Purification and characterization of heparan sulphate proteoglycan from bovine brain

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A heparan sulphate proteoglycan was purified from adult bovine brain tissues and its structure was characterized. The major heparan sulphate proteoglycan from whole bovine brain had a molecular mass of > 200 kDa on denaturing SDS/PAGE and a core protein size of 66 kDa following the removal of glycosaminoglycan chains. Fractionation on DEAE-Sephacel showed that this proteoglycan consisted of three major forms having high, intermediate and low overall charge. All core proteins were identical in size and reacted with heparan sulphate proteoglycan-stub antibody and an antibody made to a synthetic peptide based on rat glypican. The three forms of proteoglycans had identical peptide maps and their amino acid compositional analysis did not match any of the known glypicans. The internal sequence of a major peptide showed only 37.5% sequence similarity with human glypican 5. The glycosaminoglycan chain sizes of the three forms of this proteoglycan, determined after β-elimination by PAGE, were identical. The disaccharide compositional analysis on the heparan sulphate chains from the three forms of the proteoglycan, determined by treatment with a mixture of heparin lyases followed by high-resolution capillary electrophoresis, showed that they differed primarily by degree of sulphation. The most highly sulphated proteoglycan isolated had a disaccharide composition similar to heparan sulphate glycosaminoglycans found in brain tissue. Based on their sensitivity to low pH nitrous acid treatment, the N-sulphate groups in these proteoglycans were found to be primarily in the smaller glycosaminoglycan chains. The heparan sulphate proteoglycans were also heavily glycosylated with O-linked glycans and no glycosylphosphatidylinositol anchor could be detected.

Key words: glycan analysis, glycosaminoglycan, glypican, heparin, prion.

INTRODUCTION

Heparan sulphate (HS) proteoglycans (PGs) are a family of macromolecules found in virtually all tissues in a wide variety of species [1–3]. These macromolecules are comprised of a core protein to which one or more HS glycosaminoglycan (GAG) chains are attached. Much of the biological activity of HSPGs is determined by the binding of a variety of proteins to these HS GAG chains [1,2]. HS GAG chains are involved in regulation of DNA replication, producing neurites in neuronal cells, lipid metabolism, cell growth, basement-membrane permeability and cancer metastasis [1]. Specific sequences in these GAG chains are believed to be responsible for the binding of growth factors [4–6], adhesion molecules [7] and protease inhibitors [8].

While there are many different forms, there are two major families of integral-membrane cell-surface HSPGs [3]: the syndecans [9,10] and the glypicans [11]. The syndecan family has four subfamilies and glypican family has five subfamilies [12]. HSPGs from these two families have been purified from a variety of species and cell types using a number of standard biochemical techniques [13–15]. Whereas a number of PG core proteins have been cloned [16–18], considerably less success has been reported on establishing the fine structure of the HS GAG chains associated with these PGs [1,2]. Glypican HSPGs have also been found in brain and nervous tissues [11,19–24] and are involved in their development [11,25] and regeneration [7,25]. The glypican family of PGs has a characteristic glycosylphosphatidylinositol (GPI) anchor and 14 conserved cysteine residues forming the tertiary structure of its core protein [11]. Glypican-2 (cerebroglycan) is only expressed in nervous tissues [26]. This PG is unique to the developing nervous system and is expressed predominantly during neuronal differentiation [26].

Because of the localization of specific HSPGs in nervous tissues, pathological conditions relating to their presence or absence have become an area of increased interest [27]. Progressive dementia, accompanied by the deposition of insoluble protein plaque within the brain, is associated with a number of neurological degenerative disorders, including Alzheimer's disease [28] and prion-based diseases [29]. HSPGs have been implicated in the deposition of amyloid in Alzheimer's disease [30,31] and the deposition of abnormal prion protein in scrapie infection and other prion diseases [32,33]. Recently, bovine spongiform encephalopathy ('BSE'), a prion-based infection associated with 'mad cow disease', has been the source of increasing public concern [34,35]. In an effort to gain an improved understanding of bovine spongiform encephalopathy, our laboratory has undertaken to purify the major HSPG found in adult bovine brain tissue, identify its core protein and examine its HS GAG chains.

EXPERIMENTAL

Materials

Fresh bovine brains were purchased from a local slaughter house. Guanidinium chloride, piperazine/HCl, *N*-ethylmaleimide, PMSF, trichloroacetic acid, chondroitin lyase ABC, Coomassie Brilliant Blue R-250, trifluoroacetic acid, Sepharose

Abbreviations used: HS, heparan sulphate; PG proteoglycan; GAG, glycosaminoglycan; CE, capillary electrophoresis; GPC, gel permeation chromatography; ΔUAp, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; GlcNp, 2-amino-2-deoxyglucopyranose; GlcAp, glucopyranosyluronic acid; IdoAp, idopyranosyluronic acid; Ac, acetate; S, sulphate; PIM, porcine intestinal mucosa; GPI, glycosylphosphatidylinositol; AGA, monopotassium, 7-amino-1,3-naphthalenedisulphonic acid; MMCO, molecular mass cut off.

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CL-6B, Sephadex G-200, CHAPS, SDS protein standards, CNBr-activated Sepharose 4B, 4-chloronaphthol, octyl-Sepharose 4B (fast flow), *myo*-inositol, *scyllo*-inositol, Alcian Blue and Alcian Yellow were obtained from Sigma (St. Louis, MO, U.S.A.). Monopotassium 7-amino-1,3-naphthalenedisulphonic acid (AGA) was purchased from Aldrich (Milwaukee, WI, USA). Heparin lyases and HSPG-stub antibody were purchased from Seikagaku America, Inc. (Rockville, MD, U.S.A.). Heparin, low-molecular-mass heparin and HS were from Celsus Laboratory (Cincinnati, OH, U.S.A.). Heparin and HS disaccharide standards were obtained from Grampian Enzymes (Aberdeen, Scotland). The ultrapure grade of urea was purchased from Life Technologies (Grand Island, NY, U.S.A.). Constant-boiling 6 N HCl and silylation agent (Tri-Sil®) were from Pierce (Rockford, IL, U.S.A.). Triton X-100, EDTA and polyethylene glycol were from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Flame seal ampoules were from Wheaton (Millville, NJ, U.S.A.). Dialysis membrane for desalting was from Spectrum Medical (Huston, TX, U.S.A.) and the pressure filtration apparatus and membranes were from Amicon (Danvers, MA, U.S.A.). DEAE-Sephacel matrix was from Pharmcia Biotech (Uppsala, Sweden). Bio-Gel P-2, Affigel-15 and goat anti-(rabbit IgG)–horseradish peroxidase conjugate were from Bio-Rad (Richmond, CA, U.S.A.). The sequencing grade modified trypsin was from Promega (Madison, WI, U.S.A.). Immobilon-N membrane was from Millipore (Bedford, MA, U.S.A.). Proteinase K from Boehringer Mannheim (Mannheim, Germany). All other reagents used were of analytical grade. Antibodies reactive with rat glypican were a generous gift from Dr. R. Margolis (New York University Medical Center, New York, NY, U.S.A.). Bovine articular decorin was a gift from Dr. L. Rosenberg (Montifiore Medical Center, New York, NY, U.S.A.).

