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Trypsin-Like Enzyme from *Streptomyces 771*

Purification and Properties of Native and Immobilized Enzyme

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ABSTRACT

Electrophoretically homogenous proteolytic enzyme with molecular weight 31,500 and pI 3.75 was obtained from a culture medium of *Streptomyces 771* by chromatography on *N*-benzyl chitin adsorbent, subsequent chromatography on CM-cellulose, and preparative isofocusing and chromatography on Sephadex G-75. The enzyme hydrolyzes *N*-benzoyl-DL-arginine-*p*-nitroanilide *N*-benzoyl-DL-lysine-*p*-nitro- anilide

N-benzoyl-DL-arginine ethyl ester, and Na-caseinate. It also exhibits pronounced thrombolytic activity. The activity of the enzyme was suppressed by soya bean inhibitor, but remained unaffected by chelating agents and phenylmethylsulfonyl fluoride. The enzyme was immobilized on aldehyde dextran, and some kinetic parameters of the immobilized enzyme were determined. The thrombolytic activity of native and immobilized enzyme was studied as well.

Index Entries: Proteolytic enzyme, from Streptomyces 771; chromatography, of a proteolytic enzyme; thrombolytic activity, of a proteolytic enzyme; soya-bean inhibitor, of proteolytic enzyme; immobilization, of a trypsin-like enzyme; aldehyde dextran, immobilization of a proteolytic enzyme on; dextran, immobilization of a proteolytic enzyme on; streptomyces 771, a trypsin-like enzyme from; trypsin-like proteolytic enzyme, from streptomyces 771.

INTRODUCTION

Trypsin-like enzyme (TLE) from *Streptomyces griseus* is the best studied TLE (*1–4*), and even its primary structure has been determined (*5*). There are some reports on isolation and characterization of TLE from *Streptomyces paramomycinus* (*6*), *Streptomyces fradiae* (*2,3,7*) and *Streptomyces erythreus* (*2,3,8*). *Streptomyces 771* is also known to produce TLE (*9*).

Data on the purification and the properties of native TLE from *Streptomyces 771* and that of enzyme immobilized on aldehyde dextran are presented in this work.

MATERIALS AND METHODS

Materials

The following chemicals were used: Sephadex G-25, G-75 (Pharmacia, Sweden), *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*-benzoyl-DL-lysine-*p*-nitroanilide (BLPNA), *N*-benzoyl-DL-arginine ethyl ester (BAEE) (Merck, West Germany), CM-cellulose (Whatman, England), cytochrome c, chymotrypsinogen A, ovalbumin, bovine serum albumin, servalytes, pH 3–5 (Serva, West Germany), soya-bean trypsin inhibitor (STI) (Sigma, USA), Na-caseinate (Chemical Plant, Olaine, USSR). Other chemicals were of analytical grade.

Recovery of Crude Enzyme

Crude enzyme was obtained from culture liquid of *Streptomyces 771* (*9, 10*) by filtration, $(NH_4)_2SO_4$ precipitation (60% saturation), centrifugation, dialysis and freeze-drying.

Preparation of N-benzylchitin Adsorbent

The adsorbent was obtained by coupling benzyl chloride to native chitin in the presence of NaI (*11*). The number of benzyl groups coupled was 50–60 μmol/g of adsorbent. The adsorbent was used for hydrophobic chromatography.

Determination of Tryptic Activity

Tryptic activity was measured by the method (*12*) using BAPNA or BLPNA. The solution of BAPNA (43.5 mg) or of BLPNA (40.7 mg) in 5 mL of dimethylsulfoxide was diluted to the final volume of 100 mL by 0.05*M* Tris-HCl buffer, pH 8.2, containing 0.02*M* $CaCl_2$ (final concentration of substrate, $10^{-3}M$). A 2.5 mL vol of substrate solution was thermostated for 10 min at 30°C and then 0.1 mL of enzyme solution was added. After the incubation (20 min) the reaction was stopped by adding 1 mL of 30% acetic acid in dioxane. In the control tube, the enzyme was introduced after the addition of 30% acetic acid in dioxane. Optical density was measured at 410 nm. One activity unit was defined as the amount of enzyme that hydrolyzes 1 μmol of substrate in 1 min at 30°C, pH 8.2.

Protein Determination

Protein was determined by the method of Lowry et al. (*13*).

Determination of Proteolytic Activity

Proteolytic activity was measured by the modified Anson's method (*14*) in 0.1*M* universal buffer, pH 7.5, using 2% Na-caseinate as a substrate. Activity unit was defined as the amount of enzyme capable to convert Na-caseinate into a product not precipitated by CCl_3COOH in the quantity equivalent to 1 μmol of tyrosine in 1 min at 30°C. The amount of tyrosine was determined by the reaction with Folin reagent (*14*).

