

**Understanding the Catalytic Machinery of Chondroitinase ABC I  
in Processing Dermatan Sulfate**

By

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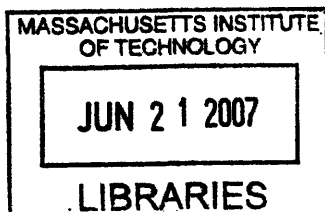
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## ABSTRACT

In recent studies related to injury to the central nervous system, researchers have found that galactosaminoglycans can serve as inhibitors to neuron regeneration. The chondroitinase enzyme family is comprised of several bacterial lyases known to dissolve galactosaminoglycans in the extracellular matrix. Although several studies have shown the benefit of using chondroitinase enzymes for treatment, there is much to learn about its enzyme-substrate complex. For the purpose of this research, we focus on the processing of two key galactosaminoglycan substrates, chondroitin-6-sulfate and dermatan sulfate. Through a systematic approach, we investigate the active site of chondroitinase ABC I with biological and structural studies. We demonstrate that calcium, a divalent ion, potentially increases the activity of chondroitinase ABC I when processing dermatan sulfate. From this we gain insight into the structural make-up of the chondroitinase ABC I enzyme, allowing us to optimize our approach for targeting inhibitory substrates that prevent regeneration in the central nervous system.

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# Table of Contents

<b>1.0 Introduction</b> .....	4
<b>2.0 Experimental Apparatus</b> .....	5
2.1 Materials .....	5
<b>3.0 Experimental Procedure</b> .....	5
3.1 Mutagenesis of Chondroitinase ABC I .....	5
3.2 Protein Expression and Purification .....	5
3.3 Fluorescence Spectrometry .....	6
3.4 Calcium and Terbium Effects as Divalent Ions .....	6
3.5 Chondroitinase ABC I Activity Assessments .....	7
<b>4.0 Results</b> .....	7
4.1 Salt versus Calcium Effects .....	7
4.2 Chondroitinase ABC I Analysis .....	9
4.3 Terbium Fluorescence Examination .....	11
4.4 Calcium Effect on Dermatan Sulfate Depolymerization .....	13
4.5 Calcium Coordination Mutagenesis Studies .....	14
<b>5.0 Discussion</b> .....	17
<b>6.0 Acknowledgement</b> .....	18
<b>References</b> .....	19
<b>Appendix</b> .....	22

## 1.0 Introduction

Injury to the spinal cord following trauma triggers a cascade of biological events that unfold within seconds and that proceed for months or even years. The events affect three major bodily systems: the nervous system, the immune system, and the vascular system. These systems interact dynamically as they respond to injury. Although some injurious responses heal and promote the recovery of function, others leave a wave of tissue damage that expands well beyond the original site of injury (15).

The efforts of our study focus on the tissue response at the site of the injury. The conventional view is that damage to neurons in the central nervous system cannot be repaired. In recent years, however, several studies suggest that some regeneration and recovery might be possible. In a recent study, mice demonstrated partial functional recovery weeks after having its spinal cord severed (1). These mice were treated with a complex series of therapies to encourage neural regeneration at the site of the damage. The proposed mechanism of action at work here is believed to involve the targeting of inhibitory proteoglycans that physically block neural path finding processes (12). This phenomenon, notably, is in play during neural development (1).

One potential strategy for addressing trauma to the central nervous system could include the removal of chondroitin sulfate proteoglycans within the glial scar (12, 15). This might be accomplished enzymatically by a number of enzymes, including bacterial chondroitinases. Bacteria uses these lyases in order to process polysaccharides from plant tissues for the purpose of nutrition (2). In order to effectively destroy these inhibitors, we must first understand the machinery of these enzymes through careful characterization.

It is the purpose of this study to probe one important aspect of these enzymes. Specifically, we will look into the involvement of the divalent ion, calcium in the catalytic machinery of chondroitinase that could play a role in the enzymes mode of cation, thereby making it more useful in therapy (24). Understanding the enzyme's catalytic machinery would pave the way toward engineering a therapeutically useful enzyme for spinal cord injury (15).

## **2.0 Experimental Apparatus**

### ***2.1 Experimental Materials***

The required substrates, both dermatan sulfate and chondroitin sulfate, were purchased from Sigma. The oligonucleotides were purchased from Invitrogen. The QuikChange Site-Directed Mutagenesis Kit was purchased from Stratagene. The QIAprep Spin Miniprep Kit was purchased from Qiagen. The protein concentrations were measured using the Bio-Rad Laboratories Bradford assay kit. Chelex resin for purification of the protein was also purchased from Bio-Rad. The terbium used in fluorescence experiments was purchased from Aldrich. All other materials are from common sources in a biological engineering laboratory.

