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ORIGINAL ARTICLE

Overexpression and Characterization of Recombinant Glutamate Decarboxylase from *Thermococcus kodakaraensis* KOD1

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Abstract Glutamate decarboxylase (GAD) (EC 4.1.1.15) catalyzes decarboxylation of glutamic acid to produce gamma-aminobutyric acid (GABA). A putative *gad* gene (tk1814) from an archaeon *Thermococcus kodakaraensis* KOD1 was cloned and transformed into *Escherichia coli* to produce a bulk amount of recombinant GAD. Activity of the purified GAD was optimal at 90°C and pH 8.0. Optimal concentration of substrate for conversion into gamma-aminobutyric acid by recombinant GAD was 50 mM monosodium glutamate. Recombinant GAD was confirmed to be monomeric, and its activity was greatly inhibited by various salts such as sodium chloride, Tris-HCl, and sodium phosphate. *K*m, *V*max, and *K*cat values were 9.92 mM, 153.8 μmol min^{-1} mg^{-1}, and 6.613×10^3 min^{-1} respectively.

Keywords gamma-aminobutyric acid · glutamate decarboxylase · *Thermococcus kodakaraensis* KOD1

Introduction

γ-Aminobutyric acid (GABA) is a non-protein amino acid widely distributed in microorganisms, plants, and animals (Ueno, 2000), as well as in hydrothermal systems (Svensson et al., 2004). GABA eminently contributes to physiological functions such as neurotransmission, induction of hypotension, and diuretic and tranquilizer effects (Ting Wong et al., 2003). GABA is also employed for its positive effects in treatment of insomnia, depression, autonomic disorders (Okada et al., 2000), chronic alcohol-related symptoms, and stimulation of immune cells (Oh et al., 2003). Moreover, GABA has anti-inflammation and fibroblast cell proliferation activities that increase the curative processes in serious injuries and wounds (Han et al., 2007). It is also a strong stimulator of insulin from the pancreas and successfully prevents diabetic conditions in humans (Hagiwara et al., 2004). Recent studies have suggested that GABA could improve the concentrations of plasma-growth hormone and the rate of protein synthesis in the brain (Tujioka et al., 2009), as well as inhibit small airway-derived lung adenocarcinoma (Schuller et al., 2008). GABA is present at very low concentrations in the temporal cortex and cerebellum of patients with Alzheimer's disease (Seidl et al., 2001). Furthermore, in microbes such as bacteria and fungi, GABA has a very prominent role in pH tolerance and ATP production (Small and Waterman, 1998).

Due to contribution of GABA to enhance physiological functions and its potential role as a bioactive component in foods items and pharmaceuticals, the development of functional foods containing GABA has been extensively studied. Food items containing GABA, such as tea (Abe et al., 1995), red mold rice (Rhyu et al., 2000), germinated wheat (Nagaoka, 2005), soy product (Aoki et al., 2003; Tsai et al., 2006) and rice germ (Zhang et al., 2006) have been developed. The utilization of GABA-containing foods has been investigated to reduce high blood pressure in humans (Inoue et al., 2003). Glutamate decarboxylase (GAD: EC 4.1.1.15), which catalyzes the decarboxylation reaction of L-glutamic acid to produce GABA, has been used extensively in pharmaceuticals and food industry (Abe et al., 1995). GAD can be produced by numerous microorganisms including bacteria (Capitani et al., 2003), fungi (Su et al., 2003) and yeasts (Masuda et al., 2008).

Heat stability is major consideration of most enzymes used in the various industries. Because most of the identified GADs were mostly isolated from mesophiles, no GAD from extremophile was reported except for an enzyme from *Pyrococcus horikoshii* (Kim et al., 2009). *Thermococcus kodakaraensis* KOD1 is a Gram-

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negative archaeon belonging to the extreme thermophilic family *Thermococcaceae,* and can survive at temperatures up to 100°C (Atomi et al., 2004). In order to extend knowledge of thermostable GAD, a gene encoding GAD (TK1814) was cloned, expressed, and purified in *Escherichia coli.* The present study is concerned with the design and calibration of a recombinant GAD-producing system to achieve goals such as the production of highly heat stable GAD for industrial use. especially for pharmaceutical and fermentation industries, and the establishment of easy approaches to produce recombinant GAD in bulk amounts.

