PURIFICATION AND CHARACTERIZATION OF PROTEINS, INCLUDING PROCOLLAGEN TYPE III, IN SALT EXTRACTS OF FETAL BOVINE SKIN*

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Received January 6,1977

SUMMARY. The nature of high-molecular-weight proteins in salt extracts of fetal bovine skin was investigated. A series of DEAE cellulose ion-exchange columns separated the mature collagen from the high molecular weight proteins and also separated the high molecular weight proteins from each other. The following proteins were isolated: (a) a very high molecular weight protein which appears to be aggregated mature collagen; (b) two high molecular weight proteins of slightly faster mobility on SDS polyacrylamide gels, one of which is collagen-like and one of which is not; and (c) a type III procollagen, purer than those previously reported in the literature. These latter three proteins were characterized by amino acid analysis, SDS polyacrylamide gel electrophoretic mobility, collagenase sensitivity, and CNBr peptide patterns from SDS-PAGE.

There are at least four types of collagen. Type I is found predominantly in skin, bone, and tendons. Type II is found in cartilage. Type III is found in skin, arteries and uterine tissue, but is referred to as fetal collagen. Type IV is found in basement membranes. The amino acid composition of the four collagens differ somewhat from one another.

Collagens are synthesized as a precursor molecule, procollagen, with N- and C-terminal extensions of non-collagen-like peptides. The isolation of a type III procollagen and its partly-hydrolyzed product, p-collagen, have been reported by Byers et al. (1).

METHODS. Neutral-salt extracts of fetal-calf skin: Neutral-salt-soluble collagen was prepared from frozen fetal-calf skin (Pel-Freez, Bio-Animals, Inc., Rogers, Ark.). After grinding, the skin was extracted in a solution of 0.05 M EDTA, 10^{-5} M PMSF, and 10^{-3} M pMB, pH 7.5 until a colorless supernatant was obtained. The washed skin was then extracted three times in the

* Supported by USPHS, NIH Grant Nos. AM-17720, GM-00035 and HD-00669.
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Abbreviations: EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; pMB, p-(hydroxymercuri)-benzoic acid; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; NSS, neutral salt solutions.

ISSN 0006-291X

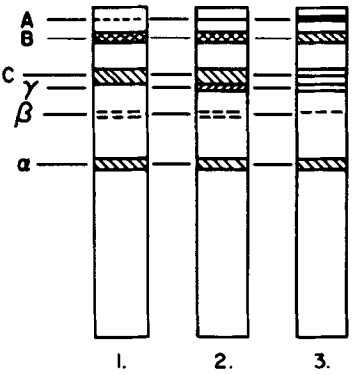

Figure 1. SDS-PAGE of salt extracts of fetal-calf skin. This figure represents the various fractions found in the sequential extractions of skin in neutral solutions of 0.5 M NaCl after precipitation with 16% KCl and resolution. The numbers under each pattern represent the order of extraction.

same wash buffer but containing 0.5 M NaCl. The supernatant solutions were adjusted to 16% KCl (w/v). The precipitate obtained was redissolved in 0.05 M potassium phosphate, 0.05 M EDTA, 10^{-5} M PMSF, 10^{-3} M pMB, pH 7.5 and stored at 4° C.

DEAE cellulose chromatography: A 5.0 x 10 cm column of Whatman DE-32, DEAE cellulose was prepared and equilibrated at 4° with a buffer containing 0.02 M NaCl, 0.03 M Tris, 2 M urea, pH 7.5 (1). The collagen preparations from the first and second skin extracts were pooled and dialyzed against equilibration buffer. The sample was pumped into the column (60 ml/hr) and 20 min fractions were collected. The protein was eluted at 90 ml/min with a linear salt gradient from 0.02 M to 0.5 M NaCl in the Tris-urea buffer. The total volume was 2 liters. After the gradient elution, one liter of column buffer containing 1.0 M NaCl was pumped through the column.

SDS gels (4%) were run on selected uv absorbing fractions according to Furthmayr and Timpl (2). The uv absorbing fractions containing procollagen and higher molecular weight components were pooled separately and dialyzed against the column starting buffer.

The procollagen pool was rechromatographed on a 2.5 x 10 cm column of DE-32 equilibrated as above and the protein was eluted with a linear salt gradient from 0.02 M to 0.2 M NaCl over a total volume of 800 ml. SDS gels were run on selected uv absorbing fractions. Based on the gel patterns, the protein fractions were pooled and stored. The protein eluting with 1 M NaCl was rechromatographed, with a linear salt gradient from 0.02 M to 0.5 M NaCl over a total volume of 800 ml followed by 400 ml of the starting buffer containing 1.0 M NaCl.

Collagenase digestion was performed on samples dialyzed against a solution containing 0.02 M NaCl, 0.03 M Tris (pH 7.5) and 2 M urea. To each 0.2 mg sample, 2 units of collagenase (Advance Biofacture, Inc.) in 0.025 M Tris (pH 7.4) plus 0.33 M calcium acetate were added. Digestion was monitored by SDS-PAGE.

