Lima Bean Trypsin Inhibitor. Limited Proteolysis by Trypsin and Chymotrypsin*

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ABSTRACT: Lima bean trypsin inhibitor inhibits both trypsin and chymotrypsin. Gel filtration studies show that the sites for trypsin and chymotrypsin binding are different and independent. Incubation of the inhibitor with catalytic amounts of trypsin at low pH results in a product which is still active but less efficient in inhibiting trypsin. Upon treatment of this modified inhibitor with carboxypeptidase B there is a release of ~0.3 mole/mole of new carboxy-terminal lysine with a concomitant reduction of the trypsin inhibitory activity to 70% of the original value. The chymotrypsin inhibitory activity of the inhibitor is unaffected by the above treatments. Furthermore, incubation of the inhibitor with catalytic amounts of chymotrypsin at acid pH results in a loss of ~70% of the original chymotrypsin inhibitory activity without affecting the trypsin inhibitory activity. After reduction and alkylation, the chymotrypsin-modified inhibitor can be separated into two peptides by gel filtration on Sephadex G-25; together these peptides account for the amino acid composition of the native inhibitor. Characterization of these peptides by end-group methods shows that the main site of chymotryptic action, under the conditions used, is a leucyl–seryl peptide bond located 29 residues in from the carboxy-terminal end of the inhibitor molecule. The sequence surrounding this sensitive bond is -Thr-Leu-Ser-Ile-Pro-. On this basis we propose that a lysyl–X peptide bond is the active site against trypsin and a leucyl–seryl peptide bond is the active site against chymotrypsin in lima bean trypsin inhibitor.

Limited proteolytic cleavage of soybean trypsin inhibitor and chicken ovomucoid by catalytic amounts of trypsin at acid pH results in the hydrolysis of a single arginyl–isoleucyl peptide bond in the former and a single arginyl–alanyl bond in the latter (Ozawa and Laskowski, Jr., 1966). Based on these observations and a literature survey of chemical modification studies on several other naturally occurring trypsin inhibitors, it was proposed (Ozawa and Laskowski, Jr., 1966) that these trypsin inhibitors contain either an arginyl–X or a lysyl–X trypsin-sensitive bond in their active site. More recent studies on the partial proteolysis of several other trypsin inhibitors have confirmed the existence of arginyl–X (Rigbi and Greene, 1968; Hochstrasser and Werle, 1969) and lysyl–X (Kress and Laskowski, Sr., 1968) active sites. A comprehensive study of 19 trypsin inhibitors by chemical modification (Fritz *et al.*, 1969) showed that their inhibitory activity could be abolished by modification of lysine residues in some and by modification of arginine residues in the others. Therefore the requirement of either an arginine or a lysine residue in the active site of trypsin inhibitors is well established (Haynes and Feeney, 1968a,b; Fritz *et al.*, 1969). However, whether or not the cleavage of the active-site peptide bond is a necessary intermediate in the formation of an enzyme–inhibitor complex as originally proposed (Finkenstadt and Laskowski, Jr., 1965) or is merely incidental, is still a matter of debate (Haynes and Feeney, 1968a,b).

Some naturally occurring inhibitors have dual inhibitory activities with independent inhibitory sites for trypsin and chymotrypsin. They have been termed "double headed" (Rhodes *et al.*, 1960). Turkey ovomucoid (Stevens and Feeney, 1963), lima bean trypsin inhibitor (Haynes and Feeney, 1967), and Bowman–Birk soybean inhibitor (Birk *et al.*, 1967) have all been cited as examples of double-headed inhibitors. In the case of the Bowman–Birk inhibitor it has also been shown that partial proteolysis with trypsin followed by carboxypeptidase B hydrolysis results in loss of trypsin inhibitory activity without affecting chymotrypsin inhibitory activity, while partial proteolysis with chymotrypsin results in loss of chymotrypsin inhibitory activity without any effect on the trypsin inhibitory activity (Birk *et al.*, 1967; Frattali and Steiner, 1969).