Materials and Methods

Cloning and Expression. A gene sequence encoding GAD of *T. kodakaraensis* KOD1 (TK1814) was obtained from the National Center for Biotechnology Information (NCBI) database. 1,155-bp of the DNA fragment of the gene was obtained from a DNA-synthesizing company (GenScript Co., Piscataway, NJ). The *gad* gene fragment was ligated into the pET21a(+) expression vector (Novagen, Madison, WI), and the generated plasmid pETK1814 was transformed into *E. coli* Rosetta (DE3) (Novagen).

Expression and purification of recombinant GAD. Recombinant *E. coli* Rosetta (DE3) containing pETK1814 was inoculated into Luria-Bertani (LB) medium in the presence of 100 mM of ampicillin. When the culture reached an A600 of 0.5, protein expression was induced by incubation in the presence of 1 mM isopropyl-thio-β-D-galactopyranoside for 4 h, after which the cells were harvested. The cell pellet was resuspended in lysis buffer containing 20 mM sodium phosphate (pH 7.4), 0.5 M NaCl and 25 mM imidazole. After sonication, the cell debris was removed by centrifugation at 4°C and the soluble crude extract injected into Ni-NTA-packed column (Qiagen Inc., Valencia, CA). Following washing with 10 column volumes of lysis buffer, all subsequent elution steps were carried out at 25°C. The bound protein was eluted with 2 column volumes of 100, 200, 300, and 500 mM and 1 M imidazole in elution buffer containing 20 mM sodium phosphate (pH 7.4) and 0.5 M NaCl. The protein in the eluted fractions was visualized with sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), and protein concentrations were measured using Bradford dye (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Gel filtration chromatography was carried out on a Superdex-200 HR 10/30 column (GE Healthcare Biosciences, Little Chalfont, UK) equilibrated with buffer containing 50 mM Tris-HCI (pH 7.4) and 1 mM dithiothreitol (DTT). The eluent was monitored at 280 nm. The standard proteins (GE Healthcare Biosciences) and their molecular weights were: catalase, 232 kDa; aldolase, 158 kDa; BSA, 67 kDa; ovalbumin, 43 kDa, and myoglobin, 17 kDa. Protein samples were flash-frozen in liquid nitrogen and stored at –70°C.

GAD activity test. Activity of GAD was determined by the method of Li using pre-staining paper chromatography (Li et al., 2009). The purified enzyme (0.27 mg/mL) was incubated with 50 mM L-glutamate and 1 mM pyridoxal-5'-phosphate (PLP) in 0.1 M HEPS buffer, pH 8.0 at 90°C for 1 h. After incubation, the reaction was stopped by placing the reaction mixture in ice for 10 min, and the concentration of GABA was analyzed by pre-staining paper chromatography. Each 2 μL of samples were spotted onto the filter paper (No. 2, Whatman, Little Chalfont, UK) and developed at 30°C with *n*-butyl alcohol : acetic acid : water (5 : 3 : 2) containing 1.2% (w/v) ninhydrin. After development, the paper was dried for color yield at 70°C in the convection oven. The GABA spots on the paper were carefully cut out and eluted with 5.0 mL of 75% alcohol (v/v): 0.6% cupric sulfate (w/v) (38 : 2, v/v) at 40°C for 1 h, and the absorptions were read at 512 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of GABA in 1 min.

