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New insights into the action of bacterial chondroitinase AC I and hyaluronidase on hyaluronic acid

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ABSTRACT

Hyaluronic acid (HA), a glycosaminoglycan, is a linear polysaccharide with negative charge, composed of a repeating disaccharide unit [→4)-β-D-glucopyranosyluronic acid (1 → 3)-β-D- *N*-acetyl-D-glucoaminopyranose (1 →]$_n$ ([→4) GlcA (1 → 3) GlcNAc 1 →]$_n$). It is widely used in different applications based on its physicochemical properties associated with its molecular weight. Enzymatic digestion by polysaccharides lyases is one of the most important ways to decrease the molecular weight of HA. Thus, it is important to understand the action patterns of lyases acting on HA. In this study, the action patterns of two common lyases, *Flavobacterial* chondroitinase AC I and *Streptomyces* hyaluronidase, were investigated by analyzing HA oligosaccharide digestion products. HA oligosaccharides having an odd-number of saccharide residues were observed in the products of both lyases, but their distributions were quite different. Chondroitinase AC acted more efficiently at the β 1–4 glycosidic bond linking GlcNAc and GlcA. Oligosaccharides, having an even number of saccharide residues, and with an unsaturated uronic acid (4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid, △UA) residue at their non-reducing end represent the major product. A minor amount of oligosaccharides having an odd number of saccharide residues resulted from the irregular terminal residues of HA substrate chains. Hyaluronidase showed a more complicated product mixture. Its minimum recognition and digestion domain is HA heptasaccharide and it could cleave both β 1–4 and β 1–3 glycosidic linkages. The HA oligosaccharides, generated with a 2-acetamido-2,3-di-deoxy-β-D-*erythro*-hex-2-enopyranose (△HexNAc) at the non-reducing end, are believed to be unstable and undergo breakdown immediately after their generation, and the oligosaccharides with △UA residue at the non-reducing end are formed. Thus, oligosaccharides having both an even and odd-number saccharide residues with a △UA residue at their non-reducing ends, represent the major products of hyaluronidase acting on HA.

1. Introduction

Hyaluronic acid (HA), a weakly acidic linear polysaccharide, is composed of a repeating disaccharide unit [→4)-β-D-glucopyranosyluronic acid (1 → 3)-β-D- *N*-acetyl-D-glucoaminopyranose (1 →]$_n$ ([→4) GlcA (1 → 3) GlcNAc 1 →]$_n$) (Kakizaki, Ibori, Kojima, Yamaguchi, & Endo, 2010). It is abundant in the extracellular matrix and is ubiquitously distributed in soft connective tissues and the body fluids (Zhao et al., 2016). It participates in and regulates many cellular events and is involved in physiological and pathophysiological processes (Mello, De Groot, Li, & Jedrzejas, 2002; Oommen, Duehrkop, Nilsson, Hilborn, & Varghese, 2016). HA can be prepared by extraction from animal tissues, such as cockscomb (Chun, Koob, & Eyre, 1988), or through bacterial fermentation of *Streptococcus zooepidemicus* (Vazquez, Montemayor, Fraguas, & Murado, 2010), or enzymatically using biosynthetic enzymes prepared from *Pasturella multocida* (Chu, Han, Guo, Fu, Liu, & Tao, 2016). HA is also known for its high viscosity, elasticity, and negative charge. It is extensively used in cosmetic and clinic, such as wrinkle filler and in dentistry (Choi, Seok, Kwon, Kwon, & Kim, 2016; Maytin, 2016). Furthermore, HA of different molecular sizes exhibits different physicochemical properties, bioactivities and is used for different applications (Britton, Ibberson, Horswill, & Cech, 2015; Bystricky, Machova, & Kolarova, 2002). Enzymatic degradation is one of the most important ways to prepare HA with different molecular weights (MWs) (Li, Kelly, Lamani, Ferraroni, & Jedrzejas, 2000; Stern, Kogan, Jedrzejas, & Šoltés, 2007). Several enzymes serve as the tools to degrade HA. Mammalian hyaluronidase, such as bovine testicular hyaluronidase, reportedly hydrolyzes β-1,4 glycosidic

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linkages, generating saturated HA oligosaccharides with glucuronic acid at the non-reducing end (Blundell & Almond, 2006; Chen et al., 2008; Kakizaki et al., 2011). Leech hyaluronidase is also hydrolase, but cleaves the β-1,3 glycosidic bond, yielding saturated HA oligosaccharides with glucuronic acid at the reducing end (El-Safory, Fazary, & Lee, 2010; Yuan et al., 2015). Microbial hyaluronidase, chondroitinase AC and chondroitinase ABC are lyases, which degrade HA by cleaving β-1,4 glycosidic bond through a β-elimination reaction and generate even-number oligosaccharides with an unsaturated uronic acid, 4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid (△UA) at the non-reducing end (Baker, Dong, & Pritchard, 2002; El-Safory et al., 2010; Li, Chen, & Yuan, 2015; Lv et al., 2016; Namburi, Berteau, Spillmann, & Rossi, 2016). These enzymes are also used as analytical tools to characterize the structures of HA. Thus, it is important to understand the action patterns of those enzymes.

However, some experimental phenomena observed in our lab are challenging the well-known action patterns of HA lyases. Unsaturated oligosaccharide products having both an even number and odd number of saccharide residues are observed in the products digested with HA lyases, such as chondroitinase AC and microbial hyaluronidase. In addition, similar phenomenon had been observed in one of the previous reports (Price, Baker, Chisena, & Cysyk, 1997). The hyaluronidase from *Streptomyces* was reported to generate unsaturated oligosaccharides with an even number of saccharide units accompanied by minor amounts oligosaccharides having an odd number of saccharide units. This did not attract wide attention as a potential hydrolase impurity in commercial enzyme was thought to be the reason for these minor products.

