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Note

Continuous-flow analysis of proteoglycans during gel filtration using guanidine hydrochloride as an eluent

BHANDARU RADHAKRISHNAMURTHY*, NEIL JEANSONNE and GERALD S. BERENSON
Departments of Medicine and Biochemistry, Louisiana State University Medical Center, 1542 Tulane Avenue, New Orleans, LA 70112 (U.S.A.)
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Gel filtration is one of the procedures used for the study of molecular sizes of proteoglycan subunits and aggregates[1]. The technique has also been used for the preliminary fractionation and purification of proteoglycans from crude extracts of tissues by 4.0 *M* guanidine hydrochloride[2,3]. Fractions of gel filtration are monitored for proteoglycans usually by uronic acid analysis from a carbazole reaction[4,5]. As salts interfere in the analysis, the fractions have to be dialyzed exhaustively against distilled water or a low ionic concentration of sodium acetate prior to analysis. The dialysis of fractions and manual analysis of uronic acid are time consuming.

This report describes a procedure using an orcinol–sulfuric acid reaction in the Technicon sugar chromatography system. A continuous-flow analysis of proteoglycans during elution by guanidine hydrochloride solutions from gel filtration columns was developed, and its application to studies of proteoglycans under associative and dissociative conditions is described.

EXPERIMENTAL

Materials

Sepharose CL-4B was purchased from Pharmacia (Piscataway, NJ, U.S.A.). Orcinol was obtained from Nutritional Biochemical Corp. (Division of ICN, Cleveland, OH, U.S.A.), reagent-grade sulfuric acid from DuPont (Wilmington, DE, U.S.A.) and guanidine hydrochloride from U.S. Biochemical Corp. (Cleveland, OH, U.S.A.).

Protease inhibitors, ε-aminocaproic acid, N-ethylmaleimide, ethylenediaminetetraacetic acid (EDTA) and *p*-chloromercuricbenzoic acid were purchased from Sigma (St. Louis, MO, U.S.A.) and benzaminidine hydrochloride from Aldrich (Milwaukee, WI, U.S.A.).

Glucuronolactone used as a standard was obtained from Corn Products Refineries (New York, NY, U.S.A.). Bovine nasal cartilage proteoglycan aggregate (A_1) and subunit (A_1D_1) were available in the laboratory from other studies.

Extraction of proteoglycans

Proteoglycans were extracted from bovine nasal cartilage by 4.0 *M* guanidine

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hydrochloride in 0.05 *M* sodium acetate (pH 6.5) in the presence of protease inhibitors, 0.1 *M* ε-aminocaproic acid, 0.005 *M* N-ethylmaleimide, 0.005 *M* benzamidine hydrochloride, 0.01 *M* EDTA and 0.001 *M* *p*-chloromercuricbenzoic acid[6]. Extraction was carried out with stirring at 4°C for 48 h. The extract was clarified by centrifugation at 6000 *g*. A portion of the supernatant was dialyzed against 9 volumes of 0.05 *M* sodium acetate (pH 6.5) containing protease inhibitors for 16 h at 4°C to facilitate proteoglycan aggregation. The dialyzed extract was fractionated by gel filtration on a Sepharose CL-4B column using 0.4 *M* guanidine hydrochloride in the presence of protease inhibitors as eluent.

To the proteoglycan aggregate eluted from the column in the excluded volume solid guanidine hydrochloride to a concentration of 4.0 *M* and protease inhibitors were added, and the solution was allowed to stand at 4°C for 16 h. The dissociated proteoglycans were fractionated on Sepharose CL-4B column using 4.0 *M* guanidine hydrochloride as eluent.

Electrophoresis

Electrophoresis of proteoglycans was performed on cellulose acetate strips in pyridine–formate buffer as previously described[7]. The proteoglycans were isolated on the strips by staining with alcian blue.

Analytical methods

The carbazole reaction of uronic acids was performed by the procedure of Dische[4] and the orcinol–sulfuric acid reaction by the manual procedure according to Kesler's[8] method.

