vAL-1, a novel polysaccharide lyase encoded by chlorovirus CVK2

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Abstract Cell wall materials isolated from *Chlorella* cells were degraded by the polysaccharide-degrading enzyme vAL-1 encoded by chlorovirus CVK2. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analyses of the degradation products (oligosaccharides) revealed major oligosaccharides contain unsaturated GlcA at the reducing terminus, and a side chain attached at C2 or C3 of GlcA(C4 = C5), which mainly consisted of Ara, GlcNAc and Gal. The results indicated that vAL-1 is a novel polysaccharide lyase, cleaving chains of β- or α-1,4-linked GlcAs. The unique structures of *Chlorella* cell wall were also revealed. Studies on the complicated structures of naturally occurring polysaccharides will be greatly facilitated by using vAL-1 as a tool in structural analysis.

Key words: *Chlorella* cell wall; Chlorovirus; Polysaccharide lyase; Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; vAL-1

1. Introduction

Large icosahedral, dsDNA-containing viruses that infect certain strains of the unicellular green alga *Chlorella* (chlorovirus or *Chlorella* virus; Phycodnaviridae) [1] are ubiquitous in natural environments [2,3]. In a normal lytic cycle, virus particles attach to the surface of host *Chlorella* cells and degrade the cell wall at the point of attachment; the viral core is then released into the host cytoplasm, leaving an empty capsid on the cell wall [2]. Within 6–8 h post infection, mature, DNA-filled viral progenies exit the cells after cell lysis. Both the initial and final stages of the viral replication cycle obviously require cell wall-degrading activities, but little is known about the nature and origin of the enzymes that degrade the cell wall.

A common characteristic of virus-sensitive *Chlorella* strains is a rigid cell wall containing uronic acids and glucosamine in addition to other sugars such as glucose, rhamnose, galactose, xylose, arabinose, and mannose [4–7]. Interestingly, a lipopolysaccharide-like component was also reported for the host *Chlorella* cell wall [8]. On the genome of *Paramecium bursaria Chlorella* virus (PBCV-1), the prototype of the Phycodnaviridae, several open reading frames (ORFs) were identified to encode polysaccharide-degrading enzymes [9], including a chitosanase [10,11], two chitinases [11,12], and β-1,3-glucanase [13]. However, cells of *Chlorella* strain NC64A, the predominant laboratory host strain, did not show any significant morphological change when treated with these enzymes [12]. Therefore, other potential enzymes encoded by chloroviruses may be involved in host cell wall degradation. Recently, Sugimoto et al. [14] found that a 349-aa ORF of chlorovirus CVK2 isolated in Kyoto, Japan encodes an actual cell wall-degrading enzyme, vAL-1. vAL-1 was found to be homologous to the unknown ORFs, A215L of PBCV-1 and CL-2 of CVN1, which was previously reported as alginate lyase [15]. vAL-1 is not packaged in the viral particles so that it likely has a role in the digestion of the cell wall before viral release at the final stage of infection [14].

In this study, cell wall material (CWM) isolated from *Chlorella* cells was degraded by vAL-1 and the degradation products (oligosaccharides) analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Structures of the oligosaccharides indicate that vAL-1 is not an alginate lyase but a novel endotype polysaccharide lyase. Very unusual sugar chains of the *Chlorella* cell wall are revealed.

2. Materials and methods

2.1. Cells and virus

Cells of *Chlorella* strain NC64A [16] were cultured in a modified Bold's basal medium (MBBM) as described previously [17]. *Chlorella* virus CVK2 was isolated from natural water in Kyoto, Japan by plaque-forming assays, and the production and purification of *Chlorella* viruses were performed as described previously [3]. For cell wall preparation, *C. vulgaris* C-135 and *C. prototechoides* 211-6 were obtained from the algal culture collection of the Institute of Molecular and Cellular Biosciences, University of Tokyo and the Algal Culture Collection, Plant Physiology Institute, University of Göttingen, respectively. All of these strains were cultured in MBBM at 25°C in light.

2.2. Preparation and purification of a GST-vAL-1 fusion protein

vAL-1 protein was formed in cells of *Escherichia coli* BL21 as a glutathione *S*-transferase (GST) fusion protein (GST-vAL-1) with a plasmid pGEX-4T-3-vAL-1 as previously described [14]. The hybrid protein was purified by affinity chromatography on a glutathione-Sepharose 4B column (Amersham Bioscience, Piscataway, NY, USA). After digestion with thrombin, the vAL-1 fragment (38 kDa) was separated by affinity chromatography and for cell wall degradation experiments.

