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Acid Proteinases from Calf Lymph Nodes

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Acid proteinases were isolated from calf lymph nodes using acid extraction, ammonium sulphate and acetone precipitation, followed by ion exchange chromatography on CM-cellulose and gel chromatography on Sephadex G-100. Cathepsin D (E. C. 3.4.23.5) is present in lymph nodes. It has the molecular weight of 39 000 as determined by gel filtration on Sephadex G-100. Cathepsins B1 and B2 (E. C. 3.4.22.1) were also isolated. Molecular weights of 22 000 and 51 000 were determined for cathepsins B1 and B2, respectively. Calf lymph nodes contain also another proteinase which degrades haemoglobin at an optimum at pH = 3.0. This proteinase has a molecular weight of 14 000 as was determined by gel filtration. Its activity is not inhibited with 0,25 μM pepstatin which inhibits 90% of the cathepsin D activity.

INTRODUCTION

Lymph nodes play an important role in the defense mechanisms of mammalian organisms. It is supposed that there exists a cooperative action of immuno competent lymphocytes, macrophages and other cells of the reticuloendothelial system[1]. The biochemical events occuring in an immunological response are still obscure. It is confirmed, however, that the overall protein turnover is often enhanced[2–5] and we suppose that intracellular proteinases play in addition to their normal role in cell metabolism also a specific role in the physiological function of lymphoid cells.

There is not much known about the intracellular proteinases of the lymph nodes. In their recent report Fräki *et al.*[6] stressed the qualitative differences between thymus and lymph nodes of rats with respect to the presence of acid, neutral and alkaline proteinases. The differences are probably due to different amounts of T and B lymphocytes in both organs. They found higher acid proteolytic activity in lymph nodes than in the thymus. From the thymus larger amounts of neutral and alkaline proteolytic active extracts were obtained. Bowers *et al.*[7] confirmed the presence of cathepsin D like proteinase in several types of lysosomes in lymphoid tissues of rat thymus, spleen and lymph nodes. The cathepsins B and C were supposed to be present in mitochondrial fractions of the rat lymph nodes extract, as found by Stein and Fruton[8]. They reported that the acid proteolytic activity, measured on haemoglobin and serum albumin, was present in the two different subcellular fractions. The two acid proteolytic activities differed also with respect to substrate affinity and cysteine activation requirement.

New cathepsin species were found in the lymphoid tissue of the rat by Yago *et al.*[9] The authors separated two peaks of the cathepsin activity which was assayed on acid denatured haemoglobin at pH = 3.6. The extracts of thoracic duct lymphocytes were run into a column of Sephadex G-100 and the molecular weights of the two cathepsins were 45 000 and 80 000. Both proteinases were very sensitive to pepstatin inhibition.

In this work we wanted to characterize acid proteinases from calf lymph nodes, which had not been isolated yet, and to determine some of their properties.

EXPERIMENTAL

Reagents and sources of supply

CM cellulose was obtained from Sigma, St. Louis, USA; Sephadex G-100, medium, from Pharmacia, Uppsala, Sweden; Blue dextran 2000 and standard proteins for molecular weight determination, such as egg albumin (M_r = 45 000), horse myoglobin (M_r = 17 800), cytochrome c (M_r = 12 400), DNP-L-alanin (M_r = = 255.5) from Serva, Heidelberg, Germany; we used dialysing tubes Kalle Aktiengesellschaft, kal 28, Wiesbaden, Germany. Haemoglobin was prepared in our laboratory; α-*N*-benzoyl-D,L-arginine amide (Bz—Arg—NH_2), dithiothreitol (DTE), bovine serum albumin (Krist. reinst 98%), monoiodoacetic acid and monoiodoacetamide were obtained from Serva; human serum albumin (grade III) from Sigma, gamma-globulin (fr. II) and fibrinogen (plasmin free, fr. I) from Pentex Miles Laboratories, Kankakee, USA; casein type Hammarsten and ethylenediaminetetraacetic acid (EDTA) was the product of BDH, Ltd. Poole, England. We obtained p-chloromercuribenzoate (PCMB) from Nutritional Biochemicals Co., Cleveland, USA; $MgCl_2$ from Riedel de Haën A. G., Hannover,, Germany and $HgCl_2$, $CoCl_2$ and other chemicals of analytical grade from Kemika, Zagreb, Yugoslavia.

