Collagen polymorphism in mature rabbit cornea

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Sequential extraction of mature rabbit corneal stroma with NaCl-Tris buffer and acetic acid solubilized only 12% of the total corneal collagen. Pepsin (E:S 1:10; 4° C, 48 hr) in 0.4M acetic acid solubilized 91% to 95% of the total collagen in the residue. Approximately 68% of the solubilized material could be precipitated at 2.5M NaCl and a further 3% to 9% at 3.5M NaCl. The collagenous material precipitating at 2.5M NaCl contained α, β, γ, and some higher molecular weight components and had a CNBr profile similar to bovine type I skin collagen. It had an hydroxylysine/lysine (OHLys/Lys) ratio of 0.43, similar to that of skin collagen, but unlike skin collagen was 52% glycosylated. Although the 3.5M NaCl precipitate had a CNBr peptide profile similar to that of type I collagen, it contained two additional collagen chains of molecular weight approximately 140,000 and 100,000 daltons, had an OHLys/Lys ratio of 0.62, and was 66% glycosylated. Individual chains were separated from the collagen precipitates by gel electrophoresis, and the additional collagen chains were shown to be carbohydrate rich. These additional collagen chains may be derived from one or more molecular species which are physiologically important in the maintenance of the unique organization of corneal collagen.

Key words: rabbit cornea, collagen polymorphism, gel electrophoresis, CNBr peptides, collagen glycosylation

Collagen is the main structural component of the cornea.[1] At present, four different types of collagen molecules are known to exist in vertebrate tissues. These types are determined genetically and differ in the amino acid sequence of their constituent α chains.[2]

Limited pepsin digestion has been used to solubilize tissue collagens and thus render them amenable to further study.[3, 4] Differential precipitation of collagen from these digests has provided significant information on the polymorphism of tissue collagens.[5] Although it has been known for some time that pepsin will solubilize a large proportion of the collagen fibrils of the rabbit cornea,[6] the polymorphic nature of the solubilized material has not been investigated.

The present communication describes our studies on differential salt fractionation of pepsin-solubilized rabbit cornea.

Materials and methods

Preparation of rabbit stroma. Frozen mature rabbit corneas (Pel-Freez Bio-Animals, Inc., Rogers, Ark.) were thawed at 4° and scraped carefully with a scalpel on both surfaces to remove epithelium and endothelium. After Descemet's membrane had been stripped from the stromal layer, the corneas were chopped fine with scissors and disintegrated in a stainless steel hammer mill.[7] The homogenized tissue was extracted for two 24 hr periods at 4° C with IM NaCl–0.05M Tris, pH 7.5. The residue was dialyzed exhaustively against water to remove NaCl, suspended in

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Table I. Sequential extraction of mature rabbit cornea

Fraction	*Hydroxyproline dissolved (% of total)*
1.0M NaCl–0 05M Tris pH 7.5 buffer	2.5
0.5M acetic acid	9.5
Insoluble residue	88.0

0.5M acetic acid, and further extracted in this solvent for two 24 hr periods. The insoluble fraction was lyophilized.

Preparation of pepsin-solubilized stromal collagens. The insoluble stromal material was resuspended in 0.4M acetic acid and incubated with pepsin (Sigma Chemical Co., St. Louis, Mo.) at an enzyme:substrate ratio of 1:10 for two successive 24 hr periods at 4° C.[8] Repepsinization (see Results) was carried out under the same conditions. The pH of the combined supernatants was raised to 8.0 by addition of 2M Tris and collagen precipitated by dialysis against 5 to 10 vol of 0.05M Tris containing progressively higher concentrations of NaCl: 2.0M, 2.5M, 3.5M, and 4.5M.[8]

Chemical analysis. Total carbohydrate content of collagen samples was obtained by the anthrone method with the use of a galactose standard.[9] Further samples were hydrolyzed in 6M HCl or 4M methanesulfonic acid containing 0 to 2% 3-(2-aminoethyl)indole (Pierce Chemical Co., Rockford, Ill.) under nitrogen in sealed glass test tubes at 107° C for 24 hr.[10] Hydroxyproline was estimated in samples of the hydrolysate,[11] and amino acid analysis was obtained by means of a Beckman 121M autoanalyzer with system AA computing integrator (Beckman Instruments, Inc., Palo Alto, Calif.)[12] Hydroxylysine glycosides were estimated from alkaline hydrolysates of collagen precipitates on the long column of the AutoAnalyzer.[13]

Gel electrophoresis. Samples of pepsin-solubilized collagens were suspended in Biophore sodium dodecyl sulfate (SDS) buffer (Bio-Rad Laboratories, Richmond, Calif.) containing 0.04M dithiothreitol (DTT), 0.001M disodium EDTA, 6% sucrose, and tracking dye. The samples were denatured for 3 min at 100° C and resolved on 4% or 7.5% polyacrylamide gels, at concentrations of approximately 20 μg/gel (for protein determination) or 100 μg/gel (for glycoprotein determination).[14] Other samples were run in the absence of DTT.

