Purification of human procollagen type III N-proteinase from placenta and preparation of antiserum

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Procollagen type III N-proteinase, of M_r about 70000, was detected in human placental tissue and purified from this source more than 5800-fold. It was found to be a glycoprotein, which was bound to both concanavalin A–Ultrogel and heparin–Sephacryl affinity columns. Binding to a type III pN-collagen–Sephacryl affinity column was used as the final step in purification. The purified enzyme accepted only native type III procollagen or [14 C]carboxymethylated type III pN-collagen as its substrate; type I, type II and type IV procollagen and heat-denatured type III pN-collagen were not cleaved by the enzyme. Antibodies against this purified enzyme protein raised in rabbits demonstrated a high inhibitory effect on the enzyme activity. Immunoblotting of the denatured protein and immunoelectrophoresis of the native enzyme showed only one major antigenic component, again with an M_r of about 70000. The antibodies cross-reacted with the enzyme preparation from foetal-calf aorta smooth-muscle cells.

INTRODUCTION

The extracellular processing of interstitial procollagens to collagen is known to occur via enzymically catalysed reactions (Tuderman *et al.*, 1978; Tuderman & Prockop, 1982; Njeha *et al.*, 1982; Halila & Peltonen, 1984). Cleavage of the N-terminal propeptide seems to take place soon after secretion in the case of type I procollagen (Fessler & Fessler, 1979) and type II procollagen (Uitto, 1977), but at a much lower rate with type III procollagen (Fessler *et al.*, 1981). In the case of this collagen type, some N-propeptides seem to remain uncleaved, especially in embryonic tissues, even when the molecules are assembled into fibres. Type III pN-collagen molecules can be detected in tissues in the form of thin fibrillar structures, typical of type III collagen (Fleischmajer *et al.*, 1981, 1983). This finding has led to speculations that the N-terminal propeptides may serve to control the size of the newly formed collagen fibres.

The biological significance of the removal of the N-terminal propeptide in the case of type I procollagen is obvious in such genetic disorders as dermatosparaxis in cattle and sheep and Ehlers–Danlos syndrome type VII in man, in which this reaction is defective, resulting in thin and irregular type I collagen fibres (Lenaers *et al.*, 1971; Lichtenstein *et al.*, 1973; Steinmann *et al.*, 1980; Byers *et al.*, 1981). The process of the removal of N-propeptide from type III procollagen has not been so well studied, although this process is also thought to be defective in dermatosparactic animals (Nusgens *et al.*, 1980; Shinkai & Lapière, 1983).

In order to study the processing of type III collagen further in embryonic tissues and in human genetic disorders, we have purified an enzyme responsible for processing type III pN-collagen to type III collagen from

human placenta close to homogeneity and have raised antibodies against this enzyme protein.

MATERIALS AND METHODS

Materials

Various analytical chemicals were purchased from Merck (Darmstadt, Germany). Iodo[14 C]acetamide and [14 C]proline were from The Radiochemical Centre (Amersham, Bucks., U.K.). CNBr-activated Sepharose 4B, Protein A–Sepharose and heparin–Sepharose 6B were from Pharmacia Chemicals (Uppsala, Sweden), and concanavalin A–Ultrogel and Ultrogel AcA-44 were from LKB (Orsay, France). The nitrocellulose membrane and the equipment for transblotting the proteins were from Bio-Rad Laboratories (Richmond, CA, U.S.A.), pig antiserum to rabbit IgG and horseradish peroxidase–anti-(horseradish peroxidase) antibody complex were from Dako Immunoglobulins (Copenhagen, Denmark), and the biotin–avidin-sensitized peroxidase–anti-peroxidase staining kit was from Vector Laboratories (Burlingame, CA, U.S.A.). The liquid-scintillation reagent (Lumagel) was purchased from Lumac (Schaesberg, The Netherlands). Haemocyanin was from Calbiochem (La Jolla, CA, U.S.A.), and Freund's complete and incomplete adjuvants were from Difco Laboratories (Detroit, MI, U.S.A.).

