Antitrypsin Site of Lima Bean Protease Inhibitor†

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ABSTRACT: The antitrypsin site of lima bean protease inhibitor has been determined by partial proteolysis with catalytic amounts of porcine trypsin at acid pH. This treatment results in a mixture containing approximately 30% native inhibitor and 70% modified inhibitor in which one lysine–X peptide bond has been cleaved. Removal of the new carboxy-terminal lysine residue from the modified inhibitor with carboxypeptidase B results in loss of trypsin inhibitory activity without affecting its chymotrypsin inhibitory activity. After reduction and alkylation, the trypsin-modified inhibitor can be separated into two peptide fractions by ion-exchange chromatography on DEAE-Sephadex and high-voltage paper electrophoresis; together these peptides account for the entire amino acid composition of the native inhibitor. Characterization of the peptides by end-group methods shows that the site of tryptic action, under the conditions used, is a lysyl–seryl peptide bond located 28 residues in from the amino-terminal end of the molecule in the sequence Thr-Lys-Ser-Ile-Pro. This finding lends strong support to our hypothesis that the antitrypsin and antichymotrypsin sites of lima bean protease inhibitor are located in homologous regions of the polypeptide chain and have probably evolved by gene duplication.

Lima bean protease inhibitor (LBI)[1] strongly inhibits both trypsin and chymotrypsin by the formation of complexes with these enzymes at different and independent combining sites (Haynes and Feeney, 1967; Krahn and Stevens, 1970, 1971). In previous studies we have determined the antichymotrypsin site of LBI (Krahn and Stevens, 1970) and the complete amino acid sequence of LBI component IV (Tan and Stevens, 1971a,b). A striking feature of the primary structure of LBI is the occurrence of a repetitive sequence, with residues 50–61 clearly homologous to residues 23–34. The antichymotrypsin site, Leu^{55}-Ser^{56}, is located approximately in the middle of one of these homologous regions and the corresponding positions in the other region are occupied by Lys^{28}-Ser^{29} (Tan and Stevens, 1971b; Stevens, 1971). This, together with the knowledge that the antitrypsin site of LBI contains a Lys–X peptide bond (Krahn and Stevens, 1970), has led us to put forward the hypothesis that Lys^{28}-Ser^{29} may be the antitrypsin site of LBI and that the two active sites of this double-headed inhibitor are located in two homologous regions of the protein molecule (Tan and Stevens, 1971b).

Previous attempts to locate the antitrypsin site of LBI by partial proteolysis with bovine trypsin (Krahn and Stevens, 1970) were unsuccessful because the reaction mixture contained about 70% native inhibitor and only 30% modified inhibitor with the Lys–X bond cleaved (Krahn and Stevens, 1970). In the present study, we use porcine rather than bovine trypsin for the partial proteolysis of LBI at acid pH according to the technique pioneered by Laskowski and coworkers (Ozawa and Laskowski, 1966; Laskoswki, 1970). In this manner we were able to produce enough of the trypsin-modified inhibitor to be able to characterize it and to identify Lys^{28}-Ser^{29} as part of the antitrypsin active site. The results of this study, therefore, offer substantial support for our previous hypothesis (Tan and Stevens, 1971b).

Materials and Methods

Materials. Lima bean trypsin inhibitor (LBI-1EA) was obtained from Worthington (Freehold, N. J.) and further purified as described by Jones *et al.* (1963). For these studies we used purified component IV[2] in the terminology of Jones *et al.* (1963). Three times crystallized porcine trypsin (4-36-564 Batch UK6) was obtained from Miles Laboratories, Inc. (Kankakee, Ill.). Bovine trypsin (TRL-OFA), bovine chymotrypsin (CDI-7CG), and carboxypeptidase B (COB-DFP 9DA) were all obtained from Worthington (Freehold, N. J.). Sephadex G-15, G-25, and DEAE-Sephadex A-25 were obtained from Pharmacia Fine Chemicals (Montreal, P.Q.) and Bio-Gel P-10 from Bio-Rad Labs (Richmond, Calif). Benzoyltyrosine ethyl ester and *p*-toluenesulfonyl-arginine methyl ester were purchased from Mann Research Labs (New York, N. Y.). Guanidine hydrochloride, β-mercaptoethanol, iodoacetic acid, and iodoacetamide were obtained from Sigma Chemical Co. (St. Louis, Mo.). Phenylthiohydantoin (sequenal grade) was obtained from Pierce Chemical Co. (Rockford, Ill.). All other chemicals were reagent grade or better.

