Journal of Chromatography, 426 (1988) 162–168
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4058

Note

Column liquid chromatographic separation of collagen α-chain polymers on sepiolite

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(Received November 11th, 1987)

Collagen, the main component of connective tissue, consists of a family of closely related but genetically distinct proteins; of these, the so called types I and III, which occur in skin and the former also in bones, are the most common [1]. Each molecule of these two types is composed of three polypeptide chains wound in a triple helical conformation. In tissues, individual polypeptide chains undergo polymerization reactions which, after denaturation, lead to the occurrence of α-chain dimers (β-chain), trimers (γ-chain) and even more polymerized fractions (δ-polymers). The proportion of non-polymerized and polymerized collagen α-chains changes, e.g. with ageing [2], in favour of the polymerized chains. Also, there are pathological situations in which the final degree of collagen polymerization is either increased (diabetes) [3] or decreased (lathyrism [4], dermatosparaxis [5]). The determination of the proportion of collagen polymers is therefore important in studies of the ethiopathogeny of these disorders and for the evaluation of connective tissue ageing.

Currently the most common methods of determining the proportion of individual collagen chain polymers are polyacrylamide gel electrophoresis [6,7] and gel permeation chromatography [8]. Each of these approaches has its advantages and drawbacks. Polyacrylamide gel electrophoresis in both acidic and alkaline buffers offers a good possibility of differentiating between α_1(I), α_2(I), α_1(III) and β-polypeptide chains; the fraction of higher polymers hardly penetrates the 7.5% polyacrylamide gel, rendering quantitation impossible. Gel permeation chromatography on agarose (Bio-Gel A-5 m) offers a good resolution of higher polymers from the α-fraction, but the resolution between γ- and δ- polymers is usually poor. Another disadvantage is the time needed for such a separation, which may well extend to several days. On the other hand, the amount of the protein

0378-4347/88/$03.50

that can be separated can be easily 50 mg. Evidently these procedures are favoured for preparative proposes.

There is scattered information in the literature on separating collagen polymers by high-performance procedures [9,10]; thus collagen monomers, dimers and trimers (the last type being constituted mainly from disulphidically bonded type III α-chains) can be separated successfully on Separon, a styrene–divinylbenzene polymer within 30 min [9]. Alternatively, it is also possible to use reversed-phase chromatography on a macroporous C_{18} silica gel [10], with which about 50 min are needed to separate α-chain monomers, dimers, and trimers. Fallon et al. [11] used a cyanopropyl-bonded sorbent for the separation of human type I, II and III collagens; the separation, however, was incomplete and could not separate polymers beyond collagen α-chain trimers. Similar separations were obtained with phenyl-bonded phases (Bakerbond diphenyl [12]) and a linear gradient of 0.1% trifluoroacetic acid–acetonitrile within less than 1 h. Again, the separation of individual α-chain polymers was incomplete and no attempts to separate α-chain polymers higher than trimers were reported. By using C_{18} PEP-RPC HR 515 (Pharmacia) reversed phase (0.1% trifluoroacetic acid–acetonitrile gradient), Bateman et al. [13] were able to obtain an incomplete separation of α-chain monomers up to trimers (including type III collagen); no resolution of higher polymers was observed. However, this method is suitable for the separation of collagen cyanogen bromide fragments or peptides obtained by trypsin digestion.

A recent report [14] describing the sorption properties of sepiolite, a magnesium silicate with only a minor aluminium component, prompted our efforts to use this material as a sorbent for a better chromatographic resolution of collagen chain polymers (including α-chain polymers higher than trimers). It was demonstrated that type I collagen from calf skin interacts with magnesium silicate with the formation of a collagen–clay complex that can be separated by centrifugation. This interaction occurs primarily with high-molecular-mass aggregates of collagen, as lathyritic collagen, which is devoid of α-chain polymers, interacts to a lesser extent.

