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

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A chymotrypsin-like enzyme (EC 3.4.21.20) was isolated from bovine granulocytes, and purified 14-fold by affinity chromatography on 4-phenylbutylamine Affi-gel. The molecular weights of the isoenzymes were estimated as 16 000, 19 300 and 24 000 by SDS-polyacrylamide gel electrophoresis. A Michaelis constant of 2 mM and a catalytic constant of 34.6 s^{-1} were determined with Bz-Tyr-OEt. The esterolytic activity of the enzyme could be inhibited both by PMSF and by TPCK. It was also inhibited by chymostatin (K_i = 0.11 μg/ml) and by the cytosol inhibitor of the bovine granulocyte (K_i' = 1 μM). The chymotrypsin-like enzyme of the bovine granulocyte shares a number of the kinetic properties common to the chymotrypsin-like enzyme of the human granulocyte. The two granulocytes showed nearly identical chymotrypsin-like enzyme activitities per cell.

Introduction

The primary function of the neutrophilic granulocyte is phagocytosis, i.e., the killing of microorganisms. For this purpose the cell contains antimicrobial systems in which strongly cationic, chymotrypsin-like isoenzymes (EC 3.4.21.20) have been detected [1–5]. The enzyme has been purified by affinity chromatography, and its properties have been compared to those of the other serine protease of the granulocyte, the elastase-like enzyme, using synthetic substrates and inhibitors as well as natural protease inhibitors [2–5].

Unexpectedly, no chymotrypsin-like enzyme has been detected in the horse blood granulocyte [6], although the cytosol has been found to contain a protein with a strong chymotrypsin-inhibiting effect [7].

The elastase-like enzyme of the bovine granulocyte has been studied earlier [8]. The present paper describes the isolation and characterization of the chymotrypsin-like enzyme of the bovine granulocyte, and the effects of various protease inhibitors are also discussed.

Materials and Methods

Chemicals. Affi-gel 10 was purchased from Bio-Rad Lab. (Richmond, CA, U.S.A.), Bz-Tyr-OEt and crystalline egg-white lysozyme from Calbiochem (San Diego, CA, U.S.A.). Crystalline α-chymotrypsin, trypsin (both from bovine pancreas), as well as Hammarsten casein were obtained from Merck (Darmstadt, F.R.G.), 4-phenylbutylamine (98% pure) from Aldrich-Europe (Beerse, Belgium). Soybean trypsin inhibitor, bovine serum albumin, ovalbumin and TPCK were supplied by Serva (Heidelberg, F.R.G.). Chymostatin was generously gifted by Professor H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan).

Protein content. This was determined according to the method of Lowry et al. [9].

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; Bz-Tyr-OEt, *N*-α-benzoyl-tyrosine ethyl ester; Me_2SO, dimethyl sulfoxide; TPCK, tosyl-phenylalanyl-chloromethyl ketone; SDS, sodium dodecyl sulfate.

Proteolytic activity. Activity was determined by the method of Dubin et al. [6] with casein substrate in 100 mM phosphate buffer (pH 7.4). $\Delta A_{280}^{1cm} = 0.1$ change in absorbance measured after 60-min incubation at 37°C this was taken as a unit proteolytic activity.

Esterolytic activity. This was assayed at 37°C in 100 mM Tris-HCl buffer (pH 7.4)/5% Me_2SO, by recording the absorbance at 256 nm. As substrate, Bz-Tyr-OEt was applied at a final concentration of 500 μM. $\Delta A_{256}^{1cm} = 0.01$ absorbance change occurring in 1 min was taken as a unit esterolytic activity.

Gel electrophoresis. Electrophoresis was performed according to the method of Riesfeld [11] in 10% polyacrylamide gels at 5 mA/tube. The gels were stained with 1% Amido black 10 B in 7% acetic acid.

Molecular weight. M_r was determined by gel electrophoresis according to Weber and Osborn [12] at 8 mA/tube using 10% polyacrylamide gels in the presence of 0.1% SDS. The gels were stained with 2.5% Coomassie brillant blue R-250 in an acetic acid/methanol mixture. As reference, lysozyme (M_r 14 600), soybean trypsin inhibitor (21 500), trypsin (24 000), chymotrypsin (25 000), ovalbumin (43 000) and bovine serum albumin (68 600) were used.

