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Isolation and characterization of proteoglycans from different tissues

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ABSTRACT

Two chromatographic procedures for the isolation and purification of proteoglycans (PG) and their related glycosaminoglycan (GAG) peptides are described. PG from human aorta were isolated from tissue extract by sequential ion-exchange, size-exclusion and hydroxyapatite chromatography. Final purification of samples was achieved by chromatography on Mono Q. Homogeneity of samples was demonstrated by Western blot analysis of biotin-labelled compounds prior to and after enzymatic digestion and dual-wavelength detection in size-exclusion chromatography. The purity of samples obtained by the procedure described was sufficient for protein sequence analysis. GAG preparations of bovine trachea cartilage were purified by the sequential use of strong anion-exchange supports. Molecular weight distribution and sensitivity to treatment with glycan-specific enzymes was shown by size-exclusion chromatography.

INTRODUCTION

Proteoglycans (PG) play an important role in fundamental biological processes such as cell–cell interaction, proliferation and differentiation and in stabilizing the structure of tissue matrices [1–3]. They represent a family of macromolecules consisting of a core protein with at least one glycosaminoglycan (GAG) chain covalently attached. Physico-chemical properties, *e.g.*, polydispersity and charge density, are mainly given by the length of and charge distribution within the GAG chain. The negative charges are due to sulphate ester moieties and/or carboxyl functions in the disaccharide units of the GAG chains. These features may be used for the separation of different PG or GAG peptides from complex mixtures [4]. Here we report on the use of several chromatographic procedures for the isolation and purification of different PG or their related GAG peptides from tissue extracts.

EXPERIMENTAL

Sample materials

The preparation of human aortae and extraction of PG were performed as described previously [5]. Bovine tracheal cartilage was a generous gift from Professor H. W. Stuhlsatz (Aachen, F.R.G.).

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Chemicals

Sepharose Q, Sepharose CL-4B, Mono Q HR 10/10, Mono Q HR 5/5, TSK G 3000 SW, TSK G 4000 SW, TSK G SWP, preformed gels for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (8–25% gradient) and low-molecular-weight standards were obtained from Pharmacia–LKB (Freiburg, F.R.G.). Hydroxyapatite (Bio-Gel HTP) and Zeta-Probe blotting membranes were obtained from Bio-Rad Labs. (Munich, F.R.G.). High-molecular-weight standards and 3-[3-(cholamidopropyl)dimethylamino]-1-propanesulphonate (CHAPS) were purchased from Sigma (Deisenhofen, F.R.G.). Chondroitin sulphate lyase AC (E.C. 4.2.2.5), chondroitin sulphate lyase ABC (E.C. 4.2.2.4), heparinase [heparin lyase (E.C. 4.2.2.7)], heparitinase [heparan sulphate lyase (E.C. 4.2.2.8)] and keratanase (E.C. 3.2.1.103) were purchased from Seikagaku (Tokyo, Japan). Sulphosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin) was obtained from Pierce (Munich, F.R.G.). All remaining reagents (Merck, Darmstadt, F.R.G.) were of analytical-reagent grade.

Apparatus

Chromatography was performed either on an LC 31 A chromatography system from Bruker (Bremen, F.R.G.) or on a fast protein liquid chromatography (FPLC) system from Pharmacia (Freiburg, F.R.G.). For multi-wavelength detection, a diode-array UV–VIS spectrophotometer (Waters Assoc., Eschborn, F.R.G.) was used. The radioactivity was measured with an LKB-Wallach scintillation counter (Type 1217 Rack Beta) using Lumagel (Lumac, Basle, Switserland) as scintillator. Ultrafiltration was performed by using YM-2 or YM-5 membranes (Amicon, Witten, F.R.G.). Amino acid and amino sugar analysis were performed after hydrolysis of sample with 3 *M* hydrochloric acid at 105°C for 15 h using an LKB Alphaplus amino acid analyser.

Isolation and purification of GAG

Papain digestion was performed as described [6]. Briefly, enzyme treatment of cartilage preparation was carried out in 10 m*M* sodium phosphate buffer containing 0.15 *M* sodium chloride and 5 m*M* cysteinium chloride (pH 6.2).

A Sepharose Q column (15 cm × 2.6 cm I.D.) was prepared according to the manufacturer's instructions. Column equilibration was performed with 10 m*M* sodium phosphate buffer containing 0.15 *M* sodium chloride (pH 6.2). The papain-treated sample was applied and absorption of the eluted compounds was monitored at 214 nm. Bound material was eluted with three linear gradients (flow-rate 1 ml/min) performed from 150 mM to 3 M NaCl in starting buffer (80 ml of 0.15–0.5 *M* NaCl, 80 ml of 0.5 *M* NaCl, 120 ml of 0.5 *M* NaCl to 3 *M* NaCl). Buffer exchange of GAG-containing fractions was performed by ultrafiltration (YM-2 membrane). For further purification, sample was applied to a Mono Q HR5/5 column, equilibrated with 40 m*M* sodium phosphate (pH 6.5). Bound compounds were eluted with three linear gradients (*i.e.*, 16.5 ml of 0 to 0.45 *M* NaCl, 20 ml of 0.45 to 1.2 *M* NaCl and 20 ml of 1.2 to 3 *M* NaCl; flow-rate 0.5 ml/min). The eluted compounds were detected by measuring their absorbance at 214 nm. Homogeneity of the samples was checked by size-exclusion chromatography on a TSK G 3000 SW column (600 mm × 7.5 mm I.D.), equipped with a TSK G SWP precolumn (75 mm × 7.5 mm I.D.). The column was equilibrated with 0.3 *M* NaCl in 10 m*M* NaH_2PO_4–Na_2HPO_4 and eluted compounds were detected by measuring their absorbance at 206 nm.

