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Human Placental Cathepsin B1

ISOLATION AND SOME PHYSICAL PROPERTIES

By ARNOLD A. SWANSON, BILL J. MARTIN and SAM S. SPICER
Departments of Biochemistry, Ophthalmology and Pathology, The Medical University of South Carolina, Charleston, S.C. 29401, *U.S.A.*

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A reproducible procedure for the isolation, from human placenta, of a cathepsin B1 in a homogeneous state, demonstrated by electrophoretic, ultracentrifugal and enzymic criteria, was carried out. The pH optimum was near pH5.5. The placental enzyme catalysed the release of acid-soluble u.v.-dense products from haemoglobin and myoglobin. It was inhibited by heavy metals and several compounds which react with the thiol groups. The optimum temperature was between 37° and 42°C. The molecular weight of the enzyme was calculated to be 24250.

Cathepsin B (EC 3.4.22.1) has been identified from lysosomal fractions in many tissues by Bouma & Gruber (1964), Greenbaum & Fruton (1957) and Tallan *et al.* (1952), including spleen, heart, liver, brain, lung, muscle, kidney, testis, adrenal and thymus tissues. According to the above investigators the enzymes are associated with the phagocytic cells of the spleen and play a vital role in the breakdown of phagocytosed material.

Apart from being a hydrolytic enzyme, cathepsin B may also function as a transferase, although according to Fujie & Fruton (1958) and Durell & Fruton (1954) it is less efficient as a transferase than as a hydrolytic enzyme. Barrett (1973) isolated cathepsin B1 from normal human liver tissue and showed hydrolysis with several *N*-substituted arginine naphthylamides as substrates for the enzyme.

Otto (1971) separated cathepsins B1 and B2 from bovine spleen and detected their occurrence in numerous tissues of the rat.

Although lysosomes have not yet been isolated from placental tissues, histochemical enzyme studies by Fox & Kharkongor (1969, 1970) showed the presence of acid phosphatase, leucine aminopeptidase and non-specific esterase, as well as fifteen other enzymes that were selected for study on intact human placental villi and on the cells grown in culture. Their conclusion was that the enzyme content of the ovoid cells was similar to that of the Hoffbauer cells and that the Hoffbauer cells of the human placenta have a high content of hydrolytic enzymes. Therefore it would appear that cathepsin B1 (and possibly B2) is a major source of proteolytic activity in the placenta. The isolation of human placental cathepsin B1 with α-*N*-benzoyl-α-arginine amide as a routine substrate for the enzyme is described in the present report.

Experimental Procedures

Materials

Full-term placentae were obtained from the obstetrics and Gynecology Department of the Medical University of South Carolina immediately *post partum*, frozen within 15–30min in liquid N_2 and stored at −80°C until used. Substrates used in the enzymic assays were obtained as follows: haemoglobin substrate powder, from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; crystalline bovine serum albumin, from Armour Laboratories, Chicago, Ill., U.S.A.; horse heart myoglobin, from Mann Research Laboratories, New York, N.Y., U.S.A.; α-*N*-benzoyl-α-arginine amide and other synthetic peptides, from Sigma Chemical Co., St. Louis, Mo., U.S.A. The various sizes of Sephadex and DEAE-Sephadex A-50 were obtained from Pharmacia, Uppsala, Sweden; the reagents for polyacrylamide-gel electrophoresis were purchased from Canalco, Rockville, Md., U.S.A., and Eastman Organic Chemicals, Rochester, N.Y., U.S.A.

Methods

Enzyme assay. Cathepsin B1 was assayed with 20mM-α-*N*-benzoyl-α-arginine amide in 0.1M-acetate buffer, pH5.3, containing 1mM-2-mercaptoethanol (Otto, 1967).

Protein concentrations were determined by the procedure of Lowry *et al.* (1951) with crystalline bovine plasma albumin as the standard. Specific activities are expressed as enzyme units/mg of protein.

Electrophoretic analyses. Polyacrylamide-disc electrophoresis (Canalco Co.) was performed by the procedures developed by Ornstein (1964) and Davis (1964).

Concentration of protein samples. Protein samples were concentrated at 4°C by using Amicon Diaflo ultrafiltration apparatus.

