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Purification and some properties of buffalo spleen cathepsin B

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Abstract. Purification of cathepsin B from buffalo-spleen, a hitherto unstudied system has been achieved by a simple procedure developed by incorporating suitable modifications in the existing methods for isolation of the enzyme from other sources. The purified enzyme has a molecular weight of 25 KDa and its Stokes radius was found to be 2·24 nm. Effects of several reducing agents, urea and thiol-protease inhibitors such as leupeptin and antipain, have been studied and the data unequivocally support the contention that the buffalo-enzyme is similar to cathepsin B from other tissues with respect to these properties.

Keywords. Lysosomal proteases; cathepsin B; buffalo-spleen; hydrodynamic properties.

Introduction

Cathepsin B (EC3·4·22·1) is the best known and characterized of the numerous lysosomal cysteine proteinases (Barrett, 1977). The enzyme is considered to play a key role in intracellular protein degradation (Huisman *et al.,* 1974; Dean, 1976; Dunn and Aronson, 1977), modifications of several proteins (Graf and Kennessey, 1976; Bond and Barrett, 1980) and several pathological conditions (Poole *et al.,* 1978; Stracher *et al.,* 1978; Recklies *et al.,* 1980; Sher *et al.,* 1981; Honn *et al.,* 1982; Burnett *et al.,* 1983; Ostensen *et al.,* 1983). The enzyme is also a dipeptidylcarboxy-peptidase which releases dipeptides sequentially from the carboxyl ends of poly-peptides (Aronson and Barrett, 1978; Nakai *et al.,* 1978; Bond and Barrett, 1979). The exact role(s) of cathepsin B *in vivo,* however, still remains speculative.

One of the major factors that makes the studies on cathepsin B difficult is un-availability of the pure enzyme in sufficient quantities. Several tissues of limited availability have been used as the sources for cathepsin B (Barrett and Kirschke, 1981) but it is only recently that the purification of the enzyme has been described from tissues easily available in large quantities (Takahashi *et al.,* 1984; Fazili and Qasim, 1986). In the present study we have chosen buffalo-spleen as the source for cathepsin B. A simple procedure for the isolation of the enzyme has been developed and some of its molecular properties are reported.

Materials and methods

Materials

Spleens of freshly slaughtered buffaloes were collected from the local slaughter

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Abbreviations used: BANA, Nα-Benzoyl-DL-arginine-*β*-naphthylamide; DMSO, dimethylsulphoxide; DTT, dithiothreitol; SDS, sodium dodecyl sulphate; BSA, bovine serum albumin; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); PAGE, Polyacrylamide gel electrophoresis.

house and were stored at freezing temperature until used. Antipain, Nα-benzoyl-DL-arginine-*β*-naphthylamide (BANA), dimethylsulfoxide (DMSO), dithiothreitol (DTT), guanidine hydrochloride, 2-mercaptoethanol, sodium dodecyl sulphate (SDS), bovine serum albumin (BSA) (lot 80F-0508), α-chymotrypsinogen (lot 29C-8010), cytochrome *C* (lot 124F-7155), myoglobin (lot 61F-7035), and ovalbumin (lot 23F-8175) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Blue Dextran 2000, DEAE-cellulose and Sephadex G-75 and G-100 were the products of Pharmacia, Sweden. 5, 5′-Dithio-bis-(2-nitrobenzoic acid) (DTNB), reduced glutathione and thioglycerol were procured from Sisco, Bombay. All other reagents used were of analytical grade.