Preparation of substrate

Type I and type III pN-collagens were extracted from foetal-calf skin by repeated salt fractionation as described by Lapière *et al.* (1977), Nusgens & Lapière (1979) and Nusgens *et al.* (1980). The substrate was then

Abbreviations used: pN-collagen, collagen precursor containing the N-terminal propeptide but not the C-terminal propeptide; pC-collagen, collagen precursor containing the C-terminal propeptide but not the N-terminal propeptide; N-proteinase, enzymic activity that removes the N-terminal propeptides from procollagen; C-proteinase, enzymic activity that removes the C-terminal propeptides from procollagen.

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carboxymethylated with iodo[^{14}C]acetamide as described for type I pN-collagen (Nusgens & Lapière, 1979). The specific radioactivity of the end product was 1.4×10^6 c.p.m./ μmol of hydroxyproline (Kivirikko *et al.*, 1967). The purity of the end product was confirmed by SDS/polyacrylamide-gel electrophoresis before and after digestion with chymotrypsin (1 mg/ml) and trypsin (0.1 mg/ml).

Human type III procollagen, chick type II procollagen and chick type I procollagen were prepared as described previously (Smith *et al.*, 1972; Dehm & Prockop, 1972; Hoffman *et al.*, 1976; Uitto, 1977; Uitto *et al.*, 1980). Type IV procollagen prepared from mouse EHS tumour organ cultures (Tryggvason *et al.*, 1980) was kindly given by Dr. Karl Tryggvason. For assay of enzyme activity against denatured pN-collagen, type III pN-collagen was denatured by reduction and alkylation by the method of Engel *et al.* (1977). This substrate was further denatured by heating for 3 min at 80 °C and cooled again immediately before use.

Assay of enzyme activity

A new method for quantifying the enzyme activity developed in our laboratory has been described elsewhere (Halila, 1985). The assay was carried out at a total volume of 140 μl , which contained 0.1–50 units of enzyme activity, 15 μg of type III pN-collagen, 2 mM- CaCl_2 and 0.15 M- NaCl in 0.1 M-sodium cacodylate buffer, pH 7.4. The control samples contained all the components of the reaction except the enzyme. The reaction products were confirmed by polyacrylamide-gel electrophoresis with a 4–10% gradient in the separation gels (King & Laemmli, 1971). The gels were impregnated with dimethyl sulphoxide/2,5-diphenyloxazole, dried under vacuum and exposed to X-ray films (Bonner & Laskey, 1974; Laskey & Mills, 1975).

Purification of the enzyme

Human placentas were obtained from the Department of Obstetrics and Gynaecology, Oulu University Central Hospital, from normal deliveries. The foetal membranes and umbilical cord were removed and foetal blood from the placental vessels was rinsed away as well as possible under cold tap water. Serum components and other water-soluble material were removed by cutting the placental tissue into small pieces and rinsing these with 5 ml of deionized water/g of tissue. The insoluble material was then suspended in 5 ml of 0.5% Triton X-100 / 2 M-KCl / 0.1 M-sodium cacodylate buffer (pH 7.4)/g of tissue at 4 °C and homogenized with a Teflon/glass homogenizer at 100 rev./min for 5 min. The extract was stirred for 2 h and centrifuged at 8000 g for 1 h, and the supernatant was recovered. The pellet was resuspended twice in an equal volume of the extraction buffer, homogenized, stirred overnight and centrifuged at 8000 g for 1 h. The pooled supernatants were either precipitated by adding 390 mg of solid $(\text{NH}_4)_2\text{SO}_4$ /ml or in some experiments diluted 1:1 with 0.1 M-sodium cacodylate buffer, pH 7.4.

