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HISTIDINE-DIRECTED ENGINEERING OF GLYCOSAMINOGLYCAN LYASES FOR SELECTIVE CHONDROITIN SULFATE AND ALGINATE CONVERSION

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ABSTRACT

Targeted engineering of glycopeptidase activity and substrate specificity by histidine is a targeted approach. In this study, wild-type and engineered GAGase variants were assessed to their differential conversions of chondroitin sulfate C and alginate since secondary enzymatic activity data. The analysis comprised time-series activity profiles, concentration-response measurements, replicated absorbance at 232 nm and substrate-selectivity indices. The chondroitin sulfates were more active, while the alginate-associated cumulative activity was noticeably lower in GAGase III-H188A and GAGase III-H188N, suggesting that residue H188 plays a significant role in the recognition or productive binding of alginate. GAGase III-H398A, in contrast, showed a decrease in chondroitin sulfate activity and an increase in alginate-associated response, resulting in an almost equal selectivity profile between the two substrates. 232 nm replicated measurements showed significant chondroitin sulfate activity in both H188A and H188N, while concentration-response analysis showed different responses of alginate to H188A and H188N. Many histidine-introduced variants were more sensitive to chondroitin sulfate than were alginates. The patterns emphasize that the residue substitutions can lead to the alteration of the discrimination of the substrate without, however, eliminating catalytic competence under similar conditions. In summary, these results show that individual histidine residues have different position-specific effects on catalytic selectivity and show that it is possible to rationally design polysaccharide lyases with selective bioconversion and controlled production of oligosaccharides.

Keywords: Glycosaminoglycan lyases; Histidine-directed engineering; Chondroitin sulfate; Alginate; Substrate selectivity

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1. Introduction

Glycosaminoglycan lyases are increasingly valued biocatalysts of the specific depolymerization of complex acidic polysaccharides such as chondroitin sulfate and structurally related substrates. These enzymes break down glycosidic bonds with β-elimination to yield unsaturated oligosaccharides of biological, pharmaceutical or industrial importance. Chondroitin sulfate is a sulfated glycosaminoglycan that has been found in abundance in the extracellular matrix, in the signalling pathway, and in therapies, and alginate is an anionic polysaccharide used extensively in food, biomedical, agricultural and bioprocessing systems. This has led to the increased interest in the development of lyases that possess better catalytic efficiency, substrate specificity, and operational stability (Cheng et al., 2020; Barzkar et al., 2022).

The alginate lyases have been well characterized in marine and microbial origins, with significant differences in catalytic mechanism, substrate specificity, thermal properties and product spectra (Arntzen et al., 2021; Nguyen et al., 2021). Some enzymes have endolytic or exolytic activity and are specific for mannuronate-rich, guluronate-rich or mixed alginate regions (Xue et al., 2022; Li et al., 2022). Large-scale studies have also revealed that there are often multiple lyases with complementary functions in alginate-degrading systems to efficiently convert heterogeneous polymeric substrates (He et al., 2022). This functional diversity has led to the emergence of recombinant, immobilized, and engineered enzymes for the production of oligosaccharides in industry and other applications (Kaur et al., 2024; Li et al., 2023).

Simultaneous progress has resulted in increased knowledge about chondroitin sulfate lyases and their structure and determinants. Microbial chondroitin AC and ABC lyases serve as valuable models for investigating the recognition, catalytic specificity, and product formation of glycosaminoglycans (Fan et al., 2022; Song et al., 2021). Sulfation pattern, polymer conformation, enzyme family and architecture of the active sites are key factors in the degradation of chondroitin sulfate, where it is known that the substrate selectivity plays a central role in the catalytic activity (Wang et al., 2023). The last few years have seen an enhanced involvement of the study of polysaccharide lyases (PL) family 35, with the discovery of some enzymes with extremely wide activity towards structurally distinct molecules, such as chondroitin sulfates and alginate-related polymers (Wei et al., 2024). Structure-function analysis of these enzymes has also revealed the relationship between the catalytic residues and the binding pocket for the various sugar chains that are recognized and cleaved, despite their high chemical diversity (Wei et al., 2025).

In this context, histidine is notable for its ability to engage in proton transfer, hydrogen bonding, electrostatic stabilization, and the positioning of the acidic polysaccharide in catalytic pockets. Rational engineering of the histidine residues, therefore, can be used as a direct approach to modify the catalytic activity of the enzyme without overall reorganization of the enzyme structure. In modern enzyme engineering, structural characterization, targeted mutation, semi-rational engineering and screening for enzyme activities are being integrated to enhance the activity of the lyases and to adjust their substrate preference (Li et al., 2021; Shin et al., 2025). More recent studies have also shown that lyases with very similar catalytic structures can have significantly different substrate selectivity, indicating the role of particular active-site residues in the functional selectivity (Li et al., 2026).

