Full Length Research Paper

Cloning and characterization of a thermostable 2-deoxy-D-ribose-5-phosphate aldolase from *Aciduliprofundum boonei*

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Analysis of the presumptive 2-deoxy-D-ribose 5-phosphate aldolase gene from *Aciduliprofundum boonei* revealed an open reading frame (ORF) encoding 222 amino acids, which was subcloned and then expressed in *Escherichia coli.* The recombinant DERA protein was purified to apparent homogeneity. The enzyme activity was optimal at pH 7.0 and 80°C. For 2-deoxyribose-5-phosphate, the apparent K_m was calculated to be 0.12 ± 0.01 mM. No loss of activity was observed after incubation at 80°C for 10 min. The enzyme was extremely stable over a wide range of pH levels. Moreover, the thermophilic enzyme also showed tolerance to acetaldehyde, which retained more than 70% activity after exposure for 4 h to 250 mM acetaldehyde at 25°C.

Key words: 2-deoxy-D-ribose 5-phosphate aldolase (DERA), thermophiles, aldo condensation.

INTRODUCTION

Aldolases are used for stereospecific carbon-carbon bond formation, one of the most important transformations in industrial organic syntheses (Kim et al., 2009). According to the chemical mechanism, aldolases are divided into two classes: Class I aldolases are cofactor-independent and catalyzes carbon-carbon bond cleavage via the formation of a Schiff base intermediate with the sugar; Class II aldolases are dependent on a divalent metal ion that acts as a Lewis acid and activates the donor substrate (Morse and Horecker, 1968; Rutter, 1964; Siebers et al., 2001; Sauve and Sygusch, 2001). Deoxyribose 5-phosphate aldolase (DERA, EC 4.1.2.4), one of the class I aldolases, constitutes the only member of the acetaldehyde-dependent aldolase family and is a powerful tool to generate chiral centers in the acetaldehyde adducts, which is the unique side chains of statins (Jennewein et al., 2006).

Although, DERAs from many microorganisms such as *Bacillus cereus, E. coli K12, Klebsiella pneumoniae, Lactobacillus plantarum, Salmonella typhimurium, Streptococcus mutans GS-5, Yersinia sp. EA015, Aeropyrum pernix, Pyrobaculum aerophilum* and *Thermotoga maritime*, have been studied (Gijsen and Wong, 1994; Sgarrella et al., 1992; Horinouchi et al., 2003; Pricer et al., 1960; Hoffee, 1968; Han et al., 2004; Kim et al., 2009; Sakuraba et al., 2003, 2007), it is generally limited by the poor resistance to a high concentration of aldehydes in industrial applications. Recently, enzymes isolated from microorganisms in extreme conditions have showed unique features. For example, they are extremely thermostable and usually resistant to chemical denaturants such as detergents, chaotropic agents and organic solvents (Burton et al., 2002; Cowan, 1997; Egorova and Antranikian, 2005; Antranikian et al., 2005; Hao and Berry, 2004). Therefore, it's a practical strategy to obtain new DERAs from thermophilic microorganisms for industrial applications.

The hyperthermophilic *A. boonei* was isolated in 2006 from deep-sea vent of the Pacific coast of South America (Reysenbach et al., 2006). The whole genome was sequenced, annotated and analyzed in 2008 (Reysenbach and Flores, 2008). In this report, we cloned, expressed and characterized the DERA from thermophilic *A. boonei*. Purified recombinant DERA showed thermo-

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Abbreviations: DERA, 2-Deoxy-D-ribose 5-phosphate aldolase; **DRP,** 2-deoxy-D-ribose- 5-phosphate; **ORF,** open reading frame.

stability and resistance to a high concentration of acetaldehydes which could be further used as the biocatalyst for industrial applications.

MATERIALS AND METHODS

The pET-303/CT-His vector was obtained from Novagen (Madison, WI, USA). The *E. coli* strain BL21-CodonPlus (DE3)-RIL was purchased from Stratagene (La Jolla, CA). 2-deoxy-D-ribose-5-phosphate (DRP), triose-phosphate isomerase (TPI) and glycerol-3-phosphate dehydrogenase (GPD) 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 *A. boonei* was obtained from EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/Access/). The DNA encoding full length DERA was synthesized by Sangon (China) and abbreviated as $DERA_{Abo}$. The restriction sites of *Xba* I and *Xho* I were introduced at the 5' end and 3' end, respectively.

