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Attempts to express recombinant Baker's yeast glutamate decarboxylase in *Saccharomyces cerevisiae* and *Escherichia coli*

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GABA-synthesizing enzyme, GAