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STUDIES ON THE INTERACTION OF LIMA BEAN PROTEASE INHIBITOR WITH TRYPSIN AND CHYMOTRYPSIN

by

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By: John Krahn

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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To Sally

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ABSTRACT

The mode of interaction of lima bean protease inhibitor with trypsin and chymotrypsin was investigated. As a preliminary step the exact stoichiometry of interaction of the inhibitor with these enzymes was examined. Binary complexes of the inhibitor and either trypsin or chymotrypsin as well as the ternary complex consisting of inhibitor and both enzymes could be demonstrated. It was therefore concluded that it could react simultaneously and independently with these two enzymes in a 1:1 molar ratio.

Treatment of lima bean inhibitor with catalytic amounts of either trypsin or chymotrypsin at acid pH produced the trypsin- or chymotrypsin-modified lima bean inhibitor respectively. These enzyme-modified inhibitors were each found to have a specific peptide bond cleaved. These bonds are referred to as the trypsin reactive site and the chymotrypsin reactive site. The trypsin reactive site was identified as the Lys^{28}-Ser^{29} peptide bond whereas the chymotrypsin reactive site was identified as the Leu^{55}-Ser^{56} peptide bond. These enzyme reactive sites are located in homologous regions of the protein.

The trypsin inhibitory activity of lima bean protease inhibitor was found to conform to the "reactive site model" for protein proteinase inhibitors as proposed by Laskowski and Sealock (1971). Incubation of lima bean inhibitor with catalytic amounts of trypsin at pH 3.1 results in cleavage of the Lys^{28}-Ser^{29} peptide bond to form the trypsin-modified inhibitor. This trypsin-modified inhibitor is still fully active although it reacts with trypsin more slowly than native inhibitor. Treatment of the trypsin-modified lima bean inhibitor with carboxy-

peptidase B results in the release of the reactive site lysine with concomitant loss of trypsin inhibitory activity. The trypsin reactive site peptide bond is involved in hydrolysis equilibria and at pH 3.1 the equilibrium mixture consists of 90% cleaved and 10% intact molecules. Rapid dissociation of the complex made from trypsin and the trypsin-modified inhibitor results in a native inhibitor indicating that the trypsin active site comes into intimate contact with the inhibitor reactive site in the trypsin-inhibitor complex.

The chymotrypsin inhibitory activity of lima bean inhibitor has been examined in less detail but it also seems to conform to the "reactive site model" for protein proteinase inhibitors.

Comparison of the four genetic variants of lima bean inhibitor shows minor differences with respect to their trypsin inhibitory properties but major differences in their chymotrypsin inhibitory properties are apparent.

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ABBREVIATIONS

BBI	Bowman-Birk inhibitor from soybeans
BPTI	Basic pancreatic trypsin inhibitor
BTEE	Benzoyltyrosine ethyl ester
COA	Carboxypeptidase A
COB	Carboxypeptidase B
CMRM-LBI	Chymotrypsin modification reaction mixture of lima bean inhibitor containing native LBI and LBI'_c
CT	Chymotrypsin
DEAE	Diethylaminoethyl
DIP	Diisopropylphosphate
EDTA	Ethylenediaminetetraacetic acid
LBI	Lima bean inhibitor (native)
LBI I-IV	LBI variants I-IV
LBI'_c	Chymotrypsin-modified lima bean inhibitor
LBI'_t	Trypsin-modified lima bean inhibitor
LBI-CT, LBI-T	1:1 molar complex between LBI and trypsin or chymotrypsin, respectively
LBI-T-CT	Ternary complex between LBI, trypsin and chymotrypsin
PSTI	Pancreatic secretory trypsin inhibitor
RCAM	Reduced and carboxamidomethylated
RCM	Reduced and carboxymethylated
STI	Soybean trypsin inhibitor
STI*	Trypsin-modified STI
T	Trypsin
TAME	Toluenesulfonylarginine methyl ester
TMRM-LBI	Trypsin modification reaction mixture of lima bean inhibitor containing native LBI and LBI'_t

TLCK	N-tosyl-L-lysylchloromethyl ketone
TPCK	N-tosyl-L-phenylalanylchloromethyl ketone

MATERIALS

Lima bean inhibitor (LBI) was obtained from Worthington (Freehold, N.J.), and further purified by the method of Jones et al. (1963). The particular lot number used will be identified in each experiment.

Bovine trypsin (TRL 8CA, TRL OFA, and TRL 2DA), bovine chymotrypsin (CDI 7CG and CDI 8GA), carboxypeptidase A (COADFP 9DA), carboxypeptidase B (COBDFP 9DA and COBDFP 1CA), and leucine aminopeptidase (LAPC 9EB) were all purchased from Worthington (Freehold, N.J.).

Three times crystallized porcine trypsin (4-36-564 Batch UK6) was obtained from Miles Laboratories, Inc. (Kankakee, Ill.)

Aminopeptidase M (Rohm and Haas) was purchased from Henley and Co., Inc., (New York, N.Y.).

Malic dehydrogenase, BTEE, guanidine hydrochloride, iodoacetic acid, and iodoacetamide were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Ribonuclease A, chymotrypsinogen A, TAME, and BTEE were purchased from Mann Research Laboratories (New York, N.Y.).

Sephadex (G-series and DEAE-) was purchased from Pharmacia Fine Chemicals (Montreal, P.Q.).

Bio-Gel (P-series) and DEAE-Cellulose (Cellex D) was purchased from Bio-Rad Laboratories (Richmond, Calif.)

Dithioerythritol and sequenal grade phenylisothiocyanate were purchased from Pierce Chemical Co. (Rockford, Ill.).

All other chemicals were reagent grade or better.

INTRODUCTION

Naturally occurring inhibitors of proteolytic enzymes are widely distributed in plant and animal tissues, fluids and secretions. Although the details of the physiological function of these inhibitors are certainly not clearly understood, it is generally accepted that they are concerned with limiting the area affected by and the duration of the various proteolytic processes. It has long been established that the mechanism of inhibition involves the formation of a specific complex between the enzyme inhibited and the inhibitor, which itself is a protein. The exact bonds and forces involved in complex formation between enzyme and inhibitor are at present not completely known and the elucidation of the mechanisms by which these complexes are formed has been the object of considerable scientific effort over the past decade. In recent years proteolytic enzymes like trypsin and chymotrypsin have been extensively studied and their amino acid sequences and three dimensional structures are known. Considerable insight into their mode of action has also been gained by combined chemical, kinetic, and X-ray diffraction studies. These studies strongly indicate that catalysis of peptide bond cleavage involves the formation of a covalent enzyme-substrate intermediate thought to be either an acyl-enzyme or a tetrahedral intermediate.

Since the protein proteinase inhibitor and the enzyme are of equal interest in the inhibition phenomenon, considerable attention has also been given to the structure and mode of action of these naturally occurring inhibitors. There are essentially two schools of thought with respect to the mechanism of action of these inhibitors. Laskowski and

coworkers feel that, at least in the case of trypsin inhibitors, inhibition involves the cleavage of a specific enzyme sensitive bond in the reactive site of the inhibitor, followed by the formation of a covalent enzyme-inhibitor complex possibly through an acyl bond between the active site serine of the enzyme and the newly formed carboxyl-terminal residue in the inhibitor. The second school of thought led by Feeney and coworkers believes that peptide bond cleavage is not central to the mechanism of inhibition and that complex formation is only the result of non-covalent interactions between complementary sites on the enzyme and the inhibitor. The "reactive site" model for the mechanism of inhibition, as proposed by Laskowski and coworkers, is mainly based on the results of extensive studies with Kunitz soybean trypsin inhibitor, an inhibitor of molecular weight 22,000 g/mole which is essentially specific for trypsin but has weak inhibitory activity against chymotrypsin. In view of the fact that the model has been proposed as a general model for all protein proteinase inhibitors of serine proteases, it was felt to be important to extend the studies to other naturally occurring inhibitors. As a result, the present investigation is concerned with lima bean protease inhibitor, a protein of molecular weight 9,000 g/mole which inhibits both trypsin and chymotrypsin independently. Lima bean inhibitor is available from commercial sources in crude form and can be easily purified. At the time this investigation was initiated, the determination of the amino acid sequence of lima bean inhibitor had already been started by another student in this laboratory.

I. Purpose of this Study

The purpose of this study includes: (1) the determination of the stoichiometry of interaction of lima bean inhibitor with the proteases trypsin and chymotrypsin (2) the determination of the reactive sites of this inhibitor for trypsin and chymotrypsin and their localization within its amino acid sequence (3) a study of the mechanism of inhibition with the object of determining whether or not it conforms to the "reactive site" model as proposed by Laskowski and coworkers.

II. General Approach to the Investigation

To determine the stoichiometry of interaction of lima bean inhibitor with the proteases trypsin and chymotrypsin, characterization of the isolated inhibitor-protease complexes was undertaken. The major part of this study centers on the modification of lima bean inhibitor by partial proteolysis with either trypsin or chymotrypsin at acid pH. Characterization of the trypsin- and chymotrypsin-modified inhibitor enabled identification of the "reactive site peptide bonds" as well as determination of their location in the primary structure of the inhibitor. The enzyme-modified inhibitors as well as the native inhibitors were examined in various ways in an attempt to determine whether or not lima bean inhibitor conformed to the "reactive site" model for the mechanism of action of protease inhibitors.

REVIEW OF THE LITERATURE

I. Organization

Especially in the last 15 years, the nutritional, pharmacological, mechanistic and general aspects of protein proteinase inhibitors have been the subject of many review articles (Green and Neurath, 1954; Laskowski and Laskowski, 1954; Desnuelle, 1960; Pusztai, 1967; Vogel et al., 1968; Feeney and Allison, 1969; Steiner and Frattali, 1969; Laskowski, Jr., 1970; Kassel, 1970a-d; Burck, 1970; Laskowski and Sealock, 1971). The proceedings of the First International Research Conference on Proteinase Inhibitors held in Munich in November, 1970, have been published in book form[1] and, as such, this book is a comprehensive compilation of reports of recent investigations involving protein proteinase inhibitors.

Since this thesis is mainly concerned with the interaction of lima bean inhibitor with trypsin and chymotrypsin, the literature review will concentrate on the proposed mechanisms of action of proteinase inhibitors. A general introduction will describe in some detail those inhibitors that are referred to in the discussion of the mechanism of inhibition. To facilitate an understanding of the mechanism of action of proteinase inhibitors, a knowledge of the enzyme that is inhibited is essential and for this purpose, a brief discussion on the mode of action of serine proteases is included.

---

[1] Proc. Int. Res. Conf. on Proteinase Inhibitors, (H. Fritz and H. Tschesche, eds.), Walter de Gruyter and Co., Berlin, 1971.

The literature on lima bean inhibitor and Bowman-Birk soybean inhibitor is reviewed in detail. It has become apparent in recent years that lima bean inhibitor and Bowman-Birk soybean inhibitor are homologous proteins. Since most of the data contained in this thesis have been published previously (Krahn and Stevens, 1970, 1971, 1972a,b, 1973; Stevens, 1971) and have in turn stimulated research on the mechanism of action of Bowman-Birk inhibitor, it was decided that this review should discuss only those articles on the Bowman-Birk inhibitor published prior to 1970; more recent articles on the Bowman-Birk inhibitor are more appropriately discussed in the General Discussion.

II. General Introduction

For the purposes of this review, the definition of a protein proteinase inhibitor will be that proposed by Laskowski and Sealock (1971) namely "a protein which may associate reversibly with one or more proteinases to form complexes of discreet stoichiometry in which all the catalytic functions of the proteinase are competitively inhibited". The above definition excludes such phenomena as antigen-antibody interactions and non-specific inhibition by non-protein inhibitors such as heparin (an acid polysaccharide) or synthetic, low molecular weight inhibitors.

The molecular weight of protein proteinase inhibitors ranges from 5,000 to 60,000 g/mole with the majority of inhibitors having a molecular weight of less than 20,000. Most of these inhibitors are very stable proteins and, with the exception of the high molecular weight serum inhibitors, contain cysteine in disulfide linkages; in

some inhibitors the content of disulfides is higher than that of any other known proteins. Some of the inhibitors are glycoprotein in nature.

Originally protein proteinase inhibitors were believed to be highly specific in that an individual inhibitor was thought to inhibit only one enzyme. Vogel et al. (1968) have listed 25 proteolytic enzymes for which inhibitors exist. These enzymes have different specificities and thus were thought to require unique inhibitors. The most common enzymes included in Vogel's list were chymotrypsin, trypsin, elastase, plasmin and kallikrein. Since the review of Vogel et al. (1968) the list of enzymes inhibited by natural proteinase inhibitors has been extended to include cocoonase (Hixon and Laskowski, Jr., 1970a), leucocyte neutral proteinase (Koj et al., 1972), and acrosomal proteinase (Polakoski et al., 1971; Zaneveld et al., 1972). Since proteinase inhibitors, as a class, inhibit such a wide spectrum of proteases the nomenclature and identification of the individual inhibitors has created problems. Different workers may assay for the inhibition of different enzymes so that the identity of like inhibitors often escapes immediate notice. It has been recognized recently that in some instances a single inhibitor is capable of inhibiting several enzymes and much of the confusion has been swept away. The inhibition of more than one enzyme molecule by a single inhibitor can theoretically occur in three possible ways namely (1) at the same site, (2) at distinct, nonoverlapping sites or (3) at distinct overlapping sites. This third possibility has not yet been demonstrated. The basic inhibitor from bovine pancreas has been shown to inhibit trypsin, kallikrein and

chymotrypsin at the same site (Laskowski and Sealock, 1971; Rigbi, 1971). Inhibitors like turkey ovomucoid and lima bean inhibitor inhibit both trypsin and chymotrypsin through distinct and independent sites and hence have been termed "doubled-headed" inhibitors (Haynes et al., 1967; Rhodes et al., 1960). Inhibitors which on a molar basis inhibit more than one mole of the same enzyme are also known. Mung bean inhibitor for example has two sites for the inhibition of trypsin (Laskowski and Sealock, 1971) whereas potato inhibitor I has four sites for the inhibition of chymotrypsin (Melville and Ryan, 1970). These inhibitors are termed multi-site inhibitors to distinguish them from double-headed inhibitors which inhibit two enzymes at different sites.

III. Review of the Properties of Some Proteinase Inhibitors

A. Inhibitors of Animal Origin

1. Distribution

Inhibitors of proteolytic enzymes are widely distributed in the animal kingdom especially in mammals. In these they appear in various tissues, secretions, and body fluids (Vogel et al., 1968). The trypsin inhibitors from various bovine and porcine organs as well as from the bovine pancreatic exocrine secretion have been the most extensively studied. In the bovine species, trypsin inhibitors have been found in pancreatic juice, pancreas, lungs, parotid glands, colostrum, lymph nodes, spleen, liver (Vogel et al., 1968), and pituitary gland (Lisowski and Sawaryn, 1972). Trypsin and chymotrypsin inhibitors have been found in the submandibular glands of dogs and cats. All mammals, including man, have inhibitors in the seminal vesicles and in semen (Vogel et al., 1968). In addition, trypsin inhibitors are

found in the mucous membrane of respiratory tissue in man and other mammals, in rabbit skin, and in the placenta, amniotic fluid, heart, lung, and brain of various species (Vogel et al., 1968). In man, proteinase inhibitors have so far been found in urine (Vogel et al., 1968), sperm (Koj et al., 1972; Zaneveld et al., 1972), tears (Kueppers, 1971), cervical secretion (Haendle et al., 1970), bronchial secretion (Hochstrasser et al., 1972), sperm plasma (Fink et al., 1971a) and nasal secretion (Hochstrasser et al., 1971). Human, bovine and ovine blood sera also contain proteinase inhibitors which are glycoprotein in nature and are found in the post-albumin region. They are included in the region from the α_1- to the α_2-globulin fractions with 90% occurring in the α_1 fraction (Vogel et al., 1968).

Although most inhibitors of animal origin known today are from mammals, inhibitors have also been found in other animals. Avian egg white contains trypsin and chymotrypsin inhibitors which are largely associated with the ovomucoid fraction. These inhibitors are glycoproteins which range in molecular weight from 28,000 - 46,000 g/mole (Vogel et al., 1968; Feeney, 1971a). The pancreas of the African lung fish also contains an inhibitor (Reeck et al., 1970). In nematodes, proteinase inhibitors were first postulated around the turn of the century (Vogel et al., 1968), but it is only since 1960 that they have actually been isolated from roundworms (Peanasky and Laskowski, 1960; Fraefel and Acher, 1968; Kucich and Peanasky, 1970; Peanasky and Abu-Erreish, 1971). More recently, inhibitors have also been discovered in the leech (Fritz et al., 1971) and in the snail, Helix pomatia (Tschesche et al., 1972).

2. Basic Pancreatic Trypsin Inhibitor (BPTI)

Kunitz and Northrup (1936) were the first to isolate this inhibitor from bovine pancreas and also crystallized its 1:1 stoichiometric complex with trypsin. It is known as basic pancreatic trypsin inhibitor (BPTI) because of its very high isoelectric pH. The purification procedures and properties of BPTI have recently been reviewed by Kassel (1970a). BPTI inhibits trypsin (Wu and Laskowski, 1955), and trypsin-like enzymes such as kallikrein (Werle and Appel, 1959), thrombin (Ferguson, 1942) and a subfraction of pronase (Trop and Birk, 1970); chymotrypsin is also inhibited but much more weakly than the trypsin-like enzymes (Wu and Laskowski, 1955). No mammals except ruminants appear to have BPTI in their pancreas or in other organs.

The inhibitor contains 58 amino acid residues, has a molecular weight of 6513 g/mole and an isoelectric pH greater than 10. It consists of a single polypeptide chain containing three disulfide bridges. Its amino acid sequence was the subject of simultaneous research and publication by several laboratories (Chauvet et al., 1964, 1966a, b, c, 1967; Kassel et al., 1964, 1965a, b, 1966; Dlouhà et al., 1965, 1966). Since BPTI is a small and well characterized protein which specifically interacts with trypsin, it provides an excellent system for the study of protein-protein interaction. For this reason, BPTI has recently been the object of an X-ray diffraction study resulting in the elucidation of its three dimensional structure (Huber et al., 1970, 1971a, b; Rühlmann et al., 1971). The research groups of Huber (Munich) and Blow (Cambridge) are now collaborating on a model-building study of the

interaction between bovine BPTI and trypsin or chymotrypsin (Blow et al., 1972) and the actual crystallographic analysis of the enzyme-inhibitor complex is in its initial stages (Cole and Parthasarathy, 1972).

3. Inhibitors Similar to Basic Pancreatic Trypsin Inhibitor (BPTI)

As early as 1929, inhibitors of kallikrein (a proteolytic enzyme that releases kallidin and bradykinin from the serum α_2-globulin fraction) were found in bovine lymph nodes, parotid gland, spleen, liver, pancreas and lungs (Vogel et al., 1968). Later it was noticed that this kallikrein inhibitor from bovine organs also inhibits trypsin and to a lesser degree chymotrypsin. For this reason the identity of the kallikrein inhibitor from bovine organs with BPTI was suspected. On the basis of either peptide mapping or partial and complete sequence determination, BPTI has been shown to be identical to kallikrein inhibitor from the following bovine organs: parotid gland, lungs (Anderer, 1965a, b), ovaries (Chauvet and Acher, 1972), and liver (Chauvet and Acher, 1970). Most probably all of the kallikrein-trypsin inhibitors distributed in bovine tissues are identical to BPTI (Kassel, 1970a). A trypsin inhibitor from ovine lung appears identical to bovine BPTI at least according to amino acid composition, electrophoretic behaviour and inhibitory properties (Fritz et al., 1970).

It is interesting to note that an inhibitor related to BPTI has been isolated from bovine colostrum (Čechová et al., 1969). Although this colostrum inhibitor shows more than 40% sequence homology with BPTI it differs from BPTI in that it is a glycoprotein (Čechová et al., 1971).

4. Pancreatic Secretory Trypsin Inhibitor (PSTI)

In 1948 Kazal et al. first isolated an acidic trypsin inhibitor from bovine pancreas. This acidic inhibitor has been purified to homogeneity (Greene et al., 1966; Fritz et al., 1966; and Cerwinsky et al., 1967). Greene (1968) proved that this inhibitor is identical to the one found in the exocrine secretion of the pancreas and hence this inhibitor is known as pancreatic secretory trypsin inhibitor (PSTI). Burck (1970) has reviewed the isolation of PSTI from the pancreas and its exocrine secretion as well as some of its properties. PSTI inhibits bovine trypsin by the formation of a 1:1 molar complex (Greene et al., 1966; Burck et al., 1967), but does not inhibit kallikrein or chymotrypsin (Greene et al., 1966; Burck et al., 1967; Fritz et al., 1966). It consists of a single polypeptide chain of 56 amino acids (Cerwinsky et al., 1967), the sequence of which has been determined (Greene and Bartelt, 1969; Greene and Giordano, 1969). The sequence of PSTI bears no relation or resemblance to that of BPTI.

Pancreatic secretory inhibitors homologous to bovine PSTI have been found in swine (Greene et al., 1968; Tschesche et al., 1969), cats (Tschesche and Kupfer, 1971), dogs (Fritz et al., 1966) and sheep (Hochstrasser et al., 1969a). Porcine pancreas and its exocrine secretion contain two similar inhibitors, I and II (Greene et al., 1968; Tschesche et al., 1969). Inhibitor I has 56 amino acids and a molecular weight of 6,024 g/mole. Its amino acid sequence has been determined (Tschesche and Wachter, 1970; Bartelt and Greene, 1971) and the disulfide bridges have been assigned (Greene and Guy, 1971). Inhibitor II is identical to I in every respect except for the

deletion of the first four amino-terminal residues (Greene et al., 1968; Tschesche et al., 1971). Tschesche et al. (1971) have shown that sheep PSTI has only one amino acid sequence difference when compared to bovine PSTI whereas porcine inhibitor I shows 12 sequence differences when compared to bovine PSTI (Bartelt and Greene, 1971). Greene and Pubols (1971) have reported the isolation of a human PSTI consisting of 56 amino acids. This inhibitor is the only PSTI found so far capable of inhibiting human as well as bovine and porcine trypsin. Bovine and porcine PSTI are completely inactive against human trypsin. Because of this difference in specificity and since there are probably only about 20 differences in amino acid sequence between human and porcine PSTI this system offers an interesting model for the study of structure-function relationships in proteins.

5. Inhibitors from Avian Egg White

The isolation and properties of egg white inhibitors have been reviewed by several authors (Vogel et al., 1968, Kassel, 1970b; Feeney, 1971a). Three distinct inhibitors of proteolytic enzymes have been identified in avian egg white namely: ovomucoid, ovoinhibitor, and a ficin-papain inhibitor (Feeney, 1971a). The ficin-papain inhibitor has been isolated only from chicken (Fossum and Whitaker, 1968) and will not be discussed in this review.

Chicken ovomucoid, which is quantitatively the most important egg white inhibitor, has been the most extensively studied. Rhodes et al. (1960) found that ovomucoids from different species have gross physical and chemical similarities but differ in their specificity. They are glycoproteins containing about 20-25% carbohydrate material

and have molecular weights around 28,000 g/mole. They all lack tryptophan but have a high cystine content. Chicken ovomucoid inhibits bovine and turkey trypsin (on a 1:1 molar ratio) but does not inhibit human trypsin nor chymotrypsin from various species. Some ovomucoids (Peking duck, goose, emu) inhibit two moles trypsin per mole ovomucoid (Kassel, 1970b) while others such as turkey ovomucoid are double-headed in that they can inhibit both trypsin and chymotrypsin independently and simultaneously on a 1:1 molar enzyme-ovomucoid ratio. Golden pheasant ovomucoid on the other hand, only inhibits chymotrypsin. Those ovomucoids which inhibit chymotrypsin also inhibit subtilisin (Feeney, 1971a) and elastase (Gertler and Feinstein, 1971), possibly at the same site on the ovomucoid.

Ovoinhibitor, the minor inhibitor of egg white, is also a glycoprotein but contains only 10% carbohydrate and has a molecular weight of 46,000 g/mole. Its amino acid composition is vastly different from that of ovomucoid but there are similarities in composition between ovoinhibitors from different avian species (Feeney, 1971a). The ovoinhibitors have a relatively wide spectrum of inhibition in that they inhibit bovine trypsin and chymotrypsin, subtilisin, and the alkaline proteinase from A. oryzae (Feeney, 1971a). Chicken ovoinhibitor has been shown to inhibit two moles of chymotrypsin and two moles of trypsin separately and independently. The chymotrypsin and subtilisin inhibition most probably occur at the same site on the ovoinhibitor. Recently it has been shown that chicken ovoinhibitor can also inhibit porcine elastase and on the basis of competition studies it was suggested that this inhibitor is triple-headed in that it can inhibit trypsin,

chymotrypsin, and elastase simultaneously and independently (Gertler and Feinstein, 1971).

B. Inhibitors of Plant Origin

1. Distribution

Proteinase inhibitors were discovered in plants as early as 1938 when Read and Haas found inhibitory activity in soybeans. All other Leguminosae thus far examined contain proteinase inhibitors. Representatives of the Solanacae (potatoes), the Graminae (wheat and barley) and the Chenopodiaceae (beets) have also been found to contain proteinase inhibitors (Vogel et al., 1968). Soybeans contain at least two distinct inhibitors namely: (1) Kunitz soybean trypsin inhibitor and (2) Bowman-Birk soybean protease inhibitor. To date, the Kunitz soybean trypsin inhibitor is the most extensively studied plant inhibitor and it has also been used to formulate a general model for the mechanism of action of proteinase inhibitors (Laskowski and Sealock, 1971). Recently, however, considerable effort has also been taken in the study of the other soybean inhibitor as well as inhibitors from lima beans, potatoes, peanuts, corn, barley and from a large variety of beans. Soybean trypsin inhibitor (Kunitz) will be discussed below since it is central to any discussions involving the mechanism of action of inhibitors. Since Bowman-Birk soybean inhibitor and lima bean inhibitor have been shown to be homologous, a separate section comparing these inhibitors is included.

2. Soybean Trypsin Inhibitor (STI)

STI is generally known as Kunitz soybean inhibitor since Kunitz (1945, 1946, 1947) was the first to isolate, crystallize,

and characterize this inhibitor. The original purification procedure has been modified and improved (Kassel, 1970c). STI has 196 amino acid residues and is a single polypeptide chain with two disulfide bridges (Wu and Scheraga, 1962). Its amino acid sequence has recently been determined by Ikenaka et al. (1971) and the position of the two disulfide bridges was previously determined by Brown et al. (1966). The molecular weight of 21,500 obtained by ultracentrifugal analysis agrees with the molecular weight of 21,700 calculated from the amino acid composition (Wu and Scheraga, 1962). STI strongly inhibits bovine, human, salmon, sting ray, barracuda and turkey trypsins whereas bovine and chicken chymotrypsins are inhibited to a lesser degree, (Kassel, 1970c); it is also a good inhibitor of cocoonase (Hixon and Laskowski, 1970a). The inhibition of trypsin by STI is stoichiometric at a 1:1 molar ratio of STI to trypsin. Recent evidence indicates that STI inhibits trypsin, cocoonase and chymotrypsin at the same site on the inhibitor (Hixon and Laskowski, 1970a; Bidlingmeyer et al., 1972).

3. Lima Bean Inhibitor (LBI)

A crystalline preparation from lima beans capable of inhibiting trypsin was first prepared by Tauber et al. (1949). In 1952, Fraenkel-Conrat et al. reported the preparation of an amorphous lima bean trypsin inhibitor of much higher specific activity than Tauber's original preparation. Since that time several groups have reported that the partially purified preparation of lima bean inhibitor could be further fractionated into 4-6 biologically active components by ion-exchange chromatography on DEAE-cellulose or DEAE-Sephadex (Jirgensons et al., 1960; Jones et al., 1963 Haynes and Feeney, 1967;

Kassel, 1970d). These components are all similar but not identical as judged by amino acid composition, biological activity (Jones et al., 1963; Haynes and Feeney, 1967) and comparison of tryptic peptide maps (Stevens, 1971). Table I shows the amino acid composition of the four components of LBI isolated by Jones et al. (1963). All are devoid of methionine and tryptophan and have a high content of acidic amino acids and cysteine. Since no free sulfhydryl groups can be detected, they contain 6-8 disulfide bridges. Stevens (1971) compared the tryptic peptide maps of the four components of LBI and found only minor differences, indicating that they are probably also very similar in amino acid sequence. It is now generally accepted that these various components of LBI do not arise as artifacts during purification but are probably of genetic origin (Haynes and Feeney, 1967). For this reason the original nomenclature of "LBI components I-IV" as suggested by Jones et al. (1963) will be changed throughout this thesis to the more appropriate lima bean inhibitor variants I-IV (LBI I-IV).[2] Ferdinand et al. (1965) have noticed that the same LBI variants from different commercial preparations of LBI are not always identical but often show significant differences in their amino acid composition.[3]

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[2]Although as many as six variants of LBI were reported by Haynes and Feeney (1967), most commercial preparations obtained from Worthington in the crude form regularly contain only four variants.

[3]The nomenclature of LBI as variants I-IV is not meant to convey the idea that only four variants of LBI exist, but instead refers only to the numerical order in which they are eluted from DEAE-cellulose under conditions specified by Jones et al. (1963).

TABLE I

AMINO ACID COMPOSITIONS OF LBI I-IV[a]

Amino Acid	LBI I	LBI II	LBI III	LBI IV
Aspartic acid	12	14	13	13
Glutamic acid	6	5	7	7
Glycine	1	0	1	1
Alanine	3	3	4	3
Valine	1	1	1	1
Leucine	3	3	3	3
Isoleucine	4	4	5	4
Serine	12	12	15	13
Threonine	4	3	5	5
Half-cystine	12	14	16	14
Proline	6	6	7	7
Phenylalanine	1	1	2	2
Tyrosine	1	1	2	1
Histidine	5	3	6	6
Lysine	4	4	4	4
Arginine	2	2	2	2

[a]obtained from Jones *et al.* (1963).

All variants of LBI have similar trypsin inhibitory activities and inhibit trypsin in a 1:1 molar ratio (Jones et al., 1963). In 1964, Ryan and Clary noticed that preparations of LBI also inhibit chymotrypsin quite strongly although at that time it was not clear whether or not both inhibitory activities could be ascribed to a single inhibitor. Haynes and Feeney (1967) found that the complex formed from LBI and trypsin could still inhibit chymotrypsin, indicating that LBI is double-headed and able to inhibit trypsin and chymotrypsin simultaneously and independently. This study also showed that the inhibition of chymotrypsin was weaker than the trypsin inhibition and varied from one variant to another. Haynes and Feeney (1967) reported that LBI did not inhibit pronase at all, but using the same casein hydrolysis assay system, Trop and Birk (1970) were able to inhibit 80% of the protease activity of pronase with LBI. Coan and Travis (1971) reported that LBI is a stronger inhibitor both of human trypsin and chymotrypsin than STI, BPTI or chicken ovomucoid.

The molecular weight of LBI was first determined by Fraenkel-Conrat et al. (1952) by ultracentrifugal measurements (10,000 g/mole) and also by osmotic pressure measurements (9,400 g/mole). The molecular weights of the four variants of LBI as calculated from their amino acid compositions are 8,408, 8,291, 9,892, and 9,423 g/mole. Haynes and Feeney (1967), using ultracentrifugation, estimated the molecular weight of their variant VI to be 16,200 g/mole and suggested that this high value was due to self-association. This self-association phenomenon was further investigated by Sakura and Timasheff (1971) who found that while LBI I and II do not associate at neutral or acid pH,

LBI III and IV do associate at low protein concentration to form dimers while trimers appear at higher inhibitor concentrations. Similar self-association properties have been observed for Bowman-Birk soybean inhibitor (Millar et al., 1969) as well as for the inhibitors from corn, rye, wheat, peanut, and potato (Hochstrasser et al., 1969b, 1970).

The amino acid sequence of LBI IV has been determined by Tan and Stevens (1971a, b) and will be discussed in the section dealing with comparison of LBI and the Bowman-Birk soybean inhibitor. Since all evidence to date indicates that sequence differences between different LBI variants are only minor, the present investigation will assume that data obtained using LBI I-IV can be interpreted on the basis of the sequence of variant IV.

4. Bowman-Birk Inhibitor (BBI) from Soybeans

In 1946 Bowman discovered an inhibitor in soybeans distinct from the Kunitz STI previously described. It was largely ignored until the early sixties when it was purified more extensively (Birk, 1961; Birk et al., 1963). This inhibitor has also been called the acetone-insoluble factor, inhibitor AA, soybean trypsin and chymotrypsin inhibitor, and 1.9S inhibitor (Yamamoto and Ikenaka, 1967). At present, it is generally referred to as Bowman-Birk inhibitor (BBI) as suggested by Frattali (1969). BBI inhibits both trypsin and chymotrypsin but Frattali (1969) has claimed that the inhibition is not stoichoimetric. Careful examination of his data indicates that up to approximately 70% inhibition, the degree of inhibition obtained is directly proportional to the amount of inhibitor present, and extrapolation of the linear portion of the inhibition curve to zero

enzyme activity shows that both trypsin and chymotrypsin react with BBI in a 1:1 molar ratio of enzyme to inhibitor. The inhibition of these two enzymes is simultaneous and independent and therefore BBI can be classified as a double-headed inhibitor (Birk et al., 1967). Birk et al. (1963), by sedimentation velocity studies, estimated the molecular weight of BBI to be 24,000 g/mole. Kakade et al. (1970) using gel filtration got an estimate of 8,250 g/mole. Millar et al. (1969) explained the discrepancy by demonstrating that BBI participates in a concentration dependent monomer-dimer-trimer equilibrium and they estimate the molecular weight of the monomer to be approximately 9,000 g/mole. This estimate agrees well with the minimum molecular weight of 7,975 g/mole as calculated by Frattali (1969) from the amino acid composition. Table II shows the amino acid composition of BBI and indicates the absence of tryptophan and glycine. BBI contains one methionine residue and 14 cysteine (present as disulfide bridges) and a high proportion of acidic residues. Its amino acid sequence has recently been determined (Odani et al., 1971, 1972, Odani and Ikenaka, 1972) and will be discussed in the next section.