The conserved residues in $DERA_{Abo}$ was identified by aligning the amino acid sequences of DERAs from representative thermophilic organisms *A. pernix*, *P. aerophilum* and *T. maritime* with CLUSTALX. The accession numbers of the sequences were as follows: *A. boonei,* B5IEU6; *A. pernix*, Q9Y948; *P. aerophilum*, Q8ZXK7 and *T. maritima*, Q9X1P5, which were obtained from UniProtKB /TrEMBL Database.

Protein expression, purification and molecular mass determination

The expression vector carrying desired DERA sequence was transformed into the *E. coli* strain BL21-CodonPlus (DE3)-RIL. The transformant in culture medium containing 100mg/L ampicillin was grown to OD_{600} = 0.8. Then IPTG was added to the final concentration of 1.0 mM. After induction at 37°C for 8.5 h in the conical flask, the bacteria were collected and lysed. The supernatant was incubated with Ni-NTA agarose (Qiagen, Hilden, Germany) for 1 h at 4°C, and the mixture was 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 His-tag 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, then freeze-dried. Enzyme powders were stored at -20°C. The results were analyzed by 12% SDS-PAGE and the protein concentration was determined by the Bradford method with bovine serum albumin as the standard.

The molecular mass of the purified enzyme was determined by analytical gel filtration on a Superdex 200 column (2.6 × 62 cm; Amersham Biosciences) and pre-equilibrated with 50mM 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 bovine serum albumin (68 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 and then DERA was removed using a centrifugal filter device (Microcon YM-10; Millipore). Reduction was carried out at 25°C for 30 min. The reaction mixture in a total volume of 400 µl, contained 100 mM sodium acetate buffer (pH 5.5), 0.1 mM NADH, 0.4 mM DRP, 11 U triose-phosphate isomerase, 4 U glycerol-3-phosphate, and various concentrations of $DERA_{Abo}$. The change in absorbance of NADH was monitored at 340 nm ($\varepsilon = 6.22$ mM^{-1} cm^{-1}).

The unit (U) activity was defined as the amount of DERA required to catalyze the cleavage of 1 µmol of DRP per minute. Kinetic properties of DERA were examined in 100 mM sodium acetate buffer (pH 5.5). Five concentrations of DRP, ranging from 0.04 to 0.4 mM were used to determine reaction rates. The apparent Michaelis-Menten constant for DRP cleavage reactions was determined from the double reciprocal Lineweaver-Burk plots of the reaction rate.

Effects of pH on enzyme activity and stability

The buffers used to determine the effects of pH were sodium acetate (0.1 M, pH 3.0 to 6.0), imidazole-HCl (0.1 M, pH 6.0 to 7.5), triethanolamine-HCl (0.1 M, pH 7.5 to 8.5), glycine-NaOH (0.1 M, pH 8.5 to 11.0) and Na_2HPO_3-NaOH (0.1 M, pH 11.0 to 13.0). The optimum pH for DERA was determined by analysis of DRP cleavage in pH from 4.0 to 10.0. The effect of pH on enzyme stability was determined by comparing the relative activity of the enzyme (at 0.5 mg/ml) incubated in pH ranging from 2.0 to 13.0 at 50°C for 30 min.

Effects of temperature on enzyme activity and stability

The optimum temperature of DERA was measured using the DRP cleavage assay with a slight modification. After the reaction mixture containing 100 mM sodium acetate buffer (pH 5.5), 0.4 mM DRP, and a known concentration of DERA was incubated in the range of 30 to 100°C for 1 min, DERA was removed. The reduction reaction was then carried out at 25°C for 30 min in the presence of TPI and GPD and the decrease of 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, and the residual activities were assayed.

Effects of acetaldehyde on enzyme stability

To examine the effect of acetaldehyde on enzyme stability, 0.5 mg/ml DERA in 10 mM Tris-HCl (pH 7.0) containing 50, 150 and 250 mM acetaldehyde, respectively was incubated at 25°C 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 100 mM sodium acetate (pH 5.5) and the residual activity was analyzed.