The protein extract was then passed through a 10–20 ml column of concanavalin A–Ultrogel in 1 M-KCl/0.1 M-sodium cacodylate buffer, pH 7.4, at 4 °C. The flow rate was 5 ml/h or less. In the case of non-concentrated material, 10 ml of the concanavalin A–Ultrogel was added to the extract, and the proteins were allowed to attach to the gel for 24 h on a magnetic

stirrer. The column was then washed with 200 ml of equilibration buffer and eluted with 0.3 M-methyl α -mannoside in the same buffer. Fractions of volume 3 ml were collected.

The fractions with the highest specific activity were pooled and dialysed against the running buffer of 0.1 M-KCl/0.1 M-sodium cacodylate buffer, pH 7.4. The sample was then passed through a 4 ml column of heparin–Sephadex 6B that had been equilibrated with the same buffer. After 50 ml of running buffer the column was eluted with 200 ml of a gradient of 0.1–1 M-KCl in 0.1 M-sodium cacodylate buffer, pH 7.4, at 4 °C. Fractions of volume 2 ml were collected.

The fractions with the highest specific activity were pooled, dialysed and passed through a 3 ml column of type III pN-collagen coupled covalently to CNBr-activated Sepharose 4B (Halila & Peltonen, 1984). The flow rate was 2 ml/h. After a washing, the enzyme was eluted with 1 M-KCl/0.1 M-sodium cacodylate buffer, pH 7.4, at 4 °C. The fractions with the highest specific activity were again pooled and precipitated with $(\text{NH}_4)_2\text{SO}_4$. The pellet was suspended in 0.5–2 ml of 0.1 M-sodium cacodylate buffer, pH 7.4, at 4 °C and dialysed against the same buffer.

One unit of enzyme activity in various purification steps was defined as the amount of enzyme present in 1 mg of original cell extract (Tuderman *et al.*, 1978; Tuderman & Prockop, 1982; Halila & Peltonen, 1984).

Preparation of antiserum against type III procollagen N-proteinase

Antibodies against the purified enzyme were prepared in rabbits. For this 50 μg of enzyme protein mixed with an equal volume of Freund's complete adjuvant was injected intracutaneously at several sites. A booster of 50 μg of enzyme mixed with Freund's incomplete adjuvant was administered 2 weeks later. To improve the immune response this enzyme was linked to haemocyanin in the presence of glutaraldehyde (Avrameas & Ternynck, 1969), and the remaining four boosters consisted of this complex mixed in Freund's incomplete adjuvant. The appearance of an immune response was followed by inhibition of type III N-proteinase activity and by dot-blotting as described by Sternberger & Jeppesen (1983). A week after the last injection the rabbit was bled, and the serum collected was frozen in small portions at –20 °C. The IgG fraction from part of the antiserum was purified by chromatography on Protein A–Sephadex (Hjelm *et al.*, 1972). After a washing with 100 ml of phosphate-buffered saline the IgG fraction was eluted with 5 column volumes of 0.1 M-sodium citrate/0.05 M-phosphate buffer, pH 3.0. The 1 ml fractions were immediately neutralized with 150 μl of 1 M-Tris. The final concentration of IgG was 1.5 mg/ml.

Specificity of the antiserum against type III procollagen N-proteinase

The enzyme was blotted on to a nitrocellulose membrane by a modification of the methods described previously (Towbin *et al.*, 1979; Vartio *et al.*, 1982). Electrophoresis was carried out as described by King & Laemmli (1971). The separating and stacking gels were prepared from 6% polyacrylamide. The proteins were transferred to nitrocellulose filters in 0.04 M-sodium phosphate buffer, pH 6.5, at 100 mA and 8 V for 16 h. When indicated, the membrane was stained with heparin

Fig. 1. Chromatography of type III N-proteinase activity on a type III pN-collagen-Sepharose affinity column

Chromatography was performed as described in the text. Fractions of volume 2 ml were collected. $\times$, Concentration of protein; $\circ$, enzymic activity. The arrow indicates the initiation of elution with 1 M-KCl/0.1 M-sodium cacodylate buffer, pH 7.4.

