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Purification and characterization of cathepsin B from buffalo (*Bubalus bubalis*) lung

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Abstract

In an attempt to improve the level of purity of buffalo lung cathepsin B, we have modified the earlier procedure by incorporating CM- Sephadex C-50 ion exchange chromatography and rechromatography on Sephacryl S-200 column. Appearance of a single band on SDS-PAGE under reducing and non-reducing conditions showed that the enzyme exists in a single chain molecule. Molecular weight of purified cathepsin B was determined to be 23800 and 25400 by SDS-PAGE and gel filtration, respectively. The enzyme, a glycoprotein, showed activity against α-N-benzoyl-DL-arginine-2-naphthylamide and α-N-benzoyl-DL-arginine-4-nitroanilide. The physico-chemical properties of the enzyme were similar to the properties reported for cathepsin B from other sources. However, the NH_2-terminal amino acid residue of the enzyme was found to be Ala as against Leu reported from other sources. The enzyme was activated by various thiol reducing reagents and inhibited by cysteine protease inhibitors and denaturing agents. The hydrodynamic behaviour of cathepsin B which includes Stokes radius (2.29 nm), frictional ratio (1.19) and intrinsic viscosity (3.08 ml/gm) suggested that the native enzyme conformation is compact and globular.

Keywords: Lysosomal protease, cathepsin B, buffalo lung, properties, hydrodynamic behaviour.

Introduction

Cathepsin B (EC 3.4.22.1), lysosomal in origin, belongs to papain-like proteases (Turk et al. 2001). The lysosome has a large number of thiol proteases which are responsible for turnover of intracellular proteins (Barrett and Kirschke 1981). Along with regulation of intracellular protein degradation, cathepsin B is also involved in processes such as neuropeptide and hormone processing (Reiser et al. 2010), insulin from proinsulin in pancreas and albumin from proalbumin in liver (Smith and Van Frank 1975), degradation of collagen type IV and X in osteoclasts and fibronectin (Sirens et al. 1995), trafficking of TNF alpha containing vesicles to plasma membrane (Ha S-D[1] et al. 2008), functioning of Toll-Like receptor-9 (Matsumoto et al. 2008), selection of peptide-MHC II complexes (Zavasnick- Bergent and Turk 2007), apoptosis of $CD4^+$T cells harbouring human immunodeficiency virus (Laforge et al. 2007), tumor progression and metastasis (Kirkegaard and Jäättelä 2009), muscular dystrophy (Sher et al. 1981), emphysema (Burnett et al. 1983), and activation of trypsin from trypsinogen in acute and chronic pancreatitis (Chen and Ferec 2009).The exact function(s) and mechanism of action of cathepsin B are, however, largely unknown. Cathepsin B has been isolated from bovine pancreas (Bansal and Kidwai 1979), goat brain (Kamboj et al. 1990) and spleen (Agarwal and Khan 1987), parathyroid gland of porcine (MacGregor et al. 1979), human liver (Barrett and Kirschke 1981), buffalo liver (Fazili and Qasim 1986), spleen (Ahmad et al. 1989) and kidney (Lamsal et al. 1997). Since the enzyme plays an important role in lung diseases (Mariana et al. 2011), prompted us to choose water buffalo (*Bubalus bubalis*) lung as a source of cathepsin B. The isolation and its essential physicochemical properties such as subunit structure and hydrodynamic properties have not been characterized. Precise viscosity data revealing the overall native conformation and shape of protein molecule are yet to be obtained for any one of the lysosomal cathepsins thus far. The present study will not only help us to obtain purify enzyme in sufficient quantity by a simple and rapid procedure, but also add to the existing knowledge on the hydrodynamic behaviour of buffalo lung cathepsin B for the first time. Further, the enzyme appears to be a single chain molecule in contrast to other mammalian cathepsin B.

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Materials and Methods

Fresh lungs of slaughtered buffaloes were collected from the local slaughter house and were stored at freezing temperature until used.

