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INTERACTION OF TRYPSIN-LIKE PROTEASE FROM *STREPTOMYCES GRISEUS* WITH AN IMMOBILIZED INHIBITOR FROM KIDNEY BEAN

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Summary

An immobilized double-headed inhibitor from *Phaseolus vulgaris* L. selectively binds the trypsin-like enzyme produced by *Streptomyces griseus*. Binding takes place at pH 8.0, and at pH 2.0 the protease can be quantitatively released from the complex. Purified by affinity chromatography, the trypsin-like enzyme is homogeneous according to polyacrylamide gel electrophoresis and ultracentrifugation data.

Physico-chemical and enzymic properties of the enzyme are identical to those exhibited by the enzyme purified by ion-exchange chromatography. Chymoelastases from *Str. griseus* as well as the subtilisin-like enzyme do not interact with an immobilized inhibitor. In solution, the inhibitor from *P. vulgaris* gives a stable ternary complex with bovine trypsin and chymotrypsin, whereas with an immobilized inhibitor the trypsin, if present, tends to displace chymotrypsin in an chymotrypsin inhibitor complex. This evidence suggests that immobilization results in considerable changes in inhibitor properties.

Introduction

The proteolytic system of the microorganism *Streptomyces griseus* consists of both endo- and exo-peptidases [1]. Among the endo-peptidases, one can detect at least four serine proteases. Two of these are chymoelastases; the third is a subtilisin-like enzyme; all three enzymes hydrolyze Ac-Tyr-OEt. The fourth protease, by its substrate specificity, response to inhibitors and physico-chemical properties, is very like bovine trypsin [2—5]. An investigation of this trypsin-like protease from *Str. griseus* is very interesting from the point of view of the evolution of protease and might also be practically significant for the specific hydrolysis of proteins [6,7].

Str. griseus trypsin was earlier shown to be inhibited by a number of naturally-occurring inhibitors of proteases [8,9]. The purpose of this work was to

study the interaction of *Str. griseus* proteases with an immobilized protease inhibitor from kidney bean (*Phaseolus vulgaris* L.).

Materials and Methods

The commercial Pronase preparation was a product of Kaken Chemical Co., Ltd., Tokyo. Protein inhibitor was isolated from *P. vulgaris* L. by a procedure described elsewhere [9]. Trypsin from Spofa was recrystallized from $MgSO_4$ solution and freeze-dried; affinity chromatography has shown it to contain 80% of active enzyme. α-Chymotrypsin obtained from Olaine Chemical Plant and was before use subjected to chromatography on CM-Sephadex C-50 [10].

Ac-Tyr-OEt and Bz-Arg-OEt were purchased from Reanal. Sepharose 4B, AH-Sepharose, CM-Sephadex C-50 were products of Pharmacia Fine Chemicals, Uppsala.

Preparation of immobilized inhibitor

The inhibitor isolated from *P. vulgaris* was immobilized by coupling the protein to AH-Sepharose in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide [11]. The procedure was as follows. AH-Sepharose was suspended in 0.5 M NaCl and washed with water. The protein (0.1 g) was dissolved in water, pH 5.7, and the solution was mixed with AH-Sepharose suspension containing 1.25 g of dry material. Carbodiimide (50 mg) dissolved in 2 ml of water at pH 4.5 was added to mixture, which was then stirred for several hours. A constant pH of 5.7 during coupling was controlled using a pH-stat.

The immobilized inhibitor was packed into a chromatographic column (1.5 × 4.5 cm), and the column was successively washed with water, 0.05 M Tris · HCl/0.2 M KCl at pH 8.0, and 0.2 M KCl at pH 2.0. The capacity of inhibitor-Sepharose column was estimated by adsorption of bovine trypsin and chymotrypsin. For capacity determination some excess of trypsin and chymotrypsin in Tris · HCl at pH 8.0, 0.02 M $CaCl_2$/0.2 M KCl was passed through the column. The bound enzymes were subsequently eluted with 0.2 M KCl at pH 2.0. All procedures were carried out at 2°C.

The amounts of eluted trypsin and chymotrypsin were determined by absorbance determinations at 280 nm. The coefficients used for concentration calculation were 0.67 for trypsin and 0.50 for chymotrypsin.

Ovomucoid, a specific trypsin inhibitor from chicken egg white, was prepared according to the method of Lineweaver and Murray [12]. It was then immobilized by a conventional procedure using Sepharose 4B activated by cyanogen bromide [13].

