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STUDIES ON THE INTERACTION OF LIMA BEAN PROTEASE INHIBITOR WITH CHYMOTRYPSIN

by

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ABSTRACT

The mode of interaction of lima bean protease inhibitor (LBI) with chymotrypsin was investigated. Treatment of LBI with chymotrypsin at acid pH produced the chymotrypsin-modified LBI with the peptide bond between positions 53 and 54 cleaved. The sample of LBI was microheterogeneous with either leucine or phenylalanine at position 53.

The chymotrypsin inhibitory activity of LBI is in agreement with the "reactive site model" for proteinase inhibitors advanced by Laskowski and Sealock (2). The chymotrypsin-modified inhibitor is formed on incubation of LBI with catalytic amounts of chymotrypsin. The chymotrypsin-modified inhibitor is completely active although it inhibits the enzyme more slowly than does the native inhibitor. Treatment of the chymotrypsin-modified inhibitor with catalytic amounts of carboxypeptidase A leads to release of phenylalanine and leucine with complete loss of inhibitory activity.

The chymotrypsin-sensitive peptide bond in LBI is involved in hydrolysis equilibria and at pH 3.1 the equilibrium mixture contains 83% cleaved and 17% intact molecules. Rapid ("kinetic control") dissociation of the enzyme-inhibitor complex results in native inhibitor; this demonstrates that the chymotrypsin active site is in close contact with the inhibitor reactive site in the enzyme-inhibitor complex.

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LITERATURE REVIEW

1 General Introduction

The existence of naturally occurring inhibitors of proteolytic enzymes was recognized around the turn of the century (1). It is widely believed that protease inhibitors represent a mechanism for metabolic regulation of proteases, but their precise physiological role and mode of action are still not yet adequately understood. It is well established that inhibition involves formation of a specific enzymically inactive complex between enzyme and inhibitor and these complexes have been studied extensively as models of protein-protein interactions.

Naturally occurring protease inhibitors are proteins and have molecular weights of between 5,000 and 60,000 daltons; they competitively inhibit the target enzyme by forming complexes with it. These complexes are usually quite stable. Many protease inhibitors are resistant to denaturation by heat or denaturing agents, and if denatured, resume native conformation and regain activity when the denaturing agent or condition is removed.

There are several reasons for the detailed examination of such enzyme-inhibitor interactions as models of protein behaviour. Among these are the simple stoichiometry generally encountered, the reversibility of most of the reactions involved, the physical and chemical stability of the inhibitors themselves, and a measurable biological activity. More recently, the demonstration of sequence homology among several inhibitors has also given promise of fresh insight into the evolutionary development of proteases and their inhibitors (2), and

studies on amino acid replacements have led to an increased understanding of the role of specific amino acid side chains for the mechanism of action of these proteins.

Protease inhibitors are very common throughout the plant kingdom, and ubiquitous among the legumes. The seeds of many plants often contain particularly large quantities of protease inhibitors, although they are by no means restricted to the seeds. It is noteworthy that in many plants the inhibitors present do not appear to act against the indigenous proteases. It has been suggested, rather, that the inhibitors in the seeds and leaves of these plants function instead as a defence mechanism against predatory insects (3).

Inhibitors are also widely found in the animal kingdom, particularly among mammals. Here they are especially found in bodily fluids and secretions, and their presence has raised the question of their possible function in metabolism. In the case of trypsin and chymotrypsin inhibitors found in mammalian pancreatic juice, it appears that the inhibitor serves to inhibit the activation of zymogens before their arrival in the small intestine (4). It is known that the activation of zymogens is due to the action of trypsin-like enzymes and chymotrypsin-like enzymes; presumably the inhibitor present in pancreatic juice protects the zymogens from premature activation.

The distribution and properties of some naturally occurring protease inhibitors is summarized in Table 1.

Because of their interest as models of heterologous protein-protein interactions, the specificity of association of proteases with their inhibitors has been the subject of extensive study during recent years. The original assumption that a single inhibitor was capable

Table 1

The Distribution and Properties of Some Naturally Occuring Protease Inhibitors[1]

	Kunitz (Pancreatic) Inhibitor	Kunitz (Soybean) Inhibitor	Bowman-Birk (Soybean) Inhibitor	Lima Bean Inhibitor
Source	bovine, ovine pancreas	seed	seed	seed
Molecular Weight	8,000	22,000	8,000	9,000
Enzymes Inhibited:				
Bovine Trypsin	+++	+++	+++	+++
Human Trypsin	+++	+	+++	+++
Bovine α-Chymotrypsin	++	+	++	++
Human Plasmin	+++	++	+	+
Human Thrombin	-	-	-	-
Kallikrein	++	-	-	-
K_{assoc} (M^{-1}), With Trypsin, pH 4.0	4×10^8	9×10^4		
Stability to Denaturing Conditions:				
3% trichloroacetic acid	stable	stable	stable	stable
90° for 5 minutes	stable	stable	stable	stable
Reduction	unstable	unstable	unstable	unstable
Recovery on Reoxidation	+	+	+	+

[1] Data from (2) and sources cited therein.

of inhibition of only one enzyme is now recognized to be erroneous; it is now understood that in some instances more than one enzyme molecule may be inhibited by the same inhibitor molecule. This could arise in any of three possible ways:

(1) Non-simultaneous inhibition of more than one protease may occur at the same site. This possibility has been demonstrated in the case of bovine pancreatic trypsin inhibitor, which can inhibit either trypsin or chymotrypsin, and in the case of soybean trypsin inhibitor, which can inhibit either trypsin, coccoonase, or chymotrypsin (5). In either case inhibition of the protease molecule occurs via association at the same site on the inhibitor.

(2) Non-simultaneous inhibition of more than one protease could theoretically occur at distinct but overlapping sites. This possibility has not as yet been reported.

(3) Simultaneous inhibition of more than one protease may occur at distinct nonoverlapping sites. Several examples of this are known, and these may be classified either as "multi-site" inhibitors, which inhibit more than one molar equivalent of the same enzyme through association at two or more sites, or as "multi-headed" inhibitors, which inhibit molar equivalents of two or more proteases. Bowman-Birk protease inhibitor from soybeans (6), turkey ovomucoid (7), and lima bean protease inhibitor (8), for instance, inhibit under optimal conditions equimolar amounts of trypsin and chymotrypsin, and therefore are double-headed inhibitors. Such inhibitors as potato inhibitor I (9) can, on a molar basis, inhibit 4 moles of chymotrypsin, and hence are referred to as multi-site inhibitors.

Several good reviews, bearing on the many facets of the study of

protease inhibitors, have appeared recently (1, 10-16). Protease inhibitors have been the subject of two international conferences (Munich, 1970 and Grosse Ledder, 1973) and the proceedings of these conferences have been published (17, 18) and can be consulted for recent information. The work presented in this thesis is primarily concerned with the mode of interaction between enzyme and inhibitor, and this review will therefore concentrate on that aspect.

The organization of this review is as follows: section 2 will discuss the mode of action of trypsin inhibitors in general, and section 3 will review double-headed inhibitors, especially lima bean protease inhibitor and Bowman-Birk protease inhibitor from soybeans.

2 Mechanism of Action of Trypsin Inhibitors

a Introduction

In this section we will focus primarily but not exclusively on Kunitz soybean inhibitor and bovine pancreatic trypsin inhibitor.

b Early Studies

The crystallization of a protease-protease inhibitor complex was first successfully accomplished by Kunitz in 1936 (19); the inhibitor is generally referred to as Kunitz soybean inhibitor. Complex formation was detected by loss of enzyme activity. The crystals so isolated consisted of equimolar amounts of enzyme and inhibitor, and were devoid of net enzyme or inhibitor activity. Kunitz' subsequent studies of the inhibitor (20-22) were directed towards the characterization of the inhibitor and of its complex with trypsin. The mechanism of complex formation itself was at that time poorly understood. Kunitz himself, on the basis of the loss of 1 mole of titrable amino groups per mole of complex formed, favoured an explanation involving classical

acid-base interactions only.

Chemical modification of specific amino acids and studies on the modified proteases and inhibitors have yielded information on the interaction of trypsin inhibitors and trypsin. In all trypsin inhibitors studied to date it has been found that inhibitory activity is lost upon modification of either lysine* or arginine* residues. The trypsin inhibitors can thus be classified as either "lysine" inhibitors or "arginine" inhibitors depending on which residues are essential for activity (23). Since trypsin specifically cleaves peptide bonds the carbonyl of which is supplied by either an arginine or a lysine residue, it was tempting to speculate that a specific Arg-X or Lys-X peptide bond is required for activity of the inhibitor. Initial experiments, however, involved the modification of <u>all</u> arginine or lysine residues in the molecule and therefore no such conclusion can be drawn.

In an elegant extension of the above studies, Chauvet and Acher (24) demonstrated that prior complexation of bovine pancreatic trypsin inhibitor with trypsin protected a single lysine residue from modification. This amino acid, lysine 15, was otherwise susceptible to chemical modification. Since complexation with trypsin prevented modification, it was concluded that lysine 15 was in close contact with the enzyme within the complex. Conversely, chemical modification of lysine 15 prevented complexation. Modification of lysine or arginine residues other than lysine 15 was without consequence as far as the inhibitory

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* Lysine residues are chemically modified by guanidination, polyalanylation, or maleylation, all at the ϵ-amino group of lysine; arginine is unaffected by these treatments. Either 2,3-butanedione or 1,2-cyclohexanedione can be used for chemical modification of the guanidinium group of arginine (23).

capacity is concerned.

This study and similar studies with other inhibitors reinforced the conclusion that, for trypsin inhibitors at least, there is a single Lys or Arg residue essential for inhibitory activity.

c Partial Proteolysis Studies

(i) Partial Proteolysis

In 1965 Finkenstadt and Laskowski (25) described experiments concerning the release of protons following addition of trypsin to soybean trypsin inhibitor. These workers added a molar amount of enzyme to inhibitor at pH 3.75 and measured the very rapid release of hydrogen ion, which produced an overshoot before reaching a lower, steady-state level at which 2 protons were released per mole of inhibitor. Alternatively, when the soybean trypsin inhibitor was preincubated with catalytic amounts of trypsin at acid pH, the overshoot in release of protons was not observed, although the same net release of protons occurred within 10 minutes. Carboxypeptidase B treatment inactivated the trypsin pre-treated inhibitor but not the native inhibitor. The interpretation offered for these results was that tryptic treatment resulted in the cleavage of a peptide bond in the inhibitor -- probably a Lys-X or and Arg-X bond -- thus exposing a new carboxyterminal amino acid. Removal of the new terminal amino acid by carboxypeptidase B results in loss of activity. This would satisfactorily explain why trypsin-treated inhibitor, but not native inhibitor, was susceptible to carboxypeptidase B treatment.

Cleavage by trypsin is proteolysis, and would involve formation, at least as an intermediate, of a covalent bond between trypsin and inhibitor. At the same time, assumption of this intermediate would,

given the proper kinetic conditions. (e.g., rapid formation of complex between enzyme and inhibitor), require an overshoot in that intermediate, which is a possible explanation of the observed overshoot in proton release.

On the basis of these experiments Finkenstadt and Laskowski proposed that the trypsin-inhibitor reaction was characterized by cleavage of a single scissile peptide bond. The complex formed between enzyme and inhibitor could dissociate to either native inhibitor (I) or modified inhibitor (I*), in which the sensitive peptide bond is cleaved:

$$E + I \rightleftharpoons EI \rightleftharpoons E + I^*.$$

At equilibrium at pH 3.75 dissociation to I* rather than to I is favoured, so that in an equilibrium mixture [I*] is larger than [I]:

$$K_{hydrolysis} = \frac{[I^*]}{[I]} > 1.$$

(ii) The Reactive Site Model

Based on studies of the mechanism of action of serine proteases, it was generally accepted that tryptic hydrolysis involved formation of a covalent intermediate with an acyl bond between the carbonyl group of the scissile peptide bond and the hydroxyl moiety of the active site serine of trypsin (26). As outlined above it was suggested by Finkenstadt and Laskowski that the stable complex formed between trypsin and Kunitz soybean trypsin inhibitor contains a covalent bond between the active site serine of the enzyme and the newly formed carbonyl residue; this is the type of acyl bond believed to be an intermediate in the mode of action of serine proteases.

More recent data on the mechanism of action of proteases have allowed a better understanding of their mechanism of action, and this has in

turn encouraged further studies on the enzyme-inhibitor interaction itself. Current explanations of the mechanism of protease inhibition centre at least in part on the specificity of protease action. The explanation advanced by Laskowski and colleagues (1) is that protease inhibition proceeds by cleavage of an enzyme-susceptible peptide bond in the inhibitor followed by formation of a covalent enzyme-inhibitor bond, thereby producing the stable enzyme-inhibitor complex. The specific scissile bond so cleaved is referred to as the "reactive site" bond, and it has been identified in several inhibitors. The presumptive covalent bond present in the intermediate is seen in this model as a prerequisite for the complex, although secondary forces are also important to the observed stability of the complex. An alternative explanation of enzyme-inhibitor interaction is the "non-bond splitting" model as proposed by Feeney and coworkers (27), according to which the initial cleavage of the reactive site peptide bond is not essential to complex formation; rather, complex formation is intrinsically the result of secondary interactions between complementary regions on the enzyme and inhibitor. Thus, the "non-bond splitting" model postulates the primary importance of non-covalent interactions between enzyme and inhibitor in formation of the complex.

