STUDIES ON THE PHYSICO - CHEMICAL AND BIOLOGICAL PROPERTIES OF BUFFALO *(Bubalus bubalis)* KIDNEY CATHEPSIN B.

By

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CERTIFICATE

Certified that the thesis entitled "STUDIES ON THE PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF BUFFALO *(Bubalus bubalis)* KIDNEY CATHEPSIN B" submitted by Mr. **Madhab Lamsal** for the award of the degree of DOCTOR OF PHILOSOPHY in Biochemistry of the North-Eastern Hill University Shillong, embodies the record of original investigations carried out by him under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of Ph.D degree. This work has not been submitted for any degree of any Univesity.

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ABSTRACT

Owing to their ubiquitous distribution in biological tissues and fluids and probable participation in different physiological and pathological processes, proteolytic enzymes have been the subject of critical investigation for the past few decades. It is now well established that all the lysosomal proteinases characterized till date belong to one or other of the four classes: cysteine, aspartic, serine or metalloproteinases. Cysteine proteinases, characterized by the presence of an essential cysteine residue at the active site, are involved in lysosomal as well as extra-lysosomal protein degradation and turnover. A number of proteinases with acidic pH optima, known as "cathepsins" are found in the lysosomal system. Of these, cathepsins B,H,L,S and M of the cysteine group, cathepsin D of aspartyl group and cathepsin G of the serine group constitute the chief proteinases which have been studied in details.

Cathepsin B (EC 3.4.22.1) is the most well characterized lysosomal cysteine proteinase belonging to the papain superfamily. Under normal conditions, this enzyme participates in vital physiological processes that involve cellular protein turnover. However, in cases where the enzyme becomes deregulated, cathepsin B has been shown to be associated with a number of diseased states.

In the present study, a simple procedure for simultaneous purification of cathepsins B and H from buffalo kidney has been developed. Purified buffalo kidney cathepsin B was found to be homogeneous on the basis of charge as well as size.

Molecular weight of the purified enzyme both from gel filtration and SDS-PAGE studies was found to be very close (26 kDa) to what has been reported for this enzyme from other sources. The stokes radius was calculated to be 2.32. However, electrophoretic studies both in absence and presence of SDS resulted into single prominent protein band suggesting that the enzyme lacked subunit structures and/or isozymes.

Buffalo kidney cathepsin B had an isoionic pH of 5.1, which was well within the range (4.8-5.3) reported for this enzyme from other sources.

One of the striking differences between buffalo kidney cathepsin B and those isolated from other sources was the presence of alanine as the NH_2- terminal amino acid residue in the former as against leucine in others. However, the COOH-terminal amino acid residue was found to be threonine which was the same as reported for cathepsin B from other tissues/sources. This may either be attributed to simple species dependence or more significantly to the possible differential post translational processing of the enzyme in the kidney tissues.

The total free sulfhydryl groups contents were found to be

0.6 and 1.6 mol/ mol of proteins under native and denaturing conditions respectively, suggesting their partial burial under the native conditions.

The glycoprotein nature of the enzyme was confirmed with the total content of about 3.6 (glucose equivalent). This value was significantly lower than what is found in cathepsin B from buffalo spleen or porcine spleen and could possibly be responsible for the altered hydration and catalytic and immunological characteristics of the buffalo kidney enzyme.

Amino acid composition of buffalo kidney cathepsin B showed close similarities with its counterparts from other sources like bovine, porcine, human and rat tissues with an exception to serine. The extinction coefficient ($E^{1\%}_{1cm}$) for this enzyme was found to be 16.78, which was comparable to the values determined for the enzyme from other sources.

