Recombinant Expression, Purification, and Biochemical Characterization of Chondroitinase ABC II from Proteus vulgaris*5

Vikas Prabhakar, Ishan Capila, Venkataramanan Soundararajan, Rahul Raman, and Ram Sasisekharan

From the Department of Biological Engineering, Harvard-MIT Division of Health Sciences & Technology, and the Koch Institute for Integrative Cancer Research Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Chondroitin lyases (or chondroitinas) are a family of enzymes that depolymerize chondroitin sulfate (CS) and dermatan sulfate (DS) galactosaminoglycans, which have gained prominence as important players in central nervous system biology. Two distinct chondroitinase ABC enzymes, cABCII and cABCII, were identified in Proteus vulgaris. Recently, cABCII was cloned, recombinantly expressed, and extensively characterized structurally and biochemically. This study focuses on recombinant expression, purification, biochemical characterization, and understanding the structure-function relationship of cABCII. The biochemical parameters for optimal activity and kinetic parameters associated with processing of various CS and DS substrates were determined. The profile of products formed by action of cABCII on different substrates was compared with product profile of cABCII. A homology-based structural model of cABCII and its complexes with CS oligosaccharides was constructed. This structural model provided molecular insights into the experimentally observed differences in the product profile of cABCII as compared with that of cABCII. The critical active site residues involved in the catalytic activity of cABCII identified based on the structural model were validated using site-directed mutagenesis and kinetic characterization of the mutants. The development of such a contaminant-free cABCII enzyme provides additional tools to decode the biologically important structure-function relationship of CS and DS galactosaminoglycans and offers novel therapeutic strategies for recovery after central nervous system injury.

Chondroitin sulfate (CS) and dermatan sulfate (DS) belong to a family of galactosaminoglycans (GalAGs) known as galactosaminoglycans (GalAGs). GalAGs are linear polysaccharides of 1→4-linked repeating disaccharide units. The disaccharide units consist of a uronic acid (α-L-iduronic acid; IdoA) or β-D-glucuronic acid (GlcA) linked 1→3 to a β-D-N-acetyl-galactosamine (GalNAc). Each disaccharide unit can additionally possess variations in the form of sulfation at the 2-O and 3-O positions of the uronic acid and 4-O and 6-O positions of the GalNAc (1). GalAG depolymerizing chondroitinases have been classified broadly into three subfamilies. Chondroitinase AC depolymerizes chondroitin-4-sulfate (C4S) and chondroitin-6-sulfate (C6S), whereas chondroitinase B depolymerizes dermatan sulfate as its sole substrate (2–9). Chondroitinase ABC has the broadest substrate specificity in that it depolymerizes both CS and DS substrates (10–12). Chondroitinases have been employed in attempts to promote functional locomotor recovery following trauma to the central nervous system (13–15). The application of cABCII at the site of central nervous system injury is believed to prune CS chains from proteoglycans localized to the glial scar. The absence of these CS chains, inhibitory to axon regeneration, facilitates neural outgrowth and reconstruction of damaged tissue. However, the use of chondroitinas as therapeutics is limited by the lack of availability of pure and contaminant-free enzyme. Further, chondroitinase enzymes are often difficult to handle, because of thermal instability and spontaneous proteolysis, as reported by various groups (12, 16, 17).

Chondroitinase AC (cAC) and chondroitinase B from Pedobacter heparinus have been characterized extensively in terms of their enzymatic activity and substrate specificity. The crystal structure and co-crystal structure of chondroitinase B with its DS substrate together with site-directed mutagenesis of its putative active site residues provided detailed insights into its substrate processing and also revealed a calcium-dependent catalytic activity (3, 6, 8). The co-crystal structures of cAC with different CS and DS oligosaccharide substrate complexes led to the proposal of multiple scenarios in which the active site residues contributed to the catalytic activity of the enzyme (7). The crystal structure of another cAC from Arthrobacter aurescens and its co-crystal structure with CS substrates provided molecular insights into the active site of this enzyme and also its exolytic mode of action compared with the endolytic mode of cAC from P. heparinus (4).

Two distinct broad substrate specificity GalAG-degrading chondroitinase ABC lyases, cABCII and cABCIII, have been identified in Proteus vulgaris (12). In fact, the ability of the conventional enzyme known as “chondroitinase ABC” to catalyze...
the complete depolymerization of GalAG substrates to disaccharides is actually the result of the joint action of cABCI and cABCII. Recently, cABCI from *P. vulgaris* was cloned, recombinantly expressed, and characterized biochemically in terms of its active site and the role of divalent cations in processing CS and DS substrates (10, 18).

Building on our previous efforts, the present study describes the cloning, recombinant expression, and biochemical characterization of cABCII from *P. vulgaris*. Using an efficient system of *Escherichia coli*-mediated expression and purification, recombinant cABCII was obtained, and the conditions for its optimal activity were examined. The kinetic parameters and mode of action of the enzyme were characterized using CS and DS substrates. In contrast to cABCI, which is predominantly an endolytic enzyme, cABCII is an exolytic enzyme that cleaves the substrate only at the nonreducing end of the polysaccharide. Furthermore, cABCII was able to efficiently cleave a DS tetrasaccharide that was resistant to cleavage by cABCI (10).

The structural basis for the role of the active site residues in enzymatic activity and exolytic processing of cABCII was further elucidated using homology-based structural models of the enzyme-substrate complexes constructed using the cABCI and cAC crystal structures (4, 7, 11) as templates. The establishment of a contaminant-free recombinant cABCII and detailed characterization of its structure-function relationship enables the elucidation of the mode of action of the enzyme were characterized using CS and DS substrates (10, 18).

### EXPERIMENTAL PROCEDURES

**Isolating Chondroitinase ABC II from *P. vulgaris***—Genomic DNA was isolated from cultures of *P. vulgaris* (ATCC 6896) using a DNeasy purification kit (Qiagen). The primers were designed based on the available sequence of the gene for both the full-length and mature versions (19). Forward primers were designed so as to incorporate an NdeI restriction site; the reverse primer was designed to incorporate BamHI and XhoI restriction sites. This allowed cloning into a pET-28a vector.

A DNeasy purification kit (Qiagen) was used with plasmid DNA as template with an extension time of 3 min. The PCR product was ligated into a TOPO TA cloning kit (Invitrogen) and transformed into DH5a *E. coli* cells. Plasmid DNA was isolated, and the cABCII gene was excised by exploiting the NdeI and XhoI restriction sites. The excised gene was ligated into similarly digested pET28a. These ligation products were transformed into DH5a *E. coli* cells. Plasmid DNA isolated from the colonies was screened by restriction digestion for incorporation of the cABCII gene. Sequencing was also undertaken to confirm incorporation of the gene. *E. coli* cells (BL21(DE3)) were transformed with plasmid DNA for expression.

**Protein Preparation**—Recombinant cABCII was expressed in *E. coli* using an adapted version of a previous approach (5, 18). The pET28a expression system contains an inducible T7 promoter, as well as an N-terminal six-histidine tag for facile purification.

Cultures of Luria-Bertani broth containing kanamycin were inoculated, with 1 mM isopropyl-β-D-thiogalactopyranoside in mid-log phase (\(A_{600} \sim 0.8\)), and incubated at room temperature overnight. Centrifugation was used to harvest the cells, and the supernatant was discarded. The cell pellet was kept on ice and resuspended in 50 mM Tris, 250 mM NaCl, 10 mM imidazole, pH 7.9 (binding buffer), and then lysed by sonication. Soluble protein was collected by centrifugation at 15,000 × *g* for 15 min at 4 °C. The soluble lysate was sequentially filtered through a 0.8-μm membrane and then a 0.45-μm membrane. A 5-ml Hi-Trap Metal Chelate column (GE Healthcare) was prepared by charging with 200 mM NiSO₄ and treatment with binding buffer. The protein was loaded onto the column, washed with a buffer containing 100 mM Tris, 250 mM NaCl, and 50 mM imidazole, and eluted into a similar buffer with increased imidazole (250 mM). The six-histidine tag was removed using a thrombin capture kit (Novagen) as previously described (20). The presence and purity of the proteins was assessed by standard methods using SDS-polyacrylamide gel electrophoresis. Protein concentration was measured using the Bradford assay (Bio-Rad) with bovine serum albumin (Sigma) as a standard.

**Site-directed Mutagenesis**—A QuikChange site-directed mutagenesis kit (Stratagene) was used with plasmid DNA template to induce mutations in the cABCII clone. As previously described (18), plasmid denaturation and annealing of custom-crafted complementary oligonucleotide primers were used to introduce mutations. The primers were designed as follows (all primers are read in the 5’→3’ orientation): the 5’ I23T primer had the sequence GCC ATC AGC GTG TTG TGT I23T; and the 3’ extension primer had the sequence GCC ATC AGC GTG TTG I23T (reverse primer). It should be noted that for the truncated gene an additional methionine was introduced into the primer sequence to allow for translation of the protein product. This causes an increment in the numbering of the residues by one for the final protein product thus produced. PCR was run using genomic DNA as template with an extension time of 3 min. The PCR product was ligated into the pCR 4-TOPO vector using the product was ligated into the pCR 4-TOPO vector using the TOPO TA cloning kit (Invitrogen) and transformed into TOP10 E. coli cells. Plasmid DNA was isolated, and the cABCII gene was excised by exploiting the NdeI and XhoI restriction sites. The excised gene was ligated into similarly digested pET28a. These ligation products were transformed into DH5a *E. coli* cells. Plasmid DNA isolated from the colonies was sequenced by restriction digestion for incorporation of the cABCII gene. Sequencing was also undertaken to confirm incorporation of the gene. *E. coli* cells (BL21(DE3)) were transformed with plasmid DNA for expression.
Chondroitinase ABC II from P. vulgaris

