ISOLATION OF A PEPTIDE CONTAINING FUCOSE, MANNOSE AND HEXOSAMINE FROM METRIDIUM DIANTHUS COLLAGEN*

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A homogeneous peptide containing 8% of 3- and 4-hydroxyproline and 30% of bound carbohydrate (consisting of fucose, xylose, mannose, galactosamine, and glucosamine) was isolated from a collagenase digest of sea anemone (Metridium dianthus) collagen by gel filtration, DEAE-cellulose chromatography, and high voltage paper electrophoresis.

The question of a covalent linkage existing between collagen and heteropolysaccharide, acid polysaccharide, or glycoprotein is one that has stimulated considerable controversy during the past decade. Much of the evidence suggesting such a linkage has come from the studies of Dische, Kefalides, Spiro, and Winzler on the chemistry of the basement membrane from kidney glomeruli and of Mashburn and Hoffman on the proteinpolysaccharide from bovine nasal cartilage (6-10). In this paper we present the first direct evidence for the covalent linkage of heteropolysaccharide to collagen.

Preparation of Collagen. The body-wall of Metridium dianthus (obtained from Marine Biological Laboratories, Woods

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Hole, Mass.) was freed of adhering visceral and epithelial tissues, minced, and extracted with NaCl, Na_2HPO_4, citrate, and acetic acid solutions as described by Rubin et al. (11). The insoluble residue (drained weight, 0.6 kg) was suspended in 5 l of 0.1% acetic acid containing 20 mg of pepsin (Worthington), and digested for 48 hours at 4°. Over 90% of the residue was solubilized and further purified by 3 precipitations with 10% KCl (11). The product was exhaustively dialyzed against 0.1% acetic acid, and lyophilized, yielding 10 g of collagen.

Characterization of Collagen. The collagen had an $[\alpha]_{365}^{4-22}$ of -1280° (c 0.6, 20 mM acetic acid). It exhibited a helix-coil phase transition (T_m=24.5°) with $[\alpha]_{365}^{26-40}$ falling to -440°. Very similar physical properties have been reported for acetic acid-soluble collagen from the coelenterate Actinia equina (12). The amino acid composition of our product agreed with previously published values for several coelenterate collagen preparations (12-14). The collagen contained 8% carbohydrate, determined by g.-l.c. (15), consisting of 0.3% fucose, 0.02% xylose, 0.35% mannose, 3.2% galactose, 3.8% glucose, 0.1% galactosamine, and 0.4% glucosamine. Arabinose was not detected (16).

Collagenase Digestion and Fractionation. Collagen (44 g in 2 l of 0.1M Na_2HPO_4, pH 7.3) was digested with Cl. histolyticum collagenase[1] (5 mg, Worthington, 230 units/mg) for 24 hours at 10°. The digest was dialyzed against water (24 hours, 10°) and the retentate (20 g, dry weight) fractionated in two portions on a large P-100 column (see Fig. 1). The fractions indicated by the bar in Fig. 1 were then chromatographed as described in Fig. 2. The fractions indicated by the bar in Fig. 2 were pooled and further purified by preparative paper electrophoresis on Whatman

[1] This lot of collagenase contained no peptidase activity when tested against casein or denatured hemoglobin.

