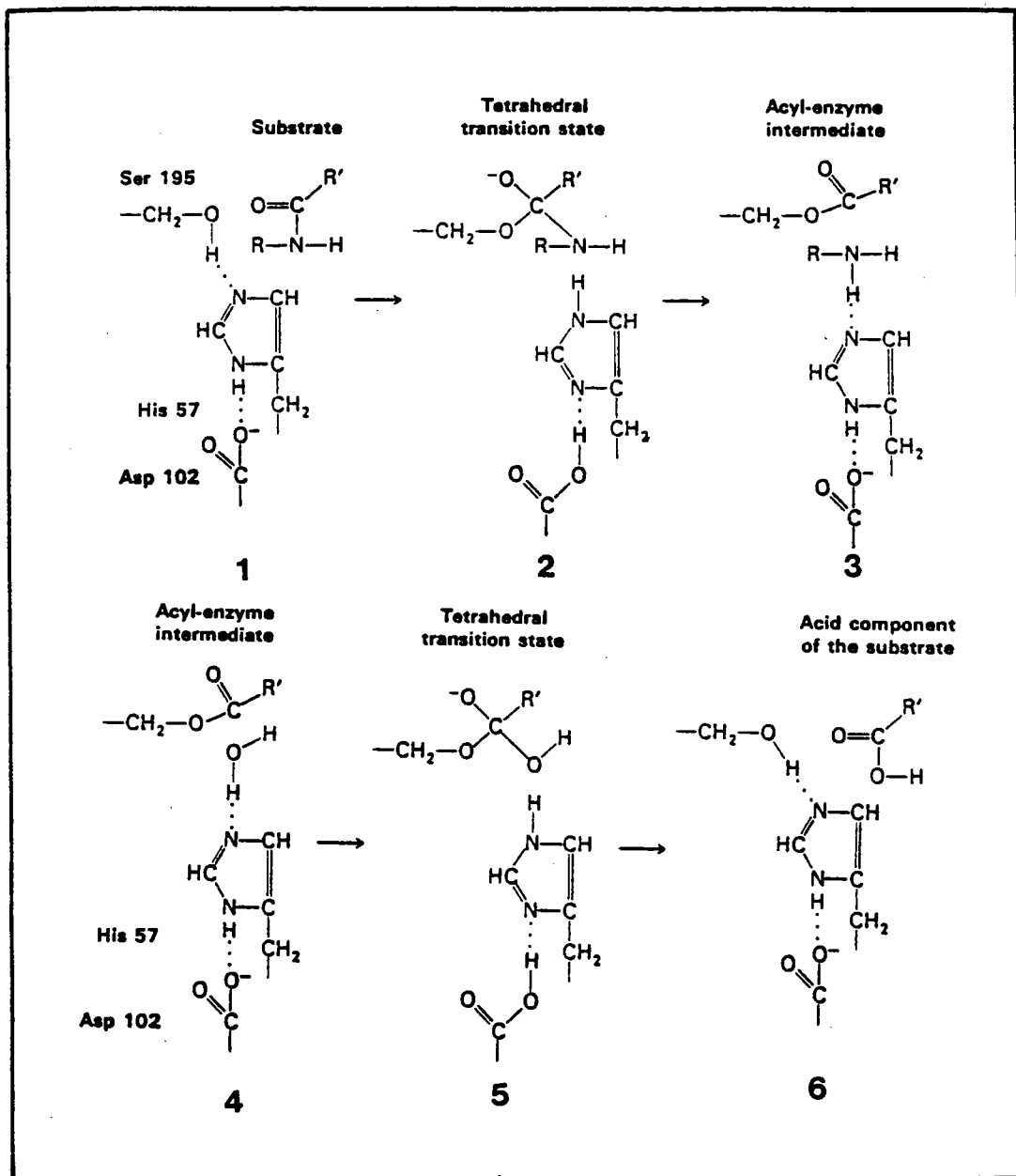


Fig. 4. The sequence for trypsin-catalyzed hydrolysis of a peptide bond.

involves transfer of a proton from Ser 195 to His 57 and the formation of a covalent bond between the carbonyl carbon of the substrate peptide and Ser 195. This is the tetrahedral intermediate (#2 in Fig. 4). The tetrahedral form collapses to form the acyl enzyme intermediate (#3) with the subsequent release of product 1. Deacylation occurs by the reverse mechanism whereby water attacks the carbonyl carbon of the acyl enzyme intermediate to form a second tetrahedral intermediate (#5) which then collapses to regenerate the enzyme and the second product, the free acid.

Studies on the BPTI-trypsin complex have demonstrated that polypeptide inhibitors bind to the catalytic and not to an allosteric site on their target protease. Evidence to support this is derived from several studies (a) selective modification of the active-site Ser 195 of trypsin by DFP or the active site His 57 by tosyl-L-lysyl chloromethylketone abrogate binding of the inhibitor (60), (b) the association rate constant of BPTI-trypsin interaction is dependent on the ionization of a group with pK_a of 7.05, presumably His 57, (55), and (c) x-ray crystallographic studies of the BPTI-trypsin complex confirm the binding of the polypeptide inhibitor to the active site of trypsin (58).

The major structural determinants of the pear-shaped BPTI molecule are two β -sheet regions, extending from Ala 16 to Gly 36, which are linked by a disulfide bond at the top of the molecule (see Fig. 5). The complex of BPTI with trypsin is mushroom-shaped with trypsin forming the "head" and BPTI forming the "stalk". In the

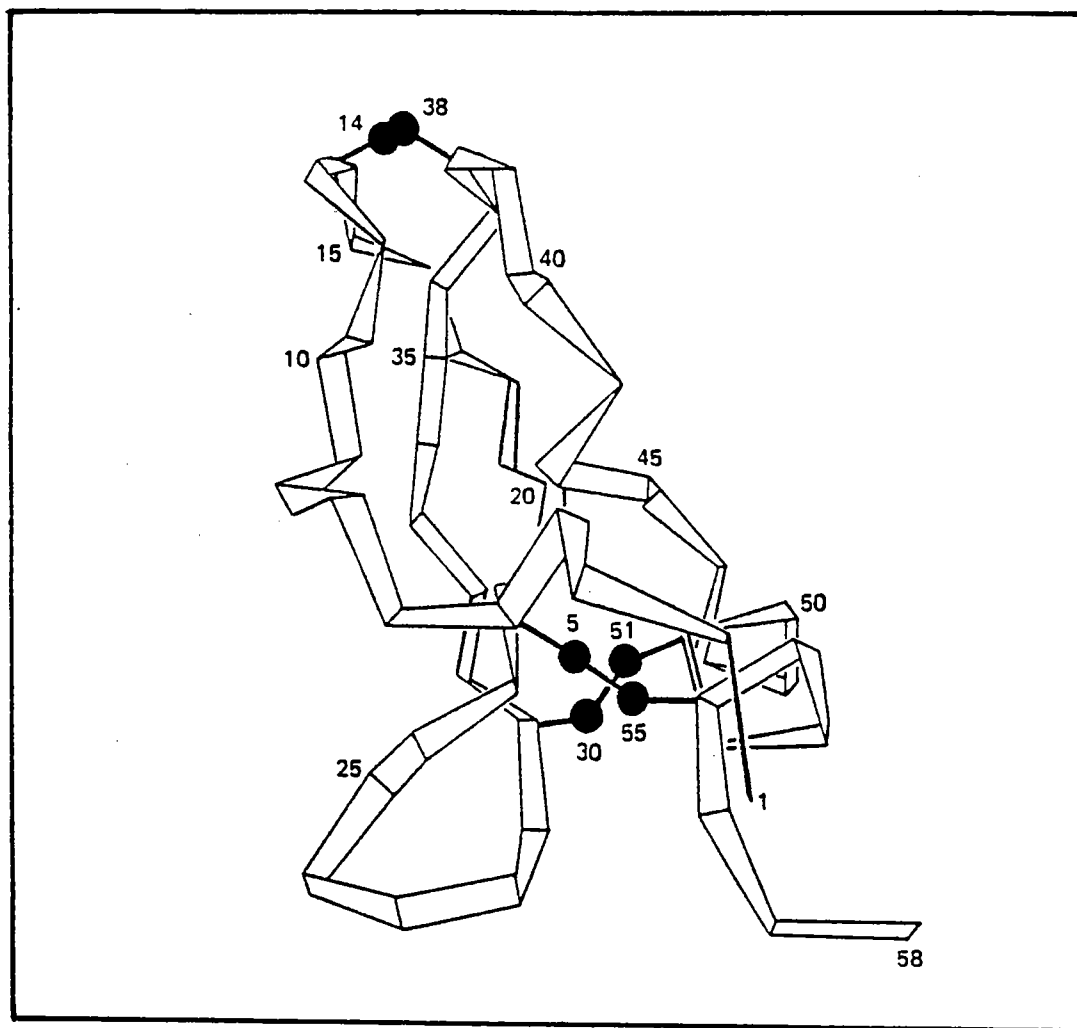


Fig. 5. Diagram illustrating the polypeptide backbone of BPTI.

representation of Fig. 5, the trypsin "head" is positioned on top of the depicted BPTI "stalk", covering, among other amino acids, the disulfide bond between cysteines 14 and 38. The complex formation is accompanied by very little structural change in the three dimensional structure of either trypsin or the inhibitor. The BPTI-trypsin complex is tightly packed (57), and exclusion of water molecules at the contact surfaces of trypsin and the inhibitor greatly enhances the stability of the complex.

Two segments of the BPTI polypeptide chain are intimately associated with trypsin (61). The first segment extends from Gly 12 to Ile 18 and the second segment extends from Tyr 35 to Arg 39. Table 3 depicts the most important amino acids in contact between trypsin and BPTI, and the type of bonding between the interacting amino acids. There are 10 hydrogen bonds formed between BPTI and trypsin. In addition, more than 200 van der Waals interactions are established.

The interactions of the major contact residues between BPTI and trypsin are illustrated in Fig. 6. The contact residues of the BPTI molecule fit precisely within the catalytic site of trypsin. The specificity pocket of trypsin, which is formed by residues 189-194 and 214-219 accommodates Lys 15 of BPTI. The ϵ -amino group of Lys 15 of BPTI hydrogen bonds with the β -carboxyl group of Asp 189 of trypsin. The polypeptide sequence Pro 13-Cys 14-Lys 15 of BPTI forms a short β -sheet-like structure with the segment Ser 214-Trp 215-Gly 216 of trypsin. The residues Ala 16-Arg 17-Ile 18 of BPTI are close to Tyr 39-His 40-Phe 41 of trypsin. The α -amino group of Arg 17 forms a hydrogen bond with the carbonyl oxygen of Phe 41, and the guanidinium

Table 3.

List of some of the residues involved in
trypsin-BPTI interaction

		BPTI													
		GLY	PRO	CYS	LYS	ALA	ARG	ILE	VAL	TYR	GLY	GLY	CYS	ARG	
		12	13	14	15	16	17	18	34	35	36	37	38	39	
TRYPSIN	TYR 39							V							
	HIS 40						H								
	PHE 41					V	H,V								
	CYS 42					V									
	HIS 57			V	V	V					V	V	V		
	CYS 58					V									
	TYR 59														
	LYS 60							V							
	SER 96														
	ASN 97													H,V	
	THR 98													V	
	LEU 99		V											V	V
	TYR 151							V		V					
	GLN 175														H
	ASP 189					S									
	SER 190					H,V									
	CYS 191					V									
	GLN 192	H	V	V	V										
	GLY 193					H,V	V	V							
	ASP 194														
	SER 195			V	H,V	V									
	SER 214					H									
TRP 215		V	V	V											
GLY 216		H,V													

H-Hydrogen bond; S-Salt bridge; V-van der Waals contacts

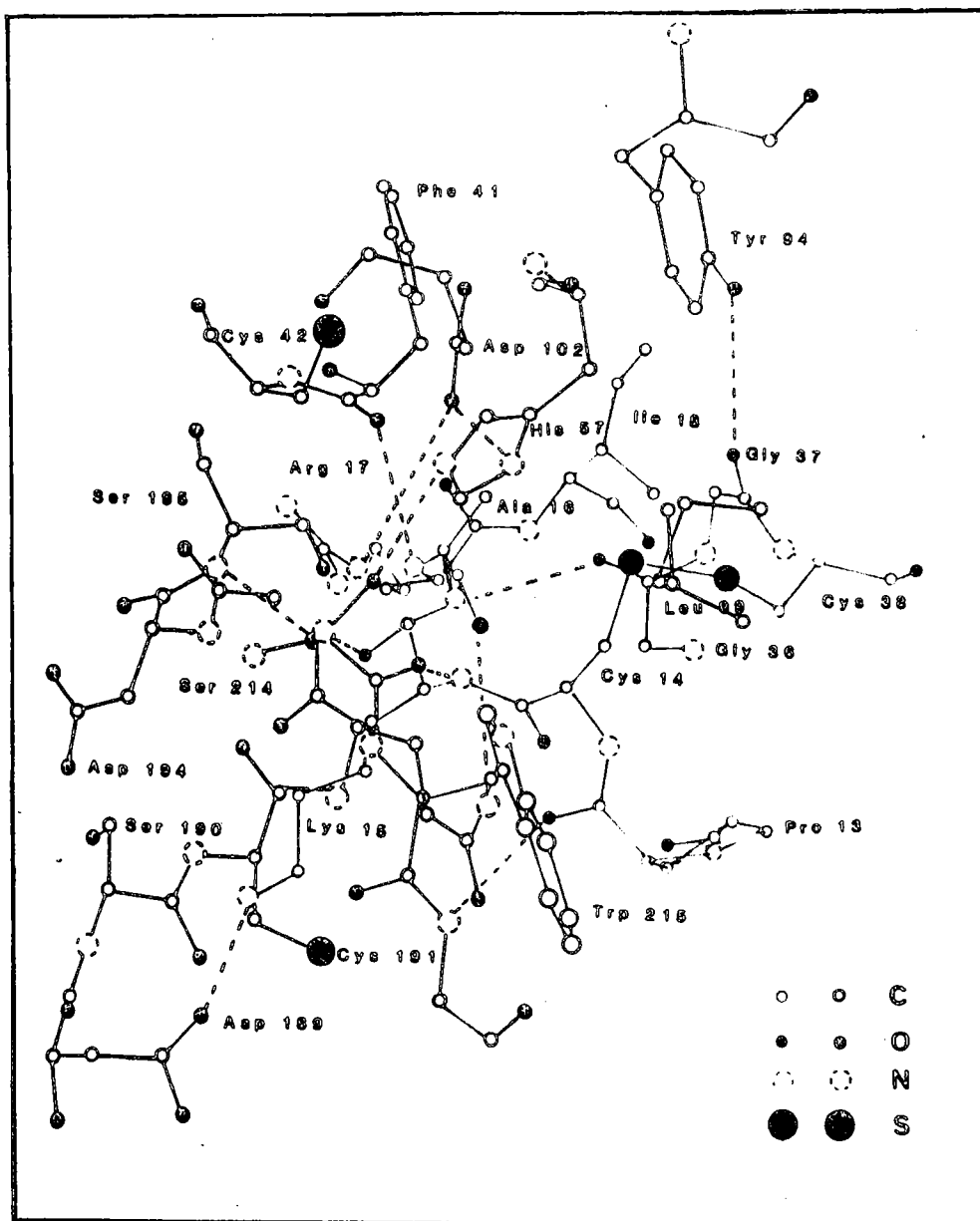


Fig. 6. Schematic drawing illustrating the major contact residues between BPTI and the active site region of bovine trypsin. (BPTI amino acid residues are colored red).

group of Arg 17 forms a hydrogen bond with the carbonyl oxygen of His 40. The polypeptide segment of BPTI from Gly 36 to Cys 38 lies close to His 57 of trypsin and establishes several van der Waals contacts with the latter residue. This portion (i.e. Gly 36 to Cys 38) of the inhibitor restricts the flexibility of the imidazole ring of His 57 of trypsin which must occur during the course of trypsin catalysis.

In the BPTI-trypsin complex, the peptide bond of BPTI between Lys 15 and Ala 16, which corresponds to the scissile peptide bond of a protein substrate, is oriented such that the γ -oxygen of Ser 195 of trypsin lies almost perpendicular above the plane of the Lys 15 carbonyl carbon. This arrangement favors a nucleophilic attack by Ser 195 along the tetrahedral bonding direction of the Lys 15 carbonyl carbon. However, in contrast to substrate catalysis (cf. trypsin catalytic mechanism on page 20), a tetrahedral intermediate does not form. Instead, the Lys 15 carbonyl carbon is distorted midway between trigonal and tetrahedral conformations. The stable complex formed between BPTI and trypsin represents an intermediate stage of catalysis and is "frozen" by the constraints imposed by the enzyme and the inhibitor.

The focus of the present study was the purification and characterization of two relatively heat stable trypsin inhibitors, trypsin inhibitors A and B, which occur in the hemolymph of the tobacco hornworm Manduca sexta. Both trypsin inhibitors A and B have striking amino acid sequence homology to BPTI and also exhibit similar physicochemical characteristics. Hence, it was reasonable to expect that the interaction of these inhibitors with trypsin would be similar to that of the well-characterized BPTI-bovine trypsin complex. Therefore, the

structure and properties of trypsin inhibitors A and B of Manduca sexta were compared with BPTI in order to identify and define the possible structure-function parameters of the Manduca sexta inhibitors.

CHAPTER II

EXPERIMENTAL PROCEDURES

A. Materials

Tritiated iodoacetic acid (specific activity 50-100 mCi/mmole) was obtained from Amersham (Arlington Heights, IL) and diluted with recrystallized sodium iodoacetate to 2 mCi/mmole. Aldrich Chemical Company (Milwaukee, WI) supplied triethylamine, L-Dopa and glutaraldehyde. Electrophoretic grade acrylamide, N, N'-methylenebis acrylamide, sodium dodecyl sulfate, Biolyte (ampholyte 3-10) and coomassie blue dye reagent concentrate for protein estimation, by the method of Bradford were products of Bio-Rad Laboratories (Richmond, CA). Dithio-threitol, p-nitrophenyl p'-guanidinobenzoate, Azocoll, and thermolysin were from Calbiochem (La Jolla, CA). Electran (brand name of low molecular weight standards) was obtained from Gallard-Schlesinger (New York, NY). Millipore Corporation supplied the 0.22 m MF type filters. Subtilisin BPN' was purchased from Nagase Chemical Company, Osaka, Japan. Sephadex G-75 (superfine), Sepharose 4B, Sephadex G-100 (superfine) and blue dextran were obtained from Pharmacia Chemical Company (Piscataway, NJ). The silver stain kit used for protein staining polyacrylamide gels was manufactured by Polysciences (Warrington, PA). Sigma Chemical Company (St. Louis, MO) supplied the following: N-benzoyl-DL-arginine p-nitroanilide (BAPNA), glutaryl-L-phenylalanyl-p-nitroanilide (GPNA), 4-methylumbelliferyl p-trimethylammonium cinnamate (MUTMAC), sodium iodoacetate, guanidine hydrochloride, laminarin, tris, trypsin, ribonuclease A, chymotrypsinogen, pepsin, horse heart myoglobin, Aprotinin,

papain, hemoglobin, plasmin, casein, thrombin, and fibrinogen. Dialysis tubing (Spectraphor M.W. cut off 3,500) was supplied by A.H. Thomas and Company (Philadelphia, PA). Chymotrypsin, L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (trypsin-TPCK), and porcine elastase were products of Worthington (Freehold, NJ). All other chemicals were of reagent grade.

B. Methods

B.1. Assays and techniques

(a) Assay for trypsin inhibitory activity

Inhibitory activity toward trypsin was measured and quantified using BAPNA as the trypsin substrate. The assay mixture consisted of 0.1 M tris-HCl buffer pH 8.3, 1 mM BAPNA, 2 μ g of bovine trypsin, 20 mM CaCl₂ and the inhibitor in a total reaction volume of 1.0 ml. Typically the mixture was incubated at 35° C for 30 minutes, the reaction terminated by the addition of 0.5 ml of 30% v/v acetic acid, and the liberated p-nitroaniline was measured at 410 nm. Under the conditions of the assay the amount of p-nitroaniline liberated was linear for at least 45 minutes. The percentage inhibition (%I) was calculated using the formula

$$\%I = \left(1 - \frac{A_I}{A_0}\right) \times 100$$

where A_I and A_0 represent the absorbance in the presence and the absence of the inhibitor respectively. The concentration of the inhibitor measured in these assays was chosen to give values that were between 0.5 and 0.7 A_0 . Under the given conditions one unit of inhibitor activity was defined as the amount of inhibitor that caused a 50% inhibition of the hydrolysis of BAPNA by 2 μ g (ca. 50 pmoles) of trypsin.

(b) Protein determination

During purification of the trypsin inhibitors A and B, protein was estimated by the coomassie blue dye binding method of Bradford (62) using bovine serum albumin as the standard. However, this method could not be employed with the purified inhibitors because they were found to bind the dye weakly. Therefore, quantitation of the purified inhibitors was performed on a amino acid analyzer.

(c) SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate slab gel electrophoresis was performed according to the method of Laemmli (63) except that the resolving gel consisted of a gradient between 10-20% acrylamide and the stacking gel was 1% acrylamide.

Samples were prepared for electrophoresis by boiling in 0.15 M tris-HCl buffer pH 6.8, containing 5.7% SDS, 30% glycerol, and 14% β -mercaptoethanol. Bromophenol blue (0.005%) was added as the tracking dye. The gels were electrophoresed at 50 V for 15 hours using cold tap water to cool the electrophoresis unit. Cyanogen bromide cleaved fragments of horse heart myoglobin (Electran brand M.W. standards) were used as molecular weight markers. The gels were stained in the following manner: Gels were fixed in 50% methanol, 12% acetic acid for 90 minutes, followed by 3 x 20 minute washes in a mixture of 10% ethanol and 5% acetic acid and 2 x 10 minute washes with deionized water. In order to enhance the silver reaction with the inhibitors, the gels were pretreated with 10% glutaraldehyde for 90 minutes followed by 6-8 rinses with deionized water and then stained in 1% ammoniacal silver

nitrate solution for 20 minutes. The gels were washed with deionized water until the washings were negative for silver ions and the protein bands were developed by immersing the gel in a 0.02% citric acid solution containing 0.18% formaldehyde.

(d) Determination of isoelectric point by isoelectrofocusing

The isoelectric point of each inhibitor was determined using 10% polyacrylamide gels (10 cm x 8 cm x 1.5 mm) in the presence of pH 3-10 polyelectrolytes. Approximately 15 μ g of each inhibitor was electro-focused at 200 V and 7^o C overnight, the gels were sliced into 6 mm horizontal sections and the pH of each slice was measured using a surface electrode. Each slice was extracted with 20 mM tris-HCl buffer containing 50 mM NaCl pH 7.5 and the inhibitory activity towards trypsin was measured.

(e) Assessment of complement Factor B cleavage by immunoelectrophoresis

One percent agarose slabs were cast on clean dry 35 mm x 75 mm microscope slides. Two sample wells were punched on each agarose slab and each well was filled with one of the experimental mixtures (obtained by incubating complement factor B or fresh frozen human serum with different reagents as described on page 51) and electrophoresed at 7.5 mA per slab for about 5 hours in 0.1 M veronal buffer, pH 8.6 containing 5 mM EDTA. Following electrophoresis, a central trough was excised between the two wells and filled with 200 μ l of rabbit antifactor B antiserum. Precipitin bands were allowed to develop overnight at room temperature in a humidity chamber.

(f) Amino acid analysis of trypsin inhibitors A and B

Samples of trypsin inhibitors A and B were dialysed against water, separately dispensed into clean pyrex tubes and lyophilized. Two hundred microliters of 5.75 N HCl was added, and the samples were hydrolyzed in vacuo at 110° C for 24 hours. The hydrolysates were dried in vacuo over phosphorous pentoxide and they were analyzed for amino acid content by the method of Jones et al. (64). Cysteine was quantitated as cysteic acid after performic acid oxidation (65).

(g) Reduction and S-carboxymethylation of trypsin inhibitors A and B

One milligram samples of inhibitor A or B were dissolved in 1 ml 0.5 M tris 7.5 M, guanidinium chloride, pH 8.0, containing 0.01 M DTT, and allowed to reduce under nitrogen for 1 hour at room temperature. Two hundred and fifty microliters of 2- [³H] iodoacetic acid solution (250 µCi, specific activity 2.0 mCi/mmoles) was added, the samples were flushed with nitrogen and S-carboxymethylation performed at room temperature for 30 minutes. The [³H]-S-carboxymethylated inhibitors were dialysed against water using 3,500 M.W. dialysis tubing until less than 50 cpm of radioactivity were detected per ml of water and then were lyophilized.