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Abbreviations: ABEE, 4-aminobenzoic acid ethyl ester; CWM, cell wall material; GST, glutathione *S*-transferase; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; ORF, open reading frame; PA, pyridylamino; PSD, post-source decay; TLC, thin-layer chromatography

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2.3. Preparation of CWM of Chlorella NC64A cells and detection of degradation products

Liter cultures of *Chlorella* cells grown in MBBM (4×10^7 cells/ml) were harvested by centrifugation at $1000\times g$ for 10 min at 4°C, washed twice with distilled water, and homogenized in a mortar and pestle under liquid nitrogen for 1 h. The homogenate was suspended in 20 ml of 1% sodium dodecyl sulfate (SDS), vortexed for 3 min at 4°C, and centrifuged at $10\,000\times g$ for 15 min at 4°C. The upper white layer of the precipitate was recovered with 20 ml of 1% SDS. This extraction was repeated several times, and the CWM was resuspended and washed twice in 5 ml distilled water. After dialysis in Seamless cellulose tubing (pore size 12–14 kDa; Nacalai Tesque, Kyoto, Japan) against distilled water, the CWM preparations were used as a substrate for vAL-1 digestion.

Three-hundred microliters of 100 mM phosphate buffer, pH 7.0, containing 3.3 mg/ml substrate was incubated with purified vAL-1 (20 μg protein) at 37°C for different periods of time. The degraded products (10–20 μg) generated by the enzyme reactions from various substrates were analyzed by thin-layer chromatography (TLC) on a silica gel 60 plate (Whatman, Kent, UK) using *n*-butanol/acetic acid/water (3:1:1) as the eluant. Sample bands were visualized by spraying with orcinol/sulfuric acid and subsequent heating at 110°C for 5 min.

2.4. Sugar composition analysis of the cell wall degradation products and detection of unsaturated uronic acids

Cell wall degradation products separated by TLC were collected from silica gel plates and extracted with distilled water. After hydrolysis with 4 M trifluoroacetic acid, monosaccharides were labeled with 4-aminobenzoic ethyl ester (ABEE) using an ABEE labeling kit (Seikagaku, Tokyo, Japan) according to the manufacturer's instructions. ABEE-converted monosaccharides were analyzed by reversed-phase high performance liquid chromatography (HPLC) with a Honenpak C18 column (75 mm×4.6 mm i.d.) (Seikagaku) [18]. Samples were applied to the column at a flow rate of 1.0 ml/min with 0.2 M potassium borate buffer (pH 8.9) containing 7% acetonitrile as a solvent at 45°C and detected by UV absorbance at 305 nm. A mixture of monosaccharides with glucuronic acid (GlcA), galacturonic acid (GalA), galactose (Gal), mannose (Man), glucose (Glc), arabinose (Ara), ribose (Rib), *N*-acetylmannosamine (ManNAc), xylose (Xyl), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), rhamnose (Rha), 2-deoxyglucose (dGlc), *N*-acetylgalactose (GalNAc), glucosamine (GlcN), galactosamine (GalN), and mannosamine (ManN) at 2 nmol each was used as standard. The identification of 4-deoxy-L-*threo*-hex-4-enopyranuronic acid (Δ4GlcA) was performed by comparison of peaking patterns with 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-0S, Kanto Kagaku, Tokyo, Japan) as a standard. After hydrogenation by a standard method [19], samples were hydrolyzed and ABEE-labeled before HPLC analysis as above. Unsaturated uronic acids produced by the lyase reaction were detected by a periodic acid/thiobarbituric acid reaction according to Weissbach and Hurwitz [20].