Enzyme Purification

Freeze-dried crude enzyme (10 g) dissolved in 400 mL of 0.01*M* universal buffer (acetic, boric, and phosphoric acids, plus NaOH), pH 3.2, containing 0.002*M* calcium acetate, was applied to a 4.5 × 60 cm column packed with 140 g of *N*-benzyl chitin adsorbent and equilibrated with the same buffer. The majority of inactive proteins and other proteolytic enzymes were removed from the column by eluting with the starting buffer. However, TLE was retarded on the column and was eluted with the same buffer later. Fractions containing TLE were combined, concentrated to 70–80 mL and applied to a 3.0 × 12 cm CM-cellulose column

equilibrated with the buffer described above, pH 4.3 (step gradient). Inactive proteins and pigments were not retarded by the column; TLE was eluted with the same buffer, pH 5.0. Fractions containing TLE were combined and further purified by preparative isoelectric focusing according to the method of Westerberg and Swenson (*15*). Isoelectric focusing was carried out in a 440 mL column with 1% servalytes, pH 3–5, in a 5–50% sucrose gradient at 4°C for 90 h (1000–1500 V). Fractions of 4 mL volumes were collected. Optical density at 280 nm, pH, and TLE activity were determined in each fraction. Sucrose, servalytes, and traces of large molecular weight protein were separated by gel filtration on Sephadex G-25 and then on Sephadex G-75. Fractions with TLE were freeze-dried. All purification steps were performed at 4°C. Purified TLE in air-dried stat can be stored at the room temperature, in solution, at 4°C.

Disc Electrophoresis in Polyacrylamide Gel

Molecular weight and homogeneity of TLE were determined by disc electrophoresis in SDS-polyacrylamide gel in Tris-glycine buffer (*16,17*). Mercaptoethanol (5%) and SDS were used to dissociate disulfide bonds. Cytochrome c, MW 12,400; chymotrypsinogen A, MW 25,000; ovalbumin, MW 45,000; and bovine serum albumin, MW 68,000 were used as standard proteins.

Effect of Specific Inhibitors on TLE

The solution of TLE in 0.002*M* calcium acetate was incubated for 10 min at 30°C in the presence of appropriate inhibitor (10^{-2}—$10^{-4}M$) and after incubation proteolytic and tryptic activities were measured.

Influence of Metal Ions on Tryptic Activity

Aqueous solutions, of $ZnCl_2$, $CoCl_2$, $FeCl_2$, $CuCl_2$ and NH_4Cl, each at $2 \times 10^{-2}M$ were mixed with the solution of TLE in 0.002*M* calcium acetate in the ratio 1 : 1 and incubated for 30 min at 30°C. T LE activity was measured after the incubation.

Amino Acid Analysis

The amino acid composition of TLE was determined by the method of Moore and Stein (*18*) using an LKB-3201 amino acid analyzer. Protein hydrolysis was carried out in 6*N* HCl at 110°C for 24 h. Cystine and methionine were determined as cystic acid and methionine sulfone, respectively, after oxidation according to Moore's method (*19*).

Immobilization of TLE on Aldehyde Dextran

Aldehyde dextran, MW 35,000–50,000, containing 20 oxidized cycles per 100 native units, was prepared by the method described in ref. (*20*).

A 0.5 mL vol of aldehyde dextran solution in water was mixed with 1.5 mL of 0.1*M* phosphate buffer, pH 8.3; 0.5 mL of $5 \times 10^{-5}M$ enzyme solution was added, and the mixture was incubated for 3 h at 4°C. The final concentration of aldehyde dextran in the mixture was (2.0–3.0) $\times 10^{-4}M$.

The enzyme preparation was isolated by gel filtration on Sephadex G-75 and equilibrated with 0.02*M* phosphate, pH 8.3.

Determination of Catalytic Activity of TLE.

The catalytic activity was determined on the pH-stat TTT-Id (Radiometer, Copenhagen, Denmark). For this purpose 4.95 mL of $10^{-2}M$ BAEE solution in 0.1*M* KCl were mixed with 0.05 mL enzyme solution, and the kinetic curve was recorded at room temperature for the pH 7.8 mixture.

The concentrations of native enzyme and modified enzyme in the pH-stat cuvet were $10^{-7}M$ and $4.11 \times 10^{-7}M$, respectively. Those of BAEE were 10^{-4}, 2×10^{-4}, 3×10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3}, and $10^{-2}M$.