Effect of temperature and pH on GAD activity. The optimum temperature of GAD activity was determined during 1 h exposure of 0.36 U of purified enzyme per 1 mL reaction mixture to a temperature range of 60 to 99°C in 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.0) with 50 mM of glutamate and 1 mM of PLP. The thermal stability of the GAD was determined by incubating 0.36 U of purified enzyme per 1 mL PLP in a temperature range of 70 to 90°C up to 16 h, and the residual activity of the enzyme was measured at 90°C. The optimum pH of GAD activity was determined within a range of pH 2.5 to 12.0 in 0.1 M pyridine-HCl buffer (pH 2.0–5.0), 0.1 M citrate-phosphate buffer (pH 5.0–6.0), 0.1 M HEPES-NaOH (pH 6.0–9.0), 0.1 M Glycine-NaOH buffer (pH 9.0–10.0), and 0.1 M sodium phosphate-NaOH (pH 10.0–12.0) supplemented with 50 mM of glutamate and 1 mM PLP at 90°C. The pH stability of the enzyme was determined by incubation with each buffer at various pH ranges for 1 h at 4°C, and its residual activity was measured.

Determination of Enzyme Kinetics. The kinetic behavior of GAD was determined by measuring the initial rate of L-glutamic acid conversion under the conditions of 0.1 M HEPES buffer (pH 8.0) at 90°C for 1 h incubation. Various substrate concentrations of L-glutamate were incubated with the recombinant GAD, and the reaction was stopped after 10, 20, 30, 40, 50, and 60 min to ensure measurements in the linear range. The enzyme K_m, V_{max}, and K_{cat} values were calculated directly using a Lineweaver-Burk plot.

Conversion Rate Calculation of L-Glutamate to GABA. To estimate the conversion rate of monosodium glutamate (MSG) to GABA, 36 units of the enzyme (27 mg) were added to 100 mL of reaction mixture containing a series of different substrate concentrations (10, 50, and 100 mM MSG) along with 0.1 M HEPES buffer (pH 8.0) and 1 mM PLP. The incubation temperature was 80°C, and the incubation time lasted up to 24 h.

Effects of PLP and Salts on GAD Activity. To determine the effects of different concentrations of salts and PLP on GAD activity, the purified recombinant GAD was incubated with substrate solution containing 0.1 M HEPES buffer (pH 8.0), 50 mM L-glutamic acid, and various concentrations of (0–1 mM) PLP (Dutyshev et al., 2005) at 90°C for 1 h. To investigate the effect of various salts on the GAD activity, reaction mixture