(iii) Properties of the Reactive Site

The existence of a reactive site in trypsin inhibitors was first proposed in 1965 (25) and since that time many studies have been concerned with the determination of its properties. One early report from Laskowski's laboratory (28) outlines experiments permitting identification of the reactive site. In this work native and trypsin-modified inhibitors were subjected to Sephadex gel chromatography separations

after reduction and alkylation. In the case of native inhibitor, such treatment resulted in a single polypeptide chain, but with trypsin-modified inhibitor, two polypeptides were obtained: the sum of their amino acid compositions accounted for that of the native protein. This constituted a demonstration that treatment of the inhibitor with catalytic amounts of trypsin resulted in selective cleavage of only one peptide bond. Characterization by end-group analysis allowed identification of this susceptible bond as that between arginine[64] and isoleucine[65].

The two peptides resulting from partial proteolysis of the inhibitor can only be separated after reduction and alkylation of the disulfide bonds. It was therefore concluded that the susceptible arginine-isoleucine bond was located in a disulfide loop.

On the basis of these experiments Ozawa and Laskowski proposed the reaction scheme shown in Figure 1. The model explains their experimental data, and suggests that the trypsin-susceptible Arg-Ile bond is involved in complex formation. It also serves as an inherently simple conceptual basis for the understanding of trypsin-inhibitor interactions: the reactive site of all trypsin inhibitors would consist of a trypsin-sensitive Lys-X or Arg-X peptide bond; after selective cleavage of the peptide bond an acyl bond between the reactive site serine of the enzyme and the new carboxyl group of the inhibitor could be formed (Figure 2).

Studies on partial proteolysis of trypsin inhibitors thus led to the same conclusions as experiments on the chemical modification of specific amino acids in inhibitors. In both cases it could be shown that, for any given trypsin inhibitor, one specific amino acid, always either a lysine or an arginine, is physically close to the enzyme in

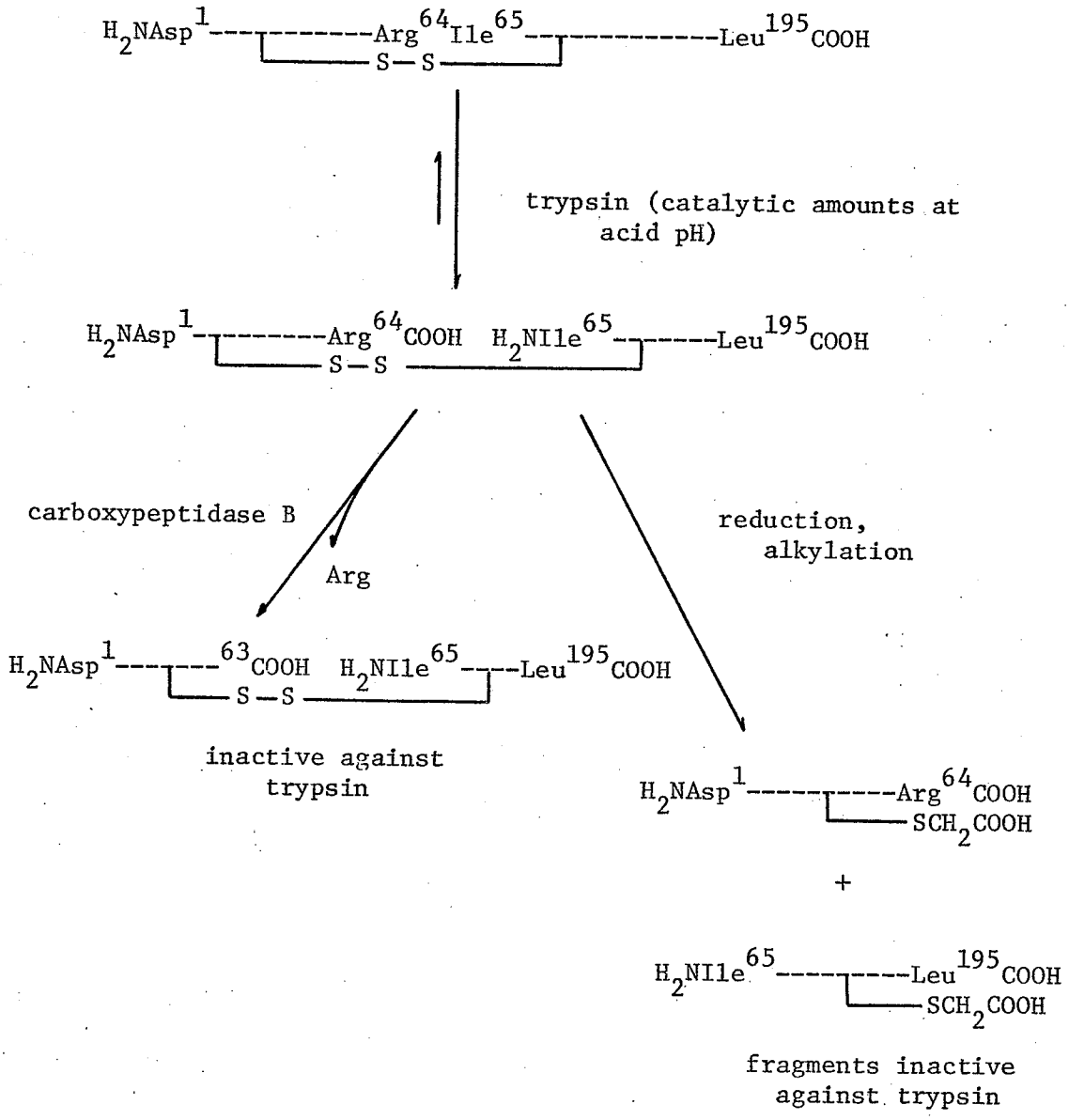
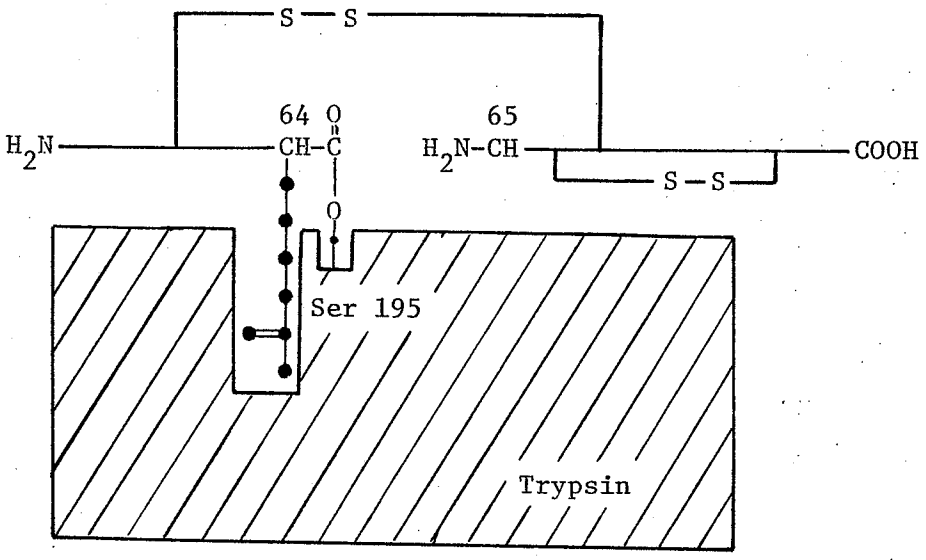


Figure 1

Determination of the Trypsin-Sensitive Bond in Soybean Trypsin Inhibitor

Figure 2

The Proposed Acyl Bond In The Trypsin-Soybean Trypsin Inhibitor Complex



the stable complex, and that under conditions of partial proteolysis, the reactive site peptide bond involving that amino acid is cleaved. Furthermore, the reactive site amino acid is itself essential for inhibitory activity. Modification of the reactive site amino acid, or removal of it by carboxypeptidase B treatment of the protease-modified inhibitor, renders the inhibitor inactive.

As these conclusions were found to hold for most trypsin inhibitors studied, including those from widely diverse biological sources, it was assumed as a working hypothesis that a general mechanism featuring these points was common to all trypsin-inhibitor interacting systems.

(iv) Kinetic Control Dissociation

The hydrolysis constant, K_{hyd}, governing the $I \rightleftharpoons I^*$ equilibrium is pH dependent and large at acid pH. Hence the conditions used by most experimenters to convert $I \longrightarrow I^*$ involve incubation of native inhibitor with catalytic amounts of trypsin at acid pH; the resulting equilibrium is established very slowly. The final reaction mixture consists of both I and I*, with I* predominating at all pH values.

Hixson and Laskowski (29) described experiments in which the conversion of $I^* \longrightarrow I$ could be observed directly, and which therefore established true reversibility. From stopped-flow measurements of the interaction of trypsin with the soybean trypsin inhibitor it was known that the minimal mechanism of interaction was

$$E + I \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} L \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} C \underset{k_3}{\overset{k_{-3}}{\rightleftharpoons}} L^* \underset{k_4}{\overset{k_{-4}}{\rightleftharpoons}} E + I^*$$

wherein L and L* are loose, non-covalent intermediates formed between trypsin and either native (I) or modified (I*) inhibitor, and C is the

complex between enzyme and inhibitor. Extremes of dilution or of pH, or addition of denaturing agents, result in dissociation of the complex;

$$E + I \longleftarrow C \longrightarrow E + I^*.$$

Since $K_{hyd} > 1$ at all pH, I* will predominate in any equilibrium mixture of I and I*. However, if the dissociation is rapid and re-association of enzyme and inhibitor is prevented by denaturing the enzyme, a kinetic rather than an equilibrium distribution of products is obtained; furthermore, if indeed the same stable complex is formed either from I or I* then the product distribution should be the same whether one starts with I or I*. If $k_{-2} \gg k_3$, rapid dissociation should form I in preference to I*. This expectation was experimentally realized by Hixson and Laskowski in the following manner. An equilibrium mixture of I and I* (in which $[I^*] > [I]$) was incubated with an equimolar amount of trypsin to allow maximum complex formation. Rapid acidification of the complex resulted in dissociation of the complex and precipitation of trypsin, and native inhibitor (I) in excess over modified inhibitor (I*) could be demonstrated in the reaction mixture. Thus net peptide bond synthesis has been achieved in a previously-equilibrated mixture of I and I*. This experimental strategy is termed "kinetic control dissociation".

Since the result of kinetic control dissociation is net peptide bond synthesis, the reversibility of the proteolytic reaction with an inhibitor is effectively demonstrated.

(v) Replacement of the Reactive Site Amino Acid

Sealock and Laskowski (30) reported a very interesting study of the importance of the reactive site amino acid. These workers first prepared soybean des-Arg^{64} trypsin inhibitor and then replaced the

missing amino acid with lysine (Figure 3) in the following manner: native soybean trypsin inhibitor was subjected to partial proteolysis at acid pH, and carboxypeptidase B was utilized to remove the reactive-site arginine^{64}. The resulting des-Arg^{64} STI* is inactive against trypsin. Carboxypeptidase B was then placed in solution with purified des-Arg^{64} STI* and with a large excess of lysine. A peptide synthetic reaction by carboxypeptidase B is of course highly unfavourable, but the reaction can be "driven" by subsequent complex formation with trypsin (and, therefore, removal of reaction products), and peptide bond synthesis becomes favoured as part of the overall net reaction. The resultant complex was treated with denaturing agents and Lys^{64}-Ile^{65} inhibitor was obtained by kinetic control dissociation as previously described. Hence the reactive site arginine had been enzymatically replaced with lysine, and the native "arginine inhibitor" had become a "lysine inhibitor". Both inhibitors effectively complex with trypsin with resulting loss of proteolytic activity. The kinetics of interaction were different for the two inhibitors, as might be expected for a replacement involving so sensitive a position.

