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CATALYTIC AND PHYSICO-CHEMICAL CHARACTERISTICS OF GOAT SPLEEN CATHEPSIN B

Sudhir K. Agarwal*, Santanu D. Choudhury#,
Madhav Lamsal# and M. Yahiya Khan
Department of Biochemistry, Dr. Ram Manohar Lohia Avadh University, Faizabad 224 001 and #Department of Biochemistry, North Eastern Hill University, Shillong 793 022 (INDIA)

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SUMMARY

To improve the level of purity of cathepsin B, we have modified the published procedure [Agarwal, S.K. and Khan, M.Y. (1987) Biochem. Int. **15**, 785-792] by incorporating CM-Sephadex ion exchange chromatography and chromatofocusing. The enzyme thus isolated could be resolved into one 26 kDa major and a minor 27 kDa protein bands on SDS-PAGE. The two components, however, could not be separated by gel filtration and they eluted, in a single peak corresponding to a molecular mass of 28.1 kDa . Among the various substrates tested, Z-Phe-Arg-MCA with a K_m of 0.058 mM and hemoglobin with a K_m of 1.449 μM were the most preferred synthetic and protein substrates respectively. It was found to be a glycoprotein with an acidic pI of 4.8. The enzyme was activated by various thiol-reducing reagents and inhibited by cysteine proteinase inhibitors, divalent cations, lysyl group modifiers, anti-inflammatory drug and denaturing agents. The hydrodynamic behaviour of cathepsin B suggested a compact and globular conformation. Immunodiffusion studies with anti-goat cathepsin B indicated a tissue/species dependence.

Key-words: Protease, cathepsin B, properties, tissue/species dependence.

Abbreviations used: BSA, bovine serum albumin; IAA, iodoacetic acid; IAM, iodoacetamide; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; BAPNA, ∝-N-benzoyl-DL-arginine-4-nitroanilide; BANA, ∝-N—benzoyl-DL-arginine-2-napthylamide; DTT, 1,4-dithiothreitol; GdnHCl, guanidine hydrochloride; MCA, 7-amino-4-methylcoumarin; Z-Arg-Arg-MCA, benzoyloxycarbonyl-arginyl-arginine-MCA; Z-Phe-Arg-MCA, benzoyloxycarbonyl-phenylalanyl-arginine-MCA; EDTA, ethylenediamine tetra acetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBE-94, polybuffer exchanger-94; TCA, trichloroacetic acid; FCA, Fruend's Complete Adjuent; IFA, Fruend's Incomplete Adjuent.

* Author to whom correspondence be addressed.

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INTRODUCTION

Lysosomal cysteine proteinases are an important group of active enzymes involved in intracellular protein catabolism (1-4). Amongst these, cathepsin B (EC 3.4.22.1) is the most thoroughly investigated enzyme. The enzyme possesses both endopeptidase (1,5) and carboxypeptidase activities (4,6,7). It is considered to play an important role in the processing of several proteins and in some physiological and pathological conditions, such as muscular dystrophy, muscle wasting, tumour invasion, cartilage destruction and inflammation (2,4,8). The exact function(s) and mechanism of action of cathepsin B are however, largely unknown.

Cathepsin B has been isolated and characterized from various mammalian species and tissues (1-4). Spleen, which is histologically a complex organ consisting of many different cells with varying degrees of differentiation (9), has been used as a source for the purification of cathepsin B from bovine (10), rat (11), porcine (9) and buffalo (12). Earlier, we have isolated partially-purified cathepsin B from goat (*Capra hircus*) spleen (13) and studied few of its properties (13,14). In the present report we have extended our purification procedure and characterized the enzyme in detail with a special emphasis on its catalytic behaviour.

EXPERIMENTAL

Materials

Spleens from freshly slaughtered goats were collected from local slaughter houses on ice and were frozen immediately at -4°C before use. Leupeptin, antipain, pepstatin A, indomethacin, maleic anhydride, succinic anhydride, IAA, IAM, TPCK, BAPNA, BANA, cysteamine, DTT, carboxypeptidase A and GdnHCl were obtained from Sigma Chemical Co., U.S.A. Z-Arg-Arg-MCA, Z-Phe-Arg-MCA and MCA were purchased from Peptide Institute, Inc. Osaka, Japan. Hemoglobin was isolated from goat blood. All other chemicals were of analytical grade or best commercially available.

