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*Full Length Research Paper*

# Characterization of thermostable 2-deoxy-D-ribose-5-phosphate aldolase with broad temperature adaptability from *Thermococcus onnurineus* NA1

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**The 2-deoxy-D-ribose-5-phosphate aldolase gene from *Thermococcus onnurineus* NA1 was sub cloned, over expressed in *Escherichia coli* and purified to apparent homogeneity. Analysis of the sequence of gene revealed an n of 681 base pairs encoding 227 amino acids predicted to yield a protein having molecular mass 25.3 kDa. The enzyme activity was optimal at 60°C and showed broad temperature adaptability, retaining more than 30% of its maximum activity when assayed at 10 to 75°C. The recombinant protein was heat stable; no activity loss was observed even after incubation at 80°C for 10 min. In addition, the thermophilic enzyme showed a remarkable resistance to acetaldehyde; it retained more than 45% activity after exposure for 20 h to 250 mM acetaldehyde at 25°C.**

**Key words:** 2-Deoxy-D-ribose-5-phosphate aldolase, thermostable enzyme, broad temperature adaptability.

## INTRODUCTION

Aldolases are a specific group of lyases that catalyze the reversible stereo selective addition of a donor compound (nucleophile) onto an acceptor compound (electrophile). These enzymes are categorized based on their requirement for nucleophiles. So-called class I aldolases need no cofactor, as their reaction mechanism of donor activation involves the formation of a Schiff base at a conserved lysine residue in the active site of the enzyme. Class II aldolases are dependent on a metal that acts as a Lewis acid and activates the donor substrate (Samland and Sprenger, 2006). 2-deoxy-D-ribose 5-phosphate aldolases (DERA; EC 4.1.2.4) are class I aldolases active in DNA salvage pathways in many microorganisms; these aldolases catalyze reversible aldol reactions between natural acetaldehydes and D-glyceraldehyde 3-phosphate to form 2-deoxyribose-5-phosphate (DRP) (Machajewski and Wong, 2000). The ability of DERAs to generate chiral centers in the resulting aldol adducts makes them powerful tools for syntheses of rare sugars or sugar-derived compounds (Barbas et al., 1990). An important application of this enzyme is found in the synthesis of epothilone A and statins, which use cheap starting materials such as acetaldehyde (Samland and Sprenger, 2006).

To date, DERAs from microorganisms, including *Bacillus cereus*, *Escherichia coli K12, Klebsiella pneumoniae, Lactobacillus plantarum, Salmonella typhimurium, Streptococcus mutans GS-5, Yersinia sp. EA015, Aeropyrum pernix, Pyrobaculum aerophilum*, *Thermotoga maritima* and *Hyperthermus butylicus,* have been studied (Pricer and Horecker, 1960; Hoffee, 1968; Sgarrella et al., 1992; Gijsen and Wong, 1994; Horinouchi et al., 2003; Han et al., 2004; Sakuraba et al., 2007; Kim et al., 2009; Wang et al., 2010). Thermostable

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**Abbreviations: DERA,** 2-Deoxy-D-ribose-5-phosphate aldolase, **DRP,** 2-deoxy-D-ribose-5-phosphate; **ORF,** open reading frame; **TPI,** triose-phosphate isomerase; **GDP,** glycerol-3-phosphate dehydrogenase; **PEG,** polyethylene glycol; **DMSO,** dimethyl sulfoxide.

enzymes, isolated from thermophilic microorganisms, always exhibit higher stability to organic solvents, acidic and alkaline pH and detergents. These characteristics are of great advantage for industrial applications (Vieille et al., 1996). One serious drawback in the application of thermophilic enzymes is the low catalytic activity at moderate or lower temperatures. They are generally barely active at room temperature at which the mesophilic or psychrophilic counterparts show high catalytic activity (Shoichet et al., 1995). The hyperthermophilic *T. onnurineus* NA1 was isolated in 2006 from a deep-sea hydrothermal vent region in the PACMANUS field (3°14'S, 151°42'E) of the East Manus Basin (Bae et al., 2006). The whole genome was sequenced, annotated and analyzed in 2006 (Lee et al., 2008). In this report, we cloned, expressed, purified and biochemically characterized the DERA from *T. onnurineus* NA1. Purified recombinant DERA showed broad temperature adaptability, heat stability and tolerance to high concentration of acetaldehyde. We anticipate that *T. onnurineus* NA1 aldolase will be an attractive enzyme for use as a biocatalyst for industrial applications.