All the substrates used were highly purified and other chemicals were of analytical grade. The L-Leu-2-naphthylamide, L-Arg-2-naphthylamide, Z-Arg-Arg-2-

naphthylamide, α-N-benzoyl-DL-arginine-2-naphthylamide BANA), α-N-benzoyl-DL-arginine-4-nitroanilide (BAPNA), leupeptin, antipain, pepstatin A, α-N-tosylphenylalanine chloromethyl ketone (TPCK), β-naphthylamine, ethylenediamine tetra acetic acid (EDTA), sodium azide, urea, dithiothreitol (DTT), guanidine-HCl (GdnHCl), α-chymotrypsinogen, bovine serum albumin (BSA), ribonuclease A, myoglobin, cytochrome C, ovalbumin, β-mercaptoethanol, dimethylsulfoxide (DMSO) and reduced glutathione were purchased from Sigma Chemicals Co. St. Louis, Missouri, USA. Blue Dextran 2000, Sephadex G-75, CM-Sephadex C-50 and Sephacryl S-200 were purchased from Pharmacia, Sweden. Iodoacetic acid (IAA), iodoacetamide (IAM) and sucrose were the product of Sisco, Bombay. Other daily routine chemicals used in this study were generally of AR grade.

Purification of cathepsin B

All the steps of purification were carried out at 4°C by incorporating suitable modifications in the procedure described earlier (Fazili and Qasim 1986). One kilogram soft mass obtained from two buffalo lungs was homogenized for 15 min in 200 ml of 3% sodium chloride containing 1 mM EDTA, pH 1.8 and then stirred for 5-6 hrs. The pH of resulting homogenate adjusted to 3.8 (acid extraction) and stirred for 2 hrs. The content was then centrifuged at 14,000 rpm for 15 min and the clear supernatant thus obtained was subjected to ammonium sulphate fractionation. Precipitate at 40-75% $(NH_4)_2SO_4$ fraction was collected and dissolved in minimum amount of cold water, then dialysed against 0.05 M sodium acetate buffer, pH 5.0 containing 1 mM EDTA and 0.02% sodium azide. The sample was again centrifuged at 14,000 rpm for 5 min and chromatographed on a Sephadex G-75 column (3.5x85 cm) equilibrated with above buffer. Enzymatically active fractions, checked by BANA hydrolase activity, were pooled out and concentrated via sucrose bed method. The concentrated sample applied to ion-exchange chromatography on CM Sephadex C-50 column (2.2x85 cm) equilibrated with 0.02 M sodium acetate buffer, pH 4.8 containing 1mM EDTA, 0.02% sodium azide and 1.4 mM 2-mercaptoethanol. After washing the column extensively with above buffer to remove unabsorbed proteins, the bound fraction of cathepsin B was eluted with the same buffer, pH 5.6. The enzyme was finally purified by chromatography on Sephacryl S-200 column (3.5x67 cm) equilibrated with 0.05 M sodium acetate buffer, pH 5.0 containing 1 mM EDTA and 0.02% sodium azide. Polyacrylamide gel electrophoresis (PAGE) was performed on 10% acrylamide gels and pH 8.3 (Davis et al. 1964). SDS-PAGE of standard proteins and of cathepsin B was also done on 10% gels in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.2% SDS by the method of Weber and Osborn (1969). The marker proteins (with their molecular weights in parentheses) were: BSA (68000), ovalbumin(43000), α-chymotrypsinogen (25700), myoglobin (17200), ribonuclease A (13700) and cytochrome C (11700).

Enzyme assay

The activity of enzyme measured both spectrophotometrically (Martinek et al. 1964) as well as fluorometrically (Barrett 1980). In case of spectrophotometric analysis, BANA was used as a substrate; the assay mixture contains 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). This mixture was incubated for 30 min at 37°C to activate enzyme and the reaction was initiated by the addition of 0.5 ml of substrate (1mg/ml) which was prepared by first dissolving 10 mg BANA in 0.3 ml of DMSO, followed by dilution to the desired concentration in appropriate buffer. After incubation with substrate for 30 min at 37°C, 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. One unit activity for BANA was defined as the amount of enzyme required to release 1 µmole of 2-naphthylamine per min at 37°C.

