Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 338 (2003) 2101–2104
CARBOHYDRATE RESEARCH
www.elsevier.com/locate/carres

Chondroitin *O*-methyl ester: an unusual substrate for chondroitin AC lyase

Fikri Y. Avci,[a] Toshihiko Toida,[b] Robert J. Linhardt[a,*]

[a] *Department of Chemistry, Rensselaer Polytechnic Institute, Troy, NY 12180-3590, USA*
[b] *Department of Bioanalytical Chemistry, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba 263-8522, Japan*

Received 27 May 2003; accepted 23 June 2003

Abstract

Chondroitin *O*-methyl ester was depolymerized by chondroitin AC lyase (EC 4.2.2.5) from *Flavobacterium heparinum*. The major product isolated from the depolymerization reaction was found to be methyl α-L-*threo*-hex-4-enopyranosyluronate-(1→4)-2-acetamido-2-deoxy-α,β-D-galactopyranoside.

Keywords: Chondroitin *O*-methyl ester; Chondroitin AC lyase; *Flavobacterium heparinum*

1. Introduction

Chondroitins are linear, polydisperse, glycosaminoglycans (GAGs) with a repeating core of disaccharide structure comprised of a D-glucopyranosyluronic acid (GlcA*p*) or L-idopyranosyluronic acid (IdoA*p*) glycosidically linked to 2-acetamido-2-deoxy-D-galactopyranose (Gal*p*NAc) residue. They are the major polysaccharide component of extracellular matrix proteoglycans. The major classes of the chondroitins are: chondroitin, →3)Gal*p*NAc(1→4)GlcA*p*(1→; chondroitin 4-sulfate (CS-A), →3)Gal*p*NAc4S(1→4)GlcA*p*(1→; dermatan sulfate (CS-B), →3)Gal*p*NAc4S(1→4)IdoA*p*(1→; and chondroitin 6-sulfate (CS-C) →3)Gal*p*NAc6S(1→4) GlcA*p*(1→. Enzymatic degradation of CS can occur through two possible mechanisms, hydrolysis catalyzed by hydrolase and β-elimination catalyzed by lyases. While polysaccharide hydrolases have been studied intensively, and reaction mechanisms of these enzymes are well characterized,[1,2] the reaction mechanisms for polysaccharide lyases are relatively less understood.

Chondroitin AC lyase (EC 4.2.2.5), from *Flavobacterium heparinum*, degrades chondroitin, chondroitin 4-sulfate (CS-A), chondroitin 6-sulfate (CS-C), and hyaluronic acid, →3)GlcpNAc(1→4)GlcAp(1→.[3] The mode of action for this enzyme has been established as random endolytic.[3–5] Dermatan sulfate containing IdoA*p* has an inhibitory effect on chondroitin AC lyase.[6] While there is no absolute requirement of a metal ion for chondroitin AC lyase activity, various mono- and di-valent metals have been shown to influence enzyme activity.[3]

The current study demonstrates that chondroitin AC lyase from *Flavobacterium heparinum* can also act on chondroitin *O*-methyl ester. The discovery of this new substrate is significant in that the negatively charged carboxyl group of GlcA*p* in the natural substrate is replaced with a neutral carboxyl methyl ester, clarifying the role of negative charge at this site in the substrate on enzyme activity.

2. Experimental

2.1. Preparation of chondroitin *O*-methyl ester

Chondroitin *O*-methyl ester (C-OMe) was prepared using a procedure first described by Kantor and Schubert.[7] The sodium salt of chondroitin sulfate from

* Corresponding author. Tel.: +1-518-276-3404; fax: +1-518-276-3405.
E-mail address: linhar@rpi.edu (R.J. Linhardt).

