



A simple purification procedure of buffalo lung cathepsin H, its properties and influence of buffer constituents on the enzyme activity

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ABSTRACT

Background: Cathepsin H (E.C.3.4.22.16) belongs to a family of lysosomal cysteine protease which regulates diverse normal biological processes mainly in intracellular proteolysis.

Methods: Purification of cathepsin H from an unstudied system i.e. buffalo lung has been achieved by a simple process developed after incorporating appropriate alteration in the available methods for isolation of the enzyme from other sources. The use of DEAE-Cellulose and SP-Sephadex C-50 helped in better and simultaneous separation of cathepsin B and H up to homogeneity.

Results: The SDS-PAGE result showed buffalo cathepsin H to be a single-chain molecule having MW, NH₂- and COOH- terminal residues of 25.4 kDa, Lys and Val respectively. The enzyme was a glycoprotein with pI of 6.2; it hydrolyzed Leu-NA ($V_{max}/K_m = 301.6$) as the most efficient substrate followed by Arg-NA, Arg-Arg-NA and BANA. Buffalo enzyme showed maximum activity at 36 °C, pH 6.75 and at a buffer concentration of 2×10^{-3} M. **Conclusion:** Catheptic activity was found to be quite stable at least for 20–30 min between pH 4.5–7.0, buffer concentration of 1×10^{-2} to 4×10^{-2} M and the temperature resistance up to 36 °C. The effects of various substances present in the buffers routinely used for the assay of catheptic activity revealed that the activity of buffalo lung cathepsin H depends not only qualitatively but also quantitatively on the constituents of assay buffer.

General significance: This study seems to provide valuable information regarding the biochemistry of cathepsin H in general as well as influence of buffer constituents on enzyme activity and physiological role in particular.

1. Introduction

Mammalian tissues consist a number of cysteine proteases, such as cathepsin B, H, L and S, which function under acidic conditions and are generally assumed to be located in the lysosomes [1]. On the basis of amino acid sequence similarities, they are considered as members of the papain superfamily [2,3]. In spite of structural similarity [4–6], they exhibit a great diversity of proteolytic activities [3]: cathepsin B shows both endopeptidase [1] and carboxypeptidase [7] activities, cathepsin L and S split peptide substrates exclusively at endoproteolytic cleavage sites [8], whereas cathepsin H possesses endopeptidase as well as aminopeptidase activities [1,9,10]. Of these proteinases, cathepsin H (EC 3.4.22.16) is a relatively copious proteinase distributed ubiquitously in tissues and implicated in intracellular protein degradation [11,12]. Many emerging facts stated that the expression level of cathepsin H is elevated during disease states, including tumour metastasis, breast carcinoma, melanoma and prostate cancer [13–15]. The enzyme also shows their contribution in the pathogenesis of numerous

diseases such as parasitic [16], bacterial [17], fungal [18] viral [19], cardiovascular [20] and neurodegenerative disorder [21]. A specific physiological function of cathepsin H has been identified in the processing of pulmonary surfactant protein B in alveolar type II cells [22]. However, the precise role(s) of cathepsin H *in vivo* still remains speculative.

Cathepsin H has been purified from human liver [23], kidney [24], brain and meningioma [25], rat spleen [26] and liver [27], bovine spleen [28] and brain [29], porcine spleen [30], rabbit lung [31] and goat liver [32], and the molecular mass of the enzyme has been reported in the range of 25000–30000 [1,6,33]. Most of the earlier purifications of cathepsin H started from tissue sources of limited availability. Since for our studies we need sufficient quantities of the enzyme, we have selected water buffalo (*Bubalus bubalis*) lung as the source of cathepsin H. The isolation and properties of cathepsin H have not been reported from this source. Since the enzyme is extremely sensitive to pH and temperature [34], and having high dependence of its activity on a number of compounds, the experimental results on

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cathepsin H tend to vary from one laboratory to other [23–31]. Unfortunately, little attention has been given to this problem. Therefore, we describe here not only a simple procedure of purification and some properties of buffalo cathepsin H, but also a systematic study on the effects of various substances present in the buffers routinely used for the assay of catheptic activity.

2. Materials and methods

Lungs of freshly slaughtered buffaloes were collected in ice from the local slaughter house and were stored at subzero temperature until used. L-Leu-2-naphthylamide (Leu-NA), L-Arg-2-naphthylamide (Arg-NA), Z-Arg-Arg-2-naphthylamide (Arg-Arg-NA), α -N-benzoyl-DL-arginine-2-naphthylamide (BANA), β -naphthylamine, ethylenediamine tetra acetic acid (EDTA), sodium azide (NaN_3), urea, Commais brillant blue R-250, azocasein, bovine serum albumin (BSA), β -mercaptoethanol, sodium dodecyl sulphate (SDS) and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. St. Louis, Missouri, USA. Blue Dextran 2000, Sephadex G-75, SP-Sephadex C-50 and DEAE Cellulose were purchased from Pharmacia, Seweden. Sucrose, silver nitrate and other products were of Sisco, Bombay. Protein markers ranging from 10 kDa to 180 kDa used for the molecular mass determination were from Thermo Fisher Scientific, Lithuania. Other routine chemicals including $(\text{NH}_4)_2\text{SO}_4$, NaCl, KCl, NH_4Cl , MgCl_2 , CaCl_2 and BaCl_2 used in this study were generally of analytical grade.