Fluorometric assay was done with substrates such as BANA, L-Leu-2-naphthylamide, L-Arg-2-naphthylamide and Z-Arg-Arg-2- naphthylamide. 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 product 2-naphthylamine released from the above substrates was monitored for 30 min on excitation and emission wavelengthsof 335 and 410 nm, respectively (Barrett 1980). Protein in crude and purified samples was estimated by the method of Bradford (1976) using BSA as standard or by spectrophotometric method using specific extinction coefficient $(E_{1cm}^{1\%})$ of 16.8 determined for buffalo cathepsin B (Lamsal et al. 1997).

Determination of molecular parameters

Molecular parameters such as molecular mass, stokes radius and frictional ratio, were obtained from the gel filtration behaviour of cathepsin B on an analytical Sephadex G-100 column. The void volume and total volume of the column were determined respectively with the help of Blue Dextran 2000 and K_4FeCN_6. The marker proteins with their Stokes radii in parentheses (Tanford et al. 1974) were BSA (3.50 nm), ovalbumin (3.00 nm), α-chymotrypsinogen (2.20 nm), myoglobin (1.90 nm), ribonuclease A (1.75 nm) and cytochrome C (1.70 nm).The diffusion coefficient, D, for buffalo lung enzyme was calculated from Stokes radius of the enzyme using the equation (Andrews, 1970),

$$D = kT / 6\pi\eta a \quad \text{..... (1)}$$

Where k is Boltzmann constant, η is the coefficient of viscosity of the medium i.e 0.02 M sodium phosphate buffer, pH 6.5 and T is the absolute temperature. Another hydrodynamic parameter namely frictional ratio, f / f_0, was obtained from the following equation (Tanford 1961),

$$f / f_0 = a / (3\overline{v_2}M / 4\pi N)^{1/3} \text{..... (2)}$$

where N is Avogadro's number and terms a, $\overline{v_2}$ and M represent the Stokes radius, partial specific volume and the molecular weight of the protein, respectively. The value of $\overline{v_2}$ was taken to be 0.742 (Fazili and Qasim, 1986). The viscosity of buffalo lung enzyme in 0.02 M sodium phosphate buffer, pH 6.5 was measured in a Scott Gerate (type 51300) viscometer having a flow time of about 430 s for 4 ml distilled water at 25°C with a precision of better than 5%. The reduced viscosity, η_{red}, was determined as described by Tanford (1961) 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.

Chemical analyses

The NH_2- and -COOH terminal amino acid residues were identified by thin layer chromatography following the

published procedure (Needleman 1970). 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 (1959). Tryptophan residues were determined colourimetrically (Needleman 1970). Carbohydrate content of the enzyme was determined according to the procedure of Dubois et al. (1956).

Effect of various compounds

The effect of various thiol reducing agents, peptidyl inhibitors, IAA, IAM, TPCK, GdnHCl and urea on the activity of cathepsin B were studied by measuring the activity in usual manner except that the enzyme was incubated with the described compound for 30 min prior to assay of its activity.

Results and Discussion

To get the homogenous preparation and to increase in the yield and fold purification, we experimented with various combinations of gel filtration and ion-exchange chromatography to isolate cathepsin B from buffalo lung. The purification steps summarized in Table 1 showed an improvement in fold purification of the enzyme from 109, 168 and 191 respectively for buffalo spleen (Ahmad et al. 1989), kidney (Lamsal et al. 1997) and liver (Fazili and Qasim 1986) cathepsin B to 225. Similarly the yield of the enzyme was also found to be increased 2-7 times from the earlier purification of buffalo cathepsin B. As can be seen in Fig. 1, a chromatogram containing a number of peaks was obtained on Sephadex G-75 column, when the ammonium sulphate precipitated protein was applied to the column. A significant amount of BANA hydrolase activity obtained between fractions 40-46 (indicated by horizontal bar) were pooled and subjected to PAGE. As many as, six protein bands were observed (Fig. 1, inset (a)). The enzyme thus obtained was purified on CM-Sephadex C-50 column followed by Sephacryl S-200 column chromatography. A striking feature of the buffalo lung enzyme is its elution from 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 (Takahashi et al. 1984) eluted at pH 5.6 in the present study. This might be attributed to the difference in the nature and extent of glycosylation (Natowiez et al. 1982) and amino acid composition of the enzyme from the two sources. K_m determined with the purified preparation of the enzyme against BANA and BAPNA was 2.48 and 1.55 mM respectively which were the same reported earlier (Barrett and Kirschke 1981; Turk et al. 2001).