## **3.0 Experimental Procedure**

### ***3.1 Mutagenesis of Chondroitinase ABC I***

The development of stock recombinant chondroitinase ABC I and the methods used to prepare the enzyme mutants are followed according to the procedure set by Prabhakar et al in their study “Chondroitinase ABC I from *Proteus vulgaris*.” (3) The primer sequences for the mutants can be found in Appendix A of this paper.

### ***3.2 Protein Expression and Purification***

The recombinant expression and purification of chondroitinase ABC I was previously described (3) To ensure that we were using a pure protein, we used gel electrophoresis analysis and Simply Blue SafeStain to examine our stock. The electrophoresis was accomplished using Invitrogen NuPAGE 12% Bis-Tris gels and the XCell SureLock Mini-Cell. We measured the protein content of our solution using a Bradford assay kit. Bovine serum albumin dye from Sigma was mixed into the solution using standard pipette methods. We eliminated the chance for structural defects in the

new mutant enzymes by comparing its structure to recombinant chondroitinase ABC I. Comparisons were accomplished through structural characterization with circular dichroism. The circular dichroism spectra were recorded at 25 degrees Celsius on an Aviv 202 circular dichroism spectrophotometer, with Quartz cuvettes holding the stock at a length of 0.1 cm from the scan. To ensure an accurate reading, three scans were done for each stock and averaged for a final reading.

### ***3.3 Fluorescence Spectrometry***

In order to monitor the activity of chondroitinase ABC I, we employed the use of terbium to act as an analog to the calcium ion (15). The terbium titrations were accomplished by adding aliquots of terbium stock solution in 10mM MOPS and 0.1 M KCl buffer at pH 7.0 with .005 mM chondroitinase ABC I. Again, accurate readings were of chief importance, so all non-terbium solutions were filtered in a chelating column to remove any impurities. The fluorescence measurements were recorded on a Cary Eclipse fluorescence spectrophotometer. Excitation wavelengths were calibrated either at 488 nm to excite the terbium or 280 nm to excite tyrosine residues on the protein. Emission wavelengths were held at 545 nm. The terbium was mixed into the solution contained within a quartz cell. Prior to taking the measurement, the new solution was allowed to come to equilibrium for 15 minutes. All measurements were taken at 90 degrees as suggested by Shriver et al in their study. (6)

### ***3.4 Calcium and Terbium Effects as Divalent Ions***

It has been proposed that divalent ions such as calcium, and its analog, terbium, could significantly affect the activity on chondroitinase ABC I. To measure this, chondroitin 6 sulfate was dissolved to 1 mg/mL in a buffer of 50 mM Tris at pH 8.0. In the buffer were 10 mM concentrations of four different divalent ion salts (CaCl<sub>2</sub>, MgSO<sub>4</sub>, MnCl<sub>2</sub>, and ZnCl<sub>2</sub>). Recombinant chondroitinase ABC I was then added to the solution and activity was measured each minute by the change in its absorbance of light at 232 nm. All activity assessments were completed with a SpectraMax 190 using 96-well quartz

plates as described by Prabhakar et al. (2) The experiment was conducted at an optimal temperature of 37 degrees Celsius. Chondroitinase activity was calculated on the basis of the initial rate of product formation.

### ***3.5 Chondroitinase ABC I Activity Assessments***

To ensure that we were working with active protein, both recombinant and mutant chondroitinase ABC I were evaluated with two substrates (chondroitin-6-sulfate and dermatan sulfate). The kinetic analysis was conducted in a similar manner to the divalent ion analysis, where absorbance at 232 nm was measured every 2 seconds for 2 minutes. The reaction consisted of 1 microL of 0.2 microg/microL of chondroitinase ABC I added to 249 microL a solution containing chondroitin-6-sulfate or dermatan sulfate in buffer #2 of Appendix A. The reaction took place in a 100 well plate, with each well containing a different concentration of substrate. The substrate concentration ranged from 0.1 to 5 mg/mL.

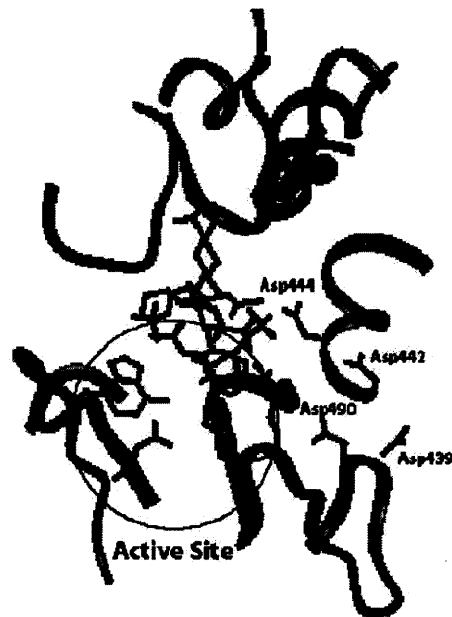
The data was analyzed for initial reaction rate ( $v_0$ ) by calculating the slope of substrate degradation over the first 20 seconds.  $V_{max}$  and  $K_m$  were calculated from the standard formula for Hanes plot analysis. For calculations, we assumed a molar absorptivity coefficient ( $\epsilon$ ) for the processed substrate of  $3800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ . Optical path length for each well was .904 cm (18). To ensure accuracy of our results, all values were averaged from three separate trials.