2.1. Isolation and purification of cathepsin H

Isolation of buffalo lung cathepsin H was carried out at 4 °C by incorporating proper modifications in the procedure described earlier for cathepsin B [35]. One kilogram soft tissue mass obtained after cleaning (involving removal of connective tissues, membranes and fats) 1.2 Kg of the buffalo lung was homogenized for 15 min in 200 ml of 3% NaCl solution containing 1 mM EDTA, pH 1.8 and finally stirred for 5–6 h. The pH of the resulting homogenate was adjusted to 3.8 by the addition of chilled HCl (4 N) and it was left for 8 h under continuous stirring. A clear supernatant was obtained after centrifugation of the content at 14,000 rpm for 15 min, which was further subjected to $(\text{NH}_4)_2\text{SO}_4$ precipitation. The fraction obtained at 40–75% saturation was collected and dissolved in least amount of cold water, then dialyzed against 0.05 M sodium acetate buffer, pH 5.0 having 1 mM EDTA and 0.02% NaN_3 . The sample was again centrifuged at 14,000 rpm for 5 min and gel filtered on a Sephadex G-75 column (3.5 × 85 cm) equilibrated with the above buffer. Enzymatically active protein fraction was pooled out, concentrated via sucrose bed method and was subsequently chromatographed on DEAE-Cellulose ion exchange column (2.5 × 20 cm) equilibrated with 0.01 M sodium phosphate buffer, pH 6.4 containing 1mM EDTA and 0.02% NaN_3 . The column was extensively washed-out with the same buffer and the bound fraction eluted with 0.01 M sodium acetate buffer, pH 5.1 having 1mM EDTA, 0.02% NaN_3 and 0.4 M NaCl was found to be cathepsin B. The unbound fraction which shows the activity of cathepsin H was finally purified by chromatography on SP-Sephadex C-50 (2.5 × 22 cm) equilibrated with 0.02 M sodium acetate buffer, pH 4.8 containing 1 mM EDTA and 0.02% NaN_3 . The column was eluted stepwise by increase in pH. Cathepsin H fractions eluted at pH 6.0 with 0.4 M NaCl were collected and used after dialysis with the equilibrated buffer for further study.

2.2. Gel electrophoresis

Non-denaturing electrophoresis was performed on 10% acrylamide gels essentially by the method of Davis [36]. SDS-PAGE of standard marker proteins and of cathepsin H was also performed on 12% acrylamide gels in Tris-glycine buffer, pH 8.3, containing 1% SDS using a procedure of Laemmli [37]. First the protein solution was denatured by heating at 90–100 °C for 5–10 min in the presence of 6% SDS. After

treatment with β -mercaptoethanol and sucrose (final concentration 2% and 40% respectively), 60–100 μg of protein was loaded on the gel and electrophoresed about 6 h by applying a current of 6mA/lane. The protein bands was stained either in 0.25% solution of Commais brilliant blue R-250 prepared in 7% glacial acetic acid and 50% methanol [36,37] or in 0.1% solution of silver nitrate in deionized water as described by Blum et al. [38]. Relative mobility of SDS-protein complex was determined for cathepsin H as well as marker proteins by the standard procedure as described earlier [39].

2.3. Assay of cathepsin H

Enzyme activity was measured either by discontinuous photometric method [40] or by continuous fluoremetric procedure [41]. The spectrophotometric analysis was performed by using BANA/Leu-NA as a substrate. The assay mixture having 0.1 ml of enzyme solution and 0.4 ml activation buffer (0.02 M sodium phosphate buffer, pH 6.5 containing 2 mM each of 2-mercaptoethanol and EDTA) was incubated for 30 min at 37 °C to activate the enzyme. The substrate solution was prepared after taking 10 mg of BANA in 0.3 ml of DMSO, followed by dilution to the preferred concentration in a particular buffer. The reaction was started by the subsequent addition of 0.5 ml of substrate having final concentration of 1mg/ml. After incubation with substrate for 30 min at 37 °C, the reaction was blocked by adding up of 0.5 ml of 4 N HCl and the product was estimated by diazotization and coupling of released β -naphthylamine with N-1-naphthylethylenediamine dihydrochloride followed by colour intensity measurement at 540 nm [40]. One unit activity of cathepsin H was explained as the quantity of enzyme required to liberate 1 μM of 2-naphthylamine per h at 37 °C.

Fluorometric assay was done with substrates such as BANA, Leu-NA, Arg-NA and Arg-Arg-NA. The mixture of 1.9 ml activation buffer and 0.1 ml enzyme solution incubated at 37 °C for 30 min, thereafter enzymatic reaction was started by adding 1 ml of substrate solution (0.1%). The released 2-naphthylamine from the above substrates was monitored for 30 min on excitation and emission wavelengths of 335 and 410 nm, respectively [41]. The rectification in emission intensity of β -naphthylamine was necessary to perform due to pH changes [1] and it was usually done by measuring the emission intensity of standard β -naphthylamine at the preferred pH.

2.4. Kinetic studies

The values of K_m and V_{max} of buffalo lung cathepsin H for the synthetic substrates were computed from the least square analysis of the data plotted according to the method of Lineweaver and Burk [42], using the general equation,

$$1/V = K_m/V_{max} (1/[S]) + 1/V_{max} \quad (1)$$

The 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 and 80% saturation.

2.5. Chemical analyses

Carbohydrate content of the enzyme was monitored through the procedure of Dubois et al. [43]. The amino- and carboxyl terminal amino acid residues were identified by thin layer chromatography following the published procedure [44]. Protein in crude and purified samples of cathepsin H was estimated by the method of Bradford [45] using BSA as standard or by spectrophotometric method using specific extinction coefficient ($E_{1cm}^{1\%}$) of 18.6 determined for buffalo cathepsin H (unpublished data).

2.6. Effect of buffer components

The activity of cathepsin H was measured at 37 °C in different molarities of buffer systems at pH 6.5, containing 2mM each of EDTA and 2-mercaptoethanol. The effects of various buffer components viz. EDTA, 2-mercaptoethanol, monovalent and divalent neutral salts on the activity of cathepsin H were also studied in 0.02 M sodium phosphate buffer, pH 6.5. In this case, cathepsin H was incubated with the respective compound for 30 min prior to assay of the enzyme activity.