Subsequent to the original enzymatic replacement of arginine^{64} with lysine, Laskowski and coworkers have described additional experiments involving replacement of arginine^{64} and isoleucine^{65} (31). In these studies, arginine has been replaced with glycine, alanine, and leucine. Of particular interest is the observation that Phe^{64}-STI retains the ability to inhibit trypsin, yet is not a chymotrypsin inhibitor. Conversely, Trp^{64}-STI is a good chymotrypsin inhibitor, but does not associate with trypsin to any appreciable extent. It is most interesting that Phe^{64}-STI is a trypsin inhibitor, as this represents the first trypsin

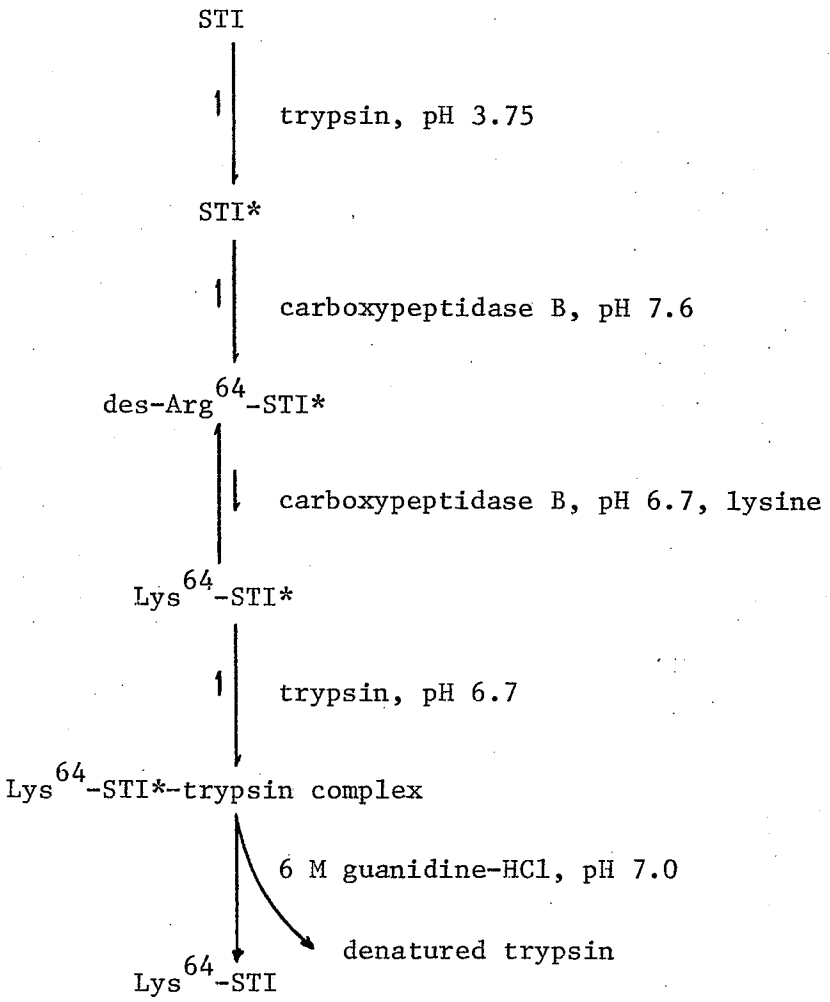


Figure 3

Enzymatic Replacement of Arginine^{64} of Soybean Trypsin Inhibitor (STI)

inhibitor having an amino acid other than lysine or arginine in its reactive site. At the same time, the failure of Phe^{64}-STI to inhibit chymotrypsin is somewhat paradoxical, as the inhibition of chymotrypsin by Trp^{64}-STI adequately shows the "acceptability" to chymotrypsin inhibition provided position 64 is occupied by a residue compatible with the specificity of chymotrypsin. Since Phe-X bonds are normally cleaved by chymotrypsin the failure of Phe^{64}-STI to inhibit the enzyme is puzzling. Resolution of this point must await further examination of the effects of amino acid replacement in protease inhibitors, and of the requirements at the complementary specificity site of chymotrypsin.

Both kinetic control dissociation and enzymatic amino acid replacement at the reactive site thus provide additional evidence of intimate involvement of the reactive site amino acid with the protease with which it complexes.

(vi) The Acyl-Enzyme Intermediate

Laskowski and coworkers, on the basis of kinetic evidence, have repeatedly suggested that the stable enzyme-inhibitor complex involves a covalent bond between enzyme and inhibitor (1). As mentioned above, the minimal mechanism of interaction of protease and inhibitor was at one time thought to be

$$E + I \rightleftharpoons L \rightleftharpoons C \rightleftharpoons L^* \rightleftharpoons E + I^*.$$

In such a scheme C is the only possible covalent enzyme-inhibitor complex. As most workers considered the acyl-enzyme intermediate a necessary intermediate in enzyme-inhibitor complex formation, C would be that acyl-enzyme complex.

d X-Ray Diffraction Studies

X-Ray diffraction Studies of the crystalline enzyme-inhibitor complex-

es formed with trypsin and either bovine pancreatic trypsin inhibitor (32, 33) or Kunitz soybean trypsin inhibitor (34) have demonstrated that these complexes are characterized by a tetrahedral adduct involving the active-site serine of the enzyme and the carbonyl carbon of the reactive site amino acid of the inhibitor (Figure 4). This result, together with knowledge of the mechanism of action of serine proteases (35) indicates that the minimal mechanism of interaction must be

$$E + I \rightleftharpoons L \rightleftharpoons T \rightleftharpoons A \rightleftharpoons T^* \rightleftharpoons L^* \rightleftharpoons E + I^*,$$

where T is the tetrahedral adduct formed between protease and native inhibitor, T* the tetrahedral adduct formed between protease and modified inhibitor, and A is the acyl intermediate. The stable complex is merely the tetrahedral adduct T instead of the acyl enzyme as originally proposed by Laskowski. The mechanism of interaction is, however, identical to that of proteolysis, a result anticipated by many workers, notably Laskowski and his colleagues (1).

In the stable enzyme-inhibitor complex, numerous hydrogen bonds, van der Waals contacts, and hydrophobic interactions are found between trypsin and the active-site region of the inhibitor. These enzyme-inhibitor interactions, together with the closeness of the fit between enzyme and inhibitor, exclude water from the vicinity of the reactive site tetrahedral intermediate, thereby preventing hydrolytic breakdown of the covalent intermediate bond.

Both bovine pancreatic trypsin inhibitor and soybean trypsin inhibitor form tetrahedral intermediates with the enzyme and hence have the same mode of binding. Information obtained from x-ray diffraction studies thus essentially corroborates the reactive-site model of Laskowski and implies a universal mechanism for interaction of protease with inhibitor.

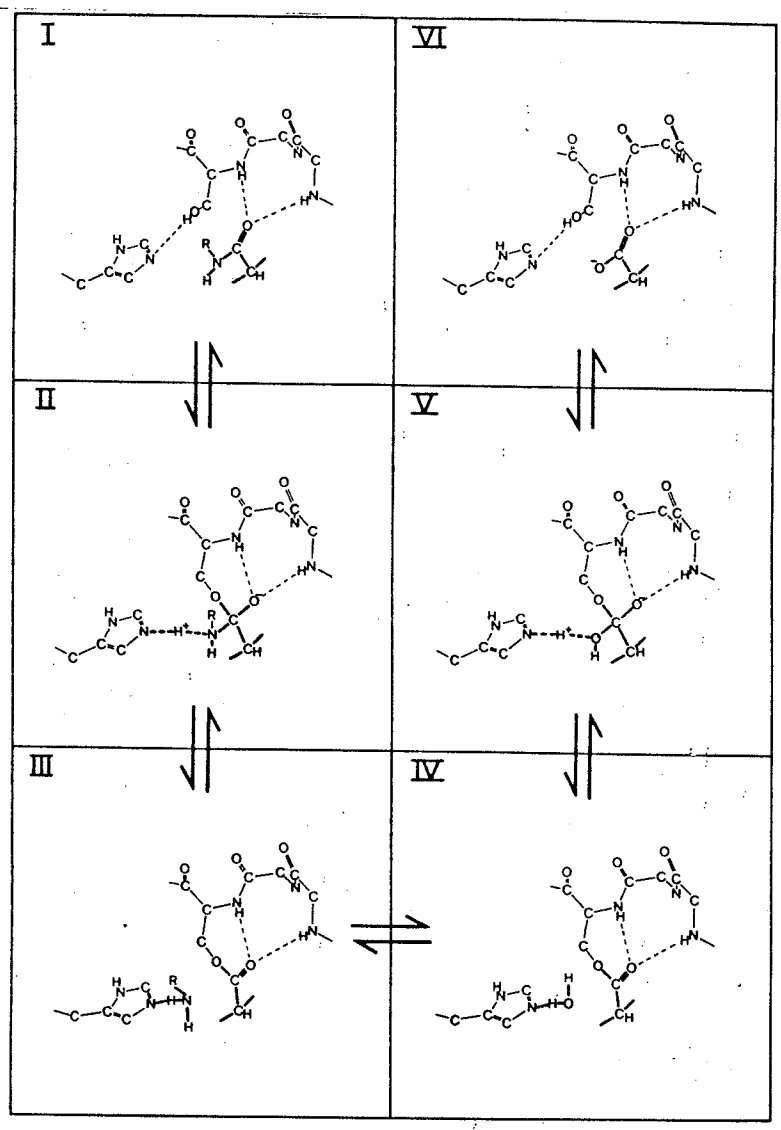


Figure 4

The Proposed Sequence of Stereochemical Steps in the Hydrolysis of an Amide Bond by Chymotrypsin[1]

1 From (35).

3 Double-Headed Inhibitors

a Examples

Examples of double-headed inhibitors identified to date include certain avian ovomucoids (7), lima bean inhibitor (8), and Bowman-Birk protease inhibitor (6). Each of these inhibitors possesses independent sites for the inhibition of two separate proteases simultaneously.

Some double-headed inhibitors may remain undetected due to the failure of researchers to assay for inhibitory activity against certain proteolytic enzymes. It is therefore possible that additional examples of double-headed inhibitors will be discovered as the existence of independent sites on the same inhibitor is demonstrated.

For the purposes of this review we will focus on lima bean protease inhibitor (LBI) and also Bowman-Birk soybean protease inhibitor (BBI) because of its relationship with LBI.

b Double-Headed Nature of LBI

(i) Physical Demonstration of Independent Sites

Haynes and Feeney (36) showed that lima bean protease inhibitor inhibits both trypsin and chymotrypsin. Isolation of the enzyme-inhibitor complexes by Krahn and Stevens (37) demonstrated that the stoichiometry of interaction for both trypsin and chymotrypsin with LBI was 1:1. It was also possible to isolate a ternary complex composed of molar amounts of LBI, trypsin, and chymotrypsin. In this ternary complex there was no net residual tryptic, chymotryptic, or inhibitory activity. This then proved that there were separate and independent sites on LBI for inhibition of trypsin and chymotrypsin.

Seidl and Liener (38) demonstrated that Bowman-Birk protease inhibitor also possessed independent loci for the inhibition of trypsin and

chymotrypsin, and that BBI also forms stoichiometric binary or ternary complexes with these enzymes.

(ii) Partial Proteolysis Studies

Early studies by Ozawa and Laskowski (28) on the effects of tryptic treatment of STI led to the suggestion that a lysyl-X or arginyl-X peptide bond was present in all naturally occurring trypsin inhibitors and was particularly susceptible to tryptic scission. Subsequent to this Laskowski developed the reactive site model (1) which further postulated the existence of a hydrolysis equilibrium for the scissile bond. Studies designed to test these suggestions using LBI as a model were reported by Krahn and Stevens (39, 40). Consistent with Ozawa and Laskowski's data for tryptic treatment of STI, it was found that tryptic treatment of LBI led to cleavage of a single peptide bond located within a disulfide loop; this peptide was later identified as Lys^{26}-Ser^{27} (40, 41). Trypsin-modified LBI (LBI'_t) is held together by a cystine bridge; if this disulfide bond is reduced and alkylated, two peptides result. The total amino acid content of the two small peptides is identical to that of LBI.

LBI thus conforms to the reactive site model both with respect to the presence of a scissile bond and a hydrolysis equilibrium governing cleavage of this bond. Similar studies involving the chymotrypsin reactive site of LBI showed that this site is analogous to the trypsin reactive site of LBI and allowed the scissile peptide bond to be identified as Leu^{53}-Ser^{54} (39, 40).

(iii) The Primary Sequence of LBI

The primary sequence of LBI was reported by Tan and Stevens (41, 42) and is shown in Figure 5. Of particular interest is the homology shown between the sequences surrounding the two reactive-site peptide bonds.

Figure 5

The Primary Sequence of LBI Lot OIA Variant IV

H_2N-Ser-Gly-His-His-Glu-His-Ser-Thr-Asp-Glx-Pro-Ser-Glx-Ser-Ser-Lys-

Pro-Cys-(Cys, Asx)-His-(Cys, $\frac{\text{Leu}}{\text{Ala}}$, Cys)-Thr-Lys-Ser-Ile-Pro-Pro-Gln-

Cys-Arg-Cys-$\frac{\text{Thr}}{\text{Ser}}$-Asp-$\frac{\text{Leu}}{\text{Phe}}$-Arg-Leu-Asp-Ser-Cys-His-Ser-Ala-Cys-Lys-

Ser-Cys-Ile-Cys-Thr-Leu-Ser-Ile-Pro-Ala-Gln-Cys-Val-(Cys, $\frac{\text{Thr}}{\text{Asx}}$, Asx)-

Ile-$\frac{\text{Asx}}{\text{Thr}}$-Asp-Phe-Cys-Tyr-Glu-Pro-Cys-Lys-Ser-Ser-His-Ser-Asp-Asp-Asp-

Asn-Asn-Asn-COOH

Data reported in (55). Positions for which two amino acids are listed represent positions known to be microheterogeneous.

These two regions are compared in Figure 6. It can be seen that there is extensive homology in the sequences from positions 22 to 34 and 49 to 61. Nine of the 13 positions are identical; of the 4 replacements, one is a conservative replacement of isoleucine for leucine, and another is the replacement of trypsin reactive-site lysine (Lys^{26}) for chymotrypsin reactive-site leucine (Leu^{53}). Since the primary amino acid sequence is commonly accepted as the predominant determinant of secondary and tertiary structures, it seems likely that these two reactive site sequences manifest three-dimensional homology as well. Stevens (43) and Tan and Stevens (42) suggested that the two independent sites arose as a result of gene duplication, and speculated that the sequences were located in similar disulfide loops.

