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PROTEOLYSIS OF PROCOLLAGEN I

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1. Digestion of procollagen I with trypsin, pepsin or pronase performed at 20°C causes the release of acidic non-collagenous fragments and hydroxyproline-rich fraction. Enzymatic proteolysis performed at 41°C (above the temperature of denaturation) results in degradation of procollagen I to low-molecular peptides.

2. The hydroxyproline-rich fraction obtained by limited proteolysis of procollagen I with pepsin (at 20°C) contains a material corresponding to α and β subunits of tropocollagen. Reduction of the hydroxyproline-rich fraction released by trypsin or pronase (at 20°C) causes the appearance of polypeptides similar to pro-α subunits.

The existence of an extracellular precursor of collagen is well documented. The precursor, procollagen, is converted to tropocollagen by the proteolytic action of an enzyme(s) referred to as procollagen peptidase(s) (for review see: Schofield & Prockop, 1973; Bornstein, 1974; Mazanowska, 1975).

Procollagen has been reported to exist in various molecular forms of different molecular weight. In addition to procollagen composed of pro-α₁ and pro-α₂ chains of molecular weight about 105 000 - 130 000 daltons per chain (Bellamy & Bornstein, 1971; Lapierre et al., 1971; Goldberg et al., 1972; Smith et al., 1972; Tanzer et al., 1974), much larger molecules have been isolated (Church et al., 1971; Bąkowski & Mitchell, 1973; Monson & Bornstein, 1973; Park et al., 1975). Murphy et al. (1975) suggested that pro-α chains represent degradation products of the primary form of procollagen, which is considerably larger. Church et al. (1971) demonstrated the existence of a very high-molecular-weight (500 000 - 600 000 daltons) form of procollagen. They proposed to designate this form of collagen precursor the procollagen I, while smaller intermediates identified by other workers would be designated procollagen II, III etc. Bąkowski & Mitchell (1973) isolated procollagen I from serum-free fibroblast culture medium; its molecular weight determined by sedimentation equilibrium was about 540 000 daltons, and the isoelectric point 4.0, i.e. much lower than the alkaline pl of tropocollagen. Procollagen I
contained a significant amount of cysteine, a higher amount of acidic amino acids and a lower quantity of glycine and imino acids as compared with tropocollagen. Procollagen I exhibited a sharp thermal transition point at 39°C, indicating that the collagenous region of the molecule is in the triple helical configuration.

The experiments to be described were performed to find out whether proteolysis of procollagen I could yield subunits similar to pro-z chains reported by other authors.

**MATERIALS AND METHODS**

*Chemicals.* Eagle’s minimal essential medium (MEM) and calf serum were the products of Serum and Vaccine Factory (Lublin, Poland); β-aminopropionitrile fumarate was obtained from Aldrich Chem. Corp. (Milwaukee, Wis., U.S.A.); [5-3H]proline (25 Cl/nmole), from the Radiochemical Centre (Amersham, Bucks., England); PCS scintillation fluid from Amersham/Searle Corp. (Arlington Heights, Ill., U.S.A.); trypsin, bovine, 2× cryst., from Koch-Light Lab. (Colnbrook, Bucks., England); pronase, B grade, and bacterial collagenase, essentially free of protease, from Calbiochem (Los Angeles, Calif., U.S.A.); sodium dodecyl sulphate (SDS) from B.D.H. (Poole, England); CM-cellulose CM-11 from Whatman (Maidstone, Kent, England); agarose for gel electrophoresis from J. Baker Chemicals B.V. (Deventer, Holland); Bio-Gel A-0.5 m (exclusion limit for globular proteins about 400 000 daltons) from Bio-Rad (Richmont, Calif., U.S.A.); 2-mercaptoethanol from Organica (Belgium).

*Lathyrnic rat skin collagen* was kindly supplied by Dr. Hector Aquilar (Dept. of Biochemistry, Vanderbilt University, Nashville, Tenn., U.S.A.). Lathyrnic collagen consists of non-polymerized tropocollagen molecules.

*Cell culture and preparation of procollagen I.* Human embryo skin fibroblasts were grown in Legroux culture flasks (50 cm²) at 37°C in 20 mL of MEM supplemented with 15% heat-inactivated calf serum. From contact-inhibited fibroblast cultures, containing about 60 000 cells/cm², the medium was removed and replaced with 20 mL of fresh MEM without serum but supplemented with 50 µg/mL of β-aminopropionitrile fumarate and 50 µg/mL of ascorbic acid and 5 µCi/mL of [5-3H]proline. After 24 h the medium was removed, dialysed against 0.02 M Tris-HCl - 0.5 M NaCl, pH 7.4, to eliminate the non-incorporated radioactivity, concentrated by vacuum dialysis at 4°C, and procollagen I was purified as previously described (Baňkowski & Mitchell, 1973), dissolved in the dialysis buffer and stored at 4°C.

