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CATHEPSINS B1 FROM HUMAN FETAL MEMBRANES

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Summary

Cathepsins B1 (EC 3.4.22.1) were isolated from fetal membranes of human placenta, i.e. amnion and chorion-decidua. Purification of the enzymes was achieved by the freezing-thawing technique, ammonium sulphate fractionation and Sephadex gel filtration. Cathepsis B1 separated either from amnion or from chorion-decidua exhibited optimum activity at pH 6.2, and an optimum temperature between 42—45°C. They were inhibited by heavy metals, and compounds which react with the thiol groups. Isoelectric focusing demonstrated three isoenzymes of cathepsin B1 originating from chorion-decidua, while only one band was found for the enzyme from amnion.

Introduction

Human fetal membranes are derived primarily from the embryo. Their main layers are the amnion and the chorion although fragments of maternal decidua remain attached after delivery. With the complex morphology of fetal membranes, the variety of active transport processes and syntheses, and presence of several enzymes [1—3], the vital significance of the membranes is demonstrated and the proper development of the fetus is highly dependent on their biological functions.

The aim of the present paper has been the isolation and characterization of cathepsins B1 (EC 3.4.22.1) from fetal membranes and comparison of their properties with those of the enzyme of placenta isolated and described by Swanson et al. [4]. It seemed probable that differences in anatomical and morphological structure as well as in biological functions might be reflected in tissue specificity of the enzyme.

Abbreviations: BANA, α-*N*-benzoyl-DL-arginine-β-naphthylamide; EDTA, ethylenediaminetetraacetic acid; $HOHgBz^-$, *p*-hydroxymercuribenzoate sodium salt; $ClHgBz^-$, *p*-chloromercuribenzoate sodium salt; Tos-LysCH_2Cl, L-1-chloro-3-*p*-tosylamido-7-amino-2-heptanone; Tos-PheCH_2Cl, L-1-chloro-3-*p*-tosylamido-4-phenyl-2-butanone.

Materials and Methods

Fetal membranes

Human fetal membranes, i.e. amnion (placental and parietal) and chorin-decidua (extraplacental portion) were separated from placentas obtained from healthy women who delivered naturally on term and did not experience any complication of pregnancy, labour or during post-delivery period. Tissues were washed free of adherent blood with distilled water, dried on filter paper and stored at −20°C until required.

Chemicals

Crystalline bovine serum albumin, Tween 20 and *N'*,*N'*-methylene bisacrylamide were obtained from Koch-Light Laboratories, England. α-*N*-benzoyl-DL-arginine-β-naphthylamide hydrochloride (BANA), DL-cysteine hydrochloride were from Reanal, Hungary. L-1-chloro-3-*p*-tosylamido-4-phenyl-2-butatone, L-1-chloro-3-*p*-tosylamido-7-amino-2-heptanone and Coomassie brilliant blue G-250 were purchased from Serva, Germany. *N*,*N*,*N'*,*N'*-tetramethylenediamine, and acrylamide were products of Fluka AG, Switzerland. Iodoacetic and *p*-chloromercuribenzoic acid were from Chemapol, Czechoslovakia. Sephadex G-75 and G-100 were products of Pharmacia Fine Chemicals, Sweden. Fast Garnet GBC was from G.T. Gurr, U.K. *p*-Hydroxymercuribenzoate sodium salt was purchased from Sigma, U.S.A., and iodoacetamide from Merck, Germany. Ampholine (pH 4—6) was from LKB, Sweden.

Polyacrylamide gel electrophoresis

Electrophoresis was performed by the procedure according to Maurer [5] using gel concentration 7.5% in β-alanine-acetic acid buffer, pH 4.5, the current 3 mA per tube, for 2 h at 4°C. Protein in the gels was stained with Coomassie brilliant blue G-250, and cathepsin B1 activity was located as described by Barrett [6]: the gels were incubated for 30 min at 40°C in 5 ml of 0.1 M phosphate buffer containing 1.33 mM EDTA, 2.67 mM cysteine, and 4.0 mg of BANA dissolved in 100 μl of dimethyl sulphoxide. β-naphthylamine liberated within the gels by the action of cathepsin B1 was visualized by transferring the gels to the solution of Fast Garnet GBC (1 mg/ml) in 5 mM *p*-chloromercuribenzoate, 25 mM EDTA for 15 min at room temperature.

