THE INTERACTION OF GUANIDINATED LIMA BEAN INHIBITOR WITH TRYPSIN

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1. Introduction

It is now well established that most, or all, of the protein inhibitors of trypsin contain a peptide bond which is susceptible to proteolytic attack by this enzyme [1–3]. The resultant modified inhibitor, which now contains a split peptide bond, can still combine with trypsin, although usually at a reduced rate. Removal of the new COOH-terminal group by treatment with carboxypeptidase B, or substitution of the new α-amino group results in a total loss of activity [1], suggesting that the susceptible peptide bond is directly involved in the formation of the stable complex [1].

As would be expected from the known specificity of trypsin, the susceptible bonds which have been identified fall into two classes according to whether the COOH-terminal group is lysine or arginine. Lima bean inhibitor (LBI) belongs to the class of lysine inhibitors, and the critical peptide bond has been identified as Lys–Ser [4]. The proteolysis is reversible and proceeds only to an equilibrium mixture in which native and modified inhibitor coexist [1]. The position of equilibrium depends upon external conditions and the nature of the inhibitor.

Rapid dissociation of the complex formed with trypsin by either native or modified inhibitor invariably results in liberation of the native inhibitor with the critical peptide bond intact [1, 3]. This may be interpreted to indicate that either the intact peptide bond is present in the stable complex or else that the dissociation is subject to kinetic control, with the rate of dissociation into the native form being very much faster than that of the competing dissociation to yield the modified form.

From the results summarized above it has been concluded that the susceptible peptide bond is directly involved in interaction with the catalytic site of trypsin and that the same stable complex is attained with both native and modified inhibitor [1, 5]. It has been inferred further that the normal course of proteolysis of a peptide bond is in this case perturbed so that the enzyme–substrate complex is stabilized to an abnormal degree.

An important remaining question is whether the critical peptide bond is intact or split in the stable complex. This is directly related to the problem of the mode of stabilization of the complex. If the bond is split then it is reasonable to postulate that the complex is stabilized by formation of an acyl bond between the new α-carboxyl group and the serine of the active site [1, 2]. If, however, the peptide bond can be shown to be intact, then this model must clearly be discarded. The plausible alternatives include the 'tetrahedral' complex, which has been proposed as an intermediate in the enzyme-catalyzed hydrolysis of peptides [6].

Evidence bearing directly on this question is scanty. The observation that any substitution of the new α-amino group of modified soya bean inhibitor totally eliminates activity is of course consistent with the tetrahedral model, but might also merely reflect a steric blockage of formation of the acyl bond [7].

It has recently been reported that guanidination of LBI fails to remove inhibitor activity and, in fact, increases the rate of combination [8]. This treatment converts lysine to homoarginine [9]. Since LBI belongs to the lysine class of trypsin inhibitors, it is probable that the critical Lys–Ser peptide bond of this inhibitor is thereby converted to a Homoarg–Ser bond.

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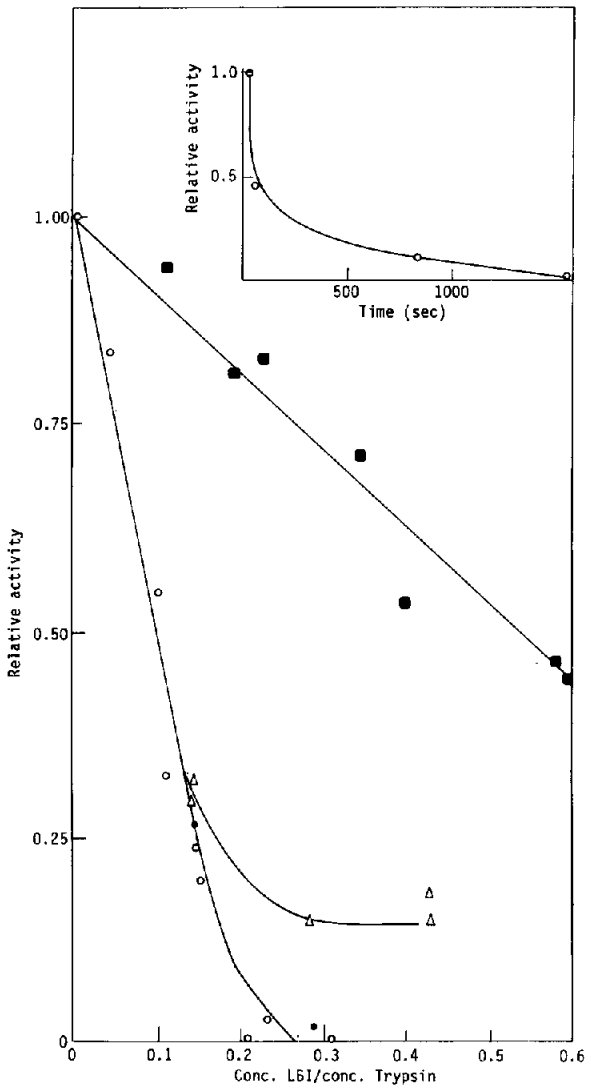

