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Purification and some Properties of Proteinases from Calf Thymus

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Calf thymus was used for the investigation of intracellular proteinases. The tissue was homogenized, centrifuged at a low speed and applied on DEAE-cellulose. Active fractions were collected and their proteolytic activity toward different protein substrates was tested. It was found that several proteinases are present in thymus; cathepsin D being the most abundant acid proteinase. The activitiy at neutral *p*H was ascribed to fibrinogen degrading proteinases.

INTRODUCTION

Several tissues have already been studied for cathepsins in our laboratory[1–3]. In this paper experiments on thymus are described. Thymus was chosen for investigation of cathepsins because its protein turnover is very intensive and it can therefore be expected that various proteolytic enzymes are present. Thymocytes are the main cell population in young thymus and so this organ represents very pure lymphatic tissue.

Only a few experiments have been done on the isolation of thymus proteinases. Furlan *et al.*[4] purified and described some properties of neutral protease from calf thymus nuclei. Some further studies of this enzyme have been done by Bartley and Chalkley[5]. There are also some reports on the proteolytic activity of crude thymus homogenates degrading nucleohistones, protamines and other substrates[6–8]. We decided to study the proteolytic activities in thymus toward various protein substrates.

EXPERIMENTAL

Calf thymus was obtained from the slaughter house. Immediately after killing the animal, the thymus was put on ice and brought to the laboratory. It was freed of fat and connective tissue and a 50% homogenate was prepared in 0.2% NaCl solution using a Waring blendor. The homogenate was six times repeatedly frozen in liquid nitrogen and thawed in order to achieve complete destruction of subcellular particles and the release of proteinases. The homogenate was centrifuged 30 min. at 1,500 rpm. (45,000 gmin.) in a Sorvall RC2 centrifuge. The crude extract was dialyzed against 0.05 *M* potassium phosphate buffer *p*H 8.0. The dialyzed extract was applied on DEAE-cellulose (Serva, Germany), equilibrated with the same buffer. Elution started with 0.05 *M* phosphate buffer *p*H 8.0. When the first peak was eluted a stepwise elution with the same buffer containing 0.1 *M* and 1.0 *M* NaCl was performed. The flow rate of the buffer was 30 ml./h. and fractions of 4 ml. were collected. Proteins in eluted fractions were determined by measuring the optical density at 280 mμ and proteolytic activity was measured according to the method of Anson[9].

The following proteins were used as substrates: native bovine hemoglobin (prepared in the laboratory) as a 2% solution of *p*H 3.5 and 7.5; bovine serum albumin

(Serva, Germany) as a 2% solution of *p*H 3.0 and 6.5; bovine *alpha*-globulin (Serva, Germany) as a 2% solution of *p*H 3.5 and 6.5; bovine *gamma*-globulin (Sigma, USA) as a 2% solution of *p*H 3.0 and 7.0; fibrin bovine (Sigma ,USA) as a 2% homogenized suspension of *p*H 3.5, 5.0 and 7.0; bovine fibrinogen (Serva, Germany); bovine fibrinogen plasmin free (Pentex, USA, Miles Lab., USA).

Acetate buffers were used in the acid *p*H range (3.0—6.0) and phosphate buffers in the neutral and alkaline *p*H range. Times of hydrolysis ranged from 30 min. to 24 h. Eluted fractions containing proteolytic activity were pooled and concentrated on LSG 60 ultrafilter (Membranfilter Gesellschaft, Germany) under nitrogen pressure. The obtained ultrafiltrates were chromatographed on Sephadex G-50 and the molecular weights of proteins were determined with chymotrypsinogen, cytochrome C, DNP-L-α-alanine and Dextran Blue (Serva, Germany) as standards. Solutions that did not pass the membrane were termed supernatants. These were further purified on Sephadex G-100 using 0.1 *M* NaCl as the eluent.

RESULTS AND DISCUSSION

The elution pattern of neutral thymus extract from DEAE-cellulose is shown on Fig. 1. It can be seen that two pronounced protein peaks were eluted whereas the measurement of proteolytic activity showed three active peaks which were concentrated on LSG 60 ultrafilter. By this procedure high and low molecular weight proteins were obtained in supernatants and ultrafiltrates, respectively.

