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# Molecular Properties and Enhancement of Thermostability by Random Mutagenesis of Glutamate Dehydrogenase from *Bacillus subtilis*

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**The *rocG* gene encoding glutamate dehydrogenase from *Bacillus subtilis* (Bs-GluDH) was cloned, and expressed at considerable magnitude in *Escherichia coli*. The recombinant Bs-GluDH was purified to homogeneity and has been determined to have a hexameric structure ($M_r$ 270 kDa) with strict specificity for 2-oxoglutarate and L-glutamate, requiring NADH and $NAD^+$ as cofactors respectively. The enzyme showed low thermostability with $T_m = 41\,°C$ due to dissociation of the hexamer. To improve the thermostability of this enzyme, we performed error-prone PCR, introducing random mutagenesis on cloned GluDH. Two single mutant enzymes, Q144R and E27F, were isolated from the final mutant library. Their $T_m$ values were 61 °C and 49 °C respectively. Furthermore, Q144R had a remarkably high $k_{cat}$ value ($435\,s^{-1}$) for amination reaction at 37 °C, 1.3 times higher than that of the wild-type. Thus, Q144R can be used as a template gene to modify the substrate specificity of Bs-GluDH for industrial use.**

**Key words:** glutamate dehydrogenase; *Bacillus subtilis*; thermostability; random mutagenesis; catalytic activity

Glutamate dehydrogenases (GluDHs) are a broadly distributed group of enzymes that catalyze the reversible oxidative deamination of L-glutamate to 2-oxoglutarate (2-OG) and ammonia.[1)] The GluDHs identified in lower eukaryotes or in prokaryotes usually act with one particular coenzyme ($NAD^+$ or $NADP^+$), whereas those of higher eukaryotes have a dual coenzyme specificity, ($NAD(P)^+$, EC 1.4.1.3).[2)] NAD-dependent GluDH (EC 1.4.1.2) is mainly involved in 2-OG production (glutamate catabolism), and NADP-dependent GluDH (EC 1.4.1.4) in glutamate production (ammonia assimilation) in microorganisms.[3)]

One of the major goals of our studies has been to understand the structural basis of amino acid substrate specificity in amino acid dehydrogenases, and to apply that knowledge to the engineering of novel substrate specificities. GluDH is one of the enzymes that offer information concerning enzymological properties and the relationships between structure and function. The extremely small equilibrium constant of GluDH allows it to act as a useful catalyst in the analysis of amino acids, 2-oxoacids, and ammonia, which are important tools in clinical chemistry, bioprocess control, and nutrition studies.[4)] NAD-dependent GluDH might be of special preference for industrial use, because $NAD^+$ and NADH are much cheaper than $NADP^+$ and NADPH respectively.[5)] In addition, 2-OG/GluDH is a promising method for regenerating NAD(P) in most enzyme-catalyzed reactions requiring the $NAD(P)/NAD(P)H^+$ system.[5)]

Recently, a thermostable NAD-dependent GluDH of the archeaeal species *Pyrobaculum islandicum* (Pi-GluDH) has been cloned, but this enzyme was expressed in inactive form in *E. coli* and required high-temperature heat treatment to obtain an active form.[6)] Moreover, certain properties related to low activity at normal room temperature of those archeal GluDHs were big obstacles to their use as industrial enzymes. Hence, we searched for a mesophilic NAD-dependent GluDH gene by Pi-GluDH homology search. In mesophilic NAD-dependent enzymes, GluDH from *Bacillus subtilis* (Bs-GluDH) showed the highest similarity against Pi-GluDH.

The bacterium *Bacillus* is very diverse in nature in its evolutionary sphere. The true existence of GluDH in the *Bacillus* species came to light a few decades ago. The complete *B. subtilis* genome sequence comprises two genes, *rocG* and *gudB*, resulting in GluDHs of 424 and 426 amino acid long peptides respectively.[7,8)] The genes are 74% identical to each other and resemble 28–51%

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*Abbreviations*: GluDH, glutamate dehydrogenase; 2-OG, 2-oxoglutarate; IPTG, isopropyl thio-β-D-galactoside

the GluDHs of *Clostridium symbiosum*,[9] *C. difficile*,[10] and *Pyrococcus furiosus*,[11] *Thermococcus profundus*,[12] and are similar to many other hexameric GluDHs. RocG is the major catabolic GluDH, while GutB is intrinsically inactive.[13] Recent study of *rocG* gene regulation associating catabolite repression and derepression on the expression of GluDH has been reported and it shows the role of GluDH in the deamination reaction.[14] The balance between the rates of glutamate synthesis and degradation is maintained by a series of regulations of the Roc pathway,[15] and RocG activity can be viewed as the final step in the use of arginine, ornithine, or purine as a carbon and nitrogen source.[16]

We have cloned *rocG* from *B. subtilis*, expressed it in *E. coli*, and characterized the molecular properties of Bs-GluDH. The present paper reports the low thermostability of recombinant Bs-GluDH and enhancement of thermostability using error-prone PCR.

## Materials and Methods

*Strains, plasmids, and culture conditions.* The bacterium *B. subtilis* ISW1214 (hsrM leuA8 metB5; $Tet^s$) was supplied by Takara (Ohtsu, Japan). *E. coli* MV 1184 [*ara*, Δ(*lac-proAB*), *rpsL*, *thi (F80lacΔM15)*, *Δ(srl-recA) 306*:: Tn*10(tet$^r$)/F′[traD36*, *proAB*$^+$, *lac I$^q$*, *lacZΔM15*] (Takara) was used as the host strain. The pT7 Blue T-vector (Novagen, San Diego, CA, U.S.A.) was used for to clone the *rocG* gene encoding Bs-GluDH, and plasmid pUC18 (Takara) was used for overexpression of *rocG*. Recombinant strains were grown in Luria-Bertani (LB) broth supplemented with 50 μg·ml$^{-1}$ ampicillin. When necessary, 0.5 mM isopropyl thio-β-D-galactoside (IPTG) was added to the media.