(15 units/ml) in 0.01 M-HCl followed by 0.02% Toluidine Blue in 0.01 M-HCl after blotting (Vartio *et al.*, 1982). The cut wells were then stained by the peroxidase-antiperoxidase staining method (Vartio *et al.*, 1982). Pre-immune serum from the same rabbit was used as the control serum. Non-specific binding of immunoglobulins was diminished by the method of Zeller *et al.* (1983). The cross-reaction with the enzyme in smooth-muscle cell extract was studied by dot-blotting as described by Sternberger & Jeppesen (1983).

Immunoelectrophoresis was carried out as described by Laurell (1965), at 20 mA and 130 V at room temperature for 3 h. Antiserum and pre-immune serum from the same rabbit were then added and precipitation of the antigen-antibody complex was allowed to proceed overnight at room temperature.

Other assays

The K_m value of the enzyme was determined from double-reciprocal Lineweaver-Burk plots with four different concentrations of enzyme and substrate. The protein concentration was measured by using the Bio-Rad protein assay kit or by the method of Lowry *et al.* (1951),

as modified by Dulley & Grieve (1975), with bovine serum albumin as standard. In samples without Triton X-100 the protein concentration could be assayed by measuring their absorbance at 230 nm.

RESULTS

Purification of enzyme activity

The initial steps in the purification of type III procollagen N-proteinase were extraction with high salt concentrations and detergent and chromatography on concanavalin A-Ultrogel. The human placental enzyme differed from the bovine enzyme in this last-mentioned step in that all of the enzyme activity was bound to the column under the conditions described above and could be eluted with 0.3 M-methyl α -mannoside, compared with the extraction of about 60% of the bound activity from foetal-calf aortic smooth-muscle cells (Halila & Peltonen, 1984). The concanavalin A-bound enzyme activity eluted with methyl α -mannoside was then purified more than 20-fold in a column of heparin-Sepharose, with a recovery of about 84%. The final purification step was with the type III pN-collagen covalently linked to Sepharose 4B (Fig. 1). This purification scheme, summarized in Table 1, resulted in an enzyme preparation that resulted in only one major band in SDS/polyacrylamide-gel electrophoresis (Fig. 2). The purification coefficient was about 6000 when compared with high-salt/Triton-extract of placenta, the total purification being even higher because of the partial solubility of proteins from placenta in the extraction scheme. The glycoprotein character of the enzyme protein possibly disturbed the staining, since only comparatively large amounts ($> 20 \mu\text{g}$) of the purified enzyme protein could be detected with the staining techniques applied.

The purified human enzyme preparation did not cleave heat-denatured [^{14}C]carboxymethylated type III pN-collagen, [^{14}C]carboxymethylated type I pN-collagen or type I, type II and type IV procollagens studied by enzyme assays and SDS/polyacrylamide-gel electrophoresis (Fig. 3). The K_m value for this human enzyme was $2 \mu\text{M}$ of [^{14}C]carboxymethylated type III pN-collagen. The pH optimum for the enzyme was pH 7.5, and the effects of the inhibitors tested, differing from those on the previously described bovine enzyme (Halila & Peltonen, 1984), are given in Table 2. Surprisingly, the human enzyme activity was not particularly affected by various concentrations of CaCl_2 . Further, EDTA required at

Table 1. Purification of type III procollagen N-proteinase

For experimental details see the text.

