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Note

Chromatographic separation and quantification of type I and type III collagens

J. CHANDRARAJAN* and LEROY KLEIN

Departments of Orthopaedics and Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio (U.S.A.)

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Several genetic variants of the connective tissue protein collagen are known [1]. Types I, II, and III collagens are collectively designated as interstitial collagens and type IV collagen is designated as basement membrane collagen. Type I collagen is the predominant type, and is found in tendon and bone, while cartilage collagen is termed type II. Types I and III collagens are found together in several tissues (skin, intestine, liver, lung, aorta, and uterus). The functional significance of collagen polymorphism is not known. However, changes in the relative distribution in tissues of the different collagen types with age [2], and in several diseases [3] have been documented.

Type III collagen molecule contains about 30% more hydroxyproline than type I collagen molecule [3]. Therefore, total collagen determination for tissues containing both types I and III collagens cannot be made accurately using hydroxyproline values unless the relative proportion of the two collagens in the specimen is known [3]. Separation procedures [2, 4] involving differential salt precipitation of pepsin-solubilized collagens, followed by molecular sieve and/or ion-exchange chromatography, result in very pure fractions of collagen types suitable for structural studies. However, because of the low recoveries of collagen, which in certain tissues may be as low as 20% [2], the above methods are not suitable for quantitative investigations involving analysis of multiple tissue samples of limited availability. Recently we reported a simple procedure for the separation of types I and III collagens by differential renaturation [5]. About 85% of the pepsin-solubilized hydroxyproline was recovered as collagens by this procedure. The present paper describes the chromatographic separation and quantification of types I and III collagens with 96—100% recoveries.

*Address for correspondence: Dr. J. ChandraRajan, 511 Wearn Building, University Hospitals, Cleveland, Ohio 44106, U.S.A.

EXPERIMENTAL

Pepsin solubilization of collagens

Skin (1 g) from a 1-week-old rat was finely minced with scissors, extracted overnight with chloroform—methanol (2:1, v/v) and then with methanol for 6 h at 4°. The defatted tissue was suspended (10 mg/ml) in 0.5 *M* acetic acid. Crystalline pepsin (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was added at a concentration of 1 mg/ml [4] and the mixture was stirred for 24 h at 8°. The pepsin digest was centrifuged at 30,000 *g* for 1 h at 4°. The skin residue was redigested overnight with additional pepsin and centrifuged as before. The supernatants were pooled and dialyzed overnight against 4 l of 0.02 *M* Na_2HPO_4 and the resultant collagen precipitate was separated by centrifugation at 30,000 *g* for 1 h at 4°.

Denaturation of pepsin-solubilized collagens. The collagen precipitate was suspended (2—10 mg/ml) in 2 *M* guanidine·HCl containing 0.05 *M* Tris·HCl (pH 7.5), and was denatured by heating to 45° for 30 min. The resultant solution was centrifuged at 30,000 *g* for 40 min at 22° and the supernatant containing the denatured collagens was obtained after filtration through glass wool.

Chromatographic separation of types I and III collagens. Two glass columns (1.6 cm diameter) were connected via a flow adapter and packed with Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A.) to provide a total bed length of 210 cm. A 5-ml aliquot of the denatured collagen solution was applied to the molecular sieve column which was equilibrated and eluted with 2 *M* guanidine·HCL containing 0.05 *M* Tris·HCL (pH 7.5), at 22°, with a flow-rate of 20 ml/h. The absorbance of the eluent at 230 nm was continuously monitored with a Fracto-Scan ultraviolet monitor (Buchler Instrument, Nuclear Chicago, Fort Lee, N.J., U.S.A.) and fractions of 3 ml were collected automatically. The ultraviolet-absorbing fractions for each peak were pooled, dialyzed against deionized water until free of salt, and then lyophilized.

Determination of relative purity of collagen fractions

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of the collagen samples was carried out in 5% cylindrical gels (85 × 7 mm), containing 0.3% N,N′-methylene-bisacrylamide [5]. Gel and electrode buffers were 0.1 *M* Tris—borate (pH 8.5) containing 0.1% sodium dodecyl sulfate (SDS). The gels were pre-electrophoresed at 2 mA/gel, 50 V, for 60 min. Collagen samples (25—100 μg) were incubated at 60° for 5 min with gel buffer containing 1% SDS and 1 *M* urea. Aliquots of the samples were also reduced with dithiothreitol (DTT) and applied to the gels. After electrophoresis at 2 mA/gel, 50 V, for 190 min, the gels were stained with Coomassie Blue R-250 [6]. All gels after removal of excess stain were scanned at 550 nm in a Gilford recording spectrophotometer equipped with a linear gel transport.

