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Original Research Article

Progressive Study on the Physiological Role and Catalytic Properties of Buffalo Lung Cathepsin B

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ABSTRACT

Physiological and catalytic properties of cathepsin B from water buffalo (Bubalus bubalis) lung, hitherto unstudied source/tissue, have been reported. The activity of the enzyme was optimal at physiological temperature (37°C) and the enzyme could withstand temperature shocks up to 37°C for 10-30 min without any significant loss of activity. Moreover the maximum activity was observed at pH 7.0 and the enzyme was fairly stable between pHs 3.5-6.75 for at least 20 min. Cathepsin B was most active at $2.5x10^{-3}$ M buffer concentration and lost its activity substantially as the buffer concentration was raised above optimum value. As the enzyme was highly stable between salt concentrations of $1.5x10^{-2}$ M to $3.5x10^{-2}$ M, it is recommended to store the enzyme in 0.05 M sodium phosphate or other buffer of equivalent ionic strength. The buffalo lung enzyme hydrolyzed Z-Phe-Arg-MCA (V_{max}/K_m=13.21) was found to be the most efficient substrate followed by Z-Arg-Arg-MCA, BANA and BAPNA. The preferred protein substrate for the enzyme is found to be haemoglobin. The enzyme also hydrolyzed other protein substrates such as bovine serum albumin, ovalbumin and casein, but to lesser extents. In contrast to prevailing opinion, it was concluded that cathepsin B can act for a limited period even at physiological temperature, pH and salt concentration before it is inactivated.
Keywords: Lysosomal cysteine proteinase, Buffalo lung, Cathepsin B, Physiological parameters, Protein substrates.

INTRODUCTION

Lysosomes contain a considerable number of proteases. Among them the best known are the cathepsins. The cathepsin family contains mainly cysteine (Cys) proteases, although Cathepsins A and G are serine proteases and Cathepsins D and E are aspartic proteases. [1,2] They are all members of the papain family, which belongs to the clan CA (cathepsins) of cysteine proteases. To check uncontrolled proteolytic activity, cathepsins are synthesized as inactive zymogens. The processing of the inactive zymogen into a catalytically active enzyme usually occurs within the lysosome by other active proteases or by autocatalysis under certain specific conditions, such as low pH or the presence of glycosaminoglycans. [3] Although, the major regulators of cathepsin activity are endogenous protein inhibitors such as stefins, cystatins etc., the activity of some cathepsins can also be lost by degradation or by oxidation of the Cys residue in the catalytically active site. [4]

Besides their main function in turnover of intracellular proteins, lysosomal cysteine cathepsins play significant roles in a variety of physiological processes such as Ag processing, bone remodelling, wound healing, prohormone and proenzyme activation and other pathological disorders. [1,3,5-7] Of these proteinases, although

cathepsin B [EC 3.4.22.1] is the most abundant and the most thoroughly studied enzyme, its mechanism of action at molecular level is not yet understood clearly. Cathepsin B acts both as endopeptidase and dipeptidyl carboxypeptidase. [8,9] Since the enzyme is very sensitive towards temperature (unstable on storage at 40°C and above) and acts optimally in a slightly acidic medium, [5,10,11] its involvement in protein degradation under physiological conditions has been questioned. However, our knowledge of the significance of the existence of cathepsin B is still incomplete and a systematic study under appropriate conditions, especially with respect to its action in vivo, is required to resolve the ambiguity.

In the present report we have studied the dependence of the activity and stability of cathepsin B from buffalo lung on various physiological parameters. Our results clearly indicate that the catheptic activity persists for a limited period even at physiological pH and temperature before the enzyme is inactivated. Likewise, the enzyme was found to be more stable at relatively low ionic strengths. These data, therefore, assume special significance not only in that the first report of its kind as it may affect the inferences made in earlier studies on cathepsin B employing buffers of very high ionic strength, [5] but also that the enzyme might play a role in intracellular protein degradation.

