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Note

High-performance gel permeation chromatography of collagens

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Gel permeation chromatography appears to be a widely used tool in the characterization of different collagen polypeptide chains [1,2]. Considerable progress in the efficiency of separation of interstitial collagens was achieved by introducing reversed-phase high-performance liquid column chromatography (HPLC). Fallon et al. [3] used bonded cyanopropyl support columns for the separation of human type I, II and III collagens. In a previous report [4] we attempted to separate collagen type I and III polypeptide chains by means of HPLC using Separon HEMA 1000 Glc gel. Though a good quality of separations was achieved, the process was not governed solely by gel permeation as long as molecular entities of identical relative molecular mass were separated. This may be of advantage in the separation of certain collagen mixtures, but causes considerable difficulties when gel permeation separations are used for the investigation of complex mixtures of different collagen polypeptide chains and their fragments. We attempted, therefore, to abolish the secondary interactions during separation as much as possible and to establish a high efficiency procedure in which gel permeation would be the only mechanism involved.

EXPERIMENTAL

Chromatographic techniques

Chromatography was carried out using a Pye-Unicam liquid chromatograph LC 20 equipped with UV detector LC 3 set at 230 nm. A stainless-steel column, 500 × 8 mm, packed with Separon HEMA 1000 Glc (12– 17 μm; Laboratory Instrument Works, Prague, Czechoslovakia) was used. The apparatus was operated at a flow-rate of 1.5 ml/min (1.5 MPa overpressure). The sensitivity of the detector was set at 0.04, chart speed was 0.25 cm/min. The whole separation lasted less than 30 min. Several solvent systems were checked to obtain maximum separation according to relative molecular mass of individual polypeptide chains. Finally, isocratic elution with a solution containing 0.2 M NaCl–2M urea–0.05 M Tris · HCl buffer (pH 7.5) was used.

Collagen standards

Samples of individual types of collagen polypeptide chains were prepared by established methods from rat skin, bovine renal glomerular basement membrane and mouse EHS sarcoma. Detailed procedures can be found in the original literature listed in Table I.

In order to avoid problems arising from the high UV absorbancy of mercaptoethanol in UV light, S—S bonds were cleaved, where necessary, with concentrated formic acid, and to that 30% hydrogen peroxide was added to a final concentration of 2%. The reaction mixture was left for 2 h at room temperature and then loaded onto the column.

Individual isolated collagen polypeptide chains were checked for purity by their amino acid composition [10], sodium dodecyl sulphate (SDS)—polyacrylamide slab gel electrophoresis [11], and those originating from basement membranes also by immunoprecipitation test [12].

RESULTS AND DISCUSSION

The applicability of the present procedure is demonstrated by the following examples. Clear-cut separations are obtained with α-chain polymers (Fig. 1) and rapid information can be obtained about S—S bond cleavage in collagen type III (Fig. 2). The separation efficiency is sufficient to distinguish between α_1 (IV) and α_2 (IV) collagen polypeptide chains, a result that has not before been visualized by gel permeation chromatography (Fig. 3).

A general picture of the retention times of individual collagen species and some of their fragments is given in Table I. The retention times decrease with increasing relative molecular mass; however, the decrease is not strictly linear in the logarithmic scale as would be expected (Fig. 4). No separation of collagen polypeptide chains of identical relative molecular mass but originating from different collagen types was observed. It has been communicated before that sorption plays an important role in high-efficiency gel permeation chromatography [4]. Indeed, in our previous communications we have observed the separation of, for example, α_1 (I) and α_1 (III) on the same sorbent that has been used in the present experiments when isocratic elution with 0.05 M Tris · HCl, pH 7.5 (2 M with respect to urea) was used. This was ascribed either to differences in
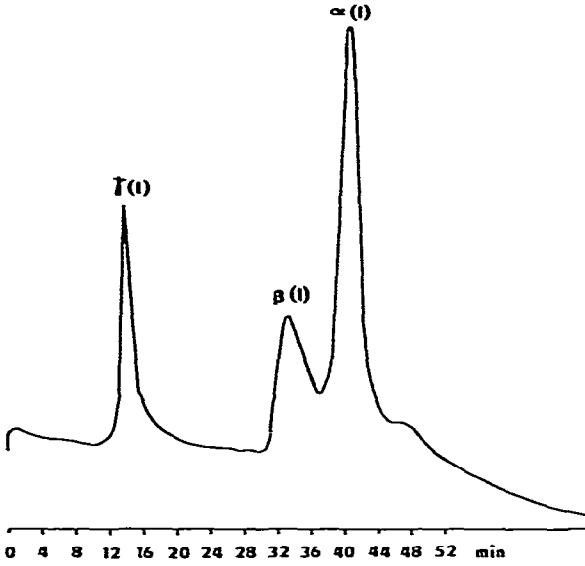
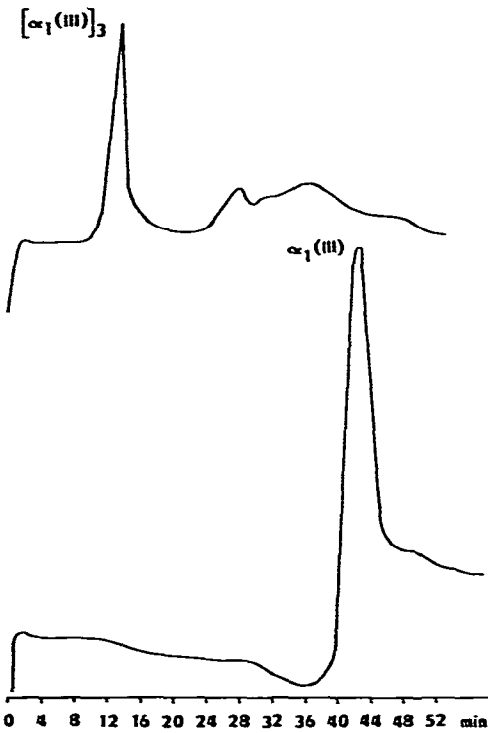Fig. 1. HPLC separation of collagen type I α-chain polymers.