Chromatographic procedures. Sephadex G-75 and G-100 and DEAE-Sephadex A-50 were dispersed in water and allowed to swell. After the fine particles had been decanted several times, the gel was poured into a chromatographic column (2cm × 25cm). The gel was then equilibrated in the first buffer to be used in the elution procedures. The effluents were collected as described in the legends to the figures. The E_{280} was measured with a Gilford model 2400 automatic recording spectrophotometer.

Ultracentrifugal analysis. Approximate molecular weights were determined by approach-to-equilibrium studies which were done in a Spinco model E analytical ultracentrifuge by the procedure described by Archibald (1947). The sedimentation-velocity measurements were done at 40°C and 56000rev./min by using a double-sector cell with an aluminium-filled Epon centrepiece.

Experimental and Results

Enzyme purification

Cathepsin B1 was purified from human placenta by a modification of the procedures of Greenbaum & Fruton (1957) and Otto (1967). All steps of the purification procedure were carried out at 4°C unless otherwise indicated. Approx. 200g of placenta was minced and then homogenized in 1000ml of 0.1M-Tris–HCl buffer, pH7.0, containing 1mM-EDTA. After standing overnight, the homogenate was centrifuged at 13000*g* for 30min.

Step 1: *treatment with acid.* The supernatant solution (975ml) was adjusted to pH3.8 with 1M-HCl by slow addition with constant stirring. After standing for 2h, the suspension was centrifuged at 3400*g* for 10min.

Step 2: *$(NH_4)_2SO_4$ precipitation.* $(NH_4)_2SO_4$ (240g) was added to the supernatant (765ml) with constant stirring to give a 60%-satd. solution. After standing for 60min, the mixture was centrifuged (25000*g* for 10min) and the supernatant was discarded. The precipitate was dissolved in 0.1M-Tris–HCl buffer, pH7.0, to a final volume of 45ml, and dialysed overnight against four 1-litre changes of buffer.

Step 3: *DEAE-cellulose chromatography.* The material from Step 2 (78ml) was added to a 2cm × 25cm column of DEAE-cellulose prepared as described under 'Chromatographic procedures'. The column was developed by discrete additions of NaCl (0.1–1.0M) to obtain preliminary enrichment of protein (Sober *et al.*, 1965). The column was washed with each buffer until the E_{280} readings of eluate fractions had returned to baseline values. The fractions under the peak (Fig. 1) were combined and dialysed against six 5-litre changes of 1mM-Tris–HCl buffer, pH7.0, containing 0.5mM-EDTA. This material was concentrated to 75ml in Amicon ultrafiltration apparatus operated at 4–6°C and 345kPa (50lbf/in²).

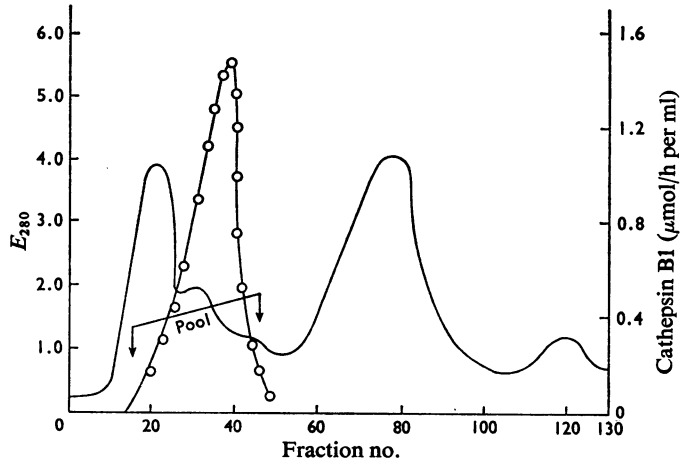


Fig. 1. *Chromatogram of the dialysed $(NH_4)_2SO_4$ fraction of the crude human placental cathepsin B1 on DEAE-Sephadex A-50 in 0.1M-Tris–HCl buffer, pH7.0*

A sample (48ml) was applied to a column (2cm × 25cm). The sample was eluted with NaCl (0.1–1.0M) at approx. 35ml/h into 5ml fractions. ——, E_{280}; ○, cathepsin B1 activity. The major proteolytic fraction contained an esterase, which was measured with *p*-nitrophenyl acetate, together with a small amount of proteinase activity measured with bovine plasma albumin.