Inhibitor Assays. The inhibitory activities of native and modified LBI against bovine trypsin and chymotrypsin were determined by the spectrophotometric method previously described (Rhodes *et al.*, 1957, 1960). *p*-Toluenesulfonyl-arginine methyl ester and benzoyltyrosine ethyl ester were used as synthetic substrates for trypsin and chymotrypsin, respectively. The substrate–indicator solution was either added immediately after mixing of enzyme and inhibitor or

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[1] Abbreviations used are: LBI, lima bean protease inhibitor; LBI′, trypsin-modified lima bean protease inhibitor; CM-Cys, carboxymethylcysteine; RCAM, reduced and carboxamidomethylated.

[2] It has been our experience that there is considerable variation in different commercial preparations of LBI. As shown in Table II, the amino acid composition of the component IV isolated in this study differs slightly from that on which the amino acid sequence studies were performed (Tan and Stevens, 1971b).

TABLE I: Inhibitory Activities of Trypsin-Modified LBI.

Sample[a]	% Residual Inhibitory Act.[b] *vs.* Trypsin Preincubation: None	Trypsin Preincubation: 15 min	Chymotrypsin Preincubation: None
LBI, control	67	100	100
LBI, COB	67	100	100
LBI_t'	25	100	100
LBI_t', COB	23	38	100

[a] LBI, COB: control LBI treated with carboxypeptidase B for 2.5 hr; LBI_t': trypsin-modified LBI; LBI_t', COB: trypsin-modified LBI treated with carboxypeptidase B for 2.5 hr. [b] The inhibition obtained with 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.

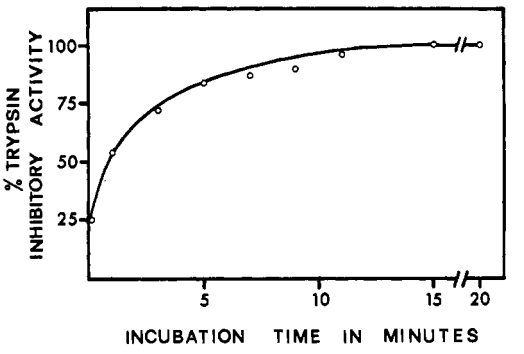

FIGURE 1: Time dependency of the regeneration of the trypsin inhibitory activity of LBI_t' by incubation with near molar amounts of trypsin. A mixture containing approximately 450 μg of bovine trypsin and 150 μg of LBI_t' in 10 ml of Tris buffer, 0.006 M, pH 8.2, was incubated at room temperature. At appropriate time intervals samples were taken out and then residual trypsin activity was determined as described under Methods.

after previous incubation of the enzyme–inhibitor mixture at room temperature for the appropriate time.

Detection of Proteins and Peptides. Column effluents were monitored by the absorption at 280 nm or, in the case of peptides, by the ninhydrin method after alkaline hydrolysis of 200-μl samples (Hirs *et al.*, 1956).

Amino Acid Analyses. Samples containing 0.05–0.2 μmole of protein or peptide were hydrolyzed with 6 N HCl at 110° in sealed evacuated tubes for at least 22 hr. To protect carboxymethylcysteine and tyrosine against destruction, 2 μl of thioglycollic acid and 50 μl of 5% phenol were added per ml of 6 N HCl as described by Howard and Pierce (1969). The analyses were carried out on the Spinco 120C 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; amino acid analysis of the residual peptides allowed identification of specific residues by the subtractive method.

Hydrolyses of Peptides with Enzymes. Hydrolyses of peptides and proteins with carboxypeptidase B were carried out as previously described (Stevens *et al.*, 1967).

