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Isolation and Some Physical and Chemical Properties of Elastase and Cathepsin G from Dog Neutrophils

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Abstract—A new method for isolation of leukocyte serine proteinases has been developed. Elastase (EC 3.4.21.37) and cathepsin G (EC 3.4.21.20) have been isolated from dog neutrophils and purified to homogeneous state. The results of inhibitor analysis indicate that the enzymes belong to the group of serine proteinases. Some physical and chemical characteristics of the purified enzymes have been determined. The molecular weights of the enzymes are 24.5-26 kD for the elastase and 23.5-25.5 kD for the cathepsin G. The cathepsin G is a glycoprotein, while the elastase molecule lacks carbohydrate components. The cathepsin G exhibits a broad pH optimum of catalytic activity in the range of 7.0-9.0; the pH optimum for the elastase is 8.0-8.5. The Michaelis constant of the elastase for N-t-Boc-L-alanine *p*-nitrophenyl ester is 0.10 mM; the Michaelis constant of the cathepsin G for N-benzoyl-L-tyrosine ethyl ester is 0.42 mM.

Key words: dog, neutrophil, serprocidins, elastase, cathepsin G

Interest in nonspecific cell and humoral factors providing defense of an organism against infections (inborn immunity system) has greatly increased in recent years. Neutrophilic granulocytes, or neutrophils, play a significant role in the reactions of inborn immunity. Mammalian neutrophils are highly differentiated cells that are specialized in defense reactions involving phagocytosis or release of antimicrobial compounds into the medium [1-3]. Lysosome granules of neutrophils contain a number of biologically active molecules—myeloperoxidase, lactoferrin, lysozyme, cationic peptides (for example, defensins), and serprocidins. The group of serprocidins includes proteins of azurophilic granules of neutrophils exhibiting microbicidal properties. These proteins can be either serine proteinases or their structural homologs lacking the enzymatic activity. The main representatives of this group are elastase (EC 3.4.21.37) and cathepsin G (EC 3.4.21.20) [4, 5]. The physiological role of serprocidins is not restricted to their participation in killing of microorganisms. Moreover, it was shown that their antimicrobial activity did not depend significantly on proteolytic activity [6-8]. The proteins of this group play an important role in a number of physiological and pathophysiological processes such as phagocytosis, chemotaxis, complement system activation, and the regulation of blood pressure, blood vessel permeability, blood coagulation, and intracellular interactions [4, 5, 9-11].

The physiological role of serprocidins has not been completely explored. Their functions in the body are multiform, and their understanding requires further intensive investigations of their physical, chemical, structural, and catalytic properties. Comparative investigations are of special importance. Thus, the investigation of the structure and functions of elastase and cathepsin G from different sources is also important. Most works on these enzymes have been performed on elastase and cathepsin G from human neutrophils. The goal of this study was to investigate elastase and cathepsin G from dog neutrophils and to compare the parameters of the enzymes with those of elastase and cathepsin G from human neutrophils. There are very few works on isolation of elastase and cathepsin G from dog neutrophils, and the data on their physical and chemical properties are incomplete and contradictory [12-14].

MATERIALS AND METHODS

Leukocytes of 16-18-h dog exudate were used in the experiments (neutrophils constituted 80-90% of the

Abbreviations: CTAB) cetyltrimethylammonium bromide; NBA) N-t-Boc-L-alanine *p*-nitrophenyl ester; BTEE) N-benzoyl-L-tyrosine ethyl ester.

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cells). The exudation was induced by anesthetized intraperitoneal injection of saline solution containing 0.3% starch.

Elastase and cathepsin were separated by a modification of the method of Desser et al. [15].

The enzymes were extracted from the leukocytes with 0.05 M sodium acetate buffer, pH 5.0, containing 0.3% cetyltrimethylammonium bromide (CTAB) using a glass–Teflon homogenizer. The homogenate was centrifuged for 30-60 min at 25,000*g*. The pellet was collected, and the extraction procedure was repeated. Thus, 10 sequential extracts were prepared.

Ion-exchange chromatography was performed on a CM-cellulose column (2.4 × 25 cm) equilibrated with buffer containing 0.05 M sodium acetate and 0.05 M NaCl, pH 5.0. The proteins were eluted at 20 ml/h with 0.05 M sodium acetate buffer, pH 5.0, containing various NaCl concentrations (0.1 and 0.2 M). Finally, a linear gradient of NaCl (0.2-1.2 M) was run through the column.

