Isolation and characterization of cathepsin B from rabbit testis*

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Summary. Cathepsin B (EC 3.4.22.1) has been purified from rabbit testes to apparent homogeneity by chromatography on DE-52, affinity chromatography on organo-mercurial agarose and subsequent gel filtrations on Sephadex G-75. The enzyme is composed of a single polypeptide of M_r 23 000. Thiol blocking agents and leupeptin abolished the activity of the enzyme completely. The enzyme showed maximum activity at pH 6.0 and 43°C, required 2 mM-cysteine for the optimal activity and had a K_m 1.45×10^{-3} M using Z-Arg- β -naphthylamide as the substrate. However, Z-Arg-Arg- β -naphthylamide was 12 times more sensitive as a substrate than was Z-Arg- β -naphthylamide. Rabbit testicular cathepsin B hydrolysed intact proteins. An endogenous inhibitor isolated from the rabbit testes inhibited purified Cathepsin B.

Introduction

Since it was discovered two decades ago that the acrosomal proteinases and hyaluronidase of the mammalian spermatozoon remove the cumulus, the corona and the zona pellucida of rabbit ova (Srivastava *et al.*, 1965a, b), the following acrosomal enzymes have been studied; acrosin (McRorie & Williams, 1974), alkaline proteinase (Ninjoor & Srivastava, 1985), aryl sulphatase (Yang & Srivastava, 1974), corona-penetrating enzyme (Zaneveld & Williams, 1970), β -N-acetyl hexosaminidase (Farooqui & Srivastava, 1980), hyaluronidase (Yang & Srivastava, 1975), neuraminidase (Srivastava & Abou-Issa, 1977), p₂-pentapeptidase (Lessley & Garner, 1984) and phospholipase C (Sheikhnejad & Srivastava, 1986). Acrosin has been extensively investigated due to its attributed role as the zona lytic enzyme (McRorie & Williams, 1974). Cathepsins, another group of enzymes, reside within lysosomes and participate in intracellular digestion of macromolecules and in enzyme activation and inactivation (Barrett, 1980). As sperm acrosomes are synonymous with lysosomes (Hartree, 1975) it seems that cathepsins contained in acrosomes and testicular lysosomes may have an essential role in fertilization. Little work has been done on cathepsins of testicular or acrosomal origin, but cathepsin D has been purified from the rabbit testes and may be involved in the activation of proacrosin (Srivastava & Ninjoor, 1982). We now describe the isolation and characterization of cathepsin B (EC 3.4.22.1) from rabbit testes.

Materials and Methods

Materials

Frozen testes, devoid of tunica albuginea and blood vessels, from mature rabbits were purchased from Pel-Freeze Biologicals, Inc., Rogers, AR. Tos-Lys CH₂Cl ketone (TLCK), phenylmethane sulphonyl fluoride (PMSF),

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benzamidine, pepstatin, leupeptin, *p*-hydroxymercuri-benzoate (PHMB), iodoacetamide, Z-Arg- β -naphthylamide, Z-Arg-Arg- β -naphthylamide, Arg-*p*-nitroanilide, Z-*p*-Leu-nitroanilide, Arg- β -naphthylamide, Leu- β -naphthylamide, benzoylarginine ethyl ester (BAEE), benzoyl tyrosine ethylester (BTEE), L-trans-epoxysuccinyl leucylamido (4-guanidino) butane (E-64), bovine serum albumin, bovine haemoglobin and azocasein were the products of Sigma Chemical Co., St Louis, MO. Azocoll (50–100 mesh) was procured from Calbiochem, La Jolla, CA, and Affigel-501 (organo mercurial agarose) and reagents for electrophoresis were obtained from Biorad Laboratories, Richmond, CA. Molecular weight markers and gel filtration media were from Pharmacia Fine Chemicals, Piscataway, NJ. Ep459 and 475 were gifts from Dr K. Hanada, Taisho Pharmaceutical Co., Saitama, Japan. All other reagents were of analytical grade.

