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ACID SULPHYDRYL PROTEASE FROM CALF LYMPH NODES

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Summary

A new sulphydryl acid protease has been isolated from calf lymph nodes. Having a molecular weight of 14 000 ± 1500, it was separated from cathepsin D (EC 3.4.23.5) on a column of Sephadex G-100. Hemoglobin hydrolysis was optimal at pH 3.0. It was not inhibited by pepstatin. Sulphydryl effectors characteristically influence this activity.

Introduction

The acid proteolytic activity of the spleen, thymus and lymph nodes differ from each other in regard to the intracellular distribution of acid proteinases as described by Bowers et al. [1] as well as with respect to the specificity and pH optima as indicated by Fräki et al. [2]. The presence of acid proteinase in bovine tonsils was proposed by Hess [3]. Stein and Fruton [4] reported that at least two acid proteolytic activities with different subcellular location, substrate affinity and cysteine requirement were present in rat lymph nodes. The two observed proteinases, however, were not efficiently purified. Using the isolation procedure described in this report, cathepsins with amidase activity toward α-*N*-benzoyl-L-arginine amide (Bz-Arg-NH_2), representing probably the action of cathepsins B1 and B2 (EC 3.4.22.1) and cathepsin D (EC 3.4.23.5) have been separated. In addition to this a new sulphydryl protease with some enzymatic properties similar to those of cathepsin D but with lower molecular weight was found. Some characteristics of this protease are described.

Methods

Purification procedure

Fresh calf lymph nodes were brought on ice from the slaughter house. A 50% aqueous homogenate was prepared from approximately 1500 g of minced

tissue using a Waring blendor homogenizer. It was centrifuged at 5000 × *g* for 90 min in a Sorvall RC 2 refrigerated centrifuge. The supernatant was acidified with HCl to pH 3.5 and centrifuged at 5000 × *g* for 20 min. To the supernatant so obtained, solid ammonium sulphate was added to 80% saturation, the precipitate being collected by centrifugation at 5000 × *g* for 10 min. The latter was then suspended in water and dialyzed against it for 20 h. Following this, proteases were precipitated with 30—60% acetone, then dissolved in 0.01 M acetate buffer pH 5.5 and run into a column of CM cellulose (Sigma, U.S.A.), equilibrated with the same buffer. Three proteolytically active peaks were eluted from the column as shown in Fig. 1. The first proteolytic peak was not separated from the amidase activity peak. They were pooled together, concentrated by lyophilization and run into a column of Sephadex G-100 (Pharmacia, Sweden). The other two proteolytically active peaks were also investigated as described elsewhere [5]. The protein content in eluted fractions was followed either by measuring the absorbance at 280 nm with a Unicam spectrophotometer SP 500 or determined by using the modified Kjeldahl method [6].

Assay of enzymes

The acid proteolytic activity was measured by Anson's procedure [7]. 2% hemoglobin solution in 0.1 M acetate buffer, pH 3.5 or 3.0, was used as a substrate. 0.4 ml of enzyme preparation were incubated with 2 ml of the substrate solution at 37°C for 10 min. The reaction was stopped with 4 ml of 5% trichloroacetic acid. To 2 ml of the digestion filtrate 4 ml of 0.5 M NaOH and 1.2 ml of the Folin Ciocalteau reagent were added. The colour difference between samples and blanks was read within 5 min at 750 nm (ΔA_{750nm}). Blanks were prepared so that trichloroacetic acid was added to the enzyme