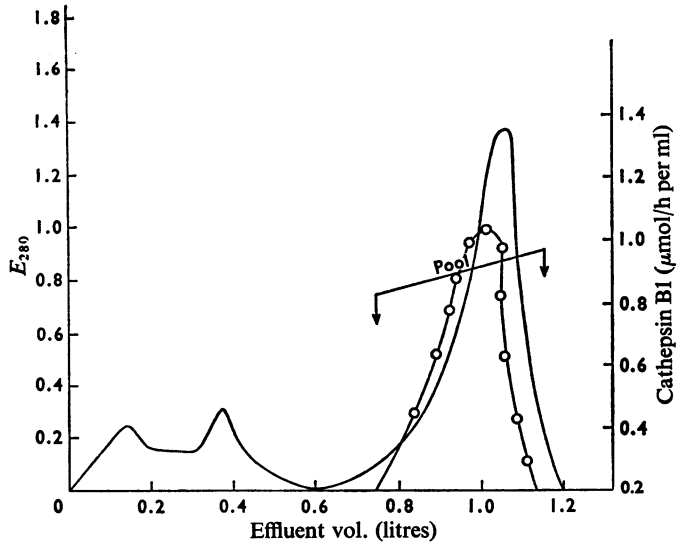


Fig. 2. *Chromatograms of the partially purified placental cathepsin B1 on Sephadex G-75*

——, E_{280}; ○, cathepsin B1 activity. Elution was with buffer at pH 7.6 (40mM-Tris–HCl–1mM-2-mercaptoethanol). The cathepsin B1 peak contained a very small amount of residual proteinase activity, which was measured with bovine plasma albumin.

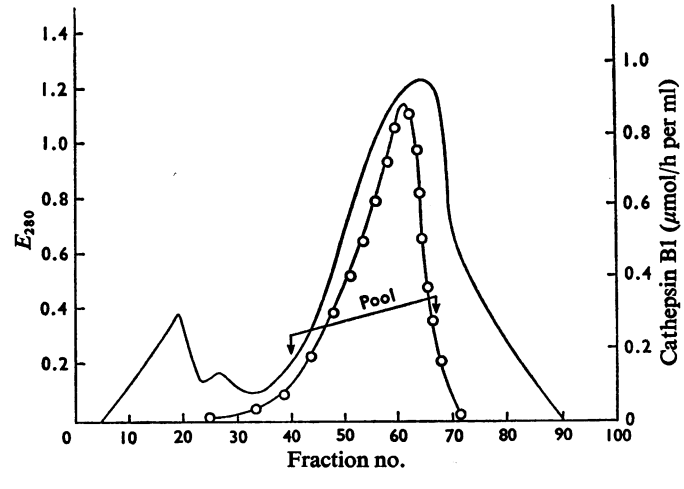


Fig. 3. *Rechromatography on Sephadex G-100*

This chromatogram represents the final step in the isolation experiment summarized in Table 1. The sample was eluted in 1mM-Tris–HCl buffer, pH 7.0, and E_{280} was measured in every other tube. The column (4.25cm × 60cm) had an elution rate of 20ml/h into fractions containing 5ml. ——, E_{280}; ○, cathepsin B1 activity.

Table 1. *Purification of human placental cathepsin B1*

See the text for experimental details and definitions of enzyme units.

Fraction	Volume (ml)	Protein (mg/ml)	Cathepsin B1 Activity (μmol/h per ml)	Cathepsin B1 Recovery (%)	Cathepsin B1 Specific activity (μmol/h per mg)
Crude extract	975	22.40	4.28	100	0.191
Acid fraction	765	16.65	4.40	81	0.264
60%-satd.-$(NH_4)_2SO_4$ fraction	78	18.25	21.84	41	1.197
DEAE-Sephadex fraction	75	6.31	9.96	18	1.578
Sephadex G-75 fraction	50	1.78	14.21	17	7.98
Sephadex G-100 fraction	18	0.69	34.65	15	50.217

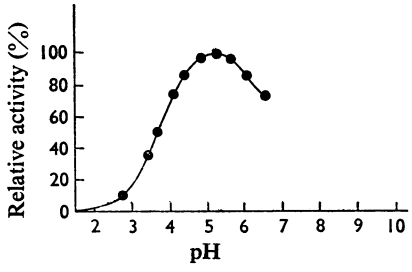


Fig. 4. *Effect of pH on the activity of human placental cathepsin B1*

The relative activity values are expressed as a percentage of the activity at pH 5.0–5.3. Conditions of the experiment are described in the text.

