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ISOLATION OF RABBIT TESTICULAR CATHEPSIN D AND ITS ROLE IN THE ACTIVATION OF PROACROSIN

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Cathepsin D was purified 900-fold with 30% recovery from rabbit testes using pepstatin bound Sepharose affinity chromatography. The enzyme is homogeneous as observed by acrylamide gel electrophoresis. The heat stable enzyme exhibits an apparent molecular weight of 42,000 with identical subunits of 20,000. Purified cathepsin D catalyses the conversion of proacrosin to acrosin.

Cathepsin D is a lysosomal acid proteinase which participates in the catabolism of proteins, peptides and proteoglycans in various tissues. No other proteolytic enzyme except acrosin, a serine proteinase, has been studied extensively in testes and spermatozoa of several mammalian species (1). There have been some sporadic reports on the occurrence of acid proteases in boar and mouse spermatozoa (2,3) and on Cathepsin D of rat testes (4). However, the acid proteinase from testes or sperm acrosomes has not been purified and characterized yet.

The present communication describes a rapid affinity chromatographic method for the purification of Cathepsin D from rabbit testes and presents evidence to show that Cathepsin D catalyses conversion of proacrosin to acrosin.

Materials and Methods

Concanavalin A (Con A)-Sepharose, AH-Sepharose 4B, Sephadex and molecular weight determination kit were obtained from Pharamacia Fine Chemicals (Piscataway, N.J.). Pepstatin, bovine hemoglobin type II, bovine serum albumin and benzoyl arginine ethyl ester (BAEE) were purchased from Sigma Chemical Company (St. Louis, Mo).

Frozen rabbit testes (Pel-Freeze Biologicals, Inc.) were thawed and tunica albuginea with its blood vessels removed. The tissue was scissor-minced and homogenized in 0.5 M KCl in a Waring Blender for 2 min. The homogenate was filtered through four layers of surgical gauze and the filtrate centrifuged at 25,000 g for 30 min. The supernatant was adjusted to pH 3.4 with 5 M HCl and the resultant precipitate removed by centrifugation at 17,000 g for 20

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min. The supernatant was concentrated by ultrafiltration and dialysed against 50 mM Tris-HCl buffer containing 0.5 M NaCl, pH 7.2. All operations were carried out at 4°C.

The dialysed extract was chromatographed on a Con-A Sepharose column and the enzyme eluted with 0.2 M methyl mannoside. Cathepsin D active fractions were pooled, dialysed against 0.1 M acetate buffer pH 3.8 containing 0.5 M NaCl, and applied to a pepstatin--Sepharose column (5). The column was washed with the above buffer and the enzyme eluted with 0.1 M $NaHCO_3$, pH 7.2 containing 0.5 M NaCl at a flow rate of 10 ml/h in 3 ml fractions. The active fractions were pooled, dialysed against 0.05 M acetate buffer pH 3.8 and concentrated by ultrafiltration. The purity of the enzyme was determined by the polyacrylamide disc gel electrophoresis (6). Unstained gels were sliced into 15-20 segments and the enzyme activity assayed. Estimation of molecular weight was performed by gel filtration on Sephadex G-100 and also by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (7).

Hemoglobin splitting activity of Cathepsin D was determined at pH 3.8 by the modified Anson's method (8). The proteolytic end products and protein were determined by Miller's method (9) using DL-tyrosine and crystalline bovine serum albumin as standards respectively. Low concentrations of protein in purified preparations were based on absorbance at 280 nm assuming 1 OD_{280}/cm equals 1 mg. The unit of enzyme activity was expressed as nmoles tyrosine liberated/min. Proacrosin was prepared from fresh rabbit testes as described by Huang-Yang and Meizel (10). The crude proacrosin was dialyzed against 1 mM HCl and freeze dried. Acrosin activity was determined spectrophotometrically by following the rate of hydrolysis of BAEE at 253 nm in presence of 0.1 M Tris-HCl buffer containing 0.05 M calcium chloride, pH 8.5. One unit of acrosin activity is defined as the quantity of enzyme required to hydrolyse 1 µmole of BAEE per min. Activation of the zymogen was carried out as described (10). The incubation of proacrosin was conducted with 0.1 M acetate buffer pH 3.8 in the presence or absence of Cathepsin D.

Results and Discussion

Since the specific activity of Cathepsin D in the acrosomal extract of rabbit sperm (11) was about the same as of the crude testicular extract, the enzyme appears to be of acrosomal origin.

