Molecular Insight into the Role of the N-terminal Extension in the Maturation, Substrate Recognition, and Catalysis of a Bacterial Alginate Lyase from Polysaccharide Lyase Family 18

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Molecular Insight into the Role of the N-terminal Extension in the Maturation, Substrate Recognition, and Catalysis of a Bacterial Alginate Lyase from Polysaccharide Lyase Family 18

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Background: The maturation and catalysis mechanisms of the PL18 alginate lyases have not yet been reported.

Results: The N-terminal extension in the precursor of PL18, aly-SJ02, helped the catalytic domain fold correctly. Key residues for substrate recognition and catalysis were determined.

Conclusion: The catalytic mechanism of aly-SJ02 is proposed.

Significance: This study provides the foremost insight into maturation and catalysis of PL18 alginate lyases.

Bacterial alginate lyases, which are members of several polysaccharide lyase (PL) families, have important biological roles and biotechnological applications. The mechanisms for maturation, substrate recognition, and catalysis of PL18 alginate lyases are still largely unknown. A PL18 alginate lyase, aly-SJ02, from Pseudoalteromonas sp. 0524 displays a β-jelly roll scaffold. Structural and biochemical analyses indicated that the N-terminal extension in the aly-SJ02 precursor may act as an intramolecular chaperone to mediate the correct folding of the catalytic domain. Molecular dynamics simulations and mutational assays suggested that the lid loops over the aly-SJ02 active center serve as a gate for substrate entry. Molecular docking and site-directed mutations revealed that certain conserved residues at the active center, especially those at subsites +1 and +2, are crucial for substrate recognition. Tyr353 may function as both a catalytic base and acid. Based on our results, a model for the catalysis of aly-SJ02 in alginate depolymerization is proposed. Moreover, although bacterial alginate lyases from families PL5, 7, 15, and 18 adopt distinct scaffolds, they share the same conformation of catalytic residues, reflecting their convergent evolution. Our results provide the foremost insight into the mechanisms of maturation, substrate recognition, and catalysis of a PL18 alginate lyase.

Alginate, which accounts for ~40% of the dry weight of the biomass of brown algae, is an important component of marine carbon sink (1) and plays important roles in the marine ecosystem. Alginate from seaweed is also widely utilized in the food, chemical, and pharmaceutical industries as a stabilizing, thickening, or emulsifying reagent because of its ability to form gels (2, 3). Furthermore, alginate is also synthesized as an exopolysaccharide by certain bacteria (4). The alginate biofilm produced by Pseudomonas aeruginosa is an important virulence factor during lung infections in cystic fibrosis patients (5). Alginate is a natural linear polysaccharide composed of (1,4)-linked β-D-mannurionate and its C5 epimer, α-L-guluronate. These uronic acids are arranged in a homopolymeric β-D-mannuronate block, a homopolymeric α-L-guluronate block, or heteropolymeric blocks with a random arrangement of both monomers (6).

Alginate lyases catalyze the degradation of alginate by a β-elimination of the 4-O-glycosyl bond to form a double bond between C4 and C5, producing 4-deoxy-L-erythro-hex-4-ene pyranosyluronate at the nonreducing end of the resulting oligosaccharides (7). These lyases are classified into three groups based on their substrate specificity: homopolymeric β-D-mannuronate block lyases (EC 4.2.2.3), homopolymeric α-L-guluronate block lyases (EC 4.2.2.11), and bifunctional lyases. Based on their amino acid sequence similarities, alginate lyases fall into seven polysaccharide lyase (PL) families (PL 5, 6, 7, 14, 15, 17, and 18) in the Carbohydrate-Active enZYmes (CAZy) database (8). Alginate lyases have important roles in algae (4, 9) and in marine carbon recycling. In addition, alginate lyases have important biotechnological and pharmaceutical applications (4, 10, 11).

To date, the three-dimensional structures of nine alginate lyases from families PL5, 7, 14, 15, 17, and 18 have been deter-
mined and deposited into Protein Data Bank (PDB). Based on these structures, the mechanisms for substrate recognition and catalysis of alginate lyases in PL5, 7, 14, and 15 have been elucidated. The catalytic triad, Tyr, His and Asn (Gln), is highly conserved in both PL5 and PL7 (12, 13). However, the PL5 alginate lyase A1-III utilizes Tyr246 as both the proton acceptor and proton donor during catalysis (14), whereas the PL7 alginate lyase A1-II’ uses His19 as the proton acceptor and Tyr284 as the proton donor (13, 15). Similar to A1-II’, the PL15 alginate lyase Atu3025 also uses His111 as the catalytic base and Tyr265 as the catalytic acid, although its overall structure is quite different from A1-II’ (16). The catalytic mechanism of the PL14 lyases is still largely unknown, although the structure of the PL14 lyase vAL-1 has been determined. vAL-1 exhibits endolytic activity at pH 7.0 and exolytic activity at pH 10.0, because the electric charges at the active site greatly influence the substrate binding mode (17).