Gel filtration

Gel filtration of proteoglycans was performed by a previously described automated procedure using the Technicon sugar chromatography system[9]. A chromatographic column (100 × 1.5 cm I.D., 3400 series; Glenco Scientific, Houston, TX, U.S.A.) filled with Sepharose CL-4B was used. Samples of proteoglycans containing 100–200 μg of uronic acids were injected into the column and eluted with solutions of 0.4 or 4.0 *M* guanidine hydrochloride containing protease inhibitors. The rate of elution (0.7 ml/min) was kept constant by negative pressure, *i.e.*, by passing the column outlet tube through the proportioning pump manifold of the AutoAnalyzer before connecting it to the manifold sample inlet[9]. The sizes of the pump tubing, colorimeter and recorder setting were the same as those used previously[9].

RESULTS AND DISCUSSION

In earlier studies[10] we observed a linear relationship between the concentration of hexuronic acid and the color absorbance of orcinol–sulfuric acid rection; the reaction was found to be suitable for monitoring hexuronic acids during ion-exchange chromatography of glycosaminoglycans (GAG). The ion-exchange column was eluted with a sodium chloride–magnesium chloride gradient (0.2–3.0 *M*) and although at low salt concentrations there was no inhibition of color intensity, at concentrations above 1.5 *M* of the salts there was a reduction in color absorbance by about 30%.

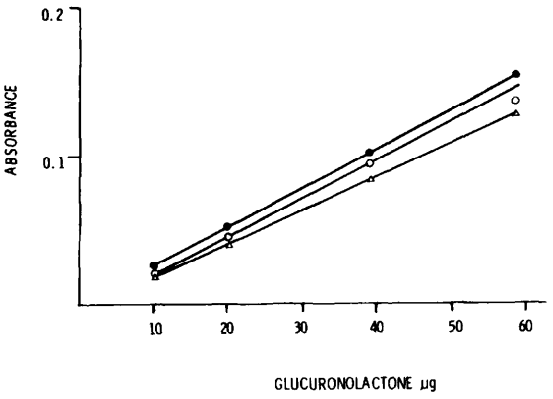

Fig. 1. Orcinol–sulfuric acid reaction of glucuronolactone in the presence of 4.0 *M* guanidine hydrochloride with (○) and without (△) protease inhibitors. ●, Distilled water.

In this study, the effect of different concentrations of guanidine hydrochloride (0.4–4.0 *M*) with and without the presence of protease inhibitors on the orcinol–sulfuric acid reaction of hexuronic acid was investigated. There was no suppression of color intensity even at 4.0 *M* guanidine hydrochloride concentration (Fig. 1) (this concentration of guanidine hydrochloride is generally used for extraction of proteoglycans and for dissociation of isolated proteoglycan aggregates). However, when protease inhibitors are included in the reaction medium along with high concentrations of guanidine hydrochloride, an approximately 10% decrease in absorbance occurred at 60 µg/ml uronic acid concentration (Fig. 1).

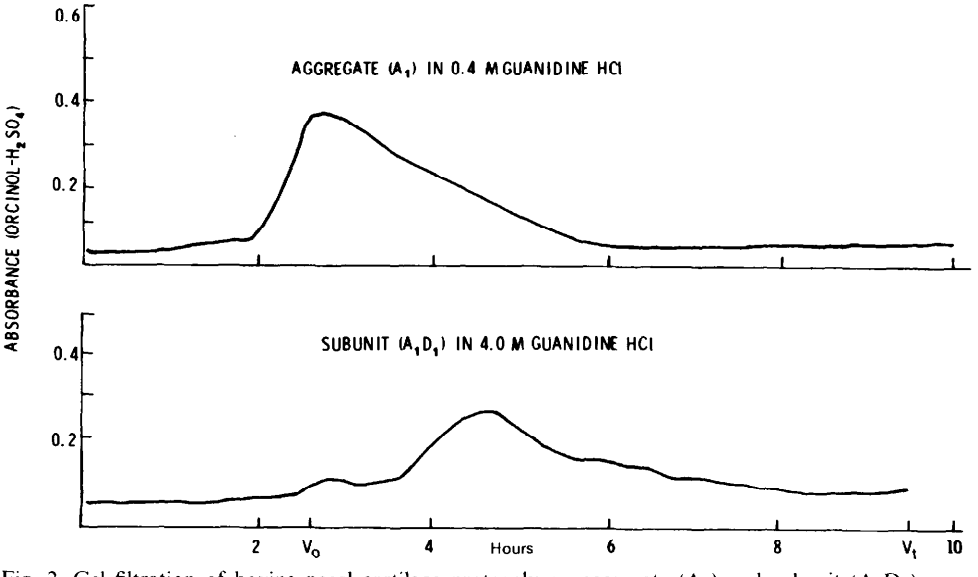

Fig. 2. Gel filtration of bovine nasal cartilage proteoglycan aggregate (A_1) and subunit (A_1D_1) on a Sepharose CL-4B column. The column was eluted with 0.4 or 4.0 *M* guanidine hydrochloride with protease inhibitors. A small amount of aggregate may be noted in the subunit chromatography. V_0 = void volume; V_t = total volume of the column.

