Crystal Structure of Family 14 Polysaccharide Lyase with pH-dependent Modes of Action

Kohei Ogura, Masayuki Yamasaki, Takashi Yamada, Bunzo Mikami, Wataru Hashimoto, and Kousaku Murata

From the 6Division of Food Science and Biotechnology and the 5Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Gokasho, Uji 611-0011 and the 8Department of Molecular Biotechnology, Graduate School of Advanced Science of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8530, Japan

The Chlorella virus enzyme vAL-1 (38 kDa), a member of polysaccharide lyase family 14, degrades the Chlorella cell wall by cleaving the glycoside bond of the glucuronate residue (GlcA) through a β-elimination reaction. The enzyme consists of an N-terminal cell wall-attaching domain (11 kDa) and a C-terminal catalytic module (27 kDa). Here, we show the enzyme characteristics of vAL-1, especially its pH-dependent modes of action, and determine the structure of the catalytic module. vAL-1 also exhibited alginate lyase activity at alkaline pH, and truncation of the N-terminal domain increased the lyase activity by 50-fold at pH 7.0. The truncated form vAL-1(S) released di- to hexasaccharides from alginate at pH 7.0, whereas disaccharides were preferentially generated at pH 10.0. This indicates that vAL-1(S) shows two pH-dependent modes of action: endo- and exotype. The x-ray crystal structure of vAL-1(S) at 1.2 Å resolution showed two antiparallel β-sheets with a deep cleft showing a β-jelly roll fold. The structure of GlcA-bound vAL-1(S) at pH 7.0 and 10.0 was determined: GlcA was found to be bound outside and inside the cleft at pH 7.0 and 10.0, respectively. This suggests that the electric charges at the active site greatly influence the binding mode of substrates and regulate endo/exo activity. Site-directed mutagenesis demonstrated that vAL-1(S) has a specific amino acid arrangement distinct from other alginate lyases crucial for catalysis. This is, to our knowledge, the first study in which the structure of a family 14 polysaccharide lyase with two different modes of action has been determined.

The Chlorella virus enzyme vAL-1 (38 kDa), a member of polysaccharide lyase family 14, degrades the Chlorella cell wall by cleaving the glycoside bond of the glucuronate residue (GlcA) through a β-elimination reaction. The enzyme consists of an N-terminal cell wall-attaching domain (11 kDa) and a C-terminal catalytic module (27 kDa). Here, we show the enzyme characteristics of vAL-1, especially its pH-dependent modes of action, and determine the structure of the catalytic module. vAL-1 also exhibited alginate lyase activity at alkaline pH, and truncation of the N-terminal domain increased the lyase activity by 50-fold at pH 7.0. The truncated form vAL-1(S) released di- to hexasaccharides from alginate at pH 7.0, whereas disaccharides were preferentially generated at pH 10.0. This indicates that vAL-1(S) shows two pH-dependent modes of action: endo- and exotype. The x-ray crystal structure of vAL-1(S) at 1.2 Å resolution showed two antiparallel β-sheets with a deep cleft showing a β-jelly roll fold. The structure of GlcA-bound vAL-1(S) at pH 7.0 and 10.0 was determined: GlcA was found to be bound outside and inside the cleft at pH 7.0 and 10.0, respectively. This suggests that the electric charges at the active site greatly influence the binding mode of substrates and regulate endo/exo activity. Site-directed mutagenesis demonstrated that vAL-1(S) has a specific amino acid arrangement distinct from other alginate lyases crucial for catalysis. This is, to our knowledge, the first study in which the structure of a family 14 polysaccharide lyase with two different modes of action has been determined.

Chlorella viruses belong to a group of large nucleocytoplasmic DNA viruses and infect unicellular eukaryotic green algae of the genus Chlorella in a manner similar to bacteriophages. The infection cycle is as follows: (i) attachment of the virus to a host cell, (ii) digestion of the cell wall by viral enzymes, (iii) insertion of viral DNA into the host cell, and (iv) release of amplified viruses out of the host cell (1–3). Several proteins involved in the cycle have been identified. The surface protein Vp130 encoded by the Chlorella virus CVK2 attaches to cell wall of the Chlorella strain NC64A (4). In the process of viral DNA insertion, the host cell is rapidly depolarized by a virus-encoded K⁺ channel protein (5–7). Among the four stages of the infection cycle, the digestion of the cell wall in the second and final stages is rather unclear.

Chlorella possesses rigid and indigestible cell walls composed of neutral sugars, uronic acids, glucosamines, and proteins. The cell wall of Chlorella NC64A contains glucose and its derivatives, which comprise 51% of the total sugar content (8). Research on another strain, Chlorella Pbi, showed that uronic acid comprises 15–20% of the total sugar content in this strain (9). The protein vAL-1 produced by the Chlorella virus CVK2 was identified to be a cell wall material (CWM)-degrading2 enzyme (10). vAL-1 binds to Chlorella NC64A, recognizes α- or β-glucuronate (GlcA) in the CWM, and cleaves its glycoside bonds through β-elimination; in other words, vAL-1 acts as a CWM lyase (11) (Fig. 1A). Because the enzyme is expressed at the final stage and not packaged in the viral particle, it is probably crucial for cell wall digestion in the final stage of the infection cycle.