The amino acid sequence of BBI has also been determined (44, 45), and is shown in Figure 7. There is extensive homology between LBI and BBI. It can readily be seen that here too there is considerable sequence homology between the inhibitory regions located between positions 5 to 22 and 31 to 49. Allowing for one deletion, that of Ser^{38}, seven of the 18 positions are identical between the two sequences, and 3 more involve conservative replacements only. Of especial interest is the replacement of lysine at position 61, the trypsin inhibitory site amino acid, for leucine at position 43, the amino acid in the chymotrypsin inhibitory site. This is the same reactive site replacement previously found in LBI.

(iv) Importance of Disulfide Bonds in LBI

Studies on the reduction and reoxidation of the disulfide bonds in LBI have been reported by Stevens and Doskoch (46). Complete reduction of all 8 bonds was associated with loss of inhibitory activity, whereas reoxidation resulted in a 100% recovery of the chymotrypsin

Figure 6

The Homologous Regions of LBI Lot 0IA Variant IV

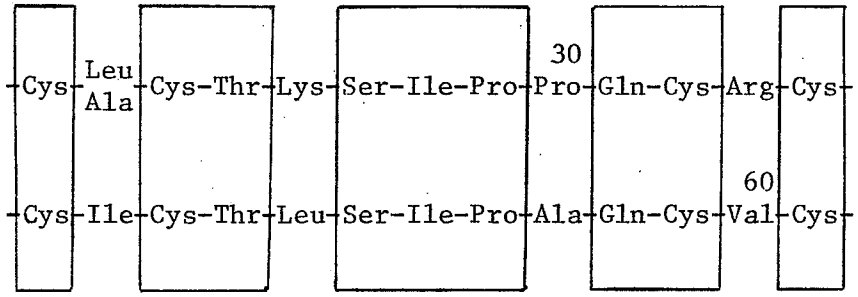


Figure 7

The Primary Sequence of BBI

10

H_2N-Asp-Asp-Glu-Ser-Ser-Lys-Pro-Cys-Cys-Asp-Gln-Cys-Ala-Cys-Thr-Lys-

20 30

Ser-Asn-Pro-Pro-Gln-Cys-Arg-Cys-Ser-Asp-Met-Arg-Leu-Asn-Ser-Cys-His-

40 50

Ser-Ala-Cys-Lys-Ser-Cys-Ile-Cys-Ala-Leu-Ser-Tyr-Pro-Ala-Gln-Cys-Phe-

60

Cys-Val-Asp-Ile-Thr-Asp-Phe-Cys-Tyr-Glu-Pro-Cys-Lys-Pro-Ser-Glu-Asp-

70

Asp-Lys-Glu-Asn-COOH

Data reported in (50).

inhibitory activity and 50% recovery of the trypsin inhibitory activity. In contrast, all but one of the disulfide bonds are protected against reduction when the inhibitory is first complexed with trypsin. The stabilization of 7 of 8 bonds in the trypsin-inhibitor complex is not yet adequately understood, but it is clear that at least one of these 7 disulfide bonds is necessary for chymotrypsin inhibitory activity, and presumably for trypsin inhibitory activity as well.

Similar studies have been carried out on BBI (47) and in addition Ikenaka and coworkers (48-50) have been able to determine the exact location of its seven disulfide bonds as shown in Figure 8. Ikenaka and his colleagues have suggested that this arrangement of bridges, together with the established sequence homology between the trypsin inhibitory region and the chymotrypsin inhibitory region, constitute a strong implication that the tertiary structure of BBI is symmetrical, with the two inhibitory regions having similar conformations.

Ikenaka's group (49, 50) has also successfully cleaved the Bowman-Birk inhibitor with cyanogen bromide and pepsin into two polypeptides (Figure 9), one of which is active against trypsin and the other of which is active in inhibiting chymotrypsin.

c Full Characterization of the Trypsin Reactive Site of LBI

A full characterization of the trypsin reactive site was undertaken by Krahn (51, 52) in order to explain in detail the trypsin inhibitory behaviour of LBI. The aim of this investigation was to determine whether LBI conforms to the "reactive-site" model proposed by Laskowski and coworkers. This study included a determination of the stoichiometry of interaction (37), a determination of the reactive sites against trypsin (40) and chymotrypsin (39), a study of the LBI$\rightleftharpoons$LBI$'_t$ reaction

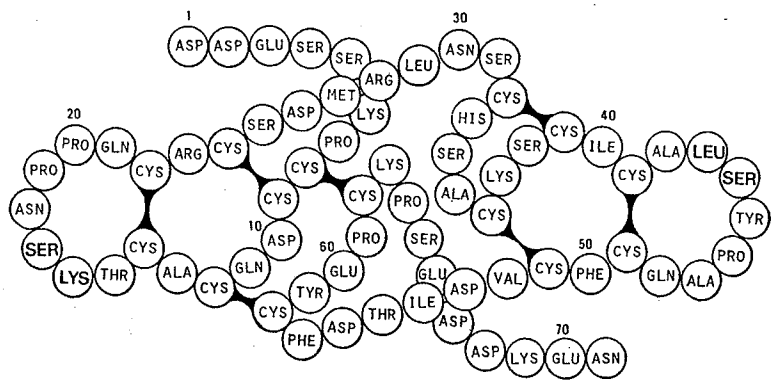


Figure 8

The Complete Covalent Structure of BBI[1]

1 From (50).

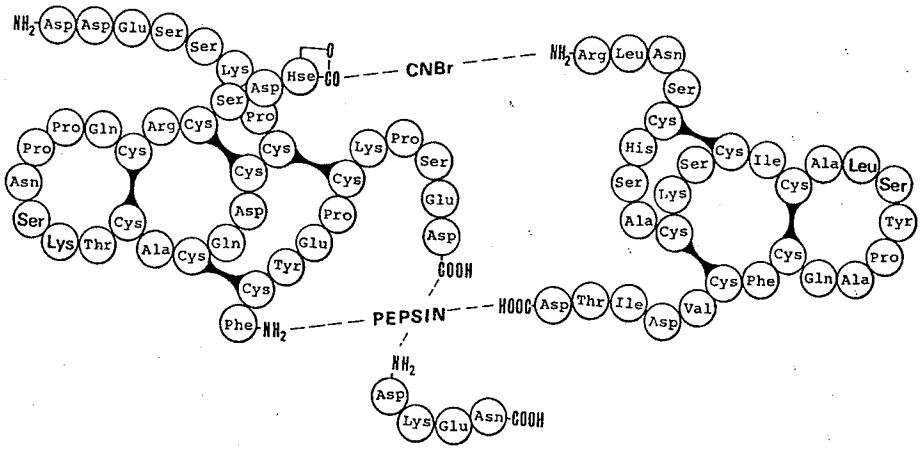


Figure 9

The Scission of BBI into Two Inhibitory Fragments[1]

The two protease inhibitory sites are shown in boldface type. The sites of cleavage shown are conjectural.

1 From (50).

(51, 52), and a study of the kinetic control dissociation of the complexes of trypsin with LBI and LBI'_t (51, 52). All of these studies have been discussed above.

d The Problem of Multiple Molecular Forms of LBI

Ion exchange separation of LBI results in 4 or more apparently homogeneous fractions (53). These fractions possess nearly identical biological activity (54) and similar but not identical molecular weight and amino acid composition (51, 54, 55). The amino acid sequences of at least two of these sequences are very similar (55) and it seems likely that further similarities exist. For a number of reasons several variants (including variants from more than one commercial preparation) have been used in inhibition studies.

METHODS

1 Purification of Lima Bean Protease Inhibitors I-IV (LBI I-IV)

Commercial preparations of LBI were obtained from Worthington Biochemical Corp. (lot numbers 2IA and 2EA) and further purified by the procedure of Jones et al (53). Gel filtration on Sephadex G-75 using 10% acetic acid as the eluant gave approximately 90% recovery of active material in a single peak, and 10% of contaminating material of higher molecular weight which did not contain any trypsin or chymotrypsin inhibiting material (Figure 10).

The active material from the Sephadex G-75 column was further fractionated by chromatography on DEAE-cellulose as shown in Figure 11. Fractions eluted before application of the gradient were devoid of significant inhibitory activity. The four fractions possessing inhibitory activity were pooled as indicated and individually rechromatographed in the same DEAE-cellulose system; they were then characterized by amino acid analysis and by protease inhibitory activity.

It is known that inhibitors prepared from separate commercial preparations often show small differences in amino acid composition and biological activity (54, 51). For this reason the inhibitor fractions from each commercial lot were kept separate, and they are identified in individual experiments. The amino acid compositions of the different preparations are summarized in Table 2.

2 Enzyme and Inhibitor Assays

The enzyme assay used was a modification of the procedure of Rhodes et al (56, 57). Ester hydrolysis at pH 8.2 in a weakly buffered system leads to the release of hydrogen ion. This change can be observed using

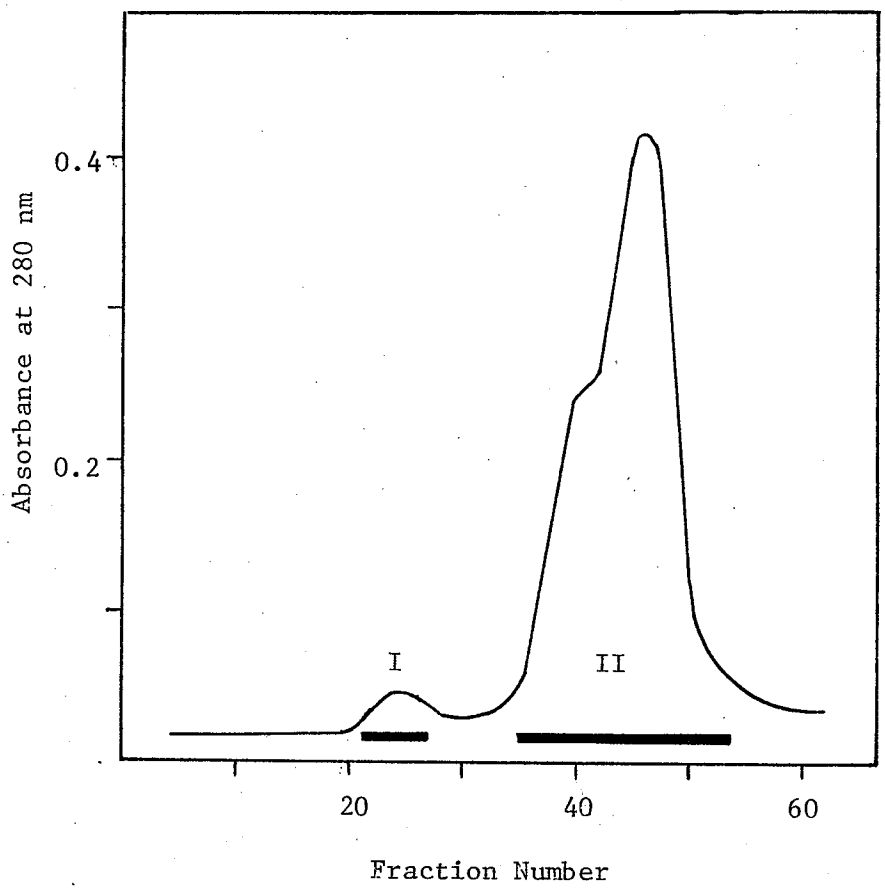


Figure 10

Gel Filtration of Commercial LBI (Worthington LBI 2IA) on Sephadex G-75

A column of Sephadex G-75 (2.5 x 90 cm) was equilibrated and developed with 0.1 M NH_4HCO_3. The sample, 100 mg of crude LBI 2IA, was dissolved in 2.8 ml of 0.1 M NH_4HCO_3. The column was operated at a flow rate of 50 ml/hr and 6.4 ml fractions were collected. The column eluant was monitored for protein at 280 nm. Fractions indicated by solid bars were pooled and lyophilized.