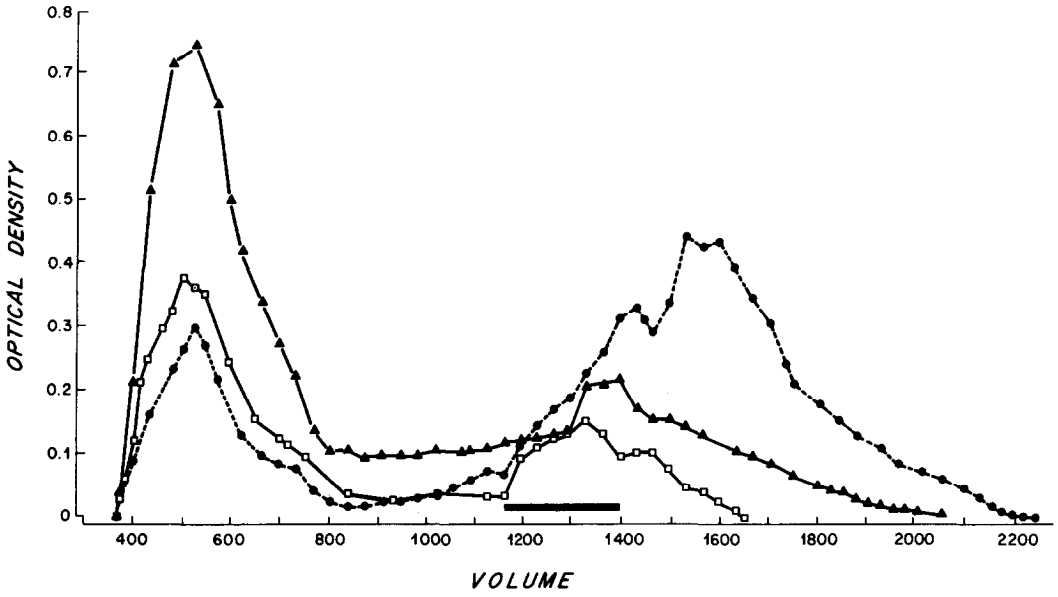

Fig. 1. P-100 Gel Filtration of Collagenase-Digested Collagen. Following collagenase digestion, the non-dialyzable fraction (10 g in 75 ml of 1% acetic acid) was chromatographed on a column (27 cm^2 x 80 cm) of Bio-Gel P-100 (50-100 mesh), eluting with 1% acetic acid. Fucose (□——□) was estimated by the method of Dische and Shettles (17); hexose (○---○) with the anthrone reagent (18); 280 nm absorbancy is indicated by (△——△). The fractions indicated by the bar were pooled and lyophilized, yielding 1.05 g. This procedure was repeated and the combined product (2.1 g) fractionated on DEAE-cellulose as described in Fig. 2.

3mm paper using 50mM sodium acetate, pH 8.5, at 120 volt-cm^{-1} for 120 min. Four ninhydrin-positive areas were detected on guide strips and labeled A through D. The area corresponding to Peptide C was eluted from the paper and chromatographed on Bio-Gel P-30 as described in Fig. 3. Peptide C displayed a homogeneous pattern by gel filtration, with constant ninhydrin-positive/protein values across the entire peak. Peptide C was also homogeneous by high voltage paper electrophoresis (see Fig. 4).

Results and Discussion. Peptide C is a glycopeptide containing 30% carbohydrate and 47 amino acids (see Table I). Its molecular weight was estimated to be between 5,000 and 10,000

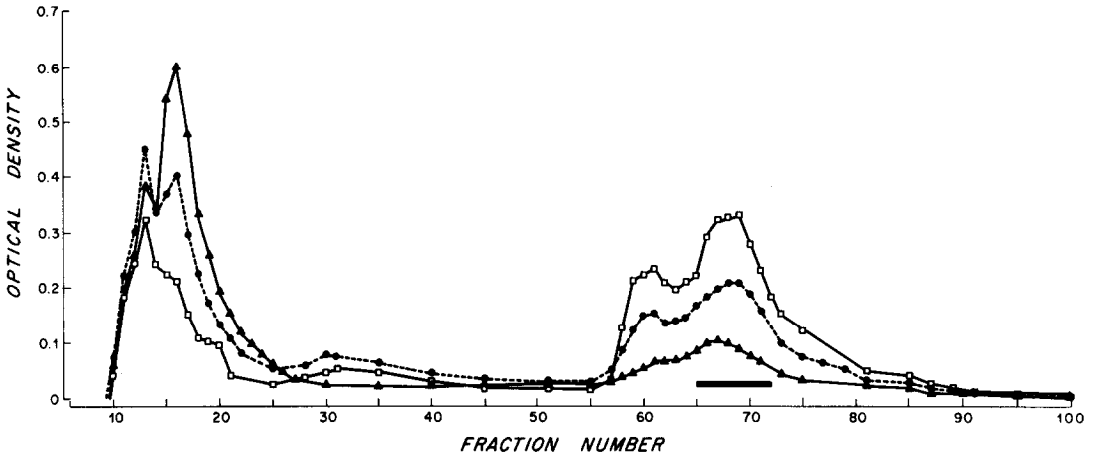

Fig. 2. DEAE-Cellulose Chromatography. The sub-fraction from Fig. 1 (2.08 g in 50 ml of 1mM sodium acetate buffer, pH 5.0) was applied to a column (7 cm^2 x 40 cm) of DEAE-cellulose (Whatman, microgranular) and eluted with a linear gradient (one liter of 1mM sodium acetate buffer, pH 5.0, versus one liter of the same buffer made 25 mM with NaCl). Fractions (20 ml) were collected and assayed as described in Fig. 1. The symbols are also the same as in Fig. 1. The fractions indicated by the bar were pooled, desalted on Bio-Gel P-2, and lyophilized, yielding 212 mg. This fraction was further purified by paper electrophoresis as described in the text.