0008-6215/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0008-6215(03)00348-3

bovine traechea, (Sigma Chemical, St. Louis, MO) a mixture (1.24/1) of CS-A/CS-C,[8] was converted to the potassium salt by ion-exchange on Dowex 50WX8-100 (Sigma). The resulting potassium salt (2.5 g) was treated with 400 mL of acidic MeOH, prepared by adding AcCl (5 mL) to MeOH (1 L) and allowing the mixture to react for 3 days at room temperature. Neither the CS starting material nor product, C-OMe, are soluble in MeOH. The reaction mixture was stirred for 1 day under an inert Ar atmosphere, centrifuged, and the solid was recovered. The recovered solid was then treated in the same way twice more with acidic MeOH. After the reaction was performed three times, the recovered solid product, C-OMe, was dissolved in 50 mL of water and dialyzed.

2.2. Chondroitin AC lyase-catalyzed depolymerization of chondroitin *O*-methyl ester and recovery of oligosaccharide products

C-OMe (100 mg) was dissolved in 50 mM Tris–HCl–sodium acetate buffer (4 mL) at pH 8. Chondroitin AC lyase (1.4 U) (Sigma) was added to the reaction medium, and the mixture was incubated for 24 h at 37 °C. Progress of the enzymatic depolymerization reaction was followed by measuring the absorbance of the sample at 232 nm. The resulting oligosaccharide products were fractionated on a Bio-Gel P4 column (2.5 cm × 80 cm; BioRad, Hercules, CA) at 0.7 mL/min. The eluent was monitored at 232 nm, and fractions corresponding to disaccharides and tetrasaccharides (Fig. 1), that eluted last from the column were collected.

2.3. Determination of molar absorbtivity of chondroitin disaccharide methyl ester

The absorbtivity coefficients (ε) for ΔUA*p*(1→3)Gal*p*-NAc (where ΔUA*p* is α-L-*threo*-hex-4-enopyranosyluronate), ΔUA*p*(1→3)Gal*p*NAc4(or 6)S and ΔUA*p* OMe ester (1→3)Gal*p*NAc at neutral pH in water were determined to be 4387, 3409 and 2818 M^{-1} cm^{-1}, respectively. Each disaccharide was prepared through a range of concentrations, and the absorbance at 232 nm was measured. The slope of the graphs of absorbance measured versus molar concentration gave the absorbtivity coefficients.

2.4. Determination of the kinetic constants for chondroitin AC lyase acting on chondroitin *O*-methyl ester

The Michaelis–Menten constants (K_m) and the maximum velocities (V_{max}) of chondroitin AC lyase were measured for three substrates: CS (from bovine traechea), C-OMe, and chondroitin (prepared by treating C-OMe with 0.1 M aqueous NaOH at room temperature for 48 h, followed by neutralization with hydrochloric acid and dialysis). Each substrate was used at seven concentrations (5, 10, 20, 40, 60, 80, 100 μM) in 50 mM Tris–HCl–sodium acetate buffer (pH 8.0). Chondroitin AC lyase (10 mU) was added, and the reaction was incubated at 37 °C. Beginning at initiation of the enzymatic reaction, the change in absorbance at 232 nm was measured over 3 min, and the linear region corresponding to the 10% of total depolymerization was used to calculate initial velocity of the reaction. The initial velocity determinations at different concentrations were inserted into Michaelis–Menten equation to determine V_{max} and K_m of the enzyme for each substrate pair.

3. Results

Chondroitin methyl ester was prepared in good yield (~80%) from CS. Analysis of the enzymatic depolymerization products showed >70% of the carboxyl groups had been methylated and <5% sulfo group per disaccharide unit remained. Chondroitin AC lyase digestion of C–OMe resulted in formation of disaccharides ~60%, tetrasaccharides ~20%, and higher oligosaccharides ~20%. ^1H NMR (Table 1) and ^1H–^1H COSY NMR (Fig. 2) analyses (collected on a Bruker AMX-600 MHz instrument in D_2O, with a chemical shift value of 4.76 for HOD) and high-resolution ESI mass spectrometric analysis (HR ESIMS: *m*/*z* 416.1148 $[M+Na]^+$; Calcd *m*/*z* 416.1169 $[M+Na]^+$) of the major fraction obtained on size-exclusion chromatography revealed that it was a disaccharide having the