*Digestion of procollagen I with proteases, and molecular sieve chromatography of proteolysis products.* Procollagen I solution was diluted to the concentration of 10 µg/mL and treated for 4 h with collagenase, pepsin, trypsin or pronase (at 20°C or 4°C, as indicated), final concentration of the enzymes being 20 µg/mL. Digestion with collagenase was performed in 0.02 M Tris-HCl - 0.5 M NaCl - 0.0025 M CaCl₂ - 0.01 M H₂O₂, pH 7.4; digestions with pepsin, trypsin and pronase were carried out, respectively, in 0.5 M acetic acid, pH 2.9; 0.2 M ammonium bicarbonate, pH 8.2, and 0.02 M Tris-HCl - 0.5 M NaCl, pH 7.4. After incubation, the reaction
mixtures (4.0 ml each) were cooled to 4°C (the pepsin hydrolysate was neutralized) and submitted to molecular sieve chromatography on Bio-Gel A-0.5 m column.

_Determination of the hydroxyproline to proline ratio._ Procollagen I or peak fractions obtained from the Bio-Gel column were evaporated in vacuum at 80°C. The dry residues were dissolved in 6 M-HCl and hydrolysed at 124°C for 16 h. The hydrolysates were evaporated in vacuum and the dry residues dissolved in water. Hydroxyproline was separated from proline by paper chromatography as described by Tomaszewski & Gilatowska (1972), the appropriate spots were cut out, the radioactivity was determined and the Hyp/Pro ratio was calculated. No radioactivity was detected on the chromatograms except at the spots of these imino acids.

_Molecular weight determination._ SDS-polyacrylamide-gel electrophoresis was performed as described by Weber & Osborn (1969). Prior to application to the gel, the sample was denatured and reduced by treatment with 1% SDS and 1% 2-mercaptoethanol at 37°C for 3 h. The gels containing lathyric collagen were stained with 1% Amido Black and destained with 7% acetic acid. The gels containing radioactive material were sliced, eluted with 1 ml of 1 M-NH₄OH and counted as described by Church et al. (1971).

_Other methods._ CM-cellulose chromatography was performed by the method of Piez et al. (1963). Agarose-gel electrophoresis was performed as described by Bańkowski & Mitchell (1973). Prior to electrophoresis, samples of high molecular weight were dialysed against the upper electrode buffer, whereas those of low molecular weight were evaporated in vacuum and dissolved in the buffer. Radioactivity was measured with Nuclear Chicago Isocap 300 scintillation counter using PCS as a scintillation fluid.

**RESULTS**

The procollagen I isolated and purified from culture medium of human skin fibroblasts incubated in the presence of [5-³H]proline, was eluted from Bio-Gel in the void volume of the column, and its hydroxyproline to proline ratio was 0.40 (Fig. 1A). Proteolysis of procollagen I with trypsin at 20°C for 4 h resulted in its partial degradation; the hydroxyproline-poor fragment was digested but the collagenous part of procollagen molecule was resistant to trypsin. This fraction, also eluted in void volume of the column, had the Hyp/Pro ratio 0.61 (Fig. 1B), i.e. higher than that of intact procollagen I and comparable to that reported for human skin collagen: 0.73 (Fleischmajer & Fishman, 1965). Neither prolongation of trypsin digestion to 6 h nor a 4 h digestion at 30°C changed the elution position of the hydroxyproline-rich fraction. Incubation at 41°C (i.e. above the temperature of procollagen I denaturation) resulted in its total degradation to low-molecular products (Fig. 1C).

Similar results were obtained on proteolysis with pepsin and pronase at 20° and 41°C. On the other hand, on treatment of procollagen I with bacterial collagenase at 20°C, the hydroxyproline-rich fragment was digested to low-molecular products eluted in the total volume of the column, whereas the hydroxyproline-poor fragments were eluted between void and total volumes (Fig. 2).
On agarose-gel electrophoresis at alkaline pH, procollagen I migrated toward the anode (Fig. 3A). The hydroxyproline-rich fraction obtained by limited tryptic hydrolysis at 20°C, lost the anionic character of procollagen I and, under the conditions applied, remained near the point of application. Only a small part of this material (probably non-digested procollagen I) migrated to the anode (Fig. 3B). The proteolysis product of the hydroxyproline-poor fraction had the same mobility as procollagen I (Fig. 3C).