Qiagen miniprep kit. Each clone was sequenced to confirm the presence of the desired mutation. Plasmid DNA was used to transform BL21 (DE3) E. coli. In addition to the mutants described above, other mutations in residues believed to be important for enzyme activity were also made. The primer sequences for each of the mutants are listed below. The H344A mutant primers have the sequences 5’-CGA GGA AGT GGT TAT CAA ATT ATT ACT GCT GTT GGT TAC CAA ACC-3’ and 5’-GGT TTT GTA ACC AAC AGC AGT AAT AAT TTG ATA ACC ACT TCC TGC-3’. The H453A mutant primers have the sequences 5’-CT GAT GGT TCT ATT TTT GCC CAT TCA CAA CAT TAC CCC GC-3’ and 5’-GC GGG GTA ATG TTG TGA ATG GGC AAA AAT AGA ACC ATC AG-3’.

The H454A mutant primers have the sequences 5’-C AAA TCT GAT GGT TCT ATT TTT CAC GCT TCA CAA CAT TAC CCC GC-3’ and 5’-GGC GGT CTA ATG TTG TGA AGC GTG AAA AAT AGA ACC ATC AGA TTT G-3’. The H457A mutant primers have the sequences 5’-CAC CAT TCA CAA GCT TAC CCC GCT TAT CAA GAT AAA GAT GC-3’ and 5’-GGC ATC TTT TTG AGC ATA AGC GGG GTA AGC TTG AGT-3’. The Y461A mutant primers have the sequences 5’-CA CAA CAT TAC CCC GCT GCT AAA GAT GCA TTT GGT GG-3’ and 5’-CC ACC AAA TGG ATC TTT AGC TTC ATG AGC AGC AGG GTA ATG TTG TG-3’. The R514A mutant primers have the sequences 5’-GTT GTG GTA TTA AGT GTA GGT C-3’ and 5’-G GCAA CAC ACC ACC ACC ACC ACC ACC-3’.

The E609A mutant primers have the sequences 5’-AGT GTT TAT CTT GTT GGT AAT GCT AGC TAT GAA AAT AAC AAC CGT-3’ and 5’-ACG GTT ATT TTC ATA ATC GTA AGC ATT ACC AAC AAG ATC ACA GC-3’.

Composition Analysis of Products from cABCII Processing of CS and DS Substrates—To investigate the composition of the final products of cABCII digestion, capillary electrophoresis was performed as previously described (6). Substrates included C6S from shark cartilage (Sigma), DS from porcine intestinal mucosa (Sigma), and C4S from sturgeon notochord (Seikagaku). Exhaustive overnight digestions of substrate by cABCII were analyzed using a Hewlett Packard three-dimensional capillary electrophoresis instrument with an extended path length cell. A voltage of 30 kV was applied using reverse polarity. Oligosaccharides were injected into the capillary using hydrodynamic pressure. The were detected using an ultraviolet detector set to 232 nm.

Biochemical Characterization of Chondroitinase ABC II Activity—Substrates (C6S and DS) were dissolved at 1 mg/ml in various buffers to determine the relative effects of pH, temperature, ionic strength, and sodium acetate concentration. The enzyme activity, as previously described (18). Chondroitin from shark cartilage (Seikagaku), hyaluronan from human umbilical cord (Sigma), heparin (Celsius), heparan sulfate (Celsius), and keratan sulfate (Sigma) were also used in these studies. For activity experiments, 2 μl of enzyme was placed in 248 μl of 50 mM Tris/HCl, pH 8.0, and reacted with 1 mg/ml substrate at 37 °C (0.25 mg/ml for hyaluronan). Product formation was monitored as an increase in absorbance at 232 nm as a function of time in a SpectraMax 190 (Molecular Devices) 96-well quartz format. For kinetic assays, 1 μl of enzyme (0.2–1.0 μg/μl) was added to 249 μl of a solution containing a GalAG substrate. Substrate concentration ranged from 0.1 to 5 mg/ml. Product formation was monitored by measuring the absorbance at 232 nm every 2 s. Kinetic parameters were determined using the initial rate of product formation and calculated as previously described based on Michaelis-Menten and Hanes techniques (18).

