

Expression and Characterization of Recombinant *Serratia liquefaciens* Nucleases Produced with *Baculovirus*-mediated Silkworm Expression System

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Abstract *Baculovirus-Bombyx mori* protein expression system has mainly been used for translation of eukaryotic proteins. In contrast, information pertaining to bacterial protein expression using this system is not sufficient. Therefore, recombinant nucleases from *Serratia liquefaciens* (rSINucAs) were expressed in a *Baculovirus-B. mori* protein expression system. rSINucAs containing the native signal peptide (rSINucA-NSP) or silkworm 30-K signal peptide (rSINucA-30K) at the NH₂-terminus were constructed to enable secretion into the extracellular fraction. Both rSINucA-30K and rSINucA-NSP were successfully secreted into hemolymph of *B. mori* larvae. Affinity-purified rSINucAs showed high nuclease activity. Optimum pH was 7.5 and half of maximum activity was maintained between pH 7.0 and 9.5. Optimum temperature was 35 °C. rSINucAs showed sufficient activity in twofold-diluted radioimmunoprecipitation assay buffer and undiluted, mild lysis buffer. Genomic DNA of *Escherichia coli* was efficiently digested by rSINucAs in the bacterial lysate. The results in this study suggest that rSINucAs expressed by the *Baculovirus-B. mori* protein expression system will be a useful tool in molecular biology. Functional recombinant protein of bacteria was produced by *Baculovirus-B. mori*

protein expression system. This system may be highly suitable for bacterial extracellular protein secreted via Sec pathway.

Keywords *Serratia liquefaciens* · *Baculovirus-Bombyx mori* protein expression system · Recombinant nuclease · Sec secretion pathway · Signal peptide

Introduction

The genus *Serratia*, a member of Family Enterobacteriaceae, comprises a group of bacteria that are phenotypically and genetically related [16]. Within the genus *Serratia*, *S. marcescens* is one of the most studied species. *S. marcescens* secretes a number of enzymes including chitinase [35], proteases [5, 6], lipase [21], and nuclease [9]. *S. marcescens* is an opportunistic pathogen and infects a wide range of hosts. The proteins secreted by this species have been studied with regard to pathology and toxicology, and some have been shown to function as virulence factors.

The virulent strain of *S. marcescens* produces ~10 times more protease in culture than the less-virulent strain [28]. When injected into the corneas, the protease has been demonstrated to induce severe lesions, similar to those caused by live bacteria. Lysenko [30] purified chitinase from *S. marcescens* culture, which was then toxic to *Galleria mellonella* larvae. To the best of our knowledge, there is no experimental evidence to show that serratial lipase and nuclease are virulence factors; however, it is suggested that microbial lipases may function as virulence factors [43]. Furthermore, *Streptococcus pyogenes* nuclease A functions as a virulence factor in a mouse infection model [19]. Recently, nuclease from *Aeromonas hydrophila* evades innate immune defenses of the host [25]. Thus,

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certain extracellular proteins produced by *S. marcescens* may be potential virulence factors.

Several enzymological studies have been conducted to clarify the pathological and biological significance of these enzymes. The results showed that some bacterial enzymes also have industrial applications or are useful as a tool in other scientific studies [18]. Among serratial enzymes, *S. marcescens* nuclease (NucA) is widely used in scientific study. NucA is often used to eliminate nucleic acid contamination from purified proteins, commonly from recombinant DNA products, or to reduce viscosity for subsequent processing steps. High viscosity of bacterial lysate caused by released nucleic acid would otherwise hinder subsequent purification steps. Use of nuclease is an inexpensive and effective method to remove the contaminating nucleic acid [3].

It is known that *Serratia* species, with the exception of *S. fonticola*, produce nuclease [16]. Study of other serratial nucleases, however, is insufficient in comparison with that on *S. marcescens* NucA. In a previous study, *Serratia liquefaciens* isolated from an antlion has been shown to be pathogenic to insects, *Bombyx mori*, and *Periplaneta americana* [10]. *S. liquefaciens* is also shown to be closely related to *Serratia proteamaculans* and *Serratia grimesii* [17], and relatively far from *S. marcescens* in phylogenetic trees based on 16S rRNA gene [1, 8] and housekeeping genes [1].

While studying the pathogenesis of *S. liquefaciens* Kuo1-1, we previously determined the draft genome sequence using strain FK01, a nalidixic acid mutant of Kuo1-1 [27, 45]. In the sequence (BAZB00000000), *nucA* gene homolog (*slnucA*, locus tag: SLIQ_04890) was found in scaffold 01 (DF820427). Subsequently, we planned to demonstrate the expression of recombinant *S. liquefaciens* nuclease (rSlNucA).

In this study, rSlNucA was produced using the *Baculovirus-B. mori* protein expression system. This system is widely used for expression of the recombinant protein [29]. This system previously enabled the expression of several useful proteins such as bacterial peptide-*N*-glycosidase F [31], urease B subunit and heat shock protein A subunit [47], fungal peroxidase fusion protein [20], arthropodic endo- β -1,4-glucanase [24], insect ferritin light chain [23], endo- β -*N*-acetylglucosaminidase H [33], DNA methyltransferase DNMT-1 [32], fish tributyltin-binding protein type 1 [41] and type 2 [40], plant mismatch endonuclease [34], and human defensins [14]. *S. marcescens* NucA is also expressed by *E. coli* [13]. Although experimental evidence is unavailable, rSlNucA can be successfully expressed in the *E. coli* system owing to its sequence similarity. The expressed rSlNucA is found in the *E. coli* inclusion body, which is dissolved by urea to obtain the *S. marcescens* NucA enzyme. The purification procedure for

soluble protein is simpler and faster than that for insoluble protein and is subsequently, cost effective. Therefore, in this study, we attempted to express rSlNucA as a soluble protein in hemolymph using the *Baculovirus-B. mori* protein expression system.

Baculovirus-B. mori protein expression system is mainly used for production of eukaryotic proteins; however, information regarding bacterial protein expression is limited [31, 47]. Although the *E. coli* expression system is a useful and widely used method, certain drawbacks remain, even with regard to bacterial protein expression. For example, if the expressed recombinant protein is toxic to *E. coli*, it may result in low yield or low activity by mutation. In this study, rSlNucA was expressed using this system as an example of bacterial proteins and was subsequently characterized.

