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WAYS OF COLLAGEN SEPARATION IN PATHOLOGICALLY ALTERED TISSUE

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SUMMARY

A system of chromatographic methods using two successive DEAE-cellulose chromatographic steps and two successive separations on Bio-Gel A-1.5 m has been worked out for the separation of individual collagen types. The success of the procedure is based on the preliminary removal of proteoglycans during the first DEAE-cellulose run. Alternatively it is possible to replace chromatographic steps, following the removal of proteoglycans, with fraction precipitation.

INTRODUCTION

Collagen is an example of a polymorphic protein; at least five different collagen types* have been described in human tissues. Different tissues contain different collagen types and frequently various collagen types occur side by side. In healthy subjects the proportions of individual collagen types are subject only to slight variations during ontogeny. On the other hand, gross changes have been observed under pathological circumstances. For example, in inflamed tissues usually the amount of collagen type III increases [1], in osteoarthrotic cartilages, besides the usual type II, types I and III are also present [2]. Altered proportions of individual collagen types also occur in inborn errors of collagen metabolism.

Despite the common skeleton of the collagen molecule individual collagen types differ in details in the amino acid composition, and in mechanical, deposition and transport properties. Currently five different collagen types are recognized. Of these only collagen type I is partially soluble; all the other collagen types can be brought into solution only after a brief proteolytic

*Since the nomenclature of collagen types and polypeptide chains that consitute the tropocollagen triple helix is rather complex, the reader not familiar with this is referred to ref. [3].

cleavage. This fact determines both some properties of the tissue and the methods of analysis. Immunofluorescence techniques that are capable of differentiating individual collagen types have the advantage of high sensitivity and allow the topographic mapping of the tissue, but they do not allow quantitative evaluation. Fraction precipitation is a simple method, which, however, allows the separation of only some collagen types. Therefore, chromatographic methods alone or in combination with other separation procedures are the methods of choice in the evaluation of healthy and diseased connnective tissue. At present there is not a single chromatographic procedure that offers complete separation of all collagen types. The main aim of this communication was to make a rational selection of chromatographic methods in order to obtain information about the content of individual collagen types in a particular tissue sample.

A system of chromatographic methods has been applied to kidney collagen as the demonstrative example because this tissue contains all collagen types, with the exception of collagen type II, in a reasonable amount.

EXPERIMENTAL

Collagen preparation

Since, apart from a small amount of collagen type I, all collagen types in adult tissues are insoluble, for the isolation of individual collagen types pepsin digestion has to be carried out on the tissue homogenate pre-extracted with citrate buffer of pH 3.7 (collagen:pepsin = 20:1, w/w; 24 h; 25°; pH 2.0 adjusted with hydrochloric acid, repeated once with a double amount of pepsin) [4]. To the pepsin digest sodium chloride was added to produce a final concentration of 0.9 *M* to precipitate collagen. The crude precipitate was then purified by extraction with 0.1 *M* NaCl in 0.05 *M* Tris—HCl buffer (pH 7.5). The collagen solution was then dialysed against 1.0% acetic acid to remove NaCl, and lyophilized.

Removal of proteoglycans by DEAE-cellulose chromatography [5]

In order to make the chromatographic system work, we have found this step essential for further collagen fractionation as proteoglycans, due to their interaction with collagen, interfere with the separation of individual collagen types. Lyophilized collagen was dissolved in 0.2 *M* NaCl in 0.05 *M* Tris—HCl (pH 7.5) and applied to a DEAE-cellulose column (Whatman DE-52, 30 × 5 cm; amount loaded 200 mg), which was equilibrated with the same buffer and cooled to preserve the collagen triple helix. Optimum flow-rate was 100 ml/h. The effluent was monitored by UV absorbance at 230 nm. When no further UV-absorbing material was detected in the effluent, the eluting solvent was changed abruptly to 1.0 *M* NaCl in 0.05 *M* Tris—HCl (pH 7.5) and elution was continued with this buffer until an additional peak was eluted from the column. The first fraction containing purified collagen was freed from non-volatile solutes by dialysis against 1.0% acetic acid and lyophilized [3]. The other (proteoglycan) fraction was discarded.