In this paper we give conclusive evidence for the double-headed nature of lima bean trypsin inhibitor. We also show the existence of a lysyl–X peptide bond in its active site against trypsin and identify a leucyl–seryl peptide bond in the active site against chymotrypsin. This leucyl–seryl chymotrypsin-sensitive peptide bond is located 29 amino acid residues from the carboxy-terminal end of the molecule in the sequence -Thr-Leu-Ser-Ile-Pro-.

Materials and Methods

Materials. Lima bean trypsin inhibitor (LBI 8KB and LBI 8DA) was obtained from Worthington (Freehold, N. J.). The exploratory experiments on partial proteolysis with trypsin and chymotrypsin were carried out with this material without further purification; the gel filtration studies on LBI[1] and its complexes were carried out with material further

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[1] Abbreviations used are: LBI, lima bean trypsin inhibitor; LBI_t',

purified by gel filtration on Sephadex G-75 (Jones *et al.*, 1963); the experiments on the localization and identification of the chymotrypsin-sensitive bond were carried out with LBI further purified by gel filtration and ion-exchange chromatography (Jones *et al.*, 1963). Component 3 (in their nomenclature) was used. Trypsin (TRL 8CA), chymotrypsin (CDI 7CG), leucine aminopeptidase (LAPC 9EB), carboxypeptidase A (COADFP 9DA), and carboxypeptidase B (COBDFP 9DA) were all obtained from Worthington (Freehold, N. J.). Aminopeptidase M was purchased from Henley and Co., Inc., (New York, N. Y.). BzTyrOEt and *p*-toluenesulfonyl-ArgOMe were purchased from Mann Research Laboratories (New York, N. Y.). Sephadex G-15, G-25, and G-75 were purchased from Pharmacia Fine Chemicals (Montreal, P. Q.). Guanidine hydrochloride, β-mercaptoethanol, and iodoacetamide were obtained from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were reagent grade or better.

Inhibitor Assays. The trypsin and chymotrypsin inhibitory activities of native and modified LBI were determined by the spectrophotometric method previously described (Rhodes *et al.*, 1957, 1960) with BzTyrOEt and *p*-toluenesulfonyl-ArgOMe as synthetic substrates for chymotrypsin and trypsin, respectively, and using a Coleman-Hitachi 124 recording spectrophotometer. The substrate–indicator solution was either added immediately after mixing of enzyme and inhibitor or else after a 15-min preincubation of the enzyme–inhibitor mixture. The results are expressed as a percentage of the inhibitory activity of native LBI determined with the 15-min preincubation assay.

Gel Filtration. Columns (2.5 × 90 cm) of Sephadex G-15, G-25, and G-75 were prepared according to the instructions given by the manufacturer.

Detection of Proteins and Peptides. Column effluents were monitored for protein by measuring the absorption at 280 mμ and for peptides samples of 200 μl were removed from alternate tubes and analyzed by the ninhydrin method after alkaline hydrolysis (Hirs *et al.*, 1956).

Amino Acid Analysis. Samples containing 0.05–0.2 μmole of protein or peptide were hydrolyzed with 6 N HCl at 110° in sealed, evacuated tubes. The analyses of native LBI were done in duplicate after 24-, 51-, and 75-hr hydrolysis. All other analyses were carried out, after 24-hr hydrolysis, on the Beckman-Spinco 120C automatic amino acid analyzer by the method of Spackman *et al.* (1958) as outlined in the Spinco manual.

Edman Degradation. The Edman degradation procedure was identical with that described by Kasper and Smith (1966) with the exception that the phenylthiohydantoin derivatives were not identified but the results are based on the subtractive method.

Hydrolysis of Peptides with Enzymes. Hydrolysis of peptides with carboxypeptidase A and leucine aminopeptidase were carried out as previously described (Stevens *et al.*, 1967). Aminopeptidase M hydrolysis was performed as described by Evans *et al.* (1968).

