# INTERACTION OF BASIC PANCREATIC TRYPSIN INHIBITOR WITH TRYPSINOGEN

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## 1. Introduction

The basic pancreatic trypsin inhibitor (BPTI) inhibits a number of alkaline proteases. The most stable complex which the inhibitor forms is that with trypsin. The dissociation constant of this complex at pH 7.8 is $2 \times 10^{-10}$ [1]. The complex of BPTI with chymotrypsin is far less stable [2]. Green [1] has shown that the binding affinity of BPTI for active trypsin is not affected by the presence of a 30-fold excess of DIP-trypsin (di-isopropylphosphoryltrypsin) and that a specific interaction between DIP-trypsin and BPTI is not detectable by sedimentation analysis.

In this study an effort was made to determine by the method of gel filtration on Sephadex G-50 the binding affinity of trypsinogen, DIP-trypsin, chymotrypsinogen and DIP-chymotrypsin for BPTI. The formation of the complex was observed only in the case of trypsinogen.

## 2. Materials and Methods

Trypsinogen was obtained from beef pancreas according to Balls [3] and was twice recrystallized. DIP-derivatives of trypsin and α-chymotrypsin were obtained from commercial crystalline products (Léčiva, Prague) by the reaction with di-isopropylphosphofluoridate [4] and were twice recrystallized. Chymotrypsinogen (Léčiva, Prague) was crystallized seven times with ammonium sulfate and twice with ethanol [5]. BPTI was prepared by the method of Dlouhá et al. [6].

*Preparation of complexes.* The zymogen or the inhibited enzyme (12 mg) was dissolved in 0.5 ml of 0.01M $(NH_4)_2CO_3$ at pH 8.6 and 12 mg of BPTI dissolved in an equal volume of the same buffer was added. The reaction mixture was set aside for 15 min at 25° and then passed through a Sephadex G-50 column under the conditions given in the legend to fig. 1. The course of the separation was examined spectrophotometrically and by testing the inhibitory activity of aliquots of individual fractions.

The activity of BPTI was assayed by a modification of the method of Nagel et al. [7] and the inhibition of tryptic hydrolysis of benzoyl-arginine-*p*-nitranilide hydrochloride (BAPA) by BPTI was determined. A 100 μl aliquot of each fraction was withdrawn, mixed with 100 μl of 5% trichloroacetic acid and allowed to stand 5 min. To this mixture, 2.5 ml of buffer (0.1M Tris HCl – 0.02M $CaCl_2$, pH 7.8) and 100 μl of a trypsin solution containing 40 μg of the enzyme in 0.001M HCl – 0.02M $CaCl_2$ were added. After 5 min of incubation at 24°, 1.2 mg of BAPA in 100 μl of formamide was added. The reaction mixture was incubated 10 min at 37°, acidified with 0.5 ml of 30% acetic acid, and its absorbance at 405 nm determined. The inhibitory activity was expressed as the difference in tryptic activity of the standard and of the assayed fraction. These values were expressed in μg of inhibited trypsin with the aid of a calibration curve for the standard.

*N*-terminal amino acids were determined by the method of Sanger [8] after 16-hour hydrolysis of the derivative.

## 3. Results

As is apparent from fig. 1A, the inhibitory activity after the interaction of a molar excess of BPTI with trypsinogen was found both in fractions corres-