2.7. Statistical analysis

Data were analyzed for statistical significance using either one way analysis of variance (ANOVA) or Student's *t*-test.

3. Results

3.1. Purification of cathepsin H

For the isolation and purification of cathepsin H from various sources, a number of methods have been developed [23–32]. However, there is a scope to device methodology suitable for a particular source in terms of simplicity, reproducibility and better yield. We, therefore, developed a procedure in this study that fulfills the above criteria while dealing with buffalo lung as the source of cathepsin H. Apart from other significant changes made in the purification method of cathepsin H [24,30], we have included SP-Sephadex over CM-Sephadex chromatography due to better separation of the enzyme with the former media. The different steps involved in purification scheme of cathepsin H is summarized in Table 1. Upon gel filtration on Sephadex G-75 column (3.5 × 85 cm), the ammonium sulphate precipitated protein at step 3 of Table 1 was eluted in 5 protein peaks with 0.05 M sodium acetate buffer, pH 5.0, containing 1mM EDTA and 0.02% NaN₃ (Fig. 1). A significant amount of BANA hydrolase activity obtained between elution volume of 200–240 (indicated by horizontal bar) were combined and electrophoresed on polyacrylamide gels. As many as 5 protein bands were appeared (see Fig. 1, inset). The enzyme thus obtained was subjected to ion-exchange chromatography on DEAE-Cellulose column. The cathepsin H obtained as unbound fractions at this stage was further purified on SP-Sephadex C-50 column (2.5 × 22 cm) equilibrated with 0.02 M sodium acetate buffer, pH 4.8 containing 1mM EDTA and 0.02% NaN₃. The protein peak eluted with 0.4 M NaCl in the operating buffer, pH 6.0 showed optimal activity against Leu-NA and/or Arg-NA (specific substrates for cathepsin H) whereas the fractions eluted at pH 5.6 and pH 6.0 did not show any considerable amount of activity (see Fig. 2). The active fractions had specific activity of 11.95 units/mg of protein (Table 1) which remained unaltered up on rechromatography on the ion-exchange column.

The purified preparation of buffalo lung cathepsin H was found to be homogeneous both with respect to charge (see inset of Fig. 2) as well as size as it showed a single symmetrical protein peak fully

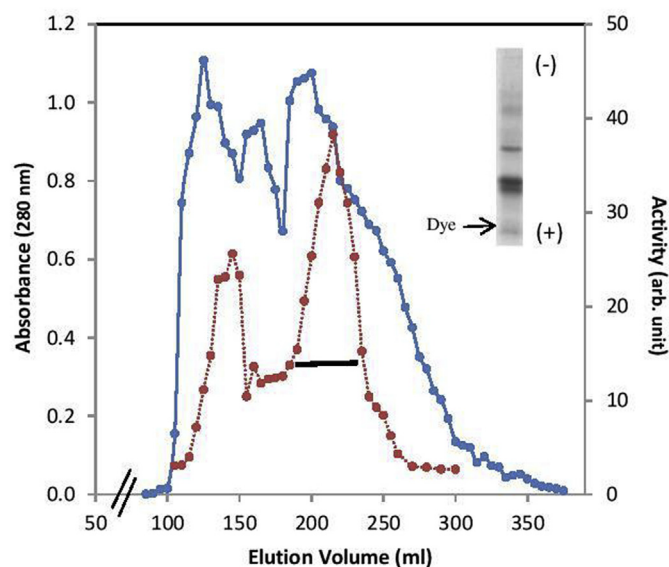


Fig. 1. Gel-filtration of (NH₄)₂SO₄ fractions (40–75%) of buffalo lung cathepsin H on Sephadex G-75 column (3.5 × 85 cm) equilibrated with 0.05 M sodium acetate buffer, pH 5.0 containing 1 mM EDTA and 0.02% NaN₃. About 100 mg of protein applied to the column was eluted at a flow rate of 20ml/h. Protein fractions (5ml) were monitored at 280 nm (—) and assayed for BANA hydrolase activity (... ..). Inset shows the PAGE pattern of pooled enzyme fractions (as shown by horizontal bar) in 10% acrylamide gel using 0.02 M Tris-glycine buffer, pH 8.3.

superimposable with its activity peak on Sephacryl S-200 column (curve not shown for brevity). The presence of other phylogenetically and catalytically related cathepsins like B, L, S and D [1,30,46,47] in our preparation were ruled out after checked out the activity with Arg-Arg-NA (specific substrate of cathepsin B), azocasein with 3.0 M urea (substrate for cathepsin L) and in presence of pepstatin A (a potent inhibitor of cathepsin D). Failure of detectable action of cathepsin H on the above substrate and indifference towards pepstatin A confirmed the absence of these related cysteine or aspartyl proteinases [1,30] in the preparation. Further, the enzyme preparation also free from cathepsin S, was supported by obtaining an irreversibly inactivated enzyme on exposure to alkaline pH and lack of activity below pH 4.5 [48,49].

3.2. Storage of the enzyme

The activity of cathepsin H was completely preserved for several months by storing the enzyme in portion of 100 μl at –20 °C in 0.02 M sodium acetate buffer, pH 4.8 containing 1mM EDTA and 0.02% NaN₃ at concentrations higher than 1mg/ml. The thawed sample was used on the same day. More than 50% of the enzyme activity was, however, abolished within a month if the pH were as high as 6.0.

