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SEPARATION OF TYPE III COLLAGEN FROM TYPE I COLLAGEN AND PEPSIN BY DIFFERENTIAL DENATURATION AND RENATURATION

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SUMMARY: Types I and III collagens were solubilized from fetal human skin by limited digestion with pepsin and precipitated by dialysis against 0.02 M Na_2HPO_4. Heat denaturation of the collagens in 2 M guanidine-HCl, pH 7.5, resulted in the precipitation of the contaminant pepsin which could be removed by centrifugation. Renaturation of the denatured collagens by dialysis against deionized water at 22° for 2 hours selectively precipitated the type III collagen fibrils. Type I collagen remained in solution. The simplicity and high recovery (77%) make this a suitable approach for the rapid estimation of type III collagen in small tissue samples.

INTRODUCTION: It is now well established that there are at least four genetically determined collagen types, each differing in the primary structure of their α chains (1). Type I collagen is the predominant type, consisting of two α1(I) and one α2 chains, and is found in skin, tendon and bone. Type II collagen $[\alpha 1(II)]_3$ is found mainly in cartilages, while basement membrane collagen $[\alpha 1(IV)]_3$ is type IV. Type III collagen $[\alpha 1(III)]_3$ is found in most connective tissues, always in association with type I collagen. It is a major component in blood vessels and fetal skin. The relative proportions of types I and III collagens within a tissue vary with age (2) and in diseases such as atherosclerosis (3), pulmonary fibrosis (4), hepatic fibrosis (5), Ehlers-Danlos Syndrome Type IV (6), osteogenesis imperfecta (7) and rheumatoid arthritis (8).

Types I and III collagens can be solubilized in good yield from skin and other tissues by limited proteolysis with pepsin (2, 9). From the resulting digest, pepsin and the collagen types I and III are separated from each other during several steps of differential salt precipitation at neutral pH (2, 9). However, this procedure has been found to be irreproducible (10). The multiple steps of isolation reduces

Abbreviations: SDS, sodium dodecyl sulfate; DTT, dithiothreitol; α, collagen peptide chain of molecular weight 95,000; β, $[\alpha]_2$; γ, $[\alpha]_3$.

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the final yield (2) and the separated collagen types I and III have a relatively high degree of cross-contamination (5). Complete separation of type III collagen from type I requires chromatography of the denatured collagens (9, 11, 12). The present paper describes a simple alternative procedure for the separation of type III collagen from type I collagen and pepsin, taking advantage of the differences in the denaturation and renaturation characteristics of these three proteins. The small number of steps in the present approach results in improved recoveries of the pepsin-solubilized types I and III collagens from human skin without any significant cross-contamination.

MATERIALS AND METHODS:

Pepsin solubilization of collagen: Human skin (1g) obtained from a 28 week-old fetus after autopsy was stripped of fat, finely minced, extracted overnight with chloroform-methanol (2:1 v/v) and then with methanol for 6 hours at 4°. The defatted tissue was suspended in 100 ml of 0.5 M acetic acid. Pepsin (Worthington Biochemical Corp., N. J.) was added at a concentration of 1 mg/ml (9) and the mixture was stirred for 24 hours at 8°. The pepsin digest was centrifuged at 30,000 x *g* for 1 hour at 4°. The skin residue was redigested overnight with additional pepsin and centrifuged as before. The supernatants were pooled and dialyzed 18 hours against 4 liters of 0.02 M Na_2HPO_4 and the resultant collagen precipitate was separated by centrifugation at 30,000 x *g* for 1 hour at 4°.

Denaturation of pepsin-soluble collagen: 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 minutes. The resultant solution was centrifuged at 30,000 x *g* for 40 minutes at 22° and the supernatant containing the denatured collagen was obtained after filtration through glass wool.

Renaturation of denatured collagen: An aliquot of the denatured collagen solution was diluted to a final concentration of about 250 µg/ml with deionized water and dialyzed twice against 4 liters each of deionized water for a total of 2 hours at 22°. The collagen fibrils precipitated during the 2 hour dialysis were separated by centrifugation at 30,000 x *g* for 40 minutes at 4°. The supernatant was dialyzed twice against 4 liters each of deionized water for a total of 16 hours at 4° and lyophilized.

