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# Role of arginine 292 in the catalytic activity of chondroitin AC lyase from *Flavobacterium heparinum*

Ishan Capila [a], Yi Wu [b], David W. Rethwisch [b], Allan Matte [c], Miroslaw Cygler [c], Robert J. Linhardt [a,b,d,*]

[a] *Department of Medicinal and Natural Products Chemistry, University of Iowa, Iowa City, IA 52242, USA*
[b] *Department of Chemical and Biochemical Engineering, University of Iowa, Iowa City, IA 52242, USA*
[c] *Biotechnology Research Institute, 6100 Royalmount Avenue, Montreal, Quebec, Canada H4P 2R2*
[d] *Department of Chemistry, University of Iowa, Iowa City, IA 52242, USA*

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**Abstract**

Chondroitin AC lyase (chondroitinase EC 4.2.2.5), an eliminase from *Flavobacterium heparinum*, cleaves chondroitin sulfate glycosaminoglycans (GAGs) at 1,4 glycosidic linkages between *N*-acetylgalactosamine and glucuronic acid residues. Cleavage occurs through β-elimination in a random endolytic action pattern. Crystal structures of chondroitin AC lyase (wild type) complexed with oligosaccharides reveal a binding site within a narrow and shallow protein channel, suggesting several amino acids as candidates for the active site residues. Site-specific mutagenesis studies on residues within the active-site tunnel revealed that only the Arg to Ala 292 mutation (R292A) retained activity. Furthermore, structural data suggested that R292 was primarily involved in recognition of *N*-acetyl or *O*-sulfo moieties of galactosamine residues and did not directly participate in catalysis. The current study demonstrates that the R292A mutation affords ~ 10-fold higher $K_m$ values but no significant change in $V_{max}$, consistent with hypothesis that R292 is involved in binding the *O*-sulfo moiety of the saccharide residues. Change in chondroitin sulfate viscosity, as a function of its enzymatic cleavage, affords a shallower concave curve for the R292A mutant, suggesting its action pattern is neither purely random endolytic nor purely random exolytic. Product studies using gel electrophoresis confirm the altered action pattern of this mutant. Thus, these data suggest that the R292A mutation effectively reduces binding affinity, making it possible for the oligosaccharide chain, still bound after initial endolytic cleavage, to slide through the tunnel to the catalytic site for subsequent, processive, step-wise, exolytic cleavage. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Arginine 292; *Flavobacterium heparinum*; Chondroiotin AC lyase

## 1. Introduction

Glycosaminoglycans (GAGs) are polydisperse, sulfated copolymers of hexosamine and uronic acid residues isolated from a wide variety of animal tissues. GAGs from vertebrate sources have average molecular weights of 10,000–30,000 Da. Polysaccharide lyases (EC 4.2.2.-) are eliminases that cleave acidic polysaccharides, such as GAGs, at specific glycosidic linkages [1]. These enzymes include the heparin and chondroitin sulfate lyases that are isolated from *Flavobacterium heparinum*, *Arthrobacter aurescens* and *Proteus vulgaris* [2]. Bacterial polysaccharide lyases presumably serve a role in the initial catabolism of GAGs [1]. Heparin and chondroitin sulfate lyases have found many applications, including the determination of GAG structure [3], the preparation of new therapeutic agents from GAGs [4,5], the analysis of GAGs found in tissues and biological fluids [6,7], and the removal of GAGs from the circulation [8].

Degradation of GAGs occurs through two possible mechanisms, hydrolysis and β-elimination. Polysaccharide hydrolases are presently better understood than the lyases, and their reaction mechanisms are well characterized [9,10]. The polysaccharide lyases have been characterized with

*Abbreviations:* Chondroitin AC lyase (*F*), chondroitin (4- or 6-) sulfate lyase from *Flavobacterium*; Chondroitin AC lyase (*A*), chondroitin (4- or 6-) sulfate lyase from *Arthrobacter*; EDTA, ethylenediaminetetracetic acid; GAGs, glycosaminoglycans; O.D., optical density; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonylfluoride; SDS-PAGE, sodium dodecyl sulfate-PAGE; TEMED, *N,N,N′,N′* -tetramethylenediamine

* Corresponding author. Department of Chemistry, University of Iowa, Iowa City, IA 52242, USA. Tel.: +1-319-335-8790; fax: +1-319-335-6634.
*E-mail address:* robert-linhardt@uiowa.edu (R.J. Linhardt).

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respect to their substrate specificity and mode of action [11], but the reaction mechanism at the molecular level is relatively less understood. Polysaccharide lyases cleave the (1 → 4) bond on the nonreducing end of an uronic acid residue, affording an unsaturated product. A possible mechanism for the β-elimination reaction has been proposed [12]; however, the details of this mechanism in the enzyme setting have not been fully elucidated. A number of important studies have extensively examined the critical residues involved in binding [13–16] and catalysis [16–20] in the related heparin lyase family. Based on the structures of enzyme–substrate or product complexes, mechanisms have also been recently proposed for pectate lyase C [21], chondroitinase B [22], and hyaluronate lyase [23]; however, these require further validation.

Chondroitin AC lyase (EC 4.2.2.5) from *F. heparinum* (chondroitinase AC) is composed of two domains, with the catalytic machinery mainly residing within the N-terminal domain. The role and function of the C-terminal, β-sheet domain of chondroitinase AC is presently unknown. The enzyme has a molecular weight of 74 kDa and in mature form consists of 673 amino acids. Chondroitin AC lyase degrades chondroitin, chondroitin-4-sulfate (chondroitin sulfate A), chondroitin-6-sulfate (chondroitin sulfate C), and, to a lesser extent, hyaluronic acid. The mode of action of this enzyme on chondroitin substrates has been established as random endolytic [11]. Dermatan sulfate has been demonstrated to inhibit *F. heparinum* AC lyase ($K_i \sim 0.2$ μM) with respect to chondroitin-6-sulfate as substrate [24].