Table 1

Purification of buffalo lung cathepsin H.^a

| Step | Total protein (mg) | Protein yield (%) | Total enzyme activity ^b (units) | Specific activity (units/mg) | Activity Yield (%) | Fold purification |
|---|--------------------|-------------------|--|------------------------------|--------------------|-------------------|
| Crude extract | 50090 | 100 | 2890 | 0.06 | 100 | 1 |
| Acid extraction | 2355 | 4.70 | 880 | 0.37 | 30.5 | 6.2 |
| (NH ₄) ₂ SO ₄ fraction (40–75%) | 541 | 1.08 | 338 | 0.63 | 11.7 | 10.5 |
| Gel-filtration on Sephadex G-75 | 241 | 0.48 | 273 | 1.13 | 9.4 | 18.8 |
| DEAE-cellulose ion-exchange chromatography | 30.5 | 0.06 | 198 | 6.49 | 6.9 | 108.2 |
| SP-Sephadex ion-exchange chromatography | 8.2 | 0.02 | 98 | 11.95 | 3.4 | 199.2 |

^a Data obtained with 1.2 kg of buffalo lung.

^b Enzyme activity was assayed in 0.02 M sodium phosphate buffer, pH 6.5 containing 2mM each of EDTA and 2-mercaptoethanol using L-leucine-2-naphthylamide as a substrate.

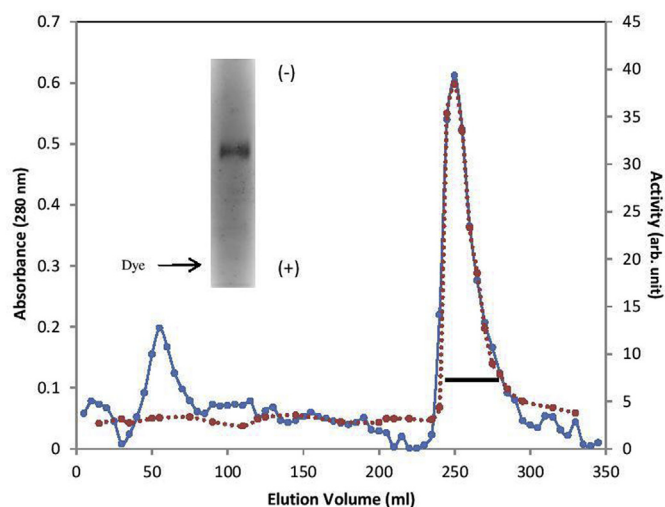


Fig. 2. Ion-exchange chromatography of buffalo lung cathepsin H on SP-Sephadex C-50 column (2.5 × 22 cm), pre-equilibrated with 0.02 M sodium acetate buffer, pH 4.8 containing 1 mM EDTA and 0.02% NaN₃. About 40mg of protein applied in the column was eluted step-wise at pH 5.6, 6.0 and finally using 0.4 M NaCl at pH 6.0. Protein concentration was measured at 280 nm (—) and enzyme activity was monitored with Leu-NA (.....). Horizontal bar shows the cathepsin H active fractions pooled for rechromatography. Inset shows the PAGE pattern of purified enzyme at pH 8.3 on 10% gel.

3.3. Molecular properties

Purified cathepsin H emerged as a single band on gel electrophoresis in presence of SDS under reducing and non-reducing conditions (see Fig. 3 lane B & C). To check any other protein band (not detectable by

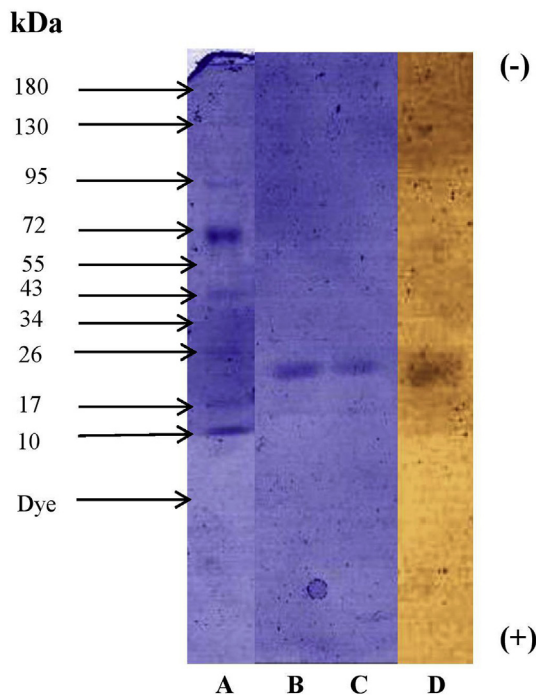


Fig. 3. SDS-PAGE pattern of marker proteins (A), cathepsin H in absence (B) and in presence (C) of 2-mercaptoethanol stained either by Commassie brilliant blue R-250 or by silver nitrate (D) in 12% polyacrylamide gels. About 50–60 μg of protein was electrophoresed at pH 8.3, I = 0.02 with a current of 6 mA per lane. Arrows indicate the position of markers. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

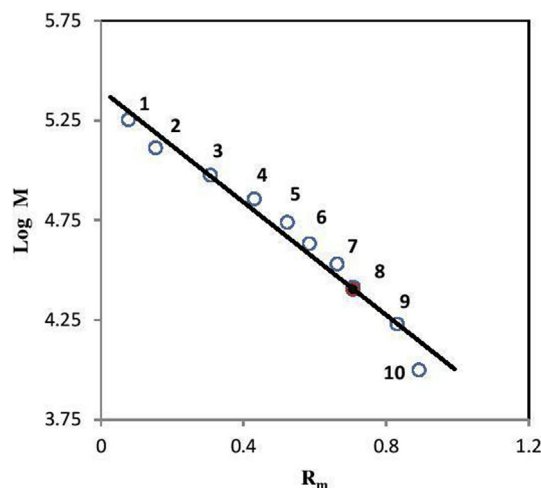


Fig. 4. The relative mobility of SDS-protein complexes of marker proteins in SDS-PAGE plotted against the logarithm of the molecular weights. The molecular weights of marker proteins were: 1. 180,000; 2. 130,000; 3. 95,000; 4. 72,000; 5. 55,000; 6. 43,000; 7. 34,000; 8. 26,000; 9. 17,000 and 10. 10,000. The filled symbol indicates the position of cathepsin H.