Polysaccharide lyases are classified into 21 families (PL-1 to 21) on the CAZy data base (12). The CWM lyase vAL-1 is classified into the family PL-14 on the basis of the homology of the C-terminal domain (27 kDa); the N-terminal domain (11 kDa) is specific for Chlorella virus proteins. Besides vAL-1, alginate lyases from Chlorella virus CVN1 and the eukaryote Halotis discus are also included in the PL-14 family (13–16). Alginate is a polysaccharide consisting of L-mannuronic acid and its C5 epimer L-guluronic acid (17) (Fig. 1, A and C). The three-dimensional structure has not been determined for any family PL-14 lyase, and the structural determinants involved in CWM and alginate degradation remain to be identified.

Polysaccharide-degrading enzymes have two modes of action, i.e. endo- and exotype. For exotype enzymes, the steric restriction and/or hindrance at the subsite are crucial for enzyme activity. The flexible loop, one of the structural determinants in exotype activity, plays an important role in recognizing the terminal saccharide (18–20). However, there is, to 2

Chlorella viruses belong to a group of large nucleocytoplasmic DNA viruses and infect unicellular eukaryotic green algae of the genus Chlorella in a manner similar to bacteriophages. The infection cycle is as follows: (i) attachment of the virus to a host cell, (ii) digestion of the cell wall by viral enzymes, (iii) insertion of viral DNA into the host cell, and (iv) release of amplified viruses out of the host cell (1–3). Several proteins involved in the cycle have been identified. The surface protein Vp130 encoded by the Chlorella virus CVK2 attaches to cell wall of the Chlorella strain NC64A (4). In the process of viral DNA insertion, the host cell is rapidly depolarized by a virus-encoded K⁺ channel protein (5–7). Among the four stages of the infection cycle, the digestion of the cell wall in the second and final stages is rather unclear.

Chlorella possesses rigid and indigestible cell walls composed of neutral sugars, uronic acids, glucosamines, and proteins. The cell wall of Chlorella NC64A contains glucose and its derivatives, which comprise 51% of the total sugar content (8). Research on another strain, Chlorella Pbi, showed that uronic acid comprises 15–20% of the total sugar content in this strain (9). The protein vAL-1 produced by the Chlorella virus CVK2 was identified to be a cell wall material (CWM)-degrading2 enzyme (10). vAL-1 binds to Chlorella NC64A, recognizes α- or β-glucuronate (GlcA) in the CWM, and cleaves its glycoside bonds through β-elimination; in other words, vAL-1 acts as a CWM lyase (11) (Fig. 1A). Because the enzyme is expressed at the final stage and not packaged in the viral particle, it is probably crucial for cell wall digestion in the final stage of the infection cycle.

Polysaccharide lyases are classified into 21 families (PL-1 to 21) on the CAZy data base (12). The CWM lyase vAL-1 is classified into the family PL-14 on the basis of the homology of the C-terminal domain (27 kDa); the N-terminal domain (11 kDa) is specific for Chlorella virus proteins. Besides vAL-1, alginate lyases from Chlorella virus CVN1 and the eukaryote Halotis discus are also included in the PL-14 family (13–16). Alginate is a polysaccharide consisting of L-mannuronic acid and its C5 epimer L-guluronic acid (17) (Fig. 1, A and C). The three-dimensional structure has not been determined for any family PL-14 lyase, and the structural determinants involved in CWM and alginate degradation remain to be identified.

Polysaccharide-degrading enzymes have two modes of action, i.e. endo- and exotype. For exotype enzymes, the steric restriction and/or hindrance at the subsite are crucial for enzyme activity. The flexible loop, one of the structural determinants in exotype activity, plays an important role in recognizing the terminal saccharide (18–20). However, there is, to
the best of our knowledge, no report on a polysaccharide-degrading enzyme with both endo- and exotype activity.

This study deals with the identification of the protein module crucial for CWM/alginate degradation, characterization of peculiar pH-dependent modes of action, and determination of the x-ray crystal structure of family PL-14 enzymes. The structural determinants for enzyme catalysis and modes of action are also discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic oligonucleotides utilized for PCR were purchased from Hokkaido System Science. Restriction enzymes were purchased from New England Biolabs. TALON metal affinity resin was purchased from Clontech. Mono S 10/100 GL and Superdex peptide 10/300 GL were purchased from GE Healthcare. Factor Xa was purchased from Novagen. Sodium alginate (average molecular mass, 110 kDa; M/G ratio, 56.5%/43.5%) from *Eisenia bicyclos* and Triton X-100 were purchased from Nacalai Tesque. GlcA sodium salt monohydrate was purchased from Sigma-Aldrich.

