

Biochemical and Electronmicroscopic Investigations on *Helix pomatia* Collagen

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The skin of *Helix pomatia* was investigated by biochemical methods and electronmicroscope. Biochemical investigations showed that *Helix pomatia* collagen represents a methionine-lacking collagen resistant to CNBr-cleavage. Electron microscopic studies showed a cross striation pattern of 53–57 nm.

Introduction

The composition of all vertebrate collagens especially of the collagen types of interstitial connective tissue is closely similar. Sequence information shows a high degree of homology in the distinct polypeptides of interstitial collagens, homology both with respect to the subunits of types I, II and III, but also of each type in various vertebrate species.

In contrast, invertebrate collagens display very different amino acid compositions and no simple generalizations concerning composition have emerged from the considerable number of species studied in different phyla [1–3]. In this paper we isolated collagen from the skin of *Helix pomatia* and compared this protein with the common vertebrate collagens.

Materials and Methods

Preparation of collagen

Small skin pieces of *Helix pomatia* were treated with 0.5 M sodium acetate at 4 °C for 24 h and after washing with water the acid-soluble collagen was extracted with two changes of 0.5 M citrate buffer (pH 3.7). The insoluble residue was solubilized with

5 mg/g wet weight pepsin (2 500 U/mg; Boehringer/Mannheim Nr. 108 057) at 4 °C for 24 h. The digest was centrifuged and the pepsin-solubilized collagen precipitated from the supernatant by the addition of solid NaCl to a final concentration of 0.9 M. The precipitate was collected by centrifugation (10 min, 4 °C, 3000 × g), redissolved in 0.05 M Tris-HCl (pH 7.5) containing 1.0 M NaCl, kept for 4 days at 4 °C to inactivate the pepsin, dialyzed exhaustively against 0.1 M acetic acid, precipitated with acetone and dried in air.

Carboxymethyl cellulose chromatography

50 mg of pepsin-solubilized collagen of *Helix pomatia* were solubilized in 3% acetic acid (4 °C, 24 h) and three times dialyzed against the starting buffer (0.02 M sodium acetate, pH 4.8, containing 1.0 M urea). The solution was heated to 50 °C for 30 min and fractionation on CM 52-cellulose (Nr. 6876, Whatman) was achieved essentially as described by Miller [4] with column dimensions of 1.5 × 10 cm and a linear gradient with 250 ml of starting buffer and 250 ml limiting buffer (0.02 M sodium acetate, pH 4.8, containing 0.12 M NaCl and 1.0 M urea).

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed according to Furthmayr and Timpl [5] with 5.75% gels at 6 mA per tube for 2 h. The gels were stained

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with Coomassie Brilliant Blue R 250 and scanned at 570 nm with a Beckman Scanning Densitometer R-112.

Digestion with CNBr

Samples of 50 mg pepsin-solubilized collagen were treated with 5 mM CNBr in 10 ml 70% formic acid under N_2 at 24 °C for 4 h. The solution was then dried under reduced pressure, redissolved in H_2O and dried under reduced pressure again.

Determination of hydroxyproline

Acetone dried samples of the skin of *Helix pomatia* were hydrolyzed under nitrogen in 6 M HCl at 105 °C for 16 h. Hydroxyproline was determined by the method of Stegemann [6].

Determination of methionine

Dried samples of the skin material were dissolved in 6 M HCl and hydrolyzed under high vacuum at 110 °C for 24, 48, and 72 h, respectively. Afterwards the samples were run on an amino acid analyzer Biocal 200. The conventional program – one column – three buffer system as for protein hydrolyzates was used.

Electron microscopy

The specimen obtained from the skin of the vineyard snail was immediately fixed in 4% glutaraldehyde buffered with cacodylate buffer (pH 7.3) for 2 h followed by 1% osmium tetroxide buffered with cacodylate buffer (pH 7.3) for 90 min, dehydrated in graded concentrations of ethanol and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and then investigated in a Zeiss electron microscope (EM 9 S-2).

Results

Using the method of Stegemann [6] we found that the pepsin-solubilized protein of the skin of *Helix pomatia* contains hydroxyproline, indicating the collagenous nature of this protein.

Polyacrylamide gel electrophoresis shows only one α -chain (Fig. 1). The migration distance was found

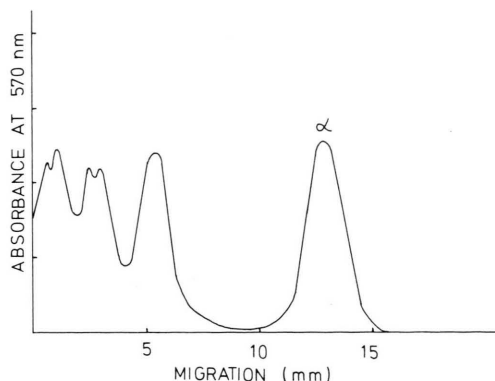


Fig. 1. Polyacrylamide gel electrophoresis of pepsin-solubilized collagen of the skin of *Helix pomatia*.

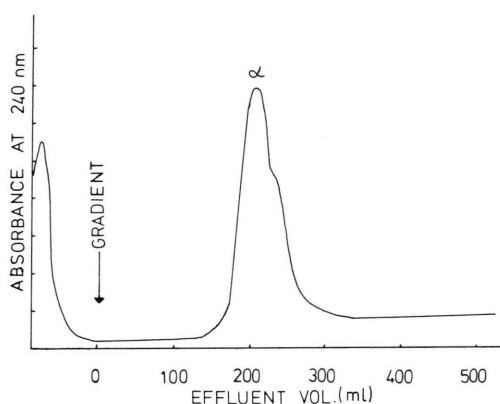


Fig. 2. CM-cellulose elution pattern of pepsin-solubilized collagen of the skin of *Helix pomatia*.

to be identical with that of the $\alpha 1$ -chains of type I or type II collagen of vertebrates, suggesting the same molecular weight. Fig. 2 demonstrates the CM-cellulose elution profile of the pepsin-solubilized *Helix pomatia* collagen. The α -chains of this collagen elute at the same position as the α -chains of type II collagen (Miller [4]).