Molecular sieve chromatography: Aliquots of the collagen fractions dissolved in 2 M guanidine-HCl containing 0.05 M Tris-HCl, pH 7.5, were warmed to 45° for 30 minutes and chromatographed (13) on a calibrated 1.6 x 115 cm column of Bio-Gel A-5m (100-200 mesh; Bio-Rad Laboratories, Richmond, Calif.). The column was equilibrated and eluted with 2 M guanidine-HCl, 0.05 M Tris-HCl, pH 7.5, at 22° with a flow rate of 10 ml/hr. Fractions of 3 ml were collected and the absorbance at 230 nm was monitored in a Gilford (Model 2400) spectrophotometer.

Polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis of the collagen samples were carried out in 5% gels containing 0.3% N,N'-methylene-bisacrylamide. Gel and electrode buffers were 0.1 M Tris-borate, pH 8.5 (14), containing 0.1% SDS. Samples (25-100 µg) were heated for 5 minutes at 60° with gel buffer containing 1% SDS and 1 M urea. Aliquots of the samples were also reduced with DTT (Calbiochem, LaJolla, Calif.). After electrophoresis (2 mA/gel, 50 V) for 190 minutes, the gels were stained with Coomassie Blue R250 (15).

Determination of hydroxyproline and proline: Aliquots of the starting material and the various fractions were hydrolyzed by autoclaving in constant-boiling HCl for 3 hours and assayed for hydroxyproline (16) and proline (17). Hydroxyproline values were used to calculate the amount of collagen in various fractions while the ratio of proline to hydroxyproline served as an additional index of the identity and relative purity of the separated types I and III collagens (13).

RESULTS:

Pepsin solubilization of collagen: More than 90% of the hydroxyproline in fetal human skin was solubilized by pepsin digestion and recovered in the collagen precipitated with 0.02 M Na_2HPO_4 (Fig. 1). SDS - polyacrylamide gel electrophoresis (Fig. 2, gels A & B) of the precipitated collagen indicated that it was composed of $\alpha 1$ and $\alpha 2$ chains of type I collagen and disulfide crosslinked γ chains of type III collagen. It was also observed that there was always a small, but significant amount of pepsin contaminant (gels A & B). The reason for the slower migration of pepsin after reduction (gel B) is not known at this time.

Differential denaturation of pepsin and collagen types I and III: Heat denaturation in 2 M guanidine-HCl containing 0.05 M Tris-HCl, pH 7.5, solubilized the precipitated collagen while the contaminant pepsin formed an insoluble precipitate. Thus, the denatured collagen could be separated from the denatured pepsin by centrifugation. The identity of the insoluble precipitate was confirmed as pepsin by SDS - polyacrylamide gel electrophoresis (not shown). Chromatography of the denatured collagens on a calibrated agarose column revealed (Fig. 3A) the presence of α and γ components. The relative proportions of the two U.V.-absorbing peaks were estimated as described earlier (13) as α(type I) = 58% and γ(type III) = 42%.

Differential renaturation of type III collagen: Denatured type III collagen molecules $[\alpha 1(III)]_3$ with intact disulfide crosslinks renature at much faster rate than $\alpha 1(I)$ or $\alpha 2(I)$ chains (18). It was also observed (13) that dialysis against water of agarose column-purified types I and III collagens produced a fibrous precipitate of the type III collagen fraction within 2 hours while the type I collagen remained essentially clear even after 16 hours. However, when denatured collagen solutions (2 - 10 mg/ml) containing both types were dialyzed against water for 2 hours at 4°, the precipitated type III collagen was contaminated with some

