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Overexpression, Purification, and Biochemical Characterization of the Thermostable NAD-dependent Alcohol Dehydrogenase from *Bacillus stearothermophilus*

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Abstract The gene ADH encoding NAD-dependent alcohol dehydrogenase from *Bacillus stearothermophilus* was cloned and overexpressed as a GST fusion protein at a high level in *Escherichia coli*. The expressed fusion protein was purified simply by glutathione affinity chromatography. GST fusion protein was then cleaved by thrombin, while soluble enzyme was further purified by glutathione affinity chromatography. The recombinant enzyme had the same elctrophoretic mobility as the native enzyme from *Bacillus stearothermophilus*. The recombinant enzyme catalyzed the oxidation of a number of alcohols and exhibited high activities towards secondary alcohols. The K_m and V_{max} values of the recombinant enzyme for ethanol were 5.11 mM and 61.35 U/mg, respectively. Pyridine and imidazole notably inhibited the enzymatic activity. The activity of the recombinant enzyme optimally proceeded at pH 9.0 and 70°C. The midpoint of the temperature-stability curve for the recombinant enzyme was approximately 68°C, and the enzyme was not completely inactivated even at 85°C. The recombinant enzyme showed a high resistance towards denaturing agents (0.05% SDS, 0.1 M urea). Therefore, due to its stability and relatively broad substrate specificity, the recombinant enzyme could be utilized in bio-industrial processes and biosensors.

Key words: Alcohol dehydrogenase, thermophilic bacterium, *Bacillus stearothermophilus*, enzymatic characterization, over-expression in *E. coli*, recombinant enzyme, simple purification, thermal resistance

Alcohol dehydrogenases (ADHs, EC 1.1.1.1) are widely distributed in nature, and are key enzymes of the primary short-chain alcohol metabolism in many organisms [3]. To date, mammalian and plant ADHs have been purified from many sources, and their properties, structures, functions, and physiological significance have been studied in detail [18, 26]. Mammalian ADHs catalyze a rate-determining step in alcohol metabolism [20, 34]. The main function of plant ADHs is to reduce acetaldehyde, so that anaerobic glycolysis produces ATP during the period of oxygen deprivation [10].

ADHs have also been found in many microorganisms. Their physiological functions are to catalyze the reversible oxidation of alcohol to their corresponding carbonyl compounds [3]. Therefore, ADHs from microorganisms attract major scientific interest for use in the synthesis or modification of high-value alcohols, including chiral alcohol [13]. In this respect, ADHs isolated from thermophilic microorganisms are advantageous over the mesophilic enzymes due to their thermal resistance and tolerance level towards the common denaturing reagents [7]. Some thermostable ADHs have been purified and well characterized from *Sulfolobus solfataricus* [29], *Thermoanaerobacter ethanolicus* [4], and *Thermoanaerobium brockii* [27]. *Bacillus stearothermophilus* is an ethanologenic facultative anaerobic thermophile with an optimal growth temperature of 55°C [9]. *B. stearothermophilus* ADHs from strains NCA 1503 and LLD-R have been isolated and the full-length gene was sequenced [11, 32, 33]. However, information regarding their biochemical properties as well as the structure and function are still lacking.

In this study, the gene encoding an NAD-dependent alcohol dehydrogenase from *B. stearothermophilus* was cloned and overexpressed as a fusion protein with glutathione *S*-transferase (GST) in *Escherichia coli*, to allow rapid purification. The recombinant enzyme was purified by GSH affinity chromatography and its enzymatic properties were compared with those enzymes from other sources.

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MATERIALS AND METHODS

Bacterial Strains, Plasmids, Enzymes, and Chemicals

Cells of *B. stearothermophilus* strain NCA 1503 (A.T.C.C. 29609) was obtained from Korean Culture Center of Microorganisms as a frozen cell paste and grown at 55°C. *E. coli* BL21 (DE3) (Novagen, U.S.A.) was used for cloning and making an overexpression of the encoding gene.

Expression vector, pGEX-KG, was obtained from the American Type Tissue Culture organization, Manassas, VA, U.S.A. Restriction enzymes and *taq* DNA polymerase were purchased from Takara Shuzo Co. (Japan). GSH-Sepharose was purchased from Pharmacia Biotech (Sweden). Alcohols, glutathione (GSH), NAD, imidazole, pyridine, and thrombin were supplied by Sigma Co (U.S.A.).

Cloning of the ADH Gene from *Bacillus stearothermophilus*

The complete coding sequence of the ADH gene from *B. stearothermophilus* was obtained directly from the genomic DNAs by PCR amplification, according to the method of Saiki [30]. Two oligonucleotide primers for the polymerase chain reaction (PCR) were designed as follows: Primer-1 (42-mer), 5'-GCTGCAGAATTCTCATGAAAGGCTGC-TGTTGT GGAACA ATTT-3'; Primer-2 (40-mer), 5'-GCGCGCAAGCTTATTAATCTACTTTTAACACGACG-CGGCC-3'. Primer-1 and Primer-2 were the 5'- and 3'-end sequences of the coding region of *B. stearothermophilus* ADH gene [32] with added *Eco*R I and *Hin*d III restriction sites, respectively. Oligonucleotide primers were synthesized by using the DNA synthesizer model 391 (Applied Biosystems, Inc.).

Construction and Transformation of Expression Plasmid pKG-ADH

The amplified DNA was digested by *Eco*R I and *Hin*d III, and ligated with the *Eco*R I/*Hin*d III site of expression vector pGEX-KG. *E. coli* BL21 (DE3) cells were transformed by the resulting plasmid of pKG-ADH.

Expression and Purification of the Enzyme

The transformed cells were grown in 1 l of LB medium containing 50 μg/ml of ampicillin at 37°C. The enzyme synthesis was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), when the cell culture reached OD_{600} of 0.4–0.5, and the culture was continued for another 6 h. The bacteria were then harvested by centrifugation at 10,000 ×g for 10 min and washed in 20 mM potassium phosphate buffer, pH 7.5 (buffer A). The cells were repelleted, then suspended in buffer A and sonicated until lysed with a ultrasonic processer (Sonics and Materials Inc., U.S.A.). The lysate was centrifuged at 45,000 ×g for 30 min at 4°C. The supernatant was loaded onto GSH-Sepharose affinity column preequilibrated with buffer A. The column was exhaustively washed with buffer A containing 200 mM potassium chloride. The enzyme was eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM GSH. Active fractions were pooled and dialyzed against buffer A. Cleavage of the fusion protein was carried out by incubation with an excess amount (a 1:50 enzyme:substrate ratio) of thrombin (Sigma Co, U.S.A.) in buffer A for 1 h at 25°C. The solution was applied to GSH-Sepharose affinity column to remove the *N*-terminal glutathione *S*-transferase, and the unbound enzyme fractions were collected and dialyzed against buffer A. The dialyzed purified enzyme was used for the next experiment. Unless otherwise indicated, all purification procedures were performed either at 4°C or on ice.

Enzyme Assay and Kinetic Studies

Enzyme activity was determined at 60°C by monitoring at 340 nm with a Jasco V-550 spectrophotometer, provided with a thermostated cell compartment (Japan). The reaction mixtures contained 1 mM NAD, 10 mM ethanol, 20 mM potassium phosphate buffer (pH 7.8), and 5–50 μl of enzyme solution in a total volume of 1 ml. Nonenzymic reaction rates served as controls, which were subtracted from the reaction rate in the presence of the enzyme. Molar absorbance coefficient of NADH was 6,220 $M^{-1} \cdot cm^{-1}$, and one unit of enzyme activity was defined as the amount of enzyme that catalyzed generation of 1 μmole of NADH per min under the above experimental conditions. Specific activity was expressed as units per milligram protein. Kinetic parameters were determined by the Lineweaver-Burk plot method [19]. The enzyme concentration used for initial-rate studies was 5–50 μg/ml, and the concentration of ethanol was varied over the range of 0.1–50 mM. All experiments were performed at least in triplicate. The protein concentration of the enzyme preparation was determined by the method of Bradford [2], using γ-globulin as a standard.

Inhibition Studies

The inhibitory effects on the enzyme activity were measured by preincubating the enzyme with each inhibitor at 60°C for 2 min and initiating the reaction by adding 10 mM ethanol. The remaining activity was determined by the above assay procedure.