Isolation and purification of proteoglycans

The 4 *M* guanidinium chloride-containing extract of human aorta intima/media preparations was dialysed against equilibration buffer as described below. The sample was applied to a Sepharose Q anion-exchange column (20 cm × 4.6 cm I.D.) equilibrated with 7 *M* urea in 0.05 *M* acetate buffer (pH 5.2) containing 0.05 *M* NaCl–0.05 *M* LiCl–0.002 *M* benzamidine hydrochloride–0.001 *M* phenylmethylsulphonyl fluoride–0.001 *M* N-ethylmaleimide–0.2% (w/v) CHAPS. The column was washed with equilibration buffer using 1.5 times the column volume. The bound material was eluted by a gradient to 4 *M* NaCl (equilibration buffer made up to 4 *M* NaCl). Gradient elution was performed at a flow-rate of 4 ml/min with two linear NaCl gradients (0.05 to 1.1 *M*, 450 ml, and 1.1 to 4.0 *M*, 450 ml). The elution of ^{35}S-labelled (sulphated) sugar compounds was monitored by scintillation counting.

Pooled fractions from the Sepharose Q chromatography were concentrated on YM-5 membranes and submitted to size-exclusion chromatography on Sepharose CL-4B. For this purpose a Sepharose CL-4B column (125 cm × 3.5 cm I.D.) was equilibrated with 7 *M* urea in 0.05 *M* acetate buffer (pH 5.8) containing 0.3 *M* NaCl–0.002 *M* benzamidine hydrochloride–0.001 *M* phenylmethylsulphonyl fluoride–0.01 *M* EDTA–0.2% (w/v) CHAPS. Chromatography was performed at a flow-rate of 0.9 ml/min. ^{35}S-containing fractions with the exception of those of the void volume were submitted to hydroxyapatite chromatography.

Hydroxyapatite chromatography was performed on a column (12 cm × 4.6 cm I.D.) equilibrated with 0.01 *M* sodium phosphate buffer (pH 6.8). After sample application the column was washed with equilibration buffer (1.2 times the column volume). Elution was performed at a flow-rate of 4 ml/min. Gradient elution was done by increasing the phosphate concentration to 0.7 *M* (pH 7.2). Two linear gradients (0.01 to 0.48 *M* phosphate, 320 ml, and 0.48 to 0.7 *M* phosphate, 100 ml) were applied. Elution of ^{35}S-labelled compounds was monitored by scintillation counting.

Final purification of PG preparations was achieved by Mono Q anion-exchange chromatography. A Mono Q column (Type HR 10/10) was equilibrated with 0.02 *M* sodium phosphate buffer (pH 4.5) containing 0.1 *M* NaCl–0.1 *M* LiCl–0.05% (w/v) CHAPS. Samples obtained after hydroxyapatite chromatography were diluted with water to a final phosphate concentration below 0.05 *M* and applied to the column. The column was washed with 45 ml of equilibration buffer. Gradient elution was performed at a flow-rate of 3 ml/min using three linear NaCl gradients (0.1 to 1.2 *M*, 240 ml, 1.2 to 2.0 *M*, 80 ml, and 2.0 to 4.0 *M*, 40 ml). Eluted compounds were detected by measuring the absorbance at 214 nm.

Homogeneity of purified PG samples was controlled by size-exclusion chromatography on TSK G 4000 SW. A column (300 mm × 7.5 mm I.D.) equipped with a TSK G SWP precolumn (75 mm × 7.5 mm I.D.) was equilibrated with 0.01 *M* sodium phosphate buffer containing 0.3 *M* NaCl (pH 6.0). Samples were applied to the column and eluted at a flow-rate of 0.6 ml/min. Absorbance was monitored at 210 and 280 nm.

Characterization of PG and GAG

Characterization of GAG samples was performed by glycan-specific digestion with either chondroitin sulphate lyase AC/ABC or keratanase as described [7,8]. GAG degradation was detected by the M_r shift using size-exclusion chromatography (TSK G 3000 SW) before and after enzymatic treatment of the sample.

PG preparations were analysed by treating biotin-labelled samples with either chondroitinase AC/ABC and/or heparinase–heparitinase [5]. After SDS–PAGE and blotting to nylon membrane the untreated and enzyme-treated samples were detected by using the avidin–peroxidase conjugate–diaminobenzidine system.

RESULTS AND DISCUSSION

Isolation and purification of glycosaminoglycan peptides from bovine trachea cartilage

For analysis of GAG chains cartilage was digested with papain; isolation of GAG peptides was performed by ion-exchange chromatography. Sepharose Q chromatography resulted in two partially separated peaks eluting between 2.1 and 3 *M* NaCl. The relative amount of total amino sugars compared with the total amount of amino acid increased with increasing ion strength in the gradient (data not shown). The first peak contained mainly peptides and only a minor part of GAG. Components eluting between 2.6 and 2.8 *M* NaCl contained GalN, indicating for chondroitin sulphate and/or dermatan sulphate, in addition to GlcN, indicative of keratan sulphate and/or oligosaccharides. Sample eluting above 2.8 *M* NaCl contained mainly GalN. In order to investigate KS-containing GAG, GlcN-containing fractions were pooled (see bar, Fig. 1). For identification of GAG chains, digestion by glycan-specific enzymes is a common procedure [9]. The presence of a special GAG can be demonstrated as a decrease in molecular weight after treatment with the respective enzyme. Size-exclusion chromatography on TSK G 3000 SW (Fig. 2) of sample 1 obtained by Sepharose Q chromatography showed the presence of at least two components of different molecular weight distribution.