Enzyme preparation

The purification of cathepsin B from buffalo spleen was performed by incorporating suitable modifications in the procedure described earlier (Fazili and Qasim, 1986). This modification primarily improved the purity of the enzyme. The soft tissue-mass (2·3 kg) obtained from 3 spleens (gross weight 3·3 kg) was homogenized for 10 min in 1·2 litre of 3% sodium chloride solution containing 1 mM EDTA and 15 mM HCl, and then stirred for 6 h at 4°C. After adjusting the pH of the resulting homogenate to 3·8, it was kept overnight at 4°C. The content was then centrifuged twice at 23,700 *g* for 15 min each at 4°C and the clear supernatant thus obtained was subjected to salt fractionation. A protein fraction precipitating between 40 and 75% $(NH_4)_2SO_4$ was collected and dialyzed against 0·05 M sodium acetate buffer, pH 5, containing 1 mM EDTA and 0·02% sodium azide. It was again centrifuged at 23,700 *g* for 5 min at 4°C and chromatographed on a Sephadex G-75 column (2·6 × 90·0cm) equilibrated with the above buffer. Enzymatically active fractions were pooled, concentrated and subjected to ion-exchange chromatography on DEAE-cellulose column equilibrated with 0·02 M Tris-acetate buffer, pH 6, containing 1 mM EDTA and 0·02% sodium azide. After washing the column extensively with the above buffer, bound fraction of cathepsin B was eluted stepwise with the same buffer containing 0·2, 0·5, and 1·0 M sodium chloride. Cathepsin B eluted at 0·2 M sodium chloride, was further purified by re-chromatography on the Sephadex G-75 column.

Enzyme assay

The activity of cathepsin B was measured at pH 6·5 and at 37°C using BANA as substrate by the procedure described earlier (Khan *et al.,* 1986). One unit of activity was defined as the amount of enzyme necessary to release 1 μM of 2-naphthylamine per h.

Protein determination

Protein was estimated by the method of Bradford (1976) with BSA as a standard.

Assay of SH-group

The sulfhydryl content of the enzyme under native conditions as well as in the

presence of 6 M guanidine hydrochloride was determined essentially by the method of Ellman (1959). The buffer used was 0·08 M Tris-HCl, pH 8, containing 0·5 mg EDTA per ml. The reaction was found to be completed within 30 min for the experimental conditions used in this study.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the absence and presence of SDS was performed according to the methods of Davis (1964) and Weber and Osborn (1969) respectively. The cross-linking of the gel in both the experiments was 10%.

Fluorescence measurements

Fluorescence was measured with 1 cm cell at 37°C with a Shimadzu RF 540, spectrofluorophotometer. The excitation and emission wavelengths were 335 and 410 nm respectively for the measurement of the enzyme activity (Khan *et al.,* 1986).

Gel filtration on Sephadex G-100

Some hydrodynamic constants were calculated from the data obtained by gel filtration on a Sephadex G-100 column (2·6 × 88·0 cm) equilibrated with 0·06 M sodium phosphate buffer, pH 6·5. The elution volume of the Blue Dextran 2000 was taken as the void volume (V_o), and the total volume (V_t) was determined from the elution volume of $K_3Fe(CN)_6$. The value of frictional ratio (f/f_0) was obtained by the procedure described in Fazili and Qasim (1986) using 0·742 as the value of partial specific volume.

Effect of various compounds

The effects of urea, antipain, leupeptin and various reducing agents 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

Purification of buffalo-spleen cathepsin B

Attempts to utilise previously published procedures for the isolation and purification of cathepsin B yielded a heterogeneous product as judged by PAGE (Otto, 1971; Fazili and Qasim, 1986). We, therefore, experimented with various combinations of gel filtration and ion-exchange chromatography to isolate cathepsin B from buffalo-spleen.The purification steps are summarized in table 1. When the ammonium sulphate precipitated protein at step 3 of table 1 was applied to a Sephadex G-75 column (2·6 × 90·0 cm) and eluted with 0·05 M sodium acetate buffer, pH 5, containing 1 mM EDTA, 5 protein peaks were found (figure 1). The fractions (indicated

Table 1. Purification of buffalo-spleen cathepsin B.

