ON THE COMPLEX FORMATION BETWEEN BASIC PANCREATIC TRYPSIN INHIBITOR AND TLCK-, TPCK-DERIVATIVES OF β-TRYPSIN

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The study of the interaction of the pancreatic inhibitor with different alkylated derivatives of α- and β-trypsin shows that: 1) TLCK-β-trypsin forms a complex with pancreatic inhibitor in tris buffer and tris-ethanol 40% system. 2) TLCK-α-trypsin and TLCK-TPCK-β-trypsin have lost their ability to complex formation with pancreatic inhibitor. TLCK-α-trypsin and TLCK-TPCK-β-trypsin are derivatives in which the "chymotryptic" active site is destroyed. The results presented in this paper prove the participation of the "chymotrypic" active site in the interaction between trypsin and pancreatic inhibitor. This is the second interaction beside that of the electrostatic bond between Asp-117 of trypsin and Lys-15 of the inhibitor which we proved earlier.

1. Introduction

The study of the complex between α-, β- and ψ-trypsin and the pancreatic inhibitor showed that only one electrostatic bond is involved in complex formation between trypsin and BPTI* [1] – the bond between Asp-177, which is a specificity site carboxyl group of trypsin, binding positively charged substrates, and Lys-15 of pancreatic inhibitor. We were able to show that hydrophobic forces play an important role in the interaction between trypsin and BPTI.

Comparison of the esterase activity of α-, β-trypsin and their alkylated derivatives demonstrated that besides the specific tryptic active site responsible for hydrolysis of positively charged substrates, there is also a second active site in the trypsin molecule which possesses a chymotrypsin like specificity [2].

In the present study we succeeded in showing that complex formation between trypsin and pancreatic inhibitor also includes the inhibition of the "chymotryptic" site of trypsin.

* Abbreviations:

BPTI	: basic pancreatic trypsin inhibitor
TPCK	: tosyl phenylalanine chloromethyl ketone
TLCK	: tosyl lysine chloromethyl ketone
Ac–Tyr–OEt	: acetyl tyrosine ethyl ester
Bz–Arg–OEt	: benzoyl arginine ethyl ester
BAPA	: N^{α}-benzoyl-*dl*-arginine-p^{1}-nitroanilide

2. Experimental procedure

2.1. *Materials*

α- and β-trypsin were isolated from commercial Worthington preparations according to the procedure of Schroeder and Shaw [3]. BPTI was purified according to the method of Dlouha et al. [4]. TLCK and TPCK were obtained from Sigma Chemical Company. Bz–Arg–OEt was a product of Fluka AG. Ac–Tyr–OEt was obtained from Schuchardt. BAPA was purchased from Mann Research Laboratories. Sephadex derivatives were obtained from Pharmacia.

2.2. *Methods*

Inhibition of trypsin by TLCK was performed according to Shaw et al. [5].