In cartilage most GAG are chondroitin sulphate and keratan sulphate [3,10].

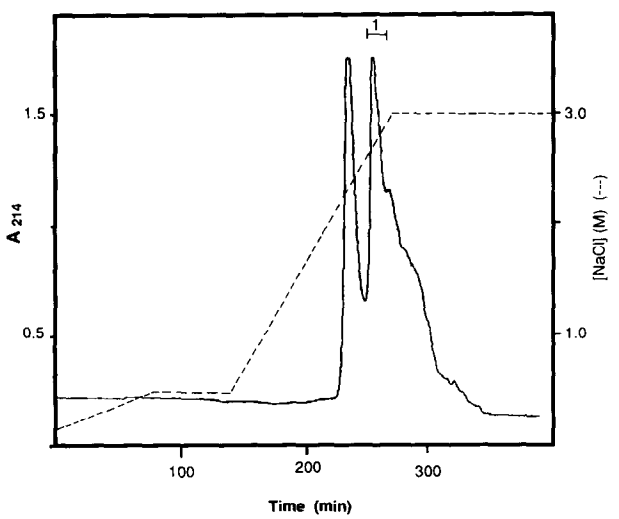

Fig. 1. Ion-exchange chromatography on Sepharose Q of GAG peptide preparation from bovine trachea cartilage. 25 ml of sample, equivalent to 2.5 g of papain-digested cartilage, were applied to a column (15 cm × 2.6 cm I.D.), equilibrated with 10 m*M* sodium phosphate buffer containing 0.15 *M* NaCl (pH 6.2). Elution was performed with linear gradients from 0.15 to 0.5 *M* NaCl and from 0.5 to 3 *M* NaCl; absorbance of eluted compounds was measured at 214 nm. Fractions were pooled as indicated (sample 1). Chromatography was carried out at room temperature.

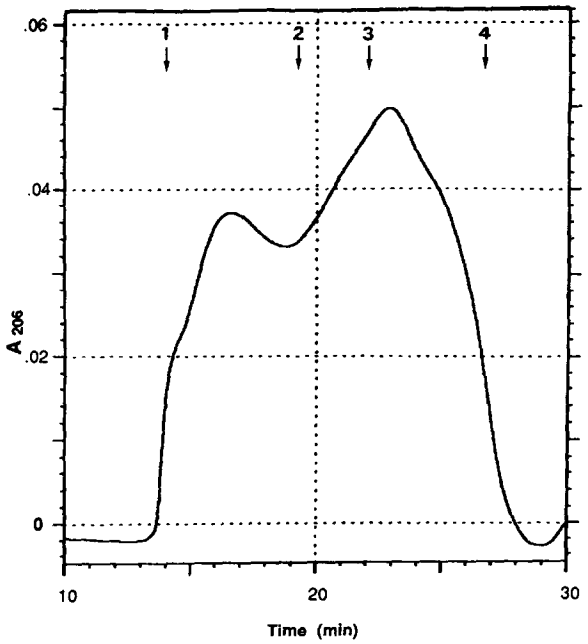

Fig. 2. Gel-permeation chromatography on TSK G 3000 SW of sample 1 prior to digestion with glycan-specific enzymes. Chromatography was performed with 0.3 *M* NaCl in 10 m*M* NaH_2PO_4-Na_2HPO_4 buffer at a flow-rate of 0.8 ml/min; elution of sample was monitored at 206 nm. Column calibration was performed by chromatography of marker proteins: 1 = thyroglobulin (669 000 dalton); 2 = immunoglobulin G (IgG) (160 000); 3 = bovine serum albumin (67 000); 4 = ovalbumin (43 000).

Aliquots of the sample were digested with either chondroitin sulphate lyase AC/ABC, keratanase or a mixture of them. The molecular weight shift obtained by enzyme treatment of the sample was determined by size-exclusion chromatography. The high-molecular-weight portion of sample is sensitive to chondroitin sulphate lyase AC/ABC (Fig. 3a), whereas the component of lower molecular weight is keratanase digestible (Fig. 3b). Incubation of the sample with both the keratanase and the chondroitin sulphate lyase AC/ABC affected the high- and low-molecular-weight components of the preparation (Fig. 3c). The peak at 22 min is due to bovine serum albumin, which is present in the enzyme preparation (Fig. 3d). Further purification was performed by high-performance anion-exchange chromatography on Mono Q (Fig. 4). The well separated major peak, eluting below 30 min, was associated with components from the initial papain digestion procedure. An extended peak, eluting in the range from 0.6 to 1.2 *M* NaCl was baseline separated from a peak eluting in the range from 1.2 to 2 *M* NaCl. Fractions were analysed for hexosamine and amino acid contents (Table I) and pooled in accordance with the molar ratio of GlcN to GalN (see bars, Fig. 4). A low GlcN/GalN ratio indicates chondroitin sulphate whereas a high ratio is indicative of keratan sulphate moieties. Aliquots of samples obtained by rechromatography on Mono Q were subjected to molcular weight distribution analysis on TSK G 3000 SW. The elution patterns were compared with those obtained from the sample prior to rechromatography on Mono Q. The second purification step resulted

in the separation of four GAG populations showing a lower diversity in molecular weight distribution (Fig. 5a and b). The purity of GAG preparations was controlled by differential enzyme digestion as described above (data not shown); no remaining contamination could be detected.

