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Biochemical characterization of the nuclease StoNurA from the hyperthermophilic archaeon *Sulfolobus tokodaii*

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ABSTRACT

The DNA nuclease gene ST2109 has been cloned from the hyperthermophilic archaeon *Sulfolobus tokodaii* and expressed in *Escherichia coli*. The recombinant protein StoNurA has been purified to homogeneity by affinity chromatography and gel filtration chromatography. Biochemical analyses demonstrated that StoNurA exhibited DNA binding and 5'–3' exonuclease activities towards ssDNA and dsDNA. The temperature and pH optima of StoNurA were determined to be 65 °C and 8.0, respectively. The activity of StoNurA was found to be dependent of Mn^{2+}, and its half-life of heat inactivation at 100 °C was 5 min. Gel filtration chromatography revealed that StoNurA could form dimers in solution. Pull-down assays also showed that StoNurA physically interacted with a DNA helicase (StoHerA). Our data suggest that NurA may play a key functional role in the processing of DNA recombinational repair.

Key words: DNA nuclease, StoNurA, dimer, interaction, recombinational repair.

INTRODUCTION

DNA nucleases can cleave the phosphodiester bonds between the sugar and phosphate moieties of DNA to give a 5'-terminal phosphate and a 3'-terminal hydroxyl group. These enzymes play crucial roles in eliminating the damaged or mismatched nucleotides generated during DNA replication, recombination and repair (Marti and Fleck 2004, Blackwood et al. 2013). They also recognize the intermediates formed during the course of DNA metabolism to facilitate the subsequent reaction steps required for the cleavage of the DNA strands. DNA nucleases are therefore indispensable to all living organisms. Based on whether they catalyze the cleavage reactions occurring at the ends or within the DNA strands, DNA nucleases can be categorized as exonuclease or endonuclease. Exonuclease DNA nucleases can be further divided into 5'- and 3'-end processing exonucleases, according to the polarity of their consecutive cleavage steps (Rangarajan and Shankar 2001). Despite their limited sequence homology, DNA nucleases share conserved minimal motifs, which usually consist of acidic and basic residues that form their active site. These active residues coordinate to catalytically divalent metal cations, which act as cofactors, such as magnesium, manganese, calcium and zinc (Chen et al. 2009, Lee et al. 2015).

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The DNA nuclease-catalyzed cleavage of phosphodiester bonds can be considered as a phosphoryl transfer to water (Nishino and Morikawa 2002). DNA nucleases employ a common three-step mechanism in their cleavage reactions (Pingoud et al. 2005, Korczynska et al. 2012), including (1) the preparation of an nucleophile attack by the base-mediated deprotonation of a water molecule [e.g., H_2O + B + R'-O5'-$(PO_2)^-$-O3'-R'' → OH^- + BH^+ + R'-O5'-$(PO_2)^-$-O3'-R"]; (2) the formation of a pentavalent transition state by the nucleophilic attack of the hydroxide ion on the phosphorous atom of a nucleotide and the subsequent Lewis acid-stabilization of the resulting pentavalent transition state bearing two negative charges [e.g., OH^- + R'-O5'-$(PO_2)^-$-O3'-R" → R'-O5'-$(PO_2OH)^{2-}$-O3'-R"]; and (3) the protonation of the 3'-OH leaving group by a general acid and its subsequent release [e.g., -O5'-$(PO_3)^{2-}$-O3' + H_2O → R'-O5'-(PO_2)-O^- + HO3'-R'' + OH^-].

Sulfolobus tokodaii is a hyperthermophilic archaeon with an optimum growth temperature of 80 °C (Suzuki et al. 2002). The sequencing of the genome of this organism was completed at the National Institute of Technology and Evaluation (Tokyo, Japan) (Kawarabayasi et al. 2001). In this paper, we report the cloning, expression and characterization of a Mn^{2+}-dependent DNA nuclease from *S. tokodaii* (StoNurA) and an evaluation of its role in the archaeal recombinational repair.

MATERIALS AND METHODS

GROWTH AND TRANSFORMATION OF THE DIFFERENT BACTERIAL STRAINS

The DH5α and BL21-CodonPlus (DE3)-RIL strains of *Escherichia coli* were purchased from Stratagene (La Jolla, CA, USA). *Sulfolobus tokodaii* was purchased from the Microbe division of the Japan Collection of Microorganisms and cultured under the condition described by Brock et al. (1972). The different strains of *E. coli* were grown at 37 °C in LB broth or on LB plates solidified with 1.5% agar. The cultures were supplemented with 100 μg/ml of ampicillin when they were grown under selection. *E. coli* was transformed using the modified $CaCl_2$ technique (Rodriguez and Tait 1983). Plasmid pET15b (Novegen) was modified with *Nco*I and *Nde*I, which were exchanged to facilitate the purification process.