Step 4: gel filtration on Sephadex G-75. The placental cathepsin B1 fraction from Step 3 was subjected to gel filtration on a column of Sephadex G-75 prepared as described above. The column (2.5cm × 50cm) was eluted at a rate of 45ml/h (Fig. 2). The pooled cathepsin B1 eluate was concentrated to 50ml.

Step 5: gradient rechromatography on Sephadex G-100. Representative separation on Sephadex G-100 is shown in Fig. 3. Chromatograms in Figs. 1, 2 and 3 depict steps in the isolation experiment summarized in Table 1. The use of Sephadex G-100 resulted in the removal of impurities, as shown by the sharp increase in specific activity and by ultracentrifugal analysis (Fig. 5).

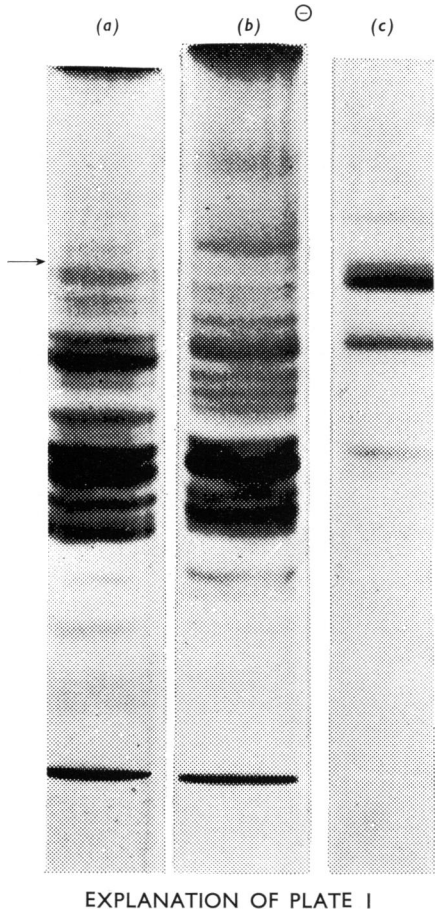
Enzymic properties of human placental cathepsin B1

pH optimum. The effects of pH on enzyme activity were studied by adjusting α-*N*-benzoyl-α-arginine amide substrate to various pH values by using a wide-range buffer which was 0.01M each in sodium acetate, borate and phosphate solutions. The placental cathepsin B1 was then assayed in the usual manner, as shown in Fig. 4. It was stable in the pH range 3.6–6.5 and the pH optimum was near pH 5.5.

Disc electrophoresis. The results of some of the isolation procedures are shown in Plate 1, in which the disc-gel-electrophoretic pattern of the crude placental extract is compared with those from one intermediate step (Step 3) and the final product purified through to Step 5. The placental cathepsin B1 behaved as an acidic protein. A single activity band, coincident with the area of protein staining with Coomassie Blue, was detected histochemically for the human placental enzyme by incubating an unstained section of the gel in a reaction mixture containing 1.3mM-α-*N*-benzoyl-L-arginine amide in 0.1M-sodium acetate buffer, pH 5.5, containing 15mM-2-mercaptoethanol and 0.06% Fast Blue BB (a diazonium salt). Simultaneous coupling readily occurred and a brick-red azo dye was precipitated *in situ*.

Specificity of placenta cathepsin B1. Although α-*N*-benzoyl-α-arginine amide was used as the substrate in our assay because it appeared to be less sensitive to interference by turbidity and coloured materials, it is not necessarily the specific substrate for cathepsin B1 (Otto, 1971). The placental cathepsin B1 readily hydrolysed natural substrates such as denatured haemoglobin and myoglobin but was devoid of activity towards bovine albumin. The behaviour of the placental enzyme with some synthetic substrates is shown in Table 2. The placental proteolytic enzyme readily cleaves peptides containing arginine, although it failed to hydrolyse other endopeptidase substrates such as benzyloxycarbonylglycyl-L-phenylalanine or benzyloxycarbonylglycyl-L-phenylalanine methyl ester and the aminopeptidase substrate, L-leucyl-β-naphthylamide.