For peptide mapping experiments, samples were reduced and S-carboxymethylated using the same protocol except that nonradioactive iodoacetic acid was used.

(h) Peptide mapping of trypsin inhibitors A and B

Approximately 450 µg of each reduced and S-carboxymethylated trypsin inhibitor was dissolved in 100 µl of 0.1 M ammonium

bicarbonate, pH 7.8, and digested for 18 hours with 1.5% (w/w) trypsin-TPCK at 37° C. At the end of the digestion, 10 µl of glacial acetic acid was added to arrest the reaction, and the samples were lyophilized. The peptide mixture was redissolved in 100 µl of 0.1% trifluoroacetic acid and injected onto a 4.6 mm x 75 mm Altex-Ultrapore RPSC C₃ column equilibrated with 0.1% trifluoroacetic acid. Peptides were separated using a gradient between 0-75% acetonitrile in 0.1% trifluoroacetic acid and the peptide fractions were collected manually by monitoring the absorbancy at 206 nm.

(i) Determination of the aminoterminal amino acid sequences of trypsin inhibitors A and B

Amino terminal amino acid sequences were obtained on 10-16 nmole of reduced and [³H]-S-carboxymethylated inhibitors using a modified Beckman 890C sequenator adapted for microsequencing (66). The phenylthiohydantoin derivatives of amino acids (PTH amino acids) were identified and quantitated using two independent reverse phase systems. The primary system utilized a 6 µm Zorbax C₁₈ HPLC column and a 12 minute 25-52% acetonitrile gradient in sodium acetate buffer pH 4.8. These identifications were confirmed using a 5 µm Ultrasphere C₁₈ column and a linear methanol gradient (20-45%) was employed. All data were quantitated with a Hewlett Packard 3390A integrator using PTH norleucine as an internal standard. S-carboxymethyl cysteines were confirmed by counting an appropriate aliquot of each cycle for radioactivity.

(j) Secondary structure predictions for trypsin inhibitors A and B using Chou and Fasman calculations

For the calculation of secondary structure, the rules of Chou and Fasman were followed (67). In this method, numerical "conformational parameters" are computed to give the probability of a particular amino acid residue being found in α -helix (P_{α}) or β -pleated sheet structure (P_{β}). Depending on the numerical value of P_{α} and P_{β} the amino acids are classified into "strong formers" (either α -helix or β -pleated sheet), "formers", "indifferent", "breakers" and "strong breakers". When four helix formers out of six residues are clustered, there is a greater probability of an α -helix structure. Propagation can then occur in both directions until it is terminated by a tetrapeptide of helix breakers. Likewise, if three out of five β -pleated sheet formers are clustered, nucleation of a β -pleated sheet may occur. As in the case of α -helical structure, the propagation of β -pleated sheet may occur in both directions until it is terminated by a tetra peptide sequence of β -pleated sheet breakers.

(k) Preparation and titration of reagents for complement factor B and factor D assays

Sixty milliliters of human venous blood was drawn and allowed to coagulate in Corex centrifuge tubes. After allowing the clot to retract at 4° C for two hours the blood was centrifuged and 5 ml portion of the clear serum was divided into 0.5 ml aliquots and frozen immediately at -70° C.

In order to titrate the alternative pathway components in fresh frozen serum, 10 μ l of freshly washed rabbit erythrocytes (3.5×10^9

cells/ml) in GVBS was incubated at 37° C for 1 hour with 25 μ l of fresh frozen serum serially diluted two fold with GVBS in a total reaction volume of 150 μ l. Ten microliters of rabbit erythrocyte (3.5×10^9 cells/ml) diluted into 140 μ l of deionized water served as the total hemolysis control. One milliliter of ice-cold GVBE was added to each incubation mixture to terminate the reaction, the cells were centrifuged at 1,200 x g and the absorbance of the supernatant was measured at 414 nm.

Factor B depleted serum (RB serum) was prepared by inactivation of fresh serum (23 ml) at 52° C for 30 minutes. Twenty five microliters of RB serum was reconstituted with 4 μ g of purified factor B and titered as described for fresh frozen serum. RB serum was then divided into 0.5 ml aliquots and frozen at -70° C.

Factor D depleted serum (RD serum) was prepared by depleting factor D on a Bio-Rex 70 ion exchange resin (68). Titration of RD serum was performed in an analogous manner to the titration of RB serum except that functionally pure factor D (Bio-Rex 70 eluate) was used to reconstitute the RD serum. For assays of factors B and D in Manduca sexta hemolymph, a 1:3 dilution of RB and undiluted RD sera were used.

B.2. Measurement of trypsin inhibitor activity in the hemolymph of Manduca sexta during larval-pupal metamorphosis

A batch of synchronously developing larvae were divided into three groups of 10 animals each and bled individually into polypropylene tubes at the fifth instar of larval development (27 days), during the early pupal stage (31 days) and during the late pupal stage (38 days). Five to twenty microliters of the crude hemolymph was used to measure trypsin

inhibitory activity, which was expressed as units of inhibitor activity/ml hemolymph.

B.3. Isolation and characterization of trypsin inhibitors from the hemolymph of Manduca sexta

(a) Collection of Manduca sexta hemolymph

The tobacco hornworm, Manduca sexta, was grown in our laboratory on a wheat-germ-agar diet as described by Yamamoto (69). The eggs were obtained from the Metabolism and Radiation Research Laboratory (Fargo, ND) or the Tobacco Research Institute (Oxford, NC). The growth temperature was maintained at $26^{\circ} \pm 1^{\circ}$ C and the larvae were kept on a photo period of 12 hours. Under these conditions the larvae grew to their maximum size (10-12 g) in about 25 days. Hemolymph was collected from the larvae during the fifth instar before they entered the wandering stage (before their dorsal blood vessel became prominent). The hemolymph to be used for inhibitor purification was obtained by cutting one of the hind legs and gently squeezing the hemolymph into an equal volume of ice-cold bleeding buffer (20 mM tris-HCl, containing 1 mM phenylthiourea and 0.02% sodium azide, pH 7.5). Between 1 and 1.2 ml of hemolymph was obtained from each animal. Hemolymph from approximately one hundred larvae were pooled, centrifuged at 4° C for 30 minutes at 15,000 rpm (SS 34 rotor) in a Sorvall RC-2B centrifuge and the clear blue-green supernatant was frozen immediately at -20° C until further use.

For studies on inhibitory properties of purified Manduca sexta hemolymph trypsin inhibitors on the activation of hemocyte prophenoloxidase and on the human alternative complement pathway, the larvae were bled into an equal volume of 10 mM cacodylate buffer, pH 7.0 containing

0.25 M sucrose and 0.1 M sodium citrate (buffer P) in order to prevent Ca^{++} -dependent preactivation of proteases secreted by hemocytes into the hemolymph. The hemolymph (40 ml) was centrifuged at 1,000 x g, the supernatant was divided into 0.5 ml aliquots and immediately frozen at -70°C . The hemocyte pellet (approximately 3×10^8 cells) was washed twice and resuspended in 3.0 ml of ice-cold buffer P lacking citrate and used in experiments to evaluate the effect of purified trypsin inhibitors A and B on the activation of hemocyte prophenoloxidase.

(b) Preparation of immobilized trypsin

Sepharose 4B was washed with deionized water and sucked "dry" on a Buchner funnel. Two hundred and twenty-five grams of Sepharose was resuspended in 225 ml of 60% acetone, chilled to -10°C and 7 g of cyanogen bromide dissolved in 10 ml of acetonitrile was added. Seventy milliliters of 1.5 M triethylamine in 60% acetone was added dropwise in order to enhance the electrophilicity of the cyanogen bromide, and thus promote the rapid activation of Sepharose (70). The mixture was stirred for 10 minutes and poured into an ice-cold mixture of 0.1 N HCl-acetone (1:1). The activated Sepharose was washed with one liter of cold 60% acetone, one liter of coupling buffer (0.1 M sodium bicarbonate containing 0.5 M NaCl, pH 8.3), resuspended in 225 ml of coupling buffer and 1.3 g of trypsin in 50 ml of coupling buffer was added. Coupling was carried out with agitation at room temperature for 3 hours and the gel was transferred to 0.2 M glycine, pH 8.0 and shaken overnight (blocking reaction). Approximately 75% of the trypsin was bound to activated Sepharose. The substituted gel was washed with 4 liters of ice cold coupling buffer, 4 liters of cold 0.1 M sodium acetate buffer containing

0.5 M NaCl, pH 4.5, and finally with two liters of 20 mM tris containing 0.5 M NaCl, pH 7.0. The trypsin-Sepharose gel was stored at 4° C in 20 mM tris-HCl buffer pH 7.0 with 0.02% sodium azide as a preservative.

(c) Protocol for the purification of trypsin inhibitors A and B from the hemolymph of Manduca sexta

During the purification of the hemolymph trypsin inhibitors all operations were performed at 0-4° C unless otherwise indicated. Crude hemolymph (315 ml) was thawed in an ice-bath and 35 ml of ice-cold 7% perchloric acid was added dropwise (final pH ca 1.5) under stirring to precipitate the acid-insoluble proteins. The milky mixture was stirred for 15 minutes and centrifuged at 27,000 x g for 30 minutes. The clear golden-yellow supernatant was passed through a pad of glass wool to remove small amounts of floating lipids and the pH readjusted to 6.0 ± 0.2 with 30% KOH. A slightly acidic pH was found to be essential at this stage for preventing the oxidation of endogenous hemolymph phenols. Two hundred and twenty-two grams of solid ammonium sulfate was added slowly to 335 ml of acid precipitated hemolymph supernatant to bring the final concentration to 90% saturation. The solution was stirred on an ice bath for 1 hour and the precipitated proteins were collected by centrifugation at 27,000 x g for 30 minutes.

The precipitate was redissolved in 20 ml of 20 mM tris-HCl buffer containing 50 mM NaCl, pH 7.5 (buffer A) and dialyzed against 4 liters of buffer A overnight. The dialysate was clarified by centrifuging at 27,000 x g for 30 minutes and loaded onto a 2 x 6.5 cm trypsin-Sepharose column in buffer A at a flow rate of 15-20 ml/hour. The column was washed successively at a flow rate of 50 ml/hour with 100 ml of buffer

A, 1 liter of 20 mM tris-HCl pH 7.5 containing 1 M NaCl (buffer B) and 300 ml of 1 mM HCl pH 3.2 \pm 0.2 (buffer D). Trypsin inhibitor activity was eluted at a flow rate of 15 ml/hour with 0.1 M HCl containing 0.75 M NaCl (pH 1.5). The eluted fractions (2 ml) were immediately neutralized by collection into 0.5 ml of 1 M tris-HCl pH 8.3 and assayed for trypsin inhibitory activity. The active fractions were pooled (40 ml) dialyzed against 4 liters of deionized water and lyophilized.

Sephadex G-75 (superfine) prepared according to the manufacturer's recommendations was packed to a bed height of 95 cm in a 1.6 cm x 100 cm Pharmacia column and equilibrated with 20 mM tris-HCl buffer pH 7.5 containing 0.5 M NaCl (buffer C). The lyophilisate from trypsin-Sepharose chromatography was redissolved in 1.5 ml of buffer C, centrifuged to remove the small amount of insoluble material and fractionated over the G-75 column at a flow rate of 11 ml/hr. The absorbance profile was monitored at 280 nm using an ISCO u.v. monitor and 5.9 ml fractions were collected. During the gel filtration, the trypsin inhibitor activity was resolved into two components which were pooled separately, dialyzed against deionized water and lyophilized. In some cases it was necessary to remove small amount of contaminants by rechromatography over the same G-75 column.

The inhibitor B preparation obtained from G-75 column was resolved by analytical isoelectrofocusing into two components with isoelectric pH of 5.3 and 7.1. However, further attempts to separate the two components by ion-exchange chromatography on DEAE-cellulose (at pH 6.4 in 10 mM phosphate buffer), and DEAE-Sephadex A 25 (at pH 6.2 in 20 mM maleate buffer) were not successful.

(d) Determination of molecular weight by gel filtration

The molecular weight of each inhibitor was estimated from their elution positions on the G-75 column after calibration with pepsin (M.W. 35,000) chymotrypsinogen (M.W. 25,000), myoglobin (M.W. 17,000), ribonuclease A (M.W. 13,700) and Aprotinin (M.W. 6,500). The void volume (V_0) was determined empirically with blue dextran. The molecular weight of each inhibitor was calculated from a plot of the log of the molecular weights of the standard proteins against their ratio of the elution volume (V_e) to the void volume.

(e) Determination of the heat stability of trypsin inhibitors A and B

A series of tightly sealed 1.5 ml eppendorf tubes each containing 27.6 pmoles of trypsin inhibitor A or 29 pmoles of trypsin inhibitor B in 100 μ l of 20 mM tris-HCl buffer pH 7.0 were treated in a boiling water bath for 5, 10, 20, and 30 minutes. The reaction tubes were rapidly cooled on ice and centrifuged in a microcentrifuge to recover any moisture that had condensed on the walls during the incubation. Twenty-five microliters of each supernatant was assayed for trypsin inhibitory activity.

(f) The effect of the reducing agent dithiothreitol on the activities of trypsin inhibitors A and B

To evaluate the effect of DTT on inhibitor activity, trypsin inhibitor A (87.6, 175.2 or 262.8 pmoles) or trypsin inhibitor B (103, 206 or 309 pmoles) were diluted into 200 μ l of 20 mM tris-HCl buffer, pH 7.5 containing 2.5 mM DTT. The tubes were flushed with nitrogen,

allowed to incubate for 60 minutes at room temperature, and 25 μ l aliquots were assayed for trypsin inhibitor activity.

(g) Determination of the inhibitory activity of inhibitors A and B towards various proteases

Representative examples of proteases from the aspartate, metallo, serine and thiol protease families were used to assess the broad range inhibitory property of trypsin inhibitors A and B. The assay conditions selected for each protease is given in Table 4. Each protease except thrombin and plasmin was preincubated for 10 minutes at 35° C with either trypsin inhibitor A (18.7 to 75.9 pmoles) or inhibitor B (20.7 to 82.9 pmoles) in 500 μ l of buffer. Five hundred microliters of the appropriate substrate was added and the incubation continued at 35° C for the desired time. Reactions were terminated, the mixtures centrifuged and the increase in absorbance of each supernatant was measured at 280 nm (hemoglobin) or 520 nm (Azocoll).

Plasmin was assayed as follows: porcine plasmin (approximately 0.38 CTA units, 23 pmoles) was preincubated with trypsin inhibitor A for 10 minutes in 500 μ l of 0.06 M sodium phosphate buffer, pH 7.4, containing 0.05 M lysine, 500 μ l of 1% casein solution in 0.06 M sodium phosphate buffer, pH 7.4, was added and incubation continued for an additional 30 minutes. The reaction was terminated and the absorbance of the supernatant was read at 275 nm following centrifugation.

Thrombin was assayed as follows: thrombin (0.2 NIH units, approx. 2.2 pmoles) was preincubated at room temperature with either inhibitor A (68 pmoles or 171 pmoles) or inhibitor B (86.5 pmoles or 173 pmoles) in a total volume of 20 μ l of 0.017 M imidazole buffer, pH 7.25. Three

Table 4

Conditions employed for the assay of proteases used in the specificity studies of trypsin inhibitors A and B.

Protease	Amount of the protease used (pmoles)	Substrate used	Buffer	Incubation time (minutes)	Reaction terminated by
Chymotrypsin	56	Hemoglobin	i+20 mM CaCl ₂	30	1.0 ml of 10% TCA
Elastase	77	Azocoll	ii	10	1.0 ml of 30% acetic acid
Papain	86	Hemoglobin	i+0.02% EDTA	30	1.0 ml of 10% TCA
Pepsin	57	Hemoglobin	0.018 M HCl	5	1.0 ml of 10% TCA
Subtilisin BPN'	73	Hemoglobin	iii	30	1.0 ml of 10% TCA
Thermolysin	58	Hemoglobin	iii	30	1.0 ml of 10% TCA
Trypsin	52	Hemoglobin	ii+20 mM CaCl ₂	30	1.0 ml of 10% TCA
Plasmin	23	Casein	iv	30	1.5 ml of 0.5 M HClO ₄
Thrombin	2.2	Fibrinogen	v	-	-

Buffer i 0.1 M tris, pH 7.5 Buffer iv 0.06 M phosphate containing 0.05 M lysine, pH 7.4

Buffer ii 0.05 M tris, pH 8.5

Buffer iii 0.05 M tris, pH 7.5 Buffer v 0.017 M imidazole, pH 7.25

hundred microliters of fibrinogen solution (0.4% in 0.017 M imidazole buffer containing 0.067% w/v CaCl_2) was added and the time at which the first clot appeared was recorded as the end point.

(h) The effect of subtilisin BPN' on trypsin inhibitors A and B

To determine if the native trypsin inhibitors A and B are susceptible to proteolytic inactivation, 93.8 pmoles of inhibitor A or 114.1 pmoles of inhibitor B were incubated for 20 minutes at 35° C in 50 mM tris-HCl buffer, pH 7.5 with 36.6, 72.7, 109.1 or 145.5 pmoles of subtilisin BPN'. At the end of the incubation, subtilisin was inactivated by boiling the mixture for 10 minutes. The tubes were allowed to cool in an ice-bath, centrifuged and 25 μl aliquots of the supernatant were assayed for trypsin inhibitor activity.

(i) The ultraviolet absorption spectra of trypsin inhibitors A and B

Trypsin inhibitor A or inhibitor B were dissolved in double distilled deionized water at a concentration of 2.307 mg/ml and 0.151 mg/ml respectively, filtered through a Millipore 0.22 μm type MF filter and the ultraviolet absorption spectra were recorded between 230 and 340 nm using a Gilford 250 spectrophotometer.

(j) Characterization of the complexes formed between trypsin inhibitor A and trypsin and chymotrypsin

To evaluate whether Manduca sexta hemolymph trypsin inhibitors A and B formed stable complexes with trypsin, samples of trypsin inhibitor A (11 nmoles) or inhibitor B (33.7 nmoles) were incubated at 35° C with 8.5 nmoles of trypsin in a total volume of 200 μl of 20 mM tris-HCl buffer, pH 7.5. After allowing 15 minutes for the association of the inhibitors with trypsin, the mixtures were chromatographed at 4° C

on a Sephadex G-100 column (0.9 x 23 cm) equilibrated with tris-HCl buffer, pH 7.5, containing 0.5 M NaCl. The absorbance of each fraction (1.4 ml) was measured at 280 nm and 25 μ l aliquots of each fraction was used to assay trypsin inhibitor activity using BAPNA as described. The Sephadex G-100 column used was calibrated with bovine serum albumin (M.W. 67,000), ovalbumin (M.W. 44,000), chymotrypsinogen (M.W. 25,000) and lysozyme (M.W. 14,400).

The stoichiometries for the association of trypsin and chymotrypsin with trypsin inhibitor A were determined by evaluating the extent of complex formation at various inhibitor concentrations using direct inhibition measurement and by the use of active site titrants. Stoichiometric studies were performed only on inhibitor A, since inhibitor B was found to resolve on isoelectrofocussing into two components with isoelectric pH of 5.4 and 7.1. Since it is not known whether both components of inhibitor B display equal affinity towards trypsin, stoichiometric studies were not performed on the inhibitor B preparation.

Trypsin (50.9 pmoles) was incubated with inhibitor A (0 to 44.2 pmoles) at 35° C for 10 minutes in 500 μ l of tris-HCl buffer (0.2 M, pH 8.3) containing 40 mM CaCl₂. Residual trypsin activity was determined by adding 500 μ l of an aqueous solution of BAPNA (final concentration 1 mM) and continuing the incubation for an additional 30 minutes. The reaction was terminated by addition of 500 μ l of 30% v/v acetic acid, and the absorbance at 410 nm was recorded. In a similar manner, 520 pmoles of chymotrypsin was preincubated with varying amounts of inhibitor A (0 to 503 pmoles) for 10 minutes at 35° C in a reaction volume of 500 μ l of tris-HCl buffer, pH 7.8, containing 20 mM CaCl₂. The

residual chymotrypsin activity was measured using glutaryl-L-phenylalanyl p-nitroanilide, GPNA (final concentration 1 mM), and the liberated p-nitroaniline was quantitated as described for trypsin.

The stoichiometry was evaluated by plotting the fractional activity, v_i/v_0 (the ratio of activity in the presence of inhibitor to the activity in the absence of inhibitor) against the molar ratio of inhibitor A to trypsin or chymotrypsin (see Results, page 71).

Active sites titrations were performed as follows: varying amounts of trypsin inhibitor A (3.13 to 15.7 nmoles) were incubated at room temperature for 5 minutes with 22 nmoles of trypsin in a total volume of 1.0 ml of 0.07 M veronal buffer, pH 8.3, containing 15 mM CaCl_2 . Ten microliters of a solution of p-nitrophenyl p'-guanidinobenzoate (NPGb, 0.01 M in dimethylformamide) was added, and the initial "burst" of p-nitrophenol released due to rapid formation of the acyl-enzyme intermediate was measured using the molar extinction coefficient for p-nitrophenol of 16,595 at 410 nm and pH 8.3 (71). Similarly, 0.286 to 1.43 nmoles of trypsin inhibitor A was permitted to complex at room temperature with 1.49 nmoles of chymotrypsin in a total volume of 1.9 ml of tris-HCl buffer, 0.05 M, pH 7.8. One hundred microliters of 0.2 mM 4-methylumbelliferyl p-N, N, N, trimethylammoniumcinnamate (MUTMAC, final concentration 10 μM) was added, and the immediate burst of fluorescence at 450 nm was recorded with excitation set at 360 nm. The amount of 4-methylumbelliferone liberated was calculated from a standard curve prepared using recrystallized 4-methylumbelliferone. Plots of the concentration of the active (i.e. uninhibited) protease against the ratio of trypsin inhibitor A to the total protease were used to calculate the stoichiometry of the reactions (see Results, page 74).

(k) Estimation of inhibition constants for the inhibition of trypsin and chymotrypsin by trypsin inhibitor A

The inhibition constants (K_i) for inhibition of trypsin and chymotrypsin by trypsin inhibitor A were evaluated by the method of Meyers-Cha (72). This method requires the initial calculation of I_{50} , the concentration of inhibitor required to cause 50% inhibition of enzyme activity, at various enzyme concentrations. In order to determine I_{50} values for trypsin inhibition, trypsin (50.9, 101.8, or 151.7 pmoles) was preincubated with 0 to 98.1 p moles of trypsin inhibitor A and residual trypsin activity was assessed using BAPNA as the substrate as described previously (page 30). The fractional activity, i.e., the ratio of the activity in the presence of inhibitor (v_i) to the activity in the absence of inhibitor (v_0), was plotted against the inhibitor concentration, and the inhibitor concentration required to produce 50% inhibition (I_{50}) was evaluated. From the I_{50} values, the inhibition constant (K_i) was obtained as described under Results (page 77).

In a similar set of experiments, chymotrypsin (0.393, 0.786, 1.179, and 1.572 nmoles) was preincubated with 0 to 1.005 nmoles of trypsin inhibitor A, and the residual chymotrypsin activity was measured using GPNA as the substrate as described above (page 45). The results were analyzed in the same way as described for trypsin (see Results, page 79).

(l) Do trypsin and chymotrypsin bind to the same site on inhibitor A?

Because trypsin inhibitor A was found to inhibit both trypsin and chymotrypsin, it was of interest to determine whether inhibitor A complexed with one protease would still inhibit the other protease.

Accordingly, two sets of reaction mixtures containing 0.57 nmoles of trypsin were preincubated for 10 minutes at 35° C with 0.37 nmoles of trypsin inhibitor A in 0.05 M tris-HCl buffer, pH 7.8. Trypsin was added in excess to ensure that inhibitor A was in complex with trypsin. After preincubation, varying amounts of chymotrypsin (0.43 to 1.7 nmoles) were added and the incubation continued for 10 minutes. One set of reaction mixtures were assayed for trypsin activity using BAPNA as the substrate and the second set of reaction mixtures were assayed for chymotrypsin activity using GPNA as the substrate. In an alternate set of experiments, 1.05 nmoles of chymotrypsin was preincubated with 0.47 nmoles of trypsin inhibitor A using the same conditions described for trypsin. At the end of the incubation varying amounts of trypsin (0.51 to 2.03 nmoles) were added and the incubation continued for 10 minutes. One set of reaction mixtures was assayed for chymotrypsin activity using GPNA as the substrate and the other set was used for trypsin assay using BAPNA as the substrate.

