Thesis for the Degree of Master of Science

Molecular characterization of an Exonuclease gene of the *Chlorella* virus SS-2

by

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February 2008

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(*Chlorella* virus SS-2에서 유래한 Exonuclease 유전자의 분자적 특성 연구)

Adviser: Prof. Gun-Do Kim

by

Sang-Eun Jung

A thesis submitted in partial fulfillment of the requirements
for the degree of

Master of Science

in Department of Microbiology, Graduate School,
Pukyong National University

February 2008

정상은의 이학석사 학위논문을 인준함

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Abstract

Chlorella viruses are large icosahedral, double-stranded DNA and plaque-forming viruses that infect certain eukaryotic *Chlorella*-like green algae. The genome of *Chlorella* virus SS-2 which had been isolated from fresh water in Korea is predicted to contain at least 373 major open reading frames (ORFs). However, the function of large numbers of these genes are not clearly understood. One of the open reading frames (ORFs) encodes a protein with some amino acids identities to other exonuclease. The *Chlorella* virus SS-2 exonuclease gene is 807 nucleotides long and encodes a polypeptide of 31 kDa. Blast search revealed that the *Chlorella* virus SS-2 exonuclease gene has high amino acid identities to the ORF A166R of *Paramecium bursaria Chlorella* virus 1 (PBCV-1, 98 %) and two ORFs of other brown algae viruses including ORF B43 of *Feldmannia irregularis* virus (FirrV, 28 %) and ORF 164 of *Ectocarpus siliculosus* virus (EsV, 27 %). The activity of *Chlorella* virus SS-2 exonuclease was investigated by using a purified recombinant protein expressed in *E. coli*. The purified recombinant protein exhibited exonuclease activity on various DNA substrates when incubated at 37 ℃, suggesting that the *Chlorella* virus SS-2 exonuclease gene encodes functional proteins. Sequence alignment of *Chlorella* virus SS-2 exonuclease revealed that they share several conserved motifs. One of these, the conserved motif D91...E111XK113 (D...EXK) in the sequence of

Chlorella virus SS-2 exonuclease, has a striking similarity to the catalytic sites of some other nucleases, including type Ⅱ restriction endonuclease, λ exonuclease and *Mut*H. The predicted secondary structures of these three residues showed high similarity to the three catalytic residues of type Ⅱ restriction endonuclease.

These results indicate that exonuclease gene of the *Chlorella* virus SS-2 was functional proteins and *Chlorella* viruses can be useful sources of protein in addition to many genes of interest.

INTRODUCTION

Chlorella viruses are large, icosahedral, plaque-forming and double stranded-DNA (dsDNA) viruses (315 to 380 kbp) (Kang *et al.,* 2005, Swaminathan *et al.,* 1996). The *Chlorella* viruses infect certain freshwater, unicellular, *Chlorella*-like green algae (e.g., *Chlorella* strain NC64A or *Chlorella* strain Pbi), which normally exist as endosymbionts in the protozoan *Paramecium bursaria*. Viruses that infect *Chlorella* NC64A (NC64A viruses) are serologically different from viruses that infect *Chlorella* Pbi (Pbi viruses). NC64A viruses neither infect nor attach to *Chlorella* Pbi, and vice versa (Nishihara *et al.,* 1998).

Chlorella viruses are known to carry multiple DNA methyltransferases (MTases) and DNA site-specific (restriction) endonucleases (REases). Although the biological role of *Chlorella* viruses restriction-modification (RM) system is not clearly understood, it has been suggested that these MTases and REases of *Chlorella* viruses could be involved in the self-protecting processes, which is similar mechanisms found in the bacterial RM system (Agarkova *et al.,* 2006, Swaminathan *et al.,* 1996, Van *et al.,* 1981, Xia *et al.,* 1986, Yang *et al.,* 2004). Similar ORFs encoding putative exonuclease genes have been found in green algae viruses. However, no detail information related their functions and biological importance are currently available.

Chlorella virus SS-2 is a member of the *Phycodnaviridae* and is classified as a chloroviruses (Cho *et al.,* 2002). The genomic DNA of *Chlorella* virus SS-2, isolated in Korea was partially analyzed but biological functions of encodes numerous coding genes are largely unknown. The viral-encoded proteins contained transcriptional and translational factor, restriction/ modification enzymes, topoisomerase, chitinase, and hyaluronan synthase (Grabowski *et al.,* 1995, Lavrukhin *et al.,* 2000, Reisser *et al.,* 1988, Van *et al.,*

1981, Xia *et al.,* 1986, Zhang *et al.,* 1992). The estimated genome of SS-2 to be 340 kbp, and over 300 kbp nucleotide sequence was analyzed except for about 20 kbp from each end of the linear DNA genome. Among the 372 open reading frames identified from PBCV-1, the prototype of *Chlorella* virus, 331 were identified from SS-2, which suggest that SS-2 is very similar to PBCV-1. However, western blot analysis of viral proteins did not show any signal with antisera against PBCV-1. It is known that this difference is due to the difference in the glycosylation of the major capsid protein (Cho *et al.,* 2002).

Recently, I sequenced the length of 310 kbp *Chlorella* virus SS-2 genome. Genomic analysis showed the one ORF revealed the similarity of amino acid with other exonuclease genes. Furthermore *Chlorella* virus SS-2 are conserved, and aligned with other nucleases conserved motifs (Goldstein *et al.,* 1998, Liu *et al.,* 1998, Martinez *et al.,* 1996). In the previous studies, little is known about the cleavage mechanism and active site for catalysis by herpesvirus DNases.

In this study, I cloned the putative exonuclease gene from the *Chlorella* virus SS-2 genome. The activity of *Chlorella* virus SS-2 exonuclease was investigated by using a purified recombinant protein in the *E. coli* expression system (Huang *et al.,* 2001). The purified recombinant protein exhibited exonuclease activity on the various DNA substrates when incubated at 37 ℃, suggesting that the *Chlorella* virus SS-2 exonuclease gene encodes functional proteins.

MATERIALS AND METHODS

Virus culture and purification

The *Chlorella* strain NC64A was kindly provided by Dr. James Van Etten in the Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska, USA. *Chlorella* strain NC64A was cultured in modified Bold's basal medium (MBBM) as described (Van *et al.,* 1985). *Chlorella* virus strains SS-2 was previously isolated from fresh water in Korea (Cho *et al.,* 2002). For infection, 100 ml culture of actively growing *Chlorella* NC64A was inoculated with virus at a multiplicity of infection of 0.01-0.001. Cells were incubated until completely lysed, and the lysate was centrifuged in a Sorvall GS-3 rotor at 5,000 rpm for 5 min at 4 °C. Triton X-100 was added to the supernatant fraction to a final concentration of 0.1 %, and the mixture was stirred for 20 min at 4 °C. Virus particles were then pelleted by centrifugation in a Sorvall T-880 rotor at 20,000 rpm for 60 min. The pellet was resuspended in 50 mM Tris-HCl, pH 7.8, and centrifuged through a 10-40 % discontinuous sucrose gradient (20,000 rpm, 20 min, 4 °C). The virus band was collected from the 30-40 % interface and then pelleted for 3 h at 27,000 rpm in a T-880 rotor. The pellet was resuspended in 50 mM Tris-HCl, pH 7.8 (Van *et al.,* 1985).

Isolation and analysis of viral genomic DNA

Isolated virus (400 µl) was mixed with 60 µl of 10 X TEN buffer (100 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1 M NaCl), 60 µl of 1 % Na-sarcosyl, 0.6 µl of 60 % (w/w) CsCl, and a trace amount of ethidium bromide. The mixture was heated at 75 °C for 15 min and then loaded onto a pre-formed 40-60 % (w/w)

CsCl gradient. The mixture was centrifuged in a Sorvall TH-641 rotor at 35,000 rpm for 18 h at 25 °C (Van *et al.,* 1983), the DNA band was collected, and the ethidium bromide was removed by butanol extraction. The DNA was precipitated with ethanol, dried, and resuspended in 1 × TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

PCR and cloning of the *Chlorella* virus SS-2 exonuclease gene

Based on DNA sequence analysis of the *Chlorella* virus SS-2, two primer that amplify the *Chlorella* virus SS-2 exonuclease were designed (Table 1). These primers were designed to create an insert with a BamHI and XhoI restriction sites. The PCR amplifications were performed in 50 μl containing 25 ng of template DNA, 10 mM of each dNTP, 20 pmol each of the primers, 2 units of Taq DNA polymerase (Koma Biotech, Seoul, Korea), 1 μl of 25 mM $MgCl_2$, and 10x reaction buffer. Thermal cycling was performed using an MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, CA, USA) with 35 cycles of denaturation at 94 °C for 30 sec, annealing at 53 °C for 30 sec, and extension at 72 °C for 1 min followed by a final extension at 72 °C for 7 min. The PCR product was electrophoresed on 1 % agarose gel, and the ethidium bromide -stained DNA was purified from the gel using a Gel Extraction kit (Bio-Rad). Gel-purified PCR product was ligated into the pGEM-T easy vector (Promega, Madison, WI, USA) and transferred into *E. coli* XL-1 blue strain. Plasmid DNAs were prepared from selected transformants, and their insert sizes were analyzed by EcoRI digestion. Plasmid with expected size inserts was sequenced, digested with BamHI and XhoI and cloned into the same sites of pET28a vector after digestion with the same enzymes, resulting pET28a-Exo (Fig.1). This expression vector was used for the expression of exonuclease protein of *Chlorella* virus SS-2.