The sample was gel filtered on a Sephadex G-75 column (2.5 × 69 cm) equilibrated with buffer containing 0.05 M sodium acetate and 1 M NaCl, pH 5.0. Before loading the column, the samples were concentrated in an ultrafiltration cell (Amicon, USA) using YM-10 membranes. The proteins were eluted with the starting buffer at 16 ml/h.

All procedures of protein isolation and purification were performed at 4°C.

Homogeneity of the preparations was estimated by electrophoresis in an acid buffer system according to Panyim and Chalkley [16]. The gel contained 12.5% acrylamide and 6.25 M urea. The electrophoresis was performed in 0.75-mm plates in a Hoefer (USA) instrument; 0.9 M solution of CH_3COOH, pH 2.2, was used as the electrode buffer. Both pre-electrophoresis and electrophoresis were performed at 150-170 V for 1-1.5 h. Carbohydrate components in proteins were revealed after the electrophoresis by staining the gel with Schiff's reagent [17].

Molecular weights of proteins were determined by SDS electrophoresis in 16% polyacrylamide gel according to Schagger and Von Jagow [18]. The electrophoresis was performed in 0.75-mm plates in the Hoefer device; run time was 1.5-2 h at 30 mA per gel. Molecular weights were also estimated by gel filtration.

The esterase activity of the elastase was determined as the rate of N-t-Boc-L-alanine *p*-nitrophenyl ester (NBA) hydrolysis according to Visser and Blaut [19]. The reaction was monitored by the change in absorption of the sample at 400 nm. Cathepsin G esterase activity was determined as the rate of N-benzoyl-L-tyrosine ethyl ester (BTEE) hydrolysis according to Hummel [20]. The reaction was monitored by the change in absorption at 256 nm. In the both cases, enzyme activity was expressed as micromoles of substrate converted per minute per mg protein. The reactions were performed at 25°C. Absorption change was measured using a Beckman DU50 spectrophotometer (USA).

Michaelis constants of the enzymes were determined using the synthetic substrates NBA and BTEE for elastase and cathepsin G, respectively. The rate of enzymatic reaction was measured at various substrate concentrations (0.048-0.240 mM in the case of NBA and 0.1-0.4 mM in the case of BTEE). The reaction was performed at 25°C and at pH values within the pH optimum. Michaelis constants were calculated using the linearization method of Lineweaver–Burk: the plot $1/v$ against $1/[S]$ (where [S] is substrate concentration and v is the rate of enzymatic reaction) yields a straight line that intersects the X-axis at the position $-1/K_m$.

Protein content in samples was determined by the Lowry method [21]. BSA solutions of known concentration were used as standards.

In this work we used CTAB, NBA, BTEE, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, leupeptin, EDTA, pepstatin, BSA, acrylamide, and protein markers for SDS-PAGE and gel filtration from Sigma (USA); SDS from Fisher (USA); CM-cellulose from Whatman (Great Britain); Sephadex G-75 from Pharmacia (Sweden). Elastase and cathepsin G from human blood leukocytes were isolated by the same procedure as described for the dog leukocyte enzymes.

RESULTS

Elastase activity was maximal in the 6-8th of the ten extracts prepared; cathepsin G activity was maximal in the 8-10th extracts. To isolate both elastase and cathepsin G, the extracts from 6th to 10th were combined and applied onto a CM-cellulose column. Then the column was washed with eluting buffer containing 0.1 M NaCl until no absorption at 280 nm was detected in the eluate. The same buffer containing 0.2 M NaCl eluted the elastase. Other proteins adsorbed to the column including cathepsin G were eluted by a linear gradient of NaCl (from 0.2 to 1.2 M) in the eluting buffer (Fig. 1). Fractions exhibiting maximal activity of elastase or cathepsin G were collected and concentrated to a volume of 2-3 ml using the ultrafiltration cell. While concentrating, the samples were freed from components of molecular weight below 10 kD. Both proteins were further purified on a Sephadex G-75 column. Figures 2 and 3 demonstrate the results of the gel filtration of elastase and cathepsin G, respectively. The described procedure of protein isolation resulted in ~30-fold purification of elastase, and more than 100-fold purification of cathepsin G with yields of the enzymes ~40% (Tables 1 and 2). Figure 4 presents the electrophoregram of the preparations.

