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CYSTATINS OF HUMAN PLACENTA*

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Two low-molecular protein fractions inhibiting cysteine proteases were isolated from human placenta by alkalization to pH 11, acetone fractionation, affinity chromatography on CM-papain-Sepharose 4B, and Sephadex G-75 gel filtration. The results of polyacrylamide gel electrophoresis, isoelectric focusing indicate that one of there fractions in a dimer of cystatins B, and another is a mixture of cystatins A and B.

Protein inhibitors of lysosomal cysteine proteases (cathepsins B, H, and L) occur in cell cytosol and body fluids (serum, urine, cerebro-spinal fluid) [1]. Taking into account the size of the molecule, two classes of inhibitors can be distinguished: α_2-macroglobulin (M_r 725 000) and cystatins (M_r 11 000 - 68 000) [2].

Cystatins constitute a superfamily of homologous inhibitors, composed of three families. The inhibitors, sometimes termed stefins, belong to the first family, they contain about 100 amino acids and have no disulphide bridges. Cystatins A and B are representatives of this group. The inibitors belonging to the second family are of a more complex structure: their polypeptide chain, composed of about 115 amino acid residues, contains two disulphide loops. Cystatin C, known also as the "γ - trace" protein of blood serum, is an example of this group. Kininogens, glycoproteins containing three copies of the sequence typical of cystatins and disulphide bridges constitute the third family [3, 4].

We have found previously [5] that a protein fraction with antipapain activity can be isolated from human placenta extract by Sephadex G-75 gel filtration. Basing on the molecular weight thermostability, pH resistance,

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and inhibition of placental cathepsins B, H, and L, this fraction was identified as a mixture of cystatins.

In this paper we describe fractionation and some properties of cystatins from normal human placenta.

MATERIALS AND METHODS

Material. Placentae from healthy women collected immediately after delivery were separated from foetal membranes, washed with water, blotted on filter paper, then frozen and kept at −20°C. Cathepsin H was isolated from human placenta according to Schwartz & Barrett [6].

Methods. The activity of papain was determined with α-*N*-benzoyl-DL-arginine-β-naphthylamide hydrochloride as a substrate [7] and that of cathepsin H with L-arginine-β-naphthylamide hydrochloride [6]. The amount of the enzyme liberating 1 μmol of β-naphthylamine from the appropriate substrate during 1 min at 40°C was defined as one unit (U).

The inhibitory activity was determined after 5 min preincubation of the enzyme with the inhibitor by the above mentioned methods, and expressed in units referring to the inhibition of the enzyme during 1 min. Since inhibition exceeding 50% showed a nonlinear character, the activity of the inhibitor was determined at concentrations causing not more than 50% inhibition of the enzyme.

Electrophoresis was carried out in 15% polyacrylamide gel in non-reducing and reducing conditions, according to Laemmli [8, 9].

Isoelectric focusing on polyacrylamide gel was performed as described by Barrett [10] except that Servalyt 4 - 9 and 3 - 7 was used instead of Ampholine.

Immunodiffusion in 1% agar gel was carried out in 0.025 veronal buffer, pH 8.2 [11].

Protein concentration was determined according to Lowry *et al.* [12] with bovine serum albumin as a standard, or by measuring the absorbance at 280 and 230 nm. $A_{1cm} = 1$ being taken as one unit.

Reagents. L-*trans*-Epoxy-succinyl-leucyl-amido(4-guanidino)-butane (E-64) was obtained from Sigma (U.S.A.). α-*N*-Benzoyl-DL-arginine-β-naphthylamide and L-arginine-β-naphthylamide were from Koch-Light (England). Immune serum against the α-inhibitor of cysteine proteases was from Calbiochem (U.S.A.). A commercial preparation of papain (Fluka A.G., Switzerland) was purified on *p*-amino-phenylmercuriacetate-Sepharose 4B [13, 14]. The purified papain was titrated with the specific inhibitor, E-64 [15]. Carboxymethyl-papain (CM-papain) was obtained by alkylation of papain with iodoacetic acid [16]. Sepharose 4B was activated as described by March *et al.* [17]. The remaining reagents were of the highest purity available.