Enzyme assays

The activity on β -naphthylamide and *p*-nitroanilide substrates was determined as described by Barrett (1977). The enzyme activity on haemoglobin, azocasein and Azocoll was measured according to Ninjoor & Srivastava (1985). Proteolysis of bovine serum albumin was measured by the absorbance of 10% trichloroacetic acid filtrate at 280 nm. The esterolytic activity on BAEE and BTEE was carried out according to Schwert & Takenaka (1955). One unit of the enzyme activity equals nmoles of naphthylamine or nitroaniline released/min from naphthylamide or nitroanilide substrates, respectively. An absorbance change of 0.001/min at 253 nm equals one unit of BAEE hydrolysis activity. One unit of haemoglobin splitting activity equals nmoles tyrosine released/min. The azocaseinolytic activity (unit) is defined as E_{366} of 1.0 in 30 min. The azocoll-splitting activity (unit) equals that amount of the enzyme which increases the A_{520} by 0.001/min. The albumin or protamine lytic activity unit equals $A_{280} \times 10^{-3}$ /min of the TCA filtrate. All assays were carried out at pH 6.0 and 43°C. Specific activity is defined as units/mg protein.

Protein estimation

Protein peaks in column fractions as well as low concentrations of proteins in purified preparations were monitored by the absorbance at 280 nm. Miller's procedure (1959) was used to measure protein content in other samples with bovine serum albumin as the standard.

Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed at pH 4.5 and pH 8.3 according to Brewer & Ashworth (1969) using 7.5% running and 2.5% spacer gels. Activity staining of the gels was carried out as described by Garner (1975). SDS gels (10%) were run according to Laemmli (1970).

Molecular weight determination

Gel filtration was carried out in a calibrated Sephadex G-75 column (60 $\times$ 3.5 cm) equilibrated and eluted with 20 mM-KH₂PO₄/Na₂HPO₄ buffer, pH 6.0, containing 0.2 M-NaCl and 1 mM-EDTA at a constant flow rate of 8.2 ml/h. The protein standards were bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), ovomucoid (M_r 29 000) and cytochrome C (M_r 12 500).

Amino acid analysis

A Beckman model 119Cl amino acid Analyzer was used for determining the amino acid content of purified cathepsin. B. Enzyme aliquants (60 μ g protein) were hydrolysed in 6 M-HCl at 110°C for 24, 48 or 72 h. Norleucine (5×10^{-4} M) was added to each sample as the standard. The amino acid content was quantified in terms of residues molecule by comparison with a standard run on the analyser.

Purification of cathepsin B

Extraction of the enzyme. Unless otherwise stated all operations were carried out at 4°C. Mature rabbit testes (275 g) were homogenized in 1 litre of 100 mM-acetic acid/sodium acetate buffer, pH 4.8, which contained 1 mM-EDTA, 10 M-PMSF, 1% (v/v) butan-1-ol and 0.5 mM-benzamidine, in a Waring blender. The homogenate was stirred for 2 h at 4°C and then autolysed overnight at 37°C. After autolysis, the sample was centrifuged at 17 000 *g* for 20 min and the sediment was resuspended in the homogenizing buffer and recentrifuged.

Ammonium sulphate fractionation. Solid (NH₄)₂SO₄ was added to the combined supernatant to 40% saturation and the precipitate was removed by centrifugation. More (NH₄)₂SO₄ was added to the supernatant to 70% saturation and the precipitate formed overnight was collected by centrifuging at 17 000 *g* for 30 min. The resultant pellet was dissolved in a minimum volume of 100 mM-sodium phosphate buffer (1 mM-EDTA, pH 6.5), and dialysed overnight with at least four changes of the same buffer.

Chromatography on DEAE cellulose. The dialysed 40–70% $(\text{NH}_4)_2\text{SO}_4$ fraction was chromatographed on a DE-52 column (30×3.5 cm), previously equilibrated with 100 mM-sodium phosphate buffer containing 1 mM-EDTA, pH 6.5. The flow rate was 30 ml/h and 3.3-ml fractions were collected. After the unbound protein was eluted in the elution buffer, a linear gradient of 0–500 mM-NaCl in the same buffer was established and the separation continued. The fractions exhibiting cathepsin B activity were pooled, dialysed against 0.05 M-acetate buffer, pH 5.0, containing 1 mM-EDTA and 200 mM-NaCl. The enzyme was concentrated by ultrafiltration on YM-10 Amicon membrane.

