The Separation of the α -Chains of Collagen by Free-Flow Electrophoresis

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1. The separation of the α -components of codfish-skin and calf-skin collagen from the higher-molecular-weight components was accomplished by gel filtration with Bio-Gel P-300 resin in 5M-lithium chloride and at pH 7.4. 2. From this fraction three distinct α -components, corresponding to the $\alpha 1$ -, $\alpha 2$ - and $\alpha 3$ -chains of collagen, were isolated by free-flow electrophoresis in 6M-urea at 4°. At the temperatures and pH values used there was no detectable degradation of the α -chains.

Considerable evidence has been presented that the structure of the collagen macromolecule can be represented by the formula $(\alpha 1)_2\alpha 2$, where $\alpha 1$ and $\alpha 2$ represent single-stranded polypeptide chains distinguished by differences in their amino acid compositions (Piez, Eigner & Lewis, 1963a). Recently the $\alpha 1$ -component of codfish skin (Piez, 1964, 1965) and calf skin (Heidrich & Wytton, 1965) has been further fractionated into an $\alpha 1$ - and an $\alpha 3$ -component. In addition to the single-stranded components (α -components), dimers (β -components), trimers (γ -components) and higher-molecular weight covalent polymers formed by intramolecular and intermolecular covalent cross-linking of the α -chains have also been observed in denatured collagens (Altgelt, Hodge & Schmitt, 1961; Piez, Lewis, Martin & Gross, 1963b; Orekhovitch & Shpikiter, 1955; Veis, Anesey & Cohen, 1960; Vies and Anesey, 1962).

Separation of the $\alpha 3$ -chain from the $\alpha 1$ -chain by chromatography of the gelatins on CM-cellulose has proved difficult, however, owing to the presence of many different dimers (β -components) and trimers (γ -components) and the degradation of components during repeated rechromatography at acid pH and at 40° (Piez *et al.* 1963a; Piez, 1965; Lewis & Piez, 1964).

In order to improve the separation of the α -chains and to prevent their degradation during the process, a method was described for the fractionation of the α -chains from the β -components, γ -components and higher-molecular-weight covalent polymers by gel filtration through Sephadex G-200 resin at neutral pH and at room temperature in 5M-lithium chloride (Francois & Glimcher, 1965). However, although it was possible by this latter

technique to isolate a fraction containing only the α -chains, separation of the $\alpha 1$ -, $\alpha 2$ - and $\alpha 3$ -components by chromatography on a variety of different CM-cellulose resin columns could only be achieved by repeated rechromatography. This was always accompanied by some degradation and a poor yield of the individual components. Since the three α -chains are distinguished by differences in their amino acid compositions, it was considered that they might be separated under milder conditions by free-flow electrophoresis. A preliminary report has already been presented (Francois & Glimcher, 1965).

EXPERIMENTAL

Preparation of collagens. The acetic acid-soluble collagens from calf skin and codfish skin were prepared from the minced tissues after the prior extraction of the neutral soluble collagens in 0.15M-NaCl and 1.0M-NaCl at pH 7.4 and at 2° (Glimcher, Francois, Richards & Krane, 1964). The collagens were purified by two precipitations with NaCl at a final concentration of 5% (w/v) and by three precipitations at low ionic strength and at neutral pH (Glimcher *et al.* 1964). All of the procedures were carried out at 2°.

Gel filtration. Bio-Gel P-300 resin (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was equilibrated for 1 week in 5M-LiCl in 0.05M-tris buffer, pH 7.4, at 30°. The small particles were removed by suction after the stirred suspension had been allowed to settle for 40–50 min. This procedure was repeated about 20–30 times. The resin was poured into two columns (3.3 cm. $\times$ 1.0 cm.) connected in series as previously described for Sephadex G-200 resin (Francois & Glimcher, 1965), except that both columns were run with upward flow to prevent packing of the gel. The gel filtration was carried out at 30°.