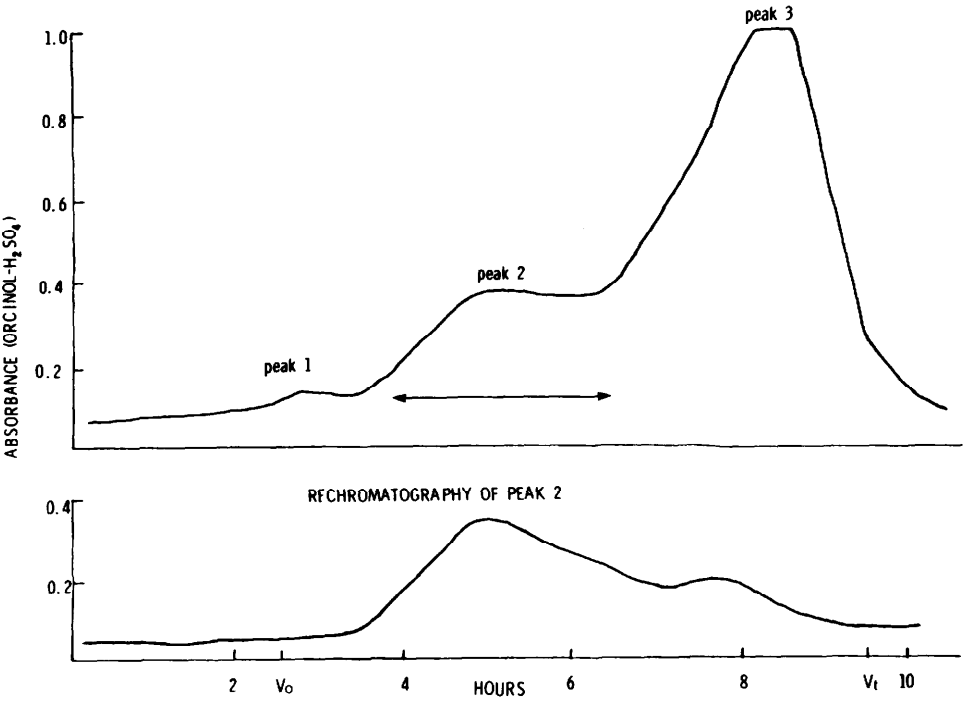

Fig. 3. Gel filtration of bovine nasal cartilage proteoglycan extract under dissociative conditions in 4.0 *M* guanidine hydrochloride. Only a small amount of material eluted in the void volume of the column. Most of the proteoglycan material was present in peak 2. Peak 3 contained large amounts of proteins with small amounts of low-molecular-weight proteoglycans or glycosaminoglycans. K_{av} of peak 2 was calculated after re-chromatography.

When known concentrations of glucuronic acid solutions in 4.0 *M* guanidine hydrochloride containing protease inhibitors were analyzed in the AutoAnalyzer, the areas of the peaks on the recorder trace were proportional to glucuronic acid concentration, suggesting the method to be suitable for monitoring proteoglycans.

Fig. 2 illustrates the gel filtration profiles of proteoglycan aggregate (A_1) and subunit (A_1D_1) isolated from bovine nasal cartilage by isopycnic centrifugation[11]. The aggregate was chromatographed in 0.4 *M* guanidine hydrochloride and the subunit in 4.0 *M* guanidine hydrochloride. Both chromatograms were developed in the presence of protease inhibitors. These observations show the orcinol–sulfuric acid reaction to be adequately sensitive for monitoring hexuronic acid during chromatography of proteoglycans.