staining with Commassie brilliant blue R-250) of low visibility, polyacrylamide gel electrophoresed under reducing condition was also stained by silver nitrate [38]. Again a single protein band was observed (lane D of Fig. 3) having identical relative mobility. The molecular weight of the enzyme was calculated from SDS-PAGE data using the relation between relative mobility and molecular weight for marker proteins (Fig. 4). After least square analysis, the relation can be articulated in terms of a straight line equation:

$$\text{Log } M = -1.415 R_m + 5.406 \quad (2)$$

The relative mobility for cathepsin H was 0.707, which upon substitution in the above equation, yielded a molecular weight value of 25,400. The total carbohydrate content of buffalo enzyme was found to be 8.7%. The NH₂- and COOH- terminal amino acid residues of buffalo lung cathepsin H were identified as Lys and Val, respectively. The observed isoelectric pH of purified enzyme was 6.2.

3.4. Catalytic properties

Table 2 summarizes the results on the kinetic studies of the purified buffalo lung cathepsin H towards the synthetic substrates. Among the four synthetic peptides tested, Leu-NA with K_m value of 0.022 (V_{max}/K_m = 301.6) was found to be the most preferred followed by Arg-NA, Arg-Arg-NA and BANA (see Table 2).

3.5. Physiological properties

All the experiments for studying physiological parameters and influence of buffer constituents on the activity of cathepsin H were performed 3–4 times independently.

Table 2

Kinetic parameters of some synthetic substrates hydrolyzed by buffalo lung cathepsin H.^a

| Substrate | K _m (mM) | V _{max} (μmole/mg/min) | V _{max} /K _m |
|------------|---------------------|---------------------------------|----------------------------------|
| Leu-NA | 0.022 | 6.903 | 301.623 |
| Arg-NA | 0.322 | 49.653 | 153.803 |
| Arg-Arg-NA | 1.120 | 7.791 | 6.956 |
| BANA | 4.320 | 27.639 | 6.398 |

^a About 20 μg cathepsin H was incubated with substrates at different concentrations and the activity was monitored fluorimetrically.

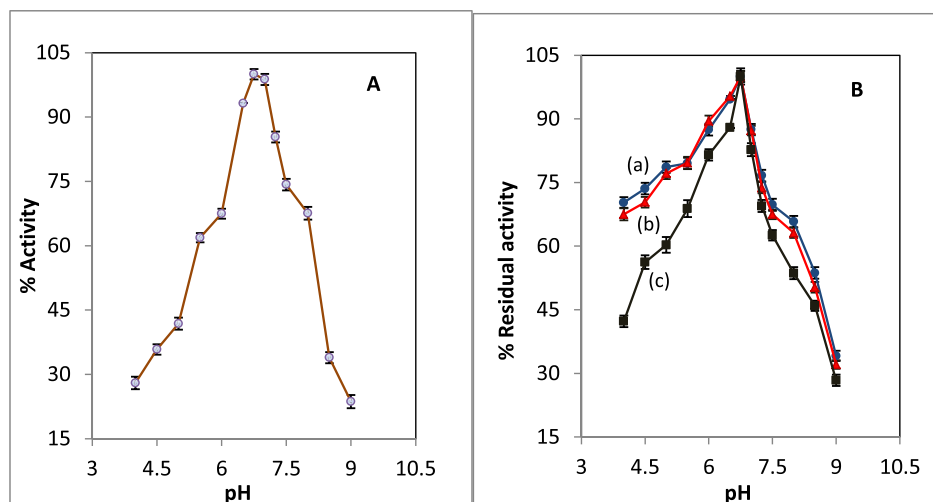


Fig. 5. pH dependence of the activity (A) and pH stability (B) of buffalo lung cathepsin H. The enzyme (0.15 mg) assay with BANA at 36 °C was made in 0.02 M sodium phosphate buffer of different pHs (A) or at pH 6.75 following exposure of the enzyme to various pHs for 10 min [B(a)], 20 min [B(b)] and for 30 min [B(c)]. The error bars represent the standard deviation of the average value for three independent experiments.

3.5.1. pH dependence and stability

The effect of pH on the activity of buffalo lung cathepsin H at 37 °C is depicted in Fig. 5A. The maximum activity of the enzyme was observed at pH 6.75; the activity decreased regularly below pH 6.75 and speedily above pH 6.75. For the purpose to study the stability of cathepsin H, the enzyme was incubated without substrate between pHs 4.0 and 9.0 at 37 °C in three different batches of 10, 20 and 30 min. The residual activity was monitored at pH 6.75 and the observations thus obtained are shown in Fig. 5B (a, b and c). The findings clearly indicate that cathepsin H is quite stable up to pH 6.75 for at least 20 min [Fig. 5B (b)]. Conversely, the enzyme exposed for 30 min was not as much as stable between pH 4.0–6.75 and very unstable above 6.75 [Fig. 5B (c)].

3.5.2. Temperature dependence and stability

The graphical result of the catheptic activity over the temperature dependence is shown in Fig. 6A. The enzyme activity was observed maximally at 36 °C and decreased rapidly above it. To check the stability of the enzyme, cathepsin H was incubated at pH 6.75 at different temperatures for 10, 20 and 30 min separately. The residual activity (in each case) was measured at 36 °C and taken as a percentage of the highest activity. The results thus obtained are presented in Fig. 6B (a, b and c). The enzyme was found to be quite stable for at least 30 min up to 36 °C [Fig. 6B (c)] but lost its activity very fast when the temperature exceed above 36 °C [see Fig. 6B (c)].