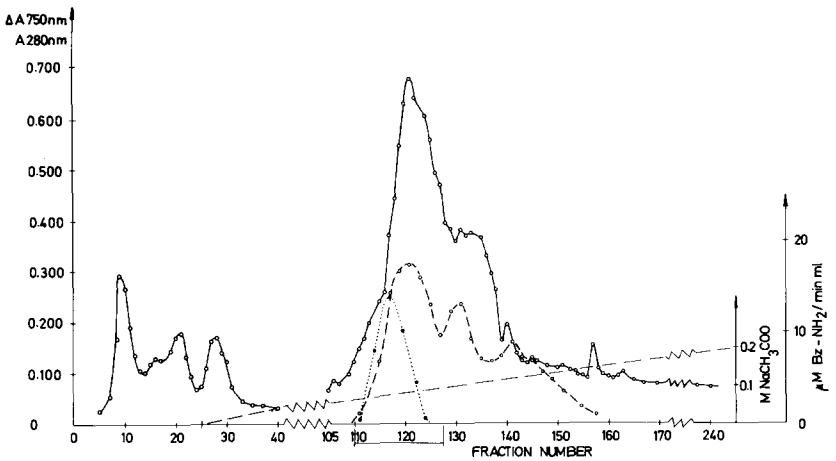

Fig. 1. Chromatography of acetone extract on CM cellulose. 28 ml of acetone extract, previously dialyzed against the elution buffer and concentrated by ultrafiltration, containing 15.8 mg protein/ml were applied on a column (5 cm × 50 cm) and eluted with 0.01 M acetate buffer pH 5.5. After the second protein peak the elution was continued using a linear gradient to 0.2 M concentration of CH_3COONa (pH 5.5). Flow rate 65 ml/h. Fractions volume: 20 ml. ———, A_{280nm}, - - - - - -, ΔA_{750nm}, acid proteolytic activity on hemoglobin at pH 3.5, · · · · · ·, amidase activity on Bz-Arg-NH_2 at pH 5.3. Fractions were pooled as indicated.

preparation prior to the addition of hemoglobin. The calibration curve was constructed relating ΔA_{750nm} values to the catheptic units. One unit of the cathepsin (A.U.) is defined as the amount which digests hemoglobin under the standard conditions at an initial rate such that there is liberated per minute an amount of split products, not precipitated by trichloroacetic acid, which gives the same colour as 1 milliequivalent of tyrosine [7]. Activity in the presence of dithiothreitol was also measured by Anson's method without the addition of Folin Ciocalteau reagent and the amount of trichloroacetic acid soluble products was determined by measuring the difference in absorbance at 280 nm (ΔA_{280nm}) between samples and blanks.

The amidase activity was determined by using Bz-Arg-NH_2 as a substrate according to Otto procedure [8].

Results and Discussion

The elution diagram from Sephadex G-100 chromatography (Fig. 2) shows a satisfactory separation of two proteolytically active peaks and two amidase peaks. Some standard proteins with known molecular weights were run on the column in order to determine the molecular weights of the most active fractions according to Whitaker's method [9]. The molecular weights of the two amidase active fractions I and III were 51 000 ± 5000 and 23 000 ± 2000, respectively. These values and the fact that amidase activity was measured toward Bz-Arg-NH_2 which is the common synthetic substrate of cathepsins B1 and B2 [10] strongly indicate that we have separated cathepsins B1 and B2 from each other. The two amidases were, however, not further characterized.

Two proteolytic activities were eluted in the region where proteins with molecular weights 39 000 ± 4000 and 14 000 ± 1500 appeared. The active fractions II and IV were concentrated and the two enzyme preparations were examined in detail. In the fraction II cathepsin D (EC 3.4.23.5) was eluted as confirmed by measuring pH optimum for hemoglobin hydrolysis, relative specificities toward several protein substrates and the influence of effectors on its proteolytic activity [5]. The second proteolytic peak pooled in fraction IV — here named cathepsin S — has some enzymatic properties similar to those of cathepsin D. The protein substrate most susceptible to acid hydrolysis was

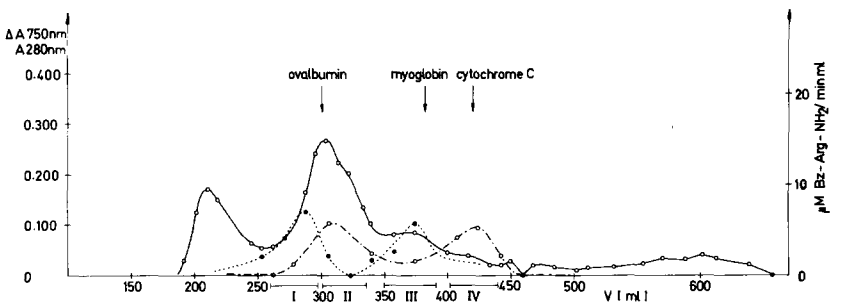

Fig. 2. Chromatography on Sephadex G-100. 4 ml of concentrated enzyme preparation containing 10.6 mg protein/ml were applied on a column (2.5 cm × 85 cm) of Sephadex G-100, medium. Elution buffer was composed of 0.15 M sodium acetate, 0.1 M NaCl, 1 mM EDTA and 0.5 mM dithiothreitol, and the pH was adjusted to 4.6. Flow rate 35 ml/h. Fractions' volume: 7—8 ml. ———, A_{280nm}, - - - - - -, ΔA_{750nm}, acid proteolytic activity, · · · · · ·, amidase activity. Fractions were pooled as indicated.