Thermoinactivation Studies

A 1 mL vol of $10^{-5}M$ enzyme solution was added to 9 mL of 0.02*M* phosphate buffer, pH 7.8, and the mixture was incubated at 37 or 50°C. At certain time intervals, 0.5 mL samples were taken and their catalytic activity was measured on a pH-stat, as described above. The concentrations of TLE in the incubation mixture of native enzyme and of TLE modified with aldehyde dextran were 10^{-6} and $4.1 \times 10^{-6}M$, respectively.

Experiments on Thrombolysis

All experiments were performed in 0.1*M* phosphate buffer, pH 7.4. A 0.5 mL vol of fibrinogen solution (5 mg/mL) and 0.2 mL of thrombin solution (2 mg/mL) were introduced into a glass tube with a porous net bottom covered with parafilm. The tube was kept at room temperature for an hour after the clot formation and then parafilm was removed and the tube was immersed in 20 mL of enzyme solution ($1.33 \times 10^{-5}M$ solution of native enzyme, and $1.1 \times 10^{-5}M$ in modified enzyme) under a constant stirring with stirrer.

The clot dissolution was followed according to the changes in optical density of the enzyme solution ($A_{280\ nm}$) and visually by a reduction of clot size.

The rate of clot lysis was determined from the slope of linear plots % ($\Delta A_t/\Delta A_{max}$) vs time, where ΔA_t is the difference in optical density of the test and control solutions at moment t.

RESULTS AND DISCUSSION

Purification of the TLE

The data on TLE purification are presented in Table 1. Purification involves four steps including chromatography on chitin adsorbent, chromatography on CM-cellulose, preparative isoelectric focusing, and gel-filtration on Sephadex G-75. During the chromatography of crude enzyme preparation on *N*-benzylchitin adsorbent, the specific adsorption of TLE takes place at pH 3.2, while several other proteases are not retarded on the column. However, along with the TLE adsorption, the adsorption of small amounts of pigments, catalytically inactive proteins and at least two other proteases takes place. That is why additional purification was performed, including chromatography on CM-cellulose. It was shown that pigments and inactive proteins were not adsorbed on CM-cellulose at pH 4.3, whereas TLE was retarded on the column and eluted with the same buffer at pH 5.0. From the data on the influence of various inhibitors on proteolytic and trypsin-like activities (Table 2) it follows that on this step the preparation of TLE contains, evidently, the admixture of metalloprotease, which can be removed by means of chromatography on biospecific adsorbent STI-Sepharose or by preparative isoelectric focusing (Fig. 1). The activity yield after chromatography was only 50%, whereas the preparative isoelectric focusing resulted in almost a quantitative yield of TLE activity. Thus, isoelectric focusing was chosen for the purification of TLE. Gel-chromatography on Sephadex G-75 was used to remove traces of high molecular weight proteins and servalytes. As a result, electrophoretically homogenous TLE was obtained (Fig. 2).

TABLE 1
Purification of TLE from *Streptomyces 771*

Step	Protein, mg	Activity, units	Specific, activity, (μ/mg)	Purification (fold)	Yield, %
Crude enzyme	19,480	168.8	0.86×10^{-2}	1.0	100
Chromatography on biospecific adsorbent	200	71.1	35.8×10^{-2}	41.3	42.2
Chromatography on CM-cellulose	49.1	62.1	126.6×10^{-2}	146.1	36.8
Preparative isoelectric focusing and chromatography on Sephadex G-75	28.3	51.2	181×10^{-2}	209	30.4

TABLE 2
The Activation of Inhibitors on Proteolytic and Tryptic Activities of TLE Obtained after the Second Step of Purification

Inhibitor	No.	*o*-Phenanthroline, $10^{-2}M$	PMSF,[a] 5×10^{-4}	Soya-bean, inhibitor, 0.1 mg/mL
Proteolytic activity, %	100	17.3	37.5	65.8
Tryptic activity, %	100	83.7	114.6	0.00

[a]Phenylmethylsulfonyl fluoride.