The purified enzyme was found to be homogenous both with respect to size as it gave a single symmetrical protein peak fully superimposable with its activity peak on Sephadex G-100 column as well as charge (Fig. 1, inset (b)). That the cathepsin B preparation obtained in this study was also free from other closely related cathepsins H and L, was confirmed by studying with L-Leu-2-naphthylamide and L-Arg-2- naphthylamide (specific substrates for cathepsin H) and azocasein with 3.0 M urea (specific substrate for cathepsin L), during enzymatic assay.

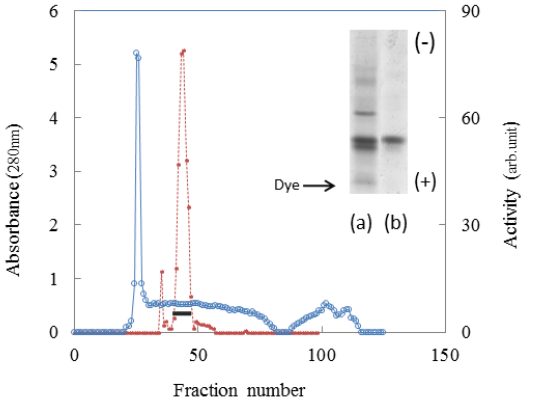

Figure 1: Elution profile of $(NH_4)_2SO_4$ fractions (40-75%) of buffalo lung cathepsin B on Sephadex G-75 column (3.5x 85 cm). About 120 mg of protein was applied to the column (equilibrated with 0.05 M sodium acetate buffer, pH 5.0 containing 1 mM EDTA and 0.02% sodium azide) and eluted at a flow rate of 15 ml/hr. Protein concentration was measured at 280 nm (——) and activity was assayed using BANA (-------). Horizontal bar indicate the fractions pooled for further purification. Inset shows the PAGE pattern of enzyme fractions pooled (a) and the purified enzyme (b) in 10% acrylamide gels using 0.02 M Tris-glycine buffer, pH 8.3.

Failure of detectable action of the enzyme on the above substrates ruled out the possible contamination of our cathepsin B preparation with these related cysteine proteinases (Barrett and Kirschke 1981; Takahashi et al. 1984).

Molecular properties

Gel electrophoresis of the purified cathepsin B in presence of SDS under reducing and non-reducing conditions yielded a single protein band. The molecular weight of the enzyme was computed from SDS-PAGE data using the relation between molecular weight for marker proteins and relative mobility (Fig. 2). After least square analysis, this can be expressed in terms of a straight line equation:

$$\log M = -1.087 R_m + 5.01 \quad \text{.....(3)}$$

The relative mobility for buffalo lung cathepsin B was 0.582, which upon substitution in the above equation, yielded a

Table 1: Purification of buffalo lung Cathepsin B[a]

Step	Total protein (mg)	Total enzyme activity[b] (units)	Specific Activity (units/mg)	Yield (%)	Fold Purification
Crude extract	60115	3616	0.06	100.0	1.0
Acid extraction	2550	1021	0.40	28.2	6.7
Ammonium Sulphate fractionation (40-75%)	602	403	0.67	11.1	11.2
Sephadex G-75 chromatography	146	331	2.27	9.2	37.8
CM-Sephadex C-50 chromatography	35	260	7.43	7.2	123.8
Sephacryl S-200 chromatograhy	10	135	13.50	3.7	225.0

[a]Data obtained with 1.5 kg buffalo lungs
[b] Enzyme activity was assayed in 0.02 M sodium phosphate buffer, pH 6.5 containing 2 mM each of EDTA and 2-mercaptoethanol using α–N- benzoyl-DL-arginine-2-napthylamide as a substrate.