Pepstatin was obtained from Dr. H. Umezawa, Tokyo, Japan.

Extraction of acid proteinases

Fresh calf lymph nodes were brought on ice from the slaughter house. The organs were freed of fascia and fat, passed once through a meat mincer and an equal volume of water was added to make a 50% hypotonic suspension which was homogenized in a Waring blendor homogenizer for 20 minutes on an ice bath. The homogenate was centrifuged at 5000 × g for 90 minutes in a refrigerated Sorvall RC2 centrifuge. The obtained supernatant was used for the extraction of proteinases. The pH was adjusted to 3.5 using 3 M HCl, and the homogenate was centrifuged at 5000 × g for 20 minutes. The proteins in the supernatant were precipitated with solid ammonium sulphate to 80% saturation and the suspension was centrifuged at 5000 × g for 10 minutes. The latter was then suspended in water and dialysed against water for 20 hours at 4 °C. Following this, proteases were precipitated with 30—60% acetone. They were then dissolved in 0.01 M acetate buffer pH = 5.5, and dialysed against that buffer overnight, and the acetone fraction was used for the separation of acid proteinases.

Separation of acid proteinases

The separation of acid proteinases was carried out on a CM-cellulose column (5 × 59 cm) using 0.01 M acetate buffer, pH = 5.5, as starting eluent. After the second protein peak the elution was continued using 1.5 l 0.01 M acetate buffer, pH = 5.5, mixing gradually with 1.5 l of the same buffer to which CH_3COONa was added to obtain a concentration of 0.2 M and the pH was readjusted to 5.5. Proteolytically active fractions were pooled, concentrated by lyophilisation to smaller volumes and dialysed against the buffer used in the next separation procedure. This was chromatographed on Sephadex G-100. Acetate buffer 0.15 M pH = 4.6, containing 0.1 M NaCl, 1 mM EDTA and 0.5 mM DTE, was used for the elution.

Protein determination

In eluted fractions from chromatographic columns the presence of proteins was followed by measuring the absorbance at 280 nm with a Unicam Spectrophotometer SP-500. In the enzyme preparations the protein content was determined by the modified Kjeldahl method[10].

Determination of molecular weight

The approximate molecular weight was determined by gel filtration on a Sephadex G-100 column (2.5 × 85 cm) using 0.15 M acetate buffer, pH = 4.6 which contained 0.1 M NaCl, 1 mM EDTA and 0.5 mM DTE as the elution buffer. The void volume (V_o) was determined with Blue dextran 2000, the elution volumes (V_e) were determined using egg albumin, myoglobin, cytochrome c and DNP-L-alanin according to the Whitaker method[11].

Disc electrophoresis

Samples containing approximately 0.2 mg of protein were run on 7.5% acrylamide gel in tris hydroxymethyl aminomethane (Tris)-glycine buffer, pH = 8.4 (0.025 M Tris, 0.2 M glycine), using 20 V, 5 mA per 1 ml of a 60 mm long gel, for 45 minutes. The Canalco equipment USA, was used. Protein staining was done with amido black for 45 minutes and destaining of gels with 7% acetic acid at 110 V for 90 minutes. Electrophoresis was carried out at room temperature.

