Biochemical characterization of a thermostable endonuclease V from the hyperthermophilic euryarchaeon Thermococcus barophilus Ch5

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Biochemical characterization of a thermostable endonuclease V from the hyperthermophilic euryarchaeon *Thermococcus barophilus* Ch5

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Abstract

Endonuclease V (Endo V) is a DNA repair enzyme that recognizes deoxyinosine and cleaves the second phosphodiester bond on the 3′ side of the deaminated base lesion. Endo V homologues are conserved in all three domains of life, bacteria, eukarya and archaea. While bacterial and eukaryotic endo Vs have been well studied, knowledge of archaeal endo Vs is limited. Here, we first characterized biochemically a thermostable endonuclease V from the hyperthermophilic euryarchaeon *Thermococcus barophilus* Ch5 (Tba endo V). The recombinant Tba endo V possesses specific endonuclease activity for deoxyinosine-containing DNA. The enzyme displayed optimal nicking activity at 70–90°C, and the enzyme retained its DNA nicking activity even after heating at 100°C for 120 min, suggesting the enzyme is a thermostable endo V. Tba endo V cleaved DNA over a wide pH spectrum ranging from 6.0 to 11.0 with an optimal pH of 8.0–9.0. In addition, Tba endo V activity was dependent on a divalent metal ion, among which Mn^{2+} and Mg^{2+} are optimal ions for the enzyme's activity. Furthermore, DNA cleavage activity of the enzyme was inhibited by high NaCl concentration. Interestingly, Tba endo V bound to all DNA substrates, however, the enzyme exhibited a higher affinity for binding to deoxyinosine-containing DNA than normal DNA. Our work provides a basis for determining the role of Tba endo V in the base excision repair pathway in *Thermococcus*, and also implicates potential applications of the enzyme in molecular biology.

Keywords: Endonuclease V; *Thermococcus barophilus*; DNA repair; DNA

1 nicking; Thermostability

Introduction

Deamination of DNA is a common lesion caused by spontaneous hydrolysis, endogenous or environmental factors as well as deaminase enzymes . Three of the four DNA bases, cytosine, adenine, and guanine, are deaminated to the corresponding base analogs uracil, hypoxanthine, and xanthine, respectively. While the rate of adenine deamination in single-stranded DNA (ssDNA) is much lower than that of cytosine deamination under physiological conditions *in vitro* , adenine deamination in double-stranded DNA (dsDNA) induced by nitrous acid occurs at a similar rate as observed in cytosine deamination . Similar to the background levels of 1–10 per 10^6 nucleotides in tissues or cells , deoxyinosine in *E. coli* and *Saccharomyces cerevisiae* cells is estimated to be 1.2 and 2.0 per 10^6 nucleotides, respectively. Since deoxyinosine has a strong ability to form mismatch, an A-T base pair would be subsequently converted to a G-C base pair during DNA replication before the deoxyinosine is repaired , potentially leading to mutations in the genome. Increased AT to GC mutations are detrimental to the cells, which may cause genome instability or cancer occurence. However, cells have evolved the corresponding excision repair pathway to counteract potential mutations generated by deoxyinosine replication. Endonuclease V (endo V) is considered to play an important role in initiating deoxyinosine repair in a base excision repair pathway.

Endo V, encoded by the *nfi* gene, was first identified in *E. coli* as an endonuclease that nicks damaged DNA and was subsequently proved to nick the second phosphodiester bond on the 3′ side of the deaminated base lesion .

Biochemical studies on *E. coliE. coli* endo V demonstrated that the enzyme is capable for recognizing all three deaminated products, deoxyuridine, deoxyinosine and deoxyxanthosine, and nicking the corresponding damaged DNA. Moreover, *E. coliE. coli* endo V displays broad substrate specificity towards apurinic/apyrimidinic (AP) site, insertion/deletion mismatches (IDM), flaps, loops and pseudo-Y DNA structures *in vitro* ; however, genetic studies revealed that the enzyme is able to protect cells from mutagenic detriments and nitrosative deamination during normal growth *in vivo* . Recently, Zhang et al. solved the crystal structure of *E. coli* endo V, demonstrating the substrate recognition and catalytic mechanism of the enzyme . In combination with biochemical, genetic and structural studies, *E. coli* endo V is proposed to recognize deoxyinosine in DNA and initiating repair pathway by hydrolyzing the second phosphodiester bond on the 3′ side of the deoxyinosine.

Endo V homologues are ubiquitously distributed in all three domains of life, bacteria, eukarya and archaea . In addition to *E. coli* endo V, the endo V homologue from the hyperthermophilic bacterium *Thermotoga maritima* has been well studied . The crystal structure of *T. maritima* endo V (Tma endo V) in complex with DNA was reported . Biochemical and structural characterization suggest that Tma endo V is a specific endonuclease for cleaving deoxyinosine-containing DNA substrate. By contrast, the mouse and human endo Vs lacked significant activity towards deoxyuridine, and displayed strong cleaving activity for inosine-containing RNA . Currently, limited studies on archaeal endo V enzymes have been reported. Similar to the mammalian endo V, the endo V from *Archaeoglobus fulgidus* only recognizes

deoxyinosine-containing DNA substrates . However, *Ferroplasma acidarmanus* endo V is fused downstream of an O6-alkylguanine-DNA alkyltransferase, and exhibits cleavage activities for DNA substrates containing uracil, hypoxanthine and xanthine bases *in vitro* . Recently, the endo V from *Pyrococcus furiosus* displays deoxyinosine endonuclease activity , but its substrate specificity remains unknown. Overall, archaeal endo V enzymes harbor diverse mechanisms for recognizing various DNA lesions; however, knowledge of these enzymes is relatively limited.

Hyperthermophilic archaea are facing severe challenges for deamination of DNA due to their inhabiting in high temperature environment . Deamination of DNA occurs at a higher rate at high temperature than at room temperature . To maintain their genome stability, hyperthermophilic archaea have evolved several repair pathways including enzymes to eliminate potentially mutational effects created by deamination of DNA. *Thermococcus* is an important branch of euryarchaea which thrive in hyperthermophilic environments. Increasing *Thermococcus* species have been isolated and studied. *Thermococcus barophilus* Ch5, which was isolated from a deep-sea hydrothermal field of the Mid-Atlantic Ridge (Logachev field chimney, 3,020 m depth) , is a new member of the piezophilic hydrothermal vent archaeal species. *T. barophilus* Ch5 has a pressure optimum of 40 Mpa and a temperature optimum of 85^{o}C . Analysis of the completed whole genome sequences of *T. barophilus* Ch5 indicated that this strain possesses an endonuclease V (Tba endo V) . In this study, the Tba endo V gene was cloned and expressed in *E. coli*. The recombinant Tba endo V was purified and biochemically characterized. We also report that Tba endo V has a

strong affinity to bind to a deoxyinosine-containing ssDNA, and specifically cleaves deoxyinosine-containing DNA. This is the first report on biochemical characterization of a thermostable endonuclease V from *Thermococcus* species.

Materials and methods

Cloning, expression and purification of *Tba* endo V

The genomic sequence of the *T. barophilus* Ch5 encodes an endonuclease V (GenBank accession number: ALM74570). The genomic DNA of the *T. barophilus* Ch5 was prepared as described by Oger et al. (2016). By using the genomic DNA of *T. barophilus* Ch5 as a template, Phusion DNA polymerase (Thermo Scientific, Waltham, MA, USA) was used to amplify the gene encoding Tba endo V in the presence of two primers (see Table 1). The amplified DNA product was cloned into vector pET-30a(+) (Novagen, Merck, Darmstadt, Germany) and the recombinant plasmid containing a sequence encoded a 6 × His-tag at the C-terminus of Tba endo V was sequenced and then transformed into *E. coli* BL21 (DE3) pLysS cells (Transgene, Beijing, China) for protein expression.

The expression strain *E. coli* harboring the recombinant plasmid was cultured into LB medium containing 100 μg/ml kanamycin and 34 μg/ml chloramphenicol at 37°C until the OD_{600} reached 0.5. And then, isopropyl thiogalactoside (IPTG) was added to the culture with a final concentration of 0.1 mM, and the culture was shaken for another 10 h at room temperature until the OD_{600} reached 1.4.