Affinity chromatography of pronase

In a routine run Pronase (150 mg) was dissolved in 50 ml Tris · HCl/5 mM $CaCl_2$ at pH 8.0 (sometimes the solution also contained 0.2 M KCl). The solution was filtered through a No. 2 fritted glass filter and was added to a column of inhibitor-Sepharose pre-equilibrated with Tris · HCl/5 mM $CaCl_2$, pH 8.0. The loaded column was washed with the same buffer as used for sample dissolution until the eluate absorption at 280 nm approached that of controls. Then the bound material was eluted with HCl, pH 2.0. Flow rate was 80 ml/h and fractions of 2.3 ml were collected.

Enzyme assays

Enzyme activity assays for Pronase enzymes were carried out using Ac-Tyr-OEt and Bz-Arg-OEt as substrates. Assays were performed in a pH-stat at 25°C, other conditions being those as described by Narahashi [1]. Concentration of *Str. griseus* trypsin in the solution was calculated from its absorbance assuming $E^{1\%}_{1cm}$ = 17.3 (at 280 nm) for pure enzyme [5]. Tryptic and chymotryptic activities were measured by conventional methods using Bz-Arg-OEt and Ac-Tyr-OEt as substrates [14].

Isoelectric focusing

Isoelectric focusing was performed in a 100 ml LKB 8100 column. A pH gradient from 3 to 10 was made using ampholine (LKB 8141). Time of each run was 42 h and temperature was 4°C.

Gel electrophoresis

Electrophoresis in polyacrylamide gel at pH 4.3 was carried out as described by Reisfeld et al. [15] using 7.5% separating gel. The electrophoresis was done at 5 mA per tube for 4 h. Gels were stained with Amido Black.

Identification of amino-terminal residues

The amino-terminal residues of the purified protein were identified by reaction with dansyl chloride. The results were analyzed by two-dimensional chromatography on polyamide sheets [16]. Solvent systems were: 1st dimension, water/90% acetic acid (20 : 3, v/v); 2nd dimension, benzene/glacial acetic acid (9 : 1, v/v).

Results and Discussion

The interaction of bovine trypsin and chymotrypsin with immobilized inhibitor from kidney bean (*P. vulgaris* L.) is illustrated in Figs. 1 and 2. Active trypsin is retained on the column at pH 8.0 and to displace it, the pH was changed

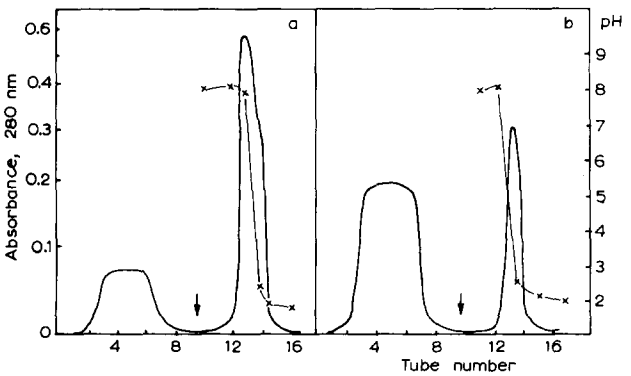


Fig. 1. Fractionation of trypsin preparations on a column with immobilized inhibitor. The column was equilibrated with 0.05 M Tris · HCl at pH 8.0/0.02 M $CaCl_2$/0.2 M KCl. (a), Trypsin (14 mg) dissolved in the above solution was immediately applied to the column; (b), Trypsin preincubated with 4 M urea for 30 min (2°C). ———, A_{280}; —X—, pH. The arrow indicates replacement of the starting buffer with 0.2 M KCl at pH 2.0.

