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CATHEPSIN H FROM HUMAN PLACENTA*

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Cathepsin H was isolated from human placenta by autolysis, acetone fractionation, and chromatography on DEAE-cellulose, Sephadex G-75, hydroxyapatite and concanavalin A-Sepharose. The enzyme gave on SDS-polyacrylamide gel electrophoresis two bands of $M\text{,}c\text{,}5\text{,}500$ and $28\text{,}500$. Two active forms of the enzyme, with $pI$ of 6.0 and 6.45, were obtained by isoelectric focusing. The enzyme is stable over the $pH$ range 5-7.5, whereas it becomes inactive on heating to 50°C. Cathepsin H of human placenta, like the enzyme from other sources, hydrolyses protein and naphthylamide substrates, showing within the latter group the strongest preference towards arginine-$\beta$-naphthylamide ($pH$ optimum 6.8). The enzyme is inhibited by the known inhibitors of cysteine proteases and by placental cystatins.

Cathepsin H (EC 3.4.22.16) belongs, together with cathepsins B, L and S, to the papain superfamily [1]. Enzymes of this superfamily, widely distributed in eukaryotic organisms, play an important role in intracellular degradation of proteins [2]. It is believed that cathepsin H acts in lysosomes mainly as aminopeptidase although, like cathepsin B, it shows also endopeptidase activity [3]. So far, in humans, the enzyme has been isolated from liver [4] and kidney [5].

The aim of the present work was to isolate cathepsin H from normal human placenta, and to study some of its properties.

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MATERIALS AND METHODS

Material. The placentae collected from healthy women immediately after normal term delivery were separated from foetal membranes, washed with water, blotted on filter paper, frozen and kept at −20°C.

Methods. The activity of cathepsin H was routinely determined at 40°C with L-arginine-β-naphthylamide hydrochloride in 37.5 mM phosphate buffer, pH 6.8, containing 3 mM cysteine [4, 6], or at 37°C with azocasein as a substrate in 50 mM phosphate buffer, pH 6.0, containing 5 mM Na₂EDTA and 5 mM reduced glutathione [7]. The amount of the enzyme decomposing 1 μmol of the substrate during 1 min was defined as one unit of its activity.

Concentration of the active enzyme in cathepsin H preparations was determined by titration with the specific inhibitor E-64 [8].

The optimum pH for cathepsin H activity with arginine-β-naphthylamide as a substrate was determined using 0.15 M citrate/sodium phosphate buffer containing 3 mM cysteine, over the pH range from 3.0 to 9.0.

To determine stability of cathepsin H, 0.2 ml of the enzyme solution in 75 mM citrate/sodium phosphate buffer of various pH was incubated for 1 h at 22°C. Then 1.8 ml of 0.1 M phosphate buffer, pH 6.8, containing cysteine was added and the enzyme activity was assayed [4].

Thermostability of cathepsin H was measured after 30 min incubation of the enzyme at temperatures ranging from 20 to 70°C.

The effect of protease inhibitors on cathepsin H was studied after 5 min preincubation; the activity was assayed with arginine-β-naphthylamide and inhibition was expressed in percent. All the inhibitors except phenylmethylsulphonyl fluoride (PMSF) and pepstatin A were dissolved in 37.5 mM phosphate buffer, pH 6.8. PMSF was dissolved in 5% ethanol, and pepstatin A in 0.1 M Na⁺H, then both inhibitors were diluted with the phosphate buffer to the appropriate concentration.

The inhibition of cathepsin H by cystatins was studied after 5 min preincubation at 40°C. The binding ratio was calculated by extrapolation of the linear part of the inhibition curve (0 - 50% inhibition). The molar concentration of cystatins was calculated assuming their Mᵣ value to be 12000 [9].

Electrophoresis in 15% polyacrylamide gel in non-reducing and reducing conditions was performed according to Laemmli et al. [10, 11]. The gels were stained with silver nitrate according to Morrissey et al. [12].

Isoelectric focusing in polyacrylamide gel was carried out as described by Barrett [13] using Servalyt 4 - 9. To locate the enzyme, gels were incubated for 5 min at 40°C in 37.5 mM phosphate buffer, pH 6.8, containing 3 mM cysteine. Then the substrate (L-leucyl-β-naphthylamide) was added to the final concentration of 1 mM. After 10 min incubation, gels were transferred to the Fast Garnet GBC staining reagent [14].
Protein concentration was determined according to Lowry et al. [15] with bovine serum albumin as a standard, or by measuring absorbance at 280 nm, taking A₁ cm = 12.2 [4].

l-trans-Epoxy-succinylleucylamido(4-guanidino)butane (E-64) was obtained from Sigma (U.S.A.). α-N-Benzoyl-DL-arginine-β-naphthylamide, l-arginine-β-naphthylamide and l-leucyl-β-naphthylamide were from Koch-Light (England), and azocasein from Serva (F.R.G.). Inhibitors of cysteine proteinases were isolated from human placenta as described previously [9].