Fig. 1. Relative activity of trypsin (4.3×10^{-5} mg/ml) in: 0.1 M Tris and 0.1 M $CaCl_2$, pH 8.0, at 25°C as a function of the weight ratio of added LBI. The trypsin contains 34% inactive material. The equivalence point occurs at a weight ratio of 0.21: (○) native LBI; (●) modified LBI (LBI–T); (△) guanidinated LBI (LBI–G); (■) modified and then guanidinated LBI (LBI–T–G). Inset: time dependence of trypsin activity for 4.3×10^{-5} mg/ml trypsin under the above conditions in the presence of 3.8×10^{-5} mg/ml modified inhibitor.

The existing evidence appears to indicate that, in the majority of cases, peptide bonds of the Homoarg–X class are resistant to the action of trypsin [10–12]. However, certain exceptions have been noted, although the cited rates of hydrolysis are relatively slow [13].

Table 1
Characteristics of modified and guanidinated LBI.

Preparation	Fraction modified	Fraction of lysine group guanidinated	Fraction of serine groups intact
LBI–G	0	0.95	1.00 ± 0.05
LBI–T	0.75	0	1.00 ± 0.05
LBI–T–G	0.75	0.95	1.00 ± 0.05

If the susceptible peptide bond of LBI, when converted to the Homoarg–X form, is indeed refractory to the action of trypsin, then it is of course unlikely that the critical bond is split in the stable complex. Moreover, if LBI were first converted to the trypsin-modified form and then guanidinated it would be unlikely that trypsin could catalyze the reformation of the peptide bond.

In view of the interest of these points with respect to the general problem of the interaction of trypsin with its protein inhibitors, the consequences of the guanidination of native and modified LBI have been reinvestigated.

2. Materials

Unfractionated LBI was purchased from the Worthington Biochemical Corporation. This consists of a mixture of the four principal variants plus one or more minor components of higher molecular weight. The latter were removed by fractionation on a 40 X 1.5 cm Sephadex G-100 column eluted with 0.3 M ammonium acetate, pH 7.0. The low molecular weight components appeared as a single effluent peak accounting for over 90% of the sample. The corresponding fractions were pooled and lyophilized.

LBI variants I–IV were isolated by DEAE chromatography, according to the procedure of Jones et al. [14]. In addition, a purified sample of component I was generously provided by Dr. L. Stevens. Carboxypeptidase B and toluene sulfonyl arginine methyl ester (TAME) were obtained from Sigma. Methyl isourea bisulfate was purchased from Aldrich. The other reagents used were reagent grade. Glass-redistilled water was used for the preparation of all solutions.