RESULTS AND DISCUSSION

Cloning, expression and purification

To over-express the PCR-amplified gene, the vector pET-303/CT-His was selected for the correct orientation of the oligonucleotide sequence and desired reading frame. The constructed plasmid $pET-DERA_{Abo}$ was confirmed by
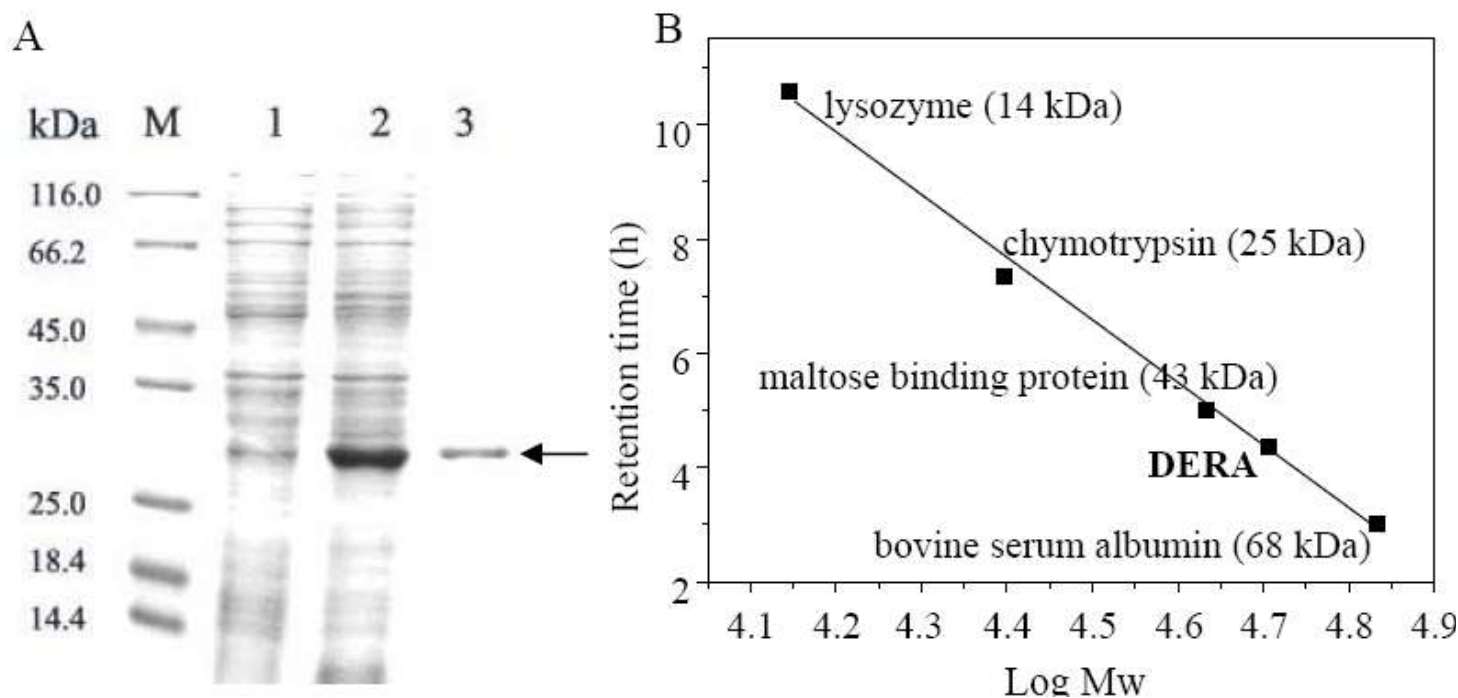
Figure 1. SDS-PAGE analysis of recombinant DERA from *A. boonei*. (A) M, molecular weight maker; Lane 1, uninduced crude extract; lane 2, induced crude extract; lane 3, purified DERA$_{Abo}$ after Ni-NTA affinity chromatography. (B) Gel filtration analysis of recombinant DERA: molecular mass of DERA was compared with the values of retention time of lysozyme (14 kDa), chymotrypsin (25 kDa), maltose binding protein (43 kDa) and bovine serum albumin (68 kDa).

DNA sequencing. Finally, the plasmid was used for the transformation of *E. coli* BL21-CodonPlus (DE3)-RIL.

The recombinant protein with His-tag was purified to homogeneity using a Ni-NTA column. The molecular weight of the protein calculated from the deduced amino acid sequence was 26.6 kDa. The apparent size of the protein was in good agreement with the calculated molecular weight (Figure 1A). The native molecular mass of the enzyme determined by gel filtration is about 53 kDa (Figure 1B), which indicated that the enzyme consists of two subunits with identical molecular mass. The DERA from *E. coli* also has a dimer structure (Protein Data Bank code 1JCL) composed of two identical subunits, which is most common for DERA (Sakuraba et al., 2003).

Multiple sequence alignment

The DERA$_{Abo}$ protein was aligned with representative DERA proteins of thermophilic microorganisms, including *A. pernix* (Q9Y948), *P. aerophilum* (Q8ZXK7) and *T. maritima* (Q9X1P5), whose catalytic sites have been confirmed. It was found that the residue Lys127 of DERA$_{Abo}$ was highly conserved in the DERAs, which was essential in forming the Schiff-base with the aldehydic substrate. Furthermore, the residues Asp92 and Lys185 were also highly conserved in DERAs (Figure 2), which were known to be important in proton relays (Sakuraba et

al., 2007).

Optimum pH and pH stability

Optimum pH and pH stability of the purified recombinant DERA$_{Abo}$ were studied using DRP as a substrate. With the standard assay method, the highest activity was obtained at pH 7.0 and more than 75% activity remained between pH 6.5 and 7.5 (Figure 3A), which is similar with DERAs from *P. aerophilum* and *T. maritima*. The stability of the enzyme after incubation at various pH values is shown in Figure 3B. After heating at pH levels ranging from 4.0 to 11.0 for 30 min at 50°C, only a few loss of activity was found. DERA$_{Abo}$ was extremely stable over a wide range of pH levels, which suggested that it will be stable in industrial processes under different pH conditions.

Optimum temperature and thermal stability

The effect of temperature on enzyme activity at pH 7.0 is shown in Figure 4. The highest activity was observed at 80°C. The activity sharply decreased below and above 80°C. The thermal stability of DERA was determined after incubation at different temperatures. After heating at 80°C for 10 min, no loss of activity was observed for DERA$_{Abo}$ (Figure 4). Even after incubation at 90°C for 10

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     A.boonei -MDAR---ELAKY--------------------IDHTNLKAFATREDIKRLCEEAKEYGFYAVCVNPYRVKDAAEFLKG  55
   T.maritime MIEYRIEEAVAKYREFYEFKPVRESAGIEDVKSAIEHTNLKPFATPDDIKKLCLEARENRFHGVCVNPCYVKLAREELEG  80
     A.pernix MPSARDILQQGLDR----------LGSPEDLASRIDSTLLSPRATEEDVRNLVREASDYGFRCAVLTPVYTVKISGLAEK  70
 P.aerophilum ---------------------------MIHLVDYALLKPYLTVDEAVAGARKAEELGVAAYCVNPIYAPVVRPLLR-  49


     A.boonei TDIKIASVVGFPLGATFTETKVQEAIMAVRNGADEIDMVMNIGAMKDGDYGFVERDIREVVEAVHPMGAKVKVIIETCYL  135
   T.maritime TDVKVVTVVGFPLGANETRTKAHEAIFAVESGADEIDMVINVGMLKAKEWEYVYEDIRSVVESVK--GKVVKVIIETCYL  158
     A.pernix LGVKLCSVIGFPLGQAPLEVKLVEAQTVLEAGATELDVVPHLSLGP----EAVYREVSGIVKLAKSYGAVVKVILEAPLW  146
 P.aerophilum -KVKLCVVADFPFGALPTASRIALVSRLAEV-ADEIDVVAPIGLVKSRRWAEVRRDLISVVGAAG--GRVVKVITEEPYL  125