The cells were collected by centrifugation (5,000 × g for 20 min at room

temperature) and resuspended with Ni column buffer A (20 mM Tris-HCl pH 8.0, 1 mM dithiothreitol (DTT), 500 mM NaCl, 50 mM imidazole and 10% glycerol). The cells were disrupted by sonication in an ice bath. After centrifugation (16,000 × g for 30 min at 4°C), the supernatant was harvested and heated at 80°C for 20 min to remove unthermostable *E. coli* proteins. The resulting supernatant was applied to a HisTrap FF column (GE Healthcare, Uppsala, Sweden) and purified with AKTA fast protein liquid chromatography (GE Healthcare) by elution with a linear gradient of 50–500 mM imidazole after centrifugation (16,000 × g for 30 min at 4°C). Fractions containing Tba endo V protein were collected and visualized by Coomassie-staining, 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The purified Tba endo V protein was dialyzed in a storage buffer containing 50 mM Tris-HCl pH8.0, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DTT and 50% glycerol, and was stored at −80°C. The protein concentration was determined using a Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

DNA cleavage assays

The p45 deoxyoligonucleotides containing I and U at the 25th base, p22, t22 and t45 deoxyoligonucleotides were synthesized at Biotech company, China. The sequences of these deoxyoligonucleotides are shown in Table 1. The deoxyoligonucleotides (p45U, p45I and p22) were labeled with Cy3 at the 5′-terminus (shown by asterisks). The Cy3 labeled deoxyoligonucleotides were annealed to the

complementary deoxyoligonucleotides to prepare ds DNA(*p22/t22, *45U/45T and *45I/45T) in an annealing buffer (20 mM Tris-Cl pH 8.0 and 1M NaCl). The endonuclease activity of Tba endo V was performed in the reactions (10 μl) containing 20 mM Tris-HCl pH 8.0, 1 mM DTT, 0.1 mg/ml BSA, 8% glycerol and the purified Tba endo V. The reactions were kept at 85°C for 20 min and stopped by the addition of 10 μl of stop solution containing 98% formamide and 20 mM EDTA. Samples were heated at 100°C for 5 min and chilled rapidly on ice for 5 min, and then loaded onto a denaturing 15% polyacrylamide gel containing 8M urea. After electrophoresis, the gels were scanned and Cy3-labelled DNA was visualized with a Molecular Image analyser (PharosFx System, BioRad). ImageQuant software was used for the quantitative analysis. The amounts of substrates and enzymes are described in the figure legend.

Assays of optimal temperature and pH for DNA cleavage

Assays to determine the optimal temperature for DNA cleavage by Tba endo V were performed in the reactions (10 μl) containing 200 nM of Cy3-labeled ssDNA (*p45I) and dsDNA (*p45I/t45), 20 mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM $MgCl_2$, 0.1mg/ml BSA, 8% glycerol and 300 nM of Tba endo V. The reactions were kept at 30, 40, 50, 60, 70, 80, 90, and 100°C for 20 min, respectively. The reactions were stopped and treated as described above.

The optimal pH for the endonuclease activity Tba endo V was examined using a pH range of 5.0–11.0 in reactions (10 μl) containing 200 nM of Cy3-labeled ssDNA (*p45I), 20 mM buffer with varied pHs, 1 mM DTT, 1 mM $MgCl_2$, 0.1 mg/ml BSA,

8% glycerol and 300 nM Tba endo V. Five different buffers (all at 20 mM concentrations) were used to prepare different pHs: acetate-sodium acetate (pH 5.0), sodium phosphate-NaOH (pH 6.0, pH 7.0, and pH 7.5), Tris-HCl (pH 8.0 and pH 8.5), Gly-NaOH (pH 9.0), and, $NaHCO_3$-NaOH (pH 10.0, and pH 11.0). The reactions were kept at 85°C for 20 min. The reactions were stopped and treated as described above.

Assays for Tba endo V Thermostability

For its thermostability assays, Tba endo V were heated at 100°C for 20, 40, 60, 90, 120, and 180 min, respectively. The heated Tba endo V were employed to examine DNA cleavage activity in the reactions (10 μl) containing 200 nM of Cy3-labeled ssDNA (*p45I), 20 mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM $MgCl_2$, 0.1 mg/ml BSA, 8% glycerol and 300 nM of the heated Tba endoV. The reactions were kept at 85°C for 20 min. The reactions were stopped and treated as described above.

Effects of divalent metal ions and NaCl on DNA cleavage

The effect of divalent metal ions on Tba endo V activity was investigated by the addition of 1 mM of Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+} and Co^{2+} (analytical purity) in the reactions (10 μl) containing 200 nM of Cy3-labeled ssDNA (*p45I), 20 mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM $MgCl_2$, 0.1mg/ml BSA, 8% glycerol and 300 nM Tba endo V. The reactions were kept at 85°C for 20 min. The reactions were stopped and treated as described above.

In addition, we determined the effects of Mg^{2+} and Mn^{2+} concentrations ranging from 10 nM to 3000 μM on Tba endo V activity in the reactions (10 μl) containing

200 nM of Cy3-labeled ssDNA (*p45I), 20 mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM $MgCl_2$, 0.1mg/ml BSA, 8% glycerol and 300 nM of Tba EndoV. The reactions were kept at 85°C for 20 min. The reactions were stopped and treated as described above.

Effect of NaCl on DNA cleavage

To determine the effect of NaCl on Tba endo V activity, DNA cleavage assays were performed using various NaCl concentrations ranging from 100 to 100 mM in the reactions (10 μl) containing 200 nM of Cy3-labeled ssDNA (*p45I), 20 mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM $MgCl_2$, 0.1mg/ml BSA, 8% glycerol and 300 nM Tba endo V. The reactions were kept at 85°C for 20 min. The reactions were stopped and treated as described above.

DNA binding by electrophoresis mobility shift assays

The DNA binding reaction mixtures (10 μl) contained 50 nM Cy3-labeled DNA substrate (*p22, *p22/t22, *p45I, and *p45/t45), 20 mM Tris-HCl pH 8.0, 1 mM DTT, 0.1 mg/ml BSA, 8% glycerol and various Tba endo V concentrations (50–3000 nM). The reactions were carried out at 25°C for 10 min. Samples were electrophoresed on a 4% native polyacrylamide gel in 0.1 × TBE (Tris-borate-EDTA) buffer. After electrophoresis, the gels were scanned and Cy3-labeled DNA was visualized with a Molecular Image analyser (BioRad). ImageQuant software was used for the quantitative analysis.

Results

Overexpression and purification of Tba endo V

The complete genome of the hyperthermophilic archaeon *T. barophilus* Ch5 has been sequenced, and was shown to encode an endonuclease V [GenBank accession number: ALM74570]. We used Clustal X V2.0 to align endonuclease V sequences, which were retrieved from the protein sequence database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/protein/). The alignment results suggested that Tba endo V shares seven highly conserved motifs with other endo Vs from archaea (*Archaeoglobus fulgidus* [GenBank accession number: KUK05292], *Pyrococcus furiosus* [GenBank accession number: WP_011012124], *Sulfolobus solfataricus* [GenBank accession number: AKA78077]), eukaryotes (*Homo sapiens* [GenBank accession number: Q8N8Q3], and *Schizosaccharomyces pombe* [GenBank accession number: Q10348]) and bacteria (*Escherichia coli* [GenBank accession number: EDV65061] and *Thermotoga maritima* [GenBank accession number: AKE27746]) (Fig. 1A). Homology analysis on endo V protein sequences demonstrated that Tba endo V is a member of the enonuclease V family that can cleave deoxyinosine-containing DNA.

To characterize the enzyme biochemically, the gene of Tba endo V was cloned into the pET-30a(+) expression vector, and expressed in *E. coli* BL21(DE3)pLys cells. The recombinant Tba endo V protein was successfully expressed as a His-tag fusion protein (Fig. 1B). After sonication, heat treatment at 85°C for 25 min and purification by affinity chromatography with a Ni column, a band of about 25 kDa that corresponded to Tba endo V protein was observed (Fig. 1B).

Cleavage of deoxyinosine-containing DNA by Tba endo V

We employed normal DNA (*p22), deoxyinosine-containing DNA (*p45I) and U-containing DNA (*p45U) as reaction substrates to perform the enzymatic analysis of Tba endo V at 85°C, which is optimal growth temperature of *T. barophilus* Ch5 . When using 50–300 nM of Tba endo V in the DNA cleavage reactions in the presence of 1 mM Mg^{2+} and 10 nM DNA (*p45I) at 85°C and at pH 8.0 for 20 min, a short DNA product was observed (Fig. 2A), however, no cleavage product was formed using the normal DNA (*p22) as a template, suggesting that the enzyme could cleave deoxyinosine-containing DNA. In addition, we found no cleavage product in the reaction with U-containing DNA (*p45U) (Fig. 2A), suggesting that Tba endo V cannot cleave U-containing DNA.