Purification steps	Total protein (mg)	Total enzyme activity (units)	Specific activity (units/mg)	Yield (%)	Fold-purification
Crude extract	148000	31620	0·214	100	1
Acid extraction	6225	17413	2·784	55	13
Ammonium sulphate fractionation	930	8316	8·932	26	42
Sephadex G-75 chromatography	271	3330	12·293	11	57
DEAE-cellulose chromatography	25·4	492	19·395	1·6	91
Sephadex G-75 re-chromatography	9·38	219	23·324	0·7	109

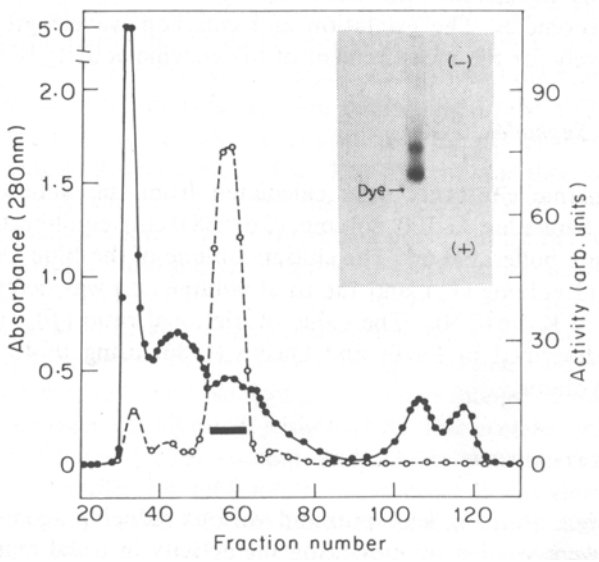

Figure 1. Sephadex gel chromatography of $(NH_4)_2SO_4$ fraction (40-75%) of buffalo-spleen cathepsin B on Sephadex G-75 column (2·6 × 90·0 cm) equilibrated with 0·05 M sodium acetate buffer, pH 5, containing 1 mM EDTA and 0·02% sodium azide. The fractions (5 ml) were read at 280 nm for protein (●), and monitored for BANA hydrolase activity (O). Horizontal bar indicate the fractions pooled for further purification. Inset shows the PAGE pattern of the purified enzyme in 10% gel using 0·02 M Tris-glycine buffer, pH 8·3.

by horizontal bar in figure 1) containing significant amount of BANA hydrolase activity were combined and subjected to ion-exchange chromatography on DEAE-cellulose column. The cathepsin B obtained at this stage was further purified by re-chromatography on Sephadex G-75 column to give the final enzyme preparation. The product of this purification method Bas found to be homogeneous with respect to size as it gave a single symmetrical protein peak fully superimposable with its activity peak on Sephadex G-100 column (2·6 × 88·0 cm). PAGE of the enzyme on 10% gels at pH 8·3 (figure 1, inset), resulted into two bands. Four minor bands

reported by Fazili and Qasim (1986) were not observed under any electrophoretic conditions used by us. Protein fractions corresponding to the two bands, presumably isozymes of cathepsin B (Otto and Reisinkonig, 1975; Takahashi *et al.,* 1986), were however, not further separated.

This procedure 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 of cathepsin B have been documented (Otto and Reisinkonig, 1975; Hirao *et al.,* 1984; Takahashi *et al.,* 1986). A lower degree of purification (109-fold) obtained 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 substrate used for its assay (Fazili and Qasim, 1986). A close examination of table 1 does indicate that relatively high specific activity obtained in the crude extract of buffalo-spleen is a characteristic feature of this tissue that might affect the final fold-purification.

Characteristics of buffalo-spleen cathepsin B

The chemical and enzymatic properties of purified enzyme were similar to those described for cathepsin B from other sources (Barrett, 1977). Under native conditions where no exogeneous thiols were added, the enzyme was found to contain about 0·3 mol of SH-group per mol of the protein. When the estimation of thiol groups was performed in the presence of 6 M guanidine hydrochloride, the SH-group content increased to about 1 mol per mol of the enzyme. The molecular weight of the enzyme, as obtained by SDS-PAGE, was found to be 23 KDa.