     A.boonei SDEEKIKACELAKKAGADFVKTSTGFGTAG---------AKVEDVKLMRSVVG---NDMGVKAAGGIHNAKQAIAMIEA  202
   T.maritime DTEEKIAACVISKLAGAHFVKTSTGFGTGG---------ATAEDVHLMKWIVG---DEMGVKASGGIRTFEDAVKMIMY  225
     A.pernix DDKTLSLLVDSSRRAGADIVKTSTGVYTKG---------GDPVTVFRLASLAKP--LGMGVKASGGIRSGIDAVLAVGA  214
 P.aerophilum RDEERYTLYDIIAEAGAHFIKSSTGFAEEAYAARQGNPVHSTPERAAAIARYIKEKGYRLGVKMAGGIRTREQAKAIVDA  205


     A.boonei GATRIGASRSVEIIETLELI---  222
   T.maritime GADRIGTSSGVKIVQGGEERYGG  248
     A.pernix GADIIGTSSAVKVLESFKSLV--  235
 P.aerophilum IGWGEDPARVRLGTSTPEALL--  226
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Figure 2. Amino acid sequence alignment of DERAs from *A. boonei* (B5IEU6), *A. pernix* (Q9Y948), *P. aerophilum* (Q8ZXK7) and *T. maritime* (Q9X1P5). Gaps, indicated by dashes, were introduced into the sequences to maximize homology. Residues important for catalysis as discussed in the text are shown in triangles.

min, the enzyme retained about 60% residual activity. These results show that $DERA_{Abo}$ is a remarkably thermal stable enzyme.

Basic kinetic constants for $DERA_{Abo}$

Initial rate kinetics for the aldol cleavage reaction with $DERA_{Abo}$ was determined with various concentrations of DRP by fitting the data to the Michaelis-Menten equation. The apparent Km value of the enzyme was 0.12 ± 0.01 mM, which is lower than that of *E. coli* DERA (Gijsen and Wong, 1994) and higher than those of *T. maritima* and *P. aerophilum* DERA (Sakuraba et al., 2003, 2007). Compared to the mesophilic DERA from Yersinia sp. with Km value of 9.1 mM, the reported thermophilic DERAs have significantly higher substrate affinity.

Effects of acetaldehyde on enzyme stability

$DERA_{Abo}$ retained more than 70% DRP cleavage activity after exposure for 4 h to 250 mM acetaldehyde at 25°C, and over 80% activity under the condition of 50/150 mM acetaldehyde (Figure 5). No further loss of activity was observed after 1 h as most of the acetaldehyde was converted to lactol. These results indicate that the enzyme was resistant to acetaldehyde of high concentration and that acetaldehyde could be used to obtain the side chain of statine with the enzymes. Thermophilic DERAs from *P. aerophilum* and *T. maritime*

also showed a significant resistance to high concentration acetaldehyde, retaining high activity after exposure for hours to 300 mM acetaldehyde. 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). The results supported 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 DERA toward high concentrations of acetaldehyde substrate.

In conclusion, we reported the cloning, expression and characterization of DERA from *A. boonei*. $DERA_{Abo}$ showed broad pH adaptability, remarkable thermostability and high stability to high concentrations of acetaldehyde. We expect that $DERA_{Abo}$ will be useful in manufacturing settings where high concentrations of acetaldehyde are necessary. Currently, we are investigating the use of $DERA_{Abo}$ in the synthesis of statin side chains and this data will be reported in due course.