Table 1. Oligonucleotide primers used for the of PCR amplification *Chlorella* virus SS-2 exonuclease

Primer	Sequence	Remarks
SS-2 ExoF	GGA TCC ATG TCG GTG TAT CCT CCA	for SS-2 forward (BamHI site)
SS-2 ExoR	CTC GAG TTA GTA TAT ATC TGT GAT	for SS-2 reverse (XhoI site)

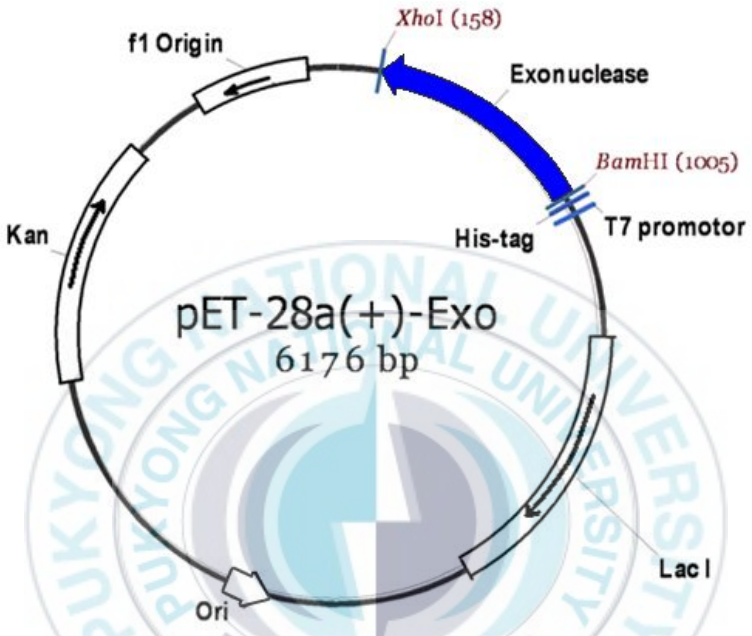


Figure 1. The pET28a(+)-Exo expression vector. The BamH I / Xho I fragment containing the SS-2 exonuclease gene was translationally fused to 6 histidine tag at the N-terminus of the fused recombinant protein.

Expression *Chlorella* virus SS-2 exonuclease in *E. coli*

The pET28a-Exo was transformed into *E. coli* cell containing a chaperone plasmid pKJE7 (Takara, Japan) (Fig. 2). Transformants were transferred in LB medium supplemented with 50 μg/ml kanamycin, 50 μg/ml chloramphenicol for plasmid selection and 0.5 mg/ml L-Arabinose for induction of the chaperone protein, and incubate at 37 ℃ until O.D 600 = 0.8. Induction was performed by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to final concentration of 0.1 mM. Then the cells were harvested by centrifugation at 1, 2, 3, 4 and 5 hours after induction. The conditions for the overexpression of the exonuclease protein were optimized.

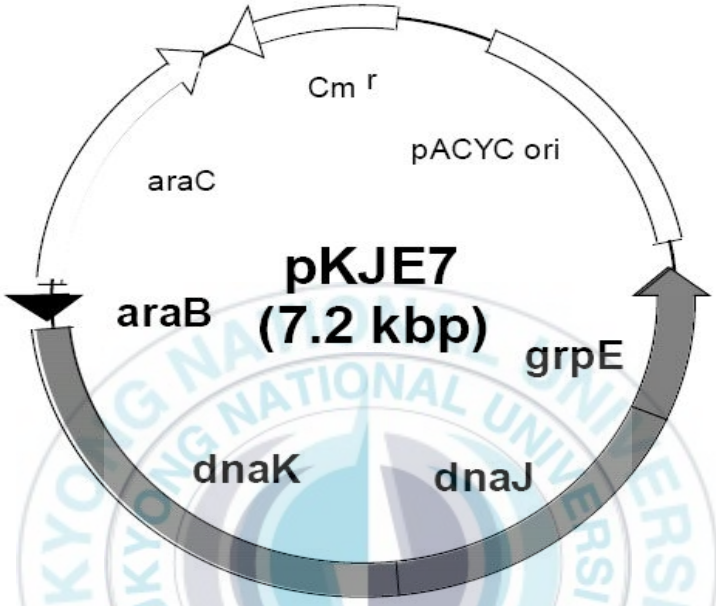


Figure 2. Chaperone Plasmid pKJE7. ori, replication origin of pACYC; Cm^r, chloramphenicol resistance gene; araB, araB promoter; dnaK, heat shock protein; dnaJ, heat shock protein; grpE, heat shock protein; araC, araC repressor gene.

Analysis of expressed protein on SDS-PAGE

The *E. coli* cells induced with 0.1 mM IPTG were centrifuged and the pellet was resuspended in 1 × sample loading buffer and heated at 100 ℃ for 3 min. The sample was incubated on ice for 2min and centrifuged 13,000 rpm for 2 min. Thirty microliters of each sample was loaded on SDS-15 % polyacrylamide gel, and electrophoresed under the constant current of 80 V for 1 hour and 130 V for 2 hours. The gel was stained with Coomassie brilliant blue and then destained with destaining buffer (40 % methanol, 10 % acetic acid)

Western blot analysis

Total proteins separated on a SDS-15 % polyacrylamide gel were transferred onto nitrocellulose membrane at 30 mA for 16 hours in transfer buffer (25 mM Tris, 192 mM Glycine, 20 % methanol). The nitrocellulose membrane was then washed three times for 5 min with TTBS buffer (20 mM Tris, pH 7.4, 0.5 M NaCl, 2.5 mM KCl, 0.05 % Tween-20) and blocked with TTBS buffer containing 5 % skim milk for 1 hour 30 min. The membrane was washed three times for 5 min with TTBS buffer and treated for 1 hour with His-Tag primary antibody (Serotec, Oxford, UK) which was diluted to 1 : 1,000 ~ 1 : 3,000 in TTBS containing 5 % skim milk. After three wash of 5 min, the membrane was treated for 1 hours with alkaline phosphatase conjugated anti-mouse IgG (sigma, USA) in TTBS containing 5 % skim milk. The membrane was washed three times for 5 min in TTBS buffer and developed with NBT/BCIP solution(Sigma, USA).

Purification procedures of exonuclease protein

Purification scheme of the expressed exonuclease protein is described in Figure 3. The cell pellet was collected by centrifugation at 4,000 x g for 20 min at 4 ℃, resuspended in 5 ml of resuspension buffer (20 mM phosphate buffer, 500 mM NaCl, pH 7.4), treated with 250 µl of 10 mM lysozyme and incubated at 4 ℃ overnight. The bacterial cells were ultra sonicated on ice for 30 sec x 6 times at 90 ~ 100 % power, centrifuged at 13,000 rpm for 20 min and the supernatant was collected. The supernatant was further filtrated using 0.22 µm filter and applied into the 1 ml of HisTrap affinity columns which was charged with Ni2+ ions (GE healthcare, Sweden) and prepared by washing with 5 ml of distilled water followed by 10 ml of binding buffer (20 mM Tris-HCl, 500 mM NaCl, and 5 mM imidazole, 1 mM NaN_3, pH 8.0). The protein was eluted with 20 ml of elution buffer (20 mM Tris-HCl, 500 mM NaCl, and 50 mM imidazole, 1 mM NaN_3, pH 8.0) and kept at -20 ℃ for further use.

The samples were dialyzed against buffer B (20 mM potassium phosphate, pH 8, 20 % glycerol, 5 mM β-mercaptoethanol (BME)) at 4 ℃ to remove imidazole and concentrated by dialysis against sucrose.

Cell culture

Medium : LB broth, Culture temperature : 37℃

Resistant marker : 50 μg/ml kanamycin, 50 μg/ml chloramphenicol

Induction agent : 0.5 mg/ml L- Arabinose

0.1 mM IPTG (final concentration)

Induction time : 3 hours

Cell lysis & purification

Centrifugation (4,000 rpm, 20 min. 4 ℃)

⇩

Pellets resuspended (cell of 1 g addition 20 ml resuspension buffer)

⇩

Sonication (for 30 sec at 90~100% power, six times)

⇩

Centrifugation (13,000 rpm, 20 min. 4 ℃)

⇩

Supernatant obtained

⇩

Affinity chromatography using a His-Trap Chelating HP column

⇩

Analysis of the purified protein

(SDS-PAGE, Western Blot)

Figure 3. Purification scheme of exonuclease protein expressed in *E. coli.*

Exonuclease activity assays

The substrate for exonuclease test was prepared by digestion of the pBluescript Ⅱ KS+ (Fermentas, USA) with EcoR I, Pst I and Dra I to create dsDNA with 5'-overhanding ends, 3'-overhanging ends, blunt ends, respectively. Also, a PCR product of 1.4 kbp was used. The standard exonuclease reactions (20 ㎕) was composed with 50 mM Tris-HCl (pH 9), 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 0.1 % BSA, 200 ng substrate nucleotide, and exonuclease protein (10 ng) (Bronstein *et al.,* 1996, Goldstein *et al.,* 1998, Sun *et al.,* 1999, Yamada *et al.,* 1993). Reactions were carried out at 37 ℃ for 20 min (unless otherwise indicated) and stopping immediately by heating at 70 ℃ for 10 min. For the pH titration experiment, the appropriate pH was attained in a buffer mixture of HCl and NaOH. For the divalent metal ion experiment, $MnCl_2$, $ZnCl_2$ or $CaCl_2$ were used in place of 10 mM $MgCl_2$ as indicated in the figure and table legends (Bronstein *et al.,* 1996, Mazur *et al.,* 2001). After the reaction was completed, the DNA mixed with ethidium bromide and separated on a 1.5 % agarose gel. The gel picture was taken by Gel Doc XR System (Bio-Rad, USA) and the intensity x area of the DNA band was analyzed by Quantity One (Version 4.6.1, Bio-Rad, USA) program. The reaction was replicated in 3 different reaction tube and the mean values were used for the analysis.

