Peptide-Bond Hydrolysis Equilibria in the Antitrypsin Site of Lima Bean Protease Inhibitor†

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ABSTRACT: It has been previously shown (Krahn and Stevens (1972), *Biochemistry 11*, 1804) that the trypsin inhibitory site of lima bean protease inhibitor contains a trypsin-sensitive lysylseryl peptide bond. We have now studied, in considerable detail, the hydrolysis equilibria governing this particular peptide bond. Incubation of lima bean protease inhibitor with catalytic amounts of trypsin at pH 3.1 for extended periods of time ultimately results in an equilibrium mixture containing ~10% native (peptide bond intact) and 90% trypsin-modified (peptide bond cleaved) inhibitor. After separating the equilibrium mixture into its components by chromatography on DEAE-cellulose, it can be shown that under identical conditions the same equilibrium mixture is obtained from pure trypsin-modified inhibitor. It can further be shown that carboxypeptidase B treatment of trypsin-modified inhibitor results in the quantitative removal of a new carboxy-terminal lysine residue (which results from the hydrolysis of the reactive site peptide bond) and a concomitant loss of its trypsin inhibitory activity; a similar treatment of native inhibitor has no effect on its biological activity and does not result in release of lysine. At near neutral pH the formation of an equimolar complex between native inhibitor and trypsin is virtually instantaneous; on the other hand the formation of the complex between trypsin-modified inhibitor and trypsin is relatively slower (10–20 min under our conditions). When the latter complex is dissociated by rapid acidification with trichloroacetic acid, one can isolate from the reaction mixture an inhibitor which behaves like native inhibitor, *i.e.*, it consists of a single polypeptide chain, it does not have a carboxy-terminal lysine, it is not inactivated by treatment with carboxypeptidase B, and it combines with trypsin almost instantaneously. These findings demonstrate that the peptide-bond hydrolysis in the trypsin-reactive site of lima bean inhibitor is a truly reversible reaction.

On the basis of experiments with Kunitz soybean trypsin inhibitor and chicken ovomucoid, Finkenstadt and Laskowski (1965) first proposed the hypothesis that the reaction between trypsin and these naturally occurring inhibitors involves cleavage of one particular trypsin-sensitive bond in the inhibitor, followed by the formation of a covalent bond between trypsin and the inhibitor (possibly an acyl bond between the active seryl of trypsin and the newly formed carboxy terminal of the inhibitor). In a subsequent paper Ozawa and Laskowski (1966) further postulated that all naturally occurring trypsin inhibitors have either an Arg-X or a Lys-X trypsin sensitive bond in their reactive site. On the basis of further kinetic and thermodynamic studies, carried out mainly with Kunitz soybean trypsin inhibitor, Laskowski and coworkers (Laskowski, 1970; Laskowski *et al.*, 1971; Laskowski and Sealock, 1971) have argued, convincingly, that the reaction between enzyme and inhibitor can best be represented by the following scheme

$$T + I \rightleftharpoons TI \rightleftharpoons T + I^*$$

where T is trypsin, I is the original intact inhibitor, TI is the trypsin–inhibitor complex, and I* is the trypsin-modified inhibitor in which the reactive site peptide bond is cleaved. Since this original proposal was formulated, considerable evidence in favor of this "reactive site model" for the mechanism of action of trypsin inhibitors has accumulated; a recent review by Laskowski and Sealock (1971) summarizes this evidence and also discusses some of the objections to this model. The active site identification of several naturally occurring inhibitors has recently been carried out either by chemical modification or by the partial proteolysis method pioneered by Ozawa and Laskowski (1966). However, extensive kinetic and thermodynamic studies of the reactions of trypsin with native and trypsin-modified inhibitors have been limited primarily to those of Kunitz soybean trypsin inhibitor. In a recent paper Kowalski and Laskowski (1972) pointed out that mere hydrolysis of a peptide bond of the inhibitor at low pH by the enzyme does not constitute proof that this peptide bond is in the reactive site of the inhibitor. Additional proof can be provided by (1) the complete inactivation of the trypsin-modified inhibitor by removal of the newly formed carboxy-terminal residue by treatment with carboxypeptidase (Finkenstadt and Laskowski, 1965) and (2) the resynthesis of the reactive site peptide bond by kinetic control dissociation of a complex formed between trypsin and trypsin-modified inhibitor (Hixson and Laskowski, 1970). Furthermore, it appears that a low K_{hyd} is also characteristic of, but not necessarily unique to, reactive site peptide bonds in proteinase inhibitors (Niekamp *et al.*, 1969; Kowalski and Laskowski, 1972); as a result, the existence of a true equilibrium between native and enzyme-modified inhibitor can therefore usually be established.