CLONING OF THE StoNurA GENE

The genomic DNA of *S. tokodaii* was prepared according to the procedure described. The gene coding for StoNurA in *S. tokodaii* was amplified using PCR with the following primers: 5'-GTGC-CGACATATGATTAAGGATGTCTATGAGC-3' (upstream) and 5'-AATTCAGGTCGACTCTA-AAAAAGTTGACTAGGCC-3' (downstream). The underlined sections of text represent the NdeI and SalI sites, respectively. The PCR product was digested with NdeI and SalI and cloned into pET15b. The resulting plasmid will be referred to hereafter as pET15b/StoNurA and used to express the N-terminal His-tagged proteins. The construction of the expression vectors for StoHerA has been described elsewhere (Zhang et al. 2008).

EXPRESSION AND PURIFICATION OF THE StoNurA

The recombinant pET15b/StoNurA plasmid was transformed into the *E. coli* BL21-CodonPlus (DE3)-RIL host. The transformed cells were grown in LB medium (500 ml) at 37 °C. When the OD_{600} reached 0.5, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. After being cultured for 4 h at 37 °C, the cells were harvested by centrifugation, resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 50 mM NaCl) and disrupted by sonication. The disrupted cells were incubated at 70 °C for 30 min and then centrifuged (12,000 ×g for 30 min) to obtain heat-stable enzymes. The supernatant was loaded on a nickel column (Invitrogen, California, USA),

which was initially washed with a buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl) containing 20 mM imidazole. The enzymes were subsequently eluted with an elution buffer (50 mM Tris–HCl, pH 8.0, 500 mM NaCl) containing 150 mM imidazole. For purification by gel filtration, the samples were dialyzed in buffer A and loaded onto a Sephacryl S-200 HR column (GE Healthcare), which had been equilibrated with buffer B (50mM Tris–HCl, pH 8.0, 200mM NaCl), and eluted with buffer B at a flow rate of 1 ml/min. The StoHerA protein was purified as described previously (Zhang et al. 2008). The protein concentration was determined according to the Bradford method.

CHEMICAL CROSS-LINKING

Cross-linking was carried out by incubating 20 μg of the enzyme in PBS containing 2% formaldehyde at room temperature for 0, 30 or 60 min. The reaction was stopped by adding glycine at a final concentration of 0.15 M. The reaction products were separated by 8% SDS-PAGE and were detected by Coomassie Brilliant Blue staining.

WESTERN BLOT ANALYSIS

Rabbit polyclonal antibodies were raised against the purified StoNurA, which was prepared according to a previously described procedure (Wei et al. 2008). Protein samples separated by SDS-PAGE were electroblotted onto a PVDF membrane and analyzed with the Enhanced Chemiluminescence system (GE Healthcare) according to the supplier's protocol.

DNA SUBSTRATES

Three oligonucleotides were synthesized and used for the preparation of the DNA substrates. A 34-nucleotide ssDNA sequence (5'-CAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGA-3') labeled with FITC at its 3' end was used as a substrate for the DNA-binding and nuclease assays, together with the corresponding 34-bp dsDNA. The dsDNA was prepared by annealing the 3'-FITC-34-mer ssDNA to the complementary 34-mer oligonucleotide (5'-TCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTG-3'). The annealing process was performed as follows: 20 pmol labeled oligonucleotide was mixed with 40 pmol unlabeled oligonucleotide in 10 μl of annealing buffer (7 mM Tris--HCl (pH 8.0) and 50 mM NaCl). The mixture was boiled for 10 min, cooled gradually to room temperature and then stored at 4 °C prior to being used.

ASSAY FOR DNA BINDING ABILITY OF StoNurA

The binding ability of StoNurA to a series of different DNA substrates was examined using a gel shift assay. StoNurA was incubated with a 0.2 pmol solution of substrate in a buffer containing 20 mM HEPES (pH 8.0), 50 mM NaCl, 0.5 mM DTT, 100 μg/ml BSA and 5% glycerol for 30 min at room temperature. The samples were then loaded on a 6% native polyacrylamide gel and run in 1×TBE buffer. After electrophoresis, the reaction products were visualized using a FluorImager 585 (GE Healthcare).