Protein or peptide bands were visualized by Coomassie blue staining and glycoproteins by the periodic acid–Schiff (PAS) reaction.[14] Gels were scanned at 600 mm (protein) and 560 mm (glycoprotein) in a Gilford spectrophotometer with scanning attachment (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

CNBr peptides. For cleavage with cyanogen bromide (CNBr),[15] samples of pepsin-solubilized collagens (250 to 500 μg) were suspended in 70% formic acid (0.5 ml.). The suspensions were flushed with nitrogen, and a weight of CNBr equal to three times the weight of the collagen sample was added. Test tubes containing the suspensions were stoppered and incubated at 30° C for 4 hr. The incubation mixture was diluted with 10 vol of water, and particulate material was removed by centrifugation at 5,000 × g for 10 min at 5° C. Peptides free of CNBr and solvent were obtained by lyophilization. Samples of these peptides (approximately 100 μg) were dissolved in sample buffer (50 μl), denatured at 100° C for 3 min, and resolved on 7.5% polyacrylamide gels.[16]

Collagen standard. Purified bovine type I collagen, prepared by limited pepsin digestion of embryonic calf-skin and fractional precipitation according to standard procedures,[8, 17, 18] was used as a standard throughout this work.

Results

Based on a hydroxyproline content of 12.9% (w/w),[19] collagen was found to account for over 80% of the dry weight of mature rabbit corneal stroma. Table I shows the relative distribution of hydroxyproline in fractions extractable from mature rabbit cornea by sequential treatment of homogenates with NaCl-Tris buffer and acetic acid. The greater bulk of the corneal collagen is insoluble in these reagents. Digestion of this insoluble residue with pepsin under conditions that maintain the helical conformation of the bulk of the collagen solubilized 90% to 95% of the hydroxyproline of the insoluble corneal material. Of the hydroxyproline, 68% was found in material which could be precipitated from solution by bringing the salt concentration to 2.5M sodium chloride. Thus approximately one third of the total hydroxyproline-containing material in the initial pepsin digest failed to precipitate under conditions known to bring type I collagen from solution.[20] A further 3% to 9% of the total hy-

droxyproline in the digest was precipitated out of solution by the addition of sodium chloride to 3.5M. About 2% of the original hydroxyproline in the digest remained in solution, even when the salt concentration was taken to 4.5M sodium chloride. Higher salt concentrations were not investigated, and no attempt was made to recover the remaining hydroxyproline-containing material from the large volumes of dialysis fluid during the salt precipitations.

Amino acid analysis of the materials precipitating at 2.5M sodium chloride and 3.5M sodium chloride revealed hydroxylysine/lysine ratios of 0.43 and 0.62, respectively. All other differences were relatively minor by comparison (Table II). Hydroxylysine glycoside analysis indicated that 52% of the hydroxylysine of the material precipitating at 2.5M sodium chloride and 66% of the hydroxylysine of the material precipitating at 3.5M sodium chloride was glycosylated. The relative levels of glucosylgalactosylhydroxylysine (GGH) and galactosylhydroxylysine (GH) are shown in Table III.

Total carbohydrate of the 2.5M sodium chloride precipitate was measured at 1.39% (w/w), whereas total carbohydrate of the 3.5M sodium chloride precipitate was 3.2% (w/w). Methanolysis of samples followed by gas chromatography of the trimethylsilyl sugars showed only glucose and galactose in significant amounts (traces of mannose and fucose were also seen). All the galactose and glucose could be accounted for in hydroxylysine-linked glycosides.