Materials and Methods

Cloning of *S. liquefaciens* Nuclease (*slnucA*) Gene

Nuclease gene (*slnucA*) was amplified from genomic DNA of *S. liquefaciens* FK01 using the primer pair, SlNucA-5-NOATG (cggtttaacaagatgtagctctggaacc) and SlNucA-3-XhoI (ggggctcagactcttgcaccatcagctc), and KOD-Plus-Neo DNA polymerase (TOYOBO, Tokyo, Japan). The amplified, complete coding region of *slnucA* (residues 1–265), including the native signal peptide (NSP) region, was cloned into the pENTRL21TEVH8STREP vector [33] that contained the attL1–attL2 sequence for Gateway technology and C-terminal His and STREP-tag.

The coding region of SlNucA (residues 21–265), not including the native signal peptide was amplified with the primer pair, SlNucA-5-NOSP (gaggcattggaatctatcgacaact-gcgcg) and SlNucA-3-XhoI (ggggctcagactcttgcaccatcagctc), and cloned into a pENTRL2130KTEVH8STREP vector that contained the silkworm 30-K (MRLTLFAFV-LAVCALASNA) signal peptides at its NH₂-terminus and COOH-terminal His and STREP-tag.

Generation of Recombinant *Baculoviruses*

Above two recombinant entry plasmids (pENTRL21NSP-SlNucA-TEVH8STREP and pENTRL2130K-SlNucA-TEVH8STREP) were transposed to the destination vector (pDEST8, Invitrogen, USA) with Gateway LR reaction as per the manufacturer protocols. The obtained transfer plasmids were transformed into *E. coli* BmDH10Bac (BmNPV expression system [36]). The SlNucA expression cassette was transferred into a mini-attTn7 target site of a *Baculovirus* shuttle vector (bacmid) via in vivo transposition mediated by Tn7 transposase.

The recombinant bacmid DNA was transfected into NIAS-Bm-oyanagi2 cells (kindly provided by Dr. Imanishi) by lipofection using the FuGENE HD transfection reagent (Promega, USA). Generation and large-scale harvest of recombinant baculoviruses were carried out as per the manufacturer protocols (Invitrogen). The viral titers were determined by the end-point dilution method [38].

Expression of rSINuCA

The expression level of rSINuCA by viral infection in NIAS-Bm-oyanagi2 cells was measured as follows: 1×10^6 cells per well in six-well tissue culture plates were infected with recombinant BmNPVs at a multiplicity of infection (MOI) values of 1. The infected cells and culture medium were collected at 4 days post-infection (dpi). After centrifugation at $1000 \times g$ for 10 min at 4 °C, the culture medium (extracellular proteins) and cells (intracellular proteins) were diluted with phosphate-buffered saline (PBS, 8.1 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , 136.9 mM NaCl, 2.7 mM KCl) and solubilized in Laemmli sample buffer, respectively. Extracellular and intracellular samples (4.5- μl culture equivalent) were separated with 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electroblotting, the recombinant proteins were detected using HisProbe-HRP conjugate (1:2000, Thermo Fisher Scientific Inc., MA, USA) as previously described [33].

With regard to rSINuCA expression levels in silkworm hemolymph, recombinant viruses were carefully injected into the hemocoel of silkworm larvae (d17 strain) on Day 2 of the 5th instar at the dose of 1×10^5 pfu per larva using a microliterTM syringe with a 30-gage needle (Hamilton Co., USA). After 4 dpi, hemolymph was collected. After centrifugation at $10,000 \times g$ for 30 min at 4 °C to remove insoluble matter, hemolymph was diluted with PBS. rSINuCA (1- μl hemolymph equivalent) was separated by 15 % SDS-PAGE and recombinant proteins were detected with HisProbe-HRP conjugate. Hemolymph collected from mock-inoculated (empty vector), and noninfected larvae were used as negative control. The experiments were repeated three times.

Purification of rSINuCA

After 4 dpi, 10 ml hemolymph, from approximately 25 silkworm larvae, was purified by two-step affinity purification using HisTrap excel (GE Healthcare Bioscience, Piscataway, NJ, USA) and StrepTrap HP columns (IBA GmbH, Germany) as previously described [33].

Concentrations of purified rSINuCA were determined using Pierce BCA protein assay kit (Thermo Fisher

Scientific Inc., MA, USA), with bovine serum albumin as a standard. The proteins were suspended in storage buffer containing PBS and then stored at -80 °C until use.

In order to confirm nuclease activity, a 10- μl reaction mixture containing rSINuCA, 20 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 7.5), 2 mM MgCl_2 , and 100 ng substrate was incubated at 37 °C for 30 min. λ DNA and *B. mori* total RNA were used as substrates in DNase and RNase activity tests, respectively. The reaction was stopped by adding 2 μl loading buffer containing 100 mM EDTA. Degradation of DNA and RNA was analyzed by agarose gel electrophoresis. Benzonase[®] (Merck Millipore Co., Darmstadt, Germany) was quantified by ImageJ using Coomassie Brilliant Blue R-250-stained SDS-PAGE gel, and was used as a positive control. The experiments were repeated at least twice.

Homomultimer Formation of rSINuCA

Ten μl of rSINuCA in storage buffer (400 ng/ μl) was mixed with an equal volume of glutaraldehyde and the mixture was incubated at 30 °C for 30 min. The cross-linking reaction was quenched by adding 2 μl of 1 M Tris–Cl (pH 8.0). The samples were added to an equal volume of 2 \times Laemmli sample buffer and a 500-ng protein aliquot was analyzed by 10 % SDS-PAGE. The experiments were repeated twice.

Characterization of rSINuCA

Standard DNase Assay

An aliquot of 100- μl reaction mixture containing 1 ng rSINuCA, 20 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 7.5), 2 mM MgCl_2 , and 50 μg DNA (salmon testes) was incubated at 37 °C for 30 min. The reaction was stopped by adding an equal volume of 5 % perchloric acid and then chilled for 20 min on ice. After centrifugation at $20,630 \times g$, at 4 °C for 5 min, an aliquot of supernatant was diluted with nine volumes of water. Absorbance at 260 nm of diluent was measured. Sample without rSINuCA was used as blank, measured in the same manner.

To characterize rSINuCA, composition of reaction or reaction conditions were changed as needed, described below. The experiments were repeated three times.