NUCLEASE ASSAYS

The standard DNA nuclease activity was evaluated in a reaction mixture (20 μl) containing 20 mM HEPES (pH 8.0), 50 mM NaCl, 5 mM $MnCl_2$, 0.5 mM DTT, 100 μg/ml BSA, 2 pmol of the substrates and the required amount of StoNurA for 30 min at 65 °C. The reactions were stopped by the addition of 5 μl of 5× stop solution (50 mM EDTA, 0.5% SDS, 25% glycerol and 0.025% bromphenol blue). The samples were loaded onto a 15% polyacrylamide gel containing 7 M urea and 1×TBE and electrophoresed for 2 h at 1000 V. After electrophoresis, the products were visualized and quantified using a FluorImager. The nuclease activity was calculated according to the reduction of the substrates, which was quantified based on the

ratio of the signal strength of products to the total signal strength of each sample.

EFFECT OF TEMPERATURES AND pH ON ENZYME ACTIVITY AND STABILITY

The pH and temperature profiles for the nuclease activity were esteblished using ssDNA as the substrate. The effect of pH on the nuclease activity was examined at 65 °C with the pH at the range of 6.5 to 9.5. The buffer used were 20 mM of HEPES buffer (pH 6.5 to 8.0), Tris-HCl (pH 8.5 to 9.0), and N-cyclohexyl-3-aminopropanesulfonic acid (CAPS, pH 9.0–9.5). The effect of temperature on the nuclease activity was examined at temperatures ranging from 45 to 90 °C in 20 mM of HEPES buffer (pH 8.0). The thermostability of the purified StoNurA was evaluated by incubating the enzyme in 20 mM of HEPES buffer (pH 8.0) at three different temperatures (80 °C, 90 °C, and 100 °C). The residual activities were assayed under the standard conditions.

EFFECT OF METAL IONS AND NaCl ON THE ENZYMATIC ACTIVITY

The effects of several different metal ions and NaCl on the nuclease activity were investigated by adding a specific metal salt (5 mM) and NaCl (0 to 300 mM) to an assay solution containing the purified enzyme (5 μg/ml) and 20 mM HEPES buffer (pH 8.0). The reaction mixture was incubated at 65 °C for 30 min. The residual enzyme activity was measured under the standard assay conditions and was initiated by addition of the substrate ssDNA. The nuclease activity of enzyme without any additives was taken as the control and the highest activity was defined as 100% activity.

PULL-DOWN ASSAY

The untagged StoHerA (300 μg) was pre-incubated with a 50% Ni-NTA agarose bead slurry in binding buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl and 15% glycerol) in a volume of 300 μl on ice for 30 min. The beads were then recovered by centrifugation at 2000 ×g for 2 min and the supernatant was used in the pull-down assays. Approximately 25 μg of His_6-tagged StoNurA immobilized on 50 μl of Ni-NTA agarose beads (slurry) was incubated with 25 μg of untagged StoHerA in 200 μl of binding buffer. The beads were washed three times with 400 μl of washing buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl and 20 mM imidazole, and eluted three times with 100 μl of elution buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl, and 500 mM imidazole). The eluent was resolved on 12% SDS-PAGE gels and stained with Coomassie Brilliant Blue.

RESULTS AND DISCUSSION

GENE CLONING, EXPRESSION, AND PURIFICATION OF StoNurA FROM *S. tokodaii*

In the *S. tokodaii* genome, the *NurA* gene encoding putative DNA nuclease is arranged in an operon-like structure with the *mre11*, *rad50* and *herA* genes (Constantinesco et al. 2002, Manzan et al. 2004, Wei et al. 2008). To evaluate the biochemical properties of StoNurA, the recombinant proteins were expressed in *E. coli* as His6-StoNurA fusion proteins (calculated to be a 38 kDa polypeptide) and purified from the soluble extracts by heat treatment, Ni-NTA affinity (Invitrogen) and Sephacryl S-200 gel filtration chromatography (GE Healthcare). The purification procedure is shown in Table I. The enzyme StoNurA was purified 4.5-fold to give a yield of 33.2% and a specific activity of 891.4 U/mg. The results of the SDS-PAGE analysis of StoNurA are shown in Fig. 1a. The protein migrated as a 38 kDa His6-StoNurA, which was in good agreement with the predicted molecular mass. To determine whether the recombinant StoNurA corresponded to the proteins produced in *S. tokodaii* cells, we prepared polyclonal antibodies using the purified recombinant StoNurA. As shown in Fig. 1b, proteins with sizes corresponding to the

recombinant StoNurA were detected in the crude cell extract of *S. tokodaii* by western blot analysis. This result indicated that the gene for StoNurA was actually expressed in *S. tokodaii*.