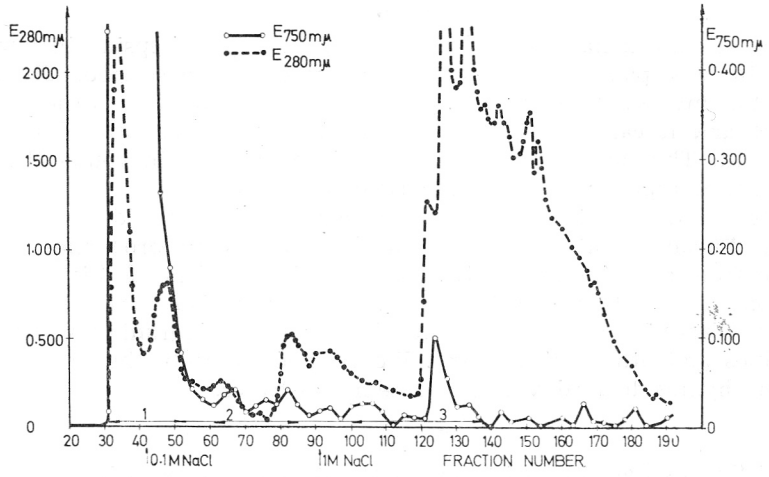

Fig. 1. Column chromatography of thymus extract on DEAE-cellulose. Dimensions of the column were 40 × 3 cm., and 0.05 *M* KH_2PO_4, *p*H 8.0 was used as eluent. Flow rate was 30 ml./h.
○——○ proteolytic activity toward hemoglobin *p*H 3.5 (in acetate buffer)
○– – –○ protein content.

Hydrolysis of various protein substrates can be used as a method for differentiation of proteinases[10]. Table I shows the hydrolysis of protein substrates by active supernatants. It can be seen that the highest hydrolysis rates toward tested protein substrates were obtained with supernatant 1; the *p*H optimum curve (Fig. 2) for hemoglobin hydrolysis by supernatant 1 shows two distinct maxima, one at *p*H 3.5 and one very low at *p*H 7.5. The proteolytic activity of the other two supernatants at the acid *p*H was also much higher than at the neutral *p*H. Hemoglobin was the most susceptible protein substrate for hydrolysis at the acid *p*H. The hydrolysis of other proteins

TABLE I

Proteolytic Activities of Supernatants 1, 2 and 3 toward Various Protein Substantes

Substrate	Supernatant 1		Supernatant 2		Supernatant 3	
	E_{750} mμ		E_{750} mμ		E_{750} mμ	
	acid *p*H	neutral *p*H	acid *p*H	neutral *p*H	acid *p*H	neutral *p*H
Hemoglobin *p*H 3.5	1.270		0.175		0.135	
Hemoglobin *p*H 7.5		0.040		0.020		0.025
Bov. ser. albumin *p*H 3.0	0.280		0.035		0.030	
Bov. ser. albumin *p*H 6.5				0.022		
Gamma-globulin *p*H 3.0	0.162		0.015			
Gamma-globulin *p*H 7.0		0.055		0.035		0.050
Alpha-globulin *p*H 3.5	0.330		0.055		0.050	
Alpha-globulin *p*H 6.5				0.017		
Fibrin *p*H 3.5			0.035			
Fibrin *p*H 5.0	0.040		0.065			
Fibrin *p*H 7.0				0.043		

Time of incubation in supernatants 1, 2 and 3 was 30 min., 120 min. and 120 min., respectively. Nitrogen concentration in supernatants 1, 2 and 3 was 2,250 γ/ml., 100 γ/ml. and 554 γ/ml., respectively. Sample volumes were 0.4 ml.

varied as shown in Table I. The relative hydrolysis rate of various substrates (Table II) is very much the same as the hydrolysis rate of these substrates by cathepsin D[3].

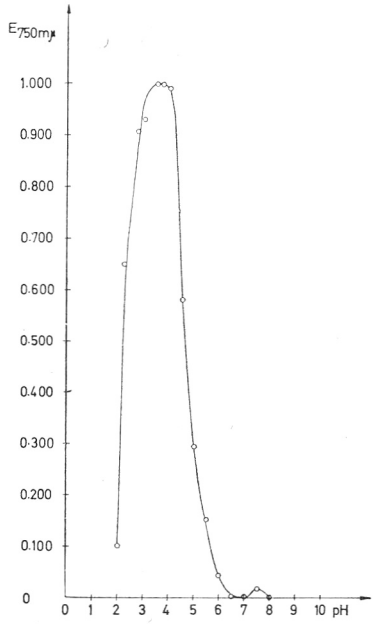

Fig. 2 *p*H optimum curve of supernatant 1 on hemoglobin substrate. 2% solutions in acetate buffer (2.0—6.0 *p*H) and phosphate buffer (6.0—8.0 *p*H) were used.