As can be seen in table 2, various reducing agents have very strong stimulatory

Table 2. Effect of various thiol-reducing compounds on the activity of buffalo-spleen cathepsin B.

Concentration (mM)	Enzyme activity (%)				
	2-Mercaptoethanol	DTT	L-Cysteine	Glutathione	Thioglycerol
0	3	2	2	2	3
1	52	90	83	60	77
2	62	100	94	77	94
3	64	96	94	85	120
4	73	88	93	68	128
6	76	80	74	68	128
8	76	79	43	51	128
10	78	74	16	26	128

Enzyme activity obtained in the presence of 2 mM DTT is taken as 100%. To 0·1 ml (0·181 mg) of the enzyme solution was added 1·9 ml of an activator solution in 0·02 M sodium phosphate buffer, pH 6·5, containing 2 mM EDTA and various concentrations of thiol-reducing compounds. After incubating the contents at 37°C for 30 min, 1 ml BANA solution (0·1%) was added and the hydrolase activity was measured fluorometrically (Khan *et al.,* 1986). The values are average of two determinations.

effect on the enzyme-activity. These results suggest that buffalo-spleen cathepsin B is a cysteine protease that requires thiol reducing compounds for its activity. Effects

of leupeptin and antipain on the activity of buffalo-spleen cathepsin B were studied at different inhibitor concentration and the results are depicted in figure 2 which

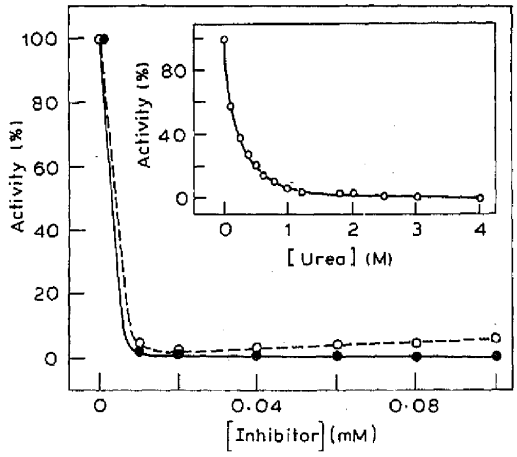

Figure 2. Influence of leupeptin (●) and antipain (O) on the activity of buffalo-spleen cathepsin B. Inset shows the dependence of the catheptic activity on urea concentration. Enzyme activity in the absence of the inhibitors was taken as 100%. A 0·02 M sodium phosphate buffer (pH 6·5) containing 2 mM each of EDTA and 2-mercaptoethanol was used as activator. Other details were the same as described in table 2.

also include the data on the influence of urea on the protease activity (figure 2, inset). It is evident from the figure that almost all the activity is lost at inhibitor concentrations of 0·01 mM and above. Out of the two inhibitors, leupeptin appears to be more effective. Buffalo-spleen cathepsin B was also found to be very sensitive towards urea (figure 2, inset). The enzyme was readily inhibited by more than 40% at urea concentrations as low as 0·1 M. The degree of inhibition was about 80% at 0·5 M urea and practically no activity was left at urea concentrations of 1 M and above. This is not in full agreement with the recent report (Fazili and Qasim, 1986) on buffalo cathepsin B from another tissue, namely liver, where 3 M urea was required to abolish the enzyme activity by more than 90%.

Above data on dependence of the activity of the buffalo-spleen enzyme on various reducing agents and inactivators are not only in agreement with the earlier reports on cathepsin B from other sources (Barrett, 1977; Bradley and Whitaker, 1986) but also rule out the presence of cathepsins H and L in our preparation. Since cathepsin H shows little sensitivity towards leupeptin, and urea has stimulatory effects on cathepsin L activity (Barrett and Kirschke, 1981), BANA hydrolase activity of our enzyme preparation, resolved into the two bands by PAGE (figure 1, inset), can only be attributed to the two isozymes of cathepsin B (Otto and Reisinkonig, 1975; Takahashi *et al.,* 1986).

