Polymeric collagen isolated from the human intestinal submucosa

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The intestinal submucosal layer has a special interest in view of its commercial use in sausage casings and also due to its importance in any study of normal and pathological changes in the human intestine. Although the submucosal layer has long been known to be a collageneous structure, no detailed report has been published for the isolation of highly pure submucosal collagen together with its chemical analysis and electron-microscopic appearance.

Earlier work from this laboratory has been concerned with the isolation and characterization of pure polymeric collagens from tendon and skin (Steven, 1964; Steven and Jackson, 1967), cornea (Freeman, Steven, and Jackson, 1968), intervertebral disc (Steven, Broady, and Jackson, 1968), and articular and intercostal cartilages (Steven, Broady, and Jackson, 1969). In each of these preparations it was necessary to pretreat the homogenized tissue with crude bacterial α -amylase or ethylenediaminetetraacetate (EDTA) in order to release the collagen fibrils from the tissue matrix in which they were embedded.

In the present study the polymeric collagen fibrils of the intestinal submucosa could be released by treatment with dilute acetic acid alone. The amino-acid composition of these polymeric collagen fibrils indicated that they consisted of at least 95% pure collagen. The purity of the polymeric collagen was further improved after treating the tissue beforehand with crude bacterial α -amylase or with EDTA.

MATERIALS AND METHODS

PREPARATION OF TISSUE Human intestines were removed as soon as possible at necropsy from subjects with no known intestinal abnormality. The tissues were carefully washed with water and the submucosal layer was scraped clean of the mucosa and muscularis layers. Segments of the submucosal layer from both the small and large intestine were removed and separately frozen in liquid nitrogen. The tissues were crushed in a stainless steel hammer mill (Steven, 1967; Walser and Bodenloss, 1954) and exhaustively washed in 0.5 M NaCl by centrifugation until the

supernatant was clear and contained no solubilized protein.

ACETIC ACID EXTRACTION Part of the homogenized tissue was suspended in 5 l. 0.2 M acetic acid and allowed to equilibrate to pH 3.2 to 3.5 over a period of one hour. The tissue was then homogenized in an Utraturax homogenizer for 10 seconds, followed by constant stirring at 4° for 18 hours. The supernatant fluid containing polymeric collagen fibrils was removed by centrifugation at 300g for 20 min at 4°C and the fibrils were collected by raising the pH to 5 to 6 with M.NaOH and stirring with a spatula (Steven, 1964; Steven and Jackson, 1967). Polymeric collagen fibrils were purified from non-collageneous proteins by redispersing in acetic acid four times followed by precipitation of the fibrils by the addition of saturated NaCl to give a final concentration of 3 to 4% w/v.

GELATIN FORMATION Acetic-acid-extracted polymeric collagen fibrils were autoclaved at pH 7.0 for six hr at 15 lb/in.²; the insoluble residue contained 94% of the initial collagen and the soluble fraction 6% respectively. The soluble fraction was purified as a gelatin after precipitation of non-collagenous impurities with 5% trichloroacetic acid and precipitation of the gelatin with 10 volumes of acetone (Jackson, 1957). The insoluble residue was autoclaved in water for a further six hr at 30 lb/in.² but failed to yield any further soluble gelatin fraction.

CRUDE α -AMYLASE TREATMENT Part of the tissue homogenate was treated with crude α -amylase, 1% w/w, as previously described for tendon and skin (Steven, 1964; Steven and Jackson, 1967), and the polymeric collagen fibrils were collected and purified as above, all steps being carried out at 4°C.

EDTA TREATMENT Part of the tissue homogenate was treated with 4% w/v EDTA pH 7.5 (Steven, 1967) and the polymeric collagen fibrils were isolated and purified as above.

AMINO ACID ANALYSIS The collagen and gelatin preparations were subjected to 24-hr hydrolysis under nitrogen in 6N HCl and amino acid analysis was carried out on the

21-hour Technicon sodium buffer system as previously described (Steven, 1967; Steven and Jackson, 1967).

ELECTRON MICROSCOPY Samples of submucosal polymeric collagen fibrils dispersed in 0.1 M acetic acid were placed on carbon-collodion-coated grids and negatively stained with 1% w/v sodium phosphotungstate pH 7.0. The grids were examined in the AEI-EM-6B electron-microscope.

RESULTS AND DISCUSSION

YIELD OF POLYMERIC COLLAGEN Virtually all the collagen of the submucosal tissue was capable of dispersion into 0.1 M acetic acid as polymeric collagen fibrils, and less than 10% remained in the insoluble residue after two extractions with acetic acid. The yield was not affected by pretreatment of the tissue with either crude α -amylase or by EDTA.

The ease with which dilute acetic acid disperses the polymeric collagen fibrils of the intestinal submucosa is in marked contrast to the difficulties encountered with other connective tissues. Skin and tendon require a pretreatment with crude bacterial α -amylase (Steven, 1964; Steven and Jackson, 1967) or EDTA (Steven, 1967) before the polymeric collagen fibrils can be dispersed in acetic acid. These pretreatments do not release pure polymeric collagen from human cartilage unless it is further treated with a proteolytic enzyme such as trypsin (Steven *et al.*, 1968 and 1969). Bone decalcified with EDTA and treated with crude α -amylase followed by trypsin cannot be dispersed in acetic

acid to yield pure polymeric collagen fibrils. It is suggested that the submucosal polymeric collagen fibrils have very little interaction with other non-collagenous components of this tissue and that they represent the least complex organization of polymeric collagen fibrils at this tissue level so far studied.