Trypsin Modification of LBI. LBI (50 mg) (Worthington) was dissolved in 4 ml of 0.1 M acetic acid–0.04 M calcium chloride and the pH was adjusted to pH 2.75 with glacial acetic acid. At zero time and again after 5 hr, 0.1 ml of trypsin (10 mg/ml in 0.004 M acetic acid–0.02 M calcium chloride) was added and the mixture was incubated at 37° for 22 hr. The pH, which did not change during the incubation, was then adjusted to pH 8.0 with 25% trimethylamine. A control sample was run under identical conditions, except that no trypsin was added. After adjusting the pH of the reaction mixture, the material was applied to a column (2.5 × 90 cm) of Sephadex G-75 previously equilibrated with 0.1 M ammonium bicarbonate. The inhibitor was eluted with the same buffer and isolated by freeze drying. To establish the effect of partial proteolysis, 5 mg of inhibitor or trypsin-modified inhibitor (LBI_t') was dissolved in 0.5 ml of 0.1 M Tris (pH 8.2). To this solution were added 50 μl of carboxypeptidase B solution (1.8 mg/ml) and the mixture was incubated at 37°. At appropriate time intervals samples for assay, chromatography, and amino acid analysis were removed. Chromatography was performed on Whatman No. 3MM paper with 1-butanol–acetic acid–water (200:30:75, v/v) as the solvent and amino acids were revealed with the ninhydrin–collidine reagent (Margoliash and Smith, 1962).

Chymotrypsin Modification of LBI. LBI (20 mg) (Worthington) was dissolved in 2 ml of 0.1 M acetic acid. This solution had a pH of 3.5. At zero time and again after 4 hr, 0.1 ml of chymotrypsin (10 mg/ml in 0.004 M acetic acid–0.02 M calcium chloride) was added and the mixture was incubated at 37° for 24 hr. The solution was then adjusted to pH 8.5 with 25% trimethylamine and brought to 4.8 ml with 0.1 M ammonium bicarbonate; 0.2 ml of carboxypeptidase A solution (4 mg/ml) was added and the mixture was incubated for 24 hr. Samples for assays, chromatography, and amino acid analyses were taken at appropriate time intervals. A control sample was run in parallel and was treated exactly the same except for the omission of chymotrypsin.

Identification of the Site of Peptide-Bond Cleavage in Chymotrypsin-Modified LBI (LBI_c'). For this experiment 110 mg of purified LBI component 3 (Jones *et al.*, 1963) was dissolved in 55 ml of 0.05 M acetic acid–0.04 M calcium chloride. The pH was adjusted to pH 3.1 with glacial acetic acid. Of this solution 50 ml (100 mg LBI) was used for partial proteolysis and 5 ml (10 mg LBI) was run through an identical scaled-down procedure as a control, treated in an identical fashion except for the omission of chymotrypsin. At zero time and again after 4 hr, 500 μl of chymotrypsin (15 mg/ml) in 0.004 M acetic acid–0.02 M calcium chloride was added and the mixture was incubated at 37° for 24 hr. A sample (100 μl) was taken at zero time and again after 24 hr for assay purposes and the rest of the incubation mixture was frozen and freeze dried. Reduction and carboxamidomethylation of native and chymotrypsin-modified LBI was carried out by a modification of the method of Hirs (1967). β-Mercaptoethanol (1 ml) (a 200–400-fold molar excess over the molarity of the disulfide bridges) was added to 5 ml of a 1–2% inhibitor solution in 5 M guanidine hydrochloride containing 0.2% EDTA and adjusted to pH 8.0 with 25% trimethylamine. During the course of the reaction, the pH was kept constant by the addition of 2 N sodium hydroxide with a Radiometer TTT11 pH-Stat. After 16 hr, 2.6 g of iodoacetamide in 1–2 ml of 1 N sodium hydroxide was slowly added in the dark and the pH was kept

trypsin-modified lima bean trypsin inhibitor; LBI_t'-COB, carboxypeptidase B treated trypsin modified lima bean inhibitor; LBI_c', chymotrypsin-modified lima bean inhibitor; RCAM, reduced and carboxamidomethylated.