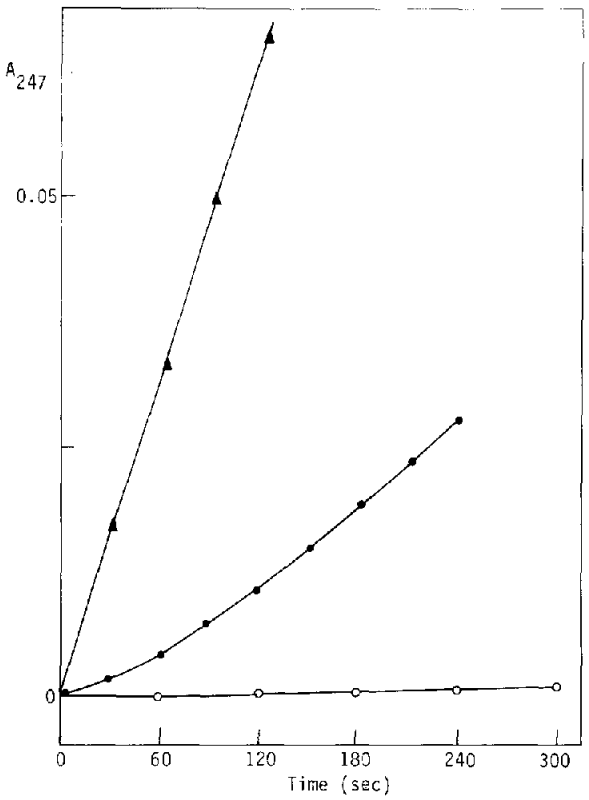

Fig. 2. Time dependence of hydrolysis of 0.001 M TAME in 0.1 M Tris and 0.1 M $CaCl_2$, pH 8.0, at 25°C, by trypsin (6.4×10^{-5} mg/ml) in the presence of 2.0 mg/ml LBI. The ordinate is the increase in absorbance at 247 nm: (△) no LBI; (○) native LBI; (●) guanidinated LBI (LBI–G).

3. Methods

Guanidination of LBI was carried out by the procedure of Hughes et al. [9]. A 1 M solution of methyl isourea bisulfate was neutralized by the addition of solid barium hydroxide and the barium sulfate precipitate removed by centrifugation. Guanidination of a 1% LBI solution was carried out in 0.5 M methyl isourea, pH 10.6, for 48 hr at 3°C. After completion of the reaction the protein was separated from excess reagent by passage through a Sephadex G-25 column, eluting with 0.3 M NH_4OAc, pH 7.0. The fractions containing protein were pooled and lyophilized.

The trypsin-modified form of LBI was prepared by incubation of a 1 mg/ml solution in 0.1 M KCl at pH 3.15 with 0.02 mg/ml trypsin for 72 hr at 25°C.

Carboxypeptidase B digestion of the trypsin-modified form was carried out in 0.1 M Tris, pH 8.0, at a carboxypeptidase level of 1 mg/ml for 24 hr at 37°C.

Amino acid analyses were carried out with a Beckman amino acid analyzer made available through the courtesy of Dr. Enrico Bucci. Protein hydrolysates for amino acid analysis was prepared by heating at 105°C with 6 M HCl in a sealed and evacuated glass tube.

Assays for trypsin activity were made using a spectrophotometric technique. Twenty microliter aliquots of solution were added to 2.0 ml of 0.001 M TAME in 0.1 M Tris, 0.1 M $CaCl_2$, pH 8.0 at 25°C, and mixed. The increase with time of absorbance at 247 nm was monitored using a Gilford spectrophotometer.

Prior to assay, trypsin and inhibitor were mixed in varying weight ratios in 0.1 M Tris and 0.1 M $CaCl_2$, pH 8.0, at 25°C. The concentration of trypsin was normally 0.05 mg/ml. Unless otherwise indicated, the mixture was allowed to stand for 15 min prior to assay.

Disc acrylamide gel electrophoresis was carried out at pH 8.7 using the Bio-Rad apparatus, usually with 7.5% polyacrylamide as the main gel. The gels were stained with Coomassie blue. The stained gels were scanned with the gel-scanning attachment of the Gilford spectrophotometer.

4. Results

The majority of the experiments reported here were made with unfractionated LBI, which had been freed from high molecular weight contaminants by gel filtration. This appears to be justified in the present case in view of the close similarity of the four variants in molecular size and amino acid composition, the identity of their susceptible peptide bonds, and the essential equivalence of the kinetics and equilibria of their interaction with trypsin [4, 8, 14].

In conformity with earlier studies [1, 4, 8], the combination of native LBI with trypsin was found to be nearly stoichiometric at pH 8 when correction was made for the fraction of inactive trypsin (fig. 1).

The modified form was produced by prolonged incubation with trypsin at pH 3.15 and 25°C [8]. The degree of conversion was assessed by treatment with carboxypeptidase B at pH 8 (see Methods) according to the procedure suggested by Krahn and Stevens [8].

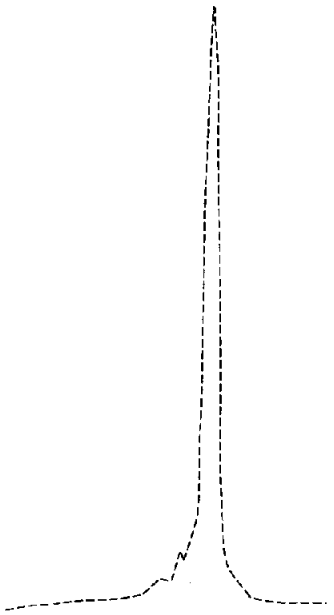

Fig. 3. (a) Scan of disc gel electrophoresis pattern of LBI–I.