## **4.0 Results**

### ***4.1 Salt versus Calcium Effects***

In order to extract significant data regarding the role of calcium in the catalytic processing of chondroitin sulfate and dermatan sulfate, steps were taken to understand all

levels of interaction between the ion and enzyme. To ensure that calcium was actually capable of binding to chondroitin ABC I, we employed fluorescence techniques to study their interaction. Terbium, which is similar to calcium in both molecular dimensions and charge, was substituted in reactions for its fluorescent properties. If the terbium interacted with the enzyme, then the ultraviolet light would excite its electrons, creating a measurable amount of fluorescence. Aside from just calcium, other divalent metal ions were tested for their interaction with chondroitinase. With calcium confirmed to interact with chondroitinase, its effects on the catalytic processing of chondroitin sulfate and dermatan sulfate were tested as well. Such a systematic approach was expected to provide insight regarding calcium's effect on the enzyme.



**FIGURE 1:** Example of active site topology of the chondroitinase ABC I active site. A galactosaminoglycan substrate is shown in the catalytic cleft of chondroitinase ABC I in close proximity to the active site residues and several aspartic acid residues. Residues Asp439, Asp442, Asp444, and Asp490 were examined through mutagenesis studies in order to inspect their potential complicity within a calcium coordination center (2).

As mentioned, one of the first steps in our analysis was to measure chondroitinase ABC I's catalytic activity with the two substrates in the presence of several different divalent ions. Table 1 shows the specific activity of chondroitinase ABC I acting on chondroitin-6-sulfate and dermatan sulfate in the presence of these various divalent ions.

In the case of chondroitin-6-sulfate, one can notice that the enzyme activity is nearly unchanged with the presence of calcium or magnesium as compared to the control (2). Manganese seemed to lower the enzyme activity while zinc clearly inhibited the enzyme. These results for manganese and zinc were expected from previous results, and gave us confidence that our activity measurements were significant.

<b>Chondroitin-6-Sulfate</b>		
<i>Divalent Ion</i>	<i>Specific Activity</i>	<i>% Change</i>
Tris pH 8	169 +/- 9	
Tris pH 8 + Ca <sup>2+</sup>	158 +/- 11	-7%
Tris pH 8 + Mg <sup>2+</sup>	201 +/- 17	19%
Tris pH 8 + Mn <sup>2+</sup>	129 +/- 6	-24%
Tris pH 8 + Zn <sup>2+</sup>	19 +/- 3	-89%
<b>Dermatan Sulfate</b>		
<i>Divalent Ion</i>	<i>Specific Activity</i>	<i>% Change</i>
Tris pH 8	9 +/- 3	
Tris pH 8 + Ca <sup>2+</sup>	231 +/- 8	2467%
Tris pH 8 + Mg <sup>2+</sup>	263 +/- 7	2822%
Tris pH 8 + Mn <sup>2+</sup>	13 +/- 8	44%
Tris pH 8 + Zn <sup>2+</sup>	20 +/- 2	122%

