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Note

Purification and Characterization of the First Archaeal Glutamate Decarboxylase from *Pyrococcus horikoshii*

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Glutamate decarboxylase (GAD) from the archaeon *Pyrococcus horikoshii* was successfully expressed and purified, with the aim of developing a hyperthermostable GAD for industrial applications. Its biochemical properties were different from those reported for other GADs. The enzyme had broad substrate specificity, and its optimum pH and temperature were pH 8.0 and >97 °C.

Key words: decarboxylase; GABA; glutamate decarboxylase; archaeon

Glutamate decarboxylase (GAD: EC 4.1.1.15) is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that belongs to the subset of PLP-dependent enzymes classified as group 2, which catalyse the irreversible α-decarboxylation of L-glutamate to yield γ-aminobutyric acid (GABA) and CO_2. GABA, which is widely distributed in nature, is a well-known inhibitory neurotransmitter in the mammalian central nervous system.[1,2] Its physiological function has been demonstrated to be the induction of hypotensive, diuretic, and tranquilizing effects. Moreover, GABA is a strong secretagog of insulin from the pancreas.[3,4] Because of these functions, we are interested in the production of GABA for industrial fields such as foods, nutraceuticals, and pharmaceuticals.

GAD has been found in animals and plants and in various micro-organisms,[5–9] but, to date, no GAD has been reported in hyperthermophilic archaea. Because of their promise for industrial use, hyperthermophilic GADs have long been anticipated. In the genome data of *Pyrococcus horikoshii*, *P. furiosus*, and *Aeropyrum pernix*, GAD homolog genes have been observed. We attempted to express GAD homologs from these hyperthermophilic archaea using an *Escherichia coli* expression system. Among them, only *P. horikoshii* GAD (referred to below as PhGAD) was expressed in *E. coli* cells.

As a genetic source of industrial enzymes, *Pyrococcus horikoshii* is a well-known hyperthermophilic archaeon. Many hyperthermophilic enzymes from this archaeon have been elucidated in the development of commercial enzymes and fundamental research. The complete sequence of the *P. horikoshii* genome has been reported by Kawarabayasi *et al.*[10,11] A PH0937 gene has been annotated as the gene encoding GAD. A BLAST search indicated that this protein sequence has high identities with those of group 2 decarboxylases of archaea such as *P. abyssi*, *P. furiosus*, and *Thermococcus kodakarensis*, but, the GAD protein has low homology to that of *E. coli* GAD, pig DOPA decarboxylase, *Neurospora crassa* GAD, and human GAD (21%, 17%, 14%, and 13% identity respectively). Multiple alignments of these protein sequences showed conserved amino acids containing a lysine residue forming an internal aldimine, which is responsible for interactions with cofactor PLP (Fig. 1).

To obtain the gene coding PH0937, we prepared genomic DNA from *P. horikoshii*, as described previously.[12] The PH0937 gene was amplified by 35 cycles of polymerase chain reaction (denaturation at 96 °C for 30 s, annealing at 50 °C for 30 s and extension at 74 °C for 1 min) using KOD DNA polymerase (Toyobo, Kyoto, Japan) with the genomic DNA as the template. The oligonucleotides 5′-GGAATTC**CATATG**AAGTTTCCCAGGATTGGAC-3′ and 5′-CG**GGATCC**TCAAGATAGGACCTCCTTTAAGTC-3′, containing the recognition sites for *Nde*I and *Bam*HI (bold), were used as primers. The PCR-amplified DNA fragment was cloned into expression vector pET11a (Novagen, Madison, WI). The resulting plasmid was introduced into *E. coli* strain Rossetta (DE3) (Novagen). The transformant was cultured in LB broth containing 100 μg/ml ampicillin at 37 °C until the absorbance at 600 nm reached 0.2, and then isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added (0.1 mM) for expression induction before cultivation at 30 °C overnight. To purify the PhGAD produced, the cultured cells were suspended in 20 mM Tris buffer (pH 7.5) and then disrupted by ultrasonication. After centrifugation (10,000 × *g*, 10 min) of the cell extract, supernatant fractions were analyzed by their enzyme activity and SDS–PAGE, but, no enzyme activity was detected in the supernatant. The expressed target protein was observed in the precipitant fraction without activity.