A number of bacterial hyaluronate lyases share significant homology to *F. heparinum* chondroitin AC lyase, having between 16% and 22% overall sequence identity [25]. Many of the identical residues conserved in the sequence alignment are found to map to a long cleft in *F. heparinum* chondroitin AC lyase, and several of these have been suggested to play a role in either substrate binding or catalysis. The crystal structures of *F. heparinum* chondroitin AC lyase complexes with bound dermatan sulfate hexasaccharide, dermatan sulfate tetrasaccharide, and hyaluronic acid tetrasaccharide have been refined at 2.0, 2.0, and 2.1 Å resolution, respectively [26]. These complexes reveal a binding site within a narrow and shallow protein channel instead of a large cleft on the surface of the protein as was originally expected [25]. Based on earlier studies and the structures of the chondroitinase AC–oligosaccharide complexes, His225, Tyr234, Arg288 and Arg292 were identified as the most likely candidates for the active site residues. Site-specific mutagenesis of these four residues within the active-site tunnel (His225Ala, Tyr234Phe, Arg288Ala, and Arg292Ala) revealed that only the Arg292Ala mutant enzyme retained activity on chondroitin sulfate [26]. Structural data suggests that Arg292 is primarily involved in recognition of *N*-acetyl and *O*-sulfo moieties of galactosamine residues, but does not directly participate in catalysis. The absence of activity with the remaining mutants is consistent with their role as key active site residues.

The purpose of the current study was to determine the effects of the Arg292Ala mutation of chondroitin AC lyase (*Flavobacterium*) on the enzyme kinetics and action pattern towards its putative natural substrate, chondroitin-6-sulfate.

## 2. Materials and methods

Chondroitin AC lyase (*F*) from *F. heparinum*, chondroitin-4-sulfate (chondroitin sulfate A; $M_r$avg = 25,000 Da) from whale cartilage and hyaluronic acid ($M_r$avg = 100,000 Da) from rooster comb, were obtained from Sigma Chemical Co. (St. Louis, MO). Chondroitin AC lyase (*A*) from *A. aurescens*, chondroitin-6-sulfate (chondroitin sulfate C; $M_r$avg = 25,000 Da) from shark cartilage and chondroitin (Na salt; $M_r$avg = 20,000 Da) were purchased from Seikagaku America (Rockville, MD). The reagents used in electrophoresis were acrylamide and *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED) and Alcian blue from Boehringer Mannheim (Indianapolis, IN), bis-*N*′,*N*′-methylenebisacrylamide from International Biotechnologies Inc. (New Haven, CT), and ammonium persulfate from MCB Manufacturing Chemists (Cincinnati, OH). Silver nitrate was from Fisher Chemical Co. (Fairlawn, NJ). SP-Sepharose Big Beads (100–300 μm) BioProcess media was from Pharmacia. Ceramic hydroxyapatite (20 μm bead diameter) was from American International Chemical Inc. (Natick, MA). Complete™ protease inhibitor cocktail tablets, as well as individual protease inhibitors, were obtained from Roche Diagnostics (Laval, Quebec, Canada). All DNA-modifying and restriction enzymes were purchased from either Amersham-Pharmacia or New-England Biolabs (Beverley, MA), and used according to the manufacturer's recommendations. All inorganics were reagent grade or better. Spectrophotometric measurements were carried out on a Shimadzu model UV-2101PC UV–Vis spectrophotometer, Shimadzu Corporation, (Kyoto, Japan). All viscometry measurements were made with a Cannon-Ubbelohde semi-microviscometer from Cannon Instrument Company (State College, PA). Gradient polyacrylamide gel electrophoresis (PAGE) analysis was performed on a 20 cm vertical slab gel (Protean™ II) from Bio-Rad, Richmond, CA.

### 2.1. Methods

#### 2.1.1. Preparation, expression and purification of chondroitin AC lyase (F) R292A mutant enzyme

The *F. heparinum* AC lyase gene with upstream Hep1 promoter was cloned as previously described [27]. For site-directed mutagenesis, the oligonucleotide primers used to introduce the Arg292Ala mutation had sequences 5′-TAGAAGGCCGCGGAGTAAGT**GCA**CCAGACATTC-TAAATAAAAAGGC-3′ and 5′-GCCTTTTTATTTA-GAATGTCTGG**TGC**ACTTACTCCGCGGCCTTCTA-3′ for the forward and reverse primers, respectively. Bases modified to obtain the desired point mutation are indicated

in bold, with those underlined indicating a silent mutation (Ser291) introduced to create an ApaL1 restriction site to be used as a diagnostic in screening. Constructs containing the mutated *cslA* gene fused with the *hepA* upstream region were introduced into *Escherichia coli* strain S17-1 and conjugated into *F. heparinum* to create strains containing the mutant genes [28]. These constructed strains were confirmed by their ability to grow on medium containing heparin as the sole source of carbon, and by their resistance to the antibiotic trimethoprim. In addition, the mutated codons were further confirmed by sequencing the mutated region amplified by polymerase chain reaction (PCR).

The Arg292Ala mutant strain was grown at 23 °C in minimal medium [28] containing 1% (w/v) semipurified heparin and 100 mg $l^{-1}$ trimethoprim antibiotic. Cells were harvested by centrifugation, resuspended in phosphate buffered saline to approximately 5 optical density (O.D.) 600 $ml^{-1}$ and lysed by sonication (45 s on, 45 s off, five cycles, 4 °C). The soluble protein fraction was isolated by centrifugation (10,000 × *g*, 4 °C, 10 min) and checked for lyase AC activity with chondroitin-4-sulfate as substrate. Expression of mutant lyase AC protein was confirmed by sodium dodecyl sulfate (SDS)-PAGE and Western blotting using polyclonal anti-lyase AC antibodies as previously described [27].