2.5. Pyridylamination of the oligosaccharides and MALDI-TOF mass spectrometry

Cell wall degradation products (oligosaccharides) separated by TLC were collected, extracted with distilled water, and purified with a cellulose cartridge glycan preparation kit (Takara Biomedicals, Tokyo, Japan). The purified oligosaccharides were pyridylaminated with a PALSTATION pyridylamination kit (Takara Biomedicals). The pyridylamino (PA)-derivatized oligosaccharides were separated by reversed-phase HPLC with a PALPAK type R column (250 mm×4.6 mm i.d.) (Takara Biomedicals). The sample was applied to the column at a flow rate of 0.9 ml/min with a solvent system of 50 mM acetic acid/triethylamine (pH 5.0) at 40°C and detected by a fluorescence detector with excitation and emission wavelengths of 320 nm and 400 nm, respectively [21]. Structural analysis of the oligosaccharides was performed using an AXIMA-CFR mass spectrometer (Shimadzu, Kyoto, Japan) equipped with a reflector. Ions formed by a pulsed nitrogen UV laser (at 337 nm) were accelerated to a kinetic energy of 20 keV. Positive-ion mode was employed and all spectra were measured in the reflector mode. 2,5-Dihydroxybenzoic acid was used as the matrix at a concentration of 10 mg/ml in 10% aqueous ethanol. Analytes were dissolved in the matrix solution, and aliquots of the resulting mixture were placed onto the target. The solvent was removed with a gentle stream of air, and the solid sample/matrix mixture was then transferred to the mass spectrometer. For the measurement of post-source decay (PSD) MALDI-TOF mass spectra, the reflection voltage was decreased in successive 20% steps, four to six segments were obtained, and 180–220 shot spectra were summed [21]. Bradykinin (757.40, MH^+) and adrenocorticotropic hormone (2465.20, MH^+) were used for mass calibration of both MALDI-TOF and PSD MALDI-TOF mass spectrometries. Errors in the mass assignment of MALDI-TOF and PSD MALDI-TOF mass spectra were less than ±0.7 Da and ±1.8 Da, respectively.

3. Results

3.1. Degradation of CWM from Chlorella cells by vAL-1

The algal lytic enzyme vAL-1 of chlorovirus CVK2 was produced as a GST fusion protein in *E. coli* harboring a recombinant plasmid pGEX-4T-vAL-1 and purified by affinity chromatography with a glutathione-Sepharose 4B column as described previously [14]. When CWM isolated from *Chlorella* strain NC64A was treated with purified vAL-1 and the resulting soluble fraction analyzed by TLC, two major bands of degradation products or oligosaccharides appeared (Fig. 1). This degradation occurred under physiological conditions and continued for more than 24 h [14]. Addition of EGTA or EDTA at concentrations up to 50 mM did not affect the degradation activity, suggesting that Ca^{2+} or other metal ions were not required for the activity. Similar oligosaccharide bands were also observed when CWM of another host, *C. prototechoides* 211-6, was treated with vAL-1, but CWM of a non-host strain, for example *C. vulgaris* C-135, gave no bands when treated with vAL-1 (Fig. 1), indicating that vAL-1 is a host-specific cell wall-degrading enzyme. Although a similar enzyme, CL-2, was previously detected in *Chlorella* virus CVN1 and supposed to be an alginate lyase [15], vAL-1 actually degraded neither alginate nor other polysaccharides containing uronic acids such as polygalacturonate, polyman-

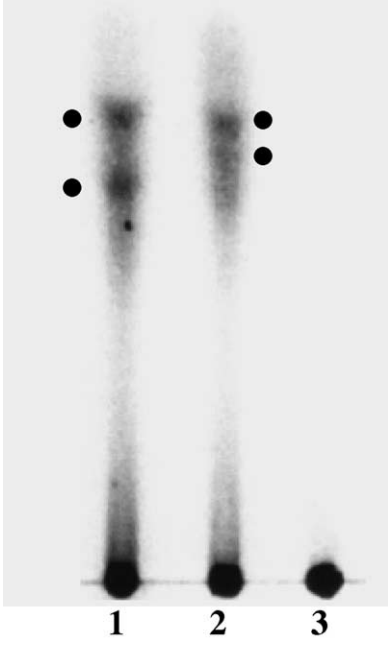

Fig. 1. TLC detects degradation products from *Chlorella* CWM treated with vAL-1. TLC was performed on a silica gel 60 plate with *n*-butanol/acetic acid/H_2O (3:1:1) as eluant. Degradation products are indicated by dots. Lanes: 1, CWM of *Chlorella* strain NC64A; 2, CWM of *C. prototechoides* 211-6; 3, CWM of *C. vulgaris* C-135.