Heat treatment at 80°C for 25 min during the purification of Tba endo V would inactivate the enzymatic activity of *E. coli* endo V; however, there was a slight possibility of *E. coli* endo V contamination, which would interfere with our observations. To rule out this possibility, we expressed the empty pET-30a(+) vector in the same expression strain as that one used for Tba endo V, disrupted the cells , and heated the supernatant. No cleavage product was observed (data not shown), when we used the heated supernatant produced from the empty vector ruling out the possibility of an *E. coli* endo V contamination during purification of Tba endo V. Furthermore, we observed that the Tba endo V is inactive to U-containing DNA, which also indirectly supports the lack of *E. coli* endo V contamination during purification of Tba endo V. Taken together, our results suggest that Tba endo V can cleave deoxyinosine-containing DNA at 85°C.

In addition, we determined the effect of Tba endo V concentration on DNA cleavage activity by the addition of varied enzyme concentration ranging from 5 nM to 500 nM. As increasing the enzyme concentration, the DNA cleavage product gradually increased. When 50 nM enzyme concentrations was employed, more than 95% of DNA substrate was cleaved (Fig. 2B), suggesting that the enzyme has strong activity for cleaving deoxyinosine-containing DNA.

Optimal reaction temperature and thermostability of Tba endo V

Since *T. barophilus* Ch5 is a hyperthermophilic archaeon and Tba endo V can cleave deoxyinosine-containing DNA at 85°C; therefore, we first determined the optimal temperature for cleaving deoxyinosine-containing DNA using Cy3-labeled p45I and p45I/t45 as DNA substrates, respectively.

When reaction temperatures increased from 30 to 100°C, Tba endo V cleaved deoxyinosine-containing DNA with varied cleavage efficiency using the Cy3-labeled ssDNA as a substrate. The cleavage efficiency of Tba endo V reached more than 95%, when reaction temperatures were 50–90°C (Fig. 3A). However, Tba endo V had reduced cleavage efficiency when the reaction temperature was less than 50°C or more than 90°C. These observations suggest that 50–90°C is the optimal temperature range for Tba endo V to cleave deoxyinosine-containing ssDNA.

In addition, the Tba endo V also displayed cleavage activity on deoxyinosine-containing ds DNA as a template over the 30 to 100°C temperature range, showing more than 95% of cleavage efficiencybetween 70 and 90°C. The Tba endo V exhibited less than 90% cleavage efficiency when reaction temperature was higher

than 90°C or less than 70°C (Fig. 3B), suggesting that 70–90°C is the optimal reaction temperature range for the enzyme to cleave deoxyinosine-containing dsDNA.

In the lower temperature range, e.g. 30–50°C, Tba endo V showed a higher cleavage efficiency for deoxyinosine-containing ssDNA than for deoxyinosine-containing dsDNA demonstrating that Tba endo V has a preference for single stranded vs. double stranded deoxyinosine-containing DNA, which cleavage may be impaired by the hydrogen bond and base stacking in the dsDNA.

To further examine the thermostability of the enzyme, Tba endo V was heated for 20–180 min at 100°C prior to activity assays. When heated for 20–90 min, Tba endo V retained more than 95% of cleavage activity (Fig. 3C). This activity decreased to 62% after 120mn of heating (Figure 3C). After 180 min, only 10% of cleavage activity reamined (Fig. 3C). Taken together, these observations suggest that Tba endo V is thermostable.

Optimal reaction pH for cleavage activity of Tba endo V

The impact of pH on the endonuclease activity of Tba endo V was assayed for pHs ranging from 5.0 to 11.0 in the standard DNA cleavage reactions. At the lowest pH (pH=5.0) and the highest pH (pH>10.0) tested, we measured a very limited cleavage efficiency (4% and ~20% respectively, Fig. 4), suggesting that low pH and high pHs are unfavorable for the enzyme to cleave DNA. Between pH=6.0 and pH=9.0, Tba endo V exhibited more than 86% cleavage efficiency with an optimal pH range between 7.5 and 9.0.

Effect of divalent metal ions on Tba endo V activity

Previous studies suggested that a divalent metal ion is required for endo Vs to cleave deoxyinosine-containing DNA. Here, we determined the effects of various divalent metal ions (Mg^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+}) on the DNA cleavage activity of Tba endo V. In absence of divalent ion (assay in the presence of 10 mM EDTA), Tba endo V displayed no cleavage activity (Fig. 5A), suggesting that a divalent metal ion is indeed required for the enzyme to cleave deoxyinosine-containing DNA. In the absence of EDTA to chelate free ions in the assays (no divalent ion lane), Tba endo V displayed 12% of cleavage efficiency (Fig. 5A), which may be caused by copurification of a divalent metal ion bound to the protein during the purification of the enzyme. Both observations suggest that Tba endo V is dependent on a divalent metal ion for its endonuclease activity.

As expected, the DNA cleavage efficiency of Tba endo V varied with the different divalent metal ions tested. In presence of 2 mM Ca^{2+}, Zn^{2+}, Co^{2+}, or Cu^{2+} no significant activity was observed. In presence of Ca^{2+}, Zn^{2+} and Co^{2+}, the Tba endo V was almost inactive, indicating that these ions could displace the ion copurified with the protein and inhibit its activity. In presence of Cu^{2+}, the activity observed was equivalent to that of the control (14% vs. 12%), indicating that this ion could probably not substitute to the native ion in the Tba endo V structure and inhibit the cleavage activity. Tba endo V was the most efficient in presence of 2 mM Mg^{2+} and Mn^{2+}. while the enzyme retained partial cleavage activity (65%) in the presence of 2 mM Ni^{++}. Thus, the order of divalent metal ions for DNA cleavage by Tba endo V would

be Mg^{2+} ≈ Mn^{2+} > Ni^{2+} > Cu^{2+} > Ca^{2+} ≈ Zn^{2+} ≈ Co^{2+}. Tba endo V exhibits cleavage activity over a large concentration of Mg^{2+} and Mn^{2+} concentrations ranging from 10 μM to 3 mM (Fig. 5B and 5C). Tba endo V displayed complete nicking activity in the presence of Mn^{2+} and Mg^{2+} concentrations equal to or higher than 10μM and 100μM respectively; At 500nM Mn^{2+}, and 10μM Mg^{2+} the activity measured was only slightly higher than controls. At high ion concentrations, Mn^{2+} begins to be less efficient in comparison to Mg^{2+}, which allows full protein activity to atl east 3mM (not shown). Thus, Tba endo V has a preference for Mn^{2+}, while at high concentrations, Mg^{2+} is prefarable.

Inhibition of Tba endo V activity by NaCl

To reveal the effect of NaCl on endonuclease activity of Tba endo V, we added various NaCl concentrations (100–1000 mM) in the DNA cleavage reactions. In absence of NaCl, Tba endo V cleaved almost completely DNA substrate (Fig. 6). As NaCl concentration increased, the amount of DNA cleavage product gradually decreased. Specifically, the addition of 500 mM NaCl to the DNA cleavage reaction enabled Tba endo V to cleave DNA with approximately 50% cleavage percent. At concentrations over 800 mM, only residual endonuclease activity comparable to controls remaiend. These results show that the endonuclease activity of Tba endo V is inhibited by high NaCl concentrations.

Kinetic assays of DNA cleavage by Tba endo V

Here, we performed kinetic assays of DNA cleavage by Tba endo V under the optimal reaction condition where 300 nM of the enzyme, 1 mM Mg^{2+} and pH 8.0 were

used. As the reaction time extended, increasing DNA cleavage product was formed by Tba endo V. When reaction time was 8 min, the cleavage percent of Tba endo V reached 92%, suggesting that the enzyme has strong endonuclease activity (Fig. 7).