Affinity chromatography. Organomercurial agarose packed in a column (20×1 cm) was regenerated by passing five bed volumes of 50 mM-acetate buffer, pH 5.0, containing 20 mM-EDTA and 10 mM- HgCl_2 . Excess HgCl_2 was washed off and the column was equilibrated with several bed volumes of 50 mM-acetate buffer containing 1 mM-EDTA and 200 mM-NaCl, pH 5.0. The enzyme from the DE-52 step was applied to the column at room temperature and the unbound protein was eluted with the equilibration buffer. When the absorbance at 280 nm reached 0.05 or less, the bound protein from the affinity column was eluted with the equilibration buffer containing 10 mM-cysteine. The flow rate was 20 ml/h and fractions of 2.5-ml were collected. The active fractions were pooled, dialysed against 0.05 M-acetate buffer, pH 5.0, containing 1 mM-EDTA and concentrated to 2 ml by ultrafiltration.

Gel filtration on Sephadex G-75. The enzyme from the affinity column was then subjected to gel filtration on a Sephadex G-75 column (3.5×64 cm) equilibrated with 50 mM-acetate buffer, pH 5.0 containing 1 mM-EDTA. The enzyme had to be chromatographed three times to remove inactive proteins at a flow rate of 15 ml/h, and 2.5-ml fractions were collected. The fractions containing the enzyme in the final step were pooled, concentrated to 5 ml and frozen at -20°C .

Results

Purification of cathepsin B

The autolysis of the testicular homogenate was a valuable step in purification of the enzyme as the yield at this stage was nearly 3 times that of the homogenate. Cathepsin H (EC 3.4.22.16) eluted from DE-52 as the unbound protein while cathepsin B emerged at a salt gradient concentration of 0.1 to 0.15 M-NaCl (Fig. 1). Both enzymes hydrolysed Z-Arg- β -naphthylamide. However, cathepsins H and B were distinguished as cathepsin H also cleaved Leu- β -naphthylamide and Arg- β -naphthylamide. The organomercurial agarose chromatography gave a sharp peak of cathepsin B (Fig. 2). Although the enzyme activity coincided with the protein peak, the preparation was not homogeneous. The enzyme had to be further chromatographed 3 times on Sephadex G-75 to obtain a homogeneous preparation. The final gel filtration yielded the enzyme with a specific activity of 1096 units/mg protein and a purification ratio of 1570-fold over the homogenate with 14% recovery (Table 1).

Properties of rabbit testicular cathepsin B

The enzyme had optimum activity at pH 6.0. Cysteine (1.3 mM) and 1 mM-EDTA were essential for the maximal activity of the enzyme. Sulphydryl reagents at >5 mM inhibited the enzyme, the maximum inhibition being by dithiothreitol followed by cysteine and then by glutathione. The enzyme was maximally active at 43°C .

Heat stability

The sensitivity of the enzyme held at different temperatures, before assay, revealed that at 45°C cathepsin B retained 100% of its activity for 30 min and gradually lost 20% of its activity in 60 min. At 50°C , the enzyme lost 50% activity in 30 min. The incubation at 55°C and 60°C abolished 75% and 95% of the activity, respectively. Exposure of the enzyme to 55°C and 60°C for more than 60 min completely inactivated the enzyme.

Storage stability

The purified enzyme was stable for up to 8 weeks when frozen in 50 mM-acetate buffer, pH 5.0, containing 2 mM-cysteine and 1 mM-EDTA. The enzyme showed 65% of its original activity after storage for 12 weeks and only 35% of activity after 16 weeks.

Fig. 1. Ion-exchange chromatography of rabbit testicular cathepsin B on DEAE cellulose. The DE-52 column (3.5×30 cm) was equilibrated with 100 mM-sodium phosphate buffer containing 1 mM-EDTA, pH 6.5 and charged with 1.1 g 40–70% ammonium sulphate fraction. Fractions of 3.3 ml were collected at a flow rate of 30 ml/h. Arrow indicates the point where the gradient of NaCl in eluting buffer was started.

Fig. 2. Affinity chromatography of cathepsin B on organomercurial agarose. Sample was applied to the column (1×20 cm) and the retained material was eluted (arrow) by 10 mM-cysteine in 50 mM-acetate buffer containing 1 mM-EDTA and 200 mM-NaCl, pH 5.0. Flow rate was 20 ml/h and the fraction volume, 2.5 ml.