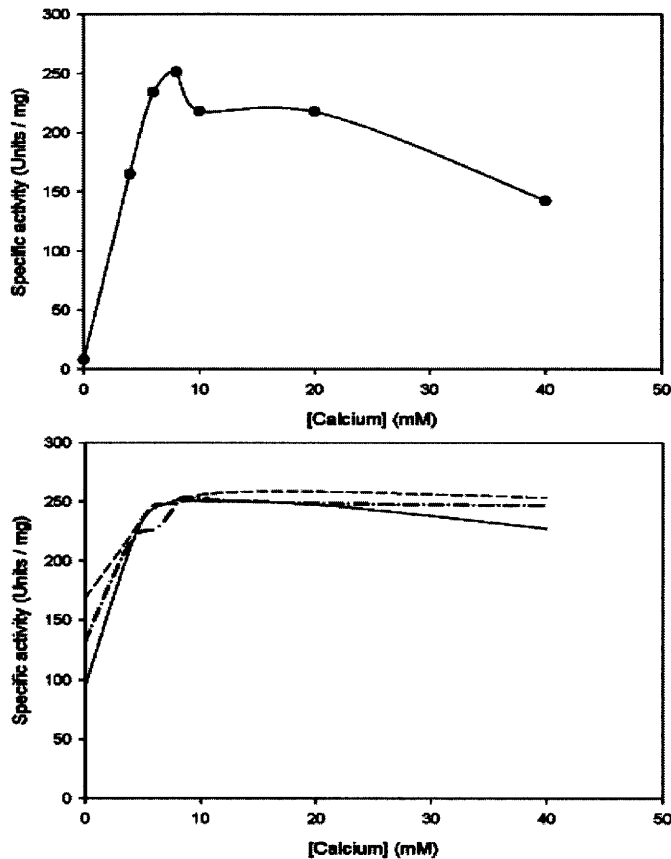
**Table 1:** The specific activity of chondroitinase ABC I on C6S and DS in the presence of divalent ions. The divalent ions are added to buffer #1 at a concentration of 10 mM. Recombinant chondroitinase ABC I was added to the solution and absorbance at 232 nm was periodically measured as mentioned earlier.

The more striking results were in the dermatan sulfate test, where the presence of calcium or magnesium had increased chondroitinase activity 2466% and 2811% respectively when compared to the control in buffer #1. Manganese and zinc did not have any significant effect on enzyme activity processing.

#### ***4.2 Chondroitinase ABC I Analysis***

Knowing that calcium had a profound effect on dermatan sulfate processing, we aimed to investigate at what concentrations it maximized chondroitinase ABC I activity.

Calcium titration experiments were performed, varying the concentration of  $\text{CaCl}_2$  from 0-40 mM. As can be observed in Figure 2, the maximal enzyme activity took place with  $\text{CaCl}_2$  in concentrations between 8-10 mM.



**FIGURE 2:** Effects of  $\text{Ca}^{2+}$  on the chondroitinase ABC I activity with the substrate dermatan sulfate. Chondroitinase ABC I activity on dermatan sulfate is increased in the presence of calcium ions in the solution (2).

The second graph is the effect of calcium on the chondroitinase ABC I-mediated depolymerization of dermatan sulfate in the presence of sodium acetate added to the solution. Sodium acetate was added to investigate whether the increased activity was due to the divalent ions like  $\text{Ca}^{2+}$  or if it was caused due to a side-effect from the presence of salt in the solution. Sodium acetate concentrations were either 25 mM (solid line), 50 mM (long dashed line), or 100 mM (short dashed line). The data clearly shows that both in the presence and absence of sodium acetate, the specific activity of chondroitinase ABC I in processing dermatan sulfate remained in the 8-10 mM  $\text{Ca}^{2+}$  concentration range. This

gives a clear indication that the effect that calcium has on the activity of chondroitinase ABC I is independent of the presence of salt in the solution.

### ***4.3 Terbium Fluorescence Examination***

The terbium fluorescence tests were designed to investigate whether recombinant chondroitinase ABC I bonded directly with the calcium, or whether the calcium had an effect on the substrate instead. The use of Terbium allows us to probe this ion-enzyme interaction, as it exhibits fluorescence capabilities (40). By allowing  $Tb^{3+}$  to interact in competition with calcium, we can determine that a successful interaction took place by judging the amount of fluorescence emitted by the sample.

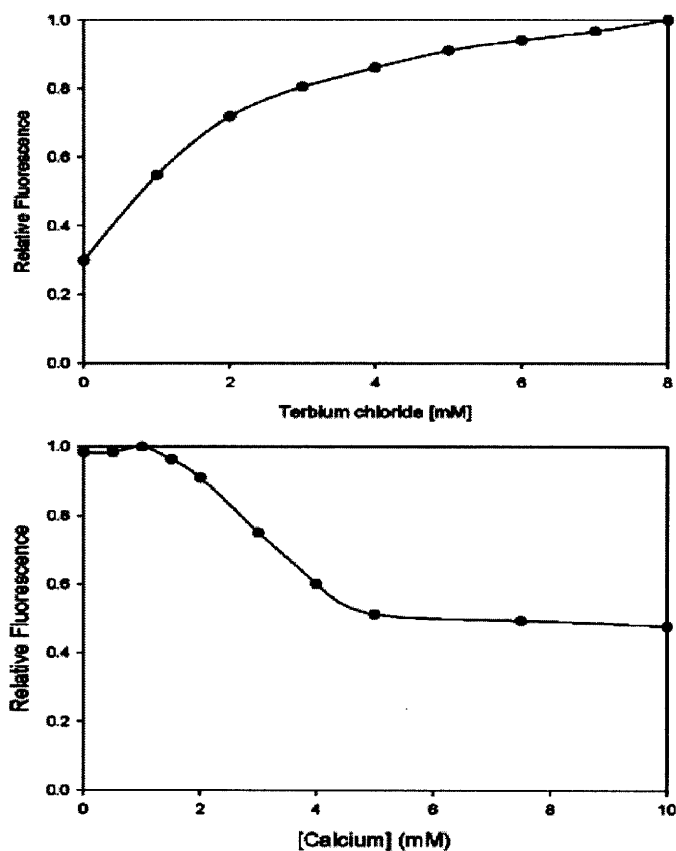
$Tb^{3+}$  is a lanthanide analog of calcium, often used to probe the nature of protein interactions with divalent ions including calcium (32). In aqueous solution,  $Tb^{3+}$  possesses an ionic radius that is very similar to that of calcium. The increased charge properties of terbium compared to those of calcium additionally confer a relatively greater affinity to calcium-binding sites for the lanthanide than for the divalent calcium (10).