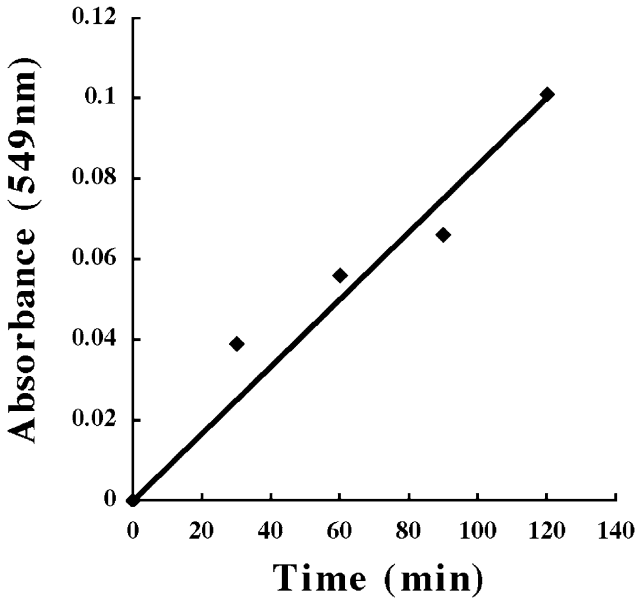

Fig. 2. Detection of unsaturated uronic acids in the cell wall degradation products by periodic acid/thiobarbituric acid reaction. CWM of *Chlorella* strain NC64A (3.3 mg/ml) was treated with purified vAL-1 (20 μg protein) at 37°C. At various times, 300-μl aliquots were removed for the assay. Absorbance at 549 nm was measured.

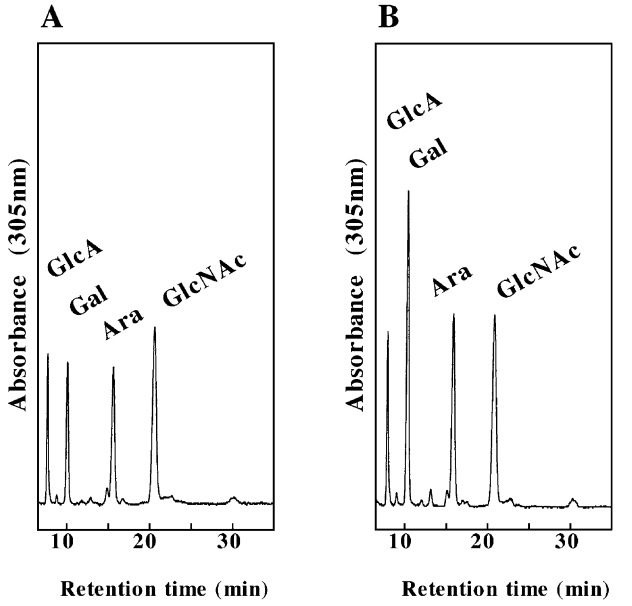

Fig. 3. Sugar composition analysis of oligosaccharides produced from NC64A CWM by treatment with vAL-1. The upper band (A) and lower band (B) separated on TLC (Fig. 1) were collected from the plate. After acid hydrolysis, monosaccharides were labeled with ABEE, subjected to reversed-phase HPLC with a Honenpak C18 column, and detected by absorbance at 305 nm. Sugars assigned to each peak are indicated.

nuronate, hyaluronan, chondroitin sulfate, and xanthan (data not shown). However, unsaturated uronic acids produced by a lyase reaction were detected by the periodic acid/thiobarbituric acid method in the degradation products of CWM treated with vAL-1 (Fig. 2), suggesting a lyase activity of vAL-1.

3.2. Sugar composition of the cell wall degradation products

Oligosaccharides were collected from the bands detected on TLC plates (Fig. 1). After complete hydrolysis, monosaccharides were labeled with ABEE and separated by HPLC. The results are shown in Fig. 3. The upper band gave four peaks with almost equal heights corresponding to GlcA, Gal, Ara, and GlcNAc, respectively (Fig. 3A). When the cell wall oligosaccharides were hydrogenated and analyzed in the same way, a new peak (with a retention time of 15.5 min) appeared and it coincided with the peak of authentic hydrogenated Δ4GlcA (data not shown). The lower band also bore four peaks assigned to the same sugars as above, but the peak of Gal was twice as high (Fig. 3B). The presence of Δ4GlcA in the degradation products again supported the lyase activity of vAL-1.

3.3. MALDI-TOF mass spectrometry of PA derivatives of oligosaccharides

Oligosaccharides collected from TLC plates were pyridylaminated and separated by reversed-phase HPLC. As shown in Fig. 4, four major peaks in each upper (A) and lower (B) band on TLC were observed in each HPLC chromatogram. In order to determine the structures of oligosaccharides, each PA-oligosaccharide was obtained by preparative HPLC and subjected to MALDI-TOF mass spectrometry and PSD MALDI-TOF mass spectrometry. The PSD MALDI-TOF mass spectra of PA-oligosaccharides are shown in Fig. 5A–F. In the mass spectrum of peak 1, sodium adduct molecular ion and fragment ions responsible for the cleavage of glycosidic bonds were observed at *m/z* 767 (M+Na), 605 (Y3+Na), 509 (C3+Na), 443 (Y2+Na), 347 (C2+Na), and 281 (Y1+Na) (Fig. 5A). These results combined with the sugar composition shown in Fig. 3A suggest that the oligosaccharide of peak 1 is characterized to be Gal-Gal-Gal-Gal-PA. In the case of peak 2, four Y-series ions were observed at *m/z* 906 (M+Na), 774 (Y4+Na), 612 (Y3+Na), 409 (Y2+Na), and 277 (Y1+Na) (Fig. 5B). The ion 277 (Y1+Na) just agreed with the PA-un-