Collagen prepared in this way is ready for isolation and quantitation of individual collagen types.

Separation of collagen type V from types I and III by DEAE-cellulose chromatography [6]

The sample of purified collagen was dissolved in 0.02 *M* NaCl in 0.05 *M* Tris—HCl, pH 7.5 (2 *M* with respect to urea) and applied to the 2.5 × 20 cm DEAE-cellulose column (Whatman DE-52), refrigerated, and equilibrated with the same buffer. When no further UV-absorbing material (220 nm) was detected, the eluting solvent was changed to a linear gradient of 0.02 *M* NaCl in 0.05 *M* Tris—HCl, pH 7.5 (2 *M* with respect to urea) to 0.3 *M* NaCl in 0.05 *M* Tris—HCl, pH 7.5 (2 *M* with respect to urea), using 500 ml of both buffers. The amount loaded was 140 mg and the flow-rate was 90 ml/h.

The first peak in this separation is formed by collagen types I and III; the peak eluted after the gradient has been introduced is mainly collagen type V.

Identification of collagen type III by agarose (Bio-Gel A-1.5 m) chromatography [7]

The identification of collagen type III is based on the presence of the disulphide bonds in this collagen type; these are absent in collagen type I. A Bio-Gel A-1.5 m column (4.5 × 150 cm) was used with 1 *M* $CaCl_2$ in 0.05 *M* Tris—HCl pH 7.5 as eluent. The UV-absorbance profile exhibits three maxima corresponding to 300,000, 200,000 and 100,000 relative molecular weights. Contrary to the previous separation procedures this one is carried out with denatured collagen. Samples were heated to 43° to ensure denaturation before application to the column. Due to the disulphide bonds, collagen type III is present in the fastest peak, where chain polymers of collagen type I are also present. Peaks with higher retention volumes contain depolymerized collagen type I.

The final proof of the presence of collagen type III was achieved by reduction and alkylation of the fast-running fraction. This was done by dissolving 20 mg of the isolate from the first agarose separation in 10 ml of 8.0 *M* urea (pH 8.0), 0.1 *M* with respect to Tris attained by the addition of Tris-free base. The reaction mixture was flushed with nitrogen, and 2-mercaptoethanol (1.42 ml/l) was added. It was left for 4 h at 37° and then the solution was made 0.02 *M* with respect to iodoacetate. The reaction mixture was left to stand an additional 45 min at room temperature in darkness [4]. After desalting by dialysis and subsequent lyophilization, the whole sample was dissolved in the eluting buffer and subjected to a second Bio-Gel A-1.5 m run. In this case a 3.0 × 150 cm column was used, with the same operating conditions as before [7].

Fraction precipitation. This was carried out by adding NaCl to 1.5 *M* concentration. The type III collagen was collected by centrifugation, dissolved in 1.0% acetic acid, the NaCl removed by dialysis and the collagen type III was lyophilized [4].

CM-cellulose (Whatman CM-52) chromatography [8]. This method was used to characterize the precipitate. The column (1.5 × 8.5 cm) maintained at 40° was equilibrated with 0.01 *M* potassium acetate and 1 *M* urea buffer (pH 4.8). The column was eluted with a linear gradient of 0—0.14 *M* LiCl in 500 ml of buffer.

Analysis of the collagenous stroma of pyelonephritic kidney

Human pyelonephritic kidney was the tissue of choice, to demonstrate the versatility of the system of chromatographic separations described above.