Modeling the Theoretical cABCII-Substrate Structural Complex—The crystal structure of cABC from P. vulgaris (Protein Data Bank code 1HNO) was used as a template to obtain the model of cABCII. Initial inspection of the sequence alignment between cABC and cABCII revealed that cABCII had multiple insertions of large loop regions as compared with cABC. In addition to cABC, the crystal structures of distinct cACs (which share the same structural fold with cABC) from P. heparinus (PhcAC; Protein Data Bank code 1CB8) and from A. aurescens (AacAC; Protein Data Bank code 1RW9) were also used to model the loop regions in cABCII that aligned with either of the cACs. The structural superimposition of cABC and these distinct cACs was obtained using combinatorial extension-Monte Carlo (CE-MC) multiple structural alignment tool (21) (supplemental Fig. S1). A homology-based structural model of cABCII was generated using the homology module of InsightII v2005 (Accelrys, San Diego, CA). The deletions in the modeled structure were closed by constrained minimization upon holding most of the structure rigid, except for regions close to the deletion site. This was followed by 300 iterations of steepest descent and 400 iterations of conjugate gradient minimization without including charges. The loops and side chains of all of the residues were then allowed to move freely by performing 500 iterations of steepest descent minimization. The refined structure was then subjected to 500 iterations of steepest descent minimization without including charges and 500 iterations of conjugate gradient minimization including charges to obtain the final predicted model of the cABCII enzyme. The final model of cABCII was validated using Whatif web-based interfaces and the Ramachandran plot explorer (supplemental Fig. S2).

A C4S Tetrasaccharide Substrate, (GlcA-GalNAc,4S)2, was docked into the putative active site of cABCII using the following approach. The SuperPose version 1.0 server (22) was used to superimpose the co-crystal structure of cAC-CS tetrasaccharide complex (Protein Data Bank code 1HMW) with the modeled cABCII structure. The CS tetrasaccharide in this cAC complex had a uronic acid with a Δ4,5 unsaturated linkage at the nonreducing end. The starting model of the C4S tetrasaccharide in the cABCII active site was therefore derived from the coordinates of a C4S hexasaccharide (Protein Data Bank code 1C4S), which was superimposed on the CS substrate in the cAC co-crystal structure. The enzyme-substrate complex was subject to minimization without charges with 400 steps of steepest descent and 600 steps of conjugate-gradient methods. This was followed by another 500 steps, each of steepest descent and conjugate-gradient methods with charges. To constrain the ring torsion angles to maintain the ring conformation of the tetrasaccharides during the process of energy minimization, a force constant of 7000 kcal/mol was utilized. To evaluate the exolytic versus endolytic propensity of cABCII in comparison...
with that of cABCI, an octasaccharide C4S substrate, (GlCAGalNAc4S)₄, was also docked in a similar fashion (described above) into putative active sites of cABCI and cABCII, respectively. The octasaccharide was generated from the coordinates of a C4S hexasaccharide (Protein Data Bank code 1C4S) by adding another, GlcA-GalNAc4S, to the reducing end of the hexasaccharide.

The viewer, builder, and discover modules of InsightII v2005 (Accelrys) were used for the visualization, structure building, and energy minimization, respectively. The AMBER force field (Amber95) provided with the Discover module was used to assign the potentials for both the enzyme and substrate. The parameters for sulfates and sulfamate groups in glycosaminoglycans described previously (23) were incorporated into this force field to assign potentials for the C4S substrates. A distance-dependent dielectric constant of 4π and scaling of 0.5 for the p1–4 cross terms were used in the discover module for the AMBER force field-based simulations according to the specifications in the InsightII manual.

RESULTS

Cloning, Expression, and Purification of cABCII—Two versions of the cABCII gene were cloned from P. vulgaris DNA: a full-length version and a truncated version that corresponds to the mature form of the enzyme, that is, without its putative leader sequence. These cABCII transcripts are ~3 kb in length—rather large, but still at an appropriate size to tolerate amplification via standard polymerase chain reaction techniques. Following cloning into a TOPO vector, the PCR product was subcloned into the pET28a expression vector. This facilitates E. coli-mediated uptake of the transcript and, ultimately, expression of the protein. Chondroitinase ABCII was expressed in E. coli as described under “Experimental Procedures.” Purification over a charged Ni²⁺ resin was possible because of the incorporation of an N-terminal His₆ tag. was induced in the log phase by the addition of isopropyl-β-d-thiogalactopyranoside. Purification of cABCII generated in excess of 50 mg of protein/500 ml of culture. SDS/PAGE analysis (Fig. 1A) confirmed the presence of highly pure cABCII at ~100 kDa, consistent with previously reported masses of the enzyme (12). Expression of the full-length cABCII clone generated a protein largely present in the insoluble fraction. The yield of soluble enzyme was greatly improved by the engineered removal of the hydrophobic N-terminal signal sequence. We then turned our attention to the truncated clones, both the original sequence and the transcript that underwent our sequential mutagenesis resolution. The recombinant protein with the sequence discrepancies was unable to effectively process GalAG substrates (Fig. 1B). The expression and purification of the modified cABCII gene, on the other hand, generated a protein product with reinvigorated functionality as demonstrated by the observed cleavage of chondroitin-6-sulfate (Fig. 1B).