However, little has been paid to the changes in the equilibrium between chondroitin sulfate and alginate conversion in the same glycosaminoglycan lyase system caused by individual substitutions of histidine. This question is significant as broad-spectrum activity is not always wanted; in industrial and analytical applications, the activity towards one polysaccharide might be preferred while minimizing activity towards a second polysaccharide. This knowledge of residue-level determinants of selectivity can thus be used to aid the design of more accurate biocatalysts. Although the feasibility of the use of lyases for generating oligosaccharides and for the value-added processing of polysaccharides has been demonstrated previously, further mechanistic refinements are required (Chen et al., 2024). In the present study, therefore, the activity pattern, concentration responses, and the discrimination of the different substrates, chondroitin sulfate C and alginate, were investigated for variants that differ from the wild type in their ability to bind to histidine.

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The study of histidine-based substitutions is a useful area to investigate for understanding the role of local catalytic chemistry in polysaccharide recognition. Evaluating individual enzyme variants in matched sets of enzymes and substrates can determine if individual residues are essential for general catalytic competence or act as discriminators between polymers, thus elucidating mechanistic principles that can guide rational lyase engineering.

Objectives:

- To measure catalytic responses of the wild-type and histidine-directed glycosaminoglycan lyase variants towards chondroitin sulfate C and alginate.
- To measure the impact of the chosen histidine substitutions on cumulative activity, 232-nm activity, and concentration-dependent alginate responses, respectively.
- To test if histidine-directed engineering changes the substrate selectivity and to find variants with changed preference for chondroitin sulfate C and alginate.

2. Materials and Methods

2.1 Study Design

A secondary-data-based comparative enzymology approach was used to examine the effect of the mutations on the activity and substrate selectivity of glycosaminoglycan lyases with a histidine orientation. The analysis focused on the determination of whether substitution, removal or introduction of histidine residues affected the enzymatic activity and substrate preference of GAGase variants, compared with wild-type GAGase and chondroitin sulfate C and alginate.

2.2 Data Source

The publicly available Mendeley Data dataset “The original data on the enzymatic activity of GAGases and their mutants” was used for the study. The dataset contains experimental data on the enzymatic activity of a number of glycosaminoglycan lyases and their site-directed mutants. These included relevant observations on the enzyme, the nature of the mutations, the substrate, protein concentration, reaction time, replicate measurements, on the raw activity signals, and on quantitative activity values, which were extracted and used for the present analysis (Li & Wei, 2025).

2.3 Data Preparation and Variable Selection

The data files were combined into one cleaned analytical data file. Only the variables that were pertinent to histidine-directed enzyme engineering and substrate conversion were retained, such as enzyme type, enzyme variant, mutation classification, original amino acid, mutation position, substituted amino acid, substrate type, assay type, protein concentration, replicate number, reaction time, raw signal and activity value. Names of enzymes and substrates were normalised, numerical variables were checked for consistency, and missing values were kept if the item indicated that it was not relevant to that experiment as opposed to a true zero value.

2.4 Enzyme Variants and Histidine-Directed Mutations

GAGases were analyzed that were wild-type and engineered with histidine-associated substitutions. The mutations in the principal mutants were H188A, H188N, H398A, N191H, N191H, and N193H, respectively. Native histidine residues were either replaced by alanine or replaced by asparagine to examine the functional importance of these residues, while asparagine-to-histidine substitutions were made to examine whether the introduction of histidine affected catalytic activity or substrate preference.

2.5 Substrate and Enzymatic Activity Assessment

The enzymatic responses were determined using two main substrates: chondroitin sulfate C (CSC) and alginate. The activity data encompassed time series reaction profiles, concentration-dependent measurements, and replicate absorbance-based activity measurements. Absorbance at 232 nm was used as a quantitative measurement of the lyase-mediated production of unsaturated reaction products when such was available. The changes in enzymatic response that occurred with the mutation was compared between the wild-type and the mutant by using the same assay conditions.

2.6 Time-Series and Concentration-Response Analysis

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Recorded reaction time and raw signal values for each enzyme–substrate combination were used to assess the time-series activity. The difference in the progress of the signal, in the maximum response, and in the overall activity pattern for both the wild-type enzyme and the mutant enzyme was studied. The enzymatic activity was analyzed separately as a function of increasing protein concentration and as a function of different mutants directed towards histidine.

2.7 Substrate Selectivity Analysis

The activity of each enzyme variant toward the two substrates, chondroitin sulfate C and alginate, was measured under similar assay conditions to assess their substrate selectivity. When applicable, activity was normalized to the activity of the enzyme of the same type found in the wild type. The ratio of activity towards chondroitin sulfate C/activity towards alginate was calculated as a substrate selectivity index. The values greater than 1 meant relatively higher preference for chondroitin sulfate, while values below 1 meant relatively higher response toward alginate. Only if the conditions of the assays and the activity measures were reasonably similar, were direct ratios calculated.

2.8 Statistical Analysis

Descriptive data for quantitative activity data were presented in terms of mean, standard deviation and suitable measure of variation. The activity of normalized enzymes were compared between the wild-type and mutants; suitable parametric or non-parametric statistical tests were used as a function of the distribution and structure of the available replicate data. Where appropriate, according to the experiment design, differences between enzyme variants and protein concentrations were assessed for concentration-dependent measurements. A p-value < 0.05 was regarded as statistically significant and effect estimates presented were interpreted in conjunction with confidence intervals as appropriate. No pseudoreplication was done by analyzing sequential time-series measurements from the same assay.