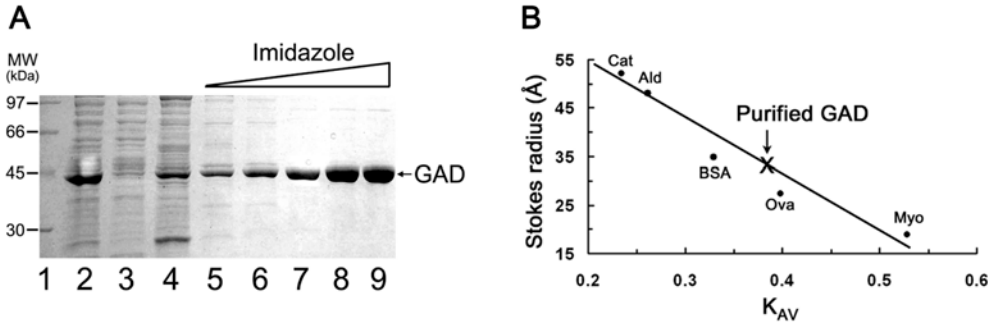

Fig. 1 Purified recombinant GAD from *T. kodakaraensis*. Panel A, 10% SDS-PAGE of His-tagged GAD protein purifying steps. Lane 1, Phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (30 kDa) as protein molecular weight markers; lane 2, crude recombinant *E. coli* extract; lane 3, unbound proteins; lane 4, washing with lysis buffer; and elutions with 100 (lane 5), 200 (lane 6), 300 (lane 7), 500 mM (lane 8), and 1 M (lane 9) imidazole. Protein position at the expected molecular mass of the 6 His-tagged tkGAD is indicated on the right. Panel B, Size exclusion chromatography. Purified protein were loaded onto a superdex-200 gel filtration column and analyzed as described under "Experimental Procedures." The stokes radius positions of catalase (Cat, 232 kDa, 52.2Å), aldolase (Ald, 158 kDa, 48.1Å), bovine serum albumin (BSA, 67 kDa, 34.9Å), ovalbumin (Ova, 43 kDa, 27.5Å), and myoglobin (Myo, 17 kDa, 19.0Å) are indicated. Elution position of the purified GAD is indicated as X.

containing 200 mM of NaCl, 200 mM Tris-HCl (pH 8.0), and 200 mM sodium phosphate (pH 8.0) was used.

Results

GAD Gene (tk1814) Cloning and Expression. The putative amino acids sequences of the GAD from *T. kodakaraensis* KOD1 shared 75% identity and 89% similarity to GAD from *P. horikoshii* (Kim et al., 2009), whereas shared only 25% identity to *Lactobaillus platarum* (Zhang et al., 2006) and *Lactococcus lactis* (Wegmann et al., 2007) (data not shown). Cloning of GAD gene of *T. kodakaraensis* KOD1 was attempted as follows. The DNA fragment of the open reading frame with an N-terminal 6-His tag sequence was synthesized *in vitro*, cloned into T-vector, and named pTK1814. After sequence confirmation, the *gad* gene fragment was sub-cloned to pET21a(+) vector using *Nde*I and *Eco*RI restriction enzyme sites, and the plasmid was named pETK1814. The plasmid was transform into *E. coli* Rosetta (DE3), and the *gad* gene was expressed by adding isopropyl-β-D-thio-galactoside (IPTG) during liquid culture.

GAD Purification and SDS–PAGE Analysis. The recombinant GAD was purified by Ni-affinity chromatography using serial concentrations of imidazole i.e., 100, 200, 300, 500 mM, and 1 M in elution buffer (Fig. 1A). All fractions eluted between 200 mM to 1 M imidazole showed significant GAD activity; the relatively pure fractions of 500 mM and 1 M imidazole were combined and desalted using dialysis tubing. SDS-PAGE profile displayed a single band near the 45-kDa marker protein, which is good match to the calculated mass, 43.37 kDa, from the sequence (Fig. 1A). Oligomerization of the recombinant GAD was determined by size exclusion chromatography using a Superdex-200 gel filtration column through which a single sharp peak was produced with a stroke radius of 33.1Å, which was slightly smaller than BSA (67 kDa) and bigger than ovalbumin (43 kDa), thus demonstrating that the recombinant GAD is a monomer (Fig. 1B).

Properties of recombinant GAD from *T. kodakaraensis*. The pH and temperature dependencies of recombinant GAD activity were examined (Fig. 2). The optimum temperature of GAD activity was determined by incubating the enzyme at temperatures ranging from 60 to 99°C for 1 h. GAD from *T. kodakaraensis* KOD1 exhibited maximum activity at 90°C, which was significantly high as compared to the GADs from other organisms but similar to that of thermophilic GAD from *P. horikoshii* (Fig. 2A and Table 1). To estimate heat stability of the purified GAD, the enzyme was incubated at 70, 80, and 90°C up to 16 h, and the remaining activity was determined (Fig. 2B). Recombinant GAD showed over 60% of remaining activity after 16 h incubation at 80°C. Its half-life at 90°C was 1 h. The effect of pH on activity of the recombinant GAD was investigated by treating the GAD at pH values ranging from 5.0 to 12.0 (Fig. 2C). The enzyme showed optimal activity at pH 8.0. Michaelis constant of the recombinant GAD for L-glutamate in 0.1 M HEPES buffer (pH 8.0) at 90°C was calculated from a Lineweaver-Burk plot. *K*m, *V*max, and *K*cat of the enzyme were calculated to be 9.92 mM, 153.8 μmol min^{-1} mg^{-1}, and 6.613×10^3 min^{-1} respectively.