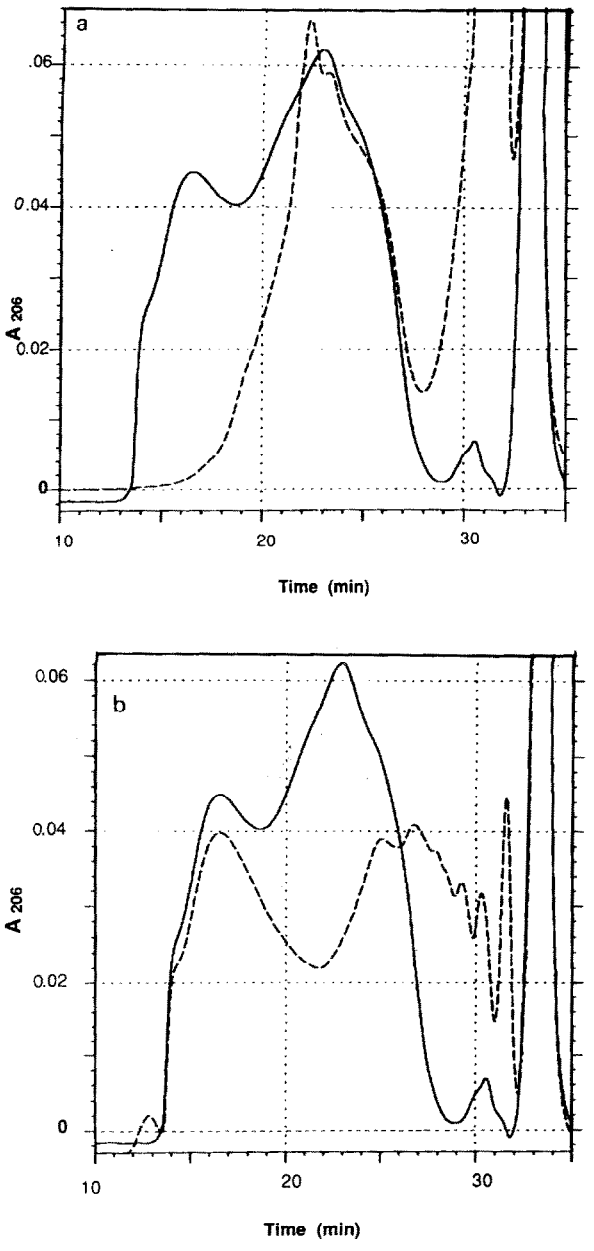

Fig. 3.

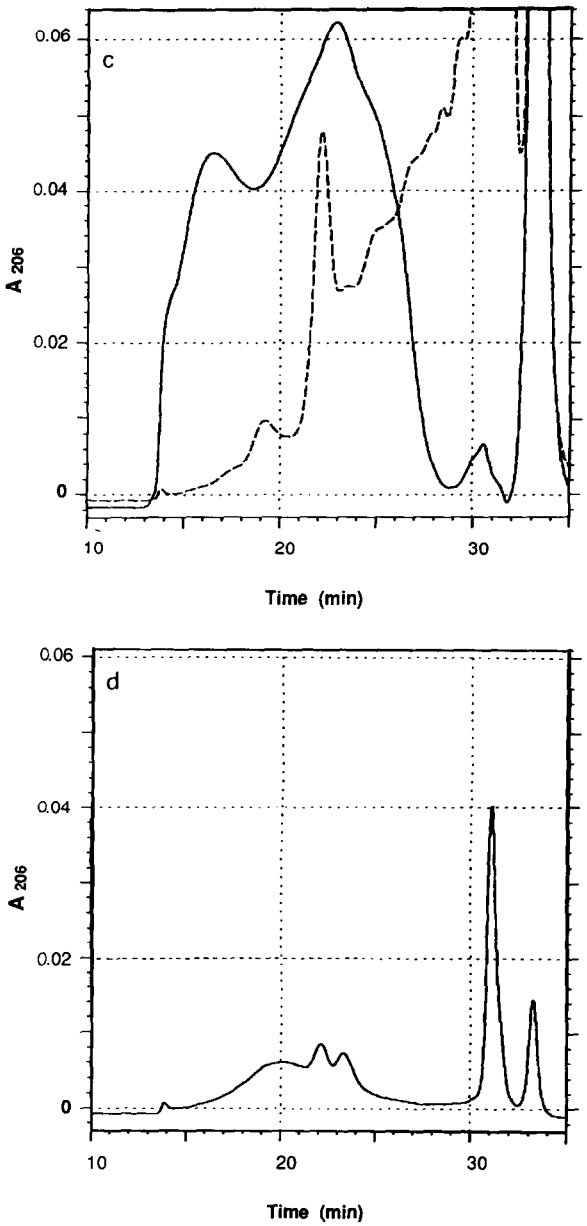

Fig. 3. Gel-permeation chromatography on TSK G 3000 SW of sample 1 prior and after treatment with glycan-specific enzymes. Conditions as in Fig. 2; in each instance 100 μl of sample (1 mg/ml) were injected. (a) Chondroitinase AC/ABC-digested (dashed line) and undigested (solid line) sample 1; (b) keratanase-digested (dashed line) and undigested sample 1 (solid line); (c) chondroitin sulphate lyase AC/ABC- and keratanase (dashed line)-treated and untreated (solid line) sample 1; (d) gel-permeation chromatography on TSK G 3000 SW of chondroitin sulphate lyase AC/ABC and keratanase preparation without addition of sample.