**Plasmid Construction**—To construct an *Escherichia coli* expression system for a fusion protein comprising glutathione S-transferase, vAL-1, and histidine-tagged sequences, the vAL-1 (1,047 bp) gene was inserted into the expression vector pET42b (Novagen) encoding glutathione vAL-1 gene (1,047 bp) was inserted into the expression vector pET21b (Novagen) encoding a histidine-tagged sequence. Detailed procedures for the plasmid constructions are described in the supplemental methods. To substitute Lys-197, Ser-209, His-213, Ser-219, Arg-221, Tyr-233, or Tyr-235 in vAL-1(S) with alanine or phenylalanine, site-directed mutagenesis was conducted using pET21b-vAL-1(S) as the template and pairs of synthetic oligonucleotides (supplemental Table S1) as primers with a QuickChange site-directed mutagenesis kit (Stratagene). The mutants were designed as K197A, S209A, H213A, S219A, R221A, Y233F, and Y235F. DNA manipulations were carried out according to the standard method (21).

**Overexpression and Purification**—*E. coli* strain BL21(DE3) was transformed with the constructed plasmids pET42b-vAL-1 and pET21b-vAL-1(S). *E. coli* cells harboring pET42b-vAL-1 were grown in Luria-Bertani medium (21), collected, suspended in 0.5 M NaCl and 20 mM Tris-HCl (pH 7.5) (buffer A) including 0.5% Triton X-100, and ultrasonically disrupted. The fusion protein consists of glutathione S-transferase, a linker including a Factor Xa site, vAL-1, and two histidine-tagged sequences in the linker and C terminus. After purification of the fusion protein on TALON metal affinity resin, vAL-1 was obtained through Factor Xa digestion and purification on a Mono S 10/100 GL column. The protein was dialyzed against 20 mM Gly-NaOH (Gly) (pH 8.4). After centrifugation at 20,000 × g and 4 °C for 20 min, the dialysate was used as purified vAL-1.

The vAL-1(S) mutants K197A, S209A, H213A, S219A, R221A, Y233F, and Y235F were also expressed in *E. coli* cells and purified similarly. Circular dichroism spectra of these mutants were measured by a J-720 spectropolarimeter (Jasco) in the range of 250−190 nm, confirming that there was no significant difference in secondary structures among vAL-1 (S) and mutants. The selenomethionine derivative of vAL-1(S) was expressed in *E. coli* B-834(DE3) cells (22). The derivative protein was purified as mentioned above for vAL-1(S).

**Assay of Protein Concentration and Enzyme Activity**—The protein concentration was determined by the bicinchoninic acid method (23) with bovine serum albumin as the standard or by measuring the absorbance at 280 nm assuming that *E* 280 = 1.5 corresponds to 1 mg/ml protein.

Alginate lyase activity was determined by the thioarbituric acid method (24). Considering that the absorbance at 549 nm of 0.01 μmol of β-formylpyruvic acid corresponds to 0.29 (25), 1 unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of β-formylpyruvic acid/min at 37 °C. The specific activity of vAL-1, vAL-1(S), and the mutants was assayed with a reaction mixture containing 1.0 mg/ml sodium alginate, 200 mM Hepes (pH 7.0) or Gly (pH 10.0), and 0.1 mg/ml enzyme. The kinetic parameters *K* *m* and *K* *cat* for vAL-1(S) were determined with several concentrations of sodium alginate (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mg/ml), 200 mM Hepes (pH 7.0), and 0.1 mg/ml enzyme. The sizes of products released from sodium alginate were measured by size exclusion chromatography. The products were prepared.
Structure and Function of Family 14 Polysaccharide Lyase

in a reaction mixture consisting of 10.0 mg/ml sodium alginate, 200 mM Hepes (pH 7.0) or Gly (pH 10.0), and 1.0 mg/ml vAL-1(S) incubated for different periods (0, 1, 2, 3, 4, 10, and 20 h). After the sample solutions were boiled for 10 min, and the supernatants were collected by centrifugation at 15,000 × g and 25 °C for 20 min to eliminate denatured vAL-1(S). Then 100 μl of supernatant was applied onto a Superdex peptide 10/300 GL column equilibrated with 20 mM potassium phosphate (pH 7.0).

The proteins in the gel were transferred to the membrane by electroblotting at a current of 2 mA/cm² for 40 min and then detected with a dye solution of Ponceau S. The N-terminal amino acid sequence of the protein corresponding to vAL-1(S) (27 kDa) was determined by Edman degradation with a Procise 492 protein sequencer. The exact molecular mass of each product was determined by gel electrophoresis.

The single anomalous dispersion method was used to determine the structure of vAL-1(S). The initial phase was determined with the SOLVE program using the anomalous data of the selenomethionine derivative (28). After density improvement by solvent flattening and histogram matching in the DM program (29), an initial model was built using the ARP/wARP program (30). The crystal structure of vAL-1(S) formed in solution A was determined by manual modeling with the Coot program (31) and anisotropic B-factor refinement with the SHELXL program (32). The crystal structures of GlcA-bound vAL-1(S) molecules at pH 7.0 and 10.0 were determined by molecular replacement with the ligand-free vAL-1(S) structure mentioned above as a search model, manual modeling with the Coot program, and refinement with the SHELXL program. The refinement statistics are shown in Table 1.

