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The hyperthermophilic α-amylase from *Thermococcus* sp. HJ21 does not require exogenous calcium for thermostability because of high-binding affinity to calcium

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The hyperthermophilic α-amylase from *Thermococcus* sp. HJ21 does not require exogenous calcium ions for thermostability, and is a promising alternative to commercially available α-amylases to increase the efficiency of industrial processes like the liquefaction of starch. We analyzed the amino acid sequence of this α-amylase by sequence alignments and structural modeling, and found that this α-amylase closely resembles the α-amylase from *Pyrococcus woesei*. The gene of this α-amylase was cloned in *Escherichia coli* and the recombinant α-amylase was overexpressed and purified with a combined renaturation-purification procedure. We confirmed thermostability and exogenous calcium ion independency of the recombinant α-amylase and further investigated the mechanism of the independency using biochemical approaches. The results suggested that the α-amylase has a high calcium ion binding affinity that traps a calcium ion that would not dissociate at high temperatures, providing a direct explanation as to why the addition of calcium ions is not required for thermostability. Understanding of the mechanism offers a strong base on which to further engineer properties of this α-amylase for better potential applications in industrial processes.

Keywords: hyperthermophilic α-amylase, *Thermococcus* sp., calcium independency

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Introduction

α-Amylases (α-1,4-D-glucan 4-glucanohydrolase, EC 3.2.1.1) are endo-acting hydrolases that randomly cleave the α-1,4-glycosidic linkages in starch (Davies and Henrissat, 1995; Pujadas and Palau, 2001). They are a group of enzymes with major industrial applications in starch-processing, brewing, alcohol production, and textiles (Gupta *et al.*, 2003; Johnson, 2013). To date, α-amylases constitute 25–30% of the commercial enzyme market worldwide (Deb *et al.*, 2013).

α-Amylases from different organisms vary on optimal temperature and pH, which determine how well they are applied in a specific industrial process. In the starch industry, α-amylase is used in the liquefaction step of starch-processing, and the ideal conditions are pH 4.5 and 105°C (Sharma and Satyanarayana, 2012). The α-amylase from *Bacillus licheniformis* (BLA) is the most thermostable α-amylase for industrial usage and it operates optimally at 90°C and pH 6, and requires the addition of calcium ion (Ca^{2+}) for thermostability (Violet and Meunier, 1989). It is currently the major α-amylase used in liquefaction during starch processing, and because of its less-ideal operating temperature/pH and the Ca^{2+} requirement, a pH adjustment and Ca^{2+} removal are necessary for the subsequent starch-processing steps. Thus, it is of great interest to develop an α-amylase with an optimal temperature and pH of 105°C and 4.5 without a Ca^{2+} requirement to reduce costs, simplify the process, and minimize the formation of high-pH by-products (Richardson *et al.*, 2002; Li *et al.*, 2016). Thermoactive α-amylases from hyperthermophilic archaea, including *Pyrococcus woesei*, *Pyrococcus furiosus*, *Thermococcus profundus*, *Thermococcus hydrothermalis*, and *Thermotoga maritima*, are targets for the development of an ideal α-amylase for the starch industry (Vieille and Zeikus, 2001). In particular, the α-amylase from *Pyrococcus woesei* (PWA), which is identical to the α-amylase from *Pyrococcus furiosus*, has been extensively studied (Koch *et al.*, 1991; Dong *et al.*, 1997; Vieille and Zeikus, 2001). Unlike BLA, PWA's thermostability is independent of exogenous Ca^{2+}, and the mechanism of this independency has been revealed by biochemical studies and crystallography (Koch *et al.*, 1991; Linden *et al.*, 2003).

We have previously described the molecular and biochemical features of an α-amylase, THJA, from the hyperthermophilic archaeon *Thermococcus* sp. HJ21 isolated from a deep-sea hydrothermal vent (Wang *et al.*, 2008). It has an optimal temperature and pH of 95°C and 5, and thermostability is independent of exogenous Ca^{2+}. These features make THJA a good candidate to be developed for the starch

industry. In this study, we analyzed THJA's amino acid sequence by sequence alignments and structural modeling, and found that THJA has a very high similarity to PWA. The gene of THJA was cloned in *Escherichia coli* and expressed, and the enzyme was purified from the catalytically active inclusion bodies. The molecular mechanism of THJA's independency of exogenous Ca^{2+} was investigated using biochemical approaches, and the results suggested high Ca^{2+} binding affinity of THJA that traps a calcium ion that would not dissociate even at high temperatures, providing a direct explanation as to why addition of Ca^{2+} is not required for THJA's thermostability.

Materials and Methods

Structural modeling and bioinformatics

Amino acid sequences of THJA and 2 other α-amylases, PWA and BLA, were aligned using MUSCLE v3.8 (Edgar, 2004). The structural model of THJA was built by standard homology modeling techniques using Modeller version 9.12 (Marti-Renom *et al.*, 2000). The crystal structure of PWA (Protein Data Bank ID 1MWO) was used as a template. Superposition of the modeled structure of THJA and the crystal structures of PWA and BLA were generated with the PyMOL Molecular Graphics System, version 1.7.2.1 (Schrödinger, LLC).

