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Some Properties of Thymus Cathepsin D

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Cathepsin D has been purified from calf thymus using ammonium sulphate precipitation and gel chromatography on Sephadex G-100. Preparative electrophoresis in polyacrylamide gel, used as the final step in the purification procedure, yielded four active forms of cathepsin D that dissociated further into several polypeptide bands in the presence of sodium dodecyl sulphate. All four forms were stable over a range of pH from 4—11. They were completely inhibited by pepstatin whereas other metal ions had no appreciable effect upon their activity.

INTRODUCTION

In our previous work[1] we found that cathepsin D was the most abundant acid proteinase present in calf thymus, using the hydrolysis of various protein substrates as the method for identification[2].

The acidic haemoglobin-splitting proteases that probably belong to the group of catepsin D[3] and E[4] have been previously demonstrated in calf thymus[5,6]. No attempt however, has been made to isolate thymus cathepsin D in a pure form. In the present work we have purified this enzyme and studied some of its biochemical properties.

EXPERIMENTAL

Calf thymus was obtained in the slaughter house and was brought to the labotory immediately after killing the animal. Fat and connective tissue were removed and thymus was frozen overnight at — 25 °C. On the following day 30% homogenate was prepared in distilled water using a Waring blendor. The homogenate was acidified to pH = 3.8 with dilute hydrochloric acid and centrifuged for 20 min at 5500 rpm (100 000 $g \cdot$ min). The proteins in the acidic supernatant were first concentrated by adding crystalline ammonium sulphate to 70% saturation, the resultant precipitate dissolved in distilled water and dialyzed. Afterwards, proteins were fractionated by precipitation with ammonium sulphate. Fraction that precipitated between 30 and 60% saturated ammonium sulphate contained the bulk of cathepsin D activity. This precipitate was dissolved in 0.1 M NaCl and dialyzed against the same solution. The enzyme sample was then applied to a Sephadex G-100 (Pharmacia, Sweden) column equilibrated with 0.1 M NaCl which was used also as eluent. The proteolytically active fractions were concentrated on UM-10 ultrafilter (Amicon, USA) and rechromatographed on a smaller column of Sephadex G-100. The last step of purification was achieved by preparative polyacrylamide gel electrophoresis in a Canalco apparatus according to the manufacturer's procedure. 2 ml of sample containing approximately 300 A. U. of cathepsin activity were applied to a preparate column of 10% acrylamide, 70 mm high. Tris glycine buffer pH = 9.5 was used for the elution. The flow rate was 60 ml/h. In the eluted fractions the presence of protein was determined by the measurement of optical density at 280

nm and proteolytic activity was measured according to the method of Anson[7] with 2% haemoglobin in acetate buffer as the substrate. The entire purification procedure was done in the cold room at 3—5 °C.

The purity of fractions through all purification procedures was followed using analytical electrophoresis (Canalco, USA) in 7.5% acrylamide gel at pH = 9.5. Gels were stained with amido black.

Polyacrylamide electrophoresis in the presence of sodium dodecyl sulphate (SDS) was run following the method of Weber and Osborn[8]. The gels were formed from solutions containing 10% acrylamide. The protein samples were dissolved in gel buffer (phosphate buffer pH = 7.4) containing 1% SDS and 0.5% 2-mercapto-ethanol. Protein standards for molecular weight determination were products of Serva, Germany. Approximate molecular weight was determined also by gel filtration method according to Whitaker[9] using the same protein standards for calibration curve.

Isoelectric focussing was performed on LKB column 8 100 at 2 °C. Ampholines for pH range 5—7 and 6—8 were used for separation which lasted 3 days at 600 V.

The effect of various substances on the enzyme was determined by mixing equal volumes of enzyme solution with 10^{-2} M solution of the substance; afterwards proteolytic activity was measured by the method of Anson, only optical density of the filtrate was measured at 280 nm without addition of Folin Ciocalteau reagent.

The effect of urea was determined by the addition of various concentrations of urea-water solution to the enzyme sample, followed by incubation for 60 min at 37 °C. Afterwards proteolytic activity toward haemoglobin was measured.

The pH stability was checked by mixing equal volumes of enzyme and buffer solution of appropriate pH (Johnson-Lindsay buffer) and incubated for 2 h at 37 °C. Proteolytic activity after incubation was measured according to the method of Anson.

Chemicals were analytical reagents purchased from BDH, England or from Serva, Germany. Haemoglobin was prepared in this laboratory according to the method of Anson. Pepstatin, a generous gift from Prof. H. Umezawa, was used without further purification.

RESULTS

Data on purification of cathepsin D from calf thymus are summarized in Table I. Using this purification procedure the yield of catheptic activity was approximately 3%. The purification factor of the major form (I) was 700.

TABLE I

Purification of Cathepsin D.