Isoelectric focusing

Isoelectric focusing in polyacrylamide gel was carried out according to Drysdale et al. [7]. Acrylamide, ampholine of pH range 4—6 and a solution of ammonium persulphate were diluted to concentrations of 4%, 2% and 0.02% respectively. After being degassed, the mixture was adjusted with *N*,*N*,*N'*,*N'*-tetramethylenediamine up to a concentration of 2%. The tubes 10 cm long and 0.5 cm diameter were used. The procedure was performed for 4 h at 4°C using 0.02 M phosphoric acid in anode and 0.01 M NaOH in cathode vessel, and a current 1 mA per tube. The gels were stained for cathepsin B1 activity in the same way as after electrophoresis. For pH determinations the gels were cut into 20 or more sections and each section was immersed in 1 ml of distilled water.

Assays

Protein concentration was determined by the procedure of Lowry et al. [8] with crystalline bovine serum albumin as the standard.

Enzyme activity was assayed according to Barrett [9] with α-*N*-benzoyl-DL-arginine-β-napththylamide hydrochloride as substrate. To an 0.5 ml sample were added 1.5 ml of 0.1 M phosphate buffer pH 6.0 with 1.33 mM EDTA, 2.67 mM cysteine, and 50 μl of substrate containing 40 mg/ml of dimethyl sulphoxide. The mixture was incubated for 1 h at 40°C, then the reaction was stopped by addition of 2 ml of the solution prepared by mixing equal volumes of sodium *p*-chloromercuribenzoate (3.57 mg/ml) and Fast Garnet GBC in 4% Tween 20 solution (0.5 mg/ml). Extinction was read at 520 nm using a Specol photocolorimeter.

Enzyme activity was expressed in units corresponding to the amount of enzyme necessary to hydrolyze 1 μmol of substrate/min. Specific activity was calculated in units/mg of protein.

Effect of potential inhibitors was investigated as follows: a solution of purified enzyme was dialysed against distilled water for 24 h at 4°C and cysteine and EDTA then were added up to concentrations of 2 mM and 1 mM, respectively. After 5 min the activated enzyme was diluted 1 : 3 (v/v) with 0.1 M phosphate buffer, pH 6.0 and cathepsin B1 activity measured in the buffer alone or containing a potential inhibitor. When studying an effect of Zn^{2+} or Ca^{2+} the phosphate buffer was replaced by 0.01 M veronal buffer pH 6.0; the effect of Tos-PheCH_2Cl was examined using 5% solution of this compound in *n*-propanol.

Results

Purification of cathepsin B1 from fetal membranes

All steps of the purification procedure were carried out at 4°C other than the thawing of tissue homogenates in step 1.

(1) Extraction. Refrigerated tissue of amnion (200 g) or chorion-decidua (140 g) were minced and then homogenized for 5 min with 2 vol. of 0.2 M acetate buffer pH 4.3 containing 1 mM EDTA. After a 1-h extraction the homogenates were submitted to twofold freezing and thawing in order to disrupt cell membranes, then they were centrifuged for 30 min at 6000 × *g* and the supernatant was used for further steps of enzyme purification.

(2) Ammonium sulphate precipitation. (a) Amnion: to 730 ml of the supernatant, solid $(NH_4)_2SO_4$ with constant stirring was added to get a 60% saturation. After standing for 1 h, the mixture was centrifuged for 15 min at 6000 × *g* and the supernatant was discarded. The precipitate was dissolved in 25 ml of distilled water and dialysed overnight against 0.5 M acetate buffer, pH 5.5 containing 0.2 M NaCl and 1 mM EDTA. (b) Chorion-decidua: to 305 ml of the supernatant, $(NH_4)_2SO_4$ was added to 40% saturation. The suspension was left for 1 h and centrifuged. The precipitate was discarded and the supernatant was adjusted with $(NH_4)_2SO_4$ to 70% saturation. The precipitate was treated in the same way as that, prepared from amnion.