DEGREE OF POLYMERIZATION OF POLYMERIC COLLAGEN

Prolonged autoclaving of collagen is known to produce peptide bond cleavage with the formation of a soluble polydisperse polypeptide fraction usually referred to as gelatin. Human skin and tendon polymeric collagen is completely solubilized after one hour's autoclaving. The marked insolubility of submucosal polymeric collagen after 12 hours' autoclaving indicates a very high degree of intermolecular cross-linking or polymerization within the polymeric collagen fibrils. These crosslinkages are stable and prevent the solubilization of polypeptide fragments resulting from peptide bond cleavage during autoclaving.

AMINO ACID COMPOSITION OF POLYMERIC COLLAGEN

The analyses presented in Table I indicate that both the crude α -amylase and EDTA-prepared polymeric collagen fibrils were highly purified. The polymeric collagen fibrils extracted with acetic acid alone contained some 6 to 7% impurity calculated on the content of hydroxyproline and proline as well as glycine residues. This impurity could be removed from the polymeric collagen by autoclaving and

TABLE I

AMINO ACID COMPOSITIONS OF POLYMERIC COLLAGENS AND DERIVED GELATIN OBTAINED FROM HUMAN INTESTINAL SUBMUCOSA¹

Preparation	Acetic-acid Extracted Polymeric Collagen	Derived Gelatin (Gel-I)	Polymeric Collagen	
			α -Amylase	EDTA
Hydroxyproline	81.3	95.1	97.1	95.4
Aspartic acid	54.2	54.1	51.4	52.4
Threonine	19.2	16.3	18.4	16.4
Serine	32.3	36.1	35.8	39.0
Glutamic acid	80.2	74.2	77.4	77.8
Proline	105.3	115.7	110.1	112.6
Glycine	313.7	339.7	315.9	315.4
Alanine	117.2	105.5	111.1	118.4
Valine	34.4	23.4	28.1	27.2
Methionine	1.9	Trace	2.1	Trace
Isoleucine	16.0	12.1	14.4	12.0
Leucine	31.5	25.0	27.1	26.7
Tyrosine	4.5	2.5	4.2	2.7
Phenylalanine	14.4	11.1	14.3	12.8
Hydroxylysine	5.3	7.5	7.2	5.7
Ornithine	2.7	2.0	1.1	Trace
Lysine	30.2	28.4	26.8	29.3
Histidine	6.0	4.7	7.2	6.0
Arginine	49.6	46.6	50.2	50.2
Total	999.9	1,000.0	999.9	1,000.0

¹Results have been expressed as residues per 1,000 total residues after allowing for hydrolytic losses (Steven and Jackson, 1967).

FIG. 1. Intestinal submucosal polymeric collagen fibrils negatively contrasted with sodium phosphotungstate (pH 7.0) $\times 50,000$, showing fibrils swollen but still maintaining 640 Å periodicity (A) and swollen stocking-like structure (B) in addition to unswollen native type collagen fibrils (C).

purifying the derived gelatin (6% of the total polymeric collagen) by trichloroacetic acid and acetone precipitation (see figures for gel-1, Table I).

The hydroxylysine content of intestinal submucosal polymeric collagen is lower than tendon and cartilage polymeric collagen (Steven *et al*, 1969). The sum of lysine plus hydroxylysine polymeric collagen obtained from these tissues is approximately constant as would be expected from the fact that hydroxylysine is derived from lysine in procollagen (Kivirikko and Prockop, 1967; Prockop and Kivirikko, 1967).

ELECTRON MICROSCOPIC EXAMINATION OF POLYMERIC COLLAGEN Examination of submucosal polymeric collagen fibrils dispersed in 0.1 M acetic acid demonstrated the typical 640 Å striated fibrils (A, Fig. 1) as well as fibrils which were partially unravelled (B, Fig. 1). The protofibrils which make up the fibril can be seen to be organized in the form of a 'stocking' type of structure, with some evidence of superhelical organization. Stirtz (1967) has also published evidence suggesting a superhelical organization of protofibrils within collagen fibrils. The swollen fibrils (B in Fig. 1) appear unable to be completely unravelled. This suggests that a limited number of interprotofibrillar bonds may be present and that these place a restriction on the dispersal of the protofibrils in dilute acetic acid.

It may be concluded that the human submucosa contains polymeric collagen fibrils which are very loosely held in the interfibrillar matrix and that dilute acetic acid readily extracts at least 90% of the polymeric collagen in the form of dispersed fibrils. The polymeric collagen fibrils are composed of a large number of superhelically organized protofibrils, whilst at the molecular level there must be a high degree of intermolecular cross-linking which is stable to autoclaving in marked contrast to other polymeric collagens so far studied.

SUMMARY

Treatment of human intestinal submucosa with dilute acetic acid allowed the dispersal of polymeric collagen fibrils which were purified by salt precipitation. These fibrils were 97% pure collagen. Treating the tissue beforehand with crude bacterial α -amylase or EDTA resulted in the dispersion of pure polymeric collagen fibrils. The amino acid composition and the electron microscopic appearance of intestinal submucosa polymeric collagen fibrils are reported.

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