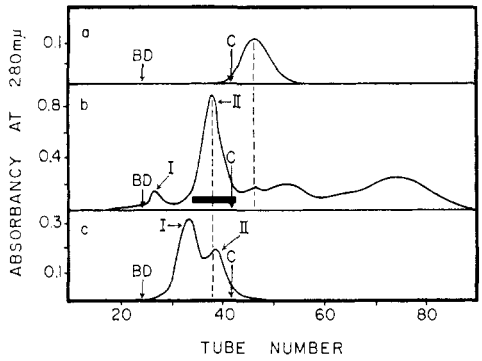

FIGURE 1: Gel filtration of LBI and its complexes on Sephadex G-75. Samples (1 ml) were run on a column (2.5 × 90 cm) of Sephadex G-75 previously equilibrated with 0.1 M ammonium bicarbonate. The eluent used was also 0.1 M ammonium bicarbonate, the flow rate 40 ml/hr and 6-ml fractions were collected. The elution volumes of blue dextran (BD) and chymotrypsin (C) are indicated by arrows. (a) 20 mg of LBI, (b) 20 mg of LBI to which 50 mg of trypsin have been added, and (c) 13.5 mg of peak II (part b) to which 3 mg of chymotrypsin has been added.

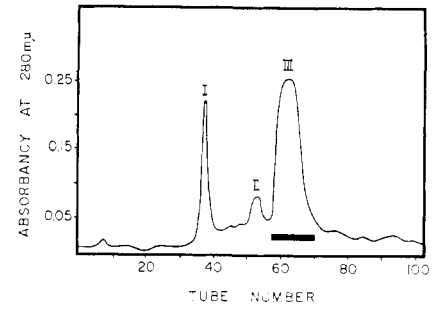

FIGURE 2: Gel filtration on Sephadex G-75 of reaction mixture containing LBI and trypsin. The sample (4.5 ml) was applied to a column (2.5 × 90 cm) of Sephadex G-75 (eluent 0.1 M ammonium bicarbonate; flow rate: 30 ml/hr; fractions: 4.8 ml/tube). Fractions were combined as indicated by bars.

constant at pH 8.0 with 4 N sodium hydroxide. When the base uptake had stopped, the reaction mixture was adjusted to pH 4.0 with glacial acetic acid. After removal of excess reagents by gel filtration on a column (2.5 × 90 cm) of Sephadex G-15, reduced carboxamidomethylated LBI_c' (RCAM-LBI_c') was separated into two peptide fractions on a column of Sephadex G-25 (2.5 × 90 cm) with 10% acetic acid as the eluent. The individual fractions were pooled, freeze dried, and characterized by amino acid analysis and end-group determination.

Results

Gel Filtration of LBI and Its Complexes. As previously shown (Haynes and Feeney, 1967), LBI is found to inhibit both trypsin and chymotrypsin. In our assay system 1 mg of inhibitor inhibits approximately 2.5 mg of trypsin and 1.0–1.5 mg of chymotrypsin.[2] Figure 1 summarizes the results of gel filtration experiments designed to demonstrate complexes between LBI and trypsin and/or chymotrypsin. On a column of G-75 LBI, trypsin,[3] and chymotrypsin have elution volumes of 275, 265, and 250 ml, respectively (Figure 1a). The material in peak II (Figure 1b) was pooled as indicated and freeze-dried. Its properties (elution volume 228 ml, no trypsin inhibitory activity, high chymotrypsin inhibitory activity) are those expected for a trypsin–LBI complex if the sites for trypsin and chymotrypsin are independent. The other peaks in Figure 1b are due to a small excess of LBI and to autolysis products. In Figure 1c peak I (elution volume 195 ml, no inhibitory activities) has the properties of a LBI–trypsin–chymotrypsin complex and peak II represents excess trypsin–LBI complex. Similar data are obtained when trypsin is added to a chymotrypsin–LBI complex (elution volume 215 ml, high trypsin inhibitory activity, no chymotrypsin inhibitory activity). These results offer substantial support to the proposal that LBI, a single molecule, inhibits both trypsin and chymotrypsin, and does so by binding them at different and independent sites.

