ISOLATION AND AMINO ACID COMPOSITION OF HUMAN PROCOLLAGEN [Pro α 1(I)]$_2$ Pro α 2 FROM SKIN FIBROBLASTS IN CULTURE

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1. Introduction

Recently, several laboratories have reported the existence of a precursor form of collagen (procollagen) obtained from human skin fibroblast cells in culture [1–5]. Most of the procollagen produced by human skin fibroblast cultures is of the form [pro α1(I)]$_2$ pro α2; however, a significant proportion is of the form [pro α1(III)]$_3$ [2,6]. The ratio of type I procollagen to type III procollagen produced by normal and dermatosparactic calf skin fibroblasts is about 3 to 1 [7,8, and unpublished]. All of the human cell lines tested in our laboratory (both normal and diseased of all ages) contain significant amounts of type III procollagen which can be separated from the type I procollagen on DEAE cellulose [2,7,8]. In this communication we describe the purification of human [pro α1(I)]$_2$ pro α2 and present its amino acid composition.

2. Materials and methods

Normal human fibroblasts were obtained from the Institute for Medical Research, Camden, New Jersey (culture designation GM-75, 10-month old male) and grown in Waymouth medium 752/1 (GIBCO) supplemented with 10% fetal calf serum and 100 μg/ml ascorbic acid, without antibiotics. Serum-free Waymouth medium supplemented with 100 μg/ml ascorbic acid and 50 μg/ml β-amino-propionitrile fumarate (βAPN) was used on the cells when procollagen purification was carried out. Radioactive labeling was carried out using [^{3}H] proline (10 μCi/ml) in modified Eagle's medium (GIBCO) minus serum, plus 100 μg/ml ascorbic acid and 50 μg/ml βAPN. Procollagen was purified from the culture medium by a four-step method as described earlier [8]. Briefly, this method involves making the harvested culture medium 30% with respect to ammonium sulfate to precipitate collagen-related proteins. This precipitate was then solubilized in phosphate buffer (pH 7.6, ionic strength 0.4) and the solution then made 18% with respect to ethanol. The precipitate from this step (containing collagen-related proteins) was then chromatographed on DEAE cellulose and the eluted procollagen peak rechromatographed on hydroxylapitite to complete the purification.

Bacterial collagenase (Sigma) was purified and used according to the method of Peterkofsky and Diegelmann [9], including the recommended levels of *N*-ethylmaleimide. Chymotrypsin treatment of isolated procollagen was as described [10]. SDS-polyacrylamide gel electrophoresis (5% polyacrylamide gels) was carried out and the stained gels monitored on a Gilford spectrophotometer exactly as described earlier [8]. Purified samples of procollagen were analyzed for amino acid composition including half-cystine and tryptophan as described [8].

3. Results and discussion

The procedure recently used in our laboratory to purify calf dermatosparactic procollagens from cell culture medium [8] has been successfully utilized to obtain a highly purified procollagen from human fibroblast culture medium. Table 1 summarizes the relative purity of human procollagen at specific steps

Table 1
Purification of culture medium [pro α1 (I)]$_2$ pro α2

Purification Step	Relative purity (1%) (C/T × 100)*
20% $(NH_4)_2SO_4$	36
18% Ethanol	52
DEAE Cellulose	92
Hydroxylapatite	98

At each purification step, a portion of each sample was examined on 5% SDS-polyacrylamide gels either before or after treatment with purified collagenase. The relative purity of the procollagens was then assayed by the method previously published [8].

* C/T × 100 = percentage of collagenous proteins in the total protein observed in gels.

in the extraction. Fig.1 demonstrates DEAE cellulose chromatography of partially purified procollagen after 18% ethanol precipitation. The three peaks were individually pooled and further characterized. The first peak (fractions 30–50) was found to be procollagen [pro α1(I)]$_2$ pro α2 at a purity of about 92%. The second peak (fractions 80–110) was procollagen [pro α1(III)]$_3$ in a considerably impure form (about 75% pure). Attempts to isolate and purify enough of this material for further analysis are presently being carried out. The third peak (fractions 115–140) was found to be a collagenous protein associated with a noncollagenous high molecular weight protein. This peak migrated as a single band on SDS-polyacrylamide gels, having an apparent mol. wt of about 400 000.