B.4. Studies on the activation of prophenoloxidase in the hemolymph of Manduca sexta

- (a) The effect of the serine protease inhibitor, diisopropylphosphofluoridate, on the appearance of phenoloxidase activity in hemolymph of Manduca sexta

To ascertain whether activation of the prophenoloxidase of Manduca sexta hemolymph was due to activity of a serine protease in a manner analogous to Bombyx mori, the effect of DFP on the activation of prophenoloxidase was studied. Five hundred microliters of hemolymph from the larvae of Manduca sexta in the final instar was mixed with 500 μ l of 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1% SDS and 10^{-2} M DFP. SDS is necessary for lysis and release of the hemocyte constit-

uents essential for prophenoloxidase activation. Five microliter aliquots were withdrawn at 5, 10, 15, 20, 25, and 30 minutes, diluted with 995 μ l of 0.1 M phosphate buffer containing 0.2% L-dopa and the absorbance increase at 475 nm due to dopachrome formation was measured. Hemolymph diluted with phosphate buffer lacking DFP served as a control. One unit of phenoloxidase activity was defined as the amount of enzyme which causes an increase in absorbance of 0.01/min at 475 nm.

(b) The effect of trypsin inhibitors A and B on the activation of prophenoloxidase in a hemocyte lysate from *Manduca sexta*

Hemocyte suspension prepared as described on page 37 was used for this experiment. Cells from a 400 μ l aliquot of the hemocyte suspension (approximately 4×10^7 cells) were pelleted and resuspended in 60 μ l of buffer G, 10 mM cacodylate buffer, pH 7.0, containing 5 mM CaCl_2 , (lysate C), or in 60 μ l of buffer G containing either 8.6 μ M trypsin inhibitor A (lysate A), or 27.4 μ M trypsin inhibitor B (lysate B). Each cell suspension was sonicated (3 x 10 second bursts), centrifuged at 12,000 x g for 3 minutes and the supernatant was used in the prophenoloxidase activation experiments described below.

Prophenoloxidase was activated and assayed as follows: 10 μ l of lysate C was incubated for 1 hour at room temperature with 0.025% laminarin and 0.05 M MgCl_2 in a total volume of 800 μ l. Prophenoloxidase in lysates A and B were activated similarly but the activation was performed in the presence of 8.6 μ M trypsin inhibitor A in the case of lysate A and 27.4 μ M trypsin inhibitor B in the case of lysate B. After incubation, 500 μ l of each reaction mixture was mixed with 500 μ l of L-Dopa (2 g/liter in buffer G) and the absorbance at 475 nm was recorded after incubating at room temperature for 3 minutes.

B.5. Does Manduca sexta hemolymph contain a system resembling the alternative complement pathway?

(a) The effect of Manduca sexta hemolymph and purified trypsin inhibitors A and B on activation of the alternative pathway of complement

The rabbit erythrocyte hemolytic assay (page 35) was used to ascertain the effect of components in the hemolymph of Manduca sexta and purified trypsin inhibitors A and B on the activity of the human alternative complement pathway. Briefly, 25 μ l of fresh frozen Manduca sexta hemolymph was incubated at 37^o C with 25 μ l of a 1:3 dilution of human serum and 10 μ l (3.5×10^7 cells) of rabbit erythrocytes in a total volume of 150 μ l. At the end of the incubation period, 1 ml of ice-cold GVBE was added, the reaction mixture centrifuged at 1,200 x g and the absorbance at 414 nm of the supernatant was determined. Controls in which GVBS replaced either hemolymph or serum were run in parallel.

To examine the effects of supernatant of heat-inactivated hemolymph and of the purified inhibitors A and B on activation of the alternative complement pathway, assays similar to the one described above was used except that fresh frozen hemolymph was replaced by the supernatant of hemolymph boiled for 15 minutes or by inhibitor A (0.43 nmoles) or inhibitor B (1.67 nmoles).

(b) Investigations to assess whether the hemolymph of Manduca sexta contains factors functionally analogous to human complement factors D and B

To assess whether Manduca sexta hemolymph contains factors capable of substituting for human factors D and B, 25 μ l of human RD or RB sera were mixed with 3.5×10^7 rabbit erythrocytes and fresh frozen Manduca

sexta hemolymph (10 μ l for RD serum and 8 μ l for RB serum) in a total reaction volume of 150 μ l. A control where GVBS replaced RD or RB serum was run in parallel. The reactions were incubated at 37 $^{\circ}$ C for 1 hour and the extent of hemolysis quantified as described.

(c) Experiments to assess human factor B cleavage by factors present in the hemolymph of Manduca sexta

It is possible that the hemolymph of Manduca sexta may contain a protease capable of cleaving human complement factor B and render it unavailable for participating in complement reactions. To assess whether such a protease is present in Manduca sexta hemolymph, 100 μ l of fresh frozen human serum containing 10 mM MgCl₂ was incubated with 10 μ l of fresh frozen hemolymph at 37 $^{\circ}$ C for 1 hour. Control reactions were run in parallel in which hemolymph was replaced by 1 mg or 10 mg/ml E.coli lipopolysaccharide in GVBS, GVBS alone, and the supernatant of hemolymph immersed in boiling water bath for 15 minutes. E.coli lipopolysaccharide is a natural activator of the alternative complement pathway which results in the cleavage of factor B to Ba and Bb fragments. In separate set of experiments, purified factor B (92.5 μ g) was incubated at 37 $^{\circ}$ C for 1 hour with 10 μ l of fresh frozen hemolymph in 100 μ l of 0.1M veronal buffer, pH 7.5 in the presence or absence of 10 mM MgCl₂. Mg⁺² ions are required for factor B cleavage by factor D. At the end of the incubation period 15 μ l aliquots of each reaction mixture was immunoelectrophoresed as described (page 32).

CHAPTER III

RESULTS

A. Measurement of trypsin inhibitory activity in the hemolymph of *Manduca sexta* during larval and pupal stages

Trypsin inhibitory activity in the hemolymph of *Manduca sexta* was found to decrease as the animals passed from the larval stage to the pupal stage (Fig. 7). The hemolymph of the early pupae had approximately 50% of the activity found in the larval hemolymph, while the hemolymph from late pupae had only a tenth of the activity of that of larvae. The total hemolymph trypsin inhibitory activity is likely to follow a similar pattern because the hemolymph volume also decreases during the pupal stage (73). Higher amount of trypsin inhibitory activity in the larval hemolymph suggests that these inhibitors have important functions in the larval hemolymph. For example, the hemolymph trypsin inhibitors could potentially regulate hemolymph prophenoloxidase activation since the latter enzyme is activated by a trypsin-like protease. Unregulated activation of prophenoloxidase can have deleterious consequences. Phenoloxidase can cause improper sclerotization thus impairing the mechanical properties of larval cuticle. Further, melanin deposition on tissues by phenoloxidase can stimulate phagocytic cells, thereby affecting tissue integrity. Larval hemolymph, in vitro, darkens slowly (due to the endogenous activation of prophenoloxidase) when compared to the pupal hemolymph suggesting an inverse relationship between hemolymph trypsin inhibitory activity and

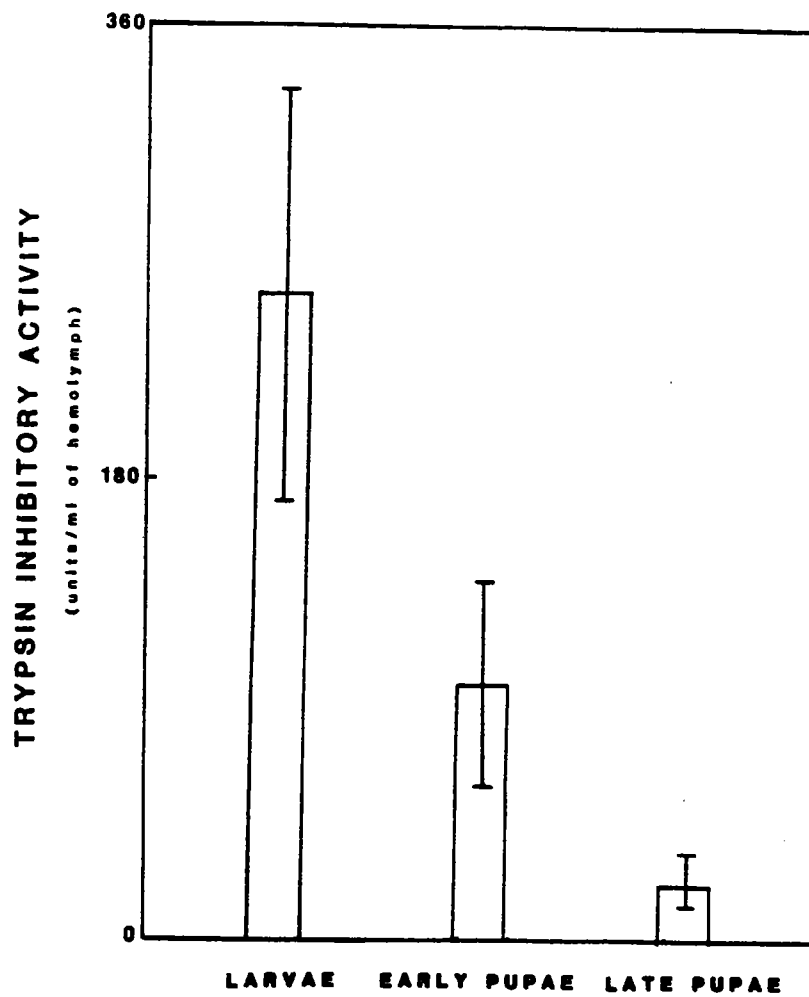


Fig. 7. Quantitation of trypsin inhibitory activity in the hemolymph of Manduca sexta during larval and pupal stages.

prophenoloxidase activation. In order to examine the effects of hemolymph trypsin inhibitors in vitro, it is necessary to purify them. Since the larval hemolymph contained higher trypsin inhibitory activity, this was chosen for inhibitor isolation.

B. Isolation and structural analysis of trypsin inhibitors from the hemolymph of *Manduca sexta*

B.1. Purification and biochemical characterization of two trypsin inhibitors from the hemolymph of the larval stage of *Manduca sexta*.

The protocol for the purification of trypsin inhibitors from *Manduca sexta* hemolymph is summarized in Fig. 8. Preliminary experiments demonstrated that the trypsin inhibitory activity in hemolymph was stable even at pH 1.0. Since addition of perchloric acid to a final concentration of 0.7% precipitated ca 90% of the hemolymph proteins while all of the trypsin inhibitory activity remained in solution acid precipitation with perchloric acid was incorporated as the initial step in the inhibitor purification scheme. Prior to trypsin-Sepharose affinity chromatography the trypsin inhibitors in the supernatant of perchloric acid-treated hemolymph were concentrated by adding ammonium sulfate to 90% saturation. *Manduca sexta* hemolymph trypsin inhibitors adsorb tightly to trypsin-Sepharose, and 0.1 M HCl containing 0.75 M NaCl (pH 1.5) was necessary for the desorption of the bound inhibitors. The trypsin-Sepharose elution profile is presented in Fig. 9. Approximately 60% of the applied activity was recovered from trypsin-Sepharose affinity gel. The reason for the relatively low yield of trypsin inhibitors is not clear. Since the trypsin-Sepharose wash fractions were not found to contain any trypsin inhibitory activity, the unre-

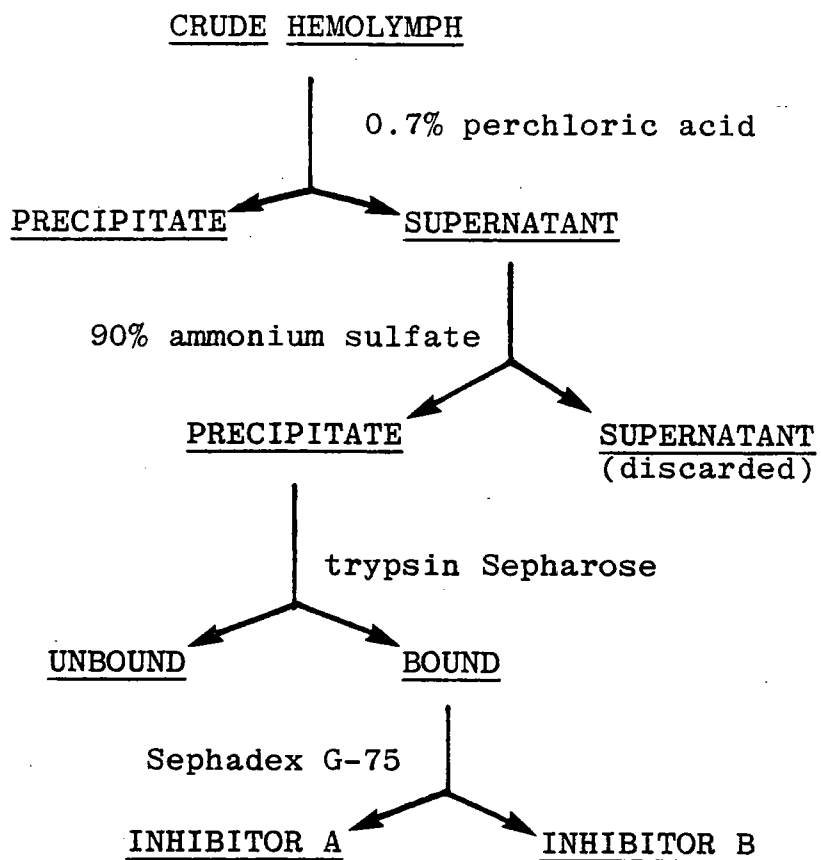
PURIFICATION PROTOCOL

Fig. 8. Flowchart illustrating the protocol adopted to purify trypsin inhibitors from Manduca sexta hemolymph.

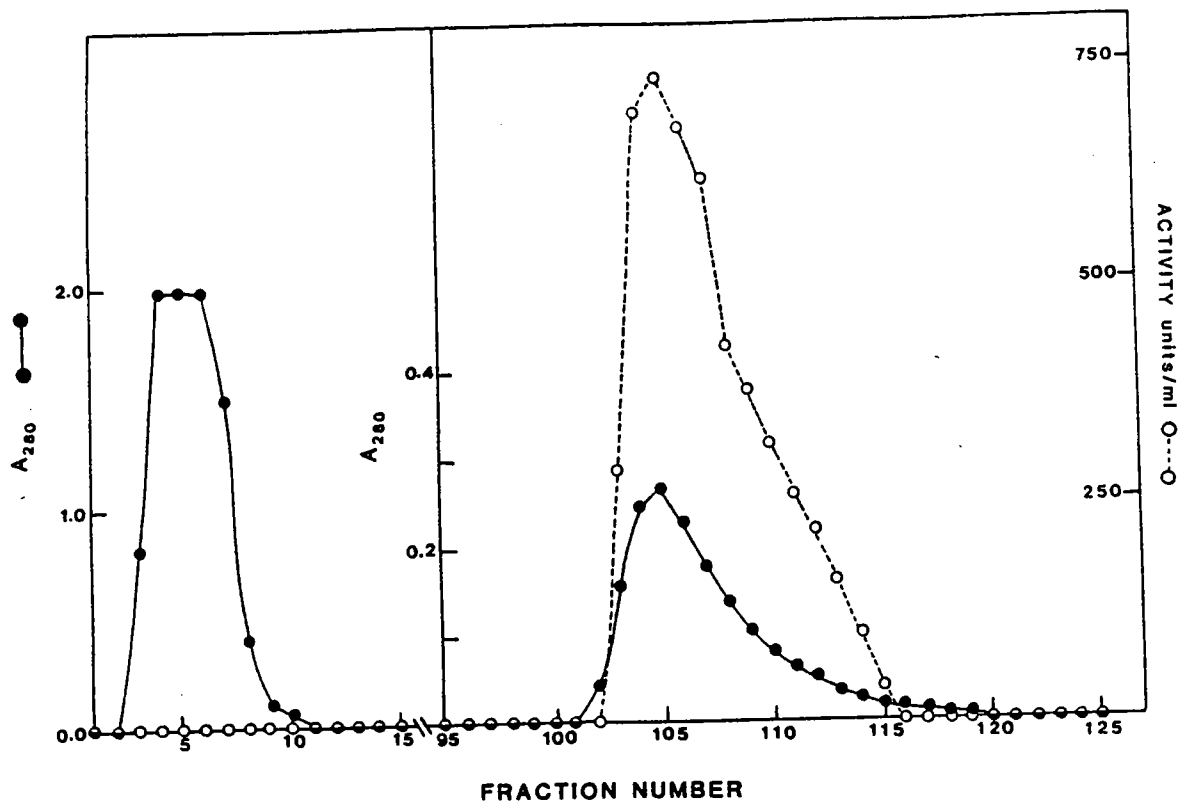


Fig. 9. Chromatographic profile of trypsin inhibitor activity on a trypsin-Sepharose affinity column.

covered trypsin inhibitory activity perhaps remains bound to the trypsin- Sepharose gel. Further attempts to recover trypsin inhibitory activity by eluting with 0.1 M HCl containing 1.5 M NaCl or 0.1 M ammonium acetate adjusted to pH 3.0 with glacial acetic acid did not increase the yield. The eluate from trypsin-Sepharose affinity chromatography was further resolved into two components with molecular weights of 13,700 and 8,000 by gel filtration on Sephadex G-75 (Fig. 10, 10a). A summary of the isolation procedure for a 315 ml volume of hemolymph preparation is outlined in Table 5.

Inhibitors A and B after gel filtration on a Sephadex G-75 column were of high purity since each gave rise to a single protein band on SDS-polyacrylamide gel electrophoresis under reducing as well as non-reducing conditions (Fig. 11 and 12). Isoelectrofocusing on polyacrylamide gels containing pH 3-10 ampholytes focused inhibitor A as a single band of isoelectric point 5.7. However, inhibitor B could be resolved into two components with isoelectric points of 7.1 and 5.3. Molecular weights estimated by SDS-polyacrylamide gel electrophoresis under non-reducing conditions gave values of 15,000 for inhibitor A and 8,500 for inhibitor B. This agrees with molecular weight estimated by gel filtration on Sephadex G-75. However on SDS-page under reducing conditions inhibitor A migrated as a single component with a calculated molecular weight of 8,300, and inhibitor B migrated with a calculated molecular weight of 9,100, suggesting that inhibitor A was composed of two identical subunits.

Purified inhibitors A and B could be stored frozen at -20° C for a minimum of six months without any detectable loss of activity. Both

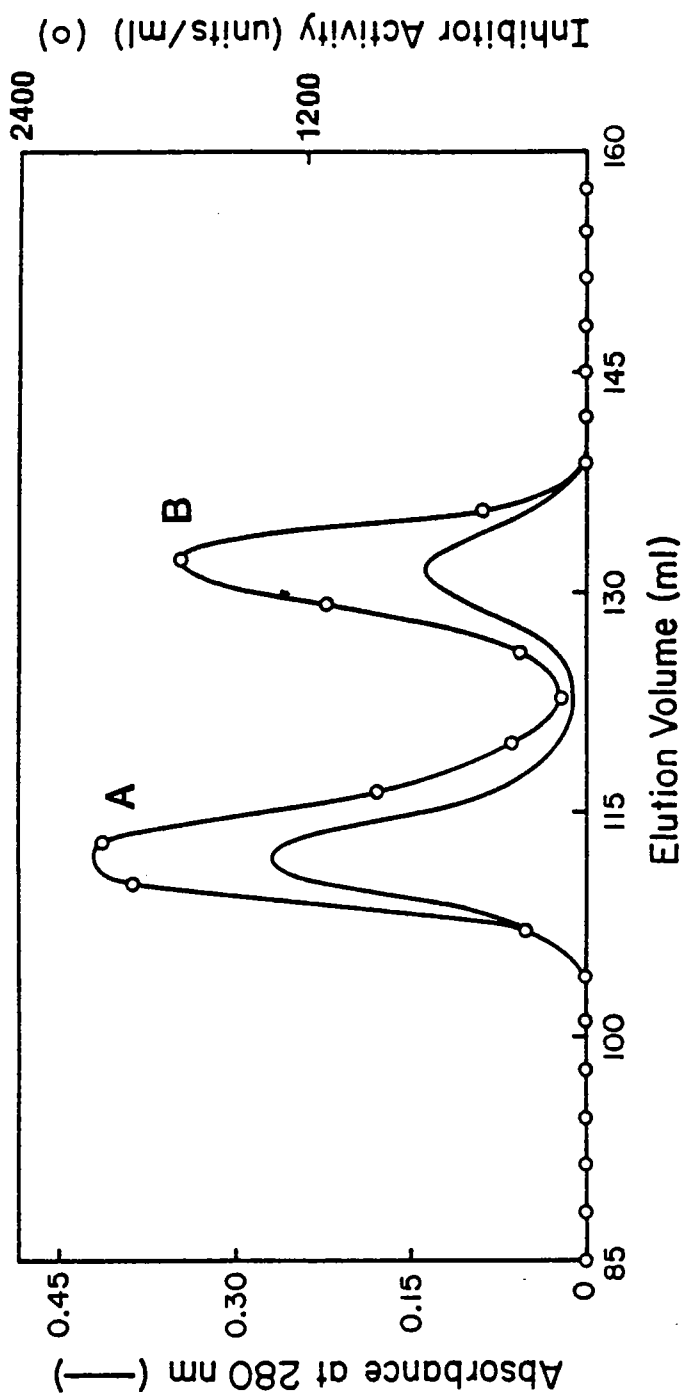


Fig. 10. Resolution of trypsin inhibitors A and B of *Manduca sexta* hemolymph by gel filtration on Sephadex G-75.

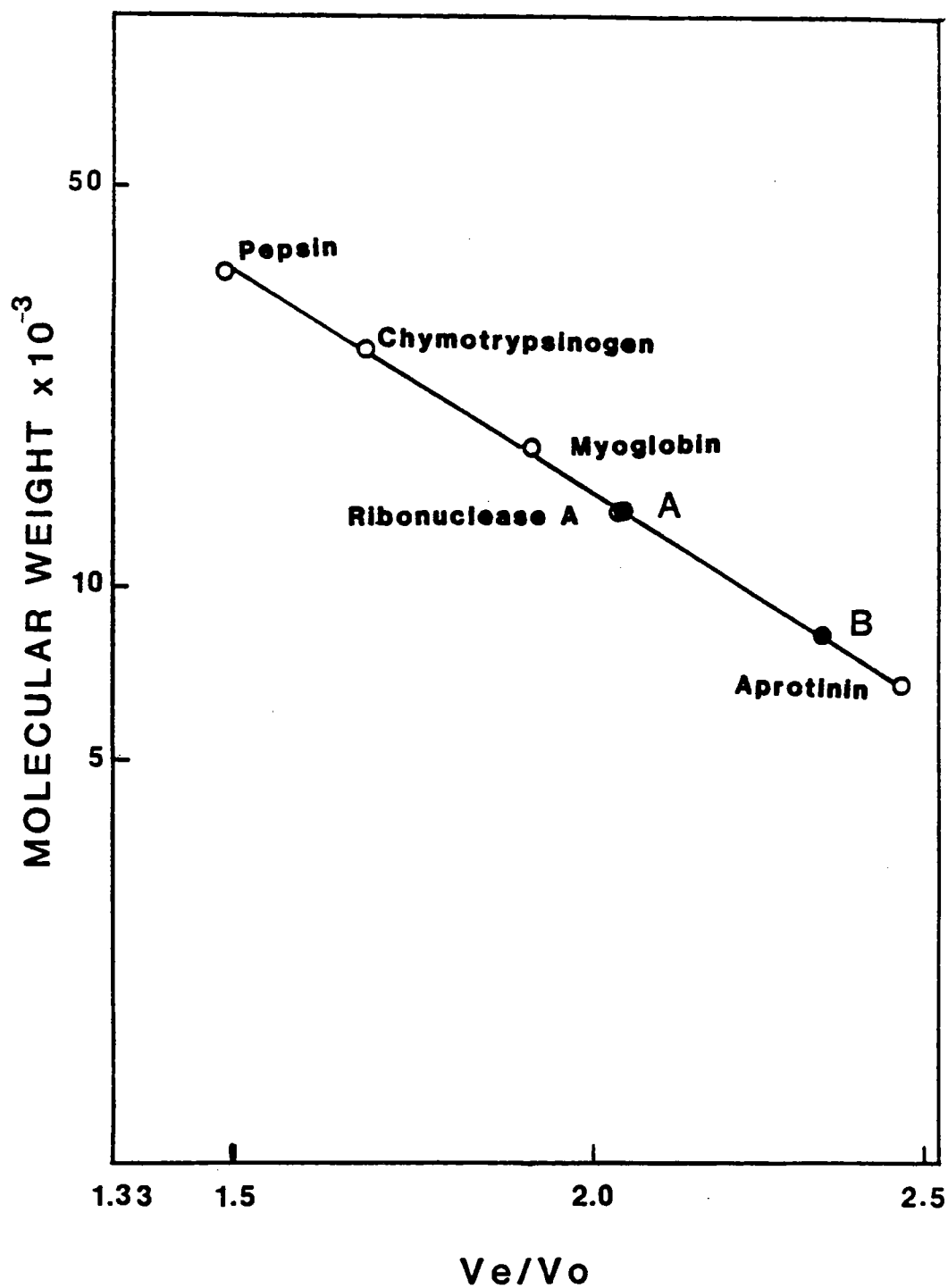


Fig.10a Molecular weight calculations of trypsin inhibitors A and B by Sephadex G-75 gel filtration.