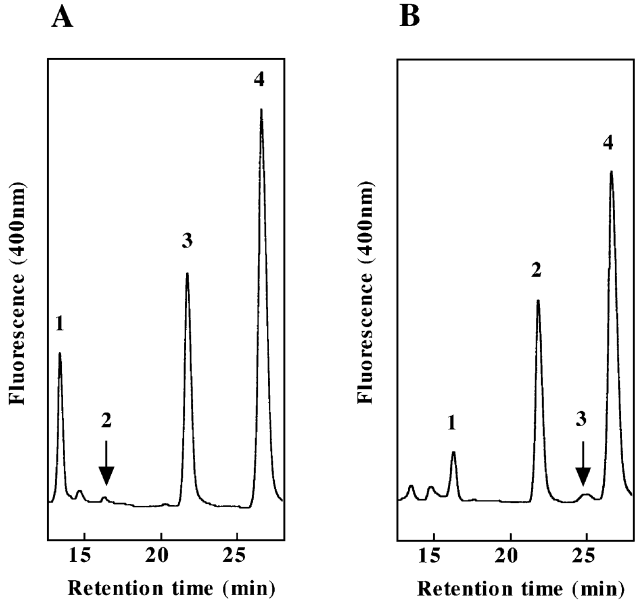

Fig. 4. Separation of PA-oligosaccharides by reversed-phase HPLC with a PALPAK type R column. The upper band (A) and lower band of Fig. 1 were collected from the TLC plate and pyridylaminated. PA-oligosaccharides were detected by a fluorescence detector with excitation and emission wavelengths of 320 nm and 400 nm, respectively. The peaks indicated by the numbers were subjected to MALDI-TOF mass spectrometry.