OLIGOMERIZATION OF StoNurA

During the gel filtration chromatography experiments, we noticed that StoNurA eluted as a peak corresponding to a molecular mass of approximately 80 kDa (Fig. 2). Since the molecular mass of the monomeric form of StoNurA is 38 kDa, we hypothesized that StoNurA could form dimers in solution. The results of cross-linking experiment with formaldehyde also verified that StoNurA could form an oligomeric structure (Fig. S1 – Supplementary Material), although the exact oligomeric status of this material was unclear and requires further investigation. These results therefore suggested that StoNurA exists as an oligomeric structure, which is consistent with the proposed mechanism that oligomerization is a major factor contributing to the thermostability of hyperthermophilic enzymes (Vieille and Zeikus 2001).

THE DNA BINDING ACTIVITY AND NUCLEASE ACTIVITY OF PURIFIED StoNurA PROTEIN

The DNA binding ability of StoNurA was examined using a gel shift assay to characterize its DNA binding activity (Fig. S2). Increasing the amount of StoNurA in the reactions led to the appearance of ssDNA and blunt-ended dsDNA as lag bands on the gels. From the unbound DNA fragment, the affinity of StoNurA for ssDNA was stronger than that for blunt-ended dsDNA (Fig. S2). These results StoNurA exhibited DNA-binding activities towards ssDNA and dsDNA, and therefore provided evidence that StoNurA could be involved in the processing of DNA recombinational repair.

The DNA nuclease activities of the recombinant StoNurA were examined in enzymatic activity assays using the ssDNA and dsDNA substrates. As

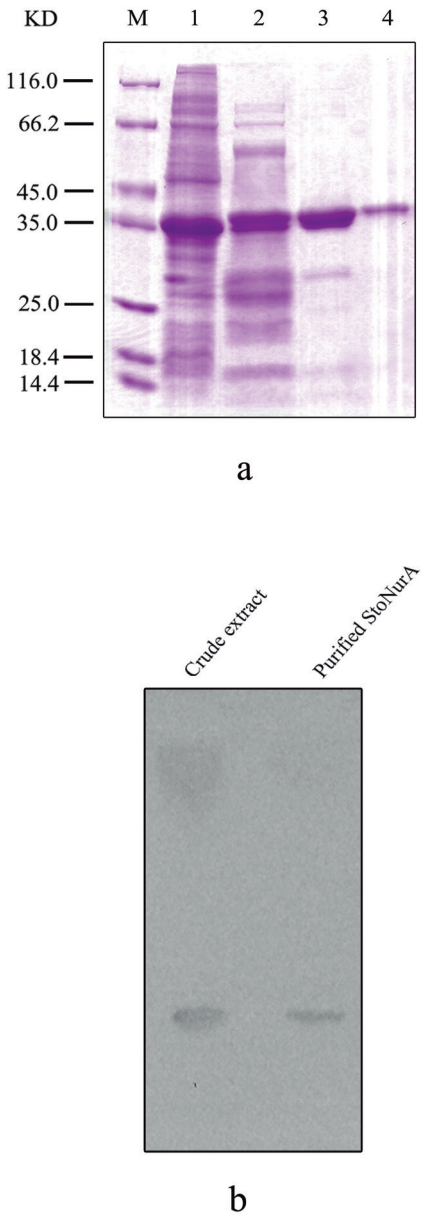

Figure 1 - (a) SDS-PAGE of StoNurA. Lanes M, molecular weight stardards; Lane 1, crude cell extract; Lane 2, supermatant after heat-treatment for 30 minutes at 70 °C; Lane 3, the partially purified enzyme after nickel column; Lane 4, the purified enzyme after gel filtration chromatography. (**b**) Identification of StoNurA in *S. tokodaii* cells. *S. tokodaii* cell extracts (100 µg) and purified StoNurA (50 ng) were seperated by 12% SDS-PAGE gels. These gels were subjected to Western blot analyses with anti-StoNurA polyclonal antibodies to detect StoNurA. (see the colors in the online version).