Trypsin Modification of LBI. In a typical experiment 100 mg of LBI component IV was dissolved in 10 ml of 0.018 M *trans*-aconitate buffer, pH 3.0, containing 0.04 M $CaCl_2$. To this solution was added 1 ml of porcine trypsin (4 mg/ml in the same buffer) and the mixture was kept at room temperature for 46 hr. A control was run under identical conditions except that no trypsin was added. The reagents were removed by gel filtration on a column (2.5 × 90 cm) of Sephadex G-25 or Bio-Gel P-10 using 10% acetic acid as the eluent and the appropriate fractions were pooled and freeze-dried. Samples of both the control and trypsin-modified LBI were assayed for their inhibitory activities against bovine trypsin and chymotrypsin and compared to native inhibitor. To establish the effect of partial proteolysis, 3 mg each of control or trypsin-modified LBI was dissolved in 0.6 ml of 0.1 M Tris buffer, pH 8.2; to this solution 50 μl of carboxypeptidase B (2 mg/ml in the same buffer) was added and the mixture was incubated at 37° for 2.5 hr. Samples were then removed for amino acid analysis and for determination of inhibitory activities.

Identification of the Site of Peptide Bond Cleavage in Trypsin-Modified LBI. Approximately 130 mg of trypsin-modified LBI was reduced with mercaptoethanol and alkylated with either iodoacetamide or iodoacetic acid in the presence of 5 M guanidine·HCl as previously described by Krahn and Stevens (1970). After removal of the excess reagents by gel filtration on a column of G-15, reduced and alkylated trypsin-modified LBI separated into its component fragments by ion-exchange chromatography on DEAE-Sephadex followed by high-voltage paper electrophoresis or by high-voltage paper electrophoresis alone. The conditions of the ion-exchange chromatography are described under results. Preparative high-voltage paper electrophoresis was performed in pyridine–acetate buffer at pH 4.7 (0.025 M pyridine–0.035 M acetic acid) as previously described (Stevens *et al.*, 1967). The resulting fragments were characterized by amino acid analysis and end group determination.

Results

Partial Proteolysis of LBI with Trypsin. As shown in Table I, and qualitatively in agreement with previous results obtained by partial proteolysis of crude LBI with bovine trypsin (Krahn and Stevens, 1970), treatment of LBI component IV with catalytic amounts of porcine trypsin at acid pH does not effect its inhibitory activity against chymotrypsin but results in an inhibitor which combines more slowly with trypsin than does native LBI. This is illustrated by the fact (Table I) that in the immediate assay LBI_t' realizes only 25% of its potential trypsin inhibitory activity (*vs.* 67% for native LBI) but it is fully active against trypsin provided the assay mixture (which contains LBI_t' and near molar amounts of bovine trypsin) has been preincubated at room temperature for 15 min. The time dependency of the complex formation between LBI_t' and bovine trypsin, as measured by inhibitory activity, is illustrated in Figure 1. It can be seen that the full trypsin inhibitory activity potential of LBI_t' is obtained after 13–15 min preincubation. From the results in Table I it can also be seen that treatment of LBI_t' with carboxypeptidase B for 2.5 hr essentially destroys its potential for the time-dependent inhibition of trypsin. On the other hand, the chymotrypsin inhibitory activity of LBI_t'