SDS polyacrylamide gel electrophoresis (SDS-Page) of the denatured chains of the 2.5M sodium chloride precipitate on 4% gels showed the presence of α, β, γ, and some higher molecular weight components. The 3.5M sodium chloride precipitate appeared to contain an additional collagen chain of molecular weight approximately 140,000 daltons. However, on 7.5% gels, it was found that the $\alpha 1$ chain could be separated into two components. Thus the 3.5M material contained two additional "pre-α" components, one of apparent molecular weight 140,000 daltons and one of apparent molecular weight 100,000 daltons. In order to verify that the purified collagens were in a native triple-helical conformation, an aliquot of the 3.5M sodium chloride precipitate was treated a second time with pepsin for 24 hr at 4°. After lyophilization, this sample was electrophoresed concurrently with the starting material. The absence of changes in the amount of collagen or the proportion of individual components demonstrated helical conformation.[21] Gels run in the absence of DTT were identical with those run in its presence. Since the mobilities of these additional chains were unchanged after repepsinization and re-

Table II. Amino acid composition of salt precipitates of pepsin-solubilized rabbit corneal collagens (in residues per 1,000 amino acid residues)

Amino acid residue	*2.5M NaCl precipitate*	*3.5M NaCl precipitate*
3-Hydroxyproline	—	—
4-Hydroxyproline	92.3	97.1
Aspartic acid	44.0	44.3
Threonine	20.0	20.2
Serine	34.7	33.3
Glutamic acid	69.7	73.7
Proline	116.1	125.0
Glycine	347.0	335.0
Alanine	108.3	102.0
Half cystine	—	—
Valine	20.6	20.7
Methionine	8.1	7.6
Isoleucine	11.2	12.1
Leucine	27.2	29.6
Tyrosine	3.6	1.7
Phenylalanine	13.2	11.7
Histidine	4.2	4.4
Hydroxylysine	9.5	13.2
Lysine	22.3	21.4
Tryptophan	—	—
Arginine	47.0	46.0

Table III. Hydroxylysine glycosides of salt precipitates of rabbit corneal collagens

	2.5M NaCl precipitate	*3.5M NaCl precipitate*
Total OHLys	9.5*	13.2
Glycosylation (%)	51.6	66.0
GGH	2.3	6.1
GH	2.6	2.6
GGH/GH	0.88	2.35

*Total residues per 1,000 amino acid residues.

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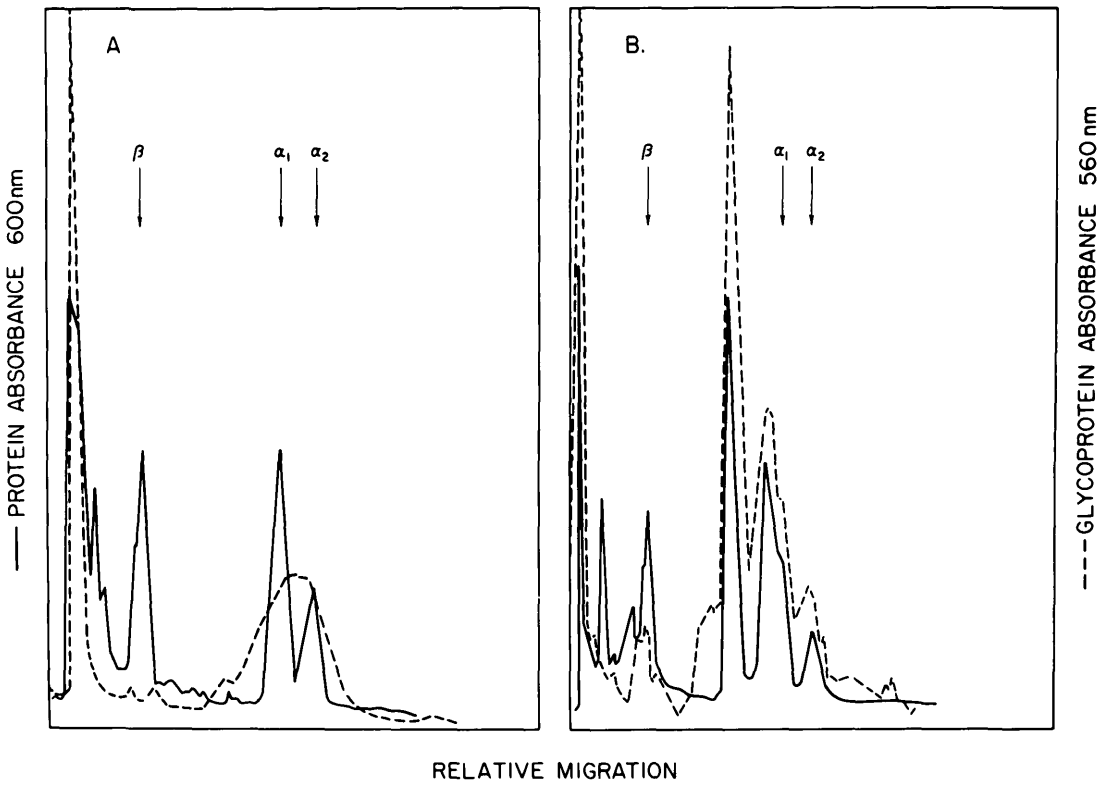