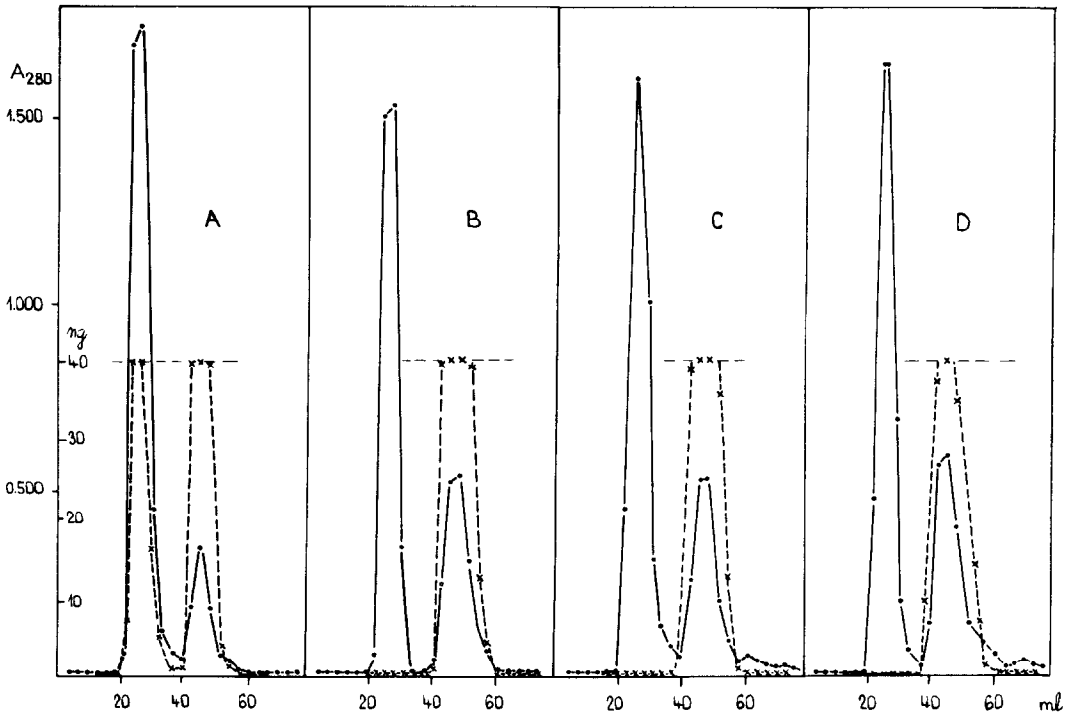


Fig. 1. Gel filtration of the mixture of BPTI and the zymogens (or inhibited enzymes) on Sephadex G-50. Molar zymogen (enzyme) to inhibitor ratio 1:4. The 49 X 1.6 cm column was eluted with 0.01M $(NH_4)_2CO_3$, pH 8.6 at a rate of 12 ml per hour. ——— absorbance at 280 nm; ------- inhibitory activity in μg of trypsin. Elution profiles obtained with mixtures of BPTI and *A*, trypsinogen; *B*, DIP-trypsin; *C*, chymotrypsinogen; *D*, DIP-chymotrypsin.

ponding to the high molecular weight component, i.e. to trypsinogen, and also in the peak representing the excess of free inhibitor. The amino acid composition of the product obtained after lyophilization of the material contained in the first peak (fig. 1A) is given in table 1. This data leads us to conclude that the trypsinogen to BPTI ratio in the complex is 1 : 1.

When the complex of trypsinogen with BPTI was subjected to qualitative *N*-terminal end-groups analysis, evidence was found of valine (*N*-terminal group of trypsinogen) and of arginine (*N*-terminal group of BPTI), in addition to traces of serine.

By contrast, in all the remaining preparations examined (as obvious from fig. 1B, C and D) the first peak corresponds to free DIP-trypsin, chymotrypsinogen, and DIP-chymotrypsin, respectively, while the inhibitory activity is shown exclusively in the second peak. In every case, in the maximum of the second peak there are fractions in which the total amount of trypsin added was completely inhibited (i.e. the upper limit of the sensitivity of the method was reached). On purpose the range of amount of trypsin added was chosen so that total absence of BPTI in the first peak in B–D be shown. Hence, these proteins do not react with the inhibitor.

## 4. Discussion

The investigation of the interaction of BPTI with trypsinogen has shown that there is a specific interaction between the two components, the molar ratio being 1 : 1. This observation cannot be accounted for by non-specific adsorption or incomplete fractionation of the reaction mixture since the excess of BPTI would have separated chromatographically (fig. 1A). If this phenomenon were to be attributed to adsorption we should meet with the same effect also in the

Table 1
Amino acid composition of trypsinogen (TG), BPTI and of their complex.

| Amino acid | BPTI | TG | Theory for TG:BPTI ratio | | Analytical values for TG-BPTI complex (peak 1, fig. 1A) |
|---|---|---|---|---|---|
| | | | 1 : 2 | 1 : 1 | |
| Lys | 4 | 15 | 23 | 19 | 20.4 |
| His | 0 | 3 | 3 | 3 | 3.4 |
| Arg | 6 | 2 | 14 | 8 | 7.5 |
| Asp | 5 | 26 | 36 | 31 | 32.0 |
| Thr | 3 | 10 | 16 | 13 | 12.4 |
| Ser | 1 | 34 | 36 | 35 | 33.6 |
| Glu | 3 | 14 | 20 | 17 | 17.3 |
| Pro | 4 | 8 | 16 | 12 | 13.0 |
| Gly | 6 | 25 | 37 | 31 | 31.2 |
| Ala | 6 | 14 | 26 | 20 | 19.5 |
| ½ Cys | 6 | 12 | 24 | 18 | 12.1 |
| Val | 1 | 18 | 20 | 19 | 16.5 |
| Met | 1 | 2 | 4 | 3 | 3.2 |
| Ile | 2 | 15 | 19 | 17 | 15.0 |
| Leu | 2 | 14 | 18 | 16 | 16.0 |
| Tyr | 4 | 10 | 18 | 14 | 13.2 |
| Phe | 4 | 3 | 11 | 7 | 6.4 |
| Trp | 0 | 4 | 4 | 4 | |