Methods

Purification of cathepsin B - Cathepsin B was isolated from goat spleens essentially by our earlier procedure (13), expect that ion-exchange chromatography (CM-Sephadex) and chromatofocusing steps were added for improving the purity of the enzyme. A column (1.6x18 cm) of CM-Sephadex C-50 equilibrated with 20 mM sodium acetate, pH 4.9 containing 1 mM EDTA and 1.4 mM 2-mercaptoethanol was used to bind the protein. It was eluted step-wise with the same buffer having the following pH values: (a) pH 4.9, (b) pH 5.6, (c) pH 6.0 and (d) pH 6.0+0.4 M NaCl. Cathepsin B which came out in the buffer (b) was pooled, dialyzed against 25 mM histidine-HCl buffer, pH 6.0 containing 1 mM EDTA and 1.4 mM

2-mercaptoethanol and applied on a column (1.2x15 cm) of PBE-94 which was eluted with diluted polybuffer 74, pH 4.0, as suggested by the manufacturer. Prior to use, enzyme preparation was routinely passed through a Sephadex G-25 column to remove the autolyzed products.

Assay - BANA hydrolyzing activity was measured fluorimetrically in 0.02 M sodium phosphate buffer, pH 6.5, containing 2 mM each of EDTA and 2-mercaptoethanol by continuously monitoring the released 2-napthylamine for 30 min at 37°C (14). In few experiments, the released 2-napthylamine was estimated by diazotization and coupling with N-1-napthylethylenediamine dihydrochloride followed by colour intensity measurement at 540 nm (15). BAPNA hydrolyzing activity was measured spectrophotometrically in the same buffer and the released nitroaniline was monitored at 400 nm. Assay with Z-Arg-Arg-MCA and Z-Phe-Arg-MCA at 37°C in 340 mM sodium acetate buffer, pH 6.5 containing 4 mM EDTA and 8 mM DTT, was done fluorometrically using excitation and emission wavelengths 370 and 440 nm respectively (4,16). When acid denatured proteins were used as the substrate, the TCA soluble peptides were estimated by the method of Moore and Stein (17).

Electrophoresis -PAGE in the absence and presence of SDS was performed according to the method of Davis (18) and Weber and Osborn (19) respectively. Isoelectric point was determined by chromatofocusing in PBE-94 (Pharmacia, Sweden) as described by the manufacturer.

Chemical analysis - Amino acid analysis of the purified cathepsin B was done using Shimadzu High Performance Liquid Chromatography, model LC-4A, using single Na^+ type cation exchange column (ISC-07/S1504 Na^+) (9).

The NH_2 - and COOH- terminal amino acid residues were identified by thin layer chromotography following the methods of Gray (20) and Narita (21) respectively. Carbohydrate content of the enzyme was determined according to the procedure of Dubois **et al.** (22). The sulfhydryl content of cathepsin B under native conditions as well as in the presence of 8 M urea was determined essentially by the method of Ellman (23).

Protein concentration - Protein concentration was measured either by the method of Bradford (24) using BSA as standard or by spectrophotometric method using specific extinction coefficient ($E^{1\%}_{1cm}$) of 15.6 (determined in this study) for goat spleen cathepsin B.

Aldolase inactivation - Rabbit muscle aldolase was incubated at 37°C with cathepsin B in 0.1 M sodium phosphate buffer (pH 6.5) containing 2 mM 2-mercaptoethanol. The molar ratio of aldolase to cathepsin B was 50:1. During incubation, aliquotes of the mixture were taken at different time intervals for the measurement of residual aldolase activity using hydrazine as described by Sigma Manual, procedure no. 752.

Determination of molecular parameters - Molecular parameters such as molecular weight, Stokes radius and frictional ratio were obtained from the gel-filtration behaviour on an analytical column (2.6x91 cm) of Sephadex G-75 at 25°C in 0.02 M sodium phosphate buffer (pH 6.5) containing 1 mM EDTA, using standard marker proteins (25). The value of frictional ratio, f/f_0, was obtained by the equation as described in Tanford (26) using 0.742 as the value of partial specific volume (12) .