Fig. 1. Scans of component chains of salt-precipitates of pepsin-solubilized rabbit collagens. **A**, 2.5M NaCl precipitate. **B**, 3.5M NaCl precipitate. For experimental procedures see text.

duction they are neither procollagens nor β-components.[21] Their collagenous nature, however, is indicated by the fact that they are destroyed, along with the regular α chains, by protease-free bacterial collagenase.[22] Although the slower moving 140,000 dalton component has a similar molecular weight to the α1(IV) chain, its insensitivity to sulfhydryl reducing agents and the absence of 3-hydroxyproline in the analyses (Table II) indicate that α1(IV) is not present.[23] Preliminary attempts to separate the chains of the 3.5M sodium chloride precipitate indicated that the additional chains had similar mobilities on carboxymethylcellulose to the recently described collagen chains X and Y synthesized by the progeny of rabbit articular chondrocytes[24] and A and B chains of fetal membranes.[25]

When the SDS-Page gels were stained for carbohydrate with the PAS stain, the 140,000 dalton component stained strongly and the pre-α component weakly, and the α1 and α2 were detectable to a limited extent (Fig. 1). These results, together with the increased amount of hydroxylysine glycosides found in the 3.5M sodium chloride precipitate by amino acid analysis suggest that the additional carbohydrate of the 3.5M sodium chloride precipitate may be due to increased levels of hydroxylysine glycosides in the additional collagen chains.

Discussion

The insolubility of corneal stroma in neutral or acid buffers has been noted previously.[29, 30] However, mature rabbit cornea is considerably more soluble than mature bovine or human cornea. A total of 12% of the total hydroxyproline is solubilized by sequential extraction of rabbit stroma compared with less than 2% of the total hydroxyproline when mature human or bovine stromas are subject to similar treatment.[31]

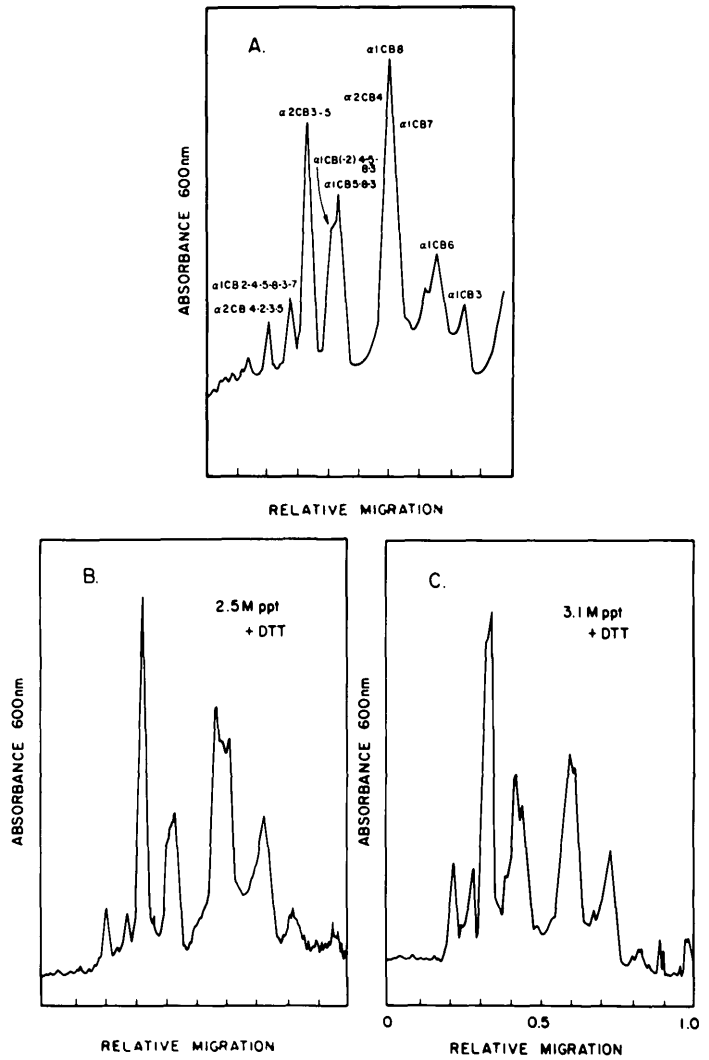

Fig. 2. Comparison scans of CNBr peptides. **A**, Bovine skin type I collagen. **B**, 2.5M NaCl precipitate. **C**, 3.5M NaCl precipitate. Identification of peptides in **A** is according to Scott and Veis.[26]

Solubility of fibrillar collagen is largely a function of the intermolecular cross-links,[32] and although the over-all appearance of the rabbit tissue is similar to that noted with other species, the increased solubility noted here is probably indicative of species specificity in corneal cross-linking. This is further evidenced by the almost complete dissolution of rabbit stromal tissue by pepsin, since both human and bovine cornea treated under the same conditions are only about 50% solubilized (I. L. Freeman, unpublished data).