EXPERIMENTAL

Chemicals

Sepiolite (magnesium silicate with only a minor aluminium component) was purchased from Tolsa (Madrid, Spain). Pepsin (recrystallized twice) was a product of Worthington Biochemical (NY, U.S.A.). Other chemicals, i.e., calcium chloride, sodium chloride, sodium hydroxide, urea, sodium acetate, disodium ethylenediaminetetraacetate, N-ethylmalimide and α-toluenesulphonyl fluoride, were of the highest available purity and were purchased from Merck (Darmstadt, F.R.G.). Bio-Gel A-5 m (200–400 mesh) was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Preparation of the sorbent

Sepiolite was first sieved through a 200–400-mesh sieve. The fraction passing through was suspended in 0.1 *M* acetate buffer (pH 3.4)–sodium acetate and left

overnight with gentle stirring. The heavy particles were left to settle and the supernatant liquid was decanted and the minor particles were collected by centrifugation at 1000 *g* for 15 min. The operation was repeated and the suspension was slurry packed (final overpressure 13 700 mPa) into a 150×6 mm I.D. stainless-steel column. After being packed, the column was conditioned by pumping acetate buffer (pH 3.4) through it for 24 h. If necessary, additional sorbent was added after this period and the column was conditioned for a further 3 h.

Collagen samples

Acid-soluble rat tail tendon collagen and fractions of the pepsin-solubilized human skin collagen were used for testing the ability of sepiolite to separate α-chain polymers. Acid-soluble rat tail tendon collagen was prepared by established methods [15]. Samples of human skin were obtained from healthy adult human subjects who had undergone various surgical procedures. The subcutaneous tissue was removed and the specimens were rinsed with 0.15 *M* sodium chloride–0.05 *M* Tris–HCl buffer (pH 7.5) at 4°C. The skin samples were then extensively minced with scissors in 0.5 *M* acetic acid (10 g of tissue per 100 ml) and homogenized with a Polytron mechanical tissue homogenizer in a cold-room. Pepsin was added to a final concentration of 100 μg/ml and the samples were incubated at 4°C for 16 h; at the end of the incubation the homogenate was centrifuged at 30 000 *g* for 60 min at 4°C. The pellet was dissolved in 0.5 *M* acetic acid and subjected to a second enzymic cleavage as described above. The pooled supernatants were adjusted to pH 8.5 with cold 1.0 *M* sodium hydroxide solution and dialysed extensively against 0.4 *M* sodium chloride–0.1 *M* Tris–HCl (pH 7.5). In order to separate genetically distinct collagen types and obtain samples with different proportions of higher α-chain polymers, the solubilized collagen was fractionated by sequential precipitations with 1.5, 2.5 and 4.5 *M* sodium chloride solution [16]. After the slow addition of solid sodium chloride to any given concentration the samples were stirred for 24 h at 4°C and centrifuged at 30 000 *g* for 60 min. The pellet was dissolved in and dialysed against 0.5 *M* acetic acid and then lyophilized.

Chromatographic procedures

Separation by sepiolite chromatography. For this separation a Toyo Soda (Tokyo, Japan) high-performance liquid chromatography (HPLC) system consisting of an AS-48 autosampler, a twin-pump 803D solvent-delivery system and a Model II variable-wavelength ultraviolet detector set at 220 nm, linked to a CP-8000 system controller and a data print-out device was used. The column was a stainless-steel 150×6 mm I.D. main column packed with sepiolite particles (15–35 μm long with a diameter of 1–3 μm). The mobile phase was 0.1 *M* sodium acetate buffer (pH 3.4). Lyophilized collagen samples were dissolved by stirring; samples were heated briefly to 100°C, cooled, centrifuged at 18 000 *g* and 10-μl aliquots were applied to the column.

The flow-rate was maintained at 1.0 ml/min, the operating pressure was about 13 700 mPa and the separation was carried out within 25 min.

Separation by agarose chromatography (comparative experiments). To prepare

collagen samples for agarose gel permeation chromatography, 50 mg of lyophilized sample were dissolved by stirring in 0.05 *M* Tris–HCl buffer (pH 7.5) containing 10 mol urea, 20 mmol of disodium ethylenediaminetetraacetate, 10 mmol of N-ethylmaleimide and 1 μmol of α-toluenesulphonyl fluoride per litre. The sample was heated for 5 min to 100 °C, centrifuged at 18 000 *g* for 10 min at 22 °C and subjected to gel permeation chromatography on a 90×2.5 cm I.D. column of 6% agarose (Bio-Gel A-5 m, 200–400 mesh), eluted with 1 *M* calcium chloride–0.05 *M* Tris–HCl (pH 7.5) at room temperature. Separation was carried out at a flow-rate of 20 ml/h and the whole run was completed within 0.5 h. The eluent was monitored on a Pye Unicam 8-200 spectrophotometer using an 80-μl flow-through cuvette.