ACKNOWLEDGEMENTS
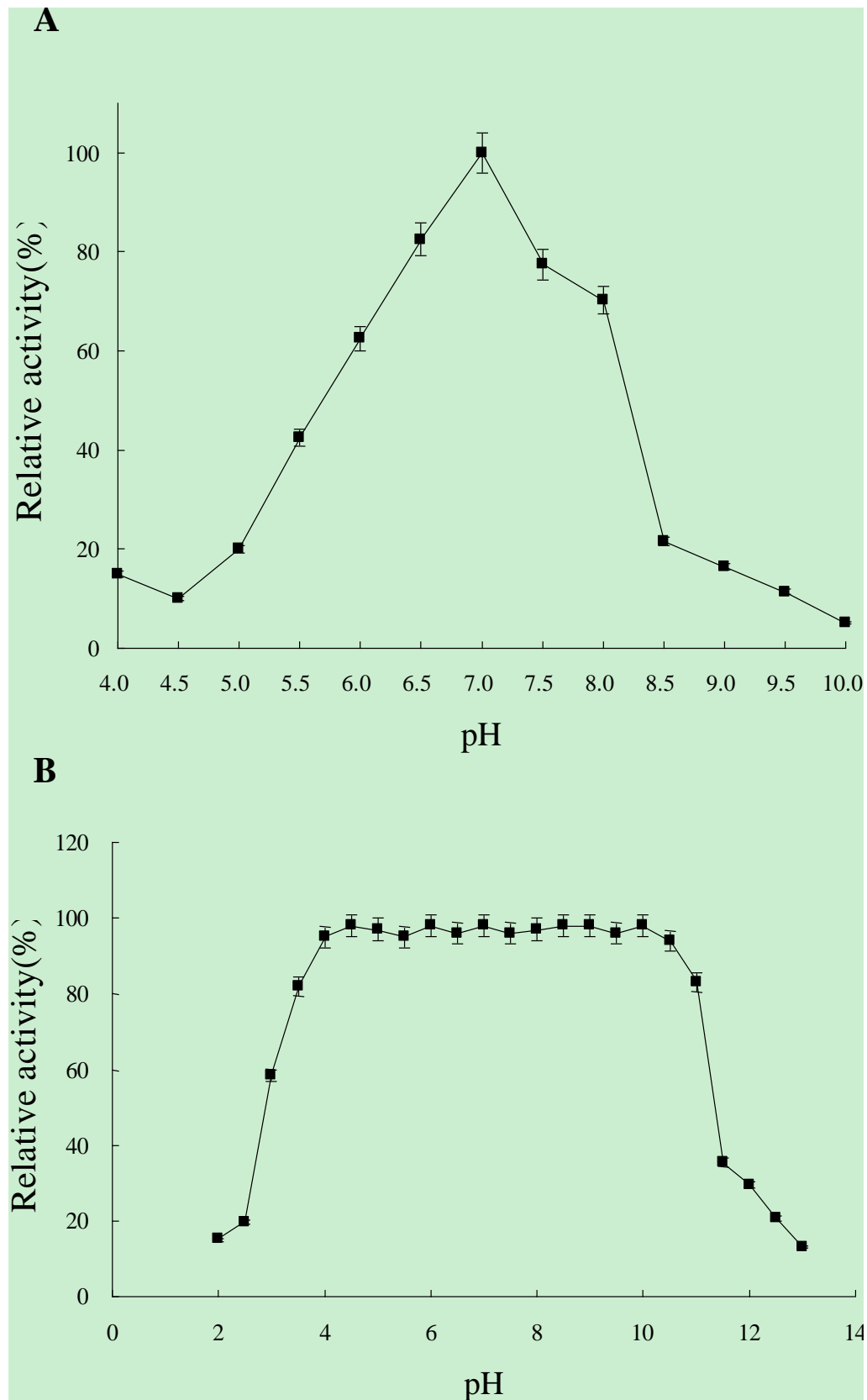
Figure 3. Optimum pH and pH stability of *A. boonei* DERA. (A) The optimum pH was performed at a variety of pH levels by determining cleavage of the DRP at 25°C. (B) The enzyme was incubated for 30 min at 50°C in buffers of various pH levels, after which the remaining activity was assayed.
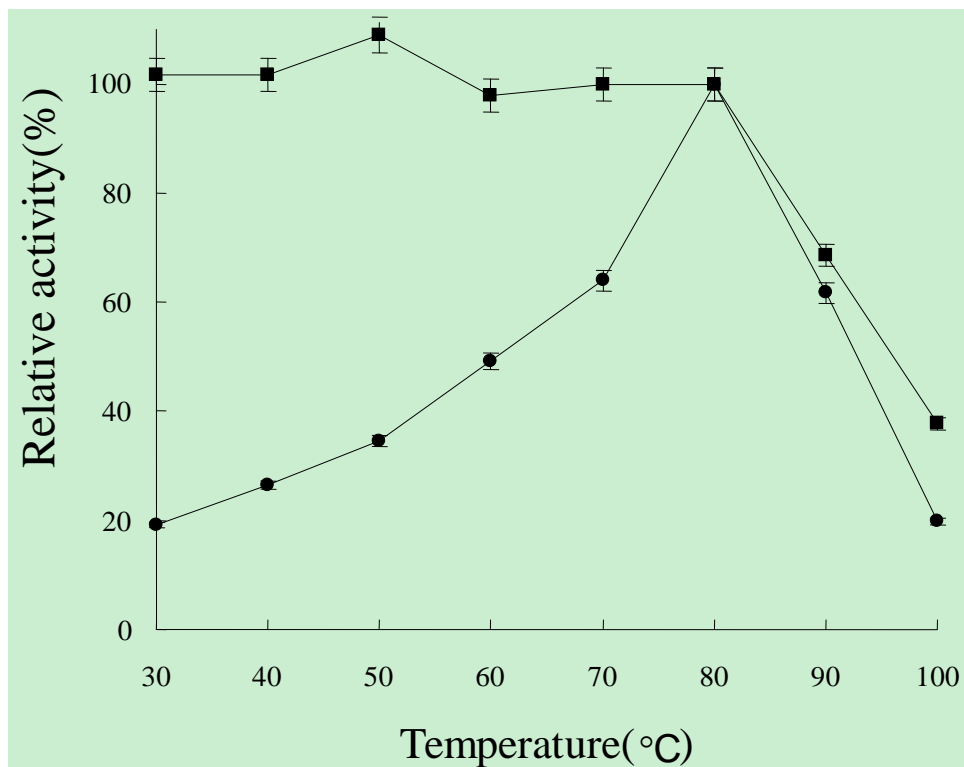
Figure 4. Effects of temperature on activity (●) and stability (■) of *A. boonei* DERA. Effect of temperature was