*Cloning of the GluDH gene from B. subtilis.* Chromosomal DNA of *B. subtilis* ISW1214 was extracted from overnight cultures according to the method of Tandeau *et al.*[17] The *rocG* gene was amplified by PCR using a Bio-Rad iCycler DNA thermal cycler. The forward and reverse primers used were 5′-CGGATCCG-TAGAGGAGAAAAAGATGTCAGCA-3′ and 5′-CA-GCTGCAGGGTGATCACCTTTCTCT-3′ respectively; the former was designed to contain the N-terminal region of the *rocG* gene from *B. subtilis* 168 (accession no. NC_000964), the SD sequence (AGGA) for the *E. coli lac* promoter, and the *Bam*HI recognition sequence, and the latter contained the C-terminal region and the *Pst*I digestion sequence respectively. The PCR thermal conditions were 94 °C for 30 s, 55 °C, 30 s, and 72 °C, 1 min through 30 cycles. The amplified PCR product was digested using *Bam*HI and *Pst*I and allowed cloning of the gene at the corresponding site in pUC18 vector. Overexpression of the GluDH coding region as cloned in the downstream region of the IPTG-inducible *lac* promoter of pUC 18 was obtained by transformation in the expression host *E. coli* MV1184, and the transformant was plated at 37 °C in LB medium containing 50 μg·ml$^{-1}$ ampicillin, 1 mM IPTG, and 40 μg·ml$^{-1}$ 5-bromo-4-chrolo-3-indolyl-β-galactoside. For large-scale growth, overexpression was induced by the addition of 0.4 mM IPTG to exponentially growing cultures of the recombinant strain. Since *E. coli* has NADP-dependent GluDH, the presence of NAD-dependent enzyme activity in transformants shows that the plasmid-encoded Bs-GluDH gene expresses an active enzyme.

*DNA manipulation and sequencing.* Isolation of plasmid DNA, restriction enzyme digestion, ligation, and transformation into *E. coli* were done according to the standard recombinant DNA manipulation techniques.[18] All restriction enzymes, DNA-modifying enzymes, and related reagents used for DNA manipulation were purchased from Takara. The sequencing was performed by the dideoxynucleotide chain-termination method employing an Applied Biosystems PRISM 3100-Avant Genetic Analyzer. Sequence data were analyzed using GENETYX 7.0 software (Software Development, Tokyo, Japan).

*Random mutagenesis and construction of mutants.* Plasmid pUC18 harboring *rocG* was used as a template in mutagenesis for error-prone PCR. Error-prone mutagenic PCR was performed using M13 primer M4 (5′-GTTTTCCCAGTCACGAC-3′) and M13 primer RV (5′-CAGGAAACAGCTATG) (Takara) as forward and reverse primer respectively, according to the method of Lenug *et al.*[19] To avoid mutational bias and to obtain both the desired level of mutation and base substitutions, the conditions used for PCR random mutagenesis were optimized. A 30-μl reaction mixture contained 3 μl $Mg^{2+}$ free 10× buffer, 1.5 mM $MgCl_2$, 4 μM dATP, 0.25 mM dGTP, 0.25 mM dCTP, 0.25 mM dTTP, 10 pmol M13 primer RV and M13 primer M4, 1 ng of template DNA, and 2.5 U of Taq polymerase (Takara). PCR was performed with an automatic thermal cycler (Bio-Rad iCycler) for 10 cycles consisting of 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min, followed by 25 cycles under the same conditions after the addition of 0.25 mM ATP only. The plasmid isolated from the thermostable mutant showing the highest activity in screening was used as the template for the next cycle of PCR random mutagenesis. Random mutagenic PCR was performed for a total of four rounds.

*Thermostability screening.* Screening of thermostable mutants was carried out by means of the active staining method using Nitro Blue Tetrazolium (NBT) and phenazine methosulfate (PMS). The 1,000–1,200 *E. coli* MV1184 colonies harboring randomly mutated *rocG* genes grown on LB agar plates containing ampicillin (50 μg/ml) and 1 mM IPTG were picked out with sterile tooth picks and inoculated into 3 ml LB broth containing 50 μg/ml ampicillin and 1 mM IPTG. After overnight incubation, the cells were collected as pellets and

suspended and lysed for 1 h at 37 °C by the addition of potassium phosphate (KP) buffer (pH 7.3) containing 0.5 mg·ml$^{-1}$ lysozyme (egg white, Wako, Osaka, Japan). To screen the thermostable mutants, the enzyme solutions were transferred to a 96-well microplate and incubated at various temperatures (60 °C, 70 °C, 75 °C, and 80 °C) for 20 min. Then the reaction mixture (100 mM Tris buffer, pH 7.7, 0.5 mM $NAD^+$, 10 mM L-glutamate, 0.15% Triton X-100, 0.3 mM NBT, and 0.1 mM PMS) was added and incubated at 37 °C for 20 min. The remaining activities of those enzymes as a function of color produced were measured with a microplate reader (Thermo Max, Molecular Devices, Sunnyvale, CA, U.S.A.).