5. Comparison of BBI and LBI

As previously described, both BBI and LBI are double-headed inhibitors and can inhibit trypsin and chymotrypsin simultaneously and independently. They have similar molecular weights and both participate in a monomer-dimer-trimer self-association. Table II compares the amino acid compositions of BBI and LBI II. Both molecules have 14 cysteine residues present as 7 disulfide linkages. Their amino acid compositions are similar; the only significant difference in the two

TABLE II

AMINO ACID COMPOSITION OF BOWMAN-BIRK INHIBITOR FROM SOYBEANS AND OF LIMA BEAN INHIBITOR II

Amino Acid	Bowman-Birk Inhibitor[a]	Lima Bean Inhibitor[b]
Lysine	5	4
Histidine	1	3
Arginine	2	2
Aspartic acid	12	14
Threonine	2	3
Serine	9	12
Glutamic acid	7	5
Proline	6	6
Glycine	0	0
Alanine	4	3
Cysteine	14	14
Valine	1	1
Methionine	1	0
Isoleucine	2	4
Leucine	2	3
Tyrosine	2	1
Phenylalanine	2	1
Tryptophan	0	0

[a] from Frattali (1969)

[b] LBI II from Jones et al. (1963)

molecules is the presence of one methionine residue in BBI whereas LBI lacks this amino acid. Odani et al. (1971, 1972) and Odani and Ikenaka (1972) have determined the amino acid sequence of BBI and compared it to the sequence of LBI IV previously determined by Tan and Stevens (1971a, b). As shown in Figure 1, 49 out of 79 positions are identical. The published amino acid sequence of LBI is presently being reinvestigated and it is not unlikely that the correction of small errors will considerably increase the degree of homology between the two proteins. It is interesting to note that the position occupied by methionine in BBI is occupied by either leucine or phenylalanine in variants of LBI. The only requirement in this position seems to be for a hydrophobic residue. Further discussion of these sequences and their implications can be found in the General Discussion where they are discussed in the context of the present investigation.

C. Physiological Importance of Protease Inhibitors

It is generally accepted that the physiological function of naturally occurring inhibitors of proteolytic enzymes is to limit the area affected by and the duration of various proteolytic processes. Although many plant and animal protease inhibitors have been isolated and characterized, the study of their physiological function has been virtually ignored. In several instances, however, very attractive and plausible hypotheses on the physiological function of protease inhibitors have been advanced. In most cases these are based on the functional role of the tissues where the inhibitor is found; in a few instances they are based on physiological studies. Four inhibitors

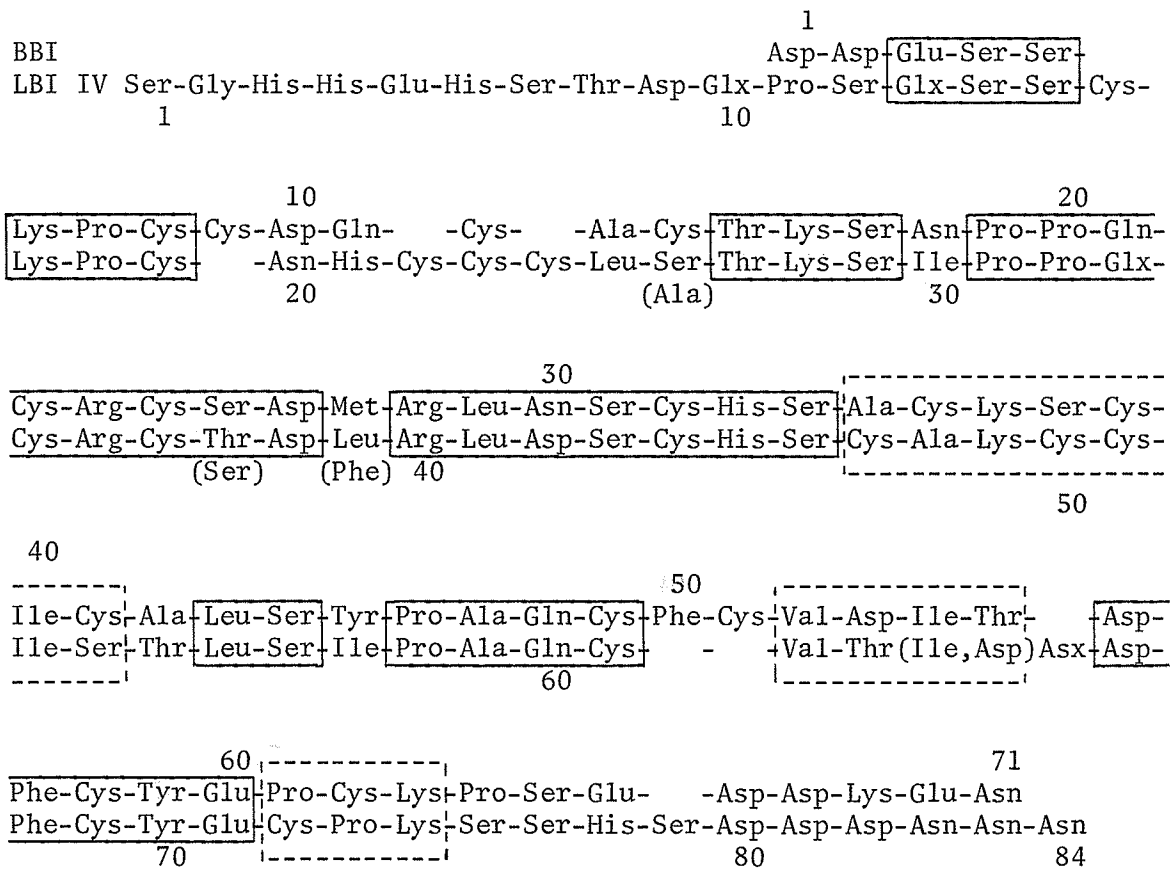

Figure 1 Amino acid sequences of LBI IV and BBI.

The top lines are the BBI sequence while the bottom lines are the LBI IV sequence. The boxes in solid lines indicate identical sequences whereas the boxes in dotted lines indicate identical amino acid composition. Figure taken from Odani et al. (1971).

for which the best understanding of function has been achieved are discussed below.

1. Pancreatic Secretory Trypsin Inhibitor

The exocrine secretion of the pancreas of most animals contains zymogens of enzymes used for the digestive process in the small intestine. This zymogen system serves to keep the enzymes inactive until they are needed. The zymogens are normally activated by trypsin (formed from trypsinogen by the action of enterokinase in the small intestine). Since PSTI is found in the pancreatic secretion of all animals thus far examined, it is generally accepted that its function is to inhibit any trypsin that is accidentally activated, thereby preventing the premature activation of the pancreatic zymogen system.

2. Colostrum Inhibitors

In many mammals, transfer of immune bodies from mother to offspring occurs via colostrum, i.e. the immune bodies are absorbed from the intestine of the suckling animal. It is thought necessary for the colostrum to have proteinase inhibitor present so that the immune bodies present in the colostrum are protected from degradation by the proteolytic enzymes of the infants' digestive system until they are absorbed intact. Circumstantial evidence supports this hypothesis but model experiments have been inconclusive (Vogel et al., 1968). It is interesting to note that in those species where immune bodies are thought to be transferred via placenta (humans) the inhibitor content of colostrum is much lower than in those species (pigs, cows) where

immune bodies are transferred via colostrum (Vogel et al., 1968).

3. Inhibitors from Ascaris Lumbricoides

Ascaris lumbricoides is a round worm parasite which lives in the intestine of its host, namely in man or pig. This environment is extremely harsh since it contains many degradative enzymes geared to break down protein, carbohydrate, fat and nucleic acids. The survival of these round worms cannot be attributed to some unique property of their cuticle but rather to the presence of trypsin, chymotrypsin, and pepsin inhibitors (Peanasky and Abu-Erreish, 1971). Whole worms do in fact inhibit these enzymes while other proteolytic enzymes such as ficin, bromelain, or papain very rapidly digest them (Vogel et al., 1968; Peanasky and Abu-Erreish, 1971).

4. Potato Inhibitors

Proteinase inhibitors from plants usually do not have the ability to inhibit plant proteinases. This has led Green and Ryan (1972) to investigate the function of these inhibitors in potatoes. These workers found that damage to the potato leaf either mechanically or by the action of potato beetles caused the concentration of potato inhibitor I (inhibits trypsin and chymotrypsin) to increase three-fold in all the leaves of the plant. Green and Ryan (1972) suggest that this phenomenon could be a potential threat to the metabolism of the insect and hence constitute a defense mechanism for the potato plant.

IV. Interaction Between Proteases and their Naturally Occurring Inhibitors

A. Mechanism of Action of Proteases

An understanding of the mode of action of proteases is a basic prerequisite to the understanding of the mechanism of action of protein protease inhibitors. This review is mainly concerned with inhibitors of serine proteases such as trypsin and chymotrypsin. It is generally accepted (Hartley and Shotton, 1971) that serine proteases from a variety of sources share a common mechanism and the present review will therefore limit itself to a brief discussion of the present state of knowledge on the substrate binding and the mechanism of action of chymotrypsin.

1. Substrate Binding to Chymotrypsin

The elucidation of the three dimensional structure of chymotrypsin (Blow, 1971) has greatly facilitated studies on the mode of binding of peptide substrates to the enzyme. These studies involve both X-ray diffraction analysis of chymotrypsin modified with site-specific inhibitors such as peptide chloromethyl ketones (Segal et al., 1971a, b) and kinetic investigation on the affinity of various peptide methyl esters for chymotrypsin (Segal, 1972). Correlation of the results of such investigations has allowed Segal to propose the mode of binding illustrated in Figure 2. The amino acid residues of the peptide substrate are designated as the P_3, P_2, P_1, and P_1' residues and the bond hydrolyzed is between P_1 and P_1'. The substrate binding site of chymotrypsin is correspondingly divided into the subsites S_3, S_2, S_1

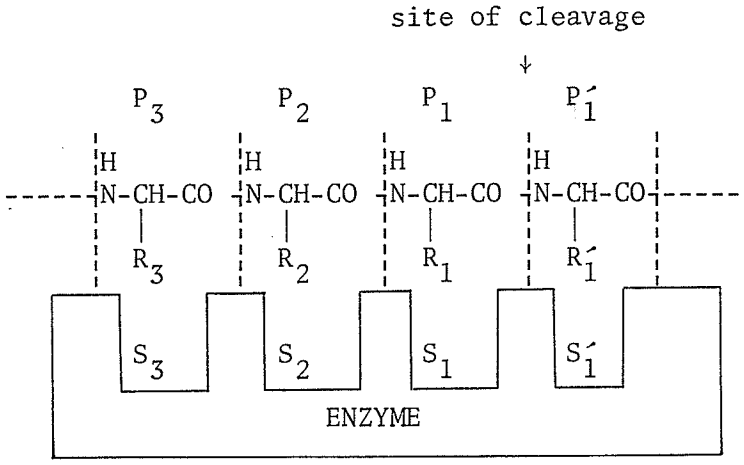

Figure 2 Substrate binding site of chymotrypsin.

P_3, P_2, P_1 and P_1' indicate the amino acid residues of the substrate at the S_3, S_2, S_1, and S_1' subsites of the substrate binding site of chymotrypsin. Peptide bond hydrolysis occurs between the P_1 and P_1' amino acid residues.

and S_1'. Subsite S_1 contains a hydrophobic specificity pocket into which fits the hydrophobic side chain (R_1) of the P_1 residue. P_1 can be tyrosine, phenylalanine, tryptophan or leucine and thus S_1 confers the "primary specificity" on chymotrypsin. In addition to this, at S_1, the acylamino group (-NH-) of P_1 is hydrogen bonded to the carbonyl group (-CO-) of serine 214 of the enzyme. At S_3, the acylamino group of P_3 is hydrogen bonded with the carbonyl group of glycine 216 of the enzyme while the carbonyl of P_3 is hydrogen bonded with the acylamino group of glycine 216 of the enzyme. Thus the segment consisting of residues 214 - 216 of the enzyme forms an anti-parallel β-pleated sheet with P_1 to P_3. Beyond P_3 no interaction between enzyme and substrate is apparent. The side chain of P_2 (R_2) may be involved in hydrophobic interaction with the enzyme at S_2. The final result of all these binding processes is that the substrate is presented in the proper fashion to the enzyme catalytic site.

2. Catalytic Mechanism of Chymotrypsin

The investigation of the catalytic mechanism of chymotrypsin for the hydrolysis of ester and peptide bonds has been carried out by various techniques. From chemical modification studies it is known that both serine 195 and histidine 57 are very important in the enzyme's catalytic mechanism. In the hydrolysis of ester substrates, enzyme intermediates in which serine 195 is acylated have been detected (Bender and Kézdy, 1965). From X-ray diffraction studies, Hess (1971) has postulated that serine 195, histidine 57, and aspartic acid 102 form a hydrogen-bonded charge relay system as shown in Figure 3. This charge relay system serves to increase the

nucleophilicity of the hydroxyl oxygen of serine 195 so that its attack on the carboxyl group of the substrate is facilitated. The

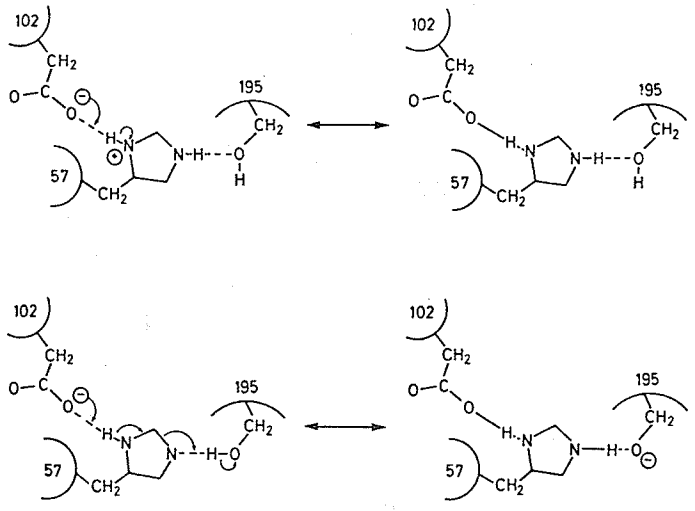

Figure 3 Canonical forms of the charge relay system in the chymotrypsin active site.

Top line, pH 4.0; bottom line, pH 8.0

steps involved in the proposed catalytic mechanism for the hydrolysis of amide substrates are illustrated in Figure 4. It seems possible that, in the Michaelis-Menten complex, the acylamino groups (-NH-) of glycine 193 and serine 195 are hydrogen bonded to the carbonyl of the amide bond being hydrolysed (Panel I, Figure 4). Panel II shows a hypothetical, tetrahedral intermediate which is already covalently linked to serine 195. Supposedly, the hydrogen-bonded charge relay system has, at this point, caused the abstraction of a proton from the hydroxyl group of serine 195 and the oxygen has formed a covalent bond

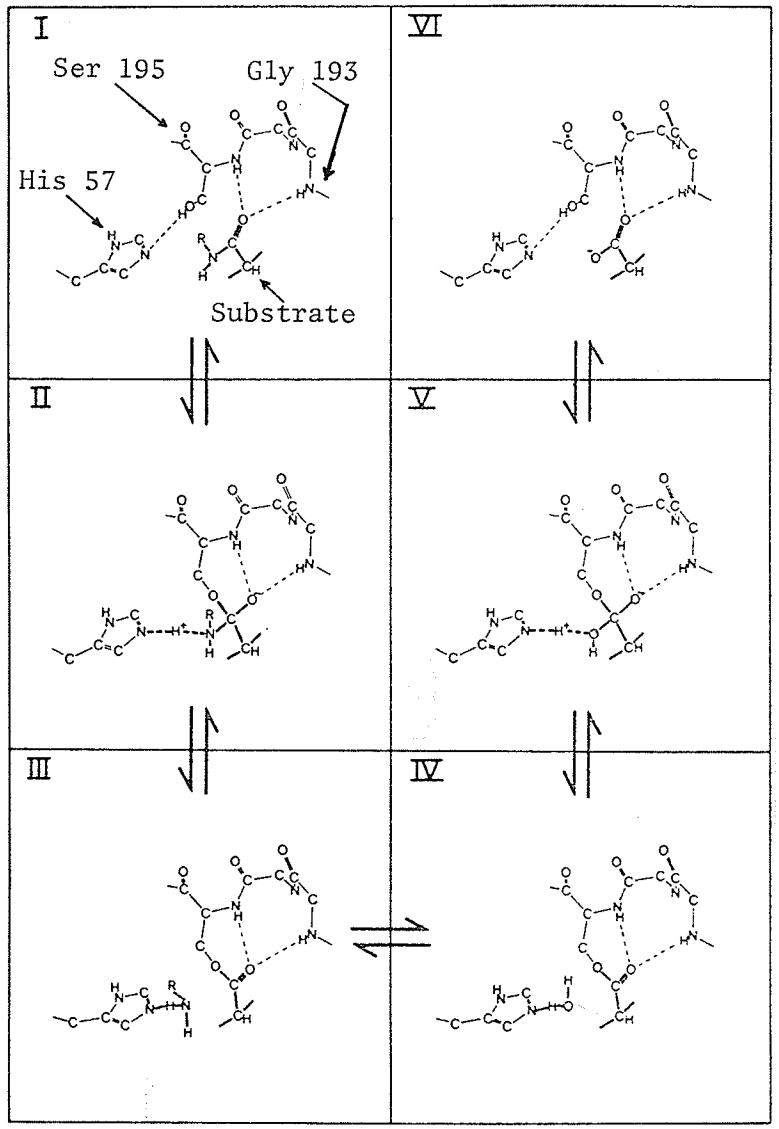

Figure 4 The proposed sequence of stereochemical events in the hydrolysis of a peptide (amide) bond by chymotrypsin.

From Henderson et al. (1972).

with the substrate carbon. The cleavage of the C-N bond is then accomplished by proton transfer from histidine 57 to the nitrogen atom of the C-N bond involved in the tetrahedral intermediate. The enzyme is now in the acylated form and has released the first product. Deacylation is simply the reverse of acylation but with the introduction of water to accomplish hydrolysis of the acyl enzyme. Hess (1971) has speculated that the hydrolysis of ester and amide bonds by chymotrypsin occurs by the same mechanism but that the rate constants for the different steps are different. In the case of ester hydrolysis, acyl enzymes are easily detected but in the case of amide hydrolysis, acyl enzymes are not detectable since the rate constants for their formation and breakdown do not favour the accumulation of detectable amounts. The feature of the above mechanism most important to our discussion is the fact that the substrate becomes covalently bound to chymotrypsin either as a tetrahedral intermediate, or as an acyl intermediate or both. It is likely that this catalytic mechanism holds true for all serine proteases and also plays an important role in the interaction of the enzymes with naturally occurring inhibitors.

B. Mechanism of Action of Proteinase Inhibitors

1. Complexation

Protein proteinase inhibitors inhibit proteolytic enzymes by forming stoichiometric complexes with them (Vogel et al., 1968; Laskowski and Sealock, 1971). These complexes are stable over a wide pH range and are physically demonstrable by such well known techniques as ultracentrifugation, gel filtration, and gel electrophoresis. The kinetics of complex formation can be followed in several ways. The

simplest is to monitor enzyme activity, while more complex methods for example utilize the difference spectra between free enzyme and inhibitor and the enzyme-inhibitor complex (Laskowski and Sealock, 1971). From slightly alkaline pH to pH 5.0 the enzyme-inhibitor complex is very stable. At pH values lower than 5, dissociation phenomena predominate and at pH 3.0, complex dissociation is generally complete (Steiner, 1972; Ishida et al., 1970).

In general, proteinase inhibitors are thought to form complexes only with active enzymes. Osuga and Feeney (1967) showed that turkey ovomucoid and potato chymotrypsin inhibitor did not form a complex with DIP-chymotrypsin[4] or with chymotrypsinogens A or B. Chicken ovomucoid, Ascaris inhibitor, and BPTI do not form complexes with DIP-trypsin although the same inhibitors show a perceptible interaction with TLCK-trypsin or photo-oxidized trypsin (Pudles and Bachellerie, 1968). Complex formation of inhibitors with inactive enzyme derivatives has been demonstrated with anhydro-chymotrypsin, tosyl-chymotrypsin, TLCK-trypsin, TPCK-chymotrypsin (Feinstein and Feeney, 1966), trypsinogen (Dlouhà and Keil, 1969), anhydro-chymotrypsin and anhydro-trypsin (Ako

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[4]DIP-trypsin and DIP-chymotrypsin are the inactive derivatives of the respective enzymes produced by reaction of diisopropylphosphofluoridate with the hydroxyl group of the enzyme active site serine residue. TLCK-trypsin and TPCK-chymotrypsin are inactive derivatives of the enzymes produced by alkylation of the imidazole side chain of the active site histidine with N-tosyl-L-lysylchloromethyl ketone (TLCK) and N-tosyl-L-phenylalanylchloromethyl ketone (TPCK) respectively. Photo-oxidized trypsin is inactive enzyme in which the active site histidine is modified. The anhydro-enzymes refer to enzymes which are chemically modified by the conversion of the active site serine to dehydroalanine rendering them completely inactive. Tosyl-chymotrypsin is the inactive derivative of chymotrypsin with a tosyl group covalently linked to Ser 195.

et al., 1972a, b). Although these data very clearly show that inactive enzyme derivatives do form complexes with various inhibitors, the actual affinity of the inhibitors for these inactive enzymes has not been determined so that a comparison of active and inactive enzymes with respect to binding affinity is not possible.

Several laboratories have demonstrated that plant inhibitors exist in solution as dimers or trimers but that upon complex formation with enzyme, these inhibitor polymers dissociate (Hochstrasser et al., 1969b, 1970; Millar et al., 1969). In one case, namely that of potato chymotrypsin inhibitor I, it has been shown that the inhibitor of molecular weight of 39,000 g/mole is composed of four subunits each of which binds one chymotrypsin molecule to form a complex with a molecular weight of 140,000 g/mole (Melville and Ryan, 1970, 1972). This is the only known inhibitor with a subunit structure which remains intact upon complex formation.

Since the three dimensional structures of both chymotrypsin (trypsin) (Blow, 1971) and BPTI (Huber et al., 1970, 1971) have been elucidated, it has recently been possible to investigate the details of their interaction by model building studies (Blow et al., 1972). These studies will be discussed in more detail later.

2. Chemical Modification Studies

Chemical modification of amino acid side chains in proteins has been used to study protein-ligand or protein-protein interactions but often has only limited value because only a small number of side chains can be separately and specifically modified. In the

case of trypsin inhibitors, however, chemical modification has been a very useful and productive technique in the study of their interaction with trypsin. All trypsin inhibitors are inactivated by modification of either their lysyl or arginyl residues (Fritz et al., 1969; Liu et al., 1968) in that some are inactivated by maleic anhydride (reacts with the ε-amino group of lysine) while others are inactivated by reaction with either 2, 3-butanedione or 1, 2-cyclohexanedione (reacts with the guanidinium group of arginine).[5] In this manner, trypsin inhibitors can be classified into one of two categories depending on whether they require lysine or arginine residues for activity. Since trypsin hydrolyzes peptide bonds at the carboxyl-terminal of arginine or lysine residues but will not cleave the same bonds when the arginine or lysine residues are modified, it has been postulated that the portion of the inhibitor responsible for the inhibitory activity resembles an actual trypsin substrate. Haynes et al. (1967) have shown that the rate of inactivation of the trypsin inhibitory activity of turkey ovomucoid upon reaction with trinitrobenzenesulfonic acid corresponds exactly to the rate of reaction of a single fast reacting lysine residue. In the cases of two different trypsin inhibitors it has been possible to identify the specific lysine residue responsible for the inhibitory activity of the inhibitor by comparing the results of chemical

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[5]In double-headed inhibitors which inhibit both trypsin and chymotrypsin these chemical modifications inactivate only the trypsin inhibitory activity and leave the chymotrypsin inhibitory activity unaffected.

modification of the inhibitor in the free form and in the trypsin-inhibitor complex. Chauvet and Acher (1967) polyalanylated the ε-amino groups of all four lysine residues in the free BPTI thereby producing an inactive inhibitor; on the other hand, when the inhibitor was modified as the trypsin-inhibitor complex, only three lysine residues of BPTI were modified and the inhibitor retained full activity. By comparative analysis of these two modified forms of BPTI by peptide mapping, Lys^{15} was identified as the protected lysine. Using similar techniques, Čechová and Musyńská (1970) showed Lys^{18} to be involved in the trypsin binding site of bovine colostrum trypsin inhibitor. It therefore appears that the activity of a trypsin inhibitor is dependent on a single amino acid side chain (lysine or arginine) with specificity for trypsin.

Table III shows all the trypsin inhibitors that have been classified to date as "lysine inhibitors" or "arginine inhibitors" by chemical modification.

In the case of "lysine inhibitors" it is of interest that chemical modifications like guanidination which allow the derivatives of the lysine side chains to retain their positive charge will not inactivate the inhibitor. Haynes and Feeney (1968) have demonstrated that both LBI and turkey ovomucoid which have been extensively guanidinated (lysine residues converted to homoarginine) retain almost full inhibitory activity. Furthermore, these guanidinated inhibitors could now be inactivated by reagents specific for guanidino groups such as 1, 2-cyclohexanedione (Haynes and Feeney, 1968). In this way they demonstrated the "transformation" of a "lysine inhibitor" to a

TABLE III

CLASSIFICATION OF TRYPSIN INHIBITORS BY CHEMICAL MODIFICATION

Lysine Inhibitors		Arginine Inhibitors	
Ovomucoid,		Ovomucoid,	
turkey	2, 4, 9	chicken	2, 4
cassowary	4, 8	Ovoinhibitor,	
penguin	4, 8	chicken	11, 13
duck	4, 8	quail	13
ostrich	8	turkey	13
rhea	8	Soybean (Kunitz)	8, 10
emu	8	Ascaris	7
Colostrum,		PSTI,	
bovine	4, 12	bovine	10
BPTI,		ovine	10
bovine	3, 6, 10	Submandibular gland,	
PSTI,		canine	10
porcine I, II	10	Seminal vesicles,	
canine	10	guinea pigs	14
feline	10	Seminal plasma,	
Beans,		porcine	14
lima	1, 4, 5, 10	Sea anemone (actiniaria)	14
soybean (BBI)	11	Wheat	10
white	10	Corn	10
Leeches	14	Rye	10
		Potato I	10
		Peanut	10
		Inter-α,	
		human serum	10

References:
1. Fraenkel-Conrat et al. (1952)
2. Stevens and Feeney (1963)
3. Kassel and Laskowski (1965b)
4. Haynes et al. (1967)
5. Haynes and Feeney (1967)
6. Chauvet and Acher (1967)
7. Pudles et al. (1967)
8. Liu et al. (1968)
9. Haynes and Feeney (1968)
10. Fritz et al. (1969)
11. Feeney and Allison (1969)
12. Muszyńská and Čechová (1970)
13. Liu et al. (1971)
14. Fink et al. (1971b)

"homoarginine inhibitor". These data are surprising since it is generally believed that peptide bonds involving a modified lysyl residue are not good trypsin substrates. Recently, however, it has been shown that a specific homoarginyl-seryl peptide bond in guanidinated, reduced and alkylated LBI is readily cleaved by trypsin (Tan and Stevens, 1971a, b). Seidl and Liener (1971a) have also shown that a homoarginyl peptide bond in Bowman-Birk inhibitor is also cleaved by trypsin.

It is known that chymotrypsin is specific for cleavage of peptide bonds involving the carboxyl group of tyrosine, phenylalanine, tryptophan or leucine and therefore by analogy it could be expected that modification of one of these side chains would inactivate chymotrypsin inhibitors. Unfortunately, no reagents specific for these side chains have as yet been devised.

3. The Reactive Site Model

a) Discovery of reactive sites in protein proteinase inhibitors

During studies on the complex formation between trypsin and soybean trypsin inhibitor, Lebowitz and Laskowski Jr. (1962) observed that, at pH values greater than 4.5, this reaction releases protons in stoichiometric amounts quickly and in a smooth, even manner. At pH values lower than 4.5, a proton overshoot is evident i.e. upon mixing of STI and trypsin there is an initial rapid release of protons followed by a slower partial uptake of the released protons. Laskowski and Sealock (1971) interpret these data to mean that two reactions are taking place upon mixing of STI and trypsin. The first reaction,

namely the association of STI and trypsin, is responsible for the fast release of protons while the second reaction, which takes up some protons, is believed to be the covalent modification of STI by the enzyme (Finkenstadt and Laskowski, Jr., 1965). Modification of STI by trypsin was not unexpected since trypsin is known to cleave peptide bonds on the carboxyl side of lysyl or arginyl residues. By incubating STI at pH 3.75 with one mole percent of trypsin, a trypsin-modified STI (STI*) was obtained (Finkenstadt and Laskowski, 1965). STI* still retains full inhibitory activity against trypsin but it combines with the enzyme at a much slower rate than does STI. Furthermore, treatment of STI* with carboxypeptidase B at neutral pH results in complete loss of its inhibitory activity whereas similar treatment of virgin STI is of no consequence. Ozawa and Laskowski (1966) were able to show that the modification in STI* consists of the cleavage of one specific peptide bond identified as Arg^{64}-Ile^{65}. The resulting two polypeptide chains are still held together by a disulfide bond and subsequent removal of the new carboxyl-terminal Arg results in an inactive product. On this basis they designated Arg^{64}-Ile^{65} as the reactive site of STI. The inhibitor with the reactive site bond intact is called "virgin" inhibitor whereas the inhibitor with the peptide bond cleaved is called the "trypsin-modified" inhibitor (STI*). Similar studies on chicken ovomucoid indicate that its reactive site is an arginyl-alanyl peptide bond (Ozawa and Laskowski, 1966). In the case of both STI and chicken ovomucoid only one of several trypsin-susceptible bonds are cleaved during incubation with catalytic amounts of trypsin at acid pH. The modified inhibitor in which a specific peptide bond is cleaved is

fully active but reacts with trypsin at a much slower rate than the virgin form. In both modified inhibitors, removal of the reactive site carboxyl-terminal residue by carboxypeptidase B renders the product inactive. On the basis of this evidence and evidence obtained by other workers by chemical modification of trypsin inhibitors (see previous section on chemical modification), Ozawa and Laskowski (1966) proposed that all trypsin inhibitors contain either a lysyl-X or an arginyl-X peptide bond at their reactive sites.

b) Detection of reactive sites

Reactive sites in protein proteinase inhibitors are generally detected by partial proteolysis of the inhibitor with catalytic amounts of the appropriate enzyme at acid pH. In analogy to the results obtained with STI and chicken ovomucoid, this treatment should result in the cleavage of the reactive site peptide bond yielding new amino- and carboxyl-terminal residues which can then be determined by classical end-group analysis. All trypsin-modified inhibitors produced in this fashion to date, have been shown to contain either lysine or arginine as the new carboxyl-terminal residue. In every case studied, the two peptide chains resulting from the partial proteolysis have been found to be held together probably by one or more disulfide bonds. Thus, to determine the location of a reactive site within the amino acid sequence of an inhibitor, the two chains of the modified inhibitor must be separated after cleavage of the disulfide linkages and characterized in terms of terminal amino acids as well as amino acid composition. Table IV shows the reactive sites of trypsin inhibitors determined in this fashion to date. The results obtained in

TABLE IV

REACTIVE SITES OF TRYPSIN INHIBITORS
DETERMINED BY CLEAVAGE WITH TRYPSIN AT ACID pH

Inhibitor	Reactive Site	Reference
BPTI[a],		
bovine	Lys^{15}-Ala^{16}	Kress and Laskowski Sr. (1968)
ovine	Lys^{15}-Ala^{16}	Laskowski and Sealock (1971)
PSTI,		
bovine	Arg^{18}-Ile^{19}	Rigbi and Greene (1969)
ovine	Arg^{18}-Ile^{19}	Hochstrasser and Werle (1971)
porcine I	Lys^{18}-Ile^{19}	Tschesche and Klein (1968)
canine	Lys -Ile	Tschesche et al. (1971)
Seminal vesicular,		
guinea pig	Arg -X	Fink et al. (1971b)
Ovomucoid,		
chicken	Arg -Ala	Ozawa and Laskowski (1966) Haynes and Feeney (1968)
STI	Arg^{64}-Ile^{65}	Ozawa and Laskowski (1966)
Lys^{64}-STI[b]	Lys^{64}-Ile^{65}	Sealock and Laskowski (1969)
Corn	Arg^{25}-Leu^{26}	Hochstrasser and Werle (1971)
Rye	Arg -Ala	Hochstrasser et al. (1969c)
Wheat	Arg -Ala	Hochstrasser et al. (1969c)
Peanut	Arg -Ala	Hochstrasser et al. (1969b)

[a]BPTI with the Cys 14-Cys 38 disulfide bond reduced and carboxamidomethylated (Kress and Laskowski, 1967, 1968).

[b]Produced by enzyme mutation as described by Sealock and Laskowski (1969) and illustrated in Figure 5.

this way are entirely consistent with those of chemical modification studies discussed earlier (See Table III). The inhibitors found by chemical modification to require lysine for activity (so-called lysine inhibitors) all have a lysyl-X reactive site whereas those requiring arginine for activity (so-called arginine inhibitors) all have an arginyl-X reactive site.

Since trypsin inhibitors have reactive sites containing either an arginyl-X or a lysyl-X peptide bond in this way reflecting trypsin specificity it could be expected that the reactive sites of chymotrypsin inhibitors would similarly reflect chymotryptic specificity. On the basis of this assumption, the reactive site of chymotrypsin inhibitors should have either a phenylalanyl-X, a tyrosyl-X, a tryptophanyl-X or a leucyl-X peptide bond. However, when the present investigation was initiated, no reactive sites in chymotrypsin inhibitors had been determined.

c) Properties of reactive sites

The amino acid sequences around many of the reactive sites listed in Table IV are known and therefore some general similarities and properties can be deduced. The reactive sites of all trypsin inhibitors contain a lysyl-X or an arginyl-X peptide bond which can be selectively hydrolyzed by trypsin at acid pH. Removal of the new carboxyl-terminal residue of the modified inhibitors results in an inactive product. The new amino-terminal residue in modified inhibitors is either Ile, Leu or Ala[6]. Kowalski and Laskowski (1972)

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[6]Bowman-Birk inhibitor and LBI have a serine in this position.

showed that chemical modification of the α-amino group of the reactive site in the modified inhibitor results in total loss of activity. To date, it appears that all reactive sites have one or more disulfide linkages which hold the two chains of the modified inhibitors together. These disulfide linkages may be essential for activity, since complete reduction results in total loss of activity whereas reoxidation in most cases restores partial or full activity (Laskowski and Sealock, 1971). In BPTI, the reactive site peptide loop is maintained by two disulfide linkages namely Cys 14-Cys 38 and Cys 5-Cys 55. The Cys 14-Cys 38 disulfide bond can be cleaved without loss of activity, but cleavage of the Cys 5-Cys 55 disulfide bond results in an inactive inhibitor. All the sequences near the reactive site so far examined are devoid of acidic residues suggesting that the presence of a negative charge in the reactive site peptide is not permissible. This hypothesis has been put to a direct test by Kress et al. (1968) using the active BPTI with the Cys 14-Cys 38 reduced. Cys 14 is only two residues removed from the reactive site Lys 16. Alkylation of Cys 14 with alkylating agents (like iodoacetic acid) which introduce a negative charge result in total loss of activity while those which introduce no charge (like iodoacetamide) result in retention of activity. Most of the reactive site disulfide loops examined contain a prolyl residue possibly to confer rigidity on the reactive site peptide (Laskowski and Sealock, 1971).

d) Peptide bond hydrolysis equilibria in the reactive site of trypsin inhibitors

Finkenstadt and Laskowski (1965) first proposed

that the reaction between soybean trypsin inhibitor (STI) and trypsin could best be described by the following reaction scheme:

$$T + STI \rightleftharpoons C \rightleftharpoons T + STI^* \quad \ldots (1)$$

in which T is trypsin, STI is virgin inhibitor, C is the trypsin-inhibitor complex and STI* is the modified inhibitor with the reactive site peptide bond cleaved. These authors demonstrated the formation of complex between STI and T as well as the formation of STI*. They also showed that STI* and T could form a complex. Subsequently Finkenstadt and Laskowski (1967), Niekamp et al. (1969), and Hixson and Laskowski (1970b) demonstrated the conversion of STI* to STI in two ways. The first method, to be discussed in more detail below, consisted of incubating STI* at acid pH in the presence of catalytic amounts of trypsin to produce STI by mass action (STI* → STI), while the second method, called kinetic control dissociation, will be discussed in a separate section (See section f). This then demonstrated all the reactions shown in Scheme (1). Virgin and modified inhibitors are in an equilibrium with each other which can be represented as follows:

$$STI \underset{\text{trypsin}}{\overset{\text{acid pH}}{\rightleftharpoons}} STI^* \quad \ldots (2)$$

Using disc gel electrophoresis, Niekamp et al. (1969) were able to distinguish between STI and STI* and conclusively prove that STI and STI* were in equilibrium with each other. They demonstrated that, at pH 4.0, both the forward and reverse reactions resulted in an equilibrium mixture containing 86±2% STI* and 14±2% STI. The forward

reaction (STI → STI*) catalyzed by 2 mole percent trypsin, reached this equilibrium position in 10 hr while the reverse reaction, catalyzed by 0.5 mole percent trypsin, reached equilibrium in 3 hr. For the hydrolysis of ordinary peptide bonds the equilibrium is highly in favor of the cleaved products, so that the peptide bond hydrolysis equilibrium in the reactive site of STI appears to be abnormal. Laskowski (1970) has shown that STI and STI* have identical conformations suggesting that the peptide bond hydrolysis equilibrium in the reactive site of soybean trypsin inhibitor is dependent on its conformation, thus accounting for its abnormal behavior. Ishida et al. (1970) noted that the rate of formation of STI* was optimal at pH 4.0 where 50% of the trypsin present is complexed with STI. Below pH 3.0, where almost no complex exists, the rate of formation of STI* is minimal, as it is at pH greater than 4.7, where all the available trypsin is complexed to STI. Laskowski and Sealock (1971) have reported the hydrolysis equilibrium constant (K_{hyd}) for the STI and STI* interconversion as a function of pH. K_{hyd} for the reaction in Scheme (2) would be:

$$K_{hyd} = \frac{[STI^*]}{[STI]} \quad \ldots . \quad (3)$$

Between pH 4.3 and 8.0 K_{hyd} has minimal values of 2 - 3. From pH 4.0 to 3.0 and from pH 8.0 to 9.0 K_{hyd} rose from 4 to 16. More extreme pH values are difficult to study due to the low activity and denaturation of trypsin at these pH values.

The study of other trypsin inhibitors from the point of view of peptide bond hydrolysis equilibria of their reactive sites has been

somewhat fragmentary. In most cases that have been studied only the forward reactions (bond cleavage) have been demonstrated. Rigbi and Greene (1968) showed that when bovine PSTI is incubated with 0.7 mole percent of trypsin at pH 3.22, a steady state plateau consisting of 30% modified PSTI and 70% virgin PSTI is attained in 4-5 hr. The pH dependence of the initial rate of this reaction gives a bell-shaped curve with a maximum at pH 2.7 and minima below pH 2.0 and above pH 5.0. For Bowman-Birk inhibitor from soybeans a steady state plateau is reached in 72 hr when 5 mg of the inhibitor are incubated with 0.1 mg of trypsin at pH 3.75. At this point about 70% of the inhibitor is modified (Birk et al., 1967). In both these cases (PSTI and BBI) a steady state plateau was attained (for the conversion of virgin to modified inhibitor) suggesting equilibrium had been reached. In all other cases where peptide bond cleavage of the reactive site has been achieved, no attempts to study equilibria were made. Similarly no chymotrypsin reactive sites have been studied in this regard.

e) Interaction of virgin and modified inhibitor with enzyme

Although the reactions described in Scheme (1) (section d) have been shown to occur, recent evidence indicates that the equation is somewhat oversimplified. Using stopped flow techniques and by taking advantage of the change in the ultraviolet absorption spectrum upon complex formation, Laskowski et al. (1971) and Luthy et al. (1973) examined the kinetics of interaction of trypsin with STI and STI*. Their evidence suggests the following reaction scheme:

$$T + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} L^* \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} T + I^* \quad . \quad . \quad . \quad (4)$$

where T is trypsin, I and I* are STI and STI*, L and L* are the loose complexes between T and I or I*, while C is the central stable complex postulated by Laskowski's group to be an acyl enzyme. This scheme is similar to Scheme (1) (section d) with the exception of the loose complexes occurring as intermediates between the stable central complex and the free forms of the enzyme and inhibitor. Both I and I* react with trypsin although the final equilibrium position between I and I* indicated by K_{hyd} is a constant that is pH dependent. Kinetic analysis has allowed some generalizations to be made about Scheme (4). The rate constants k_1 and k_{-4} are second order and probably diffusion-dependent whereas all the other rate constants are first order. Over the pH range 4.0 to 8.0, the association constants for T and I (K_L) or I*(K_{L^*}) to form the loose complexes, L and L*, are approximately equal. Since $K_L = k_{-1}/k_1$ and $K_{L^*} = k_4/k_{-4}$ the assumption $k_{-1} \simeq k_4$ and $k_1 \simeq k_{-4}$ can be made. This being so, the inhibition produced by STI must be explainable in kinetic terms in the following reactions taken from Scheme (4).

$$L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} L^*$$

Laskowski's group has proposed that C is an acyl enzyme in which the active site serine of trypsin is acylated by the P_1-residue of the inhibitor reactive site. On this basis, Laskowski et al. (1971) and Luthy et al. (1973) have compared the rate constants k_2 and k_{-3}

(supposedly acylation rate constants) with the acylation rate constants for actual substrates and found reasonable agreement. In a similar fashion, k_{-2} and k_3 would represent deacylation rate constants. The pH dependences of k_{-2} and k_3 are exactly opposite to those of actual substrates, i.e. they have very low values at neutral pH and increase dramatically as the pH decreases. This of course implies that, at neutral pH, the acyl enzyme deacylates with great difficulty thereby accounting for the inhibition phenomenon. Since k_{-2} and k_3 increase so dramatically below pH 5.0, the enzyme and inhibitor are dissociated to a large extent at lower pH values. The interconversion of I and I* at low pH is thus facilitated because any stable complex (C) formed is assured of rapid breakdown to T and I or I*. k_{-2} is much greater than k_3, a fact which is of great importance in the following discussion on kinetic control dissociation.

f) Kinetic control dissociation

The term kinetic control dissociation was first coined by Finkenstadt and Laskowski (1967). These authors and later Hixson and Laskowski (1970b) use it to describe the process whereby trypsin-inhibitor complex made either from I* or I is rapidly dissociated and the distribution of products (I and I*) is governed by the relative rates of transformation of the central complex to L or L* (the dissociation rate constants of the loose complexes are assumed equal as previously discussed). In the dissociation procedure used, trypsin denatures and precipitates out of solution. Thus the rate constants k_{-2} and k_3 [Scheme (4)] determine the distribution of I and I* upon rapid dissociation of the central complex. Since k_{-2} is much greater

than k_3, predominantly virgin inhibitor is expected. This has been demonstrated experimentally (Finkenstadt and Laskowski, 1967; Hixson and Laskowski, 1970b). These workers have subjected complexes made both from virgin STI and STI* to kinetic control dissociation. In both instances the resultant inhibitor was found to be the virgin STI. Thus the situation exists where virgin STI can be obtained from STI* by resynthesis of the reactive site peptide bond using the process of kinetic control dissociation, even though STI* is the thermodynamically favoured species. Laskowski and Sealock (1971) view this phenomenon as very clear proof that the inhibitor reactive site has been in contact with the trypsin active site, and thus is very important in the inhibition mechanism.

By kinetic control dissociation of complexes made from (1) chymotrypsin and chymotrypsin-modified Bowman-Birk inhibitor and (2) trypsin and trypsin-modified Bowman-Birk inhibitor Frattali and Steiner (1969) were able to show that the inhibitors obtained in this manner behaved electrophoretically like virgin inhibitors, suggesting that the reactive site peptide bond had been resynthesized. These authors, however, did not further characterize the inhibitors obtained from kinetic control dissociation.

No other proteinase inhibitors have been studied in this manner.

g) Enzymatic replacement of Arg^{64} in the reactive site of STI

Sealock and Laskowski (1969) in a series of elaborate experiments have replaced the arginyl residue of the reactive site of STI (Arg 64) with a lysyl residue. The method used is

illustrated in Figure 5. The first step was production of STI* followed by removal of the new carboxyl-terminal arginyl residue with carboxypeptidase B to produce des-Arg^{64}-STI*. Since these reactions are reversible, it should be possible to produce a Lys^{64}-STI* by incubating lysine, des-Arg^{64}-STI* and carboxypeptidase B. Having produced the Lys^{64}-STI* this inhibitor should now be capable of forming complex with trypsin and finally the virgin Lys^{64}-STI could be produced by kinetic control dissociation. Laskowski and Sealock (1971) have estimated the equilibrium constants for these reactions:

$$\text{Lys} + \text{des-Arg}^{64}\text{-STI*} \overset{\text{COB}}{\rightleftharpoons} \text{Lys}^{64}\text{-STI*} \qquad K = 10^{-2}\ M^{-1} \quad . \ . \ . \ (5)$$

$$\text{Lys}^{64}\text{ - STI*} + \text{trypsin} \rightleftharpoons \text{complex} \qquad K = 10^{9}\ M^{-1} \quad . \ . \ . \ (6)$$

$$\text{Thus Lys} + \text{des-Arg}^{64}\text{-STI*} + \text{trypsin} \overset{\text{COB}}{\rightleftharpoons} \text{complex} \qquad K = 10^{7}\ M^{-1} \quad . \ . \ . \ (7)$$

The reaction in Scheme (5) is in itself not favourable but when this is coupled to the complexation reaction [Scheme (6)] the overall reaction [Scheme (7)] becomes quite favourable ($K = 10^{7}\ M^{-1}$). Sealock and Laskowski (1969) have in fact coupled the two reactions and followed the progress of the reaction by monitoring STI-T complex formation by gel filtration. After ten days, when the amount of complex reached a maximum, the complex was isolated and subjected to kinetic control dissociation. The inhibitor obtained in this manner was then characterized in various ways, and shown to have lysine in its reactive site.

If the requirements for chymotrypsin reactive sites are similar to those for trypsin reactive sites, it should be possible to make a chymotrypsin inhibitor from STI by replacing Arg 64 with either tyrosine,

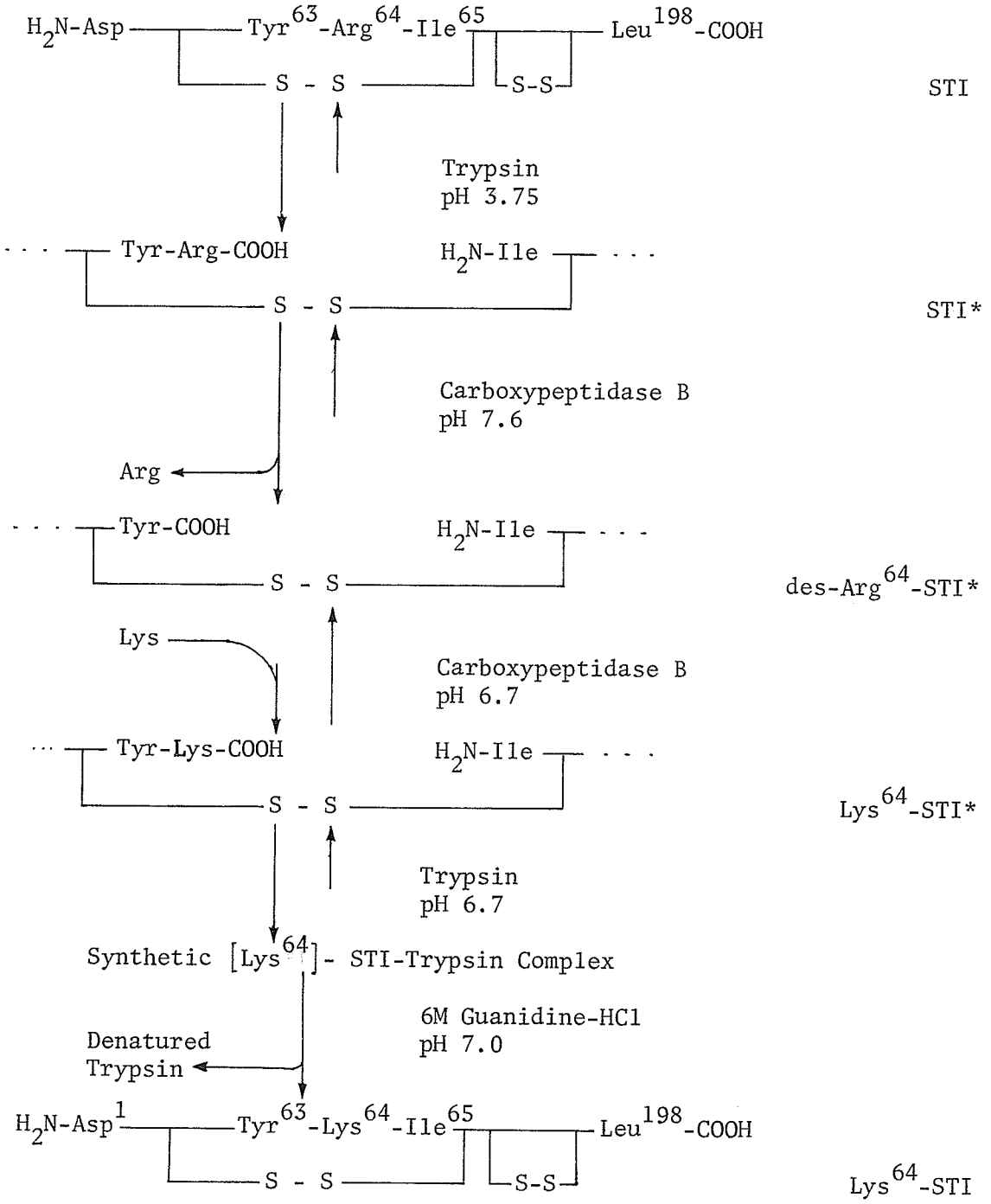

Figure 5 Enzymatic replacement of Arg^{64} in soybean trypsin inhibitor by lysine.

phenylalanine, tryptophan, or leucine in analogous experiments, but using the enzymes carboxypeptidase A and chymotrypsin. These experiments have been successfully performed by Laskowski's group but have at present not been published.[7]

h) The nature of the enzyme-inhibitor complex

(1) The acyl-enzyme hypothesis

Laskowski's group has speculated quite freely on the nature of the stable enzyme-inhibitor complex. These authors believe that the complex involves a covalent bond between enzyme and inhibitor, possibly between the hydroxyl group of the active site serine of the enzyme and the new carboxyl-terminal residue obtained by cleavage of the reactive site peptide bond of the inhibitor (Laskowski and Sealock, 1971). It is argued on mechanistic grounds, that if an acyl enzyme is indeed an intermediate in peptide bond cleavage[8] by serine proteases, an acyl enzyme must also exist at some point in the conversion of virgin to modified inhibitor. Laskowski and Sealock (1971) argue that since the stable complex occupies a central position in the reaction scheme of I and I* with trypsin and since the appropriate rate constants in this reaction scheme [Scheme (4)] agree reasonably well with acylation rate constants for actual substrates, then the stable

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[7] M. Laskowski Jr. (Personal Communication)

[8] This has been suggested by most workers in their discussion on the mechanism of action of serine proteases (Refer to the discussion on the Mechanism of Action of Proteases).

central complex must be an acyl enzyme. Up to date, however, there is no direct evidence for the existence of an acyl bond between enzyme and inhibitor. The ultimate proof will probably have to await the results of high resolution X-ray diffraction analysis of the enzyme-inhibitor complex.

(2) Model of the enzyme-inhibitor complex

The three dimensional structures of BPTI (Huber et al., 1970, 1971a, b), chymotrypsin (Blow, 1971), and trypsin (Stroud et al., 1971) have been established by X-ray diffraction studies. Chymotrypsin and trypsin are homologous proteins with similar three dimensional structures. Since BPTI inhibits trypsin, and to a lesser extent chymotrypsin [at the same reactive site (Rigbi, 1971)], the three dimensional models of these molecules provide a convenient system to study the interactions involved in the enzyme-inhibitor complex by model-building. The research groups of Huber (Munich) and Blow (Cambridge) have collaborated in this and have studied the association of BPTI with chymotrypsin (or trypsin) starting with the models of the individual molecules (Rühlmann et al., 1971; Blow et al., 1972). Their results indicate that at least 7 hydrogen bonds as well as 200 van der Waals contacts hold together these two molecules. They are listed in Tables V and VI. In their model building, Blow et al. (1972) have assumed that both molecules remain in their native conformations but have some flexibility in their extended surface side chains. The only possible mode of binding of BPTI to chymotrypsin (trypsin) was such that the reactive site peptide bond, Lys^{15}-Ala^{16}, was so situated that the side chain of Lys^{15} occupies the enzyme's

TABLE V

PROBABLE HYDROGEN BOND CONTACTS BETWEEN BASIC PANCREATIC TRYPSIN INHIBITOR AND α-CHYMOTRYPSIN[a]

Residues involved		Atoms involved		Estimated Bond lengths
Inhibitor	Chymotrypsin	Inhibitor	Chymotrypsin	(Å)
Pro-13	Gly-216	CO(13)	NH(216)	3.2
Lys-15	Ser-195	CO(15)	NH(195)	2.8
	Gly-193	CO(15)	NH(193)	3.4
	Ser-214	NH(15)	CO(214)	3.6
Ala-16	His-57	NH_2(16)	N(57)	2.9 (in acyl enzyme)
Arg-17	Phe-41	NH(17)	CO(41)	2.7
Gly-36	His-57	CO(36)	NH(57)	3.6

[a] from Blow et al. (1972).

TABLE VI

VAN DER WAALS CONTACTS BETWEEN BASIC PANCREATIC TRYPSIN INHIBITOR AND α-CHYMOTRYPSIN OR TRYPSIN[a]

Inhibitor	Residues involved Chymotrypsin (trypsin)	No. of Contacts
Pro-13	Trp-215	2
Cys-14	His-57, Ile-99(Leu), Trp-215	14
Lys-15	His-57, Ser-190, Cys-191, Met-192(Gln) Ser-195, Trp-215, Gly-216, Gly-226	35
Ala-16	Cys-42, His-57, Met-192 (Gln), Gly-193, Ser-195	15
Arg-17	His-40, Phe-41, Trp-141, Gly-142 Leu-143 (Asn), Met-192 (Gln), Gly-193, Asp-194, Ser-195	66
Ile-18	Phe-41, Cys-58	3
Ile-19	Phe-19(Tyr)	3
Gly-36	His-57	1
Gly-37	His-57	6
Cys-38	Ile-99(Leu)	3
Arg-39	Ser-96, Leu-97(Asn), Thr-98, Ile-99(Leu)	69

[a]Taken from Blow et al. (1972)

specificity pocket. The hydrogen bonds listed in Table V correspond to the binding observed for normal substrates (Segal et al., 1971a, b; Segal, 1972) with additional hydrogen bonds occurring at Arg^{17} and Gly^{36} of BPTI to Phe^{41} and His^{57} of the enzyme. Most of the van der Waals contacts are made between the general area of the enzyme active site and the inhibitor reactive site (See Table VI). As expected, the side chain of Lys^{15} of the inhibitor interacts much more favourably with the specificity pocket of trypsin than with that of chymotrypsin. The Lys^{15}-Ala^{16} reactive site peptide bond of BPTI is placed on the enzyme in the same position as the enzyme-susceptible bond of a substrate is believed to be placed; for this reason, Blow et al. (1972) believe that the probability of the formation of an acyl enzyme is high. Model building of such an acyl enzyme shows that the peptide chain of BPTI from Cys^{14} to Ala^{17} undergoes small conformational changes during the formation of the acyl enzyme. These changes seem to provide better opportunities for binding between enzyme and inhibitor. The authors, however, do not think that the acyl bond between enzyme and inhibitor affords the complex any greater stability than that already provided by secondary interactions. The contact between enzyme and inhibitor is so close that water is entirely excluded from this region (enzyme active site and inhibitor reactive site); as a result, the new amino-terminal group of the inhibitor reactive site cannot be protonated and no water is available to hydrolyze the acyl bond of the stable complex. On the basis of these data it would be predicted that, upon acid dissociation of the complex, the amino group of Ala^{16} of BPTI would take the place of the deacylating water thereby resynthesizing the reactive site peptide bond. This prediction of course is in accord with the kinetic

data of Laskowski et al. (1971) which show that STI is greatly favoured over STI* as a product of complex dissociation.

Although X-ray diffraction studies on crystals of a trypsin-inhibitor complex have already been initiated, definitive data to determine whether or not the complex is a stable acyl enzyme are not yet available (Cole and Parthasarathy, 1972).

4. The "Non Bond-splitting Model" for the Mechanism of Action of Protease Inhibitors

Feeney and coworkers have proposed a model for the mechanism of action for proteinase inhibitors which does not involve the cleavage of a peptide bond in the reactive site of the inhibitor. Their hypothesis thus directly opposes the reactive site model proposed by Laskowski and coworkers. The arguments in favour of the non bond-splitting mechanism have been summed up by Feeney (1971b) and also by Laskowski and Sealock (1971). The most convincing of these is the observation that the inhibitors are capable of forming complexes with inactive derivatives of trypsin and chymotrypsin. Therefore it appears that the hydrolysis of the reactive site peptide bond is not a prerequisite to inhibition but merely incidental to it. A corollary to this argument is that the binding between inhibitor and enzyme is the result of secondary interactions and that formation of a covalent bond between enzyme and inhibitor as proposed by Laskowski would not make an important contribution to binding. Blow et al. (1972) have suggested from their model-building studies that the formation of a covalent acyl enzyme complex would not make the complex any more stable than non-covalent interactions alone. Feinstein and Feeney (1966) showed that

the inactive enzyme derivatives TLCK-trypsin, TPCK-chymotrypsin, and anhydro-chymotrypsin can still form a complex with the appropriate proteinase inhibitor; Ako et al. (1972a, b) have shown that anhydro-trypsin and anhydro-chymotrypsin still complex all inhibitors tested; Dlouhà and Keil (1969) showed that BPTI complexes with bovine trypsinogen. These data very strongly support the argument that the binding between inhibitor and enzyme quite likely consists entirely of secondary interactions. On the other hand, all inhibitors studied to date become completely inactive when the carboxyl-terminal residue in the reactive site of the modified inhibitor is removed. This indicates that the carboxyl-terminal residue in the reactive site is required either to form an acyl bond with the active site serine of the enzyme or to align the inhibitor and enzyme properly by fitting its side chain into the enzyme's specificity pocket. At present these two sets of data are somewhat paradoxical. To resolve these difficulties, studies should be undertaken to determine degree of interaction of not only virgin inhibitors with inactive enzyme derivatives but also of modified inhibitors (reactive site peptide bond cleaved) with inactive enzyme derivatives. This could possibly shed some light on the exact role played by the reactive site residues of the inhibitors.

Another objection to the reactive site model lies in the fact that in some trypsin inhibitors it has not been possible to demonstrate the reactive site by partial proteolysis at low pH. One such inhibitor is BPTI in which the hydrolysis of the Lys^{15}-Ala^{16} reactive site can be demonstrated only after prior cleavage of the Cys 14-Cys 38 disulfide bond (Kress and Laskowski, 1967, 1968). However, Laskowski

and Sealock (1971) speculate that this phenomenon can be explained by thermodynamic considerations i.e. the highly rigid nature of the reactive site sequence of BPTI causes the intact inhibitor (reactive site peptide bond intact) to be highly favored in the peptide bond hydrolysis equilibria of the reactive site. This however is sheer speculation since the actual relation between protein structure and the equilibrium of peptide bond hydrolysis is not known.

C. Identity of Inhibitory Sites in Some Proteinase Inhibitors

Many inhibitors of proteolytic enzymes have the ability to inhibit two or more proteases (Vogel et al., 1968; Laskowski and Sealock, 1971). The possible ways in which this can occur are (1) through distinct, independent and non-overlapping sites (2) through distinct, overlapping sites or (3) through the same reactive site. The first possibility has been shown to occur with several inhibitors. Both the enzymes trypsin and chymotrypsin are independently inhibited by turkey ovomucoid (Rhodes et al., 1960), lima bean protease inhibitor (Haynes and Feeney, 1967), Bowman-Birk inhibitor (Birk, 1968) and chicken ovoinhibitor (Gertler and Feinstein, 1971). The second alternative i.e. distinct overlapping sites, although possible, is unlikely and moreover, has not been demonstrated. The third possibility, i.e. inhibition of several enzymes at one distinct reactive site has been demonstrated in several instances. Rigbi (1971) has shown that BPTI, which inhibits trypsin and to a lesser extent chymotrypsin, inhibits these enzymes at the same Lys^{15}-Ala^{16} reactive site. Soybean trypsin inhibitor inhibits trypsin, cocoonase (a trypsin-like enzyme) and to a lesser degree, chymotrypsin (Hixson and Laskowski, 1970a;

Bidlingmeyer et al., 1972) and all three enzymes are inhibited at the same reactive site namely Arg^{64}-Ile^{65}. Furthermore, it has been shown that all three enzymes can not only hydrolyze the reactive site peptide bond at acid pH but can also achieve its resynthesis by complex formation with the modified inhibitor followed by kinetic control dissociation thereby clearly indicating that all three use the same reactive site on STI. Clearly, the requirements for reactive sites on inhibitors for different proteolytic enzymes must share many structural features.

D. Temporary Inhibition

Some inhibitors of proteolytic enzymes inhibit those enzymes only for a finite time after which period the proteolytic activity of the enzyme very rapidly appears (Laskowski and Sealock, 1971). This phenomenon is observed in most pancreatic secretory trypsin inhibitors studied as well as in BPTI with the Cys 14-Cys 38 disulfide linkage cleaved. In the case of porcine PSTI, Tschesche et al. (1971) have shown that loss of inhibition is the result of degradation of the inhibitor by trypsin, i.e. through dissociation of the PSTI-trypsin complex some free trypsin becomes available for proteolysis. This free trypsin then cleaves the inhibitor at six peptide bonds thereby inactivating it. In BPTI (with the Cys 14-Cys 38 disulfide bond cleaved) temporary inhibition is explained in a similar fashion and three peptide bonds are sequentially hydrolyzed to eventually produce an inactive inhibitor (Laskowski, Sr., et al., 1971; Wilson and Laskowski, Sr., 1971). These proteolytic cleavages have pH maxima around 7.6 and thus are easily distinguishable from the

hydrolysis of the reactive site peptide bond which has a pH maximum around pH 3-4.

GENERAL EXPERIMENTAL PROCEDURES

I. Purification of Lima Bean Inhibitor

For this investigation various lots of crude commercial lima bean inhibitor were purchased from Worthington Biochemical Corporation. Since it is known that there are at least four genetic variants of LBI in most varieties of lima beans and since different varieties of lima beans may have slightly different variants it is useful to review the purification procedure of Jones et al. (1963) as it was used in this laboratory. Crude LBI was passed over Sephadex G-75 as shown in Figure 6. The inhibitor is contained in Fraction II while Fraction I is a non-inhibitor contaminating protein which was discarded. The actual amount of contaminating protein present in the different lots of crude LBI was quite variable. The inhibitor fraction obtained by gel filtration on Sephadex G-75 was further fractionated by ion-exchange chromatography on DEAE-cellulose as illustrated in Figure 7. Four inhibitor peaks were obtained representing variants I-IV (LBI I-IV). These inhibitor fractions were desalted by dialysis, lyophilized and then rechromatographed in the same system prior to use. Although all lots of crude LBI purchased from Worthington had at least four variants, the relative amounts of LBI I-IV was quite variable from one lot to another. Of somewhat greater surprise and importance was the fact that the same variants from different commercial preparations have small but significant differences in amino acid composition. For this reason, the amino acid compositions of the variants purified in this laboratory have been compiled for convenient reference and appear in the Appendix. Since the preparations of LBI used in the present studies come from

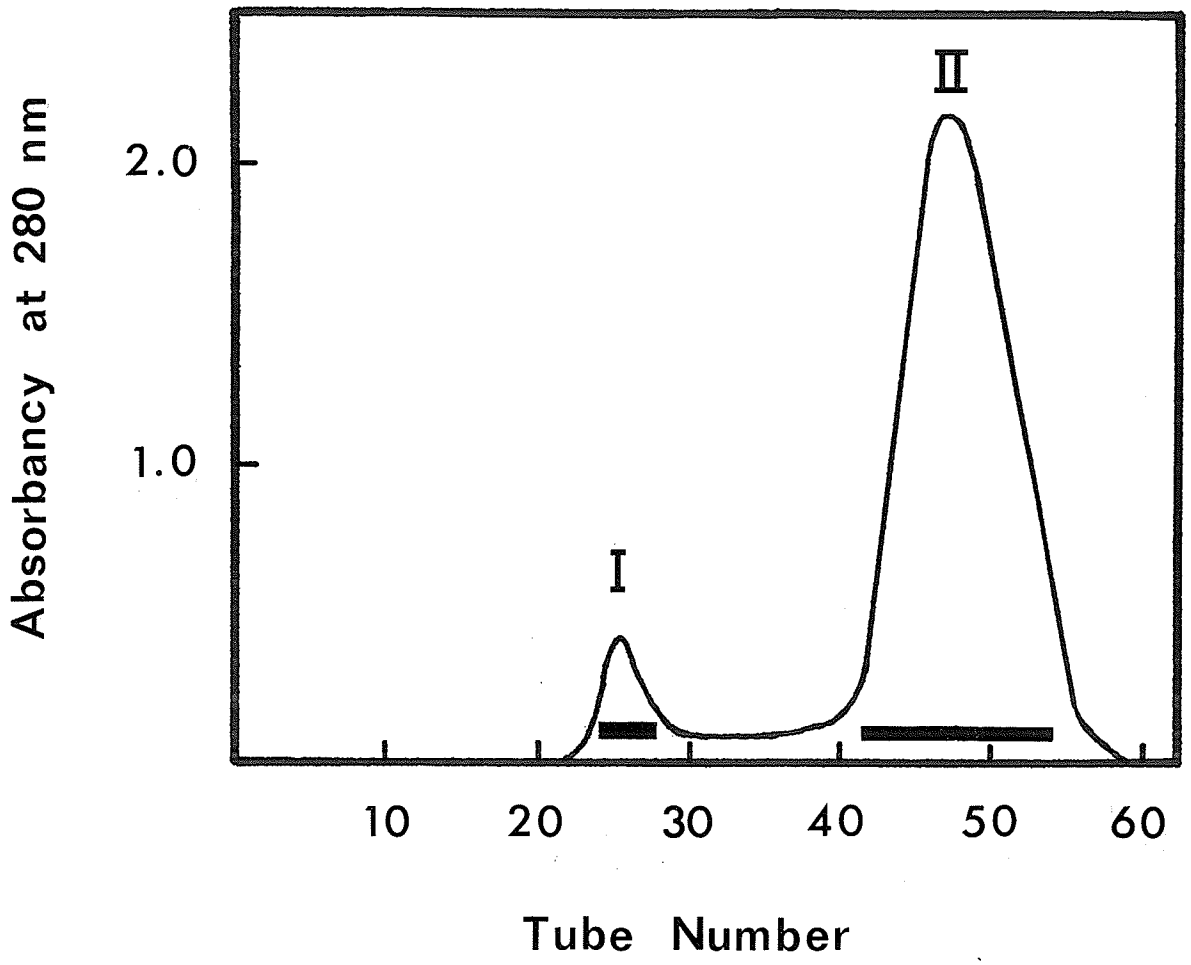

Figure 6 Gel filtration of crude commercial lima bean inhibitor on Sephadex G-75.