The data in Table 1 reveal that rabbit testicular Cathepsin D was purified 900-fold with a 31% recovery in just three steps of purification. When the

Table 1. Purification of Cathepsin D from Rabbit Testes

Fraction	Total protein (mg)	Total activity (units)	Specific Activity (units/mg protein)	Purification (fold)	Recovery (%)
Crude extract (25,000 g supernate)	2200	62,890	29	1	100
Acidification	428	58,750	138	5	93
Con A-Sepharose Chromatography	6.56	21,800	3304	116	35
Pepstatin-Sepharose Affinity Chromatography	0.80	19,700	26,076	911	31

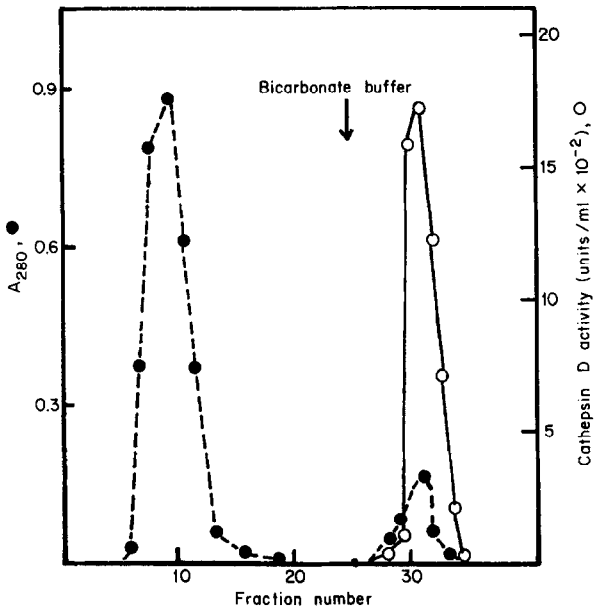

Fig. 1. Chromatography of Cathepsin D on a pepstatin sepharose column.

crude extract was autolysed overnight and then acidified, although the specific activity increased, the yield was poor. Alternatively, when the acid extract was directly chromatographed on pepstatin-Sepharose column, or chromatographed following 0-70% ammonium sulfate fractionation, a considerable improvement in the recovery was observed, but the preparation was not homogeneous. Con A-Sepharose affinity chromatography was found essential to obtain a high degree of purification (166-fold). Pepstatin, a powerful inhibitor of carboxy-proteinase, retards Cathepsin D when covalently coupled to AH-Sepharose 4B as seen from its elution pattern on affinity column (Fig. 1). The bound enzyme was eluted as a sharp peak by changing the pH from 3.8 to 7.2. The enzyme eluting from pepstatin-Sepharose affinity column revealed only one band on the polyacrylamide gel disc electrophoresis indicating that the preparation was homogeneous. When the segments of the unstained gels were examined for Cathepsin D activity, the region corresponding to the protein band exhibited peak of the enzyme activity (Fig. 2).

The apparent molecular weight of rabbit testicular Cathepsin D seemed to be 42,000 as evidenced by its elution profile on Sephadex G-100 column (Fig. 3a). However, the migration pattern of the enzyme following SDS-PAGE (Fig. 3b) in-

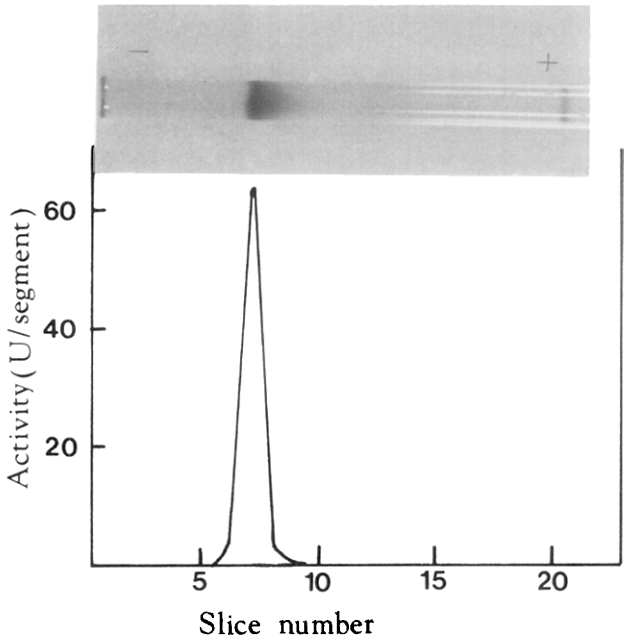

Fig. 2. Gel electrophoresis pattern of purified Cathepsin D. The lower portion shows the activity exhibited by the segments cut from the unstained gel.

dicated a molecular weight of 20,000. Since the enzyme emerges as a single band corresponding to a molecular weight of 20,000 the rabbit Cathepsin D appears to be composed of a dimer having a monomeric form of 20,000. This is in agreement with some of the reported observations on the occurrence of Cathepsin