Determination of enzymatic activities

The proteinase activity was estimated according to the modified Anson's method[12]. A haemoglobin solution (2%) in 0.1 M acetate buffer, pH = 3.5 was used as a substrate without the addition of the preservative and ammonium sulphate. The enzyme preparation (0.4 ml) was incubated at 37 °C with 2 ml of the substrate solution. The reaction was stopped with 4 ml 5% trichloroacetic acid (TCA). After 15 minutes the suspension was filtered using Selecta Nr. 589 filter paper. To 2 ml of the digestion filtrate 4 ml of 0.5 N NaOH and 1.2 ml of the Folin-Ciocaulteau reagent, dil. with 2 volumes of water, were added. The colour was read at 750 nm within 5 minutes. The colour values were corrected for the blanks which were prepared as described by Anson and the calibration curve was censtructed using tyrosine solution as standards.

In determining relative activities toward various protein substrates as well as in testing the activators and inhibitors of the proteolytic activity, the latter is measured as A (750 nm) under given experimental conditions. In the enzyme preparations the activity is determined as described by Anson and expressed in Anson units per mg of protein (A. U./mg protein).

The activity in the presence of DTE was also measured by the Anson mehod without the addition of the Folin-Ciocaulteau reagent. The amount of TCA soluble products in samples was determined by measuring the absorbance at 280 nm corrected for blanks, and expressed as A (280 nm).

The activities of the cathepsings B1 and B2 were determined using Bz—Arg—NH_2 as the substrate. The method of Otto[13] was slightly modified using 0.1 M acetate buffer, pH = 5.7, which contained 20 nM Bz—Arg—NH_2, 1 mM EDTA and 1 mM DTE, as the reaction mixture. The enzyme solution (0.1 ml) was incubated for 60 minutes at 37 °C with 1 ml of the substrate solution. 2 ml of 5% TCA were added to stop the reaction and the released NH_3 was determined with the ninhydrin reagent[14]. The amidase activity is expressed as degraded Bz—Arg—NH_2 in μmol min^{-1} ml^{-1} of the enzyme preparation[15].

RESULTS

To investigate the content of acid proteinases, the acetone extract, obtained as described above, was run into a column of CM- cellulose and eluted with the acetate buffer, pH = 5.5, and a linear gradient of sodim acetate (Fig. 1). As seen from the diagram, three proteolytically active peaks were eluted one after another with increasing ionic strength of the buffer. The fractions under