Terbium was titrated into a solution containing chondroitinase ABC I, up to a final concentration of 8 mM. Figure 2 shows the change in fluorescence over the time of the titration. It should be noted that the marginal increase in fluorescence due to terbium addition declined significantly at 8 mM, and no further titration was performed beyond this concentration. The increase in fluorescence was observed against both a direct excitation of the terbium adduct (488/nm) and at an excitation of the proximal tyrosine side chains, which results in an energy transfer to the terbium adduct (280 nm).

Because of terbium's behavior with chondroitinase, a high concentration of calcium was required to ensure a fair playing field for both ions in competition for the enzyme-ion complex (40). Both terbium and calcium compete for the same location on the enzyme, which provides an ideal environment to study the specificity occurring during binding. The test was begun by loading the solution containing the enzyme with terbium. After a short pause the fluorescence was progressively measured after the addition of calcium. As can be seen in the second figure, fluorescence eventually

decreases, giving us a clear indicator that calcium is competing with terbium for binding spots.

These results indicate that the interaction of terbium with chondroitinase ABC I is specific and that this interaction substitutes for calcium binding to chondroitinase ABC I (2). These results are consistent with previous experiments on heparinase I designed to establish terbium enzyme interaction and calcium specificity (26).



**Figure 2:** The interaction between terbium and chondroitinase ABC I was quantified through fluorescence spectrometry. Terbium, the lanthanide analogue to calcium, was observed against a direct excitation of the terbium adduct and at excitation of proximal tyrosine residues (488 and 280 nm, respectively).

As seen in graph A in Figure 2, terbium binding to chondroitinase ABC I was investigated. Fluorescence intensity increases upon titration of terbium to 5 microM

chondroitinase ABC I. Following the addition of  $TbCl_3$ , the solution was allowed to come to equilibrium, and the fluorescence at 545 nm was measured with an excitation wavelength of 280 nm. The data is presented as relative fluorescence normalized according to the peak measurement versus supplemented terbium. In graph B of Figure 2, experiments were performed to investigate whether calcium was capable of competing for chondroitinase ABC I binding sites with terbium. The terbium-enzyme complex (6 mM terbium) was titrated with increasing amounts of calcium. The sample was incubated for 15 min, and the fluorescence at 545 nm was measured. Fluorescence intensity decreased with increased calcium concentration. Relative fluorescence is presented in normalized form according to the peak fluorescence measured (40).

Graph B clearly shows that the addition of calcium does effectively compete with terbium to occupy chondroitinase ABC I binding positions. The results indicate that the interaction of terbium with chondroitinase ABC I is specific and that this interaction substitutes for calcium binding to chondroitinase ABC I. These results are consistent with previous experiments on heparinase I designed to establish terbium-enzyme interaction and calcium specificity (2).

#### ***4.4 Calcium Effect on Dermatan Sulfate Depolymerization***

With this study, we aimed to prove that chondroitinase specific activity is increased with calcium. Kinetic analysis was conducted on chondroitinase ABC I depolymerization of chondroitin-6-sulfate and dermatan sulfate. A calcium chloride concentration of 10 mM was maintained in the tests. In the test with chondroitin-6-sulfate, one can notice that neither the  $K_m$  nor  $k_{cat}$  of chondroitinase ABC I was affected by the calcium. This was not the case with dermatan sulfate, as the  $K_m$  was increased from 1.4 microM without calcium to 2.9 microM with calcium. It was similar with the  $k_{cat}$ , as it increased from 17,000  $min^{-1}$  to 33000  $min^{-1}$ . This is a clear indicator that calcium could be involved in both the structural binding and orientation of the dermatan sulfate substrate when interacting with chondroitinase ABC I (33).