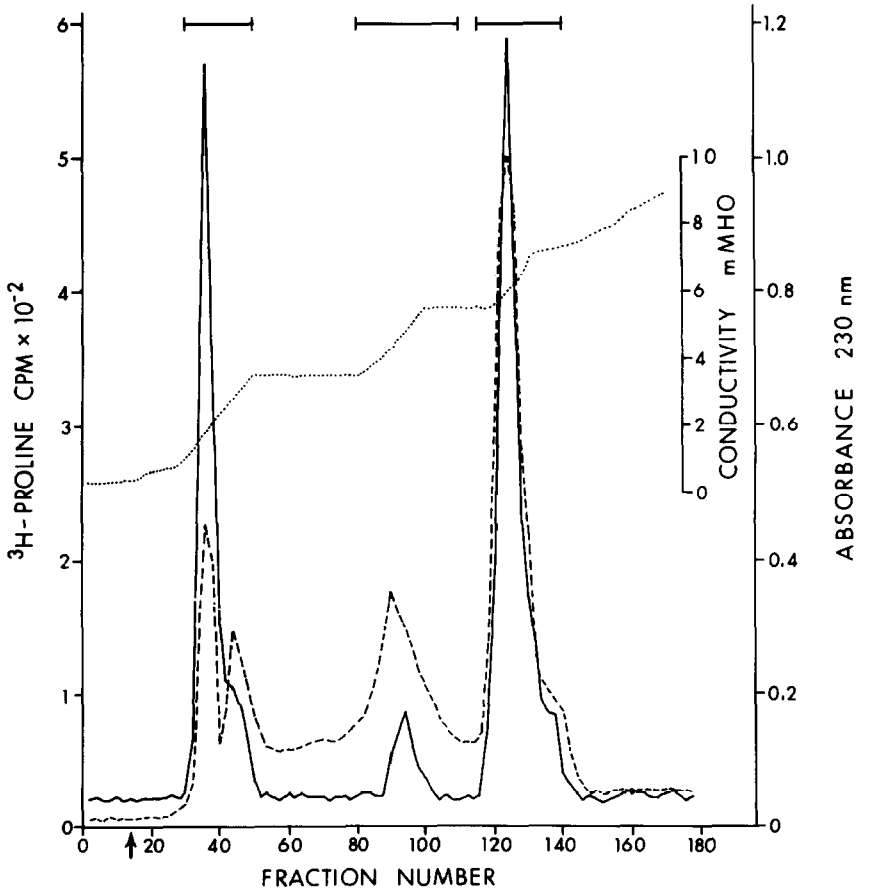

Fig.1. DEAE cellulose chromatography of partially purified procollagen obtained from the medium from human fibroblast cultures. (——) [^{3}H] proline label, and (– – –) absorbance at 230 nm were monitored. The 18% ethanol precipitate was dissolved in 0.01 M Tris–HCl (pH 7.4), 2 M urea and applied to a DEAE cellulose column [8]. The column was developed with a linear gradient of 1800 ml containing 0.3 M NaCl in the limiting buffer using a Gilson Mixograd automatic gradient former. The dotted line indicates the conductivity of the gradient formed.

Amino acid analysis of this protein fraction yielded very low hydroxyproline to proline, and hydroxylysine to lysine, ratios 0.025 and 0.011 respectively), and a low glycine content (112 residues per thousand residues). This material is similar to that reported recently by Bankowski and Mitchell [10] and appears to be collagen (or procollagen) bound in some form to a protein having a very high carbohydrate content (demonstrated by PAS-staining of polyacrylamide gels by the method of Fairbanks et al. [11], not shown).

