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2 **Biochemical characterization of a thermostable endonuclease V from the**

3 **hyperthermophilic euryarchaeon *Thermococcus barophilus* Ch5**

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Abstract

1
2 Endonuclease V (Endo V) is a DNA repair enzyme that recognizes deoxyinosine
3 and cleaves the second phosphodiester bond on the 3' side of the deaminated base
4 lesion. Endo V homologues are conserved in all three domains of life, bacteria,
5 eukarya and archaea. While bacterial and eukaryotic endo Vs have been well studied,
6 knowledge of archaeal endo Vs is limited. Here, we first characterized biochemically
7 a thermostable endonuclease V from the hyperthermophilic euryarchaeon
8 *Thermococcus barophilus* Ch5 (Tba endo V). The recombinant Tba endo V possesses
9 specific endonuclease activity for deoxyinosine-containing DNA. The enzyme
10 displayed optimal nicking activity at 70–90°C, and the enzyme retained its DNA
11 nicking activity even after heating at 100°C for 120 min, suggesting the enzyme is a
12 thermostable endo V. Tba endo V cleaved DNA over a wide pH spectrum ranging
13 from 6.0 to 11.0 with an optimal pH of 8.0–9.0. In addition, Tba endo V activity was
14 dependent on a divalent metal ion, among which Mn^{2+} and Mg^{2+} are optimal ions for
15 the enzyme's activity. Furthermore, DNA cleavage activity of the enzyme was
16 inhibited by high NaCl concentration. Interestingly, Tba endo V bound to all DNA
17 substrates, however, the enzyme exhibited a higher affinity for binding to
18 deoxyinosine-containing DNA than normal DNA. Our work provides a basis for
19 determining the role of Tba endo V in the base excision repair pathway in
20 *Thermococcus*, and also implicates potential applications of the enzyme in molecular
21 biology.

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

1 nicking; Thermostability

Introduction

1
2 Deamination of DNA is a common lesion caused by spontaneous hydrolysis,
3 endogenous or environmental factors as well as deaminase enzymes . Three of the
4 four DNA bases, cytosine, adenine, and guanine, are deaminated to the corresponding
5 base analogs uracil, hypoxanthine, and xanthine, respectively. While the rate of
6 adenine deamination in single-stranded DNA (ssDNA) is much lower than that of
7 cytosine deamination under physiological conditions *in vitro* , adenine deamination in
8 double-stranded DNA (dsDNA) induced by nitrous acid occurs at a similar rate as
9 observed in cytosine deamination . Similar to the background levels of 1–10 per 10⁶
10 nucleotides in tissues or cells , deoxyinosine in *E. coli* and *Saccharomyces cerevisiae*
11 cells is estimated to be 1.2 and 2.0 per 10⁶ nucleotides, respectively. Since deoxyinosine
12 has a strong ability to form mismatch, an A-T base pair would be subsequently
13 converted to a G-C base pair during DNA replication before the deoxyinosine is
14 repaired , potentially leading to mutations in the genome. Increased AT to GC
15 mutations are detrimental to the cells, which may cause genome instability or cancer
16 occurrence. However, cells have evolved the corresponding excision repair pathway to
17 counteract potential mutations generated by deoxyinosine replication. Endonuclease V
18 (endo V) is considered to play an important role in initiating deoxyinosine repair in a
19 base excision repair pathway.

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

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

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

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

8 Hyperthermophilic archaea are facing severe challenges for deamination of DNA
9 due to their inhabiting in high temperature environment . Deamination of DNA occurs
10 at a higher rate at high temperature than at room temperature . To maintain their
11 genome stability, hyperthermophilic archaea have evolved several repair pathways
12 including enzymes to eliminate potentially mutational effects created by deamination
13 of DNA. *Thermococcus* is an important branch of euryarchaea which thrive in
14 hyperthermophilic environments. Increasing *Thermococcus* species have been isolated
15 and studied. *Thermococcus barophilus* Ch5, which was isolated from a deep-sea
16 hydrothermal field of the Mid-Atlantic Ridge (Logachev field chimney, 3,020 m
17 depth) , is a new member of the piezophilic hydrothermal vent archaeal species. *T.*
18 *barophilus* Ch5 has a pressure optimum of 40 Mpa and a temperature optimum of
19 85°C . Analysis of the completed whole genome sequences of *T. barophilus* Ch5
20 indicated that this strain possesses an endonuclease V (Tba endo V) . In this study, the
21 Tba endo V gene was cloned and expressed in *E. coli*. The recombinant Tba endo V
22 was purified and biochemically characterized. We also report that Tba endo V has a

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

4 **Materials and methods**

5 **Cloning, expression and purification of *Tba* endo V**

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

16 The expression strain *E. coli* harboring the recombinant plasmid was cultured
17 into LB medium containing 100 µg/ml kanamycin and 34 µg/ml chloramphenicol at
18 37°C until the OD₆₀₀ reached 0.5. And then, isopropyl thiogalactoside (IPTG) was
19 added to the culture with a final concentration of 0.1 mM, and the culture was shaken
20 for another 10 h at room temperature until the OD₆₀₀ reached 1.4.