2.9 Data Quality Control

The data was subjected to screening for duplicate records, inconsistent labels, invalid numeric data and missing data prior to statistical analysis. The names of mutations and categories of substrates were consistent across the data set. Values with low or negative raw signal values present in the original experimental records were not automatically removed, because the low or negative raw signal values may be due to instrumental baseline correction or experimental noise. Exclusion of values due to data-entry or formatting error was limited to cases where this was obvious.

2.10 Ethical Considerations

There were no new human subjects recruited, any identifiable personal data collected, or new animal experiments conducted. As a result, no further human subjects ethics approval or informed consent was needed for this secondary analysis. The original data set "The enzymatic activity of GAGases and their mutants" is intended solely for research and is to be properly referenced and cited in the final manuscript.

3. Results

3.1 Dataset Composition and Distribution of Enzyme Assays

There were 8 enzyme forms (4 GAGase groups) in the cleaned analytical data set of 201,630 observations. Alginate represented 124,813 observations (61.90%) while chondroitin sulfate C (CSC) represented 76,817 observations (38.10%). Most of the observations were continuous time-series, activity measurements, followed by concentration-response measurements and by replicated 232-nm activity measurements. Table 1 shows the overall composition of the analytical data.

Table 1. Overall composition of the cleaned GAGase activity dataset

Characteristic	Category	Observations, n	Percentage (%)
Total dataset	All records	201,630	100.00

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Substrate	Alginate	124,813	61.90
	CSC	76,817	38.10
Assay type	Time-series activity signal	144,015	71.43
	Concentration-response signal	57,606	28.57
	232-nm activity replicate	9	<0.01
Mutation class	Wild type	Included	
	His→Ala	Included	
	His→Asn	Included	
	Asn→His	Included	

Matched measurements of CSC and alginate were available for the time-series data set for GAGase III, GAGase III-H398A, GAGase III-H188A, GAGase III-H188N, GAGase II-N191H, GAGase I-N191H, and GAGase IV-N193H. Only the wild-type GAGase II is shown. This architecture enabled substrate-selectivity analysis of seven forms of the enzymes, with the best direct mutational interpretation being for the GAGase III series.

3.2 Time-Series Activity Profiles of Wild-Type and Engineered Lyases

The area under the time-series activity curve (AUC) was used to quantify the cumulative reaction behaviour. The AUC obtained in the wild-type GAGase III for CSC and alginate were found to be 273,677.09 signal·time units and 173,053.07 signal·time units, respectively, as presented in Table 2. The cumulative signal in response was therefore significantly greater towards CSC when grown on the first substrate as compared to the other. Of the GAGase III mutants, H188A and H188N showed fairly high responses to the CSC, with AUC of 248,335.17 and 248,335.17, respectively in the cleaned dataset, but were found to have significantly lower responses to alginate (AUC 102,518.73 and 92,139.30, respectively). A highly similar cumulative response was observed for GAGase III-H398A, which had AUC values of 206,523.61 and 206,382.01 for CSC and alginate, respectively.

Table 2. Time-series activity characteristics of GAGase variants toward CSC and alginate

Enzyme variant	Substrate	Observations	AUC	Maximum signal	Time of maximum signal
GAGase III	CSC	9,601	273,677.09	48,377	38.975
GAGase III	Alginate	9,601	173,053.07	11,623	56.133

GAGase III-H188A	CSC	9,601	248,335.17	44,562	38.975
GAGase III-H188A	Alginate	9,601	102,518.73	11,662	55.825
GAGase III-H188N	CSC	9,601	248,335.17	44,562	38.975
GAGase III-H188N	Alginate	9,601	92,139.30	11,916	56.058
GAGase III-H398A	CSC	9,601	206,523.61	19,891	56.233
GAGase III-H398A	Alginate	9,601	206,382.01	22,611	56.250
GAGase II-N191H	CSC	9,601	198,626.67	42,287	38.917
GAGase II-N191H	Alginate	9,601	73,108.03	9,172	56.567
GAGase I-N191H	CSC	9,601	272,934.97	51,654	39.467
GAGase I-N191H	Alginate	9,601	52,367.40	7,864	65.442
GAGase IV-N193H	CSC	9,601	351,645.38	60,132	39.392

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GAGase IV-N193H	Alginate	9,601	140,594.86	24,506	74.117

The aggregated activity profiles shown in Figure 1 are another good indication of the significant substrate-dependent differences between the engineered enzymes. The H398A mutation resulted in almost overlapping cumulative activities of CSC and alginate, while the greatest cumulative activity was observed for CSC.