For the PL18 family, only the structure of the alginate lyase from Pseudoalteromonas sp. 272 (hereafter called Aly272) has been deposited in the PDB (code 1J1T). The molecular mechanisms for substrate recognition and catalysis of the PL18 alginate lyases have not been reported. In addition, the PL18 alginate lyases have a distinct structural characteristic that differs from those in the other PL families. Most PL18 alginate lyases contain an N-terminal extension in their precursors, which is excised during enzyme maturation. The function of the N-terminal extension of the PL18 alginate lyases has never been studied, but they are predicted as a carbohydrate binding module based on sequence analysis (they showed ~30% identity to the putative chitin-binding domain of Streptomyces chiminases, which belong to family CBM 16) (18, 19).

The aly-SJ02 lyase secreted by marine Pseudoalteromonas sp. SM0524 is a bifunctional alginate lyase (20). N-terminal sequence analysis suggests that aly-SJ02 is most likely an alginate lyase belonging to the PL18 family. aly-SJ02 is a highly efficient endotype alginate lyase, releasing mainly di- and trisaccharides from alginate (20). In an engineered microbial platform for direct biofuel production from brown macroalgae, aly-SJ02 is supposed to depolymerize alginate (21). In this study, using aly-SJ02 as a model of PL18 alginate lyases, the function of the N-terminal extension in enzyme maturation was investigated by biochemical and structural analyses. The molecular mechanisms for substrate recognition and catalysis were studied by molecular dynamics simulation combined with structural and mutational analyses. Based on the results, a model for the catalysis of aly-SJ02 in alginate depolymerization is proposed.

**EXPERIMENTAL PROCEDURES**

**Gene Cloning, Protein Expression and Purification, and Site-directed Mutagenesis**—Based on the N-terminal sequence of aly-SJ02 and the conserved sequence of the PL18 alginate lyases (22), the aly-SJ02 gene was obtained by PCR and thermal asymmetric interlaced PCR (23). After verification with high fidelity Fastpfu DNA polymerase (Transgen Biotech), the nucleotide sequence of this gene was submitted to the GenBank™ database under the accession number EU548077. The aly-SJ02 gene and its truncations, including the N-terminal extension (NTE, Ala32–Ser173), the catalytic domain (CATD, Thr174–Asn400), and the precursor (Aly32–Asn400), were expressed in Escherichia coli BL21 (DE3) with pET22b, pET28a, or pGEX-4T-1 (Novagen). The recombinant proteins, which were fused with either a C-terminal poly-His tag or an N-terminal GST tag, were first purified on nickel-nitrioltriacetic acid-resin (Qiagen) or glutathione S-Sepharose 4B (GS4B; GE Healthcare) resin. The proteins were then fractionated by anion ion exchange using Source 15Q and by gel filtration with Superdex 75 resin (GE healthcare). All purified proteins were immediately stored at ~80 °C until use in further assays. The site-directed mutagenesis of aly-SJ02 was performed using the typical overlap extension PCR strategy and verified by sequencing. The mutants were expressed in E. coli BL21 (DE3), and the recombinant proteins were purified following the same protocol as the wild type.

The proteins separated by SDS-PAGE were blotted on a PVDF membrane (GE Healthcare). The N-terminal amino acid sequence of the proteins were analyzed by the Edman degradation method on an ABI Procise 491 protein sequencer at Peking University (Beijing, China). The method described by Ogura et al. (15) was used to determine whether the molecules of wild-type Aly-SJ02 and the N214C/T263C mutant had thiol groups.

**Homology Modeling, Crystalization, Data Collection, Structure Determination, and Refinement**—The homology model for mature aly-SJ02 (M-CATD, Thr174–Asn400) was modeled using Modeler 9v7 (24) with the structure of Aly272 (PDB code 1J1T) as the template. A total of 1,000 models were created. The models with favorable objective function and DOPE scores were further validated using the SAVES server and the PSQ5 server. Ultimately, one model with high quality scores was selected.

The crystals of the recombinant catalytic domain (r-CATD, Thr174–Asn400) and the recombinant precursor of aly-SJ02 were obtained using the hanging drop vapor diffusion method with 2 μl of concentrated protein mixed with 2 μl of well solution. The crystal of r-CATD (10 mg/ml) was grown in 100 mM phosphate-citrate (pH 4.2) and 40% (w/v) PEG-300, whereas the crystal of the precursor (6 mg/ml) was grown in 100 mM MES (pH 7.5), 25% (w/v) PEG-4000, and 150 mM ammonium sulfate. Before data collection, the crystals were equilibrated in a cryobuffer containing equal volumes of the reservoir buffer and 20% glycerol. The data were collected at the Shanghai Synchrotron Radiation Facility, Beamline BL17U, in a 100 K nitrogen stream, and they were processed with the HKL2000 program suite (25). The final model was built automatically by ARP/wARP in the CCP4i program package (26). Refinement of the structure was performed using the programs COOT (27) and PHENIX (28). The final model was evaluated using PROCHECK, and the structures of the r-CATD crystals and the catalytic domain in the precursor crystals (P-CATD, Thr174–Asn400) were refined to high quality (Table 1). All molecular graphics were created with PyMOL. To detect the metal ions in aly-SJ02, 40 μM aly-SJ02 in 10 mM Tris-HCl (pH 8.0) and 100 mM NaCl was subjected to atomic absorption spectroscopy analysis using an atomic absorption spectrophotometer (TAS-990, PGENERAL). The calcium, copper, iron, manganese, and
Alginate Depolymerization Mechanism of PL18 Alginate Lyase

