

Purification and characterization of heparan sulfate peptidoglycan from bovine liver

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Abstract

A method for the isolation and purification of peptidoglycan heparan sulfate (HS) from bovine liver is described. Peptidoglycan HS was purified from bovine liver and its structure was characterized. The purity and identity of this HS was determined following treatment with heparin lyases by using polyacrylamide gel electrophoresis. The disaccharide compositional analysis on the peptidoglycan samples, determined by treatment with a mixture of heparin lyases followed by high-resolution capillary electrophoresis, showed that bovine liver HS was more highly sulfated than the standard porcine intestinal mucosal HS, but it contained no measurable amount of trisulfated disaccharide. One-dimensional ¹H-NMR spectroscopy was used to calculate the relative amounts of glucuronic and iduronic acid residues that were present. The presence of peptide chain in this HS was confirmed by amino acid composition analysis and its selective biotinylation and conjugation with streptavidin. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Heparan sulfate (HS) is widely distributed in animal and human tissues and has diverse roles in development, differentiation, and homeostasis. HS is a glycosaminoglycan (GAG), covalently linked to a protein core in syndecan or glypican (Fransson, 1989; Gallagher, 1989), ubiquitously expressed integral membrane and glycerol phosphatidyl inositol-linked HS proteoglycans. The protein core is biosynthesized in the endoplasmic reticulum and is glycosylated as it transits the Golgi. The HS polysaccharide is assembled by sequential action of glycosyltransferases resulting in the alternating addition of D-GlcAp and N-acetyl-D-glucosamine (GlcNpAc) resulting in a 1 → 4 linked, linear polysaccharide that varies in length from 30 to 200 disaccharides units (Gallagher, Turnbull & Lyon 1992). The chains are then modified heterogeneously and in domains through the differential action of an N-deactylase-N-sulfotransferase, C-5 epimerase and various

O-sulfotransferases. These modifications are incomplete resulting in disaccharides having both high and low content of sulfation (Fig. 1B), that are often clustered into domains (Gallagher, Lyon & Steward, 1986; Lindahl, Kusche-Gullberg & Kjellen, 1998; Maccarana, Sukara, Tawada, Yoshida & Lindahl, 1996) providing specific binding sites for a variety of proteins. Protein binding to HS may serve to sequester the protein at a particular site or to activate the protein. For example, the binding of antithrombin III (Rosenberg, Shwork, Liu, Schwartz & Zhang, 1997) to a specific pentasaccharide sequence in HS results in the activation of its anticoagulant activity (Kreuger, Pryds, Petterson, Lindahl & Salmivirta, 1999; Lindahl et al., 1998). The HS is of considerable interest because of its complexity and diversity of structure (Gallagher, Lyon & Steward, 1986; Lindahl, Thunberg, Bäckström, Riesenfeld, Nordling & Björk, 1984), which appear to be cell-specific and possibly differentiation-specific, together with their propensity for interaction with a wide range of extracellular proteins. Knowledge of the variations in composition and organization of HS from different cells and tissues is becoming increasingly important as attempts are made to elucidate the relationships between HS structure and function, i.e., the sequence-specific binding and activation