daltons by gel filtration on P-30, 7,000 daltons by quantitative Edman degradation of the N-terminus, and a multiple of 6,200 daltons as determined by its empirical formula. A two-cycle Edman degradation established the sequence of the first two amino acids from the N-terminus to be glycine and proline, respectively. The results of the Edman degradation, together with the behavior of Peptide C on electrophoresis (Fig. 4), gel filtration (Fig. 3), and DEAE-cellulose (Fig. 2), offer good evidence for the homogeneity of the peptide.

Peptide C (see Table I) contains 10 acidic residues out of a total of 47 amino acids. The basic amino acids, hydroxylysine, lysine, histidine, and arginine, are absent, as are methionine, tyrosine, and phenylalanine. The presence of cysteine is unusual;

Table I

Composition[a] of Peptide C

3-Hyp	1	Pro	4	Leu	2
4-Hyp	3	Gly	13	Fucose	0.7
Asp	7	Ala	2	Xylose	0.3
Thr	6	Cys	1	Mannose	3.5
Ser	1	Val	3	Galactosamine	0.5
Glu	3	Ile	1	Glucosamine	4.3

[a]Results are expressed as residues per 6,200 daltons. Amino acids were determined after hydrolysis in 5.7N HCl at 108° for 24 hours. Values for threonine and serine were corrected by 5% and 10%, respectively. Cystine is reported as the total of cysteic acid plus cysteine. Carbohydrates were determined by g.-l.c. of the per(trimethylsilyl)ated methyl glycosides (15).

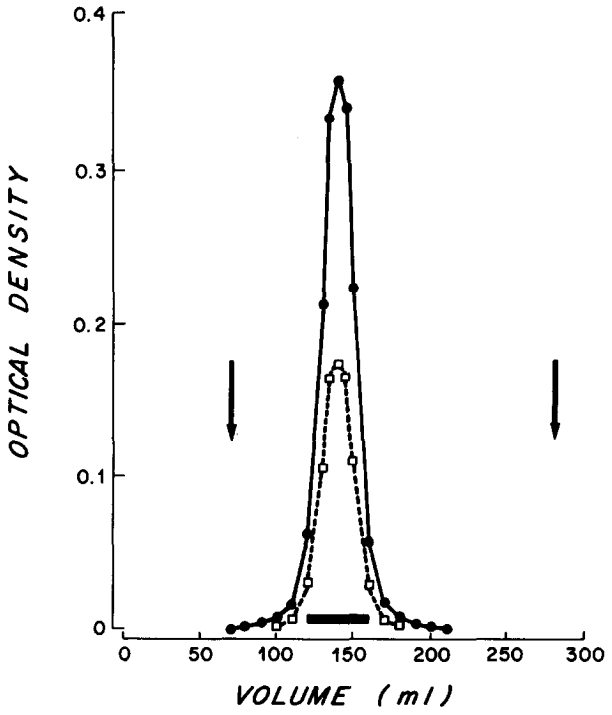

Fig. 3. P-30 Gel Filtration of Peptide C. Peptide C was chromatographed on a column (4 cm^2 x 60 cm) of Bio-Gel P-30 (100-200 mesh), eluting with 1% acetic acid. Protein was determined by the procedure of Lowry et al. (19), indicated by (□ --- □); ninhydrin-positive material (○——○) was by the procedure of Rosen (20). The arrows on the left and right of the peak indicate the elution positions of Dextran Blue 2000 and the chloride ion, respectively. The fractions indicated by the bar were pooled and lyophilized for further characterization. Yield: 59 mg.