A column (2.5 x 90 cm) of Sephadex G-75 was equilibrated and developed with 0.1M NH_4HCO_3. The sample, 500 mg of crude LBI, was dissolved in 5.4 ml of 0.1M NH_4HCO_3. The column was operated at a flow rate of 38 ml/hr and 6.0 ml fractions were collected. The column effluent was monitored for protein at 280 nm. Fractions indicated by the solid bars were pooled and lyophilized.

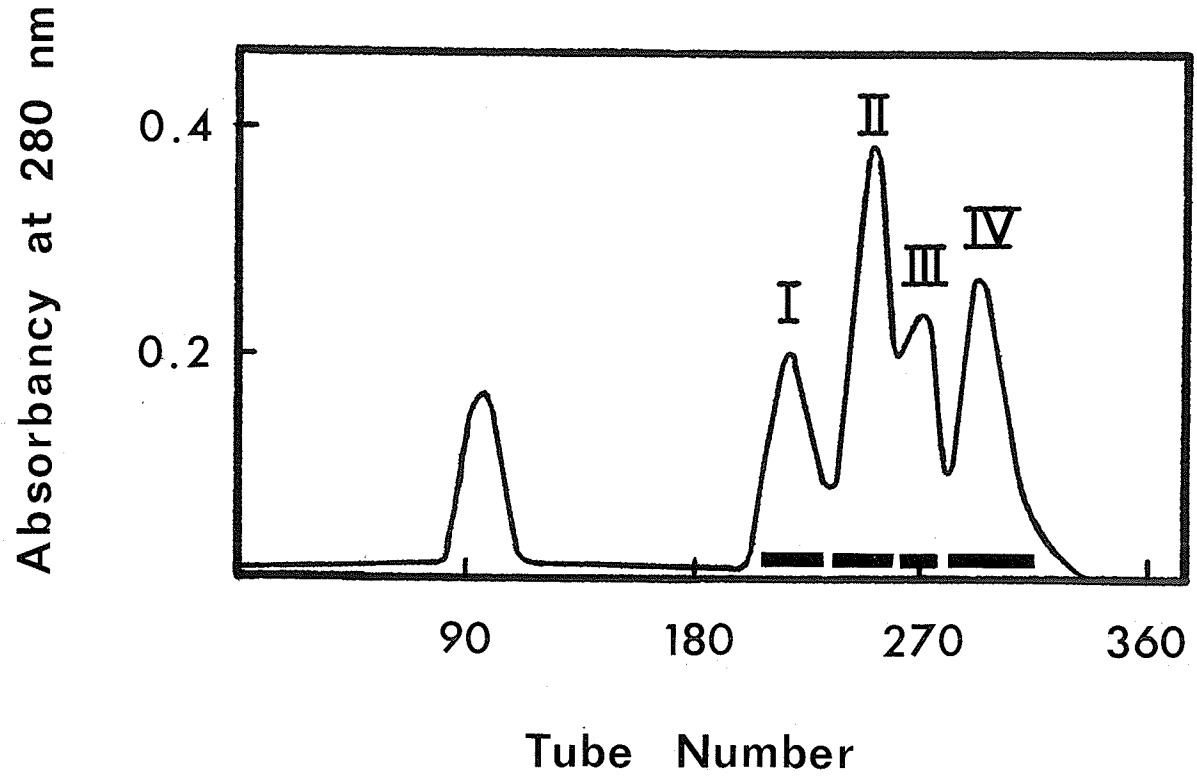

Figure 7 Ion-exchange chromatography of lima bean inhibitor on DEAE-cellulose.

A column (2.5 x 90 cm) of DEAE-cellulose was equilibrated with starting buffer, 0.01M sodium phosphate, pH 7.6. Fraction II (Figure 6) obtained by the gel filtration of 500 mg of crude LBI was dissolved in approximately 10 ml of starting buffer and applied to the column. The column was eluted with an exponential gradient of salt, 0.01M sodium phosphate and 0.4M sodium chloride, pH 7.6, applied through a 1 litre mixing chamber containing the starting buffer. The column was operated at a flow rate of 60 ml/hr and effluent fractions of 4.5 ml were collected.

many different commercial preparations, the specific preparation used for each experiment will be identified in each experimental section.

II. Inhibitor Assays

The assay system employed for trypsin and chymotrypsin inhibition in this study was that described by Rhodes et al. (1957, 1960) with slight modifications. In this system, the decrease in the esterolytic activity of a constant amount of trypsin or chymotrypsin after addition of varying amounts of inhibitor is used as a measure of the degree of inhibition. The esterolytic activity of trypsin and chymotrypsin was determined by the hydrolysis of p-toluenesulfonylarginine methyl ester (TAME) and benzoyltyrosine ethyl ester (BTEE) respectively. The hydrolysis of these substrates at pH 8.2 results in the release of hydrogen ion thereby decreasing the pH. The decrease in pH is monitored with the indicator m-nitrophenol which changes from yellow to colorless and the change can be followed spectrophotometrically at 395 nm (in this investigation a Coleman-Hitachi 124 recording spectrophotometer was used).

For the trypsin inhibitor assays, the reaction mixture in the cuvette of the spectrophotometer consisted of 0.3 ml of a solution containing 150 µg/ml of trypsin (obtained by diluting a 1.5 mg/ml solution of trypsin in 0.004M acetic acid and 0.02M $CaCl_2$ ten-fold with 0.006M Tris-HCl, pH 8.2), 0.0 to 0.7 ml of inhibitor solution in 0.006M Tris-HCl buffer, pH 8.2, and 0.7 to 0.0 ml of 0.006M Tris-HCl buffer to bring the volume to 1.0 ml. Two ml of substrate-indicator solution (0.01M TAME, 0.015M Tris-HCl and 0.012% m-nitrophenol, with a final pH of 8.2) were added to the cuvette just before starting the

recorder. The chart speed was 1/2 inch/min.

For chymotrypsin inhibitor assays, the reaction mixture in the cuvette of the spectrophotometer consisted of 0.3 ml of a solution containing 150 μg/ml of chymotrypsin (obtained by diluting a 1.5 mg/ml solution of chymotrypsin in 0.004M acetic acid and 0.02M $CaCl_2$ tenfold with 0.006M Tris-HCl, pH 8.2), 0.0 to 0.7 ml of inhibitor in 0.006M Tris-HCl buffer, pH 8.2, and 0.7 to 0.0 ml of 0.006M Tris-HCl buffer to bring the volume to 1.0 ml. To this was added 2 ml of substrate-indicator solution (0.008M BTEE, 0.0075M Tris-HCl, 0.012% m-nitrophenol, and 30% methanol with a final pH of 8.2) and the recorder started. The chart speed was 1 in/min. The residual enzyme activities were estimated by noting the change in transmittance over a fixed time interval, usually 1 min.

To determine the activity of a modified inhibitor, a standard inhibition curve was first plotted giving the residual enzyme activity of a fixed amount of enzyme as a function of the amount of native LBI added (See Figure 8, curve 1). A similar inhibition curve using the same amount of enzyme is then plotted for the modified inhibitor (Figure 8, curve 2). Finally, to determine the percent activity of the modified inhibitor, the amount of native and modified inhibitor required to produce the same amount of inhibition (in the linear portion of the inhibition curve of the modified inhibitor) is compared. Thus "a" μg of native inhibitor is as active as "b" μg of modified inhibitor. The calculation is as follows:

$$\text{ACTIVITY OF MODIFIED INHIBITOR IN \% ACTIVITY OF NATIVE LBI} = \frac{a}{b} \times 100 \quad \text{or}$$

% INACTIVATION OF INHIBITOR = 100- % ACTIVITY OF THE MODIFIED INHIBITOR

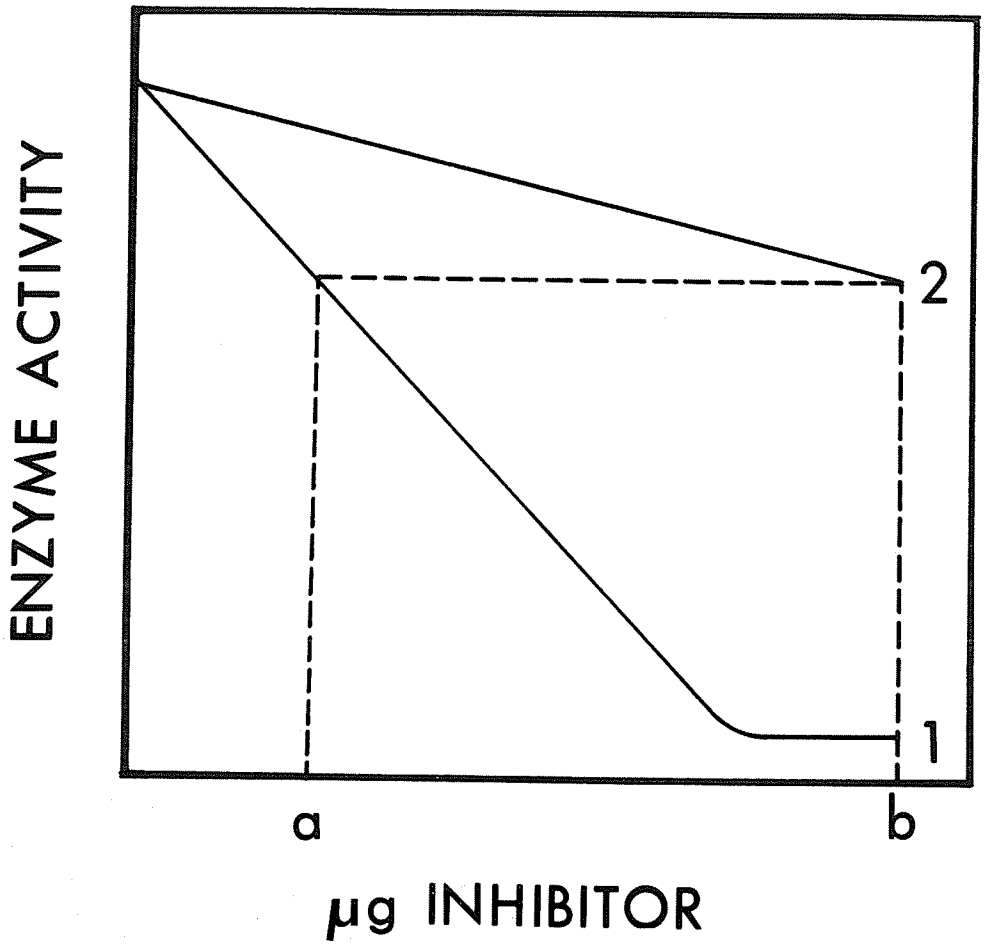

Figure 8 Inhibition curve for the enzymes, trypsin or chymotrypsin by lima bean inhibitor.

In this assay system, varying amounts of lima bean inhibitor are added to a constant amount of enzyme. (1), native, fully active LBI; (2), a partially active LBI (usually an enzyme-modified inhibitor).

The procedures used are identical for the trypsin and the chymotrypsin inhibitor assays.

The trypsin inhibitor assays were performed at 25° while the chymotrypsin inhibitor assays were performed at 37° to prevent crystallization of the substrate BTEE. Since the time of preincubation of enzyme and inhibitor prior to addition of the substrate-indicator solution was variable, the time of preincubation will be stated for each experiment.

III. Enzyme Modification of Lima Bean Inhibitor

The procedure for partial proteolysis of soybean trypsin inhibitor with catalytic amounts of trypsin at acid pH was first described by Finkenstadt and Laskowski (1965) and Ozawa and Laskowski (1966). This process yielded a trypsin-modified inhibitor in which only one specific peptide bond was hydrolyzed. These authors argued that this phenomenon had general application among protein proteinase inhibitors. For this reason, partial proteolysis of LBI by chymotrypsin and trypsin was attempted using the methods pioneered in Laskowski's laboratory.

The modification of LBI by trypsin or by chymotrypsin had common features since in both cases the inhibitor was treated at acid pH with catalytic amounts of the appropriate enzyme. Since the composition of the buffers, the pH, the protein concentration, the time of incubation, and the enzyme used were not identical in different experiments, the exact experimental conditions will be stated for each experiment. The extent of modification was in all cases determined by comparison of the activities of the native (unmodified) inhibitor and the modified inhibitor as described under Inhibitor Assays.

IV. Protein Hydrolysis and Amino Acid Analysis

Samples containing 0.05 - 0.2 μmoles of protein or peptide were hydrolyzed with 6N HCl at 110° in sealed, evacuated tubes. Where necessary, 2 μl of thioglycollic acid and 50 μl of 5% aqueous phenol were added per ml of 6N HCl to protect S-carboxymethylcysteine and tyrosine from destruction (Howard and Pierce, 1969). Routinely, the hydrolyses were carried out for 22 hr although in specific instances hydrolyses were carried out for different time periods (24, 51 and 75 hr) to allow for extrapolation of the results to zero time. The analyses were carried out in all cases on a Beckman-Spinco 120C automatic amino acid analyzer by the method of Spackman et al. (1958) as outlined in the Spinco manual.

V. End-group Analysis

A. Hydrolysis by Exopeptidases

Hydrolysis of peptides or proteins with carboxypeptidases A, B or leucine aminopeptidase were essentially carried out as previously described by Stevens et al. (1967) with modification in some instances. For hydrolysis with carboxypeptidase A or B, approximately 0.1 μmole of peptide or protein was dissolved in 0.1M NH_4HCO_3, pH 7.8, to which was added the diisopropylphosphofluoridate treated carboxypeptidase A or B. The amount of enzyme used, the temperature of incubation, as well as the time of digestion were variable and hence will be indicated for each experiment. For hydrolysis with leucine aminopeptidase, approximately 0.1 μmole of peptide or protein was dissolved in 100 μl of 0.1M NH_4HCO_3, pH 7.8, and 25 μl of 0.025M $MgCl_2$. To this were added approximately 5 units

of leucine aminopeptidase. The hydrolysis of peptide or protein with aminopeptidase M was performed as described by Evans et al. (1968). The peptide (0.1 μmole) was dissolved in 450 μl of 0.1M NH_4HCO_3, pH 7.8, and 50 μl 0.02M $MgCl_2$ and the mixture was incubated with 50 units of enzyme for varying amounts of time. The enzyme digestions were terminated by freezing in a dry ice-ethanol bath. The digests were examined either by descending paper chromatography on Whatman No. 3MM paper in a 1-butanol-acetic acid-water (200:30:75) system (usually preliminary experiments) or by amino acid analyses of the lyophilized reaction mixtures.

B. Edman Degradation

The Edman degradation procedure used was similar to that described by Kaspar and Smith (1966). The results were based solely on the subtractive method since the phenylthiohydantoin derivatives were not identified. The exact method used is described in detail by Tan (1970).

VI. Reduction and Alkylation

Reduction and alkylation of lima bean inhibitor was carried out by a modification of the method of Hirs (1967). In a typical experiment, 50-100 mg of inhibitor was dissolved in 5 ml of 5M guanidine hydrochloride containing 0.2% EDTA (previously flushed with N_2 for 20 min) and adjusted to pH 8.5 with 25% trimethylamine. Reduction was carried out for 16 hr at room temperature using a 200-400 fold molar excess (over the molarity of the disulfide bonds) of β-mercaptoethanol (2 additions of 0.5 ml each, 30 minutes apart).

During the course of the reaction, the pH was kept constant at pH 8.0 by the addition of 2N NaOH with a Radiometer TTT11 pH-stat equipped with a magnetically controlled buret. Subsequent alkylation was achieved by the slow addition of 2.6 g of iodoacetamide or the equivalent amount of iodoacetic acid (dissolved in 1-2 ml of warm 1N sodium hydroxide) to the reaction mixture and the pH was kept constant at pH 8.0 by addition of 4N NaOH. After the reaction was complete the reaction mixture was adjusted to pH 4.0 with glacial acetic acid and the reagents were removed by gel filtration on Sephadex G-25 using 10% acetic acid as eluant.

Alternatively, when working with a small amount of protein, the method of Sondack and Light (1971) employing dithioerythritol as the reducing agent (Cleland's Reagent; Cleland, 1964) was used. The inhibitor was dissolved in the reducing solution containing 1 mg/ml of EDTA, 0.1M Tris-HCl, and 0.01M dithioerythritol, pH 8.5, to an inhibitor concentration of 2 mg/ml and allowed to stand for 1 hr at room temperature at which time an equal volume of 0.2M Tris-HCl - 0.05M sodium iodoacetate, pH 8.5, was added. This reaction mixture was allowed to stand for another hour at room temperature and then the reagents were removed by gel filtration on Sephadex G-25.[9]

Alkylation of the reduced inhibitor with iodoacetate results in reduced, carboxymethylated inhibitor (RCM-LBI) whereas alkylation with

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[9]Both procedures result in complete reduction and alkylation of LBI as demonstrated by the complete conversion of cysteine to S-carboxymethylcysteine as monitored by amino acid analysis.

iodoacetamide results in reduced, carboxamidomethylated inhibitor (RCAM-LBI).

VII. High-Voltage Paper Electrophoresis

High-voltage paper electrophoresis was performed on Whatman No. 3MM paper employing a Savant tank electrophoresis system. Two different buffer systems were used: (1) 0.025M pyridine-0.035M acetic acid (pH 4.7) (2) 25 ml of 98% formic acid and 87 ml of glacial acetic acid diluted to 1 liter with H_2O (pH 1.9). The voltage applied was 53 volts per cm for 30 min. Electrophoretograms were developed with ninhydrin-collidine reagent (Margoliash and Smith, 1962). In the case of preparative high-voltage paper electrophoresis, guide strips were developed with ninhydrin to locate the peptides and the peptides located in this manner were eluted from the paper with 10 - 30% aqueous acetic acid.

VIII. Column Chromatography

Sephadex columns for gel filtration were prepared and operated according to the manufacturer's specifications. Ion-exchange columns containing DEAE-cellulose and DEAE-Sephadex A-25 were prepared and operated according to the procedures described by Peterson and Sober (1962) and Roy and Konigsberg (1972). Column effluents were monitored for protein and peptides either by measuring the absorption at 280 nm or by measuring the absorption at 570 nm after the ninhydrin reaction on alkaline hydrolysates of aliquots, according to the procedure of Hirs et al. (1956). Either all or alternate fractions were monitored in this manner. The buffer systems used, column dimensions, flow

rates, fraction sizes, and other particulars of each chromatography will be more fully described in the appropriate experiments.

THE STOICHIOMETRY OF INTERACTION OF LIMA BEAN INHIBITOR WITH TRYPSIN AND/OR CHYMOTRYPSIN

I. Introduction

It is known that lima bean inhibitor inhibits both trypsin and chymotrypsin. Haynes and Feeney (1967) showed that LBI inhibits trypsin on a 1:1 molar basis but inhibits chymotrypsin on approximately a 2:1 molar ratio. Inhibition studies to determine stoichiometry, however, are not accurate in cases where the inhibitor and enzyme interact weakly. For this reason, the present investigation has sought to isolate complexes of LBI and the enzymes and to characterize these complexes in order to establish very clearly the stoichiometry of interaction of LBI with trypsin and chymotrypsin.

II. Experimental

A. Isolation of the Enzyme-Inhibitor Complexes by Gel Filtration

In this study lima bean inhibitor (LBI 8KB) passed over Sephadex G-75 to remove contaminating protein was used. This preparation was thus a mixture of variants I-IV (Jones *et al.*, 1963). The inhibitor (30-60 mg) was first dissolved in 0.1-0.3 ml of 0.1M NH_4HCO_3 and then the appropriate amount of enzyme was dissolved into this inhibitor solution. After a 15 min preincubation at room temperature, the solution was subjected to gel filtration on a column of Sephadex G-75 under the conditions described under Results. Appropriate fractions were pooled and lyophilized.

B. Analysis of the Isolated Enzyme-Inhibitor Complexes

1. Amino Acid Composition

Protein hydrolysis (6N HCl, 110°, 22 hr, in vacuo) and amino acid analyses were performed as described under General Experimental Procedures. Analyses were done on LBI, trypsin, chymotrypsin, the LBI-trypsin and LBI-chymotrypsin complexes, and the complex formed from LBI, trypsin and chymotrypsin.

2. Molecular Weight Determination by Gel Filtration on Sephadex G-75

The molecular weights of LBI and its complexes were estimated by the method of Whitaker (1963). Solutions of reference and sample proteins (2-10 mg in 1 ml of 0.1M NH_4HCO_3) were applied to a column (1.5 x 85 cm) of Sephadex G-75 equilibrated with 0.1M NH_4HCO_3. The flow rate was 11 ml/hr and 1.3 ml fractions were collected. The void volume (Vo) was determined using a 0.1-0.2% blue dextran solution. The elution volume (Ve) for each protein is dependent on its molecular weight. The ratio Ve/Vo, for the reference proteins, was plotted against the logarithm of molecular weight, and the molecular weight of the sample protein was then obtained from its Ve/Vo ratio.

3. Inhibitor Activity

The inhibitory activities of LBI and its complexes were determined as described under General Experimental Procedures.

III. Results

A. Isolation of the Enzyme-Inhibitor Complexes

Figure 9 illustrates the isolation of LBI-enzyme complexes

by gel filtration and Table VII summarizes the inhibitory properties of these complexes. When an excess of LBI is added to trypsin (Figure 9a) or to chymotrypsin (Figure 9b) complexes are formed which can be tentatively identified by their inhibitory properties (Table VII) as being the LBI-trypsin (LBI-T) and the LBI-chymotrypsin (LBI-CT) complexes respectively. Both these complexes elute from the column prior to the excess LBI (Peak II in Figure 9a & b). When an excess of LBI-T complex is added to chymotrypsin (Figure 9c) the LBI-T-CT complex (Peak I), devoid of all inhibitory activity, is formed. Peak II, Figure 9c represents excess LBI-T complex. The LBI-T-CT complex can also be prepared by adding trypsin to the LBI-CT complex.

B. Properties of the Isolated Enzyme-Inhibitor Complexes

The inhibitory properties of the complexes have been summarized in Table VII.

1. Amino Acid Composition

Table VIII shows the amino acid analyses of LBI, T, CT, and the complexes LBI-T, LBI-CT, and LBI-T-CT. By assuming a 1:1 molar ratio of inhibitor to enzyme in the case of LBI-T and LBI-CT, and a 1:1:1 molar ratio of trypsin to inhibitor to chymotrypsin in the case of LBI-T-CT, theoretical values for the amino acid compositions were calculated for purposes of comparison with experimental values. The theoretical and experimental values agree within experimental error in all cases proving that LBI forms 1:1 molar complexes with trypsin and chymotrypsin and also forms ternary complex (LBI-T-CT) with one mole of each enzyme.

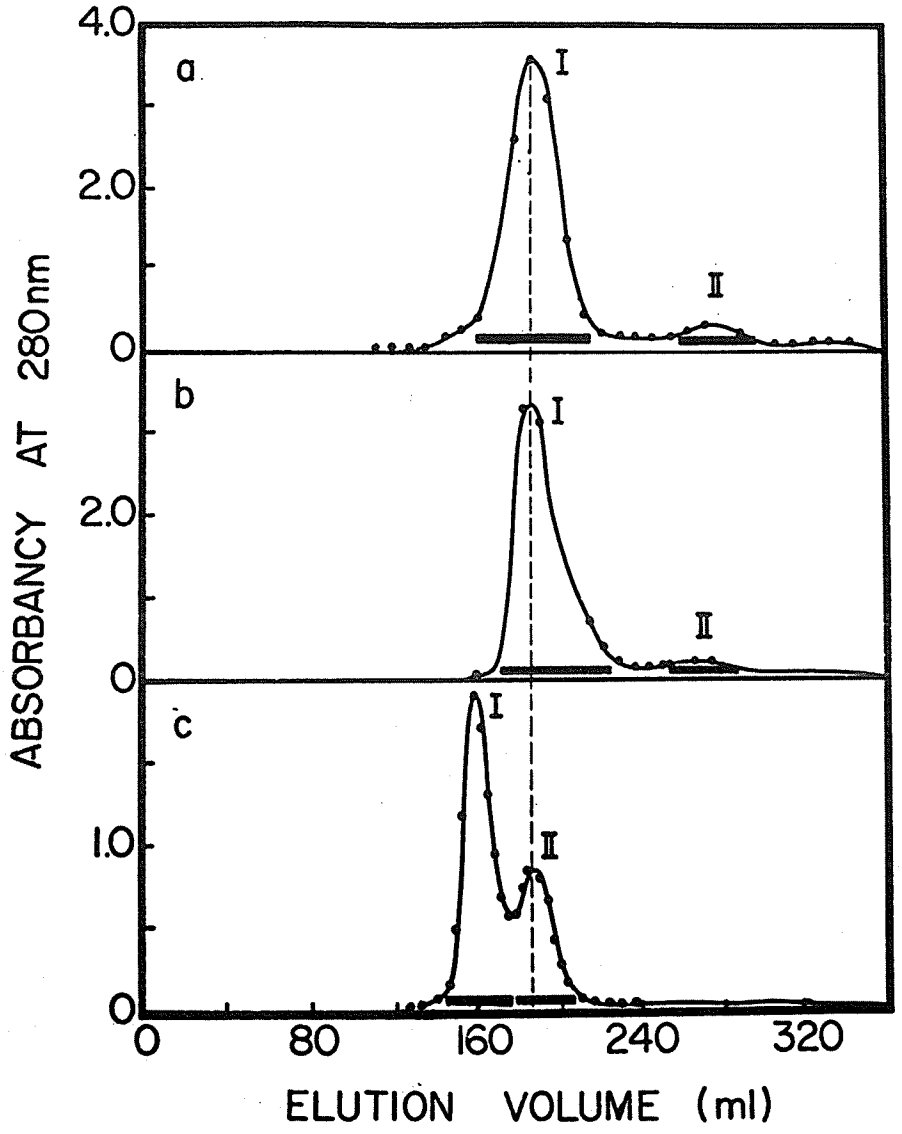

Figure 9

Figure 9 Gel filtration of the complexes of LBI with trypsin and/or chymotrypsin.

Columns (2.5 x 90 cm) of Sephadex G-75 were equilibrated and developed with 0.1M NH_4HCO_3. Samples ranged from 1-3 ml; the flow rate was 22 ml/hr and 4.5 ml fractions were collected. Protein was monitored by the absorbancy at 280 nm. (a) 40 mg of LBI to which 100 mg of trypsin have been added (b) 30 mg of LBI to which 50 mg of chymotrypsin have been added (c) 50-60 mg of peak I of Figure 9a to which 15 mg of chymotrypsin have been added.

TABLE VII

INHIBITORY ACTIVITY OF THE COMPLEXES OF LIMA BEAN INHIBITOR WITH TRYPSIN AND CHYMOTRYPSIN

Figure	Peak	Inhibitory Activity[a] vs Trypsin	vs Chymotrypsin	Tentative Identification
9a	I	-	+	LBI-T
	II	+	+	LBI
9b	I	+	-	LBI-CT
	II	+	+	LBI
9c	I	-	-	LBI-T-CT
	II	-	+	LBI-T

[a](-), lack of activity; (+), presence of activity.

TABLE VIII

AMINO ACID ANALYSES OF LBI, CHYMOTRYPSIN (CT), TRYPSIN (T) AND THE COMPLEXES OF LBI WITH TRYPSIN (LBI-T), CHYMOTRYPSIN (LBI-CT) AND WITH BOTH TRYPSIN AND CHYMOTRYPSIN (LBI-T-CT)[a]

Amino Acid	LBI	CT	LBI-CT		T	LBI-T		LBI-T-CT	
			Theory[b]	Experiment[c]		Theory[b]	Experiment[c]	Theory[b]	Experiment[c]
Lysine	4.17	14.35	18.52	19.16	14.25	18.42	17.10	32.77	34.40
Histidine	5.37	1.95	7.32	6.52	3.42	8.79	9.09	10.74	11.05
Arginine	1.95	3.10	5.05	5.05	1.92	3.87	3.82	6.97	8.08
Aspartic acid	12.80	22.90	35.70	36.20	23.25	36.05	37.90	58.95	59.30
Threonine	5.09	23.13	28.22	29.42	11.50	16.59	16.19	39.72	38.62
Serine	11.78	26.50	38.28	39.50	34.75	46.53	47.50	73.03	73.99
Glutamic acid	6.80	15.50	22.30	23.50	14.90	21.70	22.20	37.20	37.00
Proline	5.80	10.10	15.90	15.04	9.25	15.05	15.25	25.15	23.70
Glycine	1.83	24.40	26.23	27.50	27.50	29.33	28.35	53.73	52.49
Alanine	3.18	23.20	26.38	28.50	14.82	18.00	17.10	41.20	41.60
Valine	1.30	19.55	20.85	22.80	14.60	15.90	15.74	35.45	34.40
Methionine	-	1.92	1.92	1.85	1.75	1.75	1.82	3.67	2.19
Isoleucine	3.96	7.20	11.16	11.15	13.25	17.21	18.00	24.40	25.90
Leucine	2.86	20.75	23.61	25.60	14.00	16.86	17.91	37.61	35.48
Tyrosine	1.29	4.40	5.69	5.37	11.00	12.29	11.00	16.69	14.39
Phenylalanine	1.49	6.30	7.79	9.16	2.42	3.91	4.73	10.21	10.95

[a]The analyses were carried out as described in the text. The results are expressed as moles/mole and have not been corrected for destruction of residues by acid hydrolysis.

[b]The theoretical values were obtained by adding the amino acid compositions of enzyme and inhibitor, assuming the formation of 1-1 molar complexes.

[c]The experimental values were obtained by amino acid analysis of the isolated complexes.

2. Molecular Weight of LBI and the LBI-T Complex

The results in Figure 10 indicate that the apparent molecular weight of LBI is approximately 10,000 g/mole when the sample concentration is 2 mg/ml whereas it changes to approximately 18,000 g/mole when the sample concentration is 5 mg/ml suggesting that LBI undergoes a concentration dependent dimerization. The molecular weight of the LBI-T complex was calculated to be 32,800 g/mole. It should be pointed out that the elution volume of LBI-T and LBI-CT are identical (see Figure 9) and therefore their molecular weights must be identical. The molecular weight of 32,800 g/mole for LBI-T, or for that matter LBI-CT, is consistent with the formation of a 1:1 molar complex between enzyme and inhibitor assuming a molecular weight of approximately 9,000 g/mole for LBI and 24,000 g/mole for enzyme. The molecular weight of the ternary complex was not determined but its molecular weight was assumed to be the sum of the molecular weights of inhibitor and the two enzymes since it elutes from Sephadex G-75 before LBI-T or LBI-CT.

IV. Discussion

Fraenkel-Conrat et al. (1952) calculated the molecular weight of LBI to be 10,000 g/mole by osmotic pressure measurements whereas Haynes and Feeney (1967) found the molecular weight to be 16,200 g/mole by ultracentrifugation. These authors suggested that this indicated an aggregation phenomenon. The data in this study tend to substantiate this aggregation phenomenon. Millar et al. (1969) have reported that Bowman-Birk inhibitor undergoes a concentration dependent monomer-

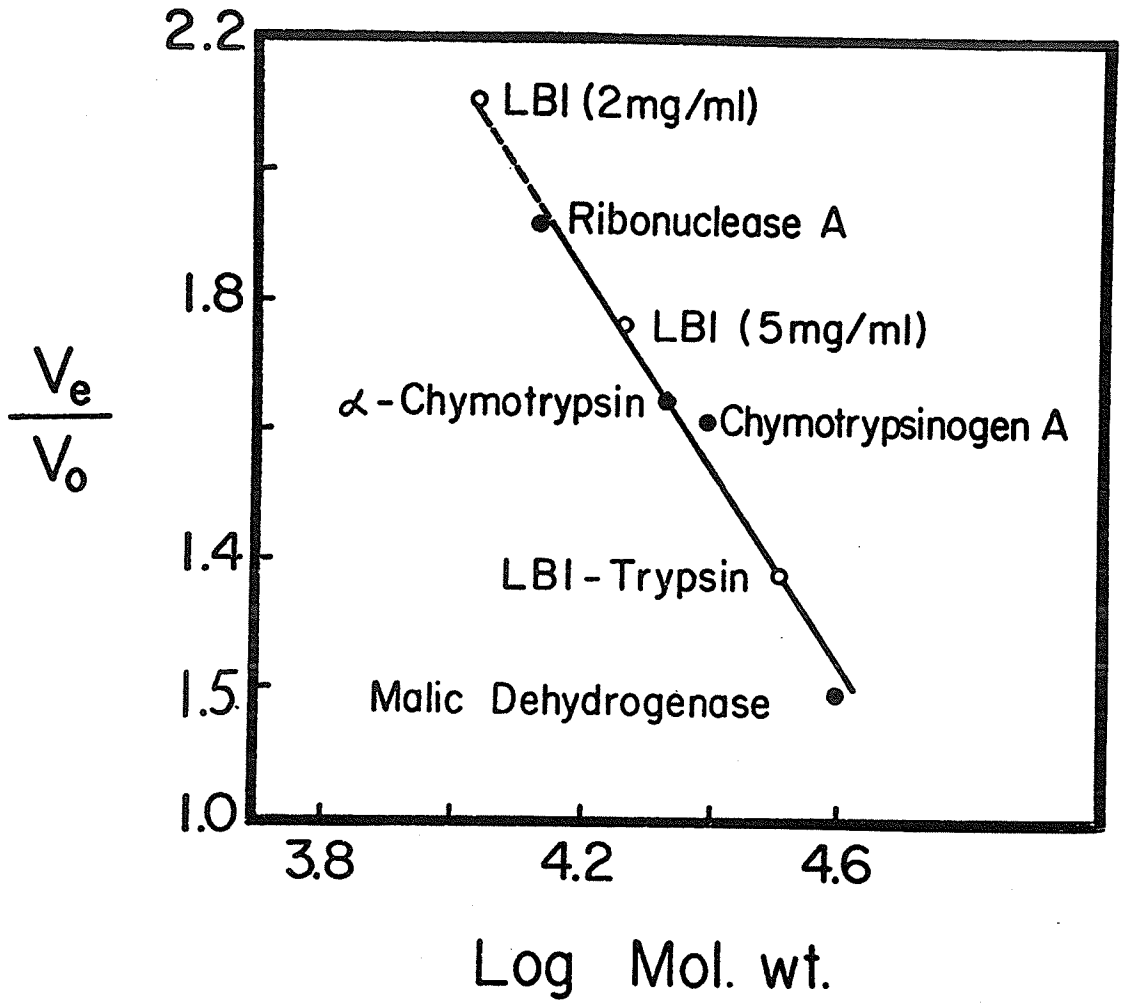

Figure 10 Molecular weight determination of LBI and the LBI-trypsin complex by gel filtration on Sephadex G-75.