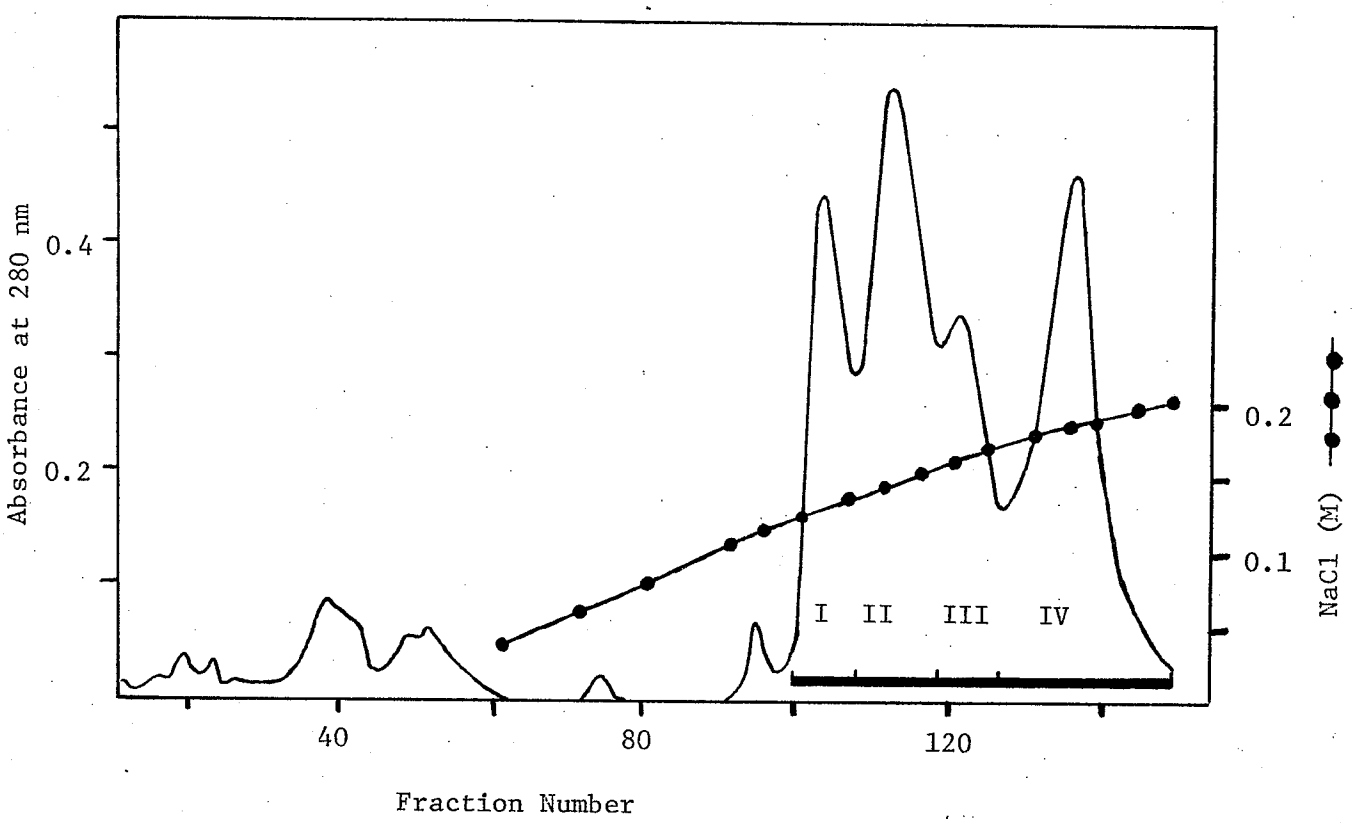


Figure 11

Ion-Exchange Chromatography of LBI 2IA on DEAE-Cellulose

A column of DEAE-Cellulose (2.5 x 90 cm) was equilibrated with starting buffer, 0.01 M sodium phosphate, pH 7.6. Peak II from the gel filtration chromatography (Figure 10) was dissolved in approximately 2 ml of starting buffer and applied to the column. The column was eluted with an exponential salt gradient to 0.01 M sodium phosphate and 0.4 M NaCl, pH 7.6, applied through a 1 litre mixing chamber containing the starting buffer. The column was operated at a flow rate of 50 ml/hr, eluant fractions of 6.2 ml were collected and pooled as indicated.

Table 2

Amino Acid Composition of Lima Bean Protease Inhibitor Lots 2IA and 2EA[1]

	2IA				2EA			
	I	II	III	IV	I	II	III	IV
Lysine	4.66	3.99	4.89	3.76	6.42	5.80	3.10	4.16
Histidine	5.91	5.78	5.49	5.92	7.31	8.85	3.23	6.83
Arginine	2.17	1.93	2.07	2.36	2.28	2.50	2.58	2.07
Aspartic Acid	13.88	13.87	14.07	13.13	18.22	19.48	9.87	16.61
Threonine	5.06	4.06	4.45	4.70	5.51	7.35	3.42	5.90
Serine	12.69	12.17	12.09	12.57	16.77	17.65	9.92	13.55
Glutamic Acid	6.69	6.83	6.22	6.29	8.02	8.66	4.17	7.95
Proline	6.18	6.05	7.60	6.08	7.55	7.91	6.25	7.26
Glycine	1.02	1.01	0.97	1.07	1.21	1.26	1.18	1.04
Alanine	2.64	3.01	3.07	2.44	3.58	3.89	2.00	2.59
Cysteine	12.79	11.67	12.37	13.24	13.48	15.40	8.28	13.47
Valine	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Methionine	-	-	-	-	-	-	-	-
Isoleucine	4.26	3.82	4.15	3.78	4.95	5.41	2.74	4.19
Leucine	3.05	3.12	2.99	3.14	4.03	3.90	2.54	3.30
Tyrosine	1.58	0.97	1.05	0.99	1.19	1.28	1.55	1.16
Phenylalanine	2.04	1.05	1.10	1.64	1.12	1.30	1.76	1.92

1 The above values were calculated assuming 1.00 valine residue per molecule (41).

m-nitrophenol as a colorimetric indicator. N^{α}-benzoyl-L-tyrosine ethyl ester (BTEE) was used as the substrate for chymotrypsin and p-toluene-sulfonyl-L-arginine methyl ester (TAME) as the substrate for trypsin.

When an inhibitor preparation is added to the assay mixture it will decrease the rate of hydrolysis of the substrate by a given amount of enzyme. This decrease can be quantitatively related to the amount of inhibitor present in the preparation.

Solutions used in the assays were as follows:

Enzyme: α-chymotrypsin (CDI 1IC) or trypsin (TRL 2DA), 15 mg in 1 ml of 20 mM $CaCl_2$ and 4 mM HAc. A 1 ml aliquot of this solution was made to 10 ml with 6.0 mM Tris buffer, pH 8.2.

Buffer: 6.0 mM Tris, pH 8.2.

Substrate-Indicator: For chymotrypsin, 8 mM BTEE in 7.5 mM Tris, pH 8.2, containing 0.012% (w:v) m-nitrophenol and 30% (v:v) methanol.
For trypsin, 10 mM TAME in 6.0 mM Tris, pH 8.2, containing 0.012% (w:v) m-nitrophenol.

Inhibitor: The typical concentration was 0.10 mg/ml, in 6.0 mM Tris, pH 8.2.

The BTEE solution (chymotrypsin substrate) must be kept stoppered and at 37° until immediately before use to prevent precipitation of BTEE. This solution is stable for at least 48 hours when so stored. Trypsin substrate TAME, buffer solution, and inhibitor solutions are stable for 48 hours at room temperature. Chymotrypsin and trypsin are also stable for several hours at room temperature; loss of activity is approximately 10% of control over 24 hours at room temperature.

The spectrophotometer used was a Coleman-Hitachi 124 recording spectrophotometer, with wavelength control set at 395 nm to observe the indicator's yellow to colourless shift on esterolysis, and chart speed at 1 cm/min. The chymotrypsin assay mixture consisted of a solution (3.0 ml total volume) prepared by first adding 2.0 ml BTEE solution,

0 - 0.7 ml buffer solution, 0.7 - 0 ml inhibitor solution, and mixing. Finally 0.3 ml of chymotrypsin solution was added, the assay mixture was again mixed, and recording begun. Thus chymotrypsin and inhibitor in this assay were not pre-mixed.

The time elapsed between addition of enzyme and the beginning of measurement was regularly observed to be 11-13 seconds.

The trypsin inhibitory assay differed slightly in that trypsin and inhibitor were mixed together first and allowed to pre-equilibrate for 90 seconds, at which time the assay was begun by addition of TAME solution. In all other respects conditions were analogous to those of chymotrypsin assays.

Enzyme activity was measured as a rate of initial hydrolysis; this rate was seen to remain constant for 20-30 seconds after beginning measurement in an assay containing no inhibitor. Inhibitor activity was calculated as a percentage decrease in enzyme activity (Figure 12).

3 Amino Acid Analysis

Samples containing not more than 0.2 μmoles of protein were hydrolyzed at 110° for 22 hours in sealed and evacuated tubes with 6 N HCl containing 0.5% phenol for protection of tyrosine residues. Analyses were carried out on a Beckman-Spinco 120C automatic amino acid analyzer according to Spackman et al (58) as described by the manufacturer.

4 Partial Proteolysis of Lima Bean Protease Inhibitor with Chymotrypsin

In a typical experiment, a lyophilized sample of LBI was dissolved in 18 mM trans-aconitate in a concentration of 1 mg/ml; the pH of such a solution is 3.1. To this was added approximately 2 mole percent of

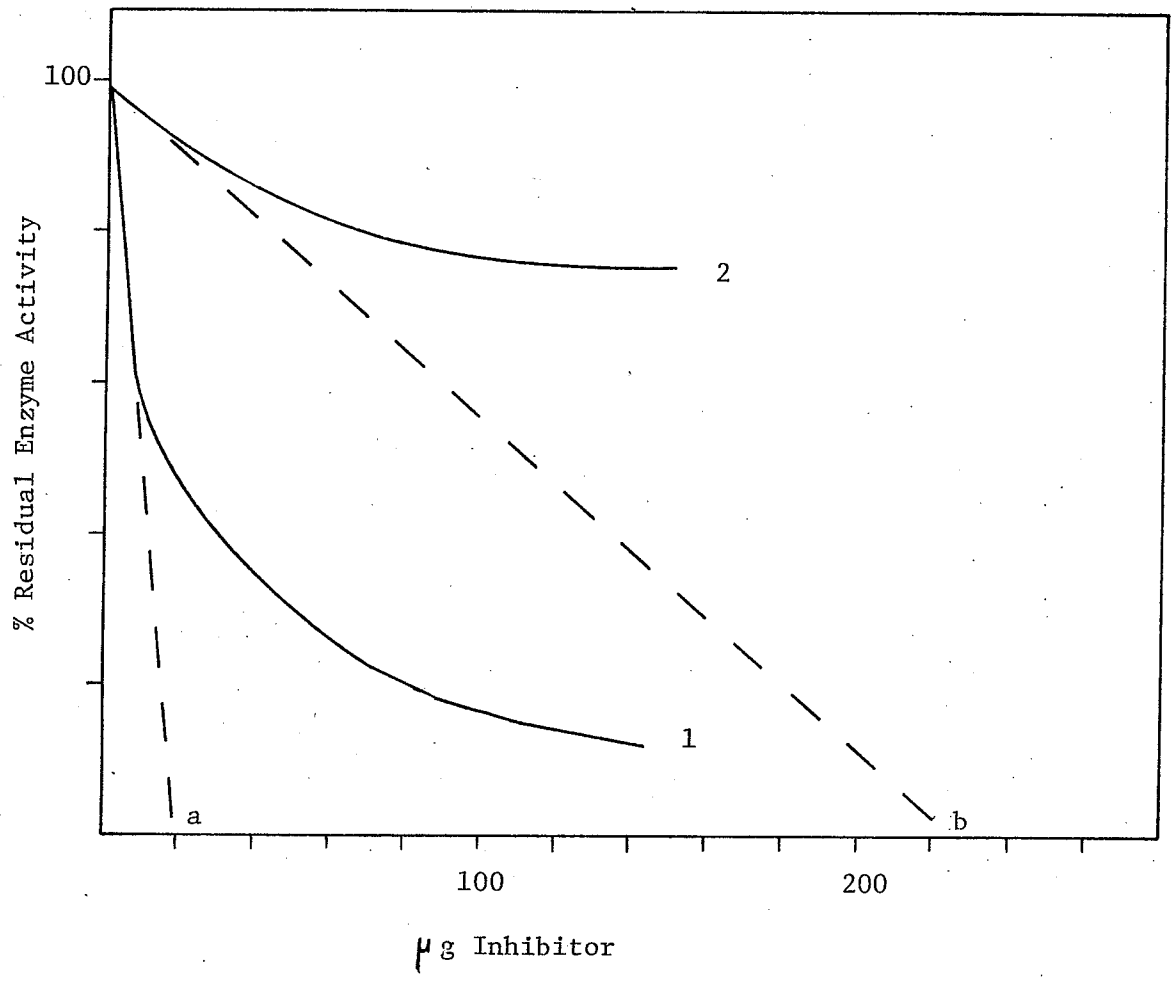


Figure 12

Inhibition of Chymotrypsin by Lima Bean Protease Inhibitor Preparations

Increasing amounts of LBI were added to 45 μg of enzyme. (1), native, fully active LBI; (2), a partially active LBI (e.g., LBI'_c).

Calculation of activity of a partially active LBI is as follows:

$$\% \text{ inhibitory activity remaining} = \frac{a}{b} \times 100\%.$$

chymotrypsin similarly dissolved in trans-aconitate. Proteolysis was allowed to proceed for 12-18 hours, at which time the reaction was stopped by freezing. The resulting reaction mixture can be characterized in several ways, as described in the Results section. Amino acid analysis and assay for trypsin inhibitory activity provided an estimate of total LBI present. Loss of chymotrypsin inhibitory activity and release of amino acids by carboxypeptidase A are two ways in which the amount of modification of the chymotrypsin inhibitory site can be monitored. In contrast, control samples which were not pre-treated with chymotrypsin had full protease inhibitory activity and released no amino acids on carboxypeptidase A treatment.