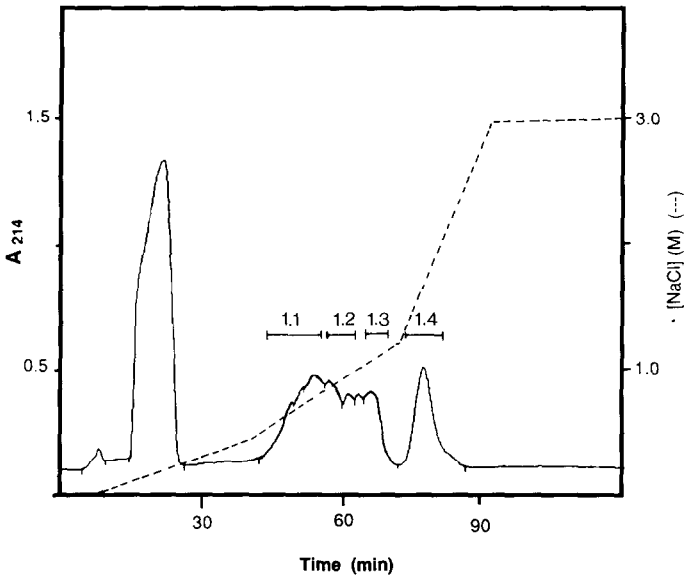

Fig. 4. Ion-exchange chromatography of sample 1 on Mono Q. 2 mg of GAG-peptide from sample 1, obtained after ion-exchange chromatography on Sepharose Q, were dissolved in 1 ml of equilibration buffer and submitted to Mono Q chromatography. The column was equilibrated with 40 m*M* sodium phosphate buffer (pH 6.5) prior to sample application. Elution was performed with three linear NaCl gradients (16.5 ml of 0 to 0.45 *M* NaCl, 20 ml of 0.45 to 1.2 *M* NaCl and 20 ml of 1.2 to 3 *M* NaCl) at a flow-rate of 0.5 ml/min; absorbance was monitored at 214 nm. Fractions were pooled as indicated by the bars (samples 1.1, 1.2, 1.3 and 1.4).

Isolation and purification of PG from human aorta

PG were extracted from human intima/media preparations in the presence of 4 *M* guanidinium chloride, protease inhibitors and CHAPS [5,11]. After changing the extraction buffer to 7 *M* urea, protease inhibitors and CHAPS anion-exchange chromatography was performed on Sepharose Q. Three distinct peaks could be separated by gradient elution (Fig. 6). The indicated peaks were submitted to size-exclusion chromatography for further purification. For none of the samples could a clear separation into different peaks be achieved (not shown); therefore, all fractions

TABLE I

MOLAR RATIO OF TOTAL AMOUNT OF AMINO ACID (aa_{tot}) AND AMINO SUGAR AND MOLAR RATIO OF GlcN TO GalN IN SAMPLE 1 AND THE DERIVED SUBFRACTIONS AFTER RECHROMATOGRAPHY ON MONO Q

Ratio	Sample				
	1	1.1	1.2	1.3	1.4
GlcN/GalN	0.40	1.20	0.25	0.04	0.06
aa_{tot}/HexN	0.59	1.03	1.26	0.51	0.46

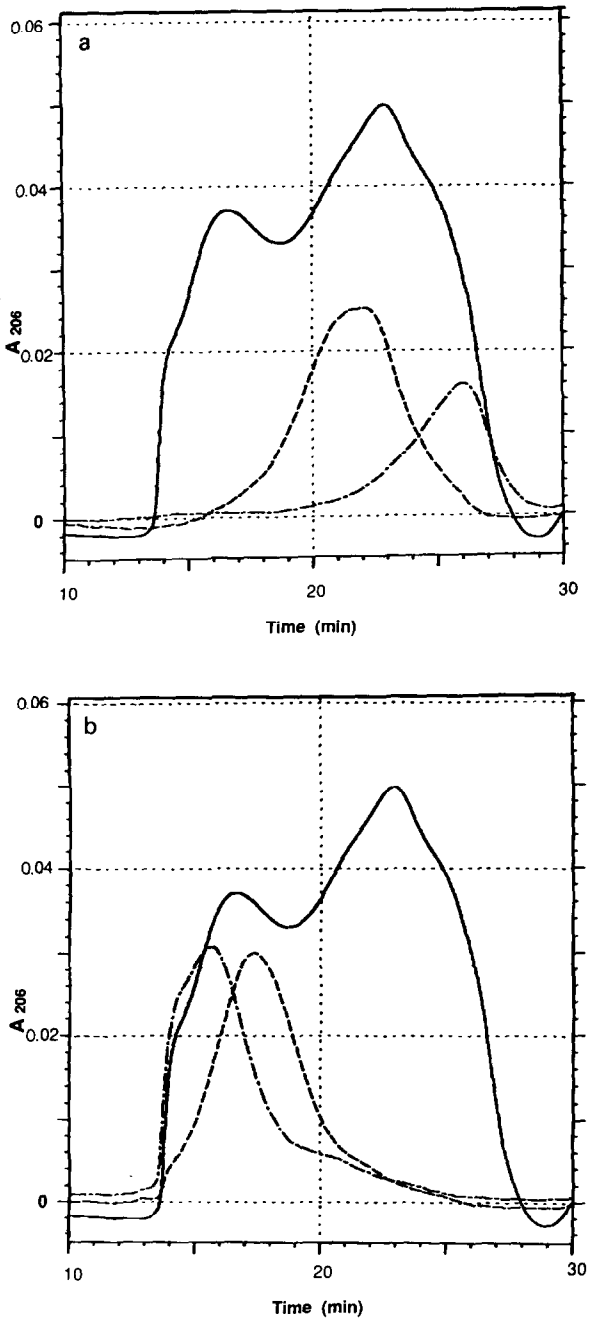

Fig. 5. Gel permeation chromatography on TSK G 3000 SW of samples obtained by rechromatography on Mono Q. Chromatograms are compared with that from sample 1 (———) of Sepharose Q chromatography. 50 μl of sample (0.4–0.8 mg/ml) were injected. Conditions of size-exclusion chromatography in Fig. 2. (a) Sample 1.1 (– · –) and sample 1.2 (– – –) in comparison with sample 1 (———). (b) Sample 1.3 (– – –) and sample 1.4 (– · –) in comparison with sample 1 (———).