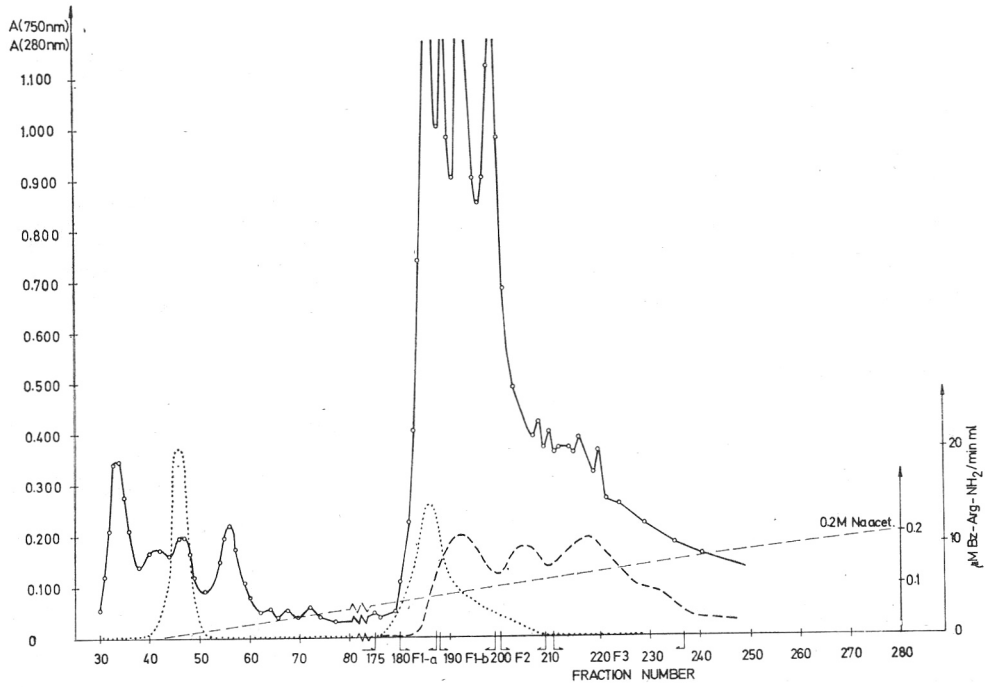

Fig. 1. Chromatography of the acetone extract on cm cellulose. 50—70 ml of dialyzed acetone extract containing about 12 mg of protein/ml were applied to column 5 × 50 cm and eluted with 0.01 M acetate buffer, pH = 5.5, and a linear gradient of CH_3COONa to concentration 0.2 M. Flow rate 60 ml/h. Fraction volume 20 ml. —— *A*(280 nm); — — — *A*(750 nm); acid proteolytic activity on haemoglobin; .... amidase activity on Bz—Arg—NH_2. Frations were pooled as indicated.

the peaks were pooled and designated F1-b, F2 and F3. The amidase activity, measured with Bz-Arg-NH_2, was eluted in two peaks; the narrow amidase activity peak, eluted at the start of the gradient elution, was not further investigated; the second amidase activity peak which was partially associated with the first proteolytically active peak, was pooled in fraction F1-a. The fractions F1-a, F1-b, F2 and F3 were concentrated by lyophilisation and further purified on Sephadex G-100. The elution buffer was 0.15 M acetate buffer, pH = 4.6, containing 0.1 M NaCl, 1 mM EDTA and 0.5 mM DTE, used also by Otto[15] for the separation of the cathepsins B1, B2 and D. The elution diagrams are presented on Fig. 2 and Fig. 3. From Fig. 2 one can see that the amidase activity which was present in fraction F-1a, was eluted in two separate peaks corresponding to proteins, with molecular weights about 51 000 and 22 000 as was determined by the Whitaker's method[11]. These values are characteristic for the cathepsins B2 and B1, respectively[15]. So we conclude that we have separated the cathepsins from each other as well as partially from the cathepsin D. They were not further investigated.

The acid proteolytic activity of all four fractions, on the other hand, appeared in the same region of the molecular weight 39 000 as can be seen from Fig. 2 and Fig. 3. Active fractions were pooled, concentrated and the enzymatic properties of these proteinases were determined. They were essentially the same and similar to those of cathepsin D (E.C.3.4.23.5) as described