The results presented here indicate that approximately two thirds of the total hydroxyproline-containing material in the initial pepsin digest could be precipitated by bringing the salt concentration to 2.5M sodium chloride, conditions which are known to precipitate type I collagen from solution.[20] The material precipitating at this salt concentration has an amino acid analysis similar to that of type I rabbit skin collagen[2] but shows the differences noted previously in terms of increased glycosylation.[29, 30] Highly glycosyl-

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ated collagens are known to have a very narrow range of fibril diameters,[30] and it is likely that hydroxylysine glycosides are important in the precise control of the diameters of corneal collagen fibrils.[29, 30]

One third of the solubilized hydroxyproline-containing material was not precipitated under these conditions. A major collagen fraction could be precipitated by bringing the salt concentration to 3.5M, but some collagen remained in solution even when the salt concentration was brought to 4.5M. These results indicate the polymorphic nature of corneal collagen and suggest the presence of molecular species other than type I collagen in the corneal stroma. This is in contrast to the work on lathyritic chick cornea,[27] where 25% of the total corneal hydroxyproline was found to be soluble in neutral salt solution and all this material was shown to be type I collagen.

The material precipitating at the higher salt concentration (3.5M) has some similarities to type I collagen in terms of its amino acid composition, but several important differences can be seen. Thus the hydroxylysine/lysine ratio indicates that the 3.5M sodium chloride precipitate contains material that is more heavily hydroxylated with respect to lysine residues than that which precipitates at 2.5M sodium chloride. In addition, a greater percentage of the hydroxylysine residues are convalently linked to carbohydrate. Table III shows that the majority of the excess glycosides of the material precipitating at 3.5M sodium chloride consist of the hydroxylysine-linked disaccharide, GGH.

When the material precipitating at 2.5M sodium chloride is compared to that precipitating at 3.5M sodium chloride, by gel electrophoresis, the former material shows a band pattern similar to bovine skin type I collagen. The latter material, however, shows the presence of two carbohydrate-rich pre-α chains of approximate apparent molecular weight 140,000 and 100,000 daltons, in addition to the pattern expected for type I collagen (Fig. 1). Thus the 3.5M sodium chloride precipitate contains two additional collagen chains which may be derived from one or more molecular species other than type I collagen. The strong affinity of these additional chains for the PAS stain and the increased amount of hydroxylysine-linked carbohydrate in the 3.5M sodium chloride precipitate, noted above, suggest that these additional collagen chains may contain increased levels of hydroxylysine glycosides.

Dische,[28] using various solubilization techniques on bovine corneal stroma, found two fractions of extractable collagen differing in their sugar content. One of these, a carbohydrate-rich collagen, may be related to the material precipitating at 3.5M sodium chloride reported here. Dische[28] suggested that carbohydrate-rich collagens may be physiologically important in the maintenance of the hexagonal lattice of the corneal fibrils. The levels of hydroxylysine glycosides in corneal collagen chains are higher than those found in any other fibrillar collagens.[29, 30] This may suggest that hydroxylysine glycosides are important in promoting collagen/glycoprotein interactions necessary for the orderly packing of corneal collagen fibrils. The material precipitating at 3.5M sodium chloride, with its increased levels of glycosylation, may be particularly important from this point of view; the additional collagen chains may represent molecular species which are physiologically important in maintaining the unique organization of the cornea.

CNBr peptide patterns of the 2.5M and 3.5M precipitate on tube gels appear to be identical to, and indistinguishable from, those of bovine skin type I standards[26] (Fig. 2). This shows either that similar sized peptides to those produced by CNBr treatment of type I collagen result from cleavage of the additional chains or that cleavage products of these additional chains do not contribute significantly to the CNBr peptide pattern. Further information will be obtained when isolation and CNBr treatment of the individual additional chains has been achieved. However, it is important to note the potential for misinterpretation of CNBr peptide patterns when additional collagens to those now well characterized are present in a mixture of collagen chains.

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