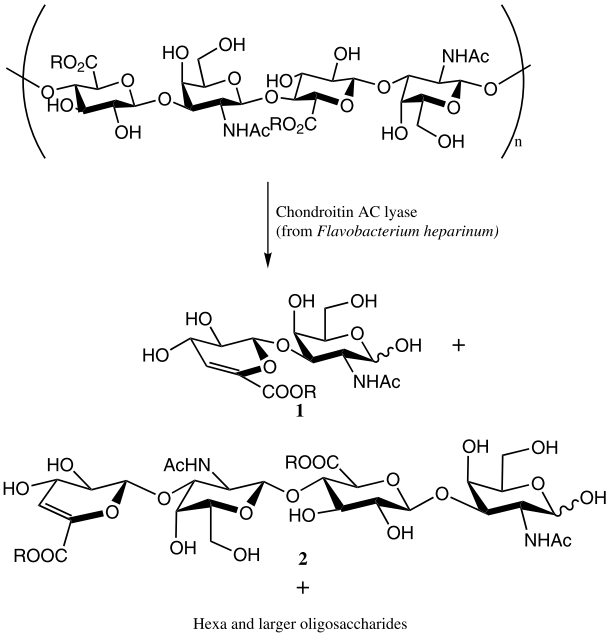

Fig. 1. Enzymatic depolymerization of chondroitin *O*-methyl ester. R = CH_3 or H.

Table 1
^{1}H NMR chemical shifts of ΔUA*p* *O*-methyl ester (1→4) Gal*p*NAc

	Proton	^{1}H Chemical shift (ppm)	
		α	β
ΔUA*p*	H-1	5.30	5.25
	H-2	3.84	3.84
	H-3	4.13	4.13
	H-4	6.25	6.25
	H-7(methyl ester)	3.82	3.82
Gal*p*NAc	H-1	5.20	4.71
	H-2	4.27	3.96
	H-3	4.08	3.90
	H-4	4.14	4.08
	H-5	4.08	4.14
	H-6	3.65–3.75	3.65–3.75

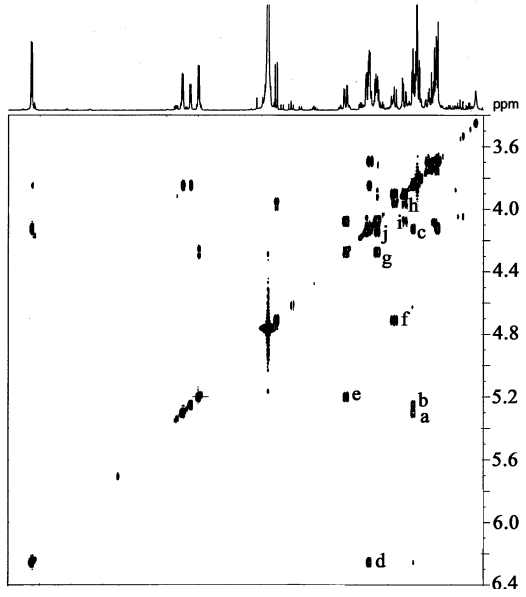

Fig. 2. NMR characterization of disaccharide using COSY spectrum with cross-peaks labeled: (a) αΔUA*p* H-1/H-2; (b) βΔUA*p* H-1/H-2; (c) α+βΔUA*p* H-2/H-3; (d) α+βΔUA*p* H-3/H-4; (e) αGal*p*NAc H-1/H-2; (f) βGal*p*NAc H-1/H-2; (g) αGal*p*NAc H-2/H-3; (h) βGal*p*NAc H-2/H-3; (i) βGal*p*NAc H-3/H-4; (j) αGal*p*NAc H-3/H-4, α+βGal*p*NAc H-4/H-5.