The AC lyase Arg292Ala mutant was purified as previously described [26]. Briefly, *F. heparinum* cells from 4 l of culture were resuspended in buffer (25 mM sodium phosphate, 100 mM NaCl pH 7) and lysed by high pressure homogenization (8000 Pa for three passes). Soluble proteins were obtained by centrifugation (10,000 × *g*, 4 °C, 30 min), protease inhibitors were added (0.5 mM of phenylmethylsulfonylfluoride (PMSF) or ethylenediaminetetracetic acid (EDTA)-free protease inhibitor cocktail tablets), and DNA was removed (1 mg/l DNAse I, 2 mM $MgCl_2$, 2–4 h, 4 °C). The protein supernatant was filtered through a 0.45 μm disk filter, diluted 1:4 (vol/vol) with 10 mM sodium phosphate buffer, pH 7 and loaded onto a SP-Sepharose Big Beads cation exchange column (1.6 × 35 cm), equilibrated with 10 mM sodium phosphate buffer, pH 7, attached to a Pharmacia FPLC system. After washing with three column volumes of equilibration buffer, bound proteins were eluted in batch with 2 column volumes of 25 mM sodium phosphate buffer, 150 mM NaCl pH 7.0 at a flow rate of 1.5 ml $min^{-1}$. Fractions containing lyase AC were pooled, diluted 1:1 (v/v) with 10 mM sodium phosphate buffer, pH 7.0 and applied to a ceramic hydroxyapatite column (1.6 × 11.5 cm) equilibrated with 10 mM sodium phosphate buffer pH 7. The column was washed with 3 column volumes of equilibration buffer, and bound proteins were eluted in a linear gradient over 12 column volumes of (10–50% v/v) 25 mM sodium phosphate buffer, 1 M NaCl, pH 7.0, followed by 2 column volumes with 100% (v/v) of the same buffer. Column fractions containing purified protein as judged by SDS-PAGE were pooled, desalted and the buffer changed to 20 mM Tris–HCl pH 8.0 by ultrafiltration using centriprep and centricon concentrators. The following protease inhibitors were added at the specified concentration: 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1μg/ml E64. Protein samples were stored at −20 °C. The mutant chondroitin AC lyases (*F*), used in this study, was homogenous; it showed no contaminating proteins on Western blot analysis using polyclonal anti-chondroitin AC lyase antibodies [26] and was sufficiently pure to be crystallized [26]. Furthermore, it should be noted that *F. heparinum* produces no other enzymes that can act on either chondroitin A or C that might interfere with these studies.

### *2.1.2. Determination of kinetic constants for enzyme–substrate pairs*

To a 1 ml quartz cuvette, 640 μl Tris–Cl/sodium acetate buffer (50 mM Tris, 60 mM sodium acetate, pH 8.0) was added. The cuvette was warmed to 37 °C (optimum temperature for chondroitin AC lyase activity) [30] in a temperature-controlled spectrophotometer. A 10 μl aliquot of chondroitin AC lyase ( ~ 10 mU enzyme) was thawed at room temperature and 90 μl of warm buffer from the cuvette in the spectrophotometer was added to it. The entire 100 μl buffer plus enzyme was transferred back immediately to the warm cuvette. The absorbance at 232 nm ($A_{232}$) was set to zero. The cuvette was removed from the spectrophotometer and 50 μl of a 20 mg/ml substrate solution was added to initiate reaction. The cuvette was sealed with parafilm and inverted once or twice to mix and then returned to the spectrophotometer. The absorbance at 232 nm was monitored as a function of time over 10 min. The enzyme activity (1 U = 1 μmol product formed/min) was calculated from the initial reaction rate ( ~ 5% reaction completion) using $\varepsilon = 3800\ M^{-1}$ for reaction products at pH 8. All of the chondroitin lyases were assayed [30] to determine their precise activity, before doing the enzyme kinetic experiments. The final absorbance value for total depolymerization of a substrate was determined and from this value an absorbance that represented 5–10% of complete substrate depolymerization was calculated (5–10% of the final $A_{232}$ value). The enzyme concentrations were then adjusted so that only 5–10% of total depolymerization would be observed at the end of a 5-min assay. The assay procedure was repeated using the optimized conditions at various substrate concentrations. Michaelis–Menten constants for chondroitin AC lyase (*F*) (wild type and R292A mutant) acting on various chondroitin substrates and hyaluronan were determined from these initial velocity measurements.

### *2.1.3. Viscosity and UV assays for chondroitin AC lyase digestion of chondroitin-6-sulfate*

Several small-scale digestions of the substrate were initially performed to ensure that the digestion reached completion within 180 min. Once the optimal ratio of substrate and enzyme was established, all of the remaining reactions were performed within the capillary viscometer. The viscometer was first cleaned, rinsed with water, then

methanol, and finally rinsed thoroughly with distilled, deionized water and air-dried. The viscometer was placed vertically in a holder within a water bath at a temperature regulated at 30 ± 0.1 °C. Assay solution was prepared that contained 8.33 mg/ml GAG substrate in a 250 mM sodium acetate, 2.5 mM calcium acetate, and 1 mM Tris–acetate buffer solution at pH 7.0. Assay solution (3 ml) was added to the viscometer and allowed to equilibrate for 30 min in a 30 °C water bath. Sufficient enzyme was then added (without diluting the substrate more than 1%) so that there was ~ 10 mU enzyme/mg substrate. The precise amount of enzyme used was adjusted based on the preliminary small-scale experiments and kinetic parameters determined previously. The digestion reaction immediately begins once the enzyme is added to the assay solution. The viscosity is measured as the time required for the level of the solution to fall between two marks above and below a bulb, divided by the time required for distilled water to fall between the same marks. This value is then multiplied by the viscosity of water at 30 °C (0.7975). The viscosity was measured prior to adding enzyme and ~ 15 times after the addition of enzyme over a 3-h period, or until the reaction was complete. At the midpoint of each viscosity measurement, two 25-μl aliquots were removed from the viscometer and quick frozen on dry ice. One aliquot was stored at − 70 °C for gradient PAGE analysis and to the second 2 ml of 30 mM hydrochloric acid was added to inactivate the enzyme and $A_{232}$ was measured.