Isolation of the bovine granulocyte cytosol inhibitor. Isolation was carried out on Sephacryl S-200 and DEAE-cellulose columns [8]. Its molecular weight was 50 000.

Human granulocyte chymotrypsin. Chymotrypsin was purified according to the method of Feinstein and Janoff [2].

Results

Extraction of granules

Granules were isolated from $2.3 \cdot 10^{10}$ bovine granulocytes, then disrupted and extracted three times in 10 mM phosphate buffer (pH 7.4)/100 mM NaCl, as reported earlier [8]. As is shown in Table I, the first fraction contained 160 mg protein, which was equal to 320 units of proteolytic activity and 37 units, i.e., 25%, of the total esterolytic activity. A further 14% of the esterolytic activity was measured in the second fraction, whereas hardly any could be detected in the third fraction which was, therefore, discarded, and the first and second fractions were collected (extract I).

The debris was extracted further in phosphate buffer containing 1 M NaCl. Thus, altogether 64% of the total proteolytic activity and 61% of the esterolytic activity could be extracted with phosphate buffer of higher ionic strength in four fractions which also contained one-third (110 mg) of the extractable protein. The four fractions were collected (extract II).

TABLE I

PROTEOLYTIC AND ESTEROLYTIC ACTIVITY IN BOVINE GRANULAR EXTRACT

Using $2.3 \cdot 10^{10}$ cells.

Extraction 10 mM phosphate buffer (pH 7.4)	Protein (mg)	Activity			
		Proteolytic (casein)		Esterolytic (Bz-Tyr-OEt)	
		(unit)	(%)	(unit)	(%)
100 mM NaCl					
fraction 1	160	320	34	37	25
fraction 2	30	12	2	20	14
fraction 3	10	–	–	–	–
extract I	190	332	36	57	39
1 M NaCl					
fraction 4	50	300	32	50	34
fraction 5	35	200	22	30	20
fraction 6	20	60	6	7	5
fraction 7	5	30	4	3	2
extract II	110	590	64	90	61

Extracts I and II contained altogether 147 units of esterolytic activity, which approximated the esterolytic activity of the human granule extract prepared by the same method. Hence, it seems obvious that nearly identical amounts of the chymotrypsin-like enzyme are present in both the bovine and human granulocytes.

Purification of the chymotrypsin-like enzyme

The enzyme was purified batchwise from extract II on 4-phenylbutylamine Affi-gel as suggested by Feinstein and Janoff [2]. 55% of the chymotrypsin-like activity was obtained in the first eluent with 10 mM phosphate buffer (pH 7.4)/1 M NaCl, and 20% Me_2SO (v/v). Thus, the enzyme was purified 14-fold, as compared to its original specific activity (Table II). After further washing, 9% of the chymotrypsin-like activity was found in the second eluent containing 100 mM arginine and 500 mM NaCl (pH 10.0), when the purification factor was 2.6-fold. Both fractions were concentrated on an Amicon PM-10 membrane, dialyzed against 10 mM phosphate buffer (pH 7.0)/300 mM NaCl, then used for further studies.

The homogeneity of the enzyme was controlled by polyacrylamide gel electrophoresis. The densitogram of extract II and that of the chymotrypsin-like enzyme eluted from the Affi-gel with phosphate buffer containing Me_2SO are presented in Fig. 1. The gel electrophoretic profile of the eluate containing arginine was not homogeneous. Near the cathode it showed several faint bands that could not be detected by densitometry.