*Enzyme purification.* The wild-type Bs-GluDH and mutant enzymes were purified to homogeneity. Unless otherwise specified, KP buffer (pH 7.3) containing 3 mM EDTA was used as the buffer throughout the purification process. The recombinant *E. coli* cells grown at 37 °C in LB medium were collected and dissolved in the buffer. The suspended cells collected from LB culture grown overnight were subjected to French Press (Ohtake, Tokyo, Japan) at 1500 kg/cm$^2$. After removing the cell debris by ultra centrifugation, the supernatant was collected and precipitated with 30–40% ammonium sulfate at pH 7.3, and left for 30 min. The precipitated enzyme was removed, collected by centrifugation, redissolved in a small amount of 0.1 M buffer, and dialyzed overnight against 5 liters of 50 mM buffer. The dialyzed sample was loaded on a DEAE-Toyopearl 650S column (2.5 × 20 cm, Tosoh, Tokyo, Japan) equilibrated with 50 mM buffer. The enzyme was eluted with a linear gradient of 0 to 0.5 M KCl in 50 mM buffer. The active fractions were combined and applied on a Butyl-Toyopearl 650M column (1.5 × 10 cm, Tosoh) equilibrated with 50 mM buffer containing 20% saturated ammonium sulfate. The enzyme was eluted with a linear gradient of 20 to 0% saturated ammonium sulfate in the buffer. The active fractions were pooled and were ready for analysis. SDS–PAGE was used to determine the purity of the protein under the conditions described by Laemmli.[20]

*Measurement of molecular mass.* The molecular mass of the native GluDH was estimated by HPLC on a gel filtration Superdex 200 HR 10/30 column (1 × 30 cm, Amersham, Biosciences, Piscataway, NJ, U.S.A.) equilibrated with 50 mM KP buffer (pH 7.3) containing 3 mM EDTA. The column was calibrated with the following proteins: yeast glutamate dehydrogenase (290 kDa), pig heart lactate dehydrogenase (142 kDa), yeast enolase (67 kDa), yeast adenylate kinase (32 kDa), and horse heart cytochrome c (12.4 kDa). The molecular mass of the subunit was estimated by SDS–PAGE. The proteins used as molecular mass standards were a mixture of phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

*$NH_2$-Terminal amino acid sequencing.* The $NH_2$-terminal sequence of purified GluDH was analyzed with a pulse liquid sequencer (Model PPSQ-10, Shimadzu, Kyoto, Japan). The enzyme was electroblotted from an SDS–PAGE gel onto a polyvinylidene difluoride membrane as described by Matsudaira.[21] The membrane was stained with Coomassie brilliant blue R250 and then used directly in the sequencer.

*Enzyme and protein assays.* The enzyme was assayed at 37 °C by the amination reaction under the following conditions: 10 mM 2-OG, 0.1 mM NADH, 100 mM Tris buffer, pH 7.3, 100 mM ammonium chloride. Activity was determined by monitoring the decrease in NADH absorbance at 340 nm (Model UV 1600, Shimadzu, Kyoto, Japan). One unit of activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of product per min at 37 °C. The deamination reaction was assayed using a mixture containing 20 mM monosodium L-glutamate, and 0.1 mM $NAD^+$ in 50 mM KP buffer, pH 7.7, by monitoring the increase in NADH absorbance at 340 nm. The apparent $K_m$ and $k_{cat}$ values were estimated from the intercepts of Lineweaver–Burk plots considering the enzyme activity measured as described above and different concentrations of substrates or cofactors.

Protein concentration was determined by the method of Bradford with a commercial assay kit (Bio-Rad, Hercules, CA, U.S.A.) using BSA as the standard.[22]

*Thermal stability.* To determine stability against thermal inactivation, the remaining activities of the purified wild-type and mutant GluDHs were measured at 37 °C after heat treatment at various temperatures ranging from 30 to 80 °C for 20 min, or at a specific interval of time (5 min).

*Homology modeling of Bs-GluDH and mutant Bs-GluDH.* A homology model of the 3D structure of the Bs-GluDH monomer was built using program MOE 2004.03: Homology Modeling (Chemical Computing Group Inc., Montreal, Canada). The Bs-GluDH model was developed from the Tl-GluDH structure (*Thermococcus litoralis*, PDB code 1BVU). There was intense interaction between the A and F subunits of homohexameric Tl-GluDH. The coordinates of both subunits were used. Five models were generated for each GluDH using complete optimization cycles, conjugate gradients, and simulated annealing. Model consistency was checked with the program PROCHECK v3.0.[23] Corrections were made with MOE rotameric libraries to avoid close contact of the side chains at other intersubunit interfaces. The quality of the model was evaluated further with the Protein Report of MOE. All other estimates of structural parameters were obtained with program MOE 2004.03

(Chemical Computing Group). The Bs-GluDH model follows the pattern of the structure, Tl-GluDH with conservation of the main features of the active-site region.

## Results

*Molecular properties of Bs-GluDH*

The gene encoding GluDH from *B. subtilis* ISW1214 was cloned, sequenced, and expressed at considerable magnitude in *E. coli*. The 1,388-bp nucleotide sequence was determined (data not shown). The ORF of the deduced nucleotide sequence composed of 424 amino acids of this enzyme differs from the GluDH of *B. subtilis* 168 (Bs168-GluDH, SWISSPROT: DHE2_BACSU, P39633) by the presence of alanine at amino acid position 324, replacing arginine in the latter. But, we found a reverse mutation (A324R) in compliance with the Bs168-GluDH amino acid sequence, and found that alanine in the 324 amino acid position has no effect on Bs-GluDH activity or stability.