Database search and sequence alignment of *Chlorella* virus SS-2 exonuclease

The nr database as well as genomic database at National Center for Biotechnology Information (NCBI) were extensively searched with the BLAST program, using independently all avaliable sequences of endonucleases and of

some representatives of alkaline exonuclease. Protein sequence alignments were conducted using Clustal X and manually adjusted on the basis of the BLAST results (Bujnicki *et al.,* 2001, Liu et al., 2003). Accession numbers in GenBank are : PBCV-1, AAC96534; *Chlorella* virus AR158, ABU43746; *Bartonella henselae str. Houston-1*, CAF27185; EsV-1-64, AAK14487; FirrV-1-B43, AAR26918; *Acanthamoeba polyphaga* mimivirus, AAV50623; *Escherichia coli*, APEC O1ABJ00523; *Bacteriophage VT2-Sa*, BAA84296; *Phage BP-4795*, CAD88815.

Prediction of secondary structure

The secondary structure elements of *Chlorella* virus SS-2 exonuclease were predicted using SWISS MODEL WORKSPACE (http://swissmodel.expasy.org/) and the elements of other nucleases were according to their X-ray crystal structure (Liu *et al.,* 2003).

Results

Amplification of *Chlorella* viruses

The virus SS-2 was amplified using *Chlorella* host NC64A. The only known host of *Chlorella* virus are the *Chlorella* strain NC64A exist as hereditary endosymbionts in green isolates of the protozoa *P. bursaria* (Swaminathan *et al.,* 1996). The *Chlorella* cells were completely lysed after 5 days. The life cycle of typical *Chlorella* virus is 480-600 min and the burst size is 200-350 PFU per cell (Van *et al.,* 1985).

Sequence analysis of *Chlorella* virus SS-2 exonuclease gene

The putative exonuclease was amplified by PCR from *Chlorella* virus SS-2 isolated in Korea. The complete nucleotide sequence of *Chlorella* virus SS-2 exonuclease is shown in Figure 4. The gene contains 807 nucleotides and encodes a protein of 268 amino acids with calculated molecular weight of 31.13 kDa. A BLAST search with the *Chlorella* virus SS-2 exonuclease gene found some amino acid identities to other genes observed in algae viruses, including ORF A166R *Paramecium bursaria Chlorella* virus-1 (PBCV-1, 98 %), ORF B43 of *Feldmannia irregularis* virus (FirrV, 28 %) and ORF 64 of *Ectocarpus siliculosus* virus (EsV, 27 %) (Fig. 5).

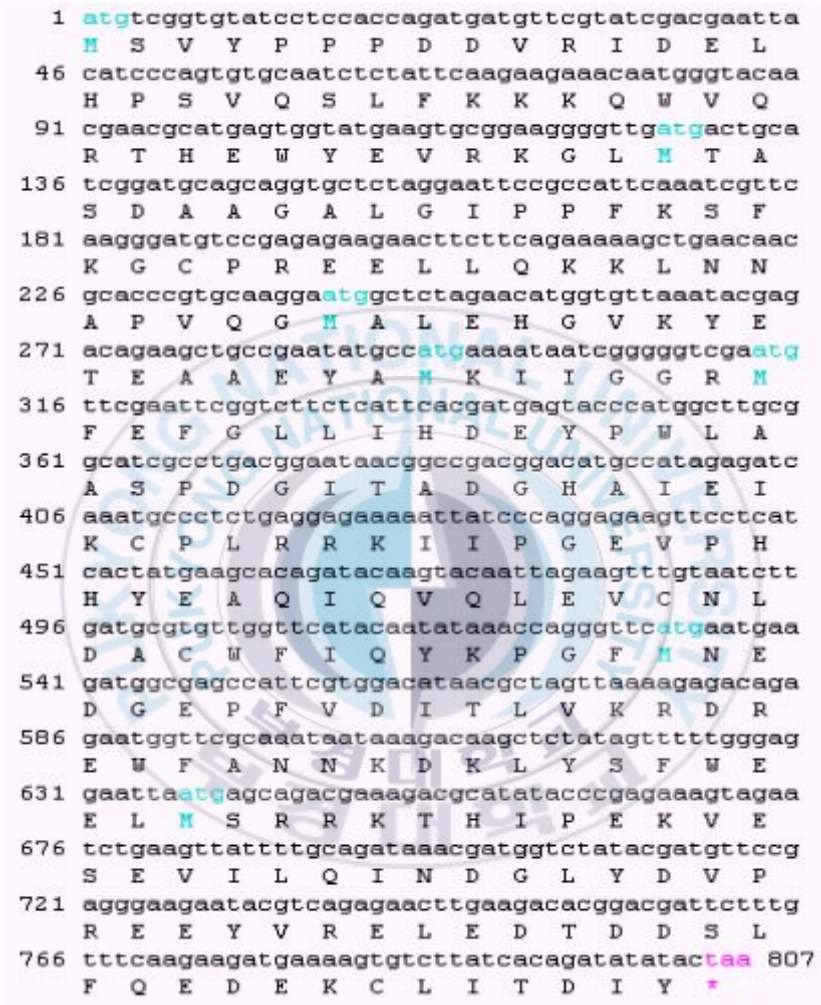


Figure 4. Nucleotide sequence of the *Chlorella* virus SS-2 exonuclease open reading frame (ORF_S) and predicted amino acids.

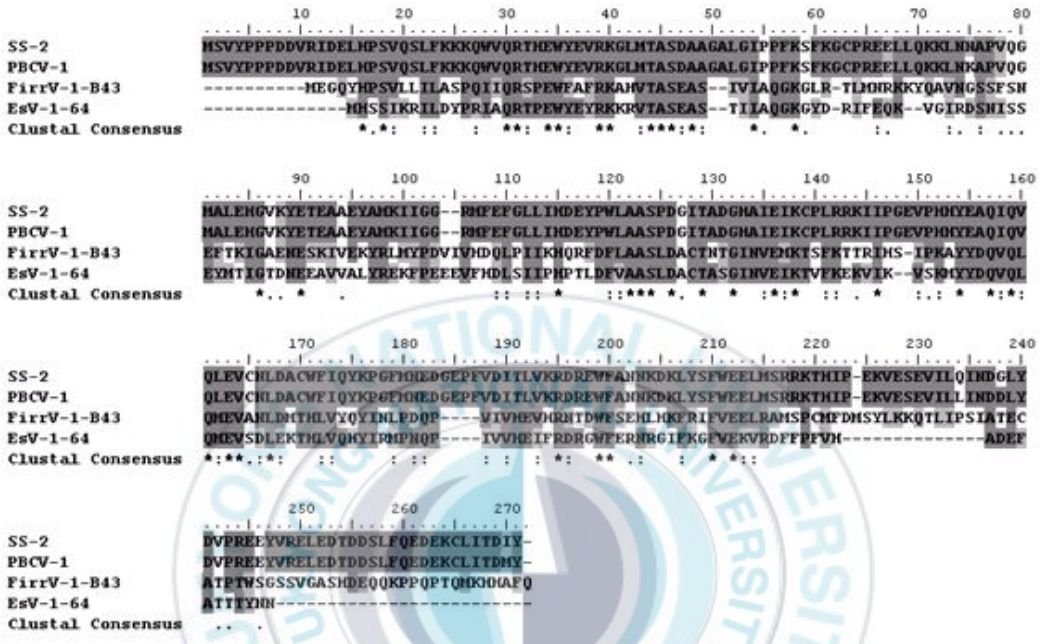


Figure 5. Alignment of deduced amino acid sequence of the *Chlorella* virus SS-2 exonuclease, FirrV-B43 and EsV-64. The identities of amino acid are designated by asterisks, strong similarities by colons and weaker similarities by full point. Identical residues are shown in black boxes, and similar residues are shaded in gray.

Isolation of *Chlorella* virus SS-2 exonuclease gene

PCR amplification with *Chlorella* virus SS-2 exonuclease specific primers produced the one band near 800 bp in 1 % agarose gel electrophoresis (Fig. 6). This band was further gel-purified and cloned into pGEM-T easy vector for sequencing. Nucleotide sequence analysis of transformants confirmed that the putative exonuclease gene from *Chlorella* virus SS-2 genome was successfully amplified through PCR and cloned into pGEM-T easy vector.

Overexpression of *Chlorella* virus SS-2 exonuclease gene in *E. coli*

In order to express the exonuclease gene in *E. coli*, the exonuclease gene was subcloned into pET28-a (+) expression vector and resulted in the pET28a-Exo plasmid which allows the expression of the recombinant protein with N-terminal fusion His-tag (Fig. 1). The plasmid was transformed into *E. coli* cells containing the chaperone plasmid pKJE7 (Fig. 2) and the expression of the recombinant exonuclease protein was induced by addition of L-Arabinose for induction of the chaperone protein and IPTG for the induction of the recombinant protein.

The expression of recombinant exonuclease protein was analyzed by 15 % SDS-PAGE (Fig. 7). The expression conditions for the recombinant exonuclease protein was measured after designated induction time; 1, 2, 3, 4 and 5 hours after addition of IPTG. As shown in Fig. 7, the optimum expression of recombinant exonuclease protein was achieved at 3 hours after IPTG induction. The optimal temperature was 37 ℃. The size of expressed recombinant exonuclease protein was approximately 40 kDa, which reflect the signal extra amino acid in the cloning vector and the 6 histidine tag at the N-terminus.

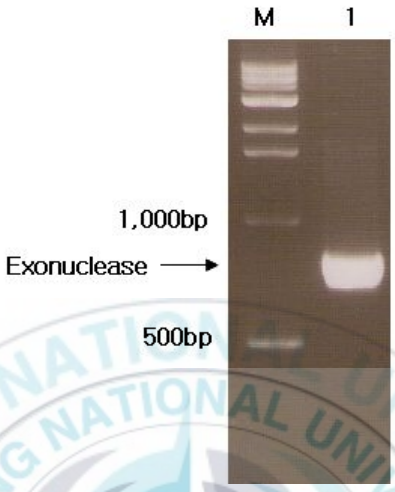


Figure 6. PCR amplification of *Chlorella* virus SS-2 exonuclease. Lane M, 1 kbp DNA Ladder; Lane 1, *Chlorella* virus SS-2 exonuclease.