Hydrodynamic properties of the enzyme

Hydrodynamic properties of buffalo-spleen cathepsin B were obtained from the gel filtration behaviour of the enzyme on a calibrated Sephadex G-100 column. Analysis

of the data by the methods of Porath (1963) and Laurent and Killander (1964) yielded straight lines (shown in figure 3) following the equations:

$$M^{1/3} = 86{\cdot}205 - 70{\cdot}274\, K_d^{1/3} \quad (1)$$

$$\text{and} \quad (-\log K_{av})^{1/2} = 0{\cdot}1837\, r + 0{\cdot}1383, \quad (2)$$

where M and r were the molecular weight and Stokes radius and were computed from above equations to be 25 KDa and 2·24 nm respectively. K_d and K_{av} represent distribution coefficient and available distribution coefficient, respectively. The value of frictional ratio corresponding to these values of molecular weight and Stokes radius was calculated by the method described by Fazili and Qasim (1986), to be 1·15. Computation of the geometric mean radius by the method of Ackers (1970) as described by Suelter (1985) yielded a value of 1·86 nm.

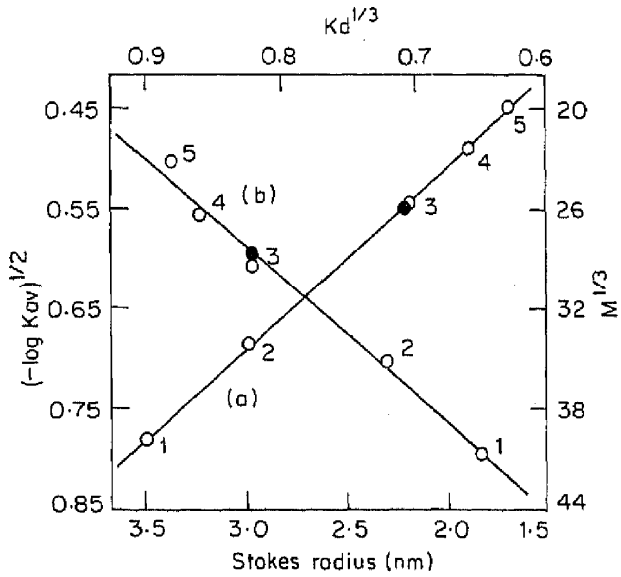

Figure 3. Treatment of gel filtration (data) (a) for the determination of Stokes radius according to Laurent and Killander (1964), and (b) for the determination of molecular weight according to Porath (1963). Straight lines (a) and (b) were drawn by the method of least squares and represent plots $(-\log K_{av})^{1/2}$ *versus* Stokes radius and $K_d^{1/3}$ *versus* $M^{1/3}$, respectively. The marker proteins used were: 1, BSA; 2, ovalbumin; 3, α-chymotrypsinogen; 4, myoglobin; and 5, cytochrome *C*. The positions of cathepsin B are indicated by filled circles.

These results suggest that although the enzyme molecule is considerably hydrated (Stokes radius is significantly higher than geometric mean radius), it exists in a compact and globular conformation since its frictional ratio is close to 1 (Yang, 1961). Furthermore, the fact that the value of molecular weight obtained by SDS-PAGE is lower (by about 2 KDa) than the value calculated from the gel filtration behaviour of the enzyme also indicate that cathepsin B is excessively hydrated under its native conditions. This should not be surprising for cathepsin B is known to contain significant amount of carbohydrate (Takahashi *et al.,* 1984; Sarfraz Ahmad and M. Yahiya Khan, unpublished results).

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