LIMA BEAN PROTEASE INHIBITOR: COMPARATIVE STUDY OF THE TRYPSIN AND CHYMOTRYPSIN INHIBITORY ACTIVITIES OF FOUR CHROMATOGRAPHICALLY PURE VARIANTS

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1. Introduction

Extensively purified commercial preparations [1] of lima bean protease inhibitor (LBI) can be further separated into at least four biologically active variants by ion exchange chromatography [2, 3]. These variants have similar but not identical amino acid compositions and tryptic peptide maps [2–4]. Previous studies [3–8] on the structure, biological activity and mode of action of LBI have been performed using either the mixture of variants or individual variant preparations. In the present study we provide the first comprehensive comparison of the inhibitory activities of four variants of LBI.

For the sake of clarity it should be recalled that LBI can inhibit both trypsin and chymotrypsin through the formation of equimolar complexes with these enzymes and that the binding sites for trypsin and chymotrypsin on LBI are distinct and independent [4–6]. We have previously identified a Lys–Ser peptide bond in the anti-trypsin site of LBI [4, 7, 8]; incubation of LBI with catalytic amounts of trypsin at acid pH results in an equilibrium mixture of native and trypsin-modified inhibitor (LBI'_t) in which this Lys–Ser peptide bond has been hydrolysed [7]. LBI'_t combines with trypsin slower than does LBI and therefore it appears less active than LBI in an assay system which does not involve preincubation between enzyme and inhibitor. Removal of the newly formed carboxyterminal lysine residue with carboxypeptidase B results in complete loss of trypsin inhibitory activity. Incubation of LBI'_t with near molar amounts of trypsin at neutral pH results in eventual complex formation and upon dissociation of the complex by acidification, native LBI with the Lys–Ser bond intact can be recovered from the reaction mixture [7, 8]. In similar fashion treatment of LBI with catalytic amounts of chymotrypsin at acid pH yields a mixture of native and chymotrypsin-modified LBI (LBI'_c) in which a specific Leu–Ser peptide bond has been hydrolyzed [4, 5]. In the LBI variant on which previous work was carried out [5] the complex formation between LBI'_c and chymotrypsin was too slow to be observed under routine assay conditions.

In the present paper four variants of LBI prepared from a commercial preparation of LBI by the method of Jones et al. [2] are compared with respect to i) the trypsin and chymotrypsin inhibitory activity of the native inhibitor, ii) the extent of modification of the inhibitor by incubation at acid pH with catalytic amounts of trypsin or chymotrypsin and iii) the kinetics of complex formation between LBI'_t and trypsin and LBI'_c and chymotrypsin. The results indicate that the four LBI variants are essentially identical with respect to their trypsin inhibitory activity but show marked differences in their affinity for chymotrypsin in either the native or the chymotrypsin-modified form.

2. Materials and methods

2.1. *Materials*

Lima bean protease inhibitor (LBI 1DA), bovine trypsin (TRL 2DA), bovine chymotrypsin (CDI 8GA) and carboxypeptidase B (COBDFP 1CA) were

obtained from Worthington (Freehold, N.J.). The inhibitor was further purified on Sephadex G-75 and separated into its four variants on DEAE-cellulose as described by Jones et al. [2]. They were named with roman numerals I through IV in order of their elution from the column. Three times crystallized porcine trypsin (4-36-564 Batch UK6) was obtained from Miles Laboratories, Inc. (Kanakakee, Ill.). 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.

2.2. *Inhibitor assays*

The inhibitory activities of LBI, LBI′$_t$ and LBI′$_c$ were determined with the spectrophotometric assay previously described by Rhodes et al. [9, 10]. Benzoyltyrosine ethyl ester and p-toluenesulfonylarginine methyl ester were used as the synthetic substrates for chymotrypsin and trypsin, respectively. The substrate-indicator solution was either added immediately after mixing of enzyme and inhibitor or, where needed, after previous incubation of the enzyme–inhibitor mixture at room temp. for the appropriate time. The amount of enzyme and inhibitor used was calculated from the weight of the dry lyophilized powder.