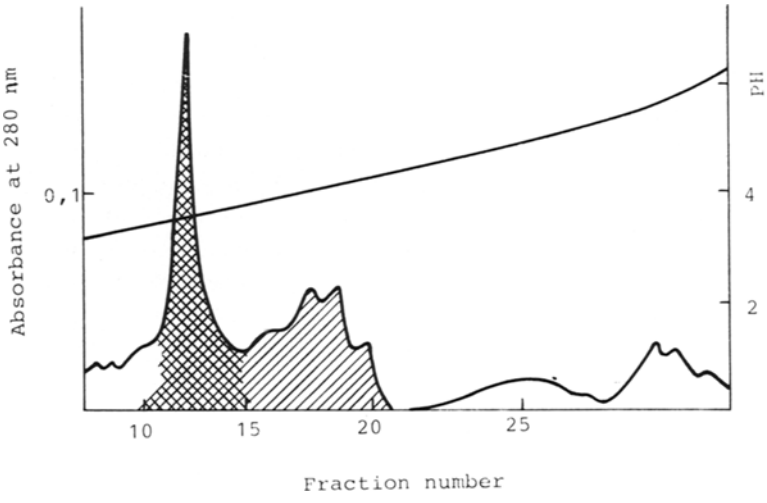

Fig. 1. Isoelectric focusing of TLE fraction: 53 mg of protein, 0.002*M* Ca acetate; 1% servalytes; pH 3–5; sucrose gradient 5–50%; −4°C, 96 h, 1000–1500 V. ▨, tryptic and proteolytic activity; ▨, proteolytic activity.

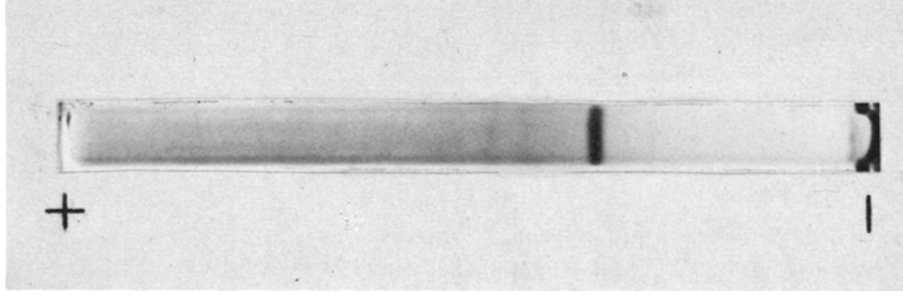

Fig. 2. SDS-electrophoresis of purified TLE.

Though the crude enzyme contained only 0.15% of TLE, the total yield of TLE was high enough (30%). The first step of purification resulted in the largest loss of activity. It may be caused by the removal of some activating substances present in a crude preparation.

Some Properties of TLE

The ability of TLE to hydrolyze BAPNA and BLPNA and to be inhibited by STI indicates that the enzyme resembles a trypsin-like protease, which is characteristic of actinomyces.

Typical inhibitors of metalloproteases like *o*-phenantroline and EDTA have only a slight effect on the tryptic activity of TLE. This leads to the conclusion that the isolated protease is not metalloprotease. Various metal ions and ammonia have only slight effects on the activity of TLE (Zn^{2+}, Mn^{2+}, Co^{2+}, NH_4^+, Cu^{2+}, Mg^{2+}). Only Fe^{2+} ions diminished the activity up to 35%.

The isoelectric point of the pure enzyme (p*I* 3.75) is close to that of TLE from *Streptomyces erythreus* [pI 4 (*8*)], but differs considerably from that of TLE from *Streptomyces griseus* and *Streptomyces fradiae* [p*I* 9 (*3*)]. The molecular weight of TLE from Sephadex G-75 gel chromatography data is 31,500.

The high content of acidic amino acids in TLE is in good agreement with the low value of its isoelectric point. A high content of dicarboxylic amino acids was also reported for TLE from *Streptomyces erythreus* (*8*). According to the cysteine content, the enzyme resembles TLE from *Streptomyces griseus*. The content of other amino acids is smaller to that of other TLE (Table 3).

Properties of Immobilized TLE

Figure 3 shows pH dependence of TLE catalytic activity in BAEE hydrolysis. At pH values exceeding 7.7, the catalytic activity reaches a plateau. Coupling of TLE to aldehyde dextran has no effect on the shape of the curve. The esterase activity of the enzyme modified by aldehyde dextran (MW 35,000–50,000) was 80% of the native enzyme (Fig. 3).

Kinetic data on catalytic hydrolysis of BAEE by native and immobilized TLE are given in Table 4. The coupling of TLE to low-molecular weight dextran resulted in slightly improved substrate binding, but k_{cat} remained unchanged.

The thermostability of native TLE at 37°C and pH 7.8 is rather high (Fig. 4). Coupling of the enzyme to support resulted in even more increased thermostability at 37 and 50°C. The values of the corresponding first-order rate constants of thermoinactivation are given in Table 4.