In the present paper we show that the lysylseryl peptide bond which we proposed in the trypsin reactive site of lima bean protease inhibitor (LBI)[1] (Krahn and Stevens, 1970; Tan and Stevens, 1971; Krahn and Stevens, 1972) meets these three criteria. Since LBI is very different from Kunitz soybean

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[1] Abbreviations used are: LBI, lima bean protease inhibitor; LBI′, trypsin-modified lima bean protease inhibitor.

inhibitor in both chemical and physical properties these results lend further support for the generality of the reactive site model of Laskowski and coworkers (Laskowski and Sealock, 1971).

Materials and Methods

Materials. Lima bean protease inhibitor (LBI 1DA), bovine trypsin (TRL 0FA), bovine chymotrypsin (CDI 8GA), and carboxypeptidase B (COBDFP 9DA) were all obtained from Worthington (Freehold, N. J.). The inhibitor was further purified as described by Jones *et al.* (1963) and all studies were performed with purified component I (terminology of Jones *et al.*). Three times crystallized porcine trypsin (4-36-564 UK6) was obtained from Miles Laboratories Inc. (Kankakee, Ill.). Sephadex G-25 and G-75 were obtained from Pharmacia Fine Chemicals (Montreal, Canada) and DEAE-cellulose (Cellex D) was purchased from Bio-Rad Laboratories (Richmond, Calif.). Benzoyltyrosine ethyl ester and *p*-toluenesulfonylarginine methyl ester were purchased from Mann Research Laboratories (New York, N. Y.). All other chemicals were reagent grade or better.

Inhibitor Assays. These were performed as previously described (Krahn and Stevens, 1972). The substrate–indicator solution was either added immediately after mixing of enzyme and inhibitor or after previous incubation of the enzyme–inhibitor mixture in the assay medium (0.006 M Tris-HCl, pH 8.2) at room temperature for the appropriate time.

Amino Acid Analysis. Samples containing 0.05–0.2 μmol of protein were hydrolyzed with 6 N HCl at 110° in sealed evacuated tubes for at least 22 hr and the analyses were carried out using a Spinco 120C analyzer by the method of Spackman *et al.* (1958) as outlined in the instrument manual.

Detection of Protein. Column effluents were monitored either by absorption at 280 nm or by the ninhydrin method after alkaline hydrolysis (Hirs *et al.*, 1956). Proteins were usually quantitated by weighing and in some cases also by amino acid analysis of a hydrolyzed sample of the protein solution. Approximate molecular weights of 10,000 g/mol for LBI and 25,000 g/mol for trypsin were used for calculating molar concentrations.

Carboxypeptidase B Digestion. Hydrolyses of proteins with carboxypeptidase B were carried out as previously described (Stevens *et al.*, 1967). Quantitation of the amino acids released by the hydrolyses was by amino acid analysis.

