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PURIFICATION AND CHARACTERIZATION OF THE ELASTASE-LIKE ENZYME OF THE BOVINE GRANULOCYTE

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Summary

The extracts of granules isolated from bovine granulocytes show elastase- and chymotrypsin-like activities, as detected with specific synthetic substrates. Extraction of these enzymes depends upon salt concentration.

In the course of the present studies a 21-fold purification of the elastase-like enzyme was achieved on a $(Ala)_3$-CH-Sepharose 4B gel. The molecular weight of the enzyme is 33 000, as determined by gel electrophoresis in the presence of sodium dodecyl sulfate.

The elastase-like activity is inhibited by phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, basic pancreatic inhibitor and by heparin at different rates. Elastatinal inhibits the enzyme competitively (K_i = 80 μM).

The cytosol of bovine granulocytes contains a protein which strongly inhibits the elastase-like enzyme of the bovine granulocyte (K_i = 0.4 nM) as well as porcine pancreatic elastase (K_i = 11 nM).

Introduction

Neutral proteases have been extracted from the granulocytes of a number of mammalian species. Three of these enzymes, an elastase-like (EC 3.4.21.11), a chymotrypsin-like (cathepsin G, EC 3.4.21.20) and a collagenase-like protease (EC 3.4.24.7) have been isolated in pure form [1]. Comparison of granulocytes of various species has pointed to differences in their protease pattern. The human elastase-like and chymotrypsin-like enzymes have been studied extensively in various laboratories [2—6]. Horse blood granulocytes have been found

Abbreviations: Bz-Arg-OEt, *N*-α-benzoyl-L-arginine-ethyl ester; Bz-Tyr-OEt, *N*-α-benzoyl-tyrosine-ethyl ester; Boc-Ala-Np, *N*-*t*-butoxycarbonyl-L-alanine-*p*-nitrophenyl ester; Suc(Ala)$_3$pNA, succinyl-L-analyl-L-alanyl-L-alanyl-*p*-nitroanilide; $(Ala)_3$-CH-Sepharose 4B, L-alanyl-L-alanyl-L-alanyl-CH-Sepharose 4B.

to contain only elastase-like isoenzymes, and in the cytosol a polyvalent protease inhibitor with a strong chymotrypsin-inhibiting effect has been found [7,8].

In the present experiments the granular elastase-like enzyme and the cytosol protease inhibitor of the bovine granulocyte were investigated, and the effects of various protease inhibitors on the isolated elastase-like enzyme were studied.

Materials and Methods

Chemicals. Affi-gel 10 was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.), human fibrinogen from Behringwerke AG (Marburg, Lahn, F.R.G.) and heparin solution from G. Richter Ltd. (Budapest, Hungary). *N*-L-benzoyl-L-arginine-ethyl ester, *N*-L-benzoyl-tyrosine-ethyl ester, crystalline egg-white lysozyme and phenylmethylsulfonyl fluoride were purchased from Calbiochem (San Diego, CA, U.S.A.). *N-t*-butoxycarbonyl-L-alanine-*p*-nitrophenyl-ester, crystalline elastase (from porcine pancreas) and L-alanyl-L-alanyl-L-alanine were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Crystalline α-chymotrypsin and trypsin (both from bovine pancreas) were from Merck (Darmstadt, F.R.G.), 4-phenylbutylamine (98% pure) from Aldrich-Europe (Beerse, Belgium), CH-Sepharose 4B from Pharmacia (Uppsala, Sweden). Soybean trypsin inhibitor, bovine serum albumin, ovalbumin and succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide were purchased from Serva (Heidelberg, F.R.G.) and *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride from Fluka AG (Buchs S.G., Switzerland). Elastatinal and basic pancreatic trypsin inhibitor were generously gifted by Professor Dr. H. Umezawa and Dr. A. Kelemen, respectively. All the other reagents were of analytical grade.