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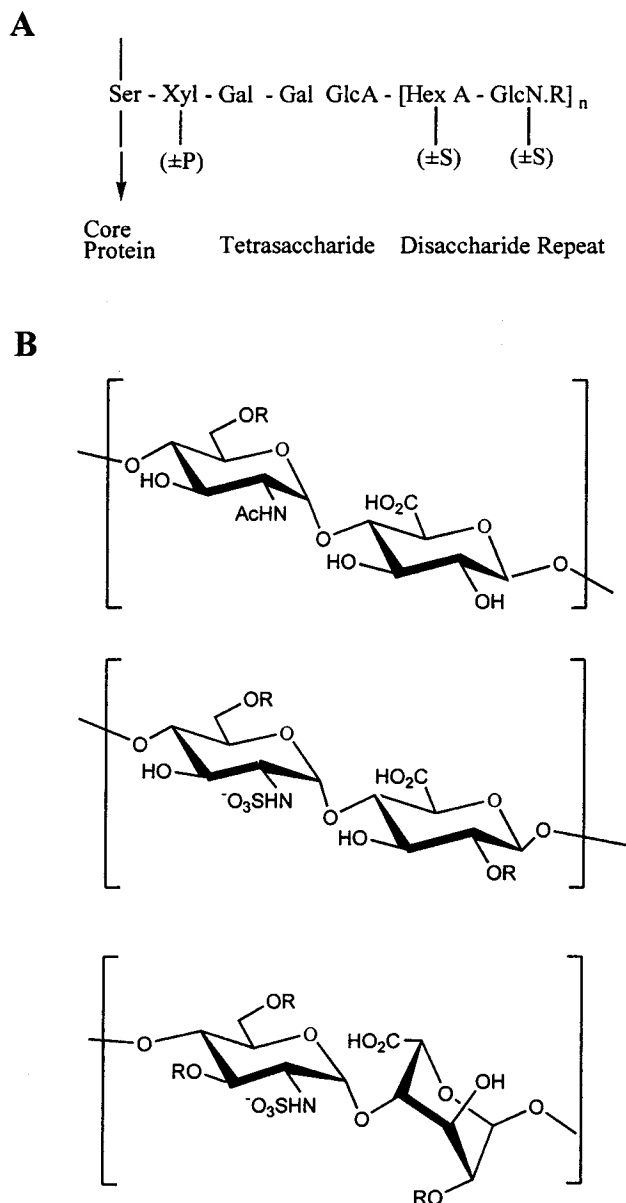


Fig. 1. (A) Protein-linkage region of HS. The hexuronate (HexAp)-glucosamine (GlcNp) disaccharide repeat of HS is contiguous with a tetrasaccharide sequence in which a terminal xylose is covalently-attached to serine (Ser) in the core protein by an *O*-glycosidic linkage. The xylose may be phosphorylated at C-2. (\pm P) = phosphate; (\pm S) = SO_3^- ; R = acetyl or SO_3^- . (B) Disaccharide sequences commonly found in HS, R = H or SO_3^- .

of proteins. In this context, the HS biosynthesized by the liver is potentially interesting because, since its early description (Oldberg, Höök, Öbrink, Pertoft & Rubin, 1977) it has been known that it has a relatively high level of overall level of sulfation (Gallagher & Lyon, 1989; Lyon and Gallagher, 1994). However, it continues to be difficult to isolate sufficient quantities to analyze using sophisticated modern spectroscopic methods, such as high field NMR. Furthermore, while the structure of HS-derived oligosaccharides containing linkage region have been determined (Toida & Linhardt, 1998), peptidoglycan (pG) HS chains

pose a challenging and attractive target for recently developed sequencing methods (Liu, Desai, Han, Toida & Linhardt, 1995; Turnbull, Hopwood & Gallagher, 1999). HS GAGs have been: isolated and purified from a large variety of animal tissues (Dietrich & Nader, 1974; Hovingh, Piepkorn & Linker, 1986; Nader & Dietrich, 1989). Peptidoglycans are typically not isolated from tissues, as the standard use of strongly basic extraction conditions, results in the β -elimination of peptide affording GAG. Moreover, the presence of the amino terminus on the peptide portion of the pG-HS makes it especially useful for its immobilization in applications such as surface plasmon resonance for HS-protein interaction studies (Hernáiz, Liu, Rosenberg & Linhardt, 2000; Hileman, Fromm, Weiler & Linhardt, 1998). The objective of this study is to prepare milligram quantities of pG HS and to introduce biotin into its peptide side-chain for future application in sequencing studies.

2. Results and discussion

Homogenization of a 2.5 kg portion of a whole bovine liver in acetone and subsequent extraction in chloroform/methanol resulted in 650 g of de-fatted tissue (Fig. 2). De-fatted tissue was subjected to proteolytic digestion and the supernatant obtained was diluted and adsorbed on to strong anion exchange (SAX) macroporous Dowex resin. After washing the resin with water, elution with 3% sodium chloride and 16% sodium chloride and precipitation with ethanol afforded a total of 10.7 g of crude anionic material.

Chondroitin sulfate, dermatan sulfate and nucleic acid contaminants were removed through exhaustive digestion with chondroitinase ABC and endonuclease. SAX chromatography of this enzyme treated sample afforded uronic acid containing fractions eluting at between 0.5 and 1.4 M sodium chloride. Highly colored and heparin lyase III resistant contaminants were removed by size-exclusion chromatography. Uronic acid-containing fractions, identified by carbazole assay (Bitter & Muir, 1962), corresponded to HS. These HS fractions eluted from the Sephadex G-150 in the included volume ($MW_{av} = 0.2$), corresponding to a molecular weight 5000–50 000. HS and heparin GAG standards eluted from this column at the same position (data not shown). The combined HS fractions were precipitated, dialyzed and freeze-dried affording 22 mg of purified HS.