Cloning, expression, and purification of THJA

The THJA gene was amplified by PCR using KOD DNA polymerase (Yugong Biolabs, Inc.) with appropriate primers from the *Thermococcus* sp. HJ21 available in the lab (Wang *et al.*, 2008). Amplified DNA fragment was digested with appropriate restriction enzymes (Yugong Biolabs, Inc.) and ligated into pET-28b vector, resulting in the fusion of a hexahistidine tag to the N-terminus of the construct. The ligated DNA was transformed into *E. coli* DH5α competent cells, and miniprep plasmid DNA was prepared by the alkaline lysis procedure (GMbiolab Co., Ltd.). The accuracy of the cloned DNA was confirmed by DNA sequencing (Synbio Technologies Co., Ltd.).

For overexpression of THJA, the plasmid was transformed into *E. coli* BL21 (DE3)-RIPL competent cells, and the cells were induced with 0.5 mM of isopropyl-β-D-thiogalactoside (IPTG) at 20°C for 16 h. The solubility of the overexpressed THJA was tested with bacterial protein extraction reagent (B-PER; Thermo Fisher Scientific Inc.). Cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C, resuspended in PBS buffer, and lysed by high pressure crushing (JuNeng Biology & Technology Co., Ltd.). The cell lysate was centrifuged at 43,000 × *g* for 30 min at 4°C to pellet the catalytically active inclusion bodies. The inclusion bodies containing the recombinant THJA enzyme were solubilized in 0.12 M of Britton-Robinson Buffer (0.2 M of NaOH, 0.04 M of phosphoric acid, 0.04 M of boric acid, and 0.04 M of acetic acid, pH 12) (Linden *et al.*, 2003), and the solution was centrifuged 3 times at 43,000 × *g* for 30 min at 4°C to remove insoluble cellular debris. The THJA enzyme in the supernatant was renatured by a gradual change of the pH of Britton-Robinson Buffer from 12 to 9 with dialysis. The renatured THJA enzyme was purified by Ni-affinity chromatography with Ni-agarose resin (Thermo Fisher Scientific Inc.) followed by size-exclusive gel filtration with the HiPrep 16/60 Sephacryl S-300 HR column on an AKTApure automated protein purification system (GE Healthcare Life Science). The Ni-affinity chromatography was performed using the following buffers: binding and wash - 0.12 M of Britton-Robinson Buffer (pH 9) with 5 mM of imidazole; and step gradient elution - 0.12 M of Britton-Robinson Buffer (pH 9) with 10–400 mM of imidazole. The gel filtration chromatography was performed using 0.12 M of Britton-Robinson Buffer (pH 9). The peak fractions were pooled and concentrated using Amicon Ultra-15 centrifugal filters (10 kDa molecular weight cut-off; Millipore, Merck KGaA) and stored at -80°C as small aliquots.

Determination of catalytic activity of THJA

The catalytic activity of THJA was determined by measuring the amount of reducing sugar released during enzymatic hydrolysis of starch (Miller, 1959; Chung *et al.*, 1995; Wang *et al.*, 2008). Briefly, 10 µl of the enzyme solution was added to 190 µl of 50 mM sodium acetate (pH 5.5) containing 1% (w/v) solubilized starch and incubated at 95°C for 15 min. The reaction was terminated by cooling on ice, and 200 µl of DNS reagent was added to the reaction mixture. The reaction mixture was boiled in a water bath for 5 min and cooled under running tap water. The color intensity of the reaction mixture was measured in an automatic microplate reader at 540 nm (Bio-Rad Laboratories, Inc.). One unit of the catalytic activity was defined as the amount of enzyme that liberates 1 µmol of reducing sugar per minute under the assay conditions with maltose as a standard. In this study, "Relative Activity" was used to compare the catalytic activities of THJA with different treatments, and the untreated THJA's activity was normalized to 100% in each set of experiments.

Examination of thermostability of THJA

To examine the thermostability of THJA, the purified enzyme was added into 0.12 M of Britton-Robinson Buffer (pH 9) to a final concentration of 3.9 µM to assemble a 50-µl reaction mixture. The mixture was covered with 20 µl of mineral oil and incubated at 90°C for up to 2 h, and the residual catalytic activity of the enzyme was measured as described above. To test the effect of Ca^{2+} on thermostability, 5 mM of the final concentration of Ca^{2+} was added into the reaction mixture.

EDTA treatment for metal chelation

For metal chelation, the purified enzyme was incubated in the 0.12 M of Britton-Robinson Buffer (pH 9) containing 2 mM of EDTA in a 50-µl reaction mixture at 70–90°C for different periods of time. After incubation, EDTA was removed from the mixture by buffer exchange to Britton-Robinson Buffer (pH 9; no EDTA) using Amicon Ultra-15 centrifugal filters (10 kDa molecular weight cut-off; Millipore, Merck KGaA), followed by activity measurement. To test whether Ca^{2+} could restore the activity, 5 mM of $CaCl_2$ was added to the solu-

bilized starch immediately before measuring the residual activity.