Isolation of granules. 20 l of citrated fresh bovine blood were centrifuged at $300 \times g$ for 30 min. The buffy coat was collected and centrifuged again. The erythrocytes, which remained in the supernatant, were disrupted by hypotonic shock treatment according to Janoff and Scherer [9]. The isolation steps were carried out at 0—4°C. After centrifugation the leukocytes were resuspended in a 340 mM saccharose solution. The cells were homogenized twice for 15 s in an MSE high-frequency homogenizer, and the homogenate was centrifuged at $300 \times g$ for 20 min. The supernatant was collected and the debris was discarded. The supernatant was centrifuged again at $15\,000 \times g$ for 20 min, and the granule-containing pellet was used in further steps. The supernatant was stored for preparation of cytosol inhibitor (see below).

Extraction of proteases from the granules. The granules, suspended in 10 ml of 10 mM phosphate buffer, pH 7.4, containing 100 mM NaCl, were disrupted by freezing in liquid nitrogen and thawing 6 times. After centrifugation at $15\,000 \times g$ for 20 min the granules were extracted twice in the same buffer according to Feinstein and Janoff [2], then the supernatants were collected. This fraction contained mainly chymotrypsin-like activity (extract I). The insoluble material was further extracted four times in 10 ml of 10 mM phosphate buffer which contained 1 M NaCl (extract II). After centrifugation the protein content and the protease activity of the collected supernatant were determined. This extract contained the bulk of elastase-like enzyme activity.

Extract II was dialyzed for 48 h against 50 mM Tris-HCl buffer, pH 8.0,

containing 50 mM NaCl. After centrifugation it was concentrated on an eluate concentrator (Amicon, Model CECl) equipped with a PM-10 membrane, and was stored at −30°C.

Isolation of a protease inhibitor from the granulocyte cytosol. The supernatant, obtained after collecting the granules, was centrifuged at 100 000 × *g* for 75 min, then dialyzed against distilled water and lyophilized. The solid material was dissolved in 100 mM Tris-HCl buffer, pH 7.5, it was then gel-filtered on a Sephacryl S-200 column (Pharmacia, Uppsala, Sweden). The active fraction was purified further by ion-exchange chromatography on a DEAE-cellulose column (Whatman DE-32). The inhibitory activity of the protein was characterized by using chymotrypsin. The protease inhibitor was found to be homogenous by polyacrylamide gel electrophoresis. Its molecular weight was 50 000, as estimated from gel-filtration data obtained by chromatography on a Sephadex G-75 column. (The details of isolation and the properties of the inhibitor will be described elsewhere.)

Protein determination was performed according to the method of Lowry and co-workers [10]. The amount of the purified elastase was determined from the absorbance at 280 nm based on $A^{1\%}_{1cm} = 9.85$ [5].

Protease activity was determined both with fibrinogen [4] and with casein substrates [7]. The amount of enzyme that caused $\Delta A^{1cm}_{280} = 0.1$ absorbance change in 60 min at pH 7.4, and 37°C was taken as unit protease activity.

Esterolytic activity. The assays were performed in 100 mM Tris-HCl buffer, pH 7.4, containing 5% Me_2SO, by determining the absorbance in a Unicam SP 500 spectrophotometer equipped with a thermostatically controlled cell holder. Bz-Tyr-OEt and Bz-Arg-OEt in 500 μM final concentration were used as substrates, and the absorbance was recorded at 256 and 253 nm, respectively.

Amidolytic activity. The chromogenic substrates were dissolved in Me_2SO. Boc-Ala-Np was applied at a final concentration of 260 μM [11,12]. The changes in absorbance were recorded at 347.5 nm in 50 mM phosphate buffer, pH 6.5. $Suc(Ala)_3pNA$ was used at a final concentration of 170 μM. The absorbance was measured at 405 nm in 100 mM Tris-HCl buffer, pH 7.4. $\Delta A^{1cm}_{\lambda} = 0.01$ absorbance change in 1 min at the given pH and 37°C was taken as unit esterase or amidase activity.

Gel electrophoresis was performed in 10% polyacrylamide gel at a current of 5 mA per tube [13]. The gel was stained with 1% Amidoblack 10 B dissolved in 7% acetic acid.