	Volume (ml)	Protein (mg/ml)	Specific activity (units/mg)	Recovery (%)
Placental extract	2000	2.1	1	100
$(\text{NH}_4)_2\text{SO}_4$ precipitate	160	14.1	1.24	66
Concanavalin A-Ultrogel eluted enzyme	20	3.65	16.9	29
Heparin-Sepharose eluted enzyme	4	0.38	384	14
Type III pN-collagen-Sepharose eluted enzyme	0.5	0.11	5800	7.5

Fig. 2. Protein staining with Coomassie Brilliant Blue (lanes b and d) and immuno-blotting of procollagen type III N-proteinase (lanes a and c) with 20 μ g of enzyme (lanes a and b) and with 50 μ g of enzyme (lanes c and d)

The antiserum was diluted 1:200, and 0.05% diaminobenzidine tetrahydrochloride in 0.05 M-Tris/HCl buffer, pH 7.6, was used as the substrate of the peroxidase-anti-peroxidase staining procedure. With non-immune serum no bands could be detected. Only one major protein band could be seen with the antiserum by this method, and no staining reaction could be seen with the same dilution of pre-immune serum (results not shown). The intense staining in the lower margin of the protein staining (lanes b and d) indicates the dye front. The positions of M_r marker proteins are shown at the left.

least 1 h preincubation before inhibiting the human enzyme activity. The second constant difference to the reported bovine type III procollagen N-proteinase was that dithiothreitol did not serve as a potent inhibitor of the enzyme (Halila & Peltonen, 1984).

Specificity of the antiserum

By Western blotting only one major band could be seen with an M_r of about 70 000 (Fig. 4), corresponding to the band registered after staining with Coomassie Brilliant Blue. In addition, only one precipitation line could be seen between the purified enzyme and the non-diluted antiserum in immunoelectrophoresis; no precipitation lines could be demonstrated when pre-immune serum from the same rabbit was studied (Fig. 4). Similarly, only one precipitation line was detected in immunoelectrophoresis of the placental homogenate.

The amount of immunoreactive enzyme protein relative to the total protein content in placental extract was clearly smaller than in the smooth-muscle cell extract (result not shown).

Less than 1 μ l of antiserum inhibited 50% of the enzyme activity, whereas no inhibition could be recorded with pre-immune serum from the same rabbit or newborn-calf serum, and about 0.7 μ g of purified IgG added to the incubation mixture inhibited 50% of the enzyme activity, whereas no inhibition could be detected by isolated IgG fraction of the pre-immune serum (Fig. 5).

DISCUSSION

The procedure described here results in extensive purification of human type III procollagen N-proteinase. The electrophoresis of the isolated enzyme protein results in one major 70 000- M_r band and thus suggests a high degree of purification. Only one single precipitation line was observed in immunoelectrophoresis and only one major band could be detected in immuno-blotting with antiserum against the enzyme.

The human enzyme purified here from placental extract shows a very close similarity in M_r and K_m value, and also in its immunological characteristics, to the enzyme activity purified from foetal-calf aortic smooth-muscle cell extract (Halila & Peltonen, 1984). However, the removal of Ca^{2+} from the incubation mixture did not inhibit the human enzyme activity, which is in contrast with the bovine enzyme or type I procollagen N-proteinase (Tuderman *et al.*, 1978; Tuderman & Prockop, 1982; Halila & Peltonen, 1984). This could possibly be explained by Ca^{2+} being more tightly bound to this human placental enzyme than to the enzyme purified from the calf aorta smooth-muscle cells. This hypothesis is supported by the fact that EDTA required at least 1 h preincubation before it inhibited the enzyme activity. There may also be some differences in the degree of glycosylation of the enzymes purified from different sources, since the human enzyme preparation showed consistent complete attachment to the concanavalin A-Ultrogel affinity column, as compared with only 50% attachment in the case of the foetal-calf enzyme preparation (Halila & Peltonen, 1984).

The antibodies against the enzyme protein, which gave a single precipitation line in immunoelectrophoresis, also strongly inhibited the enzyme activity, suggesting a strong antigenic determinant close to the active site of the enzyme or binding of the antibody molecule in such a position as to lead to the formation of a steric hindrance around the active site. The antibodies cross-reacted also with the smooth-muscle cell extract.