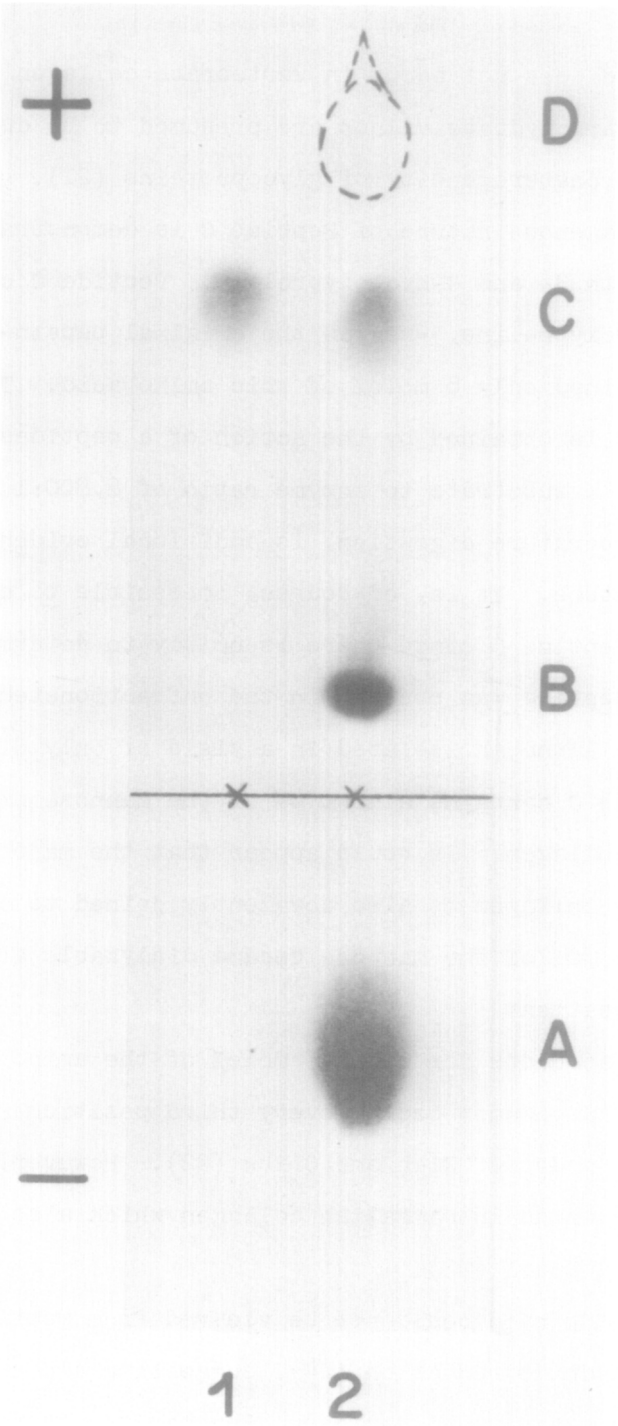

Fig. 4. High Voltage Paper Electrophoresis of Peptide C. Two mg of Peptide C (in Lane 1) were examined by paper electrophoresis on Whatman no. 1 paper using 50mM sodium acetate buffer, pH 6.8, at 75 volt-cm^{-1} for 90 min. The peptides were visualized by dipping the paper in 3% ninhydrin in ethylene glycol monomethyl ether and heating at 110° for 20 min. Lane 2 shows the pattern before electrophoretic purification.

this amino acid does not occur in vertebrate collagen (16). The non-integral carbohydrate values are presumed to be due to the well known microheterogeneity of glycoproteins (21).

The collagenous nature of Peptide C is demonstrated by the presence of both 3- and 4-hydroxyproline. Peptide C contains 8.5 mole% hydroxyproline, whereas the original pepsin-solubilized collagen contained only 6 mole% of this amino acid. The fact that Peptide C is obtained by the action of a peptidase-free collagenase at a substrate to enzyme ratio of 8,800:1 during a brief, low temperature digestion, is additional evidence for its collagenous nature. It is, of course, impossible to calculate the yield of Peptide C since there is no way to determine how much of this peptide was present in the unfractionated collagenase digest. Although isolated in a yield of only 0.13% by weight, Peptide C contains almost 4% of the mannose present in the original collagen. It would appear that the majority of the mannose in the collagen is also covalently joined to collagenous peptides since 60% of the mannose became dialyzable after collagenase treatment.

Glycine accounts for only 27 mole% of the amino acid residues and thus cannot occupy every third position as required by the helical model of Rich and Crick (22). However, there are amino acid sequences in mammalian collagen which also do not conform to this model (23).

The carbohydrate could not be cleaved from Peptide C by alkaline-borohydride reduction (24), suggesting a possible linkage *via* an asparaginyl residue. Further structural studies on Peptide C are currently in progress. We have preliminary data which suggest that there are covalent linkages between heteropolysaccharide and collagen from vertebrate skin (25).

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