with the exception of those of the void volume were pooled. Selection of the next chromatographic step was based on the finding that different GAG types show different affinities to Ca^{2+} ions [12]; in addition, hydroxyapatite has been shown to be useful for the separation of Ca^{2+}-binding proteins [13]. Therefore, PG separation of samples isolated after Sepharose CL-4B chromatography was performed on a hydroxyapatite column using phosphate gradient elution. For example, an elution pattern obtained from hydroxyapatite chromatography of a sample (Fig. 6, peak 3 after ion-exchange and Sepharose CL-4B chromatography) is shown in Fig. 7. A partially resolved peak (fractions 32–42, Fig. 7) was baseline separated from a peak eluting at high phosphate concentrations (fractions 55–58). Fractions 35–38 were pooled and submitted to high-performance anion-exchange chromatography on Mono Q.

By applying an NaCl gradient, elution of four distinct peaks could be achieved (Fig. 8). The major peak (PG-1), indicated by the bar, was selected for purity control by gel-permeation chromatography on TSK G 4000 SW. In addition to a signal in the void volumn, resulting from the high-molecular-weight portion of the PG preparation, a well defined peak was obtained. Homogeneity of the PG was demonstrated by two-wavelength detection, where a synchronous signal for absorbance at 210 and 280 nm was observed (Fig. 9). The same purification procedure was applied to another sample obtained after Sepharose Q chromatography (peak 1, Fig. 6). The result of the final purification step on Mono Q is shown in Fig. 10. Despite the compounds eluting after sample application, several baseline-separated peaks were obtained. One of them, indicated by the bar, was submitted to gel-permeation chromatography as described above (Fig. 11). Purity of the sample (PG-2) eluting near the void volume could be

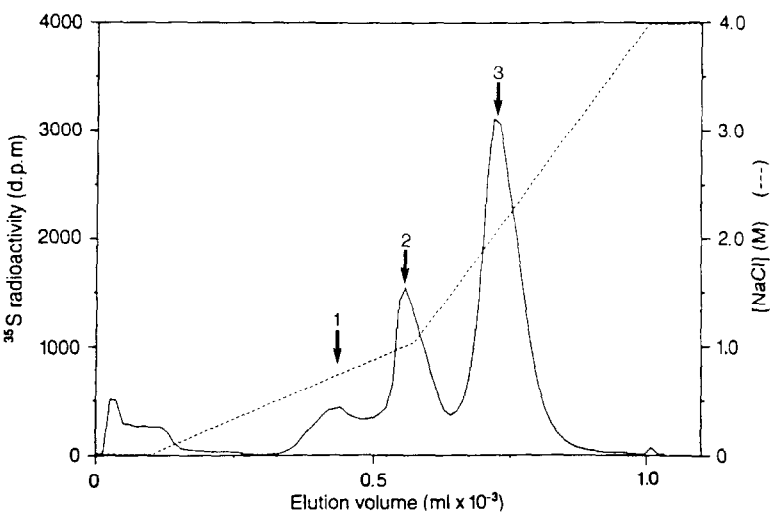

Fig. 6. Ion-exchange chromatography on Sepharose Q of PG extracted from human aorta (intima/media preparations). Prior to sample application the extraction buffer was changed to 0.05 *M* acetate buffer containing 7 *M* urea in addition to NaCl–LiCl–CHAPS and proteinase inhibitors (see Experimental). Sample application was followed by washing the column with 1.5 column volumes of starting buffer. Bound material was eluted at a flow-rate of 3 ml/min by applying two linear NaCl gradients (0.05 to 1.1 *M* and 1.1 to 4 *M* NaCl). Eluted compounds were monitored for ^{35}S radioactivity and indicated peaks were submitted to further purification procedures; the recovery of the applied activity was greater than 95%.

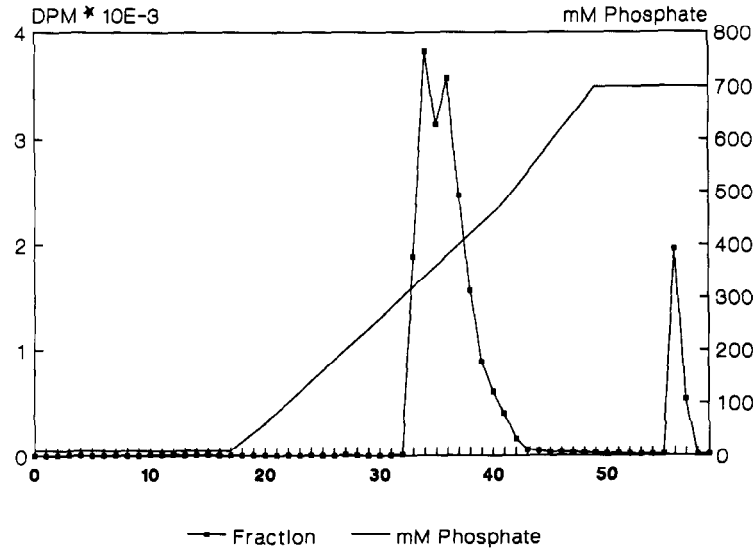

Fig. 7. Hydroxyapatite chromatography of peak 3 (Fig. 6) obtained by ion-exchange chromatography. Detailed conditions of hydroxyapatite chromatography are given under Experimental. Briefly, elution of the sample was performed by two linear gradients. Fractions (10 ml) were analysed for ^{35}S radioactivity. The recovery of the applied radioactivity was greater than 98%.