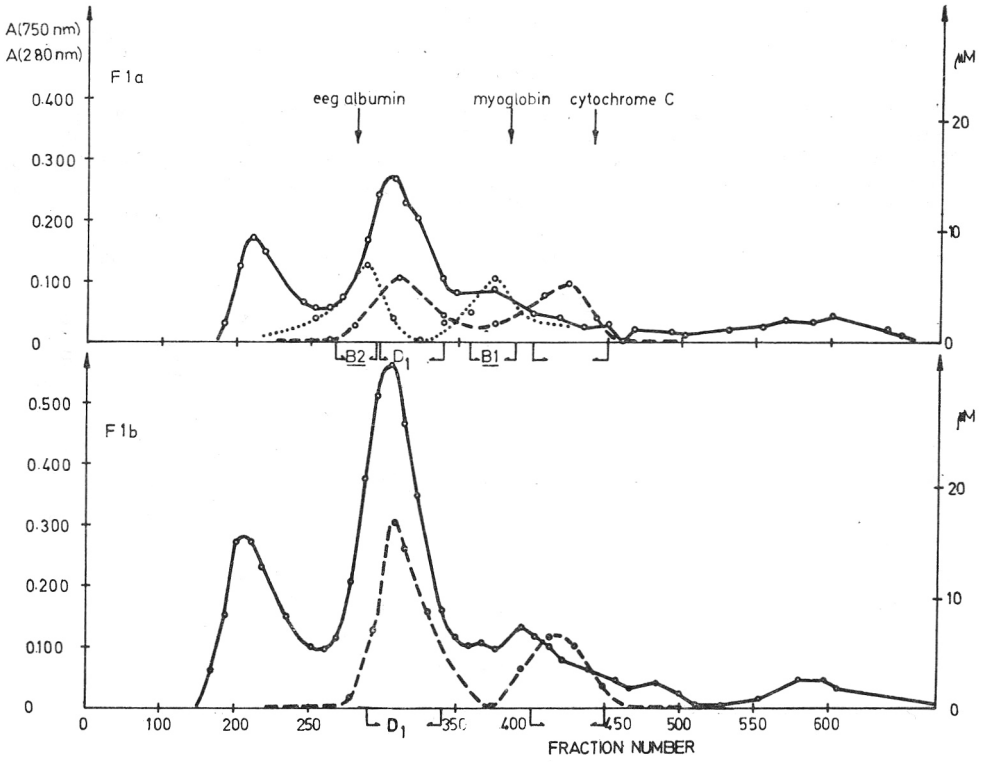

Fig. 2. Chromatography of fractions F1-a and F1-b on Sephadex G-100. 3—4 ml of concentrated fractions from CM cellulose, previously dialyzed against the elution buffer, containing about 20 mg of protein/ml were applied to a column 2.5 × 85 cm and eluted with 0.15 M acetate buffer, pH = 4.6 containing 0.1 M NaCl, 1 mM EDTA and 0.5 mM DTE. Flow rate 35 ml/h. Fraction volume 8 ml. ——— A(280 nm); — — — — 750 nm, acid proteolytic activity on haemoglobin; .... amidase activity on Bz—Arg—NH_2. Fractions were pooled as indicated. Blue dextran 2000 and some standard proteins were applied under the same experimental conditions as the enzyme preparations. Their elution volumes are indicated in the figure.

by Barrett[16]. The proteinases were successively eluted from the ion exchanger with increasing ionic stregth of the buffer, what is an indication that they differ in ionic properties and may represent multiple forms of the cathepsin D. All three proteinases degraded haemoglobin at an optimum pH = 3.5. Haemoglobin was degraded most rapidly with respect to other protein substrates. The relative specificities for some protein substrates for the three proteinases are presented in Table I. The results are in good agreement with those of Lebez *et al.*[17]. Only the enzyme preparation D_1 exhibits a somewhat greater activity toward serum albumin and fibrinogen. This could be explained by concerted action of cathepsin D and other proteases also present in the F1-a fraction (Fig. 2). We would like to mention that the enzyme preparation D_1 was obtained from fraction F1-a containing also the cathepsins B1 and B2. Concerted action of the cathepsins has been already observed by other authors[18–20]. Enzyme preparations D_2 and D_3 contained no cathepsins B1 or B2 (Fig. 3) and were also better purified. It is evident from Table II, presenting the purification of the acid proteinases, that the cathepsin D preparations were purified 1000—4300 fold.

TABLE I.

Relative Specificities for Some Protein Substrates of Cathepsin D from Calf Lymph Nodes

0.4 ml of enzyme preparation were incubated 60 min at 37 °C with 2 ml 2% protein solution or suspension in acetate buffer, pH = 3.5. During the period of incubation the rate of the reaction increased linear with the time under gven experimental conditions. The concentration of protein in enzyme preparation D_1, D_2 and D_3 are 1.07 mg protein/ml, 0.47 mg protein/ml and 0.025 mg protein/ml, respectively.

Substrate	D_1		D_2		D_3	
	activity A(750 nm)	Relative activity	activity A(750 nm)	Relative activity	activity A(750 nm)	Relative activity
Haemoglobin	0.795	(100)	0.818	(100)	1.084	(100)
Bov. ser. albumin	0.259	37	0.053	7	0.077	7
Casein	0.090	11	0.080	10	0.090	9
γ-globulin	0.034	4	0.026	3	0.044	4
Fibrinogen Plasmin free	0.188	24	0.078	10	0.021	2

TABLE II.