The first peak from DEAE cellulose (fractions 30–50) was further purified by hydroxylapitite chromatography [8], seen in fig.2. Two peaks were eluted from this column. Peak 1 (fractions 32–56) was found to be purified [pro α1(I)]$_2$ pro α2. Fig.3 shows a 5% SDS-polyacrylamide gel electrophoresis run of this material. In the upper panel (fig.3) are

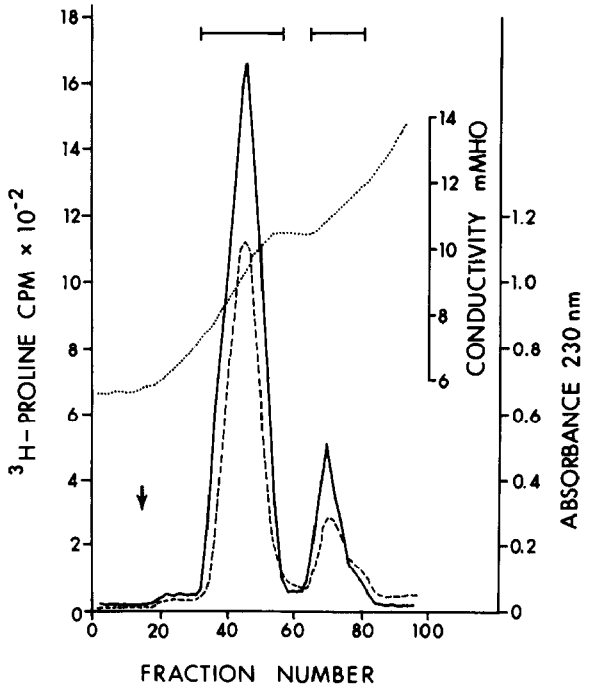

Fig.2. Hydroxylapitite chromatography of peak 1 from fig.1 (fractions 30–50). Conditions are as described [8]. The gradient was from 0.001 M sodium phosphate (pH 6.8) to 0.2 M sodium phosphate (pH 6.8) and was formed using a Gilson Mixograd automatic gradient former. The dotted line indicates the conductivity of the gradient formed. (——) [^{3}H] proline. (– – –) Absorbance at 230 nm.

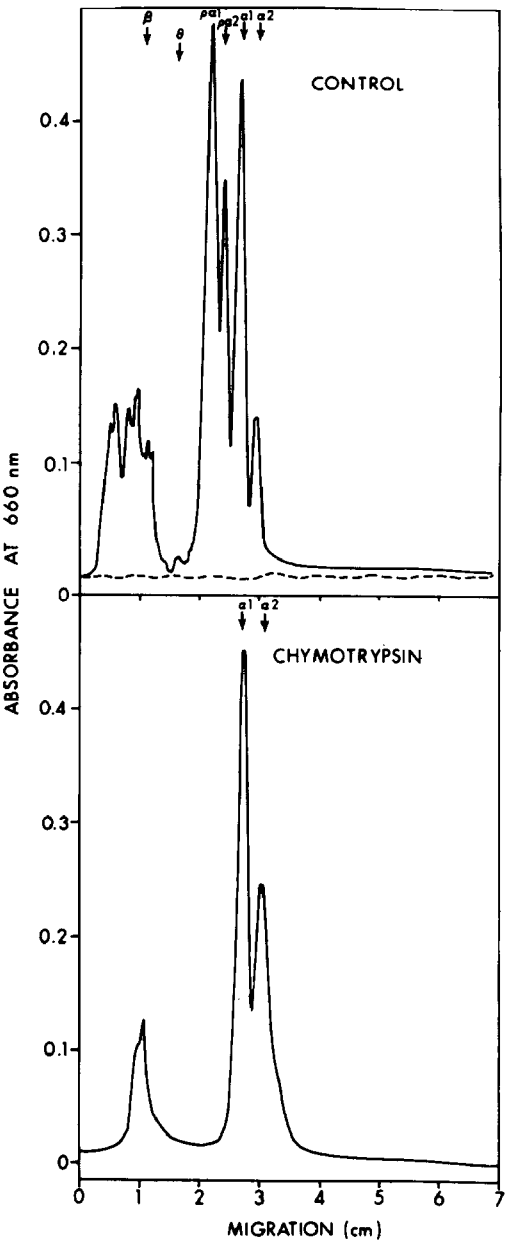

Fig.3. SDS-polyacrylamide gel electrophoresis of peak 1, fig.2 (fractions 32–56), purified [pro α1 (I)]$_2$ pro α2. Protein samples were prepared and analyzed as outlined in [8]. The gels were stained in Coomassie Blue and scanned at 660 nm using a Gilford gel scanning attachment on a Gilford spectrophotometer. The dashed line in upper panel indicates treatment with purified bacterial collagenase. Marker proteins used were culture medium calf dermatosparactic [pro α1 (I)]$_2$ pro α2 and calf skin collagen.