Studies on the Equilibrium between LBI and Trypsin-Modified LBI (LBI'_t). In a typical experiment 20 mg of LBI was dissolved in 2 ml of 0.018 M *trans*-aconitate containing 0.04 M $CaCl_2$ with a resulting pH of 3.1. To this solution was added 200 μl of either bovine or porcine trypsin (5 mg/ml in the same buffer) and the mixture was incubated at room temperature. At appropriate times 50-μl samples were withdrawn, quick-frozen in a Dry Ice–ethanol mixture, and stored at −20° until the assays for inhibitory activity were performed. The conversion of LBI'_t to LBI was carried out in an identical fashion except that the starting material in this case was purified LBI'_t (see below) and the amount of porcine trypsin used was decreased to 25% of that used in the LBI → LBI'_t conversion in order to slow down the reaction.

Carboxypeptidase B Treatment of Reaction Mixtures. From previous studies (Krahn and Stevens, 1970, 1972) it is known that the trypsin inhibitory activity of trypsin-modified but not of native LBI is inactivated by treatment with carboxypeptidase B. To determine the extent of trypsin modification of LBI as a function of time the reaction mixture samples (50 μl) were adjusted to pH ~8.0 by addition of 40 μl of 0.1 M borate, pH 10.4; to this solution 25 μl (3 mg/ml) of carboxypeptidase B was added and the mixture was incubated overnight at 37°. The samples were then diluted with 0.006 M Tris-HCl buffer, pH 8.2, to the appropriate concentration and assayed for their trypsin and chymotrypsin inhibitory activity.

Separation of LBI and LBI'_t on DEAE-Cellulose. After the conversion of LBI to LBI'_t had reached a plateau, trypsin and buffers were removed by gel filtration on Sephadex G-75, using 10% aqueous acetic acid as the eluent. After freeze-drying, the mixture of LBI and LBI'_t was dissolved in 0.01 M sodium borate, pH 8.6, and applied to a column (1.5 × 90 cm) of DEAE-cellulose previously equilibrated with the same buffer. Elution was carried out with an exponential salt gradient through a 1-l. mixing chamber (originally containing 0.01 M borate, pH 8.6), the limiting buffer containing 0.01 M borate and 0.4 M sodium chloride (pH 8.6). Appropriate fractions were pooled, dialyzed against water, lyophilized, and then subjected to rechromatography under identical conditions.

Dissociation of the Trypsin–LBI'_t Complex. LBI'_t (2 mg) was dissolved in 2 ml of 0.001 M NaOH and a slight molar excess (6 mg) of bovine trypsin was added to the solution; this mixture was allowed to stand at room temperature for 30 min and at this time 2 ml of 5% trichloroacetic acid was added and the mixture allowed to stand overnight at 0–4°. The solution was then centrifuged and the precipitate (trypsin) was discarded.[2] The supernatant containing the inhibitor was then dialyzed against water and lyophilized. The inhibitor obtained in this manner was further characterized by amino acid analysis, trypsin and chymotrypsin inhibitory activity, effect of carboxypeptidase B treatment on its biological activity, and high-voltage paper electrophoresis of the reduced, carboxymethylated inhibitor.

High Voltage Paper Electrophoresis. High voltage paper electrophoresis was carried out in Savant electrophoresis tanks using Whatman No. 3MM paper and pH 1.9 acetic acid–formic acid buffer (25 ml of 98–100% formic acid and 87 ml of glacial acetic acid to 1 l. with water). The electrophoretograms were developed with ninhydrin.

Reduction and Carboxymethylation of LBI and LBI'_t. Reduction and carboxymethylation were performed as described by Sondack and Light (1971), using dithioerythritol (Cleland, 1964) as the reducing agent. The inhibitor was dissolved in 0.1 M Tris-HCl buffer, pH 8.5, containing 0.01 M dithioerythritol and 1 mg of EDTA per ml to give a final protein concentration of approximately 2 mg/ml. The reaction mixture was allowed to stand for 1 hr at room temperature at which time an equal volume of 0.2 M Tris, pH 8.5, containing 0.05 M sodium iodoacetate was added and the mixture allowed to stand at room temperature for an additional hour. The reagents are then removed by gel filtration on Sephadex G-25. By amino acid analyses we have ascertained that these conditions result in cleavage of all the disulfide bonds in the inhibitor and quantitative conversion of cystine to *S*-carboxymethylcysteine.