3.5.3. Salt concentration dependence and stability

Fig. 7A shows the effect of salt concentration on the catheptic activity measured at 36 °C and pH 6.75. The maximal activity of cathepsin

H was expressed at about 0.002 M buffer. However, a sharp decline in the activity was observed both sides i.e. below 0.0015 M and above 0.0025 M buffer concentration. About 50% of the activity was lost when the buffer molarity was increased from 0.002 M to 0.004 M. A gradual decline in the catheptic activity was further observed as the buffer concentration was increased away from 0.004 M.

Shown in Fig. 7B (a, b and c) is the dependence of the residual activity measured at 0.002 M buffer concentration after exposing cathepsin H to the buffers of various molarity for 10, 20 and 30 min respectively. After exposing the enzyme up to 10 min, the curve [Fig. 7B (a)] was found to be almost similar to that of the dependence of the activity on salt concentration (Fig. 7A) except that an increase (~30%) in the enzyme activity was observed from 0.004 to 0.04 M. However, when the enzyme was exposed to the buffers of various molarity either for 20 or 30 min [Fig. 7B (b and c)], cathepsin H was maximally stable between 0.01 M and 0.04 M buffer concentration.

3.6. Effect of buffer constituents

The dependence and stability of cathepsin H at physiological conditions described above led us to check for the catheptic activity in different buffer systems at 37 °C and the results thus obtained are summarized in Table 3. The maximum and minimum activities were obtained with 0.02 M sodium phosphate and potassium phosphate buffers, respectively. A substantial decrease in the enzyme activity in both the buffers was observed when their concentrations were increased either to 0.05 M or 0.1 M ($p < 0.001$). However, in the case of Tris-phosphate and ammonium phosphate buffers, the activities of

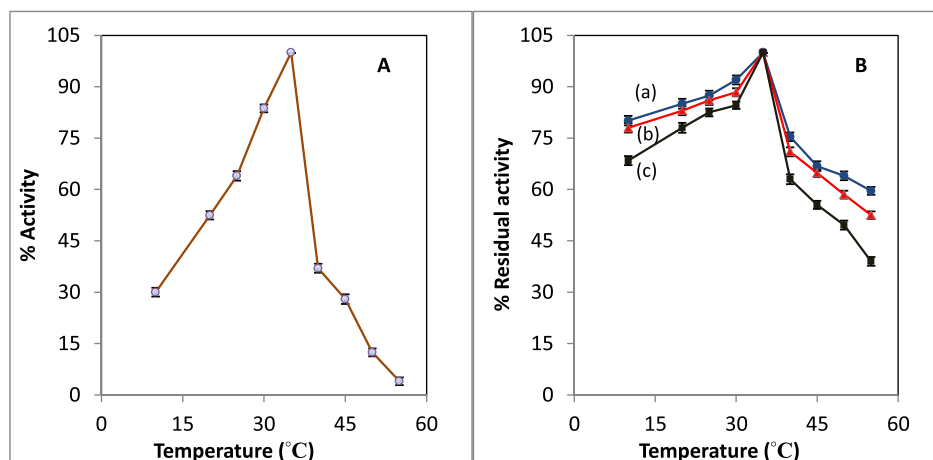


Fig. 6. Temperature dependence of the activity (A) and heat stability (B) of purified buffalo lung cathepsin H. The enzyme (0.15 mg) assay with BANA was made in 0.02 M sodium phosphate buffer, pH 6.75, at different temperatures (A) or at 36 °C prior to which the enzyme was exposed to various temperatures for 10 min [B(a)], 20 min [B(b)] and for 30 min [B(c)]. The error bars represent the standard deviation of the average value for three independent experiments.

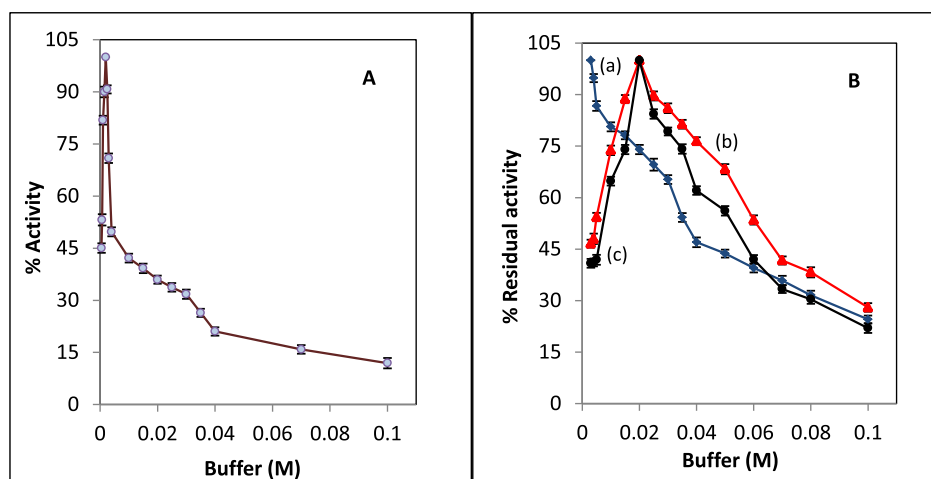


Fig. 7. Effect of buffer concentration on the activity (A) and stability (B) of buffalo lung cathepsin H. The enzyme (0.1 mg) assay with BANA at 36 °C was done in the phosphate buffer (pH 6.75) of various molarities (A) and in 0.02 M sodium phosphate buffer, pH 6.75, after exposing the enzyme to buffers of different concentrations for 10 min [B(a)], 20 min [B(b)] and 30 min [B(c)]. The error bars represent the standard deviation of the average value for three independent experiments.