**TABLE 1**

| Diffraction data                  | Parameters | P-CATD | r-CATD |
|----------------------------------|------------|--------|--------|
| **Space group**                  | P6122      | P6122  |        |
| **Unit cell**                    | 48,361, 48,361, 386,726 | 66,738, 66,738, 419,881 |
| **α, β, γ (°)**                  | α = 90°, β = 90°, γ = 120° | α = 90°, β = 90°, γ = 120° |
| Data collection                  |            |        |        |
| **Resolution limit**             | 50,000-1.65 (1.71-1.65) | 36,300-2.10 (2.16-2.09) |
| **Redundancy**                   | 19.1 (21.3) | 14.3 (15.9) |
| **Rmerge (%)**                   | 0.098 (0.569) | 0.158 (0.389) |
| Root mean square deviation from ideal geometry |                 |        |        |
| **Bond lengths (Å)**             | 0.10 Å     | 0.007 Å |
| **Bond angle (°)**               | 1.118 °    | 1.016 ° |
| Ramachandran plot (%)            |            |        |        |
| **Favored regions**              | 95.11 %    | 94.64 % |
| **Allowed regions**              | 4.89 %     | 4.69 % |
| **Overall B factors (Å²)**       | 29.539 Å²  | 21.004 Å² |

zinc in the sample were analyzed by atomic absorption spectroscopy.

**Protein Concentration Determination and Enzyme Assays**—
The protein concentration was determined by a BCA protein assay kit (Thermo). The alginate lyase activity was determined by monitoring the increase in absorbance at 235 nm (A235) that is caused by the production of unsaturated uronic acids as the lyase cleaves glycosidic bonds in the polymer chain (7). A mixture of 80 μL of buffer (50 mM Tris-HCl, pH 8.0), 100 μL of alginate substrate (10 mg/ml), and 20 μL of enzyme was incubated at 40 °C for 10 min. After incubation, the mixture was boiled for 5 min to terminate the reaction. One unit of enzyme activity was defined as the amount of enzyme that increased the absorbance at 235 by 0.1 per min. To determine the K_m value of aly-SJ02 and its mutants, the substrate concentration was varied from 0.06 to 6 mg/ml. The kinetic parameters of aly-SJ02 and its mutants were derived by a nonlinear regression fit directly to the Michaelis-Menten equation using the Origin8 Pro SR4 software.

**Model Docking and Molecular Dynamics Simulation**—
AutoDock 4.2 (29) was used to conduct aly-SJ02 and tetrasaccharide rigid body docking. The structure of the tetrasaccharide, a mannuronate tetramer, was downloaded from the PDB (code 4F13) (30). The P-CATD molecule was restrained within a grid box (70 × 55 × 46 Å) rectangular box under periodic boundary conditions. Sodium and chlorine ions were randomly dispersed, and they were located at least 5 Å from the model and at least 5 Å from one another. The entire system was minimized over the following 1 ns. Then the equilibrated system was subjected to a 50-ns MD simulation at 300 K. The MD simulation ran on 20 cores of the Inspur Tiansuo at the Supercomputing Center of Shandong University. The average wall clock time was ~2 h for a 1-ns simulation.

**Circular Dichroism Spectra**—
Wild-type aly-SJ02 and its mutants were subjected to circular dichroism spectroscopy assays at 25 °C on a J-810 spectropolarimeter (Jasco). The CD spectra of the aly-SJ02 proteins at a final concentration of 25 μM were collected from 250 to 195 nm at a scan speed of 200 nm/min with a path length of 0.1 cm. All aly-SJ02 proteins were in buffer containing 10 mM Tris-HCl (pH 8.0) and 100 mM NaCl.

**RESULTS AND DISCUSSION**

**Sequence Analysis of aly-SJ02**—The gene encoding aly-SJ02 was amplified from Pseudoalteromonas sp. SM0524. The open reading frame of aly-SJ02 is 1203 bp in length and is predicted to encode a PL18 precursor protein with 400 amino acids. According to the sequence alignment and a search in the NCBI Conserved Domain Database (32), the precursor of aly-SJ02 contains a signal peptide (Met1–Ala31), an NTE (Ala32–Gly155), a linker (Ser156–Ser173), and a PL18 CATD (Thr174–Tyr195) (Fig. 1A). The aly-SJ02 lyase secreted by Pseudoalteromonas sp. SM0524 only contains the CATD; the NTE is cleaved off at the bond between Gly157 and Ser158 in the linker (20). When the aly-SJ02 gene was expressed in E. coli, N-terminal amino acid sequencing of the recombinant aly-SJ02 showed that the NTE can be cleaved off at two sites in the linker: between Gly157 and Ser158 or between Asp160 and Gly161. It has been reported that the NTEs of other PL18 alginate lyases are also cleaved off in the linker region during enzyme maturation (19). The exact cleavage sites are different among these PL18 alginate lyases, although they share high amino acid sequence identities (more than 70% between one another) (Fig. 1B). The cleavage site in AlyPEEC is between Gly163 and Gly164 (18). However, AlyA has two cleavage sites, one between Ser147 and Gly148 and the other between Asp161 and Gly162, but the resulting 33- and 30.5-kDa forms showed little difference in enzymatic activity (19).
The PL7 alginate lyases contain three conserved motifs, SA2 (RXE), SA3 (YKAG), and SA4 (QHH), which compose the active center and are crucial for substrate recognition and catalysis (33, 34). According to sequence alignment and secondary structure prediction, the PL18 alginate lyases also have three similar conserved motifs. The conserved amino acid sequences in these motifs are RHEYK (SA2), YFKFGNYLQ (SA3), and QHH (SA4) (Fig. 1B).

