STUDIES ON PHYSICO-CHEMICAL AND KINETIC CHARACTERIZATION OF CATHEPSIN B FROM GOAT SPLEEN

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BY

SANTANU DEB CHOUDHURY

THESIS
SUBMITTED IN FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

TO

NORTH-EASTERN HILL UNIVERSITY
SHILLONG - 793 022
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ABSTRACT

Considerable research on lysosomal proteolytic enzymes over the past few decades have emphasized their wide range participation in vital physiological and pathological processes. The wide distribution of lysosomal cathepsins, a group of proteolytic enzymes, throughout most tissues, suggest that they play an important role in cellular protein turnover. Mammalian tissues are known to contain several proteinases with similar and overlapping enzymatic properties. Availability of specific substrates and inhibitors has helped in identification of more than a dozen cathepsins and related proteinases . Proteinases of lysosomal origin have been categorized into four groups, namely, cysteine, aspartic, serine and metalloproteinases. The classification is based on the nature of the most prominent functional group in the active site of the enzyme, which in the case of cysteine proteinases is an essential cysteine residue. These include cathepsins B, H, L and S.

One of the most thoroughly studied thiol proteinases, cathepsin B (EC 3.4.22.1) is essentially a carboxypeptidase which releases dipeptides sequentially from the carboxyl termini of polypeptides. However, it also exhibits endopeptidase activity with a specificity towards an arginyl-arginyl-x bond.

Apart from its involvement in various vital physiological functions such as protein turnover and processing of proteins and hormone precursors, it has been implicated in various diseases and has been reported to play significant role in tumour growth, invasiveness and metastasis.

The outcome of research on cathepsin B particularly of its isozymes, have not been unequivocal. While the enzyme was found to be homogeneous on polyacrylamide gels from some tissues, multiplicity of bands of cathepsin B preparations from other tissues have been attributed to microheterogeneity representing some related proteins and/or degradation products of the active enzyme. However the earlier reports on isozymes of cathepsin B with isoelectric pHs and molecular weights in the range of

4.5-5.5 and 24-29 kDa, respectively and our present study from goat spleen indicate that this tissue is histologically heterogeneous consisting of many different cell types with varying degrees of differentiation which may account for enzyme species with different maturational states.

In the present study goat spleen was chosen as a source and a simple procedure was developed for the isolation and purification of cathepsin B. A striking feature of the goat spleen cathepsin B is its elution by CM-Sephadex ion exchange chromatography at a relatively lower pH and ionic strength. The enzyme was eluted at pH 5.6 as against pH 6.0 and 0.1 M NaCl, required for a similar elution of cathepsin B from porcine spleen, which may be attributed to differences in the nature and extent of glycosylation and/or amino acid composition of the enzymes from the two sources.

The purified cathepsin B from goat spleen yielded two distinct protein bands on PAGE. It was therefore subjected to affinity chromatography on ConA-Sepharose 4B column. Two distinct and enzymatically active protein peaks were obtained which were named goat spleen cathepsin B-I (GSCB-I) and goat spleen cathepsin B-II (GSCB-II). GSCB-I, which did not bind to ConA-Sepharose column possibly due to differential glycosylation of the asparagine residues, constituted approximately 84% of the total enzyme activity and had a specific activity of 4.16 units/mg protein towards Z-Arg-Arg-MCA, where as GSCB-II, which was eluted with 0.5 M α-D-glucopyranoside constituted about 16% of the total activity and had a specific activity of 1.06 units/mg protein. Molecular weights of GSCB-I and GSCB-II determined by gel filtration were 28.1 kDa and 31.6 kDa, respectively and by SDS-PAGE, yielded a value of 25.7 kDa and 26.6 kDa respectively. Both enzyme fractions lacked multiple chain forms. The Stokes radius calculated for GSCB-I and II were 2.46 Å and 2.58 Å with a frictional ratio of 1.22 and 1.28 respectively, suggesting globular conformations of the enzymes under native conditions.

The experiments in this study were performed with GSCB-I (unless stated otherwise) since it was present in larger amounts and had a much higher specific activity compared to GSCB-II.

GSCB-I was found to be a glycoprotein having a carbohydrate content of 4.6%. The intrinsic viscosity of the enzyme was determined to be 3.30 ml/gm, which was well within the range (3.0-4.0 ml/gm) expected for native proteins having compact and globular conformations. NH_2- and COOH- terminal amino acid residues were found to be Leu and Thr, respectively. A total of 0.9 and 1.6 mols of thiol groups could be titrated per mol of protein in absence and presence of 8 M urea respectively, clearly showing that the free thiol group(s) in the enzyme is/are not fully exposed.

Amino acid composition of GSCB-I showed close similarities with cathepsin B from rat, bovine, human and porcine tissues, except for Ser, the amounts of which were relatively lower. Tyr and Lys were also significantly lower whereas Leu and Trp were present at relatively higher amounts. U.V. absorption spectra of the enzyme showed a maxima at 278 nm. The fluorescence emission maxima was near 340 nm which is characteristic of proteins containing significant amount of Trp residues. The specific extinction coefficient ($E^{1\%}_{1cm}$) of the enzyme was determined to be 15.64. GSCB-I had an isoionic pH of 5.12 indicating an acidic nature. This value is consistent with the values of pH range 4.8-5.3 for cathepsin B from different sources.