These parameters were routinely used to characterize samples of LBI treated by partial proteolysis.

5 Carboxypeptidase A Treatment

A 10 µl sample of carboxypeptidase A suspension (lot COADFP 7GA, 50 mg/ml) was washed with 100 µl of distilled water. After centrifugation the precipitate was suspended in 100 µl of 0.1 M NH_4HCO_3, and to this was added 10 µl of 0.1 M NaOH to make the solution basic and to dissolve the enzyme.

The sample to be treated was dissolved in 100 µl of H_2O or 0.1 M NH_4HCO_3, and to this was added 75 µl of 0.1 M boric acid and 60 µl of 0.1 M NaOH, followed by 30 µl of the prepared carboxypeptidase A solution. The pH of this solution was 7.8 and sufficiently buffered so as not to fall below 7.7 during the 12-hour hydrolysis period; carboxypeptidase A has a pH optimum of 7.6-8.0. This procedure was applied to samples of up to 1 mg of LBI (0.1 µmole).

Free amino acids resulting from carboxypeptidase A treatment were determined by amino acid analysis. Control samples, either of unmodified LBI with carboxypeptidase A treatment, or of modified LBI without carboxypeptidase A treatment, showed no released amino acids or were subtracted from experimental values.

RESULTS

1 Time Course of Peptide Bond Cleavage in the Chymotrypsin Reactive Site of LBI

a Introduction

Previous studies from this laboratory (54, 51, 39) have shown that incubation of LBI with catalytic amounts of chymotrypsin at acid pH for 24 hours resulted in a reaction mixture which had only 30% of the original chymotrypsin inhibitory activity but retained full trypsin inhibitory activity. Upon treatment of the reaction mixture with carboxypeptidase A, near equimolar amounts of leucine and threonine are released. End group studies on the peptide fragments obtained after reduction and alkylation of the reaction mixture together with knowledge of the amino acid sequence of LBI (41, 42) led Krahn and Stevens to the conclusion that the chymotrypsin reactive site of LBI is the Leu^{53}-Ser^{54} peptide bond located in the C-terminal third of the molecule in the sequence Thr-Leu-Ser-Ile (39).

The reaction mixture was believed to consist of 70% LBI'_c (peptide bond cleaved) and 30% LBI (peptide bond intact)*, but no attempt was made to determine the LBI $\rightleftharpoons$ LBI'_c equilibrium in a time course study. Furthermore a careful examination of the data shows that carboxypeptidase A treatment of the reaction mixture resulted in the release of not only Leu and Thr but also of traces of Phe (up to 20%). Their experiments were carried out on LBI variant III, the amino acid composition of which indicates a non-integral number of phenylalanine residues; this is a preliminary indication that the preparation was microheterogeneous and may

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* In subsequent discussion we will use the following terminology: LBI'_c is used to represent lima bean protease inhibitor in which the chymotrypsin reactive site peptide bond has been cleaved. The term "chymotrypsin modified LBI" will be used to describe the reaction mixture consisting of LBI'_c and native LBI.

consist of a mixture of molecules having either Phe-Ser or Leu-Ser peptide bonds at the chymotrypsin reactive site.

The purpose of this experiment then was twofold: firstly, to examine the time course of partial proteolysis and thereby determine whether an equilibrium is reached; and secondly to find out whether or not LBI could accomodate Phe-Ser as well as Leu-Ser as its chymotrypsin reactive site. Variant IV of LBI is a strong inhibitor of chymotrypsin (54) which is comparatively high in phenylalanine (Table 2) and was therefore chosen for further study.

b Experimental

Approximately 20 mg of LBI (2IA variant IV) were dissolved in 2.0 ml of 18 mM trans-aconitate, pH 3.1 and aliquots were withdrawn for trypsin and chymotrypsin inhibitory assay, carboxypeptidase A treatment, and amino acid analysis. Chymotrypsin (2 mole percent with respect to LBI) was added, the reaction mixture was incubated at room temperature, and samples were withdrawn at appropriate times over a period of 12 hours. Each aliquot consisted of 125 µl, and was divided into two portions of 75 and 50 µl and immediately frozen. The larger of the two aliquots was subsequently treated with carboxypeptidase A and analyzed for release of amino acids; the smaller, 50 µl sample was diluted in 6.0 mM Tris buffer and assayed for trypsin and chymotrypsin inhibitory activity. After 12 hours, an additional 2 percent of chymotrypsin was added and samples were also taken 4 and 11 hours after this second addition.

c Results

From the results as presented in Figure 13 it can be seen that 83% of the chymotrypsin inhibitory activity of LBI is lost after 12 hours of chymotrypsin treatment; the trypsin inhibitory activity is unaffected.

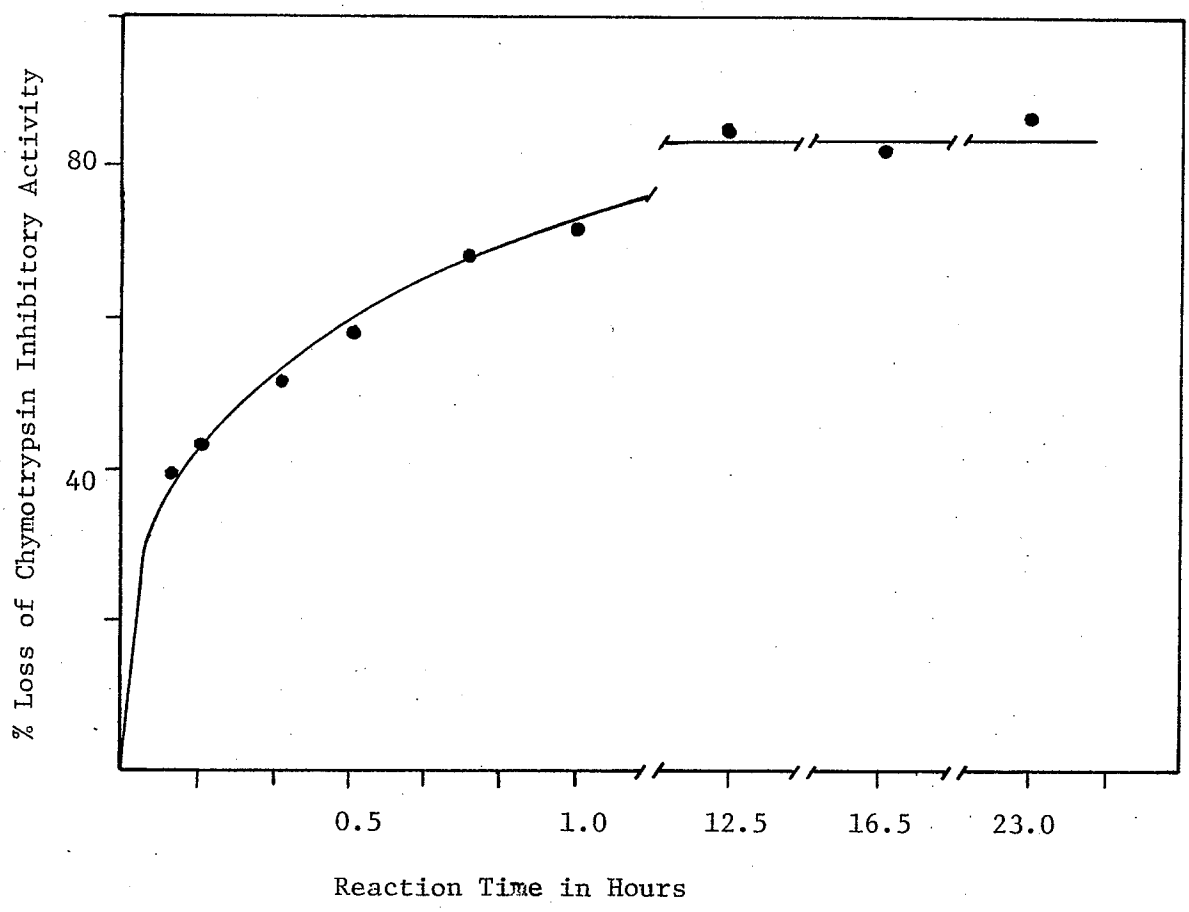


Figure 13

Inhibitory Activity of LBI as a Function of Treatment with Catalytic Amounts of Chymotrypsin

A sample of LBI was incubated with catalytic amounts (2 mole percent) of chymotrypsin at pH 3.1. Aliquots were taken at various times and assayed for trypsin and chymotrypsin inhibitory activity. Conditions are as decribed in the text.

Previous studies have already shown that this loss of activity is due to the cleavage of a single peptide bond. It can readily be seen from the graph that the equilibrium position has been attained; an additional amount of chymotrypsin added after 12 hours of incubation has no further effect on the composition of the reaction mixture in terms of the LBI'_c/LBI ratio.

Concomitant with loss of biological activity is an increased susceptibility to carboxypeptidase A, as demonstrated by release of leucine and phenylalanine (Figure 14). The combined total leucine and phenylalanine released from the sample by carboxypeptidase A treatment of the chymotrypsin-treated inhibtor (12 hours) amounts to 0.81 mole/mole of LBI. It therefore appears that the chymotrypsin-sensitive bond in this preparation of LBI exists either as Leu-Ser or as Phe-Ser, and that this bond is cleaved on incubation with chymotrypsin, thereby exposing a new carboxyl-terminal amino acid (Leu or Phe) which may be removed on carboxypeptidase A treatment. It is also evident that incubation of LBI with catalytic amounts of chymotrypsin at acid pH results in a loss of 82% of the original chymotrypsin inhibitory activity. Loss of inhibitory activity is simultaneous with scission of the chymotrypsin-sensitive peptide bond.

d Discussion

Based on the known specificity of chymotrypsin (59) one could expect the following chymotrypsin-sensitive bonds: Leu-X, Trp-X, Tyr-X, or Phe-X. Frattali and Steiner (60) proposed that the chymotrypsin-susceptible bond of LBI could be either a Trp-X or a Phe-X bond. Krahn and Stevens (39) conclusively demonstrated the existence of a Leu-X bond in the chymotrypsin reactive site of one LBI variant. The results of the

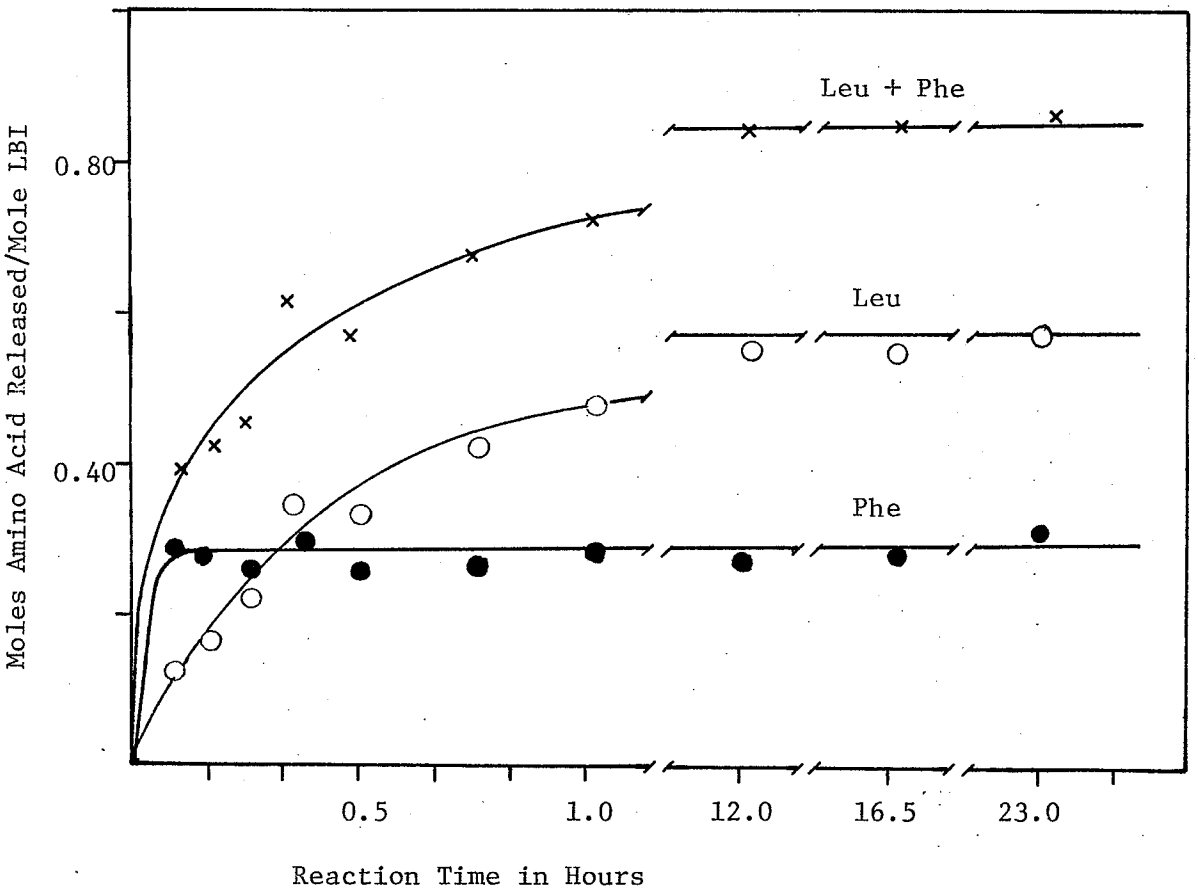


Figure 14

Release of Amino Acids by Carboxypeptidase A from LBI as a Function of Treatment with Catalytic Amounts of Chymotrypsin

A sample of LBI was preincubated with catalytic amounts (2 mole percent) of chymotrypsin at pH 3.1. Aliquots were taken at various times and subjected to carboxypeptidase A treatment, and free amino acids were determined by amino acid analysis. Conditions are as described in the text.

present study clearly show that the preparation used was microheterogeneous and consisted of variants possessing either Leu-X or Phe-X as the chymotrypsin-sensitive bond.