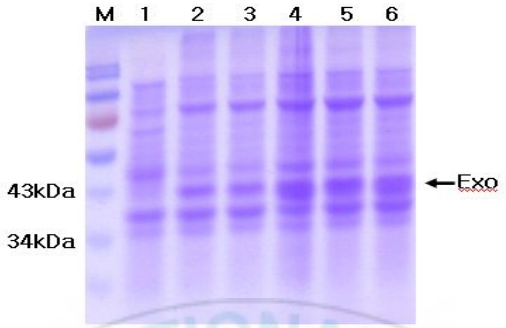


Figure 7. The expression pattern of recombinant *Chlorella* virus SS-2 exonuclease protein by SDS-PAGE. Lane M, Protein Ladder; Lane 1, no induction (control) ; Lane 2 ~ 6, proteins from induced cell after 1, 2, 3, 4 and 5 hour IPTG induction at 37 ℃.

Purification of recombinant exonuclease protein

Despite several efforts for the isolation of the recombinant SS-2 exonuclease in the *E. coli* cells including induction with different concentration of IPTG, different culture temperature and duration of induction, the protein was expressed as insoluble form, which was problem in the purification and enzyme activity test. Therefore, I used the *E. coli* cell containing a chaperone plasmid pKJE7, which are designed to enable efficient expression of multiple molecular chaperones known to work in cooperation with the folding process (Mikhailov *et al.,* 2004). The addition of chaperon protein inducer (L-arabinose) increased the expression SS-2 exonuclease (Fig. 8, lane 3). The SS-2 exonuclease protein expressed in soluble form was purified by affinity chromatography using a His-Trap chealating HP-column (GE healthcare, Sweden). The recombinant SS-2 exonuclease was eluted from the column as single peak (Fig. 9, lane 4 ~ 7) when the protein loaded on the followed by pre-washed with 60 mM imidazole. The concentration of proteins was estimated by the ND-1000 Spectrophotometer (NanoDrop Technologies, USA).

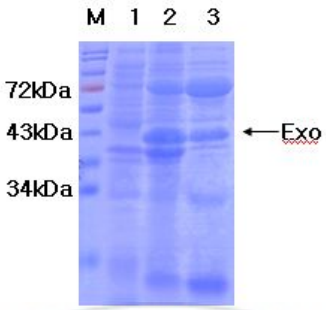


Figure 8. Expression of the *Chlorella* virus SS-2 exonuclease protein in soluble form by SDS-PAGE. Lane M, Protein Ladder; Lane 1, no induction (control); Lane 2, after 3 hours of IPTG induction at 37 ℃; Lane 3, soluble part of #2.

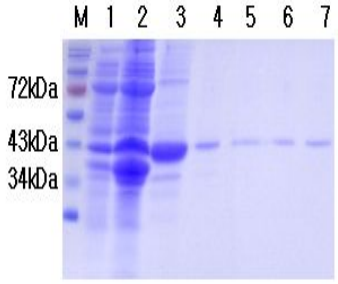


Figure 9. SDS-PAGE pattern of *Chlorella* virus SS-2 exonuclease protein by affinity chromatography. Lane M, Protein Ladder, Lane 1, no induction (control); Lane 2, after 3 hours of IPTG induction at 37 ℃; Lane 3 : soluble part of #2, Lane 4~7, purified part of #2.

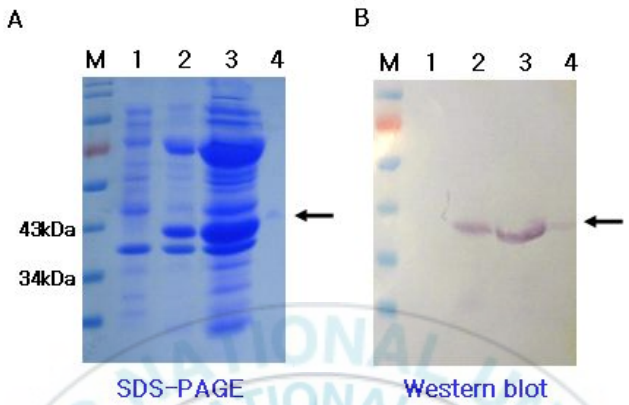


Figure 10. SDS-PAGE(A) and western blot(B) analysis of the purified *Chlorella* virus SS-2 exonuclease protein. Lane M, Protein Ladder; Lane 1, no induction (control); Lane 2, after 3 hours of IPTG induction at 37 ℃; Lane 3, soluble part of #2; Lane 4, purified part of #2.

Exonuclease activity assays.

Several experiments were performed to begin enzymatic characterization of the recombinant exonuclease protein. The *Chlorella* virus SS-2 exonuclease protein was examined its activity using various DNA samples to confirm the presence of this activity in the recombinant protein (Fig. 11). Purified His-tagged SS-2 exonuclease was found to exhibit an exonuclease activity on various DNA sample. The recombinant protein showed exonuclease activity to the DNA with 3'-recessed (5'-overhang) end (Fig. 11, lane 1 and 2), 3'-protruding (3'-overhang) end (Fig. 11, lane 3 and 4), blunt end (Fig. 11, lane 5 and 6) and PCR product of 1.4 kbp (Fig. 11, lane 7 and 8). These results showed that SS-2 exonuclease has its activity on various substrate.

In addition to its well-characterized exonucleolytic properties, a weaker endonuclease activity has been described for some preparations of alkaline exonuclease derived from HSV-1-infected cells (Bronstein *et al.,* 1996). Endonuclease activity of the SS-2 exonuclease protein was carried out by plasmid degradation assays with supercoiled covalently closed circular pBluescript II KS+ as a substrate. In the case of the supercoiled template, endonuclease activity can be analyzed by the quantification of the remaining uncut circular DNA, since nicking of a single nucleotide within the plasmid resulted in the creation of relaxed and linearized molecules; these slower-migrating forms were quickly degraded by the exonuclease activity of *Chlorella* virus SS-2 exonuclease. After 20 min incubation with the SS-2 exonuclease, the amount of both of the supercoiled DNA substrate and contaminated nicked DNA in the preparation decreased (Fig. 11, lane 11 and 12). The results indicated that *Chlorella* virus SS-2 exonuclease also has an associated endonuclease activity in addition to exonuclease activity. An analysis of reaction requirements was performed to establish optimal conditions for

Chlorella virus SS-2 exonuclease activities. There was corelation between the concentration of the enzyme and substrate degradation (Fig. 12A). The 200 ng of substrate nucleotide was invisible after 40 min incubation with 10 ng of exonuclease at 37 ℃ (Fig. 12B). The SS-2 exonuclease showed stable activity in wide range of temperature between 10 ℃ and 50 ℃ (Fig. 13A). This result is unexpected because it is known that the host *Chlorella* cell usually does not grow very well at the temperature of over 25 ℃. Also, the SS-2 exonuclease showed higher activity in the pH range of 6.5-7.5 (Fig. 13B) and 0-1 % BSA with the highest activity at 0.1 % BSA (Fig 13C).

The results shown in Fig. 14 reveal that the recombinant SS-2 exonuclease prefer Ca^{2+} and Mn^{2+} than other cationes with the highest activity at 25 mM Ca^{2+} and Mn^{2+}. In contrast, the SS-2 exonuclease showed inhibition by Na^{+} and Mg^{2+} over 5 mM. Although, the SS-2 exonuclease showed some activity in different concentration of Zn^{2+}, its activity was lower compared to the reaction in other cationes.

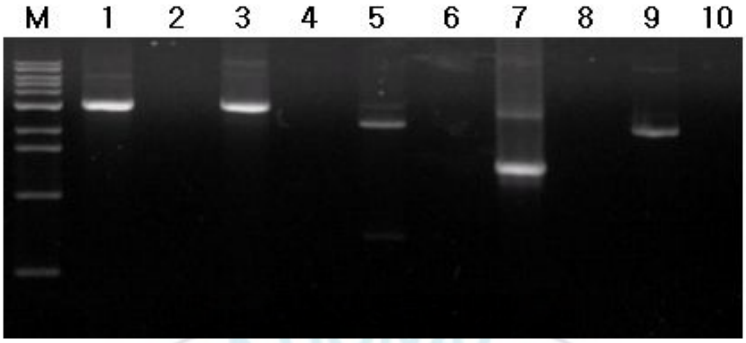


Figure 11. Nuclease activity test using various DNA templates. Lane M, Lane 1, 3, 5, 7, 9: control; Lane 2, 4, 6, 8, 10: activity of SS-2 exonuclease (37 ℃, 20 min, SS-2 exonuclease 10 ng); Lane 1~2 , EcoR I digested pBluescript Ⅱ KS+, sticky end; Lane 3~4, Pst I digested pBluescript Ⅱ KS+, sticky end; Lane 5~6, Dra I digested pBluescript Ⅱ KS+, blunt end; Lane 7~8, PCR product; Lane 9~10, circular pBluescript Ⅱ KS+.

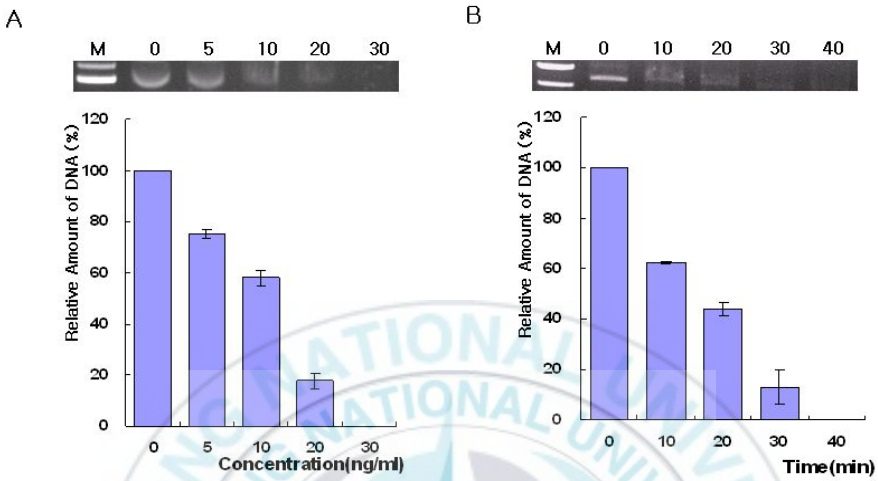


Fig. 12 Degradation of substrate DNA by recombinant SS-2 exonuclease. The mixture containing 200 ng of substrate in final volume of 20 ㎕ was incubated at 37 ℃ with different concentration of recombinant enzyme (A) or for different period with 10 ng recombinant enzyme (B).