Results

Conversion from LBI to LBI'_t. Figure 1 shows the conversion between LBI and LBI'_t using catalytic amounts of trypsin at

[2] Seidl and Liener (1971) working with Bowman–Birk soybean inhibitor used similar conditions and showed that under these conditions trypsin denatures and precipitates out of solution while the inhibitor remains soluble. Our own observations with LBI agree with their data.

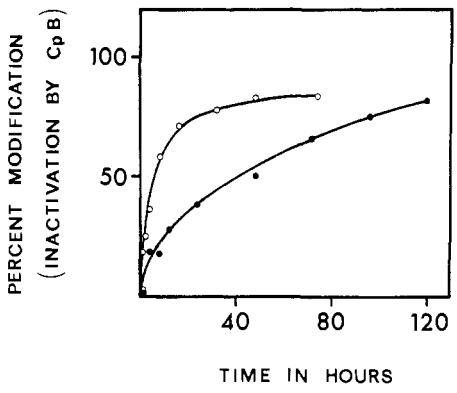

FIGURE 1: Modification of LBI by 2 mol % trypsin at pH 3.1 and 25°. The per cent modification of LBI was determined by measuring the trypsin inhibitory activity of a carboxypeptidase B treated sample in a 15-min preincubation assay. Experiment performed with: (○), porcine trypsin; (●), bovine trypsin.

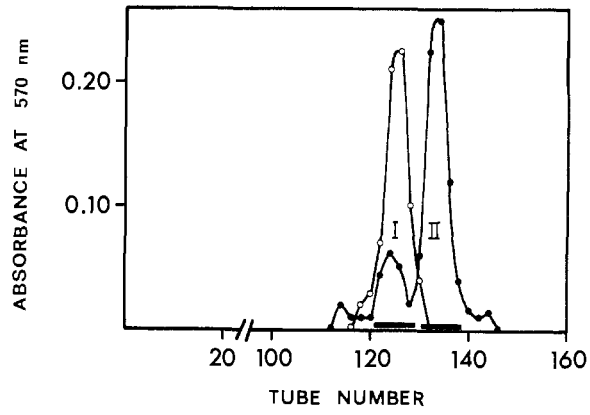

FIGURE 2: Ion exchange chromatography of native and trypsin-modified LBI. The sample (10 mg) was dissolved in 0.01 M sodium borate at pH 8.6 and applied to a column (1.5 × 80 cm) of DEAE-cellulose previously equilibrated with the same buffer. The column was eluted as described in the text; the flow rate was 29 ml/hr and effluent fractions of 4.2 ml were collected. Samples of 200 μl were taken from alternate tubes for ninhydrin determination after alkaline hydrolysis. The fractions were pooled as indicated by the solid bars, desalted, and lyophilized: (○), experiment with 10 mg of native LBI; (●), experiment with 10 mg of the reaction mixture containing both native and trypsin-modified LBI.

pH 3.1. It can be seen that the reaction approaches a steady state plateau of approximately 85% LBI_t' and 15% LBI with either bovine or porcine trypsin as the catalyst; porcine trypsin appears to be a better catalyst for this reaction than is bovine trypsin. This may be due to the greater stability of porcine trypsin under the reaction conditions. It can also be seen that the actual steady state plateau has not yet been reached even after 120 hr of incubation. In separate experiments run under identical conditions but for longer periods of time we obtain a reaction mixture containing ~90% LBI_t'; at this stage the percentage distribution of LBI and LBI_t' in the reaction mixture did not change following a doubling of trypsin and further incubation, indicating that the composition of the reaction mixture is independent of the amount of enzyme used. In order to demonstrate the existence of a true equilibrium between LBI and LBI_t' it is necessary to demonstrate that the same reaction mixture can be obtained in the reverse direction. It is therefore necessary to first obtain pure LBI_t'.