Optimum pH

The reaction was carried out using different buffer systems, including acetic acid–sodium acetate (pH 4.0–5.5), NaH_2PO_4 – Na_2HPO_4 (pH 6.0–8.0), boric acid–NaOH (pH 8.5–9.0), and NaHCO_3 –NaOH (pH 9.5–10.0).

pH Stability

rSINucA (400 ng/μl) and 100 mM buffer (acetic acid–sodium acetate at pH 4.0–5.5, NaH₂PO₄–Na₂HPO₄ at pH 6.0–8.0, boric acid–NaOH at pH 8.5–9.0, NaHCO₃–NaOH at pH 9.5–10.0) were mixed in a ratio of 1:3 and incubated at 4 °C for 18 h. An aliquot was diluted with 99-fold volume of storage buffer. The diluent (1 ng protein/μl) was used as enzyme solution.

Optimum Temperature

DNase assay was carried out at 5, 10, 15, 20, 25, 30, 35, 37, 40, 45, 50, 55, and 55 °C for determination of optimum temperature.

Heat Stability

rSINucA in storage buffer was incubated at 30, 35, 40, 45, 50, 55, 60, 65, and 70 °C for 30 min. The heat-treated protein was used as enzyme solution.

Effect of Divalent ion on DNase Activity

To determine optimum concentration of MgCl₂, reaction was supplemented with 0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 mM of MgCl₂.

Various metal ions including MgCl₂, CaCl₂, MnCl₂, CoCl₂, ZnCl₂, and BaCl₂ were added to the reaction at concentrations of 4 and 16 mM.

Effect of NaCl and KCl on DNase Activity

Reactions were supplemented with additional NaCl and KCl to be final concentrations of 6.25, 12.5, 25, 50, 100, 200, and 400 mM. Since storage buffer contained NaCl and KCl, traces of these salts (1.369 mM NaCl, 0.027 mM KCl) were also contributed by the storage buffer.

Effect of Detergents on DNase Activity

Detergents including Brij 35, Brij 58, Nonidet P-40, Triton X-100, Tween 20, Tween 80, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, sodium deoxycholate, sodium *N*-lauroylsarcosinate, and sodium dodecyl sulfate were added to reactions at the final concentrations of 0.1 and 1 %.

DNase Activity in Lysis Buffers

Nuclease is often used to reduce viscosity of the lysate during bacterial protein extraction. Therefore, two different lysis buffers: strong (RIPA buffer, 50 mM Tris–Cl, pH 8.0, 150 mM NaCl, 0.5 % sodium deoxycholate, 1 % Nonidet

P-40) and mild (1.5 % Triton X-100, 0.4 % sodium deoxycholate in PBS) lysis buffer, were used for protein extraction from whole cell and inclusion body purification, respectively, for DNase activity of rSINucA.

The reaction mixture (100 μl) containing 1 ng rSINucA, 2 mM MgCl₂, 50 μg DNA (salmon testes), and lysis buffer (series of twofold dilutions) was incubated at 37 °C for 30 min.

Analyses of DNA and Protein Prepared from Bacterial Lysate after rSINucA Treatment

E. coli BL21 DE3 (pLysS, pET16b) was cultured in LB broth containing appropriate antibiotics at 37 °C for 16 h. After centrifugation of 25 μl bacterial culture, the pellet was resuspended with half-volume of water (12.5 μl). An equal volume of 2× RIPA buffer was added to the suspension and mixed well. Subsequently, 25 μl of 4 mM MgCl₂ and 1 μl rSINucA were added. The samples were incubated at 37 °C for 30 min.

For DNA extraction, the reaction was stopped by phenol/chloroform extraction. An aliquot (10-μl culture equivalent) of the water layer after centrifugation was analyzed by agarose gel electrophoresis.

For protein extraction, reaction was stopped by adding an equal volume of 2× Laemmli sample buffer and immediately incubated at 90 °C for 3 min. The protein sample (2.5-μl culture equivalent) was analyzed by SDS-PAGE. These experiments were repeated twice.

Results

Expression and Purification of rSINucA

A phylogenetic tree based on the amino acid sequences of nucleases and multiple alignment is shown in Fig. 1. The amino acid residues contributing magnesium ion (Asn) and involved in substrate (Asp, Arg, Arg) binding were conserved in all sequences analyzed. rSINucA constructs, as shown in Fig. 2a, contained signal peptide sequence (native or 30K) at the NH₂ terminal and TEV cleavage, His-tag, and strep-tag sequences at the COOH-terminal.

Recombinant baculoviruses constructed for rSINucA expression were inoculated into NIAS-Bm-oyanagi2 cell culture. Both rSINucAs were detected in intracellular fraction but not in extracellular fraction (Fig. 2b, left). When the baculoviruses were used to infect silkworm larvae, recombinant proteins were secreted into the hemolymph (Fig. 2b, right). Fractions were analyzed by SDS-PAGE (Fig. 2c) at each purification step. As a result, rSINucAs were highly purified by two-step affinity purification.

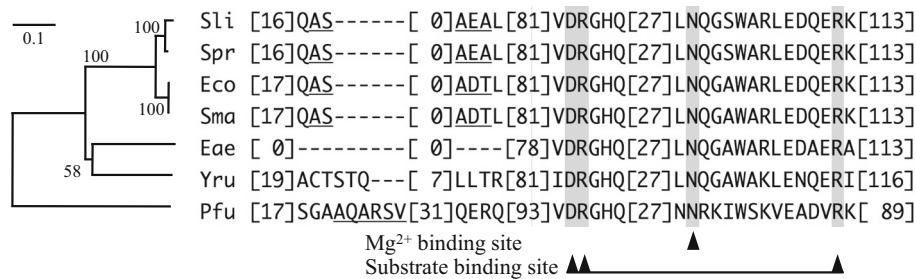


Fig. 1 Phylogenetic analysis and multiple alignments based on amino acid sequences of nucleases. In the phylogenetic tree, the number for each interior branch is the percent bootstrap value (1000 resamplings). *Pseudomonas fuscovaginae* nuclease was used as an outgroup. In alignment, *underlined letters* indicate three upstream and two downstream amino acid residues of putative cleavage sites. *Arrowheads* indicate conserved residues observed in Nuc domain.