Molecular weight was determined by gel electrophoresis [14] at a current of 8 mA per tube. A 10% polyacrylamide gel system was applied in the presence of 0.1% sodium dodecyl sulfate. The gel was stained with 2.5% Coomassie brilliant blue R-250 dissolved in acetic acid/methanol mixture. Basic pancreatic trypsin inhibitor (M_r 6500), lysozyme (14 600), soybean trypsin inhibitor (21 500), trypsin (24 000), chymotrypsin (25 000), ovalbumin (43 000) and bovine serum albumin (68 600) were used as reference proteins.

Results

Protease content of bovine granules

Table I summarizes the enzyme activities measured with various substrates.

TABLE I

PROTEOLYTIC, ESTEROLYTIC AND AMIDOLYTIC ACTIVITIES IN BOVINE GRANULAR EXTRACTS

Substrate	Total unit *	Activity					
		Extract I			Extract II		
		unit	unit/mg	%	unit	unit/mg	%
Proteolysis							
Casein	922.0	332.0	1.6	36	590.0	5.3	64
Fibrinogen	1502.0	1021.0	4.9	68	481.0	4.4	32
Esterolysis							
Bz—Arg-OEt	83.0	36.5	0.2	44	46.5	0.4	56
Bz-Tyr-OEt	147.0	57.0	0.3	39	90.0	0.8	61
Amidolysis							
Boc-Ala-Np	92.0	0	0	0	92.0	0.8	100
Suc(Ala)$_3$ pNA	4.6	0	0	0	4.6	0.04	100

* In $2.3 \cdot 10^{10}$ cells.

The data were calculated for granules obtained from $2.3 \cdot 10^{10}$ leukocytes. The total amount of extractable protein was 310 mg, two-thirds of which (200 mg) was collected in extract I, and one-third (110 mg) in extract II).

As seen in Table I, with two protein substrates, i.e., with fibrinogen and casein, different activities were measured in the two granular extracts. Extract I contained 68% of the fibrinogenolytic activity and 36% of the caseinolytic activity, whereas in extract II reverse ratios, i.e., 32% of the fibrinogenolytic and 64% of the caseinolytic activities were found.

With synthetic substrates more than half of the chymotrypsin-like and trypsin-like activity (56% and 61%, respectively) and practically the total amount of elastase-like activity, i.e., 92 Units, were found in extract II. This value, however, is still 3 times less than that which was found in a similar amount of human granulocytes isolated according to Feinstein and Janoff [2].

Purification of the elastase-like enzyme by affinity chromatography

Extract II, containing the elastase-like enzyme, was dialyzed against 50 mM Tris-HCl buffer, pH 8.0. By lowering the ionic strength the chymotrypsin-like enzyme was precipitated and the Bz-Tyr-OEt-splitting activity was thus reduced to 3% of the original. In preliminary experiments we tried to isolate the elastase-like enzyme on 4-phenylbutylamine Affi-gel and on basic pancreatic trypsin inhibitor (Trasylol® or Gordox®)-Sepharose, as suggested for the human enzyme by Feinstein and Janoff [2] and by Baugh and Travis [5], but the bovine enzyme could not be bound to either of them.

Pancreatic elastase has been purified by Katagari and co-workers [15] on an (Ala)$_3$-CH-Sepharose 4B column. 2.1 mg L-alanyl-L-alanyl-L-alanine per g CH-Sepharose has been found to bind 10 mg of pancreatic elastase. Therefore, the trialanyl-derivative of CH-Sepharose was prepared, which appeared to be a good sorbent for the bovine elastase-like enzyme.

On 36 ml of (Ala)$_3$-CH-Sepharose gel about 46 mg of granular protein were

bound using the batchwise method [16]. The protein was then eluted stepwise with different solutions, as indicated in Table II. Elution with 10 mM Tris-HCl buffer, pH 8.0, and with 50 mM Tris-HCl buffer, pH 8.0, containing 0.2—1.0 M NaCl removed one-fourth (27.7%) of the elastase-like activity, and the bulk of inactive proteins. 44% of elastase activity was obtained in a relatively pure form by using 0.1 M acetate buffer, pH 4.0, containing 1.0 M NaCl. In this step a 21-fold purification was achieved. As shown in Table II, 6.3 mg elastase-like enzyme of 6.4 U/mg specific activity was finally obtained. Elastase isoenzymes, however, could not be detected by gel electrophoresis either in the extract (Fig. 1a), or after dialysis (Fig. 1b) or after affinity chromatography (Fig. 1c).