The rate of combination of the modified inhibitor with trypsin is much slower than that of the native form (fig. 1), in conformity with the results of parallel studies upon LBI and other inhibitors [1, 3, 4]. However, at equilibrium the degree of inhibition produced by a given level of modified inhibitor is the same as for the native (fig. 1). For the modified inhibitor shown in fig. 1, the degree of conversion indicated by carboxypeptidase B treatment is 75% (table 1). While the degree of conversion is significantly less than that reported by Krahn and Stevens [8], the majority of the inhibitor is present as the modified form.

Guanidination of LBI resulted in 95% conversion of the lysine groups to homoarginine (table 1). This

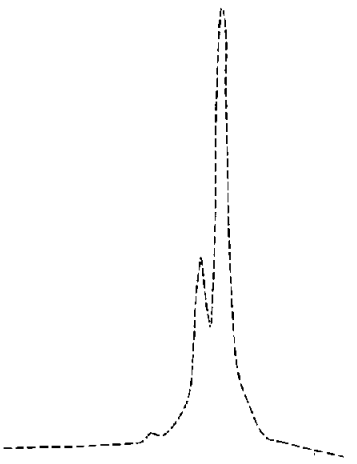

Fig. 3. (c) Guanidinated LBI–I.

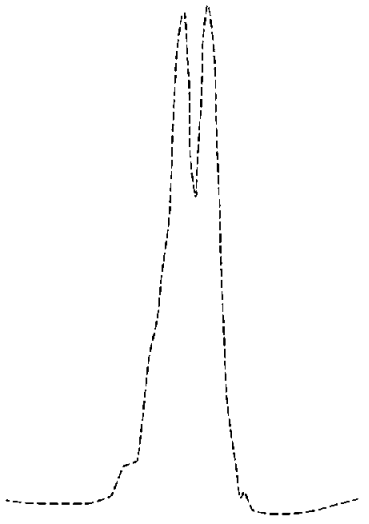

Fig. 3. (b) LBI–I after incubation with trypsin (2% of its mass) for 24 hr at pH 3.15 in 0.1M KCl at 25°C.

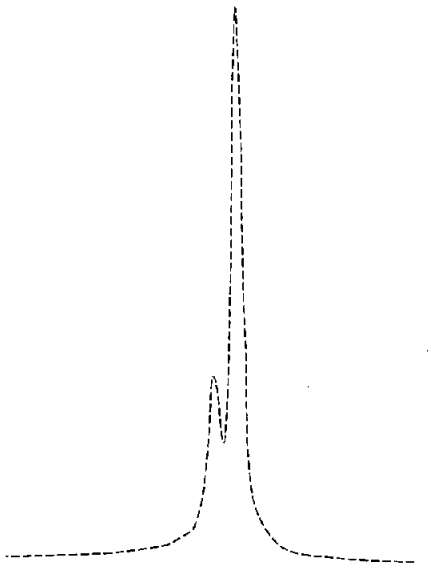

Fig. 3. (d) Guanidinated LBI–I after incubation with trypsin under above conditions for 72 hr.

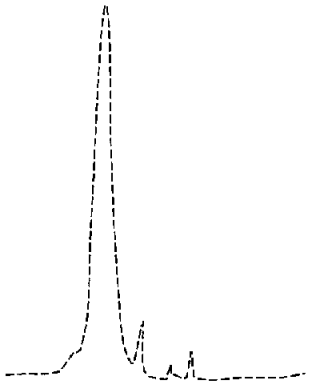

Fig. 3. (e) LBI–IV.

indicates that less than one unreacted lysine remains per molecule.

The guanidinated inhibitor shows an activity similar to that of the native if initial rates of hydrolysis are measured (fig. 1). However, the apparent degree of inhibition does not attain completion, even at large inhibitor:trypsin ratios. This appears to be a consequence of a greatly enhanced rate of dissociation of the trypsin–inhibitor complex in the presence of 0.001 M TAME, so that the observed rate increases markedly with time (fig. 2). The dissociation with time also occurs for the native inhibitor, but at a much slower rate (fig. 2). Thus the apparent residual tryptic activity observed at high inhibitor levels probably arises from the finite degree of dissociation which has occurred in the presence of TAME during the interval (30–60 sec) before data can be recorded.