Table 5

Summary of the purification of inhibitors A and B from Manduca sexta hemolymph

Step	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity units/mg	Yield (%)	Purification
1. Crude	315	39060	2325	16.8	100	-
2. Perchloric acid treatment	336	39396	146	269.8	100	16
3. Ammonium sulfate precipitation	26	29432	73.6	400.0	75	24
4. Trypsin-Sephadex chromatography	57	18412	15.6*	1180.3	47	70
5. Sephadex G-75 gel filtration						
Inhibitor A	13.6	10608	3.27*	3240.0	27	192
Inhibitor B	10.0	8000	1.05*	7610.0	20	283

* Protein content quantitated by amino acid analysis.

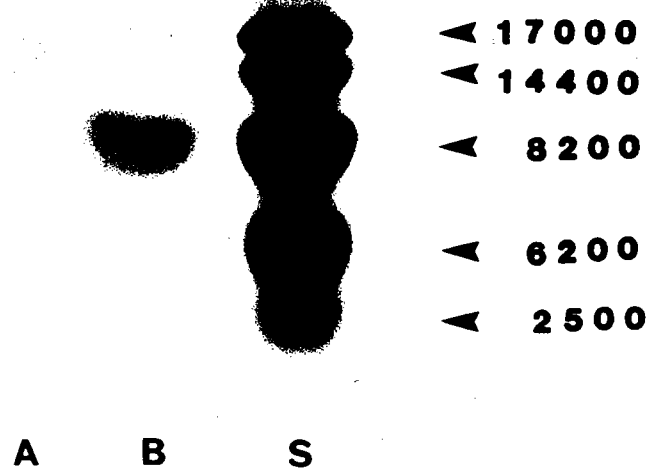


Fig. 11 SDS-Polyacrylamide slab gel electrophoresis of purified trypsin inhibitors A and B in the absence of mercaptoethanol.

A: Inhibitor A

B: Inhibitor B

S: Molecular weight standards

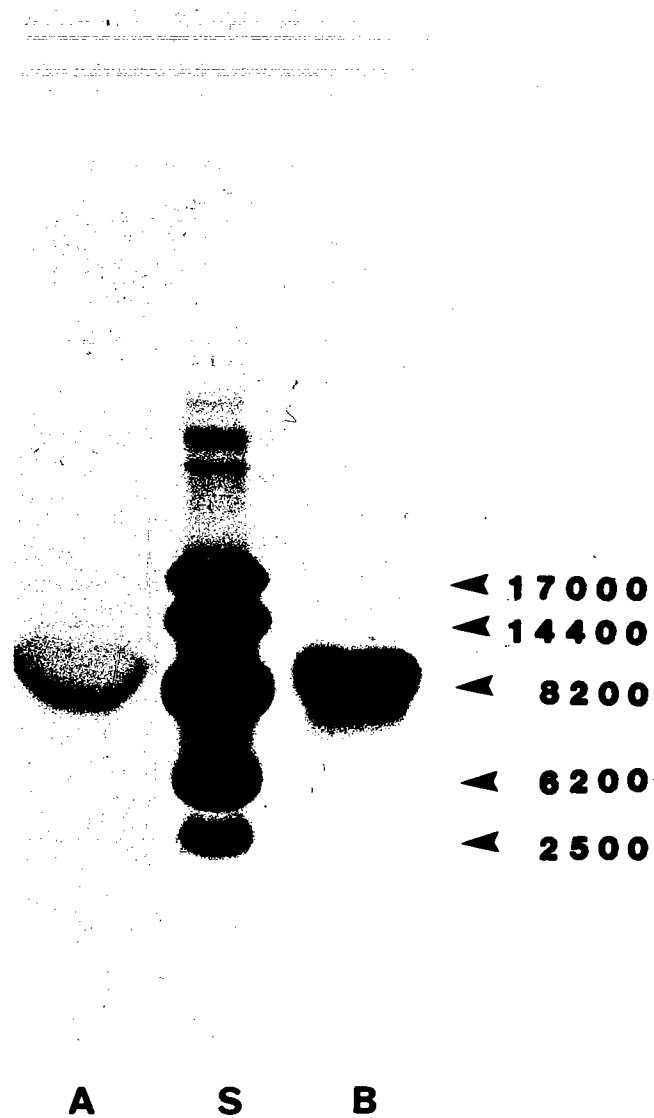


Fig. 12 SDS-Polyacrylamide slab gel electrophoresis of purified trypsin inhibitors A and B in the presence of mercaptoethanol.

A: Inhibitor A B: Inhibitor B
S: Molecular weight standards

inhibitors A and B are stable for at least 30 minutes at 100° C. However incubation of either inhibitor A or B in the presence of 2.5 mM dithiothreitol at room temperature for 1 hour resulted in the complete loss of their activity even in the absence of a denaturant.

Fig. 13 shows the effect of subtilisin BPN' on the activity of inhibitors A and B. A progressive loss of activity is observed with increasing concentrations of subtilisin BPN' indicating that inhibitors A and B are equally susceptible to proteolytic inactivation by subtilisin BPN'. Alternative possibilities such as complex formation between inhibitors A or B and subtilisin BPN' or the proteolysis of the trypsin used for quantitation of inhibitor activity by subtilisin BPN' are unlikely because subtilisin BPN' was denatured at the end of pre-incubation with inhibitors A or B by heating the reaction mixtures at 100° C for 10 minutes. The trypsin inhibitory activity of 93.8 pmoles of inhibitor A or 114.1 pmoles of inhibitor B is completely destroyed in 20 minutes by 145.5 pmoles of subtilisin BPN'.

The ultraviolet absorption spectra of both inhibitor A and inhibitor B (Fig. 14 and 15) demonstrated a characteristic protein spectrum with an absorption maximum at 277 nm.

The ability of trypsin inhibitors A and B to inhibit a variety of commercially available proteases from bacteria, plant and animal origin were evaluated. The results are tabulated in Table 6. Both inhibitor A and inhibitor B inhibit trypsin, chymotrypsin, plasmin, and thrombin. Somewhat higher concentrations of inhibitor B were required to inhibit both trypsin and chymotrypsin than were required for inhibitor A. Complete inhibition of trypsin and chymotrypsin (52 pmoles

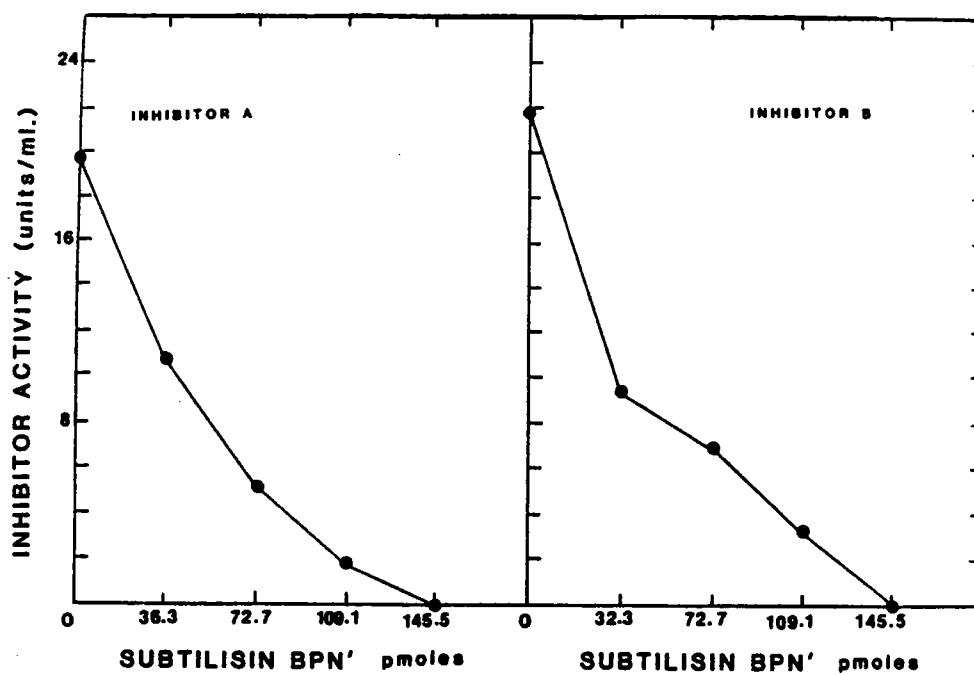


Fig. 13 The inactivation of trypsin inhibitory activity of inhibitors A and B by subtilisin BPN'.

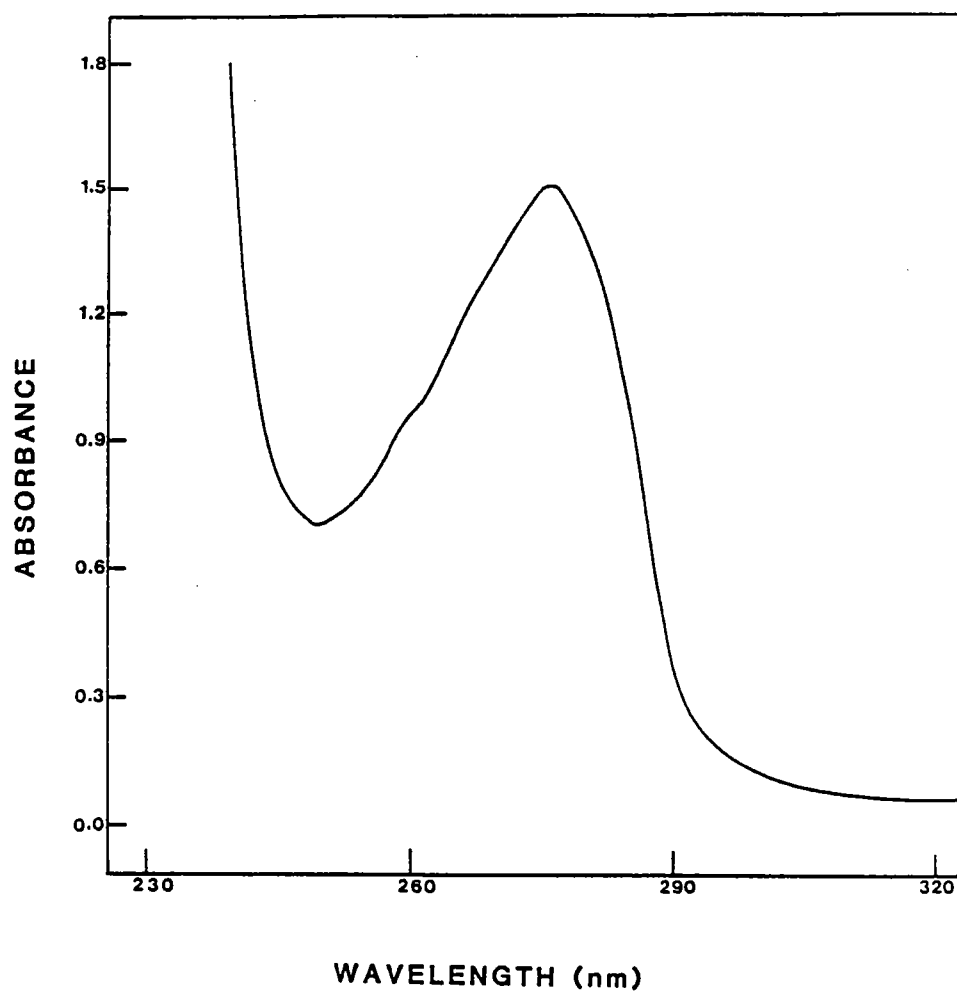


Fig. 14. Ultraviolet absorption spectrum of trypsin inhibitor A.

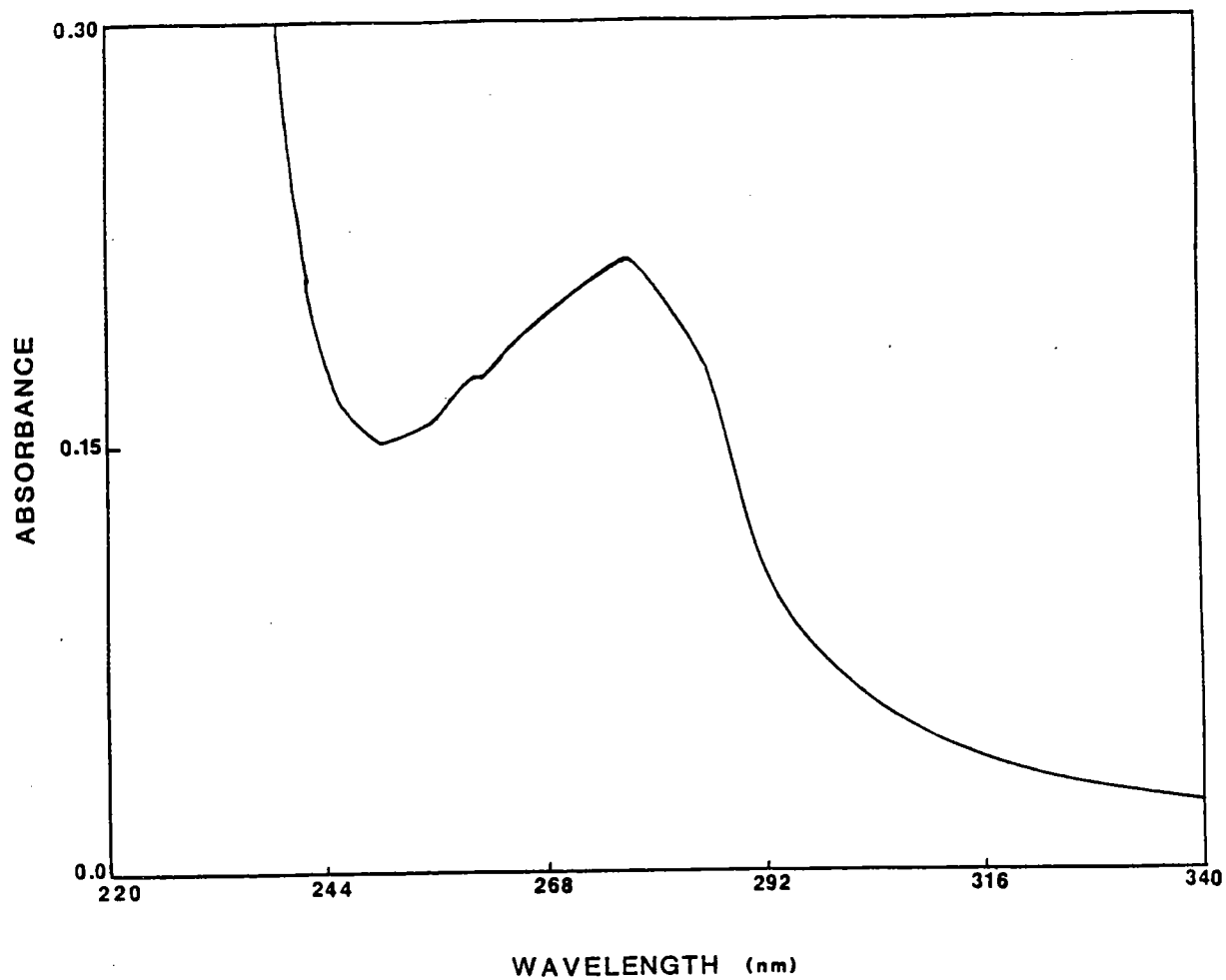


Fig. 15. Ultraviolet absorption spectrum of trypsin inhibitor B.

Table 6

Effect of trypsin inhibitors A and B on various proteases

Protease (pmole)	Activity with inhibitor A (%)*			Activity with inhibitor B (%)*		
	18.9 (pmole)	39.8 (pmole)	75.9 (pmole)	20.7 (pmole)	41.4 (pmole)	82.9 (pmole)
<u>Serine</u>						
Trypsin (52)	18.8	0	N.D.	25.0	9.4	0
Chymotrypsin (56)	14.2	0	N.D.	64.2	32.0	0
Subtilisin BPN' (73)	100	100	100	100	100	100
Elastase (77)	111	100	106	98	104	98
<u>Aspartyl</u>						
Pepsin (57)	100	104	106	100	104	98
<u>Thiol</u>						
Papain (86)	105	105	105	100	100	100
<u>Metallo</u>						
Thermolysin (58)	97	97	87	100	100	100
<u>Plasma serine proteases</u>						
	68.0 (pmole)	171.0 (pmole)		86.5 (pmole)	173.0 (pmole)	
Plasmin (23)	49.3	0		39.3	0	
Thrombin** (2.2)	50	50		50	50	

* Activity in the absence of inhibitor was taken as 100%.

** The clotting time did not increase linearly with inhibitor amount, but the amount of clot formed was less in the presence of inhibitors A and B.

each) was observed at a concentration of 39.8 pmoles of inhibitor A, whereas with 41.4 pmoles of inhibitor B 90% and 68% of the activities of trypsin and chymotrypsin, respectively, were inhibited. Plasmin (23 pmoles) was completely inhibited by 171 pmoles and 173 pmoles, respectively, of inhibitor A and inhibitor B, however, the same amount of inhibitors A and B inhibited only 50% of the activity of 2.2 pmoles of thrombin. Neither trypsin inhibitor A nor B inhibited the bacterial serine protease subtilisin BPN' or porcine elastase. Each also failed to inhibit pepsin (an aspartyl protease), papain (a sulfhydryl protease) and thermolysin (a Zn metalloprotease). Therefore, it appears that both inhibitor A and inhibitor B have specificity for chymotrypsin and trypsin-like serine proteases.

B.2 Studies on the complex formation of trypsin inhibitor A with trypsin and chymotrypsin

(a) Demonstration of the formation of complexes by trypsin inhibitors A and B with bovine trypsin.

To demonstrate that inhibitors A and B form complexes with trypsin, each inhibitor was incubated with trypsin and subjected to gel filtration on a Sephadex G-100 column. The elution profiles of inhibitors A and B and their respective complexes with trypsin are presented in Fig. 16 and 17. Inhibitor A eluted at a position corresponding to a molecular weight of ca 20,000 daltons and inhibitor B eluted with a molecular weight corresponding to ca 15,000 daltons. The molecular weights calculated for trypsin inhibitors A and B by gel filtration on G-100 column were higher than the molecular weight calculated by gel filtration on Sephadex G-75 column (M.W. of inhibitor A was 13,700 and the M.W. of inhibitor B was 8,000). The trypsin-

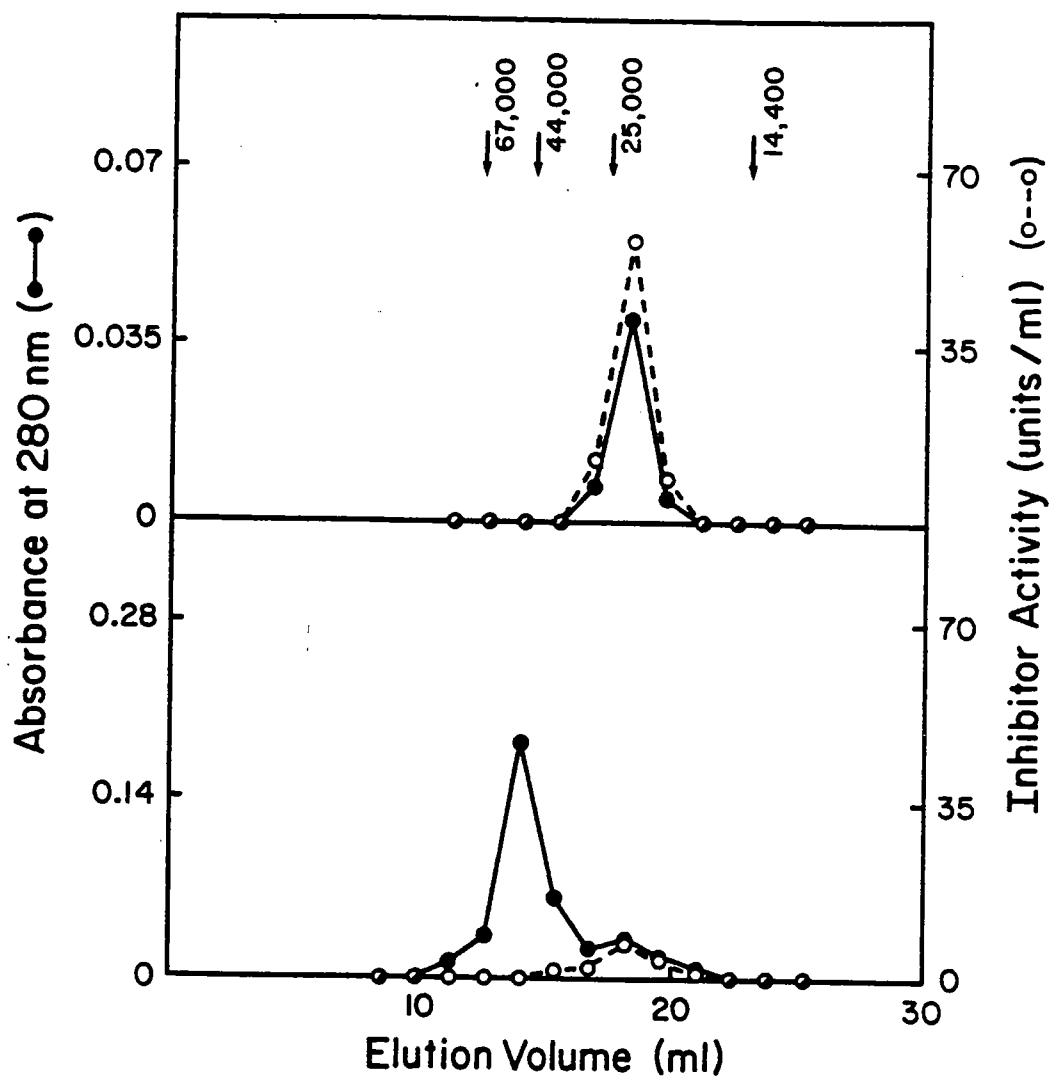


Fig. 16. Sephadex G-100 elution profile of trypsin inhibitor A and the complex formed between trypsin (8.5 nmoles) and inhibitor A (11 nmoles).