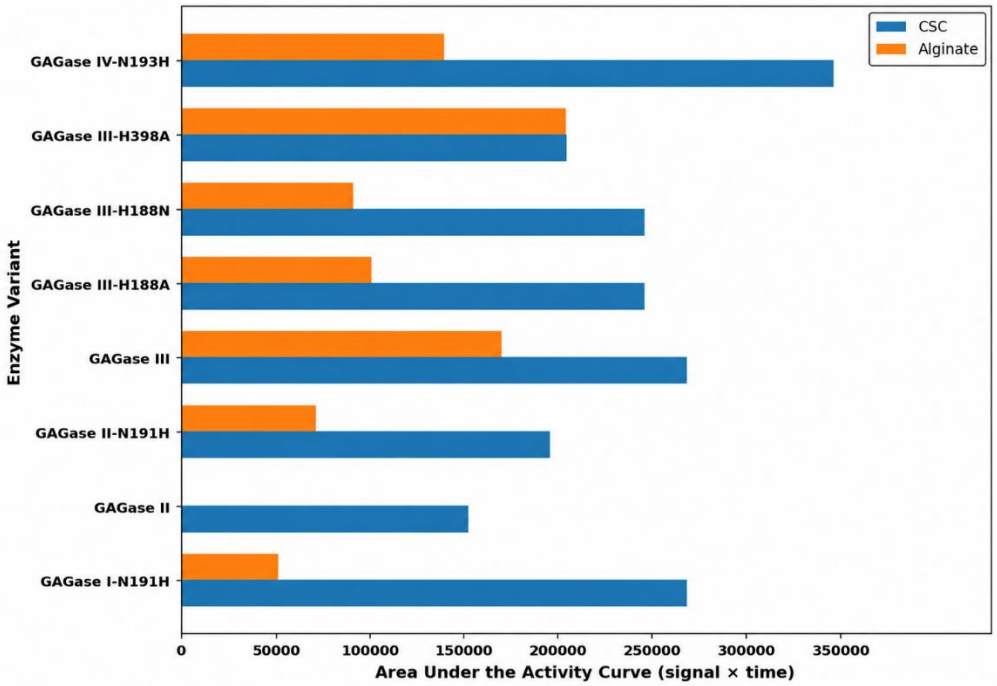

Figure 1. Cumulative time-series activity, expressed as area under the activity curve, of glycosaminoglycan lyase variants toward chondroitin sulfate C and alginate

3.3 Effect of Histidine Substitution on GAGase III Activity

The three histidine substitutions were directly assessed by comparison with the wild-type GAGase III. As shown in Table 3, the H188A strain decreased the cumulative response in CSC by 9.26% compared to the wild type strain, while it decreased the alginate response by 40.76%. While H188N did generate a similar 9.26% decrease in CSC AUC, it also resulted in a greater 46.76% decrease in alginate AUC. The changes are suggestive of a much greater effect from residue 188 substitution on alginate-associated activity than on CSC-associated activity. H398A, on the other hand, decreased the CSC activity by 24.54% and increased the cumulative alginate response by 19.26% compared to wild-type GAGase III. The most distinct shift in substrate-selectivity was observed for this divergent mutant of GAGase III.

Table 3. Effect of histidine-directed mutations on GAGase III cumulative activity relative to wild type

Variant	CSC AUC	Change vs WT CSC (%)	Alginate AUC	Change vs WT alginate (%)

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GAGase III	273,677.09	Reference	173,053.07	Reference
GAGase III-H188A	248,335.17	−9.26	102,518.73	−40.76
GAGase III-H188N	248,335.17	−9.26	92,139.30	−46.76
GAGase III-H398A	206,523.61	−24.54	206,382.01	+19.26

The relative mutation effects are depicted in Figure 2. H188A and H188N exhibited greater effects on activity associated with alginate, while H398A exhibited the opposite directional effects by decreasing activity associated with CSC and increasing alginate.