TABLE I

INFLUENCE OF SOME EFFECTORS ON HEMOGLOBIN HYDROLYSIS OF CATHEPSIN S

0.2 ml of enzyme preparation containing 0.66 mg protein/ml were preincubated with 0.2 ml of 5 mM effector solution in acetate buffer, pH 3.0, for 12 min in water bath at 37°C. Then 2 ml 2% hemoglobin solution in acetate buffer, pH 3.0, was added and incubated for 3 h at 37°C.

Added solution	$\Delta A_{750\,nm} \times 10^3$	$\Delta A_{280\,nm} \times 10^3$	% Inhibition
Acetate buffer pH 3.0	255	110	(0)
Pepstatin*	250	—	0
$CoCl_2$	175	—	31
$MgCl_2$	255	—	0
Iodoacetamide	55	—	78
Iodoacetic acid	0	—	100
$HgCl_2$	0	—	100
Dithiothreitol	—	310	(0)

* Pepstatin was added in 3 μM concentration.

hemoglobin. The hydrolysis of bovine serum albumin and human serum albumin was 50% of hemoglobin hydrolysis whereas gamma globulin was not degraded at all. The pH optimum for hemoglobin hydrolysis in acetate buffer was 3.0. The enzyme preparation of cathepsin S was not very stable, its proteolytic activity decreasing by 50% when left for 24 h at room temperature. The main difference between cathepsins D and S lies in the influence of effectors on hemoglobin hydrolysis (Table I). Pepstatin, isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methyl-heptanoyl-L-alanyl-4-amino-3-hydroxy-6-methyl heptanoic acid, which is strong inhibitor of cathepsin D [11], did not inhibit cathepsin S in 3 μM final concentration. The activity of cathepsin S was not effected by $MgCl_2$. There was 31% inhibition by $CoCl_2$, 78% inhibition by iodoacetamide and complete inhibition by iodoacetic acid and $HgCl_2$. The addition of 5 mM solution of dithiothreitol increased the activity by 300%. These reagents did

TABLE II

THE PURIFICATION OF CATHEPSINS D AND S FROM CALF LYMPH NODES

0.2 ml of enzyme preparations were preincubated with 0.2 ml of 4 μM pepstatin solution or with acetate buffer, pH 3.0, for 12 min at 37°C. Then 2 ml of 2% hemoglobin solution in acetate buffer, pH 3.0, was added and incubated for 10 min. Specific proteolytic activity (A.U./mg protein) was calculated according to Anson [7] as described.

Step of purification	Volume	Protein concentration (mg/ml)	A.U./mg protein	A.U./mg protein*	Total activity*	Purification*	Yield*
Crude homogenate	3500	79.6	0.045	0.005	1390	(1)	(100)
Supernatant 400000 $g \cdot$ min	1700	40.5	0.127	0.010	689	2	49.6
Extract, pH 3.5	650	28.0	0.254	0.030	546	6	39.3
Precipitate with $(NH_4)_2SO_4$	300	9.1	0.780	0.040	109	8	7.8
Precipitate with acetone	61	7.7	1.850	0.185	87	37	6.2
CM cellulose and gel filtration:							
fraction II (cathepsin D)	8	1.1	9.44	0.9			
fraction IV (cathepsin S)	3	0.6	0.250	0.250	0.50	50	0.03

* The activity is measured in the presence of pepstatin

not affect the activity of fraction II [5]. The results indicate that cathepsin S is a sulphydryl endopeptidase, not inhibited by pepstatin. During the purification procedure the action of both, cathepsin D and cathepsin S, was therefore measured. In order to follow the purification of cathepsin S, specific activity in the presence of $4 \cdot 10^{-6}$ M pepstatin which inhibits the action of cathepsin D [12] was determined. On the basis of these experiments the purification factor of cathepsin S was found to be 50 (Table II). The enzyme preparation, obtained after gel chromatography, was not homogenous as checked by electrophoresis on polyacrylamide gels. First, the electrophoresis at pH 8.4 was run (Fig. 3). Four protein components were found. They were extracted with water, concentrated and their proteolytic activities at pH 3.0 were measured with and without the addition of pepstatin (Table III). It is evident that component 3 must be cathepsin S because the former was not inhibited by pepstatin. Protease S in its pure form was revealed by rechromatography of component 3 under the same experimental conditions (Fig. 3). Secondly, the preparation of protease S was subjected to sodium dodecyl sulphate electrophoresis.