Viscosity measurements - The viscosity of the goat enzyme in 0.02 M sodium phosphate buffer, pH 6.5 containing 1 mM EDTA was measured in a Scott Gerate (type 513 00) viscometer having a flow time of about 430 s for 4 ml distilled water at 25°C with a precision of better than 6%. The reduced viscosity, η_{red}, was determined as described by Tanford (26) using the time of fall for the enzyme solution and the solvent in the viscometer, the density of the solvent and the partial specific volume of the enzyme.

Immunological studies - For the preparation of polyclonal antibodies, rabbits (2-3 kg of body weight) were immunized by administrating 140 µg of cathepsin B with FCA subcutaneously followed by a booster dose given at 30 days interval with 70 µg of protein with IFA. Sera was collected within four days after giving the booster dose. Immunological cross-reactivity was checked by Ouchterlony's double immunodiffusion method (27) in 1.2% agarose containing 0.02% sodium azide.

Kinetic parameters - K_m and V_{max} of the goat enzyme for synthetic as well as protein substrates were computed from the least squares analysis of the data plotted according to the method of Lineweaver and Burk (28).

RESULTS AND DISCUSSION

Inclusion of the ion exchange chromatography and chromatofocusing steps in the earlier purification scheme (13) improved the fold purification of the enzyme from 161 to 209. However, in accordance with our earlier findings (13), the enzyme preparation could be resolved on PAGE into a major component and a very minor component presumed to be a contaminant or an isozyme of cathepsin B (9,29).

A striking feature of the goat spleen enzyme is its elution from the CM-Sephadex ion exchange column at a relatively lower pH and ionic strength. The enzyme, which was obtained at pH 6.0 in presence of 0.1 M NaCl earlier (30), eluted at pH 5.6 in the present study. This might be attributed to the difference in the nature and extent of glycosylation (31) and amino acid composition of the enzyme from the two sources.

Physico-chemical characteristics

Gel electrophoresis of cathepsin B in presence of SDS and 2-mercaptoethanol yielded a major and a minor protein bands corresponding to molecular weights of 26 and 27 kDa respectively. The enzyme, however, eluted in a single peak on a calibrated gel filtration column corresponding to a molecular weight of 28.1 kDa which is found to be comparatively higher than the molecular weight of the major component on SDS-PAGE. This is more so because the enzyme is a glycoprotein having 6.3% carbohydrates which will have an enhanced tendency of hydration (26) resulting into overestimation in the molecular weight.

The various physico-chemical properties which were similar to those described for cathepsin B from other sources (29,30,32), are summarized in Table 1. In full agreement with earlier findings (12,33), the free sulfhydryl content of the enzyme increased from 0.9 in absence of urea to about 1.6 moles per mole of the enzyme in presence of the denaturant. The values of Stokes radius and intrinsic viscosity (Table 1) of the enzyme are found to be similar to the published values of these parameters for cathepsin B from other mammalian sources(12,29).

Amino acid composition

The amino acid composition of goat spleen cathepsin B compared with that of rat liver cathepsin B (11) showed higher (28%) content of Pro, Leu and Trp whereas Thr, Ser, Tyr and Lys were present in relative lesser (39%) amount (11).

Table 1 Physico-chemical properties of goat spleen cathepsin B

Property	Value
Molecular weight	
SDS-PAGE	26000-27000
Gel-filtration	28100
NH_2-terminal amino acid	Leu
COOH-terminal amino acid	Thr
Carbohydrate content[a]	6.3
Isoelectric pH (pI)	4.8
Isoionic pH	5.12
SH-group at pH 8.0[b]	0.9
SH-group at pH 8.0[b] containing 8 M urea	1.6
Stokes radius (nm)	2.46
Intrinsic viscosity (ml/g)	3.30
Frictional ratio, f/f_o	1.22

[a] Calculated as g per 100 g of the protein.

[b] Calculated as mole per mole of the protein.

