ISOLATION AND PROPERTIES OF ELASTOLYTIC ENZYME OF Actinomyces rimosus

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The elastolytic enzymes catalyze the hydrolysis of insoluble protein, of elastin clear down to low-molecular products. The elastase from the pancreas of animals has been studied in greatest detail: the mechanism of its reaction with substrates was studied and its chemical and spatial structure was established [1-3] Elastolytic activity is exhibited by some of the proteases that are produced by the microorganism Act. rimosus, used for the preparation of hydroxytetracyline [4]. Previously the protease complex from the culture fluid of Act. rimosus was isolated and purified chromatographically based on protein by passage through carboxyl resin KB-51 for 10-15 times [5]. A method for the isolation of the pure elastase of Act. rimosus and some of the properties of this enzyme are described in the present paper.

EXPERIMENTAL METHOD

The starting protease complex of Act. rimosus was obtained as described in [5]. To determine the elastolytic activity (EA) the degree of hydrolysis of the elastin obtained from the cervical ligaments of horned cattle, modified with Remazol dye (Remazol Brilliant Blue R.), and specifically RBB elastin, was measured colorimetrically by a modification of the method given in [6]. To 20 mg of the elastin in 6 ml of 0.1 M glycine buffer solution with pH 8.8, containing $1 \cdot 10^{-4}$ mole of $Ca(CH_3COO)_2$, was added 1 ml of the enzyme solution in the same buffer, the mixture was kept at 40° for 1 h with periodic shaking, 1 ml of saturated NaCl solution was added to stop the reaction, and the colored solution was filtered through filter paper and measured colorimetrically on a PEK-54 instrument at 596 nm. The amount of hydrolyzed RBB elastin was determined from a calibration curve. The amount of enzyme that converts 1 mg of the RBB elastin into solution under the described experimental conditions was taken as the EA unit. The esterase activity was determined by the action of the enzyme on a synthetic ac-ala_3-OCH_3 substrate as described in [7]. The caseinolytic activity (CA) was measured by the absorption of the digestion products of casein at 280 nm as described in [8]. A mixture of 1 ml of a 2% casein solution in 1/15 M phosphate buffer, pH 7.1, and 1 ml of the enzyme solution was heated at 40° for 10 min, after which 2 ml of a 5% trichloroacetic acid (TCA) solution was added. The trypsin and chymotrypsin activity were determined as described in [9]. The collagenolytic activity was determined viscosimetrically by the decrease in the viscosity of a 1% procollagen solution at 25° in 15 min, pH = 7.5, and E : S = 1 : 1.

The isoelectric focusing was run on an LKB-Producter instrument (Sweden). The enzyme (60 mg) was transferred to a 110-ml column; ampholytes were used as supports in a gradient of 40% saccharose solution to create a pH gradient ranging from 3.0 to 10.0. After electrophoresis for 36 h at 500 V (from cathode to anode), 1-ml fractions were collected and the pH was measured. The ampholytes were separated from the proteins by dialysis, after which the EA was determined.

* The following abbreviations were adopted in the present paper: DDP = disopropyl fluorophosphate, MPCB = mercury p-chlorobenzoate, Trilon B = disodium salt of ethylenediaminetetracetic acid, DDS = sodium dodecyl sulfate, BAEE = ethyl ester of benzoyl-L-arginine, ATEE = ethyl ester of N-acetyltyrosine, and E : S = ratio of enzyme and substrate concentrations.

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TABLE 1. Purification of Elastases of Act. rimosus

Purification step	Protein, mg	EA			CA		
		Total	Specific	Yield, %	Total	Specific	Yeild, %
Starting preparation	1000	50 000	50	100	12 000	12	100
Chromatography on CMC	100	10 800	108	21,6	400	4,0	33,3
Gel filtration on Sephadex G-75	50	7000	140	14,0	105	2,1	8,8
Rechromatography on Sephadex G-75	40	7000	175	14,0	80	2,0	6,7

The disk electrophoresis was run in 7.5% polyacrylamide gel at pH 8.7 and 4.3 [10], for 20 min at 2 mA in a tube, and for 70 min at 50 mA in a tube, at 380 V. The gel was stained with a 1% solution of Amide Black 10B in 7% CH_3COOH.