Biochemical Conditions for Optimal Enzyme Activity—After establishing the active recombinant cABCII, the reaction conditions for optimal cleavage of GalAGs were investigated. These reaction parameters included temperature, pH, ionic strength, and buffer system. For C6S substrate, cABCII demonstrated maximal processing at 37 °C, with a greater than 50% decline in activity at 42 °C (Fig. 2A). Chondroitinase ABC II similarly acted on DS substrate maximally at 37 °C, with a 50% drop at 42 °C (Fig. 2A). Activity against both substrates fell dramatically in excess of 45 °C. For both C6S and DS substrates, 37 °C was chosen as the optimal temperature for biochemical experiments.

A Tris buffer system was chosen for biochemical experiments because it permitted greater activity relative to phosphate buffer (data not shown). The recombinant enzyme demonstrated maximal activity at pH 8.0 for C6S. For DS, maximal activity occurred in the range from pH 8.0 to 8.5 (Fig. 2B).
Chondroitinase ABC II from *P. vulgaris*

**TABLE 1**

Specific activity of recombinant chondroitinase ABC II on glycosaminoglycan substrates

Specific activity was determined by monitoring the increase in absorbance at 232 nm for 5 min. The initial rate of increase in $A_{232}^{\text{nm}}$ was determined for each substrate. The enzyme activity in units (1 unit = 1 μmol product formed/min) was calculated from the initial rate using $e = 5800 \text{ M}^{-1} \text{ cm}^{-1}$ for reaction products at pH 8.0. ND, not determined.

| Substrate                  | Specific activity (milliunits/mg protein) |
|----------------------------|------------------------------------------|
| Chondroitin-6-sulfate      | 29,000                                    |
| Chondroitin-4-sulfate      | 18,000                                    |
| Dermatan sulfate           | 17,000                                    |
| Chondroitin                | 5,400                                     |
| Chondroitin sulfate D      | 4,900                                     |
| Chondroitin sulfate E      | 4,900                                     |
| Hyaluronan                 | ND                                        |
| Heparin/heparan sulfate    | ND                                        |
| Keratan sulfate            | ND                                        |

**TABLE 2**

Kinetic analysis of chondroitinase ABC II with various substrates

The values are the means of at least three experiments ± standard deviation.

| Substrate                  | $K_m$ (μM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (μM$^{-1}$ min$^{-1}$) |
|----------------------------|------------|------------------------|-------------------------------------|
| Chondroitin-6-sulfate      | 9.8 ± 2.1  | 1300 ± 113             | 132                                 |
| Dermatan sulfate           | 19.2 ± 2.9 | 1150 ± 26              | 60                                  |
| Chondroitin-4-sulfate      | 16.1 ± 4.2 | 1000 ± 60              | 60                                  |

Chondroitinase ABC II processing of GalAG substrates is greatly curtailed below pH 7.5 and above pH 8.8. For purposes of simplicity, pH 8.0 was taken as the optimal pH for biochemical studies for both C6S and DS in a Tris buffer system.

For cABCII, ionic strength proved to be an important determinant in the processing of GalAG substrates (18). The case of cABCII, ~100 mM NaCl reduced processing by 50% for both C6S and DS substrates (Fig. 2C). Processing of C6S and DS is virtually eliminated in excess of 250 mM NaCl. Furthermore, the addition of ~50 mM sodium acetate for C6S and 100 mM sodium acetate for DS activates cABCII-mediated processing (Fig. 2D). Depolymerization of GalAG substrate by cABCII is nearly completely inhibited at 500 mM sodium acetate. Therefore, unlike cABCII, which required ~50 mM of NaCl for C6S processing (and 125 mM for DS) (18), for cABCII the presence of NaCl in the buffer actually decreases the activity.

**Galactosaminoglycan Processing by cABCII**—The specific activity of the recombinant cABCII was tested against a full panel of glycosaminoglycan substrates (Table 1). The results suggest that cABCII is most proficient in degrading C6S, C4S, and DS substrates. It was not possible to detect reaction progression for hyaluronan, possibly because of low cleavage rate and nonoptimal pH for this substrate. As expected, other glycosaminoglycan families, like heparin and heparan sulfate (which contain glucosamine instead of galactosamine), were not processed by cABCII. Comparison of cABCII activity with that of cABC (18) shows that cABCII more efficiently processes C5 (~10-fold) and DS (more than 7-fold) than cABCII. Kinetic parameters for recombinant cABCII acting on C6S, DS, and C4S are summarized in Table 2. These measurements were obtained from the initial reaction rates against each substrate using both Michaelis-Menten and Hanes analysis. The turnover numbers for CABCII were experimentally determined to be 1300, 1150, and 1000 min$^{-1}$ for C6S, DS, and C4S, respectively. The catalytic efficiency was highest against C6S substrate (132 μM$^{-1}$ min$^{-1}$) and comparable for DS and C4S (both 60 μM$^{-1}$ min$^{-1}$).

