Molecular Characterization of a Nuclease Gene of Chlorella Virus SS-2

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Sequence analysis of the Chlorella virus SS-2 revealed one putative nuclease gene that is 807 bp long and encodes a 31kDa protein. Multiple sequence alignment analysis reveals the presence of highly conserved PD-(D/E)XK residues in the encoded protein. The gene cloned into an expression vector was expressed as a His-tagged fusion protein in chaperone containing pKJE7 cells. The recombinant protein was purified using a His-Trap chelating HP column and used for functional analysis. Exonuclease activity of the SS-2 nuclease was detected when the DNA substrates, such as linear ssDNA, PCR amplicon, linear dsDNA with 5'-overhang ends, 3'-overhang ends, or blunt ends were used. Covalently closed circular DNA was also degraded by the SS-2 recombinant protein, suggesting that the SS-2 nuclease has an endonuclease activity. Stable activity of SS-2 nuclease was observed between 10°C and 50°C. The optimum pH concentrations for the SS-2 nuclease were pH 6.0-8.5. Divalent ions inhibited the SS-2 nuclease activity.

Keywords : algal virus, Chlorella virus, nuclease gene, recombinant protein

The large polyhedral viruses that infect unicellular eukaryotic *Chlorella*-like green algae contain large, linear, and double-stranded (ds) genomes and are classified as members of the genus *Chlorovirus* in the family *Phycodnaviridae* (Kang et al., 2005; Van Etten et al., 1999). The most well-known example of *Chlorovirus* is the *Paramecium bursaria* Chlorella Virus-1 (PBCV-1) (Dunigan et al., 2006; Li et al., 1997; Zhang et al., 1994). Sequence analysis of the 331-kb PBCV-1 genome indicates the presence of 366 proteins encoding genes and a polycistronic tRNA encoding 11 tRNAs.

Differences in genome size among natural chlorovirus isolates have been observed (Dunigan et al., 2006; Fitzgerald et al., 2007). The genome size of the chloroviruses varies from 314 kb to 390 kb, and the serological properties differ in some isolates (Dunigan et al., 2006; Fitzgerald et al., 2007; Nishida et al., 1999). For example, Chlorella virus

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MT325, with a 314-kb genome, can infect one *Chlorella* species, *Chlorella* pbi, but does not infect other *Chlorella* species such as *Chlorella* NC64, which is the known host for the 331 kb PBCV-1 (Li et al., 1997; Fitzgerald et al., 2007). Also, spontaneous large deletion mutants (27-45 kb) have been reported in chloroviruses, suggesting that not all the genes encoded by chloroviruses are required for their replication (Landstein et al., 1995).

In 2002, we isolated 23 chlorella viruses from geographically different regions in Korea (Cho et al., 2002). Molecular characterization revealed that they were variable in both genome size (330-350 kb) and serological properties. About 95% (310 kb) of the genome of one isolate, SS-2, has been sequenced and compared to the genome of chlorella virus PBCV-1 (authors' unpublished data), and 11% of the SS-2 genes did not have any counterpart genes in PBCV-1. However, most genes (about 89%) of SS-2 have counterparts to PBCV-1 genes and share high sequence homology, ranging from 72% to 100%, at the amino acid level, implying that these proteins are important for the chlorovirus life cycle.

It is known that the virus genes involved in DNA replication or recombination are relatively well conserved among viruses. Sequence analysis of SS-2 identified one open reading frame (ORF) of 807 bp that encodes 31.1 kDa protein. This ORF shares the highest amino acid sequence similarity (96%) to the PBCV-1 A166R protein, which is proposed as a putative exonuclease gene and is assumed to play important roles in viral DNA replication and/or recombination (Fitzgerald et al., 2007). However, no functional experiments regarding this protein have been performed. In this paper, we amplified this putative exonuclease gene through PCR, cloned and expressed it in *Escherichia coli* as a recombinant protein, and tested its activities using various DNA substrates.