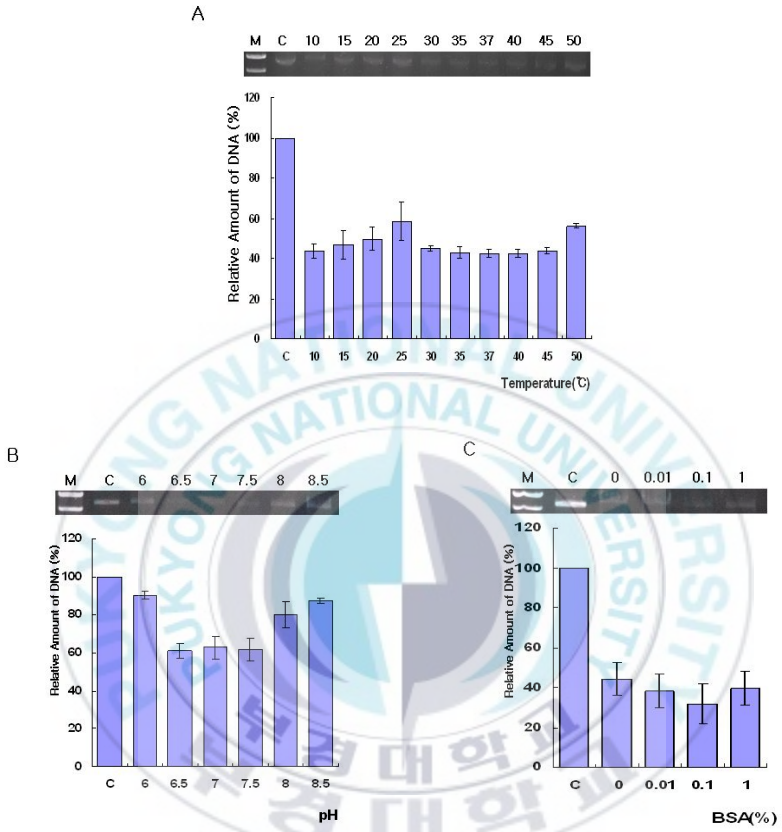


Figure 13. Effect of temperature (A), pH (B), and concentration of BSA (C) on the activity of recombinant SS-2 exonuclease. The reaction was conducted in 20 ㎕ of mixture with 200 ng substrate DNA and 10 ng recombinant enzyme at 37 ℃. The data are mean values from three independent repeats.

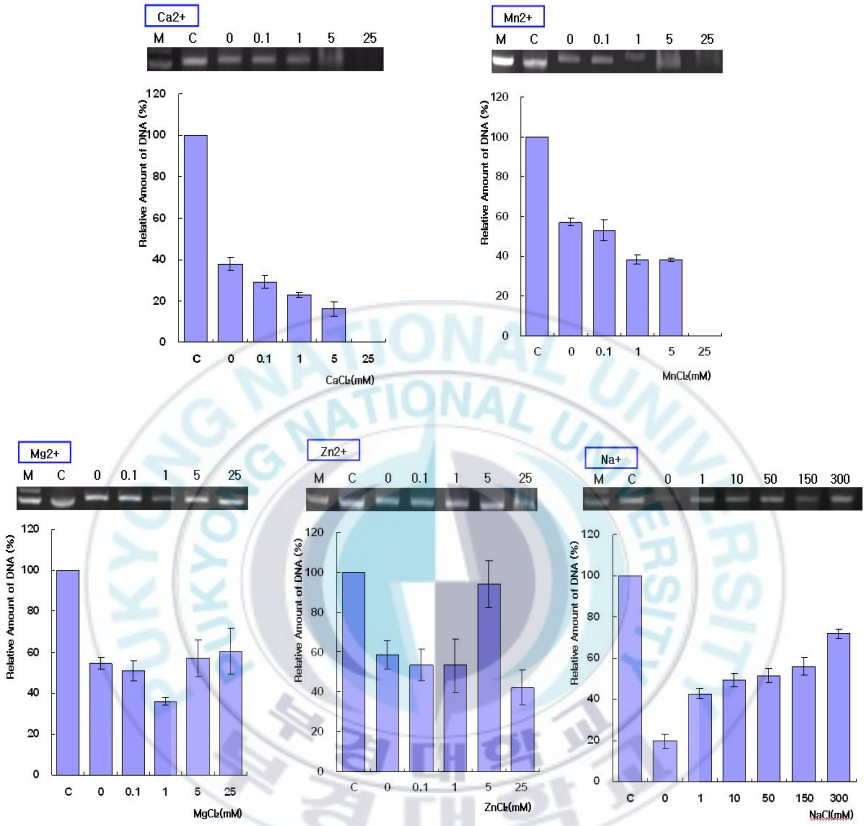
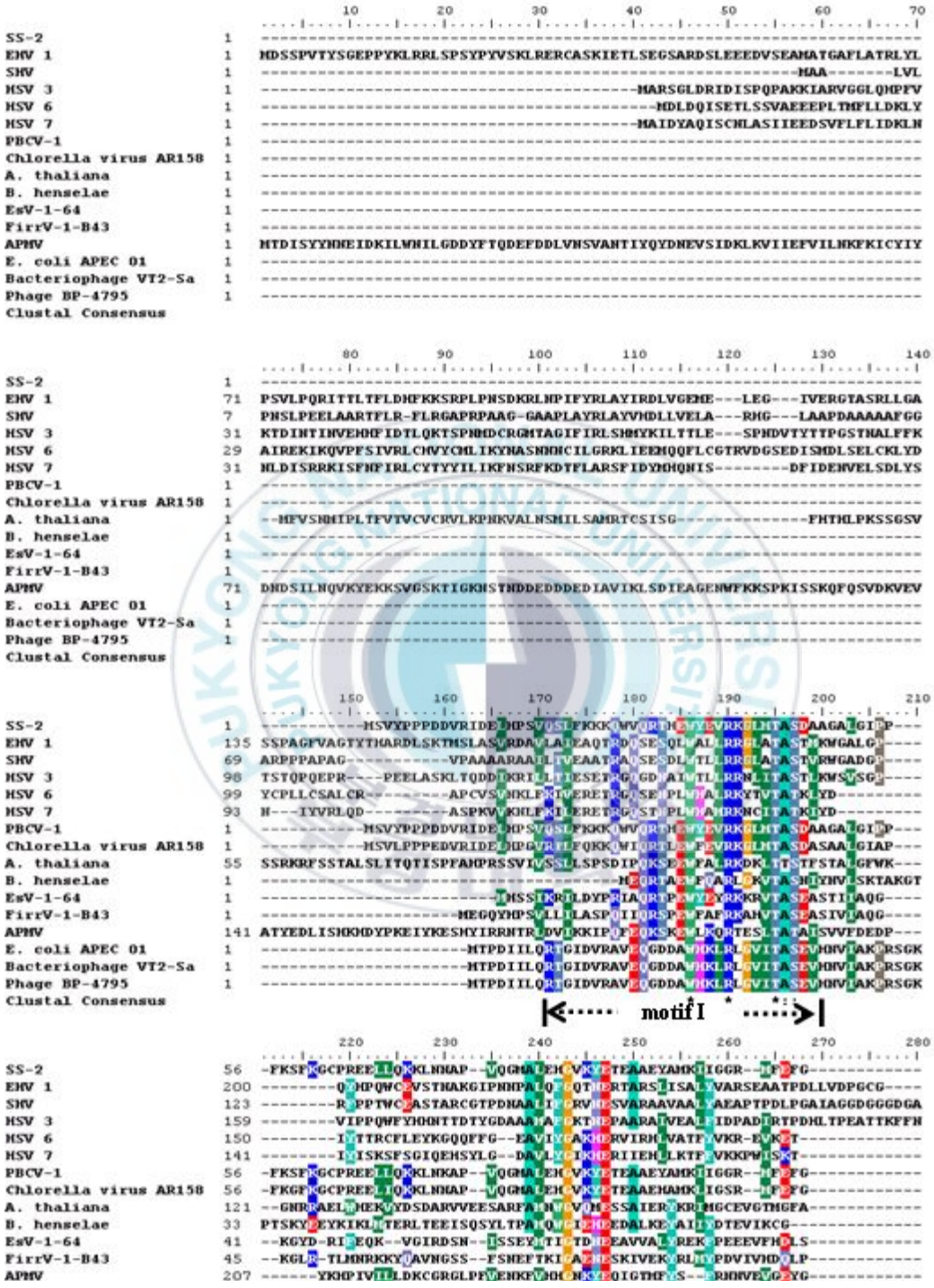
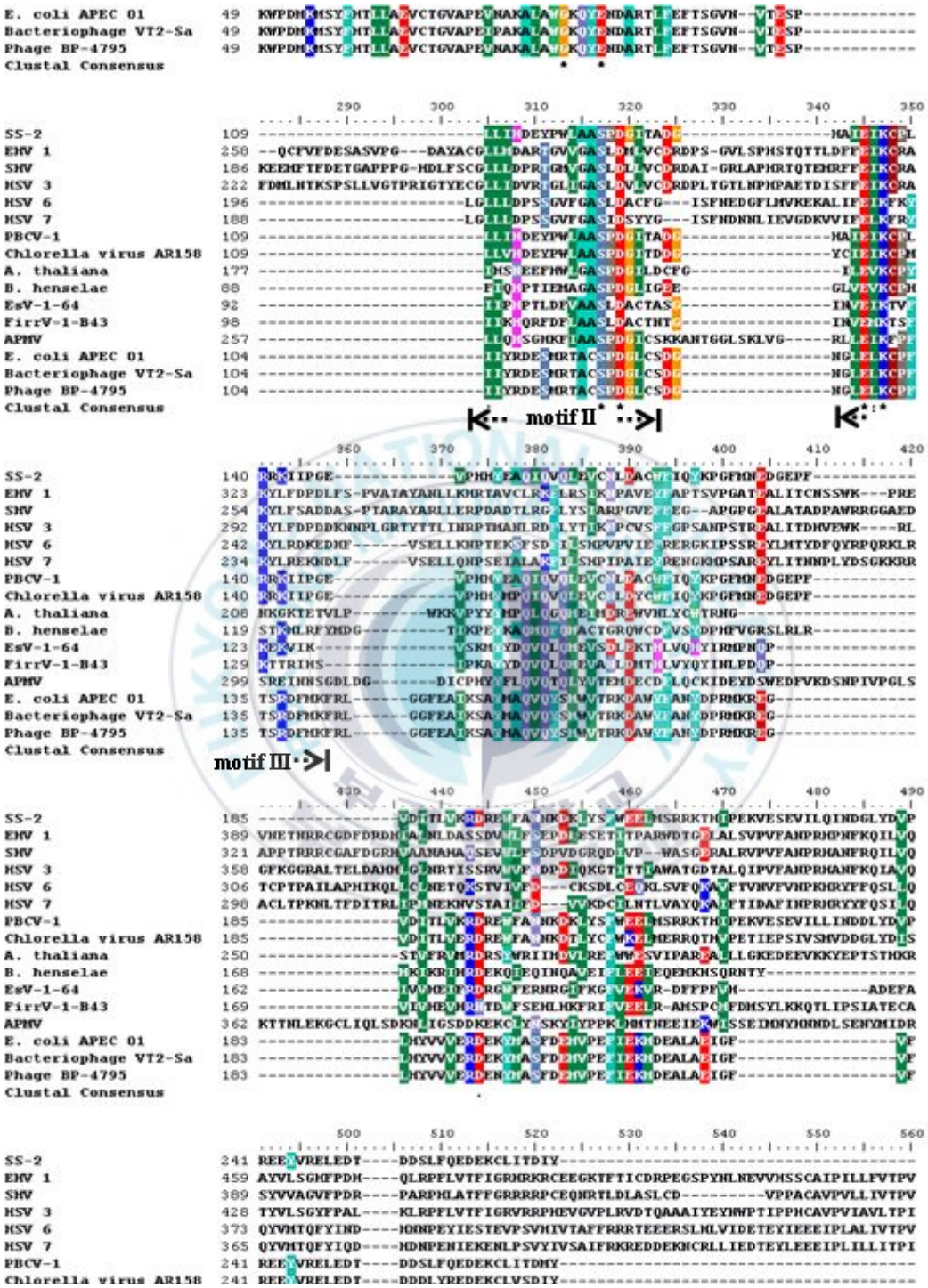