Results

Sequence analysis of THJA

The amino acid sequence of THJA was compared with that of 2 other α-amylases, PWA and BLA (Fig. 1) (Machius *et al.*, 1998; Linden *et al.*, 2003; Wang *et al.*, 2008). THJA shares a very high sequence identity with PWA, 84.3%, but a lower identity rate with BLA, only 29.8%. As α-amylases, the 3 proteins have the same 3-domain organization: the central domain A that contains the active site; domain B that forms part of the active site cleft; and the C-terminal domain C that contains a Greek key motif. Moreover, the active sites of the 3 enzymes are composed of well-conserved amino acid residues (Fig. 1, solid and hollow triangles), which is consistent with their same biological function. On the other hand, THJA and PWA, but not BLA, are well-conserved in calcium ion binding residues (Fig. 1, solid and hollow circles). The high sequence similarity of THJA and PWA, es-

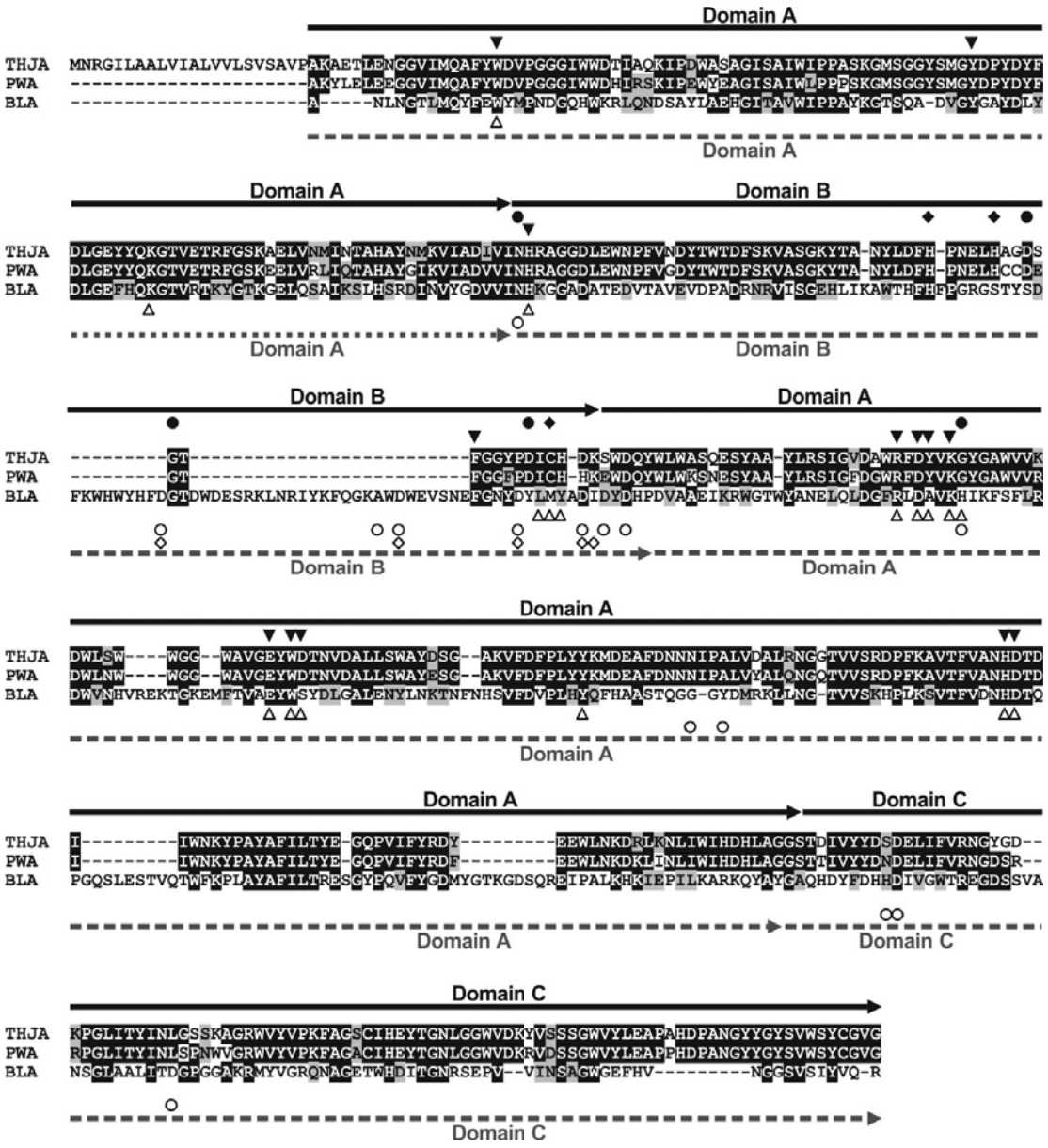


Fig. 1. Sequence alignment of THJA, PWA, and BLA. THJA, *Thermococcus* sp. HJ21 α-amylase (GenBank accession no. ABU98335); PWA, *Pyrococcus woesei* α-amylase chain A (Protein Data Bank code 1MWO); and BLA, *Bacillus licheniformis* α-amylase chain A (Protein Data Bank code 1BLI). The domains are marked by thick (THJA and PWA) or dashed (BLA) horizontal lines with arrows. The positions of amino acid residues corresponding to the active sites are marked by solid (THJA and PWA) or hollow (BLA) triangles. The calcium ion binding residues are marked by solid (THJA and PWA) or hollow (BLA) circles. The zinc ion binding residues of the (Ca, Zn) metal center of THJA and PWA are marked by solid diamonds, and the sodium ion binding residues of BLA are marked by hollow diamonds. Identical and similar amino acid residues are highlighted in black and gray in the sequences, respectively.