Capillary electrophoresis (CE) was performed using a 50 μm (inner diameter), 375 μm (outer diameter) and 62 cm long fused silica capillary from ISCO (Lincoln, NE, U.S.A.). Peptide mapping HPLC was performed on reversed phase (C_{18}) column from Vydac (Hesperia, CA, U.S.A.). Gas chromatography was performed using a capillary column DB-5 (0.32 mm × 25 m) from Alltech (Deerfield, IL, U.S.A.).

Preparation of PG from bovine brain tissues

Ten bovine brains were collected immediately after slaughter and quickly frozen on dry ice. The purification method shown in Scheme 1 was based on the previously reported purification of HSPG from liver [15]. The brains were rinsed free of extraneous blood with ice-cold acetate buffer, pH 5.0, cut into small pieces and then homogenized in 4 M guanidinium chloride, 2 % (v/v) Triton X-100, 50 mM pH 5.0 sodium acetate, 0.1 M 6-aminohexanoic acid, 20 mM benzamidine hydrochloride, 10 mM EDTA, 5 mM *N*-ethylmaleimide and 0.5 mM PMSF, to inhibit proteolysis. After stirring at 4 °C for 16 h, insoluble residues were removed by filtration. Protein was precipitated from the soluble extract with 10 % (w/v) trichloroacetic acid (30 min 4 °C) and removed by centrifuging at 2000 ***g***. After concentration of the supernatant to less than one-third of its volume by reverse osmosis using polyethylene glycol 8000, the buffer was exchanged by dialysis, using membranes having a molecular mass cut off (MMCO) of 1000 Da or 3500 Da, against 20 mM Tris/HCl, pH 8.0, containing 8 M urea, 0.15 M NaCl and 0.5 % (v/v) Triton X-100. Anionic components were adsorbed on to 350 ml DEAE-Sephacel beads, loaded into a 2.5 cm × 80 cm column, washed with 20 mM piperazine/HCl, pH 5.0, containing 6 M urea, 0.15 M NaCl and 0.5 % (v/v) Triton X-100, and PGs were then eluted (40 ml/h) using a salt gradient. Pooled fractions were concentrated 10-fold (to 15 ml) by reverse osmosis (as above) and dialysed against 50 mM Tris/HCl, pH 8.0, containing 50 mM NaCl, 0.1 % Triton X-100 and protease inhibitors. The fractions were further concentrated 30-fold using pressure filtration through an Amicon YM-10 filter (MMCO of 10000 Da) and precipitated at −20 °C for 12 h with 3 vol. of 1.3 % (w/v) potassium acetate in 95 % ethanol, in the presence of chondroitin sulphate carrier (1 μg/ml). The precipitate was recovered and redissolved in 30 mM Tris/HCl, pH 7.5, containing 4 M guanidinium chloride, 0.5 % CHAPS and separated on a Sepharose CL-6B column (1.0 cm × 107 cm) monitored by the 1,9-Dimethylmethylene Blue assay [36] and analysed on 8 % (w/v, acrylamide) SDS/PAGE.

PAGE and Western blot analysis

PGs and core protein preparations were subjected to electrophoresis in a 3.5–20 % (w/v) linear polyacrylamide gradient gel under reducing conditions and in the presence of SDS [37]. Western transfer was done on to Immobilon-N membranes, and the blots were probed with rabbit antiserum to recombinant rat glypican (obtained by thrombin cleavage of a glutathione S-transferase-fusion protein) [38] or HSPG-stub antibody [39]. Following binding of goat anti-(rabbit IgG)–horseradish peroxidase conjugate (1:1000 dilution), the bands were visualized with 4-chloronaphthol as the peroxidase substrate.

Amino acid compositional analysis

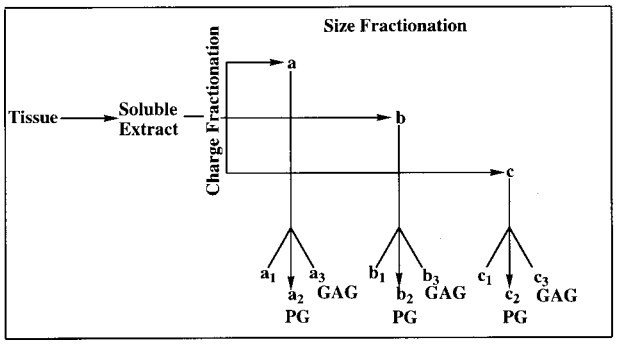
Heparin lyase III and chondroitin lyase ABC were immobilized on CNBr-activated Sepharose 4B beads [40]. The purified PGs (a_2, b_2, and c_2) were digested overnight at 37 °C with immobilized heparin lyase III and immobilized chondroitin lyase ABC in 50 mM sodium phosphate, pH 7.6. The core protein, recovered by gel permeation chromatography (GPC) through Sephadex G-25 (0.8 cm × 20 cm), was fractionated by SDS/PAGE (8 %, w/v, acrylamide), and blotted on to a membrane. The Sephadex G-25 void fraction and the blotted excised core protein were submitted for amino acid compositional analysis at the University of Iowa Protein Structure Facility.

Peptide mapping and internal sequencing

The purified PGs from intermediate molecular mass components (a_2, b_2 and c_2) from size-exclusion chromatography were treated at 37 °C for 10 h with sequencing-grade modified trypsin in 50 mM NH_4HCO_3, pH 7.8 buffer for peptide mapping. The digestion mixture was separated on a Vydac C_{18} (4.6 mm × 250 mm) column and eluted at 1 ml/min using a gradient of 0.06 % (v/v) trifluoroacetic acid in water and 0.06 % (v/v) trifluoroacetic acid in 80 % acetonitrile in water. The major peaks from a_2 were submitted for internal sequencing to the University of Iowa Protein Structure Facility.