Figure 14. Relative activity of recombinant SS-2 exonuclease in different concentration of cationes. The reaction mixture contained 200 ng of substrate DNA, 10 ng recombinant enzyme was incubated with indicated various type and concentration of cationes at 37 ℃ for 10 min.

Multiple sequence alignment and secondary structure prediction of the conserved D...EXK region of *Chlorella* virus SS-2 exonuclease

The critical amino acid residues of *Chlorella* virus SS-2 exonuclease were predicted by sequence alignment with other nucleases. Sequence alignment of *Chlorella* virus SS-2 exonuclease gene revealed similarity to other phage-related nucleases found in various organisms including *Paramecium bursaria Chlorella* virus 1, *Bartonella henselae*, *Acanthamoeba polyphaga* mimivirus, *Equid* herpesvirus 1, *Human* herpesvirus 1, *Suid* herpesvirus. It has been proposed that phage and viral exonucleases could be derived from a common ancestral enzyme. The alignment revealed that these enzymes share several conserved region (Fig. 15). Comparison of these conserved regions with those of other nucleases and with defined crystal structures revealed that a conserved motif, D91...E111XK113 in the SS-2 exonuclease, has a striking similarity to the catalytic sites of various nuclease (Fig. 15) (Bujnicki *et al.,* 2001, Rychlewski *et al.,* 2001, Knizewski *et al.,* 2007). SS-2 exonuclease proteins possess a predicted conserved core αα$\underline{\alpha}$ββα$\underline{\beta}$βα secondary structure pattern (Knizewski *et al.,* 2007). The secondary structures of this motif were predicted and indicated that D91 was located in a loop and E111 and K113 were located in a β-sheet. The predicted secondary structure involving these three residues were shown to resemble three catalytic residues of the type Ⅱ restriction endonuclease, λ exonuclease and *MutH* (Liu *et al.,* 2003). Based on these similarities, it seems possible that D91, E111 and K113 of *Chlorella* virus SS-2 exonuclease are involved in catalysis.


motif I


motif II
motif III

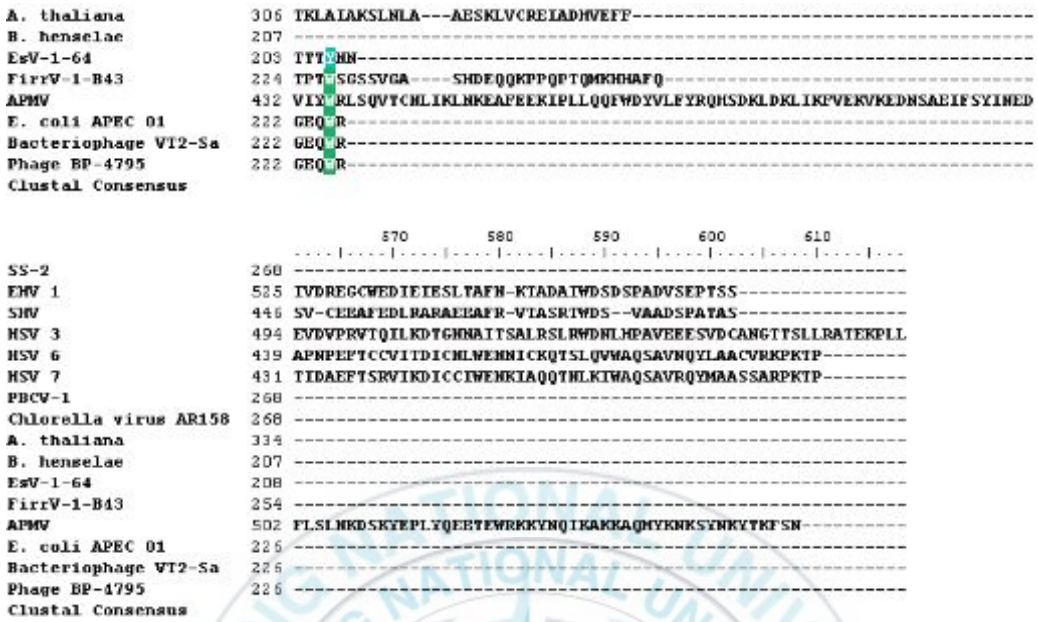


Figure 15. Amino acid sequence alignment of various nucleases. Conserved motifs are labeled according to the nomenclature described for the Herpesvirus alkaline exonuclease subfamily. Invariant residues in the conserved motifs are highlighted by asterisks and conserved residues are highlighted in color.

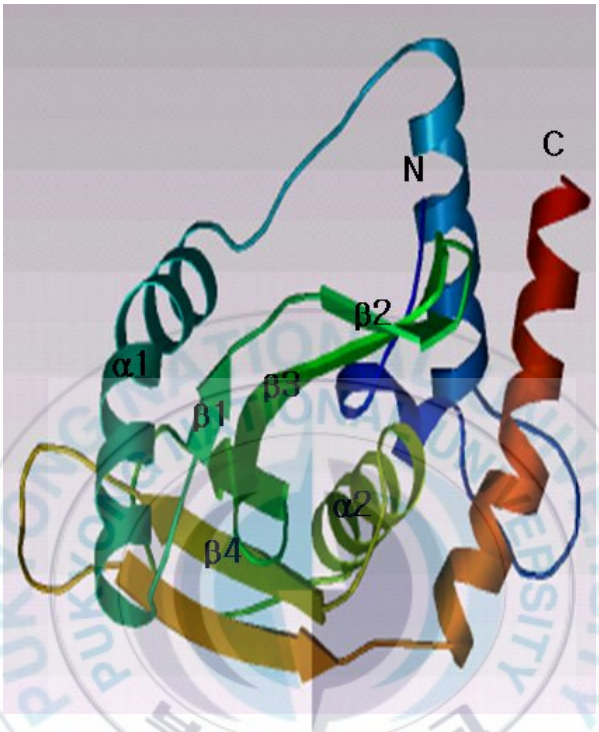


Figure 16. Predicted image of the *Chlorella* virus SS-2 exonuclease.

Discussion

This is, our knowledge, the first study reporting the expression and functional characterization of exonuclease encoded by an algal virus. The *Chlorella* virus SS-2 exonuclease was identified and characterized. In the blast search of the 300 kbp genome sequence of SS-2, I found an ORF encoding a polypeptide with significant similarity to known exonucleases (Fig. 4). Sequence analysis of the cloned gene showed that the SS-2 belong to YqaJ viral recombinase family. This protein family has been found in many different bacterial species but is of viral origin (Vellani *et al.,* 2003). The exonuclease of the *Chlorella* virus SS-2 showed 98 % amino acid identity to the exonuclease of *Paramecium bursaria Chlorella* virus 1 (PBCV-1). Also, SS-2 exonuclease showed 41 %, 28 % and 26 % amino acid sequence homology to the exonuclease of *Ostreococcus tauri, Bartonella tribocorum* and *Bartonella henselae str. Houston-1,* respectively.