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

1 temperature) and resuspended with Ni column buffer A (20 mM Tris-HCl pH 8.0, 1
2 mM dithiothreitol (DTT), 500 mM NaCl, 50 mM imidazole and 10% glycerol). The
3 cells were disrupted by sonication in an ice bath. After centrifugation (16,000 × g for
4 30 min at 4°C), the supernatant was harvested and heated at 80°C for 20 min to
5 remove unthermostable *E. coli* proteins. The resulting supernatant was applied to a
6 HisTrap FF column (GE Healthcare, Uppsala, Sweden) and purified with AKTA fast
7 protein liquid chromatography (GE Healthcare) by elution with a linear gradient of
8 50–500 mM imidazole after centrifugation (16,000 × g for 30 min at 4°C). Fractions
9 containing Tba endo V protein were collected and visualized by Coomassie-staining,
10 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
11 analysis. The purified Tba endo V protein was dialyzed in a storage buffer containing
12 50 mM Tris-HCl pH8.0, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM
13 DTT and 50% glycerol, and was stored at –80°C. The protein concentration was
14 determined using a Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

15 **DNA cleavage assays**

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

1 complementary deoxyoligonucleotides to prepare ds DNA(*p22/t22, *45U/45T and
2 *45I/45T) in an annealing buffer (20 mM Tris-Cl pH 8.0 and 1M NaCl). The
3 endonuclease activity of Tba endo V was performed in the reactions (10 µl)
4 containing 20 mM Tris-HCl pH 8.0, 1 mM DTT, 0.1 mg/ml BSA, 8% glycerol and
5 the purified Tba endo V. The reactions were kept at 85°C for 20 min and stopped by
6 the addition of 10 µl of stop solution containing 98% formamide and 20 mM EDTA.
7 Samples were heated at 100°C for 5 min and chilled rapidly on ice for 5 min, and then
8 loaded onto a denaturing 15% polyacrylamide gel containing 8M urea. After
9 electrophoresis, the gels were scanned and Cy3-labelled DNA was visualized with a
10 Molecular Image analyser (PharosFx System, BioRad). ImageQuant software was
11 used for the quantitative analysis. The amounts of substrates and enzymes are
12 described in the figure legend.

13 **Assays of optimal temperature and pH for DNA cleavage**

14 Assays to determine the optimal temperature for DNA cleavage by Tba endo V
15 were performed in the reactions (10 µl) containing 200 nM of Cy3-labeled ssDNA
16 (*p45I) and dsDNA (*p45I/t45), 20 mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM MgCl₂,
17 0.1mg/ml BSA, 8% glycerol and 300 nM of Tba endo V. The reactions were kept at
18 30, 40, 50, 60, 70, 80, 90, and 100°C for 20 min, respectively. The reactions were
19 stopped and treated as described above.

20 The optimal pH for the endonuclease activity Tba endo V was examined using a
21 pH range of 5.0–11.0 in reactions (10 µl) containing 200 nM of Cy3-labeled ssDNA
22 (*p45I), 20 mM buffer with varied pHs, 1 mM DTT, 1 mM MgCl₂, 0.1 mg/ml BSA,

1 8% glycerol and 300 nM Tba endo V. Five different buffers (all at 20 mM
2 concentrations) were used to prepare different pHs: acetate-sodium acetate (pH 5.0),
3 sodium phosphate-NaOH (pH 6.0, pH 7.0, and pH 7.5), Tris-HCl (pH 8.0 and pH
4 8.5), Gly-NaOH (pH 9.0), and, NaHCO₃-NaOH (pH 10.0, and pH 11.0). The reactions
5 were kept at 85°C for 20 min. The reactions were stopped and treated as described
6 above.

7 **Assays for Tba endo V Thermostability**

8 For its thermostability assays, Tba endo V were heated at 100°C for 20, 40, 60,
9 90, 120, and 180 min, respectively. The heated Tba endo V were employed to
10 examine DNA cleavage activity in the reactions (10 µl) containing 200 nM of Cy3-
11 labeled ssDNA (*p45I), 20 mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM MgCl₂, 0.1 mg/
12 ml BSA, 8% glycerol and 300 nM of the heated Tba endoV. The reactions were kept
13 at 85°C for 20 min. The reactions were stopped and treated as described above.