Experiment	$K_m$	$k_{cat}$	$k_{cat} / K_m$
Substrate: Chondroitin-6-Sulfate			
buffer only	2.4 +/- 0.9	22000 +/- 1200	9100
buffer + 10 mM CaCl <sub>2</sub>	2.9 +/- 0.6	20000 +/- 5000	6900
Substrate: Dermatan Sulfate			
buffer only	1.4 +/- 0.5	17000 +/- 1400	12000
buffer + 10 mM CaCl <sub>2</sub>	2.9 +/- 0.5	33000 +/- 1800	11000

**Table 2:** Recombinant chondroitinase ABC I at a concentration of 1 microg/microL was added to 249 microL of a solution containing either chondroitin-6-sulfate or dermatan sulfate in buffer #3. Calcium chloride was either included in the buffer or not at a concentration of 10 mM. Product formation was monitored by measuring the absorbance at 232 nm every 2 seconds. All values were averaged from three separate trials to ensure for accuracy.

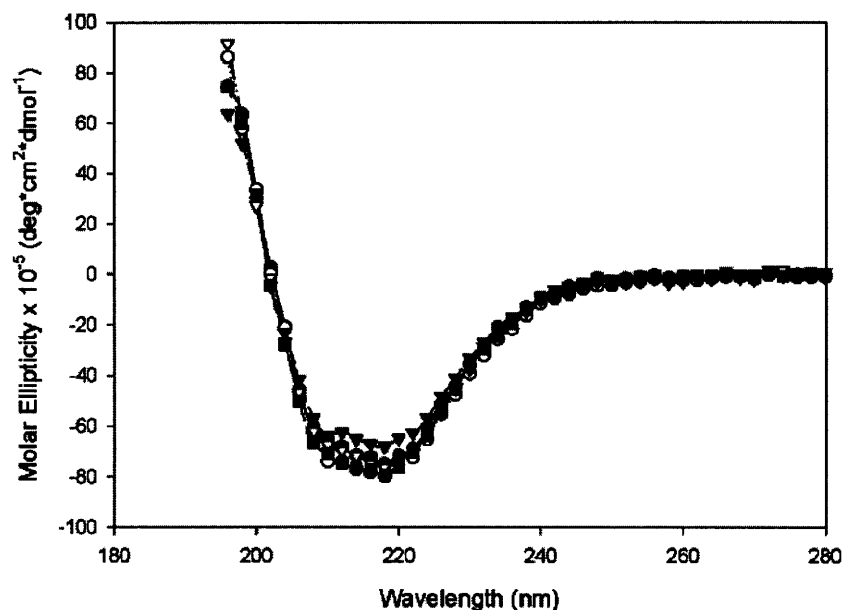
In previous studies in our lab, theoretical models were developed around the interaction between chondroitin sulfate and the enzyme. Where chondroitin sulfate would enter the enzyme's active site in a near ideal manner, it was posed that dermatan sulfate's orientation was the exact opposite of chondroitin sulfates when at the active site. Such theoretical models are supported by our previous data, showing that chondroitinase ABC I is significantly more efficient cleaving chondroitin sulfate as opposed to dermatan sulfate (33). Due to the significant rise in specific activity of the enzyme-substrate interaction in the presence of calcium, it is posed that the calcium charge binds both to dermatan sulfate and the enzyme to flip the substrate's orientation to one similar to chondroitin sulfate (35). This would explain the data we have collected and would shed new light in the role of chondroitinase ABC I in substrate processing. (24).

#### ***4.5 Calcium Coordination Mutagenesis Studies***

In order to further inspect the interaction occurring between chondroitinase ABC I and the galactosaminoglycan substrates (chondroitin-6-sulfate or dermatan sulfate),

mutagenesis studies were carried out on the enzyme's active site. There are four aspartic acid residues that we proposed as potentially involved in the depolymerization of the substrates and interaction with calcium. These residues were Asp439, Asp442, Asp444, and Asp490. As suggested by the theoretical model of the enzyme's active site, Tyr392 was also mutated to examine its potential role in the catalytic activity (35).

All the residues were replaced with alanine in chondroitinase ABC I. Since mutagenesis occasionally alters the secondary structure of the protein, we examined each of our samples with circular dichroism spectroscopy. The results are shown below in Figure 3.



**Figure 3:** CD spectra of chondroitinase ABC I and its mutants. The recombinant chondroitinase ABC I (full circle) is shown with mutants Asp439 (empty circle), Asp442 (full triangle), Asp444 (empty triangle), and Asp490 (full square). All mutants were inspected through circular dichroism for differences in its secondary structure. The proteins were concentrated and buffer-exchanged into buffer #4. Protein was analyzed using quartz cells with a path length of 0.1 cm. All proteins were measured at 0.2 mg/mL. CD spectra were recorded between 195 and 280 nm, once again using the average of three trials.