#### 2.1.4. Gradient PAGE analysis on products of enzymatic depolymerization

Lyase treated GAGs were analyzed using gradient PAGE [31]. Each sample (5 μl of 8.33 mg/ml) was removed from the freezer, immediately boiled in a 100 °C water bath for 2 min and combined with an equal volume of 50% (w/w) sucrose containing trace amounts of bromophenol blue and phenol red. Samples were loaded into a stacking gel of 5% (total acrylamide) and subjected to electrophoresis for 5–6 h at 400 V on a 12–22% linear resolving gel. Bromophenol blue and phenol red were used as indicators, and throughout the run, the heat was dissipated by cooling the apparatus with recirculating refrigerated water at 4 °C. The gel was stained and fixed with Alcian blue in acetic acid, destained with water, and stained with silver nitrate solution [7]. Oligosaccharide standards prepared from partially depolymerized bovine lung heparin [31] were analyzed together with the digestion samples.

#### 2.1.5. Digitization and analysis of PAGE data

The average of molecular weight of each digestion mixture was estimated using gradient PAGE analysis [31]. The gels were scanned and digitized. The distance that each band moved into the gel in each lane was then determined using *UN-SCAN-IT 4.3* (Orem, UT). A standard curve was prepared based on the known molecular mass of the major oligosaccharide components in the samples of bovine lung heparin partially digested with heparin lyase I (lane b in each gel) [31]. *Origin 6.0* (Northampton, MA) was used to fit the final data.

## 3. Results and discussion

### 3.1. Kinetic parameters for enzyme–substrate pairs

The enzymatic activities of chondroitin AC lyase wild type and mutant (R292A) were determined to be 1.08 and 1.5 mU/μl, respectively. Based on final absorbance values, representing totally depolymerized samples, 10 mU of each enzyme was selected for use in the initial velocity kinetic studies to determine $K_m$ and $V_{max}$ for different enzyme substrate pairs. The change in absorbance at 232 nm ($\Delta A_{232}$) as the reaction proceeded was measured over 5 min and the linear region from 0.5 to 3.0 min was used to calculate initial velocity for different enzyme–substrate pairs at various substrate concentrations. Data were collected for the action of chondroitin AC lyase (*F*) (wild type and mutant R292A) acting on chondroitin sulfate A and C, chondroitin, and hyaluronic acid. The initial velocity determinations at different substrate concentrations were used to generate Lineweaver–Burke plots (Fig. 1) from which $K_m$ and $V_{max}$ data were determined (Table 1). The $K_m$ value for the wild-type enzyme acting on chondroitin sulfate C is comparable to that reported for chondroitin AC lyase purified from *F. heparinum* [29] as well as the value recently reported for recombinant chondroitin AC lyase [32]. The $K_m$ values with chondroitin sulfates A and C are ~ 10 times higher in the case of the mutant enzyme, while the $V_{max}$ is nearly identical for both wild-type and mutant enzymes (Table 1). This suggests that the decreased substrate binding affinity of the R292A mutant enzyme is primarily responsible for reducing its catalytic efficiency (i.e., $V_{max}/K_m$). These data are consistent with hypothesis that Arg292 is involved in binding the *N*-acetyl or *O*-sulfo moieties of the saccharide residues [26]. The absence of *O*-sulfonation in hyaluronic acid and the similarity in $k_{cat}/K_M$ (< 10-fold difference) with sulfated versus nonsulfated substrates (Table 1) suggest the primary function of Arg292 is in recognition of the *N*-acetyl group, with sulfate recognition being a less critical role.

### 3.2. Viscosity profiles of enzymatic depolymerization

A characteristic feature of dilute polymer solutions is that solution viscosity is considerably higher than that of the pure solvent. Moreover, viscosity ($\eta$) provides a measure of the viscosity averaged molecular weight of linear polymers. Indeed, the molecular weight of heparin measured by sedimentation is linearly related to its viscosity in solution [33]. Thus, the change in viscosity measures the change in polysaccharide chain length and affords a measure of the average molecular weight of GAGs, such as heparin, that

