Liquid chromatography–mass spectrometry to study chondroitin lyase action pattern

Zhang, Zhenqing; Park, Youmie; Kemp, Melissa M.; Zhao, Wenjiing; Im, A-Rang; Shaya, David; Cygler, Miroslaw; Kim, Yeong Shik; Linhardt, Robert J.

Publisher's version / Version de l'éditeur:
https://doi.org/10.1016/j.ab.2008.10.014
Analytical Biochemistry, 385, pp. 57-64, 2008-10-17

Analytical Biochemistry 385 (2008) 57–64

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Liquid chromatography–mass spectrometry to study chondroitin lyase action pattern

Zhenqing Zhang [a], Youmie Park [a,b], Melissa M. Kemp [a], Wenjing Zhao [a], A-Rang Im [b], David Shaya [c], Miroslaw Cygler [c,d], Yeong Shik Kim [b], Robert J. Linhardt [a,*]

[a] Departments of Chemistry and Chemical Biology and Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 110 8th Street, Troy, NY 12180, USA
[b] Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul 151-742, Korea
[c] Department of Biochemistry, McGill University, Montreal, Que., Canada H3G 1Y6
[d] Biotechnology Research Institute, NRC, Montreal, Que., Canada H4P 2R2

ARTICLE INFO

Article history:
Received 1 August 2008
Available online 17 October 2008

Keywords:
Liquid chromatography–mass spectrometry, (LC–MS)
Chondroitin lyase
Action pattern

ABSTRACT

Liquid chromatography–mass spectrometry was applied to determine the action pattern of different chondroitin lyases. Two commercial enzymes, chondroitinase ABC (*Proteus vulgaris*) and chondroitinase ACII (*Arthrobacter aurescens*), having action patterns previously determined by viscosimetry and gel electrophoresis were first examined. Next, the action patterns of recombinant lyases, chondroitinase ABC from *Bacteroides thetaiotaomicron* (expressed in *Escherichia coli*) and chondroitinase AC from *Flavobacterium heparinum* (expressed in its original host), were examined. Chondroitin sulfate A (CS-A, also known as chondroitin-4-sulfate) was used as the substrate for these four lyases. Aliquots taken at various time points were analyzed. The products of chondroitinase ABC (*P. vulgaris*) and chondroitinase AC (*F. heparinum*) contained unsaturated oligosaccharides of sizes ranging from disaccharide to decasaccharide, demonstrating that both are endolytic enzymes. The products afforded by chondroitinase ABC (*B. thetaiotaomicron*) and chondroitinase ACII (*A. aurescens*) contained primarily unsaturated disaccharide. These two exolytic enzymes showed different minor products, suggesting some subtle specificity differences between the actions of these two exolytic lyases on chondroitin sulfate A.

Chondroitin sulfate (CS)[1] is a linear, polydisperse, microheterogeneous, anionic polysaccharide having an average molecular weight of 10,000 to 30,000 Da. Most CS is isolated from animal tissues, and unsulfated chondroitin is isolated from certain bacteria [1,2]. CS contains disaccharide repeating units composed of 2-deoxy-2-acetamido-D-galactose (GalNAc) 1→4 linked to a uronic acid such as D-glucuronic acid (GlcA) in CS-A and CS-C or L-iduronic acid (IdoA) in CS-B (Fig. 1A). Even a single type of chondroitin sulfate, such as CS-A (chondroitin-4-sulfate), has a range of molecular weights and contains sequence heterogeneity, with minor amounts of 6-sulfated, unsulfated, and disulfated disaccharide repeating units [3]. Bacterial chondroitin lyases serve a role in the initial microbial catabolism of CSs [4] and have found many applications as analytical tools in carbohydrate biochemistry [5,6], for the determination of the type of CS present in cells and tissues [7–10], for the quantitative analysis of CS [11,12], for the preparation of new therapeutic agents [1,11,12], and (most recently) in the removal of glial scar CS in the treatment of spinal cord injury [13–15].

The increased use of these enzymes, particularly for medical applications, has led to the isolation, cloning, and recombinant expression of new bacterial chondroitin lyases [16,17]. Thus, a detailed understanding of the specificity of these new chondroitin lyases is required, including both their sequence specificity and action pattern. Ultraviolet (UV) absorbance [18], viscometry [19], polyacrylamide gel electrophoresis (PAGE) [19], capillary electrophoresis (CE) [20], thin layer chromatography (TLC) [21], and high-performance liquid chromatography (HPLC) [22] have been used to determine the action patterns of chondroitin lyases. UV and viscometry are global methods to measure enzyme action patterns and are not able to monitor the formation of individual products. In contrast, PAGE, CE, TLC, and HPLC follow the formation of individual products but generally require the use of standards to identify these products. Electrospray ionization–mass spectrometry (ESI–MS) is a soft ionization method particularly useful to

* Corresponding author. Fax: +1 518 276 3405.
E-mail address: linhar@rpi.edu (R.J. Linhardt).

[1] *Abbreviations used:* CS, chondroitin sulfate; GalNAc, 2-deoxy-2-acetamido-D-galactose; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; CS-A, chondroitin-4-sulfate; UV, ultraviolet; PAGE, polyacrylamide gel electrophoresis; CE, capillary electrophoresis; TLC, thin layer chromatography; HPLC, high-performance liquid chromatography; ESI–MS, electrospray ionization–mass spectrometry; LC, liquid chromatography; RPIP, reversed-phase ion pairing; HA, hyaluronan; IPTG, isopropyl β-D-1-thiogalactopyranoside; SDS, sodium dodecyl sulfate; TBA, tributylamine; EIC, extracted ion chromatogram; dp, degree of polymerization.