**RESULTS AND DISCUSSION**

**Alginate Lyase Activity of vAL-1** vAL-1 (349 amino acid residues, 38 kDa) can be divided into two N- and C-terminal regions.
vAL-1(S) shows sequence identity with other family PL-14 lyases, the recombinant vAL-1(S) expressed in E. coli cells was purified and characterized.

vAL-1(S) showed no CWM lyase activity, possibly because of the lack of binding affinity between its N-terminal domain and CWM. In contrast, vAL-1(S) degraded alginate at pH 10.0 with a specific activity of 0.10 milliunits/mg, and its activity was comparable with that of vAL-1 (0.086 milliunits/mg). This indicates that the N-terminal domain of vAL-1 is unessential for its alginate lyase activity. Surprisingly, vAL-1(S) showed 50-fold higher alginate lyase activity at pH 7.0 (1.1 milliunits/mg) than vAL-1 (0.024 milliunits/mg). The N-terminal domain is thought to hinder the expression of alginate lyase activity at neutral pH. Unlike vAL-1, vAL-1(S) showed the highest activity at pH 7.0 (Fig. 2A, right panel). A Michaelis-Menten plot showed that the kinetic parameters $k_{cat}$ and $K_m$ of vAL-1(S) at pH 7.0 were 0.039 ± 0.002 min⁻¹ and 0.10 ± 0.03 mg/ml, respectively.

Similar truncation of the N-terminal repeated region was conducted in the family PL-7 alginate lyase A1-II', although the enzyme activity was comparable between the truncated and full-length forms (33). Although it remains unclear how the N-terminal domain hinders alginate degradation at neutral pH, two possibilities are as follows: (i) In the case of the family PL-5 alginate lyase A1-III (34), the non-catalytic lid loop over the active cleft binds to the catalytic residue in the absence of substrate. The N-terminal domain of vAL-1 may also interact at neutral pH with the active site in the absence of CWM and thereby inhibit the approach of other polysaccharides. At alkaline pH, the interaction is lowered, and the alginate molecules can be accommodated at the active site. (ii) The isoelectric point of the N-terminal domain is estimated to be considerably high (pl 10). This positive charge at neutral pH may induce the tight interaction of the N-terminal domain with highly acidic alginate rather than CWM. Because alginate is a high molecular mass polysaccharide (110 kDa), the interaction between the polysaccharide and N-terminal domain prevents another alginate molecules from binding to the active site. At alkaline pH, the interaction is reduced, and the polymer is thereby degraded by the catalytic module. Anyway, further experiments are required to clarify the reaction mechanism by full-length vAL-1.

Mode of Action—To clarify the mode of action of vAL-1(S) by which it degrades alginate, the alginate degradation profile was determined by size exclusion chromatography. The degrada-

---

**FIGURE 2. Characterization of vAL-1 and vAL-1(S).** A, effect of pH on alginate lyase activity. Left panel, vAL-1; right panel, vAL-1(S). Sodium citrate (closed square), 2-morpholinoethanesulfonic acid-NaOH (open square), Hepes (closed triangle), and Gly (open circle) buffers were utilized for the assay. B, reaction profiles of vAL-1(S). Size exclusion chromatography for products obtained from the reaction mixture of vAL-1(S) and alginate incubated for several different periods (0, 1, 2, 3, 4, 10, and 20 h) at pH 7.0 (left panel) and 10.0 (right panel). Tetra, Tri, and Di indicate alginate unsaturated tetra-, tri-, and disaccharides, respectively.
tion products were detected by monitoring the absorbance at 235 nm derived from C4–C5 double bonds of unsaturated saccharides. In the absence of vAL-1(S), alginate degradation was not observed at pH 7.0 and 10.0. In the presence of vAL-1(S), several peaks corresponding to unsaturated di-, tetra-, penta-, and hexasaccharides released from alginate were observed at pH 7.0 after reaction for 2.0 h (Fig. 2B, left panel). The amount of di-, tri-, and tetrasaccharides increased with time, and they seemed to be final products. At pH 10.0, unsaturated disaccharides were detected in the early stage (3.0 h) of the reaction. As the reaction proceeded, the amount of only disaccharides increased, and other products were scarcely detected, except a small amount of trisaccharides (Fig. 2B, right panel). The trisaccharides together with disaccharides seem to be generated from alginate pentasaccharides as the minimum substrate. A small peak corresponding to products smaller than disaccharides was observed. These products are not considered to be monosaccharides because no monosaccharides were detected by thin layer chromatography (supplemental Fig. S1).