HS can be degraded enzymatically by using bacterial heparin lyases. Heparin lyase I acts on both HS and on heparin, in contrast, heparin lyase III is specific for its action on HS and is used to confirm the presence of HS and distinguish it from heparin (LeBrun & Linhardt, 2001). A small portion of the 22 mg of recovered HS (10 μ g) was subjected to digestion with heparin lyase I and heparin lyase III. The products of the heparin lyase I and heparin lyase III digestions were analyzed by PAGE with alcian blue

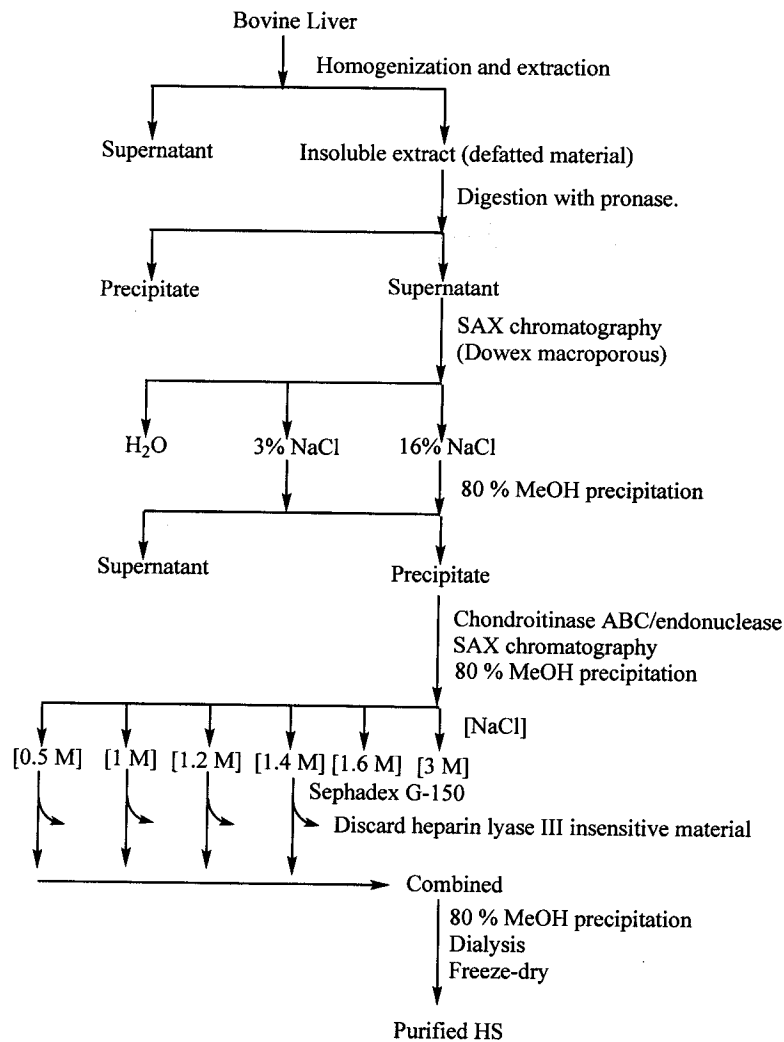


Fig. 2. Schematic summary of HS pG purification.

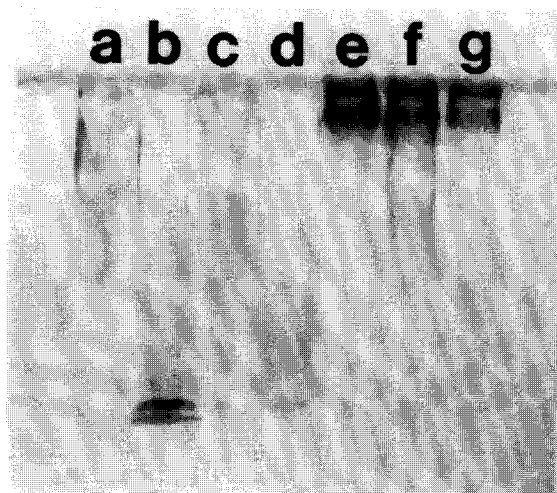


Fig. 3. PAGE analysis of bovine liver HS. Lane (a) standard porcine mucosal heparin; Lane (b) standard heparin treated with heparin lyase I; (c) standard porcine mucosal HS; (d) standard HS treated with heparin lyase III; (e) bovine liver HS; (f) bovine liver HS treated with heparin lyase I; and (g) bovine liver HS treated with heparin lyase III.