**Structural Analysis of aly-SJ02—** In addition to the intact aly-SJ02 gene, we also expressed the precursor (Ala32–Asn400) and the CATD (Thr174–Asn400) for crystallization. The crystal structure of r-CATD (the recombinant CATD) was determined to 2.1 Å, and the crystal structure of P-CATD (the CATD in the recombinant precursor) was determined to 1.7 Å. Unfortunately, the structure of the NTE (Ala32–Gly155) in the precursor could not be solved. We also could not obtain crystals of mature aly-SJ02 (the product of the intact gene expressed in *E. coli*), most likely because it was not suitably stable for crystallization. With the PL18 alginate lyase Aly272 (PDB code 1J1T) as the initial search model, the structures of r-CATD and P-CATD were solved. Two molecules were found in the asymmetric unit of r-CATD, with one molecule rotated 180° to the other (Fig. 2). Our gel filtration analysis indicated that r-CATD was present as a monomer in solution, suggesting that the r-CATD dimer observed in the structure resulted from crystal packing. The overall structures of r-CATD and P-CATD have a root mean square deviation of only 0.202 Å. Moreover, they are nearly identical to that of Aly272 (Fig. 2, B and D), because there is only one amino acid residue difference between aly-SJ02 (S173-N400) and Aly272. Because the crystal structure of mature aly-SJ02 could not be solved, the structure of mature aly-SJ02 (hereafter called M-CATD, Thr174–Asn400) was obtained by homology modeling based on the structure of Aly272.

The CATD of aly-SJ02 shows a β-jelly roll structure composed mainly of two anti-parallel β-sheets (sheets A and B) (Fig. 2C), which is a structural motif commonly shared by PL7 and PL18 alginate lyases. The SA2, SA3, and SA4 motifs form a catalytic groove. Similar to other endo-type alginate lyases (14, 34, 35), both ends of the catalytic groove of aly-SJ02 are not blocked (Fig. 2B), indicating that aly-SJ02 is an endo-type alginate lyase. This corresponds to the previous result that aly-SJ02 released di-, tri-, and tetra-saccharides from alginate (20). Two loops cover over the catalytic groove. Atomic absorption spectroscopy analysis confirmed that a Ca²⁺ ion is present in the aly-SJ02 molecule. The structural analysis showed that this metal ion is chelated by residues Asp273, Asp381, Val283, Asn286, and Ile288, as well as a water molecule in the structure (Fig. 2E). The chelation site of Ca²⁺ is far away from the active center, suggesting that this metal ion is not directly involved in the polysaccharide degrading reaction. To determine whether the Ca²⁺ has an effect on enzyme activity, we added EDTA to...
the reaction mixture to chelate the Ca$^{2+}$ from the enzyme. The deprivation of Ca$^{2+}$ from aly-SJ02 did not cause any detectable structure changes, but it resulted in a 50% reduction in enzymatic activity (Fig. 2, F and G). This outcome indicates that the Ca$^{2+}$ in aly-SJ02 is important for maintaining enzymatic activity, even though it does not directly participate in the catalytic reaction.