Table 1 shows typical results of the purification of Bs-GluDH. The enzyme was purified to homogeneity about 39-fold with about 25% recovery. We identified 15 residues from the N-terminus of the Bs-GluDH by Edman degradation. The N-terminal sequence (MSAKQVSKDEEKEAL) exactly matched the amino acid sequence deduced from the nucleotide sequence. The purified Bs-GluDH migrated in SDS–PAGE as a single band with a mass of approximately 46 kDa (Fig. 1).

The estimated molecular mass by MALDI-MS was 46,587 Da, which agrees with the molecular mass calculated from the deduced nucleotide sequence, 46,553 Da. Analysis by Superdex 200 HR 10/30 column chromatography showed that the native molecular mass of this enzyme was approximately 270 kDa. A combination of all these analyses thus conclusively reveals that the enzyme is a hexamer composed of six identical subunits, and that the subunit structure is similar to those of other 50 kDa GluDHs.[24] The optimum pHs for L-glutamate deamination and 2-OG amination were pH 7.7 and 7.3 respectively. Half-maximal activity for deamination was observed at pH 7.2 and 8.0, and that for amination was at pH 6.9 and 7.8. Bs-GluDH is very specific for both $NAD^+$ and NADH, as no activity was observed when $NADP^+$ or NADPH was used as cofactor. Specificity for other amino acids, for example

**Table 1.** Purification of Recombinant GluDH from *B. subtilis*

| Step | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Purity (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 3,735 | 42,252 | 11.3 | 1 | 100 |
| $(NH_4)_2SO_4$ fractionation | 1,332 | 20,895 | 15.7 | 1.4 | 49.5 |
| DEAE-Toyopearl | 114 | 16,040 | 140 | 12 | 37.9 |
| Butyl-Toyopearl | 24 | 10,491 | 442 | 39 | 24.9 |

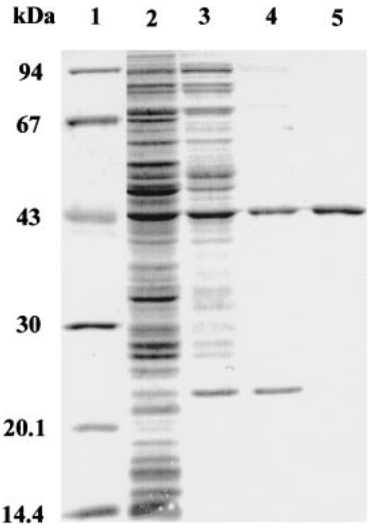


**Fig. 1.** SDS–PAGE of Purified Recombinant Bs-GluDH.
Lane1, molecular mass standard; 2, crude extract; 3, precipitation by ammonium sulfate; 4, DEAE-Toyopearl chromatography; 5, Butyl-Toyopearl chromatography.

L-aspartate, L-alanine, L-valine and L-serine, was absolutely absent, and specificity for other 2-oxoacids such as oxaloacetate, pyruvate, and 2-oxobutyrate was faint. The apparent Michaelis–Menten ($K_m$) constant of the deamination reaction was 0.34 mM for L-glutamate and 0.08 mM for $NAD^+$. The $K_m$ values of the amination reaction for 2-OG, $NH_4^+$, and NADH were 0.65 mM, 55.6 mM and 0.07 mM respectively. On the other hand, the $k_{cat}$ value of the deamination reaction was 18.1 $s^{-1}$, and the $k_{cat}$ value of the amination reaction was 342 $s^{-1}$. Thus the $k_{cat}/K_m$ of the amination reaction (535 $s^{-1}$ $mM^{-1}$) was 10-fold higher than for the deamination (53.2 $s^{-1}$ $mM^{-1}$) reaction in the case of 2-OG, suggesting that the enzyme might be turned to good use in amino acid production.

*Thermostability*

The thermal denaturation of Bs-GluDH was analyzed by measuring the remaining activity after incubation at various temperatures ranging from 30 to 50 °C at specific intervals of time (Fig. 2A). The denaturation processes up to 40 °C showed one-phase curves, while those at 45 °C and 50 °C showed two-phase curves. The $T_m$ value of Bs-GluDH was 41 °C, which means that 50% of its activity remains after incubation of the enzyme at 41 °C for 20 min. This was very low for a mesophilic enzyme. Heat inactivation was not found to be prevented by the addition of cofactor NADH (0.4 mM), even at its relatively higher protein concentration, which is dissimilar to that of alanine dehydrogenase from *Phormidium lapideum*.[25]

Stability was dependent on the protein concentration at low temperature. When different concentrations of the enzyme were incubated for 30 min at 4 °C, the enzyme showed higher stability at higher concentrations of protein (Fig. 2B), indicating that it is less prone to