## MATERIALS AND METHODS

### Materials

The pET-303/CT-His vector was obtained from Invitrogen (Paisley, UK). The *E. coli* strain BL21-CodonPlus (DE3)-RIL was purchased from Stratagene (La Jolla, CA). DRP, triose-phosphate isomerase (TPI), and glycerol-3-phosphate dehydrogenase (GDP) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of reagent grade.

### Gene cloning and Multiple sequence alignment

The nucleotide sequence encoding DERA from the thermophilic microorganism *T. onnurineus* NA1 was obtained from the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/Access/). The *T. onnurineus* NA1 DERA is abbreviated as $DERA_{NA1.}$ The DNA encoding full-length $DERA_{NA1}$ was synthesized by Sangon (Shanghai, China). An *Xba* I restriction site was introduced at the 5' end prior the initiation codon and an *Xho* I restriction site was introduced at the 3' end overlapping the termination codon. The sequences were digested with restriction enzymes (*Xba* I and *Xho* I) and ligated into the expression vector pET303/CT-His linearized with *Xba* I and *Xho* I to give an expression vector carrying the desired $DERA_{NA1}$ coding region. In order to identify the conserved residues in $DERA_{NA1}$, the amino acid sequences of DERAs from representative thermophilic organisms *A. pernix*, *P. aerophilum* and *T. maritima* were aligned by using ClustalW (http://ebi.ac.uk/clustalw/). The amino acid sequences were obtained from UniProtKB/TrEMBL Database. The accession numbers of the sequences were as follows: *A. pernix*, Q9Y948; *P. aerophilum*, Q8ZXK7; and *T. maritima*, Q9X1P5.

### Protein expression, purification and molecular mass determination

The expression vector carrying desired DERA sequence was introduced into the *E. coli* strain BL21-CodonPlus (DE3)-RIL. Cells were grown in 1 mL Luria-Bertani medium at 37°C for 1 h. The cells (10 to 200 µL) were placed on LB plates containing 100 mg/L of ampicillin and incubated at 37°C overnight to obtain transformants. The transformants were cultivated in LB medium containing 100 mg/L of ampicillin at 37°C until the optical density at 600 nm reached 0.6. Expression was induced by adding IPTG at the final concentration of 0.5 mM and cultivation was continued for an additional 5 h at 37°C. Cells were harvested by centrifugation at 15,000 x *g* for 5 min and suspended in 100 mM sodium phosphate (pH 7.5) 200 mM sodium chloride and freeze-thawed three times. Supernatant was incubated with Ni-NTA agarose (Qiagen, Hilden and Germany) for 1 h at 4°C and the mixture loaded onto a chromatography column. The column was washed with buffer containing 100 mM sodium phosphate (pH 7.5) 200 mM sodium chloride, and 10 mM imidazole. The C-terminal His-tagged DERA was eluted from the column with the same buffer containing 500 mM imidazole. The protein was dialyzed overnight against 20 mM sodium phosphate (pH 7.5) and concentrated with polyethylene glycol (PEG) 20000 and then freeze-dried. Enzyme powders were stored at -20°C. The purified enzymes were assayed by SDS-PAGE followed by Coomassie brilliant blue G-250 staining and the protein concentration was determined by the Bradford method. The molecular mass of the purified enzyme was determined by analytical gel filtration on a Superdex 200 column (2.6×62 cm; Amersham Biosciences) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl. The molecular mass of DERA was compared with the retention time of four standard proteins including lysozyme (14 kDa), chymotrypsin (25 kDa), maltose binding protein (43 kDa) and alcohol dehydrogenase (100 kDa).