Size of HS GAG chains

The size of the HS GAG chains of HSPG fractions a_2, b_2 and c_2 were analysed. The dialysed HSPG was lyophilized, β-eliminated using mild conditions [41] and then dialysed against water using a 1000 Da MMCO membrane. The resulting HS GAG was loaded on to a strong anion-exchange resin and the resin was washed sequentially with water, 3 % (w/v) NaCl and 16 % (w/v) NaCl. The 16 % NaCl fraction was dialysed against water using a 1000 Da MMCO membrane. The HS GAG chains were analysed on a 12 % (w/v) acrylamide gel together with porcine intestinal mucosa (PIM) heparin, low molecular mass heparin, a mixture of heparin oligosaccharide standards, PIM HS and



Scheme 1 Schematic of HSPG purification

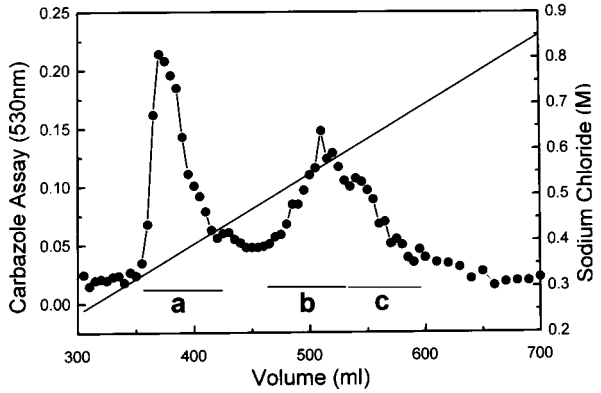


Figure 1 DEAE-Sephacel chromatography of bovine brain PG supernatant following trichloroacetic acid precipitation

The column was loaded in 20 mM piperazine/HCl, pH 5.0 buffer, containing 6 M urea, 0.15 M NaCl, 0.5% (v/v) Triton X-100 and eluted with a NaCl gradient (0.15-1.0 M) in the same buffer. Detection was by carbazole assay [55]. Fractions indicated by solid horizontal lines were collected, combined and labelled as a, b and c for further purification. Carbazole assay (●), NaCl gradient (—).

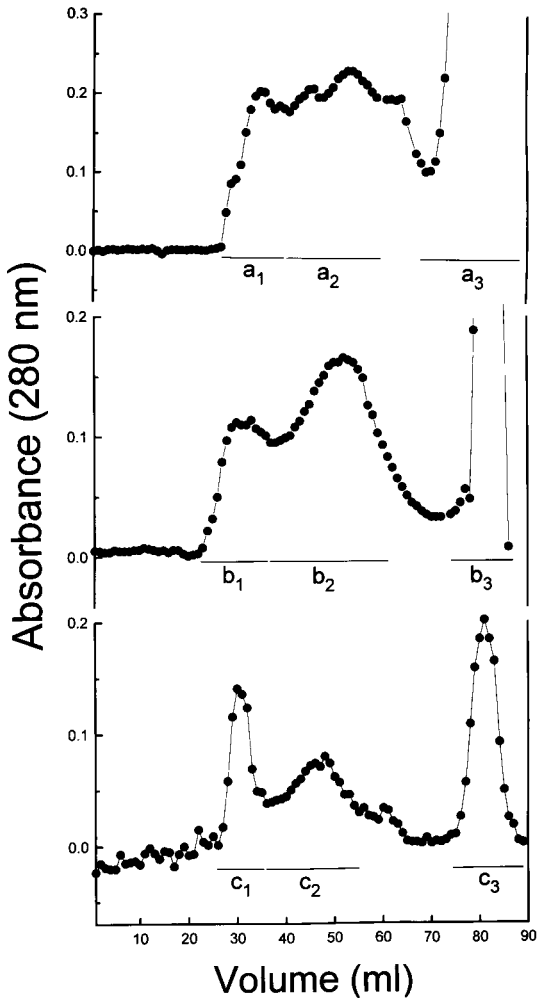


Figure 2 Sepharose CL-6B fractionation of DEAE-Sephacel fractions a (upper), b (middle) and c (lower)

The column was loaded and eluted using 30 mM Tris/HCl, pH 7.5, containing 4 M guanidinium chloride and 0.5% CHAPS at a flow rate of 4 ml/h and the eluent was monitored at 280 nm. Fractions indicated by solid horizontal lines were collected, combined and labelled as shown.

a purified, structurally characterized heparin octasaccharide [42] {ΔUAp2S(1 → [4)-α-D-GlcNpS6S(1 → 4)-α-L-IdoAp2S(1 →]$_6$4)-α-D-GlcNpS6S}. (Please refer to the abbreviations footnote for definitions of carbohydrate abbreviations.) The gel was visualized with Alcian Yellow staining followed by silver staining. One mg of β-eliminated glycan was fluorescently labelled by reductive amination with AGA as previously described [43]. Excess AGA as removed by GPC on a Bio-Gel P-2 column (1.5 cm × 45 cm). Analysis of this sample relied on GPC using a Sephadex G-200 column (detection by absorption at 247nm) and PAGE (detection by transillumination at 365 nm)[44].

Disaccharide composition analysis

The β-eliminated intermediate molecular mass components (a_2, b_2 and c_2) were each dissolved in 100 μl of 50 mM phosphate buffer, pH 7.6, and a mixture of heparin lyase I, II and III (1 m-unit each) were added together. The digestion mixtures were kept in a 37 °C water bath overnight. The enzymes were deactivated by boiling for 3 min. The CE system was operated in the reverse polarity mode by applying the sample at the cathode and running with 20 mM H_3PO_4 adjusted to pH 3.5 with 1 M Na_2HPO_4 as described previously [45]. The capillary was washed before use with 0.5 ml of 0.5 M NaOH, followed by 0.5 ml of distilled water and then 0.5 ml running buffer. Samples were applied using vacuum injection and electrophoresis was conducted at 20 kV with detection at 232 nm.

Oligosaccharide mapping

The β-eliminated GAG chains prepared from HSPG fractions a_2 and b_2 (10 μg) were subjected to nitrous acid scission [46] using freshly prepared nitrous acid [47]. The reaction was run for 1 h prior to its termination by the addition of 2 M Na_2CO_3. The products were exhaustively dialysed (1000 Da MMCO) against water and the retentate was lyophilized. Both HS GAG and nitrous acid-treated HS GAG were analysed by PAGE on a 22% (w/v) gel visualized with Alcian Blue followed by silver staining.