The depolymerization of GalAG substrates by cABCII was further scrutinized with capillary electrophoresis. These studies allow for the characterization of the final products of cABCII digestion following a 20-h exhaustive reaction at 37 °C and therefore represent an end point assay for cABCII activity. For all of the substrates examined, the product profile contains an overwhelming proportion of disaccharide products (Fig. 3). For C6S, the dominant product is a 6-O-sulfated disaccharide. For C4S, the major product is a 4-O-sulfated disaccharide. With DS, a mixture of disaccharide products includes two monosulfated species, the 4-O-sulfated DS disaccharide and the 6-O-sulfated DS disaccharide, and a doubly sulfated disaccharide, the 4-O- and 6-O-di-sulfated disaccharide. It should also be noted that the amount of disaccharide released in each of these end point assays is considerably less (as measured by the double bonds generated, i.e. $A_{232}^{\text{nm}}$) when compared with those released by cABCII processing of these substrates. Comparison of
the processing of DS by cABCI and cABCII (Fig. 3C, inset) indicated that although cABCI processing resulted in a resistant tetrasaccharide fragment (indicated by Tetra in the peak label) despite the addition of more enzyme and longer digestion period, whereas cABCII processing of DS results only in disaccharides. ΔDi4S, ΔUA-GalNAc,4S; ΔDi6S, ΔUA-GalNAc,6S; ΔDi456S, ΔUA-GalNAc,456S. Impurities in commercial substrate preparations result in the ΔDi4S peak in electrophoretogram (A) and the ΔDi6S peak in electrophoretogram (B).

**FIGURE 3. Product profile analysis of recombinant chondroitinase ABC II.** Product profiles for cABCII acting on C6S (A), C4S (B), and DS (C). Shown in inset of C is the processing of DS by cABCII. Chondroitinase ABCI is unable to cleave DS tetrasaccharide fragments (indicated by Tetra in the peak label) despite the addition of more enzyme and longer digestion period, whereas cABCII processing of DS results only in disaccharides. ΔDi4S, ΔUA-GalNAc,4S; ΔDi6S, ΔUA-GalNAc,6S; ΔDi456S, ΔUA-GalNAc,456S. Impurities in commercial substrate preparations result in the ΔDi4S peak in electrophoretogram (A) and the ΔDi6S peak in electrophoretogram (B).

**FIGURE 4.** Homology-based structural model of cABCII-C4S tetrasaccharide complex. Shown in the figure is the Cu trace (cartoon representation) of cABCII (gray)-C4S tetrasaccharide (colored by atom: carbon and sulfur, yellow; oxygen, red; nitrogen, blue) superimposed on the cABCII template (Cu trace colored pink), which was used in the homology modeling. The active site groove of the enzyme is zoomed in for clarity. The distinct additional loop regions in the active site groove of cABCII, Ile224-Thr231 and Gly914-Leu937, are colored in brick red. The critical active site tetrad residues that structurally coincide are labeled using their numbering in CABCII and the single alphabet code for clarity (Arg and His are colored blue, Glu is colored red, and Tyr is colored magenta). This figure was generated using PyMol software.

Chondroitinase ABC II from *P. vulgaris*

To understand the interactions of cABCII with its substrates, a C4S tetrasaccharide was docked into the putative active site of cABCII. Assuming that the GalNAc,4S-GlcA tetrasaccharide complex.

The analogous tetrad of residues in cABCII (that structurally coincide) is labeled using their numbering in cABCII and the single alphabet code for clarity (Arg and His are colored blue, Glu is colored red, and Tyr is colored magenta). This figure was generated using PyMol software.
residues His$^{514}$ and His$^{519}$ are proximal to the 4-sulfate group of the GlcNAc4S residue at the $-1$ subsite.

**Site-directed Mutagenesis and Kinetic Characterization of the Putative Active Site Residues**—Based on the above structural model of cABCII-CS tetrasaccharide complex, a putative catalytic tetrad, His$^{453}$, Tyr$^{460}$, Arg$^{513}$, and Glu$^{608}$, and the other residues in the active site that are positioned to interact with the substrate such as His$^{343}$, His$^{452}$ and His$^{456}$ were identified. To probe the contribution of these residues to enzymatic activity, they were mutated to alanines, and the resulting enzyme products were assayed for activity on chondroitin-6-sulfate and dermatan sulfate substrates (Table 3). The results show that mutants H453A, Y460A, R513A, and E608A all show no detectable activity on either C6S or DS, consistent with their designation as the critical catalytic tetrad required for enzyme activity. H452A shows similar $K_m$ values to the wild type enzyme but a greatly (100-fold) reduced catalytic efficiency. On the other hand, mutation of His$^{456}$ to alanine has limited effect on overall catalytic efficiency (~3-fold reduction), indicating that this residue likely does not play an important role in catalysis. Surprisingly, mutation of His$^{343}$ to alanine also yields an enzyme that shows no detectable activity on C6S and DS, suggesting that His$^{343}$ plays a critical role in the enzymatic activity in addition to the catalytic tetrad.