**N-terminal Extension-mediated Maturation of aly-SJ02**—Unlike the alginate lyases from other families, most PL18 alginate lyases have an NTE in their precursors, which is predicted as a carbohydrate binding module in the NCBI Conserved Domain Database. However, the mature aly-SJ02 enzyme and the other characterized PL18 alginate lyases all contain only a catalytic domain. Therefore, it is impossible that the N-terminal extensions function as a carbohydrate-binding module in these enzymes during catalysis. Enzymatic activity analysis showed that the activity of r-CATD toward alginate was 20% lower than that of M-CATD, which suggests that there may be some delicate differences in the structures of the active centers of r-CATD and M-CATD. Residues Arg$^{219}$, Gln$^{257}$, His$^{259}$, Tyr$^{347}$, Tyr$^{353}$, and Lys$^{349}$, located in the active center, are conserved in the alginate lyases from both PL7 and PL18 families, indicating that they may be crucial for substrate recognition or catalysis. We performed a detailed conformational comparison of these conserved residues in the active centers of r-CATD, P-CATD, and M-CATD. In P-CATD and M-CATD, these residues have the same conformation, suggesting that P-CATD folds correctly (Fig. 3A). For r-CATD, although these residues in molecule B of the asymmetric unit have nearly the same conformation as those in P-CATD (except a tiny swing between Arg$^{219}$ and Tyr$^{353}$ in the two molecules) (Fig. 3B), the conformation of molecule A differs greatly from P-CATD. The guanidino group in the side chain of Arg$^{219}$ in molecule A deviates 3.5 Å from that in P-CATD. Similarly, the imidazolyl group of His$^{259}$ in molecule A has a 4.0 Å swing (Fig. 3C). These conformational differences indicate that without the NTE, the CATD of aly-SJ02 may fold incorrectly, thereby affecting its activity toward alginate. Thus, the N-terminal extension in the aly-SJ02 precursor is likely to function as an intramolecular chaperone to help the orderly folding of the CATD. Our biochemical assays also support this hypothesis. When the NTE and the CATD were co-expressed with two different vectors in an *E. coli* cell, they formed a protein complex by interaction, which eluted as a single peak that appeared earlier than both the NTE and the CATD peaks in gel filtration (Fig. 3, D and E). In contrast, the NTE and the CATD, when expressed separately in *E. coli* cells, could not form a protein complex when they were mixed in vitro, which resulted in two peaks during gel filtration (Fig. 3E). Altogether, these results suggest that the NTE in the aly-SJ02 precursor may facilitate the folding of the CATD. After the folding of the precursor, the NTE is removed by a protease that cuts in the linker region, and the CATD becomes a mature enzyme.
Gating Function of the Lid Loops in aly-SJ02 for Substrate Entry—Alginate lyases typically have loops covering the active center, which are called the lid loops (17, 34, 36). A study on the lid loops of PL7 alginate lyase A1-II suggests that the flexibility of the lid loops is important for substrate accommodation in the active center (15). The lid loop of PL5 alginate lyase A1-III can move with a maximum distance of 13.4 Å between the open form (apo-enzyme) and the closed form (holoenzyme) in the orthorhombic crystal system (30). aly-SJ02 also has two lid loops (loop 1, Ala208–Gly217; loop 2, Ala260–Thr265) over the substrate-binding pocket (Fig. 4A). The B factor for the loop 2 region is quite high in the structure (Fig. 4A), suggesting that a disulfide bond formation, improved the mutant activity significantly. The activity of wild-type aly-SJ02 without DTT treatment is considered to be 100%.

FIGURE 3. Analysis of the function of the N-terminal extension of aly-SJ02 in enzyme maturation. A, conformational comparison of the conserved amino acid residues in the active centers of M-CATD (green) and P-CATD (blue). M-CATD was obtained by homology modeling based on the structure of Aly272 (PDB code 1J1T). The conserved residues in the two molecules share the same spatial conformation. B, conformational comparison of the conserved amino acid residues in the active centers of P-CATD (blue) and molecule A from the r-CATD asymmetric unit (red). Molecule A differs greatly from P-CATD in the conserved amino acid residue conformations. D, SDS-PAGE analysis of the co-expressed (Co-exp) NTE and CATD. In co-expression, the NTE (without His tag) and CATD (with His tag) formed a complex, which was purified by His tag affinity chromatography. The expressed NTE without the His tag could not bind nickel-nitrilotriacetic acid. E, gel filtration analysis of the interaction between the NTE and the CATD of aly-SJ02. The co-expressed NTE and CATD formed a complex that eluted in a single peak, and the separately expressed NTE and CATD did not when they were mixed in vitro.

FIGURE 4. Analysis of the gating function of the lid loops in aly-SJ02 for substrate entry. A, the B-factor for aly-SJ02 P-CATD. The B-factor for the loop 2 region is quite high in the structure. B, detailed view of the lid loops over the active center. Loops 1 and 2 cover the active center of aly-SJ02, and Asn214 from loop 1 and Thr263 from loop 2 represent the minimum distance between the lid loops. C, effect of DTT on the activity of the N214C/T263C mutant. A disulfide bond forms between loop 1 and loop 2 in the N214C/T263C mutant, resulting in inactivation of the mutant. The presence of DTT, which can reduce disulfide bond formation, improved the mutant activity significantly. The activity of wild-type aly-SJ02 without DTT treatment is considered to be 100%.