TABLE I
Purification of the nuclease StoNurA from *S. tokodaii*[a].

Step	*Total protein (mg)*	*Total activity (U)*	*Specific activity (U/mg)*	*Purification fold*	*Yield (%)*
Crude cell extract	25.8	5645	218.8	1	100
Heat treatment	15.2	4281	281.6	1.3	75.8
Ni-NTA affinity	4.6	2572	559.1	2.6	45.6
Sephacryl S-200 gel filtration	2.1	1872	891.4	4.1	33.2

[a] One unit of the nuclease activity was defined as the amount of enzyme releasing 1 μmol acid-soluble nucleotides per hour under the assay conditions. The amount of acid-soluble nucleotides was measured according to the conditions described by Curtis et al. (1966).

is shown in Fig. 3a and 3b, StoNurA successfully degraded both ss and dsDNA substrates from their 5'-ends to their 3'-ends, resulting in the release of small oligonucleotides. About 80% of the ssDNA substrate was degraded using 80 ng of StoNurA, whereas the degradation of 80% of the dsDNA substrate required 100 ng of StoNurA. This result therefore showed that StoNurA degraded ssDNA more efficiently than it degraded dsDNA, which reflected the enzymatic properties of NurA from *S. acidocaldarius* (Constantinesco et al. 2002).

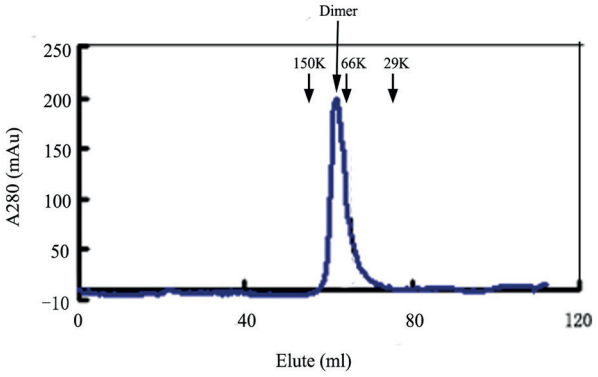

Figure 2 - Gel filtration analysis of StoNurA using Sephacryl S-200 column. The arrows indicated the eluted positions of StoNurA and protein markers (from left to the right): alcohol dehydrogenase (150 kDa, 54.12 ml), StoNurA (60.85 ml), albumin (66 kDa, 64.58 ml) and carbonic anhydrase (29 kDa, 77.54 ml). (see the colors in the online version).

EFFECT OF TEMPERATURE AND pH ON ENZYME ACTIVITY OF StoNurA

A series of detailed investigations were performed to optimize the reaction conditions for the nuclease activity of StoNurA using an ssDNA substrate. The optimal pH for StoNurA was determined to be 8.0, with only half of its maximum activity remaining at pH values in the range of 7–8.5 (Fig. 4a). The optimal temperature for the nuclease activity of StoNurA was determined to be 65 °C. StoNurA also exhibited strong activity at temperatures in the range of 55–85 °C, with half of its maximum activity remaining at 85 °C (Fig. 4b). The thermostability of StoNurA was evaluated by measuring its activity towards a ssDNA substrate after being subjected to heat treatment at three different temperatures (Fig. 4c). StoNurA retained approximately 50% of its original activity after being incubated for 60 min at 80 °C. The residual activity of StoNurA descended drastically following periods of incubation at 90 and 100 °C. The half-life of heat inactivation of StoNurA at 100 °C was about 5 min. These results show that StoNurA could maintain better catalytic properties for a considerably long reaction time at the optimal or higher temperature of reaction condition, and that this material is therefore a highly stable protein structure for temperature adaptation (Unsworth et al. 2007).