Proline:hydroxyproline ratios. Aliquots of the collagen samples were hydrolyzed in constant-boiling HCl and assayed for proline [7] and hydroxyproline [8]. The ratio of proline to hydroxyproline was used as an additional index of the identity and relative purity of the separated types I and III collagens [5, 9].

Quantification of types I and III collagens

From absorbance at 230 nm. The relative quantities of pepsin-solubilized types I and III collagens were determined by tracing the Sepharose CL-6B column elution patterns on paper, cutting out the traced outlines, and weighing the appropriate individual pieces of paper [9].

From hydroxyproline. The relative amounts of type I and type III collagens were also calculated assuming types I and III contain 13.7 and 17.9% of hydroxyproline, respectively [5, 9].

RESULTS AND DISCUSSION

Solubilization of collagen

The amount of collagen solubilized by digestion with pepsin varies with the type of tissue, age, and pathological state of the donor. We have been able to solubilize 92–95% of the total collagen expressed as hydroxyproline from young rat dermis (Fig. 1) and fetal human skin [5] after two digestions with pepsin.

Removal of pepsin

A small amount of pepsin always coprecipitated with collagen when pepsin digests of tissues were dialyzed against 0.02 *M* Na_2HPO_4 [5] (Fig. 2a). Heat denaturation in 2 *M* guanidine·HCl removed the contaminant pepsin as precipitate. Other denaturing agents such as $CaCl_2$ or urea which are used for solubilizing collagen were found to be unsuitable because they also solubilized denatured pepsin (ChandraRajan, unpublished results).

Chromatographic separation of collagen types I and III

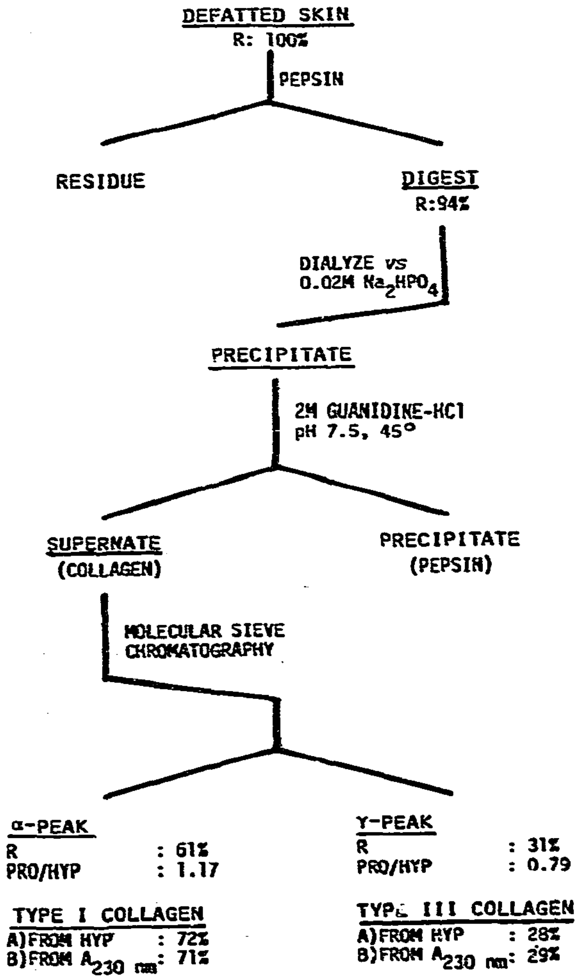
Chromatography of the denatured collagens on a calibrated Sepharose CL-6B column (Fig. 3) separated type III collagen (γ component, mol. wt. 285,000) from type I collagen (α chains, mol. wt. 95,000). The use of a longer column (210 cm) of the cross-linked agarose was necessary to obtain separations comparable to those obtained on a shorter (115 cm) column of agarose [9]. However, because of the greater stability of the cross-linked agarose in 2 *M* guanidine·HCl, and the increased flow-rate, the separation time remained essentially the same.

Characterization of the separated collagen types I and III

The identity and relative purity of each of the two chromatographic fractions were confirmed from their respective proline:hydroxyproline ratios (Fig. 1), by SDS–polyacrylamide gel electrophoresis (Fig. 2), and by cyanogen bromide peptide analysis (not shown) according to the method of Benya et al. [10].