Characterization of the bovine elastase-like enzyme

As shown in Fig. 1, after affinity chromatography the enzyme was found to be homogeneous by polyacrylamide gel electrophoresis. Its molecular weight was found to be 33 000 by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate. This value correlates with those obtained in various laboratories for human granulocyte elastase isoenzymes [1,2,5]. The K_m of the Boc-Ala-Np substrate was determined with 50 mM phosphate buffer, pH 6.5, at 37°C. The data were plotted according to Lineweaver and Burk, and a value of 500 μM was obtained. This value is in agreement with 360 μM as well as with 200 μM reported for the human granulocyte enzyme [5] and for porcine pancreatic elastase [11], respectively. The catalytic constant of the bovine granulocyte enzyme, k_{cat}, was $1 \cdot 10\ s^{-1}$, its proteolytic constant, k_{cat}/K_m, was $2200\ M^{-1} \cdot s^{-1}$. The k_{cat} of porcine pancreatic elastase has been found to be $5 \cdot 7\ s^{-1}$ [11], 5 times higher than that of the bovine enzyme.

Inhibition of the elastase-like enzyme

The experiments were carried out in the presence of 520 μM Boc-Ala-Np substrate. For comparison with the human enzyme, the concentration of the inhibitors was chosen according to Starkey [1]. As is seen in Table III, the

TABLE II

PURIFICATION OF THE ELASTASE-LIKE ENZYME

Unit elastase activity: $\Delta A_{347.5} = 0.01$/min at 37°C, pH 6.5 with Boc-Ala-Np substrate.

Steps	Protein		Enzyme activity		Yield (%)	Purification
	mg	%	unit	unit/mg		
Granules	310.0	100.0	92.0	0.30	100.0	1.0
Extract II	110.0	35.0	92.0	0.84	100.0	2.8
Dialysis	64.0	20.0	92.0	1.40	100.0	4.7
Affinity chromatography:						
36 ml $(Ala)_3$-Sepharose 4B gel						
— 100 ml of 10 mM Tris-HCl buffer, pH 8.0	18.5	6.0	11.5	0.62	12.5	2.1
— 200 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 0.2—1.0 M NaCl	21.1	6.4	14.0	0.66	15.2	2.2
— 50 ml of 100 mM acetate, pH 4.0, containing 1 M NaCl	6.3	2.0	40.3	6.40	44.0	21.0

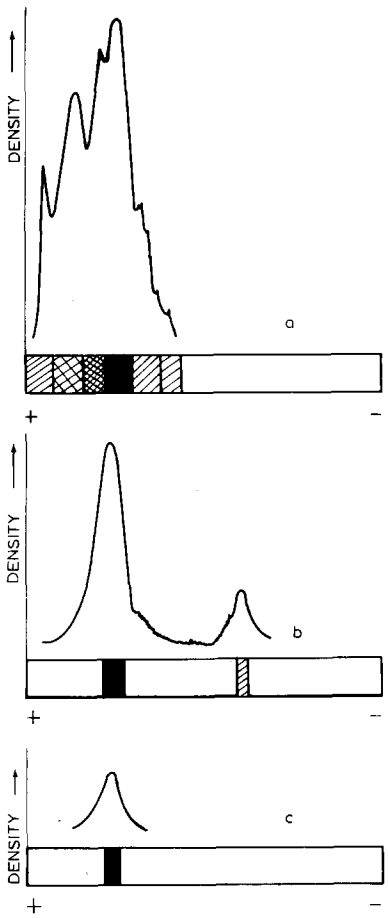

Fig. 1. Densitogram of the bovine elastase-like enzyme after gel electrophoresis in 10% polyacrylamide gel at a current of 5 mA/tube. a, 200 μg protein extracted with phosphate buffer, pH 7.4, containing 1.0 M NaCl; b, 150 μg protein after dialysis; c, 40 μg protein obtained by affinity chromatography.