**Fig. 1**
Molecular sieve chromatography on Bio-Gel A-0.5 m column of purified procollagen I and proteolysis products. The column (2.5 x 38 cm) was equilibrated (at 4°C) and eluted with 0.2 M-ammonium bicarbonate, pH 8.2. Fractions of 5 ml were collected at a rate of 15 ml/h, and radioactivity and the hydroxyproline to proline ratio determined. A, Procollagen I before proteolysis; and products of proteolysis with trypsin performed at: B, 20°C; and C, 41°C.

**Fig. 2**
Molecular sieve chromatography on Bio-Gel A-0.5 m column of the procollagen I degradation products obtained by digestion with bacterial collagenase at 20°C. For details see legend to Fig. 1.

The acidic, non-collagenous character of the hydroxyproline-poor fraction released from procollagen I by non-specific proteases, was confirmed by CM-cellulose chromatography under the conditions used by Piez et al. (1963) for separation of tropocollagen subunits. In contrast to lathyric collagen subunits, this fraction was not bound by CM-cellulose and was eluted at low salt concentration (Fig. 4B). Total amount of the hydroxyproline-rich fraction obtained by digestion of procollagen I with trypsin or pronase, was strongly bound by CM-cellulose and could not be eluted with the salt gradient used for elution of tropocollagen
Fig. 3. Agarose-gel electrophoresis of A, procollagen I; B, the hydroxyproline-rich fraction and C, the hydroxyproline-poor fraction, obtained by digestion of procollagen I with trypsin at 20°C. The fractions obtained by Bio-Gel separation were used. Conditions: Samples were mixed with sucrose (6% final concentration) and bromophenol blue, and layered on the agarose under the upper electrode buffer of 0.043 M-Tris + 0.046 M-glycine, pH 8.9. The lower chamber buffer was 0.12 M-Tris, pH 8.1. Electrophoresis was run for 2.5 h using a constant current of 3 mA per tube.

Fig. 4. CM-cellulose chromatography of A, denatured lathyric collagen; B, the hydroxyproline-poor fraction obtained by digestion of procollagen I at 20°C with trypsin; and the hydroxyproline-rich fractions obtained by digestion with C, trypsin and D, pepsin, at 20°C. Conditions: The samples were dialyzed against 0.06 M-acetate buffer, denatured by heating at 45°C for 30 min and applied on CM-cellulose column (2 x 23 cm) equilibrated with the dialysis buffer at 40°C. Elution was performed with a linear NaCl gradient (0 - 0.1 M) in 0.06 M-acetate buffer, pH 4.8, and then (arrow) with 0.4 M-NaOH + 1 M-NaCl. Fractions of 10 ml were collected and the absorbance was measured.

subunits; the elution was achieved by applying 0.4 M-NaOH + 1 M-NaCl (Fig. 4C). Of the hydroxyproline-rich fraction obtained by digestion with pepsin at 20°C, which was performed at acidic pH, about 60% was identified by CM-cellulose chromatography as a material corresponding to α and β subunits, and the remaining material was firmly bound to the column (Fig. 4D).

Treatment of procollagen I with SDS and 2-mercaptoethanol did not cause its dissociation. On SDS-polyacrylamide-gel electrophoresis, the protein migrated more slowly than γ subunits of lathyric collagen (not shown). Denaturation and reduction of the hydroxyproline-rich fraction released from procollagen I by trypsin or pronase led to the appearance of a material migrating as a somewhat diffus
band between \( \alpha \) and \( \beta \) subunits (Fig. 5). The calculated molecular weight of this material was about 125,000 - 135,000 daltons.

**Fig. 5.** Molecular weight determination of the hydroxyproline-rich fraction obtained by digestion of procollagen I with trypsin at 20°C. Electrophoresis on SDS - 3% polyacrylamide gel was applied. For details see Methods. Lathyritic collagen (\( \alpha, \beta, \gamma \) subunits) was used as a standard. Mobility of the investigated material was calculated as the ratio of protein migration distance to bromophenol blue migration distance. The procollagen I-derived material migrated as a somewhat diffuse band at the position indicated by arrows.

**DISCUSSION**

The data reported by Bańkowski & Mitchell (1973) and the above-described results point to procollagen I as a primary biosynthetic form of tropocollagen precursor. It is considerably larger than the form of procollagen which on reduction and denaturation releases pro-\( \alpha \) chains. Molecular weight of procollagen I is about 540,000 daltons (Church et al. 1971; Bańkowski & Mitchell, 1973), and the protein does not dissociate into smaller fragments under the action of 2-mercaptoethanol. The resistance of procollagen I to the action of reducing agents (2-mercaptoethanol or dithiothreitol) has been observed by Church et al. (1971).

