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Note

Comparison of the activity of two chondroitin AC lyases on dermatan sulfate

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Chondroitin sulfates are widely distributed in human and animal tissues and are the main constituents of cartilage[1]. The two major isomeric chondroitin sulfates, A (ChS-A) and C (ChS-C) are isolated as glycosaminoglycan from the proteoglycans present in tissues[2]. Structural investigations have shown that ChS-A and ChS-C both are co-polymers of D-glucuronic acid and sulfated 2-acetamido-2-deoxy-D-galactose. ChS-A primarily contains 4-sulfated 2-acetamido-2-deoxy-D-galactose residues, while ChS-C primarily contains 6-sulfated 2-acetamido-2-deoxy-D-galactose residues. Dermatan sulfate (DS) is a related glycosaminoglycan, often called chondroitin sulfate B (ChS-B), composed of 4-sulfated 2-acetamido-2-deoxy-D-galactose residues. DS differs from ChS-A and ChS-C since its primary uronic acid residue is L-iduronic acid instead of D-glucuronic acid. Despite these structural differences ChS-A, ChS-C, and DS also contain the minor uronic acid C-5 epimer (L-iduronic acid in ChS-A and ChS-C and D-glucuronic acid in DS) in their structures.

The chondroitin lyases depolymerize the ChS-A, ChS-C, and DS by an elimination mechanism into oligosaccharides containing a $\Delta_{4,5}$-unsaturated uronic acid residue at the nonreducing end[3,4] (Fig. 1). This residue exhibits an absorbance maximum at 232 nm permitting the detection of the oligosaccharide products of the chondroitin lyases using UV spectroscopy. The chondroitin lyases include ABC, AC, B, and C lyases and are microbial enzymes produced by the using ChS or DS as an inducer[3,4]. Two different chondroitin AC lyases are commercially available. Chondroitin AC lyase I (AC Flavo) is prepared from *Flavobacterium heparinum* and chondroitin AC lyase II (AC Arthro) is prepared from *Arthrobacter aurescens*. *F. heparinum* also contains a chondroitin B lyase while *A. aurescens* does not[3]. Other bacteria also reportedly produce chondroitin AC lyase including:

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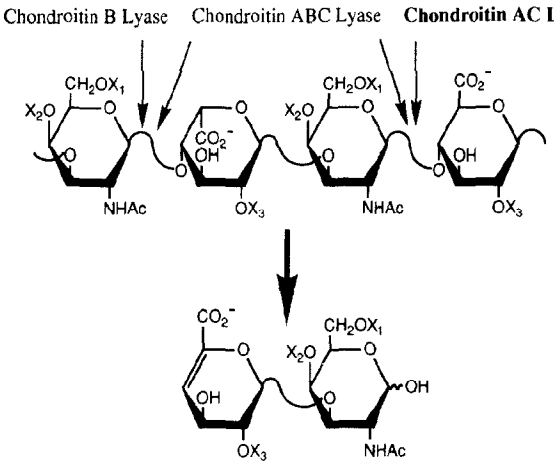

Fig. 1. A tetrasaccharide sequence common to ChS-A, ChS-C, and DS is shown. Arrows mark the sites in this sequence at which chondroitin ABC lyase, chondroitin B lyase, and chondroitin AC lyase can cleave. In this tetrasaccharide sequence, the first disaccharide residue (on the left) where $X_2 = SO_3$ and $X_1 = X_3 = H$ represents the major disaccharide repeating unit in DS. The second disaccharide residue (on the right) represents the major disaccharide repeating units in ChS-A and ChS-C where $X_2 = SO_3$, $X_1 = X_3 = H$ and $X_1 = SO_3$, $X_2 = X_3 = H$, respectively. The eight possible disaccharide products formed enzymically from these glycosaminoglycans are also shown: 1, $X_1 = X_2 = X_3 = H$; 2, $X_1 = X_3 = H$, $X_2 = SO_3^-$; 3, $X_2 = X_3 = H$, $X_1 = SO_3^-$; 4, $X_1 = X_2 = H$, $X_3 = SO_3^-$; 5, $X_1 = H$, $X_2 = X_3 = SO_3^-$; 6, $X_2 = H$, $X_1 = X_3 = SO_3^-$; 7, $X_3 = H$, $X_1 = X_2 = SO_3^-$; and 8, $X_1 = X_2 = X_3 = SO_3^-$.