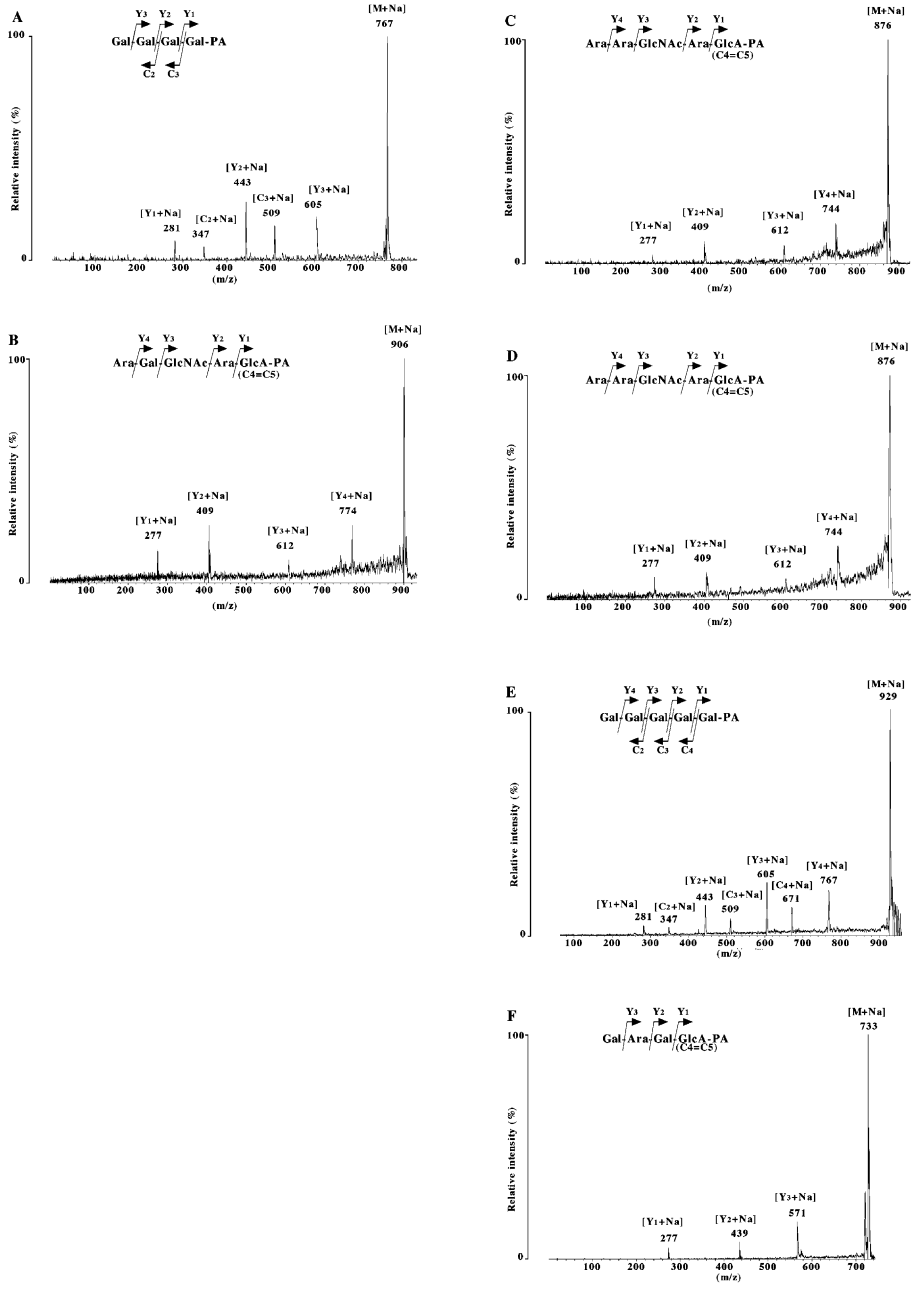

Fig. 5. PSD MALDI-TOF mass spectra of PA-derivatized oligosaccharides. The peaks 1–4 separated in Fig. 4A and the peaks 1 and 3 separated in Fig. 4B were analyzed and results are shown: A–D correspond to peaks 1–4 of Fig. 4A, respectively, and E and F correspond to peaks 1 and 3 of Fig. 4B, respectively. 2,5-Dihydroxybenzoic acid was used as the matrix at a concentration of 10 mg/ml in 10% aqueous ethanol. The fragment schema is shown according to the nomenclature of Domon and Costello [26].

saturated GlcUA mass, indicating that this oligosaccharide contained unsaturated GlcA at the reducing terminus. Considering the sugar composition, the mass values had a most likely sugar sequence for peak 2 of Ara-Gal-GlcNAc-Ara-GlcA(C4=C5)-PA. The mass spectrum for the peak 3 oligosaccharide is shown in Fig. 5C. Four Y-series ions were observed at *m/z* 876 (M+Na), 744 (Y4+Na), 612 (Y3+Na), 409 (Y2+Na), and 277 (Y1+Na). The ion 277 (Y1+Na) again coincided with the PA-unsaturated GlcA mass, indicating that this oligosaccharide also contained unsaturated GlcA at the reducing terminus. From the mass data, the peak 3 oligosaccharide was assigned to Ara-Ara-GlcNAc-Ara-GlcA(C4=C5)-PA. Fig. 5D shows the mass spectrum of peak 4. Four Y-series ions were observed at *m/z* 876 (M+Na), 744 (Y4+Na), 612 (Y3+Na), 409 (Y2+Na), and 277 (Y1+Na). Again the ion 277 (Y1+Na) corresponded to the mass of PA-unsaturated GlcA; this oligosaccharide has unsaturated GlcA at the reducing terminus. The results showed that the peak 4 oligosaccharide was Ara-Ara-GlcNAc-Ara-GlcA(C4=C5)-PA. Although the sequences of the sugars are identical between the oligosaccharides of peak 3 and peak 4, their separate patterns on HPLC (Fig. 4) might have been due to differences in the linkages of each of the sugars.