Separation of LBI and LBI_t'. Since LBI_t' is identical with LBI except for the cleavage of one peptide bond, it can be expected that it has a greater negative charge at pH 8.6, because of the presence of one extra carboxyl group. As a result it should be possible to effect a separation of these two species by ion-exchange chromatography on DEAE-cellulose at pH 8.6, using a salt gradient. The results of such a separation are illustrated in Figure 2. As expected, the native inhibitor emerges from the column first followed by the modified inhibitor which is retained more strongly. The components separated by the column were characterized by the release of lysine and the loss of trypsin inhibitory activity as a result of treatment by carboxypeptidase B. As shown in Table I such treatment with carboxypeptidase B results in the loss of 80% of the trypsin inhibitory activity of the sample applied to the column with a concomitant release of 0.8 mol/mol of lysine; this indicates that that sample contains 80% LBI_t' and 20% LBI. By the same criteria, fraction I (Figure 2) consists mainly (75%) of LBI whereas fraction II (Figure 2) is approximately 90% pure LBI_t'. By rechromatography of fraction II on DEAE-cellulose under the same conditions one obtains 98–100% pure LBI_t' (Table I).

TABLE I: Purification of LBI_t' as Monitored by Carboxypeptidase B Treatment.

Sample[a]	Lysine Released (mol/mol[b])	% Inactivation[c]
LBI–LBI_t' reaction mixture	0.80	83
DEAE-I	0.25	26
DEAE-II	0.85	93
DEAE-II (rechromatographed)	1.06	98

[a] DEAE-I and DEAE-II are the fractions obtained by ion exchange chromatography of the reaction mixture on DEAE-cellulose as illustrated in Figure 2. DEAE-II (rechromatographed) is obtained by rechromatography of fraction II under identical conditions. [b] Lysine released from the sample by treatment with carboxypeptidase B and subsequent amino acid analysis as described under Methods. [c] Per cent inactivation is calculated from the inhibitory activity of the carboxypeptidase B treated sample in a 15-min preincubation assay.

Conversion from LBI_t' to LBI. LBI_t' purified as described above was subjected to conditions similar (see Materials and Methods) to those used for the trypsin modification of LBI, *i.e.*, catalytic amounts of porcine trypsin at pH 3.1. Figure 3 illustrates that the reaction mixture becomes more resistant to loss of inhibitory activity by treatment with carboxypeptidase B as a function of time, implying that LBI_t' is being converted to LBI. The reaction is seen to approach a steady state plateau; at that stage the reaction mixture contains approximately 92% LBI_t' and 8% LBI. In a separate experiment under similar conditions but allowing the reaction to proceed for 120 hr the final position was ~90% LBI_t', 10% LBI. Within experimental error this distribution between LBI and LBI_t' is identical with that obtained in the forward direction.

Kinetics of Complex Formation between Trypsin and LBI or LBI_t'. Figure 4 compares the time dependency of the complex formation between bovine trypsin and native or trypsin-modified inhibitor as measured by the loss of enzyme activity.

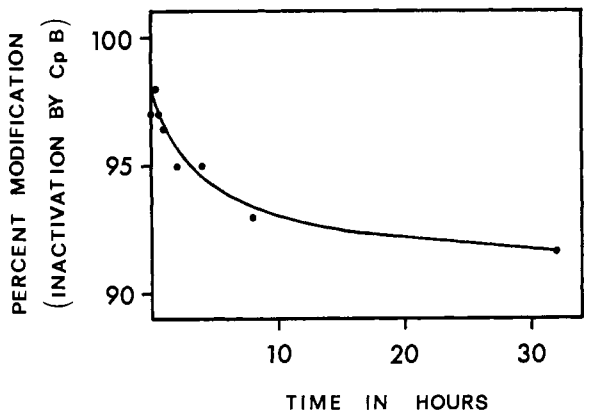

FIGURE 3: Conversion of LBI'_t to native LBI by 0.5 mol % of porcine trypsin at pH 3.1 and 25°. The per cent modification was determined by measuring the trypsin inhibitory activity of a carboxypeptidase B treated sample in a 15-min preincubation assay.