The Purification of Acid Proteinases from Calf Lymph Nodes

Step of purification	Specific proteolytic activity (A. U.*/mg protein)	Purification factor
Crude homogenate	0.01	(1)
Supernatant (400 000 g/min)	0.18	18
Extract, pH 3.5	0.43	43
Precipitate with $(NH_4)_2SO_4$ 80% sat.	0.76	76
Precipitate with acetone, 30—60%	5.12	512
CM cellulose and gel filtration:		
— enzyme preparation D_1	9.44	944
— enzyme preparation D_2	24.00	2400
— enzyme preparation D_3	43.20	4300

* Anson units $\times 10^4$

The influence of some effectors on haemoglobin hydrolysis was measured in the enzyme preparation D_1. We found that the action of the effectors is characteristic for cathepsin D (Table III). Pepstatin shows approximately 90% inhibition of the proteolysis, Co^{++} ions about 20% inhibition, while the other tested metal ions as well as thiol reagents exhibit practically no effect on the proteolytic activity. From the obtained results we conclude that this proteinase is cathepsin D, having a molecular weight of approximately 39 000. There are

TABLE III.

The Influence of Some Effectors on Acid Proteolytic Activity of Cathepsin D from Calf Lymph Nodes

0.2 ml of enzyme solution were preincubated with 0.2 ml of 5 mM effector solution in acetate buffer, pH = 3.5, for 12 min at 37 °C. 2 ml 2% haemoglobin solution was added and incubated for 60 min at 37 °C. Enzyme preparation contained 1.07 mg protein/ml.

Enzyme preparation D_1	Activity A(750 nm)	Activity A(280 nm)	Relative activity
Added solution:			
— Acetate buffer pH = 3.5	0.400	0.100	(100)
— Pepstatin*	0.090	—	12
— $CoCl_2$	0.350	—	88
— $MgCl_2$	0.400	—	100
— PCMB	0.410	—	102
— Iodoacetic acid	0.420	—	105
DTE	—	0.110	110

* Pepstatin was added in concentration 3 μM.