structure, ΔUA*p* *O*-methyl ester(1→4)Gal*p*NAc (40%). A small amount of disaccharide corresponding to ΔUA*p*(1→4)Gal*p*NAc (20%) (ESIMS: *m*/*z* 402.0 $[M+Na]^+$) was also obtained. Analysis of the tetrasaccharide showed it was a mixture of two components containing one or two methyl ester groups. The tetrasaccharide having a single methyl ester in the internal GlcA*p* residue (ESIMS: *m*/*z* 795.0 $[M+Na]^+$) was the major component with a small amount of dimethyl esterified tetrasaccharide (ESIMS: *m*/*z* 809.0 $[M+Na]^+$) also present. Analysis of the hexasaccharide showed it to be a complex mixture with both GlcA*p* and GlcA*p* OMe ester residues. The major hexasaccharide product was methyl esterified in two of the three GlcA*p* residues (ESIMS: *m*/*z* 1188.0 $[M+Na]^+$), and a minor hexasaccharide was observed that was fully methyl esterified (ESIMS: *m*/*z* 1202.0 $[M+Na]^+$). The isolation of the disaccharide methyl ester provides compelling proof of the ability of chondroitin AC lyase to act on the unnatural substrate, C-OMe. Neither the exolytic chondroitin AC lyase from *Arthrobacter aurescens* (EC 4.2.2.5) (Sigma) nor chondroitin ABC lyase from *Proteus vulgaris* (EC 4.2.2.4) (Sigma) acted on the C-OMe substrate under their optimal reaction conditions.[9] The kinetic parameters of the endolytic chondroitin AC lyase from *Flavobacterium heparinum*, acting on C-OMe, CS and chondroitin were determined and are presented in Table 2.

4. Discussion

The catalytic mechanism of chondroitin AC lyase has been proposed to involve general acid–base type catalysis in which a general base abstracts the proton from C-5 of the uronic acid, generating an enolate anion intermediate, then a general acid donates a proton to the 4-*O* glycosidic bond to enhance β-elimination[9,10] (Fig. 3). Recently, it has been suggested that this catalytic mechanism is stepwise[11] requiring the stabilization of the doubly charged enolic intermediate (one negative charge on the carboxylate and one at C-5). This might be achieved with a positively charged amino acid residue at the active site binding to the carboxylate group or through binding of a metal cation.[10,12,13] Although, in chemical terms, abstraction of an acidic proton at the α-position of the methyl ester is expected, the esterification of the carboxylate group in C-OMe might alter the interaction of anion-stabilizing elements in an enzymatic reaction, adversely impacting catalysis. Kinetic studies show that the K_m on C-OMe is comparable to that for CS-A and lower than that observed on chondroitin

Table 2
Kinetic studies on chondroitin AC lyase from *Flavobacterium heparinum*

Substrate	Average MW (g mol^{-1})	K_m (M)	V_{max} [μmol/(min mg)]
CS A	15,000	7.0	2.0
Chondroitin	10,000	63.0	3.3
C-OMe	10,000	12.0	0.3

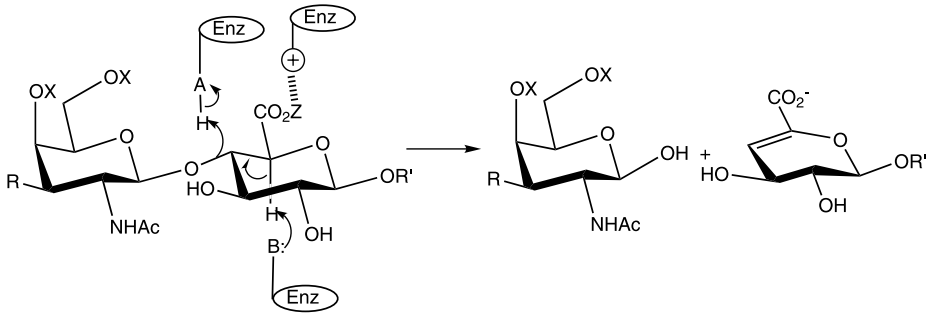

Fig. 3. Eliminative cleavage mechanism for chondroitin AC lyase. R = GlcA*p*; R′ = Gal*p*NAc; X = H or SO_3^- (where A = an acidic group on the enzyme capable of protonating the glycosidic oxygen; B = a basic residue capable of removing the acidic proton from the C-5 of the GlcA*p* residue; and + a positive amino acid residue or a metal ion capable of favorably interacting with (stabilizing) the negative charge formed at the carboxyl group (where Z = H) or unfavorably interacting with the carboxyl methyl ester (where $Z = CH_3$).