MALDI-TOF and PSD MALDI-TOF mass spectra of two of the four peaks (peaks 2 and 4) observed in the HPLC chromatogram of the lower band on TLC shown in Fig. 4B

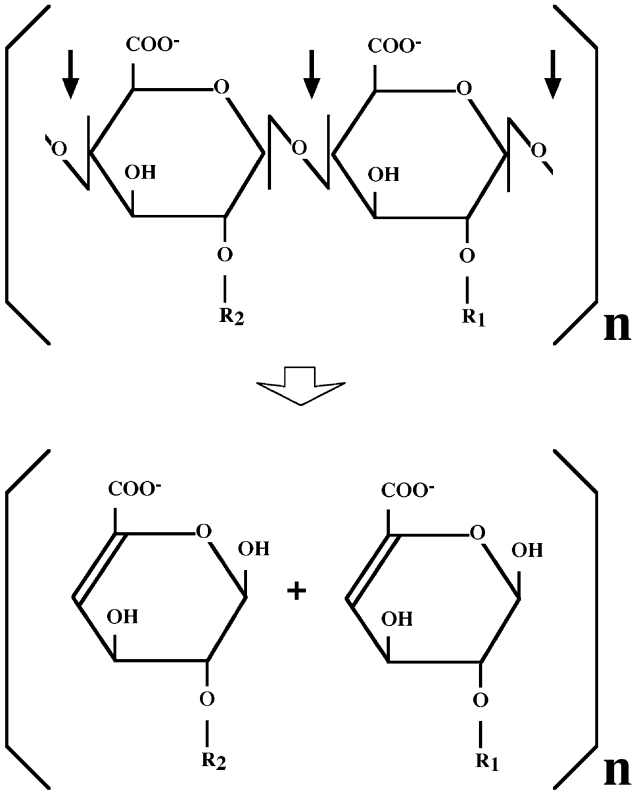

Fig. 6. Model for the polysaccharide structure of *Chlorella* cell wall and the mode of action of vAL-1 polysaccharide lyase. Thick arrows indicate the cleavage sites for vAL-1. R represents a side chain consisting of several sugars. R might be attached to C3 rather than C2. After cleavage of GlcA chains, oligosaccharides with unsaturated GlcA(C4=C5) are produced at the reducing terminus (lower part).

could not be obtained because the mass intensity of these oligosaccharides was extremely weak. On the other hand, peaks 1 and 3 showed PSD MALDI-TOF mass spectra as shown in Fig. 5E,F, respectively. These mass data combined with the sugar composition of oligosaccharides shown in Fig. 3B suggest the structures of peaks 1 and 3 to be Gal-Gal-Gal-Gal-Gal-PA and Gal-Ara-Gal-GlcA(C4=C5)-PA, respectively.

Based on these results, Fig. 6 depicts the most likely mode of action of vAL-1 polysaccharide lyase. In this model, vAL-1 cleaves β- or α-1,4-linked GlcAs, generating 4,5-unsaturated GlcA-containing oligosaccharides. Most interestingly, GlcA(C4=C5) is at the reducing terminus of every oligosaccharide product. Since vAL-1 does not work on alginate where α-1,4-linked GlcAs are included, vAL-1 seems to preferentially cleave β-1,4-linked GlcAs. A side chain of sugars indicated by R in Fig. 6 should be connected to C2 or C3 of GlcA. In the periodic acid/thiobarbituric acid reaction, GlcAs in oligosaccharides were split by periodic acid so that the side chain is likely linked to C2, as indicated in Fig. 6.

4. Discussion

In this work, the cell wall-degrading enzyme vAL-1 encoded by chlorovirus CVK2 was characterized as a polysaccharide lyase by analyzing the structures of the cell wall degradation products. Polysaccharide lyases (EC 4.2.2.X) are a group of enzymes that cleave polysaccharide chains via a β-elimination mechanism, resulting in the formation of a double bond at the newly formed non-reducing end. These enzymes are classified into 13 families based on amino acid sequence similarities (http://afmb.cnrs-mrs.fr/CAZY/PL-intro.html) [22]. The previously reported CL-2 of chlorovirus CVN1 [15] is considered to be a alginate lyase not yet assigned to a family at this site. However, as shown in this work, the vAL-1 homologous to CL-2 is not an alginate lyase but a novel polysaccharide lyase, having unique oligosaccharides as its products.