The amino acid composition, spectropolarimetric studies (Bańkowski & Mitchell, 1973) and the results of the present work confirm that procollagen I contains collagenous and non-collagenous regions. Below the temperature of denaturation, non-specific proteases digest the non-collagenous fragment of procollagen I molecule, whereas the collagenous, hydroxyproline-rich region is resistant to these enzymes. Similar resistance of native collagen was reported by Bornstein et al. (1966) and Kühn et al. (1966). The presence of hydroxyproline in low-molecular digestion products may be due to proteolysis of the procollagen which underwent denaturation during the purification procedure. Above the temperature of denaturation, the whole procollagen I molecule is digested to low-molecular peptides. Bacterial collagenase cleaves the collagenous part of procollagen I at 20°C, whereas the non-collagenous part seems to be resistant to the action of this enzyme. These observations and the previously reported circular dichroism studies (Bańkowski & Mitchell, 1973) confirm that the collagenous part of the native procollagen I is in the triple helix configuration, which makes it resistant to unspecific proteases.

The region of procollagen I sensitive to non-specific proteases contains a high amount of acidic amino acids. Its anionic character has been confirmed by agarose-gel electrophoresis at alkaline pH and CM-cellulose chromatography at pH 4.8.

Since the 2-mercaptoethanol-reduced hydroxyproline-rich fraction released from procollagen I by trypsin or pronase has a molecular weight higher than that of \( \alpha \) chains of tropocollagen and a lower Hyp/Pro ratio, this material can be considered as pro-\( \alpha \) chains described by other authors. On the other hand, pepsin releases
from procollagen I a material which after denaturation gives polypeptides similar to $\alpha$ and $\beta$ chains of tropocollagen.

It seems possible that procollagen I secreted by fibroblasts can be cleaved by extracellular proteases releasing acidic polypeptides and pro-$\alpha$ chains. The presence of acidic, procollagen-derived polypeptides in the medium of cultured cells was observed by several authors (Sherr et al., 1973; Dehm et al., 1974; Murphy et al., 1975).

Intact procollagen I does not dissociate under the action of denaturing and reducing agents; this indicates that it is synthesized as a single polypeptide chain containing alternate collagenous and non-collagenous regions (Church et al., 1971). A hypothetical model of the procollagen I structure is presented in Fig. 6. This structure is supported by the observations of Tanzer et al. (1974); they found that intermediate products of procollagen conversion to tropocollagen contain non-collagenous peptide extensions both at the amino and carboxyl terminals of tropocollagen. Park et al. (1975) suggest that the primary form of procollagen is synthesized on polycistronic mRNA.

![Diagram of procollagen structure](image)

**Fig. 6. Hypothetical model of procollagen I molecule containing alternate collagenous and non-collagenous parts.**

It is difficult to study native procollagen I because of its adsorptive properties on a variety of surfaces. The hydroxyproline-rich products obtained by limited proteolysis with trypsin or pronase are also strongly adsorbed on CM-cellulose under conditions used for separation of tropocollagen subunits, whereas the major part of the products obtained by digestion with pepsin can be eluted from the column with the salt gradient at positions corresponding to $\alpha$ and $\beta$ subunits. These observations allow to suggest that the region of procollagen I molecule responsible for the adsorption phenomenon is located in the non-collagenous regions of this protein, close to the collagenous region. The region of procollagen I molecule responsible for its adsorptive properties is split off by pepsin at 20°C, but not by trypsin or pronase. Dick & Nordwig (1966) found that stability of the secondary structure of collagen decreases at acidic pH. It seems possible that acidic medium (0.5 M-acetic acid) used for pepsin digestion causes a change in procollagen conformation which facilitates proteolysis and allows to remove a region of the molecule responsible for the adsorption phenomenon.
REFERENCES


PROTEOLIZA PROKOLAGENU I

Streszczenie

1. Trawienie prokologenu I trysąną, pepsoną lub pronazą przeprowadzone w 20°C powoduje uwalnianie kwaśnych niekolagenowych fragmentów oraz frakcji bogatej w hydroksyproolinę. Trawienie w 41°C (powyżej temperatury denaturacji) powoduje degradację prokologenu I do drobnocząsteczkowych peptydów.

2. Analiza frakcji bogatej w hydroksyproolinę, otrzymanej w wyniku ograniczonej proteolizy prokologenu I pepsoną (w 20°C), wykazuje obecność peptydów odpowiadających podjednostkom α i β (tropokologenu). Redukcja frakcji bogatej w hydroksyproolinę, uwalnianej z prokologenu I pod działaniem trysyny lub pronazy (w 20°C), powoduje pojawienie się polipeptydów podobnych do podjednostek pro-α.

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