Proteus vulgaris, Bacteroides thetaiotaomicron, and *Pseudomonas fluorescens*[5]. The specificity of a chondroitin AC lyase requires that it cleaves the $1 \rightarrow 4$ glycosidic linkage between 4- or 6-sulfated 2-acetamido-2-deoxy-D-galactose residues and D-glucuronic acid residues (Fig. 1). Previous investigators reported differences in the specificity of AC Arthro and AC Flavo[5–9]. The $1 \rightarrow 4$ glycosidic linkages between 4- or 6-sulfated 2-acetamido-2-deoxy-D-galactose residues and D-glucuronic acid residues found in DS were reportedly cleaved by AC Flavo but not by AC Arthro[8,9]. These reports conflicted with our observation that AC Arthro acted on DS samples prepared from various tissues[10].

We purified the chondroitin AC I lyase from *Flavobacterium heparinum* (AC Flavo) to homogeneity as assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE). Although *F. heparinum* also contains chondroitin B lyase, this contaminating enzyme was easily separated from AC Flavo using hydroxylapatite high-performance liquid chromatography (HPLC). A comparative study of AC Flavo prepared by our group with commercially obtained AC Flavo and AC Arthro was undertaken. Because these commercial enzyme preparations contain bovine serum albumin as an additive to protect activity, their purity cannot be assessed by SDS–PAGE. The information listed on the manufacturer's data sheets indicate that these enzymes are catalytically pure[9]. Chondroitin sulfates ChS-A, ChS-C, and DS (100 μg each) were depolymerized with the two preparations of AC Flavo and AC Arthro (15 mU) in 5 mM sodium phosphate buffer

containing 200 mM sodium chloride at pH 7.15. When ChS-A or ChS-C were treated with either of the two AC Flavo preparations or AC Arthro, oligosaccharide products containing unsaturated $\Delta_{4,5}$-uronic acid residues at the nonreducing end were observed by their absorbance at 232 (ϵ_M = 5000–6000 cm^{-1}). When DS was treated with AC Flavo and AC Arthro, a small increase in absorbance at 232 nm could also be observed. This suggested either that both AC Flavo and AC Arthro cleave a small number of sequences in DS, or that the DS preparations studied contained small contaminations of ChS-A or ChS-C glycosaminoglycans on which these enzymes could act. Since *F. heparinum* also produces a chondroitin B lyase, the results obtained for AC Flavo might also be explained by a minor B lyase contaminant capable of acting on DS. To distinguish between these possibilities, the oligosaccharide products were analyzed by strong anion-exchange (SAX)-HPLC and gradient PAGE.

SAX-HPLC analysis of oligosaccharide products.—When AC Arthro and AC Flavo treated glycosaminoglycans were analyzed by SAX-HPLC, two major peaks could be observed corresponding to disaccharides ΔUA*p*-(1 → 3)-β-D-GalN*p*Ac4S (**2**) and ΔUA*p*-(1 → 3)-β-D-GalN*p*Ac6S (**3**). (Where ΔUA*p* is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid, GalN*p* is 2-amino-2-deoxy-D-galactose, Ac is acetate, and *S* is sulfate.) When ChS-A was digested with AC Flavo or AC Arthro, the ΔUA*p*-(1 → 3)-β-D-GalN*p*Ac4S (**2**) was the major product while when ChS-C was digested with these enzymes ΔUA*p*-(1 → 3)-β-D-GalN*p*Ac6S (**3**) was the major product (Table I). Peaks corresponding to these disaccharides in the SAX-HPLC chromatograms were identified by co-injection of commercially available disaccharide standards. Quantification was based on relative peak area and is

TABLE I

Quantitative disaccharide analysis of ChS-A, ChS-C, and DS treated with chondroitin AC I and AC II lyases