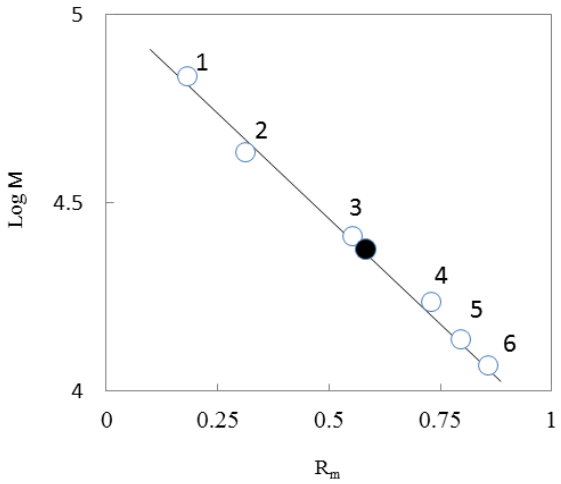

Figure 2: The relative mobility (R_m) of SDS-protein complexes of marker proteins in SDS-PAGE plotted against logarithm of the molecular weights (Log M). The marker proteins were: 1. BSA 2. ovalbumin 3. α-chymotrypsinogen 4. myoglobin 5. ribonuclease A and 6. cytocrome C. The filled symbol indicates the position of cathepsin B.

molecular weight value of 23.8 KDa. However, Sephadex G-100 column chromatography of cathepsin B preparation gave a single symmetrical protein peak corresponding to a molecular weight of 25.4 KDa (Table 2) which is found to be comparatively higher than the molecular weight of the enzyme on SDS-PAGE. The fact that the value of molecular weight obtained by gel filtration is higher (~ 2 KDa) than the value calculated from SDS-PAGE indicate that the buffalo lung enzyme is excessively hydrated under its native conditions. This is not surprising for cathepsin B because it is known to contain significant amount (3.6 to 7.6%) of carbohydrate (Takahashi et al. 1984; Ahmad et al. 1989; Lamsal et al. 1997). The various physico-chemical properties determined for the enzyme are summarized in Table 2. The total carbohydrate content of the buffalo lung enzyme was little higher (4.6%) than buffalo kidney (3.6%) but significantly lower as compared to buffalo spleen (7.0%)

Table 2: Physico-chemical properties of buffalo lung cathepsin B

Property	Cathepsin B
Molecular Weight	
SDS-PAGE	23800
Gel- filtration	25400
NH_2- terminal residue	Ala
COOH- terminal residue	Thr
Isoionic pH	5.1
Isoelectric pH	4.8 - 5.2
Carbohydrate content (%)	4.6
Tryptophan content[a]	7.8
SH- group at pH 8.0[a]	0.5
SH- group at pH 8.0 containing urea[a]	1.4
Absorption maxima (nm)	278
Emission maxima (nm)	338
Specific extinction coefficient ($E_{1cm}^{1\%}$)	16.0