The numbers in brackets indicate the number of amino acids. Abbreviations for species (accession numbers): Sli, *Serratia liquefaciens* (GAK25990); Spr, *S. proteamaculans* (WP_012006235); Eco, *Escherichia coli* (KLX16257); Sma, *S. marcescens* (M19495); Eae, *Enterobacter aerogenes* (WP_020079743); Yru, *Yersinia ruckeri* (WP_038251463); Pfu, *P. fuscovaginae* (WP_010451627)

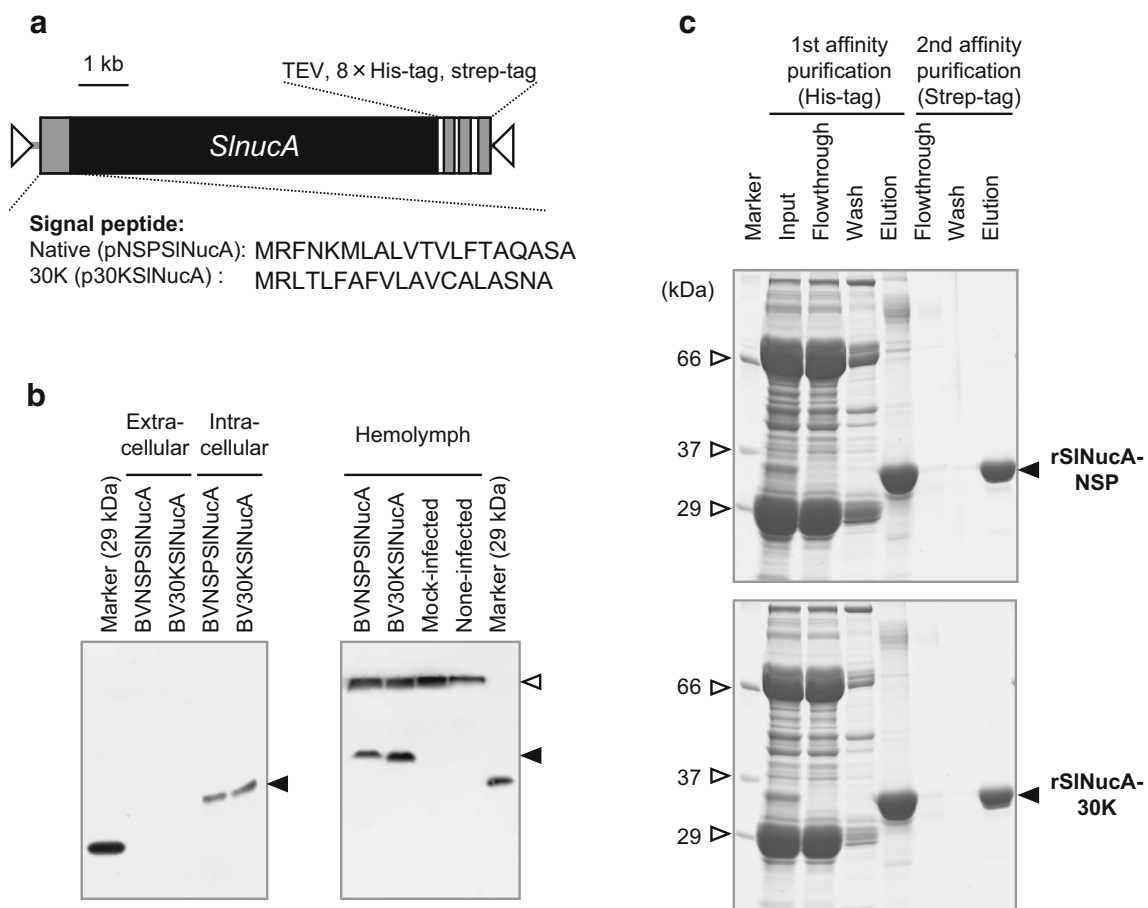


Fig. 2 Expression and purification of rSINuCA. Two nuclease genes harboring different signal peptides (**a**). Expression of rSINuCA was carried out using cultured cells (**b**, left panel) and *B. mori* larvae (**b**, right panel). *Closed triangles* indicate recombinant nuclease. *Open triangle* indicates nonspecific band originated from hemolymph

protein that reacts with HisProbe. Purification of rSINuCA containing native signal peptide (**c**, upper panel; rSINuCA-NSP) and 30K signal peptide (**c**, lower panel; rSINuCA-30K). First, rSINuCA were purified by His-tag, subsequently by Strep-tag. To detect contaminating protein, more than adequate quantity was loaded in the analysis

rSINuCA digested double-stranded DNA (λ DNA) and total RNA (*B. mori*) in a dose-dependent manner (Fig. 3). At 37 °C, 100 pg rSINuCA adequately digested 100 ng

DNA and RNA within 30 min. DNase and RNase activities were approximately 10 times lower than those of commercially available recombinant *S. marcescens* NucA,

Benzonase[®], the reason for which is unclear. This result indicated that rSINucA is a nonspecific nuclease found in a wide range of organisms such as *Bacillus subtilis*, *Azotobacter agilis*, *S. pyogenes*, *Streptomyces antibioticus*, *Drosophila melanogaster*, and *Nicotiana tabacum* [39].

Homomultimer Formation of rSINucA

To determine whether rSINucA exists as a monomer or multimer, the protein cross-linked by glutaraldehyde was analyzed by SDS-PAGE (Fig. 4). The intensity of the dimeric band increased while that of the monomeric band decreased with an increase in glutaraldehyde concentration. This result indicates that rSINucA forms homodimers under the conditions studied.

Characterization of rSINucA

Enzymatic characteristics of rSINucAs were determined. In general, the difference in native (rSINucA-NSP) or 30-K *B. mori* (rSINucA-30K) signal peptides did not affect the characteristics to a large extent.

The optimum pH for rSINucAs was 7.5, with 50 % relative activity between pH 7 and 9.5 (Fig. 5a). At pH 5.0

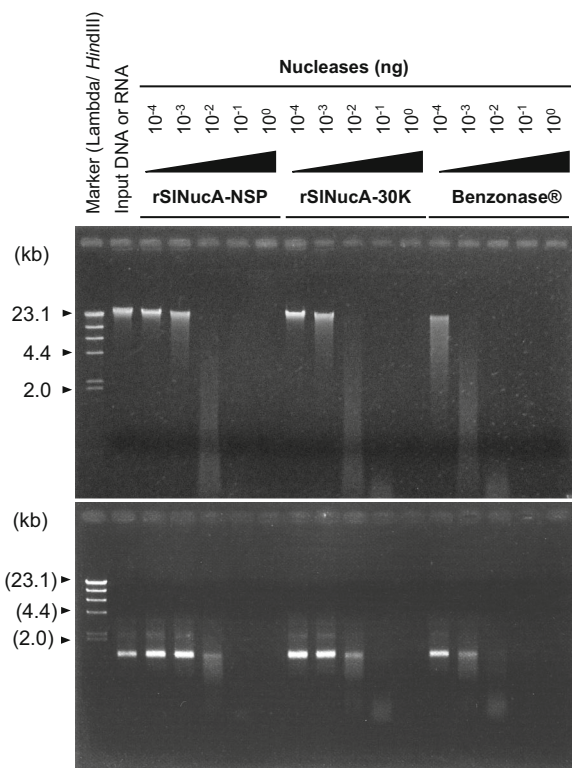


Fig. 3 Nuclease activity of rSINucA. λ DNA (upper panel) and *B. mori* total RNA (lower panel) were incubated with different doses of recombinant nuclease at 37 °C for 30 min. Benzonase[®] was used as a control. In the lower panel, values of molecular weight are indicated in parentheses, since DNA marker was used