DNA-binding of Tba endo V

In this work, we employed EMSA to determine the binding of Tba endo V to normal ssDNA and dsDNA, and deoxyinosine-containing ssDNA and dsDNA (Fig. 8). As increasing DNA substrate concentration, free DNA substrates were gradually bound by Tba endo V, suggesting that the enzyme is able to bind all four DNA substrates. When using deoxyinosine-containing ssDNA (p45I) as a substrate, the whole free DNA substrate was bound at 400 nM of Tba endo V (Fig. 8A), while at least 800 nM of enzyme was needed for complete binding to normal ssDNA (p22) (Fig. 8C). Similarly, when using deoxyinosine-containing dsDNA as DNA substrate, 800 nM of Tba endo V was required to bind all the free DNA substrate (Fig. 8B) while 2000 nM of the enzyme was required for the complete binding of the normal dsDNA substrate (Fig. 8D). These observations show that Tba endo V preferably binds ssDNA, regardless of the presence of deoxyinosine, and that it binds preferentially to deoxyinosine containing substrates regarless of their being single or double stranded. In fine, Tba endo V displays a higher affinity for single-stranded, deoxyinosine-containing DNA.

Discussion

In this contribution, we present the first biochemical characterization of a thermostable endonuclease V from te hyperthermophilic archaeon *T. barophilus* Ch5.

Here, we demonstrated that Tba endo V can cleave deoxyinosine-containing DNA at temperatures ranging from 30 to 100°C. The optimal temperature of the enzyme was 60–90°C, which is similar to those of Pfu endo V and *A. fulgidus* endo V . Tba endo V still had cleavage activity even when heated at 100°C for 120 min, suggesting that the enzyme is a thermostable endonuclease. Our results provides a support for the hypothesis that hyperthermophilic nature is a unique feature of enzymes from hyperthermophiles, as well as a necessity for these organisms often experiencing temperature above 100°C in their natural environments.

The Tba endo V cleaved only deoxyinosine, which is similar to the *A. fulgidus* endo V but contrasts with that of *E. coli*, further confirming that the cleavage spectra of endo Vs is organism specific. Similar to the *E. coli* endo V, the Tba endo V can bind unsubstituted ssDNA and dsDNA without sequence specificity. However, the Tba endo V exhibits stronger affinity for deoxyinosine-containing DNA than normal DNA as observed for human endo V, as well as a preference for single stranded targets.

The reported endo Vs are dependent on divalent metal ions, typically Mg^{2+}, Mn^{2+}, and Ni^{2+}, as metal cofactors for their endonuclease activity, with a marked preference for one divalent metal ion. In the case of the Tba endo V, we demonstrated its activity in the presence of Mn^{2+}, Mg^{2+}, and Ni^{2+}, but not in presence of Cu^{2+}, Zn^{2+}, Ca^{2+}, and Co^{2+}. Mn^{2+} and Mg^{2+} are the divalent ions allowing the complete cleaveage of deoxyinosine-containing DNA, which resembles the Pfu and human endo Vs. However, Mn^{2+} is the preferred divalent metal ion for Tba endo V, while Mg^{2+} and to

a much lesser extent, Mn^{2+}, Ni^{2+}, Co^{2+} can support the endonuclease activity of the human endo V. The optimal deoxyinosine-specific endonuclease activity of Tba endo V was observed at 10μM Mn^{2+} and 0.1 mM Mg^{2+} while 5 mM Mg^{2+} is required for *A. fulgidus* endo V to cleave deoxyinosine-containing DNA at maximum efficiency. Analyses of single-molecule studies on endo V revealed that these enzymatic activities are precursors of DNA repair and are fueled by two metal ions such as Ca^{2+} and Mg^{2+}, with the former being associated with protein binding and the latter with DNA cleavage, supporting the hypothesis that endo V has two metal binding sites to regulate its enzymatic activities . .

The Tba endo V shares several similar features with Pfu endo V, the other characterized endo V protein from the closely related hyperthermophilic archaeon *Pyrococcus furiosus*, which belongs to the same family as *Thermococcus barophilus*. Both endo V have optimal cleavage activity ca. neutral pH, and are inhibited by high NaCl concentrations. However, Tba endo V displays higher specific enzyme activity than Pfu endo V at pH 6.5. Furthermore, the Tba endo V is inhibited by much higher NaCl concentrations (400mM, Fig. 8) than the one from *Pyrococcus furiosus* (200mM).

Since the rate of deamination of DNA increases with temperature, significant amount of deoxyinosine can be generated at 85°C, which is the optimal growth temperature of *T. barophilus* Ch5. Thus, the activity of Tba endo V might be essential for mutation prevention in this organism in response to the known mutatgenic ptential of deoxyinosine. Recently, a novel endonuclease, designated as endonuclease Q

(Endo Q) was biochemically characterized in two other *Thermococcales*, *Pyrococcus furious* and *Thermococcus kodakarensis*. Similar to EndoV, EndoQ has a preference for deoxyinosine containing DNA substrate, and is abile to cleave DNA in 5' to the damage base, while Endo V is cleaving in 3'. Both enzymes have been shown ot be essential for deoxyinosine repair. We have found an homologue of endo Q in the genome of *T. barophilus* Ch5, and its biochemical characterization is under investigation in our laboratory. The analysis of the Tba endo V and endo Q will provide important information on the deoxyinosine repair mechanisms in *Thermococcales*.

Biochemical and structural data support that endonuclease V is an unusual and interesting repair enzyme, which allows its use in biotechnology. For example, the combined use of *E. coli* T/G DNA glycosylase and *E. coli* endo V has been employed to detect mutations in the BRCA1 gene . In addition, Tma endo V is used to scan mutations and amplify DNA by strand displacement in the presence of thermostable DNA ligase and DNA polymerase . Moreover, endo Vs are employed in directional cloning of DNA fragments , random mutagenesis , and DNA shuffling . Herein, we characterized the recombinant endonuclease V from the hyperthermophilic *T. barophilus* Ch5 and demonstrated that it could cleave deoxyinosine-containing DNA at high temperature, which might have a promising future in many biotechnological applications, since its use will be compatible with high temperature cycling-based approaches such as PCR.

In conclusion, we performed the first biochemical characterization of a

thermostable endonuclease V from *T. barophilus* Ch5. Tba endo V displayed deoxyinosine-containing DNA cleavage activity with the highest efficiency at 70–90°C, and cleaved DNA over a wide pH spectrum ranging from 5.5 to 10.0 with an optimal pH of 7.5–9.0. A divalent metal ion is required for the enzyme to cleave deoxyinosine-containing DNA, which Mn^{2+} and Mg^{2+} are the optimal divalent metal ions for the enzyme's activity. Furthermore, the endonuclease activity of the enzyme was inhibited by high NaCl concentration. The enzyme exhibited a strong affinity for binding to deoxyinosine-containing DNA. Our work provides a basis for determining the role of Tba endo V in the base excision repair pathway in *Thermococcus*, and also suggests potential applications of the enzyme.

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Conflict of Interests

The authors declare no conflict of interests regarding the publication of this paper.

Ethical Statement

This article does not contain any studies with human participants or animals performed by any of the authors.

References

Baumann T, Arndt KM, Muller KM (2013) Directional cloning of DNA fragments using deoxyinosine-containing oligonucleotides and endonuclease V. *Bmc Biotechnol* **13:** 81

Bazar LS, Collier GB, Vanek PG, Siles BA, Kow YW, Doetsch PW, Cunningham RP, Chirikjian JG (1999) Mutation identification DNA analysis system (MIDAS) for detection of known mutations. *Electrophoresis* **20:** 1141-1148

Cao WG (2013) Endonuclease V: an unusual enzyme for repair of DNA deamination. *Cell Mol Life Sci* **70:** 3145-3156

Dalhus B, Arvai AS, Rosnes I, Olsen OE, Backe PH, Alseth I, Gao HH, Cao WG, Tainer JA, Bjoras M (2009) Structures of endonuclease V with DNA reveal initiation of deaminated adenine repair. *Nat Struct Mol Biol* **16:** 138-143

Demple B, Linn S (1982) On the recognition and cleavage mechanism of Escherichia coli endodeoxyribonuclease V, a possible DNA repair enzyme. *J Biol Chem* **257:** 2848-2855

Feng H, Dong L, Cao WG (2006) Catalytic mechanism of endonuclease V: A catalytic and regulatory two-metal model. *Biochemistry-Us* **45:** 10251-10259

Feng H, Dong L, Klutz AM, Aghaebrahim N, Cao WG (2005) Defining amino acid residues involved in DNA-protein interactions and revelation of 3 '-exonuclease activity in endonuclease V. *Biochemistry-Us* **44:** 11486-11495