In this study, the action patterns of two common lyases, chondroitinase AC I and microbial *Streptomyces* hyaluronidase, were investigated on HA. The purities of two lyases were confirmed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The profiles, of oligosaccharide mixtures digested with these two lyases, were analyzed with ultrahigh performance size exclusion chromatography (UHPSEC) – electrospray ionization (ESI) – quadrupole/time of flight (Q/TOF) – mass spectrometry (MS). The sequences of some oligosaccharides having an odd and even number of saccharide residues were confirmed with MS/MS. In addition, several of these oligosaccharides were prepared offline, and the action patterns of these lyases were verified by analysis of the digested formed on their digestion. Based on these results, their different action patterns and their different digestion mechanisms were elucidated.

2. Experimental

2.1. Materials

Hyaluronic acid sodium salt from rooster comb was purchased from Aladdin (Shanghai China). Hyaluronidase from Streptomyces hyalurolyticus and NaBD4 (D ≥ 95%) were purchased from Aldrich. Chondroitinase AC I from *Flavobacterium heparinum* was purchased from Adhoc (Beijing China). Bio-Gel P-10 (fine) was purchased from Bio-RAD (Hercules, CA). Sephadex G10 was purchased from GE Healthcare life science (Piscataway, NJ). High-purity water (resistivity ≥ 18.2 MΩ × cm, 25 °C) was used throughout the study. All other chemicals and reagents were of HPLC grade.

2.2. Methods

2.2.1. Purity analysis of HA lyases with SDS-PAGE

The purity of two HA lyases were investigated with SDS-PAGE. It was equipped with a Mini-PROTEAN Tetra System electrophoresis apparatus (Bio-Rad, Hercules, CA), including 10 cm plates, 1 mm spacer and 0.5 cm wells. SDS-PAGE was performed with common processes (Zhao et al., 2013). The samples were denatured with the loading kit (Bio-Rad, Hercules, CA) before analysis.

2.2.2. HA digestion

HA (0.2 mg) was dissolved in 50 μL water and was treated in individual reactions with chondroitinase AC I and *Streptomyces* hyaluronidase (10 μ units), at 37 °C for 24 h with aliquots removed at various time points for analysis (5, 30 min, 1, 4, 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 and removed by centrifuge (12000 × g) for 10 min. These samples are ready for UHPSEC-ESI-Q/TOF-MS analysis.

HA (20 mg) was dissolved in 1 mL water and digested with *Streptomyces* hyaluronidase (100 μ units) at 37 °C for 4 h. The enzyme was thermally inactivated by heating at 100 °C for 10 min and removed by centrifuge (12000 × g) for 10 min. This sample is ready to prepare oligosaccharides.

2.2.3. Preparation of oligosaccharides having an odd and even number or saccharide residues

The HA oligosaccharides were prepared using size exclusion chromatography. A glass column (2.6 × 100 cm) was packed with Bio-gel P 10, which was equilibrated with 0.2 M NaCl at a flow rate of ~0.2 mL/min. The solution of HA oligosaccharides with different degree of polymerization was loaded on the equilibrated column. The process was monitored using a refractive index detector (G1362A, Agilent technology). The fractions were collected using an automatic sampler (2110, Bio-Rad, Hercules, CA) with 10 min/tube. The fractions of each peak corresponding to HA oligosaccharide were combined and then concentrated with a rotary evaporator. Desalting was performed with another glass column (2.6 × 30 cm) packed with Sephadex G10 (GE Healthcare life science, Piscataway, NJ) equilibrated with pure water. The desalted HA oligosaccharides were lyophilized. Each pure oligosaccharide is ready to be digested and analyzed (Ouyang et al., 2015).

2.2.4. Reduction of HA dp8

Purified HA dp8 (20 μg) was dissolved in 20 μL $NaBD_4$ (0.05 M) aqueous solution. The reduction was carried over at 4 °C for overnight. The reaction was stopped by adding acetic acid solution (HOAc:H_2O, 1:1, v:v) to afford the reaction solution to pH7. The reacted solution was loaded on a cation-exchange cartridge column (AG 50W, H form, Bio-Rad, Hercules, CA) to remove sodium and to afford the reduced HA dp8 (H^+ form) and boric acid. The boric acid was removed by adding methanol and blowing N_2 over the sample leaving the pure reduced HA dp8 for analysis.

2.2.5. Digestion of oligosaccharides with HA lyases

Each of the separated oligosaccharides (dp3–dp10) and reduced dp8 were treated with chondroitinase AC I and hyaluronidase, respectively. The processes were identical to that used in the HA digestions.

2.2.6. UHPSEC-ESI-Q/TOF-MS

The UHPSEC-ESI-Q/TOF-MS method was developed on an Agilent system equipped with an UHPLC (1290, dual pumps) . Data were required with MassHunter 6.0 (Agilent Technologies). Chromatograms were obtained on an ACQUITY UPLC BEH 125 size exclusion chromatography column (4.6 × 300 mm, 1.7 μm, Waters) at 20 °C. The mobile phase (A) was 50 mM ammonium acetate solution, and the mobile phase (B) was methanol. The column was equilibrated and eluted with 80% A at a flow rate of 0.1 mL/min with UV detection at 232 nm.