Characterization of the chymotrypsin-like enzyme

The activity of the enzyme was measured with

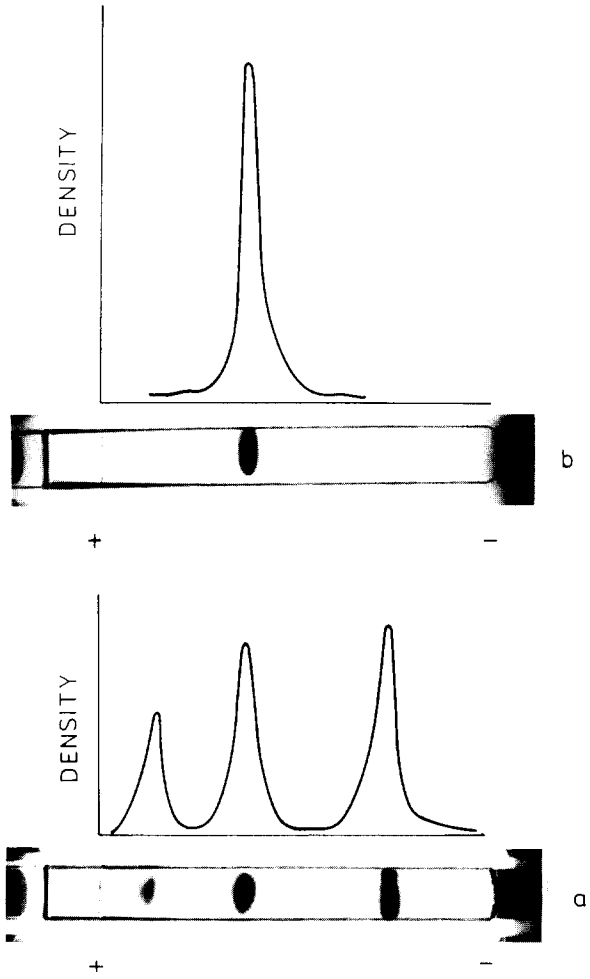

Fig. 1. Densitogram of the bovine chymotrypsin-like enzyme after gel electrophoresis in 10% polyacrylamide gels at a current of 5 mA/tube. a, 200 μg protein in extract II; b, 50 μg protein obtained by affinity chromatography.

TABLE II

PURIFICATION OF THE CHYMOTRYPSIN-LIKE ENZYME

Unit chymotrypsin activity: ΔA_{256} = 0.01/min at 37°C, pH 7.4 with Bz-Tyr-OEt substrate.

Steps	Protein (mg)	Enzyme activity		Yield (%)	Purification
		(unit)	(unit/mg)		
Granules	310.0	147.0	0.47	100.0	1.0
Extract II	110.0	90.0	0.82	61.0	1.7
Affinity chromatography	12.2	81.0	6.60	55.0	14.0
Dialysis and ultrafiltration	6.0	38.0	6.30	26.0	13.4

Bz-Tyr-OEt substrate at concentrations ranging between 100 and 700 μM in 100 mM Tris-HCl buffer (pH 7.4). The kinetic parameters were calculated according to Lineweaver and Burk [13] by the least-squares method. The Michaelis constant of the enzyme, K_m, was 2.0 mM. This value is almost identical with that reported for the human granulocyte chymotrypsin by Starkey [14]. The maximum velocity, V, was 2.25 $\mu M \cdot s^{-1}$. The SDS-polyacrylamide gel electrophoresis of the enzyme gave three bands. The corresponding molecular weights were 16000, 19300 and 24000. Consequently, an average molecular weight of 20000 was used for the calculations. Hence, the catalytic constant, k_{cat}, was found to be 34.6 s^{-1}, and the proteolytic constant, k_{cat}/K_m, 17300 $M^{-1} \cdot s^{-1}$.

Inhibition of the chymotrypsin-like enzyme

The bovine chymotrypsin-like enzyme was preincubated in 110 nM final concentration, with various inhibitors, in 100 mM Tris-HCl buffer (pH 7.4) at 37°C for 20 min, except for TPCK when the incubation time was 60 min. The remaining enzyme activity was then measured using Bz-Tyr-OEt substrate at a final concentration of 500 μM. The enzyme could be inhibited both by 1.0 mM PMSF and by 330 μM TPCK indicating that it must be a serine protease and a chymotrypsin-like enzyme.

For the calculation of the inhibitory constants, the data were plotted according to Green and Work [15] when a firm enzyme-inhibitor complex was formed. But when the complex was loose, the data were plotted according to Dixon and Webb [13].