On the basis of the information available so far concerning the enzymes removing the N-terminal propeptides from procollagen molecules, at least two distinctly different enzymes exist in vertebrates. Type I N-terminal proteinase accepts both type I and type II procollagen as its substrate and requires the native conformation of these molecules (Tuderman *et al.*, 1978; Tuderman & Prockop, 1982; Tanzawa *et al.*, 1985), whereas type III N-terminal proteinase cleaves type III pN-collagen and also requires intact triple-helical conformation in the substrate (Halila & Peltonen, 1984). The substrate alkylated in non-denaturing conditions is also cleaved, but thermal denaturation of the triple helix abolishes the

Fig. 3. Specificity of type III procollagen N-proteinase

A 50 μ l portion of the purified enzyme was incubated with type III pN-collagen, heat-denatured, reduced and alkylated type III pN-collagen and type I pN-collagen. After incubation the whole mixture was heated in SDS/polyacrylamide-gel-slab sample buffer and dialysed against the same buffer. The samples were reduced with 10% 2-mercaptoethanol before electrophoresis. Key: E, incubation of the substrate with the enzyme; C, incubation of the substrate without the enzyme. The migration positions of the standard pN α_1 (III), α_1 (III), pN α_1 (I) and α_1 (I) are indicated.

Table 2. Effect of inhibitors on type III procollagen N-proteinase

The enzyme used in the assays was type III pN-collagen-Sephadex eluted enzyme (described in the Materials and methods section). The assay has been described previously (Halila, 1985).

Compound added	Concentration (mM)	Activity (% of control)
EDTA*	2	80
	20	10
Dithiothreitol	0.2	120
	2	130
	20	140
	50	60
ZnCl ₂	0.2	60
	2	30
	20	10
CaCl ₂	0.2	100
	20	100
	—	100

* EDTA was preincubated for 1 h before the substrate was added.

cleavage completely. This enzyme, either of human or of bovine origin, does not accept type I, type II or type IV procollagen as its substrate. Type I and type III procollagen N-terminal proteinases differ also significantly in their M_r , that of type I N-proteinase being about 200000 and that of type III N-proteinase about 70000 (Tuderman *et al.*, 1978; Nussgens *et al.*, 1980; Tuderman & Prockop, 1982; Halila & Peltonen, 1984; Tanzawa *et al.*, 1985). All these findings support the hypothesis that at least two different enzymes are responsible for the

Fig. 4. Immunoelectrophoresis of procollagen type III N-proteinase

A sharp precipitation line can be seen between the electrophoresed enzyme (e) and the undiluted antiserum (arrow). No reaction could be seen between the enzyme and the non-immune serum or with a corresponding amount of bovine serum albumin (c) and antiserum.

removal of N-terminal propeptides of different procollagen types.

The preparation of antiserum against the enzyme is important for several reasons. First, the quantification of

Fig. 5. Inhibition of type III N-proteinase activity by IgG fraction purified from the specific antiserum

The IgG fraction was purified by Protein A-Sepharose chromatography as described in detail in the text. IgG was added to the assay tubes before the addition of the substrate. ○, Enzymic activity in the presence of anti-(type III procollagen N-proteinase) IgG; ×, enzymic activity in the presence of non-immune IgG of the same rabbit.

immunoreactive enzyme proteins in tissues and body fluids becomes possible. For example, the amount of immunoreactive prolyl hydroxylase has been shown to be increased in liver cirrhosis (Kuutti-Savolainen *et al.*, 1979) showing a general increase in the collagen biosynthesis. The amount of type III procollagen N-terminal propeptide is also increased in liver cirrhosis (Niemelä *et al.*, 1982). More complete studies on the metabolism of type III procollagen in fibrotic diseases are now possible with a specific antiserum. Secondly, the recognition of the nascent polypeptide chain with the antiserum is of high significance when the transcription and translation events of this enzyme are studied. The inhibitory specific antibody will also be a valuable tool in the chromosomal mapping and identification of the gene coding for this enzyme protein.

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