### Enzyme activity assay

The DERA cleavage activity was measured by following the oxidation of NADH in a coupled assay converting glyceraldehyde-3-phosphate, one of DRP cleavage products, to glycerol 3-phosphate by TPI and GDP. The DRP cleavage reaction was carried out at 50°C for 5 min, then the reduction action was carried out at 25°C for 30 min. The reaction mixture contained 50 mM triethanolamine-HCl buffer (pH 8.0), 0.1 mM NADH, 0.4 mM DRP, 11 U triose-phosphate isomerase, 4 U glycerol-3-phosphate dehydrogenase, and various concentrations of $DERA_{NA1}$. The assay was initiated by addition of DERA, and the decrease in NADH level was monitored at 340 nm using an UV-Visible spectrophotometer (Shimadzu Co., Kyoto, Japan). The unit (U) activity was defined as the amount of DERA required to catalyze the cleavage of 1 µmol of DRP per minute (Kim et al., 2009). The kinetic properties of DERA were examined in 50 mM triethanolamine-HCl buffer (pH 8.0) at 25°C. Five concentrations of DRP, ranging from 0.04 to 0.4 mM, were used to determine reaction rates. The apparent Michaelis-Menten constants for DRP cleavage reactions were determined from the double reciprocal Line weaver-Burk plots of the reaction rate.

### Effects of pH on enzyme activity and stability

The buffers used to determine effects of pH were sodium acetate (0.1 M, pH 2.0 to pH 6.0), imidazole-HCl (0.1 M, pH 6.0 to pH 7.5), triethanolamine-HCl (0.1 M, pH 7.5 to pH 8.5), glycine-NaOH (0.1 M, pH 8.5 to pH 11.0) and $Na_2HPO_3$-NaOH (0.1 M, pH 11.0 to pH 12.0). The optimum pH for DERA was determined by analysis of DRP cleavage at pHs from 5.0 to 12.0. The effect of pH on enzyme stability was determined by comparing the relative activity of the enzyme (at 0.5 mg/mL) incubated at pHs ranging 2.0 to 12.0 at 25°C for 30 min.

**Table 1.** Summary of the purification process of *T. onnurineus* NA1 DERA from 1 litre of *E.coli* BL21-CodonPlus (DE3)-RIL harboring pET−DERA.

| Step | Protein (mg) | Total activity (U) | Specific activity (U mg$^{-1}$) | Yield (%) |
|---|---|---|---|---|
| Crude cell extract | 320 | 576 | 1.8 | 100% |
| NI- NTA affinity chromatography | 45 | 207 | 4.6 | 36% |

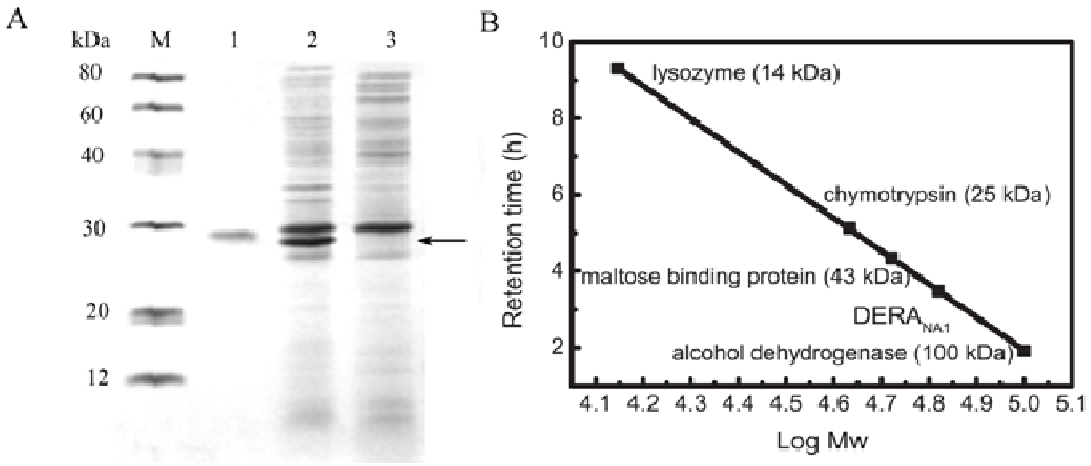


**Figure 1.** SDS-PAGE analysis of recombinant DERA from *T. onnurineus* NA1. (A) M, molecular weight marker; Lane 1, purified DERA$_{NA1}$ after NI-NTA affinity chromatography; Lane 2, induced crude extract; Lane 3, uninduced crude extract. (B) Gel filtration analysis of recombinant DERA: molecular mass of DERA was compared with the values of retention time of lysozyme (14 kDa), chymotrypsin (25 kDa), maltose binding protein (43 kDa) and alcohol dehydrogenase (100 kDa).