Nitrogen gas was used in the nebulizer at a pressure of 30 psi. The spray voltage was 3.5 kV and a flow of nitrogen of 8L/min

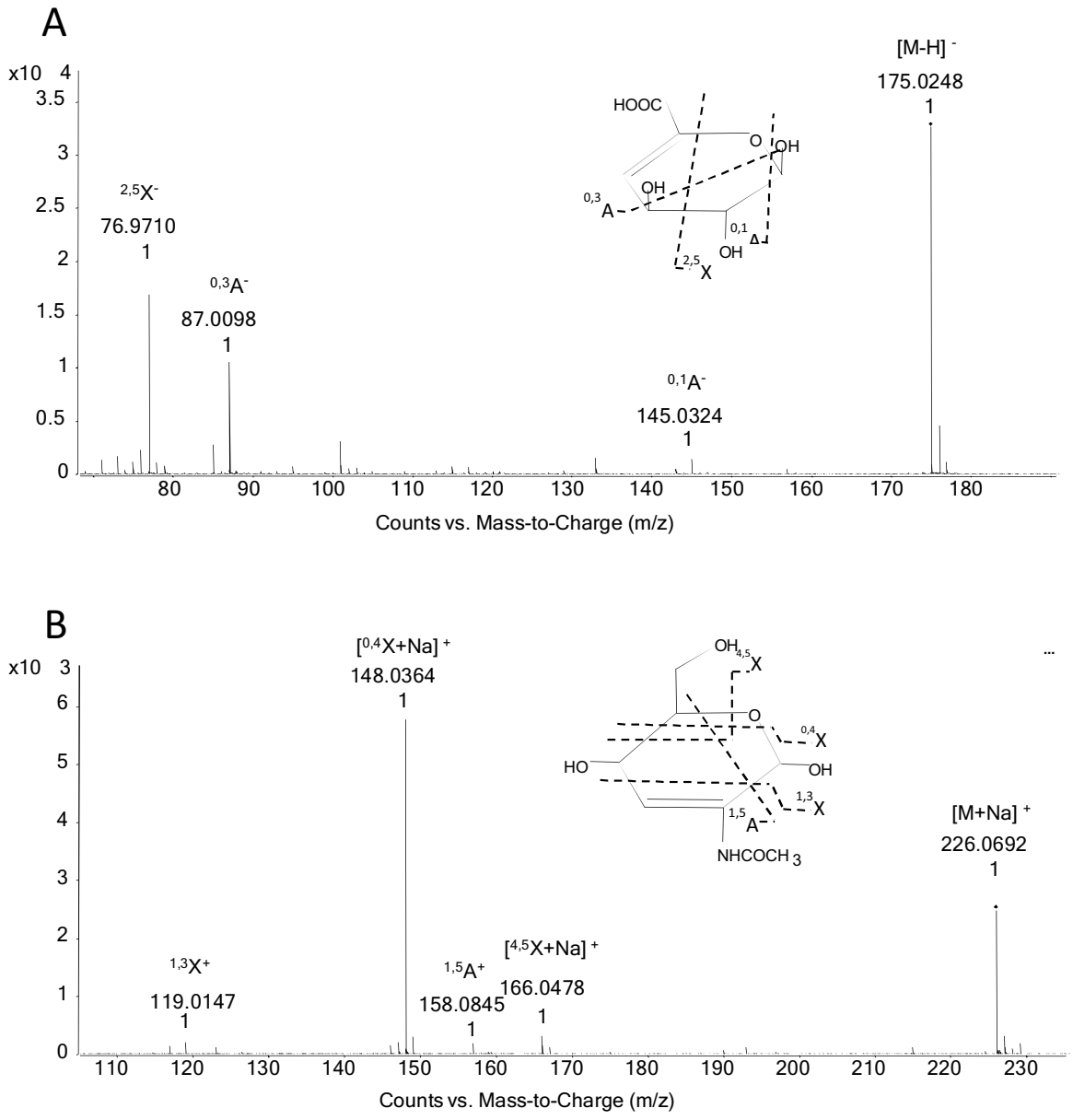

Fig. 1. MS/MS Spectra of △UA and △HexNAc.

at 350 °C assisted in drying process. Fragment voltage was set to 150 V. A full MS scan between 100 and 2000 *m*/*z* was performed. The collision-induced dissociation (CID) energy used in MS/MS to dissociate oligosaccharides was set as 10–50 V. Most of data was recorded in the negative mode. A few experiments were performed in the positive mode as required. The accuracy of MS and MS/MS are 1 ppm and 4 ppm (Yi, Ouyang et al., 2015).

3. Results and discussion

3.1. Purity analysis of HA lyases with SDS-PAGE

The SDS-PAGE analysis of chondroitinase AC I and *Streptomyces* hyaluronidase is shown in Fig. S1. A single band was observed corresponding to each enzyme. Their molecular weights, 71.1 and 38.4 kD, respectively, were consistent with previously reported values (Sutti, Tamascia, Hyslop, & Rocha, 2014; Tkalec et al., 2000).

3.2. The action pattern analysis of chondroitinase AC I

3.2.1. The analysis of digestion products from HA

The liquid chromatography-ultraviolet (LC-UV) chromatograms, of the products digested from HA with chondroitinase AC I, are shown in Fig. S2. Some oligosaccharides were observed between 24 and 36 min in the chromatogram of 5 min aliquot. This indicates that HA was digested to oligosaccharides within 5 min on treatment with chondroitinase AC I. These oligosaccharides were subsequently digested to smaller oligosaccharides and their chromatograms are shown in Fig. S2 B–E. Every peak in these chromatograms shows two oligosaccharides eluting together. They are an oligosaccharide having an even number of saccharide units, △UA (1[→3) GlcNAc (1 → 4) GlcA (1]$_n$ → 3) GlcNAc, and an oligosaccharide with an odd number of saccharide units, △UA (1[→3) GlcNAc (1 → 4) GlcA (1 →]$_n$. The degree of polymerization (dp), labeled in Fig. S2B, and the composition of these oligosaccharides were confirmed with MS (Fig. S3 and Table 1). The oligosaccharides dp2-dp6 observed in the digestion products were