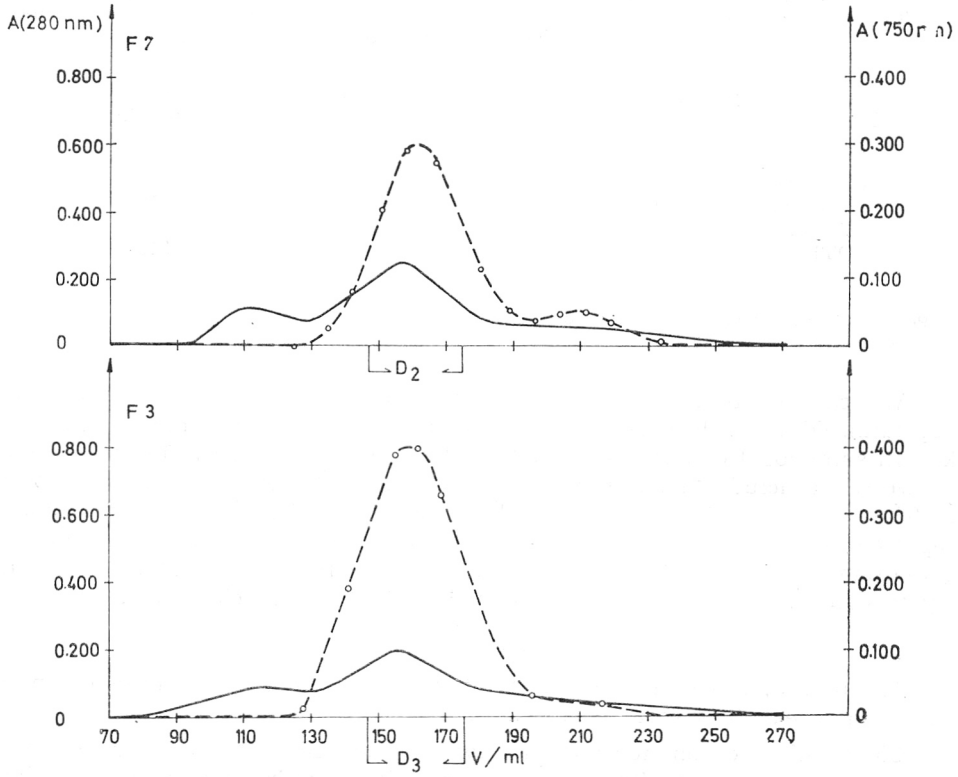

Fig. 3. Chromatography of fractions F2 and F3 on sephadex G-100. Experimental conditions were the same as described in Fig. 2.

indications that in calf lymph nodes, similar to other tissues[21,22], cathepsin D exists in more than one enzyme form. Three acid proteinases which differ in ionic properties were eluted from CM- cellulose. Having some enzyme characteristics similar to those of cathepsin D, they probably represent three multiple forms of the cathepsin D. They have been purified to different extents.

Gel filtration of fractions F1-a and F1-b (Fig. 2) showed that in addition to cathepsin D also another acid proteinase was present having a molecular weight of approximately 14 000. The acid proteolytic activity associated with the low molecular weight proteins was slightly detectable also by gel chromatography of fraction F2 but did not appear in fraction F3 (Fig. 3). Active fractions under the second proteolytic peak were pooled and concentrated by ultrafiltration using a UM 2 membrane. The specific acid proteolytic activity was determined in this enzyme preparation as well as the effect of pepstatin and DTE on the activity. The results are shown on Table IV.

TABLE IV.

Influence of Pepstatin and DTE on Proteolytic Activity of Low Molecular Weight Protease

0.2 ml of enzyme solution, containing 0.98 mg protein/ml, having specific activity 0.25 A. U./mg protein (without added activator), were preincubated with 0.2 Me of effector solution in acetate buffer, ph = 3.5, for 12 min at 37 °C. 2 ml 2% haemoglobin solution was added and incubated for 3 h at 37 °C.

Enzyme preparation: low molecular weight protease	Activity A(750 nm)	Activity A(280 nm)
Added solution:		
Acetate buffer, pH = 3.5	0.255	0.110
Pepstatin*	0.250	—
DTE**	—	0.310

* Pepstatin was added in concentration 3 μM.
** DTE was added in concentration 5 mM.

We can see that no inhibition of proteolytic activity was achieved by pepstatin. DTE which is used to activate thiol groups in the active centers of known sulphydryl cathepsins like B1[15,23], B2[24] and C[16] caused 300% activation. So we can conclude that this protease is entirely different from the cathepsin D. The occurence of the new proteolytic activity is therefore not an artefact representing the action of a fragment dissociated from cathepsin D during gel chromatography on Sephadex G-100. In our further investigations we shall try to determine the nature and specificity of this new acid protease.

DISCUSSION

The investigations on the content of the acid proteinases from calf lymph nodes have lead to several conclusions.

CM-cellulose chromatography followed by gel filtration of tissue extract has shown that no proteolytic activity is associated with proteins of molecular weight higher than 50 000 to 60 000. This is the indication that high molecular

weight cathepsin E is not present in detectable amounts in the tissue. This is also in agreement with the findings of Štefanovič *et al.*[25] who established that the cathepsin E is located mainly in macrophages and polymorphonuclear cells and only in trace amounts in lymphocytes. On the other hand, lymphocytes contain great amounts of cathepsin D. Because lymphocytes of different functional states are the main cell population of lymph nodes[6,26], our finding that cathepsin D is the main acid proteinase present in calf lymph nodes, is not surprising. This could have also been expected on the basis of the work of Bowers *et al.*[7]. Three enzyme preparations of cathepsin D have been obtained having the same molecular weight about 39 000, the same pH optimum for haemoglobin hydrolysis and the same relative specificities for protein substrates. We have observed 90% inhibition by pepstatin and a slight activation by thiol reagents in the enzyme preparation D_1. But if we had purified the preparation of cathepsin D, pepstatin should completely inhibit its activity, at the applied[27,28] concentration whereas other thiol reagents should have no appreciable effect. Reichelt *et al.*[29] reported that their pure preparation of cathepsin D was even partially (90%) inhibited by 10 mM DTE. In contrast to this, some other authors had reported[30] that the activity in crude cathepsin D preparations was activated by thiol reagents. Barrett[16] attributed this activation to the possible contamination with cathepsins B1, B2, C or some other sulphydryl carboxypeptidase or endopeptidase. So our cathepsin preparation D_1 in which the effect of activators and inhibitors was studied, could have been contaminated with other cathepsins. Concerted action of these cathepsins could also account for more extensive hydrolysis of some protein substrates other than haemoglobin if the relative specificities of all three enzyme preparations D_1, D_2 and D_3 are compared (see Table I.).