The molecular weight was determined by gel filtration as a thin layer on 14 × 16 cm plates, covered with ultrafine Sephadex G-150 [11]. The rate of the solvent front was 3 cm/h in 0.9% NaCl solution. The chromatogram was stained with bromphenol blue. As the protein standards we used highly purified collagenase of Clostridium hystolyticum (103,000), albumin serum, egg albumin, and pepsin. The molecular weight and sedimentation constant were also measured on a Spinco-E ultracentrifuge at 19,160 and 56,100 rpm. For this we took a 0.7% solution of the protein in $1 \cdot 10^{-4}$ M acetate buffer in the presence of 0.5% NaCl at 20°. The molecular weight was calculated employing the method of approaching the sedimentation equilibrium [12].

To determine the EA as a function of the pH the enzyme was dissolved in $1 \cdot 10^{-4}$ M $Ca(CH_3COO)_2$ solution, and then 0.1 ml of this solution was added to 6.9 ml of 0.2 M glycine buffer with a variable pH (from 4 to 11), and the EA was measured. To determine the CA as a function of the pH the enzyme was dissolved in 1/15 M phosphate buffer with the appropriate pH, containing $1 \cdot 10^{-4}$ M $Ca(CH_3COO)_2$, and the solution was added to a 2% casein solution with the same pH in phosphate buffer.

To determine the EA stability of the preparation as a function of the pH the enzyme was dissolved in 0.1 M glycine buffer containing $1 \cdot 10^{-4}$ M Ca^{2+}, kept at 40° for 1 h, and the EA was determined at pH 8.8. When studying the effect of inhibitors a sample of the enzyme containing the inhibitor was kept at 25° for 1 h, after which the EA was determined. A solution of the enzyme containing urea was kept at 40° for 1 h in the presence of $1 \cdot 10^{-4}$ Ca^{+}, after which the solution was diluted 100-fold and the residual EA was determined.

To study the effect of DDS on the activity of the enzyme the DDS was added to a solution of the enzyme (0.2 mg/ml in $1 \cdot 10^{-4}$ M $Ca(CH_3COO)_2$), and after 1 h the EA was determined by adding the sample containing the enzyme to RBB elastin in glycine buffer, containing the appropriate concentration of DDS. The control experiment was run under analogous conditions, but without the DDS. In the second experiment the RBB elastin was first incubated with various concentrations of DDS in 0.1 M glycine buffer, pH 8.8, for 1 h. After this the DDS was removed by washing the RBB elastin 5 times with glycine buffer minus the DDS and subsequent centrifuging. The enzyme was not treated with DDS.

The amino acid composition was determined by the standard procedure on an AAA 881 amino acid analyzer.

Isolation of Elastolytic Enzyme of Act. rimosus. The starting preparation of the enzymes of Act. rimosus was separated into the components chromatographically on carboxymethylcellulose (CMC) (Fig. 1). For this a 3.5 × 95 cm column containing CMC was equilibrated with acetate buffer (0.003 M CH_3COONa with 0.002 M $Ca(CH_3COO)_2$) pH 5.5. Then 1.2 g of the preparation, with an activity of 50 units/mg of EA and 12 units/g of CA was dissolved in 20 ml of the starting buffer, dialyzed against this buffer, centrifuged, and the clear solution was transferred to the column. After 800 ml of the buffer had been passed through the column a linear gradient of 0.125 M NaCl was supplied. The elution rate at 4° was 48 ml/h. The volume of the fractions was 12 ml. The fractions with EA were combined, dialyzed against $1 \cdot 10^{-4}$ M $Ca(CH_3COO)_2$ at 4°, pH 5.5, and dried lyophilically.

For the gel chromatography on Sephadex G-75 (medium) of the fraction with an EA (Figs. 2 and 3, Table 1) after CMC, 100 mg of the lyophilically dried fraction with an EA of 108 units/mg and a CA of 4 units/g in 5 ml of $1 \cdot 10^{-4}$ M $Ca(CH_3COO)_2$ solution, pH 5.5, was transferred to a 3 × 100 cm column, equiliberated against this solution. The elution rate at 4° was 18 ml/h.