After the above experiments were completed, Wilson and Laskowski Sr. (61, 62) published experiments demonstrating a similar microheterogeneity in the trypsin-sensitive site of garden bean inhibitor. These workers found variants of garden bean inhibitor with either Lys-X or Arg-X as the trypsin-sensitive bond. Genetic variations involving positions other than the active-site amino acid have also been found in LBI (41).

It is of interest to note that, under the conditions used, the Phe-Ser peptide bond attains maximum cleavage within 1/2 hour, whereas the Leu-Ser peptide bond requires 2 hour to reach maximum cleavage. This finding parallels that of Krahn and Stevens (39), in which the phenylalanine released on carboxypeptidase A treatment (approximately 0.2 mole/mole) was released within the first time interval studied. It would seem that the chymotrypsin specificity site has a higher affinity for the Phe-Ser reactive site than for the Leu-Ser reactive site, despite the apparently identical sequence of all other amino acids in LBI (55). Possible reasons for this difference in affinity have not been examined. It would be most interesting to investigate the kinetic consequences of this sole replacement.

Sealock and Laskowski Jr. (30) and Kowalski et al (31) have reported on the kinetic consequences of synthetic amino acid replacements in the trypsin inhibitory site of Kunitz soybean inhibitor. This line of investigation should prove most fruitful in elucidating specific structure-function relationships in this class of proteins.

2 Complex Formation Between Chymotrypsin and LBI or LBI'_c

a Introduction

It is implicit in the reactive site model proposed by Laskowski and coworkers (1, 25) that a complex can be formed between the enzyme and either native inhibitor or inhibitor modified by partial proteolysis:

$$E + I \rightleftharpoons L \rightleftharpoons C \rightleftharpoons L^* \rightleftharpoons E + I^*.$$

Preliminary investigations from this laboratory (39) using variant III led to the erroneous conclusion that chymotrypsin-modified LBI did not inhibit chymotrypsin and thus does not form a complex with it. This would be inconsistent with the Laskowski model.

Later experiments (54) using the four variants of LBI indicated that chymotrypsin-modified LBI obtained from variant IV regained its full inhibitory potential within 1 hour of incubation with molar amounts of chymotrypsin at neutral pH, whereas the chymotrypsin-modified LBI from variant I did not regain inhibitory activity even after 5 hours of incubation under identical conditions. Since it was known that LBI variant IV is a stronger inhibitor of chymotrypsin than is variant I these results were interpreted as meaning that complex formation between chymotrypsin and the chymotrypsin-modified inhibitor was easier in those cases where the chymotrypsin-modified inhibitor comes from a precursor with a stronger affinity for the enzyme. However at that time the existence of a complex formed from the chymotrypsin-modified LBI had not been physically demonstrated.

b and c Experimental and Results

(i) Complex Formation as Monitored by Assay for Residual Proteolytic Activity

Solutions containing near equimolar amounts of chymotrypsin and

native or chymotrypsin-modified LBI were preincubated at room temperature for various time periods. At appropriate time intervals 1.0 ml samples (containing 45 μg of enzyme and 18 μg of inhibitor) were withdrawn from the incubation mixture and to it were added 2.0 ml of substrate-indicator solution and the inhibitory activity of the preincubated sample determined as described under Methods. Two kinds of controls were also run: one control contained only chymotrypsin and maintained 90% of the initial esterolytic activity over a period of 30 hours; the other controls were run exactly as the samples except that the chymotrypsin-modified and native inhibitor used had been pre-treated with carboxypeptidase A as described under Methods.

The results are shown in Figure 15. Immediately after mixing enzyme and inhibitor native LBI reaches its full chymotrypsin inhibitory potential but it takes up to 6 hours of preincubation for chymotrypsin-modified LBI to reach the same chymotrypsin inhibitory activity. Furthermore, as expected, treatment with carboxypeptidase A abolishes the chymotrypsin inhibitory potential of chymotrypsin-treated LBI (by removal of the new carboxyl-terminal Phe or Leu from LBI'_c) but leaves native LBI unaffected. We interpret these results as follows: LBI'_c is capable of inhibiting chymotrypsin but the complex formed between LBI'_c and chymotrypsin is formed very slowly; removal of the new carboxyl-terminal residue prevents complex formation and results in loss of inhibitory activity.

(ii) Complex Formation Observed by Gel Filtration

In order to actually physically demonstrate the existence of a complex formed from LBI'_c and chymotrypsin, samples of LBI and of chymotrypsin-treated LBI were preincubated for 8-10 hours with equimolar amounts of chymotrypsin and subjected to gel filtration through Sephadex G-75.

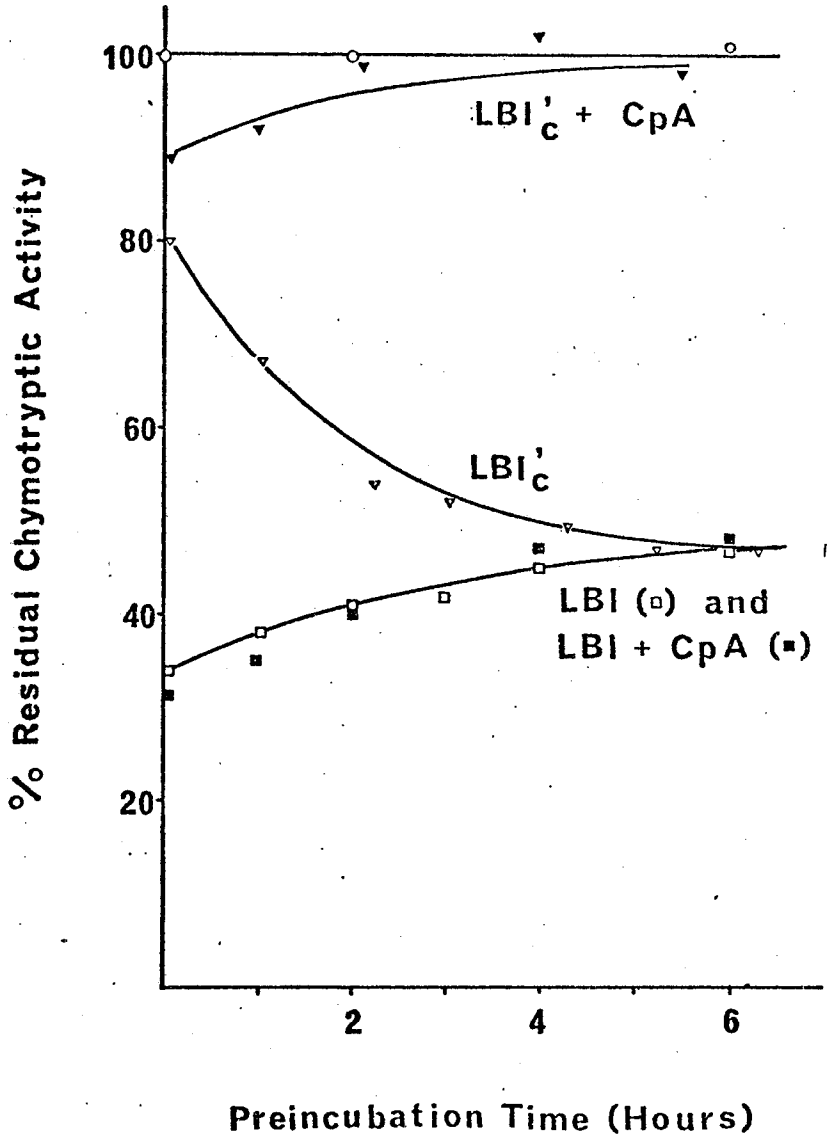


Figure 15

Complex Formation between Chymotrypsin and LBI or Chymotrypsin-modified LBI

Stoichiometric amounts of Chymotrypsin and the appropriate inhibitor were incubated and at various times aliquots were withdrawn and were examined for chymotrypsin and trypsin inhibitory activity. ○, chymotrypsin; □, LBI; ■, LBI treated with carboxypeptidase A; ▽, chymotrypsin-modified LBI; ▼, chymotrypsin-modified LBI treated with carboxypeptidase A.

As shown in Figure 16, the material obtained by preincubation of equimolar amounts of chymotrypsin and either LBI or chymotrypsin-modified LBI was eluted from the column with identical elution volumes (241 ml) which are smaller than those for either LBI (280 ml) or chymotrypsin (255 ml); this is to be expected if a complex is formed in both instances. Furthermore, as shown in Table 3, it could be shown that in both cases the amino acid composition of the material so obtained accounts, within experimental error, for the sum of the amino acid compositions of LBI and chymotrypsin as would be expected from a 1:1 molar complex. Also, in both cases, there was no net chymotrypsin, or chymotrypsin inhibitory, activity. By all criteria described above the complexes formed between chymotrypsin and either LBI or chymotrypsin-modified LBI are indistinguishable.

d Discussion

The statement of Krahn and Stevens (39) that LBI'_c is inactive against chymotrypsin was based on the results of assays which were run without prior incubation between enzyme and inhibitor. Later experiments (54) and the results of the present study indicate that the complex formation between LBI'_c and chymotrypsin is too slow to be observed under normal assay conditions. This is in contrast to the complex formation between trypsin and trypsin-modified LBI (LBI'_t) which is complete within 15 minutes (54). At present we do not fully understand this difference in affinity.

As previously described, Finkenstadt et al (63) have shown that the protease-inhibitor interaction may be written as follows:

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

in which T is the stable complex whether formed from E + I or from E + I*.

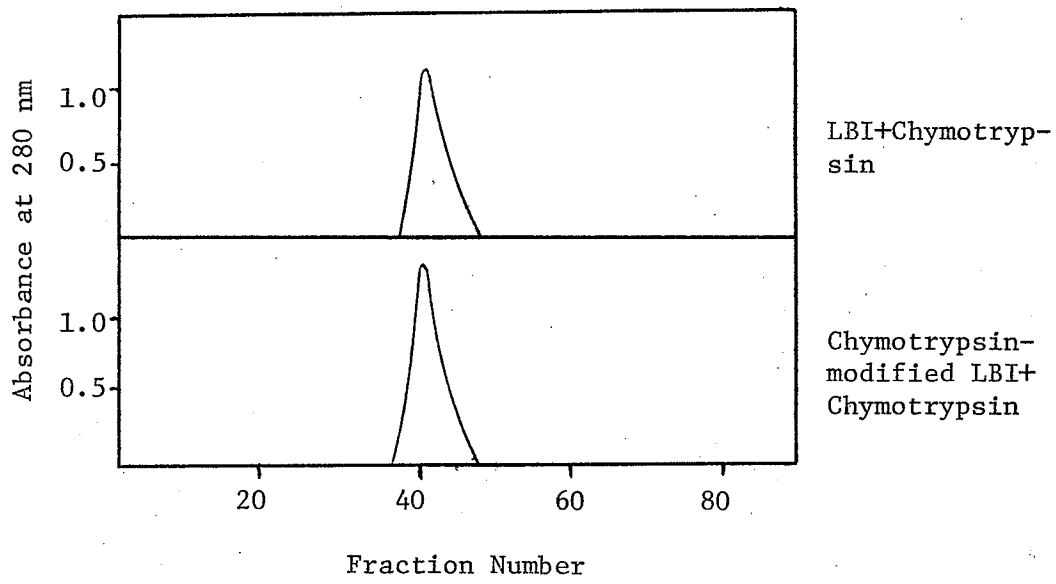


Figure 16

Gel Filtration of Chymotrypsin and LBI or Chymotrypsin-modified LBI

Stoichiometric amounts of chymotrypsin and the appropriate inhibitor preparation were preincubated at room temperature for 8 hours. The sample was dissolved in 0.1 M NH_4HCO_3 and applied to a column of Sephadex G-75 (2.5 x 90 cm) equilibrated with the same buffer. The flow rate was 50 ml/hr and samples of 5.5 ml were collected. Control samples of LBI or of chymotrypsin were run in the same system and eluted at a greater volume.