0003-2697/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2008.10.014

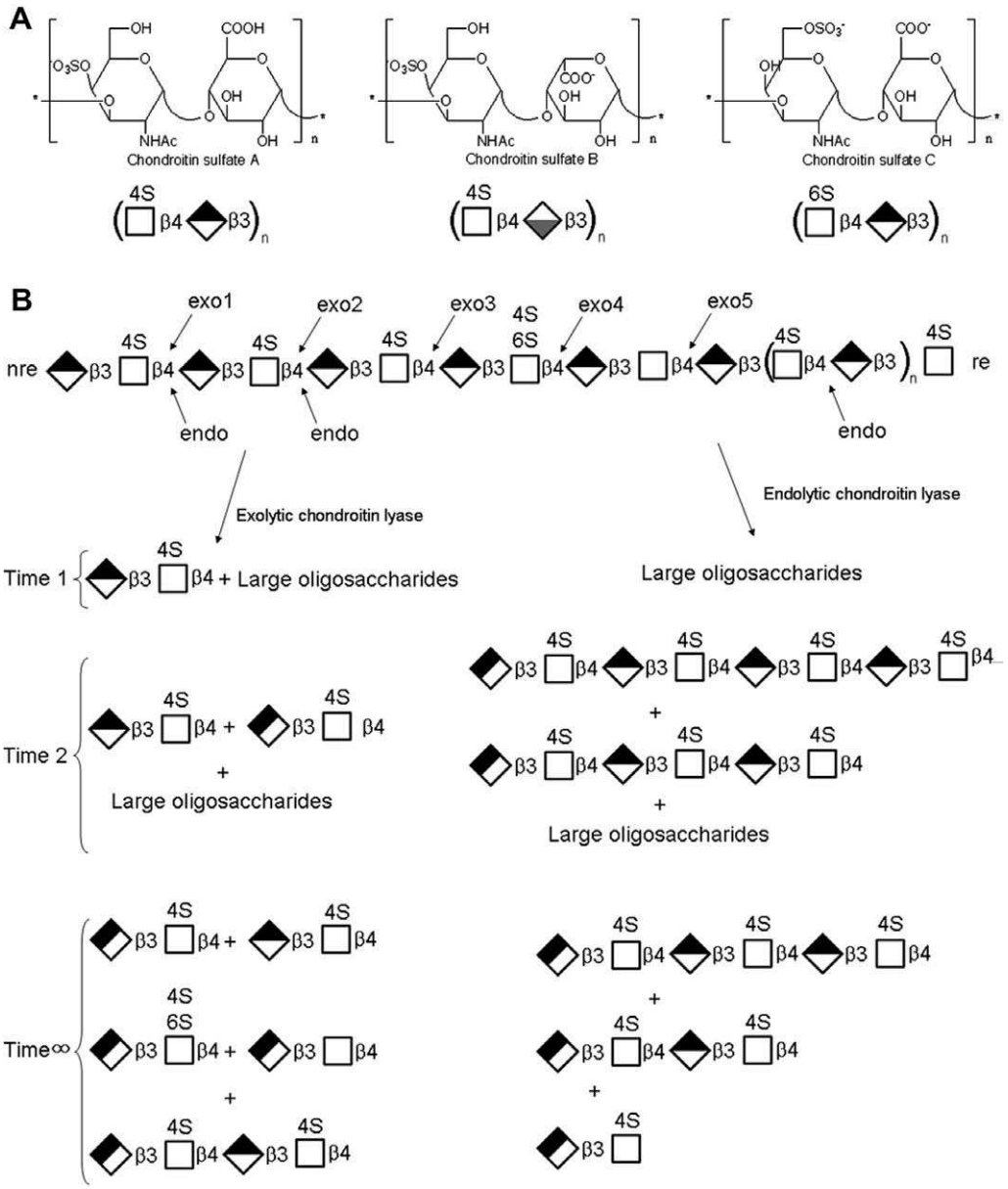

Fig. 1. Structure of CS and its cleavage by chondroitin lyase through various action patterns. (A) The chemical structure of the major saccharide residues found, CS-A, CS-B, and CS-C, where $n = 20$ to 60. The symbolic representation of these structures is also shown with □ as GalNAc, ◆ as GlcA, and ◈ as IdoA. The predominant sequences of CS-A, CS-B, and CS-C are indicated. (B) A single typical chain of CS-A is shown with some sequence heterogeneity. The nonreducing end (nre) and reducing end (re) of the chain are indicated. An exolytic enzyme cuts the chain at each cleavage site starting from the nre, whereas a random endolytic enzyme cuts the chain through the random selection of sites. Typical product distribution at time points 1 (early reaction), 2 (intermediate reaction), and ∞ (reaction completion) are shown. The symbol ◈ corresponds to unsaturated uronic acid.

monitor the formation of CS oligosaccharides [23]. Liquid chromatography (LC)–ESI–MS, relying on reversed-phase ion pairing (RPIP)–HPLC, employs volatile ion pairing reagents to successfully analyze glycosaminoglycan-derived oligosaccharides such as heparin, heparan sulfate, heparosan, and hyaluronan (HA) [24–26]. The separation profiles of enzymatic products and their structures or sequences can be deduced by LC–MS.

Chondroitin lyases originate from various microbial sources and display different specificities and action patterns (Fig. 1B) [17]. Chondroitin lyase ABC (*Proteus vulgaris,* EC 4.2.2.4), a mixture of ABC lyases I and II, acts on CS-A, CS-B, and CS-C in a predominately endolytic action pattern [19]. Chondroitin lyase AC I (*Flavobacterium heparinum,* EC 4.2.2.5) acts on CS-A and CS-C in a random endolytic action pattern [19]. Chondroitin lyase AC II (*Arthrobacter aurescens,* EC 4.2.2.5) acts on CS-A and CS-C and displays an exolytic action pattern [19,20,27–30]. The various chondroitin ABC lyases display pairwise sequence identity in the range of 23 to 31% and, therefore, have diverged substantially during evolution. Similarly, the sequence identity between the two chondroitinases AC is also only 23%. Comparison of structures of chondroitinases ABC and AC shows clear similarity of their folds in the sense that the α-helical and β-sheet domains of chondroitinase AC have counterparts in chondroitinase ABC, with the latter having an additional N-terminal β-sheet domain. However, only the α-helical domains show recognizable sequence similarity amounting to approximately 24% identity, whereas the sequences of the β-sheet domains diverged beyond recognition by programs such as BLAST.