Stability of the Enzyme

Thermal stability was determined by using the enzyme with 0.1 mg/ml protein concentration. The enzyme was incubated in buffer A at various temperatures for 1 h and then cooled in ice. The remaining activity was determined by the assay procedure. Enzyme stability towards the common denaturing reagents was determined by using 0.05% SDS, 1% triton X-100, 100 mM urea, and 200 mM guanidine-HCl: The enzyme was incubated at each concentrations of denaturing reagents for 1 h or 5 h at 4°C, and the remaining activity was determined at the end of the storage period.

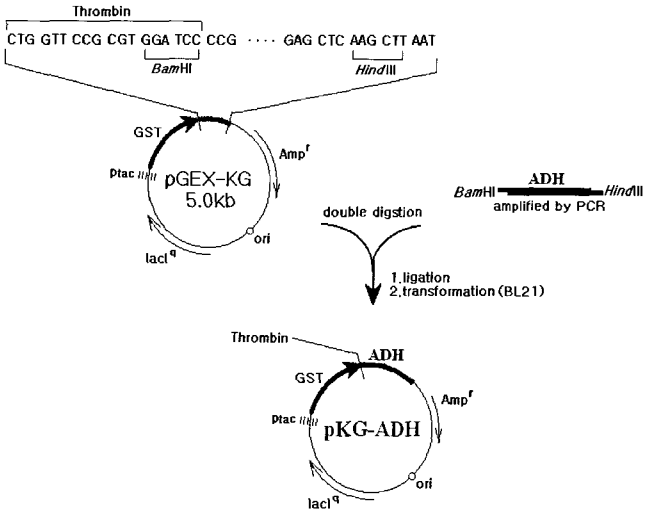

Fig. 1. Construction of pKG-ADH expression plasmids.

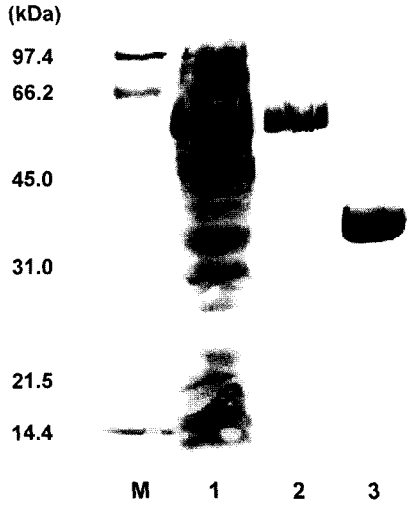

Fig. 2. Electrophoresis of the recombinant *B. stearothermophilus* alcohol dehydrogenase under denaturing conditions.
Denaturing SDS-PAGE was carried out using the method of Laemmli (1970) on 12.5% gel. Coomassie brilliant blue R-250 was used for staining. Lane M, molecular-mass standard markers; lane 1, total protein from *E. coli* cell lysates of pKG-ADH vector transformants; lane 2, the purified GST fusion protein by GSH-Sepharose affinity chromatography; lane 3, the purified enzyme by GSH-Sepharose affinity chromatography after thrombin treatment.

Electrophoresis

Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out via the method of Laemmli [16] by using the molecular weight marker protein (Bio-Rad) in 12.5% gel. Gel was stained with Coomassie Blue R-250. The molecular-mass markers were SDS molecular weight standard markers (Bio-Rad) that contained phophorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

DNA Sequence Analysis

DNA sequencing was performed by following the Sanger's dideoxynucleotide termination method [31]. *Taq* DNA polymerase and the DNA sequencer model 370A (Applied Biosystems, Inc.) were used in the process.

Results and Discussion

Construction of Expression Plasmid and its Expression in *E. coli*

The construction of expression plasmid pKG-ADH is diagrammed in Fig. 1. *B. stearothermophilus* ADH gene was amplified from the genomic DNAs by PCR and inserted into the multiple cloning site of the expression plasmid pGEX-KG by using the *Eco*R I/*Hin*d III sites. The resulting plasmid, called pKG-ADH, was used to transform *E. coli* BL21 cells, and clones with the correct plasmids were selected by restriction analysis and DNA sequencing.

To obtain active *B. stearothermophilus* ADH in a high yield, the culture and induction conditions of *E. coli* were examined [22, 25]. For the highest yield of active enzymes, 1-l cultures were grown to a cell density of 0.4-0.5 at 600 nm and then exposed to the inducer (1 mM IPTG) for 6 h. Under this growth at 37°C, 1-l of culture yielded approximately 3.5 mg of the purified active enzymes (Table 1).