Effects of inhibitors and activators on human placental cathepsin B1. In the absence of thiol compounds such as mercaptoethanol, the enzyme preparations were unstable and rapidly lost activity. Addition of mercaptoethanol did not result in an increase in activity but addition of cysteine or dithiothreitol did give an increase in activity. Thiol inhibitors such as iodoacetate, *p*-chloromercuribenzoate, *N*-ethylmaleimide and iodoacetic acid produced considerable inhibition.



EXPLANATION OF PLATE 1

Electrophoretic patterns on polyacrylamide gels of fractions obtained during the purification of the human placental cathepsin B1

Gels were stacked at pH 5.6 with samples containing 100 μg of enzyme and run at 2.5 m/gel (Ornstein, 1964; Davis, 1964). (*a*), Crude placental enzyme extract; (*b*), the active fraction from Step 3; (*c*), the final placental cathepsin B1 after Step 5. The ⊖ indicates the cathodic end of the gel. The arrow indicates the position of the cathepsin B1.

Table 2. *Action of human placental cathepsin B1 on some synthetic substrates*

Activities were determined under initial-velocity conditions at 37°C and substrate concentration of 0.01 M. Relative activities shown above are expressed as a percentage of the activity with α-*N*-benzoyl-α-arginine amide. Abbreviation: Cbz, benzyloxycarbonyl.

Substrate	Relative activity (%)
α-*N*-Benzoyl-DL-arginine *p*-nitroanilide	130
α-*N*-Benzoyl-DL-arginine β-naphthylamide	118
α-*N*-Benzoyl-L-arginine ethyl ester	90
p-Tosyl-L-arginine methyl ester	80
Benzoyl-L-lysine amide	20
Cbz-L-glutamyl-L-tyrosine	10
Cbz-glycyl-L-phenylalanine amide	0
Cbz-glycyl-L-phenylalanine methyl ester	0
L-Leucine β-naphthylamide	0
L-Alanyl-L-phenylalanine	0
L-Leucylglycine	0
Cbz-L-alanyl-L-phenylalanine	0

Table 3. *Effects of various activators and inhibitors on human placental cathepsin B1*

The enzyme was dialysed against water and assays for enzymic activity were performed as described in the text. The enzyme was incubated with the activator or inhibitor for 1 h at 37°C before assay. Results are expressed as the percentage of activity in the presence compared with the activity in the absence of added materials.

Compound	Concentration (mM)	Change in activity (%)
Dithiothreitol	0.1	130
	2	210
Cysteine	0.1	120
	5	195
Phenylmethanesulphonyl fluoride	0.1	35
	1	20
α-*N*-Tosyl-L-lysyl chloromethyl ketone	0.1	30
	1	10
N-Ethylmaleimide	0.1	20
	1	4
Iodoacetamide	0.1	15
	1	0
p-Chloromercuribenzoate	0.01	8
	1	0
Iodoacetic acid	0.1	5
	1	0
Hg^{2+}	0.05	0
Zn^{2+}	0.05	0
Cu^{2+}	0.05	0
Mn^{2+}	0.05	0
Ni^{2+}	0.05	0

Table 4. *Effect of temperature on enzymic activity*

The enzyme was incubated with substrate (α-*N*-benzoyl-α-arginine amide) at pH 6.3 for 15 min at the temperature shown. The extent of hydrolysis at 37°C was assigned a value of 100 and activities at other temperatures are expressed as relative values.

Temperature (°C)	Activity (%)
10	0
20	0
30	25
35	73
37	100
40	100
42	96
45	82
45	82
50	35
55	10
60	0
65	0
70	0

Other compounds which may interfere with the thiol group of the enzyme such as phenylmethanesulphonyl fluoride and α-*N*-tosyl-L-lysyl chloromethyl ketone produced marked inhibition. Proteolytic activity was greatly inhibited by heavy-metal ions, such as Hg^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} in concentrations of 0.05 mM, supporting the supposition that a thiol group is part of the enzyme (Otto, 1967). Table 3 lists various compounds affecting the activity of placental cathepsin B1 enzyme.