Table 3

Leu-NA hydrolase activity of purified cathepsin H in different buffer systems containing 2mM each of EDTA and 2-mercaptoethanol.

| Buffer system (pH 6.5) | Concentration (M) | Activity (%) |
|------------------------|-------------------|------------------------|
| Sodium phosphate | 0.02 | 100±1.3 |
| | 0.05 | 86.4±1.1 ^a |
| | 0.10 | 39.6±1.4 ^a |
| Potassium phosphate | 0.02 | 75.7±0.7 ^a |
| | 0.05 | 49.0± 0.8 ^b |
| | 0.10 | 26.5± 3.5 ^b |
| Tris-phosphate | 0.02 | 92.4±1.0 ^a |
| | 0.05 | 88.6±1.1 |
| | 0.10 | 85.7±0.8 |
| Ammonium phosphate | 0.02 | 87.6±0.2 ^a |
| | 0.05 | 85.7±0.3 |
| | 0.10 | 83.4±0.7 |

^a In comparison with the value for 0.02 M sodium phosphate, $p < 0.001$.

^b In comparison with the value for 0.02 M potassium phosphate, $p < 0.001$.

cathepsin H were essentially unchanged ($p > 0.01$).

Besides the buffer systems, other constituents which may influence the activity of cathepsin H are EDTA and a thiol reducing compound. So the effect of 2-mercaptoethanol and EDTA was measured separately and in combination, and the observations obtained are specified in Table 4. As can be seen, the activation with 2-mercaptoethanol agrees well with the classification of cathepsin H as cysteine protease. A decrease in

Table 4

Effect of 2-mercaptoethanol and EDTA on the Leu-NA hydrolase activity of cathepsin H.

| Additive | Concentration (mM) | Activity (%) |
|--------------------------|--------------------|------------------------|
| None (control) | – | 3.23±2.2 |
| 2-mercaptoethanol | 1 | 66.7±0.8 ^a |
| | 2 | 86.2±0.5 ^a |
| | 5 | 102.0±0.1 ^a |
| EDTA | 1 | 20.3±2.9 ^a |
| | 2 | 44.2±0.7 ^a |
| | 5 | 15.3±1.0 ^a |
| EDTA + 2-mercaptoethanol | 1 + 1 | 63.8±0.5 ^a |
| | 2 + 2 | 100.0±0.2 ^a |
| | 2 + 5 | 83.6±0.2 ^a |
| | 5 + 5 | 57.7±1.0 ^a |

^a In comparison with control, $p < 0.001$.

activity was observed when the concentration of EDTA was changed either side of 2 mM ($p < 0.001$). The activity of the enzyme was found to be optimal at the concentration of 2 mM each of EDTA and 2-mercaptoethanol.

3.7. Influence of neutral salts

The effect of various monovalent and divalent neutral salts on the activity of cathepsin H was studied in 0.02 M sodium phosphate buffer, pH 6.5, and the results are shown in Fig. 8. As can be judged from Fig. 8A, the activity of cathepsin H decreased significantly (~26%) when the salt concentration were increased from 0 to 0.1 M. A further decrease in the catheptic activity of the enzyme was found when the concentration of NaCl was raised to 0.75 M, beyond which the enzyme activity was constant [see Fig. 8A (b)]. Conversely, in the existence of KCl a decrease in the activity was only observed at a concentration of up to 0.5 M; activity increased by about 6% when KCl concentration was raised from 0.5 to 1.0 M [Fig. 8A (a)]. However, a continuous decrease (~70%) in the enzyme activity was found upon an increase in NH₄Cl concentration from 0 to 1.0 M [Fig. 8A (c)]. All the divalent cations (MgCl₂, BaCl₂ and CaCl₂) were observed to be very potent inhibitors of catheptic activity [see Fig. 8B (a, b and c)].

4. Discussion

The procedure used during isolation and purification of buffalo lung cathepsin H, was very efficient in terms of time, labour, total enzyme units and specific activity but inefficient in terms of yield and fold purification (Table 1). This is not unusual since wide variations in the degree of purification (166–5800 folds) of cathepsin H have been reported by different workers [23,24,29–31]. A lower degree of purification (199 fold) obtained in this study does not necessarily indicate the presence of impurities in our preparation since fold purification also depends, among others, on the source of the enzyme and on the use of synthetic substrates differing in specificity for the enzyme [50].

Buffalo lung cathepsin H was found to be homogeneous both with respect to size (by gel filtration and SDS-PAGE) as well as charge (by PAGE). The molecular weight of the enzyme (25,400 as determined by SDS-PAGE) under denaturing conditions in the presence and absence of 2-mercaptoethanol, show that the buffalo enzyme is a single-chain molecule. On comparison, the molecular weight of buffalo enzyme was found to be similar (25,000) with that of porcine cathepsin H [30] but significantly lower (9.2%) than human/rat (28000) enzymes [23,51]. This is not surprising because cathepsin H isolated from different species/tissues contain different kind/amount of carbohydrates [30]. The total carbohydrate content of the enzyme is little high (8.7%) but the enzyme could be specifically precipitated with concanavalin A