RESULTS

Preparation of cathepsin H. The enzyme from human placenta was prepared by the method described for human liver [4], i.e. it included autolysis, acetone fractionation, ion-exchange chromatography on DEAE-cellulose, gel filtration, hydrophobic chromatography on hydroxyapatite, and affinity chromatography on Con A-Sepharose 4B. The cationic detergent Arquad 2C-50 used in the original method was replaced by Triton X-100, and Ultragel AcA 54, by Sephadex G-75 Super Fine.

The results of preparation procedure are presented in Table 1. The cathepsin H preparation of specific activity of 0.36 units/mg was obtained with a yield of 1%. The enzyme was titrated with the specific inhibitor of cysteine proteases, E-64, and used for further experiments. For calculation of molar concentration of active cathepsin H, its molecular weight (27000) was determined by gel filtration on Sephadex G-75.

### Table 1

Isolation of cathepsin H from human placenta

At the first two steps of the preparation procedure, protein was determined according to Lowry et al. [15], and at further steps by measuring absorbance at 280 nm. The enzyme activity was determined with arginine-β-naphthylamide as a substrate.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Activity (units)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenization</td>
<td>760</td>
<td>18240</td>
<td>4.65</td>
<td>1</td>
</tr>
<tr>
<td>Autolysis</td>
<td>605</td>
<td>6897</td>
<td>2.84</td>
<td>1.7</td>
</tr>
<tr>
<td>Acetone fractionation</td>
<td>73</td>
<td>195.20</td>
<td>1.68</td>
<td>34</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>173</td>
<td>95.20</td>
<td>1.39</td>
<td>58</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>20</td>
<td>12.20</td>
<td>0.42</td>
<td>137</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>47</td>
<td>1.15</td>
<td>0.15</td>
<td>520</td>
</tr>
<tr>
<td>Con A-Sepharose</td>
<td>17</td>
<td>0.14</td>
<td>0.05</td>
<td>1420</td>
</tr>
</tbody>
</table>
Properties of placental cathepsin H. The results of polyacrylamide-gel electrophoresis show that both in non-reducing and reducing conditions the enzyme migrated as a doublet of $M_r$ 25,500 and 28,500 (Fig. 1). On isoelectric focusing two active forms of $pI$ 6.0 and 6.45 were found.

Cathepsin H determined with arginine-$\beta$-naphthylamide showed the highest activity at pH 6.8 and was stable over the pH range from 5 to 7.5. The enzyme heated for 30 min at 40°C lost 35% of its activity, and at 50°C was practically inactive.

Fig. 1. Electrophoresis of the human placenta cathepsin H: A, in 15% polyacrylamide gel in non-reducing (b) and reducing (c) conditions. As standards (a), bovine serum albumin, chicken ovalbumin, chymotrypsinogen and myoglobin were used; B, in 7% polyacrylamide gel in a pH gradient.

As shown in Table 2, cathepsin H hydrolysed endopeptidase substrates (azocasein) as well as aminopeptidase substrates. Among the naphthylamide substrates, arginine-$\beta$-naphthylamide was preferentially hydrolysed while leucyl-$\beta$-naphthylamide and benzyol-DL-arginine-$\beta$-naphthylamide showed lower affinities for the enzyme.

The effect of some potential protease inhibitors on cathepsin H from human placenta was compared with their effect on the human liver [4] enzyme (Table 3). Powerful inhibition of the placental enzyme by iodoacetic acid, 4-aminophenylmercuribenzoate (100%), and iodoacetamide (65%) confirmed its classification as a cysteine protease. Puromycin inhibited cathepsin
Table 2
Substrate specificity of cathepsin H from human placenta

The amount of the active enzyme protein was determined by titration with E-64 [8]. In the case of azocasein, for calculations the mean molecular weight of 22 000 [7] was taken.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (mM)</th>
<th>Units $\times$ mg$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine-$\beta$-naphthylamide</td>
<td>1</td>
<td>0.100</td>
</tr>
<tr>
<td>Leucyl-$\beta$-naphthylamide</td>
<td>3.4</td>
<td>0.060</td>
</tr>
<tr>
<td>Benzoyl-DL-arginine-$\beta$-naphthylamide</td>
<td>2.3</td>
<td>0.045</td>
</tr>
<tr>
<td>Azocasein</td>
<td>1</td>
<td>0.850</td>
</tr>
</tbody>
</table>

H only slightly compared with its effect on non-lysosomal leucine naphthylamidase activities reported in other tissues [4]. The effect of other inhibitors on placental cathepsin H was negligible, in contrast to the liver enzyme which was substantially inhibited by N-ethylmaleimide, leupeptin and soybean trypsin inhibitor (Table 3).