Attempted identification of a GPI anchor

Attempts to identify the presence of a GPI anchor in PG fraction a_2 closely followed a previously published method [48]. Standard solutions of *myo*-inositol and *scyllo*-inositol were similarly prepared and analysed.

RESULTS

The PG fraction of whole intact adult bovine brain tissue was isolated by homogenization of the tissue in the presence of protease inhibitors, and it was freed from extraneous protein by precipitation with cold trichloroacetic acid. This fraction was then purified by DEAE-Sephacel chromatography using a NaCl gradient elution (Figure 1). Three major peaks were observed eluting (a) at low (300–400 mM), (b) medium (500–600 mM) and (c) high (600–700 mM) NaCl concentrations. Each peak was next fractionated by size-exclusion chromatography on Sepharose CL-6B under dissociative conditions. Proteins and peptides were directly detected by measuring absorbance at 280 nm and GAG was detected by the 1,9-Dimethylmethylene Blue assay (results not shown) at an absorbance of 525 nm (Figure 2). Each charge fractionated sample showed three major molecular mass species. The high molecular mass component (a_1, b_1 and c_1) eluted near the void volume (25–35 ml, $K_{av} = 0$–0.13), the intermediate molecular mass component (a_2, b_2 and c_2) eluted in the included volume (35–55 ml, $K_{av} = 0.14$–0.48) and the low molecular mass component (a_3, b_3 and c_3) eluted at the total volume (75–90 ml, $K_{av} = 0.84$–1.0). A total of 10–15 mg of purified HSPG fractions a_2, b_2, and c_2 was recovered from 4 kg of wet bovine brain tissue. These fractions were next analysed by PAGE. The high molecular mass component (a_1, b_1 and c_1) in each chromatogram remained at the interface between the stacking gel and resolving gel, consistent with it being a high molecular mass aggregate of HSPG. Addition of SDS to this separation resulted in the movement of most of the sample into the resolving gel (migrating as a broad band of $M_r \sim 200000$), suggesting that this material was primarily a non-covalently associated aggregate of HSPG. The intermediate molecular mass component (a_2, b_2 and c_2) ran into the resolving gel, migrating slightly behind bovine articular decorin PG of M_r 180000 [49] consistent with an HSPG of $M_r \sim 200000$. Because of their polydisperse nature, only a slight difference was observed when the HSPG fractions were analysed by SDS/PAGE under both reducing and non-reducing conditions. The low-molecular-mass component (a_3, b_3 and c_3) migrated at the same position as did bovine kidney HS, suggesting that this was free GAG or peptidoglycan HS.

Analysis of the intermediate components using SDS/PAGE following treatment with immobilized heparin lyase III and immobilized chondroitin lyase ABC was performed next (Figure 3). Direct detection with Coomassie Blue visualization showed a

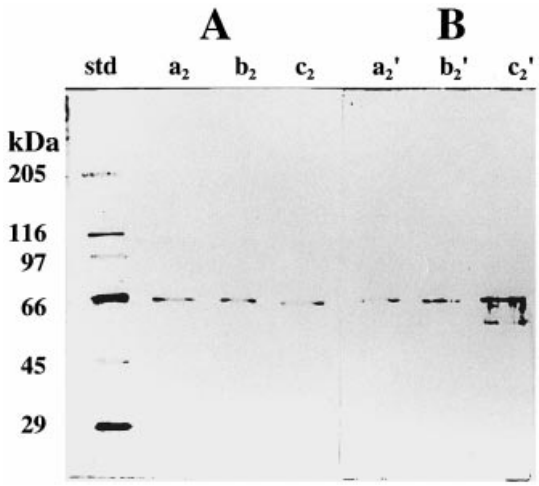


Figure 3 Analysis of PG-containing fractions a_2, b_2 and c_2 by SDS/PAGE

(**A**) shows a Coomassie-stained gel with lanes labelled as follows: molecular mass standards (std), a_2, b_2 and c_2. (**B**) shows a Western blot using the glypican antibody with lanes labelled as follows: a_2', b_2' and c_2'.

Table 1 Amino acid compositional analysis

All values are presented as mol% and the values for the c_2 core protein (both purified and blotted) were determined experimentally in the present study. The compositions for the other proteins were calculated from reported sequences (DNA Star Protein Data Bank). Cys and Trp were not determined for the c2 core protein, and for purposes of comparison the values are not presented for the other proteins (–). Abbreviations used: Chs, chondroitin; endo, endolytic; exo, exolytic.

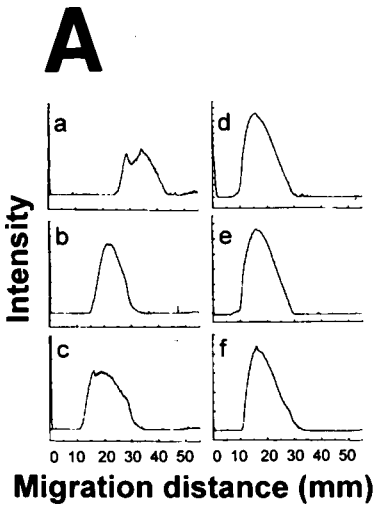
	c_2 Core (purified) protein	c_2 Core (blotted) protein	Rat brain glypican 1	Human lung glypican 1	Heparin lyase III*	Chs ABC lyase 1 (endo)*†	Chs ABC lyase 1 (exo)*†
Asx	9.78	10.38	10.92	10.72	15.0	13.5	12.5
Thr	8.88	8.95	4.33	4.74	3.9	6.4	7.5
Ser	13.95	12.74	7.84	7.84	6.0	7.4	5.8
Glx	12.81	14.12	11.75	12.16	10.6	10.9	12.5
Pro	9.12	9.64	5.15	5.36	5.5	4.4	4.1
Gly	8.10	7.13	8.25	8.04	7.9	6.6	6.1
Ala	6.87	7.52	9.69	9.07	8.2	6.9	8.6
Cys	–	–	–	–	–	–	–
Val	6.32	6.58	4.95	5.57	5.8	4.5	4.1
Met	0.49	0.35	2.27	2.06	1.1	2.3	2.5
Ile	3.22	3.05	3.51	2.68	3.8	5.7	5.0
Leu	9.18	8.94	11.55	12.16	6.6	9.8	10.6
Tyr	1.13	0.93	2.68	2.27	5.8	4.2	4.1
Phe	3.37	2.96	2.47	2.89	5.7	4.2	3.8
His	1.18	1.13	2.27	1.86	2.0	2.4	2.9
Lys	2.13	2.11	4.74	4.12	6.3	7.1	6.2
Trp	–	–	–	–	–	–	–
Arg	3.74	3.46	7.63	8.45	5.5	3.7	3.5

* Potential contaminants in c_2 core protein.