Step	Volume ml	Total units A. U.*	Specific activity A. U./mg N
Homogenate	14 000	16 300	0.21
Acid extract	10 000	14 000	0.70
Ammonium sulphate (0—70%)	518	7 000	9.40
Ammonium sulphate (0—30%)	18	35	1.60
Ammonium sulphate (30—70%)	70	4 900	73
Sephadex G-100	16	2 700	82
Sephadex G-100, rechr.	5	1 300	93
Prep. disc-el. fr. I.	17	270	140
fr. II.	16	111	74
fr. III.	13	80	60
fr. IV.	15	91	53

* Anson units × 10^4

Fig. 1 shows the elution pattern of sample chromatographed and rechromatographed on Sephadex G-100. It is evident that the majority of inactive proteins were removed by chromatography. Analytical polyacrylamide gel electrophoresis revealed however, the presence of several protein bands. Subsequent preparative electrophoresis also showed, besides inactive proteins, that at least four proteolytically active peaks *were* present in the sample (Fig. 2). The active fractions considered as multiple forms of cathepsin D were designated as I, II, III, and IV. Analytical electrophodesis of these forms showed single though rather unsharp bands (Fig. 3). In order to determine the molecular weight of these forms electrophoresis in the presence of SDS was performed.

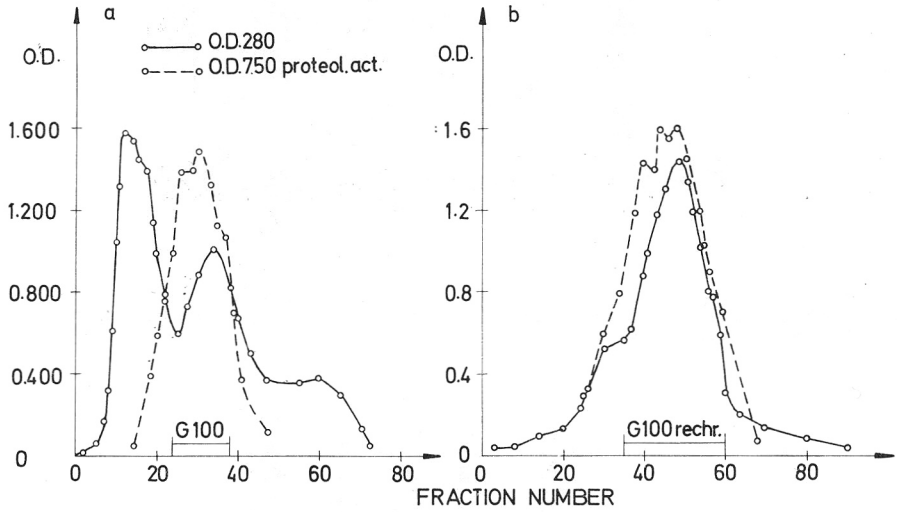

Fig. 1. Chromatography of thumus cathepsin D on Sephadex G-100:
a) approximately 2500 A. U. of enzyme was applied to a column, 70 × 6.5 cm, of Sephadex G-100 equilibrated with 0.1 M NaCl. Flow rate was 144 ml/h, each fraction contained 14.4 ml;
b) under the same conditions approximately 2700 A. U. of enzyme was rechromatographed.

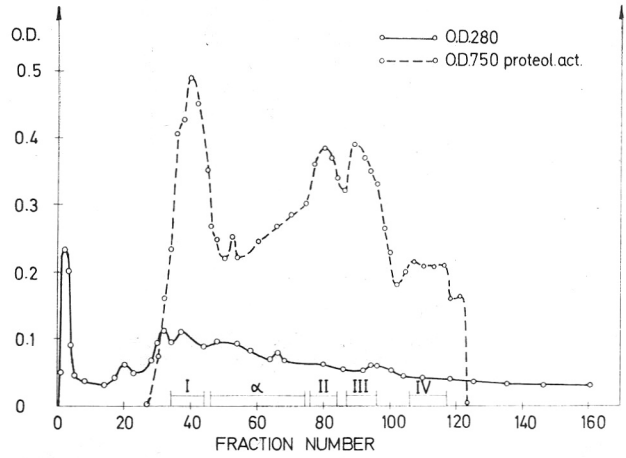

Fig. 2. Preparative polyacrylamide gel electrophoresis of cathepsin D.