The properties of the recombinant exonuclease of *Chlorella* virus SS-2 was analyzed after purification of the protein from *E. coli*. The SS-2 exonuclease degraded circular dsDNA of pBluescript Ⅱ KS+, linear dsDNA with 3'-recessed (5'-overhang) end, 3'-protruding (3'-overhang) end, blunt end and PCR product of 1.4 kbp. The 5'→3' dsDNA exonuclease activity has been reported from exonuclease Ⅲ (Hoheisel. 1993) and Baculovirus alkaline nuclease (Mikhailov *et al.,* 2003), which are active only on 5'-protruding dsDNA substrate. In contrast, the λ exonuclease (Redα) degraded 3'-protruding dsDNA from the 5' ends of dsDNA, producing 3' overhangs (Little. 1967, Mikhailov *et al.,* 2003) as SS-2 exonuclease. These results showed that SS-2 exonuclease has its activity on various double stranded (ds) DNA substrates. The SS-2 exonuclease is of interest in that it has activity on dsDNA with blunt ends, which has not been reported from any exonuclease yet. In addition to its exonuclease activity, the

expressed recombinant protein showed endonuclease activity, which has been observed from the exonucleases encoded by viruses belong to the *Herpesviridae* family (Bujnicki *et al.,* 2001).

In spite of such similarity to alkaline exonuclease, the SS-2 exonuclease showed a little difference in biochemical properties. The alkaline exonuclease showed a high optimum pH, an absolute requirement for Mg^{2+} for activity and sensitivity to high salt concentrations (Hoffmann *et al.,* 1978). In contrast, SS-2 exonuclease was active in a broad pH range from 6.5 to 7.5 with maximum activity at pH 6.5 (Fig. 13B). The SS-2 exonuclease was active between 15 °C and 45 °C with a maximum activity at 37°C. Considering that the optimum temperature for host grow is 25°C, the wide temperature range and optimum temperature of 37°C is unusual. Despite possessing the biochemical properties of other exonucleases, the SS-2 exonuclease was different in that it prefers Mg^{2+} to Ca^{2+} and Mn^{2+} as exonuclease III of *Hemophilus influenzae* (Clements *et al.,* 1978). The exonuclease of *Arabidopsis thaliana* (Plchova *et al.,* 2003) and Bacteriophage terminases (Ponchon *et al.,* 2006) are known to perper Mn^{2+} as SS-2 exonuclease. Maximum exonuclease activity of SS-2 exonuclease was observed with 25 mM Ca^{2+} and Mn^{2+}. Therefore, we may substitute Mn^{2+} and Ca^{2+} for Mg^{2+}. The exonuclease activity was enhanced with low salt concentrations as *E. coli* Endo Ⅳ exonuclease (Kerins *et al.,* 2003) and *E. coli* exonuclease X (Viswanathan *et al.,* 1999). The biochemical properties are similar to those of the Trex1 3'→5' exonuclease which prefers Mg^{2+} and Mn^{2+}, inhibited by Na^{+} and Zn^{2+}, and has optimum pH of 7.5-8.0. (Mazur *et al.,* 2001).

Database search for the conserved domain in the SS-2 exonuclease revealed even higher similarity to the PD-(D/E)XK nuclease superfamily. PD-(D/E)XK nuclease superfamily of endo- and exonuclease (ENase) group are involved in diverse biological processes such as DNA restriction and modification, DNA

repair, and recombination (Aravind *et al.,* 1999; Kovall *et al.,* 1999; Yang *et al.,* 1999, Rychlewski *et al.,* 2001). The SCOP (Murzin *et al.,* 1995) database currently groups 23 families of known structure in the restriction endonuclease-like superfamily, including 15 different restriction endonucleases (Bujnicki., 2003), holiday junction resolvases (endonuclease I, Hjc) (Hadden *et al.,* 2001, Nishino *et al.,* 2001), lambda exonuclease (Kovall *et al.,* 1997) and very short patch repair (Vsr) endonuclease (Tsutakawa *et al.,* 1999). Their function varies from repairing damaged DNA (Vsr), resolving holliday junctions (endonuclease I, Hjc), performing additional cleavage events in DNA recombination (lambda exonuclease), to protection of host organisms against foreign DNA invasion (restriction endonucleases) (Kinch *et al.,* 2005).

It has been demonstrated that SS-2 exonuclease has significant sequence homology and similar biochemical properties to those of PD-(D/E)XK nucleases, and SS-2 exonuclease was expected to have conserved catalytic residues. To identify them, I compared the catalytic residues of SS-2 exonuclease with the catalytic residues of other nucleases for which crystal structures have been determined. I found a conserved motif, D91...E111XK113, in SS-2 exonuclease, which is homologous to the catalytic centers of some nuclease, including type II restriction endonucleases, λ exonuclease (Kovall *et al.,* 1998, Kovall *et al.,* 1999). The exonuclease family of PD-(D/E)XK ENase comprises homologous proteins presently observed virtually exclusively in eukaryotic and prokaryotic viruses (Bujnicki *et al.,* 2001). Members of the PD-(D/E)XK nuclease superfamily generally have four motifs (Bujnicki *et al.,* 2001, Rychlewski *et al.,* 2001, Lukasz *et al.,* 2007). These motifs have been studied by structural and functional experiments. The SS-2 exonuclease proteins was predicted to have the conserved core ααα<u>βββαβ</u>βα secondary structure pattern. (Lukasz *et al.,* 2007). The secondary structures of this motif were predicted and indicated that D91 was located in a loop and E111 and K113 were located in a β-sheet,

similar to the location of catalytic residues of type II restriction enzymes (Liu *et al.,* 2003). Although existing restriction endonuclease-like structures retain similar active site residues within the same core fold (with αββββαβ topology) (Figure 16), they exhibit extreme structural diversity (structure comparison scores can be below threshold) (Kinch *et al.,* 2005). Therefore, further experiments are needed to confirm the roles of catalytic residues in conserved motif.

국문 초록

Chlorella virus는 진핵 *Chlorella*와 같은 녹조류를 감염시키는 바이러스로, 큰 20면체의 구조이며, 플라크를 형성하는 이중나선의 DNA 바이러스이다. *Chlorella* virus SS-2 유전체는 최소한 373개의 주요한 open reading frames (ORFs)를 포함하는 것으로 예상되며, 이들 유전자의 많은 기능적인 부분들은 명확하게 밝혀지지 않았다. *Chlorella* virus SS-2 게놈의 ORFs 중 하나는 몇몇의 다른 exonuclease와 동일한 아미노산 서열을 가진 단백질을 암호화하고 있다. *Chlorella* virus SS-2 exonuclease 유전자의 nucleotide는 807개이며, 31.13kDa의 polypeptide를 암호화하고 있다. *Chlorella* virus SS-2 exonuclease 유전자를 단백질 query를 이용한 단백질 데이터베이스 조사결과 녹조류 바이러스의 ORFs 중 하나인 *Paramecium bursaria Chlorella* virus 1 (PBCV-1, 98%)의 A166R과 높은 유사성을 보이고, 다른 갈조류 바이러스 두 개의 ORFs인 *Feldmannia irereguláris* virus (FirrV, 28 %)의 ORF B43과 *Ectocarpus siliculosus* virus (EsV, 27 %)의 ORF 164와도 유사성을 보이는 것으로 나타났다. *Chlorella* virus SS-2 exonuclease의 활성을 측정하기 위해 *E. coli* 발현시스템을 이용하여 재조합 단백질을 순수 분리하였다. 순수 분리된 재조합 단백질 exonuclease을 다양한 DNA 기질과 37 ℃에서 반응시켜본 결과 활성을 가지고 있음을 확인할 수 있었다. 따라서 우리가 획득한 *Chlorella* virus SS-2 exonuclease 유전자는 기능적인 단백질을 암호화하고 있다고 할 수 있다. 또한, 아미노산 서열의 2차 구조를 예측하고, 다른 종에서 유래된 exonuclease와 비교해보았다. 다른 종에서 유래된 exonuclease와 *Chlorella* virus SS-2 exonuclease의 서열 정렬을 통해 다수의 conserved motif을 확인하였으며, 이는 D91...E111XK113 (D...EXK)로 type Ⅱ restriction endonuclease, λ exonuclease와 *Mut*H를 포함한 몇몇 다른 nuclease의 catalytic sites로 드러난 부분이었다. 이 세부분의 2차 구조는 type Ⅱ restriction endonuclease의 세 개의 catalytic residues와 유사하였다.

이상과 같은 결과를 통하여 *Chlorella* virus SS-2에서 유래한 exonuclease는 활성을 가지며, *Chlorella* virus는 많은 중요한 유전자를 비롯하여 유용한 단백질들을 제공할 수 있는 유용한 sources임을 확인할 수 있다.

References

Agarkova IV, Dunigan DD, Van Etten JL. (2006). Virion-associated restriction endonucleases of Chloroviruses. *J. Virol.* 80(16):8114-23.

Aravind L, Walker DR, Koonin EV. (1999). Conserved domains in DNA repair proteins and evolution of repair systems. *Nucleic Acids Res.* 27(5):1223-42.

Bronstein JC, Weber PC. (1996). Purification and characterization of *Herpes simplex* virus type 1 alkaline exonuclease expressed in *Escherichia coli*. *J. Virol.* 70(3):2008-13.

Bujnicki JM, Rychlewski L. (2001). The Herpesvirus alkaline exonuclease belongs to the restriction endonuclease PD-(D/E)XK superfamily: insight from molecular modeling and phylogenetic analysis. *Virus Genes.* 22(2):219-30.

Bujnicki JM. (2000). Phylogeny of the restriction endonuclease-like superfamily inferred from comparison of protein structures. *J. Mol. Evol.* 50(1):39-44.

Cho HH, Park HH, Kim JO, Choi TJ. (2002). Isolation and characterization of *Chlorella* viruses from freshwater sources in Korea. *Mol. Cells.* 14(2):168-76.