Production of GABA from MSG by recombinant GAD. Substrate conversion rate is one of the most important factors of industrial enzymes. Evaluation of GABA production of the recombinant GAD from MSG as substrate showed when the MSG substrate concentration was 10 mM, GABA conversion rate reached to almost 75% after 24 h incubation at 80°C with 36 units of the enzyme (27 mg) per 100 mL of reaction mixture (Fig. 3). The conversion rate was maximized to 85% when 50 mM of MSG was applied for same reaction conditions after 24 h. Therefore, 1% (w/v) of substrate MSG could be easily converted into GABA with 85% purity without any other contaminant except for 10% of remaining substrate. The conversion rate was

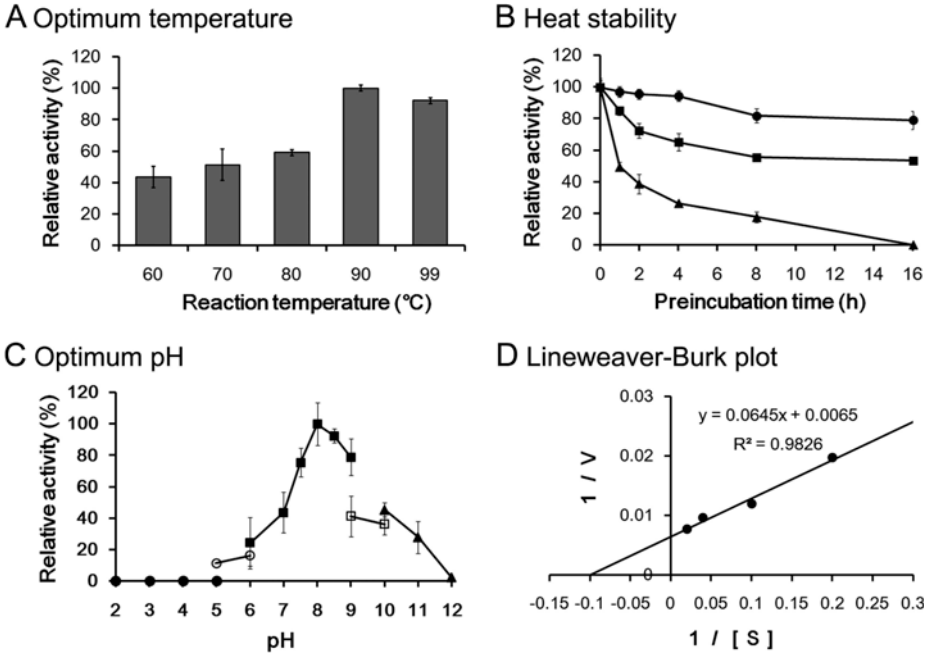

Fig. 2 Optimum temperature, heat stability, optimal pH, and Km value of recombinant GAD**.** Panel A, Optimum temperature. Enzymatic activity was measured in 0.1 M HEPES buffer (pH 8.0), 1 mM PLP, and 50 mM MSG at temperatures indicated in chart. Panel B, Heat stability. The activity of the enzyme was measured in 0.1 M HEPES buffer (pH 8.0), 1 mM PLP, and 50 mM MSG at 90°C after preincubation of GAD at 70 (●), 80 (■), and 90°C (▲) for 0–16 h. Panel C, Optimum pH. Enzymatic activity was measured in various pHs of pyridine-HCl buffer (●), citrate-phosphate buffer (○), HEPES-NaOH buffer (■), glycine-NaOH buffer (□), and sodium phosphate-NaOH buffer (▲) at 90°C for 1 h. Panel D, Evaluation of Michaelis constant of the recombinant GAD for L-glutamate in 0.1 M HEPES buffer (pH 8.0), according to Lineweaver-Burk plot.