MATERIALS AND METHODS

Lungs from the freshly slaughtered buffaloes were collected in ice from the local slaughter house and processed immediately. BANA, BAPNA, Z-Phe-Arg-MCA, Z-Arg-Arg-MCA, DMSO, casein, hemoglobin, ovalbumin, BSA, EDTA, TCA and 2-naphthylamine were purchased from Sigma Chemical Co., USA. Other chemicals were of analytical grade and were obtained from commercial sources.

Cathepsin B was isolated and purified from buffalo lungs by a modification of the method of Fazili and Qasim. [12] Protein concentration was determined by the method of Bradford, [13] with BSA as standard.

Enzyme assay

BANA (1mg/ml) was prepared by first dissolving 10 mg in 0.3 ml of DMSO, followed by its dilution to the desired concentration in appropriate buffer. The assay of the catheptic activity at different temperatures was done by activating the enzyme for 30 min in 2 ml of appropriate buffer containing 2 mM each of EDTA and β-mercaptoethanol (also see the legends to figures), and the reaction was started by the addition of 1 ml substrate solution in the same buffer. The released 2-naphthylamine was monitored continuously for 30 min by a Shimadzu spectrofluorophotometer, model RF-5301, fitted with a thermostated cell holder, using an excitation and emission wavelengths of 335nm and 410 nm, respectively. [14] The correction in emission intensity of 2-naphthylamine due to pH changes, [5] was routinely made by measuring the emission of standard 2-naphthylamine at the desired pH. BAPNA hydrolase activity was measured colourimetrically at 400 nm. Assays with 7-amino-4-methylcoumarin releasing substrates like Z-Arg-Arg-MCA and Z-Phe-Arg-MCA were done fluorimetrically. [5] The assay of cathepsin B against acid-denatured protein substrates was carried out by estimating the TCA-soluble peptides following the method of Moore and Stein. [15]

One activity unit for the synthetic peptide substrate, BANA, was defined as the amount of enzyme required to release 1μmole of 2-naphthylamine per min at 37°C.

Kinetic studies

The values of catalytic parameters (K_m and V_{max}) were computed from the least squares analysis of the best fit data plotted according to the method of Lineweaver and Burk, [16] using the general equation,

$1/\upsilon = K_m/V_{max}\,(1/[S]) + 1/V_{max}$ ... (1)

Substrate concentrations were chosen on the assumption that the initial velocity of the enzymatic reaction provided accurate values for K_m when the substrate concentration ranges between 20-80% saturation.

RESULTS

Buffalo lung cathepsin B used in this study was found to be homogenous both with respect to size as well as charge. [17] Our enzyme preparation was also free from other closely related cathepsins, H and L, was confirmed by studying the effect of leupeptin and urea on the protease activity. Total inhibition of the enzyme with urea (1M and above) eliminates the possibility of cathepsin L contamination. [5] Likewise, the enzyme was completely inhibited by leupeptin at all concentrations (0.01-1.0 mM) which ruled out the presence of cathepsin H in our preparation. [5]

Temperature optimum and stability

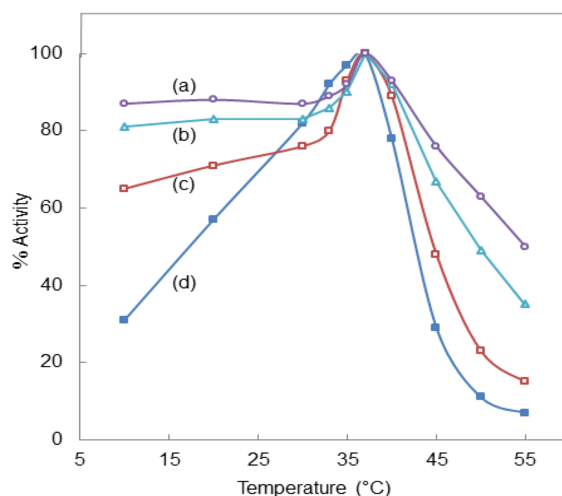

Fig. 1. Heat stability [(a)-(c)] and temperature dependence of the activity (d) of purified buffalo lung cathepsin B. The enzyme (0.15 mg) assay was made in 0.02 M sodium phosphate buffer, pH 6.5, at different temperatures (d) or at 37°C prior to which the enzyme was exposed to various temperatures for 10 min (a), 20 min (b) and for 30 min (c).

