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Solubilization of Bovine Heart-Valve Collagen

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Complete solubilization of bovine heart-valve collagen was obtained by using sequential pepsin digestion and extraction with dithiothreitol under non-denaturing conditions. Use of the reducing agent was the crucial step resulting in solubilization. These findings suggest that disulphide-bonded proteins may be in part responsible for the insolubility of bovine heart-valve collagen.

Collagen is one of the major components of the connective-tissue matrix of heart valves and it comprises approx. 50% of their dry weight (Bashey *et al.*, 1967). Complete biochemical characterization of heart-valve collagen has not been possible owing to the extreme insolubility of the tissue (Bashey *et al.*, 1972). In previous studies we found that approx. 70–80% of bovine heart-valve collagen remained insoluble even after repeated and prolonged extraction with a 20-fold excess of pepsin at 15°C (Bashey *et al.*, 1978). In the present paper we present a method that resulted in essentially complete solubilization of collagen from bovine heart valves. This method does not involve denaturing conditions and yields solubilized collagen in a native conformation. The most important step, which resulted in solubilization of most of the pepsin-insoluble collagen in the tissues, involved reduction of disulphide bonds with dithiothreitol followed by limited pepsin digestion. This method is similar to the method used by Dehm & Kefalides (1977) to solubilize anterior lens capsule basement-membrane collagen. The solubilization of large amounts of collagen after extraction with dithiothreitol suggests the possibility that reduction removed proteins that formed a disulphide-bonded highly cross-linked matrix, which was responsible for the insolubility of collagen. Solubilization of additional collagen after subsequent pepsin digestion suggests that these proteins may have also impaired the ability of the proteinase to reach the susceptible regions in collagen. Sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis of the extracts showed the presence of α_1- and α_2-chains in an approx. molar ratio of 2:1. In addition significant amounts of β, γ and higher-molecular-weight aggregates were observed. Determination of half-cysteine as cysteic acid in the extracts showed that there were 5–15 residues of the amino acid/1000 residues. It was not certain, however, if half-cysteine was present in the covalent structure of collagen or if it originated in non-collagenous components of the valves.

Experimental

Bovine hearts were obtained fresh from a local abattoir and transported to the laboratory in ice. The valves were washed several times with 0.9% NaCl, and adventitia and chordae tendinae were removed. The valves were then minced and homogenized in a buffer containing 1M-NaCl/0.02M-Tris/HCl, pH 7.45 at 4°C, and extracted three times with 20–25 volumes of the same buffer over a period of 7 days at 4°C. The remaining tissue was then similarly subjected to three extractions with 0.5M-sodium citrate followed by three extractions with 0.5M-acetic acid at 4°C. The insoluble residue remaining after NaCl, sodium citrate and acetic acid extractions was separated by centrifugation and then freeze-dried. For pepsin digestion, about 500mg of the freeze-dried insoluble residue was suspended at 100ml of 0.5M-acetic acid containing 1mg of pepsin/ml (pepsin 2×crystallized from Sigma Chemical Co., St. Louis, MO, U.S.A.) and extracted at 15°C with agitation for 72h. The pepsin-solubilized material was collected after removal of insoluble residue by centrifugation at 35000*g* for 40min at 4°C. Pepsin extraction of the residue was repeated two times under similar conditions. The residue remaining after the pepsin extractions was resuspended in a buffer containing 0.45M-NaCl/0.05M-Tris/HCl/20mM-dithiothreitol, pH 7.4 at 4°C, and extracted for 5 days in the cold. The suspension was centrifuged at 14000*g* for 60min and the supernatant containing the dithiothreitol-extracted collagen was removed. The remaining tissue was washed several times with 0.5M-acetic acid, the washes were combined with the dithiothreitol extract, and the residue was then suspended in 0.5M-acetic acid containing 1mg of pepsin/ml and extracted for 72h at 15°C. The extract was centrifuged at 14000*g* for 60min and the soluble supernatant was separated out. The remaining tissue was resuspended in 20ml of 0.5M-acetic acid and after 24h agitation at 4°C became completely solubilized.