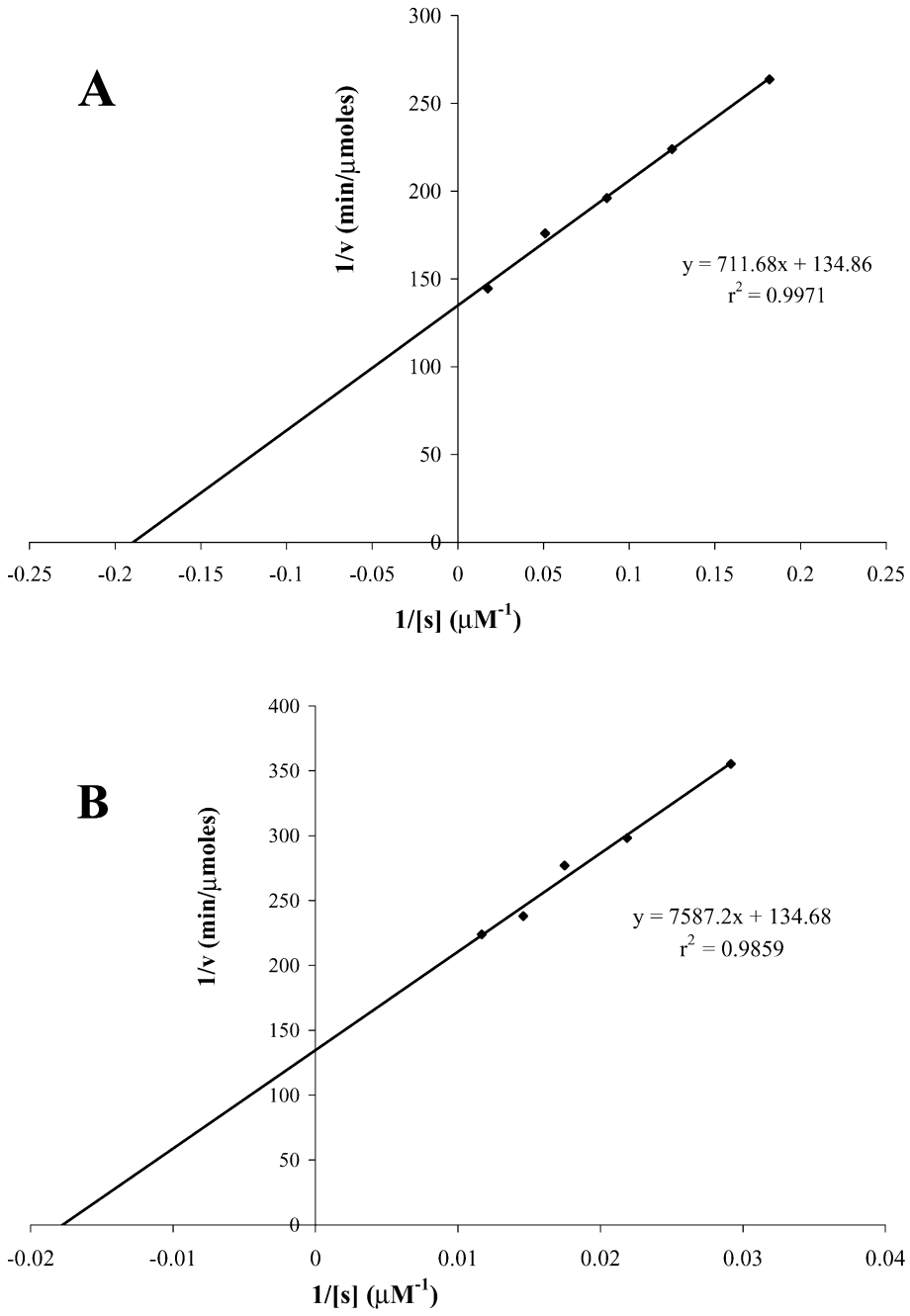


Fig. 1. Lineweaver–Burke plots used to estimate kinetic parameters ($K_m$ and $V_{max}$). (A) Chondroitin AC lyase ($F$) acting on chondroitin-6-sulfate and (B) chondroitin AC lyase (R292A mutant) acting on chondroitin-6-sulfate.

behave as Newtonian solutions at the dilute concentrations studied [34]. With each enzyme-catalyzed cleavage of a glycosidic linkage, an unsaturated uronic acid residue is formed at the nonreducing end of the product. Thus, the change in absorbance is proportional to the moles of product formed and the number of sites cleaved by the polysaccharide lyase. The depolymerization of chondroitin-6-sulfate by chondroitin AC lyases from *F. heparinum* (wild type), *F. heparinum* (mutant R292A), and from *A. aurescens* (obtained from Seikagaku America) were followed by measuring both $\Delta A_{232}$ and $\Delta\eta$.

Computer simulation studies have been used to study the action pattern of heparin lyase I acting on heparin [35]. Three theoretical plots of modeled weight average molecular weight (linearly proportional to viscosity) as a function of the moles of glycosidic linkages cleaved by heparin lyase I (linearly proportional to absorbance at 232 nm) have been described. A nonrandom exolytic action pattern, where the enzyme selects a single polysaccharide chain and removes one disaccharide unit at a time from the end of the chain, gave a linear relationship between decrease in molecular weight and number of sites cleaved. A random exolytic

Table 1
$K_m$ and $V_{max}$ for chondroitin AC lyase (wild type and R292A mutant) acting on various substrates

| Substrate | Chondroitin AC lyase (*F*) | | Chondroitin AC lyase (*F*) (R292A mutant) | |
|---|---|---|---|---|
| | $K_m$ (μM) | $V_{max}$ (μmol/min) | $K_m$ (μM) | $V_{max}$ (μmol/min) |
| Chondroitin-6-sulfate | 5.28 | 0.0074 | 56.3 | 0.0074 |
| Chondroitin-4-sulfate | 5.44 | 0.0085 | 65.1 | 0.0067 |
| Chondroitin | 32.2 | 0.015 | 119 | 0.014 |
| Hyaluronic acid | 2.86 | 0.0023 | 23.1 | 0.0083 |

action pattern, where the enzyme selects a polysaccharide chain, removes one disaccharide unit from the end of the chain, releases the chain and selects a new chain to repeat the process, gave a shallow concave curve (the rate of change in viscosity decreases throughout the reaction). A random endolytic action pattern, where the enzyme selects a polysaccharide chain, cuts any cleavable site within the chain, releases oligosaccharide product and the polysaccharide chain, and selects a new chain to repeat the process, gave a deep concave curve. Similar studies have also been reported on the chondroitin lyases acting on a variety of polysaccharide substrates [11].

Chondroitin AC lyase (*F*) acting on chondroitin-6-sulfate is known to exhibit a random endolytic action pattern [11] (Fig. 2). Based on the data obtained for this depolymerization, a plot of percent of initial viscosity versus percent of final absorbance was generated. The curve had a deep concave shape confirming the random endolytic behavior of this enzyme acting on chondroitin-6-sulfate. Chondroitin AC lyase (*A*) is known to exhibit a random exolytic action pattern on chondroitin-6-sulfate [11] (Fig. 2). This results in a change in viscosity as a function of number of sites cleaved showing a shallow concave curve [11] consistent with the expected behavior for this enzyme–substrate pair.

The viscosity profile for the R292A mutant enzyme acting on chondroitin-6-sulfate displayed a concave curve of intermediate character (Fig. 2). The shape of the curve suggests that the action pattern is neither purely random endolytic nor purely random exolytic. Thus, mutating the arginine at position 292 not only reduces substrate binding affinity (increased $K_m$) but also alters the action pattern of the enzyme on chondroitin-6-sulfate substrate.