After incubation of cathepsin B at pH 6.5 and at different temperatures for 10, 20 and 30 min separately, the residual activity (in each case) was measured at 37°C and expressed as a percentage of the maximum activity. The results thus obtained are depicted in Figure 1a,b and c. The enzyme was found to be fairly stable for at least 30 min up to 37°C (Fig. 1c) but lost its activity rapidly when the temperature exceeded 37°C. Surprisingly, the enzyme doesn't lose much of its activity beyond 37°C when it was exposed either for 10 min or 20 min (Fig.2a and b). The results on the temperature dependence of the catheptic activity are shown in Fig.1 (Fig 2d). The activity was found to be maximal at about 37°C and decreased rapidly on either side of it.

pH optimum and stability

The influence of pH on the activity of cathepsin B at 37°C is shown in Figure 2d. The activity was maximal at pH 7.0 and decreased gradually below pH 7.0 and rapidly above pH 7.0. The stability of cathepsin B towards pH was checked by incubating the enzyme without substrate at 37°C and between pHs 3.5 and 9.0 for 10, 20 and 30 min batches. The residual activity was measured at pH 6.5 in all the three experiments and the results thus obtained are depicted in Figure 2a,b and c. The curves clearly show that the enzyme is stable up to pH 6.75 for at least 20 min (Fig. 2b). However, the enzyme exposed for 30 min was less stable between pH 3.5-6.75, but highly unstable above 6.75 (Fig. 2c).

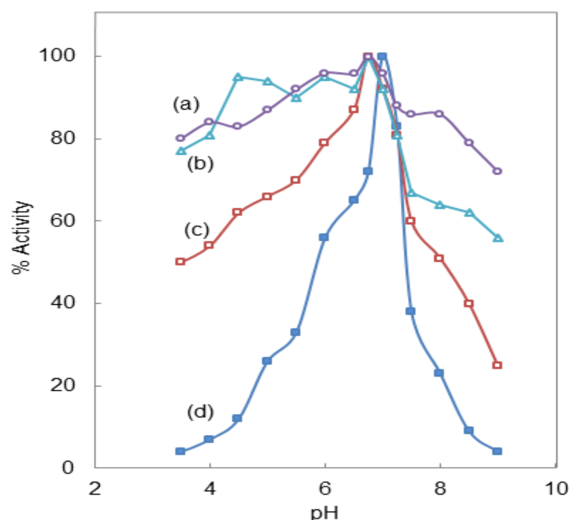

Fig. 2. pH stability [(a)-(c)] and pH dependence of the activity (d) of buffalo lung cathepsin B. The enzyme (0.15 mg) assay at 37°C was made in 0.02 M sodium phosphate buffer of different pHs (d) or at pH 6.5 following exposure of the enzyme to various pHs for 10 min (a), 20 min (b) and for 30 min (c).

Effect of salt concentration

The activity of cathepsin B was measured at 37°C, pH 6.5 and the results thus obtained are shown in Figure 3d. It is found that the maximal activity is expressed at about 2.5×10^{-3} M buffer. A sharp decline in the activity was observed both sides i.e. below 2×10^{-3} M and above 3×10^{-3} M buffer concentration. About 50% of the activity was lost when the buffer molarity was increased from 2.5×10^{-3} M to 1×10^{-2} M. A gradual decrease in activity was further seen as the buffer concentration was raised beyond 1×10^{-2} M.