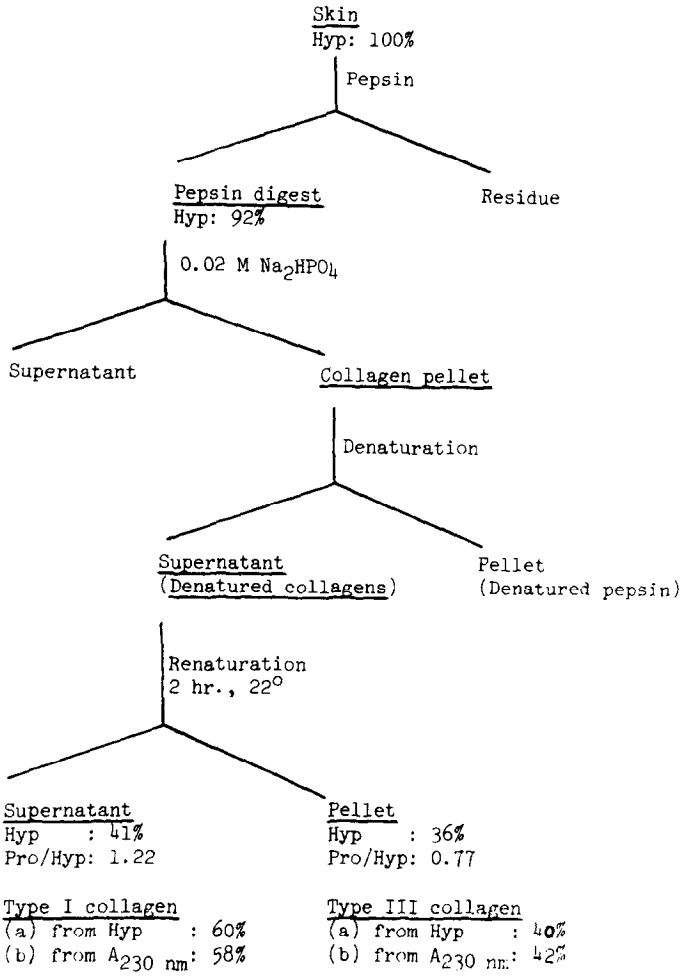

Figure 1: Estimation of recoveries and relative proportion of types I and III collagens in pepsin-solubilized human dermis. Hyp, hydroxyproline analysis was used to determine the recoveries of pepsin-solubilized collagens. Molar ratio of proline to hydroxyproline was calculated for each type of collagen as an index of its identity and relative purity. Relative amounts of types I and III collagens were determined (a), assuming types I and III containing 13.7 and 17.9% hydroxyproline respectively (13); and (b), from $A_{230\ nm}$ of α(type I) and γ(type III) fractions of Bio-Gel A-5m column (Fig. 3A).

type I. Therefore, to obtain a better separation of type III collagen from type I, different conditions of dialysis were investigated. It was found that diluting the denatured solution to contain approximately 250 µg collagen/ml and then dialyzing the dilute solution against deionized water at 22° for 2 hours resulted in the precipitation of type III collagen with less than 3% contamination with type I collagen (Fig. 2, gels C, D; Fig. 3B). The supernatant fluid was composed

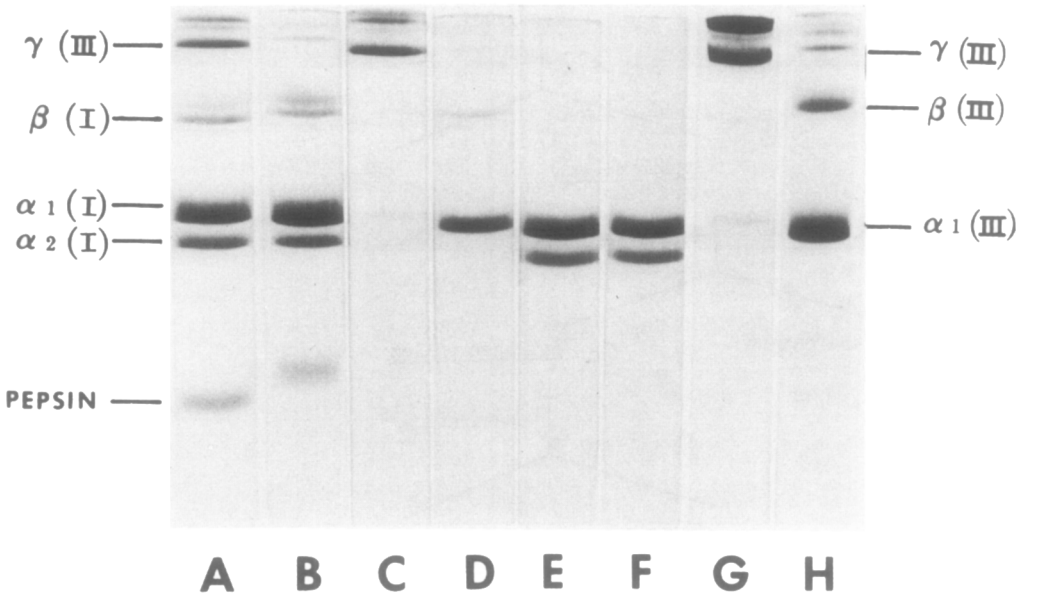

Figure 2 SDS-polyacrylamide gel electrophoresis of pepsin-solubilized collagens. A, collagens precipitated by dialysis against 0.02 M Na_2HPO_4; B, same as A, but reduced with DTT; C, type III collagen (2 hour renaturation precipitate); D, same as C, but reduced with DTT; E, type I collagen (2 hour renaturation supernatant): F, same as E, but reduced with DTT; G, reference type III collagen purified by agarose column chromatography (13); H, same as G, but reduced with DTT.