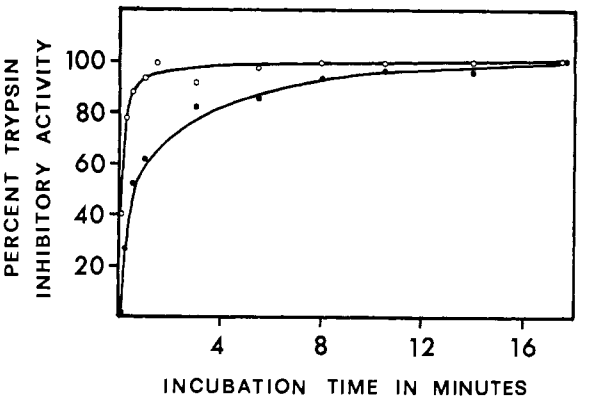

FIGURE 4: The trypsin inhibitory activity of native LBI and LBI'_t as a function of time of preincubation with near equimolar amounts of bovine trypsin. Mixtures containing approximately 45 μg of bovine trypsin and 15 μg of inhibitor in 1 ml of Tris buffer, 0.006 M, pH 8.2, were incubated at room temperature and at the appropriate times the residual trypsin activity was determined as described under Methods: (○), native LBI; (●), pure LBI'_t.

By this criterion complex formation between trypsin and LBI under our conditions is essentially complete in 60–90 sec while under the same conditions formation of the trypsin–LBI'_t complex is considerably slower (10–20 min).

Characterization of the Inhibitor Obtained by Rapid Dissociation of the Trypsin–LBI'_t Complex. Rapid dissociation of the trypsin–LBI'_t complex with trichloroacetic acid, as described in the Methods section, results in the precipitation of denatured trypsin and an inhibitor which behaves, by several criteria, like native inhibitor. As shown in Table II the inhibitor is no longer inactivated by treatment with carboxypeptidase B; also no lysine can be released by such a treatment. Positive proof of the resynthesis of the trypsin reactive site peptide bond of LBI'_t by complex formation with trypsin is offered in Figure 5. In agreement with the results of our previous studies (Krahn and Stevens, 1972), reduced and carboxymethylated LBI'_t definitely consists of two electrophoretic components, whereas reduced and carboxymethylated native LBI consists of a single electrophoretic component. The reduced and carboxymethylated inhibitor obtained by dissociating the LBI'_t–trypsin complex behaves essentially as the single electrophoretic component obtained from native LBI, although there appears to be a slight contamination with trace amounts of reduced carboxymethylated LBI'_t.

TABLE II: Trypsin Inhibitory Activity of Various Samples of LBI.

Sample	% Trypsin Inhibitory Activity[a]	
	Before Treatment[b] with Carboxypeptidase B	After Treatment[b] with Carboxypeptidase B
Native LBI	100	100
LBI'_t	100	2
Inhibitor from LBI'_t–trypsin complex[c]	100	100

[a] The inhibition obtained with native LBI in a 15-min preincubation assay was taken as 100%. [b] Samples were incubated either with or without carboxypeptidase B at 37° overnight (as described under Methods). [c] Inhibitor obtained from the LBI'_t–trypsin complex by kinetic control dissociation as described in the text.