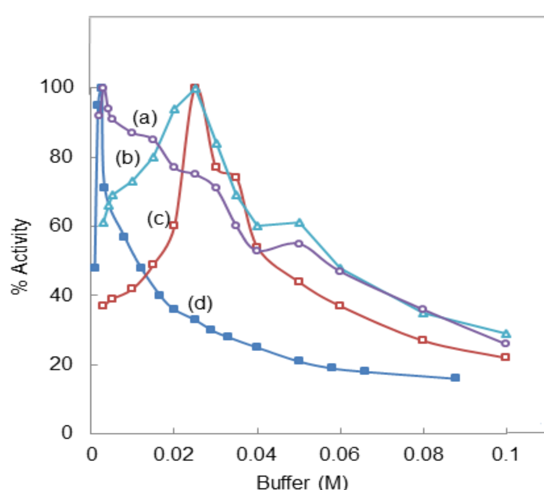

Fig. 3. Effect of buffer concentration on the stability [(a)-(c)] and activity (d) of buffalo lung cathepsin B. The enzyme (0.1 mg) assay at 37°C was done in the phosphate buffer of various molarity (d) and in 0.02 M sodium phosphate buffer, pH 6.5, after exposing the enzyme to buffers of different concentrations for 10min (a), 20 min (b) and 30 min (c).

Stability on salt concentration

The Figure 3a,b and c show the dependence of the residual activity measured at 2.5×10^{-3} M buffer concentration after exposing the enzyme to the buffers of various molarity for 10, 20 and 30 min respectively. After exposing the enzyme up to 10 min, the Curve (Fig. 3a) was almost similar to that of the dependence of the activity on salt concentration (Fig. 3d) except that an increase (~40%) in the enzyme activity was observed from 1×10^{-2} M to 6×10^{-2} M. However, when the Cathepsin B was exposed to the buffers of various molarity either for 20 min or 30 min (Fig. 3b and c), the enzyme was maximally stable between 1.5×10^{-2} M – 3.5×10^{-2} M buffer concentration.

Degradation of protein substrates

As described above the stability of cathepsin B at physiological conditions (pH, temperature and buffer concentration) led us to check for the catheptic activity against protein substrates. The results thus obtained are summarized in Table 1. Out of the four protein substrates, hemoglobin was found to be the best substrate whereas casein proved to be the least preferred substrate.

Table 1. Activities of buffalo lung cathepsin B against various protein substrates[a]

Substrate	Concentration (μg/ml)	Enzyme activity[b] (%)
Hemoglobin	75	30
	150	65
	225	100
BSA	75	18
	150	28
	225	60
Ovalbumin	75	10
	150	17
	225	32
Casein	75	8
	150	13
	225	21

[a] Enzyme concentration was 0.18 mg/ml. All the measurements were made at 37°C in 0.02 M sodium phosphate buffer, pH 6.5, and represent the mean of at least three independent experiments
[b] Enzyme activities are reported relative to that toward hemoglobin (225 μg/ml), which is expressed as 100%

Kinetic properties

Analysis of the best fit kinetic plots of cathepsin B (Table 2) revealed that lung enzyme has great catalytic potential against synthetic peptides at pH 6.5. Among the four synthetic substrates tested, Z-Phe-Arg-MCA with V_{max}/K_m value of 13.21 was found to be the most preferred followed by Z-Arg-Arg-MCA, BANA and BAPNA (see Table 2).

Table2. Kinetic parameters of buffalo lung cathepsin B

Substrate	K_m (mM)	V_{max} (μmole/mg/min)	V_{max}/K_m
BANA	2.95	0.362	0.123
BAPNA	1.90	0.060	0.032
Z-Phe-Arg-MCA	0.10	1.321	13.210
Z-Arg-Arg-MCA	0.19	0.785	4.131

DISCUSSION

There are several reports, [9,10] that cathepsin B is only active at acidic pH and

therefore, it is unlikely to play a role in protein breakdown under physiological conditions where the pH is maintained between pH 7.0-7.5. [18] Our data on buffalo lung enzyme clearly indicate that it has a pH optimum of pH 7.0 which is comparable to the value of about pH 6.7, [19] but significantly higher than pH optima (about pH 6.0) reported by others from different sources. [20-22] However, the enzyme is stable for at least 20 min up to a pH of 6.75 (Fig. 2). Similarly, our results on the thermal stability of the enzyme show that the enzyme not only has maximum activity at physiological temperature but also that it is fairly stable for at least 30 min and retains most of its activity till the temperature is raised well above 37°C (Fig. 1).