RESULTS AND DISCUSSION

As demonstrated in Fig. 1, in the first step proteoglycans were removed by the DEAE-cellulose chromatography. The fraction eluted with 0.2 *M* NaCl + 0.05 *M* Tris—HCl represents proteoglycan-free collagen, which is used for further fractionation. It should be stressed that this step is essential for subsequent isolation of collagen type III by both the appropriate chromatographic procedure and the salting-out method. In both cases, especially however in the salting out method, the presence of proteoglycans causes substantial interference and their contamination of collagen type III is always unacceptable.

The next step is the second DEAE-cellulose chromatography in which a combined peak of collagen types I and III is separated from the bulk of other collagen types (Fig. 2). It is not advisable to attempt the separation of collagen type I and III from other collagen types and proteoglycans in one chromatographic run as no clear separations of individual collagen types can be obtained under such circumstances.

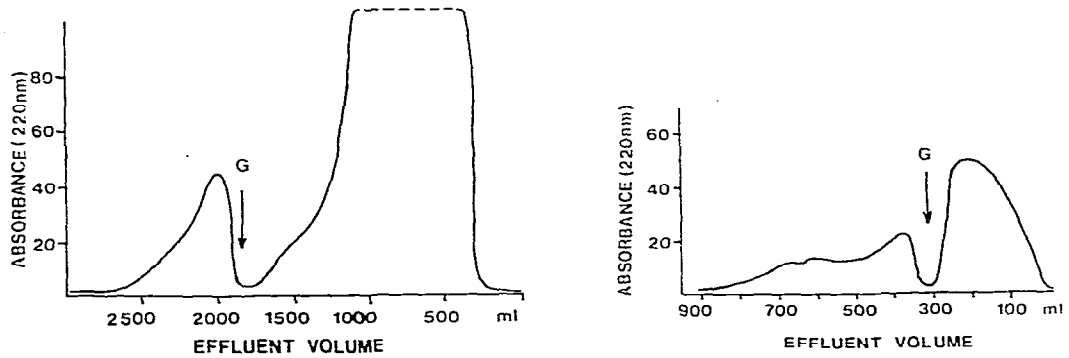

Fig. 1. DEAE-cellulose chromatography of the peptic digest used for the removal of proteoglycans. The arrow (G) indicates the change in the eluting buffer from 0.2 *M* NaCl to 1.0 *M* NaCl (0.05 *M* Tris—HCl, pH 7.5). For further details see Experimental. The peak with low retention volume represents collagen; the other represents proteoglycans.

Fig. 2. DEAE-cellulose chromatography of the purified collagen obtained by the separation shown in Fig. 1. Eluting buffer: 0.02 *M* NaCl (0.05 *M* Tris—HCl, pH 7.5, 2 *M* with respect to urea). G indicates the beginning of the linear gradient 0.02—0.3 *M* NaCl in 0.05 *M* Tris—HCl, 2 *M* with respect to urea (twice 500 ml, 2.5 × 20 cm column).

The separation of collagen types I and III is achieved by molecular sieving. The isolate from the second DEAE-cellulose separation gives on Bio-Gel A-1.5 m a dominant peak which is composed of γ-chains and higher polymers of both type I and type III collagen (Fig. 3). Minor peaks, if present at all, originate essentially from collagen type I. The material from the dominant peak is subjected to reduction and alkylation. Then the second Bio-Gel A chromatography

is carried out. The peaks with smaller molecular weight result from the depolymerized collagen type III (breakage of S—S bonds) and represent the α- and β-fractions of collagen type III. The peak that represents collagen chain polymers is formed by collagen type I and the rest of collagen type III bonded through the non-reducible cross-links (Fig. 3).

Instead of the two BioGel A chromatographic steps it is also possible to separate the collagen types I and III from the isolate of the second DEAE-cellulose run by the salting-out method. By this method collagen type III including the portion bonded with non-reducible cross-links can be isolated in reasonably pure form as shown in Fig. 4.

Collagen type I and type III were identified in all cases by amino-acid analysis and by BrCN-peptides (not shown).