† Commercially prepared chondroitin ABC lyase is comprised of an endolytic and exolytic enzyme [56].

Figure 4 Size of HS chains

The samples were subjected to PAGE (12%, w/v, acrylamide) and stained with Alcian Yellow followed by silver staining. The lanes are as follows: (a) low-molecular-mass heparin; (b) PIM heparin; (c) PIM HS; (d) HS GAG from a_2; (e) HS GAG from b_2; (f) HS GAG from c_2; (g) heparin oligosaccharide standards; (h) heparin octasaccharide.



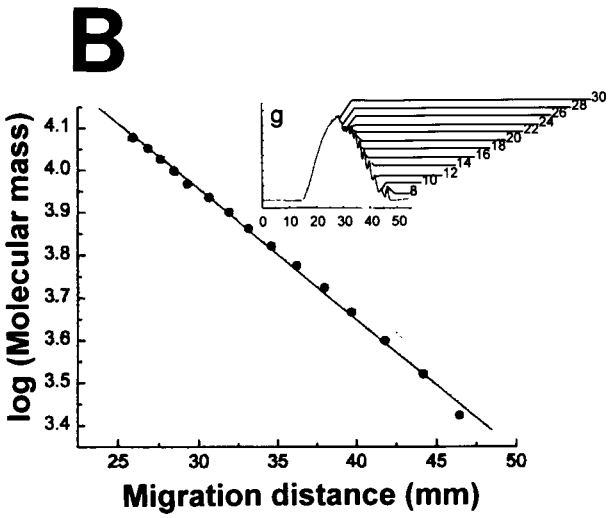


Figure 5 Staining density SDS/PAGE results (from Figure 4) as a function of migration distance

(**A**) shows a scan of the following lanes from Figure 4: (a) low-molecular-mass heparin; (b) PIM heparin; (c) PIM HS; (d) HS GAG of a_2; (e) HS GAG of b_2; (f) HS GAG of c_2. (**B**) The inset shows a scan of lane (g), heparin oligosaccharide standards, with the degree of polymerization marked. The linear relationship between the log of molecular mass and migration distance for these heparin oligosaccharide standards is also shown.

Table 2 Molecular mass determination of HS GAG chains from a_2, b_2 and c_2 compared with low-molecular-mass heparin, PIM heparin and PIM HS

Glycosaminoglycans	$M_{r,av}$ (range)
Low-molecular-mass heparin	6728 (3427–13449)
PIM heparin	15919 (8307–26884)
PIM HS	19420 (7320–35679)
HS from a_2	24413 (8561–41979)
HS from b_2	23690 (9092–38586)
HS from c_2	23547 (7498–37215)

single band at M_r 66000 for HSPGs a_2, b_2 and c_2. It should be noted that even when 10 μg of HSPG sample (an amount determined by both amino acid analysis and bicinchoninic acid assay) was loaded on to the gel it stained only faintly with silver. Western blotting and visualization with HS-stub antibody [39] (results not shown) or glypican antibody [38] (Figure 3) after transfer to nitrocellulose showed a band at the same position as the silver-stained sample.

Amino acid analysis was performed after treatment of HSPGs with immobilized heparan lyase III and immobilized chondroitin lyase ABC. The core protein was further purified by Sephadex G-25 chromatography following digestion, and the major peak was submitted for amino acid compositional analysis. The core protein was also blotted on to a PVDF membrane, and the transferred band was excised for compositional analysis. The two amino acid compositional analyses showed nearly identical results (Table 1). The composition of HSPG c_2 was higher in Thr, Ser and Pro and lower in Met, Tyr and Lys than glypican. Furthermore, contamination by the immobilized lyases used to prepare HSPG c_2 core protein could not have resulted in the observed differences. Attempts to sequence the N-terminus of this protein after transfer to nitrocellulose failed, suggesting that the N-terminus might be blocked or may have been modified during electrophoresis and electrotransfer. Treatment of the purified HSPG with sequencing-grade modified trypsin followed by peptide mapping on narrow-bore C_{18} HPLC afforded nearly identical peptide maps for a_2, b_2 and c_2. Several internal peptides were submitted for sequencing. No sequence data could be obtained from the many peptides eluting early from the column suggesting that they were glycosylated. A major peak, eluting late in the peptide map, gave a sequence QDINKYIA and a second peptide peak eluting in the middle of the peptide map showed a mixture of both a major (80%) sequence, MQPGV, and a minor (20%) sequence, FLKDQ.

The GAG chains of HSPG a_2, b_2 and c_2 were next analysed. Mild β-elimination [41] was used to obtain the intact HS GAG chains from each HSPG. The size of the HS GAG chain was analysed by PAGE (Figure 4) after β-elimination [50]. The HS GAG chain size was compared with a fully sulphated heparin octasaccharide, a mixture of heparin oligosaccharide standards, low-molecular-mass heparin, PIM heparin and PIM HS. The gel was stained with Alcian Yellow followed by silver staining and scanning. The staining density of each lane was plotted as a function of migration distance (mm) (Figure 5). Heparin oligosaccharides were used to prepare a standard curve giving a linear relationship between $\log M_r$ and migration distance (mm) as previously demonstrated by Edens et al. [50]. The average

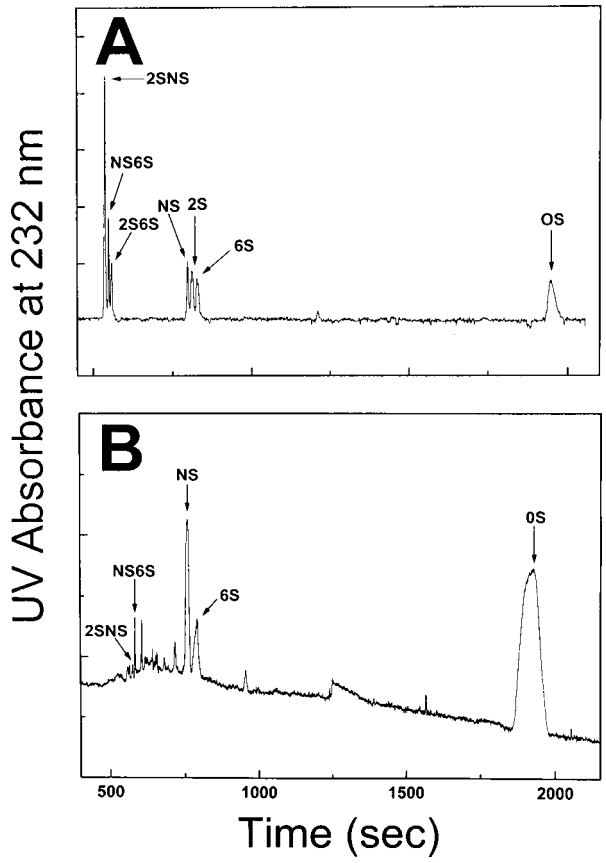


Figure 6 Capillary electropherograms of b_2 used in the disaccharide compositional analysis