Chymostatin, a competitive inhibitor of the chymotrypsin-like enzyme of the human granulocyte [3], proved to be a competitive inhibitor for the bovine enzyme as well. 65 nM enzyme were preincubated at 37°C for 5 min with 0.0–0.6 μg/ml chymostatin, then its activity was assayed with Bz-Tyr-OEt, both at 500 and 700 μM final concentrations. The value of the inhibitory constant derived from Dixon plots [13] was 0.1 μg/ml (Fig. 2), 3-times smaller than that for the human enzyme (K_i = 0.3 μg/ml).

Effect of the cytosol protease inhibitor

The bovine chymotrypsin-like enzyme was preincubated in 100 mM Tris-HCl buffer (pH 7.4) at

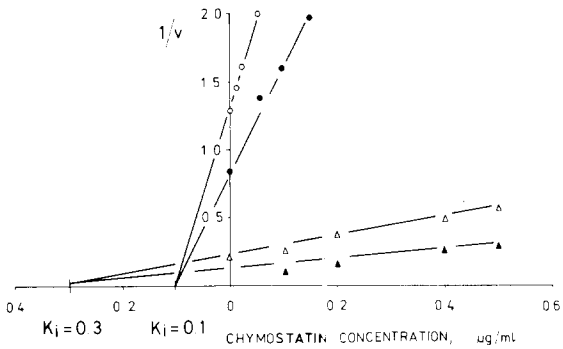

Fig. 2. Inhibition of the bovine and human granulocyte chymotrypsins by chymostatin, as plotted according to Dixon. The enzymes were preincubated at 37°C for 5 min with 0.0–0.6 μg/ml chymostatin in 100 mM Tris-HCl buffer (pH 7.4) then assayed with Bz-Tyr-OEt substrate. Bovine chymotrypsin (65 nM) at (○———○) 500 μM substrate concentration and (●———●) 700 μM substrate concentration. Human chymotrypsin (110 nM) at (△———△) 500 μM substrate concentration and at (▲———▲) 700 μM substrate concentration.

37°C for 5 min with 0.0–1.0 mM inhibitor. Bz-Tyr-OEt substrate was then added to the reaction mixture in 500 μM final concentration. The bovine granulocyte cytosol inhibitor inhibited the enzyme effectively, and an inhibitory constant of 1.0 μM was obtained.

For comparison, the effect of this inhibitor was also tested with human granulocyte chymotrypsin as well as with bovine pancreatic chymotrypsin. With the human granulocyte chymotrypsin the inhibitory constant was 6.0 μM. The bovine pancreatic enzyme was inhibited by the bovine granulocyte cytosol inhibitor 100-times more effectively than the bovine granulocyte enzyme (K_i' = 10 nM).

Discussion

The bovine and the human granulocytes contain nearly identical amounts of a chymotrypsin-like enzyme. In agreement with the results obtained by Ohlsson for the human enzyme [16], 10 mg of the chymotrypsin-like enzyme could be isolated from 10^{10} bovine granulocytes. In contrast with this, both horse [6] and rabbit blood granulocytes [17] have been found to show large differences in their chymotrypsin-like enzyme activities, as compared to

human granulocytes. The elastase-like enzyme activity of bovine granulocytes, on the other hand, has been found to be 3-times lower than that of human granulocytes [8].

The bovine and the human granulocytes also manifest several similarities in their properties, such as molecular weight, Michaelis constant and inhibition by PMSF [2,14]. Nevertheless, the two enzymes are inhibited to different degrees by other inhibitors. For example, TPCK binds more firmly to the bovine enzyme than to the human [5], whereas according to Powers et al. [10] benzoxy-carbonyl-phenylalanyl-chloromethyl ketone is effective for the human enzyme. Chymostatin is 3-times and the bovine granulocyte cytosol inhibitor is 6-times more effective for the bovine enzyme, as compared to the human. Surprisingly, the bovine pancreatic chymotrypsin is inhibited by the cytosol inhibitor 100-times more actively than the bovine granulocyte enzyme. As compared to the results of earlier studies [8], the cytosol inhibitor seems to be the most effective for the elastase-like enzyme with which it forms a much stronger complex than with the chymotrypsin-like enzyme of the same granulocyte.

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