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# SEPARATION OF TYPE IX COLLAGEN FROM OTHER CARTILAGE COLLAGENS BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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## SUMMARY

Collagen type IX was separated from other cartilage collagens (types II and XI) by hydrophobic interaction chromatography on a 25 cm×8 mm I.D. stainless-steel column packed with Separon HEMA 1000 Bio. The mobile phase was 0.84 *M* ammonium sulphate with 0.1 *M* potassium dihydrogenphosphate (pH 6.5). Under these conditions only collagen type IX was eluted from the column; it could be monitored with UV detection (218 nm) or selectively with fluorescence detection (excitation 330 nm, emission filter 389 nm). The method can be used for the isolation and quantitation of collagen type IX. The assay was linear in the range 0–10 μg, the correlation coefficient was 0.99, precision 5.5% and accuracy 13%. The detection limit was about 0.6 μg.

## INTRODUCTION

The collagens are major structural proteins of the extracellular matrix. Twelve genetically different collagen types have been described [1] and some new types are currently under extensive study. The existing high-performance liquid chromatographic (HPLC) methods for the separation of individual collagen types are focused mainly on types I, II and III and the prevailing mode of separation is reversed-phase HPLC [2–6].

Type IX collagen was isolated from chicken and mammalian cartilage in the form of fragments of high and low relative molecular mass (HMW and LMW) [7,8]. The isolation of these fragments from cartilage involves extraction with 4 *M* guanidine chloride, limited pepsin digestion and fractional salt precipitation in order to separate different collagen types [7]. The collagens are not precipitated quantitatively and the technique is very time-consuming and therefore not suitable for the determination of type IX collagen in the presence of other cartilage collagens. Therefore, we investigated the separation of HMW and/or LMW from other collagen types by a chromatographic technique.

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As type IX collagen contains fluorescence cross-link pyridinoline (derived from lysine and hydroxylysine) located in the HMW fragment [9,10] we compared UV with fluorescence detection.

## EXPERIMENTAL

### *Materials*

All chemicals were of analytical-reagent grade and were obtained from Lachema (Brno, Czechoslovakia). Collagens were solubilized from bovine and chicken embryonal cartilage by limited pepsin digestion and types II, XI and IX (fragments LMW and HMW) were obtained by fractional salt precipitation as usual [7]. Collagens were dissolved in 0.5 *M* acetic acid before chromatography.

The HPLC mobile phase was filtered through a 0.45-$\mu$m filter (Supelco, Bellefonte, PA, U.S.A.) and degassed under helium.

### *Apparatus*

The chromatographic equipment was obtained from Spectra-Physics (San Jose, CA, U.S.A.). An SP-8100 liquid chromatograph equipped with SP-8110 autosampler was connected with an SP-8440 variable-wavelength UV–VIS detector or an SP-970 fluorimetric detector. An SP-4200 computing integrator was used for plotting the chromatograms. For some experiments a Series 410 LC Bio pump from Perkin-Elmer (Norwalk, CT, U.S.A.) was used with an LC-90 Bio spectrophotometric detector and an LCI-100 computing integrator.

### *Chromatography*

A 25 cm $\times$ 8 mm I.D. stainless-steel column packed with Separon HEMA 1000 Bio (particle size 10 $\mu$m) (Laboratorní Přístroje, Prague, Czechoslovakia) was used. The mobile phase was 0.84 *M* ammonium sulphate with 0.1 *M* potassium dihydrogenphosphate (pH 6.5). A flow-rate of 0.6 ml/min generated a pressure of 4 MPa. The column temperature was ambient (ca. 25 °C). The column effluent was monitored at 218 nm with the UV detector or with the fluorescence detector using excitation at 330 nm, a 389-nm emission filter, a 7-54 excitation filter and a time constant of 2 s. The volume of the injection loop was 10 $\mu$l.

## RESULTS AND DISCUSSION

### *Chromatography*

The column was packed with Separon HEMA 1000 Bio hydrophilized polymeric hydroxyethylmethacrylate. At low concentrations of ammonium sulphate (up to 0.5 *M*) the column acts as a normal size-exclusion column (Fig. 1) and collagens are eluted with almost 100% recovery. The relative molecular mass of type II and XI collagen is approximately 300 000, whereas those of HMW and LMW fragments of type IX are 153 000 and 57 000, respectively. Therefore, types II and XI are not resolved on this column.

By increasing the concentration of ammonium sulphate above 0.5 *M* the peak areas of types II and XI begin to decrease and at 0.84 *M* ammonium sulphate the peaks of both collagen types disappear completely (Fig. 2A). Only HMW and

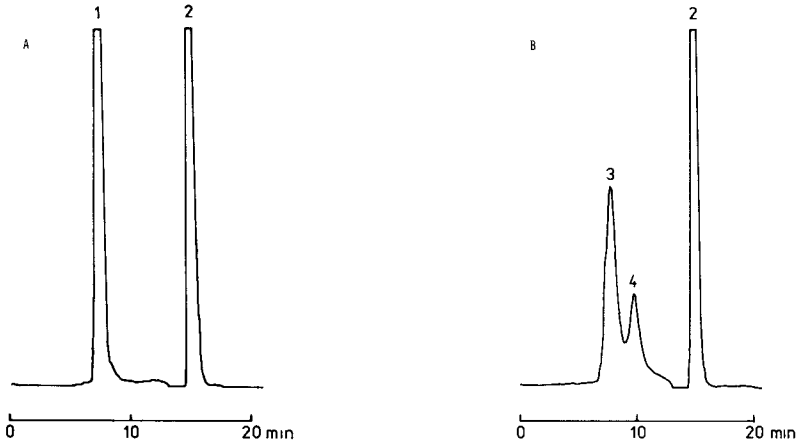


Fig. 1. Chromatography with a mobile phase composition of 0.1 *M* $KH_2PO_4$ (pH 6.5). Detection, UV (218 nm). (A) Chromatogram of type II and XI collagens. (B) Chromatogram of HMW and LMW fragments of type IX collagen isolated from bovine cartilage. Peaks: 1 = type II and XI collagen; 2 = acetic acid; 3 = HMW fragment of type IX collagen; 4 = LMW fragment.