Molecular weights of the enzymes were determined: for elastase—26 kD (estimated by gel filtration) and

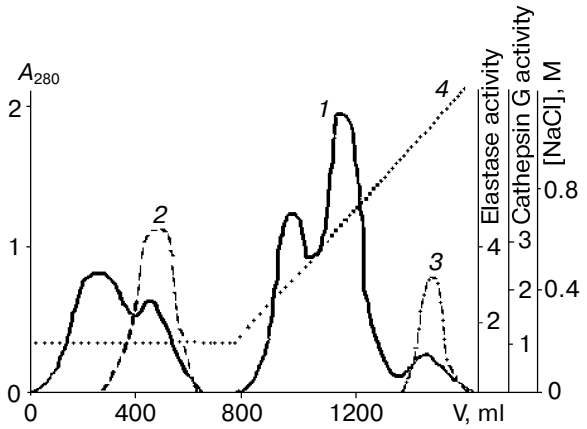

Fig. 1. CM-cellulose ion-exchange chromatography of the extract. The column (2.4 × 25 cm) was equilibrated with 0.05 M sodium acetate buffer, pH 5.0, containing 0.1 M NaCl. The proteins were eluted at 20 ml/h with the same buffer containing various concentrations of NaCl: *1*) absorption at 280 nm; *2*) elastase activity (μmol NBA/min per ml); *3*) cathepsin G activity (μmol BTEE/min per ml); *4*) NaCl concentration in the eluting buffer (M).

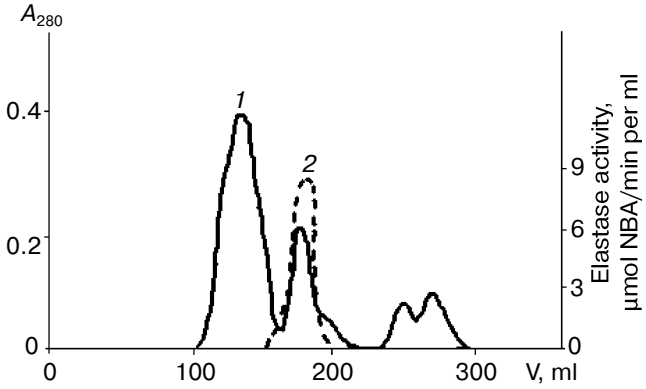

Fig. 2. Gel filtration of the elastase on a Sephadex G-75 column. The column (2.5 × 69 cm) was equilibrated with 0.05 M sodium acetate buffer, pH 5.0, containing 1 M NaCl. The enzyme was eluted with the same buffer at 16 ml/h: *1*) absorption at 280 nm; *2*) elastase activity (μmol NBA/min per ml).

24.5 kD (estimated by SDS-PAGE); for cathepsin G—23.5 kD (by gel filtration) and 25.5 kD (by SDS-PAGE). Cathepsin G was shown to be a glycoprotein, while for the elastase Schiff's reaction did not reveal a carbohydrate component.

Table 3 presents the results of inhibitor analysis. The enzymes were incubated in the presence of inhibitors for 30 min except in the case of phenylmethylsulfonyl fluoride, for which the incubation time was 20 h.

The pH optima of the enzymes were determined at 25°C using synthetic substrates—NBA (for elastase) and BTEE (for cathepsin G). The pH optima of the dog enzymes were compared to those of the human enzymes. The reactions were performed in 0.08 M Tris-HCl at pH values varying from 6.2 to 9.6. The results are presented in Fig. 5 (elastase) and Fig. 6 (cathepsin G). The dog elastase pH optimum coincides with that of the human enzyme and corresponds to the range of pH 8.0-8.5. Human cathepsin G has pH optimum at about pH 7.0, while dog cathepsin G has a broad pH optimum exhibiting virtually constant high activity in the range of pH from 7.0 to 9.0.

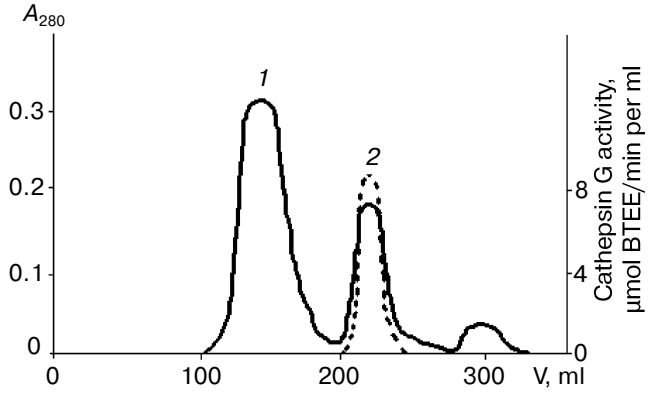

Fig. 3. Gel filtration of the cathepsin G on a Sephadex G-75 column. The column (2.5 × 69 cm) was equilibrated with 0.05 M sodium acetate (pH 5.5) containing 1 M NaCl. The enzyme was eluted with the same buffer at 16 ml/h: *1*) absorption at 280 nm; *2*) cathepsin G activity (μmol BTEE/min per ml).