For collagen determination, samples of the various extracts were dialysed and hydrolysed at 120°C in 6M-HCl for 18h. Hydroxyproline content of the samples was determined by a colorimetric assay (Woessner, 1961). Disc gel electrophoresis of the various extracts was performed on sodium dodecyl sulphate/polyacrylamide gels after denaturation of the samples at 100°C for 10min in the presence of the detergent and 1% mercaptoethanol. Electrophoresis and densitometry of electrophoretic runs was performed as described previously (Jimenez *et al.*, 1977). Chromatographic determination of half-cysteine in the extracts was performed after oxidation to cysteic acid in the presence of performic acid as described by Schram *et al.* (1954). Amino acid analysis was performed after hydrolysis of samples on a Beckman 119C amino acid analyser.

Results and Discussion

When heart valves were sequentially extracted with 1.0M-NaCl, 0.5M-sodium citrate and 0.5M-acetic acid, solvents successfully used to extract collagen from other tissues, only minimal amounts of heart-valve collagen were solubilized (Table 1). Subsequent extraction with prolonged and repeated limited pepsin digestion at 15°C was more effective and approx. 50% of the total hydroxyproline present in the valves was solubilized (Table 2). We found that optimal yield was obtained if the pepsin digestion was repeated three times. When the tissues remaining from the pepsin digestion were neutralized to inactivate pepsin and then subsequently extracted with 0.45M-NaCl/0.05M-Tris/HCl buffer containing 20mM-dithiothreitol approx. 50% of the pepsin-insoluble residue became solubilized. Repeated pepsin digestion subsequent to the extraction with reducing agent resulted in complete dissolution of the structure of the valves, which became a viscous gel-like substance. After suspension in acetic acid and subsequent agitation for 16h, essentially complete solubilization occurred. The yield of solubilized collagen at the end of the procedure was approx. 85% of the total starting insoluble collagen. The remaining 15% was probably lost during the extensive extractions. Sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis of the dithiothreitol extract and of the subsequent pepsin extract demonstrated the presence of intact collagen polypeptides, and no collagen breakdown products were observed. Densitometric analysis of electrophoretic runs of the dithiothreitol extracts (Fig. 1*a*) showed that, from the amount of protein that entered the gels, approx. 60% migrated in the position of α-chains, 11% in the position of β-chains and 15% in the position of γ-chains. The rest was composed of three minor components, one migrating between β- and α_1-chains, and two migrating slightly above γ-chains. Approx. 40–50% of the protein applied did not penetrate the gels even after prolonged electrophoresis, suggesting that this fraction contained highly cross-linked molecules.

Densitometric analysis of electrophoretic runs of the material extracted with pepsin after dithiothreitol extraction showed a similar pattern except that the component migrating between β- and α_1-chains was absent and the components migrating above γ-chains were decreased in amount (Fig. 1*b*). To explore further the role of disulphide bonds on collagen solubilization, chromatographic determination of half-cysteine as cysteic acid was performed on the extracts. It was found that the dithiothreitol extracts

Table 1. *Amounts of bovine heart-valve collagen solubilized with neutral NaCl, citrate and acetic acid solutions*
Bovine heart valves were homogenized in 1.0M-NaCl and extracted sequentially with 1.0M-NaCl/0.1M-Tris/HCl, pH7.4, 0.24M-sodium citrate and 0.5M-acetic acid as described in the text. The amount of collagen in the extracts was calculated from hydroxyproline content of the samples assuming that collagen contained 13.4% of the imino acid.

	Amount of collagen	
	(mg)	(%)
Total valve	2936.0	100
NaCl-soluble	30.35	1.03
Citrate-soluble	2.60	0.09
Acetic acid-soluble		
First extraction	4.86	0.17
Second extraction	6.46	0.22
Third extraction	2.21	0.08
Total extracted	46.48	1.58

Table 2. *Solubilization of insoluble heart-valve collagen by limited pepsin digestion and dithiothreitol extraction*
The residues remaining after neutral-NaCl and acid extraction were freeze-dried and approx. 500mg of dry tissue was used for subsequent extractions. Pepsin extraction was carried out at 15°C with 1mg of pepsin/ml in 0.5M-acetic acid. Dithiothreitol extraction was performed at 4°C with 20mM-dithiothreitol in 0.45M-NaCl/0.05M-Tris/HCl, pH7.4.