2.3. *Modification of LBI by trypsin and chymotrypsin and determination of the extent of modification*

LBI was dissolved in 0.018 M transaconitate buffer, pH 3.1 containing 0.04 M $CaCl_2$ to a final protein concentration of approx. 10 mg/ml. For the production of LBI′$_t$ the inhibitor solution was incubated with 2 mole percent porcine trypsin for 76 hr at 25°. A control was treated in identical fashion except that the trypsin was omitted. The chymotrypsin modification of LBI was carried out under the same conditions except that 1 mole percent of bovine chymotrypsin was added to the solution and the reaction mixture was incubated for 14 hr at 25°. The conditions for trypsin and chymotrypsin modification were chosen on the basis of previous experience to yield equilibrium mixtures between native and enzyme-modified inhibitor. To determine the extent of modification of the trypsin-modified inhibitor 50 μl samples (450 μg) of both control and reaction mixture were adjusted to pH 8.0 by the addition of 40 μl 0.1 M borate, pH 10.4 and after addition of 25 μl carboxypeptidase B solution (2.5 mg/ml) incubated overnight at 37° prior to assay. This treatment is known to completely inactivate LBI′$_t$ by removal of the new carboxyterminal lysine while leaving native LBI unaffected [7, 8]; any activity remaining in the reaction mixture after this treatment would be due to native LBI. Analogous treatment of the chymotryptic reaction mixture was not necessary since the complex formation between LBI′$_c$ and chymotrypsin is too slow to interfere with the assay system (see Results and discussion section).

3. Results and discussion

3.1. *Inhibitory activities of the four LBI variants*

Fig. 1 shows the trypsin inhibitory activities of native LBI and of the reaction mixture of native LBI and LBI′$_t$ after treatment with carboxypeptidase B to remove the "slow" inhibitory activity due to LBI′$_t$. It can be seen that these four variants of LBI have

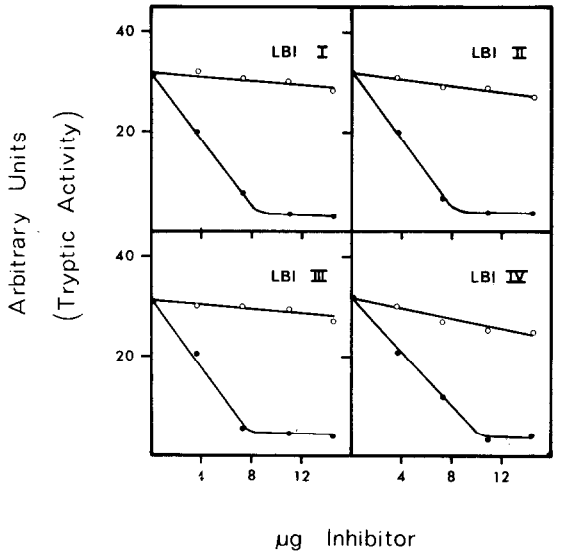

Fig. 1. Trypsin inhibitory activity of four variants of native and trypsin-modified LBI. Assays were carried out as described in the text. Increasing amounts of inhibitor were added to 30 μg of trypsin and the mixture was preincubated in the assay medium for 15 min before the addition of the substrate-inhibitor solution. In order to differentiate between LBI and LBI′$_t$ all samples were previously treated with carboxypeptidase B as described in the text. (○—○—○) Mixture of LBI and LBI′$_t$; (●—●—●) native LBI.