staining. This analysis was first performed by treating standard heparin with heparin lyase I (Fig. 3, lanes a and b) and standard HS with heparin lyase III (Fig. 3, lanes c and d). Next, bovine liver HS was analyzed directly by PAGE and was found to run as two dark bands ($MW_{avg} > 25\,000$) at the top of a 22% polyacrylamide gel (Fig. 3 lane e). Treatment with heparin lyase I (Fig. 3 lane f) showed that, while these two bands remained at the top of the gel, a portion of the sample was converted to intermediate sized products ($MW_{avg} \sim 5000$) running one-third of the way into the gel. Treatment with heparin lyase III again showed these two bands but this time with low molecular weight products ($MW_{avg} < 1,000$) running at the bottom third of the gel (Fig. 3 lane g). These analyses clearly demonstrate that the bovine liver preparation contains a heparin lyase I and III sensitive HS. It also contains a heparin lyase resistant component, corresponding to the two bands present at the top of the gel. These high molecular weight bands are not observed in GAG HS or GAG heparin. Because no base treatment was used in the preparation of this bovine liver

HS it is likely that this heparin lyase resistant material consists of peptidoglycan remnants.

A common approach for structural analysis of HS has been to characterize the disaccharide structures following its complete enzymatic depolymerization (Desai, Wang & Linhardt, 1993a,b; LeBrun & Linhardt, 2001; Yamada, Sakamoto, Tsuda, Yoshida, Sugahara, Khoo et al., 1994). The digestion of HS with an equi-unit mixture of heparin lyase I, II and III typically leads to nearly complete conversion of HS to disaccharides (LeBrun & Linhardt, 2001). These disaccharides include Δ UAp(1 \rightarrow 4)-D-GlcNpAc (0S), Δ UAp(1 \rightarrow 4)-D-GlcNpAc6S (6S), Δ UAp2S-(1 \rightarrow 4)-D-GlcNpAc (2S), Δ UAp(1 \rightarrow 4)-D-GlcNpS (NS), Δ UAp2S(1 \rightarrow 4)-D-GlcNpAc 6S(2S6S), Δ UAp(1 \rightarrow 4)-D-GlcNpS6S(NS6S), Δ UAp2S(1 \rightarrow 4)-D-GlcNpS (2SNS) and Δ UAp2S(1 \rightarrow 4)-D-GlcNpS6S (2SNS6S). The unsulfated and monosulfated disaccharides are frequently found in HS but are uncommon in heparin (Toida, Yoshida, Toyoda, Koshiishi, Imamari, Hileman et al., 1997). The disulfated and trisulfated disaccharide sequences generally occur infrequently in HS but are prominent in heparin (Pervin, Yu, Gunay & Linhardt, 1994). The disaccharide analysis was performed and each disaccharide peak was identified by co-migration with the appropriate disaccharide standard (Table 1). Bovine liver HS gave disaccharide products on exhaustive treatment with a mixture of the three heparin lyases. The major product was the unsulfated disaccharide (0S) followed monosulfated disaccharide (NS) and disulfated disaccharide (NS6S) and (2SNS). Surprisingly no trisulfated disaccharide (2SNS6S), commonly found in heparin and also reportedly found in high levels in porcine liver HS (Griffin, Linhardt, Van Gorp, Toida, Hileman, Schubert et al., 1995), was observed in bovine liver HS. Compared to standard porcine intestinal HS, bovine liver HS showed a significantly enhanced sulfation (Lyon and Gallagher, 1994), 2.5% S as compared to 1.1% S (Table 1). It should be noted that heparin lyase is an eliminase and that it affords unsaturated Δ UAp containing disaccharide products, resulting in the loss of C-5 chirality. Thus, CE disaccharide analysis provides no information on the ratio of glucuronic to iduronic acid residues in these fractions.