[a]Calculated as moles per mole of the protein

and porcine spleen (7.6%) enzymes. Determination of free thiol group(s) of buffalo lung cathepsin B yielded a value of 0.5 and 1.4 mole/mole of protein under native and denatured conditions, respectively. These values were found to be similar with the values (0.6 and 1.6) of buffalo kidney (Lamsal et al. 1997) but significantly higher than the values obtained (0.3 and 1.0) for buffalo spleen (Ahmad et al. 1989). The isoionic pH measured as 5.1 suggests that the enzyme is acidic in nature. The COOH- terminal amino acid residue of buffalo lung Cathepsin B was found to be the same i.e threonine, as reported for this enzyme from other sources (Takio et al. 1983; Fazili and Qasim 1986; Meloun et al. 1988; Ahmad et al. 1989). However, the results on the NH_2- terminal amino acid residue of buffalo lung cathepsin B were striking, alanine was observed as against leucine for this enzyme from other tissues/species (Takio et al. 1983; Takahashi et al. 1984 & 1986; Fazili and Qasim 1986). Although similar results were reported earlier for buffalo kidney enzyme (Lamsal et al. 1997), this may either be attributed to simple species dependence or more significantly to the possible post translational processing of the enzyme in the lung/kidney tissues (Hasnain et al. 1992).

Optical Properties

The UV absorption spectrum of buffalo lung cathepsin B was observed in 0.02 M sodium phosphate buffer, pH 6.5, containing 1 mM EDTA at wavelength of 220-350 nm. It absorbed maximally at 278 nm. The emission spectrum of the mammalian enzyme studied in the same buffer was found to be maximum at 338 nm (Table 2). Thus the fluorescence spectra (both excitation and emission) represent the characteristic of proteins containing tryptophan residue. For determination of specific extinction coefficient ($E_{1cm}^{1\%}$), enzyme preparation was extensively dialysed against water and then passed through a column of mixed-bed ion exchanger. The absorbance of the isoionic protein solution thus obtained was measured at 278 nm. The specific extinction coefficient was calculated by dividing the optical density of the protein solution by its weight (g/100 ml). The $E_{1cm}^{1\%}$ value of the purified lung enzyme (16.0) was comparable with the value (16.8) reported for this enzyme from buffalo kidney (Lamsal et al. 1997) but significantly different from the values (20.0 and 13.2) of human liver and buffalo spleen respectively (Barrett and Kirschke 1981; Ahmad et al. 1989) suggesting a variation in the topology of aromatic amino acid residues in the mammalian tissues/species.

Effect of reductants

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 reductants like glutathione-SH, DTT, cysteine and cysteamine. As can be seen in Fig 3, cathepsin B appears to have similar response pattern to various reducing agents. The concentration of thiol compounds required to support the maximum activity of the enzyme was, however, found to be different (see Fig 3.). Among the thiol compounds tested, DTT and glutathione-SH was most and least effective, respectively. These results suggest that buffalo lung cathepsin B is a cysteine proteinase that requires thiol reducing compounds for its activity.

Influence of inhibitors

The influence of different proteinase inhibitors on the activity of buffalo lung cathepsin B was studied and the data obtained is summarised in Table 3. Iodoacetamide inhibited the enzyme completely at 0.1 mM concentration. However, IAA

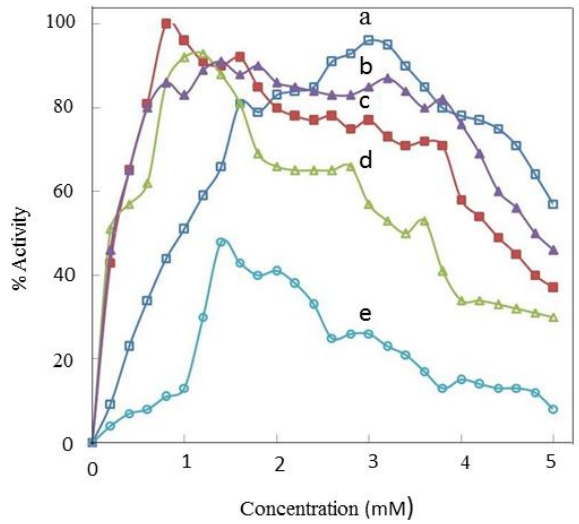

Figure 3: Effect of reductants on the activity of buffalo lung cathepsin B. The compounds are: (a) β-mercaptoethanol (b) cysteamine (c) DTT (d) cysteine and (e) glutathione-SH. Enzyme activity obtained in the presence of 0.8 mM DTT was taken as 100%.

was found to be 10 times more effective than IAM (Table 3). This extent of inhibition agrees with the earlier findings of porcine parathyroid cathepsin B (MacGregor et al. 1979). The probable reason for the effectiveness of IAA 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 (Takahashi et al. 1986), rat (Takio et al. 1983) and Human (Ritonja et al. 1985) cathepsin B. Leupeptin and Antipain, the known inhibitors of cathepsin B, showed high inhibitory activity. Almost all the activity is lost at inhibitor concentrations of 0.01 mM and above. However, Pepstatin A, an aspartate protease inhibitor, had more or less no action on the enzyme. The activity of buffalo lung enzyme was found to be very sensitive towards urea/GdnHCl. The enzyme was readily inhibited by more than 45% at urea/GdnHCl concentrations as low as 0.1 M. The degree of inhibition was about 90% at 0.5 M and practically no activity was left at urea/GdnHCl concentrations of 1 M and above. These results are found to be similar with the enzyme from goat (Agarwal and Khan 1987) or buffalo (Ahmad et al. 1989) spleens but it is not in full agreement with the report (Fazili and Qasim 1986) on buffalo cathepsin B from other tissue, namely liver, where more than 3 M

Table 3: Influence of various proteinase inhibitors on the activity of buffalo lung cathepsin B[a]

Inhibitor	Final concentration (μM)	Inhibition (%)
Leupeptin	10	100.0
Antipain	10	98.5
Pepstatin A	100	5.0
Iodoacetic acid	10	98.0
Iodoacetamide	100	94.5
TPCK	10	34.0
	100	91.5
Urea	$0.1x10^6$	44.8
	$1.0x10^6$	99.5
GdnHCl	$0.1x10^6$	45.7
	$1.0x10^6$	99.8

[a]Enzyme concentration was 30 μg/ml. The values are means of at least three determinations.

Urea was required to abolish the enzyme activity completely. TPCK showed a moderate inhibitory effect on buffalo enzyme. At 10 μM TPCK, the inhibition of buffalo lung cathepsin B was 34%; about 90% of the activity is lost when concentration of TPCK is increased up to 100 μM (Table 3) suggesting that the activity of cathepsin B may be similar to chymotryptic activity in the mode of inhibition by TPCK.

Hydrodynamic behaviour

Hydrodynamic properties of buffalo lung Cathepsin B were observed on a calibrated Sephadex G -100 column by passing marker proteins of known Stokes radii and are summarised in Table 4. Analysis of the data by the method of Laurent and

Table 4: Hydrodynamic properties of buffalo lung cathepsin B

Property	Cathepsin B
Stokes radius (nm)	2.29
Diffusion coefficient ($x\ 10^{-7} cm^2/s$)	9.57
Frictional ratio, (f/f_0)	1.19
Intrinsic viscocity (ml/g)	3.08
Equivalent hydrodynamic radius (nm)	2.28

Killander (1964) yielded straight line (shown in Fig. 4) following the equation,

$$(-\log K_{av})^{1/2} = 0.2034a + 0.2048 \quad \text{..... (4)}$$

Where K_{av} is the available distribution coefficient and a is the Stokes radius, computed with the help of above equation from its elution volume, was found to be 2.29 nm.

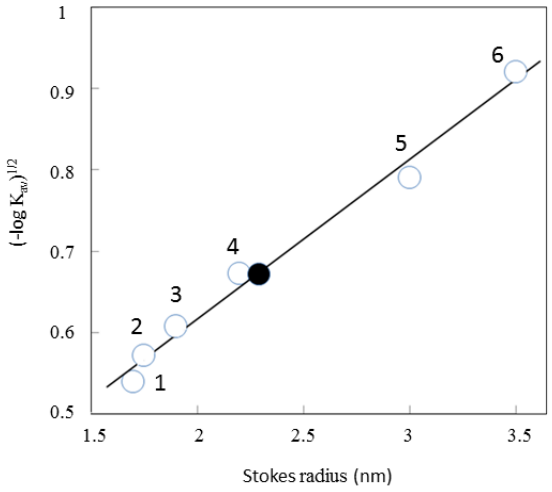

Figure 4: Treatment of gel filtration data obtained on a Sephadex G-100 column (2.5 x 80cm) in 0.06 M sodium phosphate buffer, pH 6.5 for the determination of Stokes radius according to Laurent & Killander (1964). The marker proteins used were 1. Cytochrome C 2. ribonuclease A 3. myoglobin 4. α-chymotrypsinogen 5. ovalbumin and 6. BSA. The position of cathepsin B is indicated by the filled symbol.