**Effects of temperature on enzyme activity and stability**

The optimum temperature for DERA was measured using the DRP cleavage assay with a slight modification. After the reaction mixture containing 50 mM triethanolamine-HCl buffer (pH 8.0), 0.4 mM DRP, and a known concentration of DERA was incubated at the range of 10℃ to 100℃ for 1 min, DERA was removed. The reduction reaction was then carried out at 25℃ for 30 min in the presence of TPI and GDP and the decrease in NADH was monitored. To determine the effect of temperature on enzyme stability, DERA (0.5 mg/mL) was incubated for 10 min at different temperatures, after which the residual activities were assayed.

**Effects of organic solvents and acetaldehyde on enzyme stability**

The effect of organic solvents on DERA activity was also analyzed using the standard assay method in the presence of varying concentrations of acetonitrile, dimethyl sulfoxide (DMSO), 1,4-dioxane, tetrahydrofuran, methanol, and isopropanol at 25□C. The investigation of organic solvent tolerance was executed with the standard assay method, except that varying concentrations of organic solvent was added to the standard assay buffer. The relative activity was compared with the DERA activity in the absence of the organic solvent. To examine the effect of acetaldehyde on enzyme stability, 0.5 mg/ml DERA in 50 mM triethanolamine-HCl buffer (pH 8.0) containing 50 mM, 150 mM and 250 mM acetaldehyde were incubated at 25℃ for various intervals. The acetaldehyde was removed from the enzyme solution using a centrifugal filter device (Microcon YM-10; Millipore). The resulting DERA was diluted to 0.2 mg/mL with 50 mM triethanolamine-HCl buffer (pH 8.0) and the residual activity was analyzed.

## RESULTS AND DISCUSSION

### Cloning, expression and purification

The expression vector carrying the DERA$_{NA1}$ gene was constructed by ligating the PCR-amplified gene into the plasmid pET-303/CT-His; the resulting plasmid, pET-DERA, was transformed into *E. coli* BL21-CodonPlus (DE3)-RIL cells to yield a high-level expression. The His-tagged recombinant protein was purified to homogeneity using a Ni-NTA column. The details of the purification results were shown in Table 1. The molecular weight of DERA measured by SDS-PAGE analysis was approximately 25 kDa (Figure 1a), similar to the molecular weight predicted from the amino acid sequence. The native molecular mass of the enzyme determined by gel filtration is ~ 50 kDa (Figure 1b). This indicates that the enzyme consisted of two subunits with identical molecular mass. *E. coli* DERA also has a dimer structure (Protein Data Bank code 1JCL) composed of two identical subunits, which is most common for DERA