Fig. 4 shows that each form dissociated into several bands having molecular weights from 58 000 to 14 000. Only minor changes were observed in SDS electrophoretic pattern when the protein was not reduced with 2-mercaptoethanol. The molecular weight determined by gel chromatography was

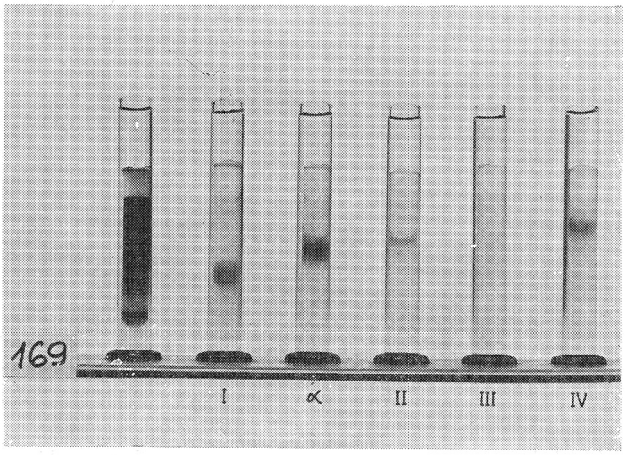

Fig. 3. Polyacrylamide gel electrophoresis of the enzyme sample after Sephadex rechromatography (left gel) and of isolated multiple forms of cathepsin D (I—IV). Gel marked α shows the pooled fractions between forms I and II containing some proteolytic activity.

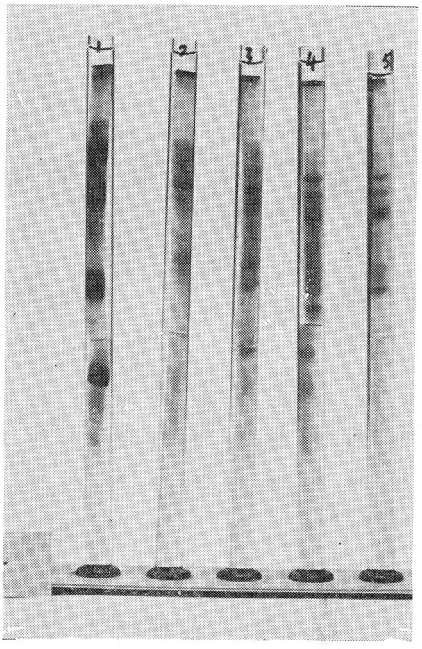

Fig. 4. Electrophoresis in sodium dodecyl sulphate-polyacrylamide gels. The standard protein mixture (left gel) contained bovine serum albumin (67 000), ovalbumin (45 000), chymotrypsinogen A (25 000) and lysozyme (14 300). Gels 2—5 show in order the pattern of forms I—IV.

39 500 ± 1500. The isoelectric points determined in the sample after Sephadex rechromatography were 7.1, 6.5, 5.6 and one below 5.

Some biochemical properties of the isolated forms were examined. The pH optimum toward haemoglobin was 3.5 for all four forms. Within the wide range of pH from 4—11 enzyme activity was practically unaffected (Fig. 5). The effect of urea was evident at concentrations higher than 2 M. At 3.5 M urea 50% of enzyme activity was still retained (Fig. 6).

Several metal ions were tested and it has been found that $CaCl_2$, $FeCl_2$, $MgCl_2$, $ZnCl_2$ as well as EDTA, cysteine and iodoacetamide at the concentration 5×10^3 had practically no effect upon enzyme activity. At the same concentration $HgCl_2$, $FeCl_3$, $AgNO_3$, $CuCl_2$, $Pb(NO_3)_2$ caused approximately 20—30% inhibition of cathepsin D activity.

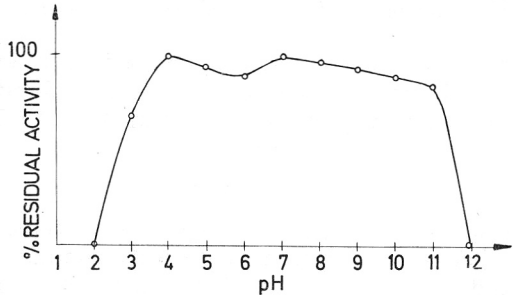

Fig. 5. Stability of cathepsin D (form I) at various pH-s at 37 °C.

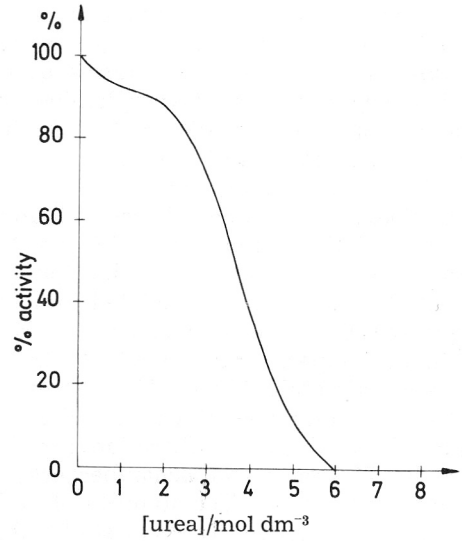

Fig. 6. Effect of urea on the activity of cathepsin D (form I).