Table 3

Amino Acid Composition of LBI, Chymotrypsin, and the LBI-Chymotrypsin Complex

	LBI[1] (found)		Chymotrypsin[2] (theor)	Complex[3] (theor)	LBI-Chym[3]	LBI'_c-Chym[3]
Lysine	3.76	(4)	14	18	19.2	19.8
Histidine	5.92	(6)	2	8	6.2	6.6
Arginine	2.36	(2)	3	5	4.8	4.8
Aspartic Acid	13.13	(13)	22	35	31.5	35.2
Threonine	4.70	(5)	22	27	29.4	27.5
Serine	12.57	(13)	27	42	50.3	38.0
Glutamic Acid	6.29	(7)	15	22	21.2	22.5
Proline	6.08	(6)	9	15	17.5	17.4
Glycine	1.07	(1)	23	24	24.6	25.9
Alanine	2.44	(2)	22	24	25.7	27.5
Valine	1.00	(1)	23	24	22.9	22.2
Methionine	-	(0)	2	2	2.2	2.2
Isoleucine	3.78	(4)	10	14	13.0	14.0
Leucine	3.14	(3)	19	22	22.6	23.8
Tyrosine	0.99	(1)	4	5	5.3	5.1
Phenylalanine	1.64	(1)	6	7	8.3	7.9

1 Data taken from Table 2 this thesis.
2 From (26).
3 Based on analyses of 22 hr hydrolysates only, hence values for serine are not extrapolated to zero time.

Since there are two additional steps in the reaction of E + I*, the attainment of equilibrium must be kinetically more complicated than is the case with E + I. This alone may explain the long time required for complex formation between chymotrypsin and LBI'_c as compared to LBI (39).

Finkenstadt et al have examined in detail the kinetics of interaction of trypsin with pancreatic trypsin inhibitor (Kunitz) and with soybean trypsin inhibitor and have concluded that the K_m of the E + I* reaction is very large compared with that of the E + I reaction. It thus appears likely that the slowness of complex formation of a protease with a modified inhibitor is a general phenomenon.

Frattali and Steiner (60) have shown that chymotrypsin-modified Bowman-Birk soybean inhibitor regains full activity after a 45 hour incubation with chymotrypsin. It therefore seems that the chymotrypsin inhibitory sites of both lima bean inhibitor and Bowman-Birk soybean inhibitor conform to the reactive site model.

3 Rapid ("Kinetic Control") Dissociation of Enzyme-Inhibitor Complex

a Introduction

As previously discussed, in the simplest version of the reaction mechanism proposed by Laskowski and coworkers the following equilibria exist:

$$I \rightleftharpoons I^* \qquad \text{(Equation 1)}$$

and

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

Equation 1 says that there is an equilibrium between native inhibitor, with the reactive site peptide bond uncleaved, and modified inhibitor,

with the reactive site cleaved. It is known that the equilibrium is catalyzed by the enzyme (in the absence of the protease the conversion of $I \rightleftharpoons I^*$ is too slow to be observed) and that the equilibrium position is pH dependent. Equation 2 says that the same central complex is obtained whether one starts with I or I*. If this central complex is quickly dissociated and the enzyme at the same time is denatured, then the product distribution will be dependent only on the relative values of k_{-2} and k_3. To check the reaction mechanism one can thus prepare complex from E and either I or I*, quickly dissociate both complexes and the ratio I/I* should be identical in both cases. This experiment, first described by Laskowski (25), has been called kinetic control dissociation because the product distribution is affected only by the relative rate k_{-2} and k_3 and no equilibrium $I \rightleftharpoons I^*$ is established. In the case of all trypsin inhibitors studied (1, 52, 63) it is a dramatic experiment because $k_{-2} \gg k_3$ and therefore one can form a complex from trypsin and trypsin-modified inhibitor, dissociate it and obtain primarily native inhibitor -- peptide bond synthesis has therefore been effected by a proteolytic enzyme. This kind of experiment has up to the present been carried out only on trypsin inhibitors.

The following experiment was begun with the aim of showing net peptide bond synthesis of the cleaved, scissile Leu-Ser or Phe-Ser peptide bond found in LBI'_c, and of showing that the distribution of products (LBI and LBI'_c) is the same for LBI isolated from complex formed using either native or chymotrypsin-modified inhibitor.

b Experimental

Equimolar amounts of chymotrypsin and either LBI or chymotrypsin-modified LBI were incubated at pH 8.2 and room temperature for 8-10 hours

to allow complete complex formation. At the end of this time the respective enzyme-inhibitor complexes were isolated by gel filtration on Sephadex G-75 as described previously (Figure 16). Aliquots were characterized by amino acid analysis and by assay. The material was then dissolved in 0.1 M NH_4HCO_3 to a concentration of 10 mg/ml. An equal volume of 5% trichloroacetic acid was added; under these conditions the complex dissociates, chymotrypsin is denatured and precipitates, while LBI remains in solution (52). The solution was allowed to stand at 4^o overnight and centrifuged. The precipitate was redissolved in 0.1 M NH_4HCO_3 and an aliquot was subjected to amino acid analysis; the amino acid composition was found to agree with that of chymotrypsin. The supernatant containing the inhibitor was dialyzed and applied to a column of Bio-Gel P-10 (1.5 x 90 cm, equilibrated with 0.1 M NH_4HCO_3) to remove the small molecular weight contaminants. The first peak obtained from this column was collected and pooled, and aliquots were taken for amino acid analysis and assay for inhibitory activity before and after treatment with carboxypeptidase A.

c Results

The results in Table 4 show that the material obtained by kinetic control dissociation of either LBI-chymotrypsin or the LBI'_c-chymotrypsin complex is indistinguishable and behaves as native LBI as judged by its chymotrypsin inhibitory activity and its resistance to carboxypeptidase A treatment. This indicates net synthesis of the scissile peptide bond in LBI'_c.

d Discussion

The results as presented indicate that the scissile peptide bond has been resynthesized as a result of complexation and kinetic control diss-

Table 4

Inhibitory Activities of LBI, Chymotrypsin-modified LBI, and Their Complexes with Chymotrypsin

Material	Protease Inhibitory Activity*	
	Chymotrypsin	Trypsin
LBI	100	100
LBI treated with Carboxypeptidase A	100	100
LBI-Chymotrypsin Complex	0	100
LBI from LBI-Chymotrypsin Complex	100	100
LBI from LBI-Chymotrypsin Complex and treated with Carboxypeptidase A	100	100
Chymotrypsin-modified LBI	15	100
Chymotrypsin-modified LBI, treated with Carboxypeptidase A	15	100
Chymotrypsin-modified LBI-Chymotrypsin Complex	0	100
LBI from Chymotrypsin-modified LBI-Chymotrypsin Complex	100	100
LBI from Chymotrypsin-modified LBI-Chymotrypsin Complex and treated with Carboxypeptidase A	100	100

* Inhibitory activities were determined by assay as described in the text. Values are $\pm$ 5%.

ociation. This interpretation is in agreement with net synthesis of the chymotrypsin-sensitive peptide bond. The conclusion is also consistent with current concepts of the mechanism of action of naturally occuring protease inhibitors, which hold that the protease-sensitive peptide bond is in close contact with the catalytic site during complex formation, and thereby resynthesizes the cleaved peptide bond during some step of complex formation.

Laskowski and coworkers have repeatedly stated (5, 64, 65) that the most effective demonstration for the presence of a reactive site on an inhibitor is complexation of protease with inhibitor and modified inhibitor, followed by kinetic control dissociation of the complexes. This statement is based on the fact that if the distribution of products is the same for complexes formed from LBI and from LBI'_c, then the sensitive peptide bond must have been in contact with the enzyme catalytic site. The conclusion then is inescapable that the peptide bond in question is the inhibitor active site.

In all cases studied to date kinetic control dissociation leads to predominantly virgin inhibitor, whereas an equilibrium distribution of products lies greatly to the side of modification. From the data presented it can be seen that the chymotrypsin-modified peptide bond in LBI'_c also conforms to this behaviour.

GENERAL CONCLUSIONS

Previous investigations have shown that LBI is a double-headed inhibitor with separate and independent sites for inhibition of trypsin and chymotrypsin, and that the stoichiometry of interaction with either of these enzymes is 1:1 in a binary complex or 1:1:1 in a ternary complex containing molar amounts of each enzyme and the inhibitor. The amino acid sequences of two variants of LBI have been established and the location of the reactive sites of this inhibitor for trypsin and chymotrypsin have been determined. The mechanism of inhibition of the reactive site for trypsin has also been studied. The results of the present investigation clearly demonstrate that LBI, insofar as its chymotrypsin reactive site is concerned, conforms to the "reactive site model" proposed as a general model of serine protease inhibitors. The characteristics of the interaction of LBI with chymotrypsin are schematically presented in Figure 17 and may be summarized as follows:

(1) The chymotrypsin reactive site can be cleaved by chymotrypsin at acid pH. This site consists of a unique peptide bond which in some inhibitor molecules is the Leu^{53}-Ser^{54} bond and in others is the Phe^{53}-Ser^{54} bond.

(2) The scissile peptide bond is involved in hydrolysis equilibrium with a $K_{hydrolysis} > 1$ at acid pH. At pH 3.1, the equilibrium mixture consists of 84% LBI'_c and 16% LBI.

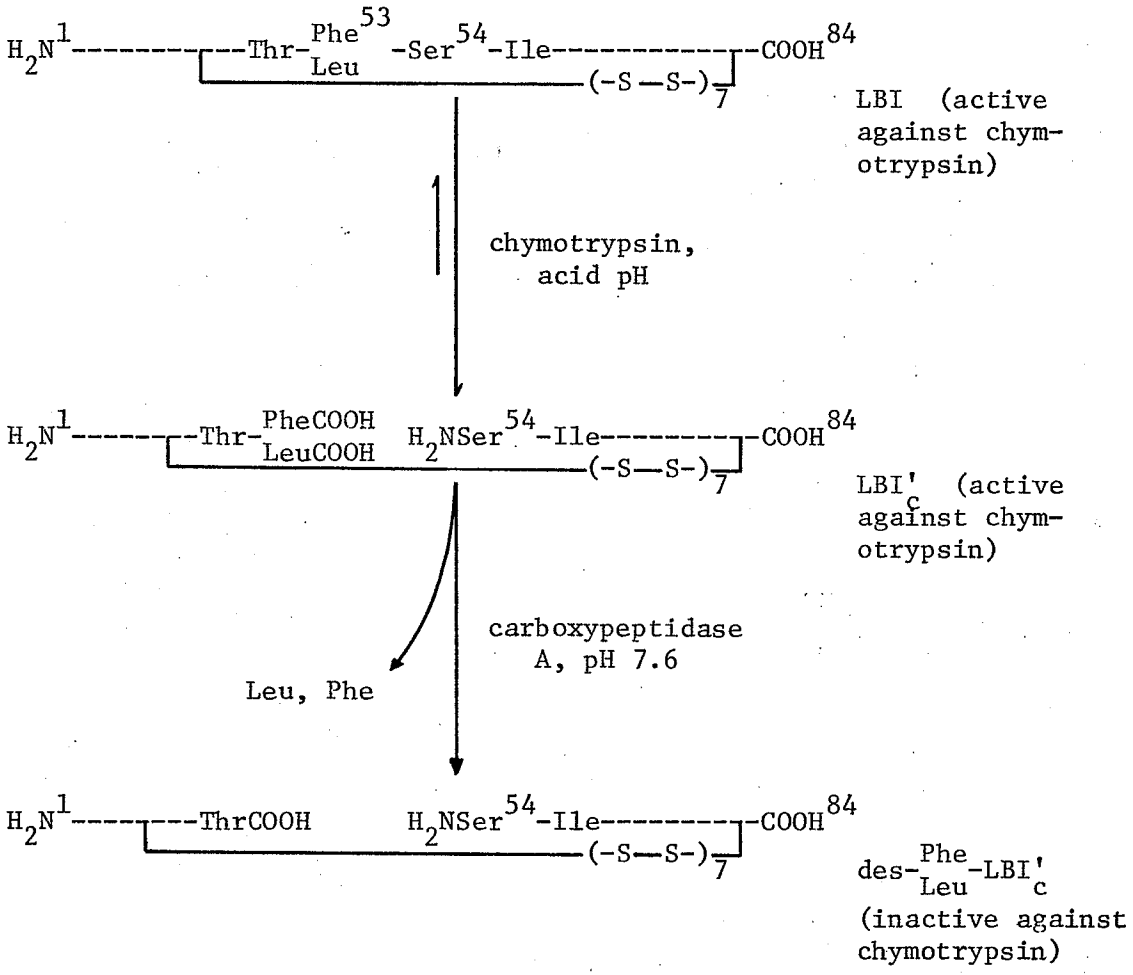
(3) LBI and LBI'_c have the same inhibitory capacity against chymotrypsin. However, at neutral pH, LBI'_c complexes with chymotrypsin much more slowly than does LBI.

(4) Removal of the reactive site amino acid (Leu or Phe) from LBI'_c by carboxypeptidase A renders the inhibitor inactive against chymotrypsin.

(5) The chymotrypsin-inhibitor complex made from either native LBI or

Figure 17

The Chymotrypsin Reactive Site of LBI and Its Peptide Bond Hydrolysis Equilibria



from chymotrypsin-modified LBI yields predominantly native LBI on rapid ("kinetic control") dissociation.

Thus the chymotrypsin reactive site of LBI shows properties consistent with those of the "reactive site" model. The trypsin reactive site of LBI, identified as the Lys^{28}-Ser^{29} peptide bond, has previously been studied in this laboratory and also conforms to the "reactive site" model (51, 52).

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