Quantitation

Quantitation was effected by integrating the peak area (220 nm absorbance). It was assumed that the molar absorption of isolated α-chains is not different from that of α-chain polymers. The results were expressed as percentages of α-chain polymers in a particular sample.

RESULTS

The separation of acid-soluble collagen and collagen fractions isolated by pepsin digestion and subsequent precipitation with 2.5 and 4.0 *M* sodium chloride on a sepiolite-packed column revealed a good separation of individual collagen α-chain polymers (Fig. 1). Attempts to separate genetically distinct α-chains (and hence α-chain polymers) were unsuccessful. Also, if 1% cetylpyridinium chloride was added to the mobile phase and if the sample before application was pre-incubated with the detergent, the separation efficiency on the sepiolite column was lost. The same occurred if the separation was carried out in the presence of 4 *M* urea (data not shown). With cetylpyridinium chloride it was virtually impossible to re-establish the separation efficiency of the column. The identity of individual peaks was assayed by agarose chromatography; accumulated samples from the sepiolite separation were dialysed against the Tris–HCl buffer (pH 7.5) (see Experimental) and processed accordingly before they were applied to the agarose column. The behaviour of α-chains and α-chain polymers was very regular; in sepiolite chromatography α-chains were eluted first, followed by β- and γ-chains, the fraction of higher polymers being eluted last. Naturally, in agarose separation the sequence was reversed, thus making the identification of individual fractions very reliable.

If quantitated by peak-area integration, our samples of acid-soluble collagen showed 60.0±1.4% of α-chains, 33.0±1.8% of β-chains, 5±0.7% of γ-components and 2.4±0.3% of higher-chain polymers (average from six assays on acid-soluble rat tail tendon collagen). This is in a very good agreement with the results of Chandrakasan et al. [17].

The recovery of collagen α-chains and their polymers was 96.5±1.2% ($n=3$) with sepiolite, compared with 94.3±1.8% ($n=3$) obtained with agarose. Recoveries were based on the comparison of absorbance of the introduced sample (230