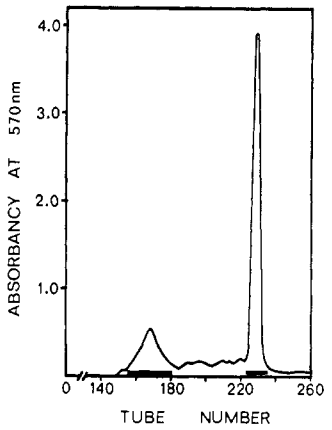

FIGURE 2: Ion-exchange chromatography of reduced, carboxamidomethylated LBI_t' on DEAE-Sephadex. The material (130 mg) was dissolved in 14 ml of 0.01 M phosphate buffer, pH 7.6, and applied to a column (2.5 × 90 cm) of DEAE-Sephadex previously equilibrated with the same buffer. The column was eluted with a linear salt gradient consisting of 1000 ml each of 0.01 M phosphate buffer, pH 7.6, and the same buffer containing 0.6 M sodium chloride. The flow rate was 24 ml/hr and effluent fractions of 3.7 ml were collected. Samples of 250 μl each were taken from alternate tubes for ninhydrin determination after alkaline hydrolysis. The fractions were pooled as indicated by the solid bars, taken to dryness by flash evaporation and subsequently desalted by gel filtration on a column of Sephadex G-25.

and the trypsin and chymotrypsin inhibitory activities of LBI are unaffected by this carboxypeptidase B treatment.

By amino acid analysis it was shown that in the case of LBI_t' the carboxypeptidase B treatment resulted in the release of 0.7 mole/mole of new carboxy-terminal lysine. In the case of the control LBI no amino acids, other than trace amounts also found in the carboxypeptidase B blank, were released after a 2.5-hr incubation with carboxypeptidase B. The data obtained both by assay of residual inhibitory activity and by amino acid analysis of the carboxypeptidase B digest of trypsin-modified LBI are in agreement with a reaction mixture containing approximately 30% native LBI and 70% LBI in which a Lys–X peptide bond has been cleaved.

Site of Peptide Bond Cleavage in LBI_t'. Gel filtration of reduced and alkylated LBI_t' 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. Attempts to separate the products by gel filtration on Sephadex G-25 or ion-exchange chromatography on Dowex 50-X2 were unsuccessful. As shown in Figure 2 reduced carboxamidomethylated LBI_t' could be separated into two ninhydrin-positive components by ion-exchange chromatography on DEAE-Sephadex under the conditions described in the figure legend. The first and most basic peak consisted of pure small fragment (see Table II); the second peak contained both uncleaved LBI and large fragment and these two components could be further separated by preparative high-voltage electrophoresis at pH 4.7. Alternatively, reduced carboxymethylated LBI_t' could also be separated into its component parts directly by preparative high-voltage paper electrophoresis at pH 4.7. As shown in Figure 3 three well-resolved, ninhydrin-positive spots, representing small fragment, large fragment, and uncleaved LBI, were obtained. Table II gives the amino acid composition of the products obtained after reduction and alkylation of trypsin-modified LBI together with the amino acid composition of native LBI-IV as determined in this

TABLE II: Amino Acid Compositions of Reduced Carboxamidomethylated LBI and of the Fragments Obtained after Reduction and Alkylation of Trypsin-Modified LBI.[a]

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

[a] Analyses were performed as described in the text. Tryptophan and methionine are known to be absent from LBI (Jones *et al.*, 1963). The results are expressed in moles per mole and were calculated on the basis of 1 residue of Ala in the case of column 1; 2 residues of Arg in the case of column 2; 2 residues of Arg and 4 residues of Ile in the case of column 4. [b] Fragments were eluted from paper with resulting high values for Gly. [c] Determined as *S*-carboxymethylcysteine. [d] Based on duplicate analyses of 24-, 48-, and 72-hr hydrolysates with values for Ser and Thr extrapolated to zero time. [e] Value taken from Tan and Stevens (1971b).

study and as determined by sequence determination (Tan and Stevens, 1971b). Comparison of the amino acid compositions of the two fragments with the published amino acid sequence of LBI (Tan and Stevens, 1971b) shows that the small fragment represents the amino-terminal end of the molecule while the large fragment represents its carboxy-terminal end. This identification is further substantiated by the results of end-group studies carried out on the two fragments. As can be seen from Figure 4, a time study of the digestion of the small fragment with carboxypeptidase B establishes its carboxy-terminal sequence as Ala-Thr-Lys, in agreement with the known sequence of residues 26–28.[3] By the substractive Edman procedure the amino-terminal sequence of the large fragment was determined as Ser-Ile-Pro in agreement with the sequence of residues 29–31 in the known sequence. On the basis of these results we identify the trypsin-sensitive bond of LBI as a lysyl–seryl peptide

[3] In the published sequence of LBI (Tan and Stevens, 1971b) position 25 is occupied by a leucine residue. The LBI variant on which the present study was carried out does not appear to have a leucine residue in that position. This discrepancy will require further investigation before any conclusion regarding the genetic differences between these two LBI variants can be drawn.