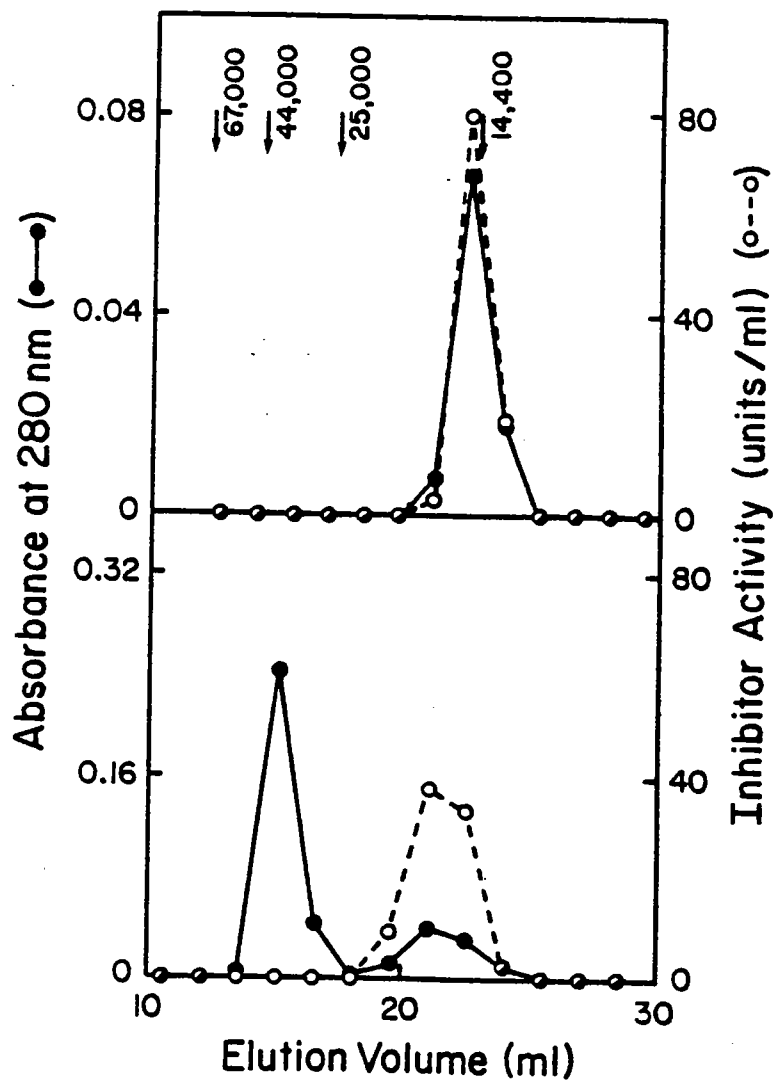


Fig. 17. Sephadex G-100 elution profile of trypsin inhibitor B and the complex formed between trypsin (8.5 nmoles) and inhibitor B (33.7 nmoles).

inhibitor A complex and the trypsin-inhibitor B complex eluted at positions of molecular weight of ca 47,000 daltons and ca 38,000 daltons respectively. Since trypsin eluted with a molecular weight of 23,000 daltons on Sephadex G-100, the molecular weights of the complexes suggest that 1:1 molar complexes are formed between both inhibitors and trypsin.

(b) Determination of the stoichiometry for the reaction of trypsin inhibitor A with trypsin and chymotrypsin.

The stoichiometries between inhibitor A and trypsin and chymotrypsin were evaluated in two ways. In the first method, 50.9 pmoles of trypsin was incubated individually with 0 to 44.2 pmoles of inhibitor A and the residual trypsin activity was quantitated using BAPNA as the substrate. Similarly, 520.0 pmoles of chymotrypsin was incubated with 0 to 503.0 pmoles of inhibitor A and the residual chymotrypsin activity was quantitated using GPNA. To obtain the stoichiometry between inhibitor A and trypsin, relative trypsin activity, i.e., the ratio of trypsin activity in the presence of inhibitor A (v_i) to the activity in the absence of inhibitor A (v_o) was plotted against the molar ratio of inhibitor A to trypsin (based on a molecular weight of 15,000 for inhibitor A and 24,000 for trypsin and chymotrypsin) (Fig. 18). Complete inhibition of trypsin was observed at an inhibitor A/trypsin ratio of 1.07 indicating a 1:1 stoichiometric complex between trypsin and inhibitor A. Similarly, a plot of the relative activity (v_i/v_o) against the molar ratio of inhibitor A to chymotrypsin (Fig. 19) showed that complete inhibition of chymotrypsin

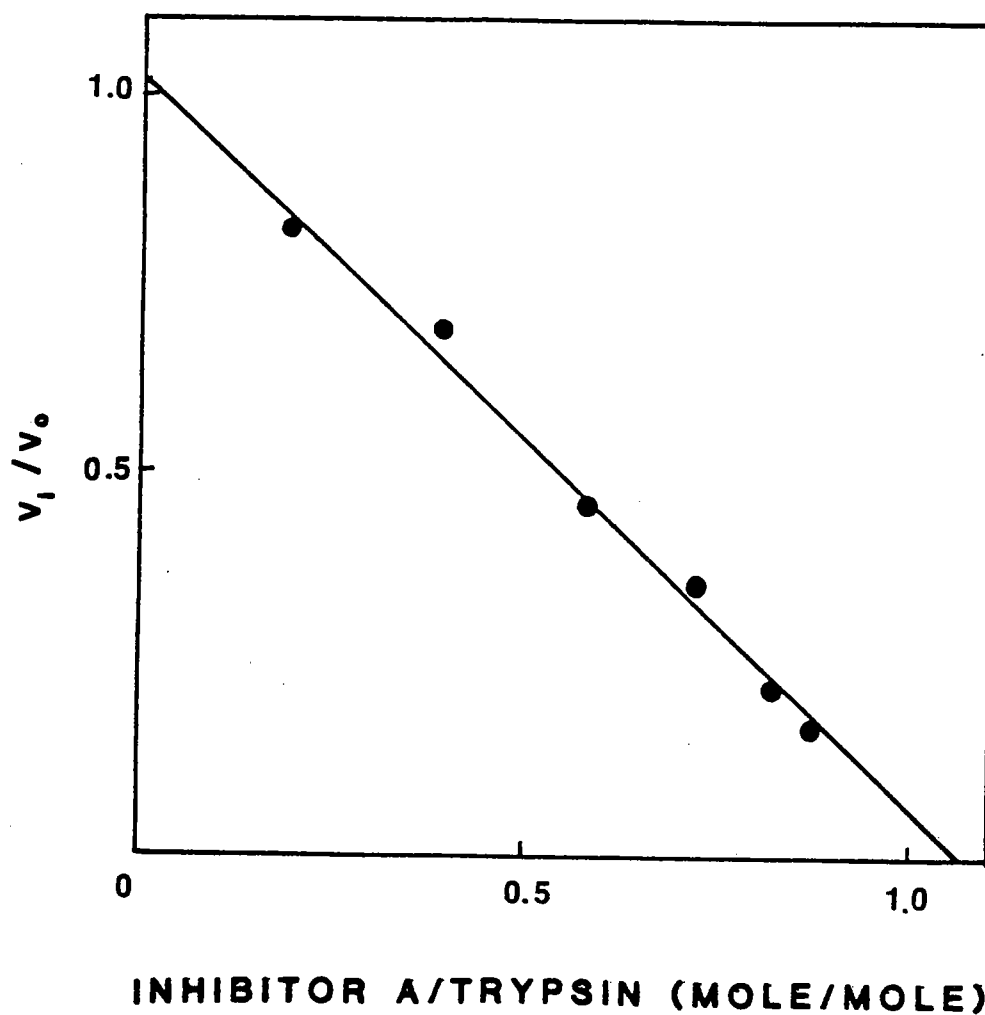


Fig. 18. Plot of v_i/v_o against the molar ratio of trypsin inhibitor A to trypsin.

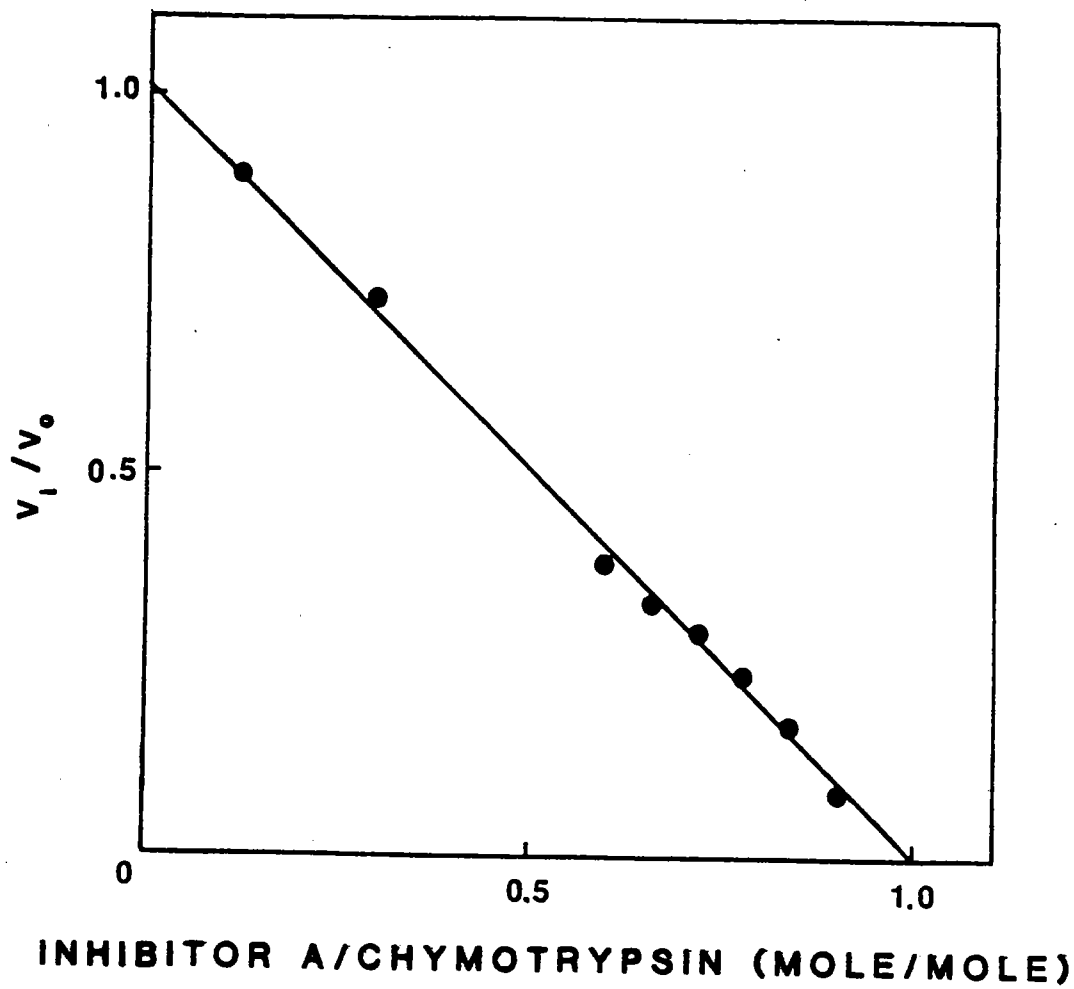


Fig. 19. Plot of v_i/v_0 against the molar ratio of trypsin inhibitor A to chymotrypsin.

was observed at an inhibitor A/chymotrypsin molar ratio of 1.0 indicating that chymotrypsin also formed a 1:1 stoichiometric complex with inhibitor A.

In an alternate method for calculating the stoichiometries, 22 nmoles of trypsin was allowed to complex individually with 3.13 to 15.7 nmoles of inhibitor A and the free trypsin was determined using the active site titrant NPGb. Likewise, 1.49 nmoles of chymotrypsin was complexed individually with 0.29 to 1.43 nmoles of inhibitor A and the free chymotrypsin was quantitated using MUTMAC as the active site titrant. The ratio of free trypsin at each inhibitor concentration with the total amount of trypsin (expressed as per cent) was plotted against the ratio of inhibitor A/trypsin (Fig. 20). The extrapolated value for 100% trypsin inhibition was calculated as 0.94 moles of inhibitor A/mole of trypsin. Likewise, the plot of free chymotrypsin at various concentrations of inhibitor A (expressed as per cent of chymotrypsin concentration in the absence of inhibitor A) against inhibitor A/chymotrypsin molar ratio (Fig. 21) gave a stoichiometric value of 0.91 for 100% chymotrypsin inhibition. Thus inhibitor A forms 1:1 stoichiometric complexes with both trypsin and chymotrypsin. On isoelectrofocusing, inhibitor B was resolved into two components with pIs of 5.3 and 7.1. Attempts to separate these components by ion-exchange chromatography on DEAE-cellulose and DEAE-Sephadex were not successful. Since it is possible that each component could have different affinity towards trypsin and chymotrypsin, stoichiometric studies and the estimations of K_i values were not performed with inhibitor B.

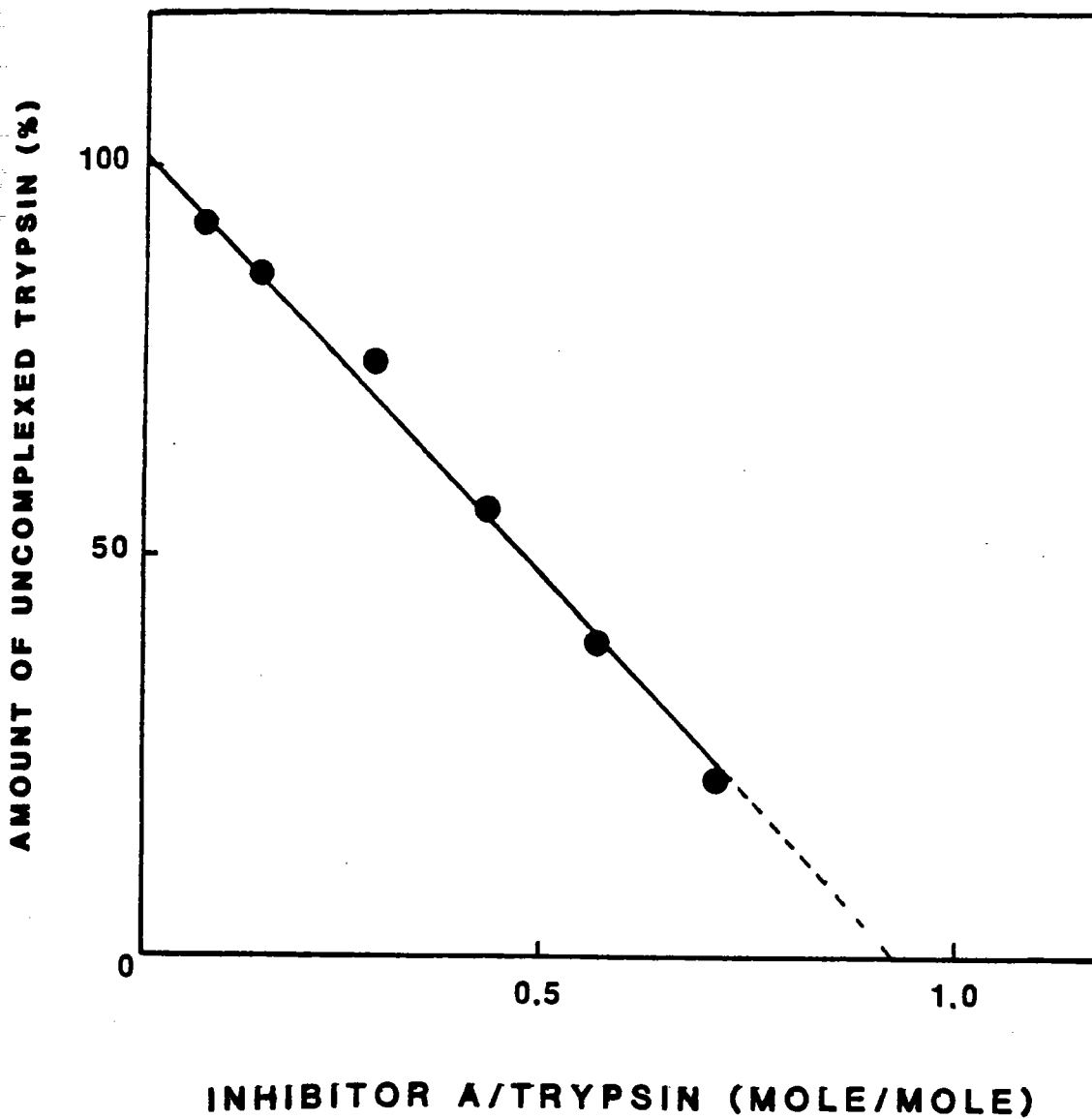


Fig.20. Plot of the concentrations of uncomplexed trypsin against the molar ratio of trypsin inhibitor A to trypsin.

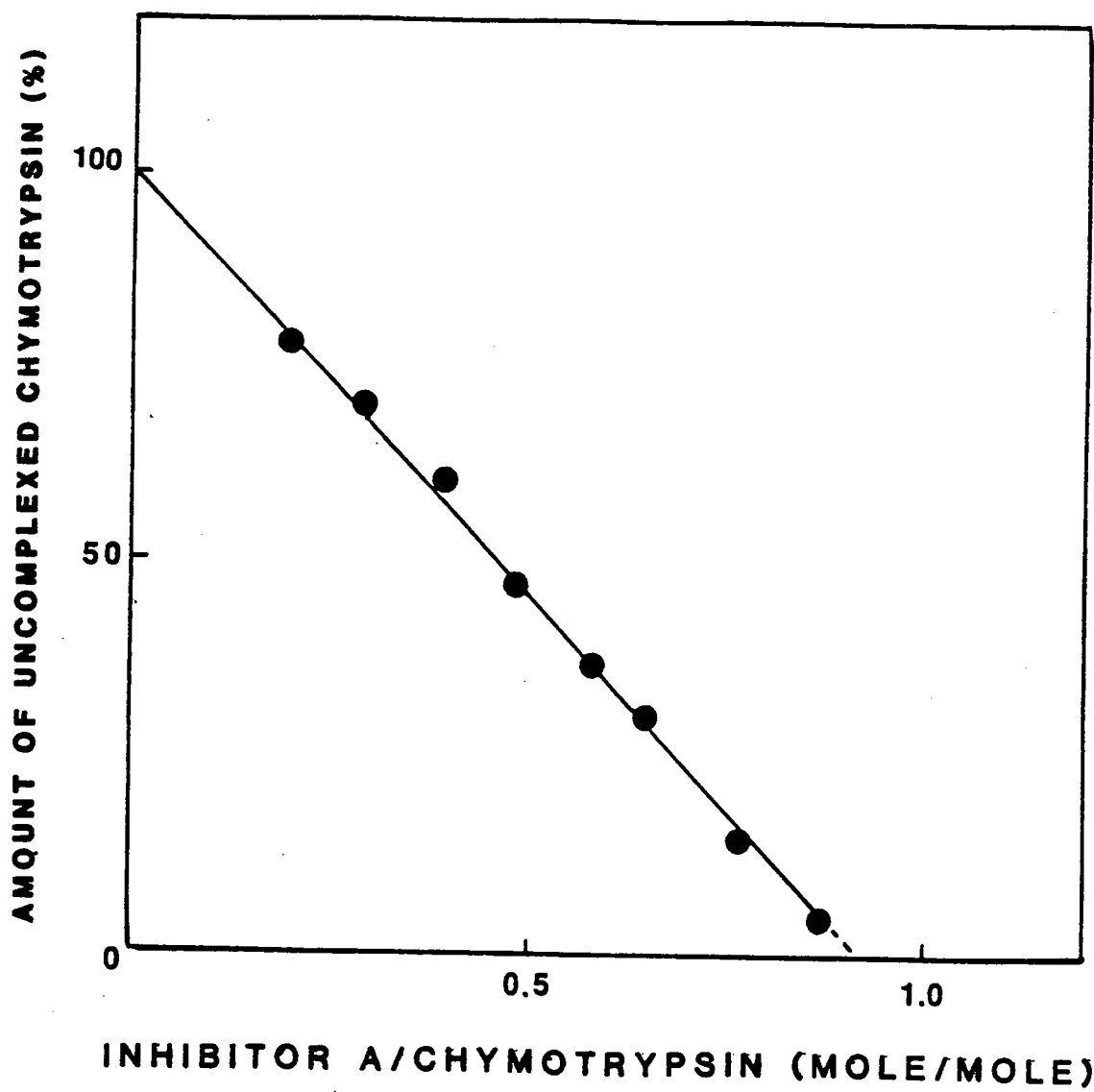


Fig. 21. Plot of the concentrations of uncomplexed chymotrypsin against the molar ratio of trypsin inhibitor A to chymotrypsin.

(c) Estimation of the inhibition constant (K_i) for the reactions of inhibitor A with trypsin and chymotrypsin

Polypeptide protease inhibitors generally exhibit high affinity for their respective proteases. As a result, most of the inhibitor added to free protease is immediately bound. The classical Lineweaver-Burk method for the estimation of inhibition constants, which assumes that a significant change in free inhibitor concentration does not occur, cannot be used for the study of such high affinity inhibitors. Goldstein (74) has derived a general equation which describes the behaviour of high affinity inhibitors (equation 1)

$$I_t = (v_0/v_i - 1) K_i + (1 - v_i/v_0) E_t \quad (1)$$

where I_t is the total inhibitor concentration, E_t is the total enzyme concentration, K_i the inhibitor dissociation constant, and v_0 and v_i are the velocities of catalytic reaction in the absence and the presence of the inhibitor, respectively. Many derivatives and transformations of this equation have been published (72, 75, 76). One such derivation which yields K_i in a simple and accurate way is that of Meyers and Cha (72, 75) (equation 2).

$$I_{50} = K_i + E_t/2 \quad (2)$$

where I_{50} is the concentration of the inhibitor which gives 50% inhibition of the enzyme activity. A plot of the I_{50} values determined at several enzyme concentrations against the total enzyme concentration yields a straight line plot with the ordinate intercept equal to K_i (72).

Fig. 22 is a plot of the I_{50} values of the reaction between trypsin and inhibitor A against the respective trypsin concentrations.

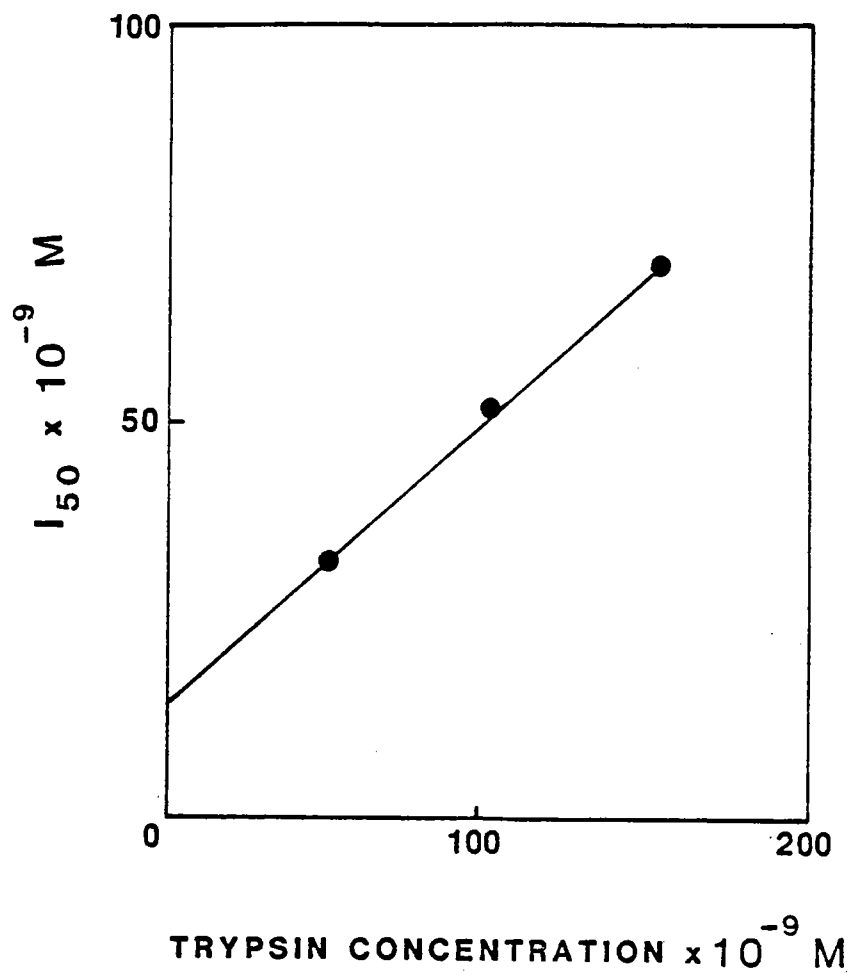


Fig. 22 Plot of I_{50} values of trypsin inhibition by inhibitor A as a function of trypsin concentration.

It was assumed that inhibitor A inhibits both trypsin and chymotrypsin competitively. From the plot, a K_i value for inhibitor A binding to trypsin was estimated to be 1.45×10^{-8} M. A similar plot of I_{50} values of the reaction between chymotrypsin and inhibitor A against chymotrypsin concentrations (Fig. 23) gave a K_i value of 1.7×10^{-8} M for inhibitor A-chymotrypsin interaction.