Gates FT, 3rd, Linn S (1977) Endonuclease V of Escherichia coli. *J Biol Chem* **252:** 1647-1653

Guo G, Weiss B (1998) Endonuclease V (nfi) mutant of Escherichia coli K-12. *J Bacteriol* **180:** 46-51

He B, Qing H, Kow YW (2000) Deoxyxanthosine in DNA is repaired by Escherichia coli endonuclease V. *Mutat Res* **459:** 109-114

Hill-Perkins M, Jones MD, Karran P (1986) Site-specific mutagenesis in vivo by single methylated or deaminated purine bases. *Mutat Res* **162:** 153-163

Hitchcock TM, Gao HH, Cao WG (2004) Cleavage of deoxyoxanosine-containing oligodeoxyribonucleotides by bacterial endonuclease V. *Nucleic Acids Res* **32:** 4071-4080

Hoeijmakers JH (2009) DNA damage, aging, and cancer. *N Engl J Med* **361:** 1475-1485

Huang J, Lu J, Barany F, Cao W (2002a) Mutational analysis of endonuclease V from Thermotoga maritima. *Biochemistry-Us* **41:** 8342-8350

Huang JM, Kirk B, Favis R, Soussi T, Paty P, Cao WG, Barany F (2002b) An endonuclease/ligase based mutation scanning method especially suited for analysis of neoplastic tissue. *Oncogene* **21:** 1909-1921

Huang JM, Lu J, Barany F, Cao WG (2001) Multiple cleavage activities of endonuclease V from Thermotoga maritima: Recognition and strand nicking mechanism. *Biochemistry-Us* **40:** 8738-8748

Kanugula S, Pauly GT, Moschel RC, Pegg AE (2005) A bifunctional DNA repair protein from Ferroplasma acidarmanus exhibits O-6-alkylguanine-DNA alkyltransferase and endonuclease V activities. *P Natl Acad Sci USA* **102:** 3617-3622

Kim YJ, Lee HS, Kim ES, Bae SS, Lim JK, Matsumi R, Lebedinsky AV, Sokolova TG, Kozhevnikova DA, Cha SS, Kim SJ, Kwon KK, Imanaka T, Atomi H, Bonch-Osmolovskaya EA, Lee JH, Kang SG (2010) Formate-driven growth coupled with H(2) production. *Nature* **467:** 352-355

Kiyonari S, Egashira Y, Ishino S, Ishino Y (2014) Biochemical characterization of endonuclease V from the hyperthermophilic archaeon, Pyrococcus furiosus. *J Biochem* **155:** 325-333

Kuraoka I (2015) Diversity of Endonuclease V: From DNA Repair to RNA Editing. *Biomolecules* **5:** 2194-2206

Lin J, Gao HH, Schallhorn KA, Harris RM, Cao WG, Ke PC (2007) Lesion recognition and cleavage by endonuclease V: A single-molecule study. *Biochemistry-Us* **46:** 7132-7137

Lindahl T (1979) DNA glycosylases, endonucleases for apurinic/apyrimidinic sites, and base excision-repair. *Prog Nucleic Acid Res Mol Biol* **22:** 135-192

Lindahl T (1993) Instability and decay of the primary structure of DNA. *Nature* **362:** 709-715

Lindahl T, Nyberg B (1974) Heat-induced deamination of cytosine residues in deoxyribonucleic acid. *Biochemistry-Us* **13:** 3405-3410

Liu B, Zhang X (2008) Deep-sea thermophilic Geobacillus bacteriophage GVE2 transcriptional profile and proteomic characterization of virions. *Appl Microbiol Biotechnol* **80:** 697-707

Liu J, He B, Qing H, Kow YW (2000) A deoxyinosine specific endonuclease from hyperthermophile, Archaeoglobus fulgidus: a homolog of Escherichia coli endonuclease V. *Mutat Res-DNA Repair* **461:** 169-177

Marteinsson VT, Birrien JL, Reysenbach AL, Vernet M, Marie D, Gambacorta A, Messner P, Sleytr UB, Prieur D (1999) Thermococcus barophilus sp. nov., a new barophilic and hyperthermophilic archaeon isolated under high hydrostatic pressure from a deep-sea hydrothermal vent. *Int J Syst Bacteriol* **49 Pt 2:** 351-359

Mi RJ, Alford-Zappala M, Kow YW, Cunningham RP, Cao WG (2012) Human endonuclease V as a repair enzyme for DNA deamination. *Mutat Res-Fund Mol M* **735:** 12-18

Miyazaki K (2002) Random DNA fragmentation with endonuclease V: application to DNA shuffling. *Nucleic Acids Res* **30**

Moe A, Ringvoll J, Nordstrand LM, Eide L, Bjoras M, Seeberg E, Rognes T, Klungland A (2003) Incision at hypoxanthine residues in DNA by a mammalian homologue of the Escherichia coli antimutator enzyme endonuclease V. *Nucleic Acids Res* **31:** 3893-3900

Oger P, Sokolova TG, Kozhevnikova DA, Taranov EA, Vannier P, Lee HS, Kwon

KK, Kang SG, Lee JH, Bonch-Osmolovskaya EA, Lebedinsky AV (2016) Complete Genome Sequence of the Hyperthermophilic and Piezophilic Archaeon Thermococcus barophilus Ch5, Capable of Growth at the Expense of Hydrogenogenesis from Carbon Monoxide and Formate. *Genome Announc* **4**

Pang B, McFaline JL, Burgis NE, Dong M, Taghizadeh K, Sullivan MR, Elmquist CE, Cunningham RP, Dedon PC (2012) Defects in purine nucleotide metabolism lead to substantial incorporation of xanthine and hypoxanthine into DNA and RNA. *Proc Natl Acad Sci U S A* **109:** 2319-2324

Pincas H, Pingle MR, Huang JM, Lao KQ, Paty PB, Friedman AM, Barany F (2004) High sensitivity EndoV mutation scanning through real-time ligase proofreading. *Nucleic Acids Res* **32**

Schouten KA, Weiss B (1999) Endonuclease V protects Escherichia coli against specific mutations caused by nitrous acid. *Mutat Res* **435:** 245-254

Schuster H (1960) [The method of reaction of desoxyribonucleic acid with nitrous acid]. *Z Naturforsch B* **15B:** 298-304

Shiraishi M, Ishino S, Yamagami T, Egashira Y, Kiyonari S, Ishino Y (2015) A novel endonuclease that may be responsible for damaged DNA base repair in Pyrococcus furiosus. *Nucleic Acids Res* **43:** 2853-2863

Taghizadeh K, McFaline JL, Pang B, Sullivan M, Dong M, Plummer E, Dedon PC (2008) Quantification of DNA damage products resulting from deamination, oxidation and reaction with products of lipid peroxidation by liquid chromatography isotope dilution tandem mass spectrometry. *Nat Protoc* **3:** 1287-1298

Turner DJ, Pingle MR, Barany F (2006) Harnessing asymmetrical substrate recognition by thermostable EndoV to achieve balanced linear amplification in multiplexed SNP typing. *Biochem Cell Biol* **84:** 232-242

van Wolferen M, Ajon M, Driessen AJM, Albers SV (2013) How hyperthermophiles adapt to change their lives: DNA exchange in extreme conditions. *Extremophiles* **17:** 545-563

Wang Z, Wang HY, Feng H (2013) A Simple and Reproducible Method for Directed Evolution: Combination of Random Mutation with dITP and DNA Fragmentation with Endonuclease V. *Mol Biotechnol* **53:** 49-54

Weiss B (2001) Endonuclease V of Escherichia coli prevents mutations from nitrosative deamination during nitrate/nitrite respiration. *Mutat Res-DNA Repair* **461:** 301-309

Weiss B (2008) Removal of deoxyinosine from the Escherichia coli chromosome as

studied by oligonucleotide transformation. *DNA Repair (Amst)* **7:** 205-212

Yao M, Hatahet Z, Melamede RJ, Kow YW (1994) Purification and characterization of a novel deoxyinosine-specific enzyme, deoxyinosine 3' endonuclease, from Escherichia coli. *J Biol Chem* **269:** 16260-16268

Yao M, Kow YW (1994) Strand-specific cleavage of mismatch-containing DNA by deoxyinosine 3'-endonuclease from Escherichia coli. *J Biol Chem* **269:** 31390-31396

