Preparation of Insoluble Collagen from Human Cartilage

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A simple technique is described for the removal of proteoglycans from human cartilage by sequential treatments with H_2O_2 followed by trypsin digestion. The resultant fibres contain no uronic acids and have the amino acid composition of highly purified collagen.

The preparation of pure collagen fibrils from human cartilage has been complicated by the fact of the strong binding of proteoglycans to the collagen of this tissue. Sajdera & Hascall (1969) employed guanidinium chloride solution to extract much of the proteoglycans from bovine nasal cartilage, leaving a partially purified denatured collagenous residue. A similar technique was used by Miller *et al.* (1969) on human cartilage: these workers solubilized only 1% of the total collagen, but reported no analyses of the purified collagenous residue. Miller (1972) published an extraction procedure for obtaining partially purified insoluble collagen from chick sternum cartilage: this collagen was still contaminated by glycosaminoglycan fractions, which appeared as major peaks on chromatography of the peptides derived from this preparation by cleavage with CNBr. After depolymerization of this insoluble preparation with pepsin Miller (1972) purified a soluble form of chick cartilage collagen.

The aim of the present study was to prepare pure collagen fibrils from human cartilage without having to resort to depolymerization, in the hope that the preparation would retain the organization of the collagen fibrils that was originally present in the intact cartilage. The technique depended for its success on the concept that selective attack on the proteoglycans by a readily diffusible chemical agent would facilitate the subsequent enzymic removal of non-collagenous components of the cartilage. Scott *et al.* (1972) published a paper on the effect of NaIO_4 on the viscosity of proteoglycans and glycosaminoglycans; a similar effect was briefly mentioned for H_2O_2 .

In the present study H_2O_2 treatment of cartilage slices was followed by trypsin digestion to remove all the proteoglycans, leaving a pure fibrous form of cartilage collagen.

Methods

Thin slices of human cartilage were removed from the femoral head as soon as possible after death. Cartilage slices (0.5 g wet wt.) were shaken with 30 ml of H_2O_2 [30% and 3% (v/v) H_2O_2 solutions were

equally effective] in the dark at 20°C for 18 h, followed by repeated washing with water and also with 1% (w/v) NaCl. The residual tissue was then digested with trypsin (EC 3.4.4.4) (twice crystallized) in 1% (w/v) NaHCO_3 (enzyme/tissue ratio approx. 1:100) for 18 h at 20°C with constant shaking. After the tissue had been washed repeatedly with water and 1% NaCl, the insoluble material was treated with 4% (w/v) EDTA, pH 7.5, at 20°C for 18 h (Steven, 1967) to remove non-collagenous proteins. The tissue was then extracted with 0.1 M-acetic acid three times, its pH was adjusted to 7.0 and it was finally washed with 1% NaCl. At this stage the whole procedure was repeated, leaving a final product with the same dimensions as the original slices but consisting of collagen fibrils only.

Uronic acid analyses were carried out by the method of Bitter & Muir (1962), and amino acid analyses were carried out on hydrolysed samples by using the JEOL JLC-6AH analyser.

Results and discussion

Uronic acid analyses indicated the complete removal of glycosaminoglycans from the tissue. Amino acid analyses of proteoglycans (Swann, 1968) and complexes of proteoglycans with collagen (Steven *et al.*, 1968) yielded a number of unidentified ninhydrin-positive peaks, which are characteristic of this type of hydrolysate. The latter are due to condensation products formed from uronic acids, amino acids and amino sugars (Muir, 1958; Muir & Jacobs, 1967). These characteristic peaks were present in the hydrolysates of the initial cartilage, but during the process of purification of the cartilage collagen described above these components were diminished in quantity and were completely absent from the final product.

The amino acid composition of three samples of insoluble cartilage collagen prepared by the H_2O_2 -trypsin techniques are presented (A, B and C) in Table 1. Sample D in Table 1 was prepared by repeated papain digestion of human cartilage at 20°C, and is included for purposes of comparison with the

Table 1. Amino acid compositions of H_2O_2 -treated (A, B and C) and papain-treated (D) human cartilage collagen

Experimental details are given in the text.