Data on titration of placental cathepsin H by the inhibitor specific for cysteine protease, E-64, and inhibition of the enzyme by endogenous cystatins are given in Fig. 2A, B. The same amount of E-64 and cystatins (1.77 and 1.78 nmol, respectively) was required for total inhibition of the enzyme activity. Since titration with specific inhibitor E-64 determined the

Table 3
The effect of inhibitors of cysteine proteinases on the activity of human cathepsin H

The activities of cathepsin H from placenta and liver were 7.2 and 8.2 milliunits.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Cathepsin H (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>placenta</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>1.0</td>
<td>65</td>
</tr>
<tr>
<td>4-Aminophenylmercuribenzoate</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>1.0</td>
<td>15</td>
</tr>
<tr>
<td>Phenylmethylsulphonyl fluoride</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>0.02</td>
<td>5</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>Puromycin</td>
<td>1.0</td>
<td>39</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>0.1</td>
<td>0</td>
</tr>
</tbody>
</table>
total number of the active enzyme molecules one may assume that inhibition of the placental enzyme by its endogenous inhibitors is of stoechiometric character.

**DISCUSSION**

Among the lysosomal cysteine proteases of human placenta, so far only cathepsin B has been isolated and characterized [16, 17]. In the present work an attempt was made to isolate cathepsin H from normal human placenta using the method described for human liver [4]. The yield of the enzyme from placenta was distinctly lower (about 20 times) than that from liver due to the presence of a large amount of other aminopeptidases in placenta [18]. In a control experiment we determined the activity with arginine-β-naphthylamide in the presence of 0.1 mM puromycin, which inhibits other aminopeptidases but not cathepsin H [4]. The result obtained showed that only 10% of the activity of the initial extract derived from cathepsin H. Accurate determination of the enzyme activity in crude tissue extracts is difficult due to the presence of endogenous inhibitors of cysteine protease.

![Graph showing inhibition of cathepsin H](image-url)

**Fig. 2.** Inhibition of cathepsin H from human placenta with the E-64 inhibitor (A) and with cystatins of human placenta (B). Arginine-β-naphthylamide served as a substrate.
The two protein bands of $M_r$ 28,500 and 25,500 separated on SDS-polyacrylamide gel electrophoresis, both in non-reducing and reducing conditions, indicate the presence of two single-chain forms of the enzyme in placenta. Schwartz & Barrett [4] found only a single form of cathepsin H of $M_r$ 28,000 in human liver. The group of Popović [19] reported recently that about 20% of cathepsin H from human kidney occurs in the two-chain form. They obtained forms of $M_r$ 24,000 and 4,000 by reduction and S-carboxymethylation followed by chromatography on Sephacryl S-200. On polyacrylamide gel electrophoresis in reducing conditions a faint band of $M_r$ about 4000 appeared, but only in some preparations [5]. Also cathepsins B and H of rat liver occur both in the single-chain and two-chain forms, in the 1:1 ratio [20].

Kominami et al. [21] demonstrated that cathepsins B, H and L in rat macrophages are synthesized as procathepsins of $M_r$ 39,000, 41,000 and 39,000, respectively. In this form 10 - 30% of the enzyme is secreted from the cells, whereas the remaining molecules undergo further transformation, first to the single-chain, and then to the two-chain forms. Processing of cathepsin in the mature single-chain form is catalysed by metallopeptidase, and proteolytic cleavage to the two-chain form, by cysteine protease [22].

The occurrence of cathepsin H in placenta in two forms and the lack of a light chain can be explained by the fact that only a part of the enzyme of $M_r$ 28,500 underwent limited proteolysis, and that the S-S linkage, which stabilized the two-chain forms, was reduced during autolysis.

On isoelectric focusing of cathepsin H from human placenta two active molecular forms were found, similarly as for the enzyme from liver [4] or kidney [19]. The nature of microheterogeneity of cathepsin H has not so far been elucidated. Usually it is ascribed to the carbohydrate moiety of the enzyme which, however, has been recognized only in rat cathepsin H [23]. In this glycoprotein 5-9 mannose residues are bound to asparagine.

Isoelectric points, pH-stability and thermostability, substrate specificity and inhibition by cystatins are similar or identical for the enzymes from placenta and liver [4]. However, there are some differences in their susceptibilities to some protease inhibitors tested, e.g. the placental enzyme is less sensitive to inhibition by leupeptin and soybean trypsin inhibitor. The differences may be related to the tissue specificity of the enzyme.

On titration of the placental enzyme with cystatins we have observed deviation from linearity when the inhibition exceeded 50%; this might suggest reversible character of the inhibition. The equimolar reaction of the placental cathepsin H with cystatins is identical with that reported for the enzyme from other sources [24].
REFERENCES


