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Notes & Tips

Thin-layer chromatography for the analysis of glycosaminoglycan oligosaccharides

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Glycosaminoglycans (GAGs)[1] are linear acidic polysaccharides found on cell surfaces and in the surrounding extracellular matrix. GAGs participate in and regulate many cellular events in physiological and pathophysiological processes, such as cell proliferation and differentiation, cell–cell and cell–matrix interactions, and viral infection, through their interaction with different proteins [1–3]. GAGs are divided into four main categories—hyaluronic acid (HA), chondroitin sulfate/dermatan sulfate (CS/DS), heparosan/heparan sulfate/heparin (HN/HS/HP), and keratan sulfate—based on monosaccharide composition and the configuration and position of the glycosidic bonds between their monosaccharides. The specificity of the interactions between GAGs and proteins results from the structural diversity of GAG type, size, saccharide composition, charge density, and sequence [4,5]. It often is necessary to determine the size and purity of GAG-derived oligosaccharides, analyze activity of enzymes acting on GAGs, and monitor the preparation of GAG-derived oligosaccharides. Polyacrylamide gel electrophoresis (PAGE) is a method used routinely for separation and analysis of GAGs and GAG-derived oligosaccharides, and the resulting gels usually are visualized by staining with Alcian blue [6–9]. Unfortunately, bands corresponding to small oligosaccharides with a low net charge show low sensitivity to Alcian blue staining, for example, HA and HN (smaller than hexasaccharide) and CS/DS and HS (smaller than tetrasaccharide). Fluorophore-assisted carbohydrate electrophoresis (FACE) affords an alternative method to analyze such small oligosaccharides [10,11]. However, both PAGE and FACE are time- and labor-consuming. In this article, we describe a newly developed thin-layer chromatography (TLC) method for the analysis of acidic GAG-derived oligosaccharides. In comparison with PAGE and FACE, this TLC method shows high sensitivity for small oligosaccharides having a low net charge and can be performed quickly and easily at a low cost. Multiple samples can be analyzed in parallel using TLC, suggesting the utility of this approach in high-throughput applications.

HN was isolated through fermentation of *Escherichia coli* K5 and purified as described previously [12]. Purified HN (500 μg/50 μl) was incubated at 37 °C for 10 h in 50 mM sodium phosphate buffer (pH 7.0) in the presence of varying amounts of heparin lyase III (1, 2, 5, 10, and 15 mU, Sigma, St. Louis, MO, USA). The reaction mixtures were heated in a boiling water bath for 10 min to thermally inactivate the enzyme, thereby halting the reaction. HA (from rooster comb) was purchased from Sigma. Chondroitin-6-sulfate, (CS-A, from bovine trachea), dermatan-4,6-disulfate (DDS, from porcine intestinal mucosa), and HP (from porcine intestinal mucosa) were purchased from Celsus (Cincinnati, OH, USA). Chondroitin ABC lyase (10, 5, and 20 mU, Seikagaku, Tokyo, Japan) was used to digest HA, CS-A, and DDS (500 μg/50 μl),

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[1] *Abbreviations used:* GAG, glycosaminoglycan; HA, hyaluronic acid; CS/DS, chondroitin sulfate/dermatan sulfate; HN/HS/HP, heparosan/heparan sulfate/heparin; PAGE, polyacrylamide gel electrophoresis; FACE, fluorophore-assisted carbohydrate electrophoresis; TLC, thin-layer chromatography; CS-A, chondroitin-6-sulfate; DDS, dermatan-4,6-disulfate; GlcA, glucuronic acid; GlcNAc, *N*-acetyl glucosamine; GalNAc, *N*-acetyl galactosamine.

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respectively, in 50 mM sodium phosphate buffer (pH 7.0) at 37 °C for 10 h. Heparin lyase I (20 mU, Sigma) was used to digest HP in 50 mM sodium phosphate buffer (pH 7.0) at 37 °C for 10 h.