of α1(I) and α2(I) chains in the ratio 2:1 which was not altered by reduction with DTT (Fig. 2, gels E &F) indicating the absence of type III collagen in this fraction. The relative proportions of the collagens were estimated by hydroxyproline analysis as approximately 60% type I and 40% type III which is in agreement with the estimation from agarose chromatography (see above) and with the previous estimate (2) based on chromatographic analysis of cyanogen bromide peptides from fetal human skin. In the present study the final recovery of hydroxyproline in the separated types was about 85% of that solubilized by pepsin (Fig. 1).

Characterization of renatured type III collagen: Type III collagen isolated by differential renaturation had disulfide crosslinks in the pepsin-resistant region of the molecule. Reductions of this collagen with DTT produced α chains of molecular weight approximately 95,000 (Fig. 2, gel D). It appeared to be essentially free from type I collagen as indicated by SDS-polyacrylamide gel electrophoresis (gels C &D), by column chromatography on agarose (Fig. 3B) and

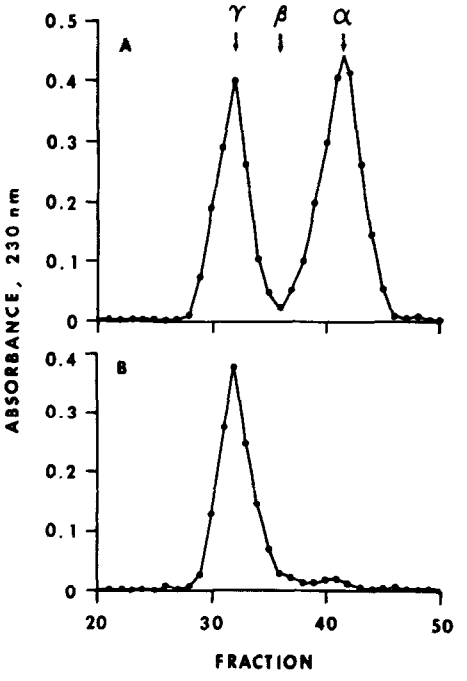

Figure 3. Bio-Gel A-5m chromatography: A, denatured types I (α) and III (γ) collagens; B, type III (γ) collagen precipitated on 2 hour renaturation. The column was equilibrated and eluted with 2 M guanidine-HCl containing 0.05 M Tris-HCl, pH 7.5. Three ml fractions each were collected and the absorbance at 230 nm was monitored.

CM-cellulose, according to Chung and Miller (9). Furthermore, the type III collagen isolated by differential renaturation had a proline to hydroxyproline ratio of 0.77 compared to 1.22 for type I collagen (Fig. 1).

DISCUSSION:

The results presented in this paper show that type III collagen can be separated from type I collagen and pepsin by making use of the differences in the denaturation and renaturation characteristics of these three proteins. Dialysis of pure pepsin against 0.02 M Na_2HPO_4 does not result in the precipitation of the enzyme (2). However, when pepsin digests containing collagen are dialyzed against 0.02 M Na_2HPO_4, a small but significant amount of pepsin always coprecipitated with the collagen fibrils. It is not clear at this time whether this pepsin is mechanically trapped within the fibrils or bound to collagen via ionic interaction. Other investigators (2, 9) have used several reprecipitation steps to remove the

remaining pepsin which reduced the recovery of collagens in certain cases (2) to about 20%. In the present study a single heat denaturation step effectively removes the contaminant pepsin, permitting essentially quantitative recovery of the collagens. The greater solubility of denatured collagens in 2 M guanidine-HCl, pH 7.5, presumably produces a 'salting-out' effect on the denatured pepsin.

The differential renaturation procedure described in this paper is for a tissue (skin) that contains only the two most common types of collagens (I, III). Preliminary results indicate that this approach could be used for the separation of type III collagen from human aorta and rat small intestine. However, the applicability of this principle for the isolation of type III collagen from tissues containing other types of collagens (II, IV) needs verification. The simplicity and high recovery make this procedure suitable for the rapid estimation of type III collagen in multiple tissue samples of limited availability.

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