Effect of rabbit testicular proteinase inhibitor on cathepsin B

A proteinase inhibitor from rabbit testes which was isolated by the acid treatment, chromatography on Sephadex G-75 and ion exchange chromatography on phenyl-Sepharose (P. N. Srivastava & V. Ninjoor, unpublished data) abolished the activity of acrosin and enhanced the activity of testicular alkaline proteinase (Ninjoor & Srivastava, 1985). The incubation of purified

Table 1. Purification of cathepsin B from rabbit testes

Fraction	Total protein (mg)	Total activity (units)*	Specific activity (units/mg)*	Purification (- fold)	Yield (%)
Homogenate	10 740	7354	0.7	1	—
Autolysate	5010	19 700	4.0	6	100
40–70% (NH ₄) ₂ SO ₄	1100	16 750	15.0	21	85
DE-52	324	11 000	34.0	50	56
Organomercurial agarose	33	9690	294.0	420	49
Sephadex G-75, III	2.5	2740	1096.0	1570	14

*Unit is defined as nmol β -naphthylamine released/min.

Table 2. Effect of various proteinase inhibitors on cathepsin B

Addition	Final conc. (mM)	Activity retained
None, control		100
EP-459	0.02	0
EP-475	0.02	0
E-64	1	0
Iodoacetamide	1	0
PHMB	2	0
Leupeptin	0.0016	6
TLCK	1	67
PMSF	0.02	79
	0.1	26
Benzamidine	1	104
Pepstatin	0.02	101

Cathepsin B (15 units) in 100 mM-phosphate buffer containing 2 mM-cysteine and 1 mM-EDTA was incubated with the inhibitor in a total volume 950 μ l at 37°C for 30 min. The assay of the retained enzyme was done at 43°C by introducing 50 μ l 50 mM Z-Arg- β -naphthylamide. When the inhibitors of sulphhydryl groups were tested, cysteine was omitted from the buffer but added before measurement of the activity.

rabbit cathepsin B (10–15 units) with 105 μ g of the inhibitor for 30 min caused an 80% reduction in the enzyme activity.

Effect of other proteinase inhibitors

The effect of various sulphhydryl blockers and other reagents on cathepsin B is summarized in Table 2. PHMB, iodoacetamide, EP-459, EP-475 and E-64 inhibited the enzyme completely at <1 mM concentration. The serine proteinase inhibitors, TLCK and PMSF, partly inhibited the activity while benzamidine had no effect. Leupeptin, the known inhibitor of cathepsin B, showed high inhibitory activity. However, pepstatin, the aspartate proteinase inhibitor, had no action on cathepsin B.

Substrate specificity

Among the *N*-blocked arginine derivatives β -naphthylamide, para-nitroanilide and the ethyl esters, the enzyme cleaved Z-Arg- β -naphthylamide most efficiently. The K_m and V_{max} , estimated from double reciprocal plots of the reaction rate versus the concentration of Z-Arg- β -naphthylamide as the substrate, were 1.45 mM and 333 nmol/mg protein, respectively. However, the enzyme

Fig. 3. Gel electrophoresis of cathepsin B under acidic conditions using 10 μ g protein.

showed markedly higher specificity towards *N*-blocked paired arginine derivative Z-Arg-Arg- β -naphthylamide which was 12 times more sensitive than Z-Arg- β -naphthylamide as the substrate. Cathepsin B did not show esterolytic activity towards Z-Arg-ethyl ester at pH 6.0. It also lacked activity towards Z-Tyr-ethyl ester, a substrate of chymotrypsin. The substrates of cathepsin H, arg- β -naphthylamide Leu- β -naphthylamide and Leu-p-nitroanilide were also not cleaved.

The specific activities of cathepsin B on intact proteins and protein derivatives, ascertained at pH 6.0, were 25 for haemoglobin, 4709 for bovine serum albumin, 1 for azocasein and 212 for azocoll. The endopeptidase nature of cathepsin B was evident by its ability to hydrolyse all the proteins except protamine sulphate.