TABLE III

EFFECT OF INHIBITORS ON THE BOVINE AND HUMAN ELASTASES

Measured with 520 μM Boc-Ala-Np substrate in 50 mM phosphate buffer, pH 6.5, at 37°C. Enzyme concentration: 200 nM.

Inhibitor	Concentration (μM)	Residual activity		
		Bovine		Human * (%)
		unit	%	
None		1.6	100	100
Phenylmethylsulfonyl fluoride	100	0	0	0
Bovine pancreatic trypsin inhibitor (Kunitz)	80	1.23	77	65
Soybean trypsin inhibitor	6	0.2	12	17
Heparin **	160	0.5	31	

* Data of Starkey [1].

** Calculations were based on an average molecular weight of 18 000.

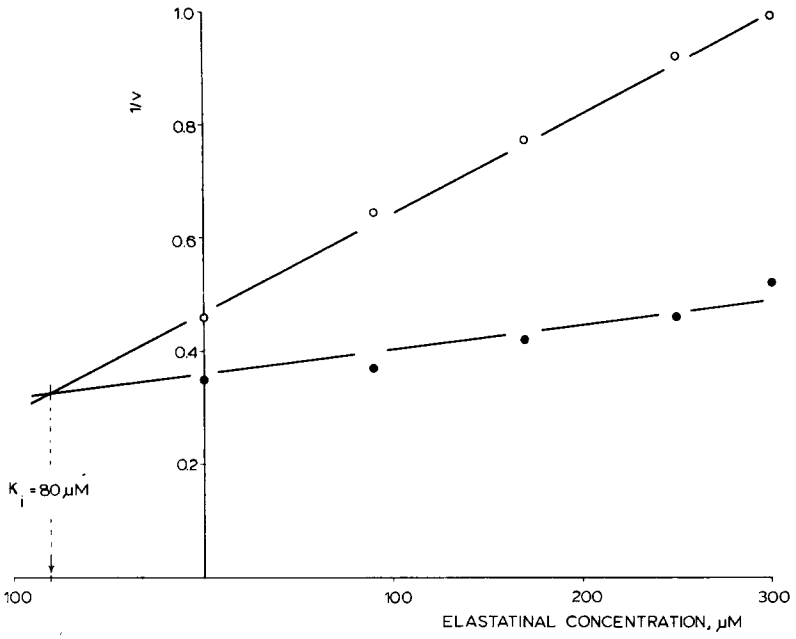

Fig. 2. Inhibition of the bovine elastase-like enzyme by elastatinal, as plotted according to Dixon. Enzyme concentration: 200 nM. Boc-Ala-Np substrate concentrations: 260 μM (o———o), 520 μM (•———•).

enzyme was inhibited readily by phenylmethylsulfonyl fluoride, i.e., by the common inhibitor of serine proteases. Of the natural compounds 6 μM soybean trypsin inhibitor appeared to have a stronger effect than 80 μM pancreatic trypsin inhibitor (Kunitz). The activity of the enzyme was inhibited also by 160 μM heparin, probably due to electrostatic interactions which may be formed between the cationic enzyme and the negatively-charged heparin.

Elastatinal has been reported to be a potent inhibitor for the human enzyme [17,18]. Therefore, the hydrolysis of Boc-Ala-Np by the bovine elastase-like enzyme was assayed in the presence of 0—300 μM elastatinal, and the data were plotted according to Dixon (Fig. 2). An inhibitory constant of 80 μM was thus obtained, which indicates that elastatinal is a good inhibitor for the bovine enzyme as well.

Effect of the cytosol protease inhibitor

The inhibitory activity of the protease inhibitor isolated from granulocyte cytosol was studied both with bovine granulocyte and with porcine pancreatic

TABLE IV

INHIBITORY CONSTANTS OF THE PROTEASE INHIBITOR (ISOLATED FROM BOVINE GRANULOCYTE CYTOSOL) WITH VARIOUS ENZYMES

Measured at 260—520 μM Boc-Ala-Np concentration in 50 mM phosphate buffer, pH 6.5, at 37°C.