**DISCUSSION**

This report is the first to describe the expression and characterization of a stable, highly active, contaminant-free recombinant chondroitinase ABC II from *P. vulgaris*. The sequence anomalies in the original clone resulted in a catalytically inactive enzyme, although these anomalies were not in the critical active site residues (Fig. 1B). It is therefore likely for these sequence differences to affect the overall stability of the enzyme, which in turn results in an inactive enzyme. Fixing these anomalies resulted in a fully active recombinant enzyme that efficiently processed both CS and DS substrates. This recombinant cABCII was examined structurally and biochemically, including reaction conditions to maximize enzyme efficacy, the product profile following digestion of GalAG substrates, kinetic analysis, substrate specificity, mode of action analysis, and structural insights into the active site-substrate interactions. Comparison of substrate processing of cABCII with that of cABCI showed that cABCII does cleave GalAG substrates at superior rates to cABCII; however, depolymerization mediated by cABCII proceeds by a course distinct from cABCI. Chondroitinase ABC I seems to prefer longer chain substrates.
and cleaves in a predominantly endolytic fashion (10, 12). Chondroitinase ABC II, on the other hand, appears to cleave shorter oligosaccharide substrates more efficiently in a predominantly exolytic fashion, resulting only in disaccharide products. The presence of these distinct cABCs offers the bacteria the ability to rapidly process GalAG substrates where cABCI would cleave the naturally occurring long GalAG polysaccharides and cABCII would then act on the smaller oligosaccharide fragments from cABCI processing to generate disaccharides that can be readily utilized for bacterial metabolism. The proposed exolytic mechanism of cABCII and its ability to efficiently process smaller GalAG substrates is also supported by the structural model of the cABCII-C4S octasaccharide complex. In this model the two extra loop regions, Ile224–Thr231 and Gly914–Leu937, in cABCI appear to constrict the active site groove specifically on the non-reducing side of the substrate. This groove constriction restricts the access of internal cleavable linkages in a long GalAG chain and thus points to a predominant exolytic mode of action for cABCII. The structural basis for differences in the activity of cABCI and cABCII is similar to the framework proposed for endolytic action of PhcAC versus exolytic action of AacAC (4, 9).

A combination of the theoretical structural model of the enzyme-C4S tetrasaccharide substrate complex and site-directed mutagenesis enabled the identification of critical residues involved in the enzymatic activity. This combined analysis further suggests the likely role of these active site residues in catalytic action of the enzyme. A tetrad of residues including His453, Tyr460, Arg513, and Glu605 is conserved in cABCI, PhcAC, and AacAC and have been shown to play a critical role in the catalytic activity of these enzymes.

FIGURE 6. Critical residues involved in catalytic action of cABCII. A shows stereo view of a C4S tetrasaccharide (carbon, cyan; oxygen, red; nitrogen, blue; sulfur, yellow) substrate docked into the active site groove of the structural model of cABCII. The groove is shown as a cartoon model generated using PyMol, and the residues that are positioned to interact with the substrate are also shown in the following colors: His, Arg, Lys (blue); Tyr (purple); and Asp, Glu (red). B shows a two-dimensional schematic of the chemical structure of C4S tetrasaccharide and its interactions with the critical residues in the active site. The sugars are numbered +2, +1, −1, and −2 from nonreducing to reducing end of the C4S substrate where the cleavage occurs between GalNAc4S and GlcA in the −1 and +1 sites, respectively. The tetrad of residues His453, Tyr460, Arg513, and Glu605 are conserved in cABCI, PhcAC, and AacAC and have been shown to play a critical role in the catalytic activity of these enzymes.
tion earlier, Glu^{608} is not directly involved in interactions with the substrate, but it is positioned to interact with both His^{453} and Arg^{513} via hydrogen bonding. Hence mutation of Glu^{608} to Ala disrupts these interactions and is therefore unfavorable for the optimal positioning of these critical residues for catalytic activity. Earlier studies (2, 10, 24) implicate distinct interactions of cABCII with CS and DS substrates. The same scenario holds good for cABCII because of the structurally conserved active site tetrad. Therefore, the proposed roles of the tetrad in catalytic activity of cABCII could potentially be interchanged to accommodate a broad range of CS and DS substrates.

In addition to the tetrad, it was surprising to note that the H343A mutant completely lost the catalytic activity toward both CS and DS substrates (Table 3). This residue is positioned on the top side of the active site groove opposite the tetrad, which is at the base of the groove (Fig. 6). In the structural model of cABCII described in this study, His^{343} is not as proximal as the tetrad to the substrate. However, the model developed in this study is based primarily on the uncomplexed cABCI enzyme. Therefore, it is possible that in the presence of the substrate the active site groove could become more “closed,” which would position His^{343} proximal to the C5 atom of the GlcA where it could play a critical role in either neutralizing the carboxylate group or stabilizing the C5 carbanion transition state.