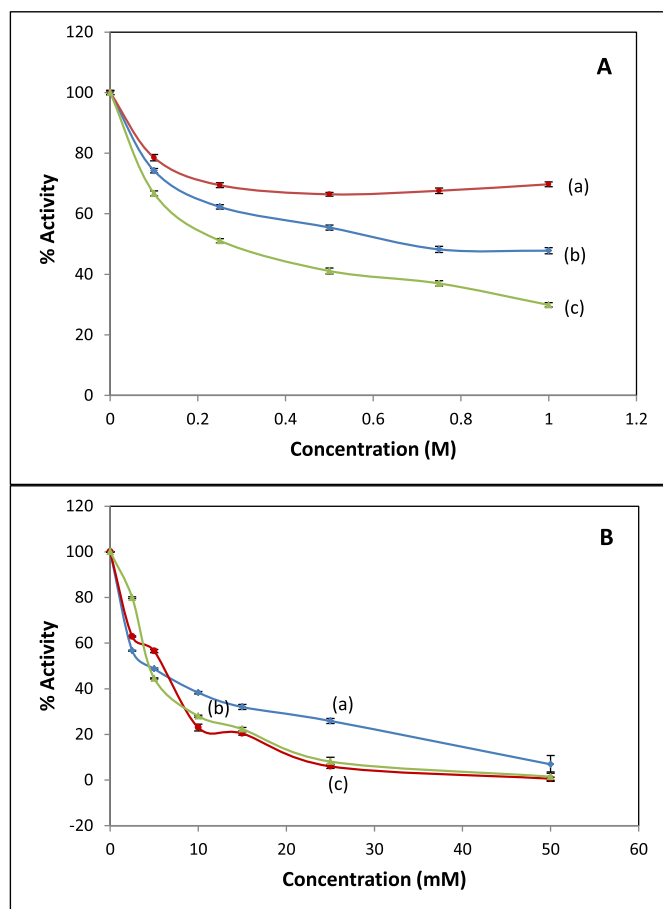


Fig. 8. Effect of monovalent (A) and divalent (B) neutral salts on the activity of buffalo lung cathepsin H. The enzyme (0.12 mg) assay with Leu-NA at 37 °C was done in 0.02 M sodium phosphate buffer, pH 6.5, at different concentrations of monovalent cations KCl [A(a)], NaCl [A(b)] and NH₄Cl [A(c)] or divalent cations MgCl₂ [B(a)], BaCl₂ [B(b)] and CaCl₂ [B(c)]. The error bars represent the standard deviation of the average value for three independent experiments.

suggesting not only its lysosomal localization but also that the carbohydrate moiety of the enzyme may contain mannose/glucose or their appropriate derivatives [34,51,52].

The results on the end group analyses of buffalo lung cathepsin H were striking, lysine was found to be the NH₂ – terminal amino acid residue as against tyrosine from other species [2]. This may either be attributed to simple species dependence or more significantly to the possible post translational processing of the enzyme in buffalo tissue [45,53,54]. However, the COOH-terminal amino acid residue was found to be same (valine) as reported for this enzyme from other sources [2]. The value of pI for buffalo lung enzyme was 6.2 which is well in agreement with the values of 6.1–6.3 for human kidney [24] and 6.0–6.4 for human liver [23] cathepsin H. However, it was found to be significantly lower than rat liver (7.1) enzyme suggesting different amino acid composition from the two sources [51]. Analysis of the best fit kinetic plots of buffalo cathepsin H revealed that lung enzyme has great catalytic potential against synthetic substrates at acidic or near neutral pH (Table 2). The enzyme showed markedly higher specificity towards synthetic peptides Arg-NA and Leu-NA; the latter ($K_m = 0.022$) was found to be 15 times more sensitive than the former ($K_m = 0.322$). Comparison of the kinetic results with similar data obtained for the bovine brain [29] and human liver [23] cathepsin H, revealed that the lung enzyme is catalytically less efficient with Arg-NA. However, data obtained for Leu-NA are consistent with those reported for cathepsin H from other sources [1,30,41].

Like other lysosomal cysteine proteinases, cathepsin H is active only

at acidic pH and irretrievably inactivated over pH 7.0 [1,34], it seems unusual that the enzyme play a role in protein breakdown under physiological conditions where the pH is maintained between pH 7.0–7.5 [55]. Our data on the enzyme from buffalo lung clearly show that it has a pH optimum 6.75 which is comparable to the value of about pH 6.8 [1,23,29] and notably high than those reported by others [24,28,31] for cathepsin H from different sources. However, the enzyme remains fully steady for a minimum of 20 min up to a pH as high as 6.75 (see Fig. 5). Likewise, our result on the thermal stability of the enzyme show that the enzyme has maximum activity at physiological temperature and it is fairly stable and retains most of its activity till the temperature is raised well above 36 °C (Fig. 6). Similarly our studies to the dependence of the activity and stability of buffalo enzyme on salt concentrations clearly indicate that cathepsin H is optimally active at low ionic strength and shows its stability relatively at higher buffer concentrations (Fig. 7). In view of the above, it can be safely concluded that the activity of cathepsin H exist for some degree of time still at physiological pH, temperature and ionic strength before the enzyme undergo inactivation.

Analysis of the study on the influence of buffer constituents and other salts (Tables 3–4 & Fig. 8) revealed that the activity of cathepsin H is not only highly sensitive towards ionic strength of the buffers but also depends on the nature of the buffer components. These observation offer a probable explanation for the inconsistency in the values of kinetic parameters of cathepsin H reported from different laboratories [1,23–30]. Therefore, appropriate care must be concerned during the selection of buffers for the assay of cathepsin H activity. Based on our results we suggest the following: (i) the activity of cathepsin H should be measured at low buffer concentration, preferably 0.02 M sodium phosphate buffer, pH 6.5 and the assay of catheptic activity at higher ionic strength, if necessary should be done in Tris-phosphate buffer, pH 6.5 (Table 3) (ii) 2mM each of EDTA and 2-mercaptoethanol should be used as activators of cathepsin H (Table 4) (iii) KCl should be used in making the salt gradient for eluting cathepsin H during its purification by ion exchange chromatography [Fig. 8A (a)] and (iv) the presence of divalent cations should be avoided during the assay of cathepsin H (Fig. 8B).

CRedit authorship contribution statement

Shalini Singh: Conceptualization, Data curation, Formal analysis, Investigation, Software, Methodology, Visualization. **Samir Sharma:** Project administration, Visualization, Writing - review & editing. **Sudhir K. Agarwal:** Project administration, Funding acquisition, Resources, Supervision, Validation.

Declaration of competing interest

The authors declare no conflict of interest.

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