Enzyme (source)	Concentration (nM)	Inhibitory constant (nM)
Elastase (procine pancreas)	70	11
Elastase-like (bovine granulocyte)	40	0.4

elastases. The concentrations of the enzymes ranged between 40 and 70 nM, and that of the cytosol inhibitor was 0—700 nM. The enzymes were preincubated for 5 min at 37°C before starting the assay by the addition of 260—520 μM Boc-Ala-Np substrate. The bovine elastase-like enzyme and porcine elastase were readily inhibited by the cytosol protease inhibitor. From the kinetic measurements the inhibitory constants were calculated (Table IV) as suggested by Green and Work [19].

The inhibitory constants of the two enzymes suggest the formation of a relatively strong bond between the enzyme and the inhibitor. For the bovine enzyme, for example, 0.4 nM was calculated. These values are similar to those found with proteases and natural inhibitors of large molecular weight [18,20].

Based on the molecular weight of the cytosol inhibitor (50 000) we may assume that the porcine pancreatic enzyme reacted with the inhibitor also at a 1 : 1 molar ratio, but the affinity of the enzyme was about two orders of magnitude lower (11 nM) than that of the bovine granular enzyme.

Discussion

The granule extract of granulocytes isolated from bovine blood shows neutral protease activity, i.e., elastase- and chymotrypsin-like enzyme activities, which can be detected by natural protease substrates such as fibrinogen and casein, and by specific synthetic substrates, respectively. In addition, the granule extract of bovine granulocytes has low trypsin-like, Bz-Arg-OEt-splitting activity as well, probably due either to the broad substrate specificity of the enzymes or to the presence of a small amount of a trypsin-like enzyme.

Comparison with the protease spectrum of human granulocytes isolated in our laboratory revealed that the elastase-like enzyme activity of human granulocytes is 3 times higher than that of bovine granulocytes. The elastase-like enzyme can easily be extracted from human granulocytes with phosphate buffer containing 0.1 M NaCl, whereas the extraction of the bovine enzyme requires 10 times higher ionic strength [2].

It is worth emphasizing that the granulocytes of a species close to the bovine, i.e., horse blood granulocytes, have been found to contain only elastase-like isoenzymes [7], and the cytosol inhibitor isolated from horse blood granulocytes has been found to possess a strong chymotrypsin-inhibiting activity [8]. In contrast, the inhibitor protein isolated from the bovine granulocyte cytosol possesses a strong elastase-inhibiting effect. The inhibitory constant is 0.4 nM, as calculated for the bovine elastase-like enzyme.

Although, both the human and the horse blood granulocytes have been found to contain elastase-like isoenzymes [1—3,5,7], in the present studies we did not succeed in detecting the presence of any isoenzymes in the bovine granulocyte.

Our previous investigations revealed marked differences between the protein substrate specificity of the granulocyte and the pancreatic elastases [21]. The human elastase-like enzyme was found to be 800-times more effective in the inactivation of the human clotting factor VIII than the related pancreatic enzymes. For the isolation of the elastase-like enzyme, however, the method described for the pancreatic elastase [15] proved to be more suitable than the

one reported for the isolation of granulocyte elastases from other species [2,5].

It is probably due to the different structure of the two elastases that the bovine granulocyte elastase could not be bound on the basic pancreatic trypsin inhibitor-Sepharose gel. This correlates with the fact that basic pancreatic trypsin inhibited the activity of the bovine enzyme by only 23%, while the human granulocyte elastase was inhibited by 65% under similar conditions.

At the same time, the molecular weight of the bovine granulocyte elastase was found to be very similar to that of the human granulocyte elastase [1,5]. Its Michaelis constant, K_m, determined with the Boc-Ala-Np substrate was only slightly different from those of the human granulocyte [5] and the bovine pancreatic elastases [11]. There was also no pronounced difference in the rates of inhibition by elastatinal between the bovine and the human enzymes.

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