TABLE II

Relative Activities of Supernatants 1, 2 and 3 toward Different Protein Substrates in Acid pH Range Compared with Cathepsin D Activity

Substrate	Supernatant 1	Supernatant 2	Supernatant 3	Cathepsin D average values**
Hemoglobin *pH* 3.5*	100%	100%	100%	100%
Bov. ser. albumin *pH* 3.0	22.5	20	23	2—13
Gamma-globulin *pH* 3.0	13	8	—	2— 5
Alpha-globulin *pH* 3.0	26	30	41	13—20
Fibrin *pH* 5.0	3	—	—	5—10

* Hemoglobin hydrolysis is arbitrarly taken as 100%.
** See reference 3.

From these findings we can conclude that supernatants contain acid as well as neutral proteinases. In supernatant 1 cathepsin D is the most abundant acid proteinase. The differences in the hydrolysis rate of *alpha*-globulin in comparison to the average values for cathepsin D in supernatants 2 and 3 could speak either for the presence of different acid proteinases or that cathepsin D, present also in these supernatant fractions, is contaminated with minor amounts of some other proteinases.

Thymus crude extract was investigated by Furlan *et al.* using fractionation on CM-cellulose[11]; their fractions also contained much higher proteolytic activity toward hemoglobin than toward other substrates. On the basis of differences in the hydrolysis rate these authors concluded that thymus contained a complex of intracellular proteinases.

Supernatant 1 was further purified on Sephadex G-100 and the elution diagram is shown in Fig. 3. Proteolytically active fractions (No. 19—29) were

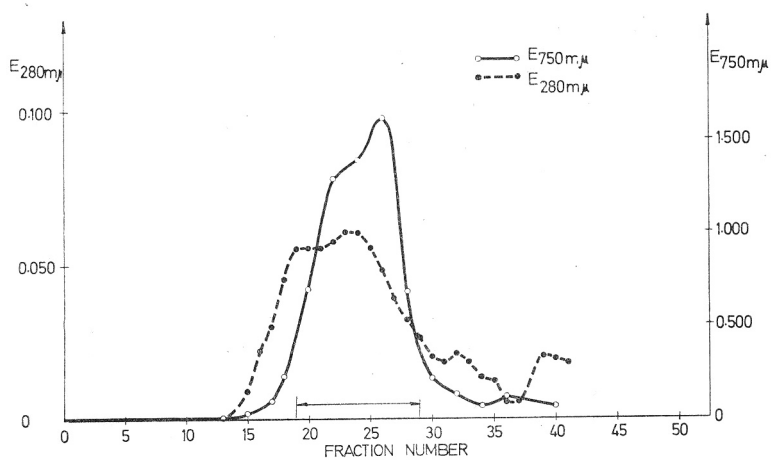

Fig. 3. Gel filtration of supernatant 1 on Sephadex G-100. Dimensions of the column were 35 × 1.5 cm., and 0.1 *M* NaCl was used as eluent. Flow rate was 10 ml./h.
O——O proteolytic activity toward hemoglobin *pH* 3.5 (in acetate buffer)
O — — — O protein content.

pooled and tested for the hydrolysis of protein substrates. The results given in Table III show that the values were approximately the same as in the unpurified supernatant 1. Proteolytic activity did not change within 3 weeks after isolation (the enzyme preparation was stored at —25°) indicating thus the stability of acid proteinase.

TABLE III

Proteolytic Activities of Supernatant 1 After Purification on Sephadex G-100

Substrate	$E_{750\ m\mu}$	Relative activities
Hemoglobin *p*H 3.5	0.580	100%
Bov. ser. albumin *p*H 3.0	0.095	14.5
Gamma-globulin *p*H 3.0	0.060	10.5
Alpha-globulin *p*H 3.5	0.150	24.5
Fibrin *p*H 3.0	0.065	11.0

Time of incubation was 20 min.; Nitrogen concentration was 189 γ/ml; Sample volume was 0.4 ml.

The activity at neutral *p*H was much lower toward all tested substrates compared with the activities at the acid *p*H, particulary toward hemoglobin (Table I). Higher than hemoglobin hydrolysis was *gamma*-globulin and fibrin hydrolysis (measured only in supernatant 2). The reason for this low neutral activity is either low content of neutral proteases in thymus or that they were inactivated during the isolation procedure. The presence of neutral proteinase bound to the nucleohistone-DNA complex was already found by the authors who purified nucleohistones from calf thymus. This enzyme hydrolyzed histones more rapidly than hemoglobin and serum albumin; below *p*H 7.0 it was almost inactive[5,12].