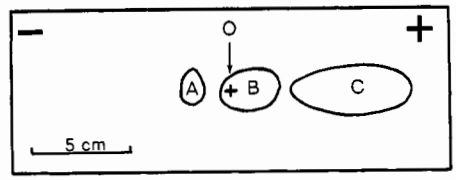

FIGURE 3: Preparative high-voltage paper electrophoresis of reduced, carboxymethylated LBI,'. The material was subjected to preparative high-voltage paper electrophoresis in pyridinium–acetate buffer, pH 4.7 at 53 V/cm for 30 min. Guide strips were stained with ninhydrin and the material was eluted with 30% acetic acid, taken to dryness by flash evaporation, and characterized by amino acid analysis after acid hydrolysis. In this way reduced carboxymethylated LBI,' could be separated into small fragment (A), uncleaved LBI (B), and large fragment (C).

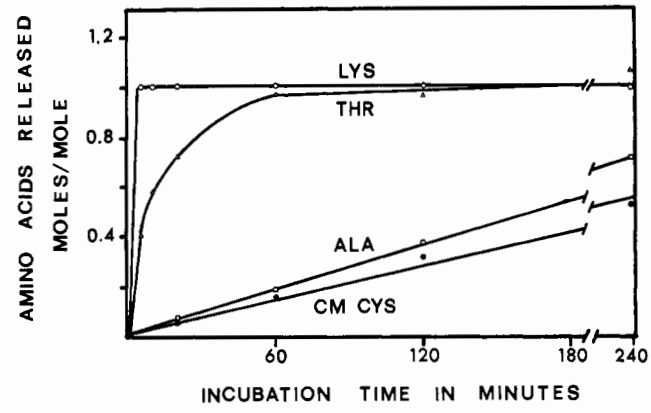

FIGURE 4: Release of free amino acids upon carboxypeptidase B digestion of the small fragment. The results are expressed as moles of amino acid released per mole of peptide. To 0.5 μmole of peptide dissolved in 625 μl of 0.1 M ammonium bicarbonate was added 25 μl of carboxypeptidase B (1.8 mg/ml), and the mixture was incubated at 20°. At appropriate time intervals samples were removed from the incubation mixture, frozen, lyophilized, and subjected to amino acid analysis. A control sample which did not contain peptide was run in an identical fashion; no amino acids were released from this control after up to 4 hr of incubation.

bond located in the amino-terminal third of the molecule in the sequence Ala-Thr-Lys-Ser-Ile-Pro.

Discussion

The results presented here are in agreement with the original hypothesis by Ozawa and Laskowski (1966) that trypsin inhibitors have a trypsin-susceptible Arg–X or Lys–X bond in their active site. Removal of the new carboxy-terminal lysine by carboxypeptidase B after cleavage of the trypsin-sensitive bond results in inactivation. Our results are also in agreement with chemical modification studies (Haynes *et al.*, 1967; Fritz *et al.*, 1969) which showed that the trypsin-inhibitory activity of LBI could be abolished by chemical modification of lysine residues. It is interesting to note that in our previous studies (Krahn and Stevens, 1970), using bovine trypsin for the partial proteolysis, we obtained a mixture of 70% native and 30% modified LBI, while in the present study, using porcine trypsin under essentially identical conditions, our reaction mixture contained ~30% native and ~70% modified LBI. The reason for this difference is at present not clear. It is obvious from thermodynamic considerations that if the same reaction is catalyzed by two different enzymes the final equilibrium attained must be the same. In these studies no attempts to reach an equilibrium mixture were made and the different reaction mixtures obtained with bovine and porcine trypsin may simply reflect differences in the rates of these two enzymes. It is also possible that there is a difference in the stability of the two enzymes and that bovine trypsin gets inactivated long before an equilibrium can be reached. Sakura and Timasheff (1971) have recently reported that using LBI fraction I (nomenclature of Jones *et al.*, 1963), they obtained a 90% conversion to modified inhibitor by incubation with catalytic amounts of trypsin.