RESULTS

Preparation of the inhibitors

For isolation of cystatins from human placenta the method described for human liver [18] was applied, except that the last step, chromatofocusing, was omitted. Frozen tissue (400 g) was homogenized (Unipan 302)

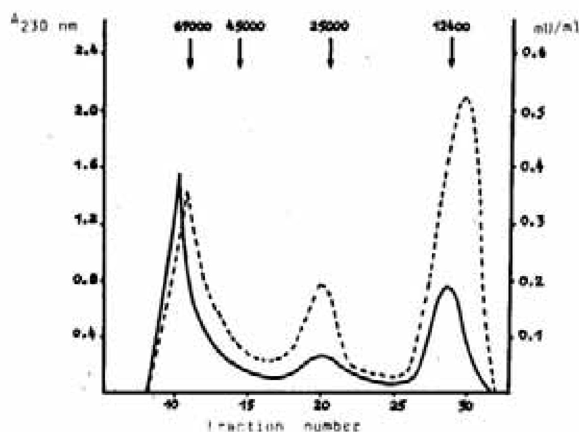

Fig. 1. The elution profiles of protein (——) and antipapain activity (- - -) of inhibitor from human placenta after chromatography on a Sephadex G-75 column (2.2 × 41 cm) equilibrated with 0.1 M phosphate buffer, pH 6.0, containing 1.33 mM Na_2EDTA. The column was calibrated with: bovine albumin, ovalbumin, chymotrypsinogen A and cytochrome *c*

in 800 ml of the mixture of 1% NaCl/3 mM Na_2EDTA/2% n-butanol for 5 min at 14 000 rev./min at 4°C. The homogenate was then centrifuged at 2 500 *g* for 30 min at 4°C. The supernatant obtained was filtered through glass wool and adjusted to pH 11 with 3 M NaOH. After 2 h incubation at 4°C, the pH was lowered to 6.5 by adding 2 M HCl, and the mixture was recentrifuged. To the supernatant, in a dry ice - acetone - water bath, an equal volume of acetone chilled to −10°C was added. The mixture was left standing for 10 min and centrifuged for 30 min at −10°C. The sediment was discarded, and the same volume of cold acetone as previously was added to the supernatant. The precipitate was dissolved overnight in 300 ml of 50 mM phosphate buffer, pH 6.5, containing 0.5 M NaCl and 0.1% Brij 35. The solution clarified by centrifugation was applied onto a CM-papain-Sepharose 4B column (4.2 × 16 cm) equilibrated with the same buffer. The proteins retained on the column were eluted with a 40 mM solution of K_3PO_4 containing 0.5 M NaCl and 0.1% Brij 35, pH 11.5. The active fraction was dialysed against distilled water adjusted with ammonia to pH 7.5, then concentrated by ultrafiltration and freeze-dried.

Table 1

Procedure for isolation of cysteine protease inhibitors from human placenta

Purification step	Volume	Protein	Activity		Purification factor	Yield
			total	specific		
	(ml)	(A_{280} units)	(units)	(units/A_{280} unit)		(%)
pH 11	780	32760.00	54.60	0.0016	1	100.00
Acetone fractionation	380	418.00	15.20	0.0360	22	28.00
CM-papain-Sepharose 4B	70	3.64	9.80	2.6900	1681	18.00
Sephadex G-75						
peak I	14	2.52	0.13	0.0520	33	0.23
peak II	20	0.14	0.40	2.8000	1750	0.73
peak III	20	0.50	7.40	14.8000	9250	13.50

At the next step the preparation of the inhibitor was subjected to gel filtration on Sephadex G-75 (Super Fine) column (2.2 × 41 cm) equilibrated with 0.1 M phosphate buffer, pH 6.0, containing 1.33 mM Na_2EDTA. The elution profile is presented in Fig. 1 and the results of the successive purification steps are summarized in Table 1.