Materials and Methods

Algal cultures and media. The *Chlorella* NC64A strain was kindly provided by Dr. Van Etten at the University of Nebraska, Lincoln, and has been continuously maintained in the laboratory by transfer into fresh modified Bold's basal medium (MBBM) and incubation at 25°C (Van Etten

et al., 1983). One chlorella virus strain, SS-2, which was previously isolated from freshwater in Korea, was used for this study (Cho et al., 2002). Actively growing host *Chlorella* NC64A was inoculated with the SS-2 virus and incubated at 25°C until complete lysis occurred. Viral DNA purification was performed following the procedures of Van Etten et al. (1983). Briefly, 1 L of viral lysate was centrifuged at 5,000 rpm for 5 min at 4°C using a Sorvall GS-3 rotor. The supernatant was transferred and centrifuged in a Sorvall T-880 rotor at 20,000×g for 1 h. The virus particles were resuspended in 50 mM Tris-HCl (pH 7.8), purified using a sucrose gradient, and dissolved in 50 mM Tris-HCl (pH 7.8). The viral genomic DNA was further purified using CsCl density gradient centrifugation and resuspended in 1X TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA].

PCR cloning of the SS-2 nuclease gene. Two primers, SS-2 F1 (5'-GGATCCATGTCGGTGTATCCTCCA-3') and SS-2 R1 (5'-CTCGAGTTAGTATATATCTGTGAT-3'), having either *Bam*HI or *Xho*I restriction sites (underlined) were designed and used for PCR amplification with purified SS-2 genomic DNA. The PCR product was ligated into the pGEM-T Easy Vector (Promega, WI, USA) and transformed into *Escherichia coli* XL-1 Blue. The transformants were identified by colony PCR, and plasmid DNA was prepared for sequencing analysis. The nuclease gene of SS-2 was cloned into the *Bam*HI and *Xho*I restriction sites of the expression vector pET28a(+) (Novagen, USA) and generated plasmid pET28a-nuclease. The pET28a-nuclease was transformed into a chaperone containing expression host pKJE7 cells (Takara, Japan) and was used for development of the recombinant protein of the SS-2 nuclease gene.

Sequence alignments of virus nucleases. The GenBank accession numbers of other nuclease sequences used for this analysis are as follow: *Paramecium bursaria* Chlorella virus PBCV-1, AAC96534; *Paramecium bursaria* Chlorella virus AR158, ABU43746; *Bartonella henselae Str.* Houston-1, CAF27185; *Ectocarpus siliculosus* virus EsV-1-64, AAK14487; *Feldmannia irregularis* virus FirrV-1-B43, AAR26918; *Acanthamoeba polyphaga* mimivirus, AAV50623; *E. coli* APEC, 01ABJ00523; Bacteriophage VT2-Sa, BAA84296; and Phage BP-4795, CAD88815. A sequence similarity search was performed using the BLAST program of the National Center for Biotechnology Information (NCBI), and multiple protein sequence alignment was conducted using the CLUSTALW program (http://www.ebi.ac.uk/clustalw).

Expression of the SS-2 nuclease gene. pKJE7(pET28a-nuclease) cells were inoculated into 5 ml of LB medium

supplemented with both 50 µg kanamycin and 50 µg chloramphenicol per ml and grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.8. For chaperone induction, L-Arabinose was added to the culture at a concentration of 0.5 mg per ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce expression of the His-tagged SS-2 nuclease. Cells were harvested at 1, 2, 3, 4, and 5 h after IPTG induction, and both their expression level and solubility were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Western blot analysis. Total proteins of IPTG-induced pKJE7(pET28a-nuclease) cells were extracted and used for Western blot analysis. The bacterial lysates from non-induced cells were also separated on SDS-PAGE and used as a negative control. The gel was blotted onto a nitrocellulose membrane using transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). The membrane was blocked with TTBS buffer [20 mM Tris (pH 7.4), 0.5 M NaCl, 2.5 mM KCl, 0.05% Tween-20] containing 5% skimmed milk, washed three times for 5 min with TTBS buffer, and incubated at room temperature for 1.5 h with anti-His-Tag antibody (Serotec, UK) which was diluted 1:3,000 in TTBS buffer with 5% skimmed milk. After three washings with TTBS, the blot was incubated at room temperature for 1 h with alkaline phosphatase conjugated anti-mouse IgG (Sigma, USA) and washed three times for 5 min with TTBS. The reaction was developed by adding NBT/BCIP solution (Sigma, USA) to the membrane.

