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PURIFICATION AND PARTIAL CHARACTERIZATION OF CATHEPSIN D FROM PORCINE (*Sus scrofa*) LIVER USING AFFINITY CHROMATOGRAPHY

Fernanda Canduri[1], Richard J. Ward[1*], Walter F. de Azevedo Jr.[1], Roseli A. S. Gomes[2] and R. K. Arni[1#]

[1] Departamento de Física, IBILCE/UNESP, 15054-000, São José do Rio Preto, S.P., Brazil. FAX 0055 017.224-8692; [2] Departamento de Bioquímica Celular e Biofísica, Faculdade de Medicina do Triângulo Mineiro, FMTM, 38015-050, Uberaba, M.G., Brazil.

[*] Current address: Department of Biochemistry, FMRP-USP, Ribeirão Preto-SP, Brazil.

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SUMMARY: Cathepsin D, a lysosomal aspartic protease, has been purified from porcine liver using a combination of pepstatin-A agarose and Affi-Gel Blue affinity chromatography, followed by size-exclusion chromatography. The purified protein consists of two polypeptide chains of 15 and 30 kDa, and has an isoelectric point of 6.8. Porcine liver cathepsin D has maximum activity at pH 2.5-3.0 as determined by its activity against hemoglobin, with a K_{cat} of 14.3 s^{-1} and a k_{cat}/K_M of 2.70 x 10^6 $s^{-1}M^{-1}$ as determined by the hydrolysis of a fluorogenic peptide substrate.

Key Words: Cathepsin D, aspartic protease, lysosomal enzyme, affinity chromatography, pepstatin A

INTRODUCTION

The aspartic protease family includes pepsin, renin, HIV protease and cathepsins D and E. Cathepsin D is the major lysosomal enzyme in liver, spleen, kidney, gastric mucous, placenta, brain and skin (1), and is synthesized as a single chain pro-enzyme. After transfer to the Golgi complex, the protein is phosphorylated (N-linked mannose-6-phosphate) and cleaved to form two chains, a light chain of 15 kDa and a heavy chain of 30 kDa (2, 6). Phosphorylation serves as a recognition signal for N-acetylglucosamine phosphotransferase (7, 8) which is the cell signal for the transport of cathepsin D to the lysosomal vesicle (9). Various pathological processes are associated with increases in lysosomal activity (3), and the elevated levels of cathepsin D in tumor cell lysosomes is a diagnostic aid of breast cancer metastases (4, 5).

To whom correspondence should be addressed.

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Cathepsin D hydrolyses the peptide bond on the N-terminal side of hydrophobic amino acids, principally Trp, Phe and Leu (10). Two aspartic acid residues (Asp33 and Asp231, in cathepsin D) are essential for the hydrolysis of the substrate (11, 12) and are structurally conserved in all the members of this family. The pH optimum for activity is between 2 and 3, which is the pH encountered in the endosomal medium (13). Pepstatin A, a peptide of 7 amino acids, is a naturally ocurring potent aspartic protease inhibitor (14). Exploiting its high affinity for pepstatin-A, we have affinity-purified and characterized the kinetics of lysosomal cathepsin D from porcine liver.

MATERIALS AND METHODS

Preparation of tissue extract: 500g of frozen porcine liver was freed of fatty tissue, cut into small cubes, the volume adjusted to 500ml with buffer (50mM NaAcetate, 150mM NaCl, 5mM EDTA, 0.2mM PMSF e 1% TRITON X100, pH 4.6) and homogenized in a Waring blender. The homogenate was filtered through a gauze mesh to separate fatty material and centrifuged at 20 000*g* for 45 min at 4°C. The pH of the supernatant fraction was adjusted to 3.5 with glacial acetic acid, and maintained at 4°C with constant agitation for 60 min. During this time further precipitation of liver proteins occurred, and a second centrifugation at 30 000*g* cleared the extract (final volume 300ml) prior to application of the supernatant fluid to the pepstatin A-affinity column.

Pepstatin A-agarose affinity chromatography: An XK16/40 column (Pharmacia, Uppsala, Sweden) was packed with 10ml of the affinity resin pepstatin A-affinity agarose (Sigma). All procedures were performed at 4°C, unless otherwise indicated. The column was equilibrated with buffer B (50mM NaAcetate, 150mM NaCl, 2mM EDTA, pH 3.5) at a flow rate of 0.5 $ml.min^{-1}$. The liver extract was applied to the affinity column at a flow rate of 0.2 $ml.min^{-1}$ using a peristaltic pump. The column was initially washed with approximately 250 ml (25 column volumes) of buffer B at a flow rate of 0.2 $ml.min^{-1}$. The column was then inverted, and subjected to a second wash at room temperature at a flow rate of 1.0 $ml.min^{-1}$ with 100ml of buffer C (Buffer B + 150mM NaCl) using an FPLC system (Pharmacia). During this second wash, the absorbance at 280 nm was monitored, and after a steady O.D. baseline was established, the column was eluted using buffer D (50mM Tris, 150mM NaCl, 2mM EDTA, pH 8.5) and a flow rate of 1 $ml.min^{-1}$. Fractions were collected over the absorbance peaks, and pooled typically giving a total volume of ~30 ml.