Partial Proteolysis of LBI by Trypsin. Figure 2 shows the elution pattern obtained when the reaction mixture of 50 mg of LBI (Worthington) and 2 mg of trypsin, incubated for 22 hr at pH 2.75 and subsequently adjusted to pH 8.0, is applied to a Sephadex G-75 column and eluted with 0.1 M ammonium bicarbonate. Assays for inhibitory activities performed on selected fractions indicate that peak I has no inhibitory activity, peak II only inhibits chymotrypsin, and peak III inhibits both trypsin and chymotrypsin. Peak I probably consists of higher molecular weight material which normally contaminates commercial preparation of LBI (Jones *et al.*, 1963). Peak II is a trypsin–LBI complex and peak III is so called trypsin-modified LBI (LBI_t'). Table I gives the inhibitory activities of LBI_t' and LBI_t' treated with carboxypeptidase B for 3 hr (LBI_t'-COB). When the assay is carried out without preincubation LBI_t' and LBI_t'-COB have a residual trypsin inhibitory activity of ~85% when compared with LBI assayed in the same way or 65–70% when compared with LBI assayed using the 15-min preincubation period. When the modified samples are assayed using the 15-min preincubation assay, LBI_t' has regained full trypsin inhibitory activity, but LBI_t'-COB is still only 70% active as compared with native LBI. Prolonging the preincubation period for up to 240 min does not alter these results. Both LBI_t' and LBI_t'-COB retain their full chymotrypsin inhibitory activity. A time course study on the carboxypeptidase B hydrolysis of LBI and LBI_t' and subsequent identification of the hydrolysates by paper chromatography showed the rapid release of considerable amounts of lysine from LBI_t' compared with trace amounts released from LBI. No other amino acids were released from either sample. Amino acid analyses of the hydrolysates after 30-min incubation showed the release of 0.2–0.3 mole/mole of lysine from LBI_t' after correction of the values for the background

[2] The amount of trypsin inhibited is in agreement with the formation of a 1:1 molar complex between enzyme and inhibitor. The amount of chymotrypsin inhibited is less than expected for a 1:1 molar complex. However, by amino acid analysis of isolated trypsin–inhibitor and chymotrypsin–inhibitor complexes it can be shown that in both cases a 1:1 molar complex is formed. That less chymotrypsin than expected is inhibited, may be due to residual chymotryptic activity in the complex (J. Krahn and F. C. Stevens, unpublished data).

[3] When trypsin is subjected to gel filtration under these conditions a complex elution pattern is obtained. This is most probably due to autodigestion (Papaioannou and Liener, 1968). The largest component, believed to be native trypsin, is eluted with an elution volume of 265 ml.

TABLE I: Inhibitory Activities of Trypsin-Modified LBI.

Sample[b]	% Residual Inhibitory Act.[a] *vs.* Trypsin Preincubation: None	Trypsin Preincubation: 15 min	Chymotrypsin Preincubation: None	Chymotrypsin Preincubation: 15 min
Native LBI	80	100	100	100
Trypsin modified	65	100	100	100
Trypsin modified and COB treated	70	70	100	100

[a] The assays are described in the Materials and Methods section. The inhibition obtained by native LBI in the 15-min preincubation assay was taken as 100%. Controls treated as described in the text behaved in all cases as native LBI. [b] The trypsin-modified sample was prepared as described under Materials and Methods. The trypsin-modified COB-treated sample was treated with carboxypeptidase B for 3 hr.

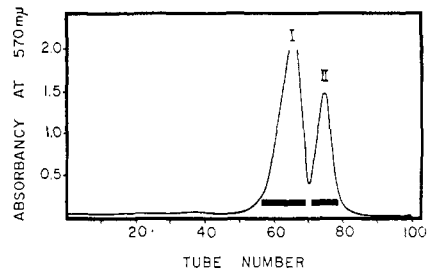

FIGURE 3: Gel filtration on Sephadex G-25 of RCAM-LBI$_c$'. The sample (4 ml) was applied to a column (2.5 × 90 cm) of Sephadex G-25 and elution was carried out with 10% acetic acid. The flow rate was 20 ml/hr, 3.3-ml fractions were collected, and 200-μl samples from alternate tubes were analyzed with the ninhydrin method after alkaline hydrolysis. Fractions were collected as indicated by the horizontal bars.

obtained from a carboxypeptidase B hydrolysate of LBI. There is no significant increase in the amount of lysine released upon prolonging the incubation to up to 6 hr. The assays (Table I) show that this release of 0.2–0.3 mole of lysine results in a loss of ~30% of the trypsin inhibitory activity without affecting the chymotrypsin inhibitory activity. In agreement with the hypothesis put forward by Ozawa and Laskowski, Jr. (1966), we think that incubation of LBI with catalytic amounts of trypsin results in an equilibrium mixture containing LBI and LBI in which a particular lysyl–X peptide bond has been cleaved. The latter reacts slower with trypsin and becomes inactive upon removal of the new carboxy-terminal lysine residue.