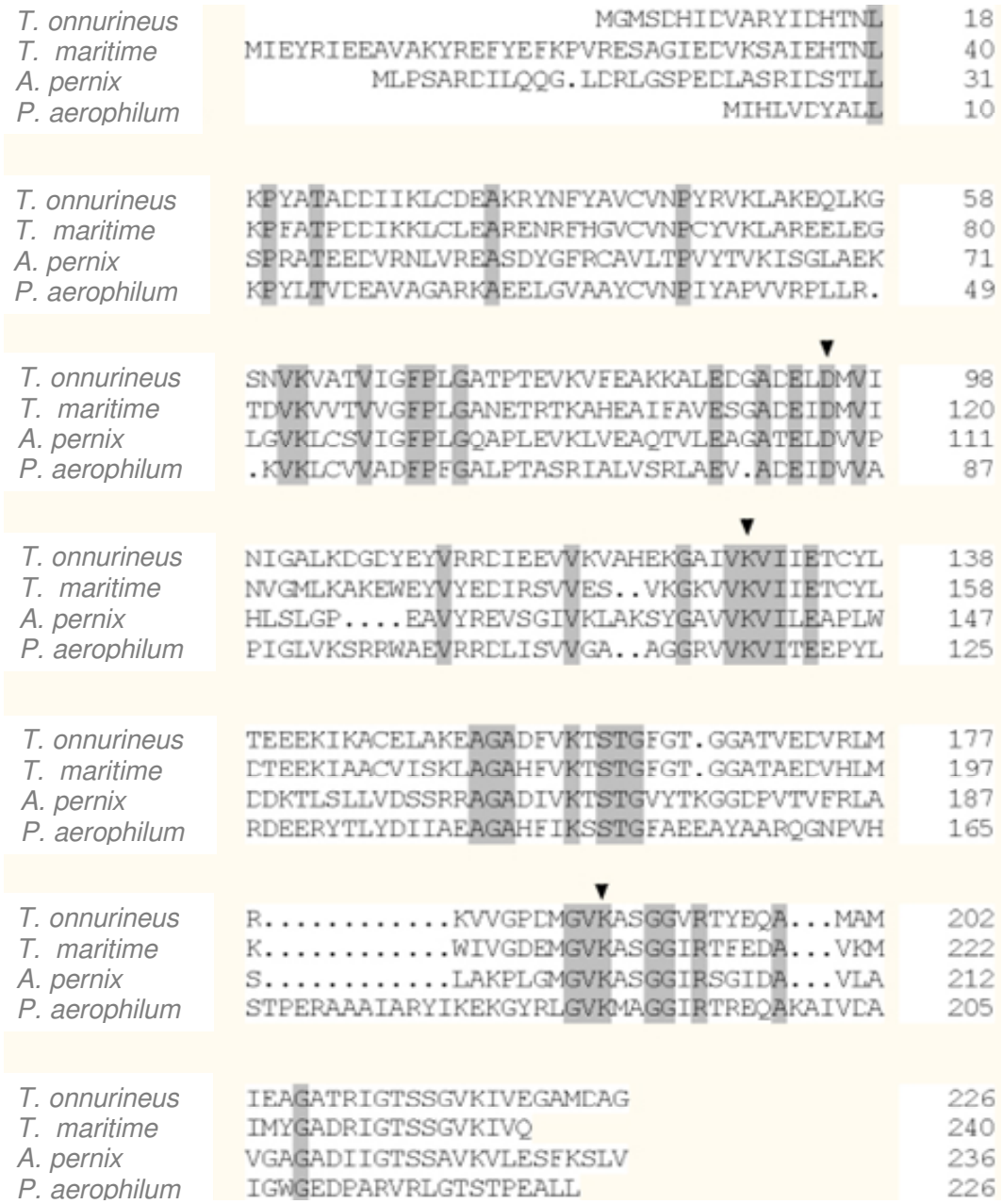


**Figure 2.** Amino acid sequence alignment of DERAs from *T. onnurineus* NA1 (B6YUB3), *A. pernix* (Q9Y948), *P. aerophilum* (Q8ZXK7), and *T. maritima* (Q9X1P5). Gaps, indicated by dashes, were introduced into the sequences to maximize homology. Identical residues are shaded in gray. Residues important for catalysis as discussed in the text are shown in triangles.

(Sakuraba et al., 2003).

## Multiple sequence alignment

The $DERA_{NA1}$ protein (A2BLE9) was aligned with representative DERA proteins of thermophilic microorganisms, including *A. pernix* (Q9Y948), *P. aerophilum* (Q8ZXK7) and *T. maritime* (Q9X1P5). A comparison of amino acid sequence of $DERA_{NA1}$ with other DERAs demonstrated that $DERA_{NA1}$ is 60, 38 and 32% identical to the sequences of *T. maritima*, *A. pernix* and *P. aerophilum*, respectively. The catalytic residue Lys130 of $DERA_{NA1}$, essential in forming the Schiff-base with the aldehydic substrate, was conserved in all DERAs. The active site residues Asp95 and Lys197, known to be important in proton relays, were also conserved in all DERAs (Figure 2) (Sakuraba et al., 2003).