Reaction product mixtures (0.3 μl of each) were loaded onto a precoated Silica Gel 60 TLC aluminum plate (3 × 5 cm, Merck, Germany) and developed with a solvent system consisting of *n*-butanol/formic acid/water (4:8:1, v/v/v). The developed plate was stained by dipping in diphenylamine–aniline–phosphoric acid reagent (1 ml of 37.5% HCl, 2 ml of aniline, 10 ml of 85% H_3PO_3, 100 ml of ethyl acetate, and 2 g of diphenylamine) for 3 s and heated at 150 °C for 10 s [13]. The TLC plate was scanned, digitized, and analyzed by UN-SCAN-IT gel scanning software (Silk Scientific, Orem, UT, USA).

HN was treated with varying amounts of heparin lyase III to obtain oligosaccharide product mixtures. The percentage completion of digestion was quantified by dividing the UV absorbance (at 232 nm) of the products prepared using a given amount of enzyme for 10 h by the UV absorbance (at 232 nm) determined at reaction completion. TLC analysis was performed to follow the enzymatic depolymerization of HN at 5, 10, 15, 30, and 50% reaction completion (Fig. 1A). The stained TLC plate was digitized to obtain a semiquantitative analysis (Fig. 1B). In the reaction mixture at 5% completion, chromatography showed that much of the product remained at the origin of TLC and that only very faint spots corresponding to smaller oligosaccharides were visible, suggesting that the product mixture contained primarily higher oligosaccharides (i.e., higher than hexadecasaccharide, >16 saccharide units). The reaction products obtained at 10 and 15% reaction completion (lanes b and c in Fig. 1A) clearly showed spots on the TLC plate corresponding to oligosaccharides having 4–14 saccharide units. At 30 and 50% reaction completion, oligosaccharides from disaccharide to tetradecasaccharide were observed as distinctive separated bands on the TLC plate (lanes d and e in Fig. 1A). Then the TLC data were digitized and intensity was plotted as a function of distance from origin (Fig. 1B). In all lanes, the band remaining at the origin corresponding to polysaccharides and oligosaccharides (>16 saccharide units) was the most intense. Whereas the bands corresponding to the oligosaccharides having 2–10 saccharide units increased, the bands associated with oligosaccharides having 12–14 saccharide units decreased with percentage digestion completion. The change in total band intensity as a function of digestion completion (Fig. 1C) confirms this trend and can be used to assess reaction kinetics. The total band intensity of disaccharide increased sharply only after the digestion completion passed 20%. Similarly, the intensity of tetrasaccharide increased sharply when the digestion completion passed 15%. The rate of increase for hexasaccharide and octasaccharide slowed after 30% digestion completion. The rate of increase for decasaccharide and dodecasaccharide decreased slightly and significantly after 30% digestion completion. The profiles of the product mixtures at different reaction completions on the TLC plate clearly demonstrate