Substitution of β-trypsin with TPCK: 2 mg of

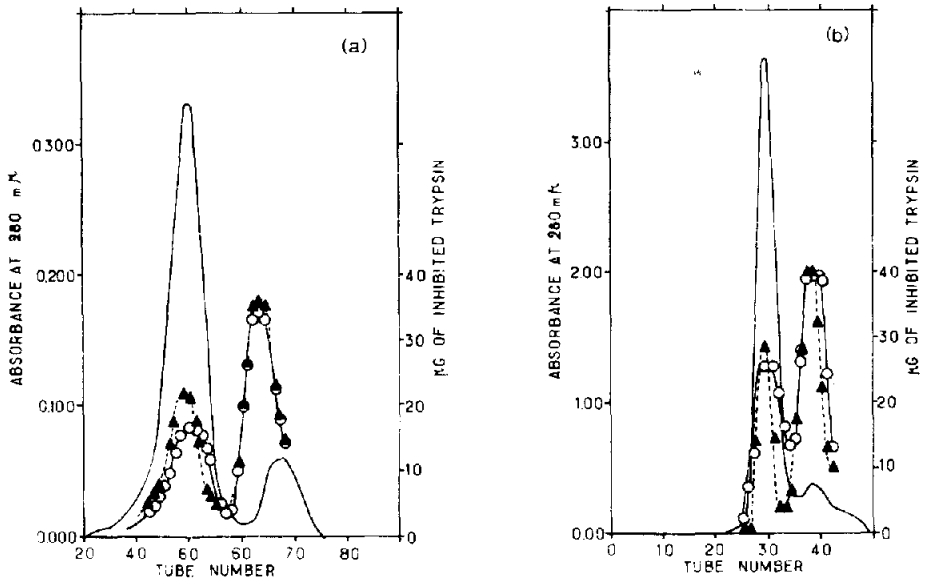

Fig. 1. Gel filtration of BPTI and TLCK-β-trypsin complex on (a) Sephadex G-75 (column 1.0 X 45 cm) in 0.05 M tris 0.02 M $CaCl_2$ buffer pH 8.3 at room temperature and (b) Sephadex G-100 (column 1.0 X 45 cm) in 0.05 M tris 0.02 M $CaCl_2$ buffer pH 8.3, 40% ethanol at room temperature. Flow rate: 1.5 ml per hr; 0.5 ml were collected per tube. o—o: inhibition of β-trypsin by the samples; ▲ - - - ▲; inhibition of β-trypsin by the samples after their treatment with 5% TCA.

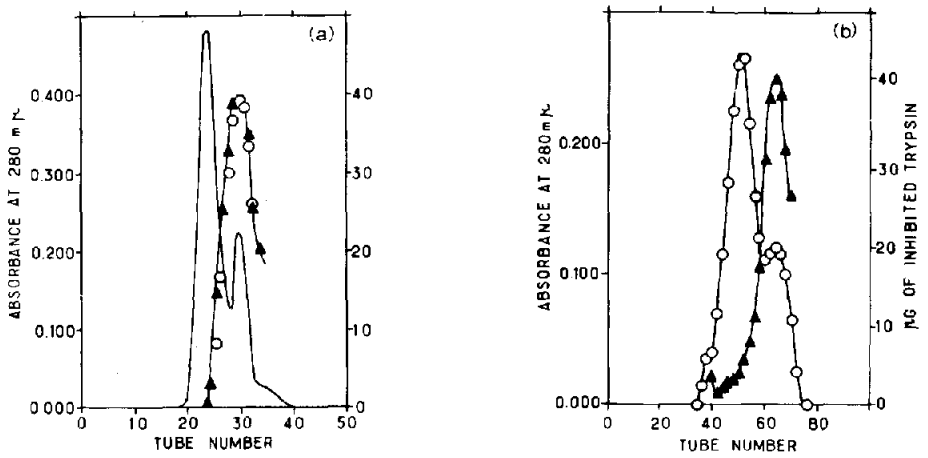

Fig. 2. Gel filtration of BTPI and TLCK-α-trypsin* mixture on (a) Sephadex G-75 (column 1.0 X 45 cm) in 0.05 M tris 0.02 M $CaCl_2$ buffer pH 8.3. o——o absorbancy at 280 nm; ▲——▲ inhibition of β-trypsin by the samples. (b) Sephadex G-100 (column 1.0 X 45 cm) in 0.05 M tris 0.02 M $CaCl_2$ buffer pH 8.3, 40% ethanol at room temperature. Flow rate: 1.8 ml per hr; 0.6 ml were collected per tube: o——o: inhibition of β-trypsin by the samples; ▲——▲: inhibition of β-trypsin by the samples after their treatment with 5% TCA.

* Gel filtration of BPTI and TLCK-TPCK-β-trypsin mixture under the conditions of fig. 2a gave identical results to fig. 2a.

β-trypsin was dissolved in 10 ml of 0.05 M tris pH 7.1 buffer containing 0.025 M $CaCl_2$ and 0.001 M benzamidine HCl. The solution was cooled in an ice-bath. Then 0.64 mg of TPCK dissolved in 0.3 ml of methanol were added with stirring. After 6 hr, the solution was dialysed against 0.01 M HCl to remove benzamidine and excess of TPCK. The volume of the sample was then reduced to 2 ml by ultrafiltration. No precipitation of the protein was observed during the entire procedure.

Molarity of active trypsin was determined by titration of the active site using the method of Chase and Shaw [6].

Esterase activity was measured titrimetrically [7] with the aid of a Radiometer type TTT 1 autotitrator fitted with a type ABU 1 autoburette. The titrations were carried out in a jacketed 5 ml vessel at 25° under nitrogen using 0.05 N NaOH. The concentration of substrate was 0.01 M. The measurements were performed in 0.05 M tris pH 8.3 buffer containing 0.02 M $CaCl_2$. Protein concentration was determined from the absorbance at 280 nm. The absorption coefficient used for the calculation was $\epsilon = 16.1$ [8]; molecular weight of trypsin was taken as 23,800. The values of the specific activity were then calculated on the basis of the molarity of active trypsin.

Tests for BPTI–trypsin complex were carried out after precipitation of the trypsin moiety by 5% trichloroacetic acid according to a method described earlier [9].