Trypsin digestion was carried out with 10 μg of trypsin per 0.25 mg of protein in 0.2 M NaCl and 0.05 M Tris (pH 7.5).

CNBr digestion was performed according to Miller et al. (3). Standards of type I and III collagens, purified according to Chung and Miller (4) were run in parallel.

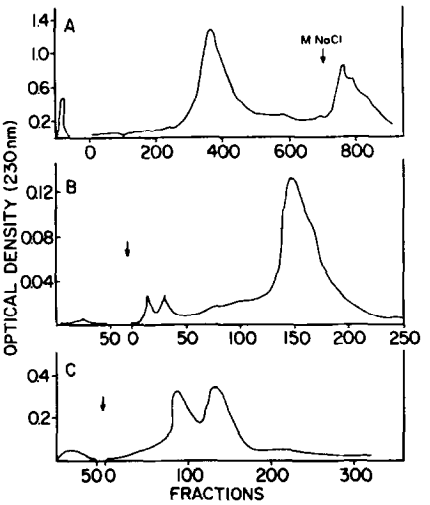

Figure 2. The results of DEAE-cellulose chromatography in the purification of various proteins. A) The elution profile of a pool of the first and second extracts of calf skin after a preliminary 16% KCl precipitation. The proteins were eluted with a 0.02 to 0.5 M NaCl linear gradient of 2 liters in 2 M urea and 0.03 M Tris, pH 7.5. The start of the gradient (zero on the abscissa) is indicated by the first arrow. The beginning of the elution with M NaCl is indicated by the second arrow. B) Rechromatography of fractions 450-600 from the column elution shown in A, above. The gradient used was 800 ml of a 0.02 to 0.2 M NaCl in the Tris-urea buffer. C) Rechromatography of fraction 720-900 from the column effluent in A, above. A linear gradient of 0.02 to 0.5 M NaCl in the urea-Tris buffer, followed by M NaCl in the same buffer.

RESULTS. Proteins in the salt extracts: Figure 1 is a representation of the SDS-PAGE patterns of the serial extracts of washed, fetal calf skin.

Type III procollagen: The first DEAE column gave the results shown in Figure 2A. SDS-PAGE indicated that the peak eluting prior to starting the gradient contained types I and III collagens. Rechromatography of fractions 450-600 gave the results shown in Figure 2B. On SDS-PAGE, the major peak indicated a relatively pure protein which, on reduction, showed a single component migrating slower than α1 of type I collagen (Figure 3). The protein was found to be collagenous in amino acid composition (Table I).

Following collagenase treatment, all protein bands disappeared, implying that the protein was reduced to small polypeptide fragments. Tryptic digestion yielded a predominance of a single band migrating to the position of α1 (I)

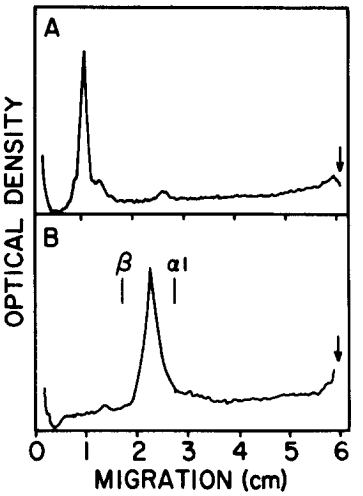

Figure 3. Absorbance scan of SDS-PAGE gels of the protein eluting from the DEAE-cellulose column in Figure 2B in fractions around number 150. A) Unreduced protein and B) protein reduced with mercaptoethanol. The positions of β_{11} and $\alpha 1$ are indicated as obtained from control runs of acid-soluble collagen.

on SDS-PAGE, together with some undigested pro-$\alpha 1$ (III). Conclusive identification of the procollagen as type III collagen was obtained by comparing its cyanogen bromide peptide pattern as determined by SDS-PAGE with those of $\alpha 1$ (I) and $\alpha 1$ (III) which were also isolated from the same tissue.

Proteins of band B (Figure 1): Two very different types of proteins in band B were isolated and partially purified. The proteins eluted with 1 M NaCl from the first DEAE-cellulose column were pooled (Figure 2A, fractions 720-900). This protein pool was rechromatographed on a second DEAE-cellulose column with a 0.02 M to 0.5 M linear salt gradient followed by 1 M salt (Figure 2C). Fractions 90 and 130 contained only one protein exhibiting the same mobility on gels (band B, Figure 1).