After verifying protein validity through the circular dichroism tests, we began activity tests to see how the mutants performed. The existing theoretical protein model predicted Asp442 and Asp444 to play the largest role in calcium coordination in the

enzyme. The other residues were expected to be too far from the binding site to have an effect on catalytic activity. In our examination, we found that the  $K_m$  changed from 1.5 microM to 1.7 microM when calcium was introduced. The  $k_{cat}$  also increased from 3300  $\text{min}^{-1}$  to 4000  $\text{min}^{-1}$ . By dividing the two, we can determine the catalytic efficiency, seen to move from  $k_{cat}/K_m$  of 2200  $\text{microM}^{-1}\text{min}^{-1}$  to 2400  $\text{microM}^{-1}\text{min}^{-1}$ . Kinetic analysis of the Asp439Ala mutant processing chondroitin-6-sulfate shows that although overall activity is reduced, it is not reduced enough to rule out that calcium had an effect on binding. As can be seen in Table 3, there was a significant change in activity in the Asp439Ala mutant's processing of dermatan sulfate, where both  $K_m$  and  $k_{cat}$  nearly double in value. This gives us clear evidence that Asp439 does not play a role in recombinant chondroitinase ABC I in calcium coordination.

<b>Mutant Kinetic Analysis: Chondroitin-6-Sulfate</b>				
mutant	+/- 10mM $\text{Ca}^{2+}$	$K_m$	$k_{cat}$	$k_{cat}/K_m$
Asp439Ala	-	1.5 +/- 0.7	3300 +/- 1300	2200
Asp439Ala	+	1.7 +/- 0.7	4000 +/- 1100	2400
Asp442Ala	-	4.0 +/- 0.9	400 +/- 200	100
Asp442Ala	+	4.5 +/- 1.1	510 +/- 240	110
Asp444Ala	+/-	no data	no data	no data
Asp490Ala	-	17.7 +/- 9.5	2100 +/- 350	120
Asp490Ala	+	14.5 +/- 1.9	1800 +/- 400	120
<b>Mutant Kinetic Analysis: Dermatan Sulfate</b>				
mutant	+/- 10mM $\text{Ca}^{2+}$	$K_m$	$k_{cat}$	$k_{cat}/K_m$
Asp439Ala	-	1.7 +/- 0.7	2300 +/- 90	1400
Asp439Ala	+	2.8 +/- 0.3	4500 +/- 340	1600
Asp442Ala	+/-	no data	no data	no data
Asp444Ala	+/-	no data	no data	no data
Asp490Ala	-	12 +/- 4.0	280 +/- 90	19
Asp490Ala	+	19 +/- 7.2	640 +/- 80	34

**Table 3:** Kinetic analysis of chondroitinase ABC I mutants in the absence and presence of calcium. The standard substrates of chondroitin-6-sulfate and dermatan sulfate were used in kinetic studies. Activity assays on mutant enzymes exhibiting considerably low activity in their reactions (no data) are listed in the table as well.

As can be seen in the table, both mutants Asp442Ala and Asp490Ala did not alter the kinetic rates among the presence of calcium in the solution. It is also noted that the rates of activity with chondroitin-6-sulfate are far lower than that of the recombinant strain. This comparison can be seen by comparing activity in Table 3 with those in previous tables. As noted in the table, Asp444Ala exhibited such low activity that it was impossible to get significant kinetic data from the reaction. This is mostly likely explained by Figure 1, where Asp444 is located in the heart of the catalytic center. Any alteration to such central residues could result in the destabilization of the active site. This would explain Asp444Ala's poor function in the reaction.

From the results, we can draw several conclusions for the residues involved in chondroitinase ABC I's active site. By mutating Asp442Ala and Asp444Ala, we noticed a significant drop in enzyme activity. It is safe to assume that both Asp442 and Asp444 may be part of the calcium coordination in substrate processing, specifically that of dermatan sulfate. Asp490 has shown an importance in calcium coordination larger than that of Asp439, but less than Asp442 and Asp444, as the data suggests. Additionally, when we mutated Tyr392, we noticed a 7-fold increase in enzyme activity when processing chondroitin sulfate when compared to dermatan sulfate processing. This could suggest that the Tyr392 residue inhibits the different substrates from entering the active site. By mutating it, we could possibly be "opening a door" to the active site on chondroitinase ABC I, making it easier for the enzyme to attach and cleave substrates.