Amino acid composition (residues/1000 total residues)

Treatment ... Preparation ...	H_2O_2 A	H_2O_2 B	H_2O_2 C	Papain D
Hyp	114.7	113.6	116.9	119.5
Asp	44.2	49.4	45.7	43.6
Thr	20.0	20.8	21.2	20.4
Ser	28.4	29.2	29.7	29.4
Glu	91.3	90.8	93.4	94.6
Pro	118.9	114.5	111.3	117.0
Gly	318.6	310.6	316.0	322.1
Ala	98.4	99.7	96.1	93.9
Val	18.0	19.5	18.0	16.8
Met*	1.9	2.9	4.5	1.6
Ile	12.1	12.4	11.2	12.4
Leu	30.0	30.3	28.0	28.8
Tyr	1.3	1.4	2.4	2.5
Phe	13.6	13.6	13.0	13.9
Hyl	18.0	19.1	19.7	15.9
Lys	19.2	20.7	20.7	18.2
His	1.8	1.7	2.4	2.2
Arg	48.8	49.8	49.7	47.4
Total	999.2	1000.0	999.9	1000.2

* Uncorrected value (see the text).

preparations obtained by the H_2O_2 -trypsin technique. The elution pattern of the hydrolysate D still contained trace amounts of impurities characteristic of proteoglycans, and for this reason collagen D is considered to be less pure than preparations A, B and C.

Tendon collagens normally contain 93 residues of hydroxyproline/1000 total amino acid residues, although partially purified chick cartilage contained 102 residues/1000 total residues (Miller, 1972). In samples A, B and C the hydroxyproline content was 114–117 residues/1000 total residues and this value might be considered to be too high, possibly as a result of oxidation of protein-bound proline to hydroxyproline during the H_2O_2 pretreatment of the tissues. Comparison of these values with the 119 hydroxyproline residues/1000 total residues found in the papain digests (D) would, however, indicate that the human femoral cartilage collagen genuinely contained rather more hydroxyproline than did tendon collagen and that these high values were not produced as a direct result of oxidation during the preparation of samples A, B and C.

Methionine is known to undergo oxidation during acid hydrolysis in the presence of O_2 to yield a number of oxidation products, including methionine

sulphoxide. Butler *et al.* (1967) calculated the methionine content of rat skin collagen α_1 -chain by summation of the methionine and methionine sulphoxide contents of selected hydrolysates in which the sulphoxide content was less than 10% of the methionine content. These authors concluded that there were 6–7 residues/ α_1 -chain.

The uncorrected values for methionine found in cartilage collagen hydrolysates A, B, C and D in Table 1 lie within the range 1.9–4.5 residues/1000 total residues; inclusion of methionine sulphoxide would increase this range to 7–9 residues/1000 total residues.

Bovine serum albumin hydrolysed in air and under N_2 yielded 12.7% and 15.9% recoveries of methionine when analysed under these conditions. Tropocollagen hydrolysates contained 0.7–1.0 residue of methionine/1000 total residues and the value was unaltered after prior treatment of the collagen with 30% H_2O_2 . In view of the low methionine contents found in cartilage collagens, tropocollagen and serum albumin hydrolysates, we have presented the actual methionine contents (Table 1) rather than attempted to adjust these values for hydrolytic losses and the conversion of part of the methionine into the sulphoxide.

It could also be argued that the combination of H_2O_2 and trypsin might result in the cleavage of peptide bonds and subsequent depolymerization of cartilage collagen. Preliminary electron-microscopic studies show no evidence of proteolytic attack on the collagen fibrils. The fibrils remain well organized, exhibiting no fraying along the fibril or frayed ends (Worrall & Steven, 1966; Milsom *et al.*, 1972). The cartilage collagen preparations A, B and C showed no instability when subjected to thermal denaturation: 96–98% of the protein was recovered in the form of an insoluble residue and only 2–4% dissolved to yield a hot-soluble gelatin fraction. Although this evidence cannot be taken to prove that no depolymerization had taken place, it is indicative that only minimal cleavage of peptide bonds had occurred. It should be pointed out that in this instance one cannot employ a suitable control of pure human cartilage collagen for comparison with the samples A, B and C, and such a sample itself might well have been expected to show a small change in thermal stability of 2–4%. The recent report that trypsin digestion of human cartilage slices with trypsin for 24 h at 37°C had no effect on the mechanical properties of cartilage collagen (Kempson *et al.*, 1973) would support the claim that trypsin has little depolymerizing action on human cartilage collagen.

This preparation is easily carried out, and we believe that the final product contains insoluble collagen of the highest purity so far obtained from human cartilage.

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