14 **Effects of divalent metal ions and NaCl on DNA cleavage**

15 The effect of divalent metal ions on Tba endo V activity was investigated by the
16 addition of 1 mM of Ca²⁺, Mg²⁺, Zn²⁺, Mn²⁺, Ni²⁺ and Co²⁺ (analytical purity) in the
17 reactions (10 µl) containing 200 nM of Cy3-labeled ssDNA (*p45I), 20 mM Tris-HCl
18 pH 8.0, 1 mM DTT, 1 mM MgCl₂, 0.1mg/ml BSA, 8% glycerol and 300 nM Tba endo
19 V. The reactions were kept at 85°C for 20 min. The reactions were stopped and
20 treated as described above.

21 In addition, we determined the effects of Mg²⁺ and Mn²⁺ concentrations ranging
22 from 10 nM to 3000 µM on Tba endo V activity in the reactions (10 µl) containing

1 200 nM of Cy3-labeled ssDNA (*p45I), 20 mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM
2 MgCl₂, 0.1mg/ml BSA, 8% glycerol and 300 nM of Tba EndoV. The reactions were
3 kept at 85°C for 20 min. The reactions were stopped and treated as described above.

4 **Effect of NaCl on DNA cleavage**

5 To determine the effect of NaCl on Tba endo V activity, DNA cleavage assays
6 were performed using various NaCl concentrations ranging from 100 to 100 mM in
7 the reactions (10 µl) containing 200 nM of Cy3-labeled ssDNA (*p45I), 20 mM Tris-
8 HCl pH 8.0, 1 mM DTT, 1 mM MgCl₂, 0.1mg/ml BSA, 8% glycerol and 300 nM Tba
9 endo V. The reactions were kept at 85°C for 20 min. The reactions were stopped and
10 treated as described above.

11 **DNA binding by electrophoresis mobility shift assays**

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

20 **Results**

21 **Overexpression and purification of Tba endo V**

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

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

22 **Cleavage of deoxyinosine-containing DNA by Tba endo V**

1 We employed normal DNA (*p22), deoxyinosine-containing DNA (*p45I) and
2 U-containing DNA (*p45U) as reaction substrates to perform the enzymatic analysis
3 of Tba endo V at 85°C, which is optimal growth temperature of *T. barophilus* Ch5 .
4 When using 50–300 nM of Tba endo V in the DNA cleavage reactions in the presence
5 of 1 mM Mg²⁺ and 10 nM DNA (*p45I) at 85°C and at pH 8.0 for 20 min, a short
6 DNA product was observed (Fig. 2A), however, no cleavage product was formed
7 using the normal DNA (*p22) as a template, suggesting that the enzyme could cleave
8 deoxyinosine-containing DNA. In addition, we found no cleavage product in the
9 reaction with U-containing DNA (*p45U) (Fig. 2A), suggesting that Tba endo V
10 cannot cleave U-containing DNA.

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

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

7 **Optimal reaction temperature and thermostability of Tba endo V**

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

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

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

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

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

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

14 **Optimal reaction pH for cleavage activity of Tba endo V**

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

22 **Effect of divalent metal ions on Tba endo V activity**

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

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

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

10 **Inhibition of Tba endo V activity by NaCl**

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

20 **Kinetic assays of DNA cleavage by Tba endo V**

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

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

4 **DNA-binding of Tba endo V**

5 In this work, we employed EMSA to determine the binding of Tba endo V to
6 normal ssDNA and dsDNA, and deoxyinosine-containing ssDNA and dsDNA (Fig.
7 8). As increasing DNA substrate concentration, free DNA substrates were gradually
8 bound by Tba endo V, suggesting that the enzyme is able to bind all four DNA
9 substrates. When using deoxyinosine-containing ssDNA (p45I) as a substrate, the
10 whole free DNA substrate was bound at 400 nM of Tba endo V (Fig. 8A), while at
11 least 800 nM of enzyme was needed for complete binding to normal ssDNA (p22)
12 (Fig. 8C). Similarly, when using deoxyinosine-containing dsDNA as DNA substrate,
13 800 nM of Tba endo V was required to bind all the free DNA substrate (Fig. 8B)
14 while 2000 nM of the enzyme was required for the complete binding of the normal
15 dsDNA substrate (Fig. 8D). These observations show that Tba endo V preferably
16 binds ssDNA, regardless of the presence of deoxyinosine, and that it binds
17 preferentially to deoxyinosine containing substrates regardless of their being single or
18 double stranded. In fine, Tba endo V displays a higher affinity for single-stranded,
19 deoxyinosine-containing DNA.