Optical properties

The specific extinction coefficient, $E^{1\%}_{1cm}$, of the enzyme was determined at 278 nm (λ_{max}) in 0.02 M sodium phosphate buffer, pH 6.5, containing 1 mM EDTA to be 15.64. This is similar (15.5) to bovine spleen cathepsin B (34) but different (13.2) from buffalo spleen cathepsin B (32). The fluorescence spectra (excitation near 280 nm and emission maximum near 340 nm) was dominated by tryptophan residue (35).

Influence of thiol-reducing compounds

In addition to 2-mercaptoethanol, which serves as an activator for cathepsin B in the usual assay, the activation of the enzyme was also studied by other thiol reducing compounds. The results thus obtained are depicted in Figure 1. All the reducing agents show very strong stimulatory effect on the enzyme activity. Among the thiol compounds tested, cysteamine was most effective and thioglycerol was the least (see Figure 1). These results indicate that goat spleen cathepsin B is a cysteine proteinase that requires thiol-reducing compounds for its activity.

Effect of various inhibitors

The influence of different proteinase inhibitors on cathepsin B was studied and the data are summarized in Table 2. Leupeptin, antipain and E-64, the well known cysteine proteinase inhibitors showed high inhibitory activity against the enzyme. However, pepstatin A, the aspartate

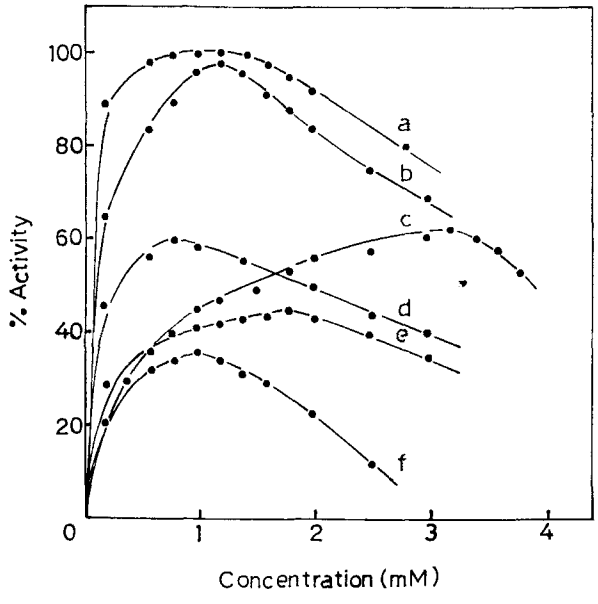

Figure 1 Effect of various reducing agents on the activity of goat spleen cathepsin B. (a) cysteamine, (b) cysteine, (c) 2-mercaptoethanol, (d) DTT, (e) glutathione-SH and (f) thioglycerol.

Table 2 Effect of various proteinase inhibitors on cathepsin B activity*

Additive	Final concentration (mM)	Inhibition (%)
Leupeptin	0.01	100.0
Antipain	0.01	100.0
E-64	0.01	100.0
Pepstatin A	0.10	2.0
$HgCl_2$	0.10	82.6
$MnCl_2$	0.01	50.7
TPCK	0.01	52.5
	0.10	100.0
Maleic anhydride	0.10	9.2
	0.40	24.7
Succinic anhydride	0.10	7.5
	0.40	18.1
Indomethacin	0.10	37.7
IAA	0.01	97.8
IAM	0.10	94.3
GdnHCl	$0.1x10^3$	50.0
	$1.0x10^3$	100.0
Urea	$0.1x10^3$	41.5
	$1.0x10^3$	96.0

* Enzyme (35 μg/ml) was incubated with the desired compounds at 37°C for 30 min prior to assay of its activity. Values are means for three independent experiments.

proteinase inhibitor, had more or less no effect on cathepsin B. Of the divalent cations, $MnCl_2$ was more potent inhibitor than $HgCl_2$. Maleic anhydride and succinic anhydride, which are used for the modification of ε-amino acid residues of proteins, showed very mild effect on the activity of cathepsin B. Only about 8% activity was abolished at a concentration of 0.1 mM modifier; the inhibition increased by about 3 times at a concentration of 0.4 mM. Indomethacin (0.1 mM), an anti-inflammatory agent, caused about 38% inhibition on goat spleen cathepsin B. This agrees well with the data (37% inhibition) of rat spleen cathepsin B which is due to a change in the conformation of the enzyme (36).