Yao M, Kow YW (1996) Cleavage of insertion/deletion mismatches, flap and pseudo-Y DNA structures by deoxyinosine 3'-endonuclease from Escherichia coli. *J Biol Chem* **271:** 30672-30676

Zhang ZM, Jia Q, Zhou C, Xie W (2015) Crystal structure of E. coli endonuclease V, an essential enzyme for deamination repair. *Sci Rep-Uk* **5**

Figure legends

Fig. 1 The genome of *T. barophilus* Ch5 encodes an endo V. A. Sequence alignment of the conserved motifs in *Tba* endo V and other endo Vs from archaea, eukarya and bacteria. Sequences of conserved motifs (I, II, III, IV, V, VI and VII) and the numbers of amino acid residues separating the motifs are indicated. The conserved amino acid residues are in bold. Tba: *Thermococcus barophilus* (GenBank accession number: ALM74570]); Afu: *Archaeoglobus fulgidus* [GenBank: KUK05292]; Pfu: *Pyrococcus furiosus* [GenBank accession number: WP_011012124]; Sso: *Sulfolobus solfataricus* [GenBank accession number: AKA78077]; Hsa: *Homo sapiens* [GenBank accession number: Q8N8Q3]; Spo: *Schizosaccharomyces pombe* [GenBank accession number: Q10348]; Eco: *Escherichia coli* [GenBank accession number: EDV65061]; Tma: *Thermotoga maritima* [GenBank accession number: AKE27746]. B. Expression and purification of Tba endo V. After IPTG induction, sonication, heat treatment (80°C for 30 min) and Ni column affinity purification, Tba endo V was purified.

Fig. 2 DNA cleavage of Tba endo V. A. DNA cleavage reactions of various concentrations (50 and 300 nM) of Tba endo V were performed in the presence of 1 mM Mg^{2+} using the Cy3-labeled p22, p45I, and p45U DNA as substrates for 20 min at 85^{o}C. B. Effect of enzyme concentrations on endonuclease activity of Tba endo V. Various Tba endo V concentrations ranging from 5 nM to 500 nM were added in the

DNA cleavage reactions using the Cy3-labeled p45I as substrate. Reaction mixtures were electrophoresed through a 15 % denaturing PAGE.

Fig. 3 Optimal temperature and thermostability of Tba endo V. A. DNA cleavage reactions of Tba endo V were performed at various temperatures (30, 40, 50, 60, 70, 80, 90 and 100°C, respectively) in the presence of 1 mM Mg^{2+} using the Cy3-labeled p45I as the substrate for 20 min. B. DNA cleavage reactions of Tba endo V were performed at various temperatures (30, 40, 50, 60, 70, 80, 90 and 100°C, respectively) in the presence of 1 mM Mg^{2+} using the Cy3-labeled p45I /t45 as the substrate for 20 min. C. To evaluate its thermostability, Tba endo V was heated at 100°C for 20, 40, 60, 90, and 120 min, respectively. DNA cleavage reactions of the heated Tba endo V were performed in the presence of 1 mM Mg^{2+} using the Cy3-labeled p45I as substrate at 85°C for 20 min. Reaction mixtures were electrophoresed through an 15 % denaturing PAGE.

Fig. 4 Effect of pH on the DNA cleavage reactions of Tba endo V. DNA cleavage assays of Tba endo V were performed using buffers at various pH levels (pH 5.0, 6.0, 7.0, 7.5, 8.0, 8.5, 9.0, 10.0 and 11.0, respectively) at 85°C for 20 min in the presence of 1 mM Mg^{2+} using the Cy3-labeled p45I as substrate at 85°C for 20 min. Reaction mixtures were electrophoresed through an 15 % denaturing PAGE.

Fig. 5 Effect of divalent metal ions on DNA cleavage reactions of Tba endo V. A. 1 mM Ca^{2+},Mg^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+} and Cu^{2+} were added to the DNA cleavage reactions catalyzed by Tba endo V using the Cy3-labeled p45I as substrate at 85°C for

20 min, respectively. B. Various concentrations of Mg^{2+} ranging from 10 nM to 2 mM were added to the DNA cleavage reactions catalyzed by Tba endo V using the Cy3-labeled p45I as substrate at 85°C for 20 min. C. Various concentrations of Mn^{2+} ranging from 10 nM to 2 mM were added to the DNA cleavage reactions catalyzed by Tba endo V using the Cy3-labeled p45I as substrate at 85°C for 20 min. Reaction mixtures were electrophoresed through an 15 % denaturing PAGE.

Fig. 6 Effect of NaCl on DNA cleavage reactions of Tba endo V. DNA cleavage reactions of Tba endo V were performed in the presence of various concentrations of NaCl ranging from 100 to 1000 mM with 1 mM Mg^{2+} using the Cy3-labeled p45I as substrate at 85°C for 20 min. Reaction mixtures were electrophoresed through an 15 % denaturing PAGE.

Fig. 7 Time course of DNA cleavage by Tba endo V. DNA cleavage reactions of Tba endo V were performed in the presence of 300 nM of the enzyme, 1 mM Mg^{2+} and pH 8.0 at various time intervals using the Cy3-labeled p45I as substrate at 85°C for 20 min. Reaction mixtures were electrophoresed through an 15 % denaturing PAGE.

Fig. 8 DNA-binding of Tba endo V. DNA binding reactions were performed in a DNA binding buffer containing 50 nM Cy3-labeled oligonucleotide DNA substrate (A: p45I; B: p45I/t45T; C: p22; D; p22/t59) 20 mM Tris-HCl pH 8.0, 1 mM DTT, 0.1 mg/ml BSA, 8% glycerol and the various concentrations (50–3000 nM) of the purified Tba endo V protein. The reactions were carried out at 25 °C for 10 min. Samples were

electrophoresed on a 4% native polyacrylamide gel in 0.1 × TBE buffer.

1 Table 1. Primers and oligonucleotides used in this study.

Name	Sequence
Tba Endo V F	5′d- GGA ATT CCA TAT GA GCA GAG CTA ATG TGA AC-3′
Tba Endo V R	5′d- CCG CTC GAG TTT TTC AGC ACC CTT TGA AAG -3′
p22	5′d- CAG TGA ATT CGA GCT CGG TAC C -3′
t22	5′d- GGT ACC GAG CTC GAA TTC ACT G -3′
p45I	5′d- CGA ACT GCC TGG AAT CCT GAC GAC ITG TAG CGA ACG ATCA CC TCA -3′
p45U	5′d- CGA ACT GCC TGG AAT CCT GAC GAC UTG TAG CGA ACG ATCA CC TCA -3′
t45	5′d- TGA GGT GAT CGT TCG CTA CAT GTC GTC AGG ATT CCA GGC AGT TCG -3′

2 The underlined nucleotides represent restriction sites. I: hypoxanthine; U: uracil.

Figure 1

A

```
Tba  15 QRKLSKR-15-AVDVSY-31-PYIPTFFFLRE-18-EGHGKAHPRGYGLASHIG-5-PTIGIAK-17-YVSVGNL-16-GYPKPL--KIAD
Afu  13 QEEMSRS-16-GVDQAF-31-PYIPTFLMFRE-21-DGSGIAHPRRCGLATYIA-5-PTVGITK-37-FISPGSY-15-GYKLPEPIRIAD
Pfu  11 QRRLSRK-15-AVDVSY-31-PYIPTFFFLRE-18-EGHGKAHPRRYGLASHIG-5-PTIGVAK-17-FVSVGNL-17-GYPKPLN--IAD
Sso  14 QFLISKN-15-GVDIAY-30-PYIPGFLFMRE-18-DGHGIAHPRKSGIAAVIG-5-PTIGVAK-30-FYSPGNK-12-GYPKV--LKIAD
Hsa  21 QARLKAH-22-GVDVSF-34-PYVSGFLAFRE-25-DGNGVLHHRGFGVACHLG-5-PCVGVAK-52-YISVGHR-16-RIPEP--VRQAD
Spo  17 QIELKGM-17-GLDISF-34-DYVPGFLSFRE-22-DGNGVLHPVGFGLACHLG-5-PVVGVAK-58-YVSIGNQ-18-RVPEP--IRQAD
Eco  10 QIELASS-16-GADVGF-33-PYIPGFLSFRE-20-DGHGISHPRRLGVASHFG-5-PTIGVAK-40-FIATGHR-15-GYRLPEPTRWAD
Tma  20 QNELRKK-16-GVDLSF-32-PYIPGLLAFRE-20-DGQGLAHPRKLGIASHMG-5-PTIGVAK-40-FVSPGHL-16-GRRIPEPTRLAH
            I        II        III             IV          V        VI         VII
```