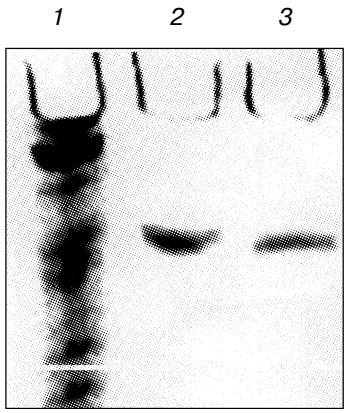

Fig. 4. Electrophoregram of elastase and cathepsin G from dog leukocytes. The gel contained 12.5% acrylamide and 6.25 M urea; the electrode buffer contained 0.9 M CH_3COOH (pH 2.2). The electrophoresis was performed in 0.75-mm plates; run time was 1-1.5 h at 150-170 V: *1*) CTAB extract containing elastase and cathepsin G; *2*) elastase; *3*) cathepsin G.

Table 1. Isolation of elastase from dog leukocytes

Purification stage	Total protein, mg	Elastase activity		Purification extent	Yield, %
		total, μmol/min	specific, μmol/min per mg protein		
CTAB extracts 1-10	2628	1021	0.39	1	100
CTAB extracts 6-10	1194	888	0.74	1.90	87
CM-cellulose ion-exchange chromatography	125	538	4.31	11.05	53
Sephadex G-75 gel filtration	39	436	11.2	28.72	43

Table 2. Isolation of cathepsin G from dog leukocytes

Purification stage	Total protein, mg	Cathepsin G activity		Purification extent	Yield, %
		total, μmol/min	specific, μmol/min per mg protein		
CTAB extracts 1-10	2628	151	0.06	1	100
CTAB extracts 6-10	1194	151	0.13	2.17	100
CM-cellulose ion-exchange chromatography	184	102	0.55	9.17	68
Sephadex G-75 gel filtration	9.7	63	6.51	108.5	42

For the dog enzymes, K_m values were found to be 0.10 mM for elastase and 0.42 mM for cathepsin G. For the human enzymes, the K_m values were 0.35 and 0.47 mM for elastase and cathepsin G, respectively.

DISCUSSION

To isolate elastase and cathepsin G from dog neutrophils, we modified the procedure of Desser et al. [15] developed for isolation of leukocyte peroxidases. At the extraction stage, the cationic detergent CTAB was used. It has some advantages in comparison with other extractants. First, CTAB extracts preferentially cationic proteins and peptides providing partial separation of proteins in the initial stage of the purification procedure. In the case of saline extractions, serine proteinases are isolated from neutrophils together with endogenous inhibitors and acid proteoglycans that significantly decrease the enzyme

Table 3. Effect of inhibitors on esterase activity of elastase and cathepsin G from dog leukocytes

Inhibitor	Concentration, mM	Remaining activity, %	
		elastase	cathepsin G
Phenylmethylsulfonyl fluoride	0.2	0	0
Soybean trypsin inhibitor	0.1	0	0
Leupeptin	0.1	100	100
EDTA	0.1	100	90
Pepstatin	0.1	85	80

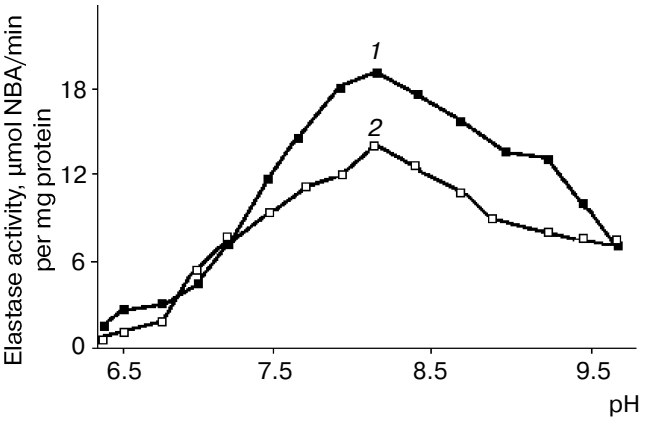

Fig. 5. Evaluation of the pH optimum of esterase activity of dog (*1*) and human (*2*) elastase using the NBA hydrolysis (μmol/min per mg protein) reaction. The activity was measured in 0.08 M Tris-HCl at various pH values at 25°C (the curve is plotted using the mean values of 5 measurements).