This understanding of the structure and mechanism of action of cABCII extends our understanding of precisely how these lyases function and how various structural features contribute to the depolymerization process. The distinct substrate processing ability of cABCII enables its use as an additional valuable resource in technologies directed at determining the fine structural elements of biologically relevant GalAGs. These enzymes may further be useful directly in strategies to interfere with GalAG function in vivo, for example, in neural regeneration therapies and other such biomedical applications.

REFERENCES
1. Sasisekharan, R., Raman, R., and Prabhakar, V. (2006) Annu. Rev. Biomed. Eng. 8, 181–231
2. Prabhakar, V., Capila, I., Raman, R., Srinivasan, A., Bosques, C. J., Pojasek, K., Wrick, M. A., and Sasisekharan, R. (2006) Biochemistry 45, 11130–11139
3. Michiel, G., Pojasek, K., Li, Y., Sulea, T., Linhardt, R. J., Raman, R., Prabhakar, V., Sasisekharan, R., and Cygler, M. (2004) J. Biol. Chem. 279, 32882–32896
4. Lunin, V. V., Li, Y., Linhardt, R. J., Miyazono, H., Kyogashima, M., Kaneko, T., Bell, A. W., and Cygler, M. (2004) J. Mol. Biol. 337, 367–386
5. Pojasek, K., Shriver, Z., Kiley, P., Venkataraman, G., and Sasisekharan, R. (2001) Biochem. Biophys. Res. Commun. 286, 343–351
6. Pojasek, K., Raman, R., Kiley, P., Venkataraman, G., and Sasisekharan, R. (2002) J. Biol. Chem. 277, 31179–31186
7. Huang, W., Boju, L., Tkalec, L., Su, H., Yang, H. O., Gunay, N. S., Linhardt, R. J., Kim, Y. S., Matte, A., and Cygler, M. (2001) Biochemistry 40, 2359–2372
8. Huang, W., Matte, A., Li, Y., Kim, Y. S., Linhardt, R. J., Su, H., and Cygler, M. (1999) J. Mol. Biol. 294, 1257–1269
9. Fethiere, J., Eggimann, B., and Cygler, M. (1999) J. Mol. Biol. 288, 635–647
10. Prabhakar, V., Raman, R., Capila, I., Bosques, C. J., Pojasek, K., and Sasisekharan, R. (2005) Biochem. J. 390, 395–405
11. Huang, W., Lunin, V. V., Li, Y., Suzuiki, S., Sugiura, N., Miyazono, H., and Cygler, M. (2003) J. Mol. Biol. 328, 623–634
12. Hamai, A., Hashimoto, N., Mochizuki, H., Kato, F., Makiguchi, Y., Horie, K., and Suzuki, S. (1997) J. Biol. Chem. 272, 9123–9130
13. Bradbury, E. J., Moon, L. D., Popat, R. J., King, V. R., Bennett, G. S., Patel, P. N., Fawcett, J. W., and McMahon, S. B. (2002) Nature 416, 636–640
14. Chau, C. H., Shum, D. K., Li, H., Pei, J., Lui, Y. Y., Wirthlin, L., Chan, Y. S., and Xu, X. M. (2004) FASEB J. 18, 194–196
15. Hartmann, U., and Maurer, P. (2001) Matrix Biol. 20, 23–35
16. Yamagata, T., Saito, H., Habuchi, O., and Suzuki, S. (1968) J. Biol. Chem. 243, 1523–1535
17. Sato, N., Shimada, M., Nakajima, H., Oda, H., and Kimura, S. (1994) Appl. Microbiol. Biotechnol. 41, 39–46
18. Prabhakar, V., Capila, I., Bosques, C. J., Pojasek, K., and Sasisekharan, R. (2005) Biochem. J. 386, 103–112
19. Ryan, M. J., Khandke, K. M., Tilley, B. C., and Lotvin, J. A. (November 10, 1994) International Patent WO 94/25567
20. Myette, J. R., Shriver, Z., Kiziletepe, T., McLean, M. W., Venkataraman, G., and Sasisekharan, R. (2002) Biochemistry 41, 7424–7434
21. Guda, C., Scheeff, E. D., Bourne, P. E., and Shindyakov, I. N. (2001) Pac. Symp. Biocomput. 6, 275–286
22. Maiti, R., Van Domselaar, G. H., Zhang, H., and Wishart, D. S. (2004) Nucleic Acids Res. 32, 590–594
23. Huige, C. J. M., and Altona, C. (1995) J. Comput. Chem. 16, 56–79
24. Shaya, D., Hahn, B. S., Bjerkran, T. M., Kim, W. S., Park, N. Y., Sim, J. S., Kim, Y. S., and Cygler, M. (2008) Glycobiology 18, 270–277