Enzyme	Substrate	Disaccharides (mol%) [a]	
		2 [b]	3 [b]
AC Flavo [c]	ChS-A	94	3
	DS	n.d. [d,e]	n.d. [d,e]
	ChS-C	15	82
AC Flavo [f]	ChS-A	90	7
	DS	n.d. [d,e]	n.d. [d,e]
	Chs-C	10	89
AC Arthro [c]	ChS-A	81	4
	DS	n.d. [d,e]	n.d. [d,e]
	ChS-C	17	83

[a] The percentage of the total peak area corresponding to each disaccharide peak was used to calculate the mol%. [b] See Fig. 1 for disaccharide structure. [c] Obtained from Seikagaku America. [d] n.d. = not detected by SAX-HPLC. [e] CZE analysis confirmed that dermatan sulfate treated with chondroitin AC I and AC II lyases resulted in small amounts of disaccharide products. [f] AC Flavo was prepared in our laboratory from *Flavobacterium heparinum*.

accurate to 5–10%. Thus, the disaccharide composition afforded by each of the enzyme preparations on a given substrate did not differ significantly. We did not observe any disaccharide peaks when the AC Arthro or AC Flavo were used to digest DS samples. These negative results could be due to the failure of AC Arthro and AC Flavo to act on DS or because the amount of disaccharide products are insufficient to be detected using SAX-HPLC.

Analysis of these mixtures by capillary electrophoresis[11] demonstrated that very small amounts of the expected disaccharide products were present, but no higher oligosaccharide products were observed. Large, highly sulfated oligosaccharides typically give broad peaks with long retention times. Thus, capillary electrophoresis is not particularly sensitive for the analysis of large oligosaccharides[12].

PAGE mapping analysis of oligosaccharide products.—ChS-A, ChS-C, and DS depolymerized with AC Flavo and AC Arthro lyases were analyzed by gradient PAGE mapping[10,13,14]. Gradient PAGE is a high-resolution, high-sensitivity method for the qualitative analysis of acidic oligosaccharides (particularly large oligosaccharides). Complete disappearance of substrate or reduction in substrate molecular size (leading to faster migration) is observed for all the AC lyase-treated glycosaminoglycans (Fig. 2 lanes 2–4, 6–8, and 10–12). ChS-A and ChS-C (Fig. 2, lanes 2–4 and 6–8) were completely converted to products by AC Arthro and AC Flavo. PAGE analysis of AC lyase-treated ChS-C clearly shows the presence of disaccharide and tetrasaccharide products (Fig. 2, lanes 6–8). PAGE analysis of ChS-A, however, shows neither substrate nor product (Fig. 2, lanes 2–4). Analysis of individual disaccharides (Fig. 1) and similar tetrasaccharides, each containing 0–3 sulfate groups, indicate that alcian/silver staining visualizes only oligosaccharides having three or more sulfate groups (data not shown). Thus, the failure to stain lanes 2–4 suggests that ChS-A has been completely converted to oligosaccharides having < 3 sulfate groups. ChS-C is apparently more highly sulfated than ChS-A since it affords some disaccharide and tetrasaccharide products that contain more than three sulfate groups. The digestion of DS by both AC Arthro and AC Flavo was clearly demonstrated from the banding pattern of oligosaccharide products on the gel (Fig. 1 lanes 10–12). PAGE analysis clearly showed that the two preparations of AC Flavo act on DS giving identical banding patterns. The AC Arthro acting on DS gave a banding pattern similar to that of AC Flavo preparations, but it converted a slightly lower percentage of DS to oligosaccharide products (Fig. 2, lane 10). The doublet of bands observed at each dp (Fig. 2, lanes 10–12) are separated by ~ 100 mass units and probably correspond to a difference of a single sulfate group[15]. Similar results were obtained when DS from Seikagaku (not shown) was used in place of the NIH standard DS (shown in Fig. 2). These gradient PAGE results confirm that DS is a substrate for the AC Arthro lyase contrary to previous reports[8,9].