From the results presented here and in agreement with previous studies, it is clear that the specific cleavage of a Lys–X peptide bond in LBI results in an inhibitor which combines more slowly with trypsin than does native LBI. Whether or not this slow complex formation between trypsin and the modified inhibitor involves the resynthesis of the cleaved peptide bond is open to discussion.

Based on the knowledge of the active sites of a small number of trypsin inhibitors, Laskowski and Sealock (1971) in a recent review have attempted to make some generalizations concerning these sites. They can be summarized as follows: (a) an enzyme-susceptible bond is required, (b) the amino-terminal residue in the cleaved site is Ile, Leu, or Ala, (c) the reactive site is in a disulfide loop, (d) there should not be any negative charges in the vicinity of the susceptible bond, (e) there should be a proline in the vicinity of the active site. Our results are in agreement with these suggestions with the exception that in our case the amino-terminal residue of the cleaved site is a serine. Birk and Gertler (1971) have recently reported that the trypsin and chymotrypsin inhibitory sites of the Bowman–Birk soybean inhibitor involve a Lys–Ser and a Leu–Ser peptide bond, respectively. We have previously also shown a Leu–Ser peptide bond in the anti-chymotrypsin active site of LBI (Krahn and Stevens, 1970). LBI and Bowman–Birk soybean inhibitor are very similar in molecular weight, amino acid composition, and biological activity (Steiner and Frattali, 1969). Unfortunately, the sequences surrounding the active sites of Bowman–Birk soybean inhibitor are not as yet known.[4]

When the data on the active sites of LBI against trypsin (this paper) and chymotrypsin (Krahn and Stevens, 1970) are extrapolated[5] to the known sequence of LBI, component IV (Tan and Stevens, 1971a,b), it becomes evident that, as previously suggested (Tan and Stevens, 1971b; Stevens, 1971), these two sites are located in two homologous regions of the sequence:

23 34
CMCys-CMCys-Leu-Ala-Thr-[Lys-Ser]-Ile-Pro-Pro-Glx-CMCys

50 61
CMCys-CMCys-Ile-Ser-Thr-[Leu-Ser]-Ile-Pro-Ala-Gln-CMCys

[4] Since the time this manuscript was first submitted for publication, the complete amino acid sequence of the Bowman–Birk inhibitor has been published (Odani *et al.*, 1971) and its trypsin reactive site has also been identified (Seidl and Liener, 1971). Both of these papers point out the striking similarities in sequence between Bowman–Birk inhibitor and LBI, particularly with reference to their proposed antitrypsin and antichymotrypsin sites.

[5] The studies on the antichymotrypsin site of LBI were carried out on LBI component III (nomenclature of Jones *et al.*, 1963) while the present studies were performed on a preparation of component IV which is slightly different from that on which the sequence studies were done. However, because of the obvious similarities in sequence in the immediate vicinity of the active sites as determined by us, we feel justified in extrapolating our results to the known sequence of LBI (Tan and Stevens, 1971b).

The trypsin-sensitive bond is located 28 residues from the amino-terminal end of the molecule while the chymotrypsin-sensitive bond is located 29 residues from the carboxy-terminal end of the molecule. Because of the sequence homology between the two active site regions it is very probable that they have arisen by gene duplication and that they would also show considerable structural similarity. A recent crystal structural analysis of the bovine pancreatic trypsin inhibitor (Huber *et al.*, 1970, 1971) showed it to be a pear-shaped molecule with the antitrypsin site freely exposed at the apex. As a working hypothesis we would like to propose that LBI, a double headed inhibitor, would contain two such exposed sites: one containing the Lys–Ser peptide bond specific for trypsin, the other containing the Leu–Ser peptide bond specific for chymotrypsin.

Acknowledgments

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