The activity of buffalo lung enzyme seems to highly dependent on the buffer concentration of the assay mixture (see Fig.3). Although the formation of enzyme-substrate complex is maximally favoured at $2.5x10^{-3}$ M buffer concentration, cathepsin B is not very stable at this ionic strength. This will lead to a sharp decrease in the enzyme activity as the molarity of buffer is increased from $2.5x10^{-3}$M to $1x10^{-2}$M at which neither the enzyme is stable nor the formation of ES complex is facilitated to the extent it was done at $2.5x10^{-3}$M buffer concentration. It is because of that at a very low buffer concentration, the enzyme structure is destabilized due to severe electrostatic repulsions among the negatively charged groups of enzyme at pH 6.5 and at a high salt concentration, the binding of the enzyme to the substrate and the stability of the ES complex may be affected. This is supported by our results on the effect of buffer concentration on the stability of cathepsin B (see Fig. 3a, b and c). The results are very interesting and tend to suggest that the enzyme is maximally stable between $1.5x10^{-2}$M-$3.5x10^{-2}$ M buffer concentration. It is to be noted that in most of the earlier studies involving cathepsin B, buffers having a high ionic strength were used, [10,23,24] which might have affected the final results. We, therefore, recommend to use 0.01-0.02 M sodium phosphate buffer for the assay of cathepsin B activity. For storage of the enzyme, however, a 0.05 M sodium phosphate or other buffer of equivalent ionic strength has been suggested.

Among the N-blocked arginine derivatives, 2-naphthylamide and para-nitroanilide, lung cathepsin B cleaved BAPNA most effectively (see Table 2). A lower K_m for BAPNA (1.90 mM) than BANA (2.95 mM) may be explained possibly due to a better fit nitroanilide in the S_1 pocket of catalytic site of the enzyme. Comparison of the kinetic results with similar data obtained for the buffalo kidney and liver cathepsin B, [12,25] revealed that the lung enzyme is catalytically less efficient with BANA. The enzyme showed markedly higher specificity towards fluorogenic substrates, Z-Arg-Arg-MCA and Z-Phe-Arg-MCA; the latter was found to be 2 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 group like phenylalanine. [26] A similar order of substrate specificity was reported for mammalian, piscean and avian cathepsin B. [27-29] However, larger K_m values of avian and piscean enzymes, [27,28] for Z-Arg-Arg-MCA and Z-Phe-Arg-MCA hydrolysis reflect species difference between avian/piscean and mammalian enymes. These results also emphasize that buffalo lung cathepsin B is more efficient towards fluoregenic substrates compared to avian/piscean enzyme.

CONCLUSION

In view of the above observations, taken together with the significant activity of the enzyme observed with various protein substrates (Table 1), indicate that the thiol protease studied by us has sufficient stability towards all the physiological variables reported here and might degrade proteins in vivo. We, therefore, suggest that the catheptic activity can persist for a limited period which could cause

proteolysis even under physiological conditions before the enzyme is inactivated. Further, catalytic data taken together; also indicate a tissue/species dependence of cathepsin B.

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Abbreviations: BANA, α-N-benzoyl-DL-arginine-2-naphthylamide; DMSO, dimethyl sulphoxide; EDTA, ethylenediaminetetraacetic acid; BAPNA, α-N-benzoyl-DL-arginine-4-nitroanilide; BSA, bovine serum albumin; TCA, trichloroacetic acid.