Discussion

Although the existence of either an Arg-X or a Lys-X peptide bond sensitive to trypsin at acid pH has been demonstrated in several protein proteinase inhibitors, it has also been pointed out (Laskowski and Sealock, 1971; Kowalski and Laskowski, 1972) that rigorous proof of the involvement of such a peptide bond in the reactive site of an inhibitor can only be ascertained by results of a series of experiments designed to test the validity of the hypothesis. This has been done in

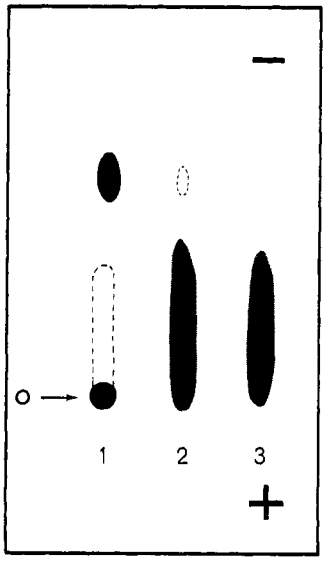

FIGURE 5: High-voltage paper electrophoresis of reduced, carboxymethylated native and trypsin-modified LBI. The material was subjected to high-voltage paper electrophoresis in formate–acetate buffer, pH 1.9, at 53 V/cm for 30 min at 10°. To visualize the protein the paper was stained with ninhydrin: (1) reduced carboxymethylated LBI'_t; (2) reduced carboxymethylated inhibitor obtained by kinetic control dissociation of the LBI'_t–trypsin complex; (3) reduced carboxymethylated LBI.

the case of the trypsin reactive site of Kunitz soybean inhibitor by Laskowski and coworkers. Because the "reactive site model" of protein proteinase inhibitors has been proposed as a general model, it is important to extend this kind of rigorous proof to other protein proteinase inhibitors. Lima bean protease inhibitor is very different from Kunitz soybean inhibitor in physical and chemical properties. Our previous studies on LBI have shown that it inhibits both trypsin and chymotrypsin at different and independent sites (Krahn and Stevens, 1971) and that the molecule contains a trypsin-sensitive Lys-Ser peptide bond (Krahn and Stevens, 1972) and a chymotrypsin-sensitive Leu-Ser peptide bond (Krahn and Stevens, 1970). These two enzyme-sensitive peptide bonds are found in two homologous regions of sequence which appear to have arisen by gene duplication (Stevens, 1971; Tan and Stevens, 1971). It should also be pointed out that recent studies on the Bowman–Birk soybean inhibitor by other workers (Frattali and Steiner, 1969; Birk and Gertler, 1971; Odani *et al.*, 1971, 1972; Odani and Ikenaka, 1972; Seidl and Liener, 1971, 1972a,b; Steiner, 1972) have shown that Bowman–Birk soybean inhibitor, as opposed to Kunitz soybean inhibitor, is homologous with and extremely similar to LBI.

The results presented in this paper show that lima bean trypsin inhibitor, at least as far as its trypsin inhibitory activity is concerned, conforms to the "reactive site model" for protein proteinase inhibitors. Because of the great similarity between LBI and Bowman–Birk soybean inhibitor the results probably also hold true for the latter.