If the modified inhibitor was then guanidinated, quite different behavior was observed (fig. 1). In this

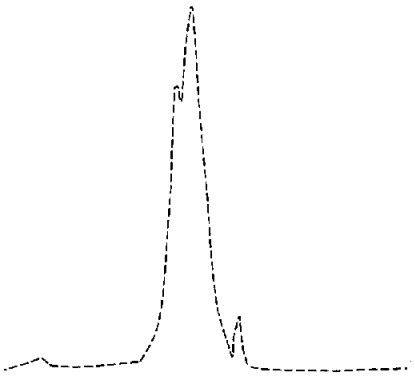

Fig. 3. (f) Guanidinated LBI–V.

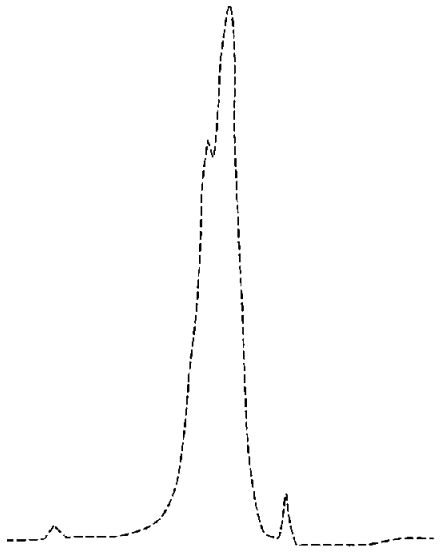

Fig. 3. (g) Guanidinated LBI–IV after incubation with trypsin under above conditions for 72 hr.

case activity was reduced by 80% (table 1), becoming essentially equivalent to that of the modified, carboxypeptidase-treated inhibitor. The degree of inhibition was not increased by allowing the trypsin–inhibitor mixture to incubate for periods up to 3 hr. It thus appears that conversion of the new C-terminal lysine to homoarginine in the modified inhibitor is sufficient to block combination with trypsin entirely or to slow the process sufficiently so that it does not occur to a significant extent over this time interval.

The reaction with methyl isourea has been reported to be entirely specific for the ϵ-amino groups of lysine [9, 15, 16]. In particular, it has been reported that α-amino groups do not react [15, 16]. This was verified in the present case by amino acid analysis of the modified guanidinated inhibitor. No loss of serine was observed (table 1), indicating that no α-amino substitution occurred.

There remains the question of whether the guanidinated inhibitor is indeed resistant to the action of trypsin. Guanidination of purified variant IV resulted in the appearance of a second component on disc gel electrophoresis, presumably reflecting the fact that conversion was not 100% (fig. 3). Incubation of the guanidinated inhibitor with 2% of its mass of trypsin for 72 hr at pH 3.15 did not alter the appearance of the pattern significantly and, in particular, did not

cause the appearance of a new component of higher mobility, as would result from the splitting of a peptide bond. In contrast, treatment of the native form of an LBI variant with trypsin under the same conditions resulted in substantial conversion to the modified form in a somewhat shorter period.

It thus appears that splitting of the susceptible peptide bond either does not occur for the guanidinated inhibitor under these conditions, or else proceeds at a rate which is much slower than that for the native inhibitor.

5. Discussion

The findings cited above have provided strong indication that conversion of the critical Lys–Ser peptide bond of LBI to Homoarg–Ser does not block the interaction with trypsin, although the rate of dissociation of the complex is considerably increased. This is the case despite the fact that efforts to detect proteolysis of guanidinated LBI by trypsin have thus far yielded only negative results.

Again it must be stressed that the statement, that proteolysis was not observed, is operationally based and may reflect nothing more than a pronounced depression of the rate of hydrolysis, as compared with that of native LBI under the same conditions. An alternative explanation might be that the equilibria are displaced as a consequence of guanidination so as to favor to an overwhelming degree the intact peptide bond. This however seems somewhat unlikely in view of the chemical similarity of homoarginine and the protonated form of lysine.

In any event it is clear that the occurrence of a split peptide bond in the trypsin complex of guanidinated LBI must be regarded with some degree of doubt.

The observation that guanidination of modified LBI blocks its activity may be interpreted to mean that either no complex with trypsin is formed, or else that the rate of combination is too slow to be measured. Since no depression of the rate of association with trypsin occurs for the guanidinated form of native LBI [8], the latter explanation is somewhat less likely.

The finding that guanidination of modified LBI abolished activity, while guanidination of the native form did not, is readily explained if the critical peptide bond is intact in the stable complex. This would be the case for a complex of the tetrahedral type. It can be reconciled with the model in which the bond is split in the complex only with the aid of special assumptions as to the magnitudes of the various rate constants.

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