B

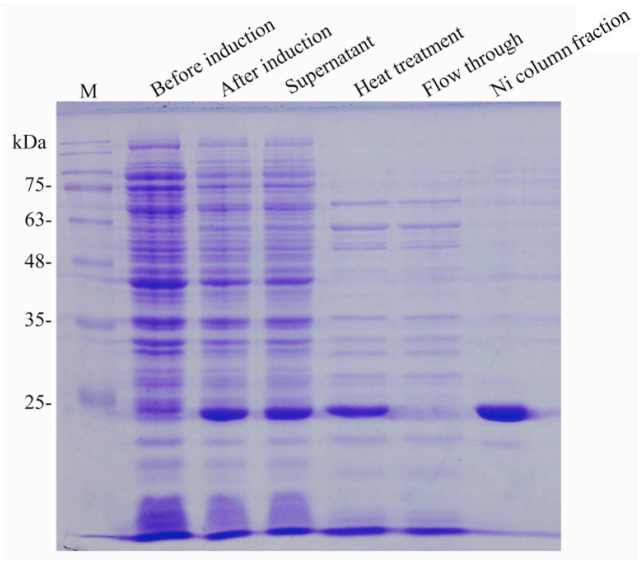


Figure 2

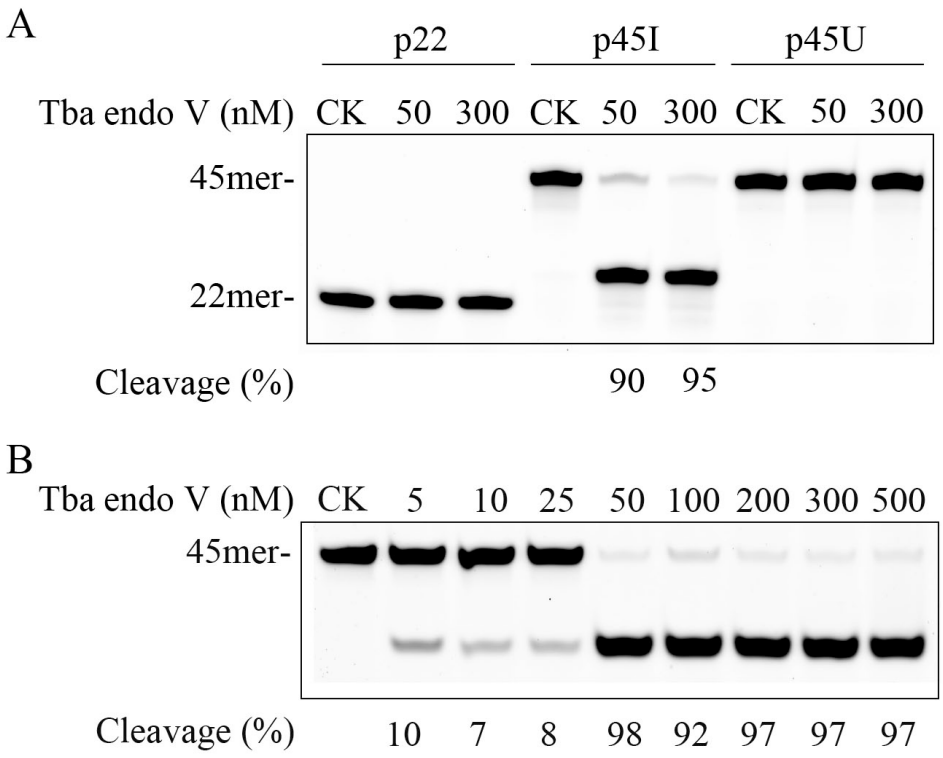
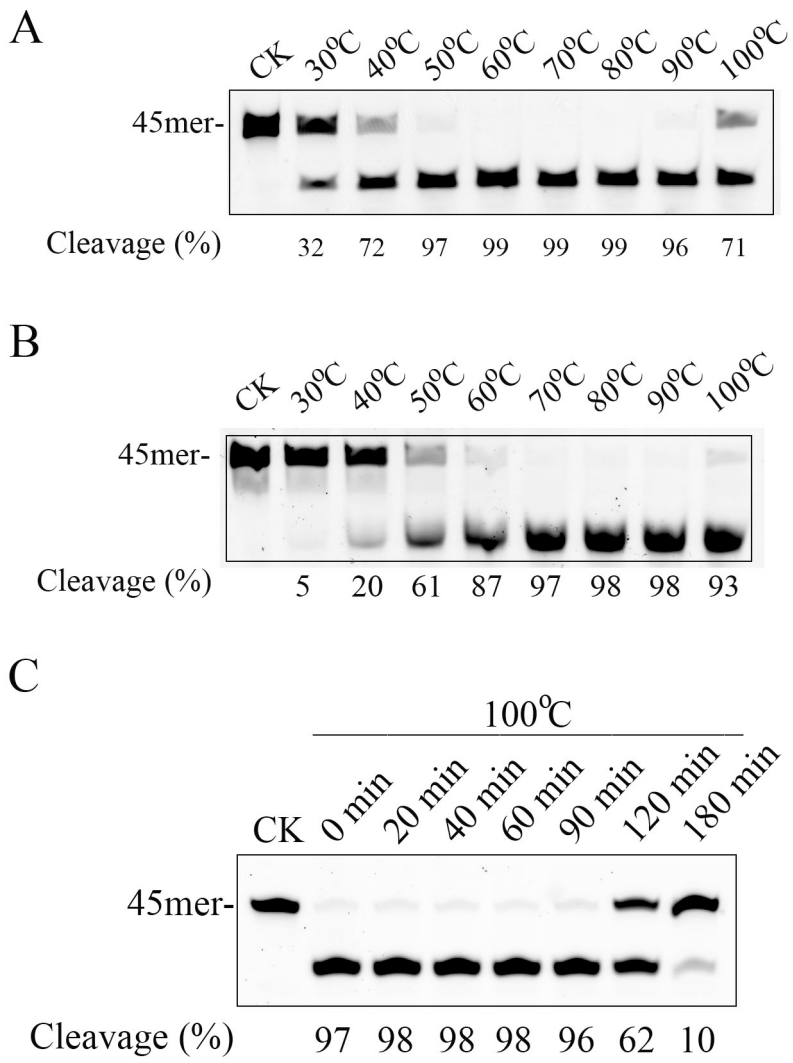
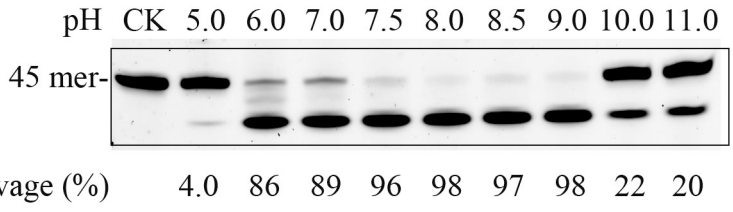

A
p22
p45I
p45U
Tba endo V (nM) CK 50 300 CK 50 300 CK 50 300
45mer-
22mer-
Cleavage (%) 90 95
B
Tba endo V (nM) CK 5 10 25 50 100 200 300 500
45mer-
Cleavage (%) 10 7 8 98 92 97 97 97