The vAL-1 protein consists of 349 aa (DDBJ accession number 044791) and is separated into three domains: a 58-aa N-terminal domain consisting of a long α-helix, 10-times repeats of a PAPK motif and a 251-aa C-terminal region with alternating α-helices and β-sheets [14]. The amino acid sequence of the 251-aa C-terminal region showed 29% identity with that of mannuronate lyase from *Turbo cornutus* [23], which is the only one in the databases showing a significant homology. Therefore, the vAL-1 lyase has very unique structural features. Functionally, it also exhibits unique characteristics including the production of oligosaccharides with an unsaturated GlcA at the reducing terminus, has no requirement for metal ions for its activity, and acts on raw CWM. vAL-1 degrades the cell wall of *Chlorella* strains [24]. In general, *Chlorella* cell walls have very complicated structures and chemical compositions, and are usually difficult for enzymes to digest [25]. According to Meints et al. [4], the cell wall of *Chlorella* strain NC64A contains Glc and Rha comprising 51% and 16% of the total sugars, respectively; with Gal, Xyl, Ara, Man, and GlcN each accounting for 5–10% of the sugars. These monosaccharides comprise by weight about 50% of the solubilized wall material. The nature of the remaining material is still not known. Uronic acids were also found to be cell wall components accounting for about 20% of the CWM [5]. In this work, a part of the *Chlorella* cell wall structure was revealed for the first time: some chains of GlcA with a side chain attached to the C2 or C3 position. The side chains consist of mainly Ara, Gal, and GlcNAc and the structure of GlcA-Ara-GlcNAc is common to most of them.

By using vAL-1 as a tool in structural analyses, studies on the complicated structures of *Chlorella* cell wall as well as other naturally occurring heterogeneous polysaccharides will be greatly facilitated.

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References

[1] Van Etten, J.L. (2000) in: Virus Taxonomy, Classification and Nomenclature of Viruses, Seventh Reports (Van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carsten, E.B., Ester, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R. and Wickner, R.B., Eds.) pp. 183–193, Academic Press, San Diego, CA.
[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] Meints, R.H., Burbank, D.E., Van Etten, J.L. and Lamport, D.T.A. (1988) Virology 164, 15–21.

[5] Kapaun, E., Loos, E. and Reisser, W. (1992) Phytochemistry 31, 3103–3104.
[6] Kapaun, E. and Reisser, W. (1995) Planta 197, 577–582.
[7] Takeda, H. (1995) Phytochemistry 40, 457–459.
[8] Royce, C.L. and Pardy, R.L. (1996) J. Endotoxin Res. 3, 437–444.
[9] Van Etten, J.L. and Meints, R.H. (1999) Annu. Rev. Microbiol. 53, 447–494.
[10] Yamada, T., Hiramatsu, S., Songsri, P. and Fujie, M. (1997) Virology 230, 361–368.
[11] Sun, L., Adams, B., Gurnon, J.R., Ye, Y. and Van Etten, J.L. (1999) Virology 263, 376–387.
[12] Hiramatsu, S., Ishihara, M., Fujie, M., Usami, S. and Yamada, T. (1999) Virology 260, 308–315.
[13] Sun, L., Gurnon, J.R., Adams, B.J., Graves, M.V. and Van Etten, J.L. (2000) Virology 276, 27–36.
[14] Sugimoto, I., Hiramatsu, S., Murakami, D., Fujie, M., Usami, S. and Yamada, T. (2000) Virology 277, 119–126.
[15] Suda, K., Tanji, Y., Hori, K. and Unno, H. (1999) FEMS Microbiol. Lett. 180, 45–53.
[16] Muscatine, L., Karakashian, S.J. and Karakashian, M.W. (1967) Comp. Biochem. Physiol. 20, 1–12.
[17] Van Etten, J.L., Burbank, D.E., Kuczmarski, D. and Meints, R.H. (1983) Science 219, 994–996.
[18] Yasuno, Y., Kokubo, K. and Kamei, M. (1999) Biosci. Biotechnol. Biochem. 63, 1353–1359.
[19] Augustine, R.L. (1965) in: Catalytic Hydrogenation: Techniques and Application in Organic Synthesis, Marcel Dekker, New York.
[20] Weissbach, A. and Hurwitz, J. (1959) J. Biol. Chem. 234, 705–709.
[21] Suzuki, M. and Suzuki, A. (2001) Biol. Chem. 382, 251–257.
[22] Coutinho, P.M. and Henrissat, B. (1999) in: Recent Advances in Carbohydrate Engineering (Gilbert, H.G., Davies, G.J., Svensson, B. and Henrissat, B., Eds.), pp. 3–12, Royal Society for Chemistry, Cambridge.
[23] Muramatsu, T., Komori, K., Sakurai, N., Yamada, K., Awasaki, Y., Fukuda, K. and Oda, T. (1996) J. Protein Chem. 15, 709–719.
[24] Chuchird, N., Hiramatsu, S., Sugimoto, I., Fujie, M., Usami, S. and Yamada, T. (2001) Microb. Environ. 16, 206–212.
[25] Yamada, T. and Sakaguchi, K. (1982) Arch. Microbiol. 132, 10–13.
[26] Domon, B. and Costello, C. (1988) Glycoconjug. J. 5, 397–409.