Purification of soluble SS-2 nuclease. The recombinant protein of pKJE7(pET28a-nuclease) was expressed as described above and used for purification of the SS-2 nuclease. One liter of cells were harvested by centrifugation at 4,000×g for 20 min at 4°C, resuspended in 5 ml of resuspension buffer [20 mM phosphate buffer (pH 7.4), 500 mM NaCl], and treated with 250 µl 10 mM lysozyme by incubation at 4°C overnight. The bacterial cells were ultrasonicated on ice for 30 s×6 times, centrifuged at 13,000×g for 20 min, and the supernatant was collected into new tubes. The supernatant was further filtered using a 0.22-µm filter and loaded onto 1 ml HisTrap affinity columns (GE Healthcare, Sweden) which had been equilibrated with the binding buffer (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, 1 mM NaN₃, pH 8.0). After loading the sample, the column was washed with 15 ml of washing buffer (20 mM Tris-HCl, 500 mM NaCl, 40 mM imidazole, 1 mM NaN₃, pH 8.0). His-tagged recombinant protein was eluted with 20 ml of elution buffer (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, 1 mM NaN₃, pH 8.0). Five-µl of eluted samples was used for SDS-PAGE electrophoresis and Western blot analysis for checking its purity. Total

purified SS-2 nuclease was finally dialyzed overnight at 4°C against 1 L of storage buffer (20 mM potassium phosphate, pH 8.0, 20% glycerol, 5 mM β-mercaptoethanol), concentrated using sucrose, and kept at −70°C until further use.

Nuclease activity assays. Standard reaction mixtures (20 μl) were composed of 50 mM Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.1% BSA, 200 ng of different substrates, and 10 ng of recombinant SS-2 nuclease. All the reactions mixtures were incubated at 37°C for 20 min and then stopped by heating at 70°C for 10 min. SS-2 nuclease activity was initially tested by incubation of linear PCR amplicon. Both DNase I (Takara, Japan) and heat-denatured recombinant SS-2 nuclease were used as controls. To test substrate specificity of SS-2 nuclease, various DNA templates were used. Linear M13 mp 18 single-stranded DNA (ssDNA) (Takara, Japan) was purchased and used as ssDNA substrate. Circular dsDNA was prepared by alkaline lysis miniprep of pBluescript II KS⁻ (Fermentas, USA) in XL1 Blue strain (Sambrook and Russell, 2001). Linear dsDNA was prepared either using 1.4-kb PCR products or digestion of circular dsDNA of the pBluescript II KS⁻ vector with the restriction enzymes, EcoRI, PstI, and DraI. The EcoRI enzyme generates 5'-overhang ends, while PstI generates 3'-overhang ends. DraI can cleave both DNA strands and generates blunt ends. To determine the optimum pH, the reactions were performed in buffers varying in pH (6.0, 6.5, 7, 7.5, 8.0, and 8.5). The effect of

temperature was analyzed by incubating the reaction mixture at 10°C, 20°C, 30°C, 40°C, and 50°C. Effects of divalent ions on SS-2 nuclease were analyzed using various concentrations of divalent ions $MnCl_2$, $ZnCl_2$, $CaCl_2$, $NaCl_2$, and $MgCl_2$. The substrate specificity and the effects of various conditions were evaluated by agarose gel electrophoresis. After reactions were completed, each sample was run on a 1% agarose gel, stained with ethidium bromide, and visualized by the Gel Doc XR system (Bio-Rad, USA). The DNA band intensity was analyzed using Quantity one (Version 4.6.1. BioRad, USA). All experiments were repeated three times, and the mean values were used for data analysis.