determined using imidazole-HCl buffer (0.1 M, pH 7.0). Thermostability was determined after incubation for 10 min at the indicated temperatures in imidazole-HCl buffer (0.1 M, pH 7.0).
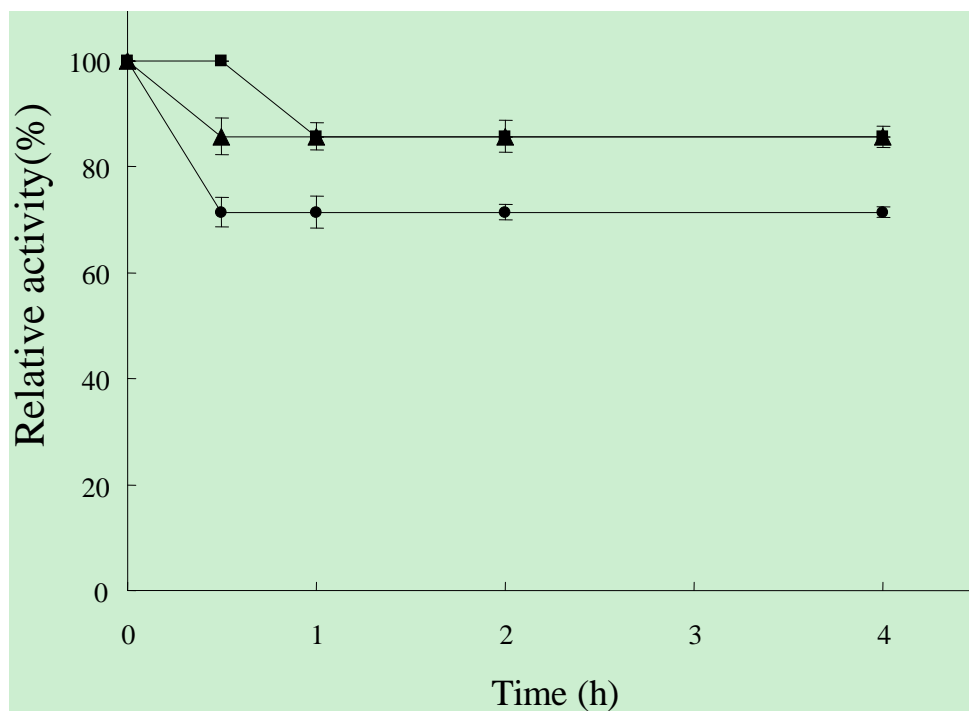
Figure 5. Effect of acetaldehyde on enzyme stability. The enzyme was incubated at 25°C in the presence of 50 mM(■),150 mM(▲) and 250 mM(●) acetaldehyde, respectively and the DRP cleavage activity was assayed at appropriate intervals.

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