Quantification of types I and III collagens

Estimation of the relative proportions of type I and type III collagens in 1-week-old rat dermis from absorbance at 230 nm or hydroxyproline content of the separated fractions gave essentially the same results (71% type I and 29% type III, Fig. 1). Because types I and III collagens contain different amounts of hydroxyproline [1], appropriate factors [5, 9] must be used for calculating the



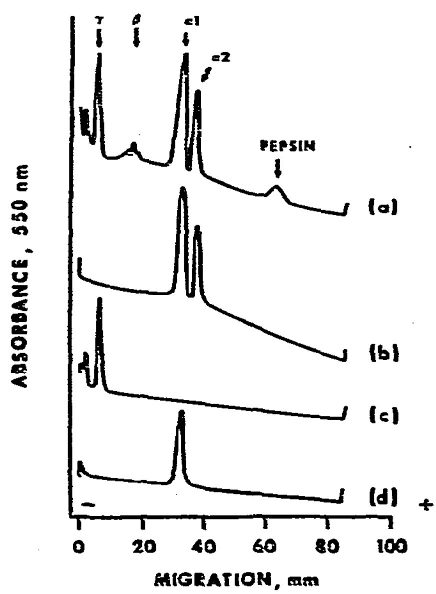


Fig. 1. Outline of the procedure for the separation and determination of relative proportions of types I and III collagens in rat dermis. R indicates recovery of collagen from defatted skin expressed as hydroxyproline. Molar ratio of proline to hydroxyproline was calculated as an index of the identity and relative purity of the collagen types. Relative amounts of types I and III collagens were determined from: (A) hydroxyproline, assuming types I and III con- [illegible] γ (type III) peaks on Sepharose CL-6B column (Fig. 3).

Fig. 2. SDS—polyacrylamide gel electrophoresis of pepsin-solubilized collagens. (a) Collagens precipitated by dialysis against 0.02 *M* Na_2HPO_4; (b) type I collagen (α peak); (c) type III collagen (γ peak) obtained after molecular sieve chromatography (Fig. 3); (d) same as (c) but reduced with dithiothreitol. The gels were stained for protein with Coomassie Blue and scanned at 550 nm.

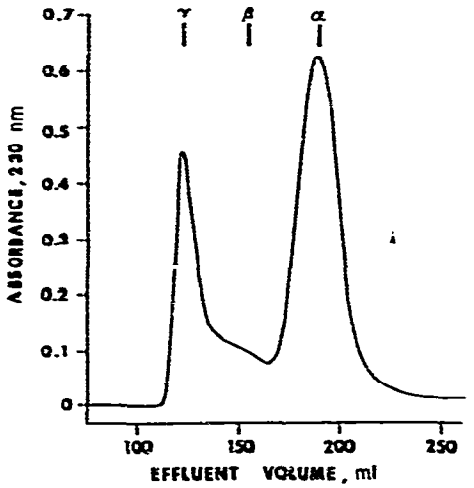


Fig. 3. Separation of type I (α) and type III (γ) collagens by molecular sieve chromatography on a Sepharose CL-6B column (210 × 1.6 cm). The sample was eluted with 2 *M* guanidine-HCl—0.05 *M* Tris-HCl (pH 7.5) at room temperature. The absorbance at 230 nm of the eluent was monitored continuously and 3-ml fractions were collected.

amounts of collagen from hydroxyproline values. Otherwise, type III collagen which contains more hydroxyproline would be overestimated. A recently reported method [11] for the quantitative assay of types I and III collagens from proline plus hydroxyproline total radioactivities would be subject to similar error because the amount of proline plus hydroxyproline for type I is 662 residues per molecule while it is 711 residues for type III collagen [1].

For the ultraviolet absorbance to be a reliable index of the amount of collagen, it was found necessary to defat the tissues prior to digestion with pepsin. Because ultraviolet-absorbing lipids elute from molecular sieve columns approximately in the region of the γ component (type III collagen), failure to remove lipids from samples would also result in overestimation of type III collagen. The success of separation and estimation of types I and III collagens using the method described by Adam et al. [12] depended on the preliminary removal of ultraviolet-absorbing proteoglycans during several steps of fractional precipitation and/or DEAE-cellulose column chromatography. No interference from proteoglycans was observed while using the present procedure for the estimation of collagen types I and III.

Recoveries of collagen

Ninety-two per cent of the defatted skin collagen (96—100% of the pepsin-solubilized collagen) expressed as hydroxyproline was recovered after molecular sieve chromatography (Fig. 1). Multiple precipitation steps to remove pepsin from pepsin-solubilized collagens usually resulted in low (20%) recoveries of collagen [2], while a single denaturation step has been shown by us [5] to remove the contaminant pepsin without loss in collagen. Therefore, the major advantage of the present procedure is the high recoveries without a reduction in relative purity of the types I and III collagen fractions.

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