Fig. 2. Chromatographic behaviour of collagen type III preparation before (upper panel) and after (lower panel) S—S bond cleavage.
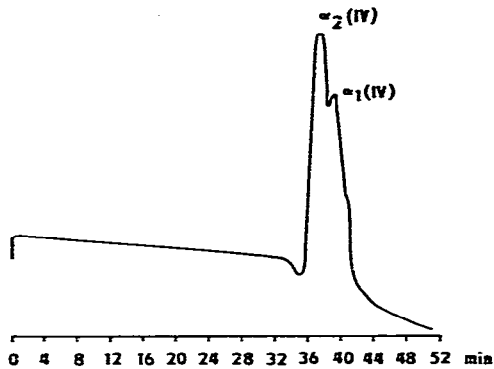
Fig. 3. Separation of α_1 (IV) and α_2 (IV) collagen polypeptide chains.

TABLE I

RETENTION TIMES OF DIFFERENT COLLAGEN CHAINS, THEIR POLYMERS AND FRAGMENTS

Retention times represent data obtained from five independent runs of the same preparation. The α_1(I) polypeptide chain served as internal standard.

Type of collagen chain	Relative molecular mass	Retention time (min)	Principle of preparation	Literature reference (description of the preparation procedure)
1 α_1(I), α_1(II), α_1(III)	100,000	42.0	Rat skin, cartilage and calf skin, limited pepsin digestion	2
2 α_1(IV)	140,000	38.5 }	Mouse tumor and human placenta, limited pepsin digestion	5
3 α_2(IV)	160,000	37.0 }		
4 β(I)	200,000	34.0 }	Human placenta, limited pepsin digestion	6
5 γ(I)	300,000	14.0 }		
6 $[\alpha_1(III)]_3$				1
7 α_1(IV) BM	160,000	37.0 }	EHS tumor, limited pepsin digestion	7
8 α_2(IV) BM	180,000	34.5 }		
9 C_1 fragment	120,000 (110,000 to 140,000)	41.0	Minces of whole placental tissues, limited pepsin digestion	8
10 C fragment	95,000	43.0		
11 50 K fragment	50,000	50.0		
12 α_{1-3}(V)	110,000	41.5	Human placenta, limited pepsin digestion	9
13 7 S	360,000	4.0 }	Mouse tumor basement membrane, limited pepsin digestion	6
14 7 S coll	225,000	29.0 }		

hydrodynamic volumes of matching collagen polypeptide chains originating from different species [1], or adsorption and/or partition interactions of the separated protein molecules [2], or, finally, to the possible weak affinity of
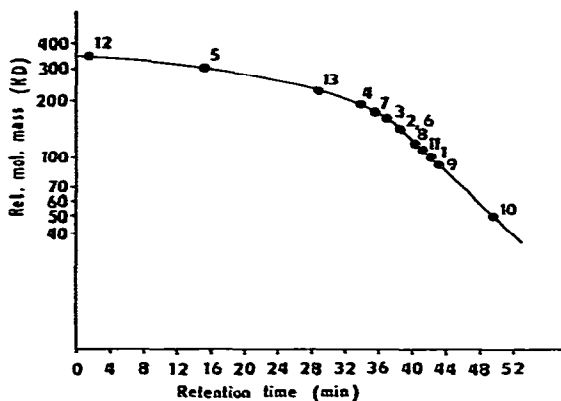
Fig. 4. Retention time vs. relative molecular mass relation for different polypeptide chains of the collagen family. Numbers correspond to listing of individual polypeptides in Table I.
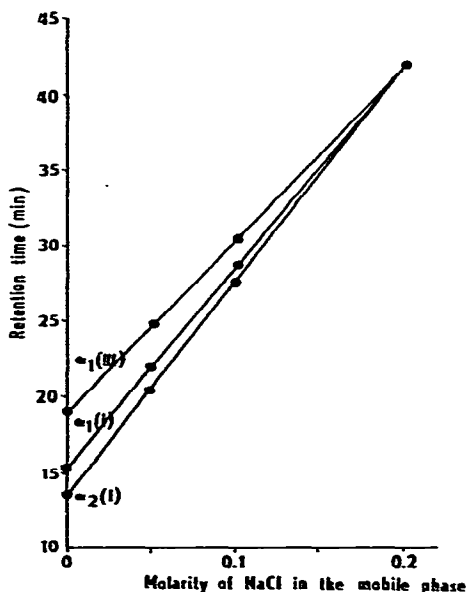

Fig. 5. Changes in retention of $\alpha_1(I)$, $\alpha_2(I)$ and $\alpha_1(III)$ with increasing NaCl molarity in the mobile phase. (Note that the three separated collagen α-chains are of identical relative molecular mass.)

collagen chains to the glucose-coated macroporous adsorbent causing respective retention differences of otherwise similar molecules.

It is now evidenced (Fig. 5) that an increase in ionic strength of the eluant is capable of completely abolishing the interspecies differences at least between collagen I, III, IV and V. The same result can be achieved either by adding sodium chloride to the eluting solvent or by increasing the concentration of the Tris buffer to 0.5 M. The separation conditions can be selected in such a way that gel permeation is the only mechanism governing the separation. Still the non-linearity of the retention time vs. log mol. wt. relation indicates that some other effects, though minimalized, still persist throughout the separation effected on Separon HEMA 1000 Glc.

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