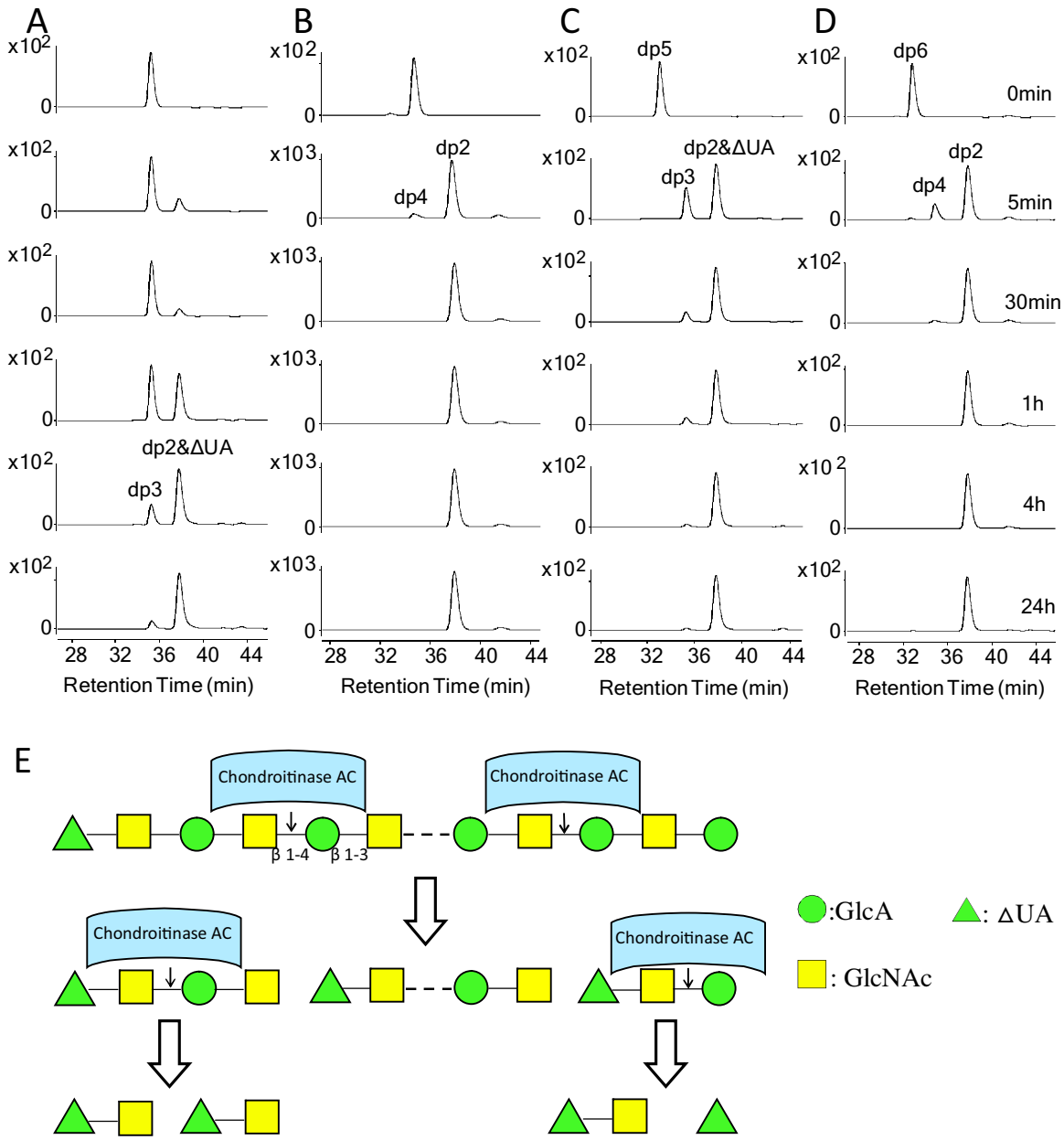

Fig. 2. The action pattern of chondroitinase AC on HA. A. chromatograms of digested oligosaccharide products from HA dp3; B. chromatograms of digested oligosaccharide products from HA dp4; C. chromatograms of digested oligosaccharide products from HA dp5; D. chromatograms of digested oligosaccharide products from HA dp6; E. Schematic drawing of action pattern of chondroitinase AC I.

sequenced using MS/MS. The assignment of molecular ions and fragment ions in MS/MS utilizes the nomenclature of Domon and Costello (Domon & Costello, 1988). Their glycosidic bond cleavage ions were unambiguously assigned in the MS/MS (Fig. S4) and are presented in Table 1. Cross-ring cleavage ions were only observed in the 1–4 linked uronic acid residues, but not in 1–3 linked GlcNAc residues. This is consistent with our previously reports (Gao, Zhang, Liu, Feizi, & Chai, 2015; Yi, Sun et al., 2015; Zhang et al., 2006), in which we concluded that the sugar residues were stabilized by the glycosylation at position 3 and few cross-ring cleavage ions could be observed with collisionally-induced dissociation (CID) MS/MS. Thus, the sequences could be confirmed for oligosaccharides with an even number of saccharide units, △UA (1[→3) GlcNAc (1 → 4) GlcA (1]$_n$ → 3) GlcNAc, and oligosaccharides with an odd number of saccharide units, △UA (1[→3) GlcNAc (1 → 4) GlcA (1 →]$_n$.