Digestion of the pepsin-treated *Helix pomatia* collagen with CNBr yields no CNBr-derived peptides. The CNBr-digested collagen of the vineyard snail displays the same polyacrylamide gel electrophoresis pattern as the undigested material shown in Fig. 3.

These results indicate the absence of methionine in the collagen molecules of *Helix pomatia*. This was confirmed by the amino acid analysis which proves

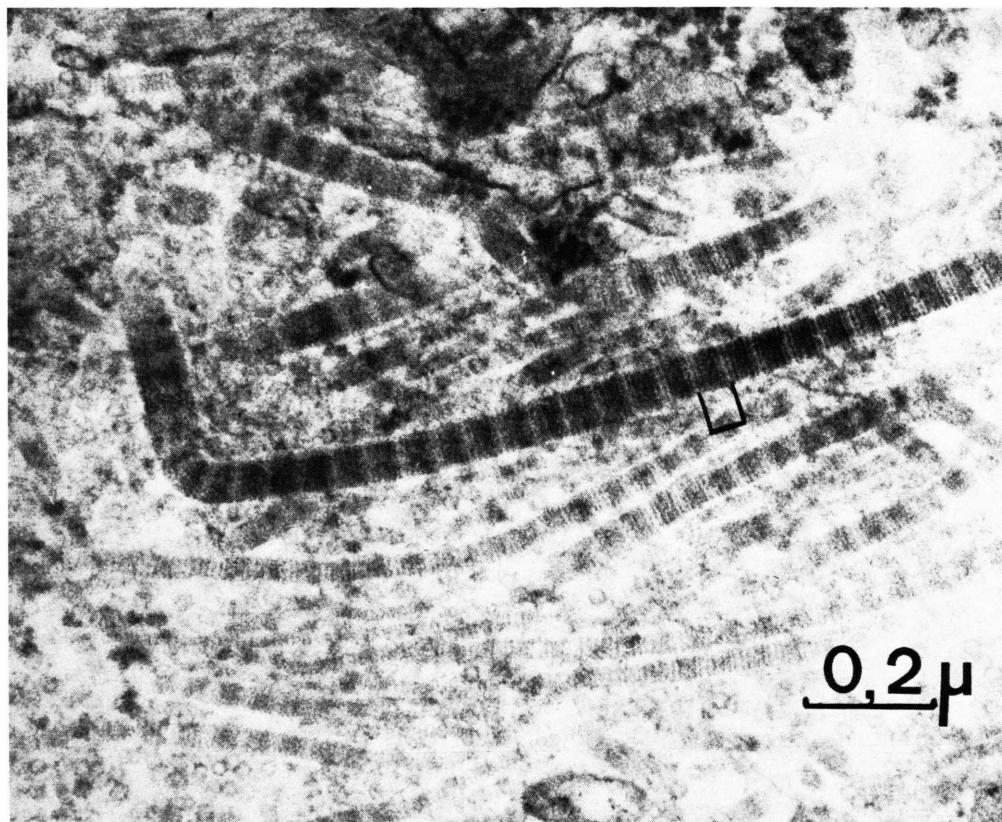


Fig. 3. Electron micrograph of vineyard snail collagen with a characteristic cross striation pattern of 53–57 nm. Magnification 84000 \times .

the absence of methionine in the skin collagen of *Helix pomatia*.

Electron microscopic investigations of the collagen of the vineyard snail show in all sections collagen fibrils which vary in diameter from 25 nm to 70 nm. In spite of the different thickness of the fibrils they all show the characteristic cross striation pattern of 53–57 nm.

Discussion

The protein of the skin of *Helix pomatia* reveals some characteristic features of vertebrate collagens. The hydroxyproline content, the migration behavior in polyacrylamide gel electrophoresis, the elution profile in CM-cellulose chromatography, and the characteristic cross striation pattern of the fibrils visible in the electron microscope display a close relation to vertebrate collagens.

In polyacrylamide gel electrophoresis *Helix pomatia* collagen shows only one α -band similar to types of vertebrate collagens which consist of molecules with three identical α -chains.

Therefore, we intended to compare *Helix pomatia* collagen with common vertebrate collagen types.

The α -chains of collagen can be cleaved at methionyl bonds by cyanogen bromide (Gross and Witkop [7]). As the α -chains of collagen contain only relatively few methionyl bonds, a defined number of CNBr-derived peptides can be obtained upon cleavage with CNBr (Bornstein and Piez [8]). In polyacrylamide gel electrophoresis these collagen peptides can be separated and each collagen type yields a characteristic peptide pattern in polyacrylamide gels. However, it was not possible to obtain CNBr-derived peptides from pepsin-solubilized collagen of *Helix pomatia* skin. Under the conditions which cleave vertebrate collagens, the α -chain of *Helix po-*

matia collagen remains stable, and in polyacrylamide gel electrophoresis the CNBr digest shows the undegraded α -chain. Therefore, we concluded that *Helix pomatia* collagen contains no methionine, which is susceptible to CNBr-cleavage. This was confirmed by amino acid analysis which revealed

that methionine was absent in the collagen molecule of *Helix pomatia*.

Our investigations suggest that despite of some characteristic similarities, *Helix pomatia* collagen does not represent anyone of the hitherto analyzed collagen types of vertebrates.

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