Partial Proteolysis of LBI by Chymotrypsin. After incubation at pH 3.5 with catalytic amounts of chymotrypsin for 21 hr and adjusting the resulting reaction mixture to pH 8.5, assays for residual inhibitory activities show that this chymotrypsin-modified LBI (LBI$_c$') is fully active against trypsin, but only retains ~30% of the original chymotrypsin inhibitory activity. The results obtained in the immediate and the 15-min preincubation assays are identical. While this work was in progress a paper appeared (Fratalli and Steiner, 1969) in which the authors show that the chymotrypsin inactivation of the Bowman–Birk soybean inhibitor is reversible upon preincubation with near equimolar amounts of chymotrypsin at neutral pH for long periods of time (up to 45 hr). When LBI$_c$' is tested for this type of reversal, it is found that preincubation under the conditions described (Frattali and Steiner, 1969) will indeed restore most of the original chymotrypsin inhibitory activity after approximately 48 hr. However, because of the time factor involved in the regeneration of this inhibitory activity, we refer to LBI$_c$' as a partially inactivated inhibitor. Incubation of LBI$_c$' with carboxypeptidase A and chromatography of samples taken at different time intervals show the immediate release of either leucine or isoleucine (these amino acids are not separated in our chromatographic system) and also small amounts of phenylalanine. A sample of the reaction mixture after a 24-hr incubation with carboxypeptidase A was subjected to amino acid analysis. The results expressed in moles per mole and corrected for the trace amounts of amino acids released under the same conditions from LBI are: Thr (0.19), Ser (0.12), Leu (0.93), Tyr (0.30), and Phe (0.32). This would indicate that the major site(s) of cleavage in LBI by incubation with catalytic amounts of chymotrypsin at low pH is at a leucyl–X peptide bond.

Site of Peptide-Bond Cleavage in LBI$_c$'. Gel filtration of reduced and carboxamidomethylated LBI$_c$' (RCAM-LBI$_c$') on Sephadex G-15 yields a single symmetrical peak at the exclusion volume of the gel. This indicates that if fragments are produced their molecular weight is probably >1500. Figure 3 shows the elution pattern obtained by gel filtration of RCAM-LBI$_c$' on a column of Sephadex G-25. Two well-separated peaks can be resolved. In the case of the control sample treated in the same way except for the omission of chymotrypsin, only a single peak is eluted from the Sephadex G-25 column. This single peak comes out in the exclusion volume. Table II gives the amino acid compositions of the small fragment (peak II of Figure 3), the large fragment (peak I of Figure 3), and the control sample (RCAM-LBI). The two fragments obtained, respectively, 29 and 69 residues long, account, within experimental error, for the amino acid composition of native and RCAM-LBI. It is not known whether the discrepancy between the cysteine values for both RCAM-LBI and the sum of the fragments on one hand and those found previously by ourselves and by Jones *et al.* (1963), on the other hand, are due to experimental error or actually reflect a true difference in components isolated from different batches of commercially available LBI. Both fragments are recovered in approximately equimolar amounts with an 80% recovery. The amino acid composition of the large fragment does not indicate contamination by native LBI. This is unexpected since the chymotrypsin-modified material retains 30% of the original chymotrypsin inhibitory activity, which now has to be attributed to LBI$_c$' which is believed to have at least one peptide bond broken but is held together by disulfide bonds. By comparison with the partial amino acid sequence which we have available for LBI component 4 (Celine G. L. Tan and Frits C. Stevens, data to be published) the small 29-residue fragment is known to represent the carboxy terminal of LBI. This identification is further substantiated by the results of end-group studies