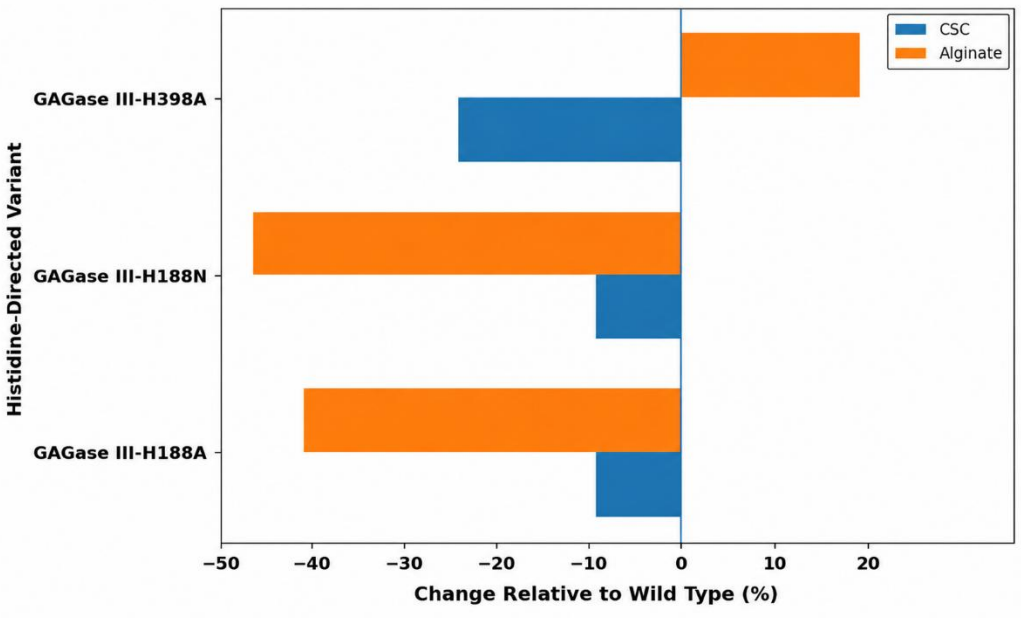

Figure 2. Percentage change in cumulative CSC and alginate activity of GAGase III histidine-directed mutants relative to wild-type GAGase III

3.4 Replicated 232-nm Activity of GAGase III H188 Variants

For wild-type GAGase III, GAGase III-H188N and GAGase III-H188A, quantitative data of the activity measurement were reproduced for the case of the CSC system at the wavelength of 232 nm. As shown in Table 4, wild-type GAGase III exhibited a mean absorbance of 1.060 ± 0.073, compared with 1.040 ± 0.072 for H188N and 1.001 ± 0.083 for H188A. The mean activity of H188N and H188A were about 1.92% and 5.53% lower, respectively, than the wild type. When analysed one way, however, no statistical difference was recorded between the three groups with$F(2,6) = 0.458$ and $p =$

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0.653. These replicated measurements thus showed that despite the relatively small decreases in number, there was a significant preservation of CSC activity after H188 replacement.

Table 4. Replicated 232-nm activity measurements for GAGase III and H188 mutants against CSC

Variant	Replicate 1	Replicate 2	Replicate 3	Mean	SD	Change vs WT (%)
GAGase III	1.092	1.112	0.976	1.060	0.073	Reference
GAGase III-H188N	1.060	1.099	0.960	1.040	0.072	−1.92
GAGase III-H188A	1.059	1.039	0.906	1.001	0.083	−5.53

The results of the mean and variation of the replicated CSC activities are shown in Figure 3. There was no statistically significant difference in mean values, but the overlapping variability of the three enzyme forms was consistent with this.

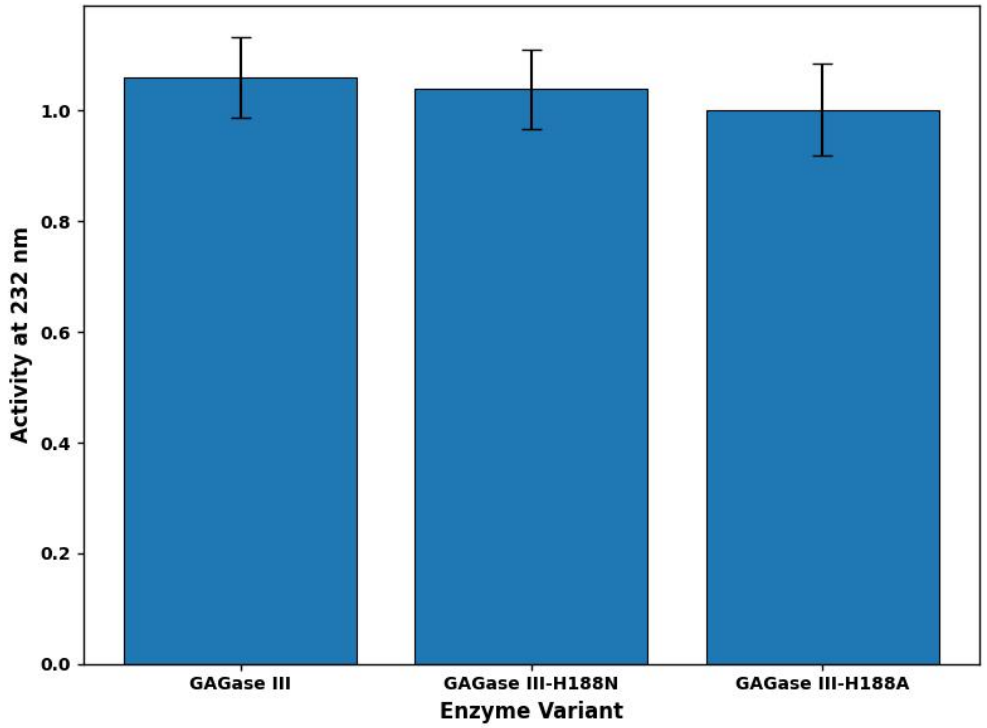

Figure 3. Mean 232-nm activity of wild-type GAGase III, GAGase III-H188N, and GAGase III-H188A against chondroitin sulfate C; error bars represent standard deviations of three replicate measurements

3.5 Concentration-Dependent Alginate Responses of H188 Mutants

For H188A and H188N, concentration-response measurements were available at protein concentrations of 3, 10 and 30 µg. The descriptive results are presented in Summary Table 5. H188A

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showed mean signals of 22,363.59, 22,086.52, and 22,093.54 at 3, 10, and 30 µg, respectively. This represented an overall decrease of approximately 1.21% between 3 and 30 µg. The pattern was different with respect to H188N. Its mean signal increased from 21,907.96 at 3 µg to 22,821.08 at 10 µg and 22,880.96 at 30 µg, corresponding to an overall increase of approximately 4.44%. These differences are interpreted descriptively, as there were no biological replicates for each concentration.