Solid circles (●) indicate reference proteins and open circles (○) indicate sample proteins. The molecular weights of the reference proteins were obtained from the Handbook of Biochemistry (1968).

dimer-trimer association process with the monomer predominating at low concentration. From inhibition data, Haynes and Feeney (1967) and Jones et al. (1963) showed the inhibition of trypsin to be on an equimolar basis if a molecular weight of approximately 10,000 g/mole was assumed for LBI. Haynes and Feeney (1967) indicated that on a similar basis the inhibition of chymotrypsin was not stoichiometric on a 1:1 inhibitor-enzyme basis. The present study clearly affirms the formation of a 1:1 molar complex between trypsin and LBI; further, it also clearly shows that LBI forms a 1:1 molar complex with chymotrypsin. The non-stoichiometric inhibition of chymotrypsin by LBI probably results from a low association constant, in which free enzyme would be available even at equilibrium. Association constants for LBI and chymotrypsin have however not been determined to date. Haynes and Feeney (1967), on the basis of inhibition data, termed LBI a "double-headed inhibitor" for trypsin and chymotrypsin meaning that the inhibitor had independent sites for the inhibition of these enzymes. The present study clearly shows that the sites for inhibition of trypsin and chymotrypsin are independent and non-overlapping as evidenced by the formation of the ternary complex (LBI-T-CT) which contains equimolar amounts of its component molecules.

After the above results were published, Seidl and Liener (1972a) isolated the complexes of Bowman-Birk soybean inhibitor (BBI) with trypsin, chymotrypsin, or with both these enzymes by gel filtration on Sephadex G-75. Characterization of these complexes by inhibitory assays, by amino acid analyses, and by molecular weight determination reveals that BBI behaves in an identical fashion to LBI.

That is, BBI forms 1:1 molar complexes with trypsin and chymotrypsin and forms a ternary complex with trypsin and chymotrypsin. BBI, thus, is also a "double-headed" inhibitor for trypsin and chymotrypsin.

STUDIES ON THE TRYPSIN INHIBITORY ACTIVITY OF LIMA BEAN INHIBITOR

I. Introduction

Ozawa and Laskowski (1966) proposed that all naturally occurring trypsin inhibitors have a lysyl-X or an arginyl-X reactive site peptide bond particularly susceptible to tryptic cleavage at acid pH. In this investigation, an attempt is made to identify the trypsin reactive peptide bond of LBI and to determine its location in the amino acid sequence. Furthermore, the peptide bond hydrolysis equilibria of this reactive site are also examined and native and trypsin-modified LBI (LBI'_t) (with the reactive site peptide cleaved) were compared in various ways. These studies were designed to determine whether LBI conformed to the "reactive site model" put forward by Laskowski. Finally, since this investigation has not concentrated on any particular LBI variant, a study was undertaken to determine the extent of the differences between LBI variants with respect to their trypsin inhibitory activity.

II. Determination of the Trypsin Reactive Peptide Bond of Lima Bean Inhibitor

A. Experimental

1. Trypsin Modification of Lima Bean Inhibitor

a) Preparation A

A commercial preparation (50 mg) of lima bean protease inhibitor (LBI 8KB) was dissolved in 4.0 ml of 0.1M acetic acid - 0.02M calcium chloride and the pH adjusted to 2.75 with glacial acetic acid. At zero time and again after 5 hr, 0.1 ml of bovine trypsin (10 mg/ml in 0.004M acetic acid-0.02M calcium chloride) were added and the

reaction mixture was allowed to stand for 22 hr at 37°. The pH of the sample was then adjusted to pH 8.0 with 25% trimethylamine. A control sample containing no trypsin was run under identical conditions. The LBI was separated from the other components of the reaction mixture by gel filtration on a 2.5 x 90 cm column of Sephadex G-75 previously equilibrated with 0.1M NH_4HCO_3 and lyophilized. This trypsin modification reaction mixture of LBI will be referred to as TMRM-LBI (PREP A).

b) Preparation B

Variant IV (LBI IV) prepared from the commercial preparation LBI 1DA (100 mg) was dissolved in 10 ml of 0.018M trans-aconitate buffer, pH 3.0, containing 0.04M calcium chloride. To this solution was added 1 ml of porcine trypsin (4 mg/ml in the same buffer) and the mixture was incubated at room temperature for 46 hr. This trypsin modification reaction mixture of LBI will be referred to as TMRM-LBI (PREP B). A control was run under identical conditions except that no trypsin was added. The reagents (salts and trypsin) were removed by gel filtration on a column (2.5 x 90 cm) of Sephadex G-25 or Bio-Gel P-10 using 10% aqueous acetic acid as the eluant.

2. Determination of the Inhibitory Activity of the Trypsin Modification Reaction Mixture of LBI (TMRM-LBI)

To establish the effect of trypsin modification on LBI, samples of TMRM-LBI and control LBI and also carboxypeptidase B treated TMRM-LBI and control LBI (see below) were assayed for their trypsin and chymotrypsin inhibitory activity as previously described

under General Experimental Procedures. In the case of the trypsin inhibitory activity assays the activity was determined either immediately after mixing of enzyme and inhibitor or after a 15 min preincubation of enzyme and inhibitor prior to addition of the substrate-indicator solution.

3. Carboxypeptidase B Treatment of the Trypsin Modification Reaction Mixture of LBI

A sample (3-5 mg) of TMRM-LBI or control LBI was dissolved in 0.5-0.6 ml of 0.1M Tris-HCl, pH 8.2, and 50 µl of carboxypeptidase B (1.8-2.0 mg/ml) were added. This mixture was incubated at 37° and at appropriate times samples were withdrawn for assays of the inhibitory activity and for the determination of the amino acids released, by paper chromatography and/or amino acid analysis.

B. Results

Table IX shows the inhibitory activities of LBI treated at acid pH with catalytic amounts of bovine trypsin for 22 hr (Preparation A) or with porcine trypsin for 48 hr (Preparation B). All samples were fully active against chymotrypsin but their ability to inhibit trypsin varied. The control LBI for both preparations was less active in a zero time preincubation assay than in a 15 min preincubation assay. Prior treatment of control LBI with carboxypeptidase B was without effect. TMRM-LBI for both preparations is less active than is control LBI in a zero time preincubation assay. However, after a 15 min preincubation, TMRM-LBI becomes as active as control LBI. Prior treatment of TMRM-LBI with carboxypeptidase B prevents it from

TABLE IX

INHIBITORY ACTIVITIES OF CONTROL LBI, LBI(COB TREATED), TMRM-LBI, AND TMRM-LBI(COB TREATED)

Sample[a]	% Residual Inhibitory Activity[b] Against			
	Trypsin Preincubation		Chymotrypsin Preincubation	
	None	15 min	None	15 min
Preparation A,				
Control LBI	80	100	100	100
TMRM-LBI	65	100	100	100
TMRM-LBI, (COB treated)	70	70	100	100
Preparation B,				
Control LBI	67	100	100	-
LBI (COB treated)	67	100	100	-
TMRM-LBI	25	100	100	-
TMRM-LBI, (COB treated)	23	38	100	-

[a] LBI (COB treated): control LBI treated with carboxypeptidase B for 2½ hr; TMRM-LBI: trypsin modification reaction mixture of LBI; TMRM-LBI (COB treated): treated with carboxypeptidase B for 2½ hr.

[b] The assays are described in the Experimental section. The inhibition obtained by control LBI (native) in a 15 min preincubation assay was taken as 100%. Controls treated as described in the text behaved in all cases like native LBI.

regaining full activity by preincubation with the enzyme. The carboxypeptidase B treated TMRM-LBI was only 70% active in the case of Preparation A and 38% active in the case of Preparation B in a 15 min preincubation assay.

A time course study of the release of amino acids from the TMRM-LBI (PREP A) by carboxypeptidase B, as determined by paper chromatography, showed the release of considerable amounts of lysine compared with trace amounts released from the control LBI. No other amino acids were released from TMRM-LBI (PREP A) in amounts comparable to the amounts of lysine released. Quantitation of the lysine released from TMRM-LBI (PREP A) showed that after correction for background values obtained from the control experiment performed with native LBI, 0.2-0.3 moles/mole were released. These values were obtained for a 30 min incubation with carboxypeptidase B and did not change significantly up to 6 hr. Quantitation of the amino acids released from TMRM-LBI (PREP B) by carboxypeptidase B showed that 0.7 moles/mole of lysine were released.

C. Discussion

The above data suggest very strongly that TMRM-LBI is a mixture of native LBI and LBI_t' (trypsin-modified LBI with the reactive site cleaved). For both preparations (A and B), TMRM-LBI is less active than control LBI in a zero time preincubation assay but regains full activity after 15 min preincubation. Treatment of control LBI with carboxypeptidase B is of no consequence whereas similar treatment of TMRM-LBI results in the release of lysine. The amount of lysine released per mole of TMRM-LBI corresponds to the amount of inactivation

produced by carboxypeptidase B treatment i.e. TMRM-LBI (PREP A) released 0.2-0.3 moles/mole of lysine and is 30% inactivated whereas TMRM-LBI (PREP B) releases 0.7 moles/mole of lysine and is 62% inactivated. The above data are consistent with the reactive site model put forward by Ozawa and Laskowski (1966) in that LBI has a peptide bond which is suceptible to tryptic cleavage at acid pH resulting in a trypsin-modified inhibitor (LBI_t') which reacts with trypsin more slowly than does native LBI and can be inactivated by removal of the new carboxyl-terminal lysyl residue. The reactive site peptide bond of LBI is thus identified as a Lys-X bond which is in agreement with the previous assignment of LBI as a "lysine inhibitor"[10] by chemical modification studies (Haynes et al., 1967; Fritz et al., 1969). LBI_t' is thus inhibitor which has been cleaved at the trypsin reactive site peptide bond (Lys-X) to produce a molecule with two polypeptide chains but which remains active towards trypsin (although it reacts with trypsin more slowly than native LBI) and chymotrypsin. The two chains of LBI_t' are probably held together by one or more disulfide bonds.

Although preparations A and B of TMRM-LBI behave similarly in their inhibitory properties and release of lysine by carboxypeptidase B, their actual degree of modification is quite different. Preparation A, produced by incubation with bovine trypsin for 22 hr is about 30% modified (30% LBI_t', 70% LBI) whereas preparation B, produced by

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[10] A "lysine inhibitor" refers to a trypsin inhibitor in which chemical modification of the ε-amino group of lysine abolishes activity.

incubation with porcine trypsin for 48 hr, is 62% modified (62% LBI_t', 38% LBI). A succeeding section studying the equilibrium between LBI and LBI_t' will show that the extent of modification of LBI is dependent on both the time of incubation as well as the trypsin used (porcine or bovine).

III. Location of the Trypsin Reactive Site in the Primary Structure of LBI

A. Experimental

1. Separation of the Components of the Reduced and Alkylated Trypsin Modification Reaction Mixture of LBI

The material used in these studies was TMRM-LBI (PREP B). As previously mentioned TMRM-LBI is believed to consist of a mixture of native LBI and LBI_t'. Reduction and alkylation of LBI is known to yield a single polypeptide chain whereas similar treatment of LBI_t' should yield two fragments. Thus, reduction and alkylation of TMRM-LBI should yield three distinct and separable peptide chains. TMRM-LBI (130 mg of PREP B) was reduced with β-mercaptoethanol and alkylated with either iodoacetic acid or iodoacetamide in the presence of 5M guanidine hydrochloride as described under General Experimental Procedures. The excess reagents were removed by gel filtration on Sephadex G-15 using 10% acetic acid as the eluant. The components of the reduced and alkylated TMRM-LBI were then separated by chromatography on DEAE-Sephadex A-25 followed by high-voltage paper electrophoresis or by high-voltage paper electrophoresis alone; experimental details are given under Results and Discussion.

2. Characterization of the Components Obtained by Fractionation of Reduced and Alkylated TMRM-LBI

The components obtained by fractionation of reduced and alkylated TMRM-LBI were characterized by their amino acid composition and by end-group analysis as previously described under General Experimental Procedures.

B. Results and Discussions

The reaction mixture obtained after reduction and alkylation of TMRM-LBI, gave a single symmetrical peak upon gel filtration on Sephadex G-15, indicating that if fragments were produced, their molecular weights were greater than 1500 g/mole. As shown in Figure 11, reduced and carboxamidomethylated TMRM-LBI can be separated into two ninhydrin-positive components by ion-exchange chromatography on DEAE-Sephadex A-25. By amino acid composition, and based upon the known amino acid sequence of LBI, peak I was recognized as the amino-terminal portion of LBI (see Table X) and, because of its relative size, was called "small fragment". Peak II could be further separated into its components by preparative high-voltage paper electrophoresis at pH 4.7. Alternatively, reduced and carboxymethylated TMRM-LBI could be directly separated into its component parts by preparative high-voltage paper electrophoresis at pH 4.7. This is illustrated in Figure 12; spots A, B, and C were shown to be small fragment, intact LBI, and large fragment respectively. Table X gives the amino acid composition of these fragments as well as that of the intact RCAM-LBI IV as determined in this study and as derived from the amino acid sequence data (Tan and Stevens, 1971b). Comparison of the amino acid

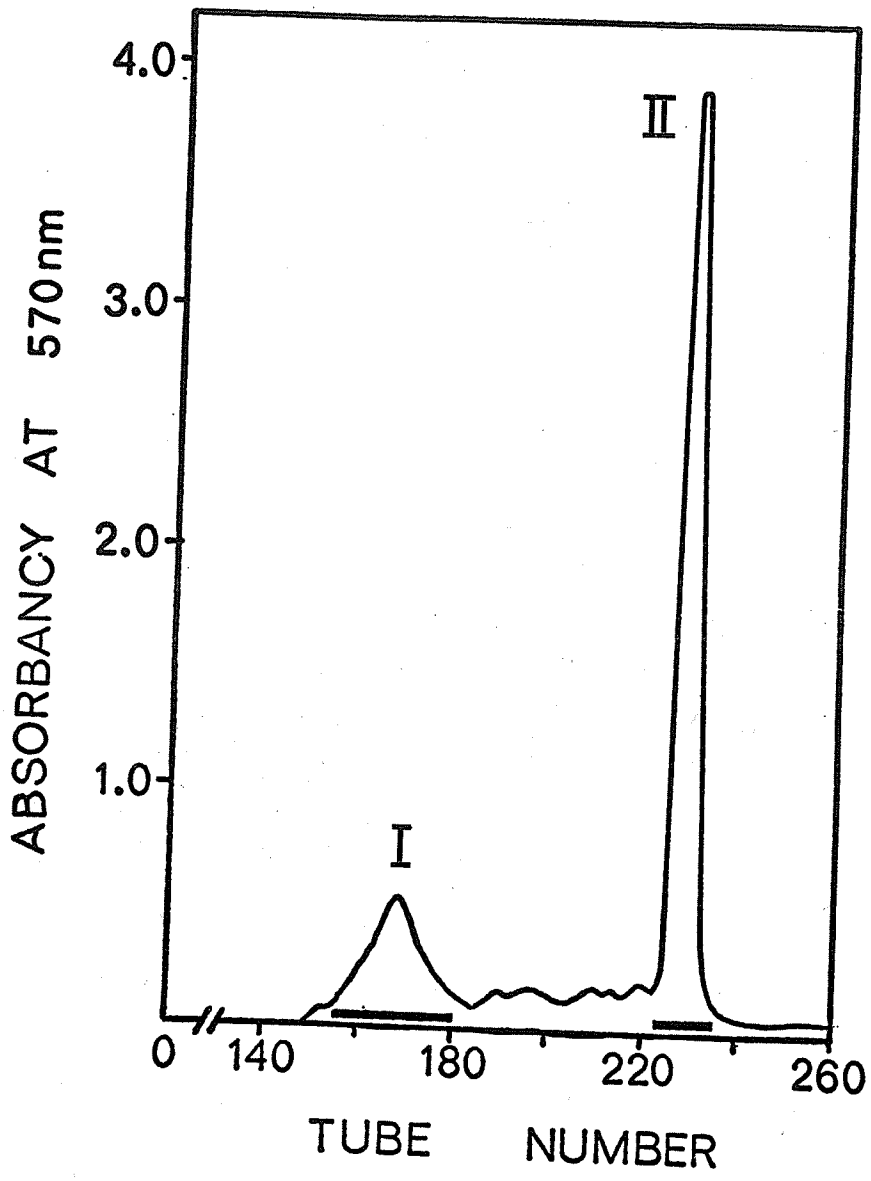

Figure 11 Ion-exchange chromatography of reduced and carboxamidomethylated TMRM-LBI on DEAE-Sephadex A-25.

Reduced and carboxamidomethylated TMRM-LBI (130 mg/14 ml starting buffer) was applied to a column (2.5 x 90 cm) of DEAE-Sephadex A-25 previously equilibrated with 0.01M sodium phosphate, pH 7.6, and eluted with a linear salt gradient consisting of 1 litre each of 0.01M sodium phosphate, pH 7.6, and the same buffer containing 0.6M NaCl. The flow rate was 24 ml/hr and fractions of 3.7 ml were collected. The fractions were pooled as indicated by the solid bars, taken to dryness by flash evaporation and desalted on Sephadex G-25.

TABLE X

AMINO ACID COMPOSITION OF REDUCED, CARBOXAMIDOMETHYLATED LBI AND OF THE FRAGMENTS OBTAINED AFTER REDUCTION AND ALKYLATION OF LBI_t[a]

Amino Acid	Fragments			Intact[d]	
	Small	Large	Sum of Both	RCAM-LBI	LBI-IV[e]
Lysine	1.60	1.50	3.10	3.56	4
Histidine	3.60	2.52	6.18	5.64	6
Arginine	0	2.00	2.00	2.10	2
Aspartic Acid	2.00	9.50	11.50	12.50	13
Threonine	1.67	2.72	4.39	4.00	4
Serine	4.00	7.97	11.97	13.00	15
Glutamic Acid	3.00	4.04	7.04	7.03	6
Proline	2.20	3.83	6.03	6.27	6
Glycine	1.33[b]	0.42[b]	1.75[b]	1.39	1
Alanine	1.00	2.10	3.10	3.14	2
Cysteine	3.47[c]	10.47[c]	13.94	14.72[c]	14
Valine	0	0.84	0.84	1.09	1
Isoleucine	0	3.49	3.49	3.91	4
Leucine	0	1.41	1.41	1.67	3
Tyrosine	0	1.02	1.02	1.27	1
Phenylalanine	0	2.23	2.23	2.51	2

[a] Analyses were performed as described in the test. Tryptophan and methionine are known to be absent from LBI (Jones et al., 1963). The results are expressed in moles per mole and were calculated on the basis of 1 residue of Ala in the case of column 1; 2 residues of Arg in the case of column 2; 2 residues of Arg and 4 residues of Ile in the case of column 4.

[b] Fragments were eluted from paper with resulting high values for Gly.

[c] Determined as S-carboxymethylcysteine.

[d] Based on duplicate analyses of 24, 48 and 72 hr hydrolysates with values for Ser and Thr extrapolated to zero time.

[e] Value taken from Tan and Stevens (1971b).

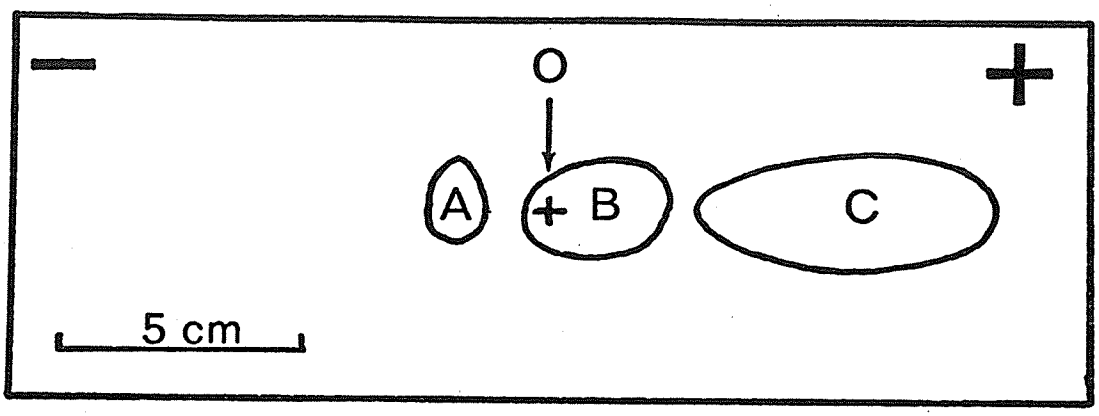

Figure 12 Preparative high-voltage paper electrophoresis of reduced and carboxymethylated TMRM-LBI.

The material was subjected to preparative high-voltage paper electrophoresis in pyridinium-acetate buffer, pH 4.7 at 53 V/cm for 30 min. Guide strips were stained with ninhydrin and the material was eluted with 30% acetic acid, taken to dryness by flash evaporation, and characterized by amino acid analysis after acid hydrolysis. In this way reduced carboxymethylated TMRM-LBI could be separated into small fragment (A), uncleaved LBI (B), and large fragment (C).

composition of the isolated fragments with the published amino acid sequence identifies the small fragment as the amino-terminal portion of the molecule whereas the large fragment represents the carboxyl-terminal portion of LBI IV. The sum of the amino acid compositions of the two fragments accounts for the entire amino acid composition of intact RCAM-LBI. To confirm identification of these fragments, the sequence of amino acids around the reactive site peptide bond was determined in terms of the carboxyl-terminal sequence of the small fragment and the amino-terminal sequence of the large fragment. The results of studies on the carboxyl-terminal sequence of the small fragment are shown in Figure 13. It can be seen that the small fragment ends in the sequence Ala-Thr-Lys. Digestion of the large fragment with aminopeptidase M for up to 16 hr, resulted in the release of only 0.3 moles/mole of serine. Serine was also confirmed as the amino-terminal residue of the large fragment by the subtractive Edman degradation procedure. These results together with the known sequence of LBI IV (see Figure 1) allows positive identification of the trypsin reactive site of LBI IV as the Lys^{28}-Ser^{29} peptide bond. It is also clear that TMRM-LBI is actually a mixture of native and trypsin-modified LBI (LBI_t'). Since LBI_t' has the Lys^{28}-Ser^{29} peptide bond cleaved it is thought to quantitatively release the carboxyl-terminal lysine upon carboxypeptidase B treatment thus accounting for the lysine released from TMRM-LBI. Final confirmation of this is shown in a later section where LBI_t' is purified from TMRM-LBI.

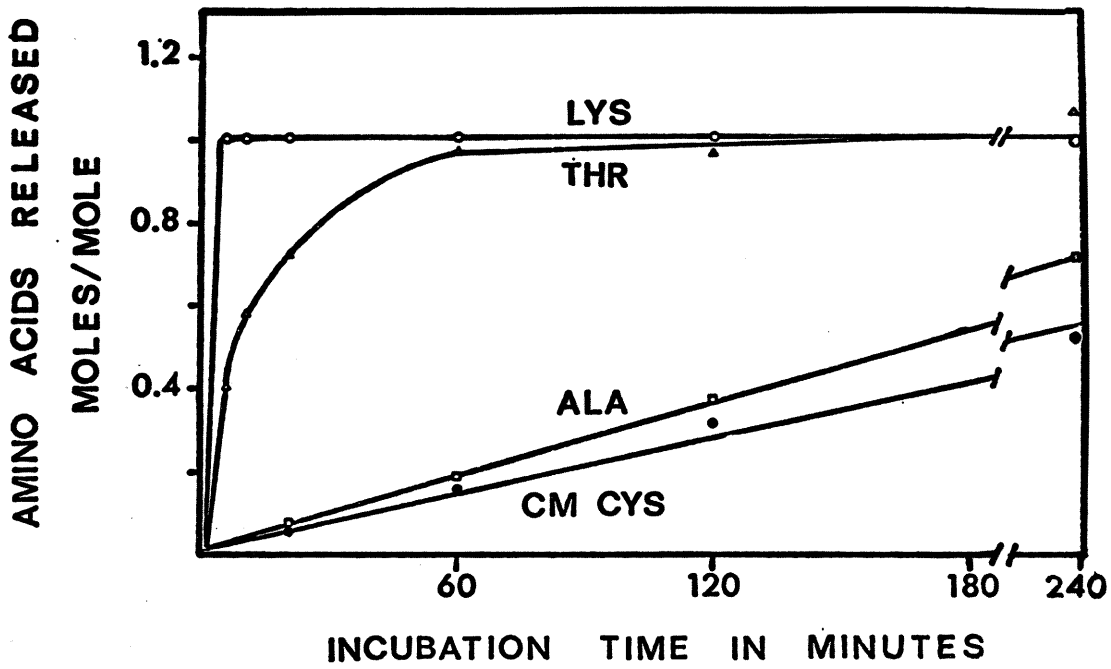

Figure 13 Time course of the release of free amino acids from the small fragment of RCAM-LBI$^{1}_{t}$ by carboxypeptidase B.

The fragment [0.5 μmoles] was dissolved in 0.1M NH_4HCO_3 and 25 μl of carboxypeptidase B (1.8 mg/ml) were added. At appropriate time intervals samples were withdrawn, quick frozen in dry ice-ethanol, lyophilized and subjected to amino acid analysis. The control sample (containing no peptide) released negligible amounts of amino acids after up to 4 hr of incubation.

IV. Studies on the Equilibrium Between Native and Trypsin-modified LBI

To show a true equilibrium between two interconvertible molecules, it is necessary to demonstrate that both the forward and the reverse reactions do in fact occur. Furthermore, it should be shown that the forward and reverse reactions converge on the same final equilibrium position as manifested in a steady-state plateau for the two interconverting species. This type of study involving LBI and LBI'_t is described below.

A. Experimental

The material used in the following experiments is LBI variant I prepared from the commercial preparation LBI 1DA.

1. The Forward Reaction (LBI → LBI'_t)

In a typical experiment LBI variant I (20 mg) was dissolved in 2 ml of 0.018M trans-aconitate buffer containing 0.04M calcium chloride resulting in a final pH of 3.1. To this solution were added 200 µl of either bovine or porcine trypsin (5mg/ml in the same buffer) and the mixture was incubated at room temperature. At the appropriate times 50 µl samples were withdrawn, quick-frozen in a dry ice-ethanol mixture and stored at -20°. To determine the relative amounts of LBI and LBI'_t present in each sample, each sample was treated with carboxypeptidase B. This treatment causes the LBI'_t present to become completely inactive towards trypsin whereas the activity of native LBI remains unaffected. To each 50 µl sample were added 40 µl of 0.1M sodium borate, pH 10.4, to bring the pH of

the reaction mixture of TMRM-LBI to approximately pH 8.0. To this solution were added 25 µl of carboxypeptidase B (3 mg/ml) and the mixture incubated overnight at 37°. A control sample was treated in identical fashion except for the omission of trypsin. Samples and control were then diluted to the appropriate concentration with 0.006M Tris-HCl buffer, pH 8.2, and assayed for their trypsin inhibitory activity in a 15 min preincubation assay.

2. Separation of LBI and LBI_t' by Ion-exchange Chromatography on DEAE-Cellulose

LBI_t' has the reactive site peptide bond cleaved and therefore has one more carboxyl group than does native LBI. At pH 8.6, LBI_t' will be more negatively charged than LBI so that their separation should be possible by ion-exchange chromatography on DEAE-cellulose. Lyophilized TMRM-LBI[11], a mixture of LBI and LBI_t', was dissolved in 0.01M sodium borate, pH 8.6, and applied to a column (1.5 x 90 cm) of DEAE-cellulose previously equilibrated with the same buffer. Elution was carried out with an exponential gradient applied through a 1 liter mixing chamber (originally containing 0.01M sodium borate, pH 8.6), the limiting buffer containing 0.01M sodium borate and 0.4M sodium chloride, pH 8.6. The effluent fractions were monitored by the ninhydrin method after alkaline hydrolysis of 200 µl aliquots of alternate fractions. As a control, a sample of

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[11]This preparation of TMRM-LBI contained approximately 80% LBI_t' and 20% LBI. Trypsin and salts had been previously removed by gel filtration

native LBI was subjected to identical DEAE-cellulose chromatography in a separate experiment. The appropriate chromatographic fractions were desalted, lyophilized and rechromatographed in the same system to achieve a higher degree of purity.

3. The Reverse Reaction ($LBI_t' \rightarrow LBI$)

The experimental conditions used to demonstrate the reverse reaction are identical to those of the forward reaction with the following exceptions: (1) the starting material was pure LBI_t' (2) the amount of porcine trypsin used as 1/4 that used in the forward reaction and (3) the control sample used was pure LBI_t' which reached full activity (equal to the activity of native LBI) after a 15 min incubation with trypsin.

B. Results and Discussion

Figure 14 shows the time course of the conversion of $LBI \rightarrow LBI_t'$ by trypsin as monitored by the % inactivation following treatment with carboxypeptidase B. Using approximately equal amounts of either porcine or bovine trypsin at acid pH, the reaction ($LBI \rightarrow LBI_t'$) approaches a steady state plateau consisting of approximately 85% LBI_t' and 15% LBI. Porcine trypsin is definitely the better catalyst for this particular reaction although no reasons for this are immediately apparent. In the reaction using bovine trypsin, the steady state plateau has not been reached even after 120 hr. In separate experiments run under identical conditions but for longer periods of time, a reaction mixture containing approximately 90% LBI_t' was obtained. At this point, further addition of trypsin (1 mg/20 mg LBI)

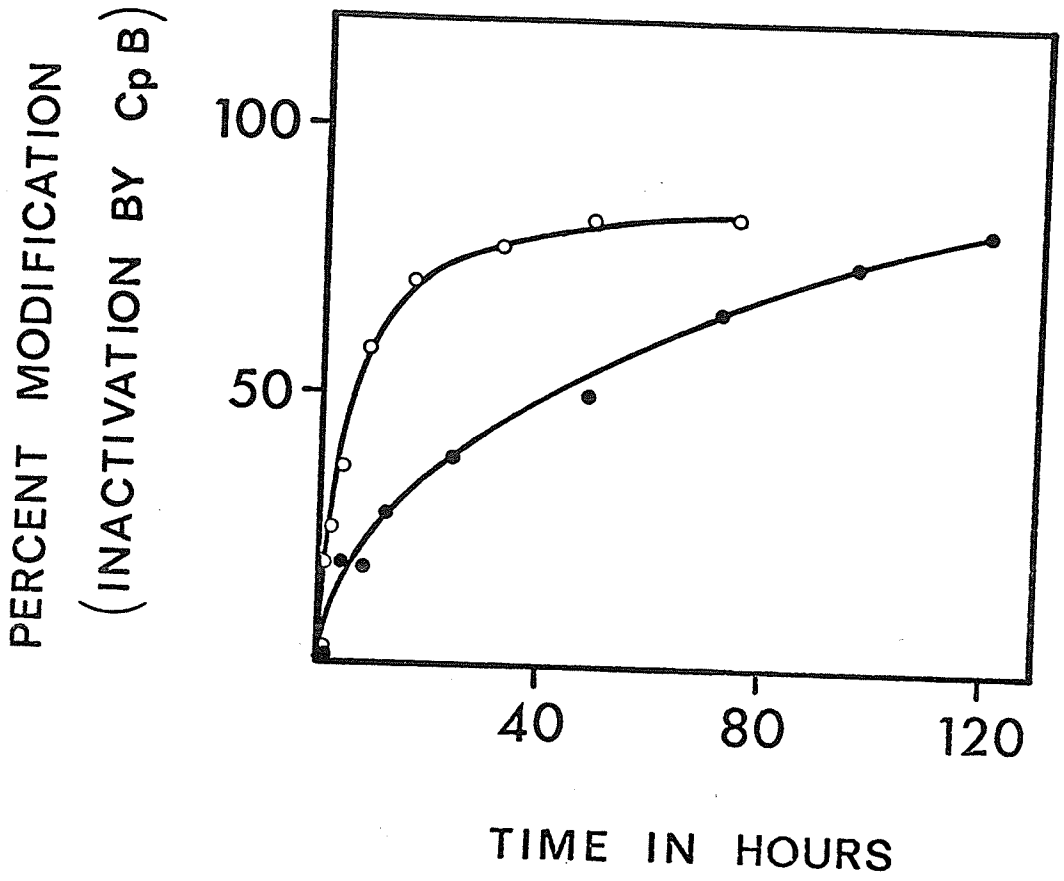

Figure 14 Trypsin modification of LBI as a function of time (LBI → LBI'_t).