Results

Amplification of the SS-2 nuclease gene. The nuclease gene of SS-2 was successfully amplified through PCR using SS-2 F1 and SS-2 R1 primers. One band near 800 bp was observed after 1% agarose gel electrophoresis (data not shown). The 800-bp PCR product was further gel-purified and cloned into the pGEM-T Easy vector for sequencing. Sequencing analysis revealed that the nucleotide sequences were perfectly matched to the original SS-2 nuclease gene, confirming that no sequence changes occurred during the PCR (data not shown). SS-2 nuclease nucleotide sequence is deposited in GenBank under accession number FJ449710. This SS-2 nuclease gene was used for cloning into the expression vector to develop a His-tag fusion recombinant
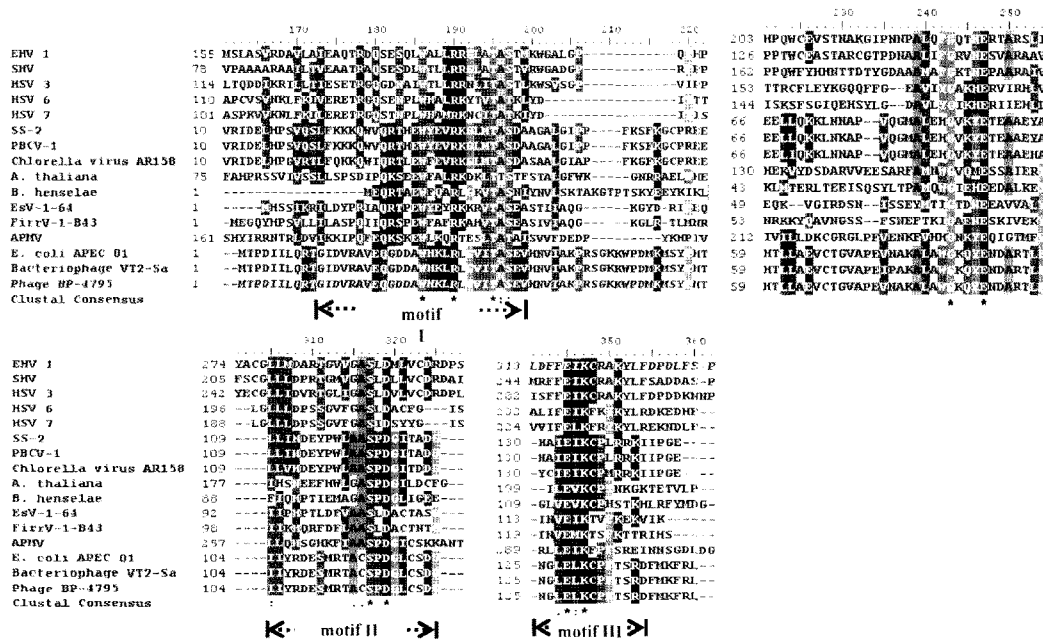

Fig. 1. Multiple sequence alignment of the SS-2 nuclease homologs. The numbers in the sequences indicate the position of residues of each nuclease. Degree of conservation and amino acid properties are marked in different colors. The three conserved motif regions are labeled according to the nomenclature described for the Herpesvirus alkaline nuclease family.
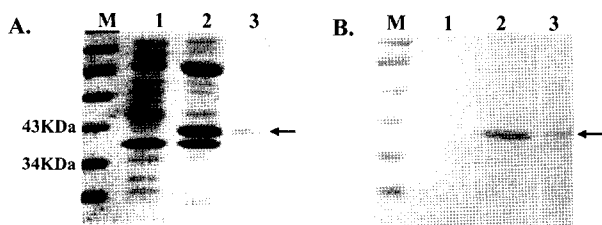
Fig. 2. Expression of the SS-2 nuclease in *E. coli* (**A**) and Western blot analysis with anti-His-tag antibodies (**B**). Lane 1, non-induced control cells; Lane 2, IPTG induction cells; lane 3, SS-2 nuclease purified using a His-Trap chelating HP column. The molecular weight marker is shown in lane M.

protein.

Sequence analysis of the SS-2 nuclease.

Blast search of SS-2 nuclease revealed sequence similarities to other nucleases in various organisms, including PBCV-1, *Bartonella henselae, Acanthamoeba polyphaga* mimivirus, equid herpesvirus 1, human herpesvirus 1, and suid herpesvirus. A multiple amino acid sequence alignment of these nucleases showed the presence of three conserved motif regions in SS-2 (I, II, and III) (Fig. 1). Highly conserved PD-(D/E)XK residues found in either motif II or III of many nucleases were also detected in D124...E134XK136 of SS-2 nuclease.

Overexpression and purification of the SS-2 nuclease.