Affi-Gel Blue affinity chromatography: 4.0 ml of Affi-Gel Blue (BioRad) was packed into a 1cm diameter column and equilibrated with 50ml of sodium phosphate 0.1M, pH 7.0. The pooled pepstatin A-affinity eluate was passed over the column at a flow rate of 2.0 $ml.min^{-1}$ with continuous monitoring of absorbance at 280nm. The eluate was collected and concentrated with centrifuge-spin filters (Centricon, 20 kDa cut-off) to a final volume of 2ml, and stored at -20°C in 50mM sodium citrate buffer pH 5.0. This second affinity purification was used to remove trace quantities of serum albumin.

Size-Exclusion Chromatography: A Superdex10/75 (Pharmacia) column (1.6 x 70 cm) was equilibrated with buffer D, and and 0.4 mg of cathepsin D was applied in a total volume of 0.5 ml. The column was eluted at 1 $ml.min^{-1}$, and the absorbance of the eluate monitored at 280 nm. This final step was used for all preparations in order to eliminate trace contaminants detected in some earlier preparations.

Electrophoresis: Sodium dodeylsulphate polyacrilamide gel electrophoresis (SDS-PAGE) was performed (15). Samples were prepared by heating at 90°C for 5 min in the presence of 2% SDS and 0.5% β-mercaptoethanol. Non-denaturing isoelectric focussing was performed (16) using an anfoline (Sigma) pH gradient of 3.0 – 10.0. Protein was visualized by silver staining.

Determination of Protein Concentration: The concentration was determined using bovine serum albumin as standard (17). In all cases, a blank sample was prepared under the same conditions, but with the absence of protein.

Determination of Proteolytic Activity: Two separate methods were used to determine proteolytic activity using as substrates denatured bovine hemoglobin and a synthetic fluorogenic peptide. The activity over a pH range of 2-8 in 0.5 unit increments was measured by the hydrolyis of denatured bovine hemoglobin (18). Due to the broad pH range tested, three buffer systems were employed; 0.2 M Glycine-HCl (pH 2.0), 0.2 M citrate-phosphate (pH 2.5-7.0) and 0.1 M Tris-HCl (pH 7.5-8.0). Kinetic parameters were measured by the hydrolysis of a synthetic fluorogenic peptide containing at the extremities the chromophore *o*-aminobenzoyl (Abz) and its quenching partner ethylenediaminidinitrophenol (Eddnp) which are separated by 8 amino acid residues including two consecutive phenyalanine residues (19). Cleavage of the peptide between these hydrophobic residues results in separation of the two peptide fragments, and a consequent dequenching of the Abz which leads to an increase in the fluorescence signal. Cathepsin D at a fixed concentration of 0.45 $\mu g.ml^{-1}$ was incubated with the fluorogenic peptide at concentrations of 0.61-32.7 μM in 2.0 ml total volume of 50 mM sodium citrate, pH 3.0. The increase in fluorescence was measured with a Hitachi F-4500 spectrofluorimeter in time-scan mode with an excitation wavelength of 320 nm, and an emission wavelength of 420 nm. The initial rates of peptide hydrolysis were calculated from the increase in the fluorescence signal (19) by the equation:

$$V_0 = I_t \,.\, [S_0] \,/\, (I^{100} - I^0).t$$

where V_0 is the initial rate of substrate hydrolysis ($nMol.s^{-1}$), S_0 is the initial substrate concentration (μM), I^0 and I^{100} are the initial and final plateux fluorescence signals respectively, I_t is the fluorescence signal at time t, and t is the time in seconds. The Michaelis-Menten constant (K_M) and maximum velocity (V_{max}) were determined using Lineweaver-Burk plots. The k_{cat} was calculated using the equation:

$$k_{cat} = V_{max}/[E]_T$$

where $[E]_T$ is the total enzyme concentration (nM).