In addition, a unique monosaccharide, △UA, eluted with HA disaccharide, △UA (1 → 3) GlcNAc, at 38 min in the chromatograms and its structure was confirmed with MS/MS analysis (Fig. 1A and Table 2). In the MS/MS of △UA, the ions at *m*/*z* 77, 87, 145 and 175 were assigned as ^{2,5}X, ^{0,3}A, ^{0,1}A and molecular ion, respectively. The double bond was assigned at bond 4 of the uronic acid as same as the common structure formed through the action of lyases (Li & Jedrzejas, 2001; Li, Kelly, Lamani, Ferraroni, & Jedrzejas, 2000; Maccari, Tripodi, & Volpi, 2004).

Furthermore, the amount of oligosaccharides having an even number of saccharide units is greater than the oligosaccharides having an odd number of saccharide units in each of the peaks based on their signal intensities in the MS spectra (Fig. S3).

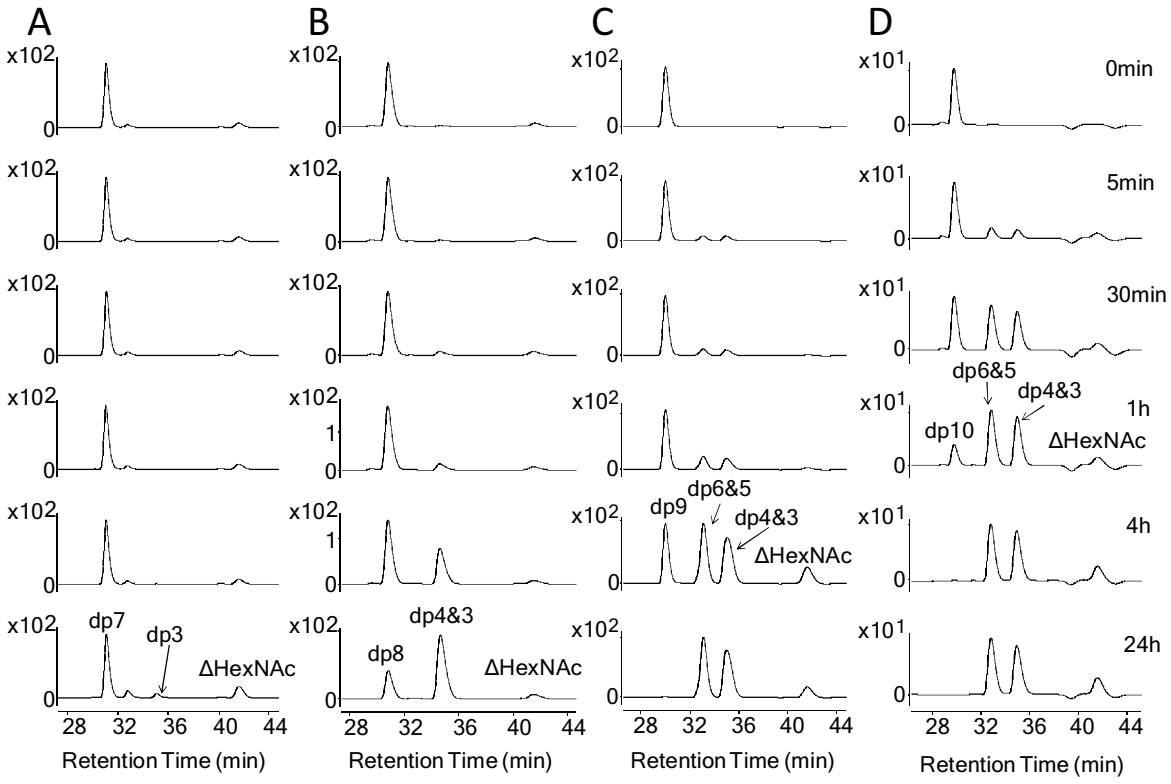

Fig. 3. Chromatograms of digested products from offline prepared HA oligosaccharides with hyaluronidase. A. chromatograms of digested oligosaccharide products from HA dp7; B. chromatograms of digested oligosaccharide products from HA dp8; C. chromatograms of digested oligosaccharide products from HA dp9; D. chromatograms of digested oligosaccharide products from HA dp10.

Table 1
Assignments of Fragment Ions in MS/MS Spectra of dp2 to dp6.

	$[M-H]^-$	$^{0,2}A_1$	$^{1,5}A_1$	$^{2,4}X_0$	B_1	C_1	Y_1^-	B_2	C_2	Y_2	$^{0,2}A_3$	$^{1,5}A_3$	C_3	Y_3	B_4	C_4	$^{0,2}A_5$	C_5
dp2	378	115	129	–	157	175												
dp3	554	–	–	131	157	175	–	360	–	396	494							
dp4	757	–	–	131	157	175	193	360	–	396	–		554					
dp5	933	–	–	131	157	175	193	–	378	396	–	510	554		739	757	873	
dp6	1136	–	–	–	157	175	–	–	–	396	–	510	554	599	739	757		933

3.2.2. *The analysis of digestion products from HA oligosaccharides*

The oligosaccharides, dp3–dp6, prepared offline were next used to investigate the action pattern of chondroitinase AC I. The sequences of these oligosaccharides were confirmed as △UA (1[→3) GlcNAc (1 → 4) GlcA (1]$_n$ → 3) GlcNAc and △UA (1[→3) GlcNAc (1 → 4) GlcA (1 →]$_n$, where n equals to 1 or 2. Their MS/MS data were consistent with their structure as shown in Fig. S4. The LC UV chromatograms of aliquots of HA trisaccharide digested with the chondroitinase AC I at different time points (0 min to 24 h) are shown in Fig. 2A. The trisaccharide of HA, △UA (1 → 3) GlcNAc (1 → 4) GlcA, was gradually (over 24 h) converted to disaccharide, △UA (1 → 3) GlcNAc, and unsaturated uronic acid (△UA). The HA tetrasaccharide digested with the same amount of chondroitinase AC I to disaccharide in shorter time than the trisaccharide, with most tetrasaccharide converted to disaccharide within 5 min (Fig. 2B). HA pentasaccharide was digested with the same amount of chondroitinase AC I to trisaccharide and disaccharide in 5 min and subsequently, the trisaccharide was gradually digested to disaccharide and △UA (Fig. 2C). HA hexasaccharide digested with the same amount of chondroitinase AC I was rapidly (5 min) converted to tetrasaccharide and disaccharide and the completely to disaccharide in 30 min (Fig. 2D).