Both AC Arthro and AC Flavo are inhibited by DS. AC Flavo acts on ChS-C ($K_m = \sim 2\ \mu M$) and is inhibited by DS ($K_i = \sim 0.2\ \mu M$) (based on a molecular weight of 20000 for both polymeric substrates)[5]. AC Arthro acts on ChS-C

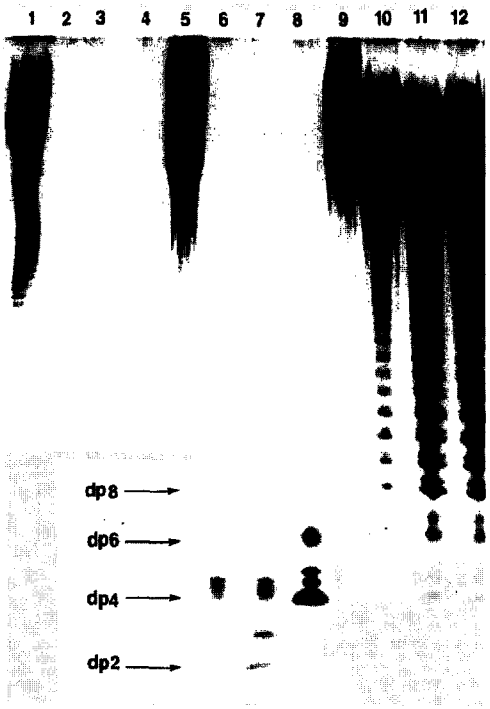

Fig. 2. Gradient PAGE analysis of ChS-A, ChS-B, and DS before and after treatment with chondroitin AC I and AC II lyases. Lane 1, untreated ChS-A; lane 2, AC Arthro treated ChS-A; lane 3, AC Flavo (commercial) treated ChS-A; lane 4, AC Flavo (our laboratory) treated ChS-A; lane 5, untreated ChS-C; lane 6, AC Arthro treated ChS-C; lane 7, AC Flavo (commercial) treated ChS-C; lane 8, AC Flavo (our laboratory) treated ChS-C; lane 9, untreated DS; lane 10, AC Arthro treated DS; lane 11, AC Flavo (commercial) treated DS; lane 12, AC Flavo (our laboratory) treated DS. Degree of polymerization (dp) is estimated based on migration with respect to heparin oligosaccharide standards[10].

($K_m = \sim 100\ \mu M$) and is inhibited by DS ($K_i = \sim 60\ \mu M$)[9]. Preliminary studies in our laboratory confirm that the action of both AC Arthro and AC Flavo on ChS-C is inhibited by DS[10]. The inhibition of these chondroitin AC lyases by DS (particularly sequences rich in L-iduronic acid) may be sensitive to the buffer system that is selected. Gradient PAGE analysis confirms that both chondroitin AC lyases catalyze the depolymerization of DS at the $1 \rightarrow 4$ glycosidic linkages between 4- or 6-sulfated 2-acetamido-2-deoxy-D-galactose and glucuronic acid when acting in 5 mM sodium phosphate pH 7.15 containing 200 mM sodium chloride. Previous studies that showed AC Arthro inactive towards DS used sodium acetate solution at pH 6.0 having an ionic strength of 70 mM. The inhibition of AC Arthro by DS is reportedly reduced in the presence of added salt[5,16]. When we examined Tris-acetate buffer (or sodium phosphate buffer in the absence of added sodium chloride), we found that the digestion of DS by AC Arthro as measured by gradient PAGE was unchanged. Thus, although the action

of both AC lyases on ChS-C is inhibited by DS, both AC lyases are capable of acting on DS.

Reports suggesting that DS was not a substrate for AC Arthro relied on HPLC disaccharide analysis[17,18] to follow enzymatic activity. As this study demonstrates, HPLC analysis is a particularly insensitive method for detection of the large oligosaccharide products afforded by the treatment of DS with AC lyases. Thus an inappropriate choice of analytical method was probably responsible for the failure of previous researchers[8,9] to observe the action of AC Arthro on DS.