20 **Discussion**

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

1 Here, we demonstrated that Tba endo V can cleave deoxyinosine-containing DNA at
2 temperatures ranging from 30 to 100°C. The optimal temperature of the enzyme was
3 60–90°C, which is similar to those of Pfu endo V and *A. fulgidus* endo V. Tba endo
4 V still had cleavage activity even when heated at 100°C for 120 min, suggesting that
5 the enzyme is a thermostable endonuclease. Our results provides a support for the
6 hypothesis that hyperthermophilic nature is a unique feature of enzymes from
7 hyperthermophiles, as well as a necessity for these organisms often experiencing
8 temperature above 100°C in their natural environments.

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

16 The reported endo Vs are dependent on divalent metal ions, typically Mg²⁺,
17 Mn²⁺, and Ni²⁺, as metal cofactors for their endonuclease activity, with a marked
18 preference for one divalent metal ion. In the case of the Tba endo V, we demonstrated
19 its activity in the presence of Mn²⁺, Mg²⁺, and Ni²⁺, but not in presence of Cu²⁺, Zn²⁺,
20 Ca²⁺, and Co²⁺. Mn²⁺ and Mg²⁺ are the divalent ions allowing the complete cleavage
21 of deoxyinosine-containing DNA, which resembles the Pfu and human endo Vs.
22 However, Mn²⁺ is the preferred divalent metal ion for Tba endo V, while Mg²⁺ and to

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

10 The Tba endo V shares several similar features with Pfu endo V, the other
11 characterized endo V protein from the closely related hyperthermophilic archaeon
12 *Pyrococcus furiosus*, which belongs to the same family as *Thermococcus barophilus*.
13 Both endo V have optimal cleavage activity ca. neutral pH, and are inhibited by high
14 NaCl concentrations. However, Tba endo V displays higher specific enzyme activity
15 than Pfu endo V at pH 6.5. Furthermore, the Tba endo V is inhibited by much higher
16 NaCl concentrations ($400mM$, Fig. 8) than the one from *Pyrococcus furiosus*
17 ($200mM$).

18 Since the rate of deamination of DNA increases with temperature, significant
19 amount of deoxyinosine can be generated at $85^{\circ}C$, which is the optimal growth
20 temperature of *T. barophilus* Ch5. Thus, the activity of Tba endo V might be essential
21 for mutation prevention in this organism in response to the known mutagenic potential
22 of deoxyinosine. Recently, a novel endonuclease, designated as endonuclease Q

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

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

22 In conclusion, we performed the first biochemical characterization of a

1 thermostable endonuclease V from *T. barophilus* Ch5. Tba endo V displayed
2 deoxyinosine-containing DNA cleavage activity with the highest efficiency at 70–
3 90°C, and cleaved DNA over a wide pH spectrum ranging from 5.5 to 10.0 with an
4 optimal pH of 7.5–9.0. A divalent metal ion is required for the enzyme to cleave
5 deoxyinosine-containing DNA, which Mn²⁺ and Mg²⁺ are the optimal divalent metal
6 ions for the enzyme's activity. Furthermore, the endonuclease activity of the enzyme
7 was inhibited by high NaCl concentration. The enzyme exhibited a strong affinity for
8 binding to deoxyinosine-containing DNA. Our work provides a basis for determining
9 the role of Tba endo V in the base excision repair pathway in *Thermococcus*, and also
10 suggests potential applications of the enzyme.

11 **Acknowledgements**

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17 **Conflict of Interests**

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

20 **Ethical Statement**

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

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16

1 **Figure legends**

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

16 **Fig. 2 DNA cleavage of *Tba* endo V.** A. DNA cleavage reactions of various
17 concentrations (50 and 300 nM) of *Tba* endo V were performed in the presence of 1
18 mM Mg²⁺ using the Cy3-labeled p22, p45I, and p45U DNA as substrates for 20 min at
19 85°C. B. Effect of enzyme concentrations on endonuclease activity of *Tba* endo V.
20 Various *Tba* endo V concentrations ranging from 5 nM to 500 nM were added in the