While the activity of cathepsin B was markedly inhibited by GdnHCl or urea, TPCK showed a moderate inhibitory effect. At 0.01 mM TPCK, the inhibition of goat enzyme was about 53%; the enzyme was fully inactivated at a TPCK concentration of 0.1 mM and above. This indicates that cathepsin B activity may be similar to chymotryptic activity in the mode of inhibition by TPCK. IAA was found to be 10 times more effective inhibitor than IAM (Table 2). This extent of inhibition agrees with the earlier findings of porcine parathyroid cathepsin B (37).

The reason for the effectiveness of iodoacetic acid may be due to a strong binding between the -ve charge of the -COOH group of IAA and +ve charge at Arg 200 located near the active site His 199 as found in porcine (9), rat (11) and Human (38) cathepsin B.

Immunological studies

The cross reactivity of the goat enzyme was studied with polyclonal antibodies raised in rabbits. Antibodies were found to cross-react distinctly with purified goat enzyme as shown in Figure 2(a). However, no antigen-antibody reaction took place (see Figure 2(b)) when buffalo kidney cathepsin B was used as an antigen. Similarly, when cathepsin H, a homologous enzyme of lysosomal cysteine proteinases, either from buffalo kidney or porcine lung was cross-reacted to anti-goat spleen cathepsin B, no precipitin arc was formed (see Figure 2(c and d)). These observations pointed out that not only antigenic determinants are dissimilar between cathepsin B and H but they are also non-identical even in the cathepsin B from different species/tissues.

Aldolase inactivation

Aldolase inactivation was studied by goat spleen cathepsin B and the results obtained are depicted in Figure 3. Only about 20% inhibition of aldolase activity was observed at aldolase to cathepsin B molar ratio of 50:1. However 65% aldolase inactivation was reported when the molar ratio of aldolase to porcine cathepsin B was 100:1(39). This indicates that goat enzyme is less effective towards aldolase inactivation than porcine cathepsin B.

Kinetic properties

K_m and V_{max} of the goat enzyme for various natural and synthetic substrates were computed from the Lineweaver-Burk plot (28) and the values are summarized in Table 3. As evident from the Table, the cathepsin B is highly active against protein substrates. Out of BSA,

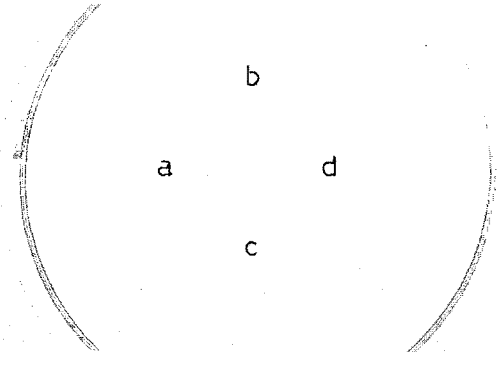

Figure 2 Ouchterlony double immunodiffusion goat spleen anti-cathepsin antiserum against cathepsin B and H. (a) cathepsin B from goat spleen, (b) cathepsin B from buffalo kidney, (c) cathepsin H from buffalo kidney and (d) cathepsin H from porcine lung.