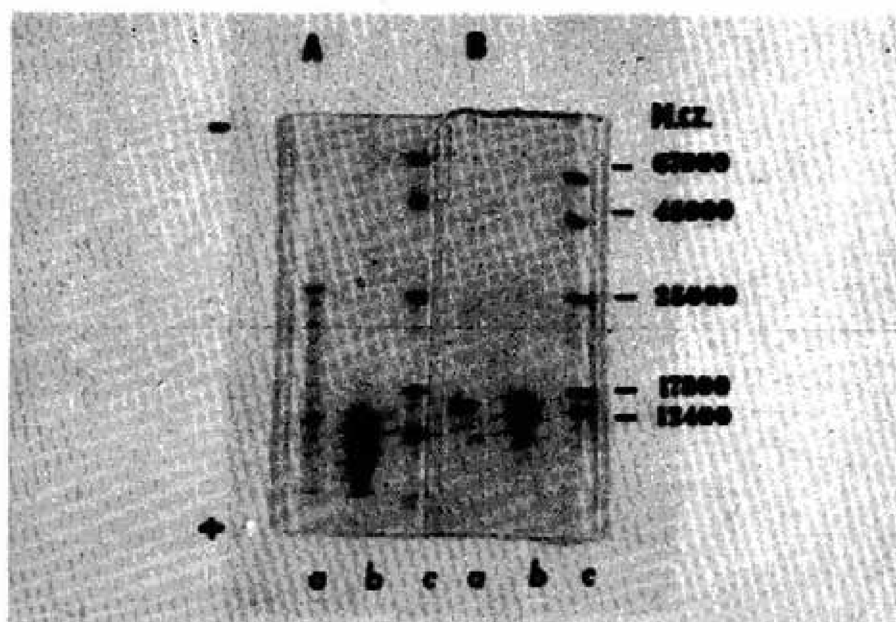

Plate 1. Electrophoresis of placental inhibitors II and III in 15% polyacrylamide gel in non-reducing (A) and reducing (B) conditions. After electrophoresis the gels were stained with Coomassie Brilliant Blue G-250. Lane a, inhibitor II; lane b, inhibitor III; lane c, standards (bovine albumin, ovalbumin, chymotrypsinogen A, myoglobin and cytochrome *c*)

After Sephadex G-75 gel filtration the antipapain activity was found in three peaks, designated as inhibitors I, II and III. Peak I, emerging at the front of the column, was not further analysed because of its high molecular weight (about 60 000) and positive reaction with the immune serum against the blood serum α-inhibitor of cysteine proteases. These properties designate its serum origin (kininogens). The activity of the inhibitor emerging in peak II, of M_r about 26 000, was obtained with a very low yield (about 1% of the total activity) varying from preparation to preparation. Therefore, our further work was concentrated on inhibitor III. This inhibitor of M_r about 12 000 was obtained with yield of about 17% and was purified about 9 000 fold.

Properties of inhibitors II and III

SDS-polyacrylamide gel electrophoresis of the inhibitors II and III is presented in Plate 1. In non-reducing conditions inhibitor II migrated as two bands, one of M_r 25700 and the other, a weaker one, of M_r 13000. Inhibitor III migrated as a doublet of M_r 13000 and 12400. In reducing

Plate 2. Isoelectric focusing of inhibitor II (a) and III (b) from human placenta, using 6.8% polyacrylamide gel and Servalyt 3 - 7 [10]

conditions, instead of the 25700 band of inhibitor II there appeared a band of M_r 112400, whereas inhibitor III showed the presence of three bands in the M_r range of 11000 - 13000.

Plate 2 presents the pattern obtained after isoelectric focusing of the two inhibitors. Inhibitor II was separated into three molecular forms of pI

Table 2

Inhibition of papain and cathepsin H by the inhibitors from human placenta

The results refer to the inhibitory activity of inhibitor II towards papain taken as 100

Enzyme	Inhibitor II	Inhibitor III
Papain	100	15
Cathepsin H	25	3.5

6.1 - 6.4, whereas inhibitor III showed the presence of additional forms of pI 5.1, 5.3 and 5.65. The molecular forms separated by isoelectric focusing were eluted from the gel with 0.1 M phosphate buffer, pH 6.0, containing 1.33 mM Na_2EDTA. All the fractions showed antipapain activity. It was found that, as compared with inhibitor II, an almost