The reaction profiles at pH 7.0 and 10.0 suggest that vAL-1(S) shows pH-dependent modes of action, i.e. the enzyme tends to act endolytically on alginate at neutral pH but exolytically at alkaline pH. The modes of action were confirmed to be influenced strictly by pH and not by the reaction components, i.e. Hepes and glycine, because similar results were obtained in 200 mM Tris-HCl (pH 7.0) and ammonia-ammonium chloride (pH 10.0). In fact, the reaction profile at pH 8.5 was a combination of those at pH 7.0 and 10.0. The reaction products at pH 8.5 comprised alginate disaccharides as well as tri- and tetrasaccharides, whereas few disaccharides were included in the reaction products at pH 7.0 under the same conditions (supplemental Fig. S1). This is, to our knowledge, the first endo and exo product corresponding to products smaller than disaccharides was detected at pH 7.0 after reaction for 2.0 h (Fig. 2B, left panel). The amount of only disaccharides increased, and other products were scarcely detected, except a small amount of trisaccharides (Fig. 2B, right panel). The trisaccharides together with disaccharides seem to be generated from alginate pentasaccharides as the minimum substrate. A small peak corresponding to products smaller than disaccharides was observed. These products are not considered to be monosaccharides because no monosaccharides were detected by thin layer chromatography (supplemental Fig. S1).

The final model of vAL-1(S) shows a glove-like β-jelly roll structure composed mainly of two anti-parallel β-sheets (sheets A and B) (Fig. 3A). Sheet A consists of seven β-strands (strand A1, residues 126–130; strand A2, 164–168; strand A3, 317–326; strand A4, 196–202; strand A5, 217–224; strand A6, 230–235; and strand A7, 254–256), and sheet B consists of six β-strands (strand B1, 108–111; strand B2, 336–346; strand B3, 177–185; strand B4, 271–278; strand B5, 291–296; and strand B6, 299–307). In addition, two small β-sheets C and D are included in the structure. Sheet C contains two strands (strand C1, 135–139; and strand C2, 142–149) and sheet D contains two strands (strand D1, 281–282; and strand D2, 286–287) (Fig. 3B). Strands A3, A4, A5, A6, and two loops (loop L1, 203–216; and loop L2, 236–253) constitute a cleft over sheet A. The DALI program (35) shows that vAL-1(S) is structurally similar to some sugar-binding or degrading proteins, with a β-jelly roll as a basic scaffold. This fold is especially common to PL-7, 18, and 20 family polysaccharide lyases (Table 2).

**Structure of vAL-1(S) in Complex with GlcA**—Because the alginate monosaccharides, D-mannuronic acid, and L-guluronic acid were not available, the structure of GlcA-bound vAL-1(S) was conducted to determine the three-dimensional structure of family PL-14 enzymes and clarify the structural determinants for the modes of action.

*Structure Determination*—Crystals of vAL-1(S) were obtained in solution A containing PEG-4000, 2-propanol, and trisodium citrate. The crystal structure of vAL-1(S) was determined at 1.2 Å resolution by the single anomalous dispersion method with an R-factor of 12.9% and a free R-factor of 15.9%.

The crystal structure of vAL-1(S) shows that it comprises 485 amino acid residues, 783 water molecules, two citrate molecules, and six glycerol molecules. Two protein molecules, A and B, are included in an asymmetrical unit. Except for the N-terminal residue in molecule A, two residues in molecule B, and the 16 C-terminal histidine-tagged residues in the two molecules, all of the coordinates were built according to the 2Fo − Fc map.

The final model of vAL-1(S) shows a glove-like β-jelly roll structure composed mainly of two anti-parallel β-sheets (sheets A and B) (Fig. 3A). Sheet A consists of seven β-strands (strand A1, residues 126–130; strand A2, 164–168; strand A3, 317–326; strand A4, 196–202; strand A5, 217–224; strand A6, 230–235; and strand A7, 254–256), and sheet B consists of six β-strands (strand B1, 108–111; strand B2, 336–346; strand B3, 177–185; strand B4, 271–278; strand B5, 291–296; and strand B6, 299–307). In addition, two small β-sheets C and D are included in the structure. Sheet C contains two strands (strand C1, 135–139; and strand C2, 142–149) and sheet D contains two strands (strand D1, 281–282; and strand D2, 286–287) (Fig. 3B). Strands A3, A4, A5, A6, and two loops (loop L1, 203–216; and loop L2, 236–253) constitute a cleft over sheet A. The DALI program (35) shows that vAL-1(S) is structurally similar to some sugar-binding or degrading proteins, with a β-jelly roll as a basic scaffold. This fold is especially common to PL-7, 18, and 20 family polysaccharide lyases (Table 2).
was determined to clarify the enzyme catalysis and modes of action. Although the vAL-1(S) crystals formed in solution A were soaked in several solutions containing GlcA, GlcA was not found bound to vAL-1(S) in the crystal structure. vAL-1(S) crystals were also obtained in solution B containing PEG-3350, ammonium formate, and Hepes (pH 7.0). The crystals, belonging to the $P_{2_1}2_12_1$ space group, were soaked in GlcA-containing solution at pH 7.0 or 10.0 and used for x-ray data collection. The crystal structures of GlcA-bound vAL-1(S) at pH 7.0 and 10.0 were determined by molecular replacement with the coordinates of ligand-free vAL-1(S) mentioned above as a search model (Fig. 4 and Table 1). No significant conformational change was found in the GlcA-bound vAL-1(S) structures at the two pH levels compared with the ligand-free model; the root-mean-square deviation is 0.25 Å for all $C\beta$ atoms between the GlcA-bound and ligand-free structures.