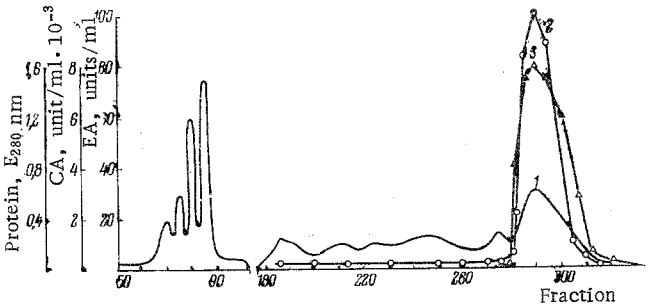

Fig. 1. Chromatography of protease preparation from Act. rimosus on CMC. Column 3.5 × 95 cm, equilibrated with acetate buffer (0.003 M CH_3COONa with 0.002 M $(CH_3COO)_2Ca$), pH 5.5. Volume of fractions 12 ml. Elution rate 48 ml/h. Here and in Figs. 2 and 3: 1) protein, $OP_{280\,nm}$; 2) EA, $OP_{595\,nm}$; 3) $OP_{280\,nm}$.

Rechromatography on DEAE-Cellulose. On a 2.2 × 40 cm column, equilibrated against 0.05 M glycine buffer, pH 7.8, was deposited 8 mg of the elastolytic enzyme with an EA of 170 units/mg, which was first dialyzed against this buffer at 4°. The enzyme was eluted with the starting buffer. Besides the fraction with an EA, which emerged together with the original volume of 18 ml, no other additional components were detected.

The L-amino acids were used in the synthesis of the ac-ala-ala-ala-OCH_3 substrate. The purity of the obtained products was checked by TLC in the systems: $H_2O-CH_3COOH-n-C_4H_9OH$ (30:10:100) (A); $sec-C_4H_9OH-3\%\ NH_3$ (100:44) (B).

Cbo-ala-ala-OCH_3 (I). To a stirred solution of 1.77 ml of isobutyl chloroformate in 15 ml of absolute $CHCl_3$, cooled to −15°, was slowly added 3 g of CbO-ala-OH and 1.85 ml of $(C_2H_5)_3N$ in 6 ml of absolute $CHCl_3$. The reaction mixture was stirred at −10° for 20 min, after which a cooled to −10° solution of 1.86 g of HCl · H-ala-OCH_3 and 1.85 ml of $(C_2H_5)_3N$ in 22 ml of absolute $CHCl_3$ was added, and the mixture was stirred for 1 h at −10°, 30 min at 0°, 1 h at 20°, and 10 min at 50°. The solution was diluted with $CHCl_3$ and washed in succession with water, 1 N HCl, 0.5 N $NaHCO_3$ and water, and dried over Na_2SO_4. After evaporation of the solvent in vacuo we obtained 39 g (76% yield) of (I), mp 105-106° (from ethyl acetate −hexane); R_f 0.91 (A, 0.84 (B).

HCl · H-ala-ala-OCH_3 (II). A solution of 3.6 g of (I) in 30 ml of CH_3OH, containing the calculated amount of HCl, was hydrogenated over Pd catalyst. After the equivalent amount of H_2 had been absorbed

TABLE 2. Properties of Elastolytic Enzyme of Act. rimosus

Elastolytic activity, units/mg	160–170
Esterase activity, units/mg	21
Caseinolytic activity, units/g	2.0
Trypsin activity, units/mg	300
Chymotrypsin activity	Absent
Collagenolytic activity	"
Molecular weight: gel filtration	28,000 ± 1000
sedimentation analysis	26.000
Sedimentation coefficient ($\cdot 10^{-13}$ s)	3.3
Isoelectric point, pH	5.6–5.8
Extinction coefficient $\varepsilon_I^{1\%}$ cm, 280 nm	18.0
Molar extinction coefficient ε_{mol} 280 nm, acetate buffer, pH 5.5	$5.02 \cdot 10^4$
pH of optimum elastolytic activity	8.85
pH of optimum caseinolytic activity	6.8–7.1
Optimum temperature, °C	37–40
pH range of pH stability	5.5–8.8