Like its counterparts from other sources/tissues, buffalo kidney cathepsin B was found to be very sensitive to parameters like temperature, pH, ionic strength and radiation. The enzyme showed maximum activity near the physiological levels of pH (6.8) and temperature (40°C). One of the striking characteristics of the kidney enzyme was that it underwent reversible inactivation at higher salt concentration or buffer strength. Hence for most enzymatic assays, the buffer strength was restricted to 20 mM, while excess salt was used for the long term storage of the

enzyme. Low doses of gamma-irradiation (2-20 Gy) had a stimulatory effect on the enzyme, whereas, higher doses (>30 Gy) led to significant loss in its activity.

Like any other cysteine proteinases, the buffalo kidney enzyme required presence of thiol reducing agents to express its optimal catalytic activity. Among the various thiol reducing agents tested, DTT and cysteamine-HCl were found to be the most and the least effective respectively. The thiol blocking compounds such as $HgCl_2$ and $ZnSO_4$, alkalyting agents like iodoacetic acid and iodoacetamide, and peptidyl inhibitors like leupeptin, antipain, chymostatin and E-64 acted as potent inhibitors of the enzyme. Pepstatin, a well known inhibitor of asparty1 proteinases, including cathepsin D, on the other hand was found to be ineffective towards the buffalo enzyme.

Denaturants such as urea and Gdn-HCl had profound effect on the activity of the kidney enzyme. More than 50% of the enzyme activity was lost at the urea concentrations >1.0 M and virtually no activity was found above 3.0 M. Reactivation studies showed that the enzyme activity could be regained only partially. This in consistence to what has been reported for its counterparts from liver and spleen. In addition, it also rules out the possibility of contamination of our cathepsin B preparation with cathepsin L.

Gdn-HCl was highly effective against the kidney cathepsin B

and it suppressed more than 50% of the enzymatic activity at a concentration as low as 0.01 M. The inactivation of the enzyme was found to be reversible for Gdn-HCl exposure up to 0.1 M. Urea and Gdn-HCl induced inactivation studies of buffalo kidney cathepsin B taken together, therefore suggest that although the concentrations of those denaturants required to fully inactivate the enzyme were too low to cause any major change in the gross structure of the enzyme, yet, the possibility of some minor "structural perturbations" around the enzyme active site effecting its activity is not ruled out.

The results on the kinetic studies of cathepsin B from buffalo kidney indicated that the enzyme has great catalytic potential against various synthetic as well as protein substrates at acidic/ or near neutral pHs. Among the four synthetic substrates tested, Z-Phe-Arg-MCA with K_m of 0.0909 mM was found to be the most preferred followed by Z-Arg-Arg-MCA (0.166 mM), BAPNA (0.1818 mM) and BANA (3.33 mM). No activity could be observed against Arg-NA, Leu-NA or Arg-MCA. These K_m were well within the range available for cathepsin B from other sources. However, the corresponding V_{max} values differed drastically from what is reported for cathepsin B from other sources.

Goat and buffalo haemoglobins with the corresponding K_m values of 1.428 μM and 2.173 μM respectively, were found to be the most preferred protein substrates for buffalo kidney cathepsin B.

Immunological studies showed that antibodies could be raised in rabbits against purified and mature buffalo kidney cathepsin B that recognizes both native as well as pH denatured cathepsin B from the buffalo kidney source. Thus, the anti-buffalo kidney cathepsin B antibody could be used as an effective tool to distinguish the enzyme from other related proteinases like cathepsins H and L from same or different sources.

A comparison of the above data on buffalo kidney cathepsin B with published results on cathepsin B from other sources thus reveals that buffalo kidney enzyme is similar to its counterparts from other sources with respect to (i) Molecular weight, (ii) hydrodynamic properties, (iii) catalytic nature, (iv) response to inhibitors and (v) optical properties, but, differ significantly with regards to its (1) NH_2- terminal amino acid residue, (2) carbohydrate contents, (3) serine contents, (4) catalytic efficiencies against various synthetic peptide and natural protein substrates including muscle aldolase, (5) immunological properties and lack of multiple chain form and/or isozyme. All these findings taken together therefore, suggests a strong species and/or tissue dependence of cathepsin B in mammalian sources.