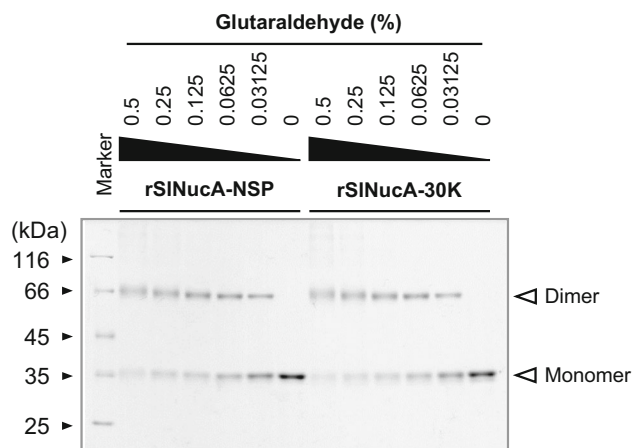


Fig. 4 Homomultimer formation of rSINucA. The recombinant proteins were cross-linked with glutaraldehyde. After quenching, the proteins were analyzed by SDS-PAGE

or lower, nuclease activity was not detected. These enzymes were highly stable within a wide range of pH at 4 °C for 18 h (Fig. 5b). They retained at least 72 % activity (rSINucA-30K at pH 4.0) or more within this range.

Optimum temperature was 35 °C (Fig. 6a). At 15 °C or lower, and 50 °C or higher, the relative activity was less than 50 %. Heat stability testing revealed that the enzymes were inactivated as temperature increased (Fig. 6b). At 55 °C or higher, the activity was less than 50 %.

When $MgCl_2$ was omitted from the reaction, nuclease activity was not detected (Fig. 7a). Until 4 mM, the activity increased in a dose-dependent manner. In the range of 1–16 mM, the relative activity was 76 % (rSINucA-30K with 1 mM $MgCl_2$) or more. Although divalent metal ions also promoted the activity partially, the effect was approximately 50 % at best (rSINucA-30K with 4 mM $CaCl_2$), in case of $MgCl_2$ (Fig. 7b).

Higher concentrations of NaCl (Fig. 8a) and KCl (Fig. 8b) inhibited nuclease activity. Since the reaction contained at least 1.369 mM NaCl and 0.027 mM KCl contributed by the storage buffer, the activity in the complete absence of these salts was not determined in this study.

Activity was promoted by supplementation of nonionic detergent at 0.1 % (Fig. 9). Although most nonionic detergent did not inhibit the activity at 1 %, Triton X-100 and Tween 20 inhibited at this concentration (less than approximately 50 %). A twitter ionic detergent, CHAPS inhibited activity of rSINucA-NSP to 50 %, but did not affect rSINucA-30K activity. Among anionic detergents, no activity was detected when 1 % sodium deoxycholate (DOC) or 0.1 and 1 % SDS were used.

The optimum pH and temperature for *S. marcescens* nuclease are 8.0–8.5 and 35–44 °C, respectively, and require Mg^{2+} , Mn^{2+} , or Co^{2+} for activity [39].

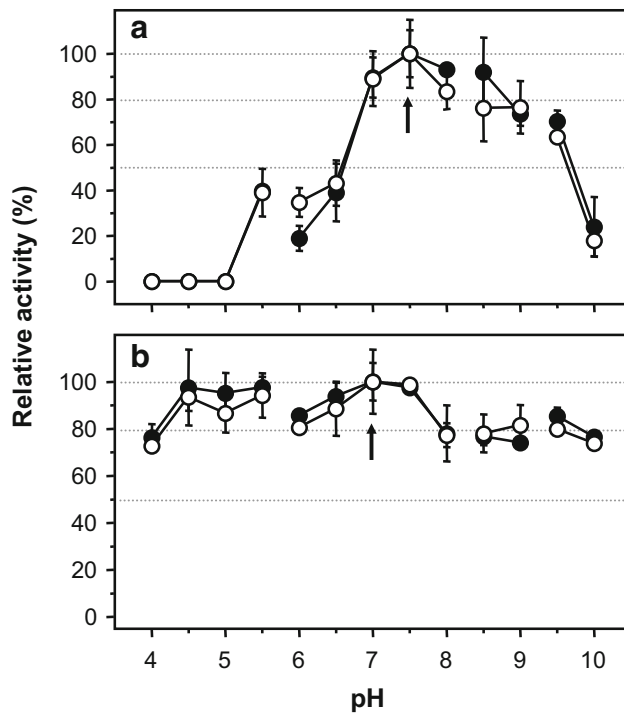


Fig. 5 Optimum pH and pH stability of rSiNucA. Relative deoxyribonuclease activities under different pH conditions (a) and stability after incubation at different pH values, at 4 °C for 18 h (b). Maximum activity in each test was defined as 100 % (arrow). Data obtained from the same pH buffer system are connected. The plots indicate average \pm standard error ($n = 3$). Closed and open circles indicate rSiNucA-NSP and rSiNucA-30K, respectively

Additionally, as per the Benzonase instruction manual, values for optimal Mg^{2+} concentration, pH, temperature, and monovalent cation concentration are 1–2 mM (effective: 1–10 mM), pH 8.0–9.0 (pH 6.0–10.0), 37 °C (0–42 °C), and 0–20 mM (0–150 mM), respectively. The enzymatic characteristics of rSiNucA were similar to those of Benzonase[®]. Although the activity was slightly low, rSiNucA is comparable to Benzonase[®] as a molecular biology tool.

DNase Activity in Lysis Buffers

DNase activity of rSiNucA in lysis buffers was measured (Fig. 10). Although relative activities in nondiluted RIPA buffer were approximately 10, 89 % or more activity was detected when RIPA buffer was diluted two- or fourfold. Nuclease activity was not inhibited in a mild lysis buffer containing Triton X-100 and DOC.