Purification of the Expressed *B. stearothermophilus* ADH

The result of purification of *B. stearothermophilus* ADH from *E. coli* BL21 containing plasmid pKG-ADH is shown in Table 1. By GSH-Sepharose affinity chromatography of the crude extract, the GST-fusion protein was purified to an electrophoretic homogeneity (Fig. 2, lane 2). After the cleavage of the fusion protein followed by GSH-Sepharose affinity chromatography, the enzyme appeared as a single band corresponding to a molecular mass of 38 kDa on

Table 1. Purification of the recombinant *B. stearothermophilus* alcohol dehydrogenase.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	495.05	402.48	1.23	100	1
GSH affinity	202.28	3.52	57.39	41	47

Purification was carried out as described in the Materials and Methods section. Enzyme activities were determined using ethanol as a substrate. Results represent experiments done in triplicate.

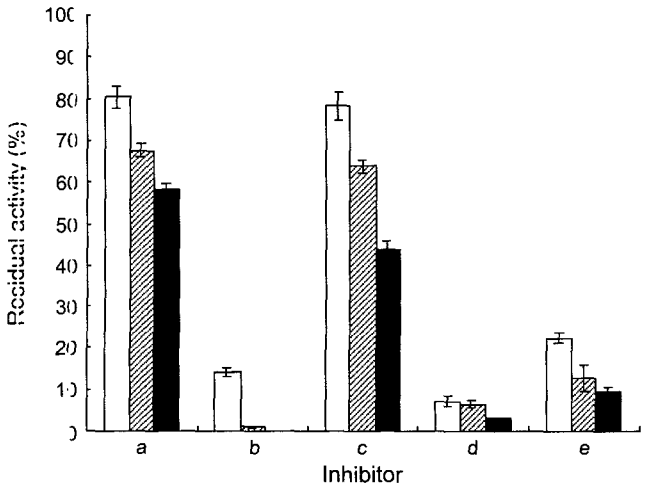

Fig. 3. Effect of inhibitors on the activity of the recombinant *B. stearothermophilus* alcohol dehydrogenase.
The inhibitors were (a) acetaldehyde, (b) imidazole, (c) pyrazole, (d) pyridine, and (e) 2,2,2-trichloroethanol. Each inhibitor was varied in the concentrations 2.5 mM (□), 5 mM (▨), and 10 mM (■). Values are Means±S.D., generally based on n≥3.

SDS-PAGE, which agreed well with the reported size of *B. stearothermopilus* ADH gene product [32] (Figs. 2, lane 3). The overall purification was estimated to be about 47-fold, and the activity recovery was 41% (Table 1). The purified enzyme at this stage was used to carry out subsequent characterizations.

The purified enzyme gave a single band on SDS electrophoresis, as shown in Figs. 2-3. Comparison of relative mobility of the enzyme with those of standard proteins indicated that a molecular mass of the recombinant *B. stearothermophilus* ADH was approximately 38 kDa by SDS-PAGE. On the other hand, the purified enzyme in gel-filtration chromatography indicated the activity eluted at a point of approximately 160 kDa. Therefore, it can be concluded that the active native form of the recombinant *B. stearothermophilus* ADH is a homotetramer of 38 kDa polypeptide. The recombinant *B. stearothermophilus* ADH seemed to be similar to *Alcaligenes eutrophus* and *Thermoanaerobacter ethanolicus* enzymes, which are homotetramers with subunits of about 40 kDa [5, 11]. On the other hand, enzymes of *Clostridium beijerinckii*, *Mycobacterium tuberculosis*, and *Thermomicrobium roseum* were homodimers with a molecular mass of approximately 80 kDa [8, 14, 36].

Catalytic Properties of the Recombinant *B. stearothermophilus* ADH

The properties of the recombinant *B. stearothermophilus* ADH were investigated with ethanol oxidation reaction. The optimum activity of the recombinant ADH on ethanol was observed at pH 9.0 and 60°C. The enzyme showed less than 40% of its maximum activity below pH 6.0. Similar results have been found with ADHs from *T. roseum* (pH 10.0) [36] and *Thermococcus* strain ES1 (pH 8.8-11.1) [21]. On the other hand, pH optima near neutrality have been observed for ADHs from *B. stearothermophilus* strain LLD-R (pH 8.0) [11], *Thermococcus zilligii* (pH 6.8-7.0) [17], and *Moraxella* sp. TAE123 (pH 7.5) [35].