(3) Filtration through Sephadex gel. (a) Amnion. Material obtained after dialysis was centrifuged for 15 min at 6000 × *g*. The supernatant was loaded on

to the Sephadex G-75 column (140 × 2 cm) equilibrated with 0.5 M acetate buffer pH 5.5 containing 0.2 M NaCl and 1 mM EDTA. Elution was achieved with the same buffer, at a flow rate of 20 ml/h. The volume of the fractions collected was 3.5 ml. Enzymatic activity was eluted from the column after large peak of higher molecular weight inactive protein. Partially purified enzyme obtained this way exhibited specific activity 0.056 units/mg of protein (Table I). (b) Chorion-decidua. Gel filtration was carried out essentially in the same way as with amnion but Sephadex G-100 was used. The enzyme preparation obtained was found to show specific activity 0.139 units/mg of protein being 30-fold purified in relation to crude homogenate. Results of the particular steps of purification of cathepsins of fetal membranes are shown in Table I.

Enzymic properties of cathepsins B1 from fetal membranes

Effect of pH and temperature on hydrolysis of BANA. Effect of pH on the enzyme activity was studied by adjusting the solution of substrate to various pH values with 0.1 M acetate and phosphate buffers containing 1.33 mM EDTA and 2.67 mM cysteine. Cathepsin B1 activity was then measured. The results obtained are shown in Fig. 1. As can be seen in Fig. 1 both cathepsins independent of the source show a pH optimum within the range of 6.0—6.2.

The effect of temperature was studied using a 15 min incubation of the enzyme with the substrate at pH 6.0 in temperatures between 6 and 60°C. Elevation of temperatures resulted in gradual increase of activities of both the cathepsins reaching a maximum at approximately 42—45°C (Fig. 2).

Stability in alkaline pH. Stability of the enzyme in alkaline pH was examined using the method of Barrett [6]. A sample of cathepsin B1 was mixed with an equal volume of 0.5 M Na_2HPO_4 (final pH 8.0) and left for 1 h at room temperature. pH of the mixture was adjusted to 6.0 by the use of 5.0 M formate buffer, pH 2.8 and activity of the enzyme was assayed. Another sample of the enzyme was treated in the same way but without exposure to alkaline pH. It

TABLE I

PURIFICATION OF CATHEPSINS B1 FROM HUMAN FETAL MEMBRANES

Tissue Step of purification	Volume (ml)	Total protein (mg)	Total activity (units)	Spec. act. (units/mg)	Degree of purification	Yield (%)
Amnion						
Homogenate	730	598.6	0.97	0.0015	—	—
Freezing and thawing	730	846.8	2.43	0.0028	1.8	100.0
$(NH_4)_2SO_4$ fractionation (60% satd.)	37	458.8	1.42	0.0085	5.7	58.3
Sephadex G-75	68	17.1	0.1	0.056	37.3	40.9
Chorion-decidua						
Homogenate	305	701.5	3.05	0.0043	—	—
Freezing and thawing	305	701.5	4.58	0.0065	1.5	100.0
$(NH_4)_2SO_4$ fractionation (40—70% satd.)	33	277.2	4.29	0.0153	3.5	93.7
Sephadex G-100	96	17.4	1.75	0.139	32.3	38.3

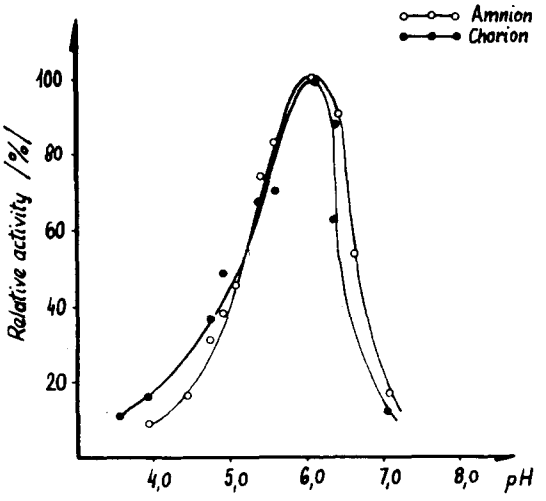

Fig. 1. Effect of pH on the activity of cathepsins B1 from human placental membranes. The relative activity values are expressed as a percentage of the activity at pH 6.0. Conditions of the experiment are described in the text.

was shown that under the conditions of the experiment neither amnion nor chorion cathepsin retained any enzymatic activity.

Effect of potential inhibitors. Preparations of cathepsin B1 from fetal membranes were dialyzed against distilled water for 24 h and were then activated with cysteine and EDTA. The final concentrations of these effectors in the incubation mixture were 166 and 83 μM, respectively. Absence of such thiol compounds as cysteine or mercaptoethanol resulted in complete loss of enzymic activity. Results of the experiment on the effects of various compounds affecting the activity of cathepsin B1 from amnion and chorion are presented in Table II.

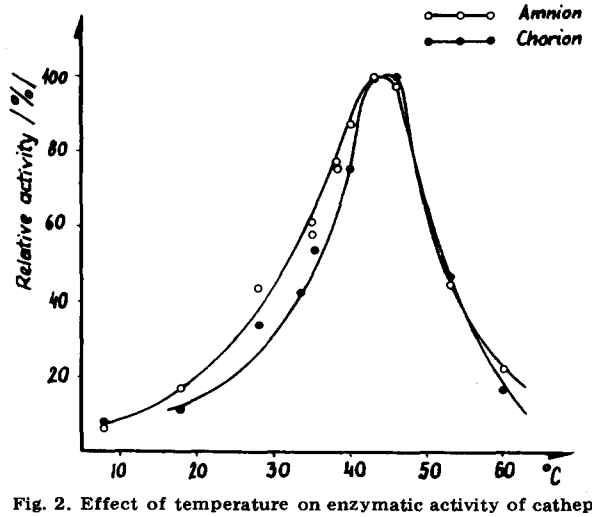

Fig. 2. Effect of temperature on enzymatic activity of cathepsins B1 from fetal membranes. The enzymes were incubated with substrate at pH 6.0 for 15 min at the given temperatures. The extent of hydrolysis at 42°C was assigned a value of 100%.

TABLE II

EFFECT OF POTENTIAL INHIBITORS ON CATHEPSIN B1 FROM HUMAN FETAL MEMBRANES

Before the assay proper, the enzymes were preincubated at pH 6.0 with each examined compound for 5 min at 40°C (see the Methods section for details). Results are expressed as the percentage of activity in the presence compared with the activity in the absence of added materials.

Compound	Final conc. (mM)	Inhibition (%)	
		Amnion	Chorion-decidua
p-Hydroxymercuribenzoate	0.1	100.0	100.0
$HgCl_2$	0.1	100.0	100.0
$HgCl_2$	0.01	39.0	44.5
$HgCl_2$ + EDTA	0.1 + 1.0	100.0	100.0
$HgCl_2$ + EDTA	0.01 + 1.0	40.0	50.0
$ZnSO_4$	0.1	95.0	100.0
$ZnSO_4$ + EDTA	0.1 + 1.0	0.0	0.0
$MnSO_4$	0.1	49.0	48.0
$MnSO_4$	0.05	24.0	24.0
$CaCl_2$	1.0	55.0	50.0
Iodoacetic acid	1.0	100.0	100.0
Iodoacetamide	1.0	100.0	100.0
Tos-PheCH_2Cl	0.1	97.0	100.0
Tos-LysCH_2Cl	0.1	91.0	100.0

As can be seen, classic inhibitors of thiol groups (iodoacetamide, iodoacetic acid, *p*-chloromercuribenzoate) produced complete loss of cathepsin activity. Other reagents which are known to react with the thiol groups of enzyme as Tos-PheCH_2Cl, Tos-LysCH_2Cl caused also considerable inhibition of both the cathepsins isolated either from amnion or from chorion. Proteolytic activity was inhibited 100% by heavy-metal ions, such as Hg^{2+} and Zn^{2+}. The Zn^{2+}-

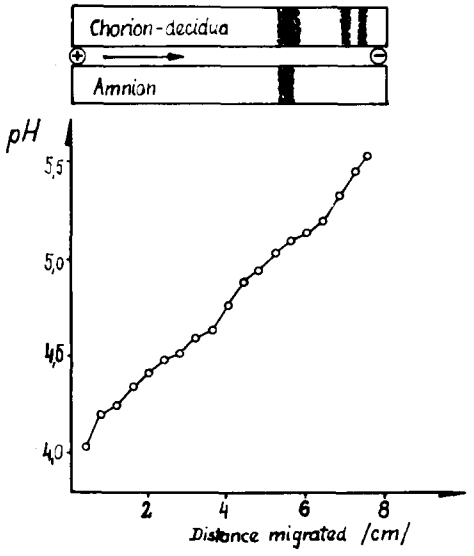

Fig. 3. Gel isoelectric focusing of cathepsins B1 from fetal membranes. 300 μg of protein was subjected to electrofocusing on polyacrylamide gel, and enzyme activity was located. For details see Methods.

dependent inhibition was abolished by the addition of EDTA resulting in a complete recovery of enzymic activity.