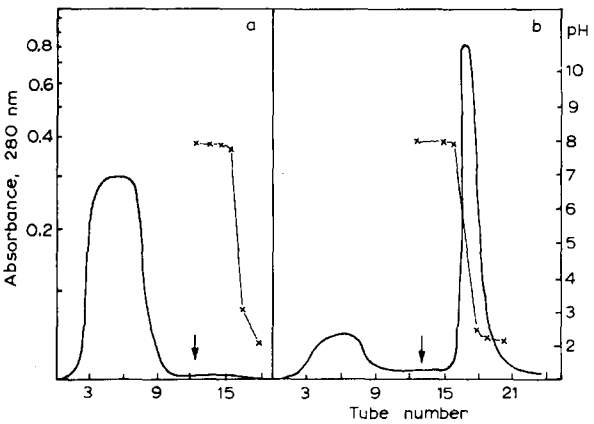


Fig. 2. Affinity chromatography of chymotrypsin on an inhibitor-Sepharose column. (a), Chymotrypsinogen A (14 mg) dissolved in 0.05 M Tris · HCl (pH 8.0)/0.02 M $CaCl_2$/0.2 M KCl was applied to a column equilibrated with the same solution; (b) Chymotrypsinogen (14 mg) preincubated with trypsin (22 : 1 w/w) during 3 h at 0°C was applied to the column. ———, A_{280}; —X—, pH. The arrow indicates replacement of the buffer with 0.2 M KCl at pH 2.0.

to 2.0 (Fig. 1a). About 20% of the initial sample, however, was not retained at pH 8.0 and emerged from the column. The amount of the material not retained at pH 8.0 was greatly enhanced after incubation in 4 M urea for 30 min (Fig. 1b). Enzymic activity determinations (Table I) showed that the material unable to bind the inhibitor at pH 8.0 had no tryptic activity. On the contrary, the protein that was adsorbed at pH 8.0 and subsequently desorbed by acidic solutions was highly active, its specific activity approaching that exhibited by highly purified trypsin preparations [17]. Chymotrypsinogen does not interact with the immobilized inhibitor at pH 8.0 and under the described conditions comes out of the column with the eluate (Fig. 2a). Also, active chymotrypsin, obtained by activation with trypsin, is quantitatively bound by immobilized inhibitor and can be eluted only with acidic (pH 2.0) solutions (Fig. 2b).

These results show that immobilized inhibitor obtained from *P. vulgaris* can

TABLE I

FRACTIONATION OF TRYPSIN BY AFFINITY CHROMATOGRAPHY ON INHIBITOR-SEPHAROSE

Enzyme preparation	Specific activity *			Yield, % (total activity)
	Starting solution	pH 8.0 eluate	pH 2.0 eluate	
Trypsin	56.7	0	67.9	84.0
Incubation with urea 10 min **	27.6	0	68.6	86.1
Incubation with urea 30 min **	17.7	0	56.6	89.0

* Units/mg protein. One enzyme unit was that activity which hydrolyzes I μmol of Bz-Arg-OEt/min.
** Trypsin (5 mg/ml) was incubated with 4 M urea in 0.05 M Tris · HCl (pH 8.0) at 20°C.

selectively bind active trypsin and chymotrypsin and thus can be used for enzyme purification.

Affinity chromatography of Pronase preparation using immobilized kidney bean inhibitor is shown in Fig. 3. The elution profile shows some of the Pronase protein to be firmly bound on the immobilized inhibitor at pH 8.0, and at pH 2.0, this material is eluted from the column. Table II shows the distribution of enzymic activities in the eluates obtained at pH 2.0. One can see that 80—90% of the initial activity towards Bz-Arg-OEt is firmly bound at pH 8.0, whereas the components hydrolysing Ac-Tyr-OEt do not interact with the immobilized inhibitor. The specific activity of material eluted from the column at pH 2.0 (Bz-Arg-OEt as substrate) is some 10—15 times higher than that of the starting Pronase preparation. This specific activity increase was particularly significant in those runs where the sample was applied to the column in solutions containing high concentrations of salt and the subsequent elution was also carried out in the presence of 0.2 M KCl (runs Nos. 3—5; Table II). This is apparently due to a decrease of nonspecific binding of protein under these conditions. Maximal activity obtained correspond to 130—140 units/mg protein. This value is near to the specific activity of the most purified preparations of *Str. griseus* trypsin (150—170 units/mg protein) obtained by ion-exchange chromatography [5]. These results show that the trypsin-like enzyme from Pronase is selectively bound with the immobilized inhibitor from kidney bean.