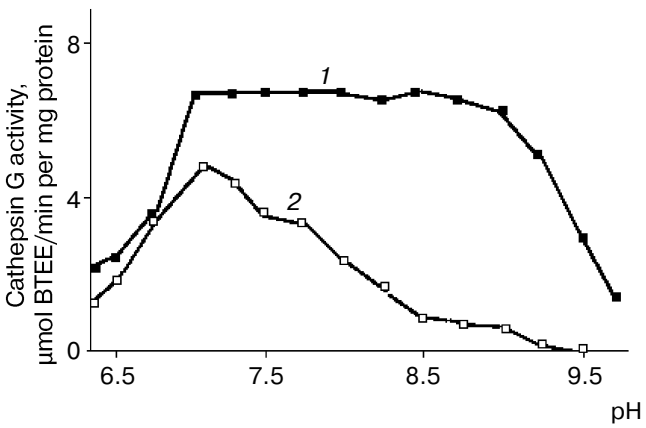

Fig. 6. Evaluation of the pH optimum of esterase activity of dog (*1*) and human (*2*) cathepsin G using the BTEE hydrolysis (μmol/min per mg protein) reaction. The activity was measured in 0.08 M Tris-HCl at various pH values at 25°C (the curve is plotted using the mean values of 5 measurements).

activity. This leads to an overvaluation of the purification extent in the following stages. The use of CTAB avoids these difficulties. Due to the high efficiency of the extraction of cationic proteins in the presence of this detergent, it became possible to omit the step of leukocyte granule isolation, although such a possibility was challenged in one of the early works on isolation of leukocyte proteinases [22]. Another feature of our method is the relatively mild extraction procedure yielding 10 consecutive extracts varying in their protein composition; this provides a partial separation of enzymes at the extraction stage. Protein distribution in the extracts indicates that cathepsin G is bound to granular matrix components more tightly than is elastase. During the ion-exchange chromatography, complete separation of elastase and cathepsin G was achieved, as well as their separation from the bulk of other proteins. During gel filtration, elastase and cathepsin G were purified to homogeneity. The procedure is rather efficient for isolation of elastase and cathepsin G in preparative amounts.

The values of molecular weights of the enzymes determined here are close to those from the literature (24.2-25.2 kD for elastase and 26.8-29.4 kD for cathepsin G) [12, 14]. Cathepsin G is shown to be a glycoprotein, while elastase lacks a carbohydrate component, also in agreement with the literature data [12].

The enzymes belong to the group of serine proteinases as shown by inhibitor analysis. The activity of the enzymes was depressed by specific inhibitors of serine proteinases—phenylmethylsulfonyl fluoride and soybean trypsin inhibitor—but virtually unaffected by other proteinase inhibitors—leupeptin (cysteine proteinase inhibitor), EDTA (metalloproteinase inhibitor), and pepstatin (aspartic proteinase inhibitor). A slight inhibiting effect of pepstatin on the activity of the both enzymes was likely due to the influence of impurities—the purity of the inhibitor preparations used was 78%.

The positions of pH optima as well as Michaelis constant values determined in this work for the human enzymes coincide with the parameters found in the literature [23-25]. An interesting feature of the isolated dog cathepsin G is its broad pH optimum (Fig. 5)—the activity remains constant in the range of pH values from 7.0 to 9.0. The broad pH optimum of cathepsin G activity is not typical for enzymes of this group isolated from other mammalian species, although the pH optimum positions can be observed both in neutral pH region (human cathepsin G) and in the weakly base region (porcine cathepsin G) [26].

According to the literature, dog neutrophil elastase significantly differs from human neutrophil elastase in some properties: it has a lower molecular weight; in contrast to human elastase, the enzyme lacks a carbohydrate component; the proteins do not exhibit cross immunoreactivity as shown by immunodiffusion [12]. This work confirms the data concerning the molecular weight and carbohydrate content. Also, the K_m value determined in this work for dog elastase (0.10 mM) differs from that obtained for human elastase (0.35 mM; the literature data [25] is consistent with our observations). This result complements data on the differences between the human and dog elastases. However, dog cathepsin G is similar to the human enzyme in its physical and chemical properties and kinetic parameters.

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