Table 1. Ca^{2+} **binding residues of the (Ca, Zn) metal center of PWA/THJA and Binding Site I of BLA**

In (Ca, Zn) metal center of PWA/THJA				In Binding Site I of BLA			
No.	Residue *(Corresponding Residue in THJA)*	Atom	Distance to Ca^{2+} (Å)	No.	Residue	Atom	Distance to Ca^{2+} (Å)
1	Asn110 *(Asn132)*	N	2.6	1	Asn104	O	2.4
2	Asp155 *(Asp177)*	O1	2.6	2	Asp194	O1	2.4
	Asp155 *(Asp177)*	O2	2.3		Asp194	O2	2.4
3	Gly157 *(Gly179)*	O	2.3				
4	Asp164 *(Asp186)*	O1	2.6	3	Asp200	O1	3.1
	Asp164 *(Asp186)*	O2	2.3		Asp200	O2	2.4
5	Gly202 *(Gly224)*	O	2.3	4	His235	O	2.4

pecially the conserved residues involving calcium ion binding, suggests that THJA and PWA may have similar thermostability and calcium ion dependency. BLA is different from PWA in that its thermostability requires exogenous calcium ions (Violet and Meunier, 1989), and indeed, the sequence analysis implied different calcium ion binding characteristics between BLA and THJA or PWA.

Structural comparison with other thermostable α-amylases

The structural model of THJA was constructed *in silico* using the crystal structure of PWA (Protein Data Bank code 1MWO) as the template (Linden *et al.*, 2003). The model showed a typical glycosylhydrolase class-13 fold with 3 domains, A-C (Fig. 2A). Consistent with the high sequence similarity with PWA, the structural model of THJA is almost identical to PWA (Fig. 2B). The (Ca, Zn) metal center that is located at the interface of domains A and B of PWA is also seen in the structural model of THJA (Linden *et al.*, 2003). Just as in PWA, the calcium ion binding site in this metal center involves 5 amino acid residues, or 7 protein ligands (Fig. 2C and Table 1). Conformations of the 5 residues of THJA and PWA are almost the same (Fig. 2C). When the THJA structural model was compared with BLA (Machius *et al.*, 1998), the results showed that the 2 enzymes share the same overall structural organization, but are quite different on the peptide chain positions (Fig. 2D). There are 3 calcium ion binding sites in the structure of BLA, 2 of which form a (Ca, Ca) me-

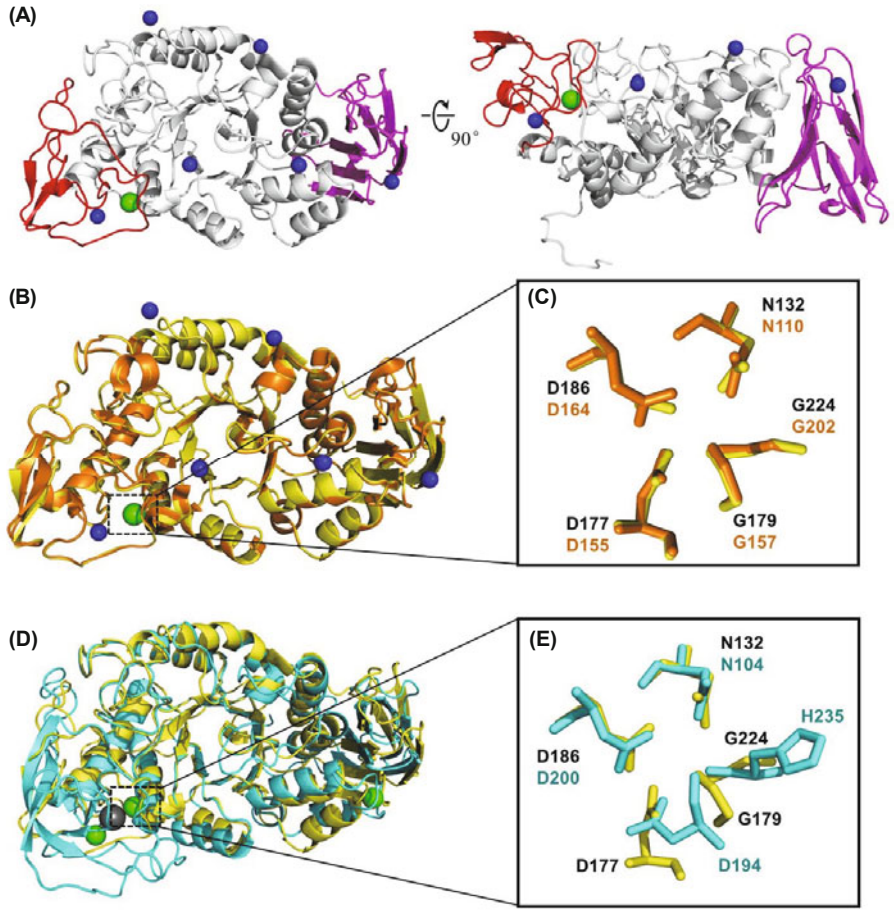


Fig. 2. Structural comparison of THJA, PWA, and BLA. (A) The structural model of THJA presented as ribbon diagrams. Domains A-C appear in white, red, and magenta, respectively. (B) Structural comparison of THJA and PWA (Protein Data Bank ID 1MWO). Superposition of THJA (yellow) with PWA (brown) is shown in ribbons. (C) Enlarged view showing the residues in the (Ca, Zn) metal center that are critical for Ca^{2+} binding. The residue numbers are indicated in black (THJA) and brown (PWA). (D) Structural comparison of THJA and BLA (Protein Data Bank ID 1BLI). Superposition of THJA (yellow) with BLA (cyan) is shown in ribbons. (E) Enlarged view showing the residues in the (Ca, Zn) metal center of THJA and the Binding Site I of BLA that are critical for Ca^{2+} binding. The residue numbers are indicated in black (THJA) and cyan (BLA). Calcium, zinc, and sodium ions appear in green, blue, and gray, respectively. In the enlarged views, the residues are shown as sticks.