(Table 2). In contrast, the V_{max} on C-OMe is significantly lower than on CS-A or chondroitin suggesting that the binding step is less adversely impacted than catalytic step by methylation of the carboxyl group. The low K_m observed for C-OMe (comparable to CS-A) might be ascribed to the contribution of hydrophobic interactions between the methyl ester of the C-OMe and the enzyme, replacing ionic interactions lost through the desulfonation and methyl esterification of the substrate. Residual free carboxylate groups flanking the methyl ester in C-OMe might also be helpful in binding the enzyme.

Studies using site-directed mutagenesis and X-ray structures of various enzyme–oligosaccharide complexes suggest that the catalytic residues of Chondroitin AC lyase include His225, Tyr234, Arg288 and Glu371.[14,15] Future studies using oligosaccharides obtained by the depolymerization of chondroitin *O*-methyl ester may help clarify whether this unnatural substrate binds in the same orientation and involves the same catalytic residues.

Acknowledgements

We thank John Snyder for his help in 2D NMR instrumentation. We also thank Dr Nathalie Karst and Tasneem Islam for their helpful discussion.

References

1. Hrmova, M.; Fincher, G. B. *Plant Mol. Biol.* **2001**, *47*, 73–91.
2. Papanikolau, Y.; Prag, G.; Tavlas, G.; Vorgias, C. E.; Oppenheim, A. B.; Petratos, K. *Biochemistry* **2001**, *40*, 11338–11343.
3. Gu, K.; Linhardt, R. J.; Laliberte, M.; Gu, K.; Zimmermann, J. *Biochem. J.* **1995**, *312*, 569–577.
4. Jandik, K. A.; Gu, K.; Linhardt, R. J. *Glycobiology* **1994**, *4*, 289–296.
5. Hiyama, K.; Okada, S. *J. Biochem.* **1976**, *80*, 1201–1207.
6. Gu, K.; Liu, J.; Pervin, A.; Linhardt, R. J. *Carbohydr. Res.* **1993**, *244*, 369–377.
7. Kantor, T. G.; Schubert, M. *J. Am. Chem. Soc.* **1956**, *79*, 152–153.
8. Mucci, A.; Schenetti, L.; Volpi, N. *Carbohydr. Polym.* **2000**, *41*, 37–45.
9. Hernáiz, M. J.; Linhardt, R. J. *Methods Mol. Biol.* **2001**, *171*, 363–371.
10. Gacesa, P. *FEBS Lett.* **1987**, *212*, 199–202.
11. Rye, C. S.; Withers, S. G. *J. Am. Chem. Soc.* **2002**, *124*, 9756–9767.
12. Gerlt, J. A.; Gassman, P. J. *J. Am. Chem. Soc.* **1992**, *114*, 5928–5934.
13. Guthrie, J. P.; Kluger, R. *J. Am. Chem. Soc.* **1993**, *115*, 11569–11572.
14. Huang, W.; Boju, L.; Tkalec, L.; Su, H.; Yang, H.; Gunay, N. S.; Linhardt, R. J.; Kim, Y. S.; Matte, A.; Cygler, M. *Biochemistry* **2001**, *40*, 2359–2372.
15. Capila, I.; Wu, Y.; Rethwisch, D. W.; Matte, A.; Cygler, M.; Linhardt, R. J. *Biochim. Biophys. Acta* **2002**, *1597*, 260–270.