## 5.0 Discussion

By studying chondroitinase ABC I, we gain valuable insight into the residues important in the catalysis of several substrates as well as gain new insight on enzyme-substrate complexes. Chondroitinase ABC I was chosen primarily for its tertiary configuration, as the active site is structurally more open for substrates to come in contact with. Other enzymes of the chondroitinase family, specifically chondroitinase AC, are thought to have a tighter structure, offering less room for substrates like chondroitin-6-sulfate and dermatan sulfate to enter the active site and position themselves for depolymerization. The increased activity of chondroitinase ABC I as compared to other

enzymes in the chondroitinase family also provide large variance in enzyme specific activity, making quantitative studies more revealing.

Through our quantitative analysis and modeling, we show that chondroitinase ABC I is capable of processing both chondroitin-6-sulfate and dermatan sulfate. It is also indicated that upon addition of calcium in solution, catalysis of dermatan sulfate is optimized. Our research also shows that calcium has no significant effect on the enzyme's ability to process chondroitin sulfate. This information shows us that with the correct concentration of calcium in solution, and galactosaminoglycan degrading enzyme's specificity can be modified to depolymerize multiple different substrates.

Correctly, recombinant galactosaminoglycan-degrading enzymes are being developed for possible medicinal purposes relevant to several issues. These include a variety of therapeutic indications (20, 36, 37) and analytical applications (10, 24, 38-41). The galactosaminoglycan-degrading enzyme studied in this paper, chondroitinase ABC I, is currently being studied to understand the full spectrum of potential substrates. By manipulating the enzyme's specificity with environmental conditions, such as calcium, this enzyme can potentially be targeted to any given issue in the body. If researchers can gain control to what substrates chondroitinase processes, then precise targeting approaches could be taken to the site of complex biological perturbations in the body. As suggested in the introduction, one such pathology is the scar tissue that develops after nerve damage. The tissue, which acts as an inhibitor to neural regeneration, can be degraded by enzymes like chondroitinase, allowing neurons to regenerate at the site of the scar.

## **6.0 Acknowledgement**

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## Appendix

Mutant primer	Sequence ( <i>Mutation</i> )
Asp439Ala	5'- GAT ATG AAA GTA AGT GCT <b>GCT</b> AGC TCT GAT CTA G-3' 5'- C TAG ATC AGA GCT AGC AGC ACT TAC TTT CAT ATC-3'
Asp442Ala	5'- GT GCT GAT AGC TCT <b>GCT</b> CTA GAT TAT TTC AAT ACC-3' 5'- GGT ATT GAA ATA ATC TAG AGC AGA GCT ATC AGC AC-3'
Asp444Ala	5'- AGC TCT GAT CTA <b>GCT</b> TAT TTC AAT ACC TTA TCT CGC C-3' 5'- G GCG AGA TAA GGT ATT GAA ATA AGC TAG ATC AGA GCT-3'
Asp490Ala	5'- CCG GGT GGT AAA <b>GCT</b> GGT TTA CGC CCT-3' 5'- AGG GCG TAA ACC AGC TTT ACC ACC CGG-3'
Tyr392Ala	5'- CC CAT CAC TGG GGA <b>TTC</b> AGT TCT CGT TGG TGG-3' 5'- CCA CCA ACG AGA ACT GAA TCC CCA GTG ATG GG-3'

### Buffers:

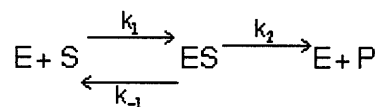
Buffer #1: 50 mM Tris at pH 8.0

Buffer #2: 50 mM Tris-HCl, 50 mM sodium acetate, and 10 mM calcium chloride at pH 8.0

Buffer #3: 50 mM Tris-HCl and 50 mM sodium acetate at pH 8.0

Buffer #4: 50 mM Sodium phosphate at pH 8.0

### Michaelis and Menten Enzyme Kinetics Equations



Rate of ES formation = Rate of ES dissolution

$$k_1 \cdot [S] \cdot [E_{\text{free}}] = k_{-1} \cdot [ES] + k_2 \cdot [ES]$$

$$k_1 \cdot [S] \cdot ([E_{\text{total}}] - [ES]) = k_{-1} \cdot [ES] + k_2 \cdot [ES]$$

$$[ES] = \frac{k_1 \cdot [E_{\text{total}}][S]}{k_1 \cdot [S] + k_2 + k_{-1}} = \frac{[E_{\text{total}}][S]}{[S] + \frac{k_2 + k_{-1}}{k_1}}$$

$$\text{Velocity} = k_2 \cdot [ES] = \frac{k_2 \cdot [E_{\text{total}}][S]}{[S] + \frac{k_2 + k_{-1}}{k_1}}$$

$$\text{Velocity} = V = \frac{V_{\text{max}}[S]}{[S] + K_M}$$