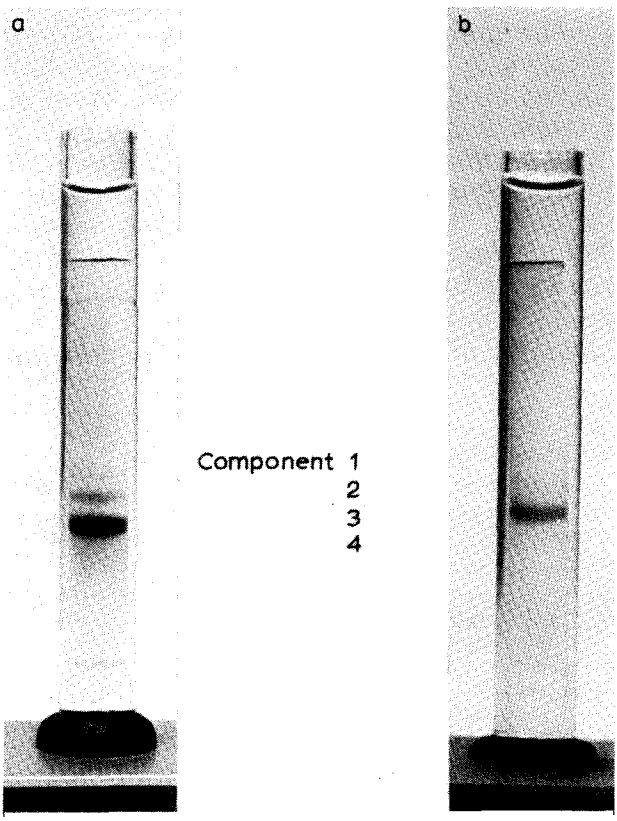

Fig. 3. a. Electrophoresis pattern of cathepsin S on polyacrylamide gel. 0.2 mg of protein per gel were applied. 7.5% polyacrylamide gel in Tris · glycine buffer, pH 8.4 (0.025 M Tris, 0.2 M glycine) was used at 20 V, 5 mA per gel length 60 mm for 45 min. Protein staining was done with Amido black and 7% acetic acid was used for destaining at 110 V for 90 min. The experiment was carried out at 0°C with Canalco (USA) apparature. b. Electrophoretic pattern of isolated component 3. Experimental conditions are the same as described under a.

TABLE III

HEMOGLOBIN HYDROLYSIS AND THE EFFECT OF PEPSTATIN ON THE HYDROLYSIS OF ELECTROPHORETICALLY SEPARATED COMPONENTS OF CATHEPSIN S

0.2 ml of eluted component was preincubated with 0.2 ml of acetate buffer (first column) or with 0.2 ml of pepstatin solution in acetate buffer in final concentration 3 μM at pH 3.0 for 12 min at 37°C. Then 2 ml 2% hemoglobin solution in acetate buffer, pH 3.0, was added and incubated for 4 h.

	$\Delta A_{750\,nm} \times 10^3$ in acetate buffer	$\Delta A_{750\,nm} \times 10^3$ with pepstatin
Component 1	76	0
Component 2	78	0
Component 3	110	125
Component 4	40	0

Besides three weak protein bands, one strong protein component of the preparation appeared which probably represented the cathepsin S. It was found to have a molecular weight 17 000 ± 1500 determined with the method of Weber and Osborn [13]. From these results it can be concluded that cathepsin S is a low molecular weight protease, thus differentiating from all known tissue proteinases, although low molecular weight is not an uncommon property for other proteinases [14].

Barrett [15] stated that the activity of carefully purified preparations of cathepsin D is not affected by cysteine or thiol blocking reagents, although it is not uncommon to find that part of the activity of crude preparations had the characteristics of a thiol proteinase [16,17]. Barrett has suggested that cathepsin B1 or some other endopeptidase with a sulphydryl requirement may be present which acts along with cathepsin D in its crude preparations. Several studies on hemoglobin hydrolysis by partially purified cathepsin preparations have suggested a synergistic action of cathepsins A, B and C on the one hand and cathepsin D on the other hand [12,18—20]. Recently Huisman et al. [12] examined the role of individual cathepsins in extract of rat liver lysosomes in the breakdown of denatured serum albumin and hemoglobin at pH 5.0. Using specific enzyme inhibitors they blocked the action of cathepsins B1, B2, C and D and the residual activity was ascribed to some thiol-dependent lysosomal endopeptidases.

Our results strongly indicate that in calf lymph nodes an endopeptidase with sulphydryl requirement is present. It differs from thiol dependent cathepsins B2 and C by its significantly lower molecular weight and from cathepsin B1 by its inability to split Bz-Arg-NH_2 (Fig. 2) and by its lower pH optimum for hemoglobin hydrolysis. This value (pH 3.0) is probably low enough to avoid any significant interference by capthepsin B1.

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