When *E. coli* cultures were induced with IPTG, the synthesis of one protein band at 40 kDa increased in cells carrying the pET28a-nuclease (Fig. 2). Also, Western blot analysis with anti-His-tag antibody detected the same sized band, indicating that the SS-2 nuclease was indeed expressed from the plasmid. No detectable band after Western blot analysis was observed in control cells. Most expressed protein was found in the insoluble cell pellet. To increase the solubility of the recombinant protein, changes of culture conditions, bacterial host strain, concentrations of IPTG, incubation temperatures, and induction times were used (data not shown). Although the majority of the SS-2 nuclease was still produced in an insoluble form, co-expression of SS-2 nuclease with molecular chaperone protein, using the chaperone-containing expression host pKJE7 cells, appeared to enhance the soluble expression. The 6xHis-tagged SS-2 nuclease protein was purified from the supernatant of chaperone-containing cells using a His-Trap chelating HP column and was used for SDS-PAGE and Western blot analysis to confirm its purity and molecular weight. Only one band of 40 kDa was observed in both SDS-PAGE and Western blot analyses, indicating that no other proteins co-purified with the SS-2 nuclease (Fig. 2). This purified SS-2 nuclease was further used for nuclease activity tests.
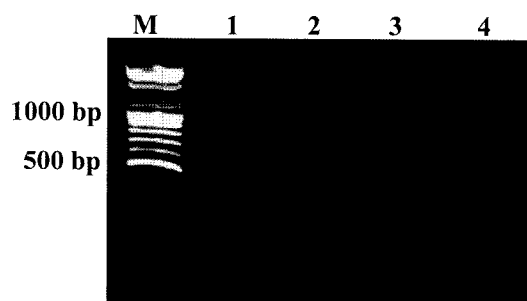

Fig. 3. Analysis of SS-2 nuclease activity by 1% agarose gel electrophoresis. PCR amplicon was treated with various enzymes including DNase I (lane 2), SS-2 nuclease (lane 3), and heat-denatured SS-2 nuclease (lane 4). Lane 1 is representing no treatment and lane M is showing standard DNA size marker.

Enzymatic characterization of the SS-2 nuclease.

Linear dsDNA templates were successfully degraded by incubation with SS-2 recombinant nuclease, but not heat-denatured SS-2 nuclease (Fig. 3). SS-2 exonuclease activities were also tested using the substrates, ssDNA, PCR products, and linear dsDNA having either overhangs (5'-overhang or 3'-overhang) or blunt ends. To test for endonuclease activity, covalently closed circular pBluescript II KS⁻ was used as substrate. All substrates treated with the SS-2 nuclease were degraded after a 20-min incubation at 37°C, suggesting that SS-2 has both exo- and endonuclease activity (Fig. 4). To examine the possibility that the nuclease activities were derived from background nucleases that had co-purified with the SS-2 nuclease, extracts from cells having only the cloning vector were prepared and tested for nuclease activity. Although weak background nuclease activity was observed in the extract, the substrates incubated with SS-2 nucleases degraded much faster (data not shown).

To find the optimal conditions for SS-2 nuclease, temperatures, pH concentration, and different divalent ions were tested. SS-2 nuclease displayed stable activity at a wide
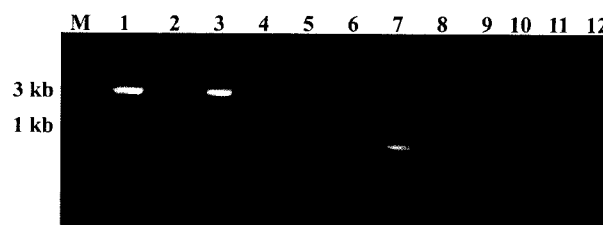