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

3 **Fig. 3 Optimal temperature and thermostability of Tba endo V.** A. DNA cleavage
4 reactions of Tba endo V were performed at various temperatures (30, 40, 50, 60, 70,
5 80, 90 and 100°C, respectively) in the presence of 1 mM Mg²⁺ using the Cy3-labeled
6 p45I as the substrate for 20 min. B. DNA cleavage reactions of Tba endo V were
7 performed at various temperatures (30, 40, 50, 60, 70, 80, 90 and 100°C, respectively)
8 in the presence of 1 mM Mg²⁺ using the Cy3-labeled p45I /t45 as the substrate for 20
9 min. C. To evaluate its thermostability, Tba endo V was heated at 100°C for 20, 40,
10 60, 90, and 120 min, respectively. DNA cleavage reactions of the heated Tba endo V
11 were performed in the presence of 1 mM Mg²⁺ using the Cy3-labeled p45I as substrate
12 at 85°C for 20 min. Reaction mixtures were electrophoresed through an 15 %
13 denaturing PAGE.

14 **Fig. 4 Effect of pH on the DNA cleavage reactions of Tba endo V.** DNA cleavage
15 assays of Tba endo V were performed using buffers at various pH levels (pH 5.0, 6.0,
16 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
17 of 1 mM Mg²⁺ using the Cy3-labeled p45I as substrate at 85°C for 20 min. Reaction
18 mixtures were electrophoresed through an 15 % denaturing PAGE.

19 **Fig. 5 Effect of divalent metal ions on DNA cleavage reactions of Tba endo V.** A.
20 1 mM Ca²⁺, Mg²⁺, Zn²⁺, Mn²⁺, Ni²⁺, Co²⁺ and Cu²⁺ were added to the DNA cleavage
21 reactions catalyzed by Tba endo V using the Cy3-labeled p45I as substrate at 85°C for

1 20 min, respectively. B. Various concentrations of Mg^{2+} ranging from 10 nM to 2 mM
2 were added to the DNA cleavage reactions catalyzed by Tba endo V using the Cy3-
3 labeled p45I as substrate at 85°C for 20 min. C. Various concentrations of Mn^{2+}
4 ranging from 10 nM to 2 mM were added to the DNA cleavage reactions catalyzed by
5 Tba endo V using the Cy3-labeled p45I as substrate at 85°C for 20 min. Reaction
6 mixtures were electrophoresed through an 15 % denaturing PAGE.

7 **Fig. 6 Effect of NaCl on DNA cleavage reactions of Tba endo V.** DNA cleavage
8 reactions of Tba endo V were performed in the presence of various concentrations of
9 NaCl ranging from 100 to 1000 mM with 1 mM Mg^{2+} using the Cy3-labeled p45I as
10 substrate at 85°C for 20 min. Reaction mixtures were electrophoresed through an 15
11 % denaturing PAGE.

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

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

1 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 <u>CCA TAT GA</u> GCA GAG CTA ATG TGA AC-3'
Tba Endo V R	5'd- CCG <u>CTC GAG</u> 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'

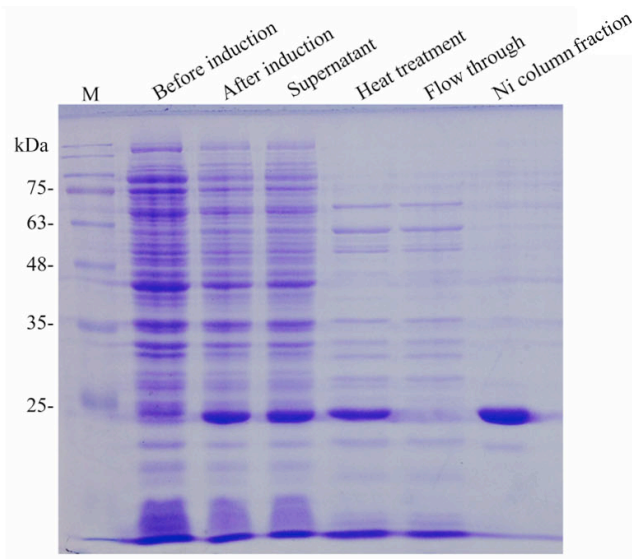
2 The underlined nucleotides represent restriction sites. I: hypoxanthine; U: uracil.