3.2.3. *Action pattern of chondroitinase AC*

Thus, it is clear that the chondroitinase AC I preferentially cleaves HA oligosaccharides to produces oligosaccharides having an even number of saccharide units (Fethiere, Eggimann, & Cygler, 1999; Lunin et al., 2004). Moreover, while it can cut small oligosaccharides, such as a HA trisaccharide, it does so with relatively lower efficiency. It has been postulated that the small amount of odd-number oligosaccharides present in the HA digestion products come from the terminals of HA chains, including those with a △UA residue. The action pattern of chondroitinase AC I acting on HA consistent with these data is shown in Fig. 2E.

3.3. *The action pattern analysis of hyaluronidase*

3.3.1. *The analysis of digestion products from HA*

The chromatographic profiles of aliquots taken at different time points of hyaluronidase acting on HA are shown in Fig. S5. At the early (5 min) time point the chromatogram shows two broad peaks at ~16 and ~20 min, corresponding to the HA polysaccharides. Additional small peaks could also be observed at 24 ~ 36 min, corresponding to oligosaccharides (Fig. S5A). After 30 min of digestion additional oligosaccharides could be clearly observed in the chromatograms (Fig. S5B). By 4 h the broad

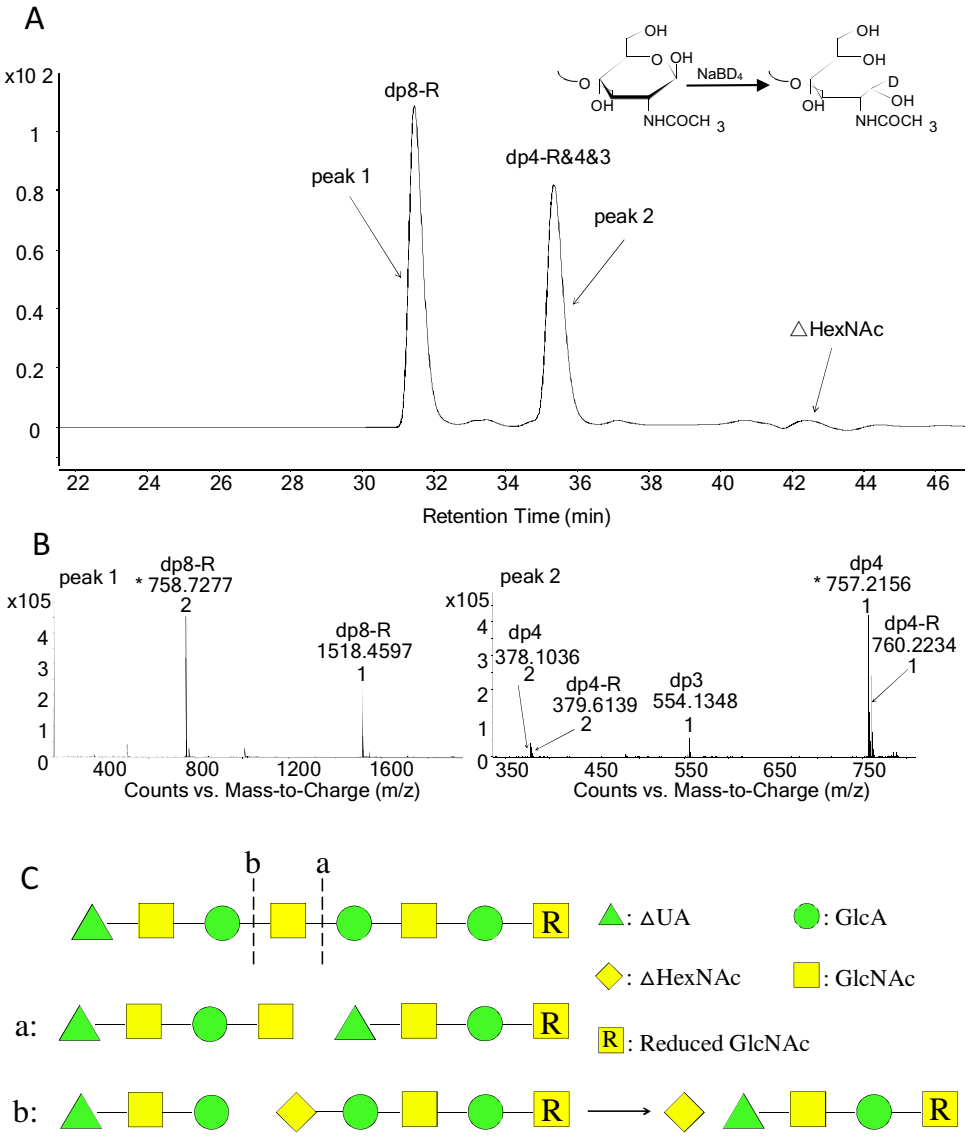

Fig. 4. Action pattern of hyaluronidase on reduced HA dp8. A. chromatogram of digested products from reduced HA dp8; B. MS spectra of corresponding chromatographic peaks; C. action pattern of hyaluronidase on reduced HA dp8.