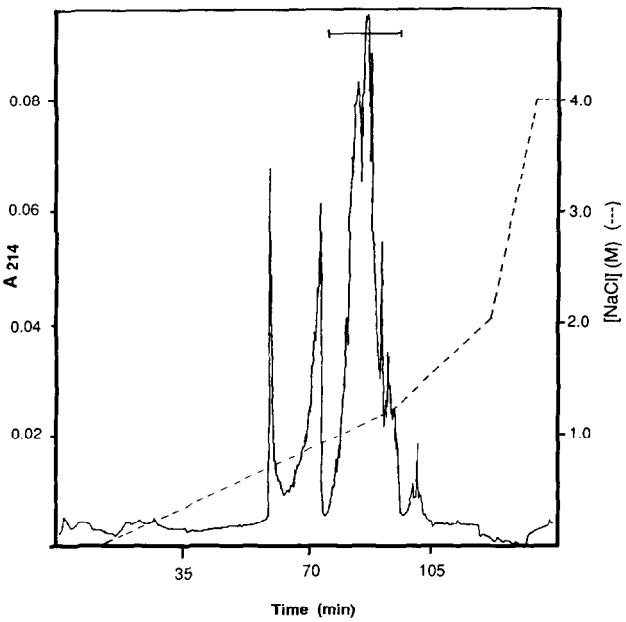

Fig. 8. Final ion-exchange HPLC on Mono Q of PG preparation purified from peak 3 (Fig. 6) of ion-exchange chromatography. Sample, isolated from peak 3 of initial ion-exchange chromatography and further purified by gel-permeation and hydroxyapatite chromatography, was applied to the column; unbound components were washed off with 45 ml of equilibration buffer before gradient elution of bound material was performed. Elution conditions are given under Experimental. Absorbance was monitored at 214 nm. Fractions were pooled as indicated by the bar (PG-1).

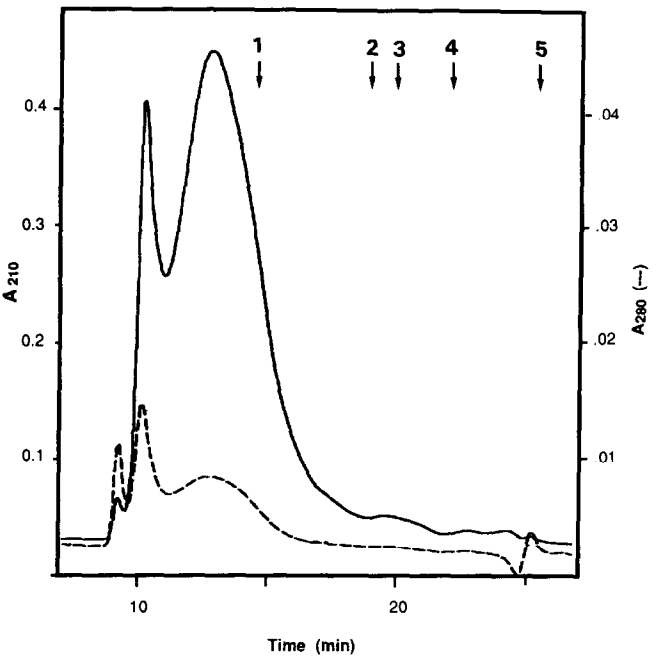

Fig. 9. Gel-permeation chromatography on TSK G 4000 SW of PG-1. 20 μl of sample (2 mg/ml) were analysed for molecular weight distribution and purity; absorbance at 210 nm and 280 nm were monitored. Elution conditions are given under Experimental. Column calibration was performed by chromatography of marker proteins: 1 = thyroglobulin (669 000 dalton); 2 = IgG (160 000); 3 = ovalbumin (43 000); 4 = myoglobin (17 000); 5 = vitamin B 12 (1300).

demonstrated. In addition, the identity and purity of the sample were evaluated by sensitive Western blot analysis (Fig. 12). The biotin-labelled sample (lane 2) was digested with glycan-specific enzymes (heparinase–heparitinase). The broad signal, ranging from the entry of the separating gel to about 180 000 dalton, disappeared after enzyme treatment, while a core protein-related signal in the region of 130 000 dalton showed up (lane 3). Amino sugar analysis showed the presence of GlcN only (data not shown), indicating KS- or HS-PG. Treatment of the sample with keratanase did not affect the staining pattern, hence the sample was identified as pure HS-PG.