Polyacrylamide gel electrophoresis. Partially purified preparations of cathepsins B1 from fetal membranes were submitted to disc electrophoresis at pH 4.5. Three protein zones stainable with Coomassie blue were detected, with one out of three showing enzymic activity.

Isoelectric focusing. Samples of cathepsins B1 from fetal membranes (0.16 units) were run in isoelectric focusing polyacrylamide gel, using ampholine pH 4—7. The distribution of activity was demonstrated according to Barrett [6]. The enzyme isolated from amnion showed one peak of activity with isoelectric point between 5.0 and 5.1. In the case of chorion-decidua enzyme three zones of activity were detected within pH range 5.0—5.5, the main zone of Ip 5.0—5.2, two other 5.4 and 5.5, respectively (Fig. 3).

Discussion

Cathepsin B1 (EC 3.4.22.1) has been identified as a lysosomal enzyme present in numerous animal tissues including placenta [4]. It has been suggested that the enzyme plays a role in modification of glucokinase, fructose diphosphate aldolase, pyruvate kinase [10] fructose-1,6-diphosphatase [11] as well as in degradation of collagen [12,13] and native albumin [14].

Swanson et al. [4] isolated cathepsin B1 from human placenta and described some properties of the enzyme which is considered a major source of proteolytic activity in the placenta. Such important processes of molecular biology as turnover of tissues and intracellular proteins, regeneration, autolysis and selective transport are related to intracellular proteolysis.

The present paper has been concerned with cathepsins B1 isolated from fetal membranes which functionally and morphologically represent quite different entities than "placenta vera"; on the other hand, amnion and chorion-decidua differ from each other in several properties, the chorion being very active in the biological mother-fetus exchange. Specific activity of cathepsin B1 in the extract from chorion was 3-fold higher than that from amnion and very similar to the specific activity of cathepsin B1 from placenta reported by Swanson et al. [4]. A similar distribution of LDH activity among fetal membranes and placenta was described by Bauer et al. [1] and Lapan and Friedman [2]. On the contrary, enzymes of Krebs cycle have been much more active in placenta than in membranes [2], which can be explained by a predominance of aerobic processes whereas in membranes anaerobic ones largely prevail.

In spite of different specific activities of cathepsins B1 from particular membranes several of their properties such as optimum pH 6.0, temperature dependence, stability in alkaline pH, effect of metal-ions were fairly comparable. The placental cathepsin B1 had somewhat lower pH optimum 5.5 and was completely inhibited by 0.05 M Mn^{2+} ions [4] while the enzyme of membranes retained under the same conditions 76% activity. The effects of pH on activity and stability closely confirm those of Barrett [6] with the liver enzyme.

Existence of three isoenzymes of cathepsin B1 from chorion differing in Ip in comparison with one band of activity in amnion was demonstrated in isoelectric focusing in polyacrylamide gel, by the use of BANA as substrate. Iso-

enzymes of cathepsin B1 isolated from bovine spleen [13,15,16] and ovine thyroid [17] were demonstrated. Barrett [6] by the use of direct staining of analytical isoelectric focusing gels for cathepsin B1 activity found six isoenzymes in human liver. It should be pointed out that this author used a more sensitive substrate than ours namely benzyloxycarbonyl-L-arginine-4-methoxynaphthylamide hydrochloride.

The nature of microheterogeneity of cathepsin B1 is not known at this time. It seems likely that these enzymatically active components found in chorion are neither artifacts resulting from the isolation procedure nor genetic variants representing particular individuals because identical isolation procedures have always resulted in one band of cathepsin B1 activity of amnion and three bands of chorion-decidua respectively. The possibility that these components represented differential oxidation states of the cysteine residues of a single enzyme species was excluded by Franklin and Mertione [15]. Moreover since cathepsin B1 is not a glycoprotein [6] the concept of microheterogeneity consisting in different contents of sialic acid as is the case with some other lysosomal enzymes, should be rejected.

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