RESULTS

Figure 1a shows the SDS-PAGE gel of the cathepsin D from porcine liver after purification using pepstatin-A and Affi-Gel blue affinity resins, and size-exclusion chromatography (SEC). In the final SEC purification step, a single peak of ~45kDa is observed

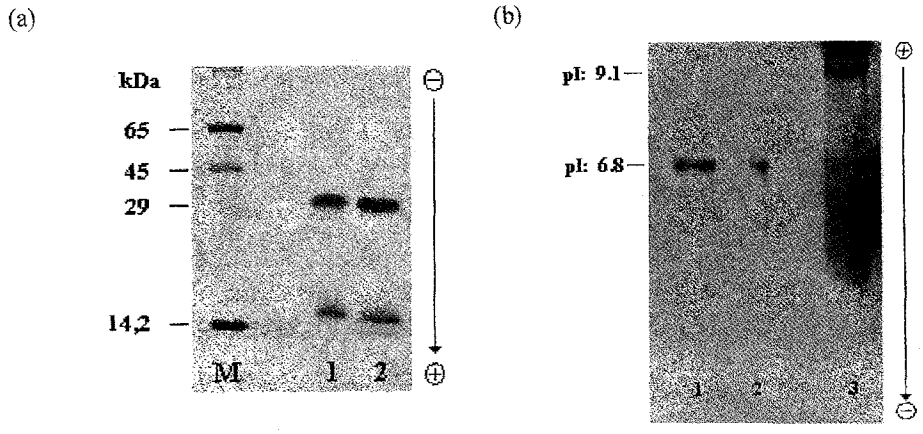

Figure 1 (a). Silver stained 12% polyacrylamide gel (SDS-PAGE) of the purified porcine liver cathepsin D (lanes 1 and 2). The molecular weights of the protein markers (lane M) are shown in kDa to the right of the gel. (b) Silver stained 7.5% polyacrylamine isoelectric focussing gel showing the purified porcine liver cathepsin D (lanes 1 and 2). Lane 3 shows the marker piratoxin I (pI=9.1).

(data not shown), however under the reducing and denaturing conditions of SDS-PAGE, two bands of 15 and 30 kDa are observed. In contrast, under the non-denaturing and non-reducing conditions of the isoelectric focussing gel presented in Figure 1b, cathepsin D focusses to a single band corresponding to an isoelectric point of 6.8. Both Figures 1a and 1b show well defined bands, with no detectable contaminants shown by the silver staining technique.

As illustrated in Figure 2, the activity tests performed over the pH range of 2-8, using denatured bovine haemoglobin as a substrate, indicate a pH optimum of 2.5-3.0 for porcine liver cathepsin D. As the pH is increased, the activity of the enzyme declines to 50% at pH values ranging from 4.0-4.5, and above pH 6.0 hydrolysis of substrate is not detected. Activity tests were also performed in 0.1 M sodium formate pH 3.0, both in the absence and presence of the inhibitor pepstatin-A. The activity decreases in proportion to the inhibitor concentration, and complete inhibition is reached at a pepstatin-A concentration of 9.5 $\mu g.ml^{-1}$ (data not shown).

The initial rates of hydrolytic activity using the synthetic fluorogenic peptide substrate are presented in Figure 3 as a Michaelis-Menten plot. These data were analysed using a hyperbolic least-squares-fit function, which yielded values for the K_M and V_{max} of 6.5 μM and 140 $nMol.s^{-1}$ respectively. The insert to Figure 3 presents the Lineweaver-Burk transformation of the data, from which were derived values for K_M of 6.6 μM and a V_{max} of 143 $nMol.s^{-1}$. Using this value of V_{max} yields a k_{cat} of 14.3 s^{-1}, and a k_{cat}/K_M ratio of 2.70 $x10^6$ $s^{-1}.M^{-1}$.

DISCUSSION

Using a combination of affinity and size-exclusion chromatography, the cathepsin D was isolated from porcine liver with a high degree of purity. The initial pepstatin-A affinity purification yielded samples containing several minor contaminants, and the subsequent Affi-Gel blue and size-exclusion chromatographic (SEC) steps successively improved the purity of the preparation. In the final SEC step, an eluted peak corresponding to a protein molecular weight 45 kDa yielded a single band of pI=6.8 using an isoelectric focussing gel. However, under the reducing and denaturing conditions of SDS-PAGE, this fraction yielded two bands corresponding to polypeptides of 15 and 30 kDa respectively.