Since inhibitor A inhibits both trypsin and chymotrypsin, two operationally distinguishable situations can arise. The proteases can either compete for the same inhibitor site, or they can be simultaneously and independently inhibited at different sites on the inhibitor. Since inhibitor A is a dimer, the latter is a distinct possibility. In order to distinguish between these two situations, reactions were carried out in which inhibitor A was fully complexed with excess trypsin and the complex was added to various amounts of chymotrypsin. The results are presented in Fig. 24. Reaction of inhibitor A (0.37 nmoles) with varying amounts of chymotrypsin (0.43 to 1.7 nmoles) resulted in the inhibition of chymotrypsin activity (control, Panel A). However, when the same experiment was performed with inhibitor A preincubated with 0.57 nmoles of trypsin to permit total complex formation, the reaction mixture demonstrated no inhibitory activity towards chymotrypsin (Panel B). In an alternate experiment, 0.47 nmoles of inhibitor A was reacted with trypsin (0.51 to 2.03 nmoles) and the residual trypsin activity was quantitated. The results, Fig. 25, Panel A, shows that the trypsin activity was inhibited. However when the same amount of inhibitor A (0.47 nmoles) was complexed with 1.05 nmoles of chymotrypsin, and then allowed to

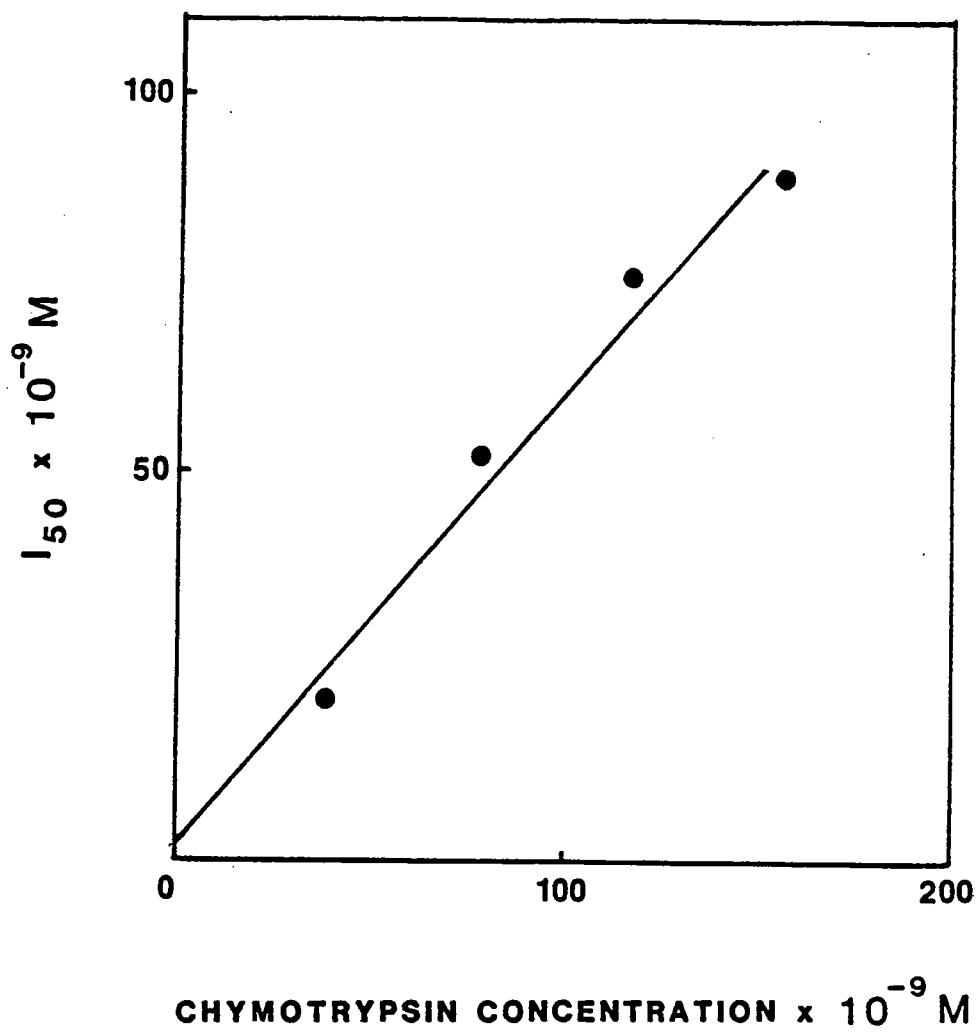


Fig. 23 Plot of I_{50} values of chymotrypsin inhibition by inhibitor A as a function of chymotrypsin concentration.

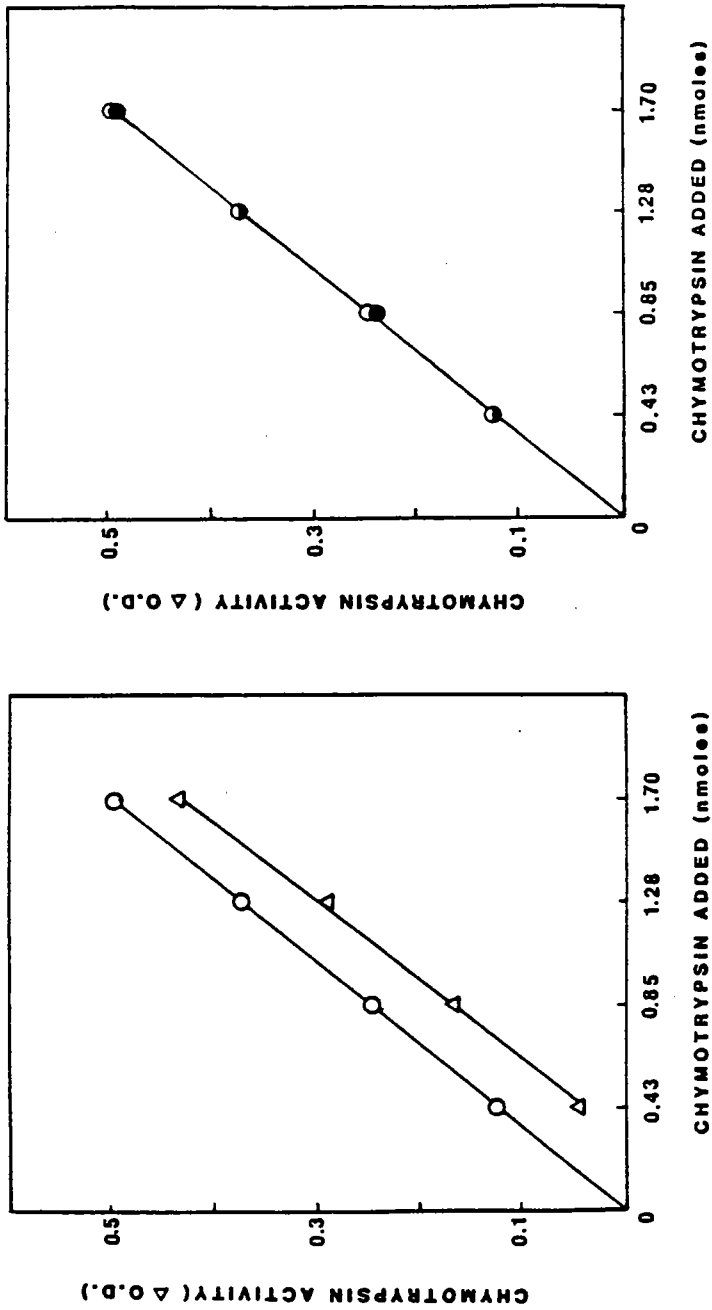


Fig. 24 Panel A: The effect of inhibitor A on the activity of chymotrypsin. ○—○ Chymotrypsin activity in the absence of inhibitor A. Δ—Δ Chymotrypsin activity in the presence of 0.37 nmolee of inhibitor A.

Panel B: The effect of 0.37 nmolee of inhibitor A in complex with 0.57 nmolee of trypsin on the activity of chymotrypsin. ○—○ Chymotrypsin activity in the absence of inhibitor A-trypsin complex. ●—● Chymotrypsin activity in the presence of inhibitor A-trypsin complex.

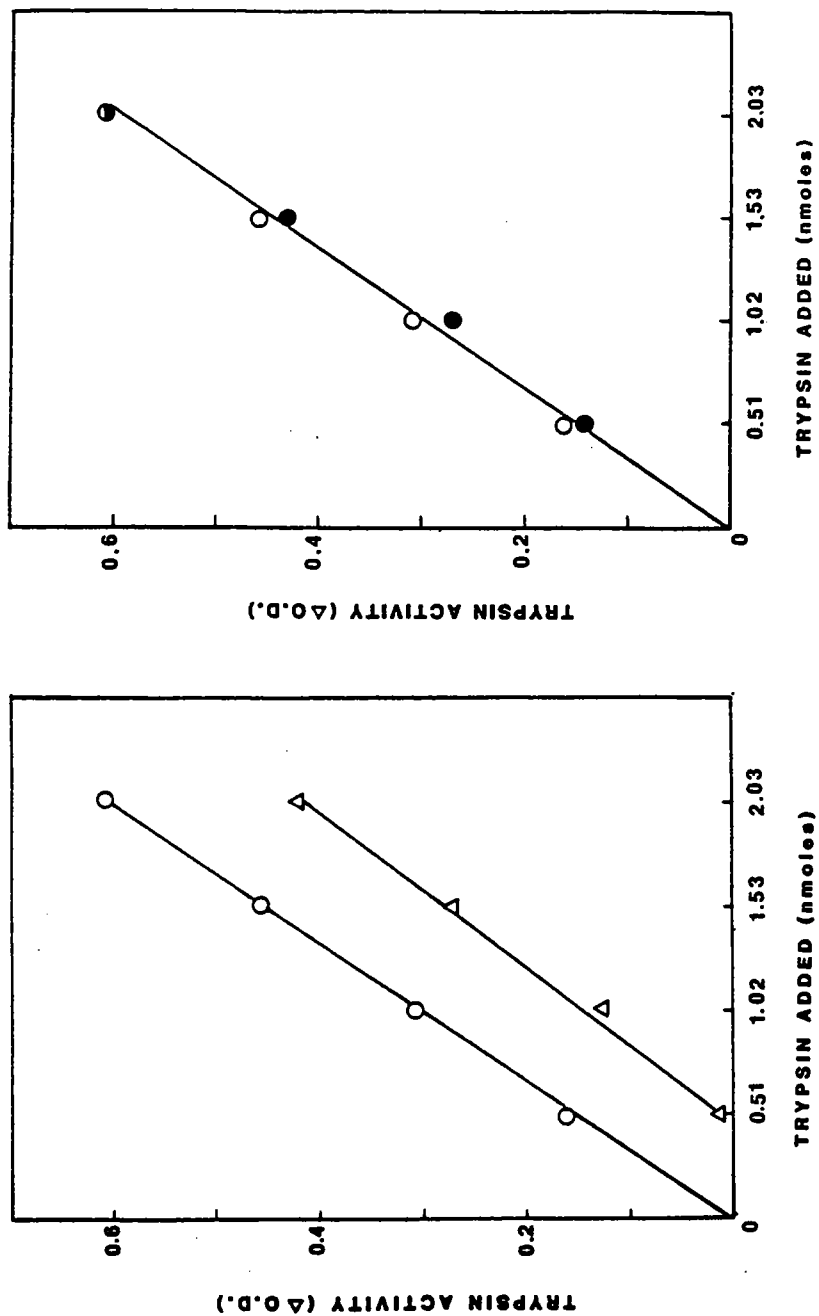


Fig. 25 Panel A: Effect of inhibitor A on the activity of trypsin. ○—○ Trypsin activity in the absence of inhibitor A. △—△ Trypsin activity in the presence of 0.47 nmoles of inhibitor A.

Panel B: Effect of 0.47 nmoles of inhibitor A in complex with 1.05 nmoles of chymotrypsin on trypsin activity. ○—○ Trypsin activity in the absence of inhibitor A-chymotrypsin complex. ●—● Trypsin activity in the presence of inhibitor A-chymotrypsin complex.

react with trypsin, no inhibitory activity towards trypsin was observed (Fig. 25 Panel B). Taken together, these results demonstrate that once a complex is formed between inhibitor A and either trypsin or chymotrypsin, the complex is not displaced by the other protease. Furthermore, these results indicate that inhibitor A does not have two independent sites for trypsin and chymotrypsin.

B.3. Amino terminal sequence analysis of inhibitors A and B from the hemolymph of Manduca sexta

(a) Amino acid composition analysis and peptide mapping of hemolymph inhibitors A and B.

The amino acid compositions of inhibitors A and B are listed in Table 7. The differences in the amino acid composition suggest that inhibitor A and inhibitor B are both unique. To further compare inhibitors A and B, each reduced and S-carboxymethylated protein was digested with trypsin-TPCK and peptide mapping was performed by HPLC. The tryptic peptide patterns obtained (Fig. 26 and 27) clearly demonstrate that inhibitors A and B are different.

(b) Amino terminal amino acid sequences of trypsin inhibitors A and B.

Knowledge of the primary structures of trypsin inhibitors A and B is essential in order for comparisons to be made with the inhibitors isolated from other sources. Such comparisons should be useful not only in understanding the physical interactions of inhibitors A and B with trypsin but also with serine proteases in general. The amino terminal amino acid sequences of inhibitors A and B were obtained using a Beckman sequenator which had been modified for protein microsequencing. Fifty-six amino terminal residues were identified for

Table 7

Amino acid compositions of trypsin inhibitors A and B.

Amino acid	Inhibitor A		Inhibitor B	
	Mole %	Residues/ mole*	Mole %	Residues/ mole*
Ala	15.65	10	11.62	7
Arg	3.27	2	3.30	2
Asn+Asp	12.87	8	10.75	7
Cys	7.93	5	8.11	5
Gln+Glu	14.59	9	16.78	10
Gly	12.58	8	13.95	9
His	0.00	0	0.00	0
Ile	1.74	1	0.83	1
Leu	6.56	4	6.20	4
Lys	4.19	3	7.44	5
Met	0.90	1	0.00	0
Phe	6.24	4	7.84	5
Pro	N.D.	-	N.D.	-
Ser	2.90	2	4.48	3
Thr	2.47	2	2.54	2
Trp	N.D.	-	N.D.	-
Tyr	6.35	4	4.88	3
Val	1.76	1	1.28	1

* Based on 2 Arg/mole

N.D. Not determined

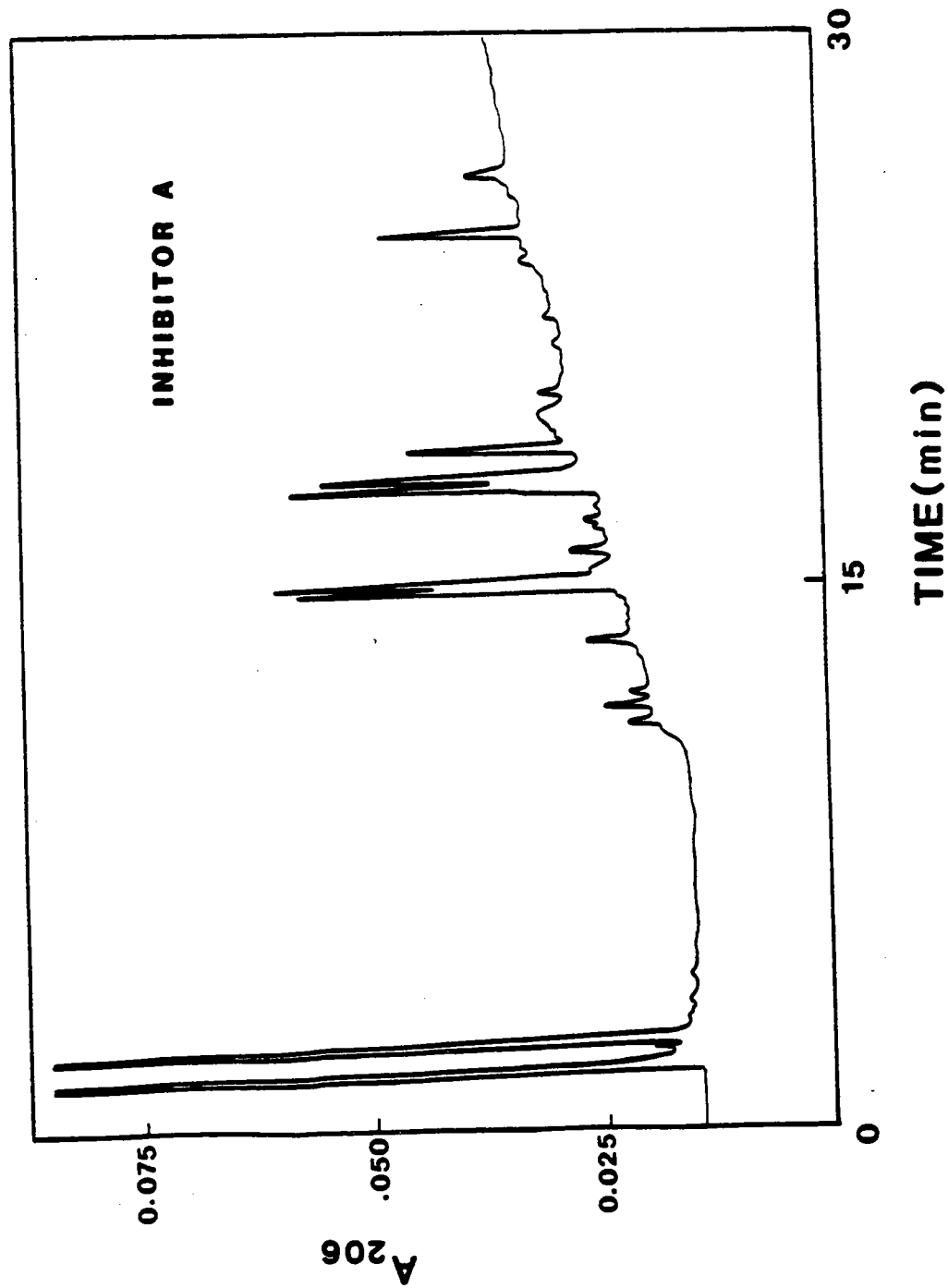


Fig. 26. Tryptic cleavage pattern for S-carboxymethylated trypsin inhibitor A.

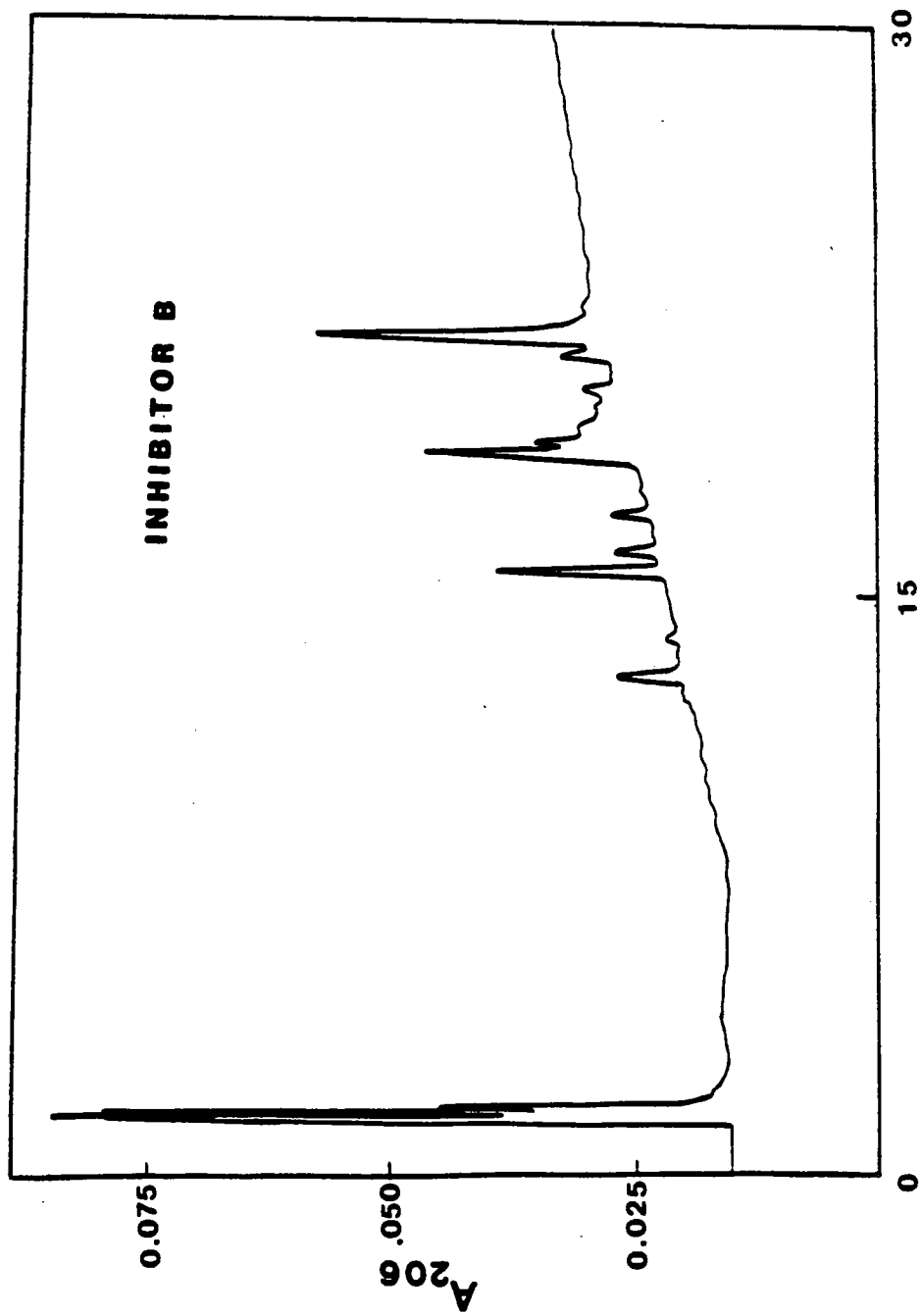


Fig. 27. Tryptic cleavage pattern for S-carboxymethylated trypsin inhibitor B.

inhibitor A and fifty-three amino terminal residues were elucidated for inhibitor B. This data is presented in Fig. 28. Interestingly, inhibitor B was heterogenous at cycle 20 (Phe and Tyr) and at cycle 33 (Thr and Gly), which is consistent with the presence of two isoelectric forms demonstrated by isoelectrofocusing.

Comparisons of the amino terminal amino acid sequences of the hemolymph inhibitors A and B with the sequences of other known serine protease inhibitors revealed that both inhibitors A and B were homologous to the BPTI family of trypsin inhibitors (Fig. 29). The conserved amino acid residues in all BPTI-like proteins have been boxed in Fig. 29. Accordingly, BPTI enumeration has been used throughout this thesis in discussions on the sequence of inhibitors A and B. When compared to BPTI, inhibitor A has 17 additional amino acid residues at the amino terminal region. Therefore, of the fifty-six known residues of inhibitor A only 39 residues correspond to BPTI-like protease inhibitors. Inhibitor A and BPTI are identical at 14 of 39 (36%) positions. If conservative substitutions are considered, nearly 50% of the amino acids are homologous. In the case of inhibitor B, 19 of the 53 residues (36%) are identical with BPTI. If conservative substitutions are considered, 50% of inhibitor B is homologous to BPTI. Inhibitors A and B share 24 of the 38 common residues.

Lys 15 has been shown to be the specificity conferring residue in BPTI (40). The peptide bond between Lys 15 and Ala 16 of BPTI has been designated as the reactive site peptide bond. Both inhibitor A and inhibitor B have Arg at the position corresponding to Lys 15 of BPTI. Since Arg and Lys are both basic amino acids which are recognized by

trypsin-like proteases, this residue, namely, Arg 15 is presumably the specificity conferring residue in both inhibitors A and B, and the peptide bond between Arg 15 and Ala 16 is the reactive site peptide bond in these inhibitors.

Crystallographic studies (77) have shown that the BPTI molecule has two β -pleated sheet regions, the first extends between amino acids 16 and 23 (BPS-I) and the second extends between residues 27 and 38 (BPS-II). In addition, the BPTI molecule also has an α -helical structure within the carboxy terminus (between residues 46 and 56) and another α -helix at the amino terminal section (between residues 2 and 7). Our calculations based on Chou-Fasman algorithms for predicting the secondary structures in proteins were able to confirm these observed secondary structures of BPTI.