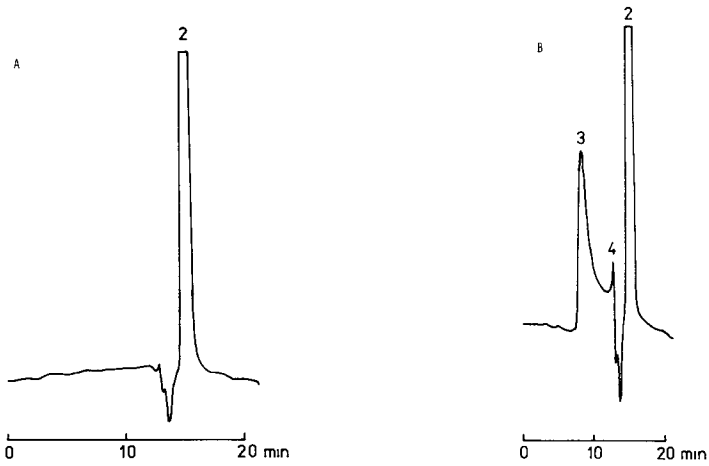


Fig. 2. Chromatography with a mobile phase composition of 0.84 *M* $(NH_4)_2SO_4$ with 0.1 *M* $KH_2PO_4$ (pH 6.5). Detection, UV (218 nm). (A) Chromatogram of type II and XI collagens. Both types are retained on the column. (B) Chromatogram of HMW and LMW fragments of type IX collagen isolated from bovine cartilage. Peaks: 2 = acetic acid; 3 = HMW fragment of type IX collagen; 4 = LMW fragment.

LMW are eluted from the column (Fig. 2B). At 1 *M* ammonium sulphate all cartilage collagens are retained on the column. This behaviour was independent of the source of the collagens; only small differences in retention times were found for mammalian and chicken embryonal cartilage, probably owing to differences in molecular size (results not shown).

There was only a slight increase in retention times with increasing concentration of the salt, from 7.5 to 7.8 min for type II collagen (0–0.5 *M* ammonium sulphate) and from 7.8 to 8.5 min and 9.8 to 13 min for HMW and LMW fragments, respectively (0–0.84 *M* ammonium sulphate). These findings suggest that

the interactions of collagen molecules with the support are mainly irreversible at high concentrations of ammonium sulphate and precipitation occurs. The different retention behaviour of collagen type IX can be explained by the hydrophilicity of this type caused by bonded chondroitin sulphate chains [11].

It must be noted that the sorbent used is intended for high-performance size-exclusion chromatography of proteins and is hydrophilized in order to minimize hydrophobic interactions. We also tried the relatively hydrophobic sorbent Separon HEMA 1000, but it retained all collagen types non-selectively at 0.4 *M* ammonium sulphate.

The attempt to separate collagens by using a decreasing gradient of ammonium sulphate concentration was unsuccessful, because adsorbed types II and XI eluted as very broad peaks. Therefore, the chromatography was performed isocratically and adsorbed collagen types II and XI were removed from the column overnight using the mobile phase without ammonium sulphate.

*Quantitation*

We developed a rapid, isocratic method for the determination of collagen type IX based on the chromatographic behaviour mentioned above. Type IX was separated from types II and XI at 0.84 *M* ammonium sulphate. A calibration graph based on peak heights of HMW was constructed. It was linear in the range 0–10 $\mu$g, the correlation coefficient was 0.99, precision 5.5% and accuracy 13%. The detection limit was about 0.6 $\mu$g.

Mixtures of cartilage collagens can be analyzed directly without any sample preparation even in solutions containing high concentrations of salts. The ions and other interferences with low relative molecular mass are separated from collagens by a size-exclusion mechanism and are eluted from the column in the void volume (13.5 min) or later if an interaction with the support occurs.

*Fluorescence detection*

The chromatogram of collagen type IX obtained with fluorescence detection is shown in Fig. 3. Only HMW was detected, because LMW does not contain fluorescence cross-link pyridinoline [10].

The chromatogram was cleaner and detection more selective, but the sensitiv-

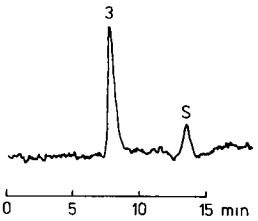


Fig. 3. Chromatogram of HMW and LMW fragments of type IX collagen isolated from bovine cartilage. Mobile phase, 0.1 *M* $KH_2PO_4$ (pH 6.5). Detection, fluorescence with excitation at 330 nm, 389-nm emission filter, 7-54 excitation filter. Peak 3 = HMW fragment of type IX collagen; S = system peak.

ity was worse because one molecule of collagen contains on average only one molecule of pyridinoline.

## CONCLUSIONS

A rapid, isocratic method for the determination of type IX collagen by hydrophobic interaction chromatography has been developed. High concentrations of salts in samples do not disturb the separation and the sensitivity of the method is satisfactory. At low concentrations of ammonium sulphate the columns acts as a size-exclusion column and may give useful information about the relative molecular mass of the sample. The method could also be used for semi-preparative purposes as several miligrams of sample can be loaded in a single run.

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