In the crystal structure of vAL-1(S) at pH 7.0, a GlcA molecule was found bound in the vicinity of A6, A7, and L2 (Fig. 4B, upper panels). Several amino acid residues interacted with GlcA through hydrogen bonds (Gln-225 O1 to O2, 3.0 Å; Gln-225 N2 to O3, 2.7 Å; Gln-258 N2 to O4, 3.1 Å; and Gly-255 N to O6A, 2.9 Å). On the other hand, in the crystal structure of vAL-1(S) at pH 10.0, a GlcA molecule was found bound in the inner part of the cleft surrounded by A4, A5, and L1 through many hydrogen bonds (Tyr-233 O1 to O2, 2.5 Å; Tyr-235 O1 to O2, 3.0 Å; Lys-197 N to O3, 2.9 Å; Gly-328 N to O3, 3.0 Å; Tyr-211 O1 to O3, 2.8 Å; Arg-221 N1 to O5, 3.0 Å; Arg-221 N2 to O5, 2.8 Å; and Arg-221 N9 to O6B, 2.9 Å) (Fig. 4B, lower panels), although the occupancy of the sugar molecule was low (0.8), and O1 was not observed in the density map (Fig. 4A, right panel). No GlcA molecule was accommodated at the position where GlcA was bound in vAL-1(S) at pH 7.0. This result indicates that the affinity level of each subsite is dependent on pH.

pH has a significant effect on the electric charge of protein molecules. At pH 10.0, the molecule surface displays the formation of a highly positively charged region composed of Lys-197, Arg-221, Tyr-233, and

---

**TABLE 2**  
Structurally related polysaccharide lyases

| PL family | Protein Data Bank code | Protein | Z value |
|-----------|------------------------|---------|--------|
| 7         | 1VAV                   | Alginate lyase PA1167 | 12.0   |
| 7         | 2CWS                   | Alginate lyase A1-II’ | 11.8   |
| 7         | 1UAI                   | Polyguluronate lyase | 10.3   |
| 18        | 1JIT                   | Alginate lyase       | 9.5    |
| 20        | 2ZZJ                   | Glucuronan lyase A   | 14.9   |

---

**FIGURE 4.** Crystal structures of GlcA-bound vAL-1(S) molecules. A, the omit maps ($F_o - F_c$) of GlcA molecules (green mesh with a contour of 3.0 o) at pH 7.0 (left panel) and 10.0 (right panel) (stereo diagram). B, hydrogen bonds between GlcA and amino acids/water at pH 7.0 (upper panels) and 10.0 (lower panels) are represented as dashed lines (stereo diagram). The figures were drawn using MolFeat (FiatLux). C, the highly positively charged region with GlcA at pH 10.0. The electric charges of the molecular surface at pH 10.0 were calculated and drawn using APBS and Pymol.
Polysaccharide lyases are characteristic in that they commonly cleave glycoside bonds through β-elimination reaction (37), although there is great diversity in the arrangement of catalytic residues crucial for the enzyme reaction. Because the family PL-14 enzymes are homologous in their primary structures, some amino acid residues conserved in this family were considered to play an important role in the catalytic reaction. Most conserved residues were found to be positioned at strands A3–A6 and the loop L1, all of which constitute the cleft (Fig. 5A). The amino acid residues Lys-197, Ser-209, His-213, Ser-219, Arg-221, Met-223, Tyr-233, Tyr-235, and Phe-325 are located on the molecular surface of the cleft (Fig. 5B). In fact, GlcA molecules are bound to residues located in the cleft. Thus, vAL-1(S) mutants with a mutation in the cleft were constructed and enzymatically characterized. Site-directed mutagenesis was performed in Lys-197, Ser-209, His-213, Ser-219, Arg-221, Tyr-233, and Tyr-235 to evaluate their contributions to the catalytic reaction and/or substrate binding. Specific activity was measured at pH 7.0 and 10.0 for K197A, S209A, H213A, S219A, R221A, Y233F, and Y235F (Table 3). All of the mutants showed lower activity than the wild-type enzyme, indicating that an active site of vAL-1(S) is present on the cleft. The mutations of Lys-197 and Ser-219 especially led to a great decrease in enzyme activity both at pH 7.0 and 10.0, suggesting that these two residues play key roles in the catalytic reaction and/or substrate binding. Although H213A, R221A, Y233F, and Y235F retained their activity to some extent at pH 7.0, these mutants showed extremely low activity at pH 10.0, suggesting that His-213, Arg-221, Tyr-233, and Tyr-235 are important for an enzyme reaction at alkaline pH.

As is the case with the family PL-14 alginate lyase vAL-1(S), family PL-7, 18, and 20 enzymes also have a β-jelly roll structure as a basic scaffold (Table 2). Previous reports propose that the family PL-7 alginate lyase A1-II’ contains a catalytic site composed of the four residues Arg, Gln, His, and Tyr (38, 39). This arrangement of four residues is also conserved in the PL-18 alginate lyase. Gln and Tyr in the structure of the family PL-20 open substrate-binding cleft, substitution of a single amino acid residue at subsite –5 changes the binding affinity and thereby influences the endo/exo mode of action (36). In the case of vAL-1(S), the substrate binding affinity at each subsite is dependent on pH and thereby determines the mode of action.