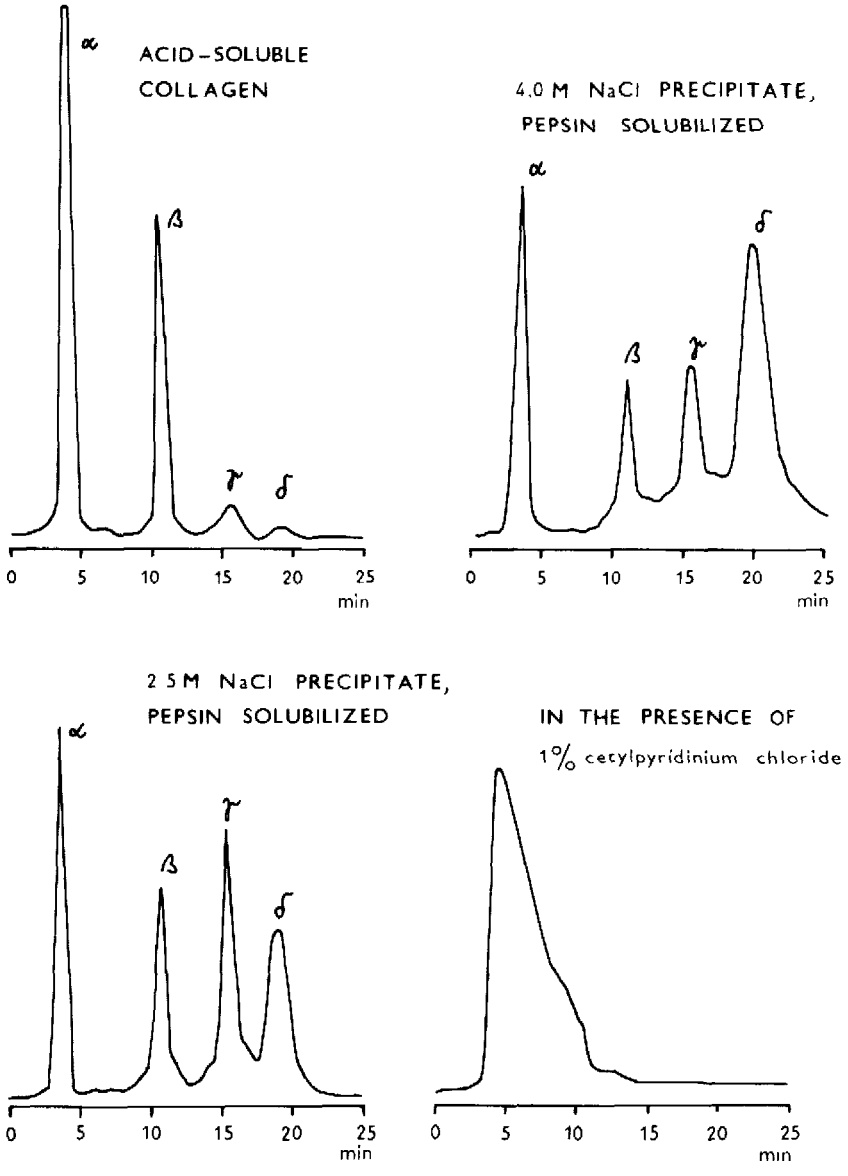

Fig. 1. Separation of acid-soluble collagen and solubilized insoluble collagen fractions on sepiolite. The bottom-right panel shows the effect of a surfactant.

nm) and with that of the sum of the eluted fractions. Calibration graphs for pure α-chain and collagen chain polymers were linear over the range 50–200 μg; the inter-assay coefficient of variation was $3.2 \pm 0.04\%$ ($n=10$).

DISCUSSION

The intriguing question to be discussed with regard to our results is the nature of the collagen–sepiolite interaction. Sepiolite is a fibrous clay mineral consisting

of talc-like ribbons, each linked to four identical ribbons to enclose longitudinal channels. Open channels run along the crystal on its surface and can accommodate exchangeable water molecules or ions. From previous studies [14], it emerges that the diameters of a rod-shaped collagen molecule and also the sepiolite channels are such that the interaction between the protein molecule and the clay can occur within these channels. It was suggested that large molecular aggregates are likely to be bound preferentially to the sorbent channels as the number of exchangeable groups is higher than in a single collagen molecule and the polymeric protein structures can be accommodated more readily in the channels of the sorbent. If the collagen polypeptide chains are denatured in the presence of cetylpyridinium chloride, the interaction with sepiolite is abolished and all material applied to the column emerges with the void volume. If in a subsequent run a sample devoid of cetylpyridinium chloride is applied to the column, the separation efficiency of the column is not recovered and it is virtually impossible to re-establish the column separation efficiency even after prolonged washing. From these results it is possible to conclude that the surfactant has a high affinity for those sites that are responsible for the sorption of collagen molecules in the sepiolite clay.

High urea concentrations (4 M) also abolish the separation efficiency of the column with similar consequences as for cetylpyridinium chloride, although in this instance it is possible to re-establish the separation efficiency of the column.

It is therefore possible to conclude that in order to separate collagen α-chain polymers effectively on the sepiolite clay, the proteins can be denatured but be present in a media from which renaturation is relatively easy; it appears feasible to assume that strong chaotropic agents prevent the accommodation of the collagenous molecules on the fibrous clay, thereby preventing the separation of α-chain polymers.

The present procedure is superior to those published previously [9–13] in offering a baseline separation of individual α-chain polymers and in separating higher-chain polymers than trimers. As indicated, the baseline separation of polymeric collagen α-chains offers a good possibility of quantitating the amount of these polymers present in pepsin-solubilized collagen structures and of relating these changes to physiological or pathological processes occurring in the extracellular matrix.

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