Additional and interesting observations can be drawn from our reinvestigation of activity of chondroitin AC lyases on DS. The percent glucuronic acid in DS can be estimated by eq. *1*. The moles of product afforded using each lyase is calculated

$$\% \text{ glucuronic acid} = \frac{\text{mol products using AC Arthro}}{\text{mol products using AC Arthro} + \text{mol products using B Flavo}} \quad (1)$$

from absorbance at 232 nm[10]. From this calculation it was determined that the NIH standard DS contains 7% glucuronic acid and 93% iduronic acid. These percentages are consistent with ones obtained for other DS samples using both enzymic methods and ^{1}H NMR spectroscopy[10]. The distribution pattern of depolymerized DS showed oligosaccharides of various molecular sizes on the gradient PAGE gel. This product distribution suggests that the glucuronic acid residues within DS (representing 7% of the total uronic acid residues) are not clustered in a single region of the polymer chain[10]. Such a clustering would result in only disaccharide and large oligosaccharide products. Instead we observe a banding pattern corresponding to oligosaccharides ranging in size from a tetrasaccharide (dp 4) to dp > 30 suggesting that the glucuronic acid residues are randomly distributed throughout the glycosaminoglycan chain. Based on an average substrate molecular weight of 20000, the action of AC lyase on 7% (randomly distributed) of the $1 \rightarrow 4$ linkages would result in an average product molecular weight of 6000 (corresponding to dp ~ 12), which is consistent with the banding pattern observed in gradient PAGE. The random distribution of glucuronic acid containing sites is also supported by SAX-HPLC and CE experiments. When AC lyase treated DS is analyzed, we see no disaccharide peak by SAX-HPLC and only very minor amounts of disaccharides by CE analysis.

The results presented cannot be attributed to a ChS contamination of DS substrate since such a contaminant would afford primarily dissacharide and tetrasaccharide products not the higher oligosaccharide products observed. Nor could these results be attributed to the presence of a chondroitin B lyase contaminant in the AC Flavo preparation (AC Arthro cannot contain chondroitin B lyase since *A. aurescens* does not produce this enzyme[3]), since the action of chondroitin B lyase on DS affords primarily disaccharide and tetrasaccharide products.[20]

In conclusion, both AC Flavo and AC Arthro acts on DS to afford primarily higher oligosaccharide products (dp 8–30). These products are consistent with the

action of these chondroitin AC lyases at glucuronate containing sites randomly distributed within the DS polymer.

EXPERIMENTAL

Materials.—ChS-A, ChS-C, commercial chondroitin lyases, chondroitin AC I lyase, AC Flavo (EC 4.2.2.5 from *Flavobacterium heparinum*), chondroitin AC II lyase, AC Arthro (EC 4.2.2.5 from *Arthrobacter aurescens*), and chondroitin B lyase (no EC number, from *F. heparinum*) were from Seikagaku American, Inc., Rockville, MD. DS was from Seikagaku, and NIH Standard DS was prepared by Mathews and Cifonelli of the University of Chicago from porcine intestinal mucosa[19]. An alternative source of chondroitin AC I lyase was purified in our laboratory from *F. heparinum.* QAE-Sephadex A-50, ChS-A for fermentation, and protamine sulfate were from Sigma Chemical Co., St. Louis, MO.

SAX-HPLC was performed on dual constametric II pumps connected through a gradient mixer from LDC-Milton Roy, Riviera Beach, FL. A fixed-loop injector no. 7125 from Rheodyne, Cotati, CA and a variable-wavelength UV-5 detector from ISCO, Lincoln, NE, were used in the system. Gradient control and data collection were monitored using an Apple IIe microcomputer running chromatochart software from Interactive Microware, State College, PA. An analytical Spherisorb SAX column (0.46 × 25 cm, 5 μm particle size) was from Phase Separations, Inc., Norwalk, CT. UV spectroscopy was done on a Shimadzu UV-160 spectrophotometer. Unsaturated chondro-disaccharide (C-kit) and unsaturated dermato/hyaluro-disaccharide (D-kit), used as HPLC standards, were from Seikagaku. The purity of these disaccharides were confirmed by capillary electrophoresis[11] and SAX-HPLC[20].

Gradient PAGE analysis was performed on a 20 cm vertical slab gel unit (Protean™ II) was from Bio-Rad, Richmond, CA. The reagents used in electrophoresis were acrylamide and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) from Boehringer-Mannheim Corp., Indianapolis, IN; bis-*N′N′*-methylenebisacrylamide from International Biotechnologies Inc, New Haven, CT; ammonium persulfate from MCB Manufacturing Chemists, Cincinnati, OH; and silver nitrate from Fisher Chemical Co., Fairlawn, NJ.