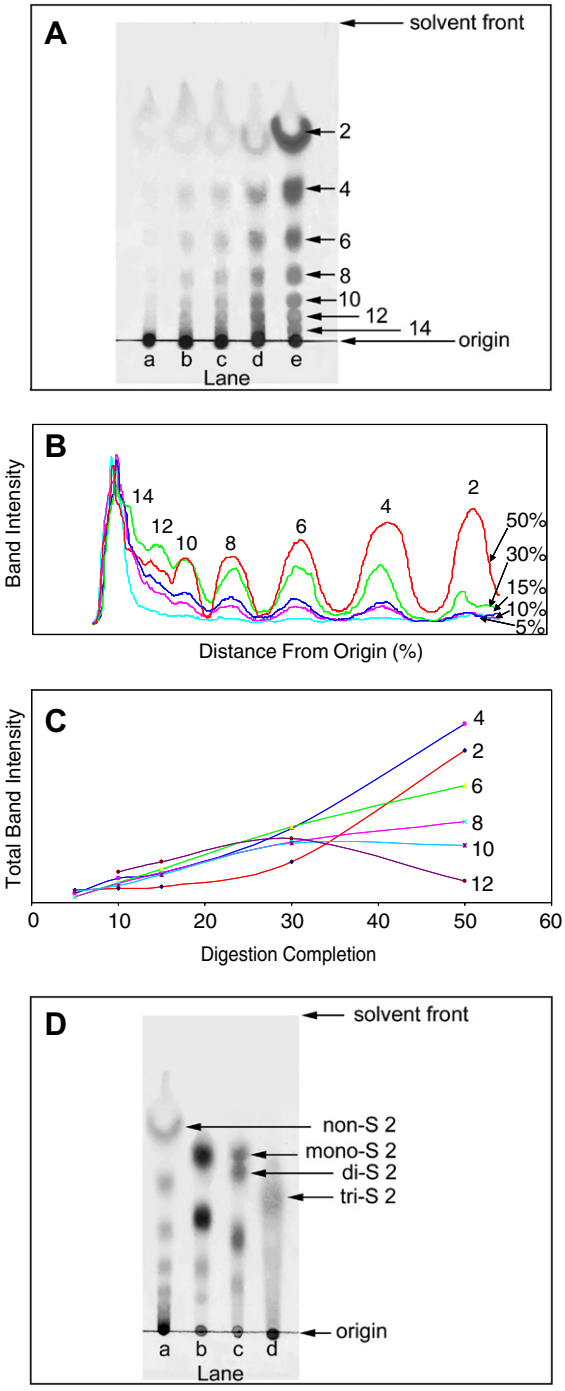

Fig. 1. (A) TLC of partially digested HN. The reaction products obtained at 5% completion (lane a), 10% completion (lane b), 15% completion (lane c), 30% completion (lane d), and 50% completion (lane e) are shown. (B) Digitized TLC. (C) Plot of total band intensity as a function of percentage digestion completion for each size oligosaccharide. (D) TLC of other GAGs digested with polysaccharide lyases: HA (lane a), CS-A (lane b), DDS (lane c), and HP (lane d). The major disaccharides found in each GAG and their numbers of sulfo groups (nonsulfated [non-S 2], monosulfated [mono-S 2], disulfated [di-S 2], and trisulfated [tri-S 2]) are indicated. Their identities were confirmed based on the use of disaccharide standards. The numbers 2–14 in all four panels (A–D) correspond to the numbers of saccharide units present in the various oligosaccharide products.

that heparin lyase III is an endolytic enzyme, confirming the results of previous methods that relied on PAGE analysis and viscometry [14]. Time course experiments using a fixed quantity of enzyme can also be conveniently monitored and analyzed by this method [15].

Next, oligosaccharide mixtures, prepared from HA, CS-A, DDS, and HP by polysaccharide lyase treatment, were analyzed by TLC plate (Fig. 1D). The analyzed HA oligosaccharide mixture contained oligosaccharides between disaccharide and hexadecasaccharide (lane a in Fig. 1D). HA is a nonsulfated GAG consisting of the repeating units →4) glucuronic acid (GlcA) (1→3) and *N*-acetyl glucosamine (GlcNAc) (1→. The absence of sulfation in HA oligosaccharides results in both enhanced migration and enhanced resolution of these oligosaccharides. HN, a second nonsulfated GAG analyzed by TLC under the same conditions, showed similarly high resolution (data not shown). Analysis of oligosaccharides prepared from CS-A (lane b in Fig. 1D) showed four spots corresponding to disaccharide, tetrasaccharide, hexasaccharide, and octasaccharide. CS-A consists of repeating units →4) GlcA (1→3) and 6-*O*-sulfated *N*-acetyl galactosamine (GalNAc) (1→. The single sulfo group at position 6 of GalNAc in CS-A oligosaccharides retards their migration on TLC as a result of their higher polarity. Analysis of DDS (prepared by chemical sulfation of dermatan sulfate at the primary 6-hydroxyl group of GalNAc, resulting in GalNAc 4S6S) is shown in lane c of Fig. 1D. Bands corresponding to disaccharide, tetrasaccharide, and hexasaccharide were observed in the TLC. The disaccharide band showed two closely spaced spots corresponding to monosulfated and disulfated disaccharides, as expected based on incomplete chemical sulfation. Analysis of heparin, a very complex GAG having variable levels of sulfation, showed a broad smear extending from the origin to disaccharide consisting primarily of a trisulfated disaccharide.