P. horikoshii is a hyperthermophilic archaeon growing optimally at a high NaCl concentration, 400 mM.[13] Although the salt concentration in its intracellular environment is not known, the related species *P. furiosus* contains a high intracellular salt concentration that resembles the optimal cultivation condition.[14] We assumed that PhGAD requires salt ions or high ionic strength and initially examined protein solubilization and enzyme activity using 400 mM NaCl. The results indicated that PhGAD was easily solubilized, and it was recovered in an activated form. Thus, this insoluble protein was confirmed to be a simple aggregated form, not an inclusion body.

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Fig. 1. Multiple Sequence Alignment of Group 2 Decarboxylases from Various Sources.

The amino acid sequences of *P. horikoshii* GAD (PhGAD, PH0937), *E. coli* GAD (EcGAD, NP_287662), pig DOPA decarboxylase (DDC, NP_999019), *Neurospora crassa* GAD (NcGAD, XP_965818), and human GAD (HsGAD65, NP_000809) were aligned with the CLUSTAL W program using default parameters. The conserved lysine residue that binds cofactor PLP is indicated by an arrow. An asterisk (*) denotes that residues at that position are exactly same. A colon (:) indicates that residues at that position are very similar. A dot (.) indicates that the residues are more or less similar.

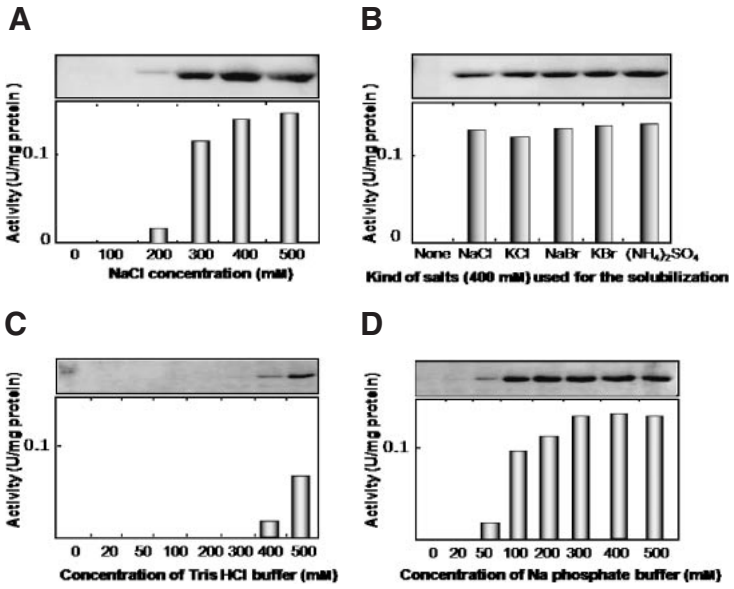

Fig. 2. Effects of Various Salt Materials and Ionic-Strength on the Solubility and Activity of Recombinant PhGAD.

A, Effects of NaCl concentration on enzymatic recovery. B, Enzymatic recovery using various salt materials. C and D, Effects of the concentrations of Tris HCl and sodium phosphate buffer on enzymatic recovery. PhGAD was eluted from the precipitate of the cell lysate using 50 mM Tris–HCl (pH 7.5) containing 0.25 mM PLP and the indicated salt. The eluted protein was analyzed by SDS–PAGE (upper column). Then the GAD activity was measured as described in the text (lower column).

Figure 2 shows the relationships between enzyme solubility and activity under various buffer conditions. Both protein solubilization and activity increased with increasing concentrations of NaCl up to 300 mM (Fig. 2A). However, GAD activity at over 500 mM decreased slightly (data not shown). We further examined enzymatic recovery of PhGAD with respect to the requirement for cations, anions, and a salt using various salt materials. No difference in enzymatic recovery was observed as compared with NaCl (Fig. 2B). Moreover, a sodium phosphate buffer with higher ionic strength was remarkably more efficient in enzymatic recovery than Tris buffer, and it showed concentration-dependent characteristics (Fig. 2C and D). Accordingly, we con-

Table 1. Substrate Specificity of PhGAD from *P. horikoshii*

Substrates	*K*m (mM)	Specific activity (*k*cat) (1/s)
L-glutamate	3.9 ± 0.4	0.26 ± 0.03
L-aspartate	1.2 ± 0.1	0.34 ± 0.02
Cysteate	2.2 ± 0.3	0.65 ± 0.07
Cysteine sulfite	32.6 ± 5.4	0.03 ± 0.00
L-tyrosine	ND	ND
D-glutamate	ND	ND
D-aspartate	ND	ND

ND, No activity was detected.

firmed that enzymatic recovery from the aggregate was affected by ionic-strength-dependent solubility, and the solubilized GAD exhibited activity.