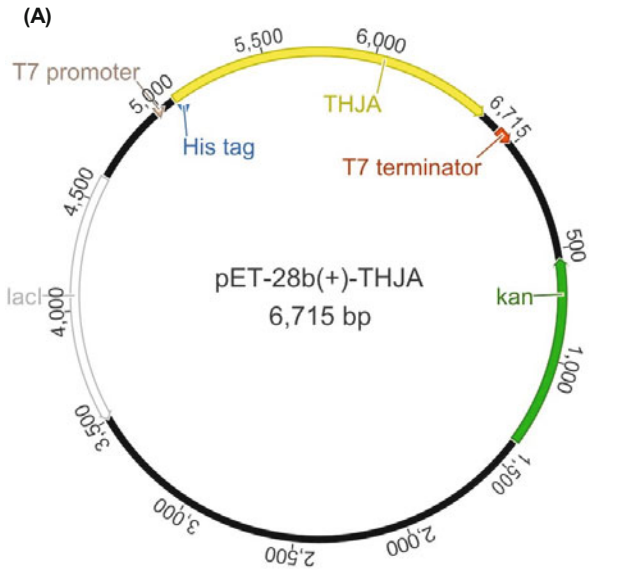


Fig. 3. Recombinant expression of THJA in *E. coli*. (A) Schematic of the THJA expression construct. The circular plasmid DNA is represented by a circle, with numbers indicating the length of the molecule (in bp); the recombinant THJA gene is represented by a yellow ribbon, and the expression direction is indicated as an arrow shape at one end of the ribbon; T7 promoter, T7 terminator, and the hexa-histidine tag (His tag) sequence at the N-terminus of the recombinant THJA gene are indicated; the kanamycin resistance gene (kan) and *lacI* gene (lacI) are represented by green and white ribbons, respectively. The construct expression of recombinant THJA enzyme is inducible with IPTG. (B) Overexpression of THJA. Before and after IPTG induction, cells from 0.1 ml of culture were pelleted by centrifugation and resuspended in SDS loading buffer, electrophoresed on a 10% SDS-PAGE gel, and stained with Coomassie Brilliant Blue. The arrow indicates the band of recombinant THJA enzyme that appeared after induction.

tal center that superimposes well with the (Ca, Zn) metal center of PWA (Machius *et al.*, 1998; Linden *et al.*, 2003). Although Binding Site I of the (Ca, Ca) metal center of BLA is considered to be conserved with the calcium-binding site of the (Ca, Zn) metal center of PWA, it is coordinated by only 4 amino acid residues, or 6 protein ligands, suggesting less binding affinity (Fig. 2E and Table 1). Moreover, only 2 of the 4 residues (N104 and D200) have the same orientations as in THJA, and the other 2 residues are positioned differently (Fig. 2E). The structural comparison indicated that THJA had a (Ca, Zn) metal center that was similar to PWA but significantly different from BLA.

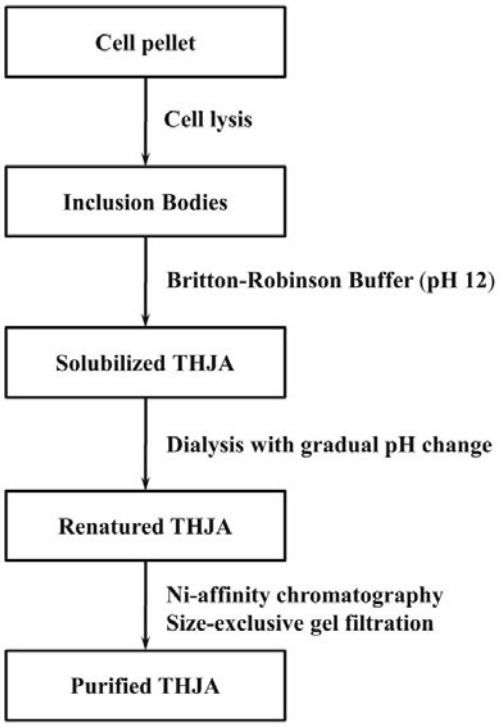
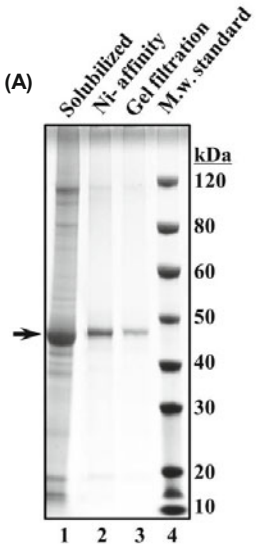


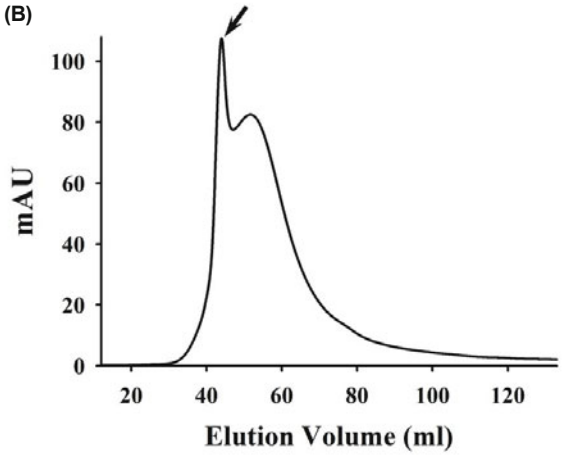
Fig. 4. Flow chart showing the combined renaturation-purification procedure used to obtain the recombinant THJA enzyme.