Gating Function of the Lid Loops in aly-SJ02 for Substrate Entry
this loop may oscillate flexibly. To investigate whether the lid loops of aly-SJ02 have a gating function in substrate entry and product release, we performed a molecular dynamics simulation for the aly-SJ02 P-CATD structure with a particular focus on these two loops. The space between loops 1 and 2 tended to oscillate in size during the 50 ns of dynamic motion (supplemental Movie S1), resulting in “open” and “closed” states. Distance measurements of the Asn214 and Thr263 side chains showed that the space between the loops can increase to 11.5 Å in the open state from 3.2 Å in the closed state, which makes it possible for an alginate molecule to enter the substrate-binding pocket of aly-SJ02. Unlike the nearly rigid body motion of the lid loop of A1-III (30), the enlargement of space between the lid loops of aly-SJ02 is mainly caused by conformational changes in the side chains of amino acids on the loops (supplemental Movie S1). As shown in Fig. 4B, the distance between Asn214 on loop 1 and Thr263 on loop 2 is the minimum between the lid loops. To further confirm the significance of the two loops for substrate entry, an N214C/T263C mutant with Asn214 and Thr263 replaced by cysteine residues was constructed to introduce a rigid interaction between loop 1 and loop 2 by the formation of a disulfide bond. No thiol groups were detected in the N214C/T263C mutant, indicating that a disulfide bond formed between the lid loops. The mutation almost completely abolished the activity of aly-SJ02, and the reduction of disulfide bond formation by the addition of DTT significantly increased the mutant activity (Fig. 4C). This result indicates that maintaining lid loop flexibility is essential for substrate entry into the substrate-binding pocket of aly-SJ02. Altogether, our results indicate that the lid loops of aly-SJ02 can alternate between open and closed states to serve as a gate for substrate entry during catalysis. In the open state, the space between the loops is large enough for alginate entry. After the substrate enters the pocket, loops 1 and 2 can move closer to one another to form the closed state, which may promote subsequent alginate lysis. As soon as the catalytic reaction is completed, the lid loops return to the open state to release the products, preparing for the next catalytic reaction.

Analysis of the Important Residues Involved in Substrate Recognition—To investigate how a substrate binds the active site of aly-SJ02, we used the program AutoDock to simulate a model of the aly-SJ02-tetrasaccharide complex without any artificial residue restrictions, except that a tetrasaccharide (a manuronate tetramer) downloaded from PDB (PDB code 4F13) was forced into the catalytic cavity of aly-SJ02. A total of 20 solutions were recorded, and only the first ranked solution, which carries the lowest binding energy reported by AutoDock, was collected for further analysis.

The subsites in aly-SJ02 are labeled with “−n” to represent those that bind the nonreducing terminus of the oligosaccharide and “+n” to represent those that bind the reducing terminus (Fig. 5A). Based on the location of the oligosaccharide in the deepest cleft in the binding pocket, the tetrasaccharide is positioned at subsites −1, +1, +2, and +3. Accordingly, the constituent alginate residues are designated as A −1, A +1, A +2, and A +3 from the nonreducing end. The cleavage would occur between A −1 and A +1 (37). Generally, the positively charged residues located in the active center of aly-SJ02 form a highly positive potential active center (Fig. 5B), which is quite adaptable for binding the negatively charged alginate and/or neutralizing the negative charge of the carboxyl groups on alginate. Uronic acid residue A −1 is accommodated at subsite −1 by residues Gln257 and Lys359. Amino acid substitution of these two residues showed that the Q355A mutant was inactive and the K364A mutant retained 85% activity, suggesting that A −1 is recognized mainly by Gln257 through a hydrogen bond with the carboxyl group and that Lys359 assisted in binding with the carboxyl group. At subsite +1, the A +1 saccharide residue is recognized by residues on SA3, SA4, L1, and L2. The carboxyl group is recognized by Gln257 and His359, O2 by Thr263, O3 by Asn216, and O4 by Thr353. Amino acid substitutions for Gln257 and His359 resulted in inactivation of the enzyme, whereas mutants of Thr263 and Asn216 still retained ~50% activity (Fig. 5C), which suggested that the conserved residues Gln257 and His359 are more important in A + 1 recognition. At subsite +2, the uronic acid residue is recognized by residues on SA2, SA3, and SA4. The carboxyl group is recognized by Arg219 and Lys349, and the O3 and O4 are recognized by His359. Substitutions of Arg219 and Lys349 generated inactive mutants, showing the importance of these residues in A + 2 recognition. At subsite +3, the carboxyl group of A + 3 is recognized by Lys223 and Tyr347. Ala substitutions of Lys223 and Tyr347 led to inactivation of the enzyme, whereas K223R and Y347F retained 13 and 76% activity, respectively. The K_m values of the active mutants above were all significantly increased (Fig. 5E), indicating that these mutations decreased the affinity of aly-SJ02 to the substrate and therefore led to a reduction in activity. Therefore, the strictly conserved residues, Arg219, Lys223 (in SA2), Gln257, His359 (in SA3), Tyr347, Lys349, and Gln355 (in SA3), mainly interact with the uronic acid carboxyl groups of the substrate. Amino acid substitutions of the conserved residues at subsites +1 and +2 resulted in inactivation of the enzyme, whereas mutations at subsites −1 and +3 still retained partial enzyme activity. These results indicate that substrate recognition in aly-SJ02 mainly happens at subsites +1 and +2, but certain secondary interactions occur at subsites −1 and +3, which is consistent with the previous result that aly-SJ02 mainly released di- and trisaccharides from alginate (20).