### 3.3. Product profiles of enzymatic depolymerization

Aliquots taken from the viscometer at various time points during the enzymatic depolymerization were analyzed together by gradient PAGE. Gradient PAGE has been used to analyze the molecular weight and polydispersity of GAGs [31] and to study the size distribution of GAG-derived oligosaccharides [36]. The gradient PAGE analysis of chondroitin AC lyase (*F*) and chondroitin AC lyase (*A*) acting on chondroitin-6-sulfate is shown in Figs. 3 and 4, respectively. The rapid initial drop in molecular weight of chondroitin-6-sulfate associated with chondroitin AC lyase (*F*) activity confirmed its action pattern as random endolytic (Fig. 3) while chondroitin AC lyase (*A*) acting on chondroitin-6-sulfate showed a considerably slower decrease in molecular weight, again suggesting an exolytic action pattern (Fig. 4).

The product profile obtained for the R292A mutant acting on chondroitin-6-sulfate (Fig. 5) is again different from the previous two profiles. While a rapid initial drop in product size as seen from gel is similar to that observed in a random endolytic action pattern, the subsequent decrease is more gradual. This is suggestive of an exolytic action pattern. These observations support the viscosity data show-

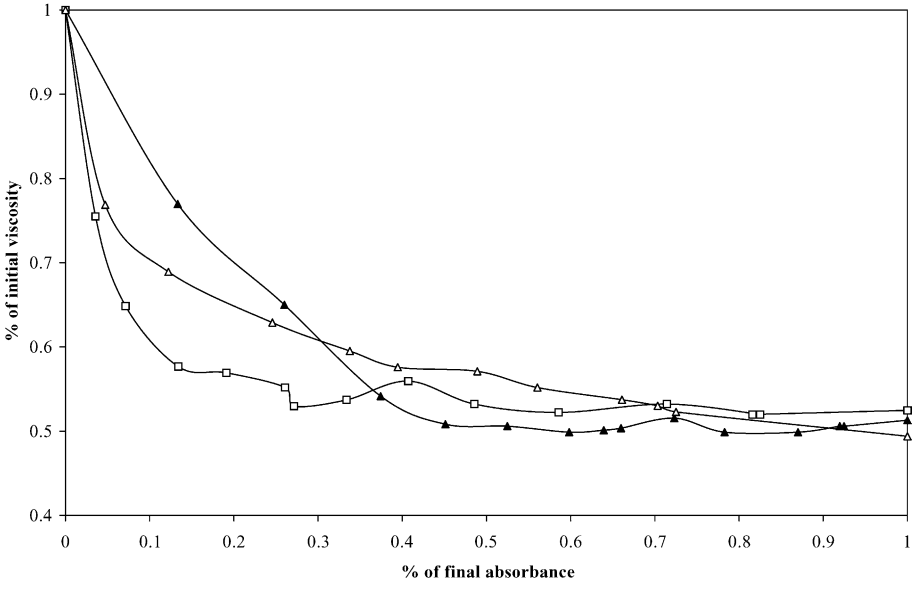


Fig. 2. Viscosity versus absorbance profiles. (□) Chondroitin AC lyase (*F*) endolytic; (△) chondroitin AC lyase (*A*) exolytic; (▲) chondroitin AC lyase (*F*) R292A mutant.


Fig. 3. Gradient PAGE analysis of chondroitin AC lyase ($F$) acting on chondroitin-6-sulfate. Lane a: tracking dyes (bromophenol blue and phenol red) and lane b: standard heparin oligosaccharide ladder to calibrate the gel. Samples taken from the viscometer containing chondroitin AC lyase ($F$) and chondroitin-6-sulfate are loaded in lanes c–m of the gel and correspond to 0%, 3%, 7%, 19%, 27%, 33%, 48%, 58%, 71%, 82%, and 100% reaction completion. The calibration curve for the standards was $y = -0.0040 \times x + 11.218$; $r^2 = 0.9991$ ($y$ = ln(molecular mass); $x$ = distance (mm)).


Fig. 4. Gradient PAGE analysis of chondroitin AC lyase (A) acting on chondroitin-6-sulfate. Lane a: tracking dyes; lane b: 4- to 6-mer heparin oligosaccharide mixture; lane c: standard heparin ladder; and lane d: 8-mer heparin oligosaccharide mixture. Samples taken from the viscometer at various time points of digestion are loaded in lanes e–n and correspond to 0%, 4%, 12%, 24%, 39%, 48%, 56%, 66%, 72%, and 100% reaction completion. The calibration curve for the standards was $y = -0.0039 \times x + 11.258$; $r^2 = 0.9982$.

ing that mutation of arginine 292 causes a change in the action pattern of this enzyme.

The gels were next scanned and digitized and the average of molecular weight of each digestion mixture was determined. Final data were fitted and the results are shown in Fig. 6B. It is difficult to acquire early time-points in these experiments due to the time required during each time-point to accurately measure viscosity. Previous studies, however, looking at change in molecular weight by PAGE alone clearly showed the expected product distribution for these early time points [11]. These data reconfirm that chondroitin AC lyase ($F$) acts on chondroitin-6-sulfate in a random endolytic action pattern while chondroitin AC lyase ($A$) acts in an exolytic pattern. It is noteworthy that at reaction completion, the gels in Figs. 3–5 show the presence of residual products larger than disaccharides. This may be the result of resistant sequences [37] or the reduced ability of lyases to act on low molecular weight substrates [38]. It should also be noted that, while the amount of these larger products looks substantial, quantitative interpretation of these data is complicated by the inability to visualize the disaccharide product by staining [37].

While the results from viscosity and gradient PAGE are not identical (Fig. 6A,B), they demonstrate that the depolymerization catalyzed by the mutant enzyme shows an action pattern that is intermediate between random exolytic and random endolytic. The difference here might result in part from SDS-PAGE measuring number-averaged molecular weight while viscosity measures the viscosity-averaged molecular weight. The viscosity-averaged molecular weight depends more heavily on high molecular weight chains [39].