Table 1 Characteristics of Glutamate decarboxylases

Origin	Subunit (kDa)	Number of subunit	Optimum temperature (°C)	Optimum pH	K_m (mM)	References
Thermococcus kodakaraensis (recombinant)	43	1	90	8.0–9.0	9.1	Present study
Escherichia coli	50	6	-	4.4	1.4	Fonda, 1985
Lactobacillus brevis (recombinant)	54	4	30	4.5–5.0	1.0	Ueno et al., 1997
Aspergillus oryzae	48	6	60	-	13.3	Kimi Tsuchiya, 2003
Pyrococcus horikoshii (recombinant)	43	1	97	8.0	3.9	Kim et al., 2009

significantly dropped when the substrate concentration was higher than 50 mM. With 100 mM MSG substrate, the rate was not over 30% after 24 h incubation.

Effect of different salts and PLP on GAD activity. Recombinant GAD activity was greatly affected by different concentration of salts such as sodium chloride (200 mM), Tris-HCl (200 mM), and sodium phosphate (200 mM), and the GAD activities remained up to 57, 42, and 63%, respectively (Fig. 4A). Furthermore the GABA production reached peak when 100 μM of PLP was applied (Fig. 4B). However, the GABA production decreased at both above and below the 100 μM PLP concentration.

Discussion

GAD has been purified from numerous mesophilic bacteria such as *Lactobacillus* (Komatsuzaki et al., 2008) and *Lactococcus* (Nomura et al., 1999). However, in the present study, a heat-stable recombinant GAD was cloned and expressed for industrial GABA production. GAD gene (tk1814) encoding GAD was isolated from a thermophilic archaean *T. kodakaraensis* KOD1 and expressed in *E. coli* Rosetta (DE3). The amino acid sequence of GAD originating from *T. kodakaraensis* KOD1 was compared with those of GADs from *L. platarum* (Galagan et al., 2003), *E. coli*

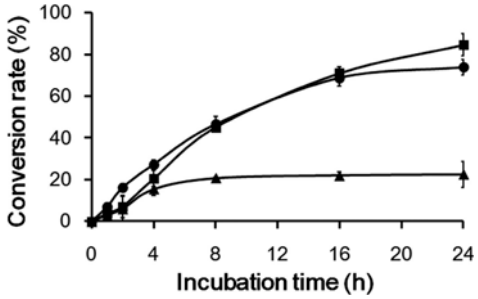

Fig. 3 GABA conversion rate by recombinant GAD catalyst. The enzyme catalytic reaction was performed in 0.1 M HEPES buffer (pH 8.0), 1 mM PLP with 10 (●), 50 (■), and 100 mM (▲) MSG mate as substrate at 80°C. GABA conversion rate was calculated by mole of GABA produced divided by initial mole of MSG.

(Welch et al., 2002), *L. lactis* (Wegmann et al., 2007), and *Neurospora crassa* (Galagan et al., 2003). The amino acid sequence of the GAD from *T. kodakaraensis* KOD1 was found to be rich with lysine residues at its active site. Lysine is a member of the ε-amino group, which often participates in hydrogen bonding and functions as a general base in catalysis (Sadoul et al., 2008). Based on these results, it could be postulated that the recombinant GAD was more heat-stable and remained more active over a wide range of temperatures as compared to the GADs from other mesophilic microbes.

The recombinant GAD expressed in *E. coli* was purified by Ni-NTA affinity chromatography. A prominent band of recombinant GAD with molecular mass of 43 kDa on the SDS-GAGE gel proved that the protein was purified to homogeneity. Size exclusion chromatography produced a sharp peak at a stroke radius 33.1Å, confirming that the recombinant GAD is monomer. In contrast to the present study, GADs originating from *E. coli* GAD (Tsai et al., 2006) and *Lactobacillus* GAD (Galagan et al., 2003) were hexamer and dimer, respectively.