Samples after 24 hour hydrolysis were analyzed. Values are as moles of amino acid. For the calculation of the number of amino acid residues (last column), the average value of leucine was taken to represent 16.0 residues (assuming a 1 : 1 TG : BPTI ratio). The lower values for valine and isoleucine are due to the presence of bonds Ile.Ile in BPTI and Ile.Val in trypsin, which are very resistant to hydrolysis. The tryptophan content of the complex was not determined.

case of DIP-trypsin, chymotrypsinogen, and DIP-chymotrypsin, whose molecular weights and primary structures are very similar. In the case of DIP-trypsin the lack of ability to form a complex with BPTI has been shown by Green [1]. The mode of interaction between trypsinogen and BPTI is therefore bonding between specific residues. The inhibitory activity assays clearly show, however, that the addition of trypsin results in the liberation of the inhibitor from the complex with trypsinogen, and that the inhibitor then inhibits the active enzyme. The binding affinity of trypsin for BPTI is therefore greater than the binding affinity of trypsinogen.

The binding of the inhibitor by trypsinogen, which is not an active enzyme, can be ascribed to the analogous three-dimensional arrangement of amino acid residues of the binding site of the zymogen and the enzyme. At the same time, the difference in the structure of trypsin and trypsinogen is more pronounced than the difference in the structure of trypsin and DIP-trypsin. During the process of zymogen activation the *N*-terminal hexapeptide Val.$Asp_4$.Lys is split off. A salt bridge is then formed between the *N*-terminal isoleucine and the aspartic acid residue no. 182, a structural rearrangement analogous to that observed in chymotrypsin [9]. By contrast, the conformational change which parallels the trypsin into DIP-trypsin conversion most likely involves only the immediate environment of the active site, i.e. of the substituted serine residue no. 183. In chymotrypsinogen, as in trypsinogen, the active serine residue is free. Both enzymes show considerable similarity in their structures. Nevertheless, neither chymotrypsinogen nor DIP-chymotrypsin reacts with BPTI.

It is known that the bond between BPTI and chymotrypsin is less stable than the bond between BPTI and trypsin [2]. So far we are lacking evidence that the combination of the inhibitor with chymo-

trypsin is due to the same amino acid residue in the inhibitor molecule, i.e. to Lys no. 15, which has been shown to interact with trypsin [10]. Different sites available for the combination with trypsin and chymotrypsin were found in the molecule of soy bean trypsin inhibitor [11]. Similarly, the enzymes may well employ other areas of contact and other amino acid residues for the binding. Since the bond between chymotrypsin and trypsin inhibitor has been found to be relatively weak, it is obvious that the bond between chymotrypsinogen and the inhibitor, if any, cannot even be perceived by our method.

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**References**

[1] N.M.Green, J. Biol. Chem. 205 (1953) 535.
[2] F.C.Wu and M.Laskowski, J. Biol. Chem. 213 (1955) 609.
[3] A.K.Balls, Proc. Nat. Acad. Sci. U.S. 53 (1965) 392.
[4] L.W.Cunningham Jr., J. Biol. Chem. 211 (1954) 13.
[5] M.Kunitz, J. Gen. Physiol. 32 (1949) 265.
[6] V.Dlouha, D.Pospisilová, B.Meloun and F.Sorm, Coll. Czech. Chem. Commun. 30 (1965) 1311.
[7] W.Nagel, F.Willig, W.Peschke and F.N.Schmidt, Z. Physiol. Chem. 340 (1966) 1.
[8] F.Sanger, Biochem. J. 39 (1945) 507.
[9] P.B.Sigler, D.M.Blow, B.W.Mathews and R.Henderson, J. Mol. Biol. 35 (1968) 143.
[10] J.Chauvet and R.Acher, J. Biol. Chem. 242 (1967) 4274.
[11] Y.Birk, A.Gertler and S.Khalef, Biochim. Biophys. Acta 147 (1967) 402.