Panel (**A**) shows heparin and HS disaccharide standards. Panel (**B**) shows the disaccharide composition of the b_2 fraction after treatment with a mixture of equal amounts (on an activity basis) of heparin lyase I, II and III. The reverse polarity mode of CE was carried out as described in the Experimental section. Abbreviations used: 0S, ΔUAp(1 → 4)-D-GlcNpAc; 6S, ΔUAp (1 → 4)-D-GlcNpAc6S; 2S, ΔUAp2S(1 → 4)-D-GlcNpAc; NS, ΔUAp(1 → 4)-D-GlcNpS; 2S6S, ΔUAp 2S(1 → 4)-D-GlcNpAc6S; NS6S, ΔUAp(1 → 4)-D-GlcNpS6S; 2SNS, ΔUAp2S(1 → 4)-D-GlcNpS.

molecular mass of each HS GAG was determined and the results are presented in Table 2.

The glycans released on mild β-elimination were reductively aminated with an AGA fluorescent tag and analysed by GPC on Sephadex G-200 and by PAGE. These analyses showed ~ 80% of the GPC-incorporated fluorescent tag was in small heparan lyase insensitive glycans ($M_r < 1000$), whereas ~ 20% was incorporated into HS GAG chains ($M_r \sim 20000$). Disaccharide compositional analysis was performed by treating the HS GAG chains released from HSPG c_2 with a mixture of heparin lyases I, II and III (of equivalent activities) followed by CE analysis (Figure 6). The resulting disaccharide composition the HS GAG chains from each HSPG is presented in Table 3 and compared with the disaccharide composition of HS GAGs from other species and tissues [51]. The degree of sulphation (sulphate/disaccharide) of the a_2, b_2 and c_2 HS GAG chains was calculated from the disaccharide analysis as, 0.14, 0.22 and 0.45, respectively (Table 4). The substantial difference in the N-sulphate content of HS GAG fractions a_2 and b_2 (Table 4) was studied by PAGE, following low pH nitrous acid treatment. Both HS GAG fractions showed only the loss of low molecular mass (< 15000) chains to form oligosaccharide products that were only faintly visible on silver staining (results not shown). No GPI anchor was detected in HSPG a_2.

Table 4 Degree of sulphation and % of total sulphates

The degree of sulphation/disaccharide is the number of sulphate groups per disaccharide repeating unit. Total 2-O-sulphates (%), 6-O-sulphates (%) and N-sulphates (%) represent the mol % of all disaccharides having 2-O-sulphate, 6-O-sulphate and N-sulphate groups.

	HSPG fraction		
	a_2	b_2	c_2
Degree of sulphation/disaccharide	0.14	0.22	0.45
Total 2-O-sulphates (%)	0.5	0.4	5
Total 6-O-sulphates (%)	5	7	20
Total N-sulphates (%)	8.5	14.4	21

DISCUSSION

Purification of the PGs of whole adult bovine brain afforded three fractions differing in charge density (labelled a, b and c). Size fractionation afforded intermediate molecular mass species (~ 200 kDa) (a_2, b_2 and c_2) that were PGs. Approx. 2.8–3.4 μg of purified HSPG (a_2, b_2, and c_2) were obtained from 1 g of wet bovine brain tissue. This value is consistent with an approximate

Table 3 Disaccharide compositional analysis

All values are presented as % (w/w) and the values for the GAG components of HSPG $a_2/b_2/c_2$ proteins were determined experimentally in the present study. The samples were treated with heparin lyase I, II and III, and the disaccharides separated and identified by CE. The compositions for the other GAGs are taken from [51]. Not detected (–).

	HSPG $a_2/b_2/c_2$*	HS GAG rat brain (1day)	HS GAG rat brain (adult)	HS GAG rabbit brain	HS GAG bovine brain	HS GAG bovine kidney	HS GAG bovine lung
ΔUAp-GlcNpAc	87/80/66	52	69	46	49	51	31
ΔUAp-GlcNpAc6S	4/6/13	7	5	7	9	18	20
ΔUAp2S-GlcNpAc	—/—/trace	–	–	–	–	–	–
ΔUAp-GlcNpS	7/13/9	18	20	21	17	12	22
ΔUAp2S-GlcNpAc6S	–	–	–	–	–	–	–
ΔUAp-GlcNpS6S	1/1/7	6	4	5	8	8	13
ΔUAp2S-GlcNpS	0.5/0.4/5	10	2	14	11	3	10
ΔUAp2S-GlcNpS6S	–	7	trace	7	6	8	4

* The peak areas of other minor peaks are not taken into account.

yield of 3.5 μg of HSPG/g of wet rat liver tissue obtained by Lyon and Gallagher [15]. Porcine brain reportedly yields 0.8 μg of HS GAG/g of wet tissue [52]. These results, together with their sensitivity to heparin lyase III, suggest that a_2, b_2 and c_2 represent the major HSPG in bovine brain.

Treatment of purified HSPG with immobilized heparin lyase III and immobilized chondroitin lyase ABC afforded core proteins of molecular mass 66 kDa. This result together with their identical peptide maps suggested that the charge differences between the three forms of this PG were associated with the GAG chains. The five known glypican family members have core protein sizes from 55–65 kDa; our core protein is very close to this size, suggesting that we have isolated a member(s) of the glypican family of PGs. After the core protein was obtained by treatment with immobilized heparin lyase III and immobilized chondroitin lyase ABC and analysed by SDS/PAGE, it was difficult to detect with silver staining (requiring ~ 10 μg of protein). This difficulty suggests that the PG was heavily glycosylated. Similar observations regarding the low staining intensity of other PGs have been reported [53]. When the purified PG was treated separately with immobilized heparin lyase III and immobilized chondroitin lyase ABC, only the heparin lyase III treatment was required to reduce it to a core protein size of 66 kDa, demonstrating that this PG has only HS chains. Both HSPG-stub antibody and glypican antibody recognized the core protein. The amino acid composition analysis obtained on the core protein did not match any of the known glypicans.