In this article, the action pattern of commercial chondroitin lyase AC II (*A. aurescens*) and commercial chondroitin lyase ABC (*P. vulgaris*) as well as two recombinant lyases expressed in *Escherichia coli* and

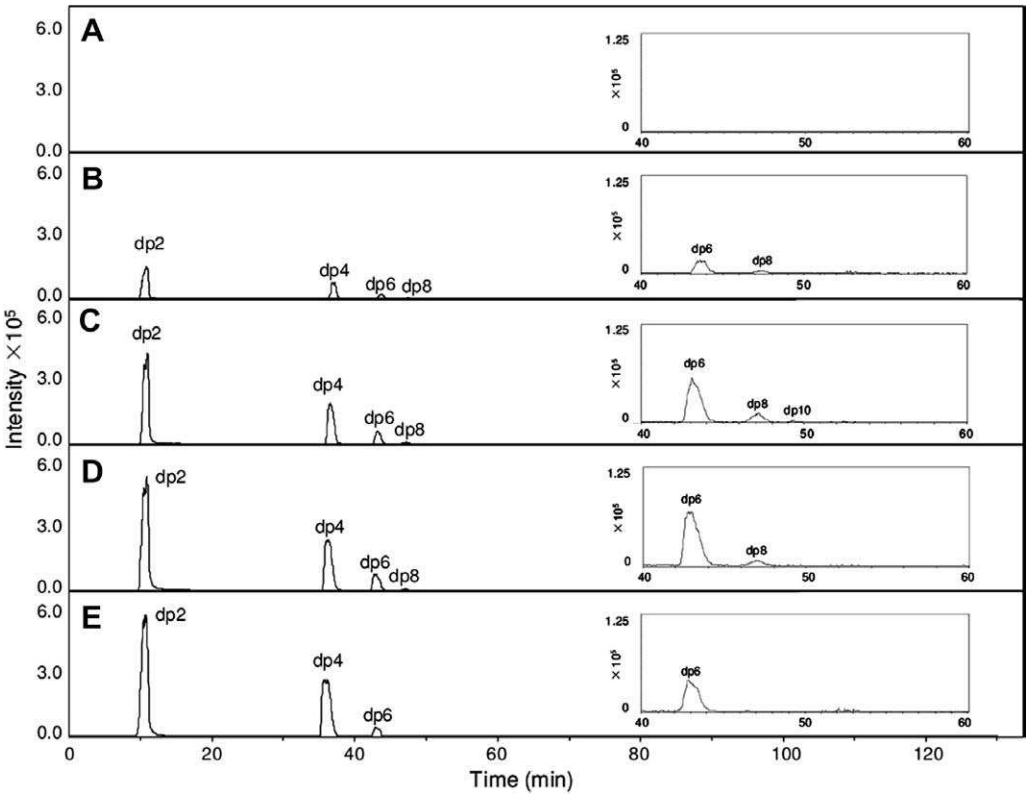

Fig. 2. EIC of products digested by chondroitinase ABC (*P. vulgaris*): (A) 0-min aliquots; (B) 10-min aliquots; (C) 30-min aliquots; (D) 60-min aliquots; (E) 120-min aliquots. Insets are 5× intensity magnification of 40 to 60 min.

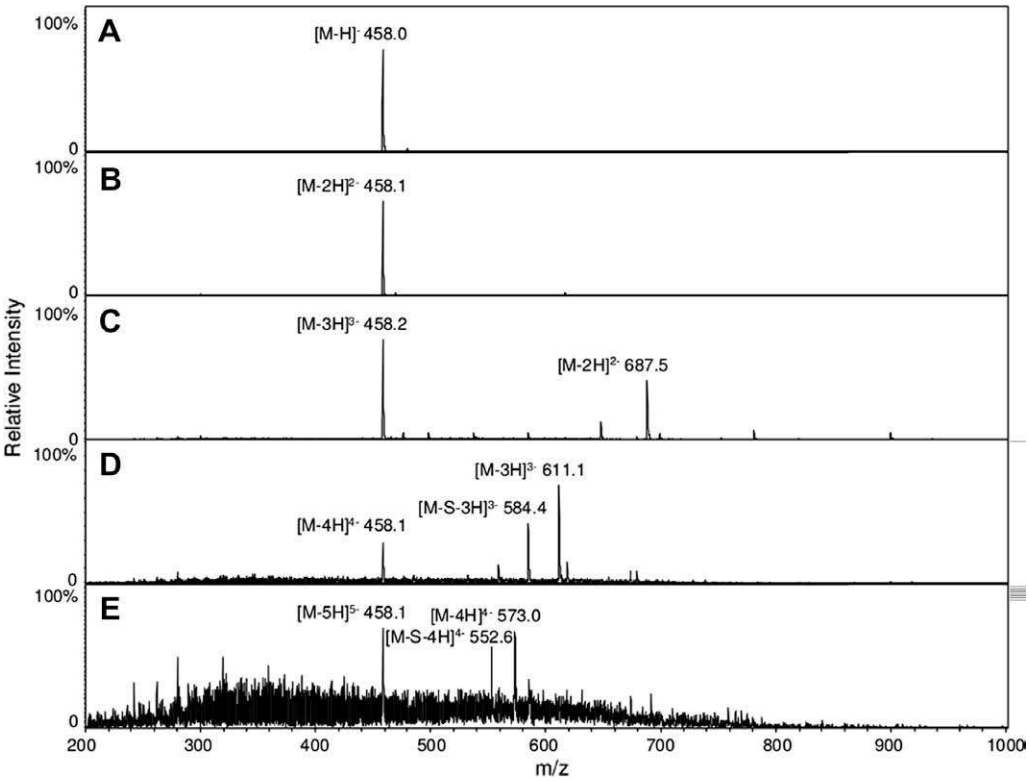

Fig. 3. Mass spectra of oligosaccharides observed in Fig. 2: (A) mass spectrum of dp2; (B) mass spectrum of dp4; (C) mass spectrum of dp6; (D) mass spectrum of dp8; (E) mass spectrum of dp10.