	Amount of collagen	
	(mg)	(%)
Starting tissue	380	100
First pepsin extraction	84.2	22.2
Second pepsin extraction	54.3	14.3
Third pepsin extraction	47.0	12.4
Dithiothreitol extraction	91.8	24.2
Pepsin extraction after reduction	49.8	13.1
Total extracted	327.1	86.1

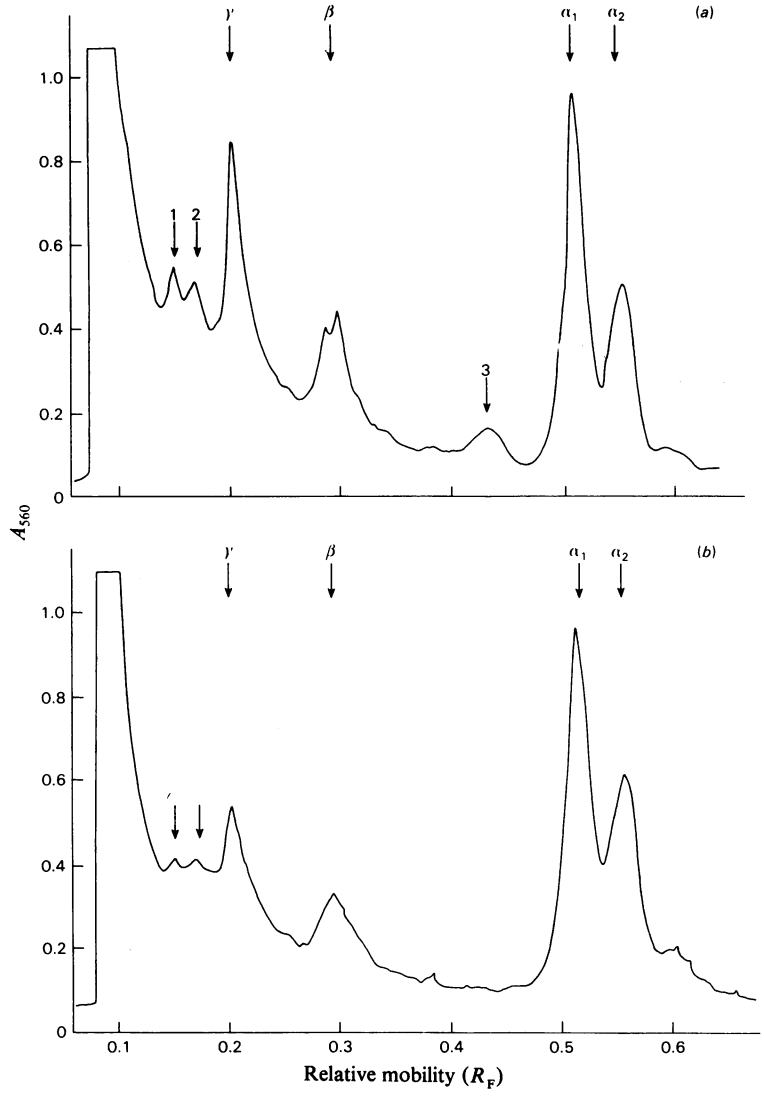

Fig. 1. *Spectrophotometric scans of sodium dodecyl sulphate/polyacrylamide gels of heart valve extracted with dithiothreitol (a) and subsequent limited pepsin digestion (b)*

Electrophoresis was performed on 5% polyacrylamide gels after heat denaturation of samples in 1% sodium dodecyl sulphate/1% mercaptoethanol. Arrows indicate the migration positions of standard α_1-, α_2-, β- and γ-chains from tendon collagen run in a parallel gel. Direction of migration was from left (cathode) to right (anode). Note the large amount of material remaining at the origin. The three minor components described in the text are labelled 1, 2 and 3.

contained 12–15 cysteic acid residues/1000 amino acid residues, whereas the pepsin extracts obtained after extraction with the reducing agent contained only 5–7 cysteic acid residues/1000 amino acid residues. Although the mechanisms responsible for solubilization of collagen after disulphide-bond reduction are not clear at present, it is possible to speculate that disulphide-bonded proteins were organized in a tightly cross-linked network around collagen molecules or fibrils and prevented their passage into the extractant solutions. It is also possible that these proteins covered the susceptible sites in the collagen telopeptides and rendered them relatively inaccessible to proteinase attack. A similar mechanism has been suggested to be responsible for the apparent larger molecular weight of a

collagenous moiety in anterior lens capsule basement membrane (Dehm & Kefalides, 1977). Although it was not possible to demonstrate the presence of half-cysteine residues in the covalent structure of the extracted collagens, it is possible that at least some disulphide bonds involved the collagen molecule proper and may have been responsible for the marked insolubility of heart-valve collagen.

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