Methods.—Preparation of chondroitin AC I lyase. Chondroitin AC I lyase was purified from *F. heparinum* by fermentation on defined medium in the presence of ChS-A[21,22]. Cell homogenization was carried out by sonication at 100 W using a 40% pulse mode for 5 min in an ice bath, and the supernatant was collected by centrifugation. Nucleic acids were removed by protamine sulfate precipitation and centrifugation at 10000*g* for 15 min at 4°C. Supernatant was passed through a QAE-Sephadex A-50 column in 50 mM sodium phosphate buffer, pH 6.8 at 4°C. Chondroitinase AC was further purified by hydroxylapatite HPLC and gel-permeation HPLC to remove a small amount of chondroitin B lyase[21,22]. The purified protein showed a single band on SDS-PAGE.

Digestion of chondroitin sulfate and dermatan sulfate with chondroitin lyases.

ChS-A, ChS-C and DS were digested by the chondroitin AC lyases using three different buffer systems and reaction conditions.

(*a*) Buffer system 1 was used to produce oligosaccharide products for gradient PAGE and SAX-HPLC analysis shown in Table I and Fig. 2. A 5-μL sample of ChS-A, ChS-C, and DS substrates (20 mg/mL) in polyethylene tubes were treated with 15 mU of chondroitin AC Arthro or AC Flavo lyases at 37°C overnight. The digestion was done in 35 μL of 5 mM sodium phosphate buffer containing 200 mM NaCl at pH 7.15. After overnight digestion aliquots were removed, 1 mL of 0.03 M HCl was added, and the absorbance was measured at 232 nm. A constant absorbance value in the presence of active enzyme demonstrated that the reaction was completed. Digestion was terminated by heating for 1 min at 100°C.

(*b*) Buffer system 2 was identical to buffer system 1 except that it contained no NaCl.

(*c*) Buffer system 3 is that described by the manufacturer[9] of AC Arthro lyase and was used only for comparison to buffer system 1. Samples (5 μL of ChS-A, ChS-C, and DS substrates (20 mg/mL) in polyethylene tubes were treated with 15 mU chondroitin AC Flavo or AC Arthro lyases at 37°C overnight. The digestion was done in 38 μL of 0.08 mM sodium acetate adjusted with acetic acid to pH 6.0 and containing 0.011% bovine serum albumin. The digestion was monitored and terminated as described above.

DS (100 μg) was treated with chondroitin B lyase (10 mU) in 50 μL of 100 mM Tris buffer and 30 mM sodium acetate buffer at pH 8.0. The digestion was incubated at 30°C for 10 h. The absorbance at 232 nm of the products formed using either B lyase or AC lyase was determined in 30 mM HCl and used in eq *1*.

Analysis of ChS and DS treated with AC lyases.—1. Gradient PAGE analysis. Both digested and undigested ChS-A, ChS-C, and DS samples were analyzed with the use of gradient polyacrylamide gel electrophoresis. Each sample (10 μL of 2 mg/mL) was dissolved in water and combined with an equal volume of 50% (w/w) sucrose solution containing traces amount of Bromophenol Blue and Phenol Red, loaded into a stacking gel of 5% (total acrylamide), and fractionated with a 12–22% linear gradient resolving gel[13,23]. Electrophoresis was performed at 400 V for 5–6 h. Bromophenol Blue and Phenol Red were used as indicators throughout the run, and the heat was dissipated using a refrigerated water circulator at 4°C. The gel was fixed with Alcian Blue dye and stained with silver nitrate solution[23].

2. SAX-HPLC analysis. The depolymerized chondroitin sulfates (1 μg) were injected onto an analytical SAX-HPLC column. Before injection, the column was pre-equilibrated with 0.05 M NaCl at pH 3.5. The samples are eluted with the use of a linear gradient of NaCl at pH 3.5 from 0.05–0.2 M over 1 h at a flow rate of 1.5 mL/min. The elution profile was monitored by the absorbance at 232 nm at 0.02 absorbance unit full scale. Between analyses, the column was washed with M NaCl for 10 min to remove the higher molecular weight oligosaccharides still bound. The disaccharides from the digested chondroitin sulfates A, B and C were identified by co-injection of ChS disaccharide standards.

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