Table 2
Assignments of Fragment Ions in MS/MS Spectra of △UA and △HexNAc.

	$[M-H]^-$	$^{2,5}X$	$^{0,3}A$	$^{0,1}A$	
△UA	175	77	87	145	
	$[M+Na]^+$	$^{1,3}X$	$[^{0,4}X+Na]^+$	$^{1,5}A$	$[^{4,5}X+Na]^+$
△HexNAc	226	119	148	158	166

peaks associated with the polysaccharide had completely disappeared (Fig. S5C). The molecular weights and the composition of oligosaccharides eluted in each peak were confirmed using MS analysis. Again, a pair of oligosaccharides, one having an even number and one with an odd number of saccharide units was observed. Moreover, a similar response for these two oligosaccharides in the MS, (Fig. S6) suggests that they are present in similar amounts. In addition, a new intense peak observed at ~42 min in the chromatogram (Fig. S5), which was assigned as unique and previously unreported 2-acetamido-2,3-di-deoxy-β-D-*erythro*-hex-2-enopyranose (△HexNAc) from the corresponding MS/MS spectrum (Fig. 1 B). MS/MS showed ions at *m*/*z* 119, 148, 158, 166 and 226 that could be assigned to $^{1,3}X$, $^{0,4}X$, $^{1,5}A$, $^{4,5}X$ and molecular ion, respectively (Table 1). No cleavage was observed through the bond between carbon 2 and 3, consistent with the presence of a 2–3 double bond in the △HexNAc structure (Fig. 1 B). In addition, the HA disaccharide, △UA (1 → 3) GlcNAc, the main product of HA digested with chondroitinase AC I, and △UA were not observed in the products of HA digestion with hyaluronidase. MS/MS analysis of the other oligosaccharide products of HA digestion with hyaluronidase showed the same sequences, △UA (1[→3) GlcNAc (1 → 4) GlcA (1]$_n$ → 3) GlcNAc and △UA (1[→3) GlcNAc (1 → 4) GlcA (1 →]$_n$, observed in the products of HA digested with chondroitinase AC I.

3.3.2. *The analysis of digestion products from HA oligosaccharides*

When isolated HA dp3-6 oligosaccharides were treated with hyaluronidase they were not cleaved further demonstrating that these were end-products and not intermediates of this reaction (Fig. S7). Hyaluronidase began to slowly (after 24 h) work only when the size of HA intermediate reached dp7 when small amount of trisaccharide and △HexNAc could be observed as products eluting at ~35.5 min and ~42 min, respectively (Fig. 3A). The HA dp8

oligosaccharide was a somewhat better substrate as it began to be digested at 30 min and was nearly converted to dp3 and dp4 (eluting together at ~35.5 min) and △HexNAc (at ~42 min) after 24 h (Fig. 3B). The digestion of HA dp9 began within 5 min and it was completely converted at 24 h to dp5 and dp6 (co-eluting at ~ 33 min) dp3 and dp4 (co-eluting at ~35.5 min) and △HexNAc (at ~42 min) (Fig. 3C). HA dp10 was completely converted to △HexNAc, dp3, 4, 5 and 6 in 4 h (Fig. 3D). MS/MS again confirmed the sequences of these oligosaccharide products to be △UA (1[→3) GlcNAc (1 → 4) GlcA (1]$_n$ → 3) GlcNAc and △UA (1[→3) GlcNAc (1 → 4) GlcA (1 →]$_n$.

3.3.3. *The analysis of digested products from reduced HA dp8*

Reduction of HA dp8 with $NaBD_4$ afforded the monodeuterated alditol (insert of Fig. 4). This monodeuterated alditol octasaccharide allows the hyaluronidase digestion products, arising from the reducing end and the non-reducing end, to be easily distinguished. The HA dp3, dp4 and reduced dp4 (co-eluting at ~35.5 min) and △HexNAc (eluting at ~42 min) were observed in Fig. 4A. The MS spectra of corresponding chromatographic peaks are shown in supplementary Fig. 4B.

3.3.4. *The action pattern of hyaluronidase*

The formation of a reducing dp4 and a monodeuterated, reduced dp4 suggests the hyaluronidase cut reduced dp8 at glycosidic linkage "a" (Fig. 4C). The formation of only a reducing dp3 in the hyaluronidase digestion products of reduced dp8, indicates that dp3 was formed from the non-reducing end of reduced dp8 by cleavage at glycosidic linkage "b", (Fig. 4C). The dp 3 cannot be from a dp4 formed from dp8 cleavage by hyaluronidase at glycosidic linkage "a". This is supported in Fig. S7 that demonstrates dp4 is stable and resistant to hyaluronidsae digestion. No pentasaccharide with GlcNAc residue at the non-reducing end and a monodeuterated alditol at the reducing end was observed. Such a pentasaccharide would be formed along with the dp 3 product if hyaluronidase had cleaved at glycosidic linkage "b" in HA dp8. However, it might be possible that the dp5 is not stable, and the GlcNAc dropped immediately after cleavage by hyaluronidase at glycosidic linkage "b".