Cloning, expression, and purification of THJA

The THJA enzyme was subject to recombinant expression in *E. coli* using the phage T7 pET overexpressing system. The coding sequence of THJA was fused into the pET-28b vector in-frame with the upstream hexahistidine tag to facilitate Ni-affinity purification of the enzyme (Fig. 3A). IPTG induction resulted in the overexpression of THJA, as indicated by SDS-PAGE showing a distinct band that appeared after induction near the position of THJA's theoretical molecular weight of 51.4 kDa (Fig. 3B). Under various induction conditions we tried, the majority of the overexpressed THJA was always in the inclusion bodies, therefore, a combined renaturation-purification procedure was performed to obtain the pure enzyme (Fig. 4). After harvesting, the cells were lysed by physical pressing and the enzyme in the inclusion bodies was solubilized in the Britton-Robinson Buffer (pH 12). Renaturation of the enzyme was achieved by extensive dialysis in the Britton-Robinson Buffer with a gradual change of pH from 12 to 9. The solubilized and renatured THJA was purified by Ni-affinity chromatography using the hexahistidine tag, followed by size-exclusive gel filtration.

After the procedure, the enzyme was purified to >80% purity (Fig. 5A). The gel filtration profile showed a relatively broad peak of the enzyme suggesting multiple oligomeric states, which was consistent with the fact that THJA had a hydrophobic surface (Fig. 5B). The broad peak was a reflection of non-specific, loose interactions between the enzyme molecules in this buffer condition. There was a small sharp fluctuation of the UV absorbance at the void volume position (arrow), but SDS-PAGE did not show any difference of the protein eluted at the volume of fluctuation. The whole peak was pooled and concentrated. From 1 L of culture, about 8 mg of the recombinant THJA could be obtained, and the specific activity was determined to be 7.05 U/mg,





(C)

Culture Volume (L)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (U/L culture)
1	56.5	8.01	7.05	56.5

Fig. 5. Purification of recombinant THJA. (A) SDS-PAGE of THJA in different purification stages. Samples of THJA after renaturation, Ni-affinity chromatography, and gel filtration were electrophoresed on a 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue. The arrow indicates the band position of recombinant THJA. (B) Gel filtration elution profile of recombinant THJA. The sharp fluctuation at the void volume is indicated by an arrow. (C) Purification summary of recombinant THJA. Results from a typical purification of 1 L of induced culture are presented.

which was equal to the specific activity of THJA produced in its natural host (Fig. 5C) (Wang *et al.*, 2008). The results indicated that recombinant expression and purification of THJA was successful.

THJA does not require exogenous Ca^{2+} for thermostability

Sequence alignment and structural modeling suggested that THJA should have similar thermostability to PWA. We tested the thermostability of THJA from the recombinant expression, and the enzyme showed good thermostability (Fig. 6).

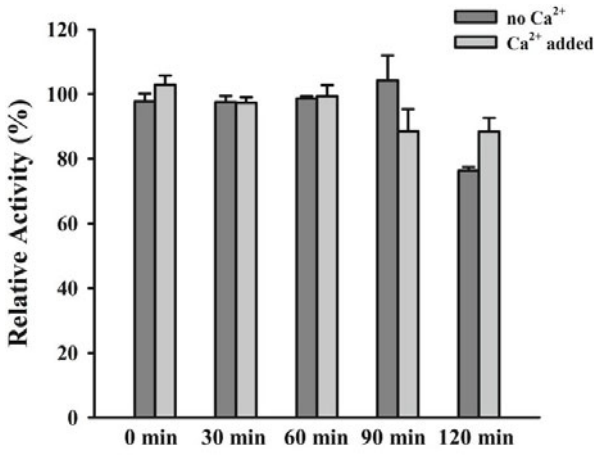


Fig. 6. THJA's thermostability was independent of exogenous Ca^{2+}. In this histogram, the relative catalytic activities of recombinant THJA (3.9 μM) treated with heat (90°C) for different time periods (min) in the absence (no Ca^{2+}) or presence (Ca^{2+} added) of exogenous Ca^{2+} are presented. The untreated THJA's activity was set as 100% of the "Relative Activity." Error bars represent the standard error from 3 independent experiments.

During the period of 2 h of incubation at 90°C, a small portion of enzymatic activity loss (~20%) was observed only at the last checkpoint. Moreover, thermostability was independent of exogenous Ca^{2+}, as the activity remained stable with or without the presence of Ca^{2+} in the reaction buffers (Fig. 6). The exogenous Ca^{2+} independency for thermostability of the recombinant THJA was consistent with that of THJA expressed in its natural host (Wang *et al.*, 2008).