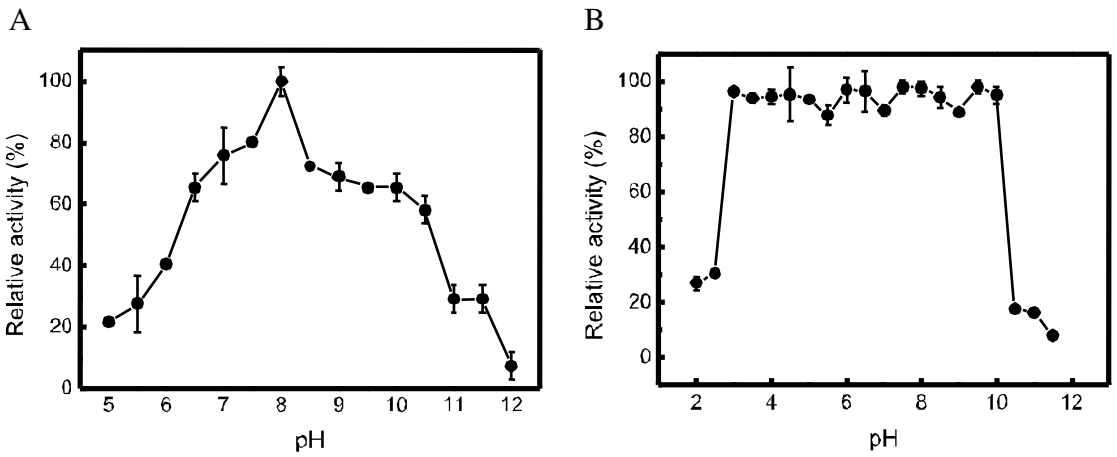


**Figure 3.** Optimum pH and pH stability of *T. onnurineus* NA1 DERA. (A) The optimum pH was tested at a variety of pH levels by measuring cleavage of the DRP at 25℃. (B) The enzyme was incubated for 30 min at 50℃ in buffers of various pH levels, after which the remaining activity was assayed. Data reported are the mean of at least two experiments.

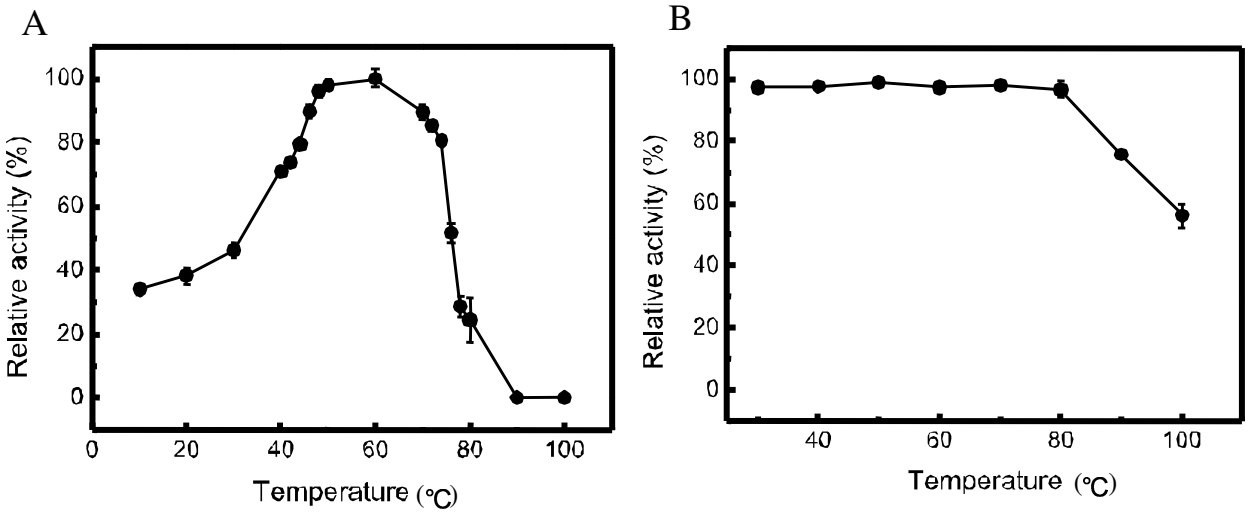


**Figure 4.** Effects of temperature on activity and stability of *T. onnurineus* NA1 DERA. (A) The effect of temperature was determined using 50 mM triethanolamine-HCl buffer (pH 8.0). (B) Thermostability was determined after incubation for 10 min at the indicated temperatures in 50 mM triethanolamine-HCl buffer (pH 8.0). Data reported are the mean of at least two experiments.