The chemical and also the physical properties of individual collagen types are different and changes in their proportion are reflected in the tissue function. It is useful to obtain information about the proportion of individual collagen

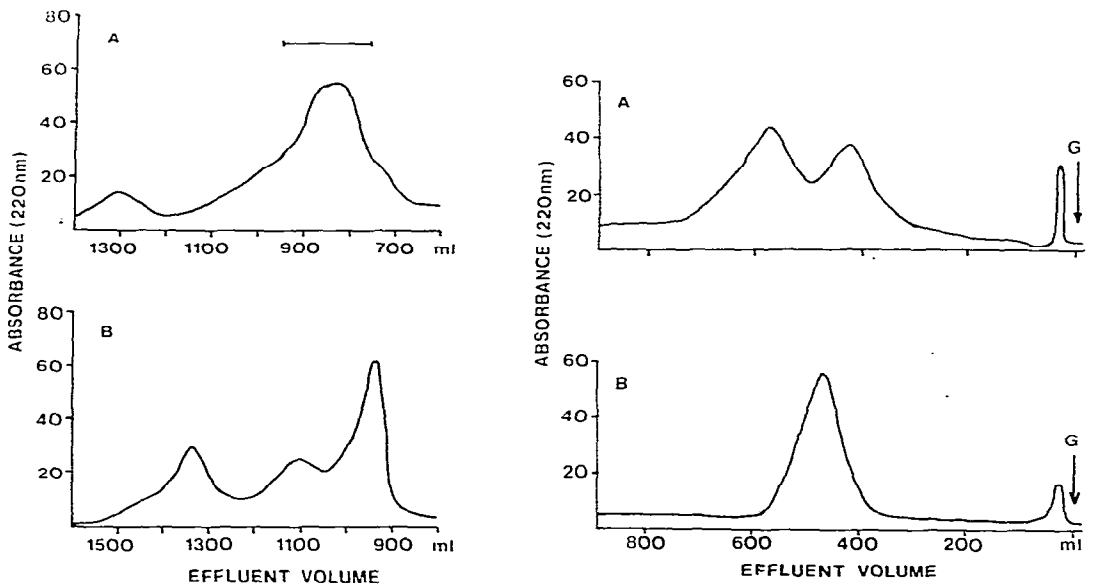

Fig. 3. Bio-Gel A-1.5 m chromatography. (A) A mixture of collagen types I and III (isolate from the second DEAE-cellulose chromatography — Fig. 2) Eluting buffer: 1 *M* $CaCl_2$ in 0.05 *M* Tris—HCl (pH 7.5) and 2 *M* urea. The dominant peak is chain polymers of collagen type I + III; the small peak contains single collagen chains (type I). (B) Rechromatography of the dominant peak from the run in (A) as indicated by the horizontal bar, after reduction and alkylation. For details see Experimental. Elution buffer identical with that of (A). Peaks from right to left: γ-fraction (chain polymers), a mixture of types I and III cross-linked with non-reducible cross-links; β-fraction (chain dimers)—collagen type III; α-chains, single polypeptide chains of collagen type III.

Fig. 4. CM-cellulose chromatography. The column (1.5 × 8.5 cm), maintained at 40°, was equilibrated with 0.02 *M* potassium acetate and 1 *M* urea buffer (pH 4.8). The column was eluted with a linear gradient of 0—0.14 *M* LiCl in 500 ml of buffer. (A) Collagen type I: the supernatant after precipitation with 1.5 *M* NaCl. (B) Collagen type III: the precipitate after precipitation with 1.5 *M* NaCl.

types in diseases affecting the connective tissue in order to understand better the pathological mechanisms involved.

For the system in which separation of individual collagen types is carried out by chromatographic methods only, the amount of collagen needed for a complete separation is of the order of 100 mg. The advantage of this procedure is that very pure collagen fractions are obtained. On the other hand, if fraction precipitation follows the preliminary removal of proteoglycans by DEAE-chromatography the amount of collagen needed can be cut down to about 10 mg. Quantitation can be done by hydroxyproline assay or by optical rotation measurement.

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