Approx. 10–12 ml. of a 2–4% (w/v) solution of gelatin was prepared from freeze-dried protein by dissolving the

material in 5M-LiCl, pH 7.4, at room temperature. The solution was clarified by centrifugation or by filtration through a fine sintered-glass filter. The protein solution was applied to the Bio-Gel P-300 resin column and eluted with 5M-LiCl, pH 7.4, at 3-4 ml./hr. Fractions (3 ml.) were collected, dialysed against water until free of LiCl and concentrated, and urea was added to give a final concentration of 6M. The presence and content of the α -, β - and γ -components of the fractions was assayed by polyacrylamide-gel electrophoresis in 6M-urea at 10° (Francois & Glimcher, 1965), by using a modification of the method of Nagai, Gross & Piez (1964) that utilizes lysine in the anode buffer (J. Gross, personal communication). This procedure separated the α 2-component from the α 1- and α 3-components. By using the results of the gel electrophoresis as a guide, a fraction containing the pure α -components was separated by the gel filtration. In some instances the fraction containing the α -components was re-run a second time. A better separation of the α -, β - and γ -components was achieved with the Bio-Gel P-300 resin than that previously reported with Sephadex G-200 (Francois & Glimcher, 1965). The fractions containing the α -components were either dialysed against water until free of LiCl or desalted on a column (5.0 cm. $\times$ 50.0 cm.) of Bio-Gel P-10 resin, equilibrated on 0.1M-pyridine-acetate buffer, pH 4.0, at room temperature, and the samples were freeze-dried.

Continuous free-flow electrophoresis. To separate the α 1-, α 2- and α 3-chains under the mildest conditions, continuous free-flow electrophoresis was carried out with a commercial model (Brinkmann Instruments, Westbury, Long Island, N.Y., U.S.A.) of the instrument described by Hannig (1961). To prevent aggregation and degradation of the α -chains, the electrophoresis was run in approx. 6M-urea at 4°.

The electrode buffer was prepared by adding 1320 ml. of distilled water and 34 ml. of trimethylacetic acid (melted at 50°) to 4100 ml. of 8M-urea. The pH was adjusted to 5.25 by the addition of 6N-NaOH and then dilute NaOH. The same chamber buffer was used for all ports. It was prepared by diluting the electrode buffer with an equal volume of 6M-urea.

A 2% (w/v) solution of the gelatin in urea was dialysed extensively at 4° against the chamber buffer and clarified by filtration through a medium-porosity sintered-glass filter. The protein solution was applied to the electrophoresis instrument at a flow rate of 1 ml./hr. at the port corresponding to tube fraction no. 34, counting from the left side, which corresponded to the cathode. The flow rate of the chamber buffer was 25 ml./hr. Electrophoresis was carried out at 2300 v and 70 ma at 4° for about 12 hr. Forty-eight fractions were collected and desalted on columns (2.0 cm. $\times$ 20.0 cm.) of Bio-Gel P-10 resin (Bio-Rad Laboratories) equilibrated in 0.1M-pyridine-acetate buffer, pH 4.0, at room temperature. The fractions were freeze-dried and redissolved in a known volume of water, and portions were used for hydroxyproline determination (Stegemann, 1958) and for polyacrylamide-gel electrophoresis. Fractions containing only a single component were pooled.

CM-cellulose chromatography. The method described by Piez *et al.* (1963b) was modified as follows. The CM-cellulose resin used was Selectacel (lot no. 1434; Schleicher and Schuell, Keene, N.H., U.S.A.). The column size was 0.9 cm. $\times$ 13.0 cm. The protein was eluted by a linear gradient of NaCl obtained by using 50 ml. in the first two

chambers of a commercial Varigrad (Phoenix Precision Instrument Co., Philadelphia, Pa., U.S.A.). For codfish-skin collagen the first chamber contained 0.08M-sodium acetate, pH 4.80, and for calf-skin collagen it contained 0.06M-sodium acetate, pH 4.80. The solution in the second chamber was the same for both cod-skin and calf-skin collagen: 0.08M-sodium acetate-0.08M-NaCl, pH 4.80. Chromatography was carried out at a flow rate of 17.5 ml./hr. Fractions (0.8 ml.) were collected and the concentration of protein was determined by measuring $E_{230\text{ m}\mu}$. The presence of α 1-, α 2- and α 3-fractions was detected by polyacrylamide-gel electrophoresis after first desalting the samples by dialysis against water. Approx. 90% of the individual peaks were used for amino acid analyses.