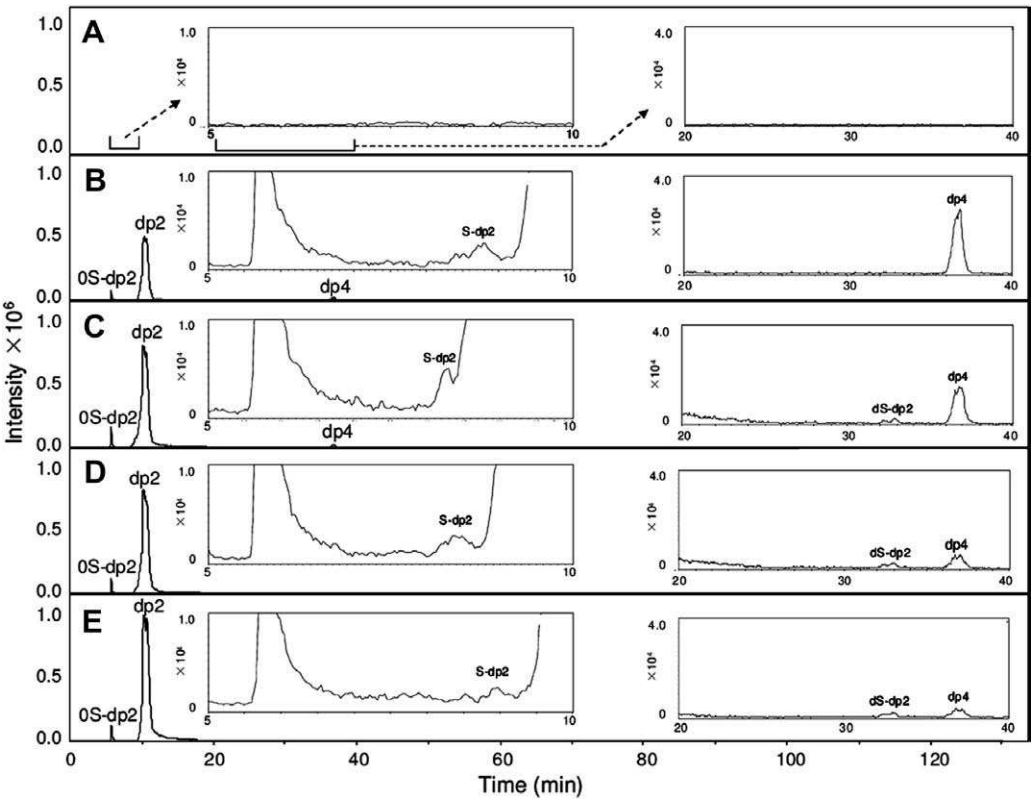

Fig. 4. EIC of products digested by chondroitinase AC II (*A. aurescens*): (A) 0-min aliquots; (B) 10-min aliquots; (C) 30-min aliquots; (D) 60-min aliquots; (E) 120-min aliquots. Insets are 100× intensity magnification of 5 to 10 min and 25× intensity magnification of 20 to 40 min.

F. heparinum, chondroitin lyase ABC (*Bacteroides thetaiotaomicron*) and AC (*F. heparinum*), respectively, are examined using LC–MS.

Materials and methods

Materials

CS-A (from bovine trachea) was purchased from Celsus (Cincinnati, OH, USA). Chondroitin lyase ABC (*P. vulgaris*) and ACII (*A. aurescens*) were obtained from Seikagaku (Tokyo, Japan).

Methods

Expression of chondroitin lyase ABC from B. thetaiotaomicron and chondroitin AC lyase from F. heparinum

The recombinant chondroitin lyase ABC (*B. thetaiotaomicron*) was expressed in *E. coli* BL21(DE3) and purified as described previously [31]. Briefly, cells expressing the enzyme were cultured in Luria broth at 37 °C supplemented with 100 μg ml^{-1} ampicillin, and protein expression was induced with 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG). Cells were disrupted by sonication and purified using a combination of DEAE (GE Healthcare, Piscataway, NJ, USA), Ni-NTA (Qiagen, Valencia, CA, USA), Mono-S HR 10/10 (GE Healthcare), and HiLoad 16/60 Superdex 200 (GE Healthcare) columns. The purity of the eluted fractions was evaluated using sodium dodecyl sulfate (SDS)–PAGE, and the specific activity was determined.

The *F. heparinum* chondroitin AC lyase was expressed and purified as described previously [32].

Digestion

CS-A (2 mg/ml) in 1 ml of 50 mM sodium phosphate buffer (pH 7.0) was treated in individual reactions with chondroitin lyases ABC (*P. vulgaris*), ACII (*A. aurescens*), and AC (*F. heparinum*) (50-μ units) at 37 °C for 3 h with aliquots removed at various time points for analysis (0, 10, 30, 60, and 120 min). Chondroitin lyase ABC (*B. thetaiotaomicron*) was used in an identical fashion except over a 24-h period, with aliquots taken at 0, 1, 5, 12, and 24 h. As soon as each aliquot was removed from an enzymatic reaction, the enzyme was thermally inactivated by heating at 100 °C for 10 min.

CS-A (2 mg/ml) in 0.1 ml of 50 mM sodium phosphate buffer (pH 7.0) was treated in reaction with both chondroitin lyases ABC (*P. vulgaris*) and ACII (*A. aurescens*) (50-μ units) at 37 °C overnight. The enzyme was thermally inactivated by heating at 100 °C for 10 min and was removed by centrifuge (12,000 g). The supernatant was freeze-dried and ready for disaccharide analysis.

LC–MS

LC–MS analyses were performed on an Agilent 1100 LC/MSD instrument (Agilent Technologies, Wilmington, DE, USA) equipped with an ion trap, a binary pump, and a UV detector. The column used was a 5-μm Agilent Zorbax SB-C18 (0.5 × 250 mm, Agilent Technologies). Eluent A was water/acetonitrile (85:15, v/v), and eluent B was water/acetonitrile (35:65, v/v). Both eluents contained 12 mM tributylamine (TBA) and 38 mM NH_4OAc with pH adjusted to 6.5 with HOAc. A gradient of 0% B for 15 min, and 0 to 100% B over 85 min was used at a flow rate of 10 μl/min. Another gradient of 0% B for 20 min and 0 to 50% B over 25 min was used for disaccharide analysis. Injected samples had a volume of 8 μl. Mass spectra were obtained using an Agilent 1100 series Classic G2445D LC/MSD trap (Agilent Technologies). The electrospray interface was set in negative ionization mode with the skimmer potential –40.0 V, capillary exit at –40.0 V, and a source temperature of 325 °C to obtain maximum abundance of the ions in a full-scan spectrum (150–1500 Da, 10 full scans/s). Nitrogen was used as a drying (5 L/min) and nebulizing (20 psi) gas. Extracted ion chromatograms (EICs) and mass