Analysis of the amino-terminal residues showed predominance of valine with trace amounts of threonine, glycine and alanine. Smillie et al. [6] have shown valine to be the amino-terminal amino acid in the *Str. griseus* trypsin. It is of interest that among the identified amino-terminal residues there is no isoleucine, which is the amino-terminal residue in chymoelastases I and II [18]. (Chymoelastases and the subtilisin-like enzyme are responsible for the bulk of Pronase activity towards Ac-Tyr-OEt). The esterase activity of the enzyme obtained by affinity chromatography was completely inhibited after 30 min incubation with 7-fold molar excess of L-1-chloro-3-tosylamido-7-amino-2-heptatone at pH 7.9. The enzymic activity was also inhibited by diisopropyl phosphorofluoridate and soybean trypsin inhibitor (Kunitz), but was not inhibited by L-1-chloro-3-tosylamido-4-phenyl-2-butanone.

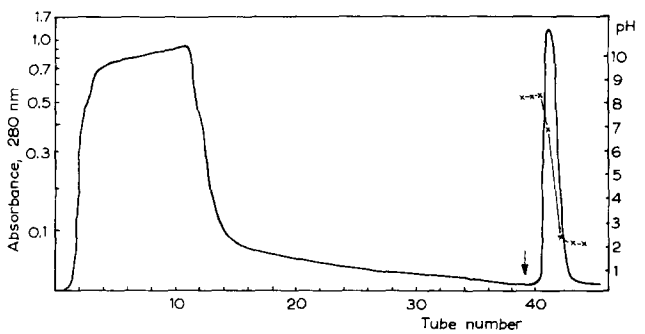


Fig. 3. Affinity chromatography of Pronase on immobilized inhibitor from kidney bean. Pronase (150 mg) was dissolved in 50 ml of 5 mM Tris · HCl (pH 8.0) buffer/5 mM $CaCl_2$/0.2 M KCl and applied to the column. The bound protein was eluted with 0.01 M HCl. ———, A_{280}; —X—, pH. The beginning of elution is indicated with an arrow.

TABLE II

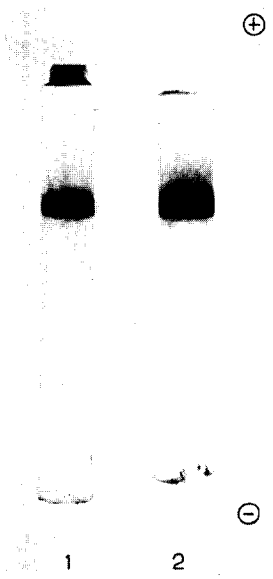
ENZYMIC ACTIVITIES OF THE PRONASE PROTEIN ELUTED WITH ACIDIC SOLUTION FROM THE INHIBITOR-SEPHAROSE COLUMN

Expt. No.	Specific activity (units/mg protein)		Total activity % *		Protein % *
	Bz-Arg-OEt	Ac-Tyr-OEt	Bz-Arg-OEt	Ac-Tyr-OEt	
1	111.6	2.3	85.4	10.2	9.6
2	94.6	—	89.4	—	10.9
3	130.5	0.98	79.3	1.77	7.5
4	135.0	1.08	81.9	1.66	7.3
5	135.0	1.00	88.0	1.50	12.0

* % of activity or total protein applied to the column.

The polyacrylamide gel electrophoresis of the purified enzyme carried out at two sample concentrations and at pH 4.3 has shown it to contain only one protein component (Fig. 4). Ultracentrifugation of purified sample has also shown it to be homogenous with a sedimentation coefficient of 2.46 S.

Isoelectric focusing of the enzyme, pretreated with diisopropyl phosphorofluoridate, has shown the sample to contain a main component of p*I* 9.2 and several minir ones, of which the more abundant one had a p*I* 7.5 (Fig. 5). It is suggested that the minor components resulted from autolysis of the enzyme,



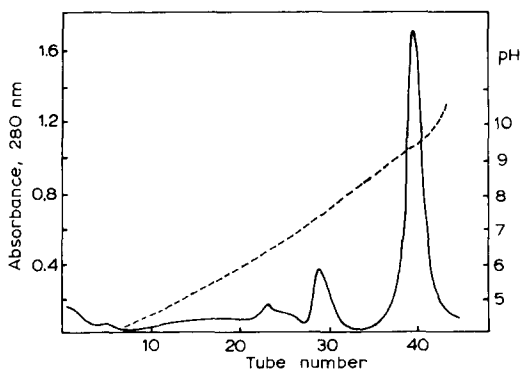


Fig. 4. Polyacrylamide gel electrophoresis of *Str. griseus* trypsin-like enzyme purified by affinity chromatography; (1) 1.32 μg of protein, (2) 2.65 μg of protein. Electrophoresis was performed at 5 mA per tube for 4 h (4°C).