Taylor and Tappel[31] have tried to identify and to separate several lysosomal carboxypeptidases from rat liver and supposed that the rate of protein hydrolysis in lysosomes was increased by their presence and that they acted in concert with endopeptidases. But there is not much known about the way of the concerted action of different intracellular endopeptidases and exopeptidases because the specificities of action of these enzymes have not been sufficiently investigated yet. The cathepsins B1 and B2 could have influenced the acid proteolysis of the cathepsin D_1 preparation. Cathepsin B1 could act as an exopeptidase or an endopeptidase and De Lummen and Tappel[24] suggested that also cathepsin B2 should be included as an endopeptidase along with cathepsins B1, D and E. pH optima of cathepsins B_1 and B_2 for hydrolyses of synthetic peptides is at pH = 5.0 to 6.0[15]. Protein substrates like haemoglobin and azo-haemoglobin were degraded by cathepsin B1 optimally at pH = 6.0, as confirmed by Barrett[23], while Swanson *et al.*[32] reported that haemoglobin and myoglobin were degraded optimally at pH = 5.5. Etherington[33] found out that collagenolytic action of cathepsin B1 was maximal at pH = 4.0 to 5.0 and Burleigh *et al.*[24] reported that the highest activity against collagen in solution, due to cathepsin B1, was at pH = 4.5 to 5.0. So without regard to the nature of action only very limited contribution of both cathepsins B1 and B2, to the acid proteolyses of various protein substrates by unpurified preparation of cathepsin D, could be expected.

On the other hand, we have found out that another endopeptidase with low pH optimum at pH = 3.0 is present in calf lymph nodes. Having the molecular weight approximately 14 000, it was separated on Sephadex G-100

from other cathepsins. It was not active against Bz-Arg-NH_2. This protease therefore does not belong to the cathepsins of the B type, although it is probably also a sulphydryl dependent protease. The nature of acid hydrolysis of haemoglobin differs, however, from that of cathepsin D, because pepstatin was not effective in its inhibition.

The experiments on isolation and characterisation of this new type of protease are in progress.

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Kisle proteinaze telečjih bezgavk

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Kisle proteinaze telečjih bezgavk smo izolirali s pomočjo kisle ekstrakcije, obarjanja z amonsulfatom in acetonom ter nadaljnjo ionsko izmenjalno kromatografijo na CM-celulozi in gelsko kromatografijo na Sephadexu G-100. Telečje bezgavke vsebujejo katepsin D. S pomočjo gelske filtracije na Sephadexu G-100 smo določili molekulsko maso 39 000. Izolirali smo tudi katepsina B1 in B2 in določili molekulski masi 22 000 za katepsin B1 in 51 000 za katepsin B2. Telečje bezgavke pa vsebujejlo še neko drugo proteinazo, ki hidrolizira hemoglobin optimalno pri pH = 3.0. S pomočjo gelske filtracije smo določili njeno molekulsko težo 14 000. Aktivnost te proteinaze ni inhibirana s pepstatinom, če smo ga dodali v koncentraciji 0,25 μM, kar je inhibiralo 90% aktivnosti katepsina D.

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