The processing of human cathepsin D from the 45 kDa pro-enzyme to the heterodimeric species is known to occur in the Golgi apparatus (2,6). In several preparations, we have observed, by the silver stained SDS-PAGE, a 45 kDa protein present in small amounts in samples taken immediately after elution of the pepstatin-A column which may correspond to the native protein. This protein species is unstable, and is not observed either in the final sample

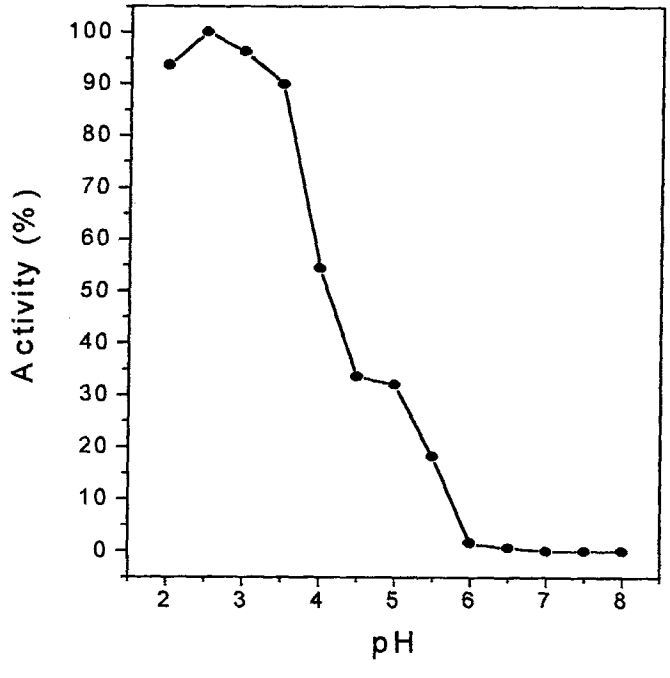

Figure 2. Activity of porcine liver cathepsin D against denatured bovine haemoglobin as a function of pH. Activity is expressed as the percentage of the maximum activity observed at pH 2.5.

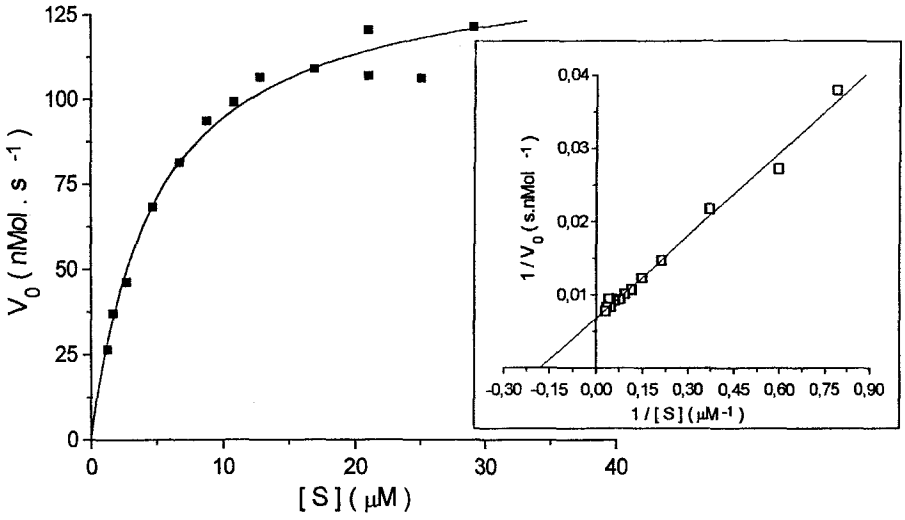

Figure 3. Michaelis-Menten plot of the initial reaction rates (V_0) measured from the increase in fluorescence due to hydrolysis of a fluorogenic substrate as a function of the substrate concentration, [S]. The data were fitted with a hyperbolic function using a least-squares-fitting routine (curve shown). The inset shows the Lineweaver-Burk transformation of the Michaelis-Menten plot, which was used to derive the values for the K_m and V_{max} of the reaction.

or after storage of the pepstatin-A column eluate at 4°C. The degradation of the purified 45 kDa species suggests that conversion of the pro-enzyme may occur through auto-hydrolysis, however proteolysis by trace contaminants of other proteases cannot be ruled out.

An optimum pH of 2.5-3.0 for the activity of the purified porcine liver cathepsin D is typical of the lysosomal aspartic proteases. The crucial role played by the aspartic acid residues in the catalytic mechanism is evident from the pH value of 4.0-4.5 for the 50% activity observed here, which approximately corresponds to the pK' of aspartic acid. The values derived for the K_M and k_{cat} are within the range of values for other aspartic proteases using the same substrate, however they are higher than those observed for human cathepsin D (19). Nevertheless, the value for k_{cat}/K_M ratio is similar for these two enzymes (2.74 x10^6 for human cathepsin D (19) versus 2.70 x10^6 for the porcine enzyme), indicating that despite the differing values of k_{cat} and K_M, the efficiencies of the two enzymes are comparable.

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