Table 1
Disaccharide compositional analysis (mole%)

Fractions ^a	HS GAG standard	HS pG bovine liver
Δ UAp-GlcNpAc (0S)	75.7	66.4
Δ UAp-GlcNpAc6S (6S)	4.4	–
Δ UAp2S-GlcNpAc (2S)	–	–
Δ UAp-GlcNpS (NS)	14.1	29.7
Δ UAp2S-GlcNpAc6S (2S6S)	–	–
Δ UAp-GlcNpS6S (NS6S)	1.4	2.8
Δ UAp2S-GlcNpS (2SNS)	1.0	1.1
Δ UAp2S-GlcNpS6S (2SNS6S)	< 1	–
Estimated% S ^b	1.1	2.5

^a Samples were treated with equi-unit of heparin lyase I, II, and III.

^b Calculated from disaccharide analysis.

¹H NMR analysis of bovine lung HS was particularly informative on the ratio of glucuronic to iduronic acid residues (Toida et al., 1997). The 500 MHz 1D ¹H-NMR spectra of bovine liver HS were acquired to analyze its structure (Fig. 4). 1D ¹H NMR gave a spectrum that was consistent with HS structure and the major assignable peaks are shown in Fig. 4. The content of IdoA was estimated at ~50% based on the anomeric signals assigned in Fig. 4. Signals associated with peptide component (–CH₂– and CH₃–) are also observed at 0.9 and 1.15 ppm.

Bovine liver HS was next analyzed by SDS/PAGE with silver staining (not shown). Silver staining of heparin and HS standards (without prior alcian blue staining (Al-Hakim & Linhardt, 1991) showed no detectable material. In contrast, bovine liver HS showed a broad smear over the same molecular weight range as observed by alcian blue staining when the gel was stained directly with silver. These results confirm the presence of an integral peptide component associated with bovine liver HS, suggesting that it contains some HS pG. The amino acid compositional analysis showed presence of various amino acid residues (Table 2). Bovine liver HS preparation showed presence of substantial amounts of Asx, Ser, Glx, Ala and Leu, not observed in the porcine mucosal HS GAG standard. Ser was observed at high levels in bovine liver HS preparation, suggesting that this is the Ser residue to which the GAG chain is attached. Other amino acid residues including Thr, Val, His, Lys and Arg were present in small amounts in

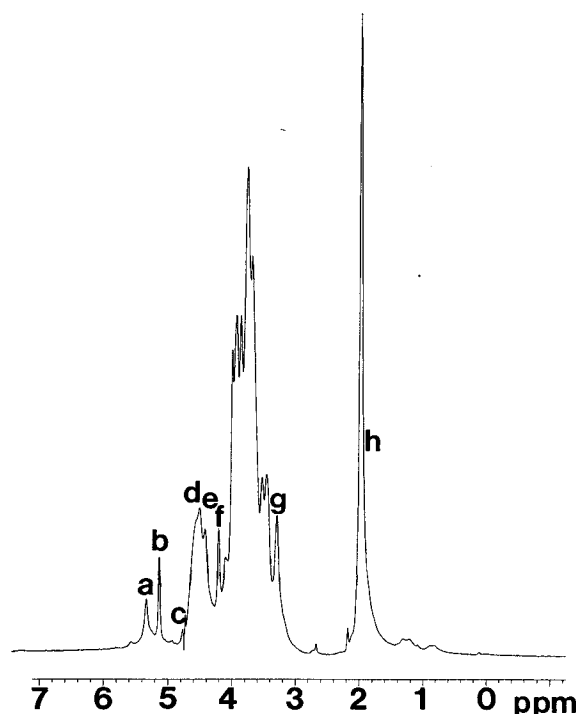


Fig. 4. Analysis of bovine lung HS by 500 MHz ¹H NMR spectroscopy. Labeled peaks are assigned as: (a) H-1 of GlcNpAc (or GlcNpS) \rightarrow GlcAp; (b) H-1 of GlcNpAc \rightarrow IdoAp; (c) H-1 of IdoAp; (d) H-1 of GlcAp; (e) H-5 of IdoAp; (f) H-4 of IdoAp; (g) H-2 of GlcAp; and (h) methyl of GlcNAc.