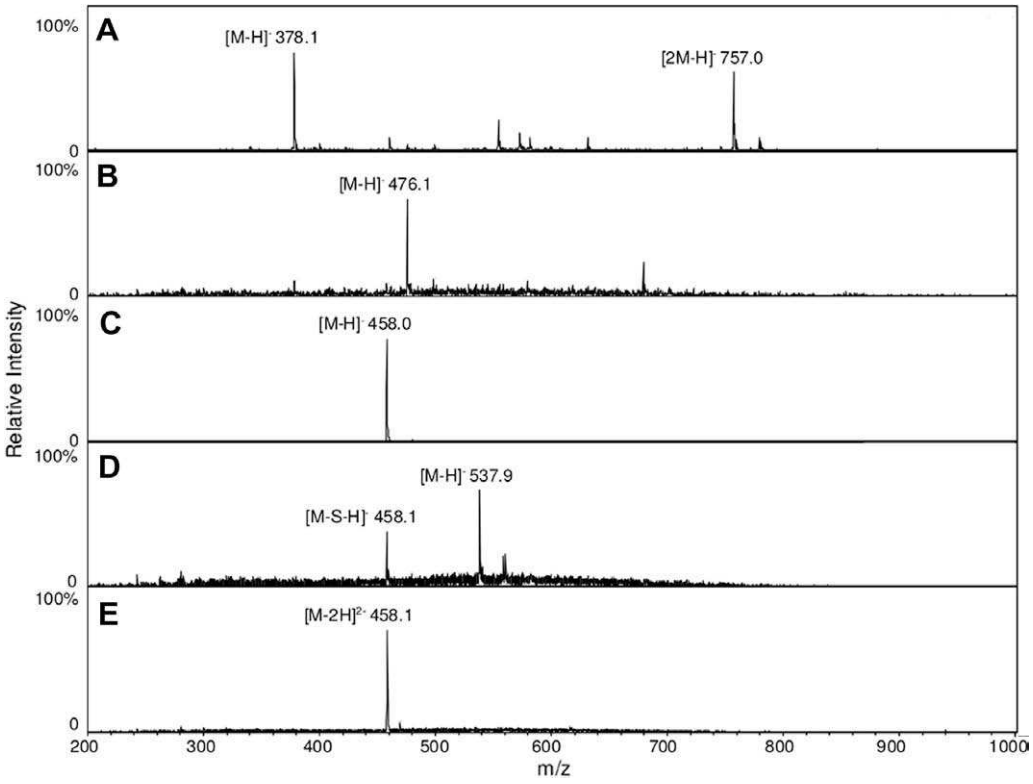

Fig. 5. Mass spectra of oligosaccharides observed in Fig. 4: (A) mass spectrum of nonsulfated unsaturated dp2; (B) mass spectrum of monosulfated saturated dp2; (C) mass spectrum of monosulfated unsaturated dp2; (D) mass spectrum of disulfated unsaturated dp2; (E) mass spectrum of unsaturated dp4.

spectra were processed using Data Analysis 2.0 software (Bruker Daltonics, Billerica, MA, USA).

Results

Chondroitin lyase ABC (P. vulgaris)

The digested CS-A was analyzed by LC–MS. Molecular weights and MS confirmed the sequence of each separated oligosaccharide product. The EICs from different digestion time aliquots of chondroitin lyase ABC (*P. vulgaris*) are shown in Fig. 2. The initial chromatogram taken prior to the addition of the enzyme (time point 0) showed no peaks corresponding to products (Fig. 2A). By 10 min, the EIC showed four peaks corresponding to a degree of polymerization (dp) 2, dp4, dp6, and dp8 (see Fig. 2B and inset). The mass spectra of these peaks (Fig. 3) indicate clearly that the products are: monosulfated unsaturated disaccharide (dp2), disulfated unsaturated tetrasaccharide (dp4), trisulfated unsaturated hexasaccharide (dp6), and tetrasulfated unsaturated octasaccharide (dp8). The molecular ion of monosulfated unsaturated disaccharide was observed at m/z 458.0, whereas the disulfated unsaturated tetrasaccharide showed a doubly charged molecular ion at m/z 458.1, the trisulfated unsaturated hexasaccharide showed both triply charged and doubly charged molecular ions at m/z 458.2 and 687.5, respectively, and the tetrasulfated unsaturated octasaccharide showed quadruply charged and triply charged molecular ions at m/z 458.1 and 611.1, respectively. A triply charged ion, corresponding to a trisulfated unsaturated octasaccharide resulting from fragmentation of this highly charged octasaccharide by cleavage of one of the unstable sulfo groups, was also observed in this spectrum. The EIC presented in Fig. 2C (inset) corresponds to the 30 min digestion and displayed an additional small peak corresponding to the molecular ion of a pentasulfated unsaturated decasaccharide having charge stages of –5 and –4 at m/z 458.1 and 573.0, respectively (Fig. 3E). Again, the loss of a single sulfo group through fragmentation was observed. The transient appearance of larger oligosaccharides such as dp10 (Fig. 2C) and the increase and subsequent decrease in dp8 (Fig. 2B–E) confirm that chondroitin lyase ABC (*P. vulgaris*) displays primarily an endolytic action pattern.