CONCLUSIONS

PG and GAG were isolated from different tissues. Well established procedures use ultracentrifugation under different conditions and subsequent chromatography on a DEAE-based anion exchanger [4,9]. As these methods are time consuming, especially for preparative isolation, an alternative method was established, based on chromatographic steps only. Separation conditions were chosen with respect to physico-chemical properties. In accordance with the high charge density within the GAG moieties and the large molecular size of PG, strong anion exchangers based on wide-pore supports were used for the separation of PG. These materials have been

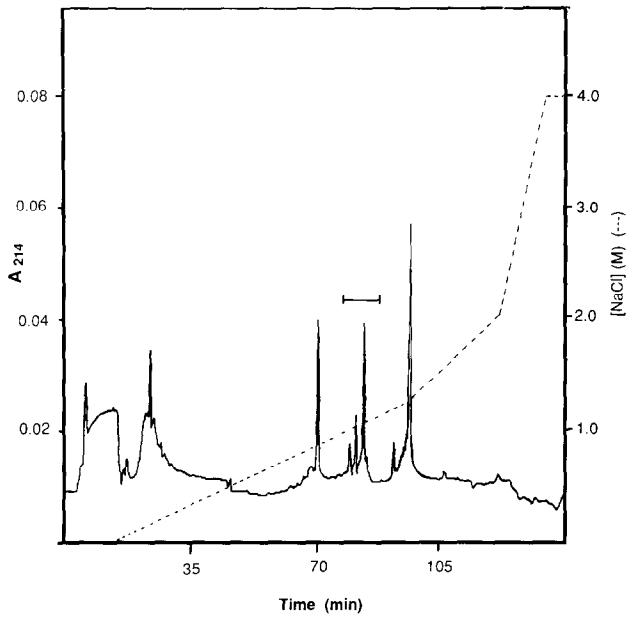

Fig. 10. Final ion-exchange HPLC on Mono Q of PG preparation isolated from peak 1 (Fig. 6) of initial ion-exchange chromatography. Sample, isolated from peak 1 of initial ion-exchange chromatography and further purified by gel-permeation and hydroxyapatite chromatography, was applied to the column; unbound components were washed off with 45 ml of equilibration buffer before gradient elution of bound material was performed. Elution conditions are given under Experimental. Absorbance was monitored at 214 nm. Fractions were pooled as indicated by the bar (PG-2).

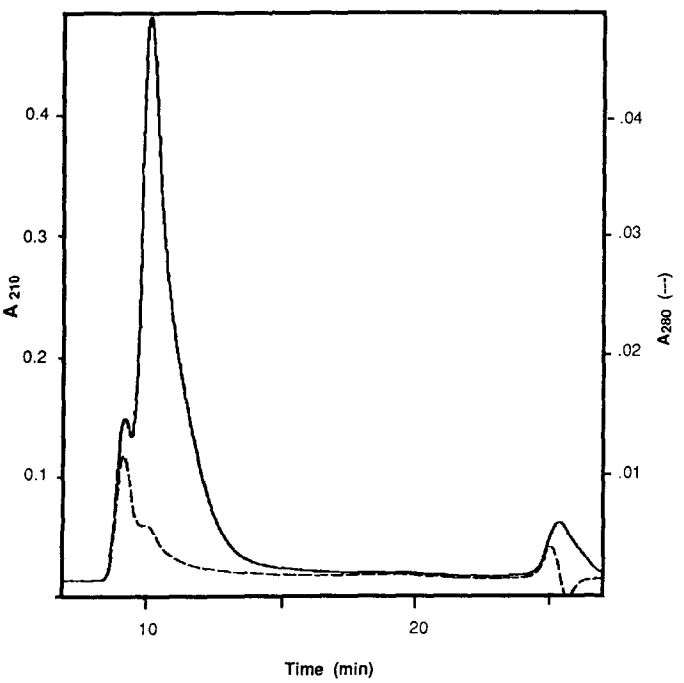

Fig. 11. Gel-permeation chromatography on TSK G 4000 SW of PG-2. 20 μl of sample (1 mg/ml) were analysed for molecular weight distribution and purity; absorbance at 210 nm and 280 nm were monitored. Elution conditions are given under Experimental.

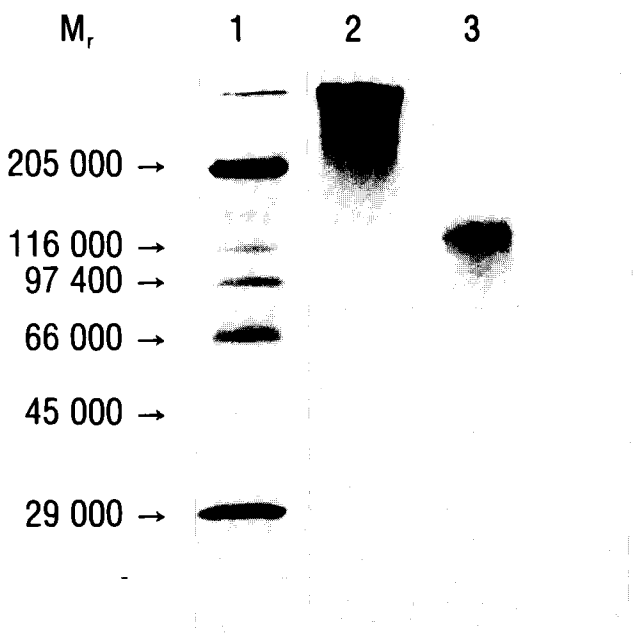

Fig. 12. Western blot of biotin-labelled PG-2 prior to and after heparinase–heparitinase digestion. Heparinase–heparitinase-digested and undigested biotin-labelled PG-2 were submitted to SDS-PAGE (8–25% gel, reducing conditions) prior to Western blotting. Lanes: 1 = molecular weight (M_r) standard; 2 = PG-2; 3 = PG-2 after heparinase–heparitinase digestion.

shown to be valuable also for the purification of GAG. Based on the Ca^{2+}-binding properties of GAG moieties, a hydroxyapatite chromatographic procedure was established. Using the protocol described, PG were isolated from complex mixtures. The purity of the isolated PG was sufficient for protein sequence analysis [14].

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