Figure 3

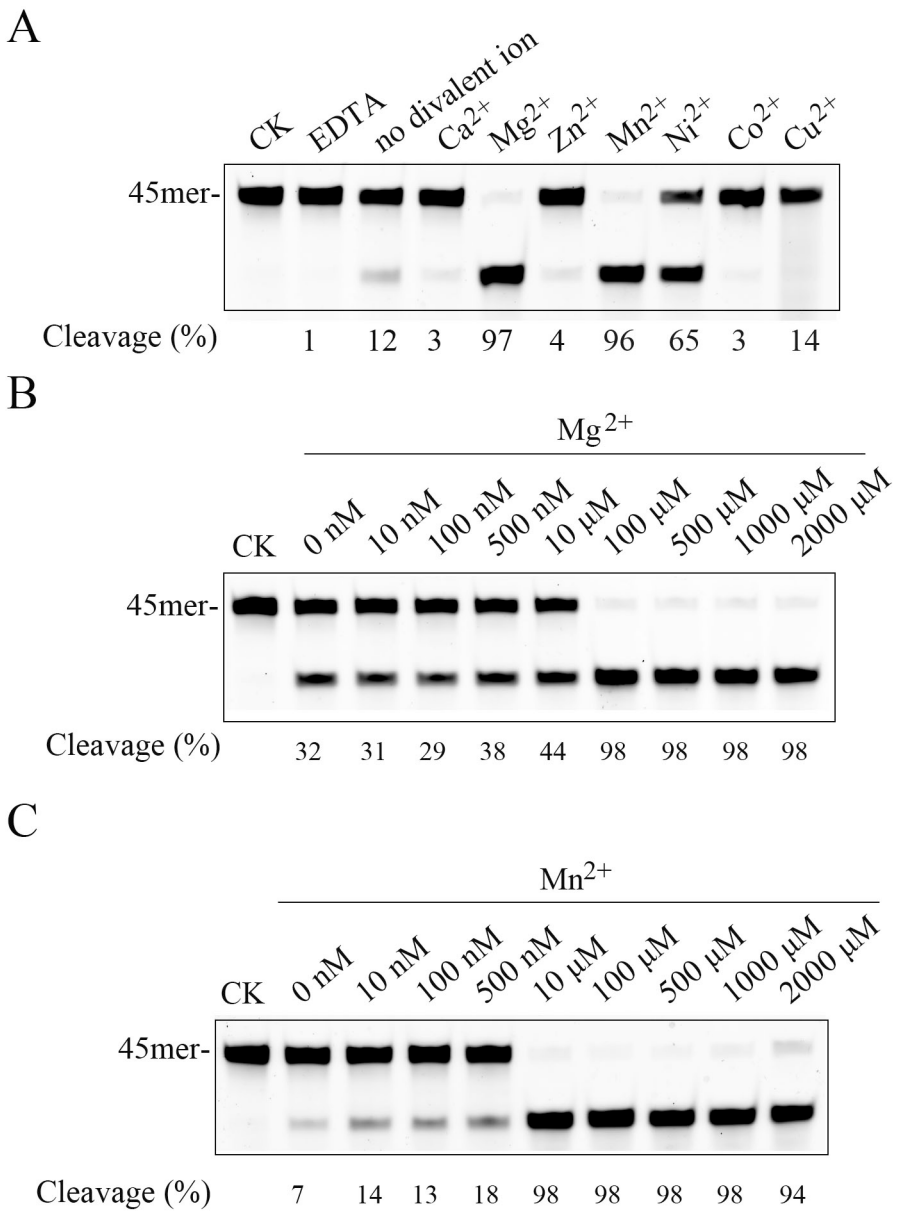

A
CK 30℃ 40℃ 50℃ 60℃ 70℃ 80℃ 90℃ 100℃
45mer-
Cleavage (%) 32 72 97 99 99 99 96 71
B
CK 30℃ 40℃ 50℃ 60℃ 70℃ 80℃ 90℃ 100℃
45mer-
Cleavage (%) 5 20 61 87 97 98 98 93
C
100℃
CK 0 min 20 min 40 min 60 min 90 min 120 min 180 min
45mer-
Cleavage (%) 97 98 98 98 96 62 10

1 Figure 4



2 Cleavage (%) 4.0 86 89 96 98 97 98 22 20

Figure 5



It is important to adress the issue of the background level here since it will bring comments from the reviewers.

1

2

3 Figure 6

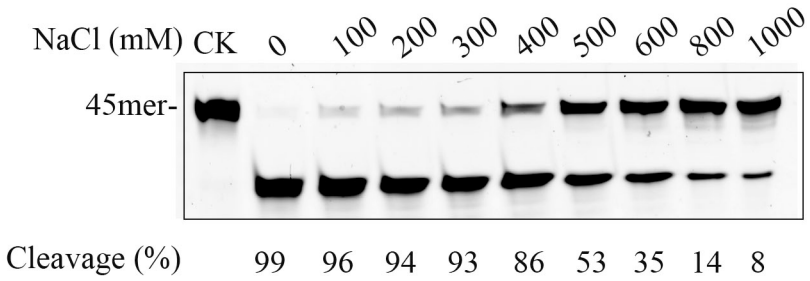


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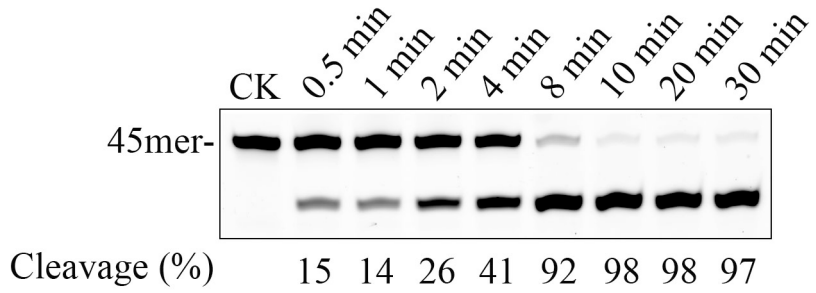
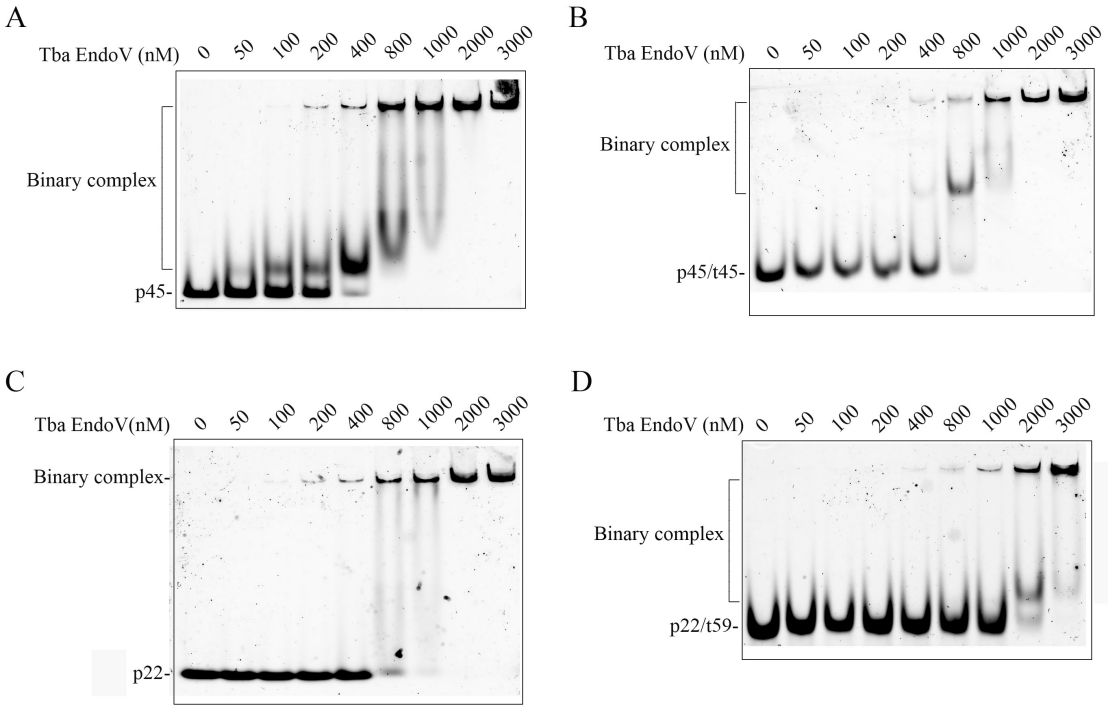
Figure 7


CK
0.5 min
1 min
2 min
4 min
8 min
10 min
20 min
30 min
45mer-
Cleavage (%)
15 14 26 41 92 98 98 97

1 Figure 8


A
Tba EndoV (nM) 0 50 100 200 400 800 1000 2000 3000
Binary complex
p45-
B
Tba EndoV (nM) 0 50 100 200 400 800 1000 2000 3000
Binary complex
p45/t45-
C
Tba EndoV(nM) 0 50 100 200 400 800 1000 2000 3000
Binary complex-
p22-
D
Tba EndoV (nM) 0 50 100 200 400 800 1000 2000 3000
Binary complex
p22/t59-

2

3