Fig. 5. Gradient PAGE analysis of chondroitin AC lyase ($F$) R292A mutant acting on chondroitin-6-sulfate. Lane a: tracking dyes and lane b: heparin oligosaccharide ladder standard. Samples taken from the viscometer at various time points of digestion are loaded in lanes c–k and correspond to 0%, 13%, 30%, 37%, 52%, 63%, 78%, 92%, and 100% reaction completion. The calibration curve for the standards was $y = -0.0051 \times x + 11.360$; $r^2 = 0.9969$.

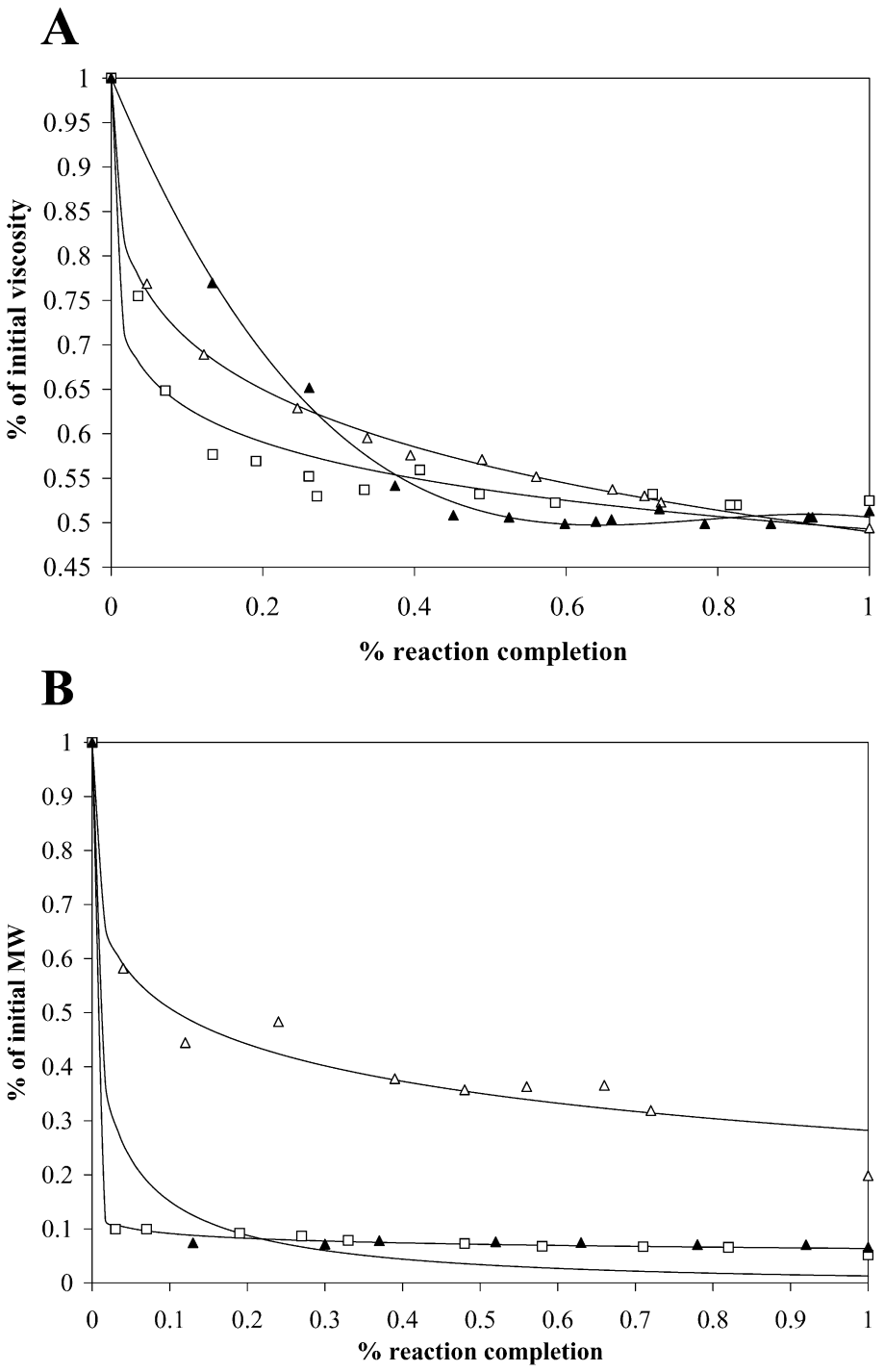


Fig. 6. (A) Percent initial viscosity versus percent reaction completion. (B) Percent initial MW versus percent reaction completion. (□) Chondroitin AC lyase (*F*) endolytic; (△) chondroitin AC lyase (*A*) exolytic; (▲) chondroitin AC lyase (*F*) R292A mutant.

### *3.4. Implications of structural changes in chondroitin AC lyase on action pattern*

The recently solved crystal structures of chondroitin AC lyase in complex with different oligosaccharides have shed some light on the active site of this enzyme and the role played by key residues in the active site [26]. The chondroitin AC lyase-oligosaccharide (dermatan sulfate hexasaccharide and chondroitin sulfate tetrasaccharide) complexes reveal binding at four subsites, −2, −1, +1, +2, within a narrow, shallow protein channel (Fig. 7). According to the nomenclature proposed by Davies et al. [40], the sugars are numbered starting from the cleavage point, with the positive numbers increasing toward the reducing end of the oligosaccharide, and the negative numbers decreasing toward the nonreducing end (nonreducing end..., −3, −2, −1, +1,