Analyses of DNA and Protein Prepared from Bacterial Lysate after rSiNucA Treatment

Nuclease is often used to digest genomic DNA during purification of recombinant protein expressed in *E. coli*.

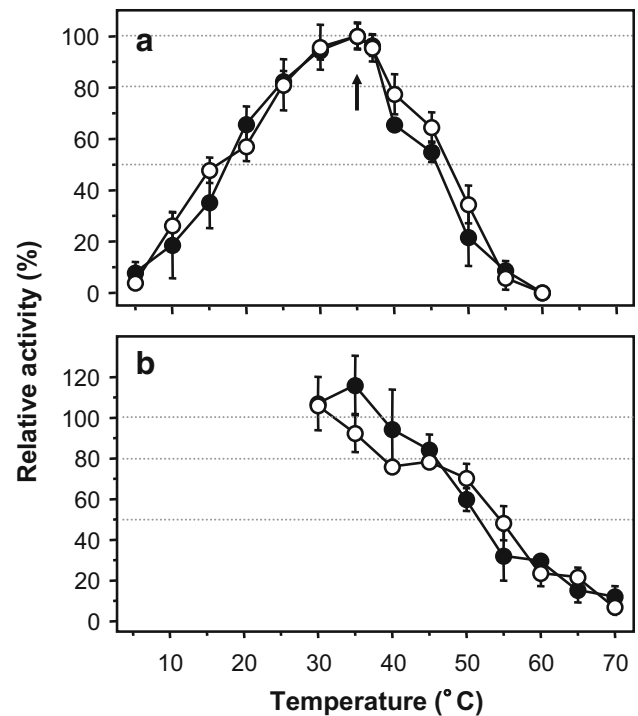


Fig. 6 Optimum temperature and heat stability of rSiNucA. Relative deoxyribonuclease activities under different temperatures (a) and stability after incubation in different temperatures for 30 min. Maximum activity in the test (a, arrow) or activity of nontreated nuclease (b) are defined as 100 %. The plots indicate average \pm standard error ($n = 3$). Closed and open circles indicate rSiNucA-NSP and rSiNucA-30K, respectively

Therefore, nuclease activity was assessed in *E. coli* lysate. Since rSiNucA was active in twofold-diluted RIPA buffer, as described above (Fig. 10), *E. coli* lysate prepared with RIPA buffer was diluted. Obvious DNA fragmentation was observed when 10 pg rSiNucA was added to 50 μ l lysate (Fig. 11a). Higher dose of rSiNucA caused further DNA degradation. Protein profiles were not affected by addition of excessive rSiNucA (Fig. 11b). This result indicates that the purified rSiNucA solution did not contain contamination by proteinase.

Discussion

Baculovirus-silkworm system is widely used for the production of recombinant eukaryotic protein. We expressed recombinant nuclease from *S. liquefaciens* to ascertain if this system is also useful for bacterial protein expression. Previously, the signal peptide of a 30-kDa protein from *B. mori* was shown to exhibit efficient secretion of recombinant proteins produced by the *Baculovirus*-silkworm expression system [15, 42]. Therefore, the recombinant protein, harboring not only native signal peptide (rSiNucA-

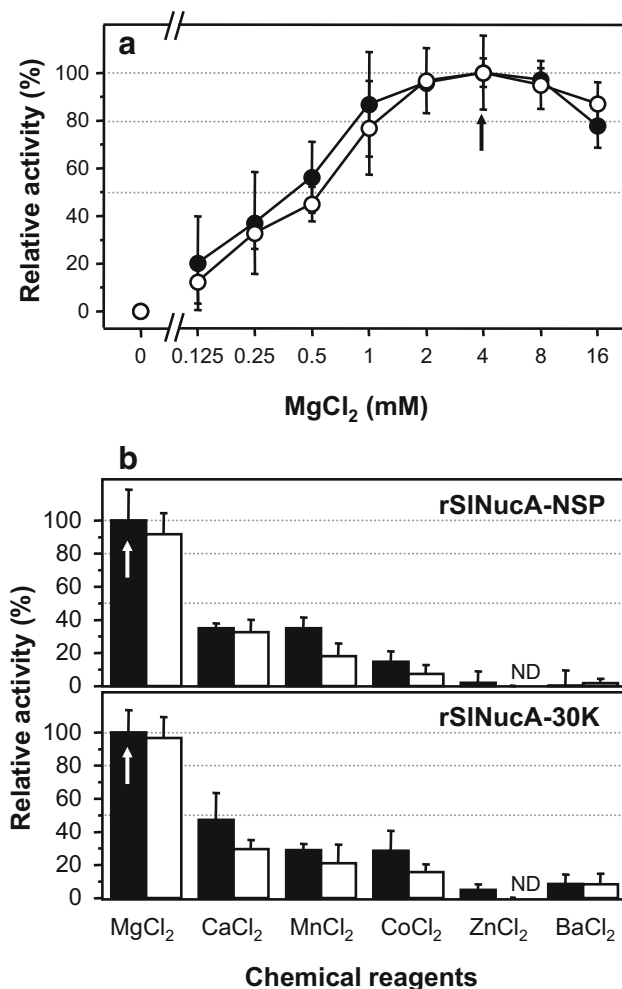


Fig. 7 Effect of divalent metal ions on deoxyribonuclease activity of rSINucA. Relative deoxyribonuclease activities with different concentrations of MgCl₂ (a) and various metal ions (b). Maximum activity in the tests was defined as 100 % (arrows). In panel A, data shown in logarithmic scale are connected, and closed and open circles indicate rSINucA-NSP and rSINucA-30K, respectively. In panel B, closed and open bars indicate 4 and 16 mM, respectively. ND means “not detected.” The data indicate average \pm standard error ($n = 3$)

NSP) but also the 30-K signal peptide (rSINucA-30K), was expressed.