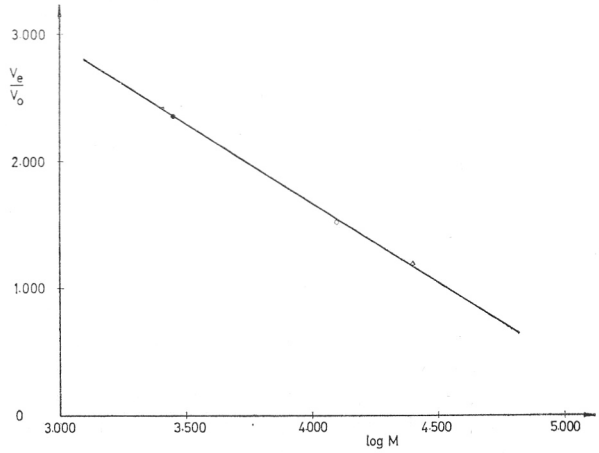

Fig. 4. Calibration curve for molecular weight determination on Sephadex G-50. Dimensions of the column were 48 × 1 cm., and 0.1 *M* NaCl was used as eluent. Flow rate was 5 ml./h.
△ chymotrypsinogen (M = 25 000)
○ cytochrome C (M = 12 400)
□ DNP-L-α-Alanine (M = 2552)
● Main protein peak of ultrafiltrate 2

Ultrafiltrates with rather low content of protein material (1% of the starting amount in crude extract) were further separated on Sephadex G-50. It was found that the molecular weight of these proteins lay between 2,000 and 5,000 (Fig. 4). No proteins with a high molecular weight were found which accounts for the effectiveness of separation by ultrafiltration. In ultrafiltrates the proteolytic activity toward protein substrates at acid and neutral *p*H was measured. Although longer times of incubation were used (up to 24 h.), no conclusion can be made about the presence of hemoglobin and serum albumin digesting proteinases because the obtained O. D. values were within experimental error. Significant hydrolysis of fibrinogen, however, was observed at *p*H 7.0. Time dependence of fibrinogen hydrolysis with ultrafiltrate 1 was followed. The high blank values could be explained by the activation of plasminogen present in the fibrinogen substrate. Therefore, in further experiments plasmin free fibrinogen was digested by ultrafiltrate 2 and a high hydrolysis was observed (Fig. 5).

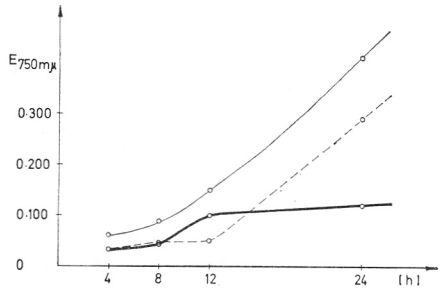

Fig. 5. Proteolytic activity of ultrafiltrate 2 toward plasmin free fibrinogen. 2 ml 2% solution of substrate in phosphate buffer (*p*H 7.0) was used with 0.4 ml. enzyme preparation (112 γ N/ml). The reaction was stopped with 4 ml. TCA and acid soluble split products were measured spectrophotometrically after the addition of Folin-Ciocalteau reagent.
O———O substrate hydrolysis
O———O enzyme and substrate hydrolysis
O———O hydrolysis due to enzyme action.

The above results indicate that in the ultrafiltrate an enzyme is present which probably causes the direct hydrolysis of fibrinogen. Similar enzymes, named cathepsins F, were found in porcine leucocytes[14].

Although still inhomogenous, our enzyme preparations show characteristic relative enzyme activities toward various protein substrates. Differences in these activities were used as a criterion for the classification of tissue proteinases. Among acid proteinases of high molecular weight cahtepsin D is the most abundant enzyme. We believe also that neutral proteinases are present in thymus having lower activity toward hemoglobin than toward some other substrates. The activity of a low molecular weight enzyme toward fibrinogen was ascribed to F type proteinase. Thus, from the obtained results we can conclude that several proteinases of high and low molecular weight are present in thymus.

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IZVLEČEK

Čiščenje in nekatere lastnosti proteinaz telečjega timusa

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Za preiskave intracelularnih proteinaz smo uporabili telečji timus. Tkivo smo homogenizirali, centrifugirali pri nizkih obratih in nanesli na DEAE celulozo. V združenih aktivnih frakcijah smo merili proteolizno aktivnost napram različnim proteinskim substratom. Ugotovili smo, da so v timusu prisotne različne proteinaze.

Med kislima proteinazami je najizraziteje zastopan katepsin D. Nevtralno aktivnost pripisujemo proteinazam F tipa.

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