Table 2
Amino Acid compositional analysis (mole%)

Residue	HS GAG	HS pG Bovine Liver
Asx	Nd ^a	9.5
Thr	Nd	8.8
Ser	Nd	13.2
Glx	Nd	11.4
Ala	Nd	9.2
Val	Nd	2.7
Ile	Nd	1.8
Leu	Nd	13.2
His	Nd	0.3
Lys	Nd	5.0
Arg	Nd	0.4

^a No detectable amino acid residue.

bovine liver HS. Our inability to detect other amino acids and the relatively low level of basic amino acids suggests that the peptide component is covalently attached rather than simply ionically bound (Hileman et al., 1998).

To further confirm the presence of a covalently attached peptide, HS pG was reacted with NHS–LC–biotin. Previous studies using an amino reactive fluorescent label, had demonstrated that such reactions could be specifically targeted to the amino group of the peptide portion of peptidoglycan component (Liu et al., 1995). Streptavidin is a non-glycosylated microbial protein (MW 60 000) that is comprised of four identical tetrameric subunits each containing a site for the high affinity ($K_d \sim 10^{-15}$ M) binding of biotin (Chen and Wold, 1986). Biotinylated HS pG was immobilized on streptavidin beads and its concentration on these beads was determined colorimetrically by dye binding assay using toluidine blue. Based on the standard curve and an average molecular weight of 15 000 for HS, we estimate that 3% at the bovine liver HS chains were biotinylated.

HS is known to contain a small number of unsubstituted amino groups in the GAG backbone that might also react with NHS-biotin (Toida et al., 1997). To confirm that the biotin was attached through peptide (Kuberan, Gunay, Dordick & Linhardt, 1999), the biotinylated HS was subjected to mild β -elimination with lithium hydroxide (Liu et al., 1995) and again tested for binding to streptavidin beads. A marked reduction in streptavidin bound HS was observed clearly demonstrating that the biotin was primarily located on a peptide *O*-linked through the Ser residue of the HS chain.

In summary, while GAGs (Dietrich & Nader, 1974; Nader & Dietrich, 1989; Hovingh et al., 1986; Toida et al., 1997) and proteoglycans (Park, Yu, Gunay & Linhardt, 1999) have been prepared in multi-milligram quantities for structural characterization studies, there are few if any similar reports for HS peptidoglycans. The current study demonstrates that it is possible to prepare HS peptidoglycan from bovine liver. Amino acid analysis confirms the presence of a high fraction of serine in these fractions consistent with the structure of these *O*-linked glycans.

Biotin could be successfully coupled to the peptide portion of bovine liver HS. Future studies will be aimed at using these HS peptidoglycans as targets for recently developed micro-sequencing techniques (Liu et al., 1995; Turnbull et al., 1999) to elucidate the structure surrounding their linkage regions and in surface plasmon resonance for HS-protein interaction studies (Hernaiz et al., 2000; Hileman et al., 1998).

3. Experimental

3.1. Materials

Fresh bovine liver was purchased from a local slaughter house. The following enzymes were obtained from the commercial sources indicated: pronase (type XXV from *Streptomyces griseus*, Sigma), alkalase (proteolytic enzyme from *Bacillus subtilis*, 2.4L, NovoNordisk, Bagsvaerd, Denmark), endonuclease (Benzonase EC 3.1.30.2, Sigma, St. Louis, MO, USA), chondroitinase ABC (chondroitinase ABC lyase from *Proteus vulgaris*, EC 4.2.2.4, Sigma), heparinase I and III (Seikagaku America Inc., Rockville, MD, USA). NHS–LC–Biotin and ImmunoPure Immobilized Streptavidin (Pierce, Rockford, IL, USA), Sephadex G-150, heparin and HS disaccharides standard, alcian blue were obtained from Sigma. Heparin/heparan sulfate disaccharide standards were from Grampian Enzymes, Aberdeen, UK. Dialysis membrane for desalting was from Spectrum Medical. HS and heparin were from Celsus Laboratory (Cincinnati, OH, USA). All other reagents used were analytical grade. UV spectroscopy was performed on a Shimadzu model UV 160 spectrometer equipped with a thermostated cell.

3.2. Isolation of peptidoglycan HS

3.2.1. Extraction

Liver tissue (2.5 kg) was cut into small pieces that were homogenized in a homogenizer in 10 l of ice-cold acetone and after decantation overnight at 4 °C, the resulting supernatant was discarded (Fig. 2). The pellet was suspended in 10 l of ice-cold chloroform/methanol (2:1) and was then re-homogenized and decanted. The extraction with chloroform/methanol was performed five times. The de-fatted material was finally washed with cold ethyl ether and dried overnight. The residue was weighed to obtain dry weight of de-fatted material (650 g).