It is known that the endoplasmic reticulum (ER) and Golgi body, which are necessary apparatus for protein secretion in eukaryotic cells, are present in the cultured cell. Therefore, we expected that at least rSINucA-30K will be detected in the extracellular fraction of cultured cells. However, neither rSINucA was detected nor abundant recombinant proteins were detected in hemolymph when *B. mori* larvae were used as the host. Although the reason is unclear, we speculate that rSINucAs may have been secreted at lower levels than the detection limit when cultured cells were used as host. In a complex multicellular organism, different cell types engage in specific functions

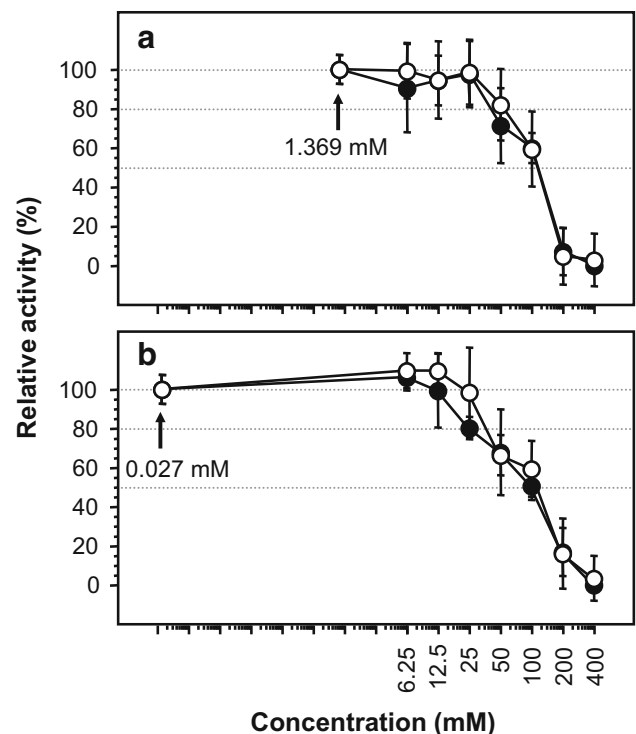


Fig. 8 Effect of NaCl and KCl on deoxyribonuclease activity of rSINucA. Relative deoxyribonuclease activities with different concentrations of NaCl (a) and KCl (b). The sample in which salt was not supplemented was defined as 100 % (arrows). Small amount of salts (1.369 mM NaCl, 0.027 mM KCl) was contributed by the enzyme stock solution. Closed and open circles indicate rSINucA-NSP and rSINucA-30K, respectively. The data indicate average \pm standard error ($n = 3$)

and as a result, the secretory output of cells and tissues varies widely [4]. rSINucAs are efficiently secreted only in *B. mori*. The difference in cellular localization of rSINucAs between cultured cells and *B. mori* larvae may be due to the systematically controlled secretion system of a multicellular organism. Therefore, using *B. mori* larvae to produce recombinant proteins may be advantageous.

rSINucA-NSP harboring native signal peptide was efficiently secreted to *B. mori* hemolymph. In gram-negative bacteria, proteins are secreted via two membranes, cytoplasmic (inner) and outer membranes. To secrete protein from cytoplasmic to the extracellular space, gram-negative bacteria possess several secretion systems, categorized Type I to VI [7]. In *S. marcescens*, NucA is secreted via Type II secretion system, a two-step process. In the first step, NucA is rapidly translocated across the cytoplasmic membrane and the signal peptide is rapidly processed. Subsequently, mature NucA is slowly secreted to the extracellular space, the second step [44]. Sec and Tat pathways are two known pathways in the first of the two-step process, and NucA is transferred by the Sec pathway [44] from the cytoplasmic to the periplasmic space. The

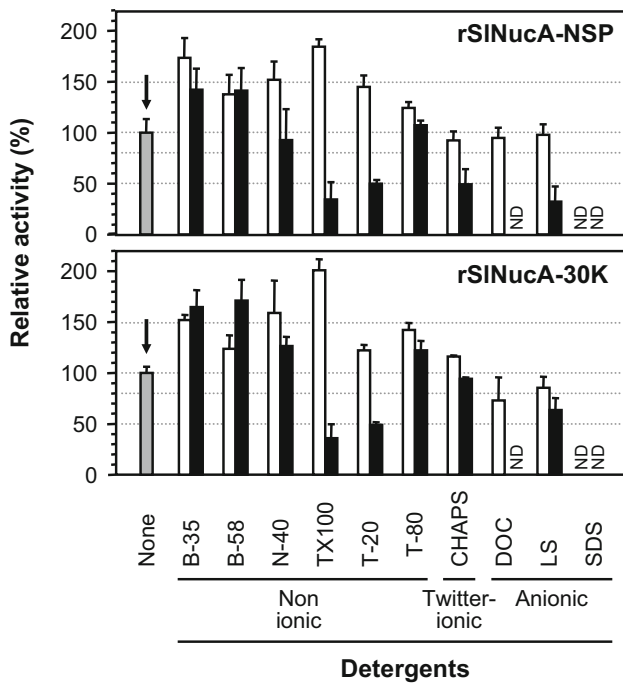


Fig. 9 Effect of detergents on deoxyribonuclease activity of rSINucA. Relative deoxyribonuclease activities with 0.1 % (open bar) and 1 % (closed bar) detergent. The sample in which detergent was not supplemented is defined as 100 % (arrows). Abbreviations for detergents: B-35 Brij 35, B-58 Brij 58, N-40 Nonidet P-40, TX100 Triton X-100, T-20 Tween 20, T-80 Tween 80, CHAPS 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; DOC, sodium deoxycholate, LS sodium N-laurylsarcosinate, SDS sodium dodecyl sulfate. The data indicate average \pm standard error ($n = 3$)

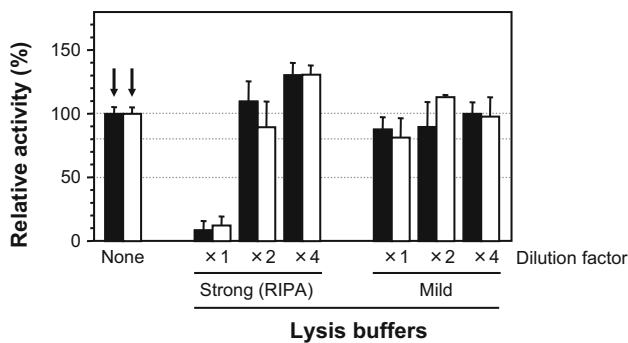


Fig. 10 Effect of lysis buffers on deoxyribonuclease activity of rSINucA. Relative deoxyribonuclease activities in different concentrations of strong and mild lysis buffers. The deoxyribonuclease activity of the sample in which lysis buffer was not supplemented, was defined as 100 % (arrows). Closed and open columns indicate rSINucA-NSP and rSINucA-30K, respectively. The data indicate average \pm standard error ($n = 3$)

signal peptide for Sec pathway typically has an average length of 20 amino acid residues with tripartite structure, i.e., a positively charged amino-terminal (n-region), a hydrophobic core (h-region), and a polar carboxyl-terminal. The overall tripartite structure is recognized by Sec