THJA becomes thermolabile when treated with EDTA/EGTA under high temperature

THJA exhibited thermostable property without the presence of exogenous Ca^{2+}. As THJA shares a high sequence and structural similarity with PWA, it could utilize a mechanism similar to PWA to remain thermostable, in which the calcium ion tightly bound to the enzyme provides the stability. To verify this hypothesis, we used EDTA to chelate out metal ions bound to THJA, and tested whether the thermostability was affected. Two sets of experiments were designed. In one set THJA was incubated in a buffer containing 2 mM of EDTA at 70°C or on ice, and the activities after incubation were compared. The results showed that just adding EDTA to the enzyme did not change its activity, but incubation of the enzyme with EDTA under a high temperature (70°C) for 20 min significantly reduced its activity (Fig. 7A). In the other set THJA was heat treated with or without the presence of EDTA for different periods of time. When no EDTA was added, the activity remained above 100%. We even observed increased activity up to 130–160% compared to that at 0 min. This is because THJA is a thermophilic enzyme, and heat treatment had an activating effect on it. With the presence of EDTA, however, THJA's activity decreased over time under the heat treatment (Fig. 7B). EGTA was also used in-

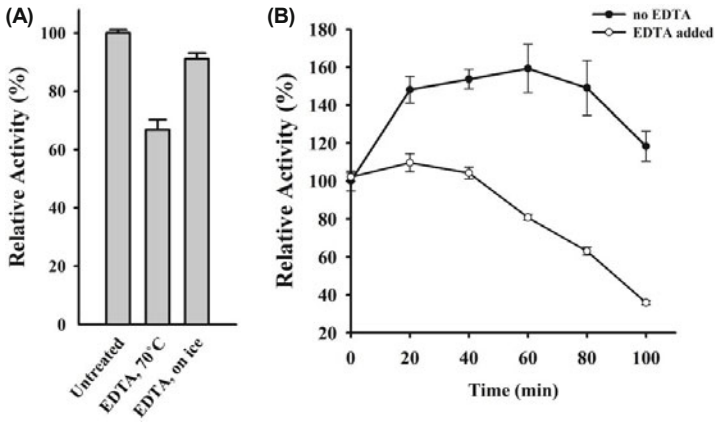


Fig. 7. THJA became thermolabile when treated with EDTA under high temperature. (A) Histogram showing the relative catalytic activities of recombinant THJA (3.9 μM) treated with EDTA either at 70°C for 20 min or on ice. (B) Curves showing the change of relative activity of recombinant THJA (3.9 μM) over time with heat treatment (70°C) in the absence (no EDTA) or presence (EDTA added) of EDTA. Error bars in this figure represent the standard error from 3 independent experiments.

stead of EDTA in the above experiments, and the results were the same (data not shown). These results indicated that THJA could not sustain a high temperature when EDTA or EGTA was present, suggesting that when the bound metal ions were chelated out, the enzyme lost its resistance to heat and became thermolabile.

Ca^{2+} could partially restore THJA's catalytic activity after EDTA/EGTA chelating

THJA's thermostability was lost when the bound metal ions were chelated out by EDTA or EGTA. To test if Ca^{2+} is a key metal ion for thermostability, we added exogenous Ca^{2+} to the enzymatic reaction after EDTA chelating. Under 80°C, EDTA chelating caused about a 50% loss of catalytic activity in 20 min (Fig. 8A). After this treatment, we removed EDTA and added exogenous Ca^{2+} (5 mM) to the reaction mixture for activity testing. We observed that the addition of exogenous Ca^{2+} could restore the activity to about 80% (Fig. 8A). On the other hand, incubation of the enzyme with EDTA at 90°C for 20 min caused a complete loss of activity, and addition of exogenous Ca^{2+} could not restore any activity (Fig. 8B). Also, EGTA was used instead of EDTA in the above experiments, and the same phenomenon was observed (data not shown). The results indicated that when the bound metal ions of THJA were chelated out, heat would cause damage to the enzyme. That the damage could be partially restored by exogenous Ca^{2+} suggested that Ca^{2+} was indeed the key metal ion for thermostability.

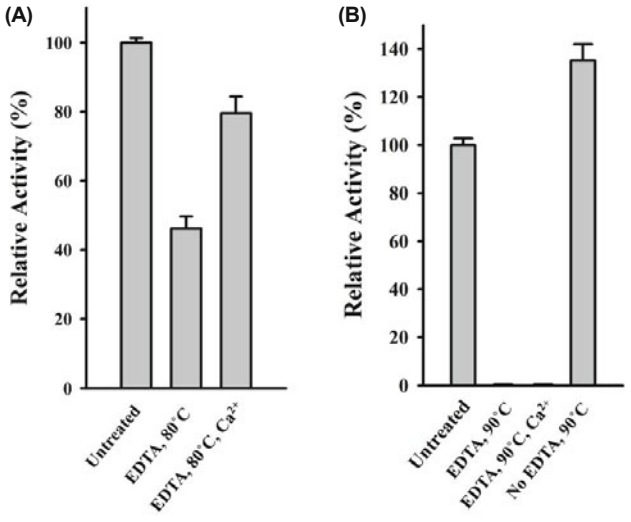


Fig. 8. Partial restoration of THJA activity by Ca^{2+}. Histograms showing the relative catalytic activities of recombinant THJA (3.9 μM) treated with EDTA for 20 min at 80°C (A) or 90°C (B) are presented. The addition of Ca^{2+} after EDTA chelating is indicated by "Ca^{2+}" in the descriptive text under each respective vertical bar. Activity of THJA treated with 90°C heating without EDTA (No EDTA, 90°C) was included in (B) as a control. Error bars in this figure represent the standard error from 3 independent experiments.