Chondroitin lyase ACII (A. aurescens)

The EICs of time aliquots of CS-A digestion with chondroitin lyase ACII (*A. aurescens*) are presented in Fig. 4. As expected, this enzyme clearly shows an exolytic action pattern. The major product of this lyase is the monosulfated unsaturated disaccharide with the peak intensity increasing continuously over the time points examined (Fig. 4). The mass spectrum of dp2 confirmed it to be the monosulfated unsaturated disaccharide at m/z 458.0 (Fig. 5C). In addition, a tiny peak, close to the monosulfated unsaturated (dp2) peak, labeled as S-dp2 was observed. This peak corresponds to a monosulfated saturated disaccharide, which afforded an m/z of 476.1 (Fig. 5B). This peak originates from the nonreducing terminal of CS-A and is often not observed by other methods because of the low amounts present and its low UV absorbance. Peaks corresponding to nonsulfated and disulfated unsaturated disaccharide could also be observed and are consistent with the sequence heterogeneity of the substrate. Disaccharide analysis of this CS-A sample showed 93% monosulfated disaccharide, 6% nonsulfated disaccharide, and 1% disulfated disaccharide. These structures were confirmed by MS (Fig. 5A and D). The action pattern of chondroitin lyase ACII (*A. aurescens*) can easily be confirmed by the appearance of increasing amounts of dp2 over time with only very minor secondary products. The intensities of the peaks (nonsulfated and disulfated unsaturated disaccharides) did not increase after 30 min digestion, suggesting that these domains are located close to

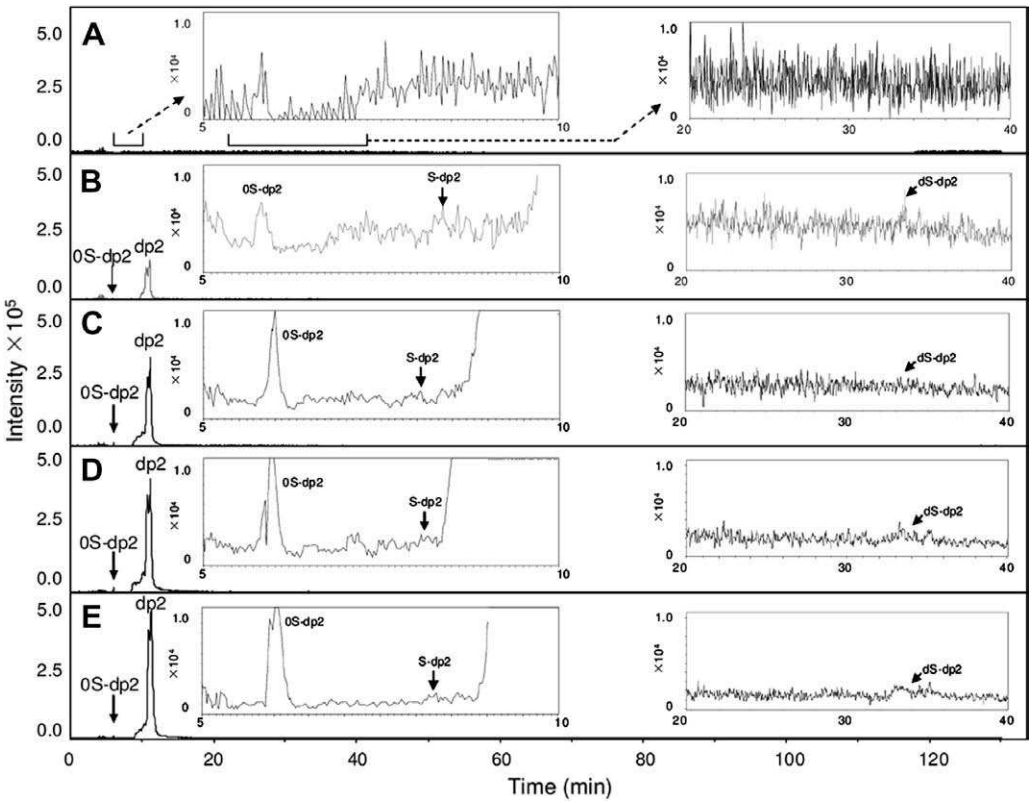

Fig. 6. EIC of products digested by chondroitinase ABC (*B. thetaiotaomicron*): (A) 0-h aliquots; (B) 1-h aliquots; (C) 5-h aliquots; (D) 12-h aliquots; (E) 24-h aliquots. Insets are 50× intensity magnification of 5 to 10 min and 50× intensity magnification of 20 to 40 min.

the nonreducing end of the CS-A chain. The disulfated unsaturated tetrasaccharide appeared most prominently in the early time points and was subsequently digested. This tetrasaccharide appears to be a side product of this exolytic lyase, which in turn can itself be used as a substrate.

Chondroitin lyase ABC (B. thetaiotaomicron)

Next, chondroitin lyase ABC (*B. thetaiotaomicron*), recombinantly expressed in *E. coli,* was examined. The monosulfated unsaturated disaccharide (dp2) was observed as a major peak in the EIC (Fig. 6). The intensity of this peak increased with digestion time. In addition, a monosulfated saturated disaccharide (S-dp2) and nonsulfated and disulfated unsaturated disaccharides (0S-dp2 and dS-dp2) were also observed in the EIC (Fig. 6), and their structures were determined based on molecular ions at *m*/*z* 476.1, 378.1, and 538.1, respectively. These results were similar to those observed for chondroitin lyase ACII (*A. aurescens*), suggesting that recombinantly expressed chondroitin lyase ABC (*B. thetaiotaomicron*) is also an exolytic enzyme. These are the first action pattern data reported for this enzyme. No unsaturated dp4 was observed among the products of this lyase, demonstrating the presence of subtle differences between the exolytic action patterns of these two lyases.

Chondroitin lyase AC (F. heparinum)

Recombinant chondroitin lyase AC (*F. heparinum*) expressed in *F. heparinum* displays an endolytic action pattern when acting on CS-A. Throughout the entire course of the reaction, dp4 was the dominant peak in the EIC (Fig. 7). Only when the reaction was completed did dp2 become the major product. Oligosaccharides (dp6, dp8, and dp10) appeared transiently throughout the time course of the reaction.

Discussion

LC–MS has been demonstrated to be a powerful method for the analysis of products afforded through the digestion of glycosaminoglycans by polysaccharide lyases. The product distribution can be profiled through LC, and the product structure can be analyzed by MS. Based on the products detected by LC–MS, the action patterns of various lyases can be confirmed.