## Optimum pH and pH stability

The properties of the purified recombinant $DERA_{NA1}$ were investigated using DRP as a substrate. Under the assay conditions used in our study, the enzyme exhibited its highest activity at pH 8.0 with more than 65% activity retained between pH 6.5 and 10.0 (Figure 3a); this pH optimum is more alkaline pH optimum than that exhibited by other thermostable DERAs. DERAs from *P. aerophilum* and *T. maritima* exhibited highest activity at pH 6.0 and 7.0, respectively. The activity of $DERA_{NA1}$ decreased sharply below pH 6.5 and above pH 10.0. The stability of the enzyme after incubation at various pH values is shown in Figure 4a. The enzyme was extremely stable over a wide range of pH levels: After heating at pH levels ranging from 3.0 to 10.0 for 30 min at 50℃, the enzyme showed no loss of activity.

## Optimum temperature and thermal stability

The effect of temperature on enzyme activity at pH 8.0 is

**Table 2.** Activity of $DERA_{NA1}$ in organic solvent.

| Organic solvent | Relative activity (%)[a] | |
|---|---|---|
| | 5% solvent | 20% solvent |
| Acetonitrile | 67 | 0 |
| Dimethyl sulfoxide | 108 | 65 |
| 1,4-Dioxane | 89 | 72 |
| Methanol | 117 | 71 |
| Isopropanol | 111 | 21 |
| Tetrahydrofuran | 107 | 0 |

[a] The activity of purified $DERA_{NA1}$ was measured in the standard assay buffer in the indicated concentration of organic solvent; activity is relative to $DERA_{NA1}$ without organic solvent.

shown in Figure 3b. The enzyme exhibited its maximum activity at 60℃ and almost similar activity at temperatures between 50 to 70℃. The activity sharply decreased above 70℃. The thermal stability of DERA was determined after incubation at various temperatures. After heating the protein at 80℃ for 10 min, no loss of activity was observed for $DERA_{NA1}$ (Figure 4b). After incubation at 100℃ for 10 min, the enzyme retained 55% its activity. These results showed that $DERA_{NA1}$ is a remarkable heat-stable enzyme. $DERA_{NA1}$ showed broad tempturature adaptation, retaining more than 30% activity between 10 and 70℃. This adaptation of $DERA_{NA1}$ resulted from its high activity at low temperature. As a thermostable enzyme from thermophilic organism, $DERA_{NA1}$ showed striking low temperature adaptation, exhibiting more than 30% of maximum activity at 10℃. Thermostable *H. butylicus* DERA showed its maximum activity at 60℃, while totally lost its activity below 20℃ (Wang et al., 2010). Mesophilic DERA from *Galactomyces geotrichum* showed a similar activity profile at low temperature (Kim et al., 2009). However, this DERA retained only about 10% of their maximum activity when assayed above 60℃, at which $DERA_{NA1}$ showed maximal activity. In addition, compared to that mesophilic DERA, $DERA_{NA1}$ showed considerably higher thermostability with more than 55% activity retained after incubation at 100℃ for 10 min. While DERA from *G. geotrichum* lost most of its activity after incubation above 50℃ (Kim et al., 2009). These combined properties of stability at high temperatures and high catalytic activity at low temperatures made $DERA_{NA1}$ an attractive ezyme for industrial applications.