Clements JE, Rogers SG, Weiss B. (1978). A DNase for apurinic/apyrimidinic sites associated with exonuclease III of *Hemophilus influenzae*. *J. Biol. Chem.* 253(9):2990-9.

Goldstein JN, Weller SK. (1998). The exonuclease activity of HSV-1 UL12 is required for in vivo function. *Virology.* 244(2):442-57.

Grabowski G, Jeltsch A, Wolfes H, Maass G, Alves J. (1995). Site-directed mutagenesis in the catalytic center of the restriction endonuclease EcoRI. *Gene.* 157(1-2):113-8.

Hadden JM, Convery MA, Déclais AC, Lilley DM, Phillips SE. (2001). Crystal structure of the Holliday junction resolving enzyme T7 endonuclease I. *Nat. Struct. Biol.* 8(1):62-7.

Hoffmann PJ, Cheng YC. (1978). The deoxyribonuclease induced after infection of KB cells by *Herpes simplex* virus type 1 or type 2. I. Purification and characterization of the enzyme. *J. Biol. Chem.* 253(10):3557-62.

Hoheisel JD. (1993). On the activities of *Escherichia coli* exonuclease III. *Anal. Biochem.* 209(2):238-46.

Huang J, Levin DB. (2001). Expression, purification and characterization of the *Spodoptera littoralis* nucleopolyhedrovirus (SpliNPV) DNA polymerase and interaction with the SpliNPV non-*hr* origin of DNA replication. *J. Gen. Virology.* 82:1767-76.

Kang M, Dunigan DD, Van Etten JL. (2005). *Chlorovirus*: a genus of *Phycodnaviridae* that infects certain *Chlorella*-like green algae. *Mol. Plant. Pathol.* 6(3):213-224.

Kerins SM, Collins R, McCarthy TV. (2003). Characterization of an endonuclease IV 3'-5' exonuclease activity. *J. Biol. Chem.* 278(5):3048-54.

Kinch LN, Ginalski K, Rychlewski L, Grishin NV. (2005). Identification of novel restriction endonuclease-like fold families among hypothetical proteins. *Nucleic Acids Res.* 33(11):3598-605.

Knizewski L, Kinch LN, Grishin NV, Rychlewski L, Ginalski K. (2007). Realm of PD-(D/E)XK nuclease superfamily revisited: detection of novel families with modified transitive meta profile searches. *BMC Struct. Biol.* 7:40.

Kovall R, Matthews BW. (1997). Toroidal structure of lambda-exonuclease. *Science.* 277(5333):1824-7.

Kovall RA, Matthews BW. (1998). Structural, functional, and evolutionary relationships between lambda-exonuclease and the type II restriction endonucleases. *Proc. Natl. Acad. Sci. USA.* 95(14):7893-7.

Kovall RA, Matthews BW. (1999). Type II restriction endonucleases: structural, functional and evolutionary relationships. *Curr. Opin. Chem. Biol.* 3(5):578-83.

Lavrukhin OV, Fortune JM, Wood TG, Burbank DE, Van Etten JL, Osheroff N, Lloyd RS. (2000). Topoisomerase II from *Chlorella* virus PBCV-1. Characterization of the smallest known type II topoisomerase. *J. Biol. Chem.* 275(10):6915-21.

Little JW. (1967). An exonuclease induced by bacteriophage lambda. II. Nature of the enzymatic reaction. *J. Biol. Chem.* 242(4):679-86.

Liu MT, Hsu TY, Lin SF, Seow SV, Liu MY, Chen JY, Yang CS. (1998). Distinct

regions of EBV DNase are required for nuclease and DNA binding activities. *Virology.* 242(1):6-13.

Liu MT, Hu HP, Hsu TY, Chen JY. (2003). Site-directed mutagenesis in a conserved motif of *Epstein-Barr* virus DNase that is homologous to the catalytic centre of type II restriction endonucleases. *J. Gen. Virol.* 84:677-86.

Martinez R, Sarisky RT, Weber PC, Weller SK. (1996). *Herpes simplex* virus type 1 alkaline nuclease is required for efficient processing of viral DNA replication intermediates. *J. Virol.* 70(4):2075-85.

Mazur DJ, Perrino FW. (2001). Excision of 3' termini by the Trex1 and TREX2 3'-->5' exonucleases. Characterization of the recombinant proteins. *J. Biol. Chem.* 276(20):17022-9.

Mikhailov VS, Okano K, Rohrmann GF. (2003). Baculovirus alkaline nuclease possesses a 5'-->3' exonuclease activity and associates with the DNA-binding protein LEF-3. *J. Virol.* 77(4):2436-44.

Mikhailov VS, Okano K, Rohrmann GF. (2004). Specificity of the endonuclease activity of the baculovirus alkaline nuclease for single-stranded DNA. *J. Biol. Chem.* 279(15):14734-45.

Murzin AG, Brenner SE, Hubbard T, Chothia C. (1995). SCOP: a structural classification of proteins database for the investigation of sequences and structures. *J. Mol. Biol.* 247(4):536-40.

Nishihara K, Kanemori M, Kitagawa M, Yanagi H, Yura T. (1998). Chaperone

coexpression plasmids: differential and synergistic roles of DnaK-DnaJ-GrpE and GroEL-GroES in assisting folding of an allergen of Japanese cedar pollen, Cryj2, in *Escherichia coli*. *Appl. Environ. Microbiol.* 64(5):1694-9.

Nishino T, Komori K, Tsuchiya D, Ishino Y, Morikawa K. (2001). Crystal structure of the archaeal holliday junction resolvase Hjc and implications for DNA recognition. *Structure.* 9(3):197-204.

Plchova H, Hartung F, Puchta H. (2003). Biochemical characterization of an exonuclease from *Arabidopsis thaliana* reveals similarities to the DNA exonuclease of the human Werner syndrome protein. *J. Biol. Chem.* 278(45):44128-38.

Ponchon L, Boulanger P, Labesse G, Letellier L. (2006). The endonuclease domain of bacteriophage terminases belongs to the resolvase/integrase/ribonuclease H superfamily: a bioinformatics analysis validated by a functional study on bacteriophage T5. *J. Biol. Chem.* 281(9):5829-36.

Reisser W, Burbank DE, Meints SM, Meints RH, Becker B, Van Etten JL. (1988). A comparison of viruses infecting two different *Chlorella*-like green algae. *Virology.* 167(1):143-9.

Rychlewski L. Bujnicki JM, (2001). Identification of a PD-(D/E)XK-like domain with a novel configuration of the endonuclease active site in the methyl-directed restriction enzyme Mrr and its homologs. *Gene.* 267(2):183-91.

Sun L, Adams B, Gurnon JR, Ye Y, Van Etten JL. (1999). Characterization of two chitinase genes and one chitosanase gene encoded by *Chlorella* virus PBCV-1. *Virology.* 263(2):376-87.

Swaminathan N, Mead DA, McMaster K, George D, Van Etten JL, Skowron PM. (1996). Molecular cloning of the three base restriction endonuclease R.CviJI from eukaryotic *Chlorella* virus IL-3A. *Nucleic Acids Res.* 24(13):2463-9.

Tsutakawa SE, Jingami H, Morikawa K. (1999). Recognition of a TG mismatch: the crystal structure of very short patch repair endonuclease in complex with a DNA duplex. *Cell.* 99(6):615-23.

Van Etten JL, Burbank DE, Xia Y, Meints RH. (1983). Growth cycle of a virus, PBCV-1, that infects *Chlorella*-like algae. *Virology.* 126:117-25.

Van Etten JL, Meints RH, Burbank DE, Kuczmarski D, Cuppels DA. and Lane, L. C. (1981). Isolation and characterization of a virus from the intracellular green alga symbiotic with *Hydra viridis*. *Virology.* 113:704-1.

Van Etten JL, Van Etten CH, Johnson JK, Burbank DE. (1985). A survey for viruses from fresh water that infect a eukaryotic *Chlorella*-like green alga. *Appl. Environ. Microbiol.* 49:1326-8.

Vellani TS, Myers RS. (2003). Bacteriophage SPP1 Chu is an alkaline exonuclease in the SynExo family of viral two-component recombinases. *J. Bacteriol.* 185(8):2465-74.

Viswanathan M, Lovett ST. (1999). Exonuclease X of *Escherichia coli*. A novel

3'-5' DNase and Dnaq superfamily member involved in DNA repair. *J. Biol. Chem.* 274(42):30094-100.

Xia YN, Burbank DE, Uher L, Rabussay D, Van Etten JL. (1986). Restriction endonuclease activity induced by PBCV-1 virus infection of a *Chlorella*-like green alga. *Mol. Cell. Biol.* 6(5):1430-9.

Xia YN, Van Etten JL. (1986). DNA methyltransferase induced by PBCV-1 virus infection of a *Chlorella*-like green alga. *Mol. Cell. Biol.* 6(5):1440-5.

Yamada T, Fukuda T, Tamura K, Furukawa S, Songsri P. (1993). Expression of the gene encoding a translational elongation factor 3 homolog of *Chlorella* virus CVK2. *Virology*. 197(2):742-50.

Yang DH, de Jong JG, Makhmoudova A, Arif BM, Krell PJ. (2004). *Choristoneura fumiferana* nucleopolyhedrovirus encodes a functional 3'-5' exonuclease. *J. Gen. Virol.* 85:3569-73.

Yang J, Malik HS, Eickbush TH. (1999). Identification of the endonuclease domain encoded by R2 and other site-specific, non-long terminal repeat retrotransposable elements. *Proc. Natl. Acad. Sci. USA.* 96(14):7847-52.

Zhang Y, Nelson M, Nietfeldt JW, Burbank DE, Van Etten JL.(1992). Characterization of *Chlorella* virus PBCV-1 CviAII restriction and modification system. *Nucleic Acids Res.* 20(20):5351-6.