Residue Glu221 at SA2, which is quite conserved in both PL7 and PL18 alginate lyases, most likely has no interaction with the substrate, because the distance between them is >5 Å. However, the mutation of Glu221 to Ala or similar residues (Asp or Gln) resulted in severe activity loss (Fig. 5D). Structural analysis showed that the distance from Glu221 to Lys349 is 2.9 Å, and that from Glu221 to Arg219 is 3.7 Å, indicating that there may be interactions between them. Therefore, Glu221 is most likely essential to maintain the conformation of Lys349 and Arg219, which are both responsible for the recognition of the carboxyl group of A + 2 (Fig. 5F).

In addition, analysis of the circular dichroism spectra of aly-SJ02 and its mutants indicated that there are no detectable structural changes in the mutants (Fig. 6). Therefore, the changes in the activity and the K_m of the mutants resulted from residue substitutions rather than from overall structural changes.
Catalytic Mechanism of PL18 Alginate Lyase aly-SJ02—Alginate lyases catalyze the degradation of alginate by a $\beta$-elimination mechanism where the glycosidic $1\rightarrow4$ $O$-linkage is cleaved between subsites $+1$ and $-1$, and a double bond forms between C4 and C5 on uronic acid A $+1$ (38). In the model of the aly-SJ02-tetrasaccharide complex, the distance between C5 of A $+1$ and Tyr$^{353}$ is 3.1 Å, and the distance between O4 of A $+1$ and Tyr$^{353}$ is 3.3 Å (Fig. 5G). Mutations of Tyr$^{353}$ resulted in inactivation of the enzyme (Fig. 5D). Therefore, residue Tyr$^{353}$ most likely functions as both a catalytic base and acid during aly-SJ02 catalysis.

In summary, our results for aly-SJ02 indicated that the lid loops are critical for substrate entry; the conserved residues at the active center, Arg$^{219}$, Lys$^{223}$, Gln$^{257}$, His$^{259}$, Tyr$^{347}$, and Lys$^{349}$, recognize and stabilize the carboxyl group of the substrate; and Tyr$^{353}$ acts as both a catalytic base and acid in catalysis. Based on our results, a model for the catalysis of aly-SJ02 in alginate degradation is proposed as follows (Fig. 7): (i) When

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**FIGURE 5.** The simulated model of the aly-SJ02-tetrasaccharide complex and mutational analysis of the conserved amino acid residues in the active center. A, schematic drawing of the sugar-binding subsites in alginate lyase. The nonreducing end of the substrate is drawn on the left, and the reducing end is on the right. The cleavage site is indicated by an arrow. B, the surface model of the aly-SJ02-tetrasaccharide complex. aly-SJ02 is shown as an electrostatic surface, and the tetrasaccharide is displayed as cyan sticks. C, the amino acid residues interacting with the tetrasaccharide in the aly-SJ02-tetrasaccharide model. The residues are presented as sticks in blue, and the tetrasaccharide is shown as lines in green. D, enzymatic activities of the aly-SJ02 mutants. The activity of wild-type aly-SJ02 is considered to be 100%. E, $K_m$ values of the mutants of aly-SJ02. The $K_m$ value of wild-type aly-SJ02 is considered to be 100%. F, interactions of Glu$^{221}$ with Arg$^{219}$ and Lys$^{349}$. The distance between Glu$^{221}$ and the substrate is 5 Å. The distance from Glu$^{221}$ to Lys$^{349}$ is 2.9 Å, and that from Glu$^{221}$ to Arg$^{219}$ is 3.7 Å, suggesting interactions between them. G, the detailed amino acid environment around the cleavage site in the aly-SJ02-tetrasaccharide model. Tyr$^{353}$ is located at an appropriate site to act as both a catalytic base and acid.
Aly-SJ02 approaches an alginate chain, the lid loops are in the open state, and the negatively charged alginate chain enters the positively charged active site. The conserved residues in the active site recognize the carboxyl groups of the substrate. After the substrate is recognized in the active site, the lid loops convert to the closed state for the subsequent catalytic reaction.

**FIGURE 6.** Circular dichroism spectra of aly-SJ02 and its mutants. There were no detectable structural changes among aly-SJ02 and its mutants.

**FIGURE 7.** Schematic diagram of the catalytic mechanism of aly-SJ02 in alginate degradation. A, the chemical equation for alginate depolymerization catalyzed by alginate lyase. B, schematic representation of the catalytic mechanism of aly-SJ02. Panel (i), aly-SJ02 approaches the alginate chain and the conserved residues in the active site recognize the carboxyl groups. Panel (ii), Tyr^353^ abstracts the proton from C5, resulting in the formation of a carboxylate dianion intermediate. The positive active center stabilizes the transition state, which is a key factor for this reaction. Panel (iii), Tyr^353^ donates a proton to the glycoside bond, and the C5 carbanion begins to form a double bond with C4. Panel (iv), the glycosidic 1–4 O-linkage between A^+1^ and A^−1^ is cleaved, and a double bond forms between C4 and C5 of A^+1^.
The strong electronic attraction of the carboxylate anion makes the proton on C5 easily attacked by a base catalyst. The general base catalyst Tyr353 abstracts the proton from C5 of the uronic acid, and a transition state forms with C5 becoming a carbanion. The positively charged residues at subsite /H11001 form a positive environment to stabilize and neutralize the carbanion. The stability of the carbanion is a key factor for this reaction. (iii) Simultaneously, protonated Tyr353, as a general acid catalyst, donates the proton to the glycoside bond, whereas the C5 carbanion begins to form a double bond with C4. (iv) The glycosidic 1–4 O-linkage between A/H11001 and A/H11002 is cleaved, and a double bond forms between C4 and C5 at A/H11001. The lid loops return to the open state, and the reaction products are released.