**Mutation in the Active Cleft—**

Polysaccharide lyases are characteristic in that they commonly cleave glycoside bonds through β-elimination reaction (37), although there is great diversity in the arrangement of catalytic residues crucial for the enzyme reaction. Because the family PL-14 enzymes are homologous in their primary structures, some amino acid residues conserved in this family were considered to play an important role in the catalytic reaction. Most conserved residues were found to be positioned at strands A3–A6 and the loop L1, all of which constitute the cleft (Fig. 5A). The amino acid residues Lys-197, Ser-209, His-213, Ser-219, Arg-221, Met-223, Tyr-233, Tyr-235, and Phe-325 are located on the molecular surface of the cleft (Fig. 5B). In fact, GlcA molecules are bound to residues located in the cleft. Thus, vAL-1(S) mutants with a mutation in the cleft were constructed and enzymatically characterized. Site-directed mutagenesis was performed in Lys-197, Ser-209, His-213, Ser-219, Arg-221, Tyr-233, and Tyr-235 to evaluate their contributions to the catalytic reaction and/or substrate binding. Specific activity was measured at pH 7.0 and 10.0 for K197A, S209A, H213A, S219A, R221A, Y233F, and Y235F (Table 3). All of the mutants showed lower activity than the wild-type enzyme, indicating that an active site of vAL-1(S) is present on the cleft. The mutations of Lys-197 and Ser-219 especially led to a great decrease in enzyme activity both at pH 7.0 and 10.0, suggesting that these two residues play key roles in the catalytic reaction and/or substrate binding. Although H213A, R221A, Y233F, and Y235F retained their activity to some extent at pH 7.0, these mutants showed extremely low activity at pH 10.0, suggesting that His-213, Arg-221, Tyr-233, and Tyr-235 are important for an enzyme reaction at alkaline pH.

As is the case with the family PL-14 alginate lyase vAL-1(S), family PL-7, 18, and 20 enzymes also have a β-jelly roll structure as a basic scaffold (Table 2). Previous reports propose that the family PL-7 alginate lyase A1-II’ contains a catalytic site composed of the four residues Arg, Gln, His, and Tyr (38, 39). This arrangement of four residues is also conserved in the PL-18 alginate lyase. Gln and Tyr in the structure of the family PL-20...
β-1,4-glucuronan lyase are well superimposed on those in family PL-7 and 18 lyases, whereas His and Arg are replaced with Ile and His, respectively (40). To examine the catalytic site in the family PL-14 alginate lyases, vAL-1(S) was superimposed on family PL-7 A1-II’. Despite the common scaffold, the arrangement of the four residues is not conserved in family PL-14 alginate lyases; vAL-1(S) has Gly, Gly, Phe, and Phe at the corresponding positions, implying that family PL-14 enzymes have a different specific catalytic site (Fig. 5A). Site-directed mutants with a low activity indicated that Lys-197, Ser-209, His-213, Ser-219 are especially crucial for the enzyme reaction at both pH 7.0 and 10.0.

In conclusion, this is the first report on the molecular identification of alginate lyase, which was shown to have two pH-dependent modes of action, and structural determination of family PL-14 enzymes. Distinct from family PL-7, 18, and 20 polysaccharide lyases, this family PL-14 lyase has a specific amino acid arrangement at its catalytic site. Further, it exhibits pH-dependent modes of action, which regulate the binding affinity level with the substrate at each subsite.

Seaweed alginate is widely utilized in the food, chemical, and pharmaceutical industries as a stabilizing, thickening, or emulsifying reagent (17), whereas the polymer produced by bacteria such as Pseudomonas aeruginosa functions as an important virulence factor during lung infections in cystic fibrosis patients (41). Thus, alginate lyases hold promise as biochemicals for processing edible seaweed alginate and removing bacterial biofilm alginate (42, 43). More recently, alginate lyases have been expected to become potential enzymes in the bioenergy generation from alginate (44). The alginate lyase with two different modes of action is applicable to various fields, and the regulation of endo/exo modes by electric charges at subsites contributes to molecular design of useful polymer-degrading enzymes.

Acknowledgments—We thank the Japan Synchrotron Radiation Research Institute, especially Drs. T. Kumasaka (Beamline BL38B1), K. Baba (Beamline BL38B1), and N. Shimizu (Beamline BL41XU), for help with collecting x-ray data.