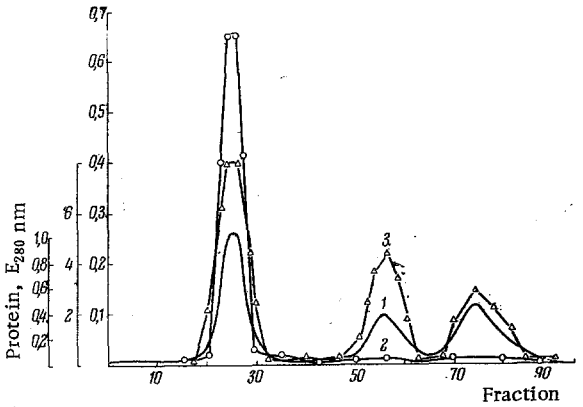

Fig. 2

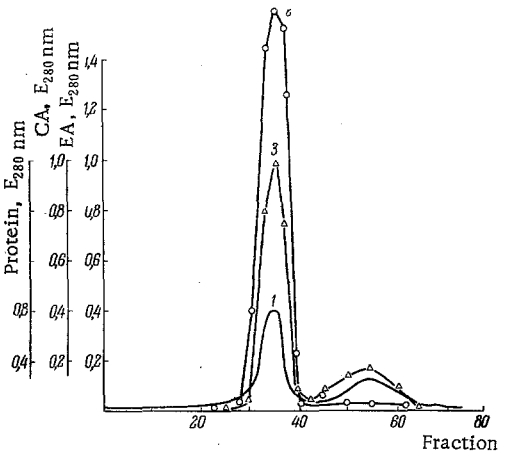

Fig. 3

Fig. 2. Gel chromatography of elastolytic fraction (after CMC) on Sephadex G-75 (medium). Column 3 × 100 cm, $1 \cdot 10^{-4}$ M acetate buffer, pH 5.5. Elution rate 18 ml/h. Volume of fractions 4.5 ml.

Fig. 3. Rechromatography of elastolytic enzyme of Act. rimosus on Sephadex G-75 (medium). 50 mg of the enzyme with an EA of 140 units/mg and a CA of 2 units/g was dissolved in 4 ml of $1 \cdot 10^{-4}$ M acetate buffer, pH 5.5. Deposited on a 2 × 70 cm column. Elution rate 8 ml/h. Volume of fractions 2 ml.

TABLE 3. Action of Inhibitors on Elastolytic Activity of Enzyme of Act. rimosus

Inhibitor	Concentration in sample, mole/liter		Activity, %
	Inhibitor $\cdot 10^3$	Enzyme $\cdot 10^4$	
Control	—	2,5	100
DPP	5	2,5	0
Trilon B	5	2,5	0
MPCB	5	2,5	100
Trypsin from soybean	3,6*	1,0*	100
Trypsin from potato	1,2*	1,0*	100

* In mg/ml.

the solution was filtered, and the solvent was evaporated in vauco. We obtained 2.4 g (75% yield) of (II), mp 108-109° (from MeOH), R_f 0.56 (A), 0.55 (B).

CbO-ala-ala-ala-OCH_3 (III). Similar to (I), from 2.5 g og CbO-ala-OH and 2.3 g of (II) we obtained 3.1 g (70.5% yield) of (III), mp 175-176° (from EtOH); R_f 0.80 (A), 0.83 (B).

HCl · H-ala-ala-ala-OCH_3 (IV). Similar to (II), from 2 g of (III) we obtained 1.4 g (94% yield) of (IV); R_f 0.45 (A), 0.51 (B).

ac-ala-ala-ala-OCH_3 (V). A solution of 1 g of (IV) in 25 ml of C_5H_5N and 0.6 ml of $(CH_3CO)_2O$ was stirred at 20° for 1 h. The solvent was removed. The precipitate was dissolved in water, cooled to 0°, excess IRA-401 AR resin (OH^-) was added the mixture was stirred at 0° for 15 min, filtered, and the solvent was evaporated in vacuo. We obtained 0.9 g (79.5% yield) of (V), mp 150-151° (from ethyl acetate – ether); R_f 0.65 (A), 0.75 (B). The data on the isolation of the purified elastolytic enzyme in steps are given in Table 1.