The amino acid composition (Table I) of one protein was similar to collagen. Surprisingly, this protein could not be digested by collagenase to any noticeable extent for times well beyond those required to digest acid-soluble collagen. Trypsin digestion was not performed due to a lack of

TABLE I

Amino Acid Analysis of Proteins Isolated from Salt Extracts of Fetal-Calf Skin[a]

Residue	Type III[b]	Type III Procollagen	Band B_1[c]	Band B_2[c]
Hydroxyproline	119	84	87	trace
Aspartic acid	47	57	67	86
Threonine	16	18	27	32
Serine	48	53	63	57
Proline	106	125	83	171
Glutamic acid	71	89	105	146
Glycine	341	309	278	201
Alanine	95	96	80	76
Valine	16	12	14	27
Half-cystine	1	3	N.D.[d]	N.D.
Methionine	8	8	9	10
Isoleucine	14	9	13	13
Leucine	18	27	51	80
Tyrosine	4	6	11	25
Phenylalanine	9	13	19	12
Hydroxylysine	7	7	6	-
Lysine	28	36	36	33
Histidine	7	6	11	6
Arginine	45	42	49	44

[a] In residues/1000 residues

[b] According to Timpl et al. ( )

[c] Both proteins migrate with band B (Fig. 1)

[d] Not determinable

material. The other protein was non-collagenous (Table I) as confirmed by trypsin and collagenase digestion.

Protein of band A (Figure 1): In the last NNS extract from the calf skin a majority of the protein seemed to be in band A (Figure 1). Attempts were made to purify and characterize this protein. This last extract was first chromatographed on DEAE-cellulose with a 0.02 M to 0.5 M salt gradient

followed by a 1 M salt wash (not shown). The only prominent protein peak in the profile was mature collagen which eluted before the gradient. Further investigation showed that when the same was fresh there was predominantly band A and that with dialysis into 2 M urea at 4° C, the quantity of band A was reduced and the amount of band B and Type I mature collagen increased.

DISCUSSION. The type III procollagen isolated in this study appeared to be quite pure based on the criterion of its behavior on DEAE-cellulose and SDS gels. Based on the gels of reduced procollagen, this material is purer than that presented by Timpl et al. (5). Their gel of reduced procollagen demonstrated a minimum of four protein bands, one of the major contaminents being mature type III collagen. In the report of Byers et al. (1), a type III precursor collagen designated p-collagen was isolated and characterized. The molecular weight of p-collagen was not determined. The only molecular weight determinations reported on mature α1 (III) were apparent molecular weights derived from SDS acrylamide gels (5,6) and agarose A-5M chromatography (7,8). In all cases, the apparent molecular weight was shown to be between 93,000 and 95,000 daltons. Based on 120 residues of hydroxyproline per thousand residues, the type III p-collagen reported by Byers et al. would have a molecular weight of approximately 100,000 daltons, even although the paper implied that it was 120,000 daltons. Type III procollagen, though shown to be present, eluded isolation.

The amino acid composition of the type III procollagen isolated in this study showed a reduced percentage of hydroxyproline. Based on this, the procollagen chain is 47% larger than a mature α chain, therefore having a molecular weight of approximately 140,000 daltons. The hydroxyproline plus proline (209 residues per thousand) for the type III procollagen reported here was significantly lower than the value of 221 previously reported. This indicated that the precursor collagen isolated in the current study was purer than any previously isolated precursor.

The collagenase digestion supported the fact that this isolated protein

was collagenous. Although the non-collagenous portions of the procollagen were not digested by collagenase, these fragments were too small to be visible on 4% SDS acrylamide gels. This phenomenon was also shown by Byers et al. (1). The trypsin digestion proved that the isolated collagenous protein was indeed a collagen precursor since, after trypsin treatment, the protein was converted to α1 (III) chains. The CNBr digest showed the peptide pattern of the freshly isolated procollagen to be identical to that of α1 (III).

All the protein peaks which were completely resolved by DEAE-cellulose chromatography (Figure 2C) demonstrated the same mobility on SDS gels (band B), thus implying that they were closely related in molecular weight and differed only in amino acid content or charge. It was found that one of the proteins was collagenous, while the other was not. The collagenous protein may be a larger collagen precursor, according to the proline plus hydroxyproline content (170 residues/1000 residues) but this was not established on the basis of questionable purity. A collagenase digestion was performed on the collagenous protein but the material was not digested. This does not refute the evidence that it was collagenous. One possible explanation is that this collagenous protein may be a larger precursor form of procollagen, and that the precursor portions were large enough to fold back and mask the collagenase-sensitive backbone of the molecule. More protein must be isolated for study before alternatives can be further explored.

The non-collagenous protein in band B was also not digested by collagenase. It was digested by trypsin, which suggests that it was definitely not collagenous. Its presence in the collagen preparation may be easily explained by the fact that the epidermis used as the starting material was a heterogeneous collection of cell types. This non-collagenous protein may be a more highly purified form of the acidic structural proteins of connective tissue described by Timpl et al. (9).

Attempts to purify the protein(s) of band A indicated that it was aggregated, mature collagen. This suggestion is based on the fact that the only

protein eluted from the DEAE cellulose column eluted with mature collagen, and that when the preparation, greatly enriched in band A, was dialyzed against 2 M urea and run on SDS-PAGE, band A decreased in intensity and the mature collagen bands increased dramatically. Aggregated collagen has been reported before in extracts of tissue by Veis and Anesey (10).

In summary, all major high molecular weight bands after SDS-PAGE of salt extracts of fetal-calf-skin collagen have been identified including pro α1 (III), purer than has been reported previously.

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