The chondroitin lyases, investigated in this study, displayed two general action patterns: endolytic and exolytic. Chondroitin lyase ABC (*P. vulgaris*) and recombinant chondroitin lyase AC (*F. heparinum*) both showed similar endolytic action patterns (Figs. 2 and 7). In contrast, although both chondroitin lyase AC (*A. aurescens*) and recombinant chondroitin ABC (*B. thetaiotaomicron*) are clearly exolytic, they displayed distinctive differences (Figs. 4 and 6). In particular, chondroitin lyase AC (*A. aurescens*) showed a transient dp4 intermediate that was not observed in chondroitin lyase ABC (*B. thetaiotaomicron*) digestion. This suggests that chondroitin lyase AC (*A. aurescens*) can skip over cleavable sites only to return and cleave these sites later during the reaction. Such subtle differences in action patterns are clearly important when using chondroitin lyases in CS sequencing studies and could easily be missed when applying global analysis, such as viscometry, or more standard methods of analysis for the study of enzyme action patterns. Furthermore, the application of LC–MS also allows the observation of minor products arising from the natural sequence microheterogeneity of CS, such as unsulfated and disulfated sequences and oligosaccharides, originating from the nonreducing terminus of the CS chain. In this study, sequence heterogeneity was observed only when using exolytic enzymes. The monosulfated disaccharide domain is the most enzymatically sensitive one [16,21] and is digested first by endolytic enzymes. In contrast, any structure present at

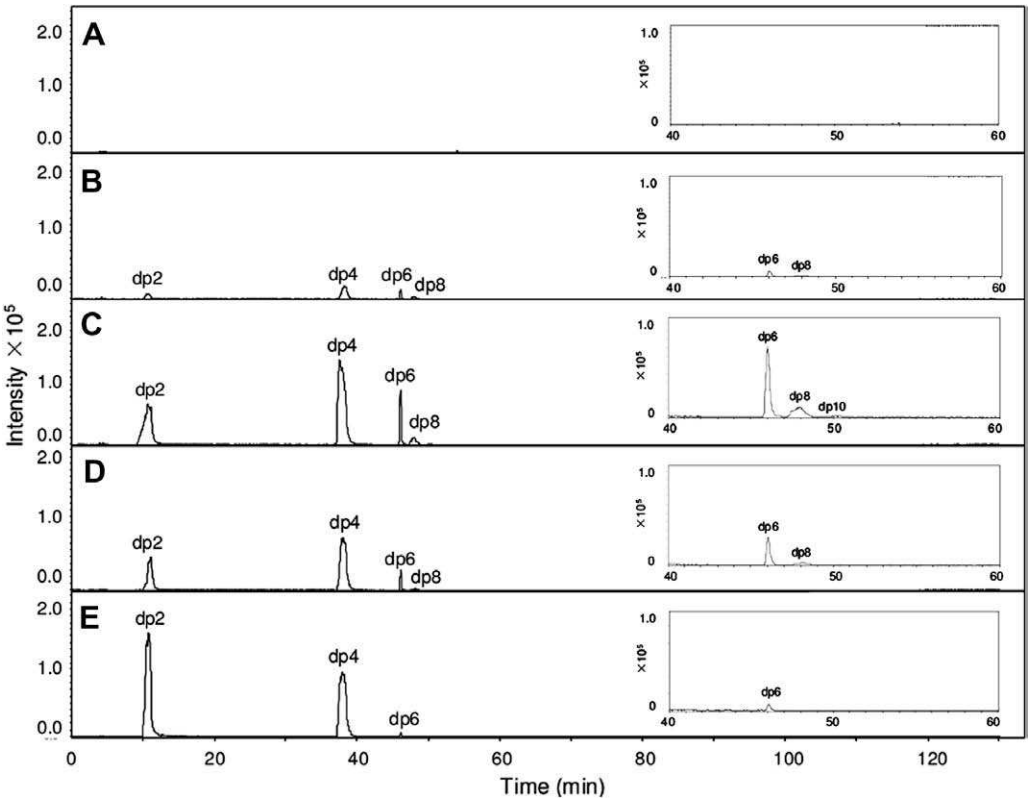

Fig. 7. EIC of products digested by chondroitinase AC (*F. heparinum*): (A) 0-min aliquots; (B) 10-min aliquots; (C) 30-min aliquots; (D) 60-min aliquots; (E) 120-min aliquots. Insets are 2.5× intensity magnification of 40 to 60 min.

the nonreducing terminus is digested first by exolytic enzymes. Product structures with different sulfation patterns have also been observed in previous studies [16,21]. In the current study, we elected to examine a single substrate type, CS-A. It is likely that CS substrates having different sulfation patterns or different domain structures will give differences in product distribution and possibly differences in action patterns. LC–MS offers an excellent approach for analysis because minor sequence heterogeneities, having a profound importance in CS biology, can be identified using this method.