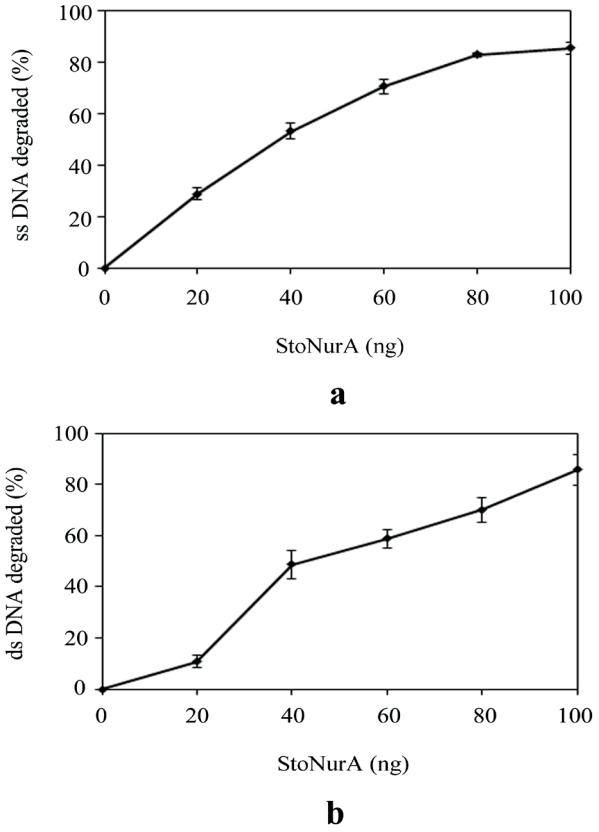

Figure 3 - Nuclease activities of StoNurA. The reaction mixtures (20 µl) contained increasing amounts (0, 20, 40, 60, 80 and 100 ng) of the StoNurA and 2 pmol ssDNA (**a**) or dsDNA (**b**). The sample were incubated at 65 °C for 30 min. The products were resolved on 15% polyacrylamide gels containing 7 M urea and visualized using a FluoImager 585. Each value was calculated based on the results of three independent reactions.

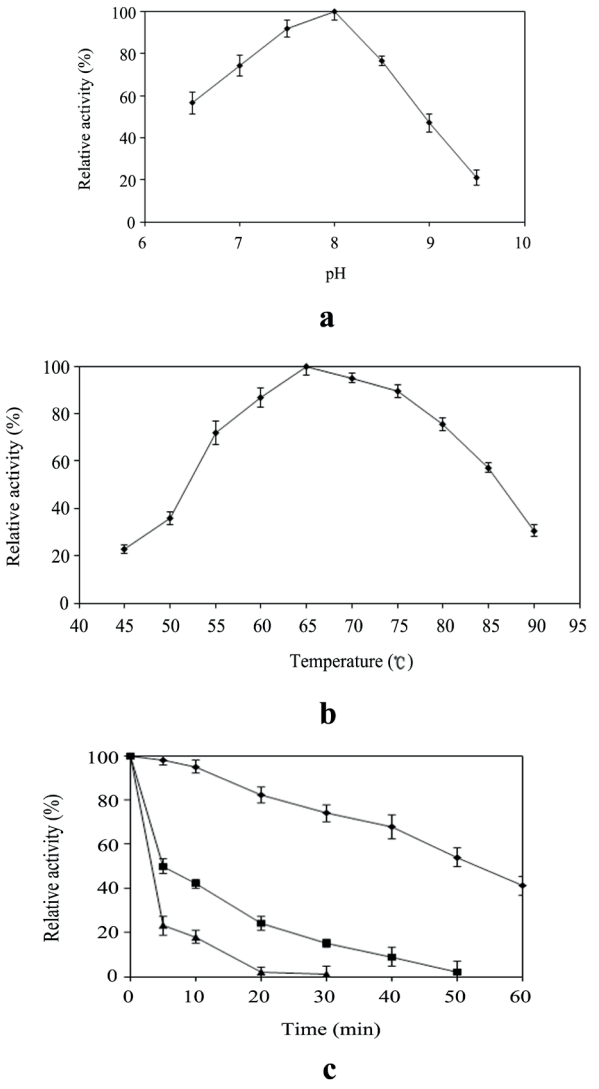

Figure 4 - Biochemical characterization of StoNurA. (**a**) Effect of pH on the purified StoNurA. Optimal pH of StoNurA at pHs ranging from 6.5 to 9.5 was measured for 30 min at 65 °C. (**b**) Effect of temperature on the purified StoNurA. Optimal temperature of StoNurA was determined with the ssDNA as substrates in 20 mM of HEPES buffer (pH 8.0) at different temperatures ranging from 45 to 90 °C. (**c**) Thermostability of StoNurA. The residual enzyme activity was measured after incubation of the purified enzyme at 80 °C (diamonds), 90 °C (boxes), and 100 °C (triangles), respectively. Each value was calculated based on the results of three independent reactions.

EFFECT OF METAL IONS AND NaCl ON THE ENZYMATIC ACTIVITY OF StoNurA

A divalent cation is indispensable to the activity of nuclease enzymes. Most nucleases use magnesium, calcium, manganese or zinc as a cofactor. Among the divalent cations tested, StoNurA required Mn^{2+} as a metal cofactor for its nuclease activity (Fig. 5a), which is consistent with other DNA nucleases reported in the literature (Marti and Fleck 2004). StoNurA appeared to be less active when Mg^{2+}, Fe^{2+}, Zn^{2+} or Ni^{2+} were used as the cofactor, while Ca^{2+} and Cu^{2+} failed to support the nuclease activity of the enzyme. The effect of the salt (NaCl) concentration on the enzyme activity was also evaluated, and the results are shown in Fig. 5b. The addition of low a concentration of NaCl (0 to 75 mM) did not significantly affect the enzymatic activity of StoNurA. In contrast, higher

concentrations of NaCl (75 to 300 mM) inhibited the enzyme activity (Fig. 5b). The low concentration of NaCl might provide the proper ionic strength to ensure the activity of the enzyme, while the high concentration of NaCl could impair the stability and conformational structure of StoNurA (Müller-Santos et al. 2009).

StoNurA INTERACTS WITH StoHerA REVEALED BY OULL-DOWN AND GEL FILTRATION ASSAY

In the genome of *S. tokodaii*, the Mre11 and Rad50 homologs are arranged in operon-like structure with a nuclease NurA and a DNA helicase HerA (Constantinesco et al. 2002, Almond et al. 2014). Previous reports have shown that the NurA and HerA proteins from *Pyrococcus furiosus* and *Sulfolobus solfataricus* can physically interact by gel filtration (Blackwood et al. 2012, Hopkins and Paull 2008). However, there have been no reports pertaining to the identity of the interactions between NurA and HerA from *S. tokodaii*. We initially used a pull-down assay to determine whether there is a physical interaction between StoNurA and StoHerA. The purified StoNurA had a 6× His tag at its N-terminal, whilst the StoHerA had no tag. StoHerA was incubated with His-tagged StoNurA for 30 min on ice and then loaded onto the Ni^{2+}-NTA affinity column. As shown in Fig. 6a, StoHerA was eluted along with StoNurA in elution buffer containing 500 mM imidazole. This result demonstrates that StoNurA forms a physical interaction with StoHerA *in vitro*. To further confirm the interaction, we carried out gel filtration using purified StoNurA and StoHerA proteins. Previous *S. solfataricus* and *Deinococcus radiodurans* studys showed that HerA and NurA interacted with a 6:2 stoichiometry (Cheng et al. 2015), which were consistent with the crystal structure of the NurA-HerA complex (Byrne et al. 2014, Rzechorzek et al. 2014). When StoNurA and StoHerA were mixed at a 3:1 molar ratio, the large complex was eluted around 40 ml on Sephacryl S-200, indicating that the two proteins formed a stable complex (Fig. 6b and Fig. S3). HerAs from *P. furiosus* and *Methanobacter thermoautotrophicus* have been reported to form a hexameric ring structure with a central hole (Manzan et al. 2004, Hopkins and Paull 2008). Based on the results of our gel filtration and pull-down experiments, we speculated that NurA and HerA could form a hexameric ring complex around DNA, which could process recombination intermediates such as Holliday junctions or D-loop structures. It will be interesting to investigate whether a ring complex of this type is formed between NurA and HerA and if