The peptide map of the PG core gave mostly peaks that eluted early from the C_{18} column. These early eluting peptides could not be sequenced. From peaks eluting late in the peptide map three internal peptide sequences were obtained. The longest of these, an 8-mer, gave only a low similarity (37.5 %) to sequences from human glypican-5. Only one match (to the variable region of mouse IgG) was found in a search of GenBank. The shorter 5-mer sequences had higher similarity not only to glypicans but to many other proteins as well.

The GAG chains on the PG were clearly demonstrated to be HS, as they were heparin lyase III sensitive, and displayed no chondroitin ABC lyase sensitivity. Mild β-elimination was used to release the O-linked glycans from the HSPG. The molecular mass of the released HS chains were calculated as $M_{r,av} = 24000$ with a range of 7500–42000. The number of GAG chains was estimated as six from the M_r of the HS chains and the observed molecular size differences between the PG and core protein. The difference in the elution position of the PG from DEAE-Sephacel was ascribed to sulphation differences in the HS GAG rather than to differences in GAG chain length. The released O-linked glycans were fluorescently labelled by reductive amination with AGA. Analysis by GPC and PAGE showed ~ 80 mol % of the labelled sample was small ($M_r < 1000$) O-linked glycans, whereas ~ 20 mol % were HS GAG chains. These results, in combination with the measured M_r of the HS GAG chains and the core protein, suggest that the HSPG contains four to six GAG chains and 16–24 small O-linked glycan chains. This is consistent with the large number of serine residues (13 mol %) found in the core protein (Table 1).

Disaccharide analysis showed that all forms of the PG contained the same disaccharide components, but that the percentage of sulphated disaccharides increased from a_2 to b_2 to c_2. The disaccharide composition of the HS GAG chains derived from a_2 and b_2 HSPG fractions was considerably higher in unsulphated disaccharide (ΔUAp -GlcNpAc) than that found in the HS GAGs of other bovine tissues and the brain tissues of other species (Table 3). HSPG fraction c_2 most closely resembled the composition of HS GAGs obtained from brain tissues. HSPG c_2 showed ΔUAp-GlcNpAc, ΔUAp2S-GlcNpS and ΔUAp2S-GlcNpS6S levels comparable to HS GAG isolated from adult rat brain (66 %, 5 %, 0 %, respectively, compared with 69 %, 2 %, trace). The levels of ΔUAp-GlcNpAc6S and ΔUAp-GlcNpS6S in HSPG c_2 are close to those of HS GAG isolated from adult bovine brain (13 % and 7 %, respectively, compared with 9 % and 8 %) The levels of ΔUAp-GlcNpS observed in all HSPG fractions (a_2, b_2 and c_2) are considerably lower than those observed in any of the brain HS GAGs. Although it is difficult to interpret the reasons for these observed differences in disaccharide compositions, one possibility is that all of the previously reported data come from total brain HS GAG. Those analyses corresponded to a diverse group of HSPGs present in whole brain tissue whereas the disaccharide composition presented in the present work comes from a single bovine brain HSPG. Of possible interest is that the c_2 form contained a trace amount of ΔUAp2S-GlcNpAc. This disaccharide could arise from either the [→4)-β-D-GlcAp2S(1→4)-β-D-GlcNpAc(1→] or the [→4)-α-L-IdoAp2S(1→4)-β-D-GlcNpAc(1→] sequence. The first of these two sequences has been previously described as one prevalent in HS GAG chains extracted from amyloid plaques [54]. HS GAG chains prepared from a_2 and b_2 through β-elimination showed similar changes on low pH nitrous acid treatment despite the nearly two-fold difference in their content of N-sulphate groups (Table 4). The disappearance of only the lower molecular mass HS GAG chains on low pH nitrous acid treatment suggests that the HSPGs contain two different types of GAG chains, those that are N-sulphate-group rich ($M_r < 15000$) and those that are N-sulphate-group poor ($M_r > 15000$). More work is required to understand the fine details of the structure of these HS GAG chains. A GPI anchor could not be detected, and it is unlikely that it could have been lost during HSPG purification, since the conditions used were very mild [41]. More likely explanations are either that the HSPG had been primarily isolated from the extracellular matrix after having been shed from the membrane, or that this HSPG is not a glypican.

In conclusion, the major HSPG in adult bovine brain tissue has been purified and the structure of its protein and GAG components partially characterized. This PG occurs in three major forms that differ in saccharide composition, primarily on the basis of sulphation level. The most sulphated form of this PG contains a trace amount of a disaccharide that corresponds to that frequently occurring in HS GAG chains extracted from amyloid plaque deposits. Work is ongoing to study the binding of prion and amyloid plaque proteins to this new bovine brain HS PG.

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REFERENCES

1 Gallagher, J. T., Lyon, M. and Steward, W. P. (1986) Biochem. J. **236**, 313–325
2 Gallagher, J. T., Turnbull, J. E. and Lyon, M. (1990) Biochem. Soc. Trans. **18**, 207–209
3 David, G. (1993) FASEB J. **7**, 1023–1030
4 Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J. and Rees, D. C. (1996) Science **271**, 1116–1120
5 Steinfeld, R., Van Den Berghe, H. and David, G. (1996) J. Cell Biol. **133**, 405–416
6 Kato, M., Wang, H., Bernfield, M., Gallagher, J. T. and Turnbull, J. E. (1994) J. Biol. Chem. **269**, 18881–18890
7 Kinnunen, T., Raulo, E., Nolo, R., Maccarana, M., Lindahl, U. and Rauvala, H. (1996) J. Biol. Chem. **271**, 2243–2248