Thiol reducing agents had a strong stimulatory effect on catheptic activity. Among the thiol modulators tested, cysteamine was most effective and thioglycerol was least effective. These results indicate differential effect of thiol group modulators in altering the activity of this enzyme.

Influences of various inhibitors on GSCB-I show divalent cations such as Mn^{++} and Hg^{++}, alkylating agents like iodoacetic acid and iodoacetamide and peptidyl inhibitors such as E-64, antipain and leupeptin inactivated the enzyme. Maleic and succinic anhydride used for modification of α-amino group of proteins showed very mild inhibitory effect. Pepstatin, a well known inhibitor of aspartyl proteinases, including cathepsin D, had no inhibitory effect on enzyme activity. Indomethacin, an anti-inflammatory agent, caused about 38% inhibition of the enzyme activity. This is in

accordance with 37% inhibition of rat spleen cathepsin B activity, which has been shown to be due to a conformational change brought about by indomethacin on the enzyme. This may be a contributory factor in reducing inflammation by lowering the rate of proteolysis.

GSCB-I was markedly inhibited by Gdn-HCl and urea. 50% inactivation of the enzyme was achieved at a urea concentration of about 0.15 M and virtually no activity was found above 1.0 M. The process of inactivation became irreversible as the urea concentration was raised beyond 2.5 M. This also ruled out the possible contamination of the cathepsin B preparation with cathepsin L as cathepsin L activity is retained even at a urea concentration of 3 M. Gdn-HCl was more effective against the enzyme and 50% inactivation was achieved at 0.12 M. The loss of catheptic activity was reversible at and below 1.8 M Gdn-HCl, but above 2.0 M concentration the inactivation process became irreversible. Since the concentrations of the denaturants are low to cause any significant conformational alterations of the enzyme, the loss of activity may be possibly due to perturbations near the environment of the enzyme active site. TPCK inhibition of the enzyme at 0.01 mM concentration was about 53% and the enzyme was fully inactivated at a TPCK concentration of 0.1 mM and above, indicating that cathepsin B activity may be similar to chymotryptic activity in the mechanism of inhibition by TPCK.

GSCB-I was found to be very sensitive to physical parameters such as pH, temperature and ionic strength. The enzyme showed highest activity at a pH of 6.8 and was fully stable for at least 20 min upto pH 7.0. The enzyme was found to have maximum activity at physiological temperature and was fairly stable and retained most of its activity till the temperature was raised above 40°C. Maximum activity of the enzyme was observed at an ionic strength of 0.22. However at higher salt concentrations, a pronounced decrease in the activity was observed. Hence, 20 mM sodium phosphate buffer or other buffers of equivalent ionic strength were used for the assay of the enzyme activity.

The kinetic parameters K_m and V_{max} of GSCB-I showed that the enzyme had greater catalytic potential at acidic and/or near neutral pHs,

for various natural and synthetic substrates. Among the synthetic substrates tested Z-Phe-Arg-MCA with a K_m of 0.07 mM was found to be most preferred followed by Z-Arg-Arg-MCA (K_m=0.22 mM), BAPNA (K_m=0.70 mM) and BANA (K_m=2.64 mM). Hemoglobin, among the natural substrates tested for GSCB-I, proved to be the most preferred with a K_m of 1.46 μM followed by casein (K_m=2.18 μM) and BSA (K_m=2.67 μM). The ability of cathepsin B to inactivate aldolase have been shown to be a characteristic feature of this enzyme and it also differentiates cathepsin B from other cathepsins. However only about 20% aldolase inactivation was observed at aldolase to GSCB-I molar ratio of 50:1, which is significantly lower than the values reported for aldolase inactivation by cathepsin B from other sources.

A comparative study of GSCB-I and II showed that inhibitors such as leupeptin, antipain, pepstatin, iodoacetic acid and iodoacetamide and denaturants such as Gdn-HCl and urea showed close similarities in their mode of inhibition of both the enzyme fractions. However kinetic parameters of GSCB-II against the substrates Z-Phe-Arg-MCA, Z-Arg-Arg-MCA and BANA showed that though the enzyme was similar in its enzymatic activity against these substrates with that of GSCB-I, it differed significantly in its K_m and V_{max} as compared to that of GSCB-I. Among the substrates tested, Z-Phe-Arg-MCA was most preferred for GSCB-II (K_m = 0.05 mM) followed by Z-Arg-Arg-MCA (K_m=0.14 mM) and BANA (K_m=1.87 mM). No activity against the substrates Leu-NA or Arg-MCA were shown either by GSCB-I or II, thus ruling out the possibility of the presence of leucine aminopeptidases or cathepsin H in the enzyme preparations.

Immunological studies revealed that polyclonal antibodies raised in rabbits against the unresolved purified goat spleen cathepsin B (enzyme preparation before the ConA-Sepharose step) reacted only with purified goat spleen enzyme (unresolved goat spleen cathepsin B, GSCB-I and GSCB-II). However, no antigen-antibody reaction took place against buffalo kidney cathepsin B or cathepsin H from buffalo kidney or porcine lung.

A comparison of the results summarized above with that of the literature available on cathepsin B from other sources show that GSCB-I is similar to cathepsin B reported from other mammalian tissues with respect to most of the physicochemical properties. However, it differed significantly with regard to the number of some of its amino acid residues and catalytic efficiencies against various synthetic and natural protein substrates including muscle aldolase.

The differences, particularly in the catalytic efficiency and the possible presence of isozymes of goat spleen cathepsin B, reflect a species and/or tissue dependence of the enzyme.