The homogeneity of the obtained enzyme was established by: a) electrophoresis in polyacrylamide gel at pH 8.3 and 4.3; b) rechromatography on DEAE-cellulose; c) sedimentation analysis (Fig. 4). Some of its properties are given in Table 2.

DPP and Trilon B completely suppress the EA of the enzyme, whereas MPCB and the trypsin inhibitor from soybean and potato are completely without effect (Table 3). Urea lowers the activity of the enzyme substantially even at a concentration of 1 M, while at 5 M the enzyme is inactivated completely. From Fig. 5 it can be seen that DDS (from 0.1 to 0.6%) activates the EA, provided the enzyme is present in the mixture with DDS and the substrate (curve 1). DDS inhibits the EA at concentrations above 0.6%. Prior treatment of the RBB elastin with DDS, and subsequent repeated washing out of the DDS, has little effect on the EA (see Fig. 5, curve 2).

The purified enzyme is stable in the pH range 5.5-8.8 in the presence of $1 \cdot 10^{-4}$ M Ca^{2+}. The optimum temperature of the EA and CA is found to be at 37-40°. The optimum pH of the CA is equal to 6.7-7.1, and a pH of 8.8 for the EA (Fig. 6). The amino acid composition of the elastolytic enzyme of Act. rimosus is given in Table 4.

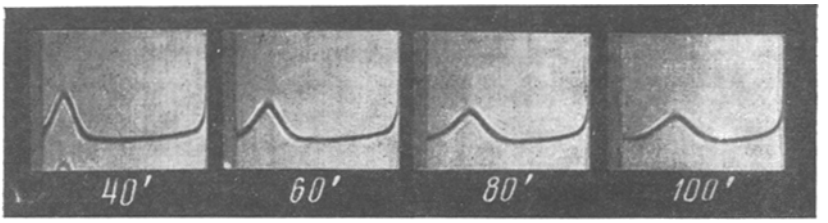

Fig. 4. Sedimentation diagram of purified elastolytic enzyme of Act. rimosus. Rotation speed 56,100 rpm, 20°.

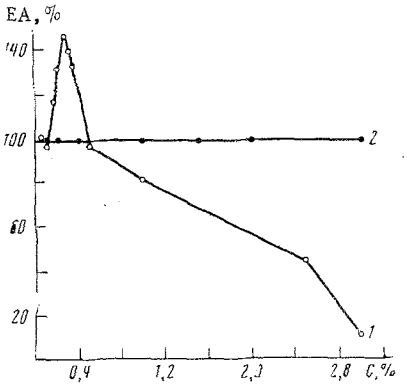

Fig. 5

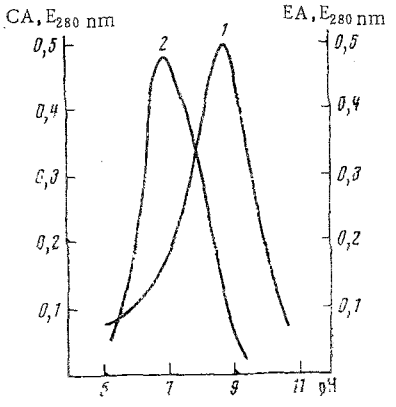

Fig. 6

Fig. 5. Effect of DDS concentration on EA of enzyme of Act. rimosus.

Fig. 6. Optimum pH of enzyme of Act. rimosus: 1) CA; 2) EA.