Based on the action pattern observed in the digestion of reduced dp8 with hyaluronidase, the phenomena observed in the digestions of HA dp7, dp8, dp9 and dp10 could be explained. The HA dp3 was observed as a major digestion product from HA dp7 suggesting hyaluronidase cleave at glycosidic linkage "a" (Fig. 5A). The dp4 intermediate having the GlcNAc residue at the non-reducing end then looses this residue as △HexNAc and the dp3 product, △UA (1 → 3) GlcNAc (1 → 4) GlcA, is formed. Two glycosidic linkages in HA dp8 can be cleaved by hyaluronidase and dp3 and dp4 were observed in the digested product, consistent with the experiments described for the hyaluronidase digestion of reduced dp8 (Fig. 4C). Three glycosidic linkages in HA dp9 could be cleaved (a, b, and c) affording △HexNAc dp3, dp4 and dp5 as products (Fig. 5 B). Cleaving glycosidic linkage "a" generates stable product dp3, △UA (1 → 3) GlcNAc (1 → 4) GlcA, and intermediate dp6, △GlcNAc (1 → 4) GlcA (1 → 3) GlcNAc (1 → 4) GlcA (1 → 3) GlcNAc (1 → 4) GlcA, which immediately generates the stable product dp5, △UA (1 → 3) GlcNAc (1 → 4) GlcA (1 → 3) GlcNAc (1 → 4) GlcA, through the breaking of glycosidic linkage "b". HA dp4 and dp5 are produced by cleaving glycosidic linkage "b". Both dp4 and dp5, having a △UA at their non-reducing end are stable products. Cleaving glycosidic linkage "c" affords the stable product dp5, △UA (1 → 3) GlcNAc (1 → 4) GlcA (1 → 3) GlcNAc (1 → 4) GlcA, and intermediate dp4, △HexNAc (1 → 4) GlcA (1 → 3) GlcNAc (1 → 4) GlcA, which forms △HexNAc and the dp3 product. Four glycosidic linkage (a, b, c, d) in HA dp10 can be cleaved (Fig. 5C). HA product dp3, △UA (1 → 3) GlcNAc (1 → 4) GlcA, and intermediate dp7 are produced through the cleavage of glycosidic linkage "a". Next, intermediate dp7 gives rise to dp6 through the breakage of glycosidic linkage "b". HA products dp4 and dp6 are produced directly by cutting HA dp10 at position "b". HA product dp5, △UA (1 → 3) GlcNAc (1 → 4) GlcA (1 → 3) GlcNAc (1 → 4) GlcA, and HA intermediate dp5, △HexNAc (1 → 4) GlcA (1 → 3) GlcNAc (1 → 4) GlcA (1 → 3) GlcNAc, are produced by cleavage of dp10 at glycosidic linkage "c". The unstable intermediate dp5 then forms product dp4 and △HexNAc through the breaking of glycosidic linkage "d". The direct cleavage of glycosidic linkage "d" in HA dp10 affords the stable products dp4 and dp6.

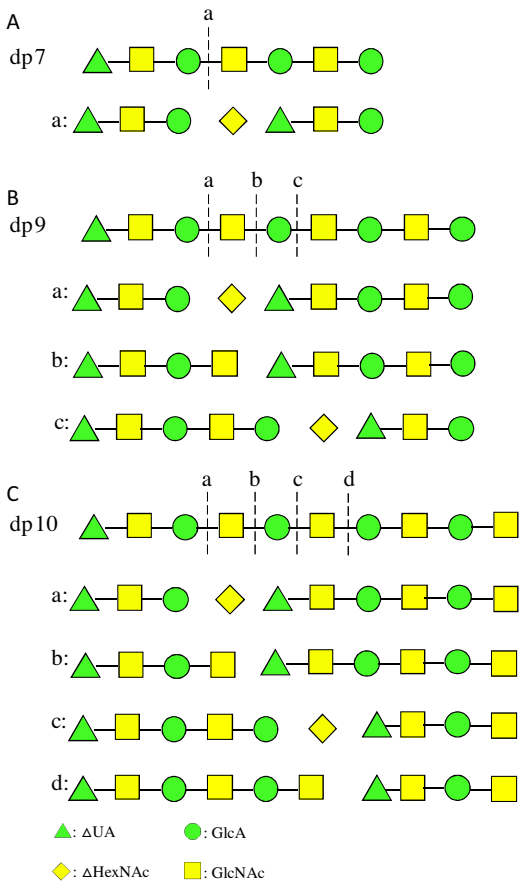

Fig. 5. Action pattern of *Streptomyces* hyaluronidase on HA. A. action pattern of hyaluronidase on HA dp7; B. action pattern of hyaluronidase on HA dp9; C. action pattern of hyaluronidase on HA dp10.

4. Conclusion

Two pure lyases show very different action patterns on digestion of HA. Chondroitinase AC I is more efficient at breaking down HA. A trisaccharide is its minimum recognition and digestion domain. The cleavage position in HA is β 1–4 glycosidic bond linked GlcNAc and GlcA, so the oligosaccharides having an even number of saccharide residues with an △UA residue at their non-reducing ends are the major products formed. Small amounts of products with an odd number of saccharide residues are also formed from the irregular termini of the HA chains, *i.e.*, a △UA product from a chain with a GlcA residue at the reducing end or a saturated product from a HA chain with a GlcNAc residue at the non-reducing end.

The minimum recognition and digestion domain of *Streptomyces* hyaluronidase is HA dp7. In the dp7 substrate four of the saccharide residues are required on the reducing end and three of the saccharide residues on the non-reducing side of the cleavage site (Fig. 5).

The most surprising finding of the current study is that *Streptomyces* hyaluronidase can cleave at both glycosidic bonds, β 1–4 linked GlcNAc to GlcA and β 1–3 linked GlcA to GlcNAc. Oligosaccharides formed with a *N*-acetylhexosamine at their non-reducing end, through the action of hyaluronidase at a β 1–3 glycosidic linkage, are unstable intermediates. This *N*-acetylhexosamine residue at the non-reducing end is lost as an unusual △HexNAc monosaccharide product immediately after the oligosaccharide is generated. Thus, oligosaccharides, with both an even and odd number saccharide residues, terminated at their non-reducing ends with △UA are major digestion products of hyaluronidase.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2016.12.010.

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