Amino acid analyses. Samples (approx. 4 mg.) of freeze-dried protein were dissolved in 1.5 ml. of 0.01N-HCl and transferred to test tubes filled with sealed connexions at their tops to which vacuum lines could be attached. An equal volume of reagent-grade 12N-HCl was added, the test tube cooled in a mixture of solid CO₂ and ethanol and connected to a vacuum line. The sample was slowly melted during which time the test tube was gently shaken. N₂ was then introduced slowly and the test tube slowly evacuated again. This was repeated several times. The sample was then frozen again and the top sealed under vacuum. Hydrolysis was carried out at 110° for 24 hr. The hydrolysates were evaporated in a vacuum desiccator over NaOH pellets until dry, and redissolved in 0.01N-HCl at a final concentration of approx. 0.6 mg./ml. and filtered. Amino acid analyses were performed on a modified commercial model (Phoenix Precision Instrument Co.) of the automatic amino acid analyser described by Piez & Morris (1960). To calculate both the major and minor amino acid constituents accurately, both large (approx. 1.0-1.5 mg.) and small (0.3 mg.) samples were analysed. The ratio of the volumes of the two samples was accurately calculated from the respective areas of the chromatographs for eight amino acids with extinction coefficients that varied between 0.8 and 0.2 on both charts. From this ratio it was possible to calculate the concentrations of all of the amino acids with precision. Triplicate analyses were performed on all of the fractions. The corrections for losses of amino acids during hydrolysis were those described by Piez, Weiss, & Lewis (1960), except that no correction was used for valine since no further release of valine occurred when samples were hydrolysed for an additional 24 hr.

RESULTS

The results of a typical gel filtration are shown in Fig. 1. The α -components are eluted at a volume equal to 1.3 times the void volume, and are well separated from the β -components, γ -components and higher-molecular-weight fractions. By using the results of polyacrylamide-gel electrophoresis of each of the individual tube fractions, samples containing only α -components could be isolated in a yield of approx. 80-85% of the total amount of α -component present in the original sample. At 30° and at pH 7.4 there was no degradation of the α -chains, as noted by polyacrylamide-gel electrophoresis.

The β -components, eluted at a volume equal to 1.11 times the void volume, could be obtained without contamination with other components by a second gel filtration. Approx. 30% of the β -components could be recovered. Samples enriched in γ -component, but containing some higher- and lower-molecular-weight species, could also be isolated by a second gel filtration.

A typical free-flow electrophoresis experiment of the mixed α -fraction of calf-skin collagen obtained by gel filtration is shown in Fig. 2. Three fractions are partially resolved by the free-flow electrophoresis technique. The most positively charged fraction corresponds to the $\alpha 2$ -component both by

Fig. 1. Elution curve of denatured calf-skin collagen filtered through Bio-Gel P-300 in 5M-LiCl at 30° and at pH 7.4. The broken lines were drawn from the results of the polyacrylamide-gel electrophoresis. The γ -fraction also contained higher-molecular-weight aggregates.

Fig. 2. Separation of the α -components of calf-skin collagen by free-flow electrophoresis in 6M-urea at 4° and at pH 5.25. The broken lines were drawn from the results of the polyacrylamide-gel electrophoresis.

CM-cellulose chromatography and by polyacrylamide-gel electrophoresis. The least positively charged component obtained by free-flow electrophoresis is eluted from the CM-cellulose in the region corresponding to $\alpha 1$ -component and moves as $\alpha 1$ -component on polyacrylamide-gel electrophoresis. The third component, with a mobility intermediate between those of the $\alpha 1$ - and $\alpha 2$ -components, but closer to the latter, was eluted somewhat later than $\alpha 1$ -component from the CM-cellulose, in the region described by Lewis & Piez (1964) and Piez (1964, 1965) corresponding to the $\alpha 3$ -component. It moved with the $\alpha 1$ -component on polyacrylamide-gel electrophoresis. The results of CM-cellulose chromatography are shown in Fig. 3. When the mixed α -fraction was chromatographed directly on CM-cellulose (Fig. 3a), the $\alpha 1$ - and $\alpha 3$ -components were not resolved. However, they could be distinguished when the pure fractions, obtained by free-flow electrophoresis, were chromatographed separately (Figs. 3c and 3d). Similar results were obtained with the collagen of codfish skin.

The amino acid compositions of the three

Fig. 3. Chromatography on CM-cellulose of the α -components of calf-skin collagen: (a) mixture of the α -components obtained by gel filtration, (b) $\alpha 2$ fraction, (c) $\alpha 3$ fraction and (d) $\alpha 1$ fraction, all obtained by free-flow electrophoresis of the mixed α -components. The $\alpha 1$ - and $\alpha 3$ -components are not resolved when the mixed α -fraction is chromatographed directly on CM-cellulose (a).

Table 1. Amino acid composition of codfish-skin and calf-skin collagens and their constituent α -chains

Amino acid composition (residues/1000 total amino acid residues)												
Calf-skin collagen					Codfish-skin collagen							
Mixed α -components	Components separated by electrophoresis only				Mixed α -components	Components separated by electrophoresis only			Components separated by electrophoresis and chromatography on CM-cellulose			
	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 1$		$\alpha 2$	$\alpha 3$	$\alpha 1$	$\alpha 2$	$\alpha 3$		
3-Hydroxyproline	1.3	1.7	1.3	1.5	Trace	Trace	Trace	0.5	0.6			
4-Hydroxyproline	98	104	98	94	59	66	53	61	61	55	61	
Aspartic acid	45.2	43.9	45.6	44.8	49.9	49.7	56	49.6	50	48.6	51	
Threonine	18.5	18.7	18.9	18.5	24	24.1	27.4	23.5	23.2	24.4	24.8	
Serine	35.9	36.3	34.4	36.6	70	66	67	69	69	67	70	
Glutamic acid	74	76	69	77	73	76	59	76	73	60	74	
Proline	121	124	112	124	92	92	95	93	93	98	94	
Glycine	330	325	333	326	351	346	346	348	354	348	344	
Alanine	116	118	107	119	108	111	190	110	110	113	111	
Valine	17.7	14.9	26.0	16.4	17.9	17.3	20.6	16.9	16.5	21.0	16.5	
Methionine	6.9	7.7	5.3	7.3	17.1	16.8	17.7	16.6	16.2	17.7	17.3	
Isoleucine	9.7	8.2	13.7	8.9	10.3	10.2	11.9	10.4	9.8	11.4	10.3	
Leucine	22.1	19.2	28.4	20.3	18.8	16.9	23.8	17.1	16.4	20.2	17.0	
Tyrosine	3.4	3.8	3.2	3.4	2.8	1.3	5.8	1.9	1.4	6.6	1.1	
Phenylalanine	12.0	11.8	13.2	11.9	11.1	12.6	7.9	12.3	13.2	8.3	14	
Hydroxylysine	6.8	5.7	9.1	6.2	6.2	5.2	9.2	5.0	6.0	9.3	5.4	
Lysine	27.9	30.9	24.1	29.9	27.9	31.3	20.8	30.7	30.2	21.8	30.5	
Histidine	3.0	2.5	5.9	2.7	7.4	5.4	10.9	6.1	5.2	12.6	5.5	
Arginine	50	47.2	51	51	51	51	55	51	49.7	56	52	

α -chains of both codfish-skin and calf-skin collagen are presented in Table 1. These include the α -chains separated by free-flow electrophoresis only, and the single chains obtained from the free-flow electrophoresis experiments after these fractions were then further purified by chromatography on CM-cellulose (Fig. 3).

DISCUSSION

Free-flow electrophoresis of the mixed α -components of collagen, when combined with a prior separation of the single α -chains from the β -components, γ -components and higher-molecular-weight polymers by gel filtration, has permitted the separation of three different α -components from the collagens of codfish skin and calf skin. The amino acid compositions and the chromatographic behaviour on CM-cellulose of the three individual fractions isolated by the free-flow electrophoresis confirm that these fractions correspond to the $\alpha 1$ -, $\alpha 2$ - and $\alpha 3$ -components described by Piez (1965) and Heidrich & Wytton (1965).

The major differences in the amino acid chromatograms of the α -chains separated by electrophoresis and these same fractions after chromatography on CM-cellulose was the presence of several small additional peaks in the latter samples. Both their number and amount were variable in different samples of the same component. This may account for the small differences noted in the amino acid compositions of the components before and after CM-cellulose chromatography.

The procedures employed in the present study have a number of advantages over the method of repeated chromatography of gelatins on CM-cellulose (Piez, 1965). First, the gel-filtration step permits the separation of the mixed α -components from the β -components, γ -components and higher-molecular-weight polymers. This allows one to begin the separation of the three α -components from each other with a mixture containing only the α -components. Moreover, the separation of the $\alpha 3$ -chain from the $\alpha 1$ -chain by free-flow electrophoresis is much improved over that obtained by chromatographing the mixed α -components directly on CM-cellulose. In all but a few instances, when a mixture of the three α -chains was chromatographed on CM-cellulose only two peaks were noted, the

first of which was broad and asymmetric and consisted of a mixture of both $\alpha 1$ - and $\alpha 3$ -components. Since all of the procedures are carried out at low temperatures and at near neutral pH (gel filtration) or at slightly acid pH (free-flow electrophoresis), there was no detectable degradation of the components during the procedures used. Further, relatively large amounts of collagen can be processed by these methods, and the pure β -components and a fraction enriched in the γ -components can also be separated during the gel-filtration step.

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