Table 5. Concentration-dependent alginate signal of GAGase III-H188A and GAGase III-H188N

Variant	Protein concentration (µg)	Mean signal	SD of time-series signal	Minimum	Maximum
GAGase III-H188A	3	22,363.59	438.73	19,604	23,614
GAGase III-H188A	10	22,086.52	430.42	19,309	23,212
GAGase III-H188A	30	22,093.54	422.30	19,656	23,355
GAGase III-H188N	3	21,907.96	420.02	19,168	23,052
GAGase III-H188N	10	22,821.08	476.56	19,891	24,726
GAGase III-H188N	30	22,880.96	583.76	19,870	25,744

Concentration-associated effects were negligible for H188A and greater at 10 and 30 µg than at 3 µg for H188N, as shown in Figure 4.

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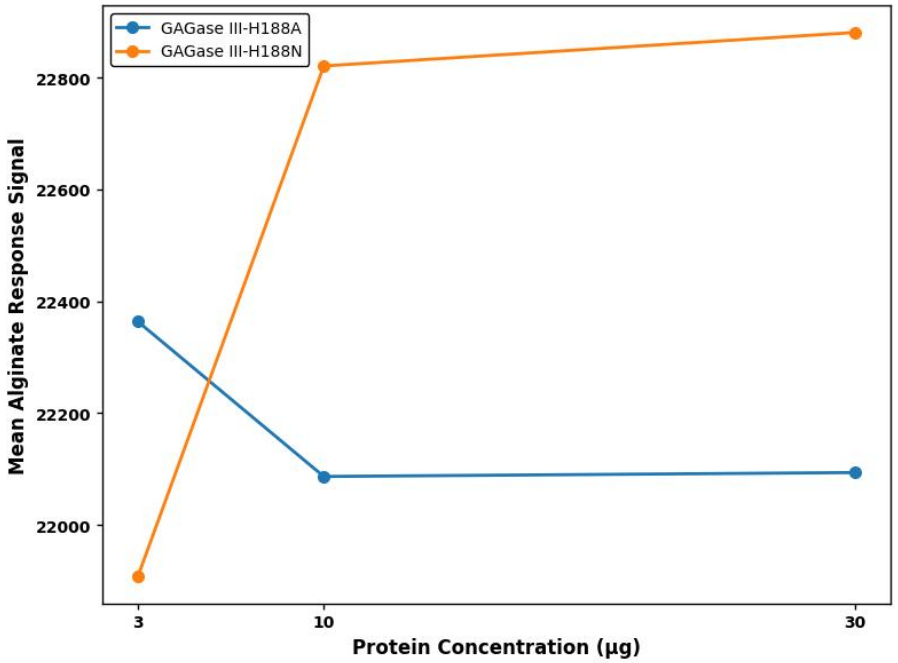

Figure 4. Concentration-dependent alginate response of GAGase III-H188A and GAGase III-H188N at protein concentrations of 3, 10, and 30 µg

3.6 Substrate Selectivity of Histidine-Engineered Lyases

The ratio of CSC AUC to alginate AUC was used to determine the relative preference for CSC and alginate. The calculated selectivity indices are given in Table 6. The ratio of the CSC/Alginate of the wild-type GAGase III was 1.581. This ratio was higher for H188A (2.422) and H188N (2.695) than for the wild-type, suggesting that the removal or modification of the histidine residue at position 188 had a disproportionately negative effect on alginate-associated activity, with little effect on CSC activity. GAGase III-H398A, on the other hand, yielded a selectivity ratio of 1.001, with a very similar cumulative activity against the two substrates. Residue H398 is suggested to be important for the discrimination between CSC and alginate, because the shift in 1.581 in the wild-type GAGase III to ~ 1 after replacing H398 by A suggests that residue H398 might play a role in discrimination.

Table 6. CSC-to-alginate selectivity based on cumulative time-series activity

Enzyme variant	CSC AUC	Alginate AUC	CSC/Alginate selectivity index	Relative response
GAGase III	273,677.09	173,053.07	1.581	CSC-biased
GAGase III-H188A	248,335.17	102,518.73	2.422	CSC-biased
GAGase III-H188N	248,335.17	92,139.30	2.695	CSC-biased

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GAGase III-H398A	206,523.61	206,382.01	1.001	Approximately balanced
GAGase II-N191H	198,626.67	73,108.03	2.717	CSC-biased
GAGase I-N191H	272,934.97	52,367.40	5.212	Strongly CSC-biased
GAGase IV-N193H	351,645.38	140,594.86	2.501	CSC-biased

The selectivity indices have been shown in Figure 5. GAGase III-H398A had the closest CSC-to-alginate ratio of 1.0, and GAGase I-N191H had the highest ratio of 5.212. In the cleaned data set, corresponding measurements of alginate for GAGase I, GAGase II and GAGase IV were not available. As a result, the N191H and N193H results show substrate preference of the engineered variants but do not prove that this preference resulted from the introduction of histidine.