TABLE II: Amino Acid Compositions of LBI, RCAM-LBI, and Fragments Obtained by Gel Filtration of Reduced and Carboxamidomethylated Chymotrypsin-Modified LBI.[a]

Amino Acid	Fragments			RCAM-LBI	LBI Component 3 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.

carried out on the two fragments. Table III gives the results of three cycles of the Edman degradation of the small fragment. Together with the results of leucine aminopeptidase hydrolysis (0.17 mole/mole of serine released in 4 hr) and aminopeptidase M hydrolysis (0.32 mole/mole of serine released in 24 hr) the data in Table III definitely established the amino-terminal sequence of the small fragment as Ser-Ile-Pro-. Figure 4 shows the results of a time study of the carboxypeptidase A hydrolysis of the large fragment and establishes its carboxy-terminal sequence as -Thr-Leu. The small amounts of phenylalanine which are also released from the large fragment are probably due to contamination by other fragments produced by nonspecific peptide-bond cleavage. On the basis of these results we identify the chymotrypsin-sensitive bond of LBI as a leucyl–seryl peptide bond located 29 residues in from the carboxy-terminal end of the molecule. The sequence surrounding this sensitive bond is -Thr-Leu-Ser-Ile-Pro-.

Discussion

We have shown that lima bean trypsin inhibitor, a molecule of mol wt <10,000, can inhibit both trypsin and chymotrypsin and does so by forming complexes with the enzymes at different and independent sites. That lima bean trypsin inhibitor is a double-headed inhibitor was first indicated by Haynes and Feeney (1967). The similarity between lima bean trypsin inhibitor and the Bowman–Birk soybean trypsin inhibitor is striking. They have comparable molecular weight, amino acid composition, and biological activity (Frattali, 1969). This similarity is further extended by comparing the results of partial proteolysis of lima bean inhibitor by trypsin and chymotrypsin described in this paper with published observations (Birk *et al.*, 1967; Frattali and Steiner, 1969) on the Bowman–Birk inhibitor.

In our attempts to characterize the active sites of lima bean trypsin inhibitor for trypsin and chymotrypsin we established, by partial proteolysis with catalytic amounts of trypsin at acid pH, the existence of at least one particular trypsin-labile lysyl–X peptide bond. That partial proteolysis did not result in extensive breakdown of the molecule is evident since the chymotrypsin inhibitory activity is unaffected by this treatment. The presence of seven to eight disulfide bonds (Jones *et al.*, 1963) in LBI could certainly account for this resistance to proteolysis and would also most probably place the trypsin-sensitive bond in a disulfide loop as has been suggested in the case of other inhibitors. The presence of a trypsin-sensitive lysyl–X bond is in agreement with the active-site proposal of Ozawa and Laskowski, Jr. (1966), in which it was proposed that the active sites of trypsin inhibitors consist of lysyl–X or arginyl–X peptide bonds. Our results on the partial proteolysis of LBI by trypsin indicate that our reaction mixture contains approximately 30% modified (lysyl–X bond cleaved) and 70% native (lysyl–X bond intact) inhibitor. In the case of Kunitz soybean inhibitor (Niekamp *et al.*, 1969) the equilibrium mixture

TABLE III: Subtractive Edman Degradation of the Small Fragment (Peak II of Figure 3).

Amino Acid	No. of Cycles Moles of Amino Acid/Mole of Peptide			
	0	1	2	3
Lysine[a]	0.99 (1)	(1)	(1)	(1)
Histidine[a]	0.96 (1)	(1)	(1)	(1)
S-Carboxymethyl-cysteine[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 (1)	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)	1.30 (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 sequence information (C. G. L. Tan and F. C. Stevens, data to be published) it is known that Lys and His are located in the carboxy-terminal-half of this fragment. Therefore the amino acid hydrolysates after Edman degradation were applied to the long column only. [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 the amino-terminal residue. During Edman degradation difficulties were encountered in the determination of *S*-carboxymethylcysteine some of which appeared on the analysis as cysteine and cysteic acid.