The percent modification of LBI was determined by measuring the trypsin inhibitory activity of a carboxypeptidase B treated sample in a 15 min preincubation assay. Experiment performed with: (o—o—o), porcine trypsin; (●—●—●), bovine trypsin.

did not change the composition of TMRM-LBI with respect to LBI and LBI'_t. In this way it was conclusively demonstrated that the conversion of LBI to LBI'_t reaches a steady state plateau.

In order to demonstrate the existence of a true equilibrium between LBI and LBI'_t it is of course necessary to show that the same equilibrium mixture can be obtained by the reverse reaction and hence it was necessary to purify LBI'_t from TMRM-LBI. Because LBI'_t has an additional carboxyl group and is therefore more negatively charged at pH 8.6, as compared to native LBI, it would be expected to bind more strongly to DEAE-cellulose, at pH 8.6, than does native LBI. This was indeed the case as shown in Figure 15. Native LBI emerges from the column first (as shown by the control experiment) followed by a large peak probably representing LBI'_t. The separated components of TMRM-LBI were characterized by the release of lysine and the loss of trypsin inhibitory activity upon treatment by carboxypeptidase B; in this manner, the purification of LBI'_t can be adequately monitored. The results shown in Table XI indicate that the reaction mixture applied to the column contained $\simeq$ 80% LBI'_t as indicated by the fact that it was inactivated 83% by carboxypeptidase B concomitant with the release of 0.8 moles/mole of lysine. By the same criteria, fraction I (Figure 15) consists mainly of native LBI (contains only 25% LBI'_t) whereas fraction II (Figure 15) is mainly LBI'_t (90% LBI'_t). Rechromatography of fraction II on DEAE-cellulose under the same conditions yields LBI'_t judged pure by these criteria. This was the material used to demonstrate the reverse reaction ($LBI'_t \rightarrow$ LBI) illustrated in Figure 16. It can be seen that the reaction mixture becomes more

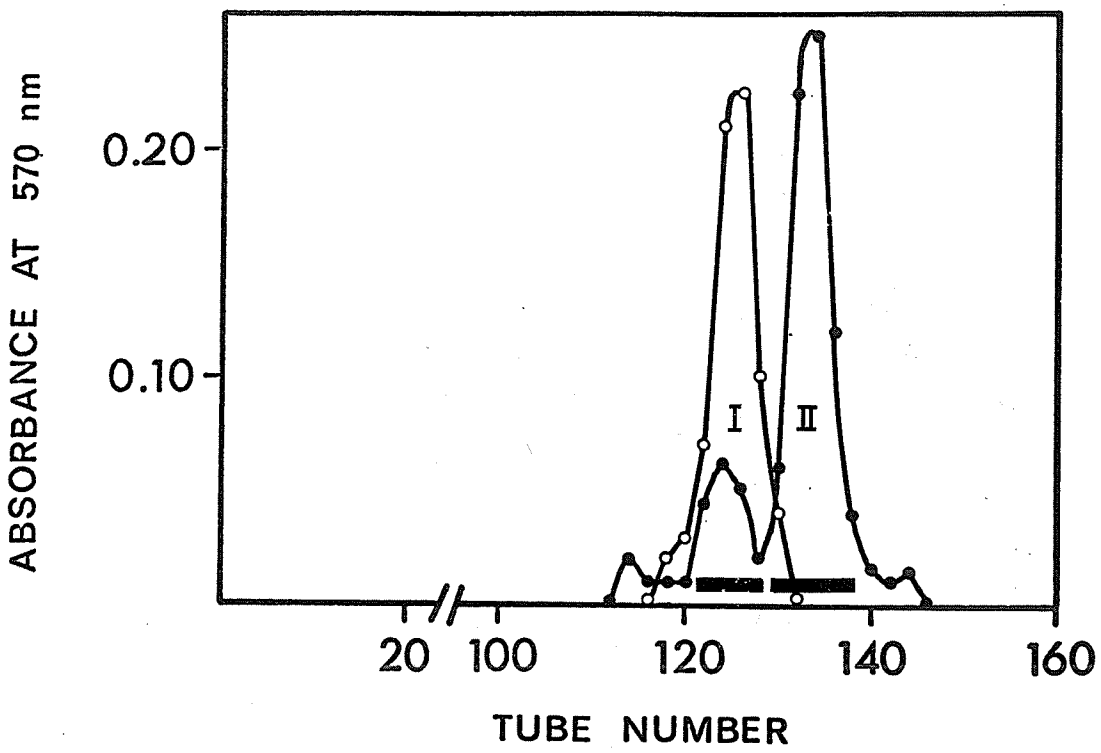

Figure 15 Separation of LBI and LBI'_t from TMRM-LBI by ion-exchange chromatography on DEAE-cellulose.

Both experiments were run under identical chromatographic conditions as described under Experimental. The flow rate was 29 ml/hr and effluent fractions of 4.2 ml were collected. The fractions were pooled as indicated, desalted and lyophilized. (o—o—o), experiment with 10 mg of native LBI; (●—●—●), experiment with 10 mg of TMRM-LBI containing LBI and LBI'_t.

TABLE XI

PURIFICATION OF LBI_t' AS MONITORED BY CARBOXYPEPTIDASE B TREATMENT

Fraction from DEAE-Cellulose[a]	Moles Lys Released per mole LBI	% Inactivation[b]
Sample applied	0.80	83
I	0.25	26
II	0.85	93
II (Rechromatographed)	1.06	98

[a]The material is that obtained from column chromatography of TMRM-LBI (a mixture of native LBI and LBI_t') as illustrated in Figure 15.

[b]% inactivation was calculated by comparing LBI_t' and the carboxypeptidase B treated sample in a 15 min preincubation assay and taking the trypsin inhibitory activity of LBI_t' as 100%. Recall that native LBI and TMRM-LBI are equally active in a 15 min preincubation assay.

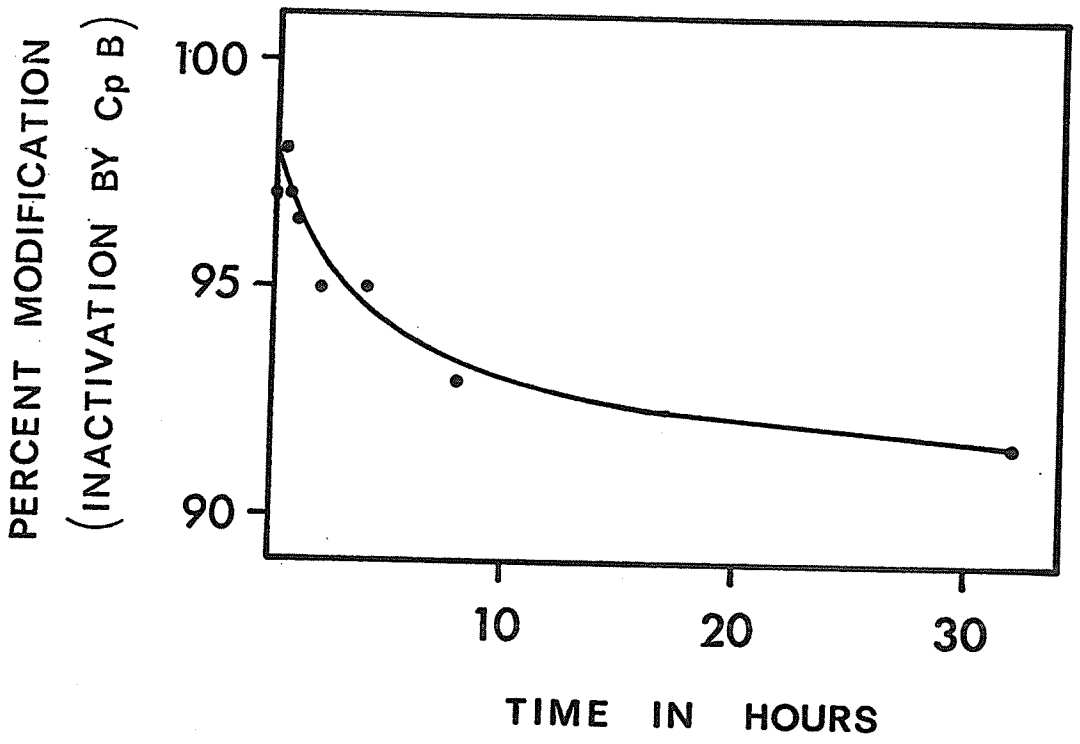

Figure 16 Conversion of LBI'_t to LBI.

The percent modification was determined by measuring the trypsin inhibitory activity of a carboxypeptidase B treated sample in a 15 min preincubation assay as described under Experimental. The reaction was catalyzed by porcine trypsin at acid pH.

resistant to the loss of trypsin inhibitory activity by treatment with carboxypeptidase B as a function of time of incubation with catalytic amounts of trypsin at acid pH; this implies the formation of LBI from LBI'_t. The conversion of $LBI'_t \rightarrow$ LBI is seen to approach a steady state plateau consisting of approximately 92% LBI'_t and 8% LBI. In a separate experiment under similar conditions but allowing the reaction to proceed for 120 hr, the final composition of the reaction mixture was approximately 90% LBI'_t and 10% LBI. Within the experimental error of this method the compositions of the forward and the reverse reaction mixtures with respect to LBI'_t and LBI are identical. These data can be summarized in the following equation:

$$\text{LBI} \underset{\text{acid pH}}{\overset{\text{catalytic amounts of trypsin}}{\rightleftharpoons}} LBI'_t$$

in which LBI represents the native inhibitor with the trypsin reactive site peptide bond intact and LBI'_t represents the trypsin-modified inhibitor with the trypsin reactive site peptide bond cleaved. It is important to note that, in either direction, the equilibrium is reached at acid pH and is independent of the amount of trypsin present. At neutral pH, on the other hand, the hydrolysis of the reactive site peptide bond cannot be detected by the methods used in this study.

V. Studies on the Reaction of LBI and LBI'_t with Trypsin

A. Experimental

For all the experiments in this section, LBI variant I, prepared from the commercial preparation LBI 1DA was used.

1. Kinetics of Complex Formation

To monitor the kinetics of complex formation between trypsin and either native or trypsin-modified LBI, near equimolar amounts of inhibitor and enzyme[12] were incubated in the assay medium (0.006M Tris-HCl, pH 8.2) at room temperature and at the appropriate intervals, samples were withdrawn and their residual trypsin activity determined. Since it has already been shown that a mixture of LBI and LBI'_t is fully active (as active as native LBI) after 15 min preincubation with enzyme, the trypsin inhibitory activity of LBI and LBI'_t after 15 min preincubation with trypsin was taken as 100% activity.

2. Rapid Dissociation of the Inhibitor-Trypsin Complex

The enzyme inhibitor complex was made by adding a slight molar excess (6 mg) of trypsin to a solution of 2 mg of inhibitor (either LBI or LBI'_t) in 2 ml of 0.001M sodium hydroxide. After 30 min at room temperature, 2 ml of 5% trichloracetic acid were added and the resulting solution was allowed to stand overnight at 4°. It was then centrifuged and the precipitate discarded. Seidl and Liener (1971b) working with Bowman-Birk inhibitor used similar conditions and showed that under these conditions trypsin denatures and precipitates out of solution while the inhibitor remains soluble. The supernatant containing the inhibitor was subsequently dialyzed against

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[12]The amount of inhibitor used was determined from an inhibition curve using a constant amount of trypsin and varying amounts of inhibitor in a 15 min preincubation assay and corresponds to somewhat less inhibitor than would be present at the equivalence point.

water and lyophilized prior to characterization.

3. Characterization of the Inhibitor Obtained by Rapid Dissociation of Complexes Prepared From Trypsin and LBI or LBI'_t

The inhibitor obtained by dissociation of the LBI-trypsin complex was characterized by amino acid analysis, determination of the trypsin and chymotrypsin inhibitory activity in a zero time preincubation assay, and release of lysine by carboxypeptidase B treatment. The inhibitor obtained by dissociation of the LBI'_t-trypsin complex was more extensively characterized. This inhibitor was treated with carboxypeptidase B as previously described and assayed for trypsin inhibitory activity in a 15 min preincubation assay. Release of lysine from this inhibitor by carboxypeptidase B was also studied by methods previously described. Finally, to determine whether the inhibitor obtained from the rapid dissociation of LBI'_t-trypsin complex was a protein consisting of one or two polypeptide chains, it was reduced and carboxymethylated according to the method of Sondack and Light (1971) as previously described under General Experimental Procedures. Native LBI and LBI'_t were similarly reduced and carboxymethylated. The three modified inhibitors namely (1) RCM-LBI (2) RCM-LBI'_t and (3) RCM-inhibitor obtained from rapid dissociation of the LBI'_t-trypsin complex, were then compared by high-voltage paper electrophoresis at pH 1.9.

B. Results and Discussion

Figure 17 shows the kinetics of complex formation between trypsin and either native or trypsin-modified LBI as measured by the

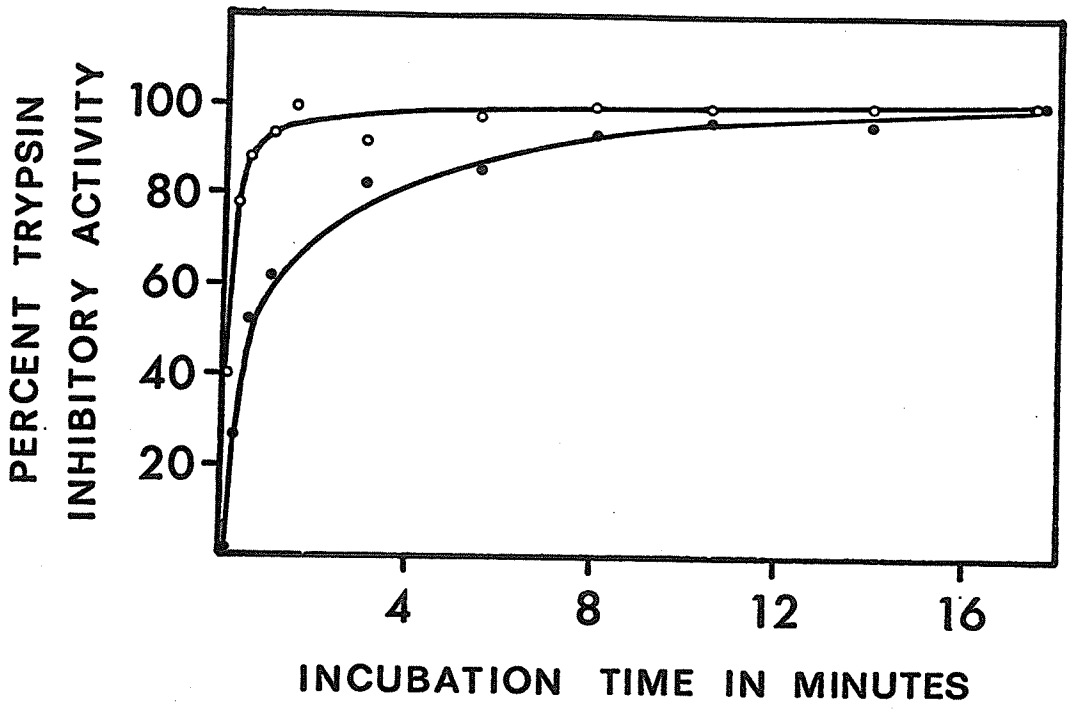

Figure 17 The kinetics of complex formation between trypsin and LBI or LBI'_t.

Mixtures containing approximately 45 µg of trypsin and 15 µg of inhibitor in 1 ml of assay medium (0.006M Tris-HCl, pH 8.2) were incubated at room temperature and at the appropriate times the residual trypsin activity was determined. (O—O—O), experiment with native LBI; (●—●—●), experiment with trypsin-modified LBI (LBI'_t).

residual trypsin activity. The formation of complex between native LBI and trypsin is complete within 90 seconds while the formation of complex between $LBI_t^!$ and trypsin requires up to 15 min to reach completion. The nature of the complexes formed from trypsin and either LBI or $LBI_t^!$ is not known. This study has examined one of the properties of these complexes by characterizing the inhibitor obtained by rapid dissociation of the inhibitor-trypsin complex. Dissociation of the complex formed between native LBI and trypsin, by addition of 5% trichloroacetic acid, resulted in a precipitate which was shown by amino acid analysis to consist of trypsin. The supernatant was found to contain free inhibitor; it was fully active against trypsin and chymotrypsin and did not release lysine upon treatment with carboxypeptidase B. This identified it as LBI with the trypsin reactive site peptide bond intact. The inhibitor obtained from the $LBI_t^!$-trypsin complex was characterized somewhat more extensively. Table XII shows the trypsin inhibitory activity of this inhibitor as compared to either $LBI_t^!$ or native LBI. Native LBI is fully active in a 15 min preincubation assay even when subjected to prior carboxypeptidase B treatment. Pure $LBI_t^!$ treated with carboxypeptidase B prior to assay is only 2% active. However, the inhibitor obtained from the $LBI_t^!$-trypsin complex is totally resistant to carboxypeptidase B inactivation, and by this criterion behaves like native LBI.

Native LBI does not release any lysine upon incubation with carboxypeptidase B whereas similar treatment of pure $LBI_t^!$ releases 1.06 moles/mole of lysine (Table XI). The inhibitor obtained from the rapid dissociation of the $LBI_t^!$-trypsin complex releases no lysine

TABLE XII

THE TRYPSIN INHIBITORY ACTIVITY OF MODIFIED LIMA BEAN INHIBITORS

Sample	% Trypsin Inhibitory Activity[a]	
	Before treatment[b] with carboxypeptidase B	After treatment[b] with carboxypeptidase B
LBI (native)	100	100
LBI'_t	100	2
Inhibitor from LBI'_t-trypsin complex[c]	100	100

[a]The inhibition obtained with native LBI in a 15 min preincubation assay was taken as 100%.

[b]Samples were incubated either with or without carboxypeptidase B overnight at 37° as described under General Experimental Procedures. The inhibitory activity was then determined in a 15 min preincubation assay.

[c]Inhibitor was obtained from the LBI'_t-trypsin complex by rapid dissociation as described under Experimental.

above control values so that by this criterion also, the inhibitor is similar to native LBI, in that the reactive site lysyl residue is unavailable for attack by carboxypeptidase B.

To further establish whether the inhibitor obtained by rapid dissociation of the LBI_t'-trypsin complex indeed consists of a single polypeptide chain, it was reduced and alkylated prior to examination by high-voltage paper electrophoresis as illustrated in Figure 18. Reduced and carboxymethylated LBI_t' (RCM-LBI_t') consists of two electrophoretic components whereas reduced and carboxymethylated native LBI (RCM-LBI) consists of only a single electrophoretic component with a mobility intermediate to that of the two components of RCM-LBI_t'. Reduced and carboxymethylated inhibitor obtained by rapid dissociation of the LBI_t'-trypsin complex has only a single electrophoretic component with mobility equal to that of RCM-LBI, indicating that this inhibitor consists of a single polypeptide chain.

By all criteria examined, the inhibitor obtained by rapid dissociation of the LBI_t'-trypsin complex behaves like native LBI. These data conclusively show that when LBI_t' is allowed to form a complex with trypsin and the resulting complex then is rapidly dissociated, the inhibitor obtained is intact implying that resynthesis of the trypsin reactive site peptide bond has been achieved enzymatically by trypsin. Hixon and Laskowski (1970b) have made similar observations in their study of soybean trypsin inhibitor and have called the process "kinetic control dissociation". These authors believe that the complexes of trypsin with either native (I) or trypsin-modified inhibitors (I_t') are identical and can dissociate to yield free trypsin

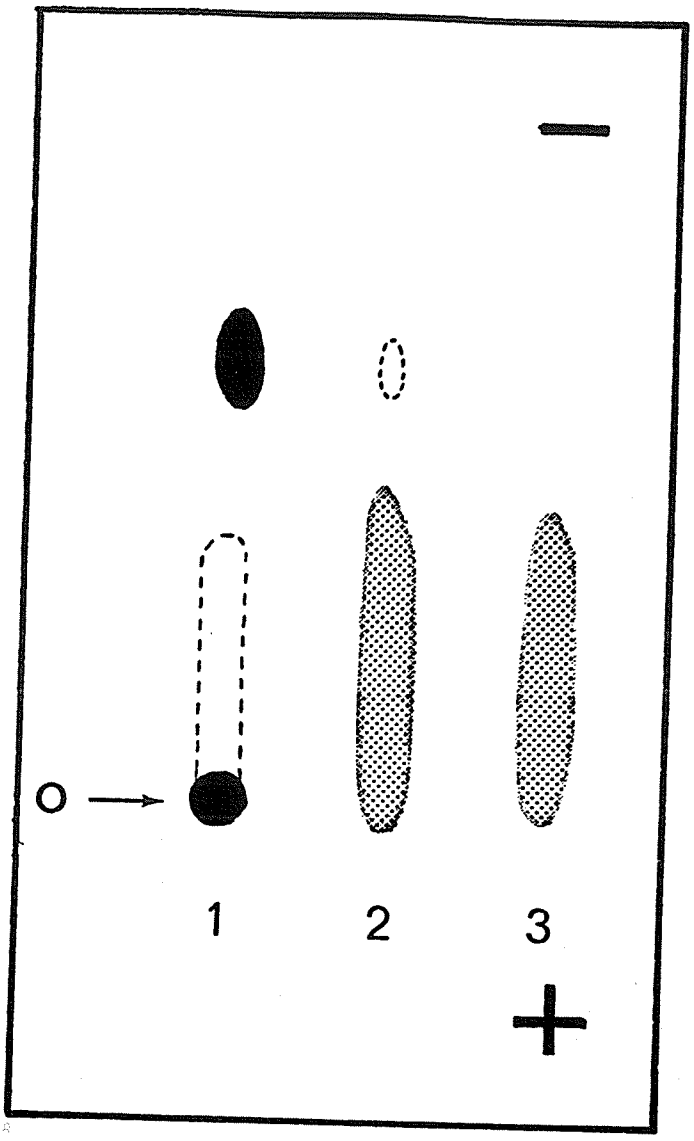

Figure 18 High-voltage electrophoresis of reduced and carboxymethylated lima bean inhibitors.

The electrophoresis was carried out at pH 1.9 in formate-acetate buffer at 53 V/cm for 30 min at 10°. O indicates the point at which the samples were applied. The electrophoretogram was stained with ninhydrin-collidine reagent : (1) LBI'_t (2) inhibitor obtained by rapid dissociation of the LBI'_t-trypsin complex (3) native LBI.

and either I or I_t'; since the rate of dissociation of the complex to trypsin and I is much greater than to trypsin and I_t', rapid dissociation results in a non-equilibrium distribution of I and I_t' with I predominating. Laskowski's group takes this phenomenon to mean that the active site of the enzyme and the reactive site of the inhibitor have been in direct contact, thereby illustrating the importance of the inhibitor's reactive site to the inhibition process.

VI. Comparative Studies on the Trypsin Inhibitory Activity of Four Variants of LBI

Since different LBI variants had been used in the studies involving the trypsin reactive site of LBI it was decided to do a comparative study of four variants. They are compared with respect to trypsin inhibitory activity, extent of modification by trypsin, and the kinetics of complex formation between trypsin and either native LBI or TMRM-LBI(mixture of LBI and LBI_t').

a. Experimental

The four variants of lima bean inhibitor (LBI I-IV) used in these studies were prepared from commercial preparation LBI 1DA.

1. Trypsin Modification of LBI I-IV

Each of the four variants was dissolved in 0.018M trans-aconitate-0.04M calcium chloride to a final protein concentration of approximately 10 mg/ml with a final pH of 3.1. To a 500 µl aliquot of each of the above solutions were added 5 µl of porcine trypsin (50 mg/ml in the same buffer) and the mixture was incubated for 76 hr at room temperature to produce a trypsin modification

reaction mixture of LBI (TMRM-LBI). On the basis of previous data this TMRM-LBI should consist of equilibrium concentrations of LBI and LBI'_t. A control sample was run under identical conditions except for the omission of trypsin.

2. Determination of the Trypsin Inhibitory Activity of LBI and TMRM-LBI

To determine the trypsin inhibitory activity of LBI (control) and TMRM-LBI, 50 µl samples of each were adjusted to pH 8.0 by the addition of 40 µl of 0.1M sodium borate, pH 10.4. To these solutions were added 25 µl of carboxypeptidase B (2.5 mg/ml) and the mixtures incubated overnight at 37°. After the appropriate dilution with 0.006M Tris-HCl, pH 8.2, the samples were assayed for their trypsin inhibitory activity in a 15 min preincubation assay. The extent of modification (the amount of LBI'_t present in TMRM-LBI) was calculated by comparing the activity of TMRM-LBI with control LBI as previously described under General Experimental Procedures.

3. Kinetics of Complex Formation Between Trypsin and LBI or TMRM-LBI Variants I-IV

The trypsin inhibitory activities of the control and the TMRM-LBI were determined as a function of preincubation time with near molar amounts of bovine trypsin in 0.006M Tris-HCl, pH 8.2, at room temperature. The degree of inhibition obtained was considered a measure of the amount of complex formed.

B. Results and Discussion

Figure 19 shows that the trypsin inhibitory activities of control LBI I-IV are all approximately equal. TMRM-LBI, of course, is much less active than control LBI, and the extent of modification (a measure of the % LBI_t' present in TMRM-LBI) of LBI variants I-IV by trypsin was calculated to be 93%, 90%,93% and 82% respectively. Whether these values represent the equilibrium concentration cannot be definitely stated since a time course study has not been done for each of the four variants.

Figure 20 shows the kinetics of complex formation between control LBI or TMRM-LBI and trypsin. A control shows that the activity of the enzyme remains constant during the period of preincubation. In the case of the native inhibitor (variants I-IV), full inhibition is attained after 60 seconds of preincubation with trypsin. The TMRM-LBI (containing approximately 90% LBI_t') of each of the LBI variants attains full activity at a slower rate than its control. TMRM-LBI of variant I attains full activity in less than 5 min, whereas the others require 15 min or more. The inhibitory activity of TMRM-LBI (Variant IV) regenerates at the slowest rate. The differences in the rate of complex formation between trypsin and the trypsin-modified variants may be a reflection of their ability to bind trypsin.

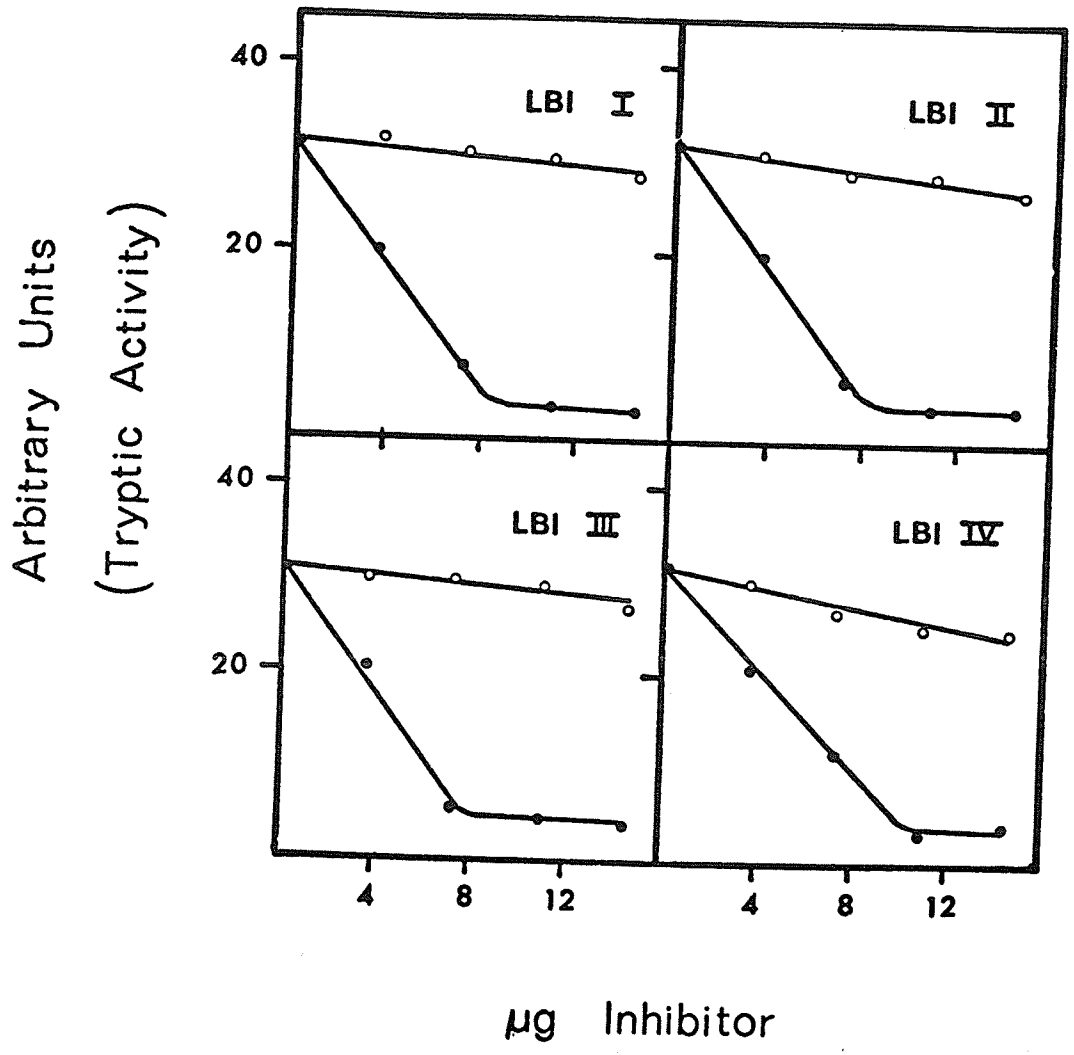

Figure 19 The trypsin inhibitory activities of the four variants of native LBI and TMRM-LBI.

Increasing amounts of inhibitor were added to 30 µg of trypsin and the mixtures were incubated in the assay medium for 15 min before the addition of the substrate-indicator solution. Both control LBI and TMRM-LBI were subjected to carboxypeptidase B treatment prior to assay, as described under Experimental. (o—o—o), TMRM-LBI (mixture of LBI_t' and LBI); (●—●—●), control LBI.

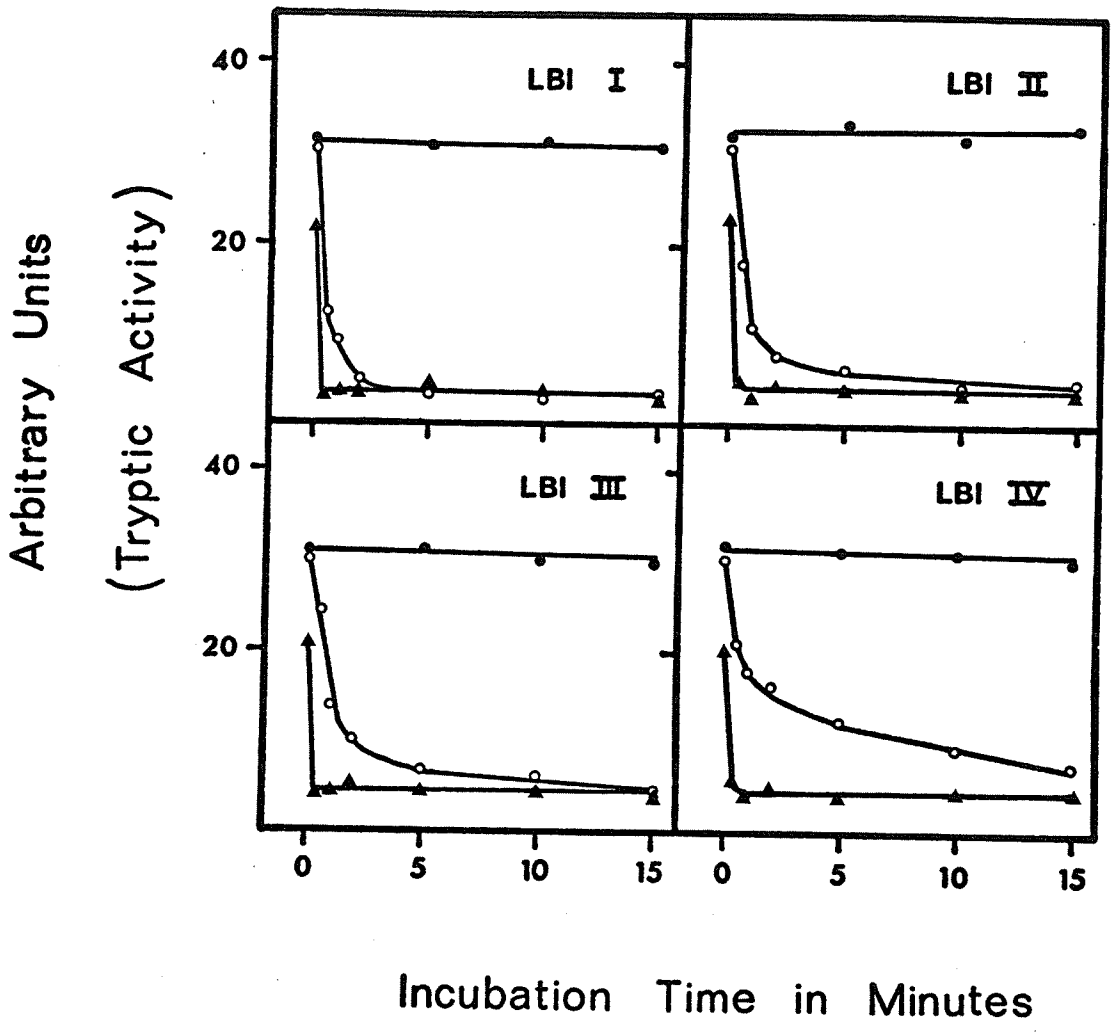

Figure 20 Time course study of the trypsin inhibitory capacity of native LBI and TMRM-LBI, variants I-IV.