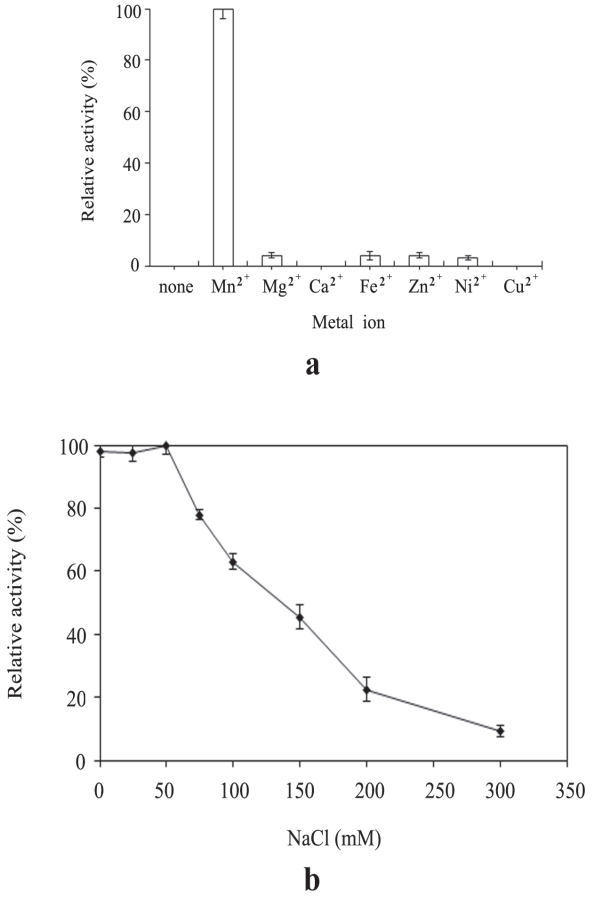

Figure 5 - (**a**) Effect of metal ions on the nuclease activity of the purified StoNurA. The concentration of all metal ions were adjusted to 5 mM. (**b**) Effect of NaCl concentration on the nuclease activity of the purified StoNurA. The nuclease activities of the treated samples were determined by the standard assay using ssDNA as the substrates. Each value was calculated based on the results of three independent reactions.

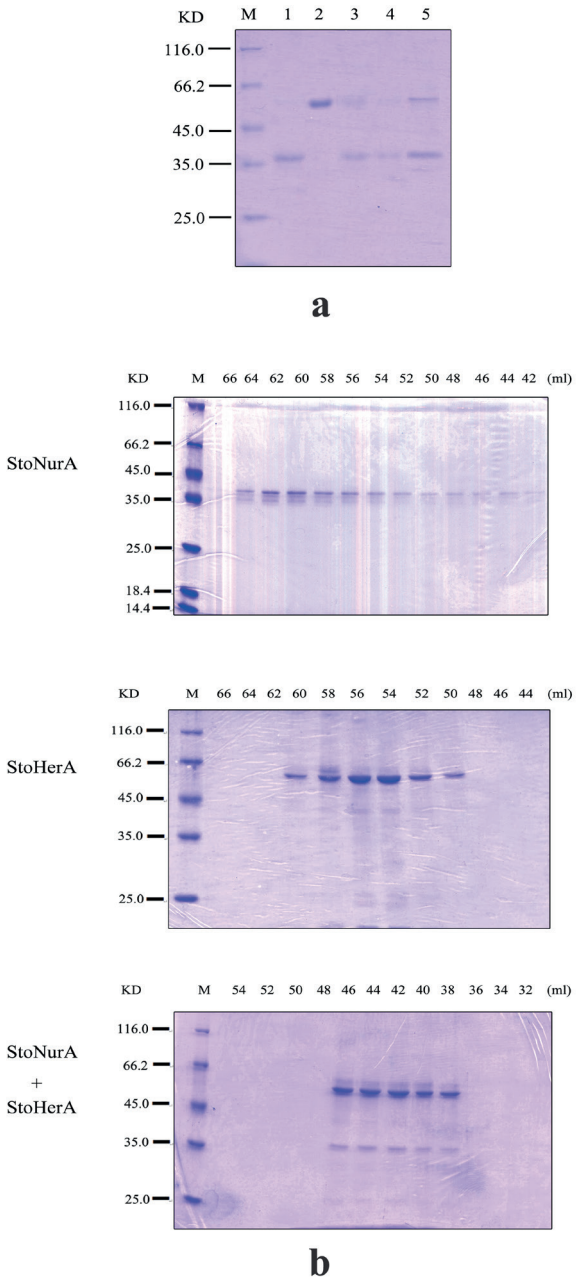

Figure 6 - (**a**) Physical interaction between StoNurA and StoHerA by pull-down assay. Lane 1, molecular size markers; Lane 2, purified His-tagged StoNurA;Lane 3, purified untagged StoHerA; Lane 4, flow-through of the mixture; Lane 5, wash with washing buffer; Lane 6, elution with buffer containing 500 mM imidazole. (**b**) Sephacryl S-200 gel filtration of purified StoHerA and StoNurA. Purified proteins were incubated alone (StoNurA: top panel, StoHerA: middle panel) or in combination (bottom panel) at 65 °C for 30 min before gel filtration, as indicated, and fractions were analyzed by 12% SDS-PAGE. Migration of protein markers are indicated. (see the colors in the online version).

the resulting NurA/HerA complex can translocate on DNA. These studies are currently underway in our laboratory and will be reported in due course.

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SUPPLEMENTARY MATERIAL

FIGURES S1, S2 and S3.