REFERENCES

1. Turk V, Turk B, Turk D. Lysosomal cysteine proteases: facts and opportunities. EMBO J. 2001; 20: 4629-4633.
2. Rossi A, Deveraux Q, Turk B, Sali A. Comprehensive search for cysteine Cathepsins in the human genome. Biol Chem. 2004; 385: 363-372.
3. Zavasnik-Bergant T, Turk B. Cysteine Cathepsins in the immune response. Tissue Antigens. 2006; 67: 349-355.
4. Turk B, Turk D, Turk V. Lysosomal cysteine proteases: more than scavengers. Biochem Biophys Acta. 2000; 1477: 98-111.
5. Barrett AJ, Kirschke H. Cathepsin B, Cathepsin H, and cathepsin L. Methods Enzymol, 1981; 80: 535-561.
6. Turk V, Stoka V, Vasiljeva O, Renko M, Sun T, Turk B, Turk D. Cysteine cathepsins: from structure, function and regulation to new frontiers. Biochim Biophys Acta. 2012; 1824: 68-88.
7. Ravish I, Neera R, Curcumin as inhibitor of mammalian Cathepsin B, Cathepsin H, acid phosphatase and alkaline phosphatise: a correlation with pharmacological activities. Med Chem Res. 2014; 23: 2847-2855.
8. Aronson NN, Barrett AJ. The specificity of cathepsin B. Hydrolysis of glucagon at the C-terminus by a peptidyldipeptidase mechanism. Biochem J. 1978; 171: 759-765.
9. Bond JS, Barrett AJ. Degradation of Fructose-1,6-bisphosphate aldolase by cathepsin B. Biochem J, 1980; 189: 17-25.
10. McDonald JK, Ellis S. On the substrate specificity of cathepsins B1 and B2 including a new fluorogenic substrate for cathepsin B1. Life Sci. 1975; 17: 1269-1276.
11. Kirschke H, Langner J, Riemann S, Wiederanders B, Ansorge S, Bohley P. Protein Degradation in Health and Disease. (D Evered and J Whelan, eds.), Amsterdam: Elsevier; 1980, 15-35.
12. Fazili KM, Qasim MA. Purification and some properties of buffalo liver cathepsin B. J Biochem. 1986; 100: 293-299.
13. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72: 248-254.
14. Agarwal SK, Khan MY. A probable mechanism of inactivation by urea of goat spleen cathepsin B. Unfolding and refolding studies. Biochem J. 1988; 256: 609-613.
15. Moore S, Stein WH. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J Biol Chem. 1954; 211: 907-913.
16. Lineweaver H, Burk D. The determination of enzyme dissociation constant. J Am Chem Soc. 1934; 56: 658-666.
17. Agarwal SK, Ukil A, Sharma S. Purification and characterization of cathepsin B from buffalo (Bubalus bubalis) lung. J Biochem Tech. 2016; 7: 1051-1057.
18. Mort JS, Recklies AD, Poole AR. Extracellular presence of the lysosomal proteinase cathepsin B in rheumatoid synovium and its activity at neutral pH. Arthritis & Rheumatism. 1984; 27: 509-515.
19. Fok AK, Paeste RM. Lysosomal enzymes of Paramecium caudatum and Paramecium tetraurelia. Exp Cell Res. 1982; 139: 159-169.
20. Otto K. Tissue proteinases. (AJ Barrett and JT Dingle, eds), Amsterdam: North-Holland Publ. Co; 1971, 1-28.
21. Barrett AJ. A new assay for cathepsin B1 and other thiol proteinases. Anal Biochem. 1972; 47: 280-293.
22. Znovar-popovic T, Lah T, Kregar I, Turk V. Some characteristics of cathepsin-B and

alpha-N-benzoylarginine-beta-naphthylamide hydrolase from bovine lymph-nodes. Croat Chem Acta. 1980; 53: 509-517.
23. Mycek MJ. Cathepsins. Methods Enzymol. 1970; 9: 285-315.
24. Turk V, Kregar I. Methods of Enzymatic Analysis. (J Bergmeyer and M Grabl, eds) Weinheim: Verlag Chemie Gmbh; 1984, Vol. V, 195-210.
25. Lamsal M, Agarwal SK, Choudhury SD, Khan MY. Purification and tissue/species dependence of the specificity of buffalo kidney cathepsin B. Ind J Biochem Biophys. 1997; 34: 461-469.
26. Takahashi T, Yonezawa S, Dehdarani H, Tang J. Porcine spleen cathepsin B is an exopeptidase. J Biol Chem. 1986; 261: 9368-9374.
27. Yamashita M, Konagaya S. Purification and characterization of cathepsin L from the white muscle of chum salmon. Oncorhynchus keta Comp Biochem Physiol 1990; 96B: 247-252.
28. Wada K, Tanabe T. Purification and characterization of chicken liver cathepsin B. J Biochem. 1988; 104: 472-476.
29. Kirschke H, Barrett AJ. Lysosomes: Their Role in Protein Breakdown. (Glaumann H and Ballard FJ, eds) New York: Academic Press; 1987, 193-238.

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