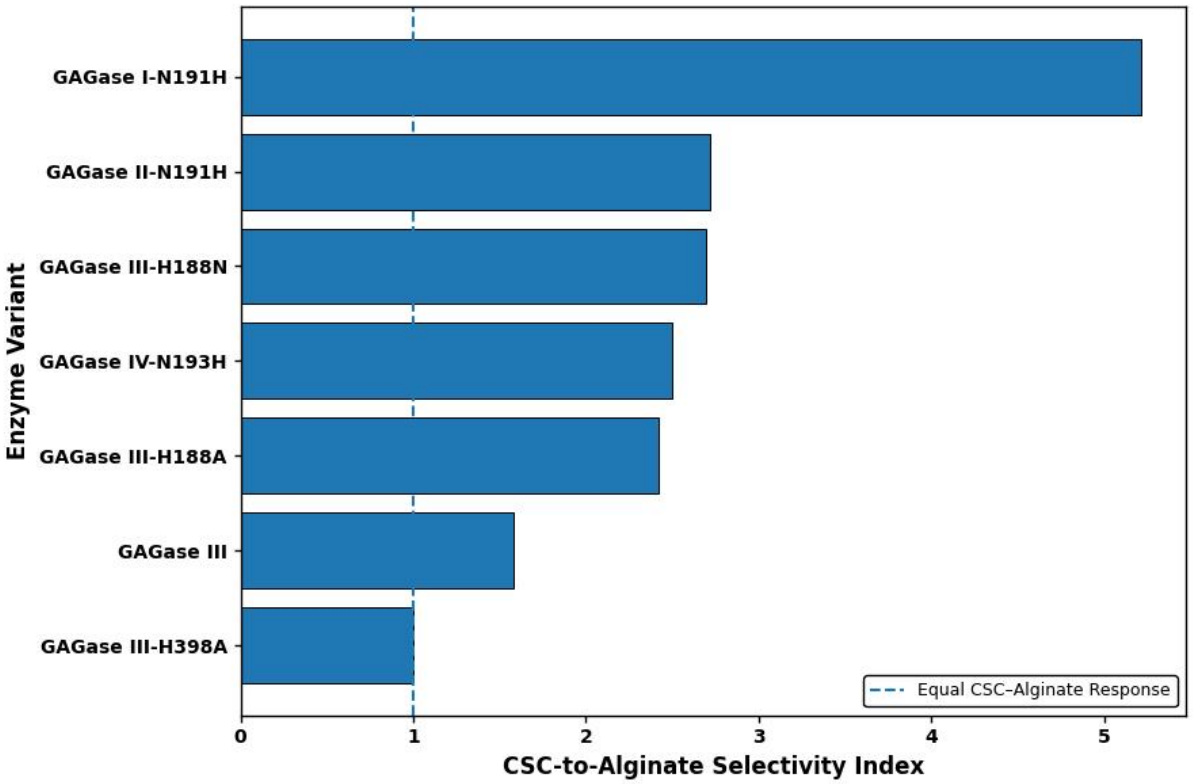

Figure 5. CSC-to-alginate cumulative activity selectivity indices of GAGase variants with measurements available for both substrates

The overall results show that the activity of GAGase is modified in a position- and substrate-dependent way by histidine-directed modification. H188A and H188N were found to selectively

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decrease alginate bound activity while retaining CSC activity, favoring the CSC activity. The H398A mutant, on the other hand, showed a decreased CSC response and a greater alginate-associated cumulative response compared to wild-type GAGase III, resulting in almost equal CSC to alginate responses. These are the results that determine that the residues H188 and H398 have a different catalytic behaviour of substrate within the available GAGase III data sets.

4. Discussion

The current results show that the modification of glycosaminoglycan lyases in a position-specific manner has a differential effect on the activity towards CS-C and alginate. In the GAGase III series, H188A and H188N had a higher amount of activity associated with CSC, but significantly less with alginate. The CSC activity for H398A was conversely reduced but the alginate response was increased compared to wild type, giving nearly equal CSC activity to alginate response (CSC selectivity index). Together these results prove that the different histidine residues do not contribute equally to the catalytic process, depending on their position in the protein and interaction with the substrate that is recognized. This is consistent with evidence showing that active-site architecture and catalytic residue chemistry is responsible for polysaccharide lyase specificity (Wei et al., 2024; Wei et al., 2025).