Criteria for homogeneity and molecular weight determination

The enzyme appeared to be pure as the activity and protein peaks from final Sephadex G-75 coincided and the specific activity across the peak fractions remained constant. Also, PAGE of the enzyme showed a single band (Fig. 3). When the unstained gels were stained for activity with Z-Arg- β -naphthylamide as the substrate and Fast Garnet GBC salt as the staining reagent, a single pink band, somewhat diffused, corresponding to the protein band in the gel stained with Coomassie blue was observed. SDS-PAGE also showed a single band at a region corresponding to M_r 23 000. The single band obtained by electrophoresis under denatured conditions indicated that cathepsin B from rabbit testes was composed of a single polypeptide chain. The molecular weight of cathepsin B was further established by its elution profile on a calibrated Sephadex G-75 column. The enzyme eluted between ovomucoid and cytochrome C which corresponded to an M_r 23 000.

Amino acid composition

The predominant amino acid residues per molecule (Table 3) were glycine followed by aspartic and glutamic acids. The enzyme molecule also possessed proline, alanine, valine and isoleucine in equal proportion.

Table 3. Amino acid composition of cathepsin B purified from rabbit testes

Amino acid	Residues/molecule	
	24 h hydrolysis	72 h hydrolysis
Aspartic acid	29.7	25.3
Threonine	13.8	11.0
Serine	18.6	14.0
Glutamic acid	28.6	22.5
Proline	14.2	12.4
Glycine	36.0	36.0
Alanine	14.0	12.7
Valine	14.2	11.8
Isoleucine	16.0	13.3
Leucine	13.3	11.6
Tyrosine	3.3	1.7
Phenylalanine	7.2	4.8
Lysine	13.2	11.4
Arginine	7.4	4.3

Elution pattern on concanavalin A-Sepharose

To ascertain the glycoprotein nature of cathepsin B, the enzyme was chromatographed on a con-A-Sepharose (1×10 cm) column equilibrated with 100 mM-phosphate buffer, pH 6.0, containing 200 mM-NaCl, and 2 mM each of CaCl_2 , MnCl_2 and MgCl_2 . Cathepsin B did not bind to the affinity matrix and therefore seemed to lack glycosyl and mannosyl residues.

Discussion

This is the first report on the homogeneous preparation of testicular cathepsin B and its properties. The molecular weight deduced for testicular cathepsin B, both by gel filtration and SDS-PAGE, was M_r 23 000 which is comparable to M_r of 24 000–28 000 for cathepsin B from other tissues (Katunuma & Kaminami, 1983). The rabbit testicular cathepsin B seemed to possess a single polypeptide chain as judged by its mobility on SDS-PAGE. However, Takahashi *et al.* (1984) have shown that cathepsin B from pig spleens is a two-chain enzyme of M_r 27 000 with a heavy chain (M_r 22 000) and a light chain (M_r 5000), while that from monkey skeletal muscle enzyme is a single polypeptide of M_r 24 000 (Hirao *et al.*, 1984).

The amino acid composition of testicular cathepsin B resembles that of the enzyme isolated from pig liver (Takahashi *et al.*, 1979), bovine lymph nodes (Popovic *et al.*, 1980), pig spleen (Takahashi *et al.*, 1984) and rat liver (Schwartz & Bird, 1977). Although high contents of glycine, aspartic and glutamic acids reduce the activity of kidney cathepsin B (Gounaris & Storter, 1982), they had no deleterious effect on the testicular enzyme.

Testicular cathepsin B resembled human (Barrett, 1973) and rat liver (Schwartz & Bird, 1977) cathepsin B in regard to its inhibition pattern by sulphydryl blockers such as PHMB and iodoacetamide and cysteine proteinase specific inhibitors EP-459 and EP-475. Although leupeptin at 1.6×10^{-6} M abolished the activity of cathepsin B, benzamidine, a powerful inhibitor of acrosin (McRorie & Williams, 1974), had no inhibitory effect on cathepsin B. The enzyme was free from cathepsin H-type activity as it failed to cleave amino acid naphthylamides and nitroanilides. The Z-Arg-ethyl ester (BAEE) used as a substrate for cathepsin B in earlier studies (Otto, 1971) is not susceptible to hydrolysis by cathepsin B of rabbit testes at pH 6.0. It is likely that the pH require-

ment for the esterolytic activity is different from that of the amidase activity. As cathepsin B promotes the conversion of proinsulin to insulin (Smith & Van Frank, 1975) and proalbumin to albumin (Quinn & Judah, 1978), it is likely that testicular cathepsin B may play a similar role in the conversion of proacrosin, phospholipase C and other sperm acrosomal proenzymes into their active forms as required during gamete interaction.

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