3.2.2. Digestion with pronase

De-fatted tissue (650 g) was digested with 1.45 g of pronase in 2.8 l of 0.1 M Tris-HCl buffer containing 2 mM CaCl₂, 3% ethanol (pH = 7). After 48 h of incubation at 50 °C the digest was heated at 100 °C for 1 h. The sample was cooled and centrifuged at 8000 rpm for 20 min and the resulting pellet was discarded. The supernatant was diluted to 6 l with the same buffer and applied to a Dowex macroporous SAX resin column (5 × 30 cm) eluted with 3

column volumes of water, 2 column volumes of 50% aqueous methanol, 3 column volumes of 3% and 16% aqueous sodium chloride solution. Fractions obtained in the 3% and 16% sodium chloride washes were precipitated with 80% of ethanol, centrifuged at 8000 rpm for 20 min, combined and freeze-dried overnight to afford 10.7 g of crude pG/GAG mixture.

3.2.3. Digestion with chondroitinase ABC and endonucleases

For degradation of nucleic acids and galactosaminoglycans, sample (10.7 g crude pG/GAG mixture) was dissolved in 300 ml of 0.05 M Tris-HCl buffer pH = 7, 1 mM MgCl₂, 3 mM sodium acetate containing 20 unit of chondroitinase ABC and 25 000 units of endonuclease and the mixture was incubated at 37 °C for 15 h and the reaction was terminated by heating at 100°C for 1 h.

The HS pG/GAG containing mixture (600 mg in 150 ml of water) was applied to a Dowex macroporous SAX resin column (5 × 30 cm) and eluted with 2 column-volumes of 0.5, 1, 1.2, 1.4, 1.6 and 3 M NaCl. Fractions obtained in each of these washes were precipitated with 80% of ethanol and then centrifuged at 8000 rpm for 20 min and freeze-dried. Four SAX fractions afforded methanol precipitates totaling 66 mg.

The four SAX fractions affording methanol precipitates were applied to a G-150 Sephadex column (1.5 × 95 cm) equilibrated with a solution of 200 mM NaCl and eluted at 0.25 ml/min. Carbohydrate-containing fractions were identified by carbazole assay (Bitter & Muir, 1962) and UV detection at 525 nm. The carbazole-positive fractions were combined, desalted by dialysis against distilled water using a molecular weight cut off dialysis tubing (MWCO 1000) and freeze-dried to afford 39 mg of carbohydrate containing sample. The combined HS fractions (Fig. 2) were dissolved in 16% sodium chloride, precipitated by adding anhydrous methanol to the final concentration of 80% (v/v), recovered by centrifugation at 8000 rpm for 20 min, dialyzed exhaustively against distilled water using 3500 MWCO membrane and freeze-dried affording 22 mg of purified HS pG.

3.3. Characterization of HS pG

3.3.1. Enzymatic depolymerization of bovine liver HS

Two small portions of the 22 mg of recovered HS (10 µg) were dissolved in 10 µl of 50 mM sodium phosphate/100 mM NaCl buffer, pH 7.1 and 50 mM sodium phosphate buffer, pH 7.6, and treated with 10 m-unit heparin lyase I and III, respectively. The reactions were incubated in polyethylene vials at 30 °C (heparin lyase I) and 37 °C (heparin lyase III) overnight. The enzymes were deactivated by boiling for 3 min and the products were analyzed PAGE (Fig. 3) and CE (Table 1).

3.3.2. PAGE analysis

Polyacrylamide gel electrophoresis (PAGE) was performed on a 32 cm vertical slab gel unit SE620, from Hoefer Scientific Instruments (San Francisco, CA), equipped with Model 1420B power source from Bio-Rad (Richmond, CA). Polyacrylamide resolving gel (14 × 28 cm, 12% acrylamide) was prepared and run as previously described (Edens, Al-Hakim, Weiler, Rethwisch, Fareed & Linhardt, 1992). The molecular sizes of the carbohydrate containing fractions were determined by comparing with a banding ladder standards prepared from heparin and using heparin/HS disaccharide standards (Edens et al., 1992).