Fig. 4. Digestion of various DNA substrates by SS-2 nuclease. Purified SS-2 nuclease was incubated with various DNA substrates including *EcR* I digested vector (lane 2), *Pst* I digested vector (lane 4), *Dra* I digested vector (lane 6), PCR product (lane 8), circular vector (lane 10), and single-stranded DNA (lane 12). Each lane with odd numbers represents the DNA substrate that was not treated with SS-2 nuclease as a control for each even number lane.
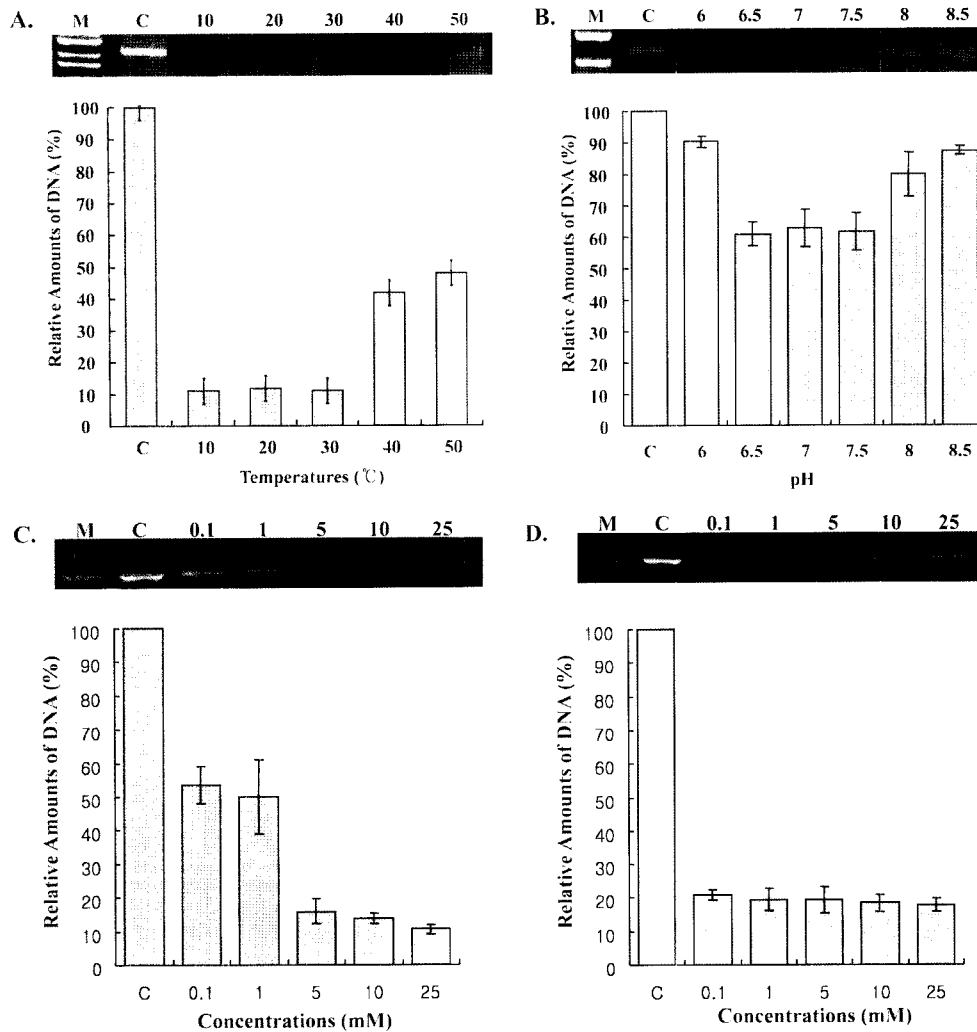
Fig. 5. Effects of temperatures (**A**), pH (**B**), Mn^{2+} (**C**), and Mg^{2+} (**D**) on the SS-2 nuclease activity. Each activity was assessed relative to the intensity of the control DNA bands, shown as C in each panel, after a 20-min incubation. The molecular weight marker is shown in lane M.

range of temperatures, between 10-50°C (Fig. 5). In contrast, SS-2 nuclease had a narrow range of pH, and the highest activity was observed between pH 6.5 and 7.5 (Fig. 5). The effects of divalent ions on SS-2 nuclease activities were quite variable and the results for Mg^{2+} and Mn^{2+} are shown in Fig. 5. Increasing concentrations of both Ca^{2+} and Mn^{2+} (up to 25 mM) enhanced SS-2 enzyme activity, while both Mg^{2+} and Zn^{2+} had only a slight stimulation effect; however, the presence of Na^{2+} strongly inhibited SS-2 exonuclease activity.

Discussion

In this study, we expressed a chorella virus SS-2 nuclease in *E. coli* and tested its activity using various DNA substrates. It is known that each individual nuclease displays a preference for specific substrates and sites. For example,

the lambda exonuclease hydrolyzes dsDNA in a 5' → 3' direction but does not digest closed circular DNA (Carter and Radding, 1971). Other nucleases, such as the alkaline nuclease (AN) encoded by *Autographa californica* multiple-capsid nucleopolyhedrovirus (AcMNPV), preferentially digest ssDNA rather than dsDNA and also possess endonuclease activity (Li and Rohrmann, 2000). Despite differences in substrate specificity, both the lambda exonuclease group and the alkaline nuclease of AcMNPV contain all three conserved motifs found in many other nucleases (Bujinicki and Rychlewski, 2001). Interestingly, our multiple amino acid sequence alignments revealed the presence of these three conserved motifs in SS-2 nuclease. However, SS-2 nuclease digested diverse DNA substrates, such as dsDNA, ssDNA, and circular DNA, indicating that SS-2 nuclease has a broader range of substrate specificity.