References

[1] N. Volpi, Chondroitin Sulfate Structure, Role, and Pharmacological Activity Advances in Pharmacology, Academic Press, San Diego, 2006 pp. 1–10.
[2] P.L. DeAngelis, N.S. Gunay, T. Toida, W-J. Mao, R.J. Linhardt, Identification of the capsular polysaccharides of type D and F *Pasteurella multocida* as unmodified heparin and chondroitin, respectively, Carbohydr. Res. 337 (2002) 1547–1552.
[3] H.E. Bulow, O. Hobert, The molecular diversity of glycosaminoglycans shapes animal development, Annu. Rev. Cell Dev. Biol. 22 (2006) 375–407.
[4] R.J. Linhardt, P.M. Galliher, C.L. Cooney, Polysaccharide lyases, Appl. Biochem. Biotechnol. 12 (1986) 135–176.
[5] M. Koketsu, R.J. Linhardt, Electrophoresis for the analysis of acidic oligosaccharides, Anal. Biochem. 283 (2000) 136–145.
[6] A. Al-Hakim, R.J. Linhardt, Capillary electrophoresis for the analysis of chondroitin sulfate- and dermatan sulfate-derived disaccharides, Anal. Biochem. 195 (1991) 68–73.
[7] M. Warda, F. Zhang, M. Radwan, Z. Zhang, N. Kim, Y.N. Kim, R.J. Linhardt, J. Han, Is human placenta proteoglycan remodeling involved in pre-eclampsia?, Glycoconj. J. 25 (2008) 441–450.
[8] S. Sakai, J. Onose, H. Nakamura, H. Toyoda, T. Toida, T. Imanari, R.J. Linhardt, Pretreatment procedure for the microdetermination of chondroitin sulfate in plasma and urine, Anal. Biochem. 302 (2002) 169–174.
[9] P. Sinnis, A. Coppi, T. Toida, H. Toyoda, A. Kinoshita-Toyoda, J. Xie, M.M. Kemp, R.J. Linhardt, Mosquito heparan sulfate and its potential role in malaria infection and transmission, J. Biol. Chem. 282 (2007) 25376–25384.
[10] A.V. Nairn, A. Kinoshita-Toyoda, H. Toyoda, J. Xie, K. Harris, S. Dalton, M. Kulik, J.M. Pierce, T. Toida, K.W. Moremen, R.J. Linhardt, Glycomics of proteoglycan biosynthesis in murine embryonic stem cell differentiation, J. Proteome Res. 6 (2007) 4374–4387.
[11] J-S. Sim, T. Toida, S.Y. Cho, D.W. Choi, S-Y. Chang, R.J. Linhardt, Y.S. Kim, Quantitative analysis of chondroitin sulfate in raw materials. Ophthalmic solutions, soft capsules, and liquid preparations, J. Chromatogr. B 818 (2005) 133–139.
[12] S. Sakai, H. Akiyama, Y. Sato, Y. Yoshioka, R.J. Linhardt, Y. Goda, T. Maitani, T. Toida, Chondroitin sulfate intake inhibits the IgE-mediated allergic response by down-regulating Th2 responses in mice, J. Biol. Chem. 281 (2006) 19872–19880.
[13] J.W. Fawcett, Overcoming inhibition in the damaged spinal cord, J. Neurotrauma 23 (2006) 371–383.
[14] N.J. Tester, D.R. Howland, Chondroitinase ABC improves basic and skilled locomotion in spinal cord injured cats, Exp. Neurol. 209 (2008) 483–496.
[15] J.E. Davies, X. Tang, J.C. Bournat, S.J. Davies, Decorin promotes plasminogen/plasmin expression within acute spinal cord injuries and by adult microglia in vitro, J. Neurotrauma 23 (2006) 397–408.
[16] D. Shaya, B.S. Hahn, N.Y. Park, J.S. Sim, Y.S. Kim, M. Cygler, Characterization of chondroitin sulfate lyase ABC from *Bacteroides thetaiotaomicron* WAL2926, Biochemistry 47 (2008) 6650–6661.
[17] R.J. Linhardt, F.Y. Avci, T. Toida, Y.S. Kim, M. Cygler, CS lyases: Structure, activity, and applications in analysis and the treatment of diseases, Adv. Pharmacol. 53 (2006) 187–215.
[18] R.J. Linhardt, J.E. Turnbull, H.M. Wang, D. Loganathan, J.T. Gallagher, Examination of the substrate specificity of heparin and heparan sulfate lyases, Biochemistry 29 (1990) 2611–2617.
[19] K.A. Jandik, K. Gu, R.J. Linhardt, Action pattern of polysaccharide lyases on glycosaminoglycans, Glycobiology 4 (1994) 289–296.
[20] T. Yamagata, H. Saito, O. Habuchi, S. Suzuki, Purification and properties of bacterial chondroitinases and chondrosulfatases, J. Biol. Chem. 243 (1968) 1523–1535.
[21] Z. Zhang, J. Xie, F. Zhang, R.J. Linhardt, Thin-layer chromatography for the analysis of glycosaminoglycan oligosaccharides, Anal. Biochem. 371 (2007) 118–120.
[22] K.G. Rice, Y.S. Kim, A.C. Grant, Z.M. Merchant, R.J. Linhardt, High-performance liquid chromatographic separation of heparin-derived oligosaccharides, Anal. Biochem. 150 (1985) 325–331.
[23] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Electrospray ionization for mass spectrometry of large biomolecules, Science 246 (1989) 64–71.
[24] B. Kuberan, M. Lech, L. Zhang, Z.L. Wu, D.L. Beeler, R.D. Rosenberg, Analysis of heparan sulfate oligosaccharides with ion pair–reverse phase capillary high

performance liquid chromatography–microelectrospray ionization time-of-flight mass spectrometry, J. Am. Chem. Soc. 124 (2002) 8707–8718.
[25] C. Thanawiroon, K.G. Rice, T. Toida, R.J. Linhardt, Liquid chromatography/mass spectrometry sequencing approach for highly sulfated heparin-derived oligosaccharides, J. Biol. Chem. 279 (2004) 2608–2615.
[26] Z. Zhang, J. Xie, J. Liu, R.J. Linhardt, Tandem MS can distinguish hyaluronic acid from *N*-acetylheparosan, J. Am. Soc. Mass Spectrom. 19 (2008) 82–90.
[27] K. Hiyama, S. Okada, Amino acid composition and physiochemical characterization of chondroitinase from *Arthrobacter aurescens*, J. Biochem. 78 (1975) 1183–1190.
[28] W. Huang, V.V. Lunin, Y. Li, S. Suzuki, N. Sugiura, H. Miyazono, M. Cygler, Crystal structure of *Proteus vulgaris* chondroitin sulfate ABC lyase I at 1.9 Å resolution, J. Mol. Biol. 328 (2003) 623–634.
[29] J. Féthière, B. Eggimann, M. Cygler, Crystal structure of chondroitin AC lyase, a representative of a family of glycosaminoglycan degrading enzymes, J. Mol. Biol. 288 (1999) 635–647.
[30] I. Capila, Y. Wu, D.W. Rethwisch, A. Matte, M. Cygler, R.J. Linhardt, Role of arginine 292 in the catalytic activity of chondroitin AC lyase from *Flavobacterium heparinum*, Biochim. Biophys. Acta 1597 (2002) 260–270.
[31] D. Shaya, B-S. Hahn, T.M. Bjerkan, W.S. Kim, N.Y. Park, J-S. Sim, Y-S. Kim, M. Cygler, Composite active site of chondroitin lyase ABC accepting both epimers of uronic acid, Glycobiology 18 (2008) 270–277.
[32] J. Féthière, B.H. Shilton, Y. Li, M. Allaire, M. Laliberté, B. Eggimann, M. Cygler, Crystallization and preliminary analysis of chondroitinase AC from *Flavobacterium heparinum*, Acta Crystallogr. D 54 (1998) 279–280.