Fig. 5. Isoelectric focusing of *Str. griseus* enzyme purified by affinity chromatography. Isoelectric focusing was performed for 42 h at 4°C. ———, A_{280}; - - - - -, pH.

since diisopropyl phosphorofluoridate inhibition was rather slow (after 2 h incubation some 20% activity remained). In the case of a preliminary treatment of the enzyme with L-1-chloro-3-tosylamido-7-amino-2-heptanone, the content of minor components was considerably reduced.

The above-described method gives a yield of 80% which is much higher than the yields obtained with ion-exchange chromatography [7,19]. A comparable high yield of trypsin-like enzyme from Pronase was also reported by Ishii et al. [20] who used affinity chromatography on an agarose derivative which was prepared by coupling a tryptic digest of salmine to cyanogen bromide-activated Sepharose 4B.

The kidney bean inhibitor is known to reduce the activity of Pronase with Ac-Tyr-OEt and Bz-Arg-OEt in the solution. Furthermore this protein is the so-called "double-headed inhibitor" [9]. It seems very probable that the trypsin-like enzyme from *Str. griseus* would bind to the same binding site on the inhibitor molecule which binds bovine trypsin. Likewise, one would expect that enzymes hydrolysing Ac-Tyr-OEt should be bound by the same site which binds bovine chymotrypsin.

However, it follows from the above that the last-mentioned enzymes do not interact with an immobilized inhibitor. One possible reason is that these enzymes interact not with the chymotrypsin binding site but with some other site on the inhibitor molecule, which gets buried or otherwise masked during immobilization. A second reason might be that, after immobilization, the inhibitor loses its ability to bind two enzymes simultaneously. To test the latter, a trypsin solution was passed through the column with immobilized inhibitor at pH 8.0 (an excess of trypsin was used to saturate all the possible trypsin binding sites). Free trypsin was removed by eluting with a pH 8.0 solution, then the column was loaded with chymotrypsin and again washed with a pH 8.0 solution to remove free chymotrypsin. Both bound trypsin and chymotrypsin were eluted with HCl at pH 2.0. To determine trypsin and chymotrypsin in the eluate separately, we made use of the capability of chicken ovomucoid to selectively bind trypsin [17]. The acidic eluate containing both trypsin and chymotrypsin was brought to pH 8.0 and applied to a second column packed with immobilized chicken ovomucoid. Chymotrypsin passed through the column without being retarded and was quantitatively determined in the eluate. The column was then eluted with an acidic solution to release trypsin. An analogous experiment has been carried out, which involved a reverse order of applying enzymes to the frist column. Chymotrypsin solution was the first to be applied to the column with the kidney bean inhibitor. Determination of bound chymotrypsin and trypsin was made using the same procedure with immobilized chicken ovomucoid.

It is clear from Table III that, if the immobilized inhibitor is first loaded with trypsin and afterwards with chymotrypsin, then the amount of bound chymotrypsin is rather low. Apparently, trypsin binding in some way reduces the ability to bind chymotrypsin. In the alternate experiment when the inhibitor is first loaded with chymotrypsin and then with trypsin, the binding of the last enzyme is not at all impaired and, further, trypsin seems to partially displace chymotrypsin. It may be suggested that trypsin and chymotrypsin have partially overlapping binding sites on the inhibitor molecule. In solution, there is no

TABLE III

CONCURRENT BINDING OF TRYPSIN AND CHYMOTRYPSIN BY THE IMMOBILIZED INHIBITOR FROM KIDNEY BEAN

Expt. No.	Order of application	Enzyme bound in mg	
		Trypsin	Chymotrypsin
1	Trypsin	25.2	—
2	Chymotrypsin	—	24.8
3	Chymotrypsin added after trypsin	25.2	8.4
4	Chymotrypsin added before trypsin	24.1	11.2

obvious competition beween the two enzymes and ternary complexes are formed.

After immobilization, some auxiliary binding groups of the inhibitor may become masked and, as a result, the competition between the two enzymes is more pronounced. Under such conditions one would expect preferential binding for that enzyme which has greater affinity to the inhibitor, or whose binding site is less disturbed by the immobilization. In our case this evidently is trypsin. It seems that a similar mechanism is operative in the case of *Str. griseus* trypsin binding with the immobilized inhibitor.

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