Although conserved motifs appear to be irrelevant to

determining substrate specificity, highly conserved residues (PD-(D/E)XK) present in either motif II or III are considered important to nuclease activity (Anderson, 1993; Kovall and Matthews, 1999). High conservation of the PD-(D/E)XK motifs has been observed in a number of nuclease families including several restriction endonucleases, DNA repair enzymes, holiday junction resolvases, and other nucleotide-cleaving enzymes (Knizewski et al., 2006; Murzin et al., 1995). These residues are thought to be involved in metal ion binding, typically Mg^{2+}, and catalytic functions (Doetsch and Cunningham, 1990; Kovall and Matthews, 1999). A number of experiments have revealed that mutation of any of these residues results in either significantly decreased or completely destroyed nuclease activity (Liu et al., 2003; Selent et al., 1992; Wolfes et al., 1986). Similarly, some mutations in highly conserved residues of SS-2 significantly altered the nuclease activity determined by gel electrophoresis, suggesting that these residues play important roles for development of nuclease activity (authors unpublished data). However, the divalent ion required for the SS-2 nuclease reaction is different from that for other known nucleases. The divalent ion typically needed for the nuclease reactions is Mg^{2+} (Doetsch and Cunningham, 1990; Kovall and Matthews, 1999), while the best divalent ion for the SS-2 nuclease reactions is Mn^{2+}. A few experiments have reported that some nucleases, such as the exonuclease of Arabidopsis thaliana, human Werner syndrome exonuclease, and phage terminases, display similar levels of enzymatic activity with either Mg^{2+} or Mn^{2+} (Plchova et al., 2003; Ponchon et al., 2006; Shen and Loeb, 2000). In addition, human Werner protein hydrolyze more efficiently stem loop DNA in the presence of Mn^{2+} rather than Mg^{2+}, indicating that the divalent ion required for enzymatic reactions might differ depending on the nuclease (Shen and Loeb, 2000).

Most research related to the nucleases of the chlorovirus group has focused on DNA site-specific restriction endonucleases (REases) (Xia et al., 1986; Xia and Van Etten, 1986; Zhang et al., 1998). These endonucleases are considered to be important because some of them are accompanied by DNA methyltransferase (MTases) genes and are expected to be restriction-modification (R/M) systems. R/M systems are defense mechanisms found in bacteria that protect bacterial genes against REases by methylation of DNA MTases, which cleave specific nucleotide sequences of DNA. Similar functions for REases and MTases found in chlorovirus groups have been proposed; however, their biological functions are not clearly understood. Deletion mutants of chlorovirus IL-3A lacking both functional DNA MTases and REases still degraded host nuclear and chloroplast DNA, suggesting that REase genes are not the only group of genes responsible for degradation of host chromo-

somal DNA (Burbank et al., 1990). Recently, Agarkova et al. (2006) reported similar results, and found that there are at least three different uncharacterized general nucleases in disrupted PBCV-1 particles, in addition to the two characterized REase activities. In this regard, it should be interesting to determine whether SS-2 nuclease is involved in host genome degradation.

Sequence comparison revealed that other chloroviruses, such as PBCV-1, MT325, and FR483, contain SS-2 nuclease homologs. Also, the Ectocarpus siliculosus virus 1 (EsV-1), which is one of the marine filamentous brown algae viruses, contains two exonuclease genes, including one homolog of the SS-2 nuclease and one unique gene. In addition, Feldmannia irregularis virus 1 (FirrV-1), infecting brown algae contains one exonuclease gene, which is the homolog of the SS-2 nuclease. These findings indirectly suggest that the nuclease gene of SS-2 could play essential roles in viral life cycles. It is noteworthy that the nuclease gene of SS-2 has been categorized in the functional group of genes involving in DNA replication, recombination, and repair (Dunigan et al., 2006; Fitzgerald et al., 2007). Because SS-2 nuclease shares some similarities with diverse nucleases, such as PD-(D/E)XK superfamily proteins, which are known to be involved in DNA repair and recombination, similar functions for SS-2 were expected. Most PD-(D/E)XK superfamily proteins have similar biochemical properties, such as degradation of DNA; however, their biological roles are quite different depending on the protein. For example, Epstein–Barr virus (EBV) DNase appears to be involved in viral DNA replication (Cheng et al., 1980), while the Herpes simplex virus type 1 (HSV-1) alkaline nuclease gene seems to be involved in viral DNA packaging, rather than viral DNA synthesis (Weller et al., 1990). Therefore, more studies to determine the roles of SS-2 nuclease gene products in the chlorovirus life cycle are required.

Acknowledgment

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