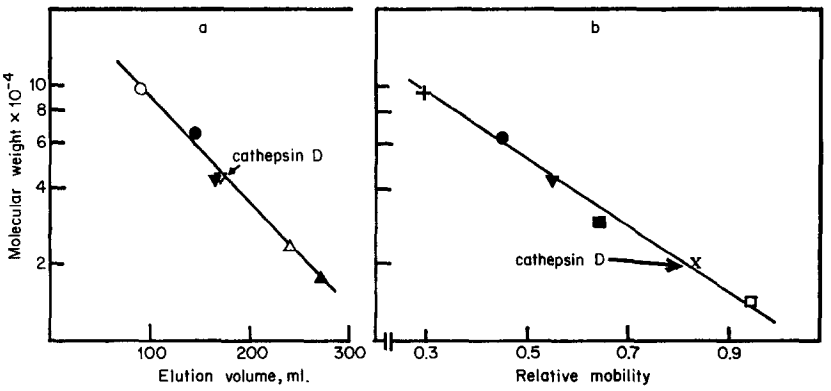

Fig. 3. a. Estimation of apparent molecular weight of Cathepsin D by chromatography on Sephadex G-100 column (2 X 95 cm). Hexokinase (M_r 102,000) ○ , bovine serum albumin (M_r = 67,000) ●, ovalbumin (M_r = 43,000) ▼ , ovomucoid (M_r = 29,000) △ , and ribonuclease (M_r = 13,700) ▲ were used as standards.

b. Estimation of molecular weight of Cathepsin D by analytical SDS-PAGE. Phosphorylase b (M_r = 94,000) +, bovine serum albumin (M_r = 67,000) ● , ovalbumin (M_r = 43,000) ▼ , carbonic anhydrase (M_r = 30,000) ■ , soybean trypsin inhibitor (M_r = 20,100) X and α-lactalbumin (M_r = 14,400) □ were employed as molecular weight markers.

Table 2. Activation of Rabbit Proacrosin by Cathepsin D

Samples	n	Acrosin (mUnits)
Proacrosin (Control)	(4)	329 ± 62
Proacrosin + Cathepsin D	(4)	1147 ± 353
Proacrosin + Cathepsin D + Pepstatin (2 mM)	(3)	212 ± 39

The reaction mixture (0.75 ml) contained proacrosin and Cathepsin D at a protein ratio of 200:1 in 0.25 ml. The rest was made up with 0.1 M acetate buffer, pH 3.8. The samples were incubated for 30 min at 37°C and acrosin activity assayed as described in text. The results represent the average ± S.E of indicated independent experiments carried out on different proacrosin preparations.

D in multiple forms and precursor species (12,13). The purified enzyme from rabbit testes shows temperature optimum at 55°C and pH optimum at 3.8. The high temperature stability of the enzyme was further ascertained by subjecting the purified preparation to varying levels of thermal treatment in the presence or absence of the substrate. The data (not shown) reveal that while Cathepsin D loses its activity completely at 70°C in the absence of the substrate, it retains 40% of activity in the presence of the substrate. The activation energy for the enzyme calculated on the basis of Arrhenius plot was 3.09 k cal/mole. Although Cathepsin D has been routinely assayed at 37°C (8) the high temperature optimum and thermal stability have been reported for a purified preparation from fish skeletal muscle (14). In frozen condition the enzyme does not lose its activity at least up to two months of storage.

The role of Cathepsin D in fertilization is not known. Acrosin which is believed to participate in sperm penetration of the ovum exists as an inactive zymogen, proacrosin, in the acrosome and testes (1). The conversion of proacrosin to acrosin takes place *in vitro* by the limited proteolysis of the zymogen accomplished by the proteolytic activity of the endogenously generated acrosin (15). Self catalysis of proacrosin to form acrosin by an intrazymogen mechanism has also been proposed (16). The results in Table 2 show that Cathepsin D generated significant amounts of acrosin from proacrosin. Addition of pepstatin abolished this activation as Cathepsin D was inactivated. Our proacrosin preparation did not autoactivate itself at pH 8.5 as described (10).

Since we used homogeneous Cathepsin D the observed activation can not be due to contamination. Involvement of an acrosomal amino proteinase, acrolysin, has been suggested in the proacrosin activation (17) but the enzyme has not yet been isolated. Cathepsin D is a carboxy proteinase having endopeptidase activity and unlike acrolysin it is neither inhibited by Zn^{2+} or phosphoramidon nor stimulated by Ca^{2+}. The gradual emergence of acrosin in the control seems to suggest that the incubation of proacrosin at the acidic pH might have partially displaced acrosin inhibitor as autoactivation is disallowed at this pH. Lysosomal Cathepsins B and D are known to promote the limited proteolysis and turnover of biologically active compounds from their precursors (18).

We suggest that the acrosomal Cathepsin D may initiate the proacrosin conversion into acrosin by providing the initial molecule of acrosin through the limited proteolysis of the zymogen intracellularly. Cathepsin D may also inactivate the acrosin inhibitor and thus allow the endogenous autoactivation of proacrosin to continue. The acidic pH within the acrosome (19) is conducive to the optimum activity of Cathepsin D. These results are of significance in the understanding of molecular basis of the acrosomal reaction and subsequent gamete interaction. We tentatively designate acrosomal Cathepsin D as, 'proacrosinin'.

Acknowledgement

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