DISCUSSION OF RESULTS

The same as pancreatic elastase, the elastolytic enzyme of Act. rimosus can be assigned to the proteases of the serine type, since DPP suppresses its activity completely [13]. However, the purified protease of Act. rimosus, which has EA, is also a metalloenzyme. The presence of Ca^{2+} ions is necessary for the manifestation of its EA and CA, since their removal from the medium leads to a comparatively rapid inactivation of the enzyme [14]. This enzyme is irreversibly inactivated under the influence of Trilon B. It should be emphasized that when the concentration of the calcium ions in the medium exceeds $1 \cdot 10^{-4}$ M the EA of the enzyme of Act. rimosus is suppressed and is a total of only 20% of the original value even at a Ca^{2+} concentration of $3 \cdot 10^{-4}$ M. Na^+ also exerts a similar effect, but the Na^+ concentration shoule be a 100 times greater than the Ca^{2+} concentration in order to cause the same inhibiting effect [14]. All of this indicates the electrostatic character of the sorption of the elastolytic enzyme of Act. rimosus on elastin, which was mentioned previously for pancreatic elastase and other elastolytic enzymes [15-17].

The elastase of Act. rimosus has approximately twice the EA that pancreatic elastase has and is capable of solubilizing 160-170 mg of elastin in 1 h at 40° when based on 1 mg of the enzyme. The purified elastase of Act. rimosus also hydrolyzes other proteins (casein). Consequently it can be regarded as a nonspecific elastase, to which also belong all of the other elastolytic enzymes of animal and microbial origin, with the exception of the elastase of Flavobacterium elastolyticum. The latter is the sole known elastase that acts only on elastin [18]. Native collagen is not hydrolyzed by the enzyme of Act. rimosus, or by the elastase of Asp. aeruginosa.

In its CA the elastolytic enzyme of Act. rimosus can be assigned to the neutral proteases. However, the maximum EA is observed in the alkaline region (pH 8.8), which is characteristic for most elastolytic enzymes [3]. The same as the elastase of animal origin, the elastolytic enzyme of Act. rimosus does not hydrolyze ATEE, which is a specific substrate of chymotrypsin. The investigated enzyme exhibits a definite esterase action on BAEE, the substrate of trypsin, and has an activity of 200 units/mg. At the same time, the highly purified pancreatic elastase, as is known, does not act on BAEE [3]. The elastolytic enzyme of Act. rimosus, the same as pancreatic elastase, exhibits esterase activity toward

TABLE 4. Amino Acid Composition of Elastolytic Enzyme of Act. rimosus

Amino acid	Number of residues per molecule (mol. wt. 28,000)	Amino acid	Number of residues per molecule (mol. wt. 28,000)
Lysine	15	Alanine	26
Histidine	5	1/2 Cystine	4
Arginine	7	Valine	15
Aspartic acid	37	Methionine	1
Threonine	23	Isoleucine	7
Serine	20	Leucine	12
Glutamic acid	12	Tyrosine	16
Proline	6	Phenylalanine	5
Glycine	32	Tryptophan	4
		Total	247

the synthetic ac-ala-ala-ala-OCH_3 substrate.

Contradictory data exist regarding the action of surface-active compounds, including DDS, on elastolytic enzymes. Most of the authors mention the inhibiting effect of DDS on the elastases from various sources [17]. The data obtained in this study show that, in certain concentrations, DDS can activate the elastolysis reaction by the proteinase of Act. rimosus. Here such action by the DDS is probably caused by specific conformational changes in the enzyme molecule involving the DDS, and is not associated with irreversible changes in the structure. Evidence is support of this is the fact that the substrate of RBB elastin that had been previously treated with DDS, after washing out the excess DDS with buffer solution is hydrolyzed at the same speed as the untreated substrate. It should be mentioned that DDS also exerts an activating effect on the EA of pancreatic elastase [19].

CONCLUSIONS

1. The elastolytic enzyme was isolated from the culture of the microorganism Act. rimosus by chromatographing the preparation on carboxymethylcellulose and subsequent double purification on Sephadex G-75.

2. Based on the data of electrophoresis in polyacrylamide gel, rechromatography on DEAE-cellulose, and also the sedimentation analysis, the elastolytic enzyme of Act. rimosus was homogeneous.

3. A number of the physicochemical properties of the elastolytic enzyme of Act. rimosus was studied.

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