The thermal stability of GAD was measured between 70 to 90°C, and the enzyme was determined to be highly stable and active. One reason for the thermal stability of the enzyme could be the high number of lysine residues in the active site. GAD activity was adversely affected by Tris-HCl, sodium phosphate, and NaCl, and decreased the catalytic activity up to 50% (Fig. 4A). In contrast, the catalytic activity of the GAD from mesophilic microbes was enhanced by these salts (Hiraga et al., 2008), which could increase the interaction among subunits of polymeric GAD by forming salt bridge and hence increasing the catalytic activity of GAD (Ueno et al., 1997). However, GAD from *T. kodakaraensis* KOD1 was proved to be monomeric in the present study; therefore, the role of hydrophobic interaction between different subunits was excluded. On another hand, GAD from *T. kodakaraensis* KOD1 has a large number of lysine residues in its active site, which are partly hydrophobic in nature (i.e. the part of the side-chain nearest to the protein main-chain) and presumably interact with lysine residues and cause enzyme inhibition (Betts and Russell, 2007). Optimal concentration of PLP was measured to determine the optimal activity of recombinant enzyme. GABA production reached peak when a 100 μM of PLP was applied (Fig. 4B).

The GABA production rate reached up to 80% when 50 mM L-glutamate was applied. However, higher concentrations of substrate suppressed enzyme activity, thereby comfirming 50 mM L-glutamate was optimum for GABA production by recombinant GAD from *T. kodakaraensis*, and further addition of substrate caused a substrate inhibition effect as described elsewhere (Abo Mohammed et al., 2009).

In the present study, a heat-stable recombinant GAD with high substrate conversion rate was successfully produced. This thermally stable GAD could be the best candidate for use in the pharmaceutical and fermentation industries and would increase production of GABA from MSG even at high temperatures at which conventional GAD have failed.

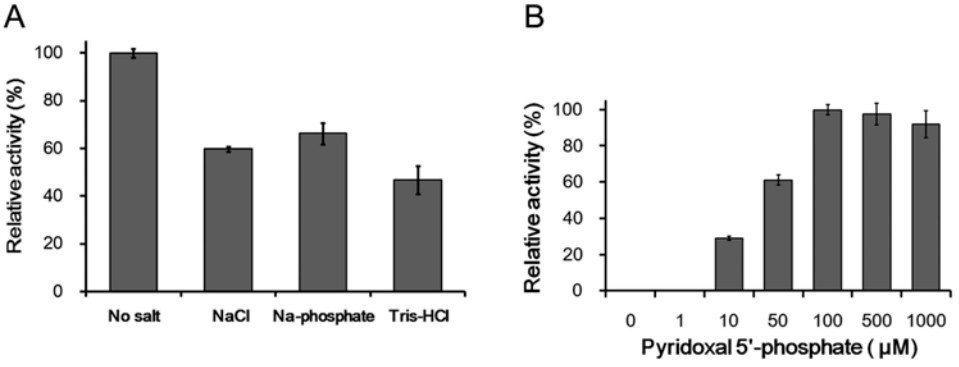

Fig. 4 Effects of salt and pyridoxal 5'-phosphate on recombinant GAD activity. Panel A, Enzymatic activity was measured in 0.1 M HEPES buffer (pH 8.0), 1 mM PLP, and 50 mM MSG at 90°C for 1 h. Additional salts, 200 mM each sodium chloride (NaCl), sodium phosphate (pH 8.0) or Tris-HCl (pH 8.0) were added in the reaction mixture. Panel B, Enzymatic activity was measured in 0.1 M HEPES buffer (pH 8.0), 50 mM MSG and 0–1,000 μM of PLP at 90°C for 1 h.

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