REFERENCES
1. Meints, R. H., Lee, K., Burbank, D. E., and Van Etten, J. L. (1984) Virology 138, 341–346
2. Van Etten, J. L., Lane, L. C., and Meints, R. H. (1991) Microbiol. Rev. 55, 586–620
3. Yamada, T., Higashiyama, T., and Fukuda, T. (1991) Appl. Environ. Microbiol. 57, 3433–3437
4. Onimatsu, H., Sugimoto, I., Fujie, M., Usami, S., and Yamada, T. (2004) Virology 319, 71–80
5. Pluggé, B., Gazzarrini, S., Nelson, M., Cerana, R., Van Etten, J. L., Derst, C., DiFrancesco, D., Moroni, A., and Thiel, G. (2000) Science 287, 1641–1644
6. Kang, M., Moroni, A., Gazzarrini, S., DiFrancesco, D., Thiel, G., Severino, M., and Van Etten, J. L. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 5318–5324
7. Gazzarrini, S., Kang, M., Van Etten, J. L., Tayefeh, S., Kast, S. M., DiFrancesco, D., Thiel, G., and Moroni, A. (2004) J. Biol. Chem. 279, 28443–28449
8. Meints, R. H., Burbank, D. E., Van Etten, J. L., and Lampert, D. T. (1988) Virology 164, 15–21
9. Kapaun, E., Loos, E., and Reisser, W. (1992) Phytochemistry 31, 3103–3104
10. Sugimoto, I., Hiramatsu, S., Murakami, D., Fujie, M., Usami, S., and Yamada, T. (2000) Virology 277, 119–126
11. Sugimoto, I., Onimatsu, H., Fujie, M., Usami, S., and Yamada, T. (2004) FEBS Lett. 559, 51–56
12. Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009) Nucleic Acids Res. 37, D233–D238
13. Suda, K., Tanji, Y., Hori, K., and Unno, H. (1999) FEMS Microbiol. Lett. 180, 45–53
14. Suzuki, K., Ojima, T., and Nishita, K. (2003) Eur. J. Biochem. 270, 771–778
15. Shimizu, E., Ojima, T., and Nishita, K. (2003) Carbohydr. Res. 338, 2841–2852
16. Suzuki, H., Suzuki, K., Inoue, A., and Ojima, T. (2006) Carbohydr. Res. 341, 1809–1819
17. Gacesa, P. (1988) Carbohydr. Polym. 8, 161–182
18. Meinke, A., Damude, H. G., Tomme, P., Kwan, E., Kilburn, D. G., Miller, R. C., Jr., Warren, R. A., and Gilkes, N. R. (1995) J. Biol. Chem. 270, 4383–4386
19. Proctor, M. R., Taylor, E. J., Nurizzo, D., Turkenburg, J. P., Lloyd, R. M., Vardakou, M., Davies, G. J., and Gilbert, H. J. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 2697–2702
20. Ochiai, A., Itoh, T., Mikami, B., Hashimoto, W., and Murata, K. (2009) J. Biol. Chem. 284, 10181–10189
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Double, S. (1997) Methods Enzymol. 276, 523–530
23. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goee, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 150, 76–85
24. Weissbach, A., and Hurwitz, J. (1959) J. Biol. Chem. 234, 705–709
25. Preiss, J., and Ashwell, G. (1962) J. Biol. Chem. 237, 309–316
26. Laemmli, U. K. (1970) Nature 227, 680–685
27. Ortwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
28. Terwilliger, T. C., and Berendzen, J. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55, 849–861
29. Cowtan, K. (1994) Joint CCP4 and ESF-EACBM Newsletter on Protein Crystallography 31, 34–38
30. Perrakis, A., Morris, R., and Lamzin, V. S. (1999) Nat. Struct. Biol. 6, 458–463
31. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 2126–2132
32. Sheldrick, G. M., and Schneider, T. R. (1997) Methods Enzymol. 277, 319–343
33. Miyake, O., Ochiai, A., Hashimoto, W., and Murata, K. (2004) J. Bacteriol. 186, 2891–2896
34. Mikami, B., Suzuki, S., Yoon, H.-J., Miyake, O., Hashimoto, W., and Murata, K. (2002) Acta Crystallogr. Sect. A 58, C271
35. Holm, L., Kääriäinen, S., Rosenström, P., and Schenkel, A. (2008) Bioinformatics 24, 2780–2781
36. Pages, S., Kester, H. C., Visser, J., and Benen, J. A. (2001) J. Biol. Chem. 276, 33652–33656
37. Linhardt, R. J., Galiher, P. M., and Cooney, C. L. (1986) Appl. Biochem. Biotechnol. 12, 135–176
38. Yamasaki, M., Ogura, K., Hashimoto, W., Mikami, B., and Murata, K. (2005) J. Mol. Biol. 352, 11–21
39. Ogura, K., Yamasaki, M., Mikami, B., Hashimoto, W., and Murata, K. (2008) J. Mol. Biol. 380, 373–385
40. Konno, N., Ishida, T., Igarashi, K., Fushinobu, S., Habu, N., Samejima, M., and Isogai, A. (2009) FEBS Lett. 583, 1323–1326
41. May, T. B., and Chakrabarty, A. M. (1994) Trends Microbiol. 2, 151–157
42. Wong, T. Y., Preston, L. A., and Schiller, N. L. (2000) Annu. Rev. Microbiol. 54, 289–340
43. Alkawash, M. A., Soothill, J. S., and Schiller, N. L. (2006) APMIS 114, 131–138
44. Hashimoto, W., Kawai, S., and Murata, K. (2009) Bioengineered Bugs, in press