We have demonstrated that there exists a true equilibrium between LBI (Lys-Ser bond intact) and LBI_t' (Lys-Ser bond cleaved) by showing that both the forward reaction (LBI $\rightarrow$ LBI_t') and the reverse reaction ($LBI_t' \rightarrow$ LBI) are possible and lead to the same equilibrium mixture of $\sim$90% LBI_t' and 10% LBI at pH 3.1 and room temperature. These results were obtained using LBI component I (terminology of Jones *et al.*, 1963); the percentage of LBI_t' in the reaction mixture reported here is considerably higher than previously reported by us (Krahn and Stevens, 1970, 1972); this discrepancy is due to the fact that in our previous studies we did not incubate the reaction mixture for long enough periods of time and as a result the reaction did not reach equilibrium. The values obtained here agree with those reported by Sakura and Timasheff (1971) for the LBI $\rightarrow$ LBI_t' conversion of LBI component I; however, a comparison between their results and ours is not really possible since their abstract does not specify the pH of the reaction mixture and does not indicate whether or not a steady state plateau had been reached. Similarly, previous studies on Bowman–Birk soybean inhibitor (Birk *et al.*, 1967; Frattali and Steiner, 1969; Odani and Ikenaka, 1972; Seidl and Liener, 1971) show up to 80% conversion from native to trypsin-modified inhibitor by catalytic amounts of trypsin at acid pH; in this case, however, no attempts were made to demonstrate that a steady state plateau had been reached or that the reverse reaction (conversion of trypsin-modified inhibitor to native inhibitor) took place under the same conditions to yield the same steady state plateau. Using Bowman–Birk soybean inhibitor it has been demonstrated (Frattali and Steiner, 1969; Steiner, 1972) that the trypsin-modified inhibitor could still form a complex with trypsin but did so much slower than native inhibitor. By polyacrylamide gel electrophoresis, Frattali and Steiner (1969) were able to demonstrate that upon rapid dissociation of a complex formed between the enzyme and the trypsin-modified inhibitor one could obtain a molecular species which behaved electrophoretically as native inhibitor. This "regenerated inhibitor" was, however, not further characterized. In the present study we have carried out similar experiments with trypsin-modified lima bean protease inhibitor. At pH 8.2 and room temperature LBI_t' reaches its full inhibitory capacity in 10–20 min. It is difficult to compare our data on LBI with those obtained with Bowman–Birk soybean inhibitor (Frattali and Steiner, 1969; Steiner, 1972) because their regeneration experiments were carried out under different conditions. By dissociation of the complex between LBI_t' and trypsin we were able to isolate from the reaction mixture a molecule which behaves identically with native LBI in its trypsin inhibitory capacity, its resistance to carboxypeptidase B digestion, and its electrophoretic behavior after complete reduction and alkylation. The latter treatment in the case of LBI_t' results in two peptides (Krahn and Stevens, 1972) as would be expected from a molecule in which one peptide bond had been cleaved but which was still held together by disulfide bonds. Laskowski and Sealock (1971) and Kowalski and Laskowski (1972) refer to this type of experiment as kinetic control dissociation; the complex is caused to dissociate in such a manner that once dissociation takes place the inhibitor can no longer recombine with the enzyme; in this way one gets a kinetic distribution of products rather than an equilibrium. The resynthesis of the cleaved peptide bond is felt to be strong evidence for the role of this peptide bond in the active site of the inhibitor (Kowalski and Laskowski, 1972). It also indicates that the peptide bond cleavage of protein proteinase inhibitors by trypsin at acid pH is not of a random nature but very specific and hence involved in some manner with the inhibition phenomenon.

Finkenstadt and Laskowski (1965) originally proposed that the trypsin inhibitor reaction consists of the cleavage of a peptide bond in the inhibitor followed by the formation of an ester bond between the active site seryl of trypsin and the newly formed carboxy terminal of the inhibitor. Considerable evidence argues in favor of peptide bond cleavage but so far it has not been possible to demonstrate the existence of a covalent bond between enzyme and inhibitor. Recent model building studies of Blow *et al.* (1972) suggest that in the association of bovine pancreatic trypsin inhibitor with trypsin and chymotrypsin the formation of an acyl-enzyme is stereochemically possible but final proof of its actual existence will probably have to await further high-resolution X-ray crystallographic studies of enzyme–inhibitor complexes.

Acknowledgments

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References

Birk, Y., Gertler, A., and Khalef, S. (1967), *Biochim. Biophys. Acta 147*, 402.

Birk, Y., and Gertler, A. (1971), *in* Proceedings of the International Research Conference on Proteinase Inhibitors, Fritz, H., and Tschesche, H., Ed., Berlin, Walter de Gruyter and Co., p 142.

Blow, D. M., Wright, C. S., Kukla, D., Ruhlmann, A., Steigemann, W., and Huber, H. (1972), *J. Mol. Biol. 69*, 137.

Cleland, W. W. (1964), *Biochemistry 3*, 480.

Finkenstadt, W. R., and Laskowski, M., Jr. (1965), *J. Biol. Chem. 240*, PC 962.

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