8 Rosenberg, R. D., Bauer, K. A. and Marcum, J. A. (1985) J. Med. **16**, 351–416
9 Bernfield, M, Kokenyesi, R., Kato, M., Hinkes, M. T., Spring, J., Gallo, R. L. and Lose, E. J. (1992) Annu. Rev. Cell Biol. **8**, 365–393
10 Salmivirta, M. and Jalkanen, M. (1995) Experientia **51**, 863–872
11 Lander, A. D., Stipp, C. S. and Ivins, J. K. (1995) Perspect. Develop. Neurobiol. **3**, 347–358
12 Rosenberg, R. D., Shworak, N. W., Liu, J., Schwartz, J. J. and Zhang, L. (1997) J. Clin. Invest. **100**, S67–S75
13 Fedarko, N. S. (1993) Experientia **49**, 369–383
14 Hascall, V. C., Calabro, A., Midura, R. J. and Yanagishita, M. (1994) Methods Enzymol. **230**, 390–417
15 Lyon, M. and Gallagher, J. T. (1991) Biochemistry **273**, 415–422
16 David, G., Lories, V., Decock, B., Marynen, P, Cassiman, J.-J. and Van den Berghe, H. (1990) J. Cell Biol. **111**, 3165–3176
17 Karthikeyan, L., Maurel, P., Rauch, U., Margolis, R. K. and Margolis, R. U. (1992) Biochem. Biophys. Res. Commun. **188**, 395–401
18 Carey, D. J., Evans, D. M., Stahl, R. C., Asundi, V. K., Conner, K. J., Garbes, P. and Cizmeci-Smith, G. (1992) J. Cell Biol. **117**, 191–201
19 Watanabe, K., Yamada, H. and Yamaguchi, Y. (1995) J. Cell Biol. **130**, 1207–1218
20 Litwack, E. D., Stipp, C. S., Kumbasar, A. and Lander, A. D. (1994) J. Neurosci. **14**, 3713–3724
21 Karthikeyan, L., Flad, M., Engel, M., Meyer-Puttlitz, B., Margolis, R. U. and Margolis, R. K. (1994) J. Cell Sci. **107**, 3213–3222
22 Chernousov, M. A. and Carey, D. J. (1993) J. Biol. Chem. **268**, 16810–16814
23 Margolis, R. K. and Margolis, R. U. (1993) Experientia **49**, 429–446
24 Ripellino, J. A. and Margolis, R. U. (1989) J. Neurochem. **52**, 807–812
25 Small, D. H., San Mok, S., Williamson, T. G. and Nurcombe, V. (1996) J. Neurochem. **67**, 889–899
26 Stipp, C. S., Litwack, E. D. and Lander, A. D. (1994) J. Cell Biol. **124**, 149–160
27 Dow, K. E., Mirski, S. E. L., Roder, J. C. and Riopelle, R. J. (1988) J. Neurosci. **8**, 3278–3289
28 Cornelli, U. (1996) in Noncoagulant Actions of Glycosaminoglycans (Harenberg, J. and Casu, B., eds.), pp. 249–279, Plenum Press, New York
29 Baldwin, M. A., Cohen, F. E. and Prusiner, S. B. (1995) J. Biol. Chem. **270**, 19197–19200
30 Snow, A. D., Mar, H., Nochlin, D., Kimata, K., Kato, M., Suzuki, S., Hassell, J. and Wight, T. N. (1988) Am. J. Pathol. **133**, 456–463
31 Williamson, T. G., San Mok, S., Henry, A., Cappai, R., Lander, A. D., Nurcombe, V., Beyreuther, K., Masters, C. L. and Small, D. H. (1996) J. Biol. Chem. **271**, 31215–31221
32 Chadha, K. C., Kazim, A. L., Parthasarathy, R., Sulkowski, E. and Tomasi, T. B. (1997) Int. J. Biochromatogr. **2**, 211–223
33 Snow, A. D., Wight, T. N., Nochlin, D., Koike, Y., Kimata, K., Dearmond, S. J. and Prusiner, S. B. (1990) Lab. Invest. **63**, 601–611
34 Patterson, W. J. and Dealler, S. (1995) J. Public Health Med. **17**, 261–268
35 Lacey, R. W. (1996) Br. Food J. **98**, 3–4
36 Chandrasekhar, S., Esterman, M. A. and Hoffman, H. A. (1987) Anal. Biochem. **161**, 103–108
37 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
38 Liang, Y., Haring, M., Roughley, P. J., Margolis, R. K. and Margolis, R. U. (1997) J. Cell Biol. **139**, 851–864
39 Gallagher, S., Winston, S. E., Fuller, S. A. and Hurrell, J. G. R. (1997) in Current Protocols in Molecular Biology, Analysis of Proteins (Ausubel, F. M., ed.), pp. 10.8.1–10.8.21, Wiley Interscience, Boston
40 Caldwell, E. E. O., Nadkarni, V. D., Fromm, J. R., Linhardt, R. J. and Weiler, J. M. (1996) Int. J. Biochem. Cell Biol. **28**, 203–216
41 Heinegard, D. (1972) Biochim. Biophys. Acta **285**, 193–207
42 Pervin, A., Gallo, C., Jandik, K. A., Han, X.-J. and Linhardt, R. J. (1995) Glycobiology **5**, 83–95
43 Park, Y., Cho, S. and Linhardt, R. J. (1997) Biochim. Biophys. Acta **1337**, 217–226
44 Liu, J., Desai, U. R., Han, X. J., Toida, T. and Linhardt, R. J. (1995) Glycobiology **5**, 765–774
45 Pervin, A., Al-Hakim, A. and Linhardt, R. J. (1994) Anal. Biochem. **221**, 182–188
46 Jayson, G. C., Lyon, M., Paraskeva, C., Turnbull, J. E., Deakin, J. A. and Gallagher, J. T. (1998) J. Biol. Chem. **273**, 51–57
47 Shively, J. E. and Conrad, H. E. (1976) Biochemistry **15**, 3932–3942
48 Ferguson, M. A. I. (1993) in Glycobiology: A Practical Approach (Fukuda, M. and Kobata, A., eds.), pp. 349–383, Oxford Univeristy Press, New York
49 Choi, H. U., Johnson, T. L., Pal, S., Tang, L.-H., Rosenberg, L. and Neame, P. J. (1989) J. Biol. Chem. **264**, 2876–2884
50 Edens, R. E., Al-Hakim, A., Weiler, J. M., Rethwisch, D. G., Fareed, J. and Linhardt, R. J. (1992) J. Pharm. Sci. **81**, 823–827
51 Tekotte, H., Engel, M., Margolis, R. U. and Margolis, R. K. (1994) J. Neurochem. **62**, 1126–1130
52 Toida, T., Yoshida, H., Toyoda, H., Koshiishi, I., Imanari, T., Hileman, R. E., Fromm, J. R. and Linhardt, R. J. (1997) Biochem. J., **322**, 499–506
53 Ripellino, J. A. and Margolis, R. U. (1989) J. Neurochem. **52**, 807–812
54 Lindahl, B., Eriksson, L. and Lindahl, U. (1995) Biochem. J. **306**, 177–184
55 Bitter, T. and Muir, H. M. (1962) Anal. Biochem. **4**, 330–334
56 Hamai, A., Hashimoto, N., Mochizuki, H., Kato, F., Makiguchi, Y., Horie, K. and Suzuki, S. (1997) J. Biol. Chem. **272**, 9123–9130

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