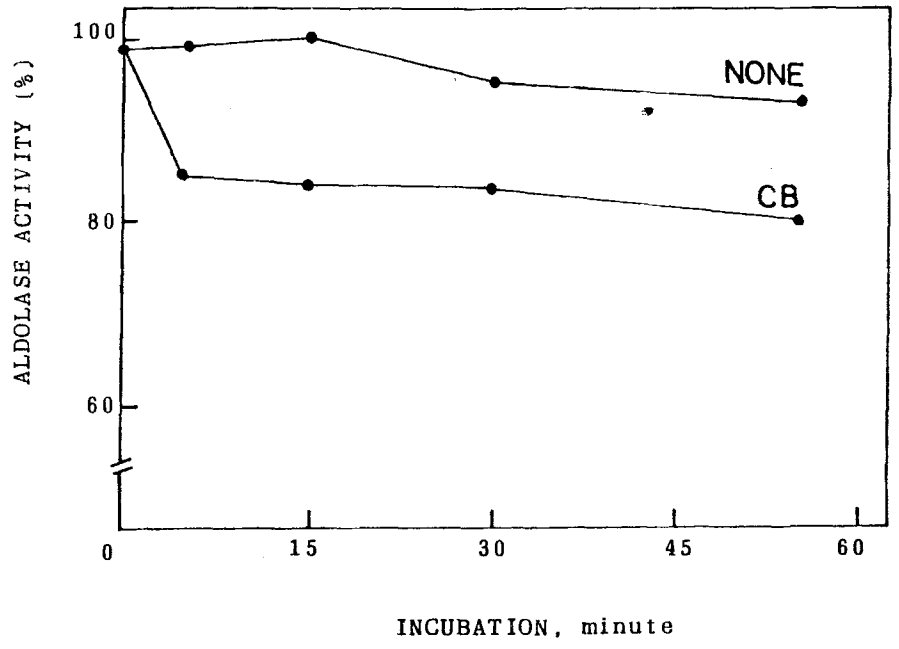

Figure 3 **Aldolase inactivation by goat spleen cathepsin B (CB).** Aldolase was incubated with goat enzyme in a molar ratio of 50:1 and the residual activity was measured by taking aliquotes at different time intervals.

Table 3 Kinetic parameters of cathepsin B from goat spleen

Substrate	Goat spleen	
	K_m	V_{max}
BANA	2.530 mM	0.562 units[a]
BAPNA	0.685 mM	0.247 "
Z-Phe-Arg-MCA	0.058 mM	1.128 "
Z-Arg-Arg-MCA	0.262 mM	0.653 "
BSA	2.632 μM	0.202 x 10^3 units[b]
Casein	2.151 μM	0.390 x 10^3 "
Hemoglobin	1.449 μM	0.667 x 10^3 "

[a] One unit represents the amount of enzyme required to release 1μ mole of product per min.
[b] One unit corresponds to the amount of enzyme required to increase the colour yield by 0.01 O.D. unit per hour under our assay conditions.

hemoglobin and casein, hemoglobin (K_m = 1.449 μM) was found to be the most preferred substrate whereas BSA (K_m = 2.632 μM) was the least favoured one. These results thus suggest that goat enzyme has a great potential to degrade proteins in vivo.

Among the N-blocked arginine derivatives, 2-naphthylamide and para-nitroanilide, goat cathepsin B cleaved BAPNA most effectively. The K_m and V_{max}, estimated from double reciprocal plots of the reaction rate versus the concentration of substrate, were 0.685 mM and 2.53 mM and 0.247 units/mg and 0.562 units/mg respectively for BAPNA and BANA. These values lie in the range of the values of these parameters reported for the enzyme from other sources (4,9,29,32). However, a lower K_m for BAPNA than BANA may be explained possibly due to a better fit of nitroanilide in the S_1 pocket of catalytic site of the enzyme. The enzyme showed markedly higher specificity towards fluorogenic substrates, Z-Arg-Arg-MCA and Z-Phe-Arg-MCA; the latter was found to be 5 times more sensitive than the former (Table 3). This is, however, explainable because S_2 pocket of catalytic site of the enzyme favours large hydrophobic groups like phenylalanine (9). A similar order of substrate specificity was reported for mammalian, avian and piscean cathepsin B (40-42). However, larger K_m values of avian and piscean enzymes (40,41) for Z-Arg-Arg-MCA and Z-Phe-Arg-MCA hydrolysis reflect species difference between avian/piscean and mammalian enzymes. These results also emphasize that goat spleen cathepsin B is more efficient towards fluoregenic substrates compared to avian/piscean enzyme.

Above results thus clearly show that cathepsin B from goat spleen is similar to its counterpart from other sources with regard to some of its properties. On the other hand, the goat enzyme differs significantly with the same proteinases from other sources with regard to other properties, especially kinetic and inhibitory. The data taken together, thus suggest a tissue/species dependence of cathepsin B.

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