A summary of the secondary structure predictions for inhibitors A, inhibitor B, and BPTI using Chou-Fasman algorithms is given in Table 8. A β -pleated sheet regions at positions corresponding to BPS-I could be predicted in trypsin inhibitor A and in trypsin inhibitor B. Neither inhibitor A nor inhibitor B were predicted to have the NH_2 -terminal α -helix present between residues 2 through 7 of BPTI. The region corresponding to this α -helix was predicted to have a β -pleated sheet structure in inhibitor A (residues -1 through 7), while inhibitor B was predicted to have an unstructured region (residues 2 through 15). The amino acid residues 45 through 54 of BPTI have been predicted to form an α -helix, the corresponding region in inhibitor B (residues 45 through 50) was also predicted to form α -helix. Predictions of the secondary structures beyond residue 30 (using BPTI enumeration, see

Table 8

Comparison of the secondary structure predictions for BPTI, trypsin inhibitor A, and trypsin inhibitor B using Chou-Fasman calculations.

Predictions for BPTI	Predictions for Inhibitor A	Predictions for Inhibitor B
	coil (-17 to -9)	
	α -helix (-8 to -2)	
α -helix (2 to 7)	β -pleated sheet (-1 to 7)	coil (2 to 15)
coil (8 to 15)	α -helix (8 to 13)	coil (2 to 15)
β -pleated sheet (16 to 23)	β -pleated sheet (14 to 24)	β -pleated sheet (16 to 24)
coil (24 to 26)	coil (25 to 30)	coil (25 to 31)
β -pleated sheet (27 to 38)	N.D.*	β -pleated sheet (32 to 40)
coil (39 to 44)	N.D.	coil (41 to 44)
α -helix (45 to 54)	N.D.	α -helix** (45 to 50)

The numbers in parentheses denote the extent of each prediction.

- * Prediction of secondary structures were not performed for residues 31 to 39 of inhibitor A because this sequence contains two undetermined amino acid residues.
- ** α -helix probably extends beyond residue 50 in inhibitor B. The extent of this α -helix could not be fully predicted due to the undetermined residue at position 52 and due to the lack of sequence data beyond residue 53.

Fig. 28) was not attempted for inhibitor A because the sequence 31-39 contains two unidentified amino acids (residues 31 and 38) and further, the sequence of inhibitor A, at present, is not known beyond the position corresponding to residue 39 of BPTI.

C. The effect of trypsin inhibitors A and B on the activation of prophenoloxidase from a hemocyte lysate of *Manduca sexta*

When crude hemolymph was treated with an equal volume of 0.1% SDS, phenoloxidase activity appeared after 10 minutes. However, if diisopropylfluorophosphate (DFP, an inhibitor of serine proteases) at a concentration of 0.01 M was added to crude hemolymph prior to adding SDS, phenoloxidase activity did not appear even after 30 minutes (Fig. 30). This indicated that serine protease(s) are involved in the activation of prophenoloxidase. Therefore, it was of interest to test whether purified trypsin inhibitors A and B would affect the activation of prophenoloxidase. A hemocyte lysate preparation was used to study the effect of inhibitors A and B on prophenoloxidase activation, because hemocytes are generally considered to be the source of prophenoloxidase (27).

Sonication of the hemocytes was observed to cause activation of prophenoloxidase, but maximum phenoloxidase activity was obtained only after incubating hemocyte lysates with laminarin and $MgCl_2$ for 1 hour at room temperature (78). In this study inhibitors A and B were added at a concentration of 8.6 μM and 27.4 μM , respectively, both during sonication of the hemocytes and during the subsequent incubation of the lysates with laminarin. The results given in Table 9 demonstrate that addition of inhibitor A caused ca. 50% reduction in phenoloxidase

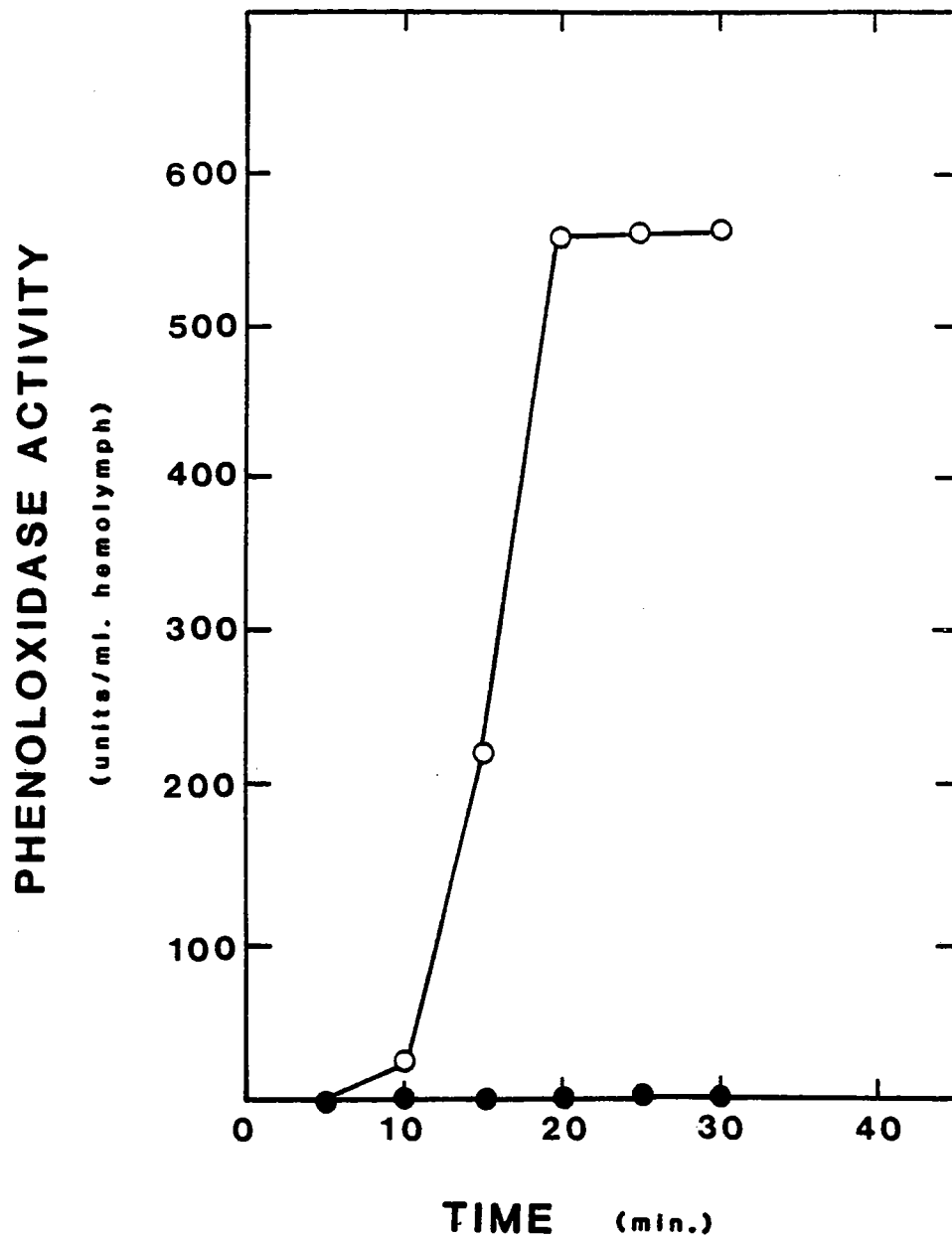


Fig. 30 The effect of DFP on the appearance of phenoloxidase activity in SDS-treated hemolymph of the larval stage Manduca sexta.

- Phenoloxidase activity in the absence of DFP
- Phenoloxidase activity in the presence of 0.01 M DFP

Table 9

The effect of trypsin inhibitors A and B on the activation of prophenoloxidase in lysates of Manduca sexta hemocytes.

Test system	Phenoloxidase activity
No inhibitors (control)	100
With inhibitor A (8.6 μ M)	52*
With inhibitor B (27.4 μ M)	110*

* % of control activity

activity. However, inhibitor B, even at three times the concentration did not diminish the appearance of phenoloxidase activity.

D. Does the hemolymph of *Manduca sexta* contain factors functionally analogous to human complement factors B and D?

Although a complement system has not been unequivocally demonstrated in insects, the general mechanism of activation of prophenoloxidase in insect hemolymph by yeast zymosan or laminarin is similar to the activation of the mammalian complement system via the alternative pathway (21, 25). Since many key biochemical pathways are evolutionally conserved, it was of interest to examine whether the hemolymph of *Manduca sexta* could functionally substitute in hemolytic assays for the factors B and D of the human alternative complement pathway.

Rabbit erythrocytes (RE) are natural activators for the alternative complement pathway and are hemolysed when added to fresh or fresh frozen serum. It was, therefore, of interest to investigate whether RE could act as an activator for factors present in *Manduca sexta* hemolymph. Incubation of RE with the hemolymph of *Manduca sexta* did not result in hemolysis suggesting that a completely functional alternative complement pathway leading to the lysis of rabbit erythrocytes was absent in the hemolymph of *Manduca sexta*. In addition, components in crude hemolymph did not appear to substitute for either factor B or factor D when assayed in sera deficient in factors B (RB) and D (RD) (Table 10). At face value these results suggested that proteases analogous to human factors B and D may not be present in *Manduca sexta* hemolymph. Interestingly, a heat labile factor present in *Manduca sexta* hemolymph abrogates RE hemolysis when

Table 10

Experiments to assess the effects of Manduca sexta hemolymph and purified trypsin inhibitors A and B on the activity of the human serum alternative complement pathway.

Experiment	Hemolysis (% of control)*
RB serum + factor B	100
RB serum + 8 μ l hemolymph	0
RD serum + factor \bar{D} (functionally pure)	102
RD serum + 10 μ l hemolymph	0
RB serum + 25 μ l hemolymph + factor B	0
RB serum + factor B + 25 μ l boiled hemolymph	96
RB serum + factor B + inhibitor A (0.43 nmoles)	99
RB serum + factor B + inhibitor B (1.67 nmoles)	98

* Hemolysis by 25 μ l of a 1:3 dilution of RB serum reconstituted with 4 μ g/ml of purified factor B was taken as 100%.

using RB serum reconstituted with purified human factor B (reconstitution of RB serum with 4 μ g/ml factor B fully restores the alternative pathway complement activity). The observed inhibition of hemolysis could be due to the inhibition of the function of one or more alternative complement pathway components by hemolymph or to the non-productive, non-lytic, fluid-phase proteolytic activation of factor B by proteases present in the hemolymph. For that matter, the hemolymph could be acting to inhibit the action of any of the complement proteins D, B, properdin, or C3 to C9 by activating non-productively. The addition of purified inhibitors A or B to the assay for the alternative complement pathway had no effect (Table 10). The amount of inhibitor A and inhibitor B added were, on a molar basis, respectively, 10 fold and 40 fold in excess of the amount of factor B present in the assay mixture. These results, therefore demonstrate that neither inhibitor A nor inhibitor B were the factors present in hemolymph which inhibited activation of the human alternative complement pathway.

To assess whether fluid phase cleavage of factor B did indeed occur on addition of Manduca sexta hemolymph, fresh frozen human serum and purified human factor B were each separately incubated with Manduca sexta hemolymph and the extent of factor B cleavage was assessed by immunoelectrophoresis (Fig. 31). Purified factor B was not cleaved by the addition of Manduca sexta hemolymph, suggesting that the Manduca sexta hemolymph does not contain proteases capable of cleaving human factor B. By contrast, factor B cleavage fragment, Bb, was observed when fresh frozen serum was incubated with Manduca sexta hemolymph suggesting that the hemolymph stimulates the initial events in the

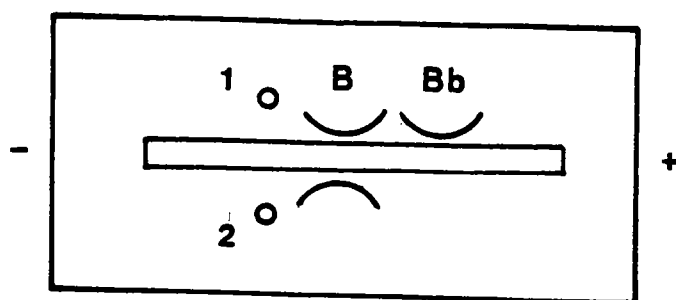


Fig. 31 Schematic diagram depicting immunoelectrophoretic assessment of human factor B cleavage.

Well 1 Fresh frozen serum incubated with hemolymph

Well 2 Fresh frozen serum incubated with GVBS

B Precipitin band of uncleaved factor B

Bb Precipitin band of Bb fragment

activation of the alternative complement pathway but does not itself provide a protease analogous to factor D which cleaves factor B. Furthermore, the factor(s) in hemolymph responsible for causing human factor B cleavage is not likely to be non-specific stimulators such as lipopolysaccharides, laminarin or zymosan because heat inactivated hemolymph did not cause cleavage of factor B in fresh frozen human serum. Another distinct possibility is that the hemolymph may provide a C3b-like cofactor which forms a stable fluid-phase complex with factor B. Factor B thus complexed may be cleaved by factor D. If the complex of the hemolymph factor and Bb is active as C3 convertase, it can form more C3b, which would combine with factor B and cause an extensive cleavage of the latter factor (see Appendix on cobra venom factor). The observation that hemolymph causes factor B cleavage only in serum and not purified factor B supports this contention. In the latter case, factor D is not present to cleave factor B in complex with the hemolymph factor. Further, since the complex is present primarily in the fluid phase and not deposited on RE, hemolysis does not ensue the activation of the complement pathway in a hemolytic assay in the presence of Manduca sexta hemolymph.

CHAPTER IV

DISCUSSION

A. Comparisons of the structures and properties of trypsin inhibitors A and B from *Manduca sexta* with bovine pancreatic trypsin inhibitor (BPTI)

Based on the observation that polypeptide protease inhibitors form strong complexes with proteases, a relatively simple protocol for the isolation of trypsin inhibitors from the hemolymph of the *Manduca sexta* larvae was developed. At least two inhibitors present in the hemolymph bound to immobilized trypsin and required 0.1 M HCl containing 0.75 M NaCl (pH 1.5) for elution. The catalytic action of trypsin is essential for the formation of BPTI-trypsin complex (79), consequently the stability of the BPTI-trypsin complex is lowest at acid pH when trypsin is catalytically not active. By analogy, the stability of the complexes between inhibitors A or B and trypsin should be lower at acid pH. In view of the stability of inhibitors A and B at acid pH, it was decided to use acid buffers for the elution of the bound inhibitors from trypsin-Sepharose. However, approximately 50% of the total trypsin inhibitory activity in crude hemolymph was lost following trypsin-Sepharose affinity chromatography. Elution with 0.1 M HCl containing 1.5 M NaCl or 0.1 M ammonium acetate adjusted to pH 3.0 with glacial acetic acid did not improve the yield of trypsin inhibitors A or B. Therefore, it appears that there may be yet other trypsin inhibitory component(s) which are bound very tightly trypsin-Sepharose. Further attempts to characterize this (these) component(s) were not performed. Both inhibitor A and inhibitor B are stained

weakly by coomassie brilliant blue G 250 or R 250 and amido black suggesting that these inhibitors do not have strong binding sites for these dyes. Since some glycoproteins also do not stain with the common protein stains (80), trypsin inhibitors A and B were subsequently tested for the presence of carbohydrates. Neither inhibitor A nor inhibitor B stained with Alcian blue or periodic acid-Schiff's base stains suggesting that they do not contain appreciable carbohydrate.

Trypsin inhibitors from Manduca sexta hemolymph share many of the properties of BPTI. For example, inhibitors A and B, like BPTI, are both heat and acid-stable and inhibit trypsin-like serine proteases. A high proportion of secondary structure (β -pleated sheet) and the presence of three intramolecular disulfide bonds are thought to be responsible for the stability of the BPTI molecule (77). Since the three half-cystine residues so far unambiguously identified in inhibitor A and the five half-cystine residues thus far sequenced in inhibitor B are found in corresponding positions in BPTI (Fig. 29), it is likely that the stability conferred by these disulfide bonds also is present in the Manduca sexta trypsin inhibitors. Moreover, the importance of exposed disulfide bonds towards the structural stability of inhibitors A and B is illustrated by the fact that reduction of either inhibitors under relatively mild nondenaturing conditions (2.5 mM DTT in 20 mM tris-HCl buffer, pH 7.5) leads to the complete loss of biological activity. By contrast, reduction of the exposed disulfide bond (between Cys 14 and Cys 38) in BPTI by sodium borohydride (81) does not affect BPTI activity. Clearly other intramolecular interactions in the molecule apart from the Cys 14-Cys 38 disulfide bond provide for the

integrity of the BPTI structure. It is, therefore, reasonable to presume that some of the interactions which provide for the integrity of the BPTI structure may not be present in inhibitors A and B.

In order to examine the possibility that inhibitor B (the lower molecular weight inhibitor) was derived from inhibitor A by limited proteolysis, each inhibitor was subjected to amino acid compositional analysis (Table 7), and tryptic peptide maps of each protein were prepared. The amino acid compositions are different and the tryptic peptide map of each inhibitor is unique. Moreover, the NH₂-terminal amino acid sequences of inhibitor A and B were different proving conclusively that each was a different protein. Nevertheless, close inspection of the amino acid sequences revealed that inhibitors A and B were homologous proteins and share 18 out of the 38 common residues (Fig. 28).

Trypsin inhibitors A and B inhibit the activities of trypsin and trypsin-like serine proteases (Table 6), but at picomolar concentrations of the proteases an order of magnitude more of inhibitors A and B are required for plasmin and thrombin inhibition than for trypsin inhibition. Weinstein and Doolittle (82) have studied the hydrolysis of a series of synthetic peptide and ester substrates by trypsin, plasmin, and thrombin and have concluded that plasmin cleaves Lys-X peptide bonds preferentially, while thrombin is selective for Arg-X peptide bonds. Trypsin hydrolyses both Lys-X and Arg-X peptide bonds. Trypsin inhibitors A and B both have Arg at the reactive site (Arg 15). Therefore, it is understandable that a relatively Lys specific protease like plasmin would have a lower affinity towards inhibitors A and B.

Among trypsin, plasmin, and thrombin, the protease which shows the highest selectivity is thrombin. Thrombin is selective for Arg-X peptide bonds and cleaves only four Arg-Gly peptide bonds in its natural substrate fibrinogen. It is noteworthy that BPTI which has reactive site Lys (Lys 15) does not inhibit thrombin. Since both inhibitors A and B have Arg-Ala peptide bond at their presumed reactive sites, it is not unexpected that these inhibitors recognize and thus inhibit thrombin. However, the peptide conformation around the reactive site in inhibitors A and B may not be conducive for the rapid interaction with thrombin as it is with trypsin. Consequently, trypsin inhibitors A and B display lower affinity for thrombin than for trypsin.

Interestingly, chymotrypsin is strongly inhibited by trypsin inhibitors A and B although chymotrypsin does not exhibit specificity for basic amino acids. Elastase, however, is not inhibited by either trypsin inhibitor A or trypsin inhibitor B. Trypsin, chymotrypsin, and elastase belong to the same family of pancreatic proteases and the active site region of all the three proteases are similar. The specificity pocket of elastase can accommodate only small aliphatic side chains and cannot accommodate the side chain of Arg 15 of inhibitors A and B. Consequently, this protease cannot bind to trypsin inhibitors A and B. By comparison, chymotrypsin has a larger specificity pocket which may accommodate larger side chains. Studies with molecular models of chymotrypsin and BPTI have shown that Lys 15 of BPTI can be accommodated within the specificity pocket of chymotrypsin provided the pK of the ϵ -amino group of Lys is lowered or the positive charge on Lys 15

is buried. Moreover, the amino acid residues of trypsin which interact with BPTI are conserved in chymotrypsin (83). Inhibitors A and B are homologous to BPTI (see below). By analogy to BPTI, it may be expected that Arg 15 of inhibitors A and B will also be accommodated within the specificity pocket of chymotrypsin. Hence, trypsin inhibitors A and B are able to inhibit chymotrypsin.

Neither trypsin inhibitor A nor trypsin inhibitor B inhibit proteases from bacteria or plants. In fact, the bacterial protease subtilisin BPN' completely inactivates trypsin inhibitors A and B. BPTI, by contrast, is not inactivated by subtilisin BPN'. However, like trypsin inhibitors A and B, BPTI also does not inhibit subtilisin BPN'.

BPTI forms a 1:1 complex with both trypsin and chymotrypsin (84). Stoichiometric calculations (see page 71) based on molecular weights of 15,000 for inhibitor A and 24,000 for both trypsin and chymotrypsin demonstrates that trypsin inhibitor A likewise forms a 1:1 complex with trypsin as well as chymotrypsin. Inhibitor A is a dimer composed of two identical subunits; the formation of 1:1 molar complexes with trypsin and chymotrypsin suggests that both the subunits are needed for complex formation. However, it is also possible that binding of a protease to a subunit renders the other subunit unavailable due to steric hindrance. As mentioned earlier, stoichiometric calculations and determination of K_i were not performed with inhibitor B, because inhibitor B appears to have two components.

The calculated inhibitory constants, K_i values, for inhibitor A interaction with trypsin and chymotrypsin are 1.45×10^{-8} M and $1.7 \times$

10^{-8} M respectively. Table 11 summarizes the K_i values for several members of the BPTI family of trypsin inhibitors. Inhibitor A has six orders of magnitude less affinity for trypsin than BPTI. When compared to other trypsin inhibitors from lower animals, inhibitor A displays approximately 1/100 the affinity for trypsin.

Inhibitor A forms a tight equimolar complex with trypsin which is not dissociated by chymotrypsin. Similarly, the chymotrypsin-inhibitor A complex cannot be displaced by free trypsin, which is in agreement with their inhibition constants. Further, binding of one protease to inhibitor A precludes the binding of the other protease thereby indicating that inhibitor A does not have independent binding site for each protease. However, the present results do not indicate whether trypsin and chymotrypsin bind to the same site on inhibitor A, or the binding sites of trypsin and chymotrypsin are close such that binding of one protease sterically hinders the binding of the other protease. X-ray crystallographic studies have shown that BPTI binds to the active site region of trypsin (58). Molecular models based on the crystal structure of chymotrypsin and the structure of BPTI (83) have shown that BPTI can bind to the active site of chymotrypsin provided the pK of the ϵ -amino group of Lys 15 is lowered or the positive charge on this residue is buried. Although inhibitors A and B have Arg 15 instead of Lys 15, other structural features of inhibitors A and B closely resemble that of BPTI (see below). Therefore, it is probable that inhibitor A binds to chymotrypsin and trypsin in a manner similar to BPTI.

Table 11

Representative inhibition constants (K_i) for trypsin inhibition by trypsin inhibitors of the BPTI (Kunitz) family.

Inhibitor	Reactive site (P_1) amino acid*	K_i (M)	Reference
BPTI	Lys 15	6.0×10^{-14}	55
Manduca sexta inhibitor A	(Arg 15)	1.45×10^{-8}	
Cow colostrum inhibitor	Lys 15	4.0×10^{-12}	91
Snail iso-inhibitors	Lys 15	10^{-9} to 10^{-11}	90
Sea anemone inhibitor II	Arg 15	3.0×10^{-10}	89
Cuttlefish iso-inhibitor L	Arg 15	2.2×10^{-10}	88
Russell viper venom inhibitor	Arg 15	7.6×10^{-10}	87

*Using BPTI enumeration, see Fig. 29.

Comparison of the amino acid sequences of trypsin inhibitor A and trypsin inhibitor B with that of BPTI (Kunitz) (Fig. 29) revealed that these inhibitors are homologous to the latter protein. Before examining the homology in detail, it is necessary to briefly review the structure of BPTI. BPTI is a pear shaped molecule which is very compact and rigid (77). The rigidity and the compactness are, in part, attributable to the three disulfide bonds present in the molecule. On account of its small size, BPTI has only a small proportion of internal residues. The aromatic residues Phe 4, Tyr 21, Phe 22, Tyr 23, Phe 33, Tyr 35, and Phe 45 (see Fig. 3) are present inside the molecule and are, therefore, important structural determinants. The side chain of Asn 43, which is also present in the molecular interior, participates in three crucial hydrogen bonds (with the carbonyl groups of Glu 7 and Tyr 23 and the amino group of Tyr 23) in the center of the molecule (85). BPTI polypeptide chain folding requires that residues at positions 12 and 37 not have a side chain. Accordingly, Gly is present at both of these positions.