A promising area for the application of immobilized proteolytic trypsin-like enzymes lies in their potential medical use. This kind of preparation can be successfully employed in thrombolytic therapy, where enzyme stability under physiological conditions becomes very im-

TABLE 3
Amino Acid Composition of TLE from Different Sources[a]

Amino acid	*Streptomyces* 771	*Streptomyces griseus* (4)	*Streptomyces fradiae* (8)	*Streptomyces erythreus* (8)	*Streptomyces paramomycinus* (6)
Aspartic acid	42	18	13	31	18
Threonine	17—18	16	19	15	17
Serine	19	14	18	9	17—18
Glutamic acid	38	17	15	23	15—16
Proline	14	8	10	11	11
Glycine	32	28	31	31	35
Alanine	29	26	24	27	23
Cystine	6	6	4	4	4
Valine	14	18	11	24	15
Methionine	6	3	3	3	2
Isoleucine	8	8	7	5	7
Leucine	17	11	12	8	11
Tyrosine	14	8	7	5	5—6
Phenylalanine	6-7	6	4	3	6
Histidine	2—3	1	1	3	1
Lysine	17	6—7	4	8	13
Arginine	8	8	8	3	10
Tryptophan	—		4	2	—

[a]Amino acid residues 299–302.

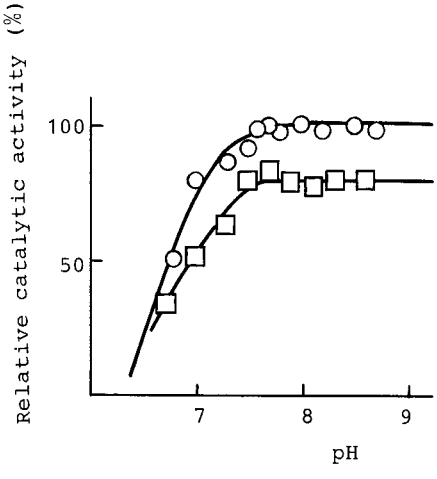

Fig. 3. pH Profiles of catalytic BAEE hydrolysis by native (○) and coupled to adelhyde dextran (□) TLE. Experimental conditions: BAEE $10^{-2}M$ in $0.1M$ KCl, $[E_{nat}] \sim 10^{-7}M$, $[E_{mod}] \sim 4.11 \times 10^{-7}M$, 20°C.

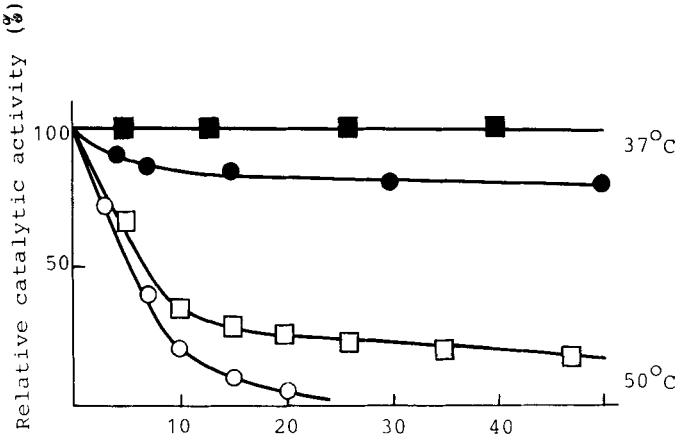

Fig. 4. Thermoinactivation of TLE preparation: ●, native and ■, coupled to aldehyde dextran, TLE at 37°C; ○, native and □, coupled to aldehyde dextran, TLE at 50°C. Experimental conditions: 0.02*M* phosphate buffer, pH 7.8, $[E_{nat}] \sim 10^{-6}$M, $[E_{mod}] \sim 4.1 \times 10^{-6}M$.

TABLE 4
Properties of Native and Dextran-Immobilized TLE from *Streptomyces 771*

Enzyme	Esterase activity,[a] %	Parameters of catalytic hydrolysis of BAEE		K_{inact} at 50°C, min^{-1}	Thrombolytic activity, %
		K_m, *M*	K_{cat}, S^{-1}		
Native TLE	100	1.82×10^{-4}	127	0.150	100
Dextran-immobilized TLE	80	0.72×10^{-4}	125	0.018	113

[a]0.1*M* KCl, pH 7.8, room temperature, $10^{-2}M$ BAEE; 0.1*M* KCl, pH 7.8, room temperature; 0.02*M* phosphate buffer, pH 7.8, 50°C.

portant. In this case it is also very important to preserve specific properties of the enzyme upon immobilization or modification. The data on thrombolysis by native TLE and TLE coupled to aldehyde dextran show (Table 4) that the thrombolytic activity of the enzyme is only slightly affected by modification.

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