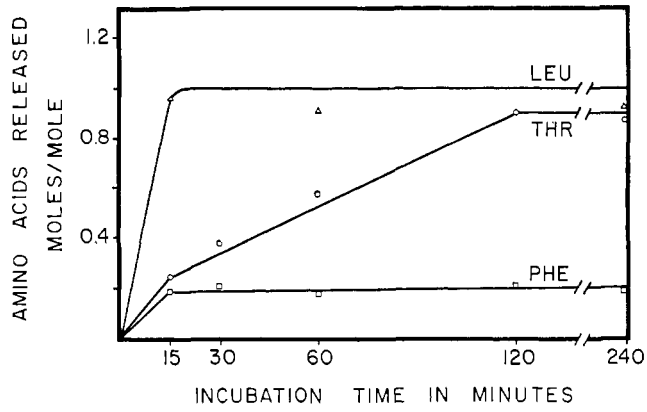

FIGURE 4: Release of free amino acids upon carboxypeptidase A digestion of the large RCAM fragment (peak I, Figure 2). The results are expressed as moles of amino acid released per mole of peptide. Details are given in the text.

contained 86% modified and 14% native inhibitor; in the case of bovine pancreatic secretory trypsin inhibitor (Rigbi and Greene, 1968) a mixture containing only about 25% modified inhibitor could be obtained under similar conditions. The latter both contain an arginyl–isoleucyl peptide bond in their active site. The difficulty in obtaining, by partial proteolysis, modified trypsin inhibitors of the lysyl-X type has been previously observed (Kress and Laskowski, Sr., 1968; Sealock and Laskowski, Jr., 1969). In fact, substitution of the active-site arginine residue by a lysine residue in Kunitz soybean inhibitor results in an active inhibitor which is converted ten times more slowly into the modified form (Sealock and Laskowski, Jr., 1969). That lima bean trypsin inhibitor requires at least one lysine residue for its trypsin inhibitory activity has been well established by chemical modification studies (Haynes and Feeney, 1967, 1968b; Fritz *et al.*, 1969). It has also been shown that the lysine residues can be converted into homoarginine residues by guanidination without seriously affecting trypsin inhibitory activity. Our results show by partial proteolysis that there is a specific trypsin-sensitive lysyl–X peptide bond in lima bean inhibitor and that removal of that lysine by carboxypeptidase B after cleavage of the bond results in inactivation as far as the trypsin inhibitory activity is concerned. Because of the difficulty in obtaining enough of the inhibitor with the lysyl–X bond broken we have been unable to characterize the product in any greater detail.

Since the anti-trypsin active site of trypsin inhibitors resembles a trypsin substrate (Arg-X, Lys-X), it is reasonable to assume, as a working hypothesis, that the active site of chymotrypsin inhibitors would resemble a chymotrypsin substrate. Based on the observation that the chymotrypsin inhibitory activity of the Bowman–Birk inhibitor is affected by incubation with catalytic amounts of chymotrypsin at low pH it was proposed, but not proven (Frattali and Steiner, 1969) that the anti-chymotrypsin active site could be in the form of a tyrosine–X or a phenylalanine–X peptide bond. Our results with lima bean trypsin inhibitor conclusively show the existence of a chymotrypsin-sensitive leucyl–seryl peptide bond located 29 residues in from the carboxy-terminal end of the molecule in the following sequence: -Thr-Leu-Ser-Ile-Pro-. Hydrolysis of this peptide bond results in loss of most of the chymotrypsin inhibitory activity of the inhibitor, without affecting its trypsin inhibitory activity. The susceptibility of certain leucyl–X peptide bonds to chymotryptic hydrolysis has been known for some time (Hill, 1965). In analogy to the case of trypsin inhibitors we propose the leucyl–seryl peptide bond, located in the sequence -Thr-Leu-Ser-Ile-Pro-, as the anti-chymotrypsin active site of lima bean inhibitor. Both the anti-trypsin and anti-chymotrypsin active sites of lima bean inhibitor therefore resemble substrates of the respective enzymes. In this way these naturally occurring inhibitors may be analogous to synthetically produced active-site-directed inhibitors which resemble substrates but form unproductive enzyme–substrate complexes.

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