1 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-GYKLEPEIRIAD
Pfu	11	QRRLSRK-15-AVDVSY-31-PYIPTFFFLRE-18-EGHGKAHPRRYGLASHIG-5-PTIGVAK-17-FVSVGNL-17-GYPKPLN--IAD
Sso	14	QFLISK-15-GVDIAY-30-PYIPGFLFMRE-18-DGHGIAHPRKSGIAAVIG-5-PTIGVAK-30-FYSFGNK-12-GYPKV--LKIAD
Hsa	21	QARLKAH-22-GVDVSF-34-PYVSGFLAFRE-25-DGNGVLHHRGFVACHLG-5-PCVGVAK-52-YISVGH-16-RIPEP--VRQAD
Spo	17	QIELKGM-17-GLDISF-34-DYVPGFLSFR-22-DGNGVLHPVGFGLACHLG-5-PVVGVA-58-YVSIQ-18-RVPEP--IRQAD
Eco	10	QIELASS-16-GADVGF-33-PYIPGFLSFR-20-DGHGISHPRRLGVASHFG-5-PTIGVAK-40-FIATGHR-15-GYRLPEPTRWAD
Tma	20	QNELRKK-16-GVDLSF-32-PYIPGLLAFRE-20-DGQGLAHPKLGIAASHMG-5-PTIGVAK-40-FVSPGHL-16-GRRIPPEPTRLAH
		I II III IV V VI VII

B



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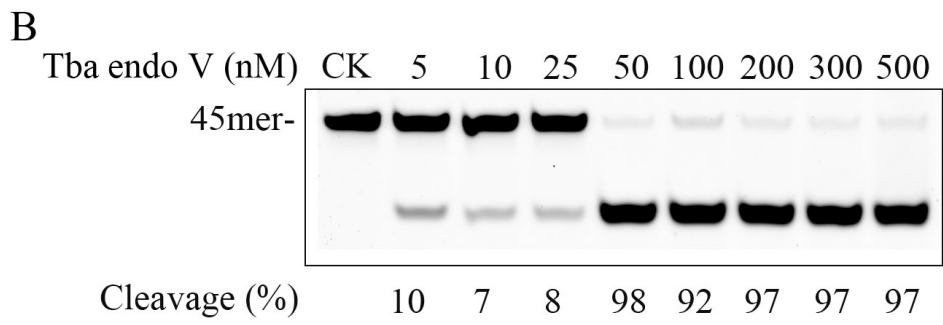
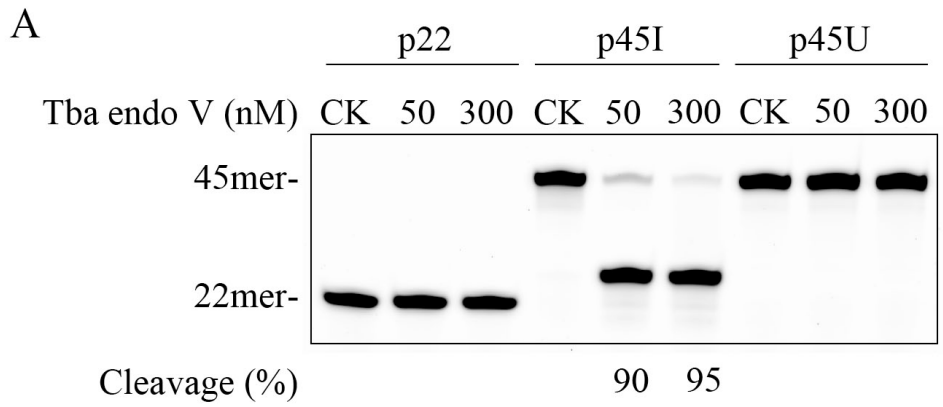
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2 Figure 2

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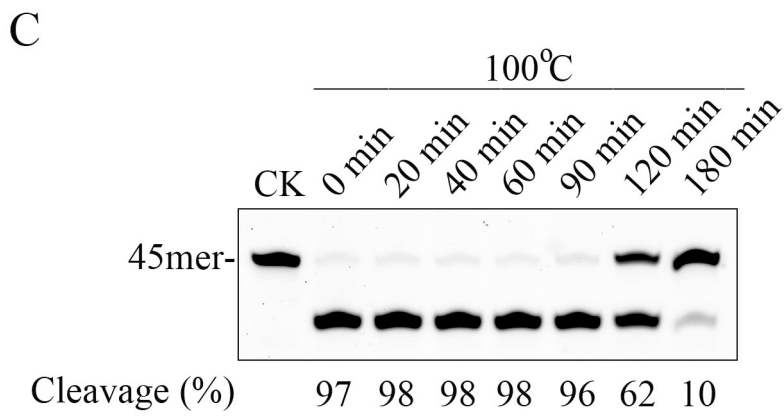
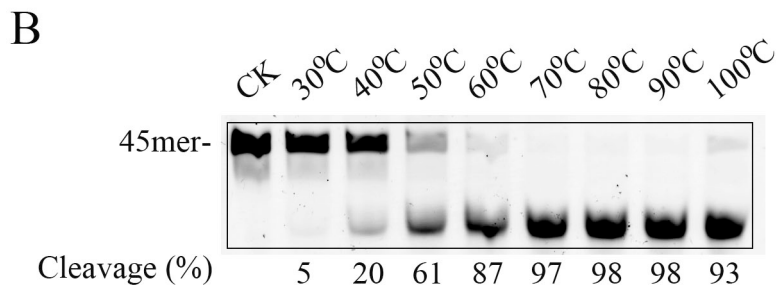
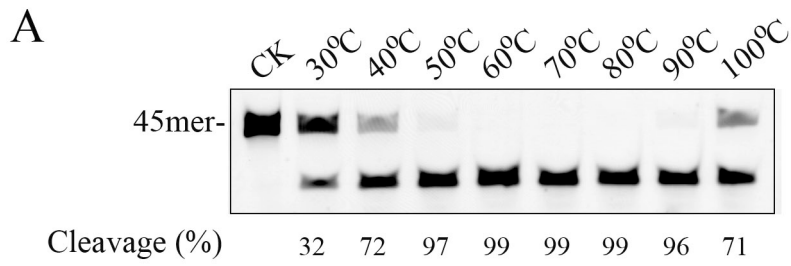
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1 Figure 3