Temperature stability of the human placental cathepsin B1. The enzyme was more stable in Tris, veronal or acetate buffers than in phosphate or borate–NaOH buffers; citrate buffers above pH 6.5 led to a rapid loss of enzyme activity in any buffer. Table 4 shows the effects of the temperature of incubation and indicates that maximum enzymic activity for a 15 min reaction is at approx. 37–42°C.

Molecular weight. Sedimentation patterns obtained by ultracentrifugal analysis are shown in Fig. 5 for human placental cathepsin B1. On the basis of the values from sedimentation and diffusion measurements, the Svedberg equation gave a mol.wt. of 24250.

Discussion

In recent years the number of publications dealing with the isolation and characterization of cathepsins has increased. It has become evident that the physiological and pathological degradation of protein *in vivo*, like its synthesis, is very interesting. These investigations lagged behind studies of protein synthesis. Such biological phenomena as turnover of tissue and

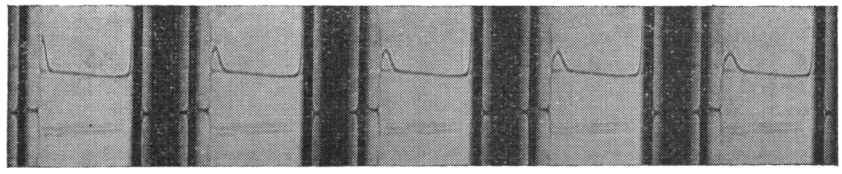


Fig. 5. *Sedimentation pattern of the isolated human placental cathepsin B1*

Schlieren images obtained during a sedimentation-velocity experiment in which the protein concentration was 8 mg/ml are shown. Exposures were made at 6 min, 18 min, 22 min and 26 min after the rotor attained 56000 rev./min. In each case, the schlieren-phase-plate angle was 70°. Sedimentation is from left to right.

extracellular protein, regeneration, autolysis and possibly selective transport are also related to intracellular proteolysis. From this viewpoint, we can see a need for intensified study of cathepsins and their role in protein metabolism.

The cathepsin B1 enzymes are ubiquitously distributed in mammalian tissues. However, it is evident that the enzyme displays a characteristic pattern of distribution among the various tissues. Although the patterns of distribution of the enzyme among different tissues are of value for purification purposes, they offer little insight into the role of the cathepsin B1 in cellular metabolism.

The placental cathepsin B1 had somewhat higher activity with α-*N*-benzoyl-DL-arginine *p*-nitroanilide and α-*N*-benzoyl-DL-arginine β-naphthylamide. These may be more specific substrates for mammalian cathepsin B1, as suggested by the studies of Otto (1971) and Barrett (1973), than the α-*N*-benzoyl-α-arginine amide used in our assays.

The requirement for mercaptoethanol for stability suggested the presence of a thiol-dependent enzyme. This observation was confirmed by the increased activity noted in the presence of dithiothreitol and cysteine, and the inhibition on addition of thiol inhibitors tends to support the claim of other investigators (Greenbaum & Fruton, 1957; Otto, 1971; Barrett, 1973).

The results presented here show that we have worked out a reproducible procedure for the isolation of a cathepsin B1 from human placenta with a high degree of purity as evidenced by electrophoretic, ultracentrifugal and enzymic criteria.

Christie (1968) used what he termed A and C esterase as a marker for cathepsins. His results shown an increase in human placental esterases suggesting an increased breakdown of materials for utilization or transport to the foetus with placental aging. Also, increased esterase activity was observed in both epithelium and trophoblasts, which may be concerned in protein metabolism.

Our determination of some physical properties appears to be the first reported for human placental cathepsin B1. None of the enzymes reported to have been found in the placenta have been characterized except arylsulphatase (Gniot & Kosnoszynski, 1972), and neither cathepsin B1 nor arylsulphatase has been assigned a specific metabolic role.

These results suggest the appropriateness of continued research on placental hydrolytic enzymes for the purpose of seeking additional information on natural substrate specificities and for further investigation of the mechanism of action of cathepsin B1 in the placenta.

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