An attempt was made to study proteoglycan extracts by gel filtration and the orcinol–sulfuric acid reaction. Gel filtration profiles of bovine nasal cartilage extract under associative and dissociative conditions are shown in Figs. 3 and 4. The extract in 4.0 *M* guanidine hydrochloride resolved into three peaks (Fig. 3). Peak 1 was very small and eluted in the excluded volume and the other two peaks in the inclusive volume. K_{av} for peak 2 was 0.24. Peak 3 was large and broad and eluted near the total volume of the column. As there was overlapping of peak 2 with peak 3, fractions corresponding to peak 2 were pooled (as shown in Fig. 3) and re-chromatographed. Peaks 2 and 3 contained alcian blue-positive materials. In electrophoresis in pyridine–

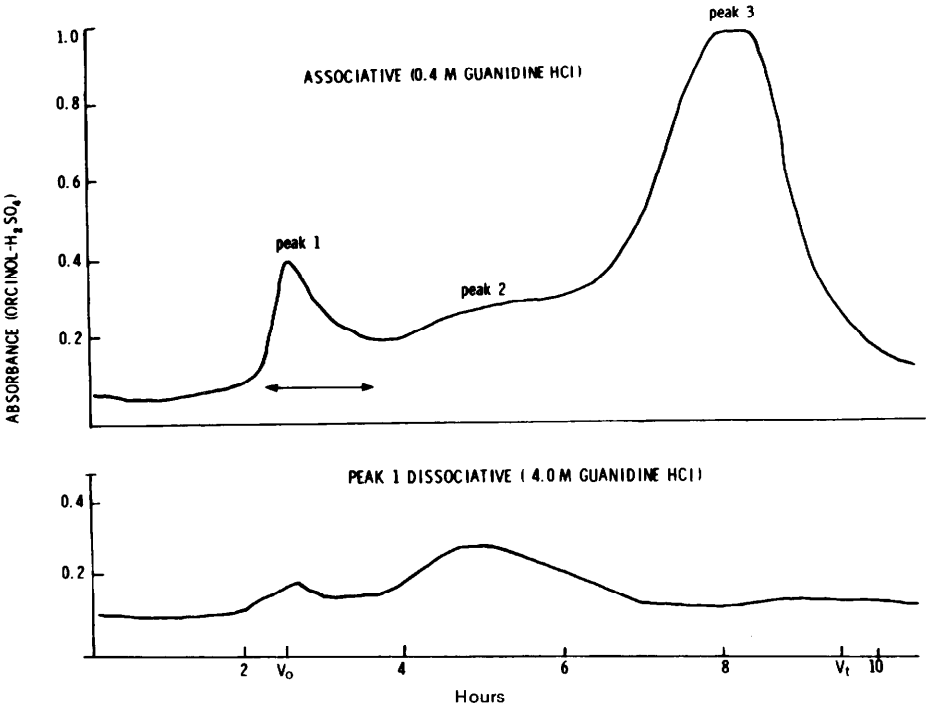

Fig. 4. Gel filtration of bovine nasal cartilage proteoglycan extract under associative conditions in 0.4 *M* guanidine hydrochloride. Fractions under peak 1, which represent aggregate, were pooled, incubated in 4.0 *M* guanidine hydrochloride to dissociate and chromatographed using the same solvent as eluent. Dissociation of aggregate can be noticed from the decrease in the area of peak 1 and the appearance of peak 2 in the chromatogram.

formate buffer both peaks moved similarly to chondroitin sulfates. Owing to limitations of materials peak 1 was not studied.

Under associative conditions in 0.4 *M* guanidine hydrochloride the proteoglycan extract was resolved into three peaks (Fig. 4). Peak 1 was considerably larger than the corresponding peak in the dissociative chromatography (Fig. 3). When the fractions under peak 1 (as shown in Fig. 4) were pooled, incubated in 4.0 *M* guanidine hydrochloride and re-chromatographed the material resolved into two peaks. One eluted in the excluded volume and the second with a K_{av} of 0.26. Both peaks showed alcian blue-positive material and similarly moved in electrophoresis like chondroitin sulfates.

It should be mentioned that the orcinol–sulphuric acid reaction is common for all reducing sugars and is not specific only for hexuronic acids. It is possible that tissue extracts used in these procedures may also contain glycoproteins in addition to proteoglycans, and monitoring fractions from gel filtration could produce spurious results. As the main purpose of this study was to determine the suitability of the orcinol–sulphuric acid reaction for monitoring proteoglycans during gel filtration, we did not attempt to purify these proteoglycan fractions further. Purification and fractionation of these proteoglycans can be achieved by cesium chloride isopycnic centrifugation.

In conclusion, the method described here affords a rapid procedure for monitoring proteoglycans during gel filtration and will significantly aid studies of proteoglycans under both associative and dissociative conditions.

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