Inhibitor A has an extra segment of 17 residues at its amino terminus which is not present in BPTI. Consequently, it is possible to compare only residues 18 through 56 of inhibitor A with BPTI. Inhibitor B, on the other hand, does not have the extra amino terminal segment, and therefore, all 53 residues of inhibitor B thus far sequenced can be compared with BPTI. A comparison of the amino acid sequences of trypsin inhibitors A and B with those of selected members of the BPTI (Kunitz) family of trypsin inhibitors is given in Fig. 29. For the

ease of comparisons, the numbering system used corresponds to that of BPTI.

Half-cystine residues are, in general, invariant among the BPTI family of trypsin inhibitors (85). The three half-cystine residues unambiguously indentified in inhibitor A and the five half-cystine residues thus far identified in inhibitor B are homologous to BPTI. The internal aromatic residues Tyr 21, Tyr 23, Phe 33, and Tyr 35 are clearly conserved in inhibitor A and inhibitor B. Phe 4 has been replaced by Ile in both inhibitors A and B, however, since Ile is also hydrophobic, this replacement is not expected to affect the local interactions in that region. The conservation of Phe 45 and Asn 43 are apparent in inhibitor B, but the lack of sequence data does not allow the comparison of these residues in inhibitor A. The strict conservation of Gly residues at 12 and 37 are evident in both inhibitor A and in inhibitor B. The aromatic residue Phe 22 however, has been replaced by Gly in inhibitor A and Ala in inhibitor B.

A comparison of the trypsin contact residues of BPTI with the homologous sequences in inhibitor A and inhibitor B deserves attention. In the BPTI molecule, the peptide segments extending from residue 12 to 18 and 35 to 39 are intimately involved in the contact with trypsin. The side chain of Lys 15 is inserted into the specificity pocket of trypsin with the ϵ -amino group hydrogen bonded to the β -carboxylate of Asp-189 of trypsin. A comparison of the amino acid residues at the trypsin contact region of BPTI with homologous sequences of inhibitor A and inhibitor B is presented in Fig. 32. Of the 12 residues compared, six residues, namely Gly 12, Cys 14, Ala 16, Tyr 35, Gly 36,

<u>Residue number</u>	<u>12</u>	<u>13</u>	<u>14</u>	<u>15</u>	<u>16</u>	<u>17</u>	<u>18</u>	<u>35</u>	<u>36</u>	<u>37</u>	<u>38</u>	<u>39</u>
BPTI	G	P	C	K	A	R	I	Y	G	G	C	R
Inhibitor A	G	V	C	R	A	L	F	Y	G	G	X	Q
Inhibitor B	G	P	C	R	A	G	F	Y	G	G	C	Q

Fig. 32 Comparison of the trypsin binding region of BPTI with the homologous regions of trypsin inhibitors A and B.

and Gly 37, are conserved in inhibitor A. Lys 15 is conservatively replaced by Arg in inhibitor A and the hydrophobic residue Ile 18 is replaced by another hydrophobic residue Phe. Since half-cystine residues are conserved among the members of the BPTI (Kunitz) family, it is likely that the amino acid at position 38 of inhibitor A (Fig. 29) be a half-cystine residue. In trypsin inhibitor B, eight of the twelve residues namely Gly 12, Pro 13, Cys 14, Ala 16, Tyr 35, Gly 36, Gly 37, and Cys 38 are conserved compared to BPTI. As found for inhibitor A, Lys 15 and Ile 18 are conservatively substituted by Arg 15 and Phe 18, respectively, in inhibitor B.

The types of interactions between the contact regions of BPTI and bovine trypsin are listed in Table 3. From the table it is clear that Lys 15 is a major contributor of polar (four hydrogen bonds) and van der Waals interactions. Lys 15 of BPTI has been replaced by Arg in both inhibitors A and B. Since Arg is also a basic amino acid, it may be expected to enter into similar interactions as Lys 15 of BPTI.

Two residues at the trypsin contact region which are strikingly different in trypsin inhibitors A and B from those of BPTI are Arg 17 and Arg 39. Arg 17 is replaced by Leu in inhibitor A and Gly in inhibitor B, while Arg 39 is replaced by Gln in both inhibitors A and B. Blow *et al.* (83) have noted that BPTI makes about 217 van der Waals contacts with trypsin and, of these ca. 60% (135 contacts) are contributed by Arg 17 and Arg 39. The absence of both of these residues from inhibitor A and inhibitor B may explain the lower K_i which was found, at least in the case of inhibitor A to be 10^{-8} M vs K_i for trypsin-BPTI interaction which is 10^{-14} M (55). In addition, Pro 13 is replaced by

Val in inhibitor A. The side chain of Pro 13 of BPTI does not enter into critical interactions with trypsin, therefore the replacement of Pro by Val may not affect the binding of inhibitor A to trypsin.

A comparison of the secondary structure predictions of inhibitor A, inhibitor B, and BPTI using Chou-Fasman calculations are tabulated in Table 8. Perusal through this table shows that the secondary structure predictions for inhibitor B is similar to that of BPTI, with the exception that the amino terminal α -helix of BPTI is replaced by a random coil. Comparison of the secondary structure predictions of inhibitor A with BPTI revealed that there are some similarities and some differences between these two proteins. The amino acid residues of inhibitor A from Ala 16 to Asp 24 was predicted to assume β -pleated sheet structure. This would correspond to the β -pleated sheet structure BPS-I (between Ala 16 and Tyr 23) of BPTI.

The amino acid sequences of inhibitor A (from Tyr -1 to Leu 7) was predicted to nucleate a β -pleated sheet, whereas the corresponding region in BPTI harbors an α -helix. Further, an α -helix was predicted from Pro 8 to Val 13 of inhibitor A. By contrast, the sequence corresponding to this α -helix of inhibitor A was predicted to be a random coil in BPTI. Pro residues, in general, disrupt both α -helix and β -pleated sheet structures in proteins. The absence of Pro 9 and Pro 13 in inhibitor A (but which are present in BPTI) may facilitate the nucleation of α -helix between Pro 8 and Val 13. An α -helix was predicted within the unique amino terminal peptide sequence of inhibitor A (between Val -2 and Ile -8).

The amino terminal region of inhibitor A appears to be highly structured with alternate α -helical and β -pleated sheet regions. One may speculate that this conformation may be necessary for the efficient interaction of inhibitor A with its unique target protease in the hemolymph.

It was pointed out earlier (page 108) that most of the critical residues essential for the folded conformation of BPTI are conserved in inhibitors A and B. This observation together with the finding that the secondary structure of inhibitor B closely resembles that of BPTI indicates that the conformation of at least inhibitor B, and probably the region of inhibitor A homologous to BPTI as well, resembles that of BPTI. Further, the conservation of the amino acid residues in trypsin inhibitors A and B at positions homologous to the trypsin binding region of BPTI indicate that trypsin inhibitors A and B probably interact with trypsin in a manner analogous to BPTI.

B. The possible biological significance of trypsin inhibitors A and B from *Manduca sexta* hemolymph

It was pointed out in the introduction section that a number of physiological processes in insects such as tissue turnover during metamorphosis, activation of hemolymph prophenoloxidase, and coagulation require the action of proteolytic enzymes. Some of these processes may involve several proteases participating in a cascade. The proteolytic activation of hemolymph prophenoloxidase, for example, has been suggested to resemble the initial events in the activation of the human complement alternative pathway (21, see also section C below). Efficient control mechanisms should exist for the regulation of hemolymph pro-

teases. The presence of a large amount of protease inhibitors in the hemolymph of all insects examined suggest that these inhibitors are important in the regulation of proteases in these animals.

Three lines of evidences suggest that trypsin inhibitors in Manduca sexta hemolymph may be important in regulating the activity of hemocyte and hemolymph prophenoloxidase. First, spontaneous activation of hemolymph prophenoloxidase in vitro is slower in the larval hemolymph than in the the pupal hemolymph, and this activity is inversely related to the amount of trypsin inhibitory activity (cf. Fig. 7). Second, the prophenoloxidase activating enzyme of a closely related species Bombyx mori has been purified and has been shown to be a serine protease with trypsin-like specificity (19). Partial purification of a prophenoloxidase activating enzyme from Manduca sexta has also been achieved and this enzyme also appears to be a serine protease with trypsin-like specificity. Since both inhibitors A and B are trypsin inhibitors, it is not unreasonable that these inhibitors could control the activity of this prophenoloxidase activating enzyme. Finally, studies reported here have shown that addition of purified inhibitor A to a hemocyte lysate decreased the rate of appearance of phenoloxidase activity (Table 9). Interestingly, inhibitor B, even at three times the concentration of inhibitor A, did not affect the activation of prophenoloxidase suggesting that inhibitor A is involved in the control of prophenoloxidase activation while inhibitor B may not be important. Although the exact reason for this selectivity is not immediately obvious, it is reasonable to assume that the selectivity is related to the structure of these inhibitors. The amino terminus of inhibitor A

has 18 additional residues not found in inhibitor B (see Fig.28) nor BPTI or other known trypsin inhibitors of the BPTI (Kunitz) class for that matter. The extra amino acid residues may be involved in the selective recognition and therefore the inhibition of one or more proteases involved in prophenoloxidase activation.

During the pupal stage, insect tissues undergo extensive turnover and proteolytic enzymes play a key role in this process (7). Protease inhibitors have been implicated in the control of metamorphosis-associated tissue proteolysis (46). The decreased activity of the trypsin inhibitors observed in the pupal stage in contrast to the larval stage is consistent with this hypothesis. It is not known if the decreased activity of the trypsin inhibitors is due to a lower biosynthetic rate or to the inhibitors being preferentially catabolized during the pupal stage so that they do not interfere with tissue proteolysis. The possibility that the hemolymph trypsin inhibitors could be taken up by the surrounding tissues and regulate intracellular proteolysis cannot be excluded. Further studies on the isolated proteases involved in tissue catabolism during metamorphosis and the effect of hemolymph protease inhibitors on these proteases will obviously be important in understanding the various control events during metamorphosis.

Apart from Manduca sexta, protease inhibitors homologous to BPTI have been isolated from such diverse sources as humans (86), snake venom (87), cuttlefish (88), and sea anemone (89). The gene coding for the BPTI (Kunitz) class of protease inhibitors thus appears to be very old and well distributed in the animal kingdom. The high degree of

amino acid sequence homology among the diverse members of the BPTI (Kunitz) family of protease inhibitors (for example see Fig.29) demonstrates that this family evolved from a common ancestor very early in evolution.

The gene coding for BPTI (Kunitz) class of protease inhibitors appears to have been retained by primitive as well as more evolved animals, suggesting that these gene products serve important function(s) in the organism. Although the precise physiological functions of this group of proteins is not clear, their common property as protease inhibitors suggests some functions in common. Accordingly, our studies have indicated that Manduca sexta trypsin inhibitor A probably plays a role in the regulation of the proteolytic activation of prophenoloxidase in Manduca sexta hemocyte lysate (see page 92).

A discussion on the homology among the Manduca sexta trypsin inhibitors A and B and BPTI (Kunitz) was presented earlier (pages 108 to 112). The BPTI molecule has a very compact and rigid conformation which will not accommodate the addition or deletion of amino acid residues (85). Consequently none of the BPTI (Kunitz) class of protease inhibitors show any deletion or addition of amino acid sequences within the BPTI (Kunitz) domain. The amino acid sequences of inhibitor A and inhibitor B obtained thus far also suggest that there may be no insertions or deletions within the BPTI (Kunitz) domain of these inhibitors.

As a general rule, during the divergent evolution of a family of proteins, the region of the polypeptide which defines the active site is conserved even though other regions of the polypeptide may undergo

considerable change (92). In accordance with this rule, most of the amino acid residues at positions homologous to the trypsin binding regions of BPTI (residues 12 - 18 and 35 - 39) are either conserved or conservatively substituted in inhibitor A and inhibitor B (page 108). Striking conservation of certain residues in the sequence presented in Fig. 29 pinpoints those residues crucial for maintaining the folded conformation. Although the amino acid sequence data is not complete, comparisons of the available amino acid sequences show that many critical residues are conserved in both inhibitors A and B. Hence, these inhibitors may be expected to assume a three dimensional structure similar to that of BPTI.

As alluded to earlier, inhibitor A has 17 additional amino acid residues at the amino terminus when compared to BPTI. These amino acid residues are clearly not critical for the protease inhibitory function, because inhibitor B, which does not possess these residues, and inhibitor A show similar inhibitory specificities towards various proteases. It is, however, possible that these additional amino terminal residues may be involved in the biological function of inhibitor A or, alternatively, may serve to modulate the affinity of inhibitor A towards proteases. For example, only inhibitor A appears to inhibit the activation of prophenoloxidase from hemocyte lysates of Manduca sexta.

In many organisms, the BPTI (Kunitz) family of protease inhibitors are present in multiple forms which differ slightly in their primary structure. For instance, from snails twelve homologous inhibitors of the BPTI (Kunitz) class have been identified (90), and sea

anemone has been shown to possess at least ten homologous BPTI-like trypsin inhibitors (89). Similarly, in this work it has been demonstrated that the hemolymph of Manduca sexta contains at least two homologous trypsin inhibitors, namely inhibitor A and inhibitor B. The strong amino acid sequence homology between inhibitor A and inhibitor B indicate that these inhibitors probably arose by gene duplication and subsequent diversification. The requirement for more than one form of inhibitor is not entirely clear; perhaps inhibitors with slightly different specificities and/or kinetic properties may be required to regulate the activity of proteases in different tissues.

C. Is there a system in Manduca sexta which resembles the vertebrate alternative complement pathway?

The vertebrate complement system is both an amplifier of specific immunity and a nonspecific effector of immune reactions (see Appendix for a description of the human complement system). A complete vertebrate type complement system has not yet been demonstrated in insects, nevertheless, a cobra-venom dependent "lytic complement-like system" capable of hemolyzing sheep erythrocytes has been shown to be present in the hemolymph of more primitive animals such as the horse-shoe crab, Limulus polyphemus (93), and in the coelomic fluid of the sea urchin Strongylocentrotus droebachiensis (94) and the star fish Asterius forbesi (93). Furthermore, known activators of the alternative pathway of complement such as fungal polysaccharides, yeast zymosan and laminarin also induce the activation of prophenoloxidase in insect hemolymph (21) and in the hemolymph of more primitive arthropods such as the horse-shoe crab (78) and the crayfish (95). In all these

cases these activators appear to activate one or more protease(s) which in turn activate the prophenoloxidase. Thus, activation of prophenoloxidase is analogous to the early sequence of events involved in the activation of the alternative complement pathway (see Appendix).

Consequently, it was of interest to examine Manduca sexta hemolymph for the presence of complement-like factors. Interestingly, factor(s) in Manduca sexta hemolymph was observed to inhibit alternative complement pathway-mediated hemolysis of rabbit erythrocytes. Since trypsin inhibitor A appears to be involved in the regulation of prophenoloxidase activation in Manduca sexta, it was initially decided to examine whether inhibitor A and/or B also inhibit the proteolytic activation of the human alternative complement pathway. Experiments were also performed to ascertain whether factors present in Manduca sexta hemolymph could directly substitute for human complement factors B and D in a rabbit erythrocyte hemolytic assay. These studies (page 94) indicated that neither inhibitor A nor inhibitor B prevented activation of the alternative complement pathway in human serum when examined in a rabbit erythrocyte hemolytic assay. Moreover, our studies also suggest that crude hemolymph does not functionally substitute for alternative complement factors B and D in the rabbit erythrocyte hemolytic assay.

D'Cruz and Day have identified protein factor(s) in the hemolymph of the silkworm Bombyx mori (96) and the armyworm Spodoptera frugiperda (97) which inhibit(s) the alternative complement pathway-mediated hemolysis of rabbit erythrocytes. The mechanism of action of these inhibitors have not been elucidated, but the authors suggest that these factors may either block the C3b receptor or acceptor sites on

rabbit erythrocytes or could compete with C3 for the C3 convertase. Koch and Nielsen (98) have demonstrated that addition of hemolymph of the snail Helix pomatia causes the cleavage of factor B in human serum and have suggested that a factor analogous to cobra venom factor (see Appendix) may be present in Helix pomatia hemolymph. These authors, however, have not determined whether the snail factor inhibits the alternative complement pathway mediated hemolysis of rabbit erythrocytes.

Several mechanisms may be suggested for the observed inhibition of the alternative complement pathway mediated hemolysis of rabbit erythrocytes caused by Manduca sexta hemolymph. First, the hemolymph may contain C3b-like (cobra venom factor-like) factor(s) which bind(s) to human factor B and causes its cleavage, thereby rendering it unavailable to participate in the lysis of rabbit erythrocytes. Secondly, hemolymph may contain protease(s) which directly cleave factor B nonproductively and thereby prevent hemolysis by the late acting complement components. Third, factors analogous to β 1H (which causes the dissociation of the C3 convertase C3bBb) or C3b inactivator (which proteolytically inactivates C3b) may exist in the hemolymph. Finally, hemolymph may contain inhibitors that are able to inhibit the activity of complement protease(s). As preliminary experiments to distinguish among these mechanisms, we tested whether Manduca sexta hemolymph contained factors capable of causing human factor B cleavage. In this experiment, fresh frozen human serum was incubated with crude hemolymph and factor B cleavage assessed by immunoelectrophoresis (page 97). The results demonstrated that factor(s) present in crude hemolymph induced

the cleavage of human serum complement component factor B into Ba and Bb fragments. Hemolymph, however, does not cleave purified factor B indicating that hemolymph does not contain non-specific protease(s) capable of cleaving factor B directly. The cleavage of factor B in serum but not purified factor B by crude hemolymph suggests that factor B probably must be complexed to C3b to be cleaved by the hemolymph factor. A factor similar to factor D will cleave factor B in the serum but not purified factor B. However, the hemolymph factor is not likely to be factor D-like because such a factor would enhance rather than inhibit hemolysis of rabbit erythrocytes in a hemolytic assay. The hemolymph factor stimulating factor B cleavage is unlikely to be a non-specific stimulator such as a polysaccharide since boiling destroys the ability of the hemolymph to induce factor B cleavage.

Taken together these findings are consistent with the working hypothesis that Manduca sexta hemolymph contains a factor or factors which cause(s) the cleavage of human complement factor B in complex with C3b. The identity of this factor is not clear. However, the presence of a C3b-like factor in the hemolymph is a possibility. Such a factor would cause factor D mediated cleavage of factor B by forming a complex with the latter factor (see Appendix on cobra venom factor). Furthermore, a C3b-like factor which is not affected by regulatory proteins such as β 1H and C3b inactivator can stimulate autocatalytic depletion of C3 and thereby inhibit complement mediated hemolysis of rabbit erythrocytes.

The present studies on the effect of Manduca sexta hemolymph on the human alternative complement pathway are in accordance with the

findings of both D'cruz and Day (96, 97) and Koch and Nielsen (98). We have demonstrated that Manduca sexta hemolymph causes both cleavage of human serum complement factor B and inhibition of the alternative complement pathway mediated hemolysis of rabbit erythrocytes. Our findings suggest that insect hemolymph (and perhaps invertebrate hemolymph in general) contains factor(s) which can initiate the vertebrate alternative complement pathway, but which does not cause hemolysis of rabbit erythrocytes.

APPENDIX

A. A short note on the human complement system

The human complement system consists of at least 20 blood plasma proteins (Table 12), whose major function is to act as an effector mechanism in immune defense against infection by microorganisms. On activation, the complement system causes the lysis of cellular antigens, attracts phagocytic cells to the site of activation and promotes phagocytosis. The initial steps in the activation of the complement system occurs by two pathways, namely the classical pathway and the alternative pathway. The classical pathway is activated mainly by the formation of specific antigen-antibody complexes, whereas the alternative pathway is activated by a variety of activators including polysaccharides, such as those present in bacterial cell walls. Figure 33 is a diagram of the activation scheme of both pathways of complement. The activation of complement by either pathway results in the formation of two complex proteases, namely the C3 convertase and the C5 convertase. The composition of the C3 convertase and the C5 convertase is different in each pathway, nevertheless, they catalyze identical reactions. In both pathways, activation of C5 is the last proteolytic step and the product C5b associates with C6, C7, C8, and C9 to form the lytic complex.

B. The initiation of the alternative complement pathway

The proteins involved in the initiation of the alternative pathway are C3, factor B, and factor D. The mechanism of stimulation and activation of the alternative complement pathway is still not clear.

Proteins of the human complement systemProteins of the classical pathway of complement

C1q

C1r

C1s

C4

C2

C3

Proteins of the alternative pathway of complementFactor \bar{D}

Factor B

C3

Properdin

Proteins involved in membrane attack

C5

C6

C7

C8

C9

Control proteinsC $\bar{1}$ inhibitor

C3b inactivator

 β 1H

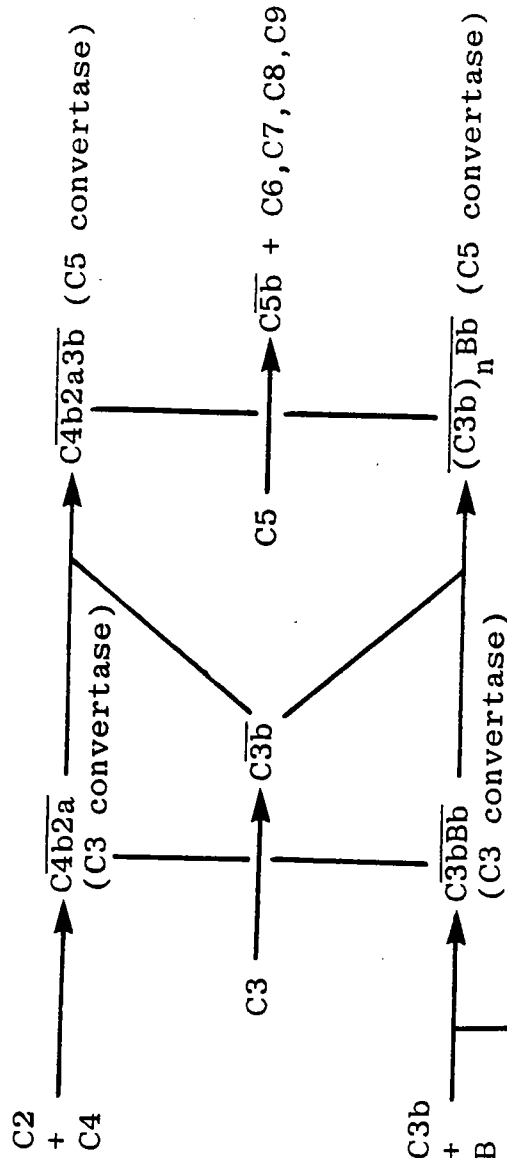
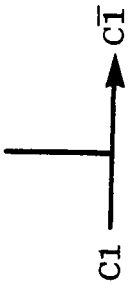
C4b binding protein

DAF (decay acceleration factor)

CR-1 (C3b receptor)

CLASSICAL PATHWAY

Ab-Ag aggregates



Factor D̄

ALTERNATIVE PATHWAY

Fig. 33. Pathways of complement activation.

The favoured mechanism involves the formation of the complex C3bB from low levels of C3b present in the serum. When complexed with C3b, factor B is cleaved by factor D into 63,000 dalton Bb and 30,000 dalton Ba fragments. The Bb stays associated with C3b and forms C3 convertase. The end product of C3 convertase, namely C3b, can then associate with C3bBb to form C3b_nBb, a C5 convertase. In addition, C3b also associates with factor B molecules and generates more C3 convertase, thus amplifying the alternative complement pathway. The C3bBb complex is relatively unstable, but, binding to another serum protein, properdin, extends the half-life of the complex. Inactivation of C3b is achieved by a serum protease, C3b inactivator, which cleaves C3b in the presence of another serum protein β 1H. Particles with repeating units, such as bacterial cell walls, afford microenvironments which protect C3b against rapid destruction by C3b inactivator. Hence, these particles serve to activate the alternative complement pathway.

C. Mechanism of action of cobra venom factor

A factor isolated from the venom of cobra (Naja naja) is functionally analogous to C3b but is resistant to inactivation by C3b inactivator. Cobra venom factor, therefore, forms a stable C3 convertase which catalyses the cleavage of C3 to C3b, and C3b in turn forms more C3 convertase. Through this autocatalytic mechanism C3 is quickly depleted. Without C3 the entire complement system is ineffective.

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