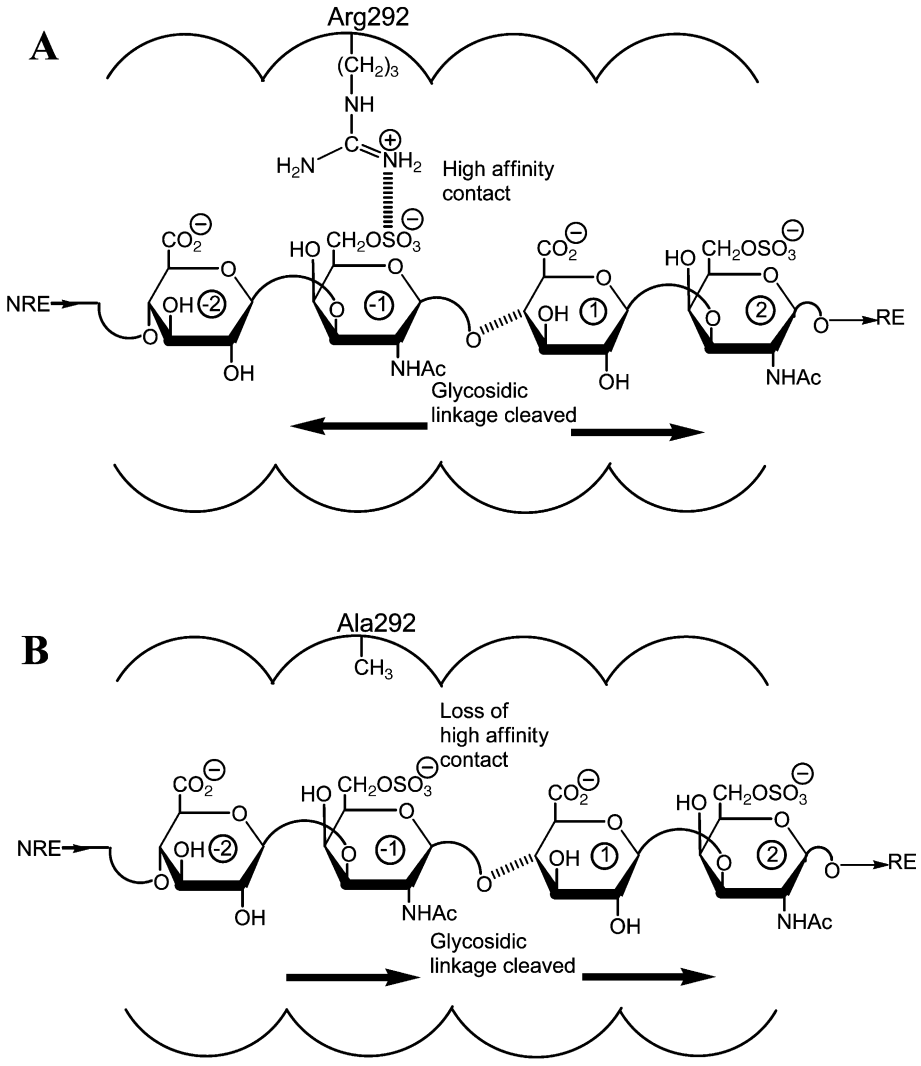


Fig. 7. A cartoon representation of the enzyme active site tunnel explaining the increase in $K_m$ and the switch in action pattern observed in the R292A mutant. (A) Chondroitin-6-sulfate tetrasaccharide bound in the active site of chondroitin AC lyase (*F*) wild type. The high affinity contact with Arg292 is shown. Bold arrows indicate the directions in which substrate and product leave after cleavage. (B) Chondroitin-6-sulfate tetrasaccharide bound in the active site of chondroitin AC lyase (*F*) R292A mutant. Loss of high affinity contact enables substrate to slide forward in the active site for further cleavage.

+2, +3, ... reducing end). The oligosaccharide is bound near the narrow end of the groove enclosed by the loops from the C-terminal domain. This association of the N- and C-terminal domains creates a short tunnel leading from the narrow end of the groove to the exterior of the enzyme. Rather unexpectedly, the dermatan sulfate hexasaccharide is found occupying this tunnel, with its reducing end protruding outside of the protein. To get into this position, the oligosaccharide either has to thread through the tunnel or the loops enclosing the tunnel have to reorient to let the oligosaccharide pass through.

Our data and previous studies on the mode of action of *F. heparinum* AC lyase show that this enzyme cleaves the chondroitin-6-sulfate chain in a random, endolytic fashion, affording a mixture of disaccharides, tetrasaccharides, and longer oligosaccharides as the final products [11]. This mode of action is surprising, given the observed tunnel-like binding sites for oligosaccharides observed in the co-crystal structure [26]. Many polysaccharide degrading enzymes having tunnel-like active sites, such as the cellobiohydrolases, exhibit an exolytic action pattern [41]. At least one enzyme, Cel48F cellulase from *Clostridium cellulolyticum*, having a 25 Å tunnel within its catalytic domain [42], shows a processive, endolytic action pattern. The behavior exhibited by these enzymes can be explained based on a tunnel containing flexible loops that can open periodically, allowing the GAG chain to slide in. The loops are then closed prior to catalysis.

A comparison of the chondroitin AC lyase–oligosaccharide complexes suggests that the oligosaccharides bind initially within the groove and slide through the tunnel, positioning at the −2 and −1 subsites at the enzyme active site. These subsites have been suggested to represent a high-affinity substrate recognition domain, while the +1 and +2

subsites represent a product release area [43]. One of the residues involved in binding the substrate in the high affinity −1 site is R292. It is possible that after cleavage, the oligosaccharides can become trapped in the high affinity −1 subsite and cannot overcome the energy barrier necessary to slide through the tunnel to the "+" subsites, preventing subsequent cleavage within the same oligosaccharide chain [26]. Thus, removing R292 effectively reduces the high affinity binding at subsite −1, making it possible for the oligosaccharide chain bound at the −1 subsite after cleavage to slide through the tunnel to the "+" subsites, leading to a subsequent, processive, step-wise, exolytic depolymerization within the same oligosaccharide chain. As a residue corresponding to Arg292 is conserved in the sequences of bacterial hyaluronate lyases [26], and has been shown to interact with the *N*-acetyl moiety of GlcNAc in the co-crystal structure of a hyaluronate lyase with hyaluronic acid disaccharide [44], it can be concluded that this residue is likely to have a similar function in the hyaluronate lyases as well.

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