In conclusion, TLC can be used to separate GAG oligosaccharides on the basis of size and disaccharides on the basis of sulfation level. Furthermore, TLC represents a quick, easy, and reliable method for the parallel analysis of GAG oligosaccharide samples and can be used to examine the active pattern of polysaccharide lyases.

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References

[1] Z.L. Wu, L. Zhang, D.L. Beeler, B. Kuberan, R.D. Rosenberg, A new strategy for defining critical functional groups on heparan sulfate, FASEB J. 16 (2002) 539–545.
[2] I. Capila, R.J. Linhardt, Heparin–protein interactions, Angew. Chem. Intl. Ed. 41 (2002) 391–412.
[3] M. Bernfield, M. Gotte, P.W. Park, O. Reizes, M.L. Fitzgerald, J. Lincecum, M. Zako, Functions of cell surface heparan sulfate proteoglycans, Annu. Rev. Biochem. 68 (1999) 729–777.
[4] K.R. Taylor, R.L. Gallo, Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation, FASEB J. 20 (2006) 9–22.
[5] R. Sasisekharan, R. Raman, V. Prabhakar, Glycomics approach to structure–function relationships of glycosaminoglycans, Annu. Rev. Biomed. Eng. 8 (2006) 181–231.
[6] R.E. Edens, A. al-Hakim, J.M. Weiler, D.G. Rethwisch, J. Fareed, R.J. Linhardt, Gradient polyacrylamide gel electrophoresis for determination of molecular weights of heparin preparations and low-molecular-weight heparin derivatives, J. Pharm. Sci. 81 (1992) 823–827.
[7] K.B. Lee, U.R. Desai, M.M. Palcic, O. Hindsgaul, R.J. Linhardt, An electrophoresis-based assay for glycosyltransferase activity, Anal. Biochem. 205 (1992) 108–114.
[8] H. Min, M.K. Cowman, Combined Alcian blue and silver staining of glycosaminoglycans in polyacrylamide gels: application to electrophoretic analysis of molecular weight distribution, Anal. Biochem. 155 (1986) 275–285.
[9] K.G. Rice, M.K. Rottink, R.J. Linhardt, Fractionation of heparin-derived oligosaccharides by gradient polyacrylamide-gel electrophoresis, Biochem. J. 244 (1987) 515–522.
[10] A. Calabro, V.C. Hascall, R.J. Midura, Adaptation of FACE methodology for microanalysis of total hyaluronan and chondroitin sulfate composition from cartilage, Glycobiology 10 (2000) 283–293.
[11] E.G. Karousou, G. Porta, G. De Luca, A. Passi, Analysis of fluorophore-labelled hyaluronan and chondroitin sulfate disaccharides in biological samples, J. Pharm. Biomed. Anal. 34 (2004) 791–795.
[12] W.F. Vann, M.A. Schmidt, B. Jann, K. Jann, The structure of the capsular polysaccharide (K5 antigen) of urinary-tract-infective *Escherichia coli* 010:K5:H4: A polymer similar to desulfo-heparin, Eur. J. Biochem. 116 (1981) 359–364.
[13] Z. Zhang, G. Yu, X. Zhao, H. Liu, H. Guan, A.M. Lawson, W. Chai, Sequence analysis of alginate-derived oligosaccharides by negative-ion electrospray tandem mass spectrometry, J. Am. Soc. Mass Spectrom. 17 (2006) 621–630.
[14] K.A. Jandik, K. Gu, R.J. Linhardt, Action pattern of polysaccharide lyases on glycosaminoglycans, Glycobiology 4 (1994) 289–296.
[15] Z. Zhang, J. Xie, J. Liu, R. J. Linhardt, LC–MS/MS to distinguish hyaluronic acid from *N* -acetylheparosan (Submitted for publication).