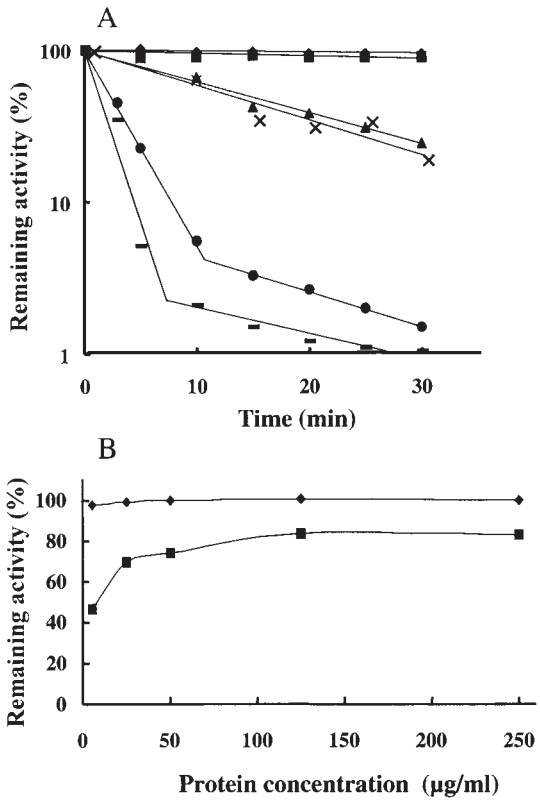


**Fig. 2.** Effect of Temperature and Various Protein Concentrations on the Activity and Stability of Bs-GluDH.

A, The activity of GluDH was measured after incubating the enzyme (10 μg·ml$^{-1}$) at various temperatures for 2.5–30 min (◆ 30 °C, ■ 35 °C, ▲ 37 °C, ✕ 40 °C, ● 45 °C, – 50 °C). B, Effect of protein concentration on Bs-GluDH activity. The remaining activity of the enzyme after incubation at 4 °C for 30 min with various concentrations of protein was estimated (◆ 0 min, ■ 30 min).

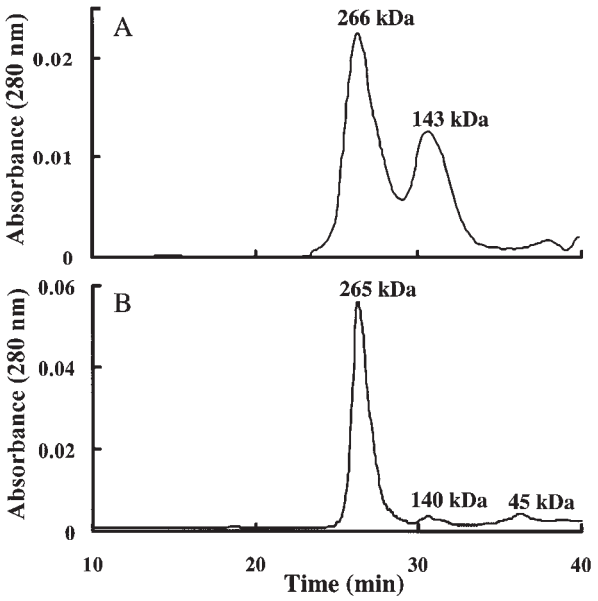


**Fig. 3.** Sequential Disintegration of Bs-GluDH Observed in Elution Profile of Gel-Filtration Chromatography after Incubation at 4 °C (A) and 45 °C (B).

The enzymes were incubated at 4 °C for 30 min at 10 μg·ml$^{-1}$ (A), and at 45 °C for 3 min at 3.05 mg·ml$^{-1}$ (B), and applied on a Superdex 200 HR 10/30 column (1 × 30 cm, bed volume: 24 ml) already equilibrated with 50 mM KP buffer containing 0.15 M NaCl. The flow rate of the buffer was 0.5 ml·min$^{-1}$, and the amount of sample applied was 200 μl. The enzyme, incubated at 4 °C for 30 min was also concentrated at 600 μg·ml$^{-1}$ by ultrafiltration (Centricon 30, Amicon), and recovered to 73% to of its original activity.

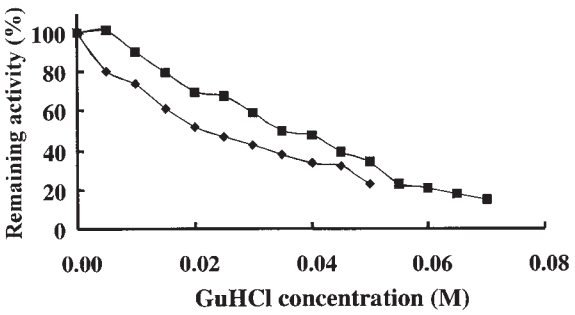


**Fig. 4.** Effect of Denaturant on Bs-GluDH Activity.

The enzyme at various concentrations of protein was subjected to a series of treatments by GuHCl, and the activity of the enzyme was measured at two concentrations of protein (◆ 10 μg ml$^{-1}$, ■ 100 μg ml$^{-1}$).

inactivation at higher concentrations of protein. The loss of enzyme activity at 4 °C at low protein concentrations was examined by gel filtration (Fig. 3A). Two protein peaks appeared that corresponded to molecular masses of 270 kDa and 143 kDa. Enzyme activity was located in the high molecular weight fraction (hexamer), while no detectable activity was found in the low molecular mass fraction (trimer). On the other hand, the enzyme incubated at 45 °C for 3 min was also applied to a gel filtration column following the same procedure, and the major peak, corresponding to the hexamer, was observed to contain 56% of its original activity, which remained in 62% of total protein (Fig. 3B). The other two minor peaks observed sequentially in the chromatogram were predicted to be trimers and monomers.

To understand the dependency of activity on protein concentration, an inactivation profile was also obtained by measuring enzyme activity in the presence of different concentrations of guanidine–HCl (GuHCl). Fifty percent activity was lost on treating the enzyme with 0.02 M and 0.035 M GuHCl, while the protein concentrations were 10 μg·ml$^{-1}$ and 100 μg·ml$^{-1}$ respectively (Fig. 4).

*Random mutagenesis of Bs-GluDH*

Wild-type plasmid containing the *rocG* gene was used as a template in the first round of mutagenic PCR to introduce random point mutation in the GluDH-encoding region. The thermostable GluDH variants expressed in *E. coli* that showed the highest activity were identified from enzyme libraries by a rapid and sensitive visual screening process. The mutant colonies, in the various rounds of random screening that exhibited the highest GluDH activity after incubation at various

elevated temperatures were selected for purification, and the purified GluDH was analyzed. In the mutant enzyme library evolved from the first round, the highest activity was recovered in the single mutant E27V (R1). Finally, the double mutants (E27V/W100R, R2) and (E27V/Q144R, R3), along with a triple mutant (E27V/Q144R/G255A, R4), were found in the 2nd, -3rd, -and 4th-round libraries respectively. All mutant enzymes had the E27V mutation.

The remaining activity of the enzymes from R1–4 was measured after treating it at various temperatures for 20 min. Although the enzymes from R1 and R2 did not show high thermostability, those from R3 and R4 were enhanced significantly. To understand the significant mutation that contributed to the thermostability of the R3 and R4 mutant enzymes, a series of single mutant was constructed taking each site of mutation. The remaining activity, measured after treating the mutant enzyme candidates at 50 °C and 60 °C for 20 min respectively, was found to be highest for Q144R, a common site of mutation in double and triple mutants evolved from 3rd- and 4th-round mutagenesis. Although the mutant candidate E27V constructed showed low thermostability, we considered it for further experimentation since it also possessed a common mutation site found in both the 3rd and 4th rounds of mutagenesis. The other two sites, W100R and G255A, neither showed significant remaining activity nor appeared to be candidates for consideration.

*Thermostabilities of mutant GluDH variants*

To understand better the significance of the mutation sites that contributed to the thermostability of R3 and R4, Gln144 and Glu27 were replaced by a number of other amino acids that gave rise to a series of single mutants (Q144R, Q144D, Q144E, Q144K, Q144H, Q144N, Q144A, Q144L, Q144C, E27F, E27K, E27R, and E27A). By a preliminary experiment on the thermostability of these mutant enzymes, it was found that Q144R, Q144C, Q144D, Q144K, E27F, E27K, and E27V showed some degree of thermostability. These mutant enzymes were purified to homogeneity and the thermostabilities of the mutant enzymes were estimated. Figure 5A shows the remaining activities after treating the mutant enzymes at various temperatures for 20 min. Table 2 shows the estimated $T_m$ values of the wild type, R4, and those mutants estimated from the curves in Fig. 5A. Among the single mutants, those which were subjected to scrupulous investigation, Q144R, Q144C, Q144D, E27F, and E27K, were found to have superior thermostability, with $T_m$ values of 61 °C, 47 °C, 43 °C, 49 °C, and 43 °C respectively.

*Steady-state kinetic parameters of wild-type and thermostable mutant GluDH variants*

The mutant enzymes Q144R and E27F, which showed enhancement of thermostabitility 20 °C and 8 °C higher than that of the wild type respectively, were further

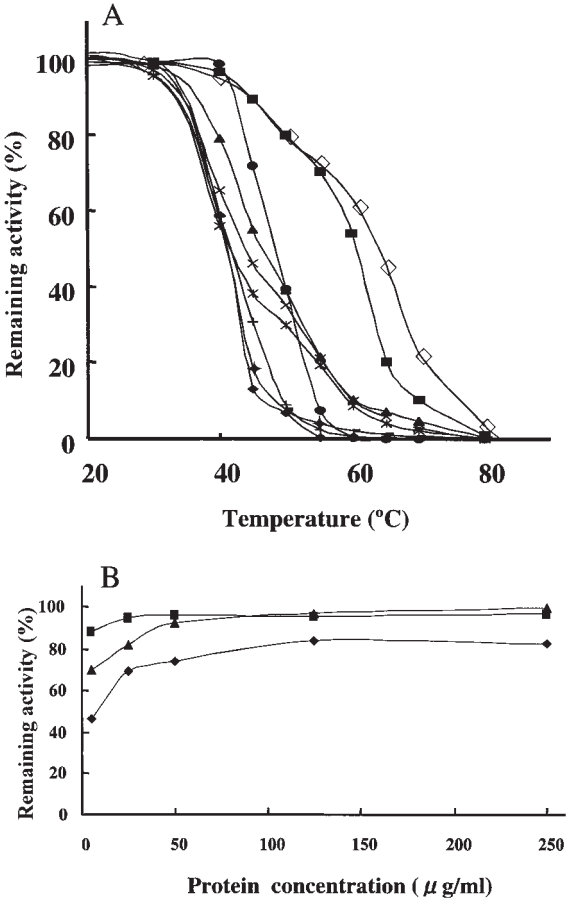


**Fig. 5.** Effect of Temperature and Protein Concentration on the Stability and Activity of Wild-Type and Various Mutant Bs-GluDHs.

A, Effect of temperature on the stability of wild-type Bs-GluDH and mutant enzymes obtained from R4 and various single mutant Bs-GluDHs. Each purified enzyme diluted to the same concentration (10 μg·ml$^{-1}$) with 50 mM KP buffer (pH 7.3) and incubated at various temperatures for 20 min. The remaining activities were expressed as percentages of the original activities after incubation of the enzymes at various temperatures (◆ Wild type, ◇ R4, ■ Q144R, ▲ Q144C, ✕ Q144D, ✳ Q144K, ● E27F, + E27K, ✦ E27V). B, Effect of protein concentration on the activity of wild-type Bs-GluDH and the single mutants Q144R and E27F (◆ Wild type, ■ Q144R, ▲ E27F).

**Table 2.** $T_m$ Values of Wild-Type, R4, and the Single Mutant Enzymes Resulting from Replacement of Glu and Gln at the 144 and 27 Positions Respectively

| Enzyme | $T_m$ | $\Delta T_m$* |
|---|---|---|
| Wild-Type | 41 | 0 |
| R4 | 63 | 22 |
| Q144R | 61 | 20 |
| Q144C | 47 | 6 |
| Q144D | 43 | 2 |
| Q144K | 40 | −1 |
| E27F | 49 | 8 |
| E27K | 43 | 2 |
| E27V | 42 | 1 |

*Difference in $T_m$ value between mutant enzyme and wild type.

**Table 3.** Steady-State Kinetic Parameters of Wild-Type, Q144R, and E27F Mutant Enzymes in Their Amination Reactions

| Enzyme | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) | | | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) | |
|---|---|---|---|---|---|---|
| | | NADH | 2-OG | $NH_4Cl$ | NADH | 2-OG |
| Wild-Type | 342 | 0.07 | 0.65 | 55.6 | 4,890 | 535 |
| Q144R | 435 | 0.41 | 1.22 | 56.8 | 1,060 | 356 |
| E27F | 344 | 0.16 | 0.93 | 52.3 | 2,150 | 270 |

All assays were performed at 37 °C and pH 7.3.

studied for their steady-state kinetic parameters. The kinetic parameters of the wild-type, Q144R, and E27F enzymes are shown in Table 3. Although the $k_{cat}$ value of E27F was almost the same as the value of the wild-type, the value of Q144R was considerably higher than that of the wild-type. But the $K_m$ values for NADH and 2-OG of the wild-type were lower than those of mutant Q144R and E27F. The $K_m$ value for 2-OG of Q144R was double and that of E27F was about one and half times higher than that of the wild-type. Therefore, the $k_{cat}/K_m$ values for 2-OG of Q144R and E27F were 67% and 50% of that of wild type respectively. The remaining activities of wild-type and mutant enzymes after incubation at 4 °C for 30 min at various protein concentrations were measured (Fig. 5B). Q144R and E27F mutant enzymes from low to high protein concentrations showed higher magnitude of remaining activities than that of wild-type.

## Discussion

Although much information has been gained about the crystal structures of GluDHs from mesophilic and thermophilic microorganisms[26,27] and an account of the high thermostability of thermophilic GluDH has been given,[27–29] relatively few successful studies have been devoted to characterizing the thermostability of GluDHs from mesophilic bacteria. The unstable character of GluDH in *Bacillus* species was reported in the past decade. Conflicting reports on the presence or absence of NAD-dependent GluDH in *Bacillus* strains a decade ago might have been due to the extremely rapid loss of activity of the enzyme in the cold.[30] Protein concentration did not affect the loss of enzyme activity of NAD-dependent GluDH from *B. cereus* DSM 31 in the cold, and no reactivation of the enzyme was observed upon incubation of cold-inactivated cell-free extract at 30 °C for 5 min.[31] On the other hand, the unfolding of the protein structure of GluDH from *B. acidocaldarius* at higher concentrations of GuHCl underwent refolding when the unfolded mixture was diluted.[32] GluDH hexamer from bovine liver, baker's yeast, and *C. symbiosum* dissociate to form trimers, which then dissociate to monomers at higher concentrations of GuHCl.[33] Dissociation to trimers is accompanied by a reversible loss of enzyme activity, but no gross structural changes can be detected by fluorescence or CD. Thus similarly to the GluDHs discussed above, the Bs-GluDH hexamer might have dissociated to trimers (first phase), and then the trimers might have dissociated to monomers (second phase), when incubated at 45 °C or 50 °C (Fig. 2A, Fig. 3B). On the other hand, the Bs-GluDH hexamer might have dissociated to trimers (one-phase process) after incubation at 4 °C at a low protein concentration (Fig. 2B, Fig. 3A).

A comparison of GluDHs between thermophiles and counter mesophiles according to multiple considerations suggests that thermostability is an attribute that arises from the subtle addition of many different contributing factors.[34,35] Rice *et al.* reported that hyperthermophilic GluDH contains a striking series of networks of ion-pairs which are formed by regions of the protein which contain a high density of charged residues.[36] Such regions are not found in the mesophilic enzymes, and the number and extent of ion-pair formation is much more limited. The ion-pair networks are clustered at both inter-domain and inter-subunit interfaces, and might well represent a major stabilizing feature associated with the adaptation of enzymes to extreme temperatures.[36] In Pf-GluDH, the most dramatic ionpair cluster lies at the interface between the two-fold axis relating dimers (dimer AF-dimer CD) in this hexameric protein, and involves the linkage of 18 charged residues.[37] On the other hand, the number of inter-subunit ion-pairs in Pi-GluDH molecules is much smaller than those in Pf-GluDH.[38] The number of hydrophobic interactions at the inter-subunit interfaces (A–B and A–F) increased and were responsible for the extremely high thermostability of Pi-GluDH.[38] Based on analyses from the homology modeling exercise, we predicted that the low thermostability of Bs-GluDH was due to small inter-subunit (A–F) interactions, particularly fewer hydrogen bonding and ion-pairing interactions.

Our experimental results indicate that the Q144R mutation in Bs-GluDH contributes greatly to the thermostability of the R4 mutant enzyme. Glutamine is a polar residue, and its replacement by arginine, a positively charged amino acid residue, probably best tones with negatively charged residue. The occurrence of prevalent ion pairing by the charged residues in the inter-subunit region contributed by the side-chain molecular structure of the arginine residue in thermophilic Pf-GluDH[36] stirred us to search for any possibility of ion pairing that might indicate a solution to the inadequate ion pairing of Cs-GluDH and the consequent relatively low thermostability. The structure of Bs-GluDH (by homology modeling) shows that amino acid sequence position 144 is near the center of the two-fold axis of Bs-GluDH dimer (A–F), which is important for the maintenance of hexameric structural integrity (Fig. 6). Although two Gln144 residues are in face-to-face position to each other, no interaction between the two residues was found. This key fact emerged from the formation of two hydrogen bonds by two-counterpart arginine residues, and also by those of Ala141 residues belonging to each subunit of the dimer, which was found

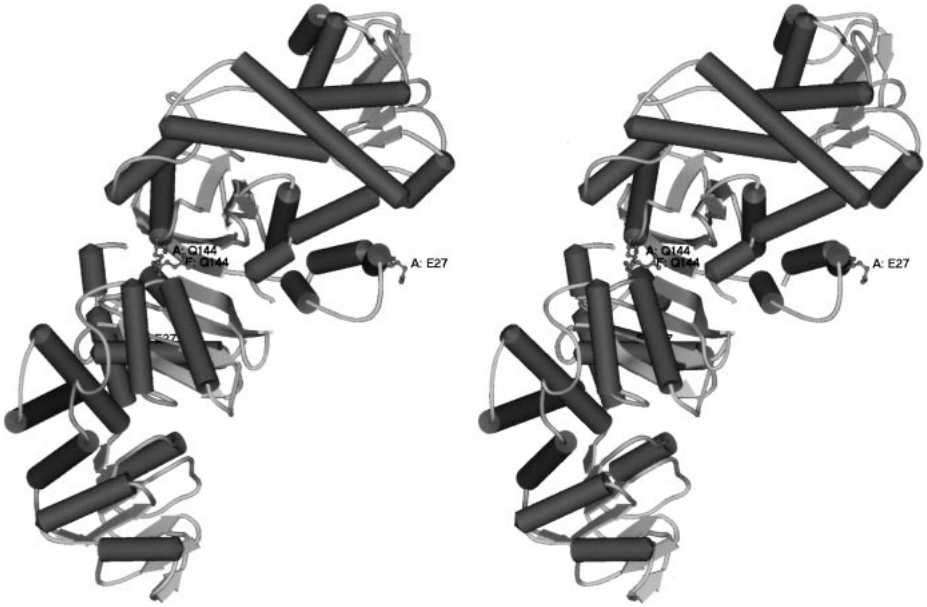


**Fig. 6.** Stereo Images of a Three-Dimensional Structural Model of Bs-GluDH Indicating the Location of Gln144 and Glu27 in the A–F Dimer of Bs-GluDH.

The 144th and 27th residues are shown as ball-and-stick models.

as the only possible prediction from our model. Although the $T_m$ of the Q144R mutant enzyme is nearly the same as that of Cs-GluDH,[36] the equivalent position of Arg144 in the Bs-GluDH Q144R mutant is Arg153 in Cs-GluDH, which, unlike the A–F dimer, makes inter-subunit interaction by hydrogen bond formation between the A–D dimer (Arg153NH1-Ile183.O and Arg153NH2-Lys182.O). However, based on our model, we expect that in the inter-subunit interaction, Arg144 forms hydrogen bonds with its identical counterpart in the A–F dimer, or it might additionally form interactions in the A–D dimer like that of Cs-GluDH, which might be detectable by a more precise model. In thermophilic proteins, the number of side-chain hydrogen bonds is increasingly high and the proportion of thermostable residues like Arg and Tyr increases with a significant decrease in thermolabile residues (Cys and Ser), as compared to their mesophilic homologs.[39] Two hydrogen bonds formed by two counterpart Arg144 residues and Ala141 probably retain the dimer resulting in a hexamer in an intervening manner. The replacement of Gln by other possible amino acids, including lysine, probably does not ensure fine-tuned hydrogen bonding interaction with the counterpart residue.

We achieved GluDH mutation that exhibited both increased activity and increased thermostability, although sometimes stabilization of an enzyme is achieved with a significant loss of catalytic activity.[40] However, the $K_m$ values for 2-OG and NADH of Q144R is two times and six times higher respectively than that of the wild type. It has been suggested that high flexibility of an enzyme is tightly correlated with increased thermolability of the enzyme. Although the reason behind the increased catalytic activity of Q144R was not clear, the high $K_m$ value for NADH of Q144R might be due to reduction of flexibility of the dimer structure.

The E27F mutation also contributes to enhancement of thermostability in Bs-GluDH. Position 27 (Fig. 6) locates near the hinge region in the interface of the two domains, containing conserved amino acid residues. The hinge region is thought to regulate the open and closed conformations of GluDH and assumed to play an important role in determining the activity and stability of the enzyme.[41] A certain level of conformational stability is compromised while the enzyme maintains its native conformation and conformational flexibility. In order to function, the regions of the GluDH enzyme structure mainly responsible for conformational change, such as the hinge region, account for the optimization of catalysis.[42] The increased thermostability of E27F and E27V might be due to the increased packing density that results in reduced cavities in the hydrophobic core near the hinge region, although no incensed interaction in the E27F subunit was found from the homology modeled structures of E27F and E27V.

Due to both the enhanced thermostability and the catalytic activity of Q144R, we concluded that the Q144R Bs-GluDH gene is suitable as a template gene for modification of its substrate specificity for industrial use by using molecular evolutionary engineering.

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