The trypsin-inhibitor solutions were incubated in the assay medium for various time intervals prior to addition of the substrate-indicator solution. (●—●—●), 30 µg of trypsin; (O—O—O), 30 µg of trypsin and 9.1 µg of TMRM-LBI; (▲—▲—▲), 30 µg of trypsin and 9.1 µg of control LBI.

STUDIES ON THE CHYMOTRYPSIN INHIBITORY ACTIVITY OF LIMA BEAN INHIBITOR

I. Introduction

This chapter examines LBI with respect to its inhibition of chymotrypsin. The chymotrypsin reactive peptide bond and its location in the primary structure of the inhibitor are determined by techniques similar to those already described in connection with the determination of the trypsin-reactive site. Peptide bond hydrolysis equilibria of the chymotrypsin reactive site are examined in a preliminary manner. Finally, since different variants of LBI are used in this study, the four variants of LBI (LBI I-IV) are compared with respect to their chymotrypsin inhibitory activity.

II. Determination of the Chymotrypsin Reactive Peptide Bond of Lima Bean Inhibitor

A. Experimental

1. Chymotrypsin Modification of LBI

a) Preparation A

In this experiment Fraction II, prepared by the gel filtration of commercial inhibitor (LBI 8KB) on Sephadex G-75, was used (See Figure 6). This preparation contains LBI variants I-IV. LBI (20 mg) was dissolved in 2.0 ml of 0.1M acetic acid resulting in a final pH of 3.5. At zero time and again at 4 hr, 100 µl of bovine chymotrypsin (10 mg/ml in 0.004M acetic acid-0.02M calcium chloride) were added and the mixture incubated at 37° for 24 hr. This solution was then adjusted to pH 8.5 with 25% trimethylamine and diluted to 4.8 ml with 0.1M ammonium bicarbonate. The resulting mixture, called

CMRM-LBI (Prep A), was then used either for carboxyl-terminal determination by treatment with carboxypeptidase A or for activity determination after the appropriate dilution. A control LBI was run in an identical fashion except for the omission of chymotrypsin.

b) Preparation B

LBI variant III (110 mg), prepared from lot LBI 8KB, was dissolved in 55 ml of 0.05M acetic acid-0.04M calcium chloride and the pH adjusted to pH 3.1 with glacial acetic acid. This solution was divided into a 50 ml (100 mg LBI) portion, used for chymotrypsin modification, and a 5 ml (10 mg LBI) portion, used as an identical scaled-down control. To the sample (50 ml, 100 mg LBI) were added 500 µl of chymotrypsin (50 mg/ml in 0.004M acetic acid-0.02M calcium chloride) at zero time and again at 4 hr. The resulting reaction mixture was incubated at 37° for 24 hr. The control was run in an identical manner except for the omission of chymotrypsin. 100 µl portions were then withdrawn from both the sample and control for activity determination after the appropriate dilution. After completion of the 24 hr incubation of both sample and control, the inhibitor was separated from the reagents by gel filtration on Sephadex G-75, followed by lyophilization. This chymotrypsin modification reaction mixture of LBI is called CMRM-LBI (Prep B).

2. Determination of the Inhibitory Activity of the Chymotrypsin Modification Reaction Mixture of LBI (CMRM-LBI)

CMRM-LBI (Preps A and B) was assayed for its trypsin

and chymotrypsin inhibitory activity as previously described. The chymotrypsin inhibitory activity of the inhibitors was determined after a zero time and a 15 min preincubation of inhibitor with chymotrypsin.

3. Carboxypeptidase A Treatment of the Chymotrypsin Modification Reaction Mixture of LBI (CMRM-LBI)

To CMRM-LBI (Prep A) were added 200 µl of carboxypeptidase A (4mg/ml) and the solution was incubated for 24 hr at 37°. At the appropriate time intervals, samples were withdrawn for the determination of the release of amino acids by paper chromatography or by automatic amino acid analysis. Control LBI (Prep A) was examined in identical fashion.

B. Results and Discussion

CMRM-LBI (Preps A and B) has full trypsin inhibitory activity. Prep A has only 30% activity against chymotrypsin and this does not increase during a 15 min preincubation assay. Paper chromatography of the carboxypeptidase A digest of CMRM-LBI (Prep A) indicated that either leucine or isoleucine (these amino acids are not separated in the chromatographic system) is released immediately. Amino acid analysis of the same digestion mixture after 24 hr showed that leucine is released in the largest quantity (0.93 mole/mole of CMRM-LBI) accompanied by the release of minor amounts of threonine (0.19), serine (0.12), tyrosine (0.30), and phenylalanine (0.32). The above data suggest that the CMRM-LBI consists of a mixture of native LBI and chymotrypsin-modified LBI (LBI'_c). LBI'_c, according to this hypothesis, should have a specific chymotrypsin reactive peptide bond

cleaved, in this case a Leu-X peptide bond, thus rendering the LBI'_c molecule less active than the native LBI. The data are consistent with this hypothesis since CMRM-LBI is 70% inactive and releases 0.93 moles leucine. The discrepancy between the amount of leucine released (0.93 moles/mole) and the per cent inactivation (70%) is probably due to errors in quantitation, since later data, obtained with LBI I (this chapter, section IV), show excellent agreement between these two values.

It is quite clear from these studies that LBI has a chymotrypsin reactive peptide bond, namely a leucyl-X peptide bond, which is particularly susceptible to chymotryptic cleavage at acid pH. Native LBI is a single polypeptide chain, whereas LBI'_c, with the chymotrypsin reactive site peptide bond cleaved, probably consists of two polypeptide chains linked by disulfide bonds.

The chymotrypsin inhibitory activity of CMRM-LBI (Prep B) was also found to be 30% of the control. This sample was used to determine the location of the chymotrypsin reactive site in the amino acid sequence of LBI.

III. Location of the Chymotrypsin Reactive Site in the Primary Structure of LBI

A. Experimental

1. Separation of the Components of the Reduced and Alkylated CMRM-LBI

CMRM-LBI (Prep B) and control LBI were reduced and carboxamidomethylated by the method of Hirs (1967) as described under General Experimental Procedures. After removal of the reagents by gel

filtration on Sephadex G-15, the reduced and carboxamidomethylated samples were subjected to gel filtration on Sephadex G-25 using 10% acetic acid as the eluant. The exact chromatographic conditions are given under Results.

2. Characterization of the Components of Reduced and Alkylated CMRM-LBI

The components, obtained by gel filtration of reduced and carboxamidomethylated CMRM-LBI, were characterized by amino acid composition and end-group analysis. For carboxyl-terminal sequence analysis, 0.5 µmoles of peptide were dissolved in 500 µl of 0.1M NH_4HCO_3 and 25 µl of carboxypeptidase A (4 mg/ml) were added. This solution was incubated at 37° and at the appropriate intervals 100 µl samples were withdrawn, quick-frozen in dry ice-ethanol and lyophilized prior to amino acid analysis. Amino-terminal sequence analysis was done by digestion with leucine aminopeptidase and aminopeptidase M or by the subtractive Edman degradation procedure; these techniques have been previously described under General Experimental Procedures.

B. Results and Discussion

Gel filtration of the reduced and carboxyamidomethylated CMRM-LBI on Sephadex G-15 yielded only a single symmetrical peak of protein at the exclusion volume of the gel indicating that if fragments are produced from LBI'_c by disulfide cleavage they have molecular weights greater than 1500 g/mole. Figure 21 shows the elution profile of the RCAM-mixture on Sephadex G-25. In this system, two ninhydrin positive peaks are well resolved; peak I appears at the

exclusion volume of the gel and must be a large fragment of LBI'_c whereas peak II is retarded by the gel and hence, must be a small fragment of LBI'_c, most probably a peptide of molecular weight less than 5,000 g/mole. In the same chromatographic system RCAM-LBI (control LBI) showed only a single symmetrical peak at the exclusion volume of the gel. Table XIII gives the amino acid compositions of the fragments obtained by gel filtration (Figure 21) as well as those of LBI and RCAM-LBI. Peak II (Figure 21), the small fragment, was found to represent the carboxyl-terminal portion of LBI on the basis of its amino acid composition and comparison with the known sequence of LBI (Tan and Stevens, 1971b). Similarly, peak I (Figure 21), the large fragment was recognized as the amino-terminal portion of LBI. The sum of the amino acid compositions of these two fragments accounts, within experimental error, for the complete LBI molecule. The large fragment elutes at the exclusion volume of the gel and thus should be contaminated by intact LBI (CMRM-LBI contains both LBI and LBI'_c) but the amino acid composition indicates no such contamination. This apparent absence of the intact LBI molecule is puzzling and cannot be explained at present.

Further evidence for the identification of the two fragments involves determination of the sequence around the chymotrypsin reactive site. The small fragment released 0.17 moles/mole of serine by leucine aminopeptidase digestion and 0.32 moles/mole serine by aminopeptidase M digestion. The results of the subtractive Edman degradation on the small fragment are shown in Table XIV. In the first cycle, both serine and S-carboxymethylcysteine are decreased;

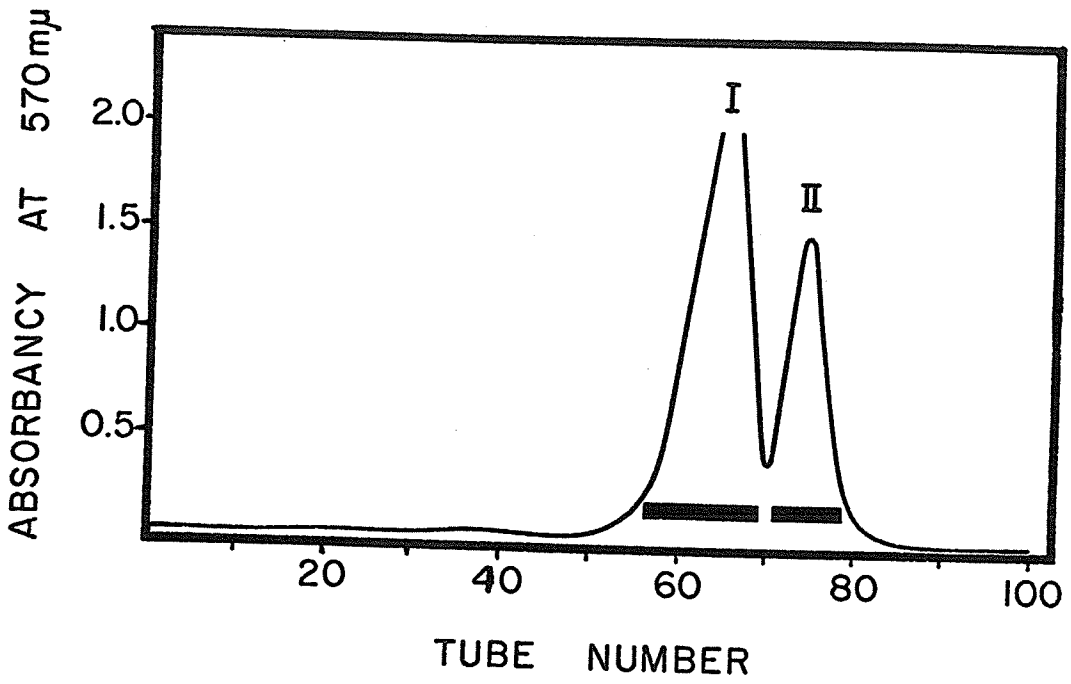

Figure 21 Gel filtration of reduced and carboxamidomethylated CMRM-LBI on Sephadex G-25.

The sample (4ml) was applied to a column (2.5 x 90 cm) of Sephadex G-25 and elution was carried out with 10% acetic acid. The flow rate was 20 ml/hr, 3.3 ml fractions were collected and 200 μl samples from alternate tubes were analyzed with the ninhydrin method after alkaline hydrolysis. Fractions were collected as indicated by the horizontal bars.

TABLE XIII

THE AMINO ACID COMPOSITION OF LBI, RCAM-LBI, AND FRAGMENTS OBTAINED BY GEL FILTRATION OF THE REDUCED AND CARBOXAMIDOMETHYLATED CHYMOTRYPSIN MODIFICATION REACTION MIXTURE OF LBI (CMRM-LBI)[a]

Amino Acid	Fragments			RCAM-LBI	LBI Variant III Best Values	
	Small	Large	Sum of Both		This Work	Lit.[b]
Lysine	0.99 (1)	4.13 (4)	5.12 (5)	4.72 (5)	4	4
Histidine	0.96 (1)	4.87 (5)	5.83 (6)	5.43 (5)	6	6
Arginine	0	2.24 (2)	2.24 (2)	2.20 (2)	2	2
Aspartic acid	8.41 (8)	6.30 (6)	14.71 (15)	15.18 (15)	14	13
Threonine	1.18 (1)	4.68 (5)	5.86 (6)	6.80 (7)	6	5
Serine	3.92 (4)	10.96 (11)	14.88 (15)	13.86 (14)	16	15
Glutamic acid	2.10 (2)	5.28 (5)	7.38 (7)	7.12 (7)	7	7
Proline	2.00 (2)	5.18 (5)	7.18 (7)	6.81 (7)	7	7
Glycine	0	0.91 (1)	0.91 (1)	1.00 (1)	1	1
Alanine	1.00 (1)	1.86 (2)	2.86 (3)	2.71 (3)	3	4
Cysteine	4.50 (4)[c]	14.31 (14)[c]	18.81 (19)	18.50 (19)[c]	16	16
Valine	0.92 (1)	0.14 (0)	1.06 (1)	1.11 (1)	1	1
Isoleucine	1.92 (2)	2.56 (3)	4.48 (4)	4.40 (4)	4	5
Leucine	0	4.02 (4)	4.02 (4)	3.69 (4)	4	5
Tyrosine	0.92 (1)	0.13 (0)	1.06 (1)	1.17 (1)	1	2
Phenylalanine	0.99 (1)	0.66 (1)	1.65 (2)	1.52 (2)	2	2
Total	29	69	98	97	94	93

[a]The analyses were performed as described in the text. Tryptophan and methionine are known to be absent from LBI (Jones et al., 1963). The results are expressed in moles per mole and are calculated on the basis of 2 residues of Arg, 1 residue of Gly, and 1 residue of Val in the case of columns 4 and 5; 1 residue of Ala in the case of column 1; 2 residues of Arg, 1 residue of Gly, and 2 residues of Ala in the case of column 2.

[b]The literature values are taken from Jones et al. (1963).

[c]These were determined as S-carboxymethylcysteine.

TABLE XIV

SUBTRACTIVE EDMAN DEGRADATION OF THE SMALL FRAGMENT
(PEAK II, FIGURE 21)

Amino Acid	Number of Cycles Moles Amino Acid/mole Peptide			
	0	1	2	3
Lysine[a]	0.99 (1)	(1)	(1)	(1)
Histidine[a]	0.96 (1)	(1)	(1)	(1)
S-Carboxymethylcysteine[b]..	4.50 (4)	3.05 (4)	2.90 (4)	2.76 (4)
Aspartic acid	8.41 (8)	8.50 (8)	8.30 (8)	8.30 (8)
Threonine	1.18 (1)	1.00 (1)	0.93 (1)	0.96 (1)
Serine	3.92 (4)	2.64 (3)	2.83 (3)	2.72 (3)
Glutamic acid.....	2.10 (2)	2.12 (2)	2.06 (2)	1.94 (2)
Proline	2.00 (2)	2.00 (2)	2.03 (2)	1.17 (1)
Alanine	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
Valine	0.92 (1)	0.94 (1)	0.63 (1)	0.63 (1)
Isoleucine	1.92 (2)	2.15 (2)	1.30 (1)	0.89 (1)
Tyrosine	0.92 (1)	0.76 (1)	0.30 (1)	lost (1)
Phenylalanine	0.99 (1)	1.00 (1)	0.97 (1)	0.89 (1)

[a]From the published amino acid sequence (Tan and Stevens, 1971b), it is known that Lys and His are located in the carboxyl-terminal half of this fragment. Therefore the amino acid hydrolysates after Edman Degradation were applied only to the long column.

[b]In the first cycle, the values for Ser and S-carboxymethylcysteine were both decreased by about one residue. The results of the experiments with leucine aminopeptidase and aminopeptidase M conclusively establish Ser as amino-terminal. During Edman degradation, difficulties were encountered in the determination of S-carboxymethylcysteine some of which appeared on the analysis as cysteine or cysteic acid.

however serine was indicated as amino-terminal by leucine amino-peptidase and aminopeptidase M digestion. In the second cycle isoleucine decreased by one residue followed in cycle three by proline. The amino-terminal sequence of the small fragment therefore starts with Ser-Ile-Pro. Since the large fragment was believed to be the amino-terminal portion of LBI, only its carboxyl-terminal sequence was examined. Figure 22 shows the time course of the release of amino acids from the large fragment by carboxypeptidase A. Leucine is released very rapidly followed by a slower release of threonine. Phenylalanine is also released rapidly and for this reason probably represents contamination produced by non-specific peptide bond cleavage. These data establish the carboxyl-terminal sequence of the large fragment as ending in -Thr-Leu.

On the basis of the above data, and the known sequence of LBI, the chymotrypsin reactive site of LBI is a Leu-Ser bond located 29 residues from the carboxyl-terminal end of the molecule. This leucyl-seryl peptide bond is located at positions 55 and 56 of the published amino acid sequence of LBI IV. The sequence surrounding this peptide bond is:

Thr-Leu-Ser-Ile-Pro

IV. Peptide Bond Hydrolysis Equilibria Involving the Chymotrypsin Reactive Site Peptide Bond

A. Experimental

1. Time Course Study on the Forward Reaction (LBI $\rightarrow$ LBI$'_t$)

The material used in this study was LBI variant I

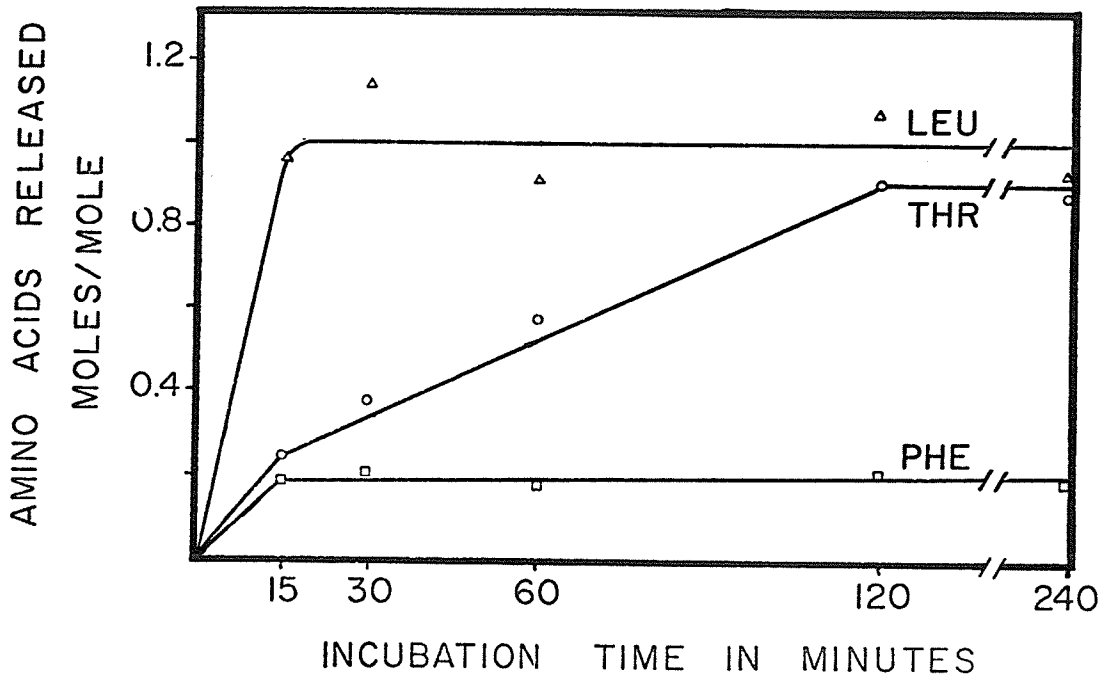

Figure 22 Time course release of free amino acids from the large fragment (peak I, Figure 21) by carboxypeptidase A.

The results are expressed as moles amino acids released per mole peptide. Details are given under Experimental. In a control experiment run under identical conditions but containing no peptide there was release of only negligible amounts of amino acids.

prepared from the commercial preparation, LBI 9JB. The inhibitor (25 mg) was dissolved in 2.5 ml of 0.018M trans-aconitate-0.04M calcium chloride buffer. 500 µl of this solution were set aside as control LBI while 25 µl of bovine α-chymotrypsin (20 mg/ml in the same buffer) were added to the remaining 2 ml. The solution was incubated at room temperature and at the appropriate time intervals 100 µl aliquots were withdrawn, quick-frozen in dry ice-ethanol, and stored at -20° until assayed. The samples were diluted and their chymotrypsin inhibitory activity directly determined. The incubation was terminated after 55 hr and the reaction mixture frozen. A portion of this CMRM-LBI had the reagents removed on Sephadex G-75 prior to carboxypeptidase A digestion whereas the remainder of the CMRM-LBI was used directly in studies on the kinetics of complex formation of CMRM-LBI with chymotrypsin.

2. Characterization of the CMRM-LBI Equilibrium Mixture

The CMRM-LBI equilibrium mixture obtained above was characterized by the release of amino acids by carboxypeptidase A digestion and also by its inhibitory activity against chymotrypsin upon preincubation with near molar amounts of chymotrypsin.

CMRM-LBI (0.055 µmoles) was dissolved in 100 µl of 0.1M ammonium bicarbonate and 30 µl of carboxypeptidase A (7.68 mg/ml) were added. This solution was incubated overnight at 37°. A control was run in identical fashion but omitting CMRM-LBI.

To determine whether the LBI'_c in CMRM-LBI was active, equimolar amounts of CMRM-LBI and chymotrypsin were incubated in 0.006M Tris-HCl,

pH 8.2. At various times aliquots were withdrawn and assayed for residual chymotrypsin activity. Solutions containing the same amount of chymotrypsin but containing native LBI or no LBI served as control experiments.

B. Results and Discussion

The experiment illustrated in Figure 23 shows that the conversion of LBI → LBI'_c, as monitored by decrease in chymotrypsin inhibitory activity, reaches a steady state plateau at 6 hr. At this point LBI has been 92% inactivated by chymotrypsin. By allowing the incubation to proceed for up to 55 hr the percent inactivation remains unchanged at 92%. Experiments performed under identical conditions but using four times as much chymotrypsin show that a steady state plateau of 90% LBI'_c and 10% LBI has already been reached after 2 hr. These results very strongly suggest that equilibrium concentrations of LBI'_c and LBI in the CMRM-LBI have been attained. It was found that carboxypeptidase A released 0.93 moles/mole of leucine from CMRM-LBI that was 92% inactivated thus clearly indicating the presence of 92-93% LBI'_c and 7-8% LBI.

Upon preincubation of a solution containing near equimolar amounts of chymotrypsin and CMRM-LBI at pH 8.2 for up to 48 hr prior to addition of substrate-indicator solution, no change in the chymotrypsin inhibitory activity of the preincubation solution was noted. Clearly then, LBI'_c variant I has no activity towards chymotrypsin (does not interact appreciably with chymotrypsin) and thus studies of the effect of removal of the chymotrypsin reactive site

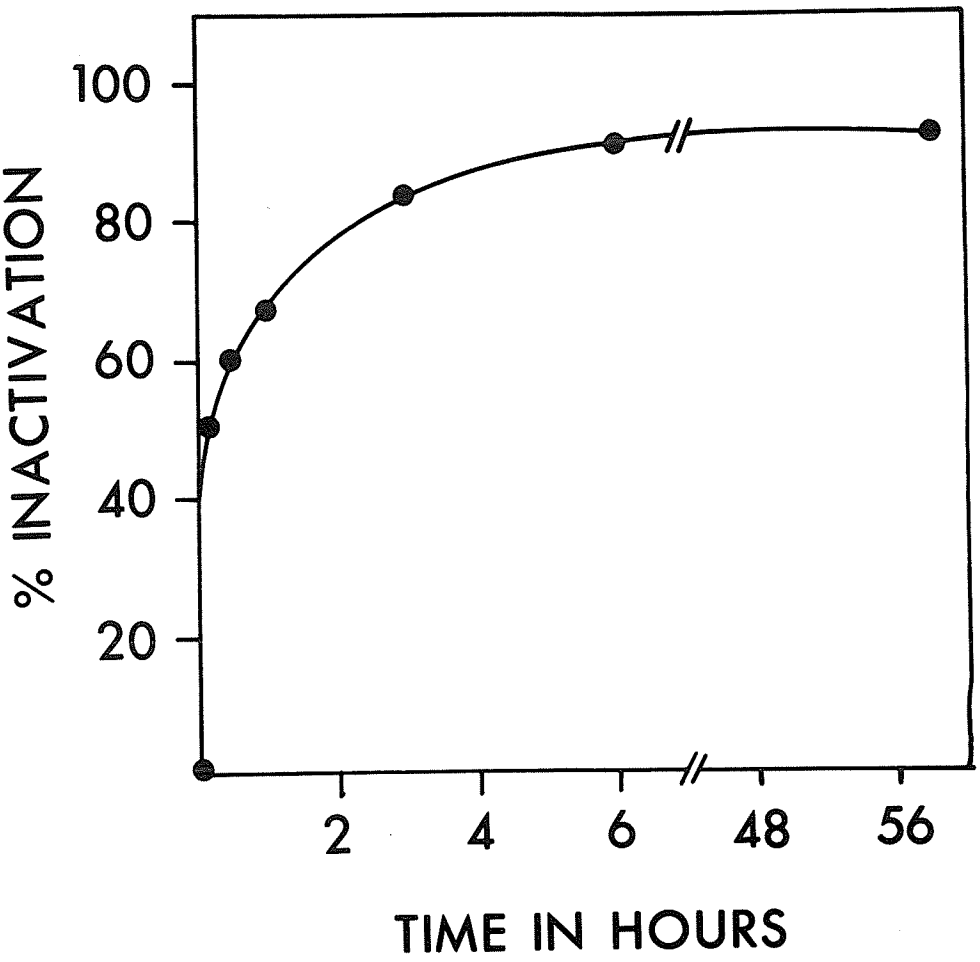

Figure 23 Chymotrypsin modification of LBI as a function of time (LBI $\rightarrow$ LBI$'_c$).

The % inactivation of LBI was determined by measuring the chymotrypsin inhibitory activity in a zero time preincubation assay.

peptide bond leucine residue of LBI'_c would be useless.[13] To illustrate the importance of the reactive site leucyl residue, an LBI'_c which regains activity in the presence of chymotrypsin at neutral pH must be obtained. Removal of the reactive site leucyl residue in such an LBI'_c by carboxypeptidase A should inactivate the inhibitor. With the view of finding such an LBI'_c, a comparative study on LBI variants I-IV was undertaken.

V. Comparative Studies on the Four Variants of Lima Bean Inhibitor: Chymotrypsin Inhibitory Activity

A. Experimental

The four variants of lima bean inhibitor (LBI I-IV) used in these experiments were prepared from the commercial preparation, LBI 1DA, by the method of Jones et al., (1963).

1. Chymotrypsin Modification of LBI I-IV

Each of the four variants of LBI was dissolved in 0.018M trans-aconitate-0.04M calcium chloride to a final concentration of approximately 10 mg/ml to give a final pH of 3.1. To 500 µl aliquots of the above solutions were added 15 µl of bovine chymotrypsin (20 mg/ml in the same buffer) and the solution was incubated for 14 hr at 25° to produce a chymotrypsin modification reaction mixture of LBI (CMRM-LBI). 500 µl aliquots of the LBI I-IV solutions were carried through

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[13] This conclusion is warranted only for LBI variant I obtained from the commercial preparation, LBI 9JB, since experiments on other LBI variants from different commercial preparations had not been studied at this time.

as controls in that they were treated in identical fashion except for the omission of chymotrypsin.

2. Determination of the Chymotrypin Inhibitory Activity of LBI and CMRM-LBI

To determine the chymotrypsin inhibitory activity of LBI (control) and of CMRM-LBI, 100 µl aliquots of each were diluted to 5.0 ml with 0.006M Tris-HCl, pH 8.2, and the solutions were assayed, without preincubation, for their chymotrypsin inhibitory activity. The extent of modification (the amount of LBI'_c present in CMRM-LBI) was calculated by comparing the activity of CMRM-LBI with control LBI as previously described under General Experimental Procedures.

3. Kinetics of Complex Formation Between Chymotrypsin and LBI or CMRM-LBI (Variants I-IV)

The chymotrypsin inhibitory activities of the control LBI and CMRM-LBI were determined as a function of preincubation time with near molar amounts of bovine chymotrypsin in 0.006M Tris-HCl, pH 8.2, at room temperature. Details are given under Results.

B. Results and Discussion

Figure 24 shows that there are differences in the chymotrypsin inhibitory activity of LBI I-IV. A good inhibitor with a large association constant should decrease the enzyme's activity in an amount proportional to the amount of inhibitor added till the equivalence point is attained. This is true only for LBI IV (Figure 24). LBI III does not produce inhibition in proportion to the amount of inhibitor added and neither do LBI II and LBI I. In the case of

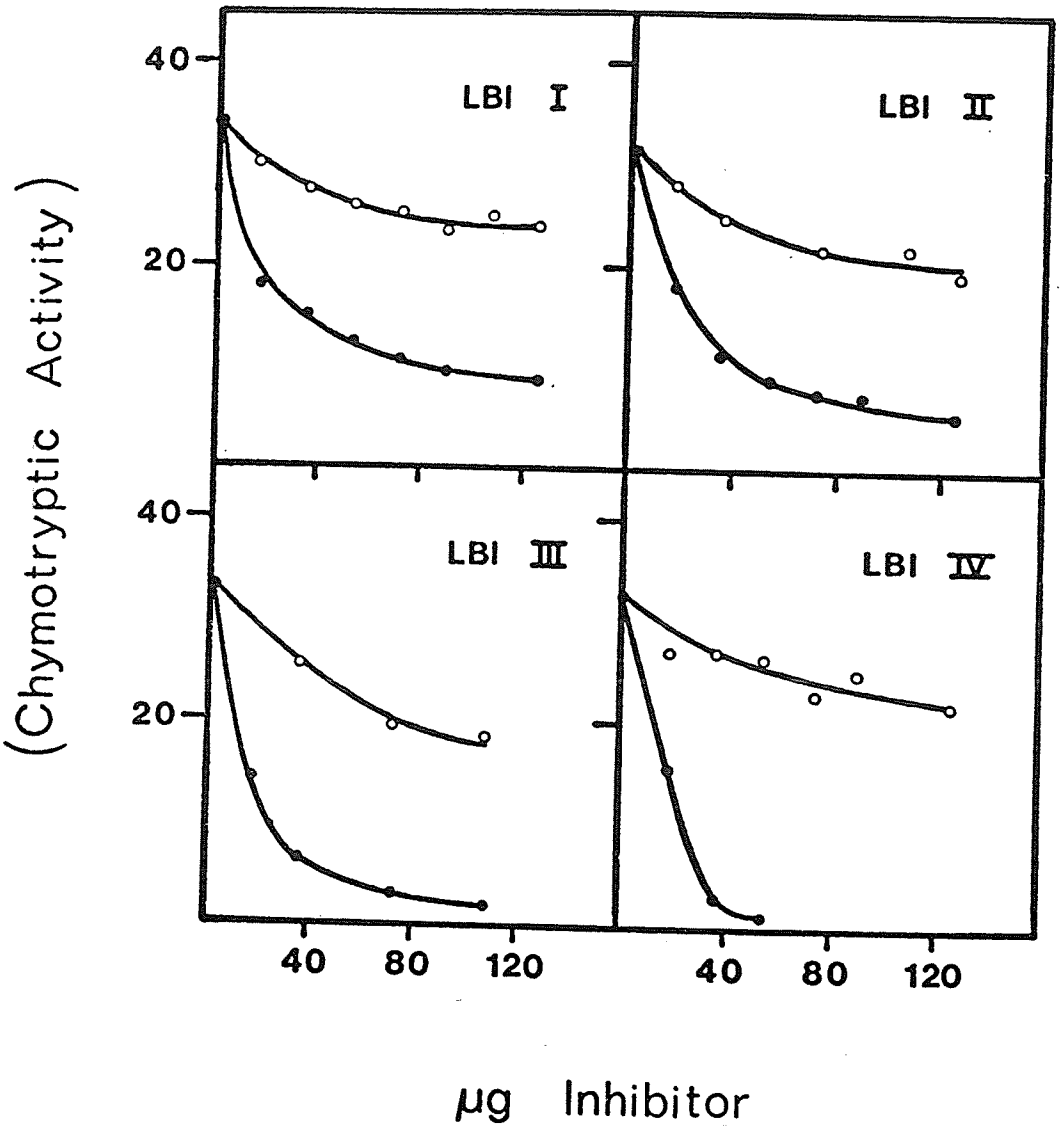

Figure 24 The chymotrypsin inhibitory activities of four variants of native LBI and CMRM-LBI.

Increasing amounts of inhibitor were added to 45 µg of bovine chymotrypsin and the assays carried out without prior preincubation of enzyme and inhibitor. (o—o—o), CMRM-LBI containing LBI and LBI'_c; (●—●—●), native LBI.

LBI I even large excesses of inhibitor produce only 60-70% inhibition. By drawing a tangent line to the inhibition curve and extrapolating this line back to zero enzyme activity the equivalence point of inhibitor to enzyme is obtained. If this is done for LBI I-IV, the equivalence points for the four variants are virtually identical. This indicates that the four variants, present in equal amounts, have the same stoichiometry of interaction with chymotrypsin but that their affinities for chymotrypsin are different. An attractive explanation of the inhibition curves would thus be that LBI I has a low affinity for chymotrypsin and that the affinity of the inhibitor for chymotrypsin progressively increases through LBI II-IV. Figure 24 also shows that the CMRM-LBIs are all much less active than the control LBI. The degree of inactivation produced in CMRM-LBI (a measure of the LBI'_c present) was estimated in all cases to be approximately 90%. In all likelihood these values represent the equilibrium position.

Figure 25 shows the kinetics of complex formation between chymotrypsin and either control LBI or CMRM-LBI. Over the 5 hr preincubation period the activity of the chymotrypsin solution decreases only slightly. All the control samples have attained full activity by the time of the first measurement at zero time preincubation and their activity stays relatively constant during the course of the experiment.

The activity of CMRM-LBI as a function of preincubation time with chymotrypsin at pH 8.2 varies from LBI I-LBI IV. CMRM-LBI (LBI I) is less active than the control (native) and remains so

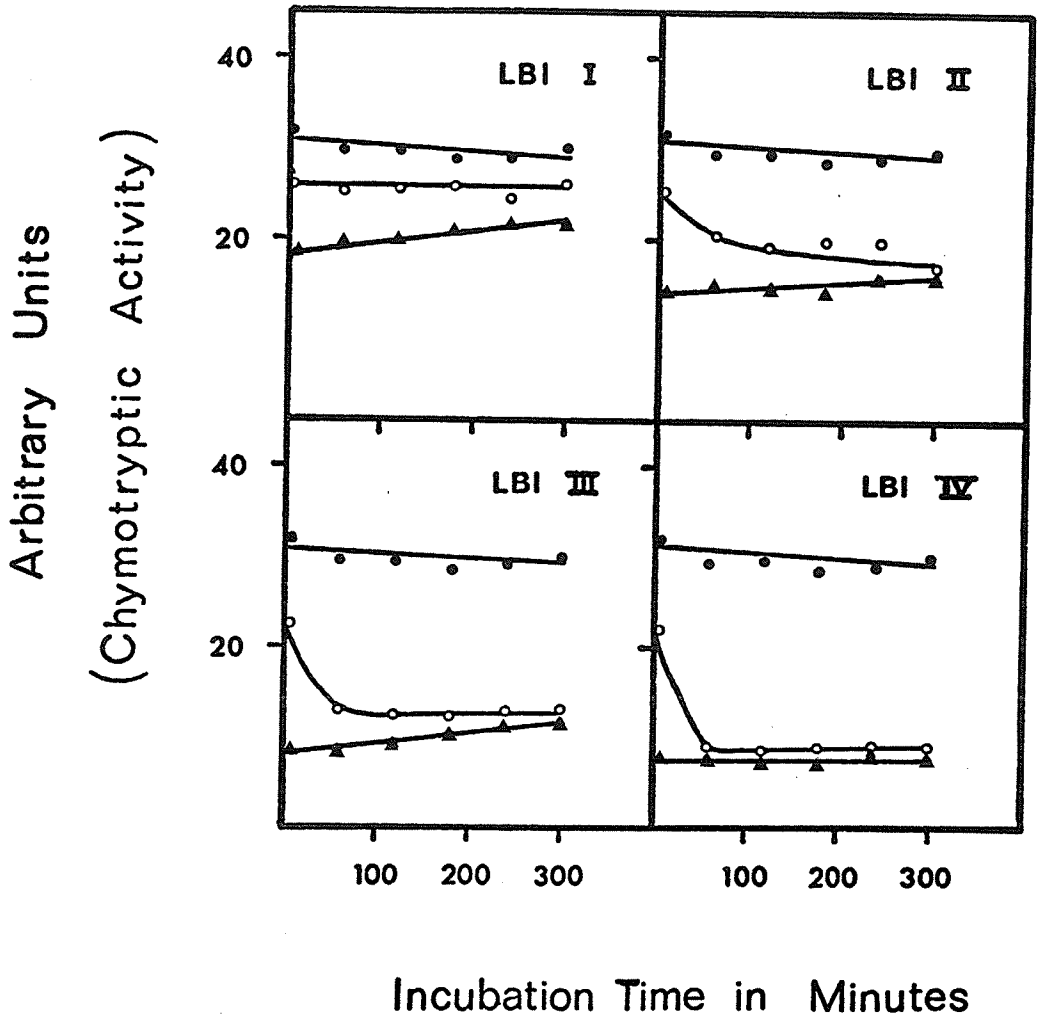

Figure 25 Time course study of the chymotrypsin inhibitory capacity of native LBI and CMRM-LBI, variants I-IV.

The chymotrypsin-inhibitor mixtures were incubated in the assay medium for varying amounts of time prior to addition of the substrate-indicator solution. (●—●—●); 45 µg of chymotrypsin alone; (○—○—○), 45 µg of chymotrypsin and 27 µg of CMRM-LBI; (▲—▲—▲), 45 µg of chymotrypsin and 27 µg of native LBI.

throughout the experiment. CMRM-LBI (LBI II), on the other hand, slowly regains activity and after 5 hr its activity is comparable to that of control LBI II. CMRM-LBI (LBI III) regains its activity faster than II and attains full activity after 2-3 hr whereas CMRM-LBI (LBI IV) is fully active after 1 hr. The time required for full recovery of chymotrypsin inhibitory activity is a measure of the time required for complex formation and thus a measure of the affinity of LBI'_c for chymotrypsin. Thus it is clear that a correlation exists between the affinity of an LBI variant for chymotrypsin and the ability of the LBI'_c to regain its activity upon incubation with chymotrypsin at neutral pH. The chymotrypsin-modified LBI (LBI'_c) of LBI I (a weak inhibitor of chymotrypsin) shows no ability to regain its chymotrypsin inhibitory activity whereas LBI'_c of LBI IV (a strong inhibitor of chymotrypsin) rapidly regains full activity. LBI II and III are intermediate to LBI I and IV both in their strength of inhibition and the ability of their LBI'_c forms to regain their activity in the presence of equimolar chymotrypsin at pH 8.2.

GENERAL DISCUSSION

Although previous investigators have shown that LBI is a double-headed inhibitor capable of inhibiting trypsin and chymotrypsin simultaneously and independently, the stoichiometry of these interactions was not well established. From inhibition studies, Haynes and Feeney (1967) estimated that LBI interacted with trypsin on a 1:1 molar basis but interacted with chymotrypsin on approximately a 2:1 molar basis i.e. two moles of LBI were required to inhibit one mole of chymotrypsin. By characterization of the isolated LBI-enzyme complexes, this investigation has clearly demonstrated that LBI interacts with both enzymes on a 1:1 molar basis. This is illustrated in Figure 26 which shows that trypsin and LBI interact on a 1:1 molar basis to form a LBI-T complex. This complex can now further react with a mole of chymotrypsin to form the ternary complex, LBI-T-CT. By changing the order of addition of the enzymes, the LBI-CT complex is formed first, followed by formation of the ternary complex. After publication of these results (Krahn and Stevens, 1971), it was shown by Seidl and Liener (1972a) that Bowman-Birk inhibitor behaved like LBI with respect to the stoichiometry of interaction with trypsin and chymotrypsin. The data of Haynes and Feeney (1967) indicating that two moles of LBI inhibit one mole of chymotrypsin are derived from inhibition studies and as such need clarification. Such studies measure enzyme activity and thus if the enzyme-inhibitor complex dissociates easily, an excess of inhibitor is needed to completely inhibit the enzyme. Although the dissociation constant for the LBI-chymotrypsin complex has not been determined, inhibition curves of chymotrypsin by LBI indicate that most LBI variants

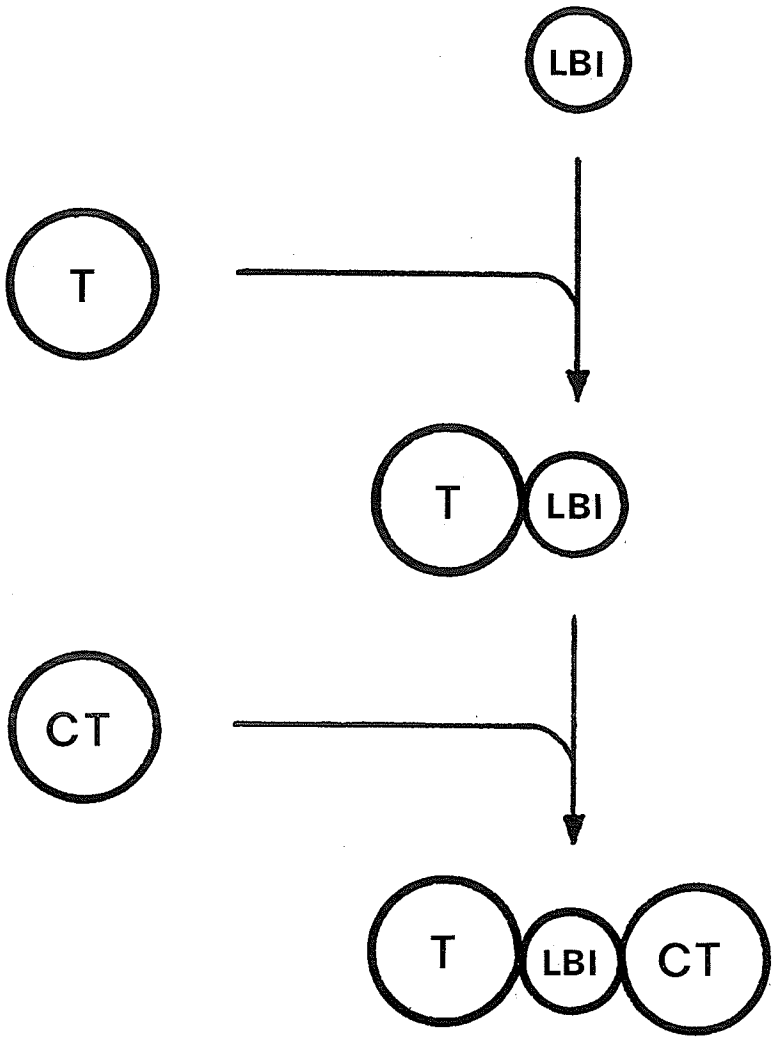

Figure 26 Interaction of lima bean inhibitor (LBI) with trypsin (T) and chymotrypsin (CT).

interact with chymotrypsin only weakly thereby explaining the results of Haynes and Feeney (1967).

The trypsin reactive site of lima bean inhibitor was found to be the Lys^{28}-Ser^{29} peptide bond while the chymotrypsin reactive site was found to be the Leu^{55}-Ser^{56} peptide bond. Examination of the sequences around these reactive sites has revealed that they are homologous. In Figure 27 it can be seen that the sequences from position 23 to 34 and 50 to 61 are homologous. In these sequences 9

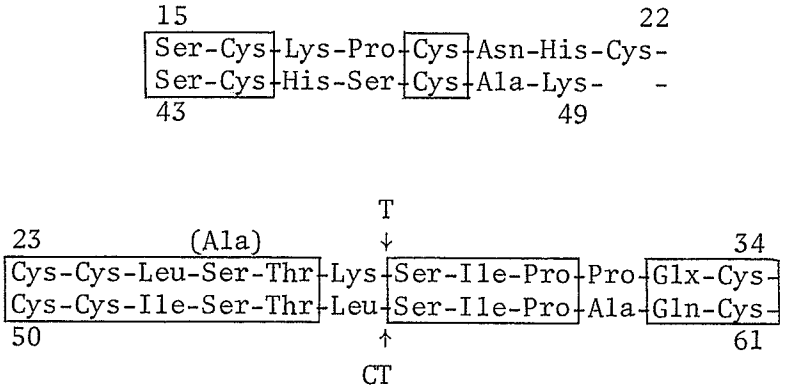

Figure 27 The homologous regions of lima bean inhibitor (LBI IV). The solid lines enclose identical sequences. T and CT indicate the trypsin and chymotrypsin reactive sites of LBI.

out of 12 positions are identical; there is a conservative replacement of isoleucine for leucine and two other replacements involve alanine-proline and leucine-lysine substitutions. Assuming only one deletion, these homologous sequences can be extended further to include residues 15 to 22 on one hand and 42 to 49 on the other hand. Since the cysteine residues of these two regions are homologous it is tempting to speculate that one is dealing with two homologous disulfide loops.

Of greatest interest is the presence of the reactive sites in these homologous regions of LBI. The chymotrypsin reactive site of LBI is the Leu^{55}-Ser^{56} peptide bond whereas the trypsin reactive site is the Lys^{28}-Ser^{29} peptide bond. It may well be that this double-headed inhibitor arose by a process of gene duplication, followed by divergent evolution, resulting in substitution of a leucine residue for a lysine residue thereby changing the specificity of the reactive site. It thus appears that the structural requirements for the trypsin and the chymotrypsin reactive site regions are very similar. The main exception is the carboxyl-terminal residue of the reactive site whose side chain is believed to fit the enzyme's specificity pocket; this residue is leucine in the case of the chymotrypsin reactive site and lysine in the case of the trypsin reactive site, in accordance with the known specificities of these enzymes. Recent crystal structural analysis of bovine basic pancreatic trypsin inhibitor (Huber et al., 1970, 1971) showed it to be a pear-shaped molecule with the trypsin reactive site exposed at the apex. It is likely that LBI, a double-headed inhibitor, has two such exposed sites: one containing the Lys-Ser peptide bond specific for trypsin, the other containing the Leu-Ser peptide bond specific for chymotrypsin. The trypsin and chymotrypsin reactive sites of the Bowman-Birk inhibitor (BBI) have been determined by Seidl and Liener (1971b, 1972b) and by Odani and Ikenaka (1972). The trypsin reactive site of BBI is the Lys^{16}-Ser^{17} peptide bond whereas the chymotrypsin reactive site peptide bond is the Leu^{43}-Ser^{44} peptide bond. As expected, these sites occupy the same relative positions as do the proteinase reactive sites of LBI and occur in two homologous regions of BBI which are identical in 9 out of 17 positions (See Figure 1).

Because the "reactive site model" of protein proteinase inhibitors has been proposed as general model and is based largely on the Kunitz soybean trypsin inhibitor it was deemed necessary to extend proof of this model to LBI which is very different from Kunitz soybean trypsin inhibitor in its physical and chemical properties. The results of this investigation show that lima bean inhibitor, at least as far as its trypsin inhibitory activity is concerned, conforms to this "reactive site model". These results are summarized in Figure 28 and can be listed in the following way:

1. The trypsin reactive site is a trypsin-susceptible bond namely the Lys^{28}-Ser^{29} peptide bond which can be cleaved by trypsin at acid pH. This agrees with the classification of LBI as a "lysine inhibitor" by chemical modification.
2. The Lys^{28}-Ser^{29} peptide bond is involved in hydrolysis equilibria with a low K_{hyd} at acid pH. A low K_{hyd} seems to be characteristic of all inhibitor reactive sites. At pH 3.1, the equilibrium mixture contains 90% LBI'_t (bond cleaved) and 10% LBI (bond intact).
3. Both LBI and LBI'_t have equal inhibitory capacity against trypsin although, at neutral pH, LBI'_t forms a complex with trypsin at a much slower rate than does LBI.
4. Removal of the reactive site lysyl residue of LBI'_t with carboxypeptidase B renders the product completely inactive.
5. The trypsin-inhibitor complex made from either native LBI or trypsin-modified inhibitor (LBI'_t) yields predominantly virgin inhibitor upon rapid dissociation. The reactive site

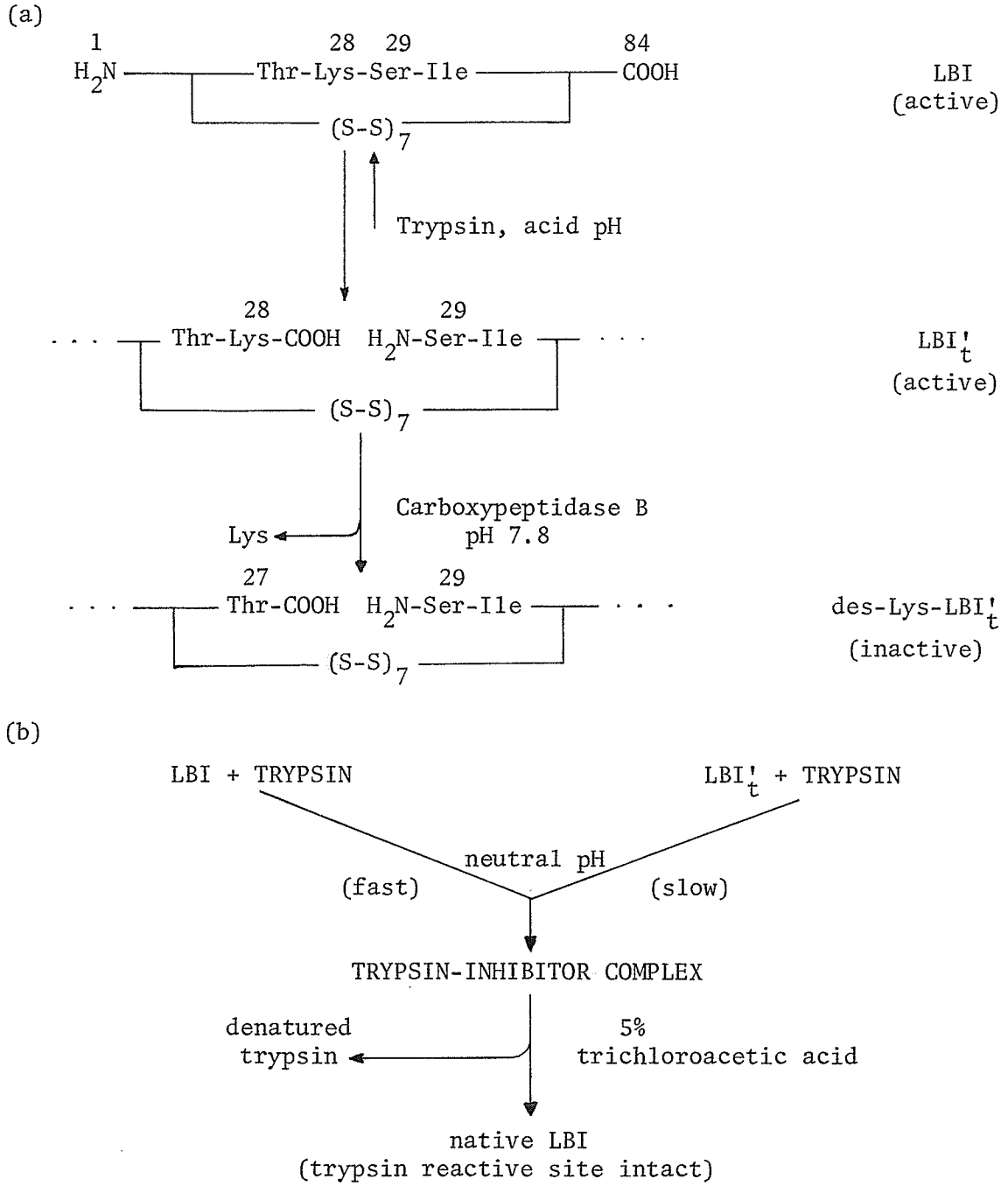

Figure 28 Reactions of lima bean inhibitor and trypsin.

(a) The trypsin reactive site of LBI and its peptide bond hydrolysis equilibria.

(b) Kinetic control dissociation.

peptide bond can be resynthesized in this manner.

These data very clearly show that the Lys^{28}-Ser^{29} reactive site of lima bean inhibitor is very important for trypsin inhibition. Furthermore, the fact that the reactive site peptide bond can be resynthesized by either mass action ($LBI_t^! \rightarrow LBI$) or by kinetic control dissociation is very good evidence for the existence of a close contact between the trypsin reactive site of LBI and the active site of trypsin; this could indicate the possible existence of a covalent enzyme-inhibitor intermediate somewhere in the reaction scheme. Thus, although the evidence shows LBI to conform to the reactive site model for proteinase inhibitors, it cannot indicate whether the stable complex is a covalent intermediate as has been suggested by Laskowski and Sealock (1971).

The chymotrypsin reactive site of LBI has been identified as the Leu^{55}-Ser^{56} peptide bond and although this reactive site has not been studied in great detail, all evidence points to the likelihood that it also conforms to the reactive site model for protein proteinase inhibitors.

Because Bowman-Birk inhibitor and lima bean inhibitor are homologous proteins with identical trypsin and chymotrypsin reactive sites, it can probably be assumed that the reactive site model for protein proteinase inhibitors also holds true for Bowman-Birk inhibitor. Frattali and Steiner (1969) have done some preliminary studies on Bowman-Birk inhibitor which suggest this to be true.

It is interesting to note that, although LBI I-IV have very similar amino acid sequences (Stevens, 1971) and trypsin inhibitory

activities, their chymotrypsin inhibitory activity varies greatly. Future studies on lima bean inhibitor could possibly focus on correlating differences in chymotrypsin inhibitory activity to actual differences in primary structure.

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APPENDIX

The Amino Acid Compositions of Four Variants Obtained From Different Commercial Preparations of Lima Bean Inhibitor

All commercial preparations of LBI were purchased from Worthington Biochemical Corporation. The inhibitor variants were prepared by the method described by Jones et al. (1963). They found all to be devoid of methionine and tryptophan.

The results in most cases are based on a single amino acid analysis of a 22 hr hydrolysate of the variant in 6N HCl. For lot 7HA the results represent averages of duplicate analyses and ½ cystine was determined as S-carboxymethylcysteine. In the case of variants obtained from both preparations 7HA and OIA the values given for Ser, Thr, and Tyr were obtained by extrapolation to zero time of values obtained for different hydrolysis times.

TABLE XV

THE AMINO ACID COMPOSITION OF LBI VARIANT I

Amino Acid	7HA	9CA	9JB	0IA	1DA	2IA	Jones et al. (1963)
Lysine	4.87 (5)	4.13 (4)	4.00 (4)	3.70 (4)	4.12 (4)	4.66 (5)	4.12 (4)
Histidine	5.60 (6)	2.96 (3)	2.88 (3)	2.59 (3)	4.58 (5)	5.91 (6)	5.12 (5)
Arginine	1.67 (2)	2.00 (2)	1.93 (2)	1.70 (2)	1.65 (2)	2.17 (2)	1.88 (2)
Aspartic acid	14.30 (14)	11.71 (12)	12.88 (13)	9.56 (10)	10.53 (11)	13.88 (14)	12.10 (12)
Threonine	5.00 (5)	2.81 (3)	3.04 (3)	3.20 (3)	3.47 (3)	5.06 (5)	4.40 (4)
Serine	12.20 (12)	8.75 (9)	10.07 (10)	10.84 (11)	9.00 (9)	12.69 (13)	11.70 (12)
Glutamic acid	6.00 (6)	4.04 (4)	5.11 (5)	4.49 (4)	5.47 (5)	6.69 (7)	5.94 (6)
Proline	5.55 (6)	4.88 (5)	5.55 (6)	5.17 (5)	4.88 (5)	6.18 (6)	5.75 (6)
Glycine	1.00 (1)	0.15 (0)	0.32 (0)	0.26 (0)	1.35 (1)	1.02 (1)	0.92 (1)
Alanine	3.45 (3)	2.88 (3)	3.19 (3)	2.82 (3)	2.88 (3)	2.64 (3)	3.05 (3)
1/2 Cystine	14.40 (14)	12.20 (12)	15.33 (15)	12.25 (12)	9.18 (9)	12.79 (13)	12.20 (12)
Valine	1.00 (1)	1.00 (1)	1.26 (1)	1.00 (1)	1.00 (1)	1.00 (1)	0.95 (1)
Isoleucine	3.87 (4)	3.79 (4)	3.70 (4)	3.87 (4)	3.85 (4)	4.26 (4)	3.83 (4)
Leucine	2.95 (3)	2.96 (3)	3.19 (3)	2.71 (3)	3.15 (3)	3.05 (3)	2.88 (3)
Tyrosine	0.80 (1)	0.88 (1)	0.51 (1)	0.85 (1)	0.47 (0)	1.58 (2)	0.93 (1)
Phenylalanine	1.02 (1)	1.04 (1)	0.81 (1)	0.97 (1)	0.82 (1)	2.04 (2)	1.02 (1)

TABLE XVI

THE AMINO ACID COMPOSITION OF LBI VARIANT II

Amino Acid	7HA	8KB	9CA	9JB	0IA	1DA	2LA	Jones et al. (1963)
Lysine	4.30 (4)	4.18 (4)	4.00 (4)	4.20 (4)	3.93 (4)	3.95 (4)	3.99 (4)	4.06 (4)
Histidine	5.85 (6)	3.52 (4)	2.89 (3)	4.60 (5)	2.94 (3)	5.10 (5)	5.78 (5)	2.97 (3)
Arginine	1.85 (2)	2.00 (2)	2.14 (2)	2.00 (2)	1.90 (2)	1.85 (2)	1.93 (2)	1.97 (2)
Aspartic acid	15.60 (16)	13.21 (13)	13.21 (13)	14.20 (14)	10.88 (11)	11.30 (11)	13.87 (14)	13.70 (14)
Threonine	5.75 (6)	3.18 (3)	3.18 (3)	3.70 (4)	3.45 (3)	3.80 (4)	4.06 (4)	3.30 (4)
Serine	13.00 (13)	10.24 (10)	10.00 (10)	10.10 (10)	11.80 (12)	9.40 (9)	12.17 (12)	11.50 (12)
Glutamic acid	6.10 (6)	5.15 (5)	4.57 (5)	6.00 (6)	4.87 (5)	5.80 (6)	6.83 (7)	5.29 (5)
Proline	5.92 (6)	5.94 (6)	5.29 (5)	6.00 (6)	5.49 (5)	5.40 (5)	6.05 (6)	5.97 (6)
Glycine	1.00 (1)	0.15 (0)	0.21 (0)	0.70 (1)	0.18 (0)	1.20 (1)	1.01 (1)	0.02 (0)
Alanine	3.06 (3)	3.03 (3)	3.00 (3)	3.20 (3)	2.92 (3)	3.00 (3)	3.01 (3)	3.15 (3)
1/2 Cystine	14.00 (14)	13.61 (14)	13.07 (13)	14.80 (15)	12.28 (12)	12.75 (13)	11.67 (12)	14.00 (14)
Valine	1.06 (1)	0.94 (1)	1.00 (1)	1.20 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
Isoleucine	4.31 (4)	3.70 (4)	3.93 (4)	4.20 (4)	3.93 (4)	3.50 (4)	3.82 (4)	3.92 (4)
Leucine	3.49 (3)	2.97 (3)	3.14 (3)	3.20 (3)	2.87 (3)	3.90 (4)	3.12 (3)	3.03 (3)
Tyrosine	0.90 (1)	0.94 (1)	0.93 (1)	0.51 (1)	1.04 (1)	1.10 (1)	0.97 (1)	0.90 (1)
Phenylalanine	1.10 (1)	1.03 (1)	1.11 (1)	1.00 (1)	1.10 (1)	1.10 (1)	1.05 (1)	1.01 (1)

TABLE XVII

THE AMINO ACID COMPOSITION OF LBI VARIANT III

Amino Acid	7HA	8KB	9CA	9JB	0IA	1DA	2IA	Jones et al. (1963)
Lysine	4.00 (4)	4.39 (4)	4.11 (4)	3.79 (4)	3.77 (4)	3.63 (4)	4.89 (5)	4.26 (4)
Histidine	5.79 (6)	6.11 (6)	4.57 (5)	2.92 (3)	3.46 (3)	5.07 (5)	5.49 (5)	5.93 (6)
Arginine	1.86 (2)	2.18 (2)	1.98 (2)	2.00 (2)	1.91 (2)	1.81 (2)	2.07 (2)	2.10 (2)
Aspartic acid	12.50 (13)	12.82 (13)	12.77 (13)	12.52 (13)	12.15 (12)	10.37 (10)	14.07 (14)	13.10 (13)
Threonine	5.56 (6)	4.89 (5)	4.48 (4)	4.16 (4)	4.00 (4)	4.22 (4)	4.45 (4)	5.00 (5)
Serine	11.25 (11)	12.89 (13)	12.89 (13)	11.25 (11)	14.66 (15)	9.96 (10)	12.09 (12)	14.50 (15)
Glutamic acid	5.66 (6)	6.69 (7)	5.77 (6)	5.63 (6)	5.92 (6)	5.93 (6)	6.22 (6)	7.10 (7)
Proline	5.55 (6)	6.36 (6)	6.11 (6)	6.37 (6)	5.70 (6)	5.45 (5)	7.60 (8)	6.99 (7)
Glycine	1.00 (1)	0.89 (1)	0.68 (1)	0.25 (0)	0.25 (0)	1.03 (1)	0.97 (1)	0.92 (1)
Alanine	2.17 (2)	3.21 (3)	2.39 (2)	2.58 (3)	2.73 (3)	2.93 (3)	3.07 (3)	3.68 (4)
1/2 Cystine	16.00 (16)	14.32 (14)	13.48 (14)	12.61 (13)	12.82 (13)	12.29 (12)	12.37 (12)	15.90 (16)
Valine	0.79 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.05 (1)
Isoleucine	3.52 (4)	4.00 (4)	3.89 (4)	4.04 (4)	3.69 (4)	2.88 (3)	4.15 (4)	4.64 (5)
Leucine	3.61 (4)	2.57 (3)	3.18 (3)	3.33 (3)	2.33 (2)	2.70 (3)	2.99 (3)	3.00 (3)
Tyrosine	0.83 (1)	1.64 (2)	0.91 (1)	0.75 (1)	1.06 (1)	0.96 (1)	1.05 (1)	1.85 (2)
Phenylalanine	1.17 (1)	1.68 (2)	1.41 (1)	1.58 (2)	1.85 (2)	1.52 (2)	1.10 (1)	1.84 (2)

TABLE XVIII

THE AMINO ACID COMPOSITION OF LBI VARIANT IV

Amino Acid	7HA	8KB	9CA	9JB A	9JB B	0IA	1DA	2IA	Jones et al. (1963)
Lysine	3.90 (4)	3.45 (3)	3.71 (4)	4.10 (4)	4.00 (4)	3.73 (4)	3.87 (4)	3.76 (4)	3.82 (4)
Histidine	5.65 (6)	6.00 (6)	5.76 (6)	3.84 (4)	6.92 (7)	4.62 (5)	6.05 (6)	5.92 (6)	6.00 (6)
Arginine	1.86 (2)	2.05 (2)	2.03 (2)	2.25 (2)	2.16 (2)	2.07 (2)	2.21 (2)	2.36 (2)	2.04 (2)
Aspartic acid	13.00 (13)	13.05 (13)	13.08 (13)	13.50 (14)	14.10 (14)	13.08 (13)	9.59 (10)	13.13 (13)	13.30 (13)
Threonine	5.32 (5)	4.45 (4)	4.82 (5)	4.36 (4)	4.75 (5)	5.00 (5)	4.04 (4)	4.70 (5)	5.00 (5)
Serine	13.75 (14)	12.10 (12)	12.68 (13)	11.85 (12)	12.10 (12)	13.30 (13)	9.46 (9)	12.57 (13)	12.70 (13)
Glutamic acid	6.89 (7)	7.65 (8)	7.61 (8)	6.20 (6)	7.42 (7)	6.39 (6)	5.33 (5)	6.29 (6)	6.99 (7)
Proline	6.02 (6)	6.80 (7)	6.18 (6)	6.65 (7)	7.25 (7)	6.05 (6)	4.82 (5)	6.08 (6)	7.05 (7)
Glycine	1.00 (1)	1.00 (1)	1.00 (1)	0.34 (0)	1.33 (1)	0.57 (1)	1.24 (1)	1.07 (1)	1.01 (1)
Alanine	2.30 (2)	3.20 (3)	2.55 (3)	2.62 (3)	3.67 (4)	2.81 (3)	2.63 (3)	2.44 (2)	2.64 (3)
1/2 Cystine	14.20 (14)	14.00 (14)	12.95 (13)	14.05 (14)	13.25 (13)	13.92 (14)	———	13.24 (13)	14.30 (14)
Valine	0.54 (1)	1.05 (1)	1.00 (1)	1.03 (1)	1.50 (2)	1.00 (1)	1.00 (1)	1.00 (1)	0.98 (1)
Isoleucine	3.72 (4)	4.15 (4)	4.21 (4)	4.20 (4)	4.34 (4)	4.03 (4)	3.03 (3)	3.78 (4)	4.12 (4)
Leucine	3.38 (3)	1.30 (1)	3.45 (3)	2.77 (3)	3.00 (3)	2.38 (2)	1.41 (1)	3.14 (3)	3.14 (3)
Tyrosine	1.05 (1)	0.95 (1)	0.89 (1)	0.89 (1)	0.58 (1)	0.98 (1)	0.69 (1)	0.99 (1)	0.97 (1)
Phenylalanine	1.36 (1)	2.75 (3)	1.68 (2)	1.81 (2)	2.00 (2)	2.23 (2)	2.06 (2)	1.64 (2)	2.10 (2)