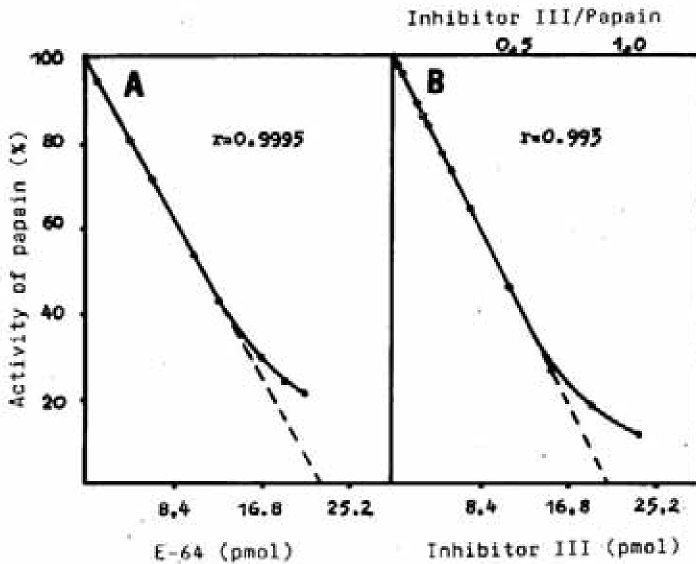

Fig. 2. Titration of papain with the inhibitor E-64 (A) and inhibitor III from human placenta (B) according to Barrett *et al.* [7] with the use of α-*N*-benzoyl-DL-arginine-β-naphthylamide as a substrate (4 μg of papain contained 22.4 pmol, i.e. 13.5% of the active enzyme)

7-fold amount of inhibitor III was required to inhibit either papain or cathepsin H from human placenta (Table 2). Inhibitor III inhibited much more effectively cathepsin than papain.

The results of titration of papain with the inhibitor E-64 and placental inhibitor III are presented in Fig. 2. Inhibition by the placental inhibitor was linear until about 70% of the activity was inhibited. The ratio of papain to inhibitor calculated from the graph was 1:1.12.

DISCUSSION

Lysosomes present in cytotrophoblast and syncytiotrophoblast of placenta take part in intracellular degradation of proteins [21]. Among the lysosomal cathepsins, the enzymes possessing cysteine in their active centers (cathepsins B, H and L) constitute an important group of both endo- and exopeptidases, inhibited by cystatins.

The procedure applied in the present work for purification of cystatins involved inactivation of endogenous proteases at pH 11 and successive elimination of kininogens (α-inhibitors of cysteine proteases) by acetone fractionation and Sephadex G-75 chromatography. As a result, two low-molecular fractions of cysteine protease inhibitors were isolated from placenta: inhibitor II and inhibitor III. Inhibitor II is probably a dimer of M_r about 26000 since on electrophoresis in reducing conditions it revealed the presence of a single band of M_r about 13000. It is worth noting that cystatin B is able to form dimers stabilized by disulphide bridges [18, 22]. Moreover, Ritonija *et al.* [20] have found that partly degraded cystatin B forms aggregates due to non-covalent interactions.

The heterogeneity of placental inhibitors in isoelectric focusing is in agreement with the reports on other cystatins [18, 23]. Heterogeneity of cystatins results from degradation of the polypeptide chain by a few, probably less than 20, amino acids, and this does not necessarily lead to a loss of the inhibitory properties. The fact that all the protein bands visualized on isoelectric focusing show antipapain activity precludes the possibility of contamintion of the preparation with other, non-inhibitory proteins. The isoelectric points of placental inhibitors indicate that inhibitor II corresponds to cystatin B, and inhibitor III is a mixture of cystatins A and B. This is supported by inhibition of papain and cathepsin H by placental inhibitors, and greater effectiveness of inhibitor III as that of inhibitor II. It is known that cystatin A inhibits more effectively papain and cathepsin H than does cystatin B [4]; and that cystatin B from human liver in the dimer form is active [18], whereas cystatin B from spleen in inactive [22]. Titration of papain with the inhibitor III from placenta proved that this inhibitor, like other cystatins has onè binding site for papain.

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