## Basic kinetic constants for DERANA1

Initial rate kinetics for the aldol cleavage reaction catalyzed by $DERA_{NA1}$ was determined at various concentrations of DRP by fitting the data to the Michaelis-Menten equation. The apparent $K_m$ value of the enzyme was 0.22±0.01 mM, which is similar to that of *P. aerophilum* DERA and higher than that of *T. maritima* (Sakuraba et al., 2007; Sakuraba et al., 2003). Compared to $K_m$ value of 9.1 mM for mesophilic DERA from *Yersinia sp.* (Kim et al., 2009), the reported thermophilic DERAs have significantly higher substrate affinity with $K_m$ values of no more than 0.25 mM.

## Effects of organic solvents and acetaldehyde on enzyme stability

The effect of organic solvents on $DERA_{NA1}$ activity was determined. $DERA_{NA1}$ showed moderate stability in the presence of organic solvents (Table 2). DMSO, Methanol and Isopropanol of 5% (v/v) slightly activated the enzyme activity. The enzyme retained over 60% activities in 20% Methanol, 1, 4-Dioxane and DMSO, but lost activity completely in 20% Acetonitrile and Tetrahydrofuran. Compared to DERA from *A. pernix*, $DERA_{NA1}$ was less stable in the presence of organic solvents. Since no data are available for stability of DERAs from other organisms in organic solvents, a further comparison of the data is not possible. $DERA_{NA1}$ retained more than 90, 60 and 45% DRP cleavage activity after exposure for 20 h to 50, 150 and 250 mM acetaldehyde, respectively (Figure 5).

A significant slow decrease of activity was observed in the middle and late period of incubation, as most of acetaldehyde was converted to lactol product catalyzed by $DERA_{NA1}$. These results indicated that the enzyme was good biocatalysts for aldol condensation reactions with high concentration of acetaldehyde. This is consistent with previous findings. Thermophilic DERAs from *P. aerophilum* and *T. maritime* also show a significant resistance to high concentration acetaldehyde, retaining high activity after exposure for 20 h. In contrast, the mesophilic counterpart DERA from *E. coli* is almost completely inactivated after exposure to acetaldehyde for 2 h under the same conditions (Sakuraba et al., 2007). Our findings, together with previous reports, support the hypothesis that thermostable DERAs are promising candidates for industrial applications (Burton et al., 2002; Cowan, 1997; Egorova and Antranikian, 2005), as the key issue faced in statin synthesis is poor tolerance of

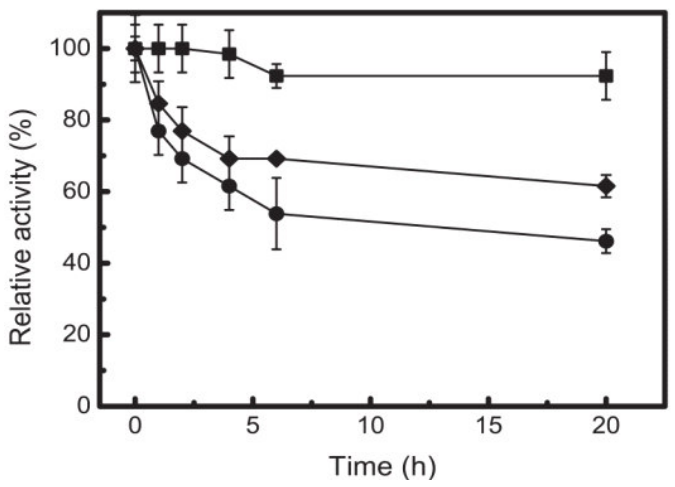


**Figure 5.** Effect of acetaldehyde on enzyme stability. The enzyme was incubated at 25℃ in the presence of 50 mM (■), 150 mM (◆) and 250 mM (●) acetaldehyde and the DRP cleavage activity was assayed at appropriate intervals. Data reported are the mean of at least two experiments.

DERA to high concentrations of acetaldehyde substrate.

In conclusion, we have reported the cloning, expression and characterization of DERA from *T. onnurineus* NA1. $DERA_{NA1}$ showed broad temperature adaptability, remarkable thermostability and high stability to acidic and alkaline pH and high concentrations of acetaldehyde. These favorable properties should make $DERA_{NA1}$ a good candidate in various industrial applications.

## ACKNOWLEDGEMENT

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