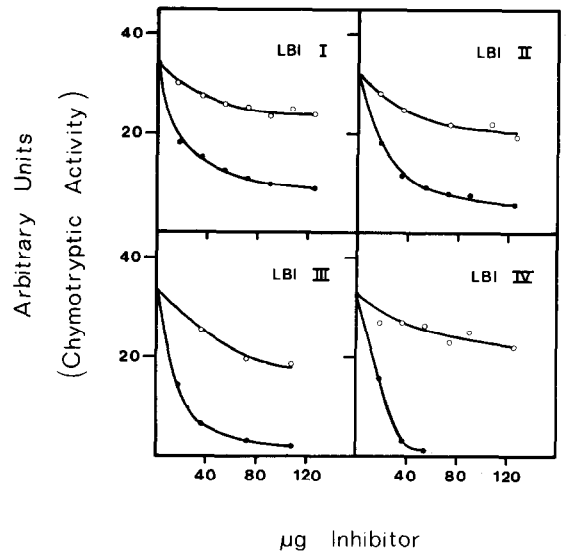

Fig. 2. Chymotrypsin inhibitory activity of four variants of native and chymotrypsin-modified LBI. Increasing amounts of inhibitor were added to 45 μg of chymotrypsin and the assays were carried out as described in the text without preincubation. (○—○—○) Mixture of LBI′$_c$ and LBI; (●—●—●) native LBI.

similar if not identical trypsin inhibitory capacities. From these results the percentage of LBI′$_t$ in the reaction mixtures and therefore the extent of modification of LBI by catalytic amounts of trypsin at acid pH can be calculated. The values for LBI variants I through IV are 93%, 90%, 93% and 82%, respectively. These values are considerably higher than those previously reported by us [5, 8]. It has been shown [7, 11] that this is due to the fact that in previous studies we did not reach the true equilibrium between LBI and LBI′$_t$. Fig. 2 shows that, even though by extrapolation it can be seen that the equivalence point may be virtually identical for the four variants, there are vast differences in their actual chymotrypsin inhibitory activity. While variant IV seems to be a strong inhibitor of chymotrypsin at near molar amounts, variant I achieves only approx. 60% inhibition even when present in a large molar excess. LBI variants II and III appear intermediate in their chymotrypsin inhibitory activity. This difference in chymotrypsin inhibitory activity of the variants probably indicates that the K_{diss} for the LBI–chymotrypsin complex is largest for LBI I and decreases progressively through variants II, III and IV. On the other hand, the extent of modification of the LBI variants by incubation with catalytic amounts of bovine chymotrypsin at acid pH appears to be identical. In every case the equilibrium mixture contains approx. 90% LBI′$_c$ and 10% native LBI.

3.2. *Kinetics of complex formation between LBI′$_t$ and trypsin and LBI′$_c$ and chymotrypsin*

It is generally accepted [12] that the inhibitory activity as measured here is actually a measure of the extent of complex formation between enzyme and inhibitor. Fig. 3 shows that while complex formation between native LBI and trypsin is complete in less than 1 min, the complex formation of LBI′$_t$ variants I through IV takes about 5–15 min. It appears therefore that the variants are almost identical with respect to i) trypsin inhibitory activity, ii) extent of modification by trypsin at acid pH as well as iii) the kinetics of complex formation between LBI′$_t$ and trypsin.

Fig. 4 on the other hand illustrates the differences in affinity for chymotrypsin between LBI′$_c$ variants.

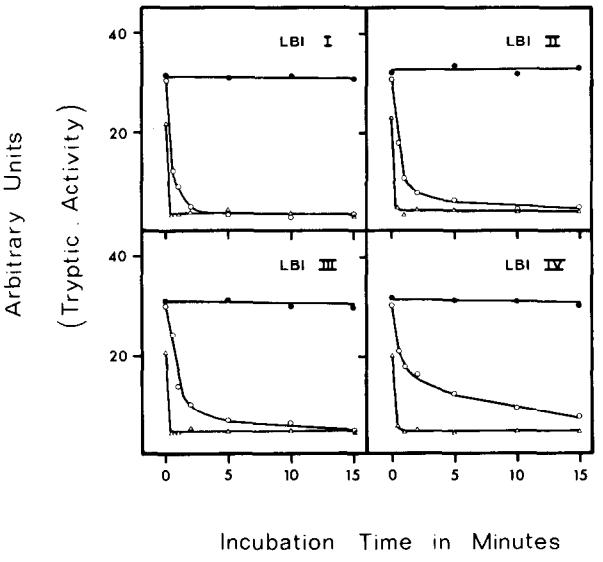

Fig. 3. Time course study of the trypsin inhibitory capacity of LBI and LBI′$_t$ variants I through IV. Assays were carried out as described in the text, but enzyme–inhibitor mixtures were incubated in the assay medium for varying amounts of time prior to addition of the substrate. (●—●—●) 30 μg of trypsin (control); (○—○—○) 30 μg of trypsin and 9.1 μg of the reaction mixture of LBI and LBI′$_t$; (△—△—△) 30 μg of trypsin and 9.1 μg of native LBI.