3. Results and discussion

The comparison of profiles presented in fig. 1a and b shows that TLCK-β-trypsin forms a complex with BPTI in tris buffer as well as in tris–40% ethanol. This means that the alkylation of His-46 with TLCK does not protect β-trypsin from the interaction with pancreatic inhibitor. The data presented in table 1 confirm the results obtained by chromatography. Complete inhibition of TLCK-β-trypsin esterase activity was observed in the presence of BPTI.

In contrast to β-trypsin, substitution of α-trypsin with TLCK leads to a complete loss of its esterase activity (table 1). Gel filtration of a mixture of TLCK-α-trypsin and a molar excess of BPTI (fig. 2) proved that no complex formation takes place between TLCK-α-trypsin and the pancreatic inhibitor. All inhibitory activity is concentrated in the second peak which corresponds to an excess of free inhibitor.

As is well known, α-trypsin differs from the β-form in the cleavage of one bond between Lys-131 and Ser-132 [3]. In our previous work we were able to show that this particular cleavage destroys the "chymotryptic" active site of the trypsin molecule [2]. We have also proved that only one electrostatic bond exists between Asp-177 of trypsin and Lys-15 of the inhibitor and that the other interactions are due to hydrophobic forces [1]. Alkylation of His-46 in α- and β-trypsin with TLCK leads to the same chemical modification resulting in the inactivation of the specific tryptic active site which is responsible for cleavage of positively charged substrates. On the other hand, the difference between α- and β-forms consists in their "chymotryptic" active site. The complex formation with pancreatic inhibitor was observed only in the case of TLCK-β-trypsin, whose "chymotryptic" active site is not touched. In contrast to the β-form, TLCK-α-trypsin has completely lost its affinity for BPTI as a result of the disarrangement of its "chymotryptic" active site.

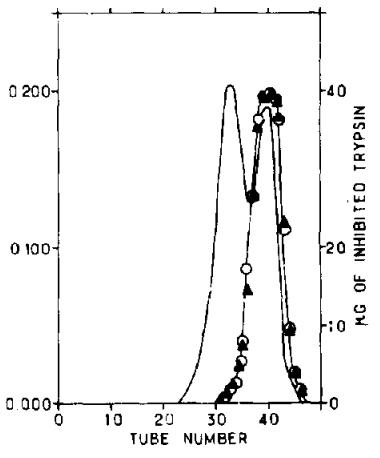

Fig. 3. Gel filtration of BPTI and TLCK-TPCK-β-trypsin mixture on Sephadex G-100 (column 1.0 × 45 cm) in 0.05 M tris 0.02 M $CaCl_2$ buffer pH 8.3, 40% ethanol. Conditions were as in fig. 1.

Table 1
Influence of BPTI on esterase activity of trypsin (TR) and its derivatives.

Trypsin and derivatives	Molar ratio BPTI/Trypsin	Tris		Tris-ethanol 40%	
		Bz–Arg–OEt S.A.	Ac–Tyr–OEt S.A.	Bz–Arg–OEt S.A.	Ac–Tyr–OEt S.A.
β-TR	6	201	30.3	196.5	18.8
	4	0	0	96.5	8.35
α-TR	0	190	15.5	116	9.0
	4	0	0	0	0
TLCK-β-TR	0	7.0	3.1	35.6	0
	4	0	0	0	0
TPCK-β-TR	0	195	4.6	208	0
	4	0	0	0	0
TLCK-TPCK-β-TR	0	0	0	0	0
	4	– *	–	–	–
TLCK-α-TR	0	0	0	0	0
	4	–	–	–	–

S.A. Specific activity was expressed in μeq/min/mg. * not determined.

The substitution of TLCK-β-trypsin with TPCK brought about the same change of enzymatic properties as in the case of TLCK-α-trypsin. As can be seen from table 1, TLCK-TPCK-β-trypsin completely lost the esterase activity. The results presented in fig. 3 prove that it also lost the affinity for complex formation with pancreatic inhibitor.

To summarize the results presented above, we can conclude that the second interaction between trypsin and pancreatic inhibitor occurs in the "chymotryptic" active site of trypsin. This is an interaction of hydrophobic character. Consequently, during the complex formation between trypsin and pancreatic inhibitor the inhibition of two active sites occurs: 1) the inhibition of the specific active site of trypsin by an electrostatic interaction between Asp-177 of trypsin and Lys-15 of the inhibitor [1], 2) the inhibition of the non-specific "chymotryptic" active site of trypsin through a hydrophobic interaction between hydrophobic residues of trypsin and the inhibitor.

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