By using this value of Stokes radius and with the help of equation (1), the diffusion coefficient of the enzyme molecule was calculated to be $9.57x10^{-7} cm^2/sec$ (Table 4). Likewise, the value of frictional ratio corresponding to the value of Stokes radius (2.29 nm) was calculated from equation (2) and was found to be 1.19. This value of frictional ratio is consistent with the

conclusion, since compact and globular proteins are reported to have values of f/f_o close to 1 (Tanford 1961; Andrews 1970).

The intrinsic viscosity of cathepsin B was determined at 25°C in 0.02 M sodium phosphate buffer, pH 6.5 from the linear plot of $(t-t_o)/t_oC$ against protein concentration. The results thus obtained are shown in Fig. 5. The intrinsic viscosity as calculated from Fig. 5 becomes 3.08 ml/g (Table 4). It should be pointed out that the intrinsic viscosity of globular proteins lies in the range 3.0-4.0 ml/g (Tanford 1961). The equivalent hydrodynamic radius which is

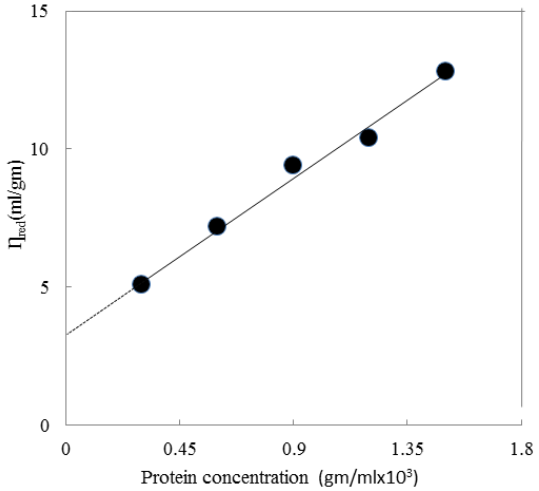

Figure 5: Reduced viscosity of purified buffalo lung cathepsin B as a function of protein concentration in 0.06 M sodium phosphate buffer, pH 6.5 at 25°C.

assumed to be equal to the Stokes radius of the enzyme was also computed from its intrinsic viscosity using the equation,

$$R_e^3 = 3M[\eta]/10N \quad \text{.....(5)}$$

Where R_e, M and $[\eta]$ represent the equivalent hydrodynamic radius, molecular weight and intrinsic viscosity of the enzyme, respectively and N is Avogadro's number. The value of R_e was thus found to be 2.28 nm (Table 4) which was identical with the value of Stokes radius (2.29 nm) determined by gel filtration.

Conclusions

The above observations taken together with other molecular and hydrodynamic properties (see Tables 2 & 4) suggested that although the enzyme molecule had a great tendency of hydration, it existed in a compact and globular conformation in the solution. Since cathepsin B obtained from various sources share many common physico-chemical and structural properties (Barrett and Kirchke 1981; Takio et al. 1983; Takahashi et al. 1984, 1986), the buffalo enzyme is likely to possess structural organization similar to that of human cathepsin B for which crystal structure revealed a disc shaped molecule with a diameter of 50 Å and a thickness of 30 Å (Musil et al. 1991). On the other hand, the buffalo enzyme differs significantly with the same proteinases from other sources with regard to other properties, especially inhibitory, NH_2-terminal residue and subunit structure. The data taken together, thus suggest a tissue/species dependence of cathepsin B.

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