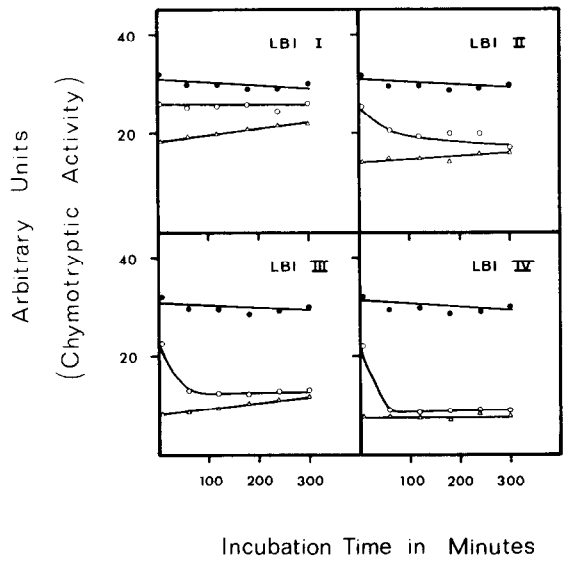

Fig. 4. Time course study of the chymotrypsin inhibitory capacity of LBI and LBI'$_C$ variants I through IV. Assays were carried out as described in the text, but enzyme–inhibitor mixtures were incubated in the assay medium for varying amounts of time prior to addition of the substrate. (●—●—●) 45 μg of chymotrypsin (control); (○—○—○) 45 μg of chymotrypsin and 27 μg of the reaction mixture of LBI and LBI'$_C$; (△—△—△) 45 μg of chymotrypsin and 27 μg of native LBI.

Whereas variant I does not appear to regain any inhibitory activity for periods up to 5 hr, variant II approaches its full potential in the same time. Variants III and IV reach their full inhibitory activity in 2.5 and 1 hr, respectively.

The time needed for regaining full inhibitory activity is a measure of the time needed for complex formation and thus a measure of the affinity between enzyme and inhibitor. In comparing the results in figs. 2 and 4 it is interesting to note that the affinity for chymotrypsin of the chymotrypsin-modified LBI variants parallels the inhibitory activity of these variants. In experiments now in progress we are trying to relate these differences in chymotrypsin inhibitory activity to structural differences between the variants.

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References

[1] H. Fraenkel-Conrat, R.C. Bean, E.D. Ducay and H.S. Olcott, Arch. Biochem. Biophys. 37 (1952) 393.
[2] G. Jones, S. Moore and W.H. Stein, Biochemistry 2 (1963) 66.
[3] R. Haynes and R.E. Feeney, J. Biol. Chem. 242 (1967) 5378.
[4] F.C. Stevens, in: Proceedings of the International Research Conference on Proteinase Inhibitors, eds. H. Fritz and H. Tschesche (Walter de Gruyter, Berlin, New York, 1971) p. 149.
[5] J. Krahn and F.C. Stevens, Biochemistry 9 (1970) 2646.
[6] J. Krahn and F.C. Stevens, FEBS Letters 13 (1971) 339.
[7] J. Krahn and F.C. Stevens, Can. Fed. Biol. Soc. 15 (1972) Abst. No. 371.
[8] J. Krahn and F.C. Stevens, Biochemistry 11 (1972) 1804.
[9] M.B. Rhodes, R.M. Hill and R.E. Feeney, Anal. Chem. 29 (1957) 376.
[10] M.B. Rhodes, N. Bennett and R.E. Feeney, J. Biol. Chem. 235 (1960) 1686.
[11] J. Krahn and F.C. Stevens (1972) in preparation.
[12] M. Laskowski, Jr. and R.W. Sealock, in: The Enzymes, Vol. III, ed. P.D. Boyer (Academic Press, New York, London, 1971) p. 375.