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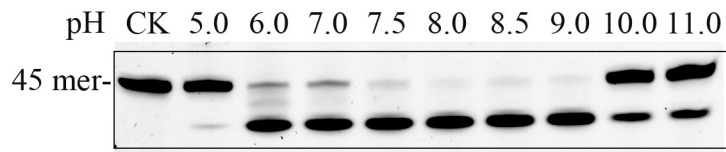
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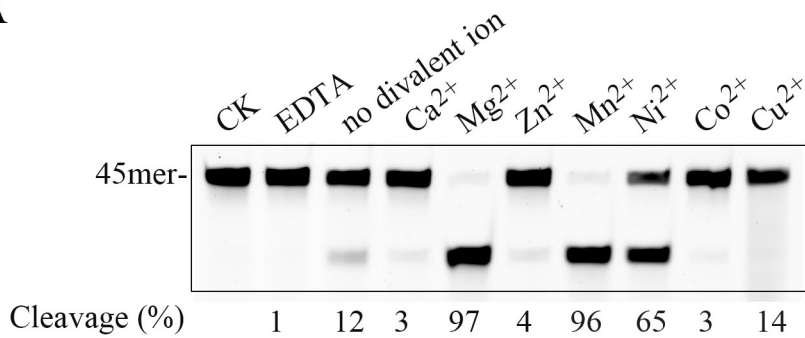
1 Figure 4



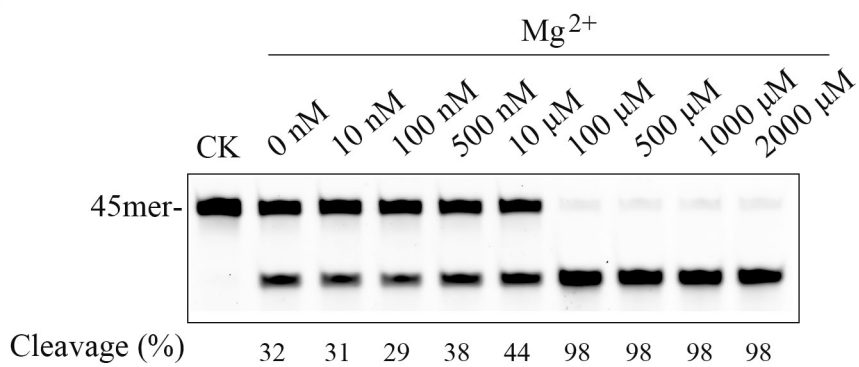
2 Cleavage (%) 4.0 86 89 96 98 97 98 22 20