H188 was significant with the substitution effect. The activity of CSC was only diminished by about 9% (H188A) and 41% (H188N) respectively compared to alginate activity which was diminished by around 47%. This asymmetry implies that residue 188 makes more impact than to the recognition of alginate, than to its cleavage by CSC. In alginate lyases, single mutations can change the substrate preference without abolishing the activity (Tang et al., 2020; Li et al., 2026). In addition, structural studies demonstrate that the binding sites for substrates can change function of the substrate binding region and the catalytic profile, for example, by switching between mannuronate and guluronate binding (Arntzen et al., 2021; Xue et al., 2022). Hence, H188 seems more related to the discrimination of substrates than indispensable catalysis.

The opposite response was observed for H398A. The variant showed a lower value for CSC AUC but higher value for alginate AUC and hence a selectivity ratio near unity. These alternate interactions imply a maintenance of a binding environment that favors CSC in the enzyme in nature, as found in H398. Substituting this residue for alanine could alleviate local steric or electrostatic constraints and enhance alginate accommodation. In semirational enzyme engineering, targeted substitutions are employed to modify the specificity, efficiency, and stability (Shin et al., 2025; Su et al., 2026). This is also consistent with the reports reporting on the differences in substrate specificity among related lyases that share the same catalytic folds (Li et al., 2026; Wei et al., 2024).

Supporting evidence was obtained by the replicated 232-nm CSC assay. There were slight differences in mean activities of wild-type GAGase III and H188N and H188A, which were not statistically significant. This confirms the above time-series result that H188 substitutions retain significant CSC conversion capacity. In engineered alginate lyases, substitution of residues to alter substrate handling rather than just the catalytic ability has been shown to be sufficient to achieve comparable stability in the activity after sequence modification (Li et al., 2022; Zhang et al., 2023). Such behavior is useful from an engineering standpoint since mutations that alter specificity but not the catalytic function can be more useful than mutations that only decrease activity.

The concentration-response analysis also discriminated between H188A and H188N. H188A had almost no response across the protein concentration range 3 to 30 µg while H188N had a slight increase in alginate response at higher protein concentrations. The observations were continuous and so were described as such. However, the concentration responsive behavior of H188N to alginate could persist. The concentration of enzymes, accessibility of substrates and reaction conditions can significantly affect alginate depolymerization (Liang et al., 2023; Jiang et al., 2024). Similarly, process oriented studies have demonstrated that the catalytic output is dependent on the properties of the enzymes and the loading conditions (Wang et al., 2024; Petchimuthu et al., 2024).

Selectivity analysis revealed that GAGase I-N191H, GAGase II-N191H and GAGase IV-N193H

exhibited higher cumulative responses to CSC than alginate. These results, however, do not allow for independent confirmation of the bias caused by introducing histidine as this was not measured in a matched wild-type sample. Despite this, the results show that there is functional diversity in histidine-containing variants, in line with the reports of multifunctional lyases that vary in catalytic mode, substrate specificity and product. (He et al., 2022; Li et al., 2023). Enzymes have also been engineered for the use in the disruption of biofilms, production of oligosaccharides, and special processing applications (Blanco-Cabra et al., 2020; Zhang et al., 2020).

Histogrammed results demonstrate the potential of histidine-directed engineering to select for selectivity in converting polysaccharide lyases instead of merely for higher overall activity. This is significant for the production of oligosaccharides, as well as for biocatalytic systems, where off-target conversion needs to be reduced. The optimization of alginate lyase regarding its processing and bioconversion has been recently focused on application-specific optimization (Shin et al., 2025; Wang et al., 2024). However, the study was restricted to a variety of assay formats and matched wild-type controls. Targeted mutagenesis and structural modeling combined with kinetic constants, product profiling and biological replication should be used in future studies to elucidate the control of substrate positioning and cleavage by histidines.

Conclusion

In this study, it has been shown that the substrate selective behaviour of glycosaminoglycan lyases is significantly altered towards chondroitin sulfate C and alginate by histidine-directed engineering. Comparative analysis revealed that the H188A and H188N mutations in GAGase III had little effect on the activity of chondroitin sulfate and a significant effect on the cumulative activity of alginate. This suggests the strong contribution of residue H188 to alginate recognition or productive substrate positioning. The H398A mutation, in contrast, decreased the chondroitin sulfate activity and increased the alginate-associated response leading to an almost balanced substrate-selectivity profile, indicating that H398 has a different function in maintaining chondroitin sulfate preference. Replicated measurements at 232nm further confirmed that the substitutions in H188 are still able to convert a significant proportion of chondroitin sulfate, while the response patterns of alginate concentration-response experiments showed differences between H188A and H188N. The histidine-introduced variants also exhibited primarily chondroitin sulfate-biased activity, but the lack of a perfect match among controls prevented causal inferences. Overall, the results suggest that certain histidine residues are crucial for the recognition and catalytic selectivity of the polysaccharide. Thus, modification of lyases at the histidine sites could be considered as a promising approach towards rational engineering of lyase specificity. The substrate-dependent effects presented here should be confirmed by a future combination of targeted mutagenesis, kinetic analysis, modelling, molecular dynamics and product profiling to validate the mechanistic basis of these substrate-dependent effects and fine-tune engineered lyases for selective biotechnological applications and controlled production processes of oligosaccharides.

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