Discussion

The α-amylase from the hyperthermophilic archaeon *Thermococcus* sp. HJ21 (THJA) belongs to class-13 of glycosylhydrolases (Davies and Henrissat, 1995; Wang *et al.*, 2008; Choi *et al.*, 2011). Because of the high thermostability, THJA is potentially applicable to the liquefaction of starch, a process that requires high temperatures of up to 100°C. At present, industrial enzymes for this process are α-amylases from *B. licheniformis* and *B. stearothermophilus* (Li *et al.*, 2016). These enzymes display full catalytic activity and stability at high temperatures only if calcium ion is added, but the addition of calcium ion is inhibitory for the subsequent starch-processing reactions. Researchers are looking for alternative α-amylases that are highly thermostable and independent of exogenous calcium ion. THJA meets this standard and could be developed to replace the current starch-processing α-amylases, such as BLA.

Sequence alignments showed very high similarity of THJA with PWA. Not only did the sequence identity reach 84%, but the amino acid residues critical for function were all conserved. There are 13 residues that compose the catalytic site of PWA (Linden *et al.*, 2003), and the exact same residues were found in the THJA sequence (Fig. 1, solid triangles). The (Ca, Zn) metal center of PWA is unique among α-amylases, and so far has been identified only in PWA and its close homolog *Thermococcus hydrothermalis* (Leveque *et al.*, 2000; Linden *et al.*, 2003). This (Ca, Zn) metal center contains a calcium ion binding site and a zinc ion binding site involving 8 residues (Linden *et al.*, 2003), all of which are

conserved in the THJA sequence (Fig. 1, solid circles and diamonds). This suggests that a (Ca, Zn) metal center is also present in THJA. Structural modeling of THJA was conducted using the crystal structure of PWA as the template. Given the high sequence identity, the model of THJA has a high confidence and shows a structure almost identical to PWA. The catalytic site and the (Ca, Zn) metal center are well conserved in the THJA structural model.

The (Ca, Zn) metal center of PWA is critical for thermostability. It has been demonstrated that once the zinc ion binding site was abolished by mutation, PWA's thermostability was lost (Savchenko *et al.*, 2002). Although a calcium ion binding site at the conserved position is common in class-13 α-amylases, the site in the (Ca, Zn) metal center of PWA is unique in that it coordinates 7 proteins ligands, while in other class-13 α-amylases only 6 or less protein ligands were involved (Kamitori *et al.*, 2002; Chai *et al.*, 2016). It is believed that the tight binding of zinc and calcium ions in the (Ca, Zn) metal center stabilizes PWA at high temperatures (Linden *et al.*, 2003). Similarly, the (Ca, Zn) metal center in THJA should ensure high-affinity binding of these metal ions that make THJA thermostable.

The gene of THJA was cloned into *E. coli* and expressed. The recombinant THJA was purified from the catalytically active inclusion bodies, which closely resembled the recombinant expression of PWA (Linden *et al.*, 2000). The purified recombinant THJA exhibited a specific activity of 7.05 U/mg that equals the specific activity of THJA from its natural host and showed thermostability that was independent of exogenous calcium ion, indicating successful recombinant expression (Wang *et al.*, 2008).

During the purification process, THJA had been dialyzed extensively against a series of buffers that contained no Ca^{2+}. Any free calcium ion or calcium ions loosely bound to the enzyme should have been removed. Still, we observed that the enzyme remained thermostable without the addition of a calcium ion. This supports the hypothesis that high-affinity binding to calcium is the reason why THJA is catalytically active at high temperatures without the addition of Ca^{2+}. To test this hypothesis, we used EDTA or EGTA to chelate out any bound metal ions from THJA and tested the thermostability. Our results showed that with the presence of EDTA or EGTA THJA cannot sustain high temperatures. Significant reduction of activity was seen when the enzyme was heated to 70°C, and higher temperatures resulted in a more severe loss of activity (Figs. 7 and 8). EDTA or EGTA, as the metal ion chelator, competitively removed metal ions from THJA and resulted in thermostability loss, indicating that the metal ions bound to the enzyme are important for the thermostability. When the metal ions were removed, the enzyme was no longer resistant to heat.

To confirm whether calcium was the metal ion critical for THJA's thermostability, we added Ca^{2+} back to the enzyme after heat treatment with EDTA or EGTA. The addition of Ca^{2+} partially restored the activity only if the enzyme was not completely inactivated by heat (Fig. 8). The results confirmed that calcium was indeed the critical metal ion for THJA to stay active against heat, and further suggested that heat would irreversibly damage the enzyme when the bound metal ions were removed.

Sequence alignments and structural modeling suggested that THJA contains the (Ca, Zn) metal center that tightly binds both calcium and zinc ions. EDTA or EGTA treatment would remove both ions from THJA, and the absence of zinc ions could be the reason why the addition of Ca^{2+} only partially restored the activity. It is logical to speculate that, calcium and zinc ions cooperatively maintain the stable conformation of the (Ca, Zn) metal center. Removing either of the ions would affect the binding of the other ion, and compromise thermostability of the enzyme. When the (Ca, Zn) metal center of THJA is intact, both the calcium and the zinc ions are tightly bound, making the enzyme thermophilic with no dependency of exogenous calcium ion.

THJA is a relatively new α-amylase found in *Thermococcus* sp. HJ21 that was discovered only in the last decade (Wang *et al.*, 2008; Ying *et al.*, 2012) and so far it is the only α-amylase from *Thermococcus* sp. strains that was found to contain the novel (Ca, Zn) metal center. The important feature, independency of exogenous calcium ions for thermostability, makes THJA a good alternative to BLA to further improve the efficiency of industrial processes in starch liquefaction. Understanding the mechanism of its independency of exogenous calcium ions offers a strong base on which to further engineer properties of THJA for better potential applications in industrial processes.

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Conflict of Interest

The authors declare they have no competing interests.

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