seen peaks containing polypeptides corresponding to α2, α1 (both 98 000 mol. wt, pro α2, pro α1 (both 120 000 mol. wt, and polypeptides with a molecular weight greater than β-chains (presumptive pro β-chains). We do not detect any polypeptide having a mobility of the θ-chain (150 000 mol. wt), which is observed in calf dermatosparactic procollagen [8] but is also lacking in normal calf procollagen (unpublished). Collagenase-treatment (dashed line, upper panel) destroys all of the polypeptides, and chymotrypsin (lower panel) seems to convert most of the polypeptides to α-sized material (with some β-sized polypeptides). Since virtually no noncollagenous stained bands were observed in SDS-polyacrylamide gels run on the hydroxylapitite chromatography-purified [pro α1(I)]$_2$ pro α2, even when the gels were grossly overloaded (100 μg protein on one gel), we concluded that the procollagen sample was pure (although the assay system, due to inherent imperfections in the gels, is only sensitive to 98% purity. The second peak (fractions 65–80) eluted from hydroxylapitite (fig.2) was found to contain no hydroxyproline or hydroxylysine and contained no collagenase-sensitive material.

Amino acid analysis (table 2) was carried out on the purified [pro α1 (I)]$_2$ pro α2 (first peak from hydroxylapitite chromatography). This material is similar in amino acid composition to other animal procollagens [8,12,13], having low glycine and hydroxyproline contents and increased tryposine, aspartic acid and hydrophobic amino acid contents, when compared to collagen. Also detected were half-cystine and tryptophan, not found in collagen but present in procollagen.

This procollagen appears to be similar to peak 1 procollagen partially purified by Smith et al. [2] who used DEAE cellulose chromatography to separate two types of procollagen. The procollagen extensively studied by Goldberg and associates [3–5,14,15] must be assumed to be a mixture of type I and type III procollagen in that no mechanism to separate the two types was employed. All human cell lines studied in our laboratory (including CRL 1121, used by Goldberg and associates) have yielded both type I and type III procollagen.

The above purification and amino acid composition of [pro α1 (I)]$_2$ pro α2 is the first presented for human procollagen. This material has an amino acid composition similar to procollagens isolated from other animal sources [8,12,13] and contains no θ-chain observed in calf dermatosparactic procollagen [7,8]. Since Lichtenstein et al. [16] have reported a human form of dermatosparaxis, it will be of interest to determine if human dermatosparactic fibroblast procollagen contains θ-chains as a diagnostic feature of the disease.

Table 2
Amino acid compositions of human skin collagen and numan (pro α1)$_2$ pro α2 purified from cell culture

Amino acid	Human fibroblast (pro α1)$_2$ pro α2*	Human skin collagen**
	Residues/1000 Residues	
Lysine	35	27
Histidine	8.1	5.4
Arginine	52	49
4-Hydroxyproline	75	91
Aspartic Acid	58	47
Threonine	28	18
Serine	47	37
Glutamic Acid	80	78
Proline	108	125
Glycine	280	324
Alanine	102	115
Half-Cystine	4.7	–
Valine	31	25
Methionine	7.1	7.0
Isoleucine	14	10
Leucine	32	25
Tyrosine	10	3.5
Phenylalanine	18	13
Hydroxylysine	8.1	5.9
Tryptophan	2.1	0

* Obtained from peak No. 1, fig.2, Hydroxylapatite purification. The values are calculated as residues per 1000 residues and are the averages of six separate determinations.

** As reported by Fleishmajer and Fishman [17].

Acknowledgements

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