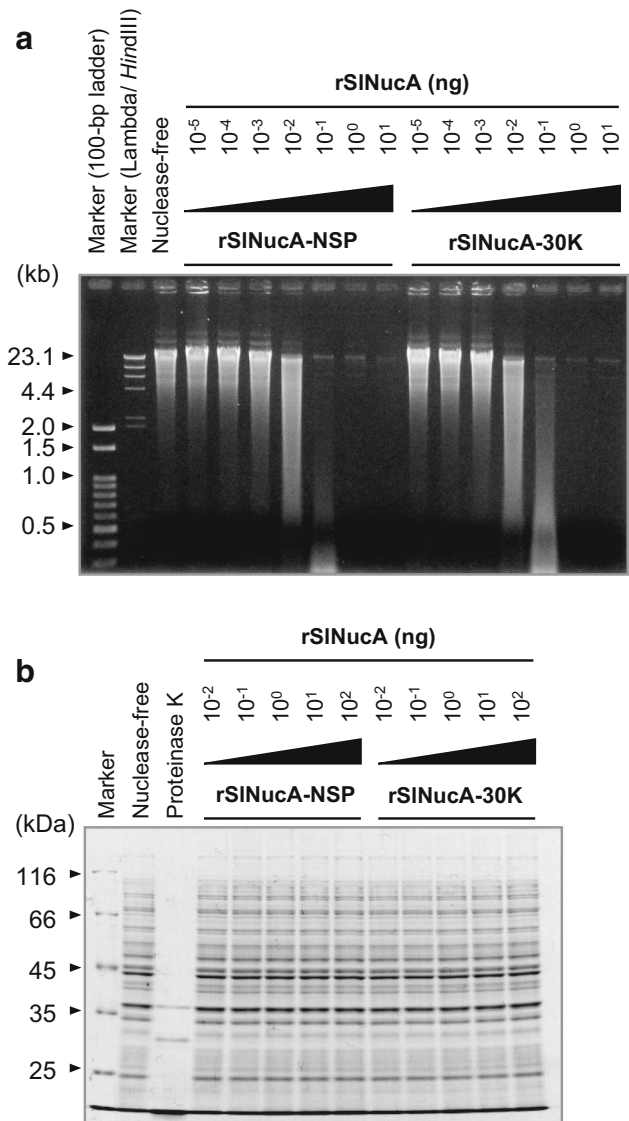


Fig. 11 Electrophoretic profiles of DNA and protein prepared from *E. coli* lysate after rSINucA treatment. Lysate of *E. coli* BL21 DE3 (pLysS, pET16b) with RIPA buffer was diluted with an equal volume of 4 mM MgCl₂. After incubation with rSINucA, DNA (a) and protein (b) were separately extracted and analyzed

components [37]. The bacterial Sec signal peptide is functionally interchangeable with the signal sequences that direct protein to the Sec systems of the thylakoid membrane and the ER [37, 46]. Based on the information available in the literature, it was estimated that signal peptide of rSINucA-NSP was functional in *B. mori* larvae; therefore, rSINucA-NSP was successfully secreted to hemolymph.

In addition, the results from this study suggest that bacterial protein secreted through Sec pathway can be secreted into hemolymph without additional signal peptide in the *Baculovirus*-silkworm system. Bagos et al. [2] reported prediction of Tat and Sec signal peptides (READ-

TAT, <http://www.compgen.org/tools/PRED-TAT>). Since it was inferred that this program is useful to estimate localization of recombinant bacterial protein expressed by *Baculovirus*-silkworm system, rSINucAs were analyzed by READ-TAT program. The prediction was as follows: rSINucA-NSP, Sec signal peptide (0.996); rSINucA-30K, Sec signal peptide (0.999). The results suggest that READ-TAT possibly predicts localization of bacterial recombinant protein expressed in the *Baculovirus*-silkworm system, and bacterial extracellular proteins secreted through Sec pathway may be highly compatible with the protein expression system. It is necessary to investigate correlation between the prediction and experimental evidence of localization using several other bacterial proteins.

S. marcescens NucA forms a dimer. Although the two subunits are functionally independent and dimer formation is not essential for its activity, dimeric forms of NucA were relatively more active than monomeric forms [12]. Variants (H184A, H184N, H184T, and H184R) of *S. marcescens* NucA were monomers, have the same secondary structure and activity as the wild-type enzyme [11]. In contrast, amino acid substitution that alters the secondary structure (S179C) is suggested to cause decrease in activity. Thus, secondary structure and dimer formation are important for optimal nuclease activity.

In the present study, artificial sequences for His-, Strep-tags, and TEV cleavage site were added at the COOH-terminus of rSINucA for effective and simple purification. It was important to ascertain whether this artificial sequence affected secondary structure and dimer formation. It was predicted by PSIPRED V3.3 [26] that the addition of the artificial sequence would not affect the intramolecular secondary structure of the other regions (data not shown). In addition, dimer formation of rSINucAs was experimentally demonstrated. Therefore, the activity of rSINucA may not presumably decrease in comparison with native SINucA. It was shown that these tags help effective and rapid purification of rSINucA. When rSINucAs are used to reduce viscosity of the solution during purification of recombinant proteins, these tags enable to separate rSINucA from other proteins. When the tags are identical between rSINucA and other proteins, the tags can be eliminated by TEV digestion.

In conclusion, rSINucA was expressed by the *Baculovirus*-silkworm system. Purified rSINucA digested DNA and RNA. Enzymatic characteristics were found to be similar to those of *S. marcescens* NucA, and it could digest *E. coli* DNA in diluted RIPA buffer. These results indicate that rSINucA could be a useful tool in molecular biology.

Although biological function of serratial nuclease is not yet well characterized, it has been shown that *Shewanella oneidensis* nuclease utilizes extracellular DNA as a source of nutrients such as carbon, nitrogen, and phosphorus [22].

In addition, nucleases of some bacterial species were shown to be potential virulence factors [19, 25]. Because it is possible that *S. liquefaciens* nuclease contributes to pathogenesis or contributes to bacterial survival in the host, this enzyme may be a potential target molecule for drug development. rSINucAs produced by the *Baculovirus*-silkworm system utilized in this study will therefore contribute to such fundamental studies. However, determination of biological and pathological function of rSINucA in *S. liquefaciens* needs further study.

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Compliance with Ethical Standards

Conflict of Interest No conflict of interest declared.

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