1 Figure 5

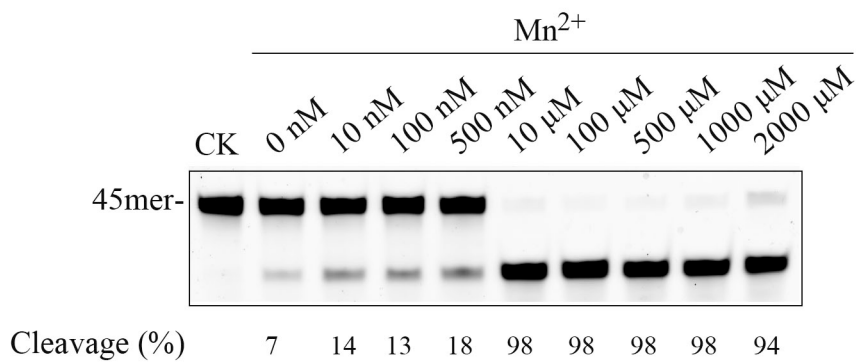
A



B



C



2

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

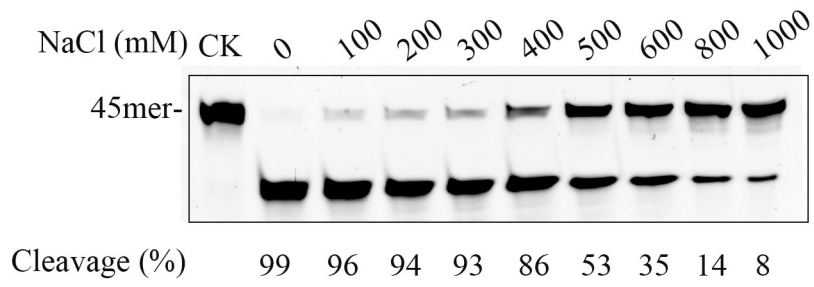
5

6

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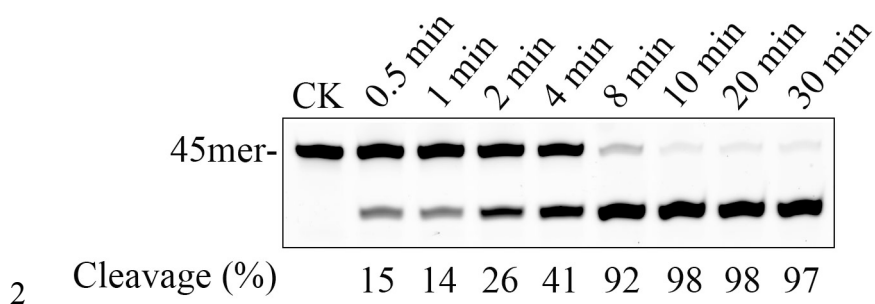
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3 Figure 6



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1 Figure 7



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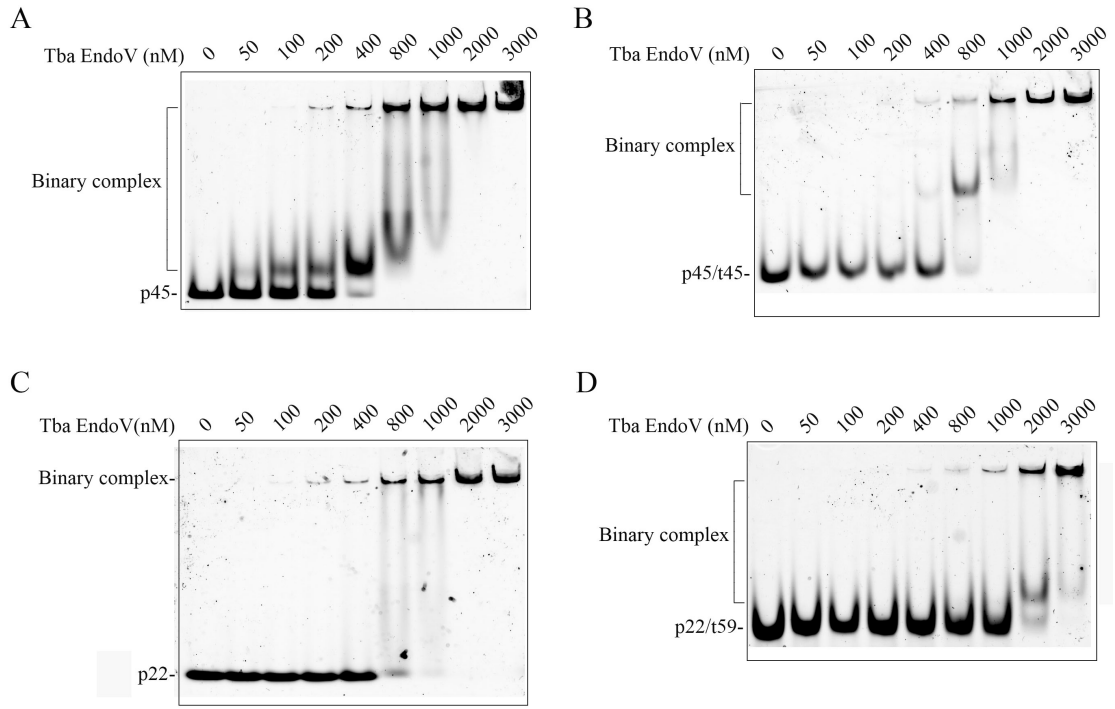
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1 Figure 8



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