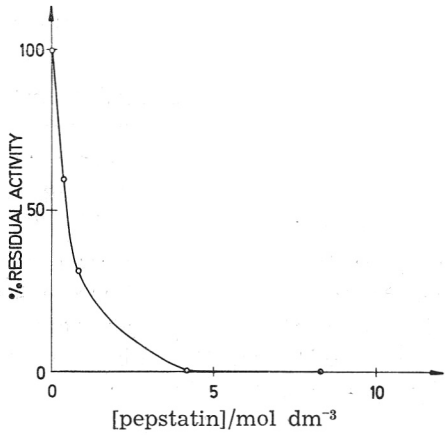

Fig. 7. Pepstatin inhibition of hemoglobin digestion by cathepsin D (form III). The concentration of cathepsin was approximately 4×10^{-8} M assuming molecular weight of 40 000.

The dependence of enzyme activity upon various concentrations of pepstatin is shown in Fig. 7. One can see that a complete inhibition with pepstatin was observed at the concentrations higher than 4×10^{-7} M. All four forms were completely inhibited by pepstatin.

DISCUSSION

Thymus is known as an organ that contains a considerable amount of cathepsin D[10]. It consists of almost homogeneous population of lymphocytes. Fräki *et al.*[11] found that phosphate buffer pH = 8 was suitable for the extraction of acidic proteases. Lebez and Turk[12] showed however, that acidic pH was more suitable for the extraction of cathepsins from bovine spleen than neutral pH, so we decided to use acid extraction.

From the results of the purification it can by seen that ammonium sulphate precipitation and gel chromatography yielded an enzyme sample that contained several protein bands. The use of preparative disc electrophoresis as the final purification step enabled us to isolate four forms of cathepsin D. Each of these forms appeared homogeneous on polyacrylamide gel electrophoresis but dissociated in the presence of SDS. The existence of multiple forms of cathepsin D has been observed by many other authors. Press *et al.*[3] described more than 10 forms of cathepsin D in bovine liver, Barrett[13] described 3 forms in chicken and human liver, and Woessner and Schamberger[14] found 12 in bovine uterus. The dissociation of forms in the presence of SDS and 2-mercaptoethanol was observed also by Sapolsky and Woessner[15] who found some forms having molecular weight of 42 000 that partly dissociated into two polypeptide bands with molecular weight 25 000—28 000 and 13 000—14 000. Quite similar bands were obtained also in our experiments except for the protein band having molecular weight of approximately 58 000. The presence of a proteolytically active protein of 28 000 molecular weight was found also by Ferguson *et al.*[16]. The molecular weight obtained by gel filtration for thymus cathepsin D is the same as observed by Woessner and Schamberger[14], Ferguson *et al.*[16] and close to the value found by Barrett[13]. The stability of cathepsin D over a wide range of pH has been noted previously by Press *et al.*[3]. Wojtowicz and Odense[17] reported an apparent activation of cathepsin D up to an urea concentration

of about 2 M but Sapolsky and Woessner[16] found sharper decrease of catheptic activity with increasing urea concentration than found in the present study. Similar effects of metal ions on the activity of cathepsin D were observed also by Woessner and Schamberger[14] and by Misaka and Tappel[18].

Inhibition of cathepsin D with pepstatin was first reported by Umezawa *et al.*[19] and later by Barrett[20] who noted this substance to be a valuable group-specific inhibitor for acid proteinases. Woessner[21] also found a complete inhibition of cathepsin D from bovine uterus and rabbit ear cartillage at 10^{-7} M concentration of pepstatin. In our experiments a 100% inhibition of haemoglobin digestion by cathepsin D was also noted.

Our results show that the isolated multiple forms of cathepsin D have probably undergone some chain scission that produces the peptide chains of various molecular weight which are evident on electrophoresis in the presence of SDS. Proteins having molecular weight higher than 40 000 are probably contaminants of multiple forms.

Further experiments will show whether this possibly limited proteolysis of the cathepsin molecule occurs during the preparation or whether multiple forms exist already *in vivo*.

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IZVLEČEK

Nekatere lastnosti katepsina D iz timusa

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Iz telečjega timusa smo očistili katepsin D z obarjanjem z amonsulfatom in z gelsko kromatografijo na Sephadexu G-100. S preparativno elektroforezo v poliakrilamidnem gelu kot končno stopnjo v procesu izolacije smo dobili 4 aktivne oblike katepsina D, ki so v prisotnosti natrijevega dodecil sulfata disociirale v več polipeptidnih lis. Vse štiri oblike so bile stabilne v območju pH 4—11. Pepstatin jih je popolnoma inhibiral, medtem ko kovinski ioni niso imeli bistvenega učinka na njihovo aktivnost.

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