In order to understand the effect of temperature on the enzyme activity, the reaction mixture was incubated at different temperatures, ranging from 25°C to 75°C. The optimum temperature of the recombinant *B. stearothermophilus* ADH was 70°C. This value was higher than those reported for mesophilic microorganism ADHs (25-40°C) [12, 23, 24, 35]. Therefore, the enzyme was quite thermophilic, in agreement with results found in ADHs from *T. roseum* (70°C) [36], *T. ethanolicus* (70°C) [28], and *Thermococcus hydrothermalis* (80°C) [1].

The substrate specificity of the recombinant *B. stearothermophilus* ADH towards several compounds is shown in Table 2. The enzyme displayed broad substrate specificity towards a number of primary, secondary, and tertiary alcohols: The enzyme exhibited high activities towards secondary alcohols. On the other hand, the enzyme showed very low activities towards 3-pentanol and 2,2,2-trichloroethanol. These results indicate that acceptability of linear alcohols to the recombinant *B. stearothermophilus* ADH depends on the size of the two substituent groups on the carbon with the alcoholic function: Activity was the highest when at least one group was methyl (2-propanol, 2-butanol, 2-pentanol), and it dramatically decreased when both substituents were larger than methyl (e.g. 3-pentanol). This substrate specificity of the enzyme was similar to those of ADHs from *B. stearothermophilus* strain LLD-R [11], *T. brockii* [15], and *T. ethanolicus* [28].

The effect of ethanol concentration on ADH activity was investigated at 60°C. The K_m and V_{max} values of the

Table 2. Substrate specificity of the recombinant *B. stearothermophilus* alcohol dehydrogenase.

Substrate	Specific activity (U/mg)	Relative activity (%)
methanol	7.79±0.85	9±1
ethanol	57.39±2.24	70±3
2,2,2-trichloroethanol	0.48±0.12	0.6±0.1
n-propanol	68.45±4.35	83±5
2-propanol	75.48±2.54	92±3
n-butanol	25.83±0.84	31±1
2-butanol	82.11±3.62	100±4
t-butanol	38.12±1.20	46±1
n-pentanol	72.45±3.45	88±4
2-pentanol	78.54±4.54	96±6
3-pentanol	0.21±0.02	0.3±0.02
isoamyl alcohol	12.75±0.74	16±1
crotyl alcohol	24.43±1.86	30±2

The values showed are Means±S.D., generally based on n≥5.

recombinant *B. stearothermophilus* ADH for ethanol were 5.11 mM and 61.35 U/mg, respectively. This K_m value of the enzyme is smaller than those of ADHs from *S. solfataricus* (17.2 mM) [6], *T. ethanolicus* 39E (25 mM) [5], and *T. roseum* (24.2 mM) [36]. Therefore, the recombinant *B. stearothermophilus* ADH has a stronger affinity toward ethanol than ADHs from *S. solfataricus*, *T. ethanolicus*, and *T. roseum*.

Several compounds were tested for their inhibitory action on the recombinant *B. stearothermophilus* ADH (Fig. 3). Two compounds (imidazole and pyridine) that have structure similar to adenine of NAD notably inhibited the enzyme. The enzyme was also inhibited by 2,2,2-trichloroethanol, a competitive inhibitor for ethanol. On the other hand, acetaldehyde, an oxidation product of ethanol by ADH was the least potent inhibitor among the substances tested.

Stability of the Recombinant *B. stearothermophilus* ADH

The thermostability of the recombinant *B. stearothermophilus* ADH was investigated by incubating the enzyme at various temperatures for 1 h (Fig. 4). The midpoint of the temperature-stability curve was approx. 68°C for the enzyme. The enzyme was fairly stable at temperature up to 60°C. Above 62°C, however, its activities declined rapidly as the temperature increased, and the enzyme was not completely inactivated at 85°C. This thermostability of the enzyme is comparable with those of ADHs purified from other thermophilic bacteria [1, 4, 11, 14, 29, 36].

The tolerance of the recombinant *B. stearothermophilus* ADH was investigated by incubation of the enzyme with denaturing agents for 1 h or 5 h (Fig. 5). The enzyme was

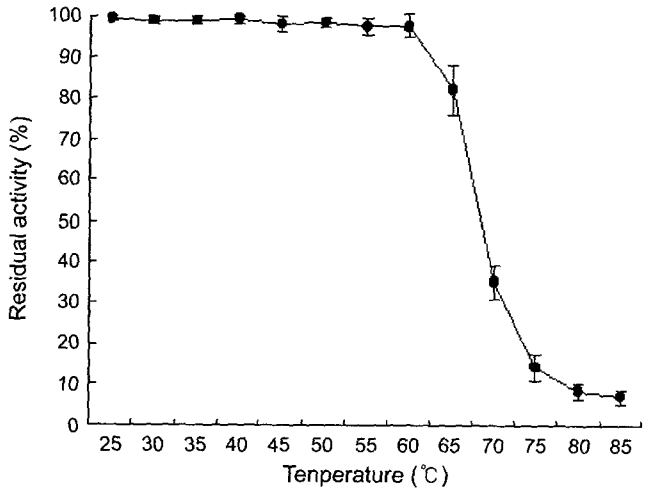

Fig. 4. Thermostability of the recombinant *B. stearothermophilus* alcohol dehydrogenase.
Remaining ADH activity after incubation for 1 h at each temperature. Values are Means±S.D., generally based on n≥3.

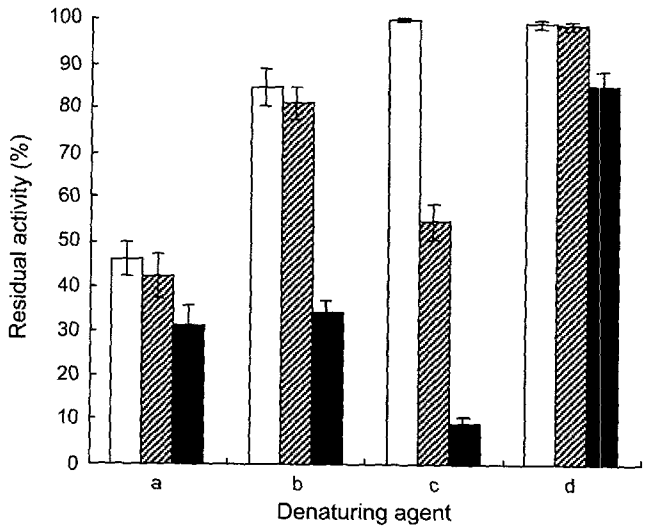

Fig. 5. Tolerance of the recombinant *B. stearothermophilus* alcohol dehydrogenase towards denaturing agents.
The activity was assayed on each denaturing agent after incubation for 1 h (▨) and 5 h (■). Controls indicate (□). The denaturing agents were; (a) 0.2 M guanidine hydrochloride, (b) 0.05% SDS, (c) 1% triton X-100, and (d) 0.1 M urea. Values are Means±S.D., generally based on n≥3.

fairly stable towards 0.05% SDS or 0.1 M urea for 1 h. On the other hand, the activity was significantly affected (more than 50% inactivation) by 0.2 M guanidine hydrochloride or 1% triton X-100. This tolerance level of the enzyme towards denaturing agents is similar to *T. roseum* ADH [36]. These results indicate that the recombinant *B. stearothermophilus* ADH is extremely stable against high temperature and denaturing reagent. In fact, this stable recombinant *B. stearothermophilus* ADH appears to represent a good model for studying the stability of ADH.

In conclusion, the recombinant pKG-ADH construct provided sufficient quantity of *B. stearothermophilus* ADH expressed in *E. coli* and allowed rapid purification by using affinity chromatography. The recombinant enzyme displayed the relatively broad substrate specificity. The recombinant enzyme showed resistance towards several denaturing agents and heating and was highly stable against high temperature. Stable ADH with broader specificity is definitely needed for industrial use in stereospecific organic synthesis and in the production of high-added-value products. In this respect, our recombinant enzyme showed an excellent and competitive characteristics when compared with other available ADHs. With the presently described recombinant *B. stearothermophilus* ADH, we are in a process to study the detailed catalytic mechanism and the structure-function relationship of this enzyme by site-directed mutagenesis and structural analysis. Finally, exploiting its stability and relatively broad substrate specificity, we plan to utilize this recombinant enzyme in bio-industrial processes and biosensors.

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