The strong electronic attraction of the carboxylate anion makes the proton on C5 easily attacked by a base catalyst. The general base catalyst Tyr353 abstracts the proton from C5 of the uronic acid, and a transition state forms with C5 becoming a carbanion. The positively charged residues at subsite /H11001 form a positive environment to stabilize and neutralize the carbanion. The stability of the carbanion is a key factor for this reaction. (iii) Simultaneously, protonated Tyr353, as a general acid catalyst, donates the proton to the glycoside bond, whereas the C5 carbanion begins to form a double bond with C4. (iv) The glycosidic 1–4 O-linkage between A + 1 and A − 1 is cleaved, and a double bond forms between C4 and C5 at A + 1. The lid loops return to the open state, and the reaction products are released.

**Structural Comparison of Alginic Lyases from Different Families**—The carbohydrate-active enzymes have huge substrate diversity in a highly selective manner using only a limited number of available folds, indicating that they were therefore subjected to multiple divergent and convergent evolutionary events (39). A phylogenetic tree was constructed for the characterized alginic lyases in families PL5, 7, 14, 15, and 18 where the sequences fell into five branches (Fig. 8A). Although the characterized PL14 alginic lyases are from eukaryota or viruses, the other characterized alginic lyases are all from bacteria. The crystal structures of these alginic lyases adopt three different scaffolds. PL5 alginic lyase A1-III has an α/α-barrel scaffold, the alginic lyases from families PL7, 14, and 18 adopt a β-jelly roll as their basic scaffold, and PL15 alginic lyase Atu3025 has an α/α-barrel + anti-parallel β-sheet-fold (Fig. 8B). Despite the distinct structural scaffolds of PL5 A1-III and PL7 ALY-1, the catalytic residues of A1-III, Tyr246, His192, Asn191, and Arg239, share a similar spatial arrangement with the ALY-1 catalytic residues, Tyr195, His119, Gln117, and Arg72 (12). We compared the active centers of the alginic lyases from families PL5, 7 (A1-II’ as the representative), 14, and 18 (Fig. 8C). The spatial arrangements of the catalytic residues in the active centers of alginic lyases from families PL 5, 7, 15, and 18 are nearly identical, whereas in the PL14 alginic lyase ALY-1 are quite different.

**FIGURE 8.** Structural comparison of alginic lyases from different PL families. A, phylogenetic tree of characterized alginic lyases. The scale bar indicates ~20% sequence difference. The alginic lyases with crystal structures are shown in red. PL14 alginic lyases shaded in green are from viruses or eukaryota. B, overall structures of the alginic lyases. PL5 alginic lyase A1-III (1HV6) is in green, PL7 alginic lyase A1-II’ (2ZAB) in cyan; PL14 alginic lyase val-1(s) (3A0N) in yellow, PL15 alginic lyase Atu3025 (3A0O) in blue, and PL18 alginic lyase aly-SJ02 (4Q8K) in pink. The putative catalytic residues are shown as red sticks. C, conformation comparison of the conserved residues in the alginic lyase active centers. The color of the conserved amino acid residues in the active center of each lyase corresponds to that of the overall structure in B. The distances between the residues are labeled. The spatial arrangements of the catalytic residues in the active centers of alginic lyases from families PL 5, 7, 15, and 18 are approximately the same, whereas those in the PL14 alginic lyase ALY-1 are quite different.
although PL14 alginate lyase vAL-1 has a similar β-jelly roll scaffold as the alginate lyases from families PL7 and 18, the arrangement of the putative catalytic residues of vAL-1 is quite different from PL7 and 18, implying that PL14 alginate lyases may utilize a different catalytic mechanism from the other alginate lyases. Because PL14 alginate lyases are from viruses or eukaryota, they may depolymerize alginate in a different manner from bacteria.

Conclusions—The alginate lyase aly-SJ02 from the marine bacterium Pseudoalteromonas sp. SM0524 is a member of the PL18 family. Mature aly-SJ02 containing only a catalytic domain displays a β-sandwich fold. The NTE in the aly-SJ02 precursor may help mediate protein folding during maturation. Structural and mutational analyses revealed that certain conserved residues at subsites +1 and +2 that stabilize or neutralize the negative charge of uronic acid are essential for substrate recognition. Moreover, these analyses indicated that Tyr353 may function as both a catalytic base and acid. A model involving a four-step elimination reaction for alginate degradation catalyzed by aly-SJ02 is proposed based on our results. Our study provides the foremost insight into the maturation, substrate recognition, and catalysis mechanisms of a PL18 alginate lyase, which are helpful for further study of the biological roles and biotechnological applications of the PL18 alginate lyases.

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