3.3.3. Disaccharide composition analysis by CE

The experiments were performed with a capillary electrophoresis PACE 5500 System (Beckman Instruments, Fullerton, CA) at a constant capillary temperature of 18 °C with a potential of -22 kV by UV absorbance at 232 nm. The electropherograms were acquired using the system Gold software package (Beckman Instruments, Fullerton, CA). Separation and analysis were carried out in a reversed polarity mode using a fused silica (externally coated except where the tube passed through the detector) capillary tube (50 µm inner diameter, 360 µm outer diameter, 57 cm long, and 49 cm effective length). Prior the every run, the capillary was conditioned with 0.5 M NaOH (1 min, 20 psi) and rinsed (1 min, 20 psi) with separation buffer (20 mM H₃PO₄ adjusted to pH 3.5 with saturated dibasic sodium phosphate). Samples were applied by pressure injection 15 s at 0.5 psi.

3.3.4. ¹H NMR analysis

For ¹H NMR spectroscopy, approximately 5 mg of each sample was freeze-dried from 0.5 ml portions of ²H₂O (99.6%, Sigma, St Louis, MO), to exchange the labile protons with deuterium. The thoroughly dried sample was re-dissolved in 0.7 ml of ²H₂O (99.6%), and transferred to the NMR tube. All spectra were determined on a Varian UNITY-500 spectrometer equipped with 5 mm triple resonance tunable probe with standard Varian software at 298 K on 700 µl samples at 0.1–0.5 mM. The HOD signal was suppressed by pre-saturation during 3 s.

3.3.5. Amino acid composition analysis

Selected fractions were submitted for amino acid compositional analysis at the University of Iowa Protein Structure Facility.

3.3.6. Biotinylation of HS pG

Sample (7.4 mg) was dissolved in 400 µl of 0.1 M sodium bicarbonate and incubated with NHS-LC-biotin (4.1 mg) dissolved in 40 µl of dimethyl formamide (DMF) at room temperature for 15 h (Kuberan et al., 1999). The

reaction mixture was dialyzed against water using 3500 MWCO membrane and freeze-dried. The product was purified by Dowex macroporous SAX resin column (1×7 cm) eluted with 3 column volumes of water, 2 column volumes of 50% aqueous methanol, 3 column volumes of 1.5% and 16% aqueous sodium chloride solution. Fraction obtained in the 16% sodium chloride washes were exhaustively dialyzed against distilled water using 3500 MWCO membrane and freeze-dried to afford 3.5 mg HS pG and biotinylated HS pG.

3.3.7. Colorimetric determination of biotinylated HS pG

Biotinylated HS pG: Streptavidin beads, (a 1:4 slurry in water) was shaken overnight at 4 °C, then washed with 2 column volumes of 1% NaCl and 4 column volumes of water. Streptavidin beads, streptavidin beads and standard HS and 0.2% NaCl in the absence of beads were used as controls in this experiment. Toluidine blue solution 2.5 ml 0.005% was pipetted into tubes with 1:4 diluted slurry of biotinylated HS pG: streptavidin beads in water containing various amounts of HS solution to prepare a standard curve over a concentration range of 20–120 µg HS. Each tube was diluted with 0.2% NaCl to a total volume of 5 ml and mixed by a vortex mixer for 30 s. Hexane (5 ml) was then added to each tube and tubes were shaken for another 30 s to separate HS-dye complex formed in standard HS solutions. Hexane was not added in tubes containing the beads since HS-dye complex formed on the gel surface. Tubes containing the beads were centrifuged 1000 rpm, 3 min the aqueous layers of all the tubes were diluted 1:10 with absolute ethanol. The absorbance was measured at 631 nm to afford a standard curve y (absorbance) = $-9.45 \times 10^{-4}x$ (µg/ml HS) + 0.24 (A 631 nm).

3.3.8. Confirmation that biotin was localized on the peptide portion of HS pG

Sample (600 µg) was dissolved in 1 ml of 0.5 M lithium hydroxide (Liu et al., 1995) (nitrogen saturated and the solution was kept at 4 °C for 48 h under nitrogen atmosphere). The reaction was stopped by addition of 0.5 N acetic acid and immediately freeze-dried. Sample was dissolved in 0.1 M sodium phosphate buffer, pH 7.0 and dialyzed against water using 1000 MWCO membrane and freeze-dried. The resulting product was again analyzed for biotin using colorimetric assay.

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