Purification of PhGAD was performed using 20 mM Tris buffer (pH 7.5) containing 0.4 M NaCl and 1 mM PLP following heat treatment (for 30 min at 85 °C). Ammonium sulfate was dissolved into the supernatant after heat treatment to 1.7 M, and applied to a HiTrap phenyl HP(Amersham, Piscataway, NJ) chromatography column equilibrated with 20 mM Tris–HCl (pH 7.5) containing 0.25 mM PLP, 0.4 M NaCl, and 1.7 M ammonium sulfate. The enzyme was eluted using a linear decrease in the ammonium sulfate concentration in the buffer from 1.7 to 0 M. The enzyme solution was collected and dialyzed against 20 mM Tris–HCl (pH 7.5) containing 0.25 mM PLP and 0.4 M NaCl. The purified enzyme ran as a single protein band as analyzed by SDS–PAGE, indicating that it had been purified to homogeneity.

The molecular weight of the enzyme, which was about 43 kDa based on DNA sequence, was estimated to be 45 kDa and 42 kDa by gel filtration and SDS–PAGE, respectively. The structure of PhGAD appears to be monomer. In contrast, most GADs reported in literature exist in a multimeric form, such as dimer or hexamer.[8,15] Only GAD from the fungus *Neurospora crassa* has been reported to be expressed in monomer form.[16]

GAD activity was measured using a modified version of the method described by Okada *et al.*[17] The reaction mixture (0.1 ml) consisted of 50 mM glutamate, 0.25 mM PLP, 0.4 M NaCl, and 0.1 M sodium phosphate (pH 8.0). The enzyme reaction was performed by incubating the mixture for 60 min at 85 °C. Then 20 µl aliquots of the mixture were dried and treated with phenyl-isothiocyanate. The resulting phenylthiocarbamoyl-gamma-aminobutyric acid was measured using a high-performance liquid chromatography system (HPLC; Tosoh, Tokyo) with a column (4.6 × 150 mm, Wakosil-PTC; Wako Pure Chemical Industries, Kyoto). For the substrate specificities of PhGAD, the decarboxylation activities toward L-aspartate, cysteinate, L-tyrosine, and cysteine sulfinate were determined by measuring the concentrations of the decarboxylation products of their respective reactions under the same reaction conditions: β-alanine, taurine, tyramine, and hypotaurine. Compared with the decarboxylation activities toward the various substrates, the specific activities toward cysteate and L-aspartate represented slightly higher values than L-glutamate; no activity against L-tyrosine was found (Table 1).

The range of pH at which PhGAD was active and stable was determined with glutamate as a substrate. GAD activity was observed at maximum at pH 8.0, and was stable at weak alkalinity, of 8.0–8.5. The optimum temperature of the enzyme was higher than 97 °C, and its activity was very stable against heat inactivation. It survived for prolonged periods at 85 °C. Its half-life at 95 °C was 3 h.

GAD enzymes have been reported from widely varied organisms, from bacteria to mammals. Bacterial and plant GADs share similar features in their biochemical properties and their functions, such as specific activity against glutamate, acidic optimal pH, and their response mechanisms to environmental stress.[8,18] In contrast to these enzymes, PhGAD showed biochemically broad substrate specificity and optimal activity under weak alkali pH conditions. Although its amino acid sequence did not share homology with that of the mammalian enzyme, these biochemical properties closely resembled those of the mammalian enzyme, which has a neutral optimal pH, and also showed activity against various substrates, such as cysteate, cysteine sulfite, and L-aspartate-like PhGAD.[19,20] As a representative function of bacterial GAD, *E. coli* GAD is known to be involved in resistance to acidic environments, but PhGAD might be not relevant to this function in view of its alkaline optimal pH. Consequently, the biochemical properties of PhGAD suggest that the enzyme participates in the metabolic pathways of various amino acids according to the internal milieu of the host cell.

This is the first report on archaeal hyperthermophilic GAD. This enzyme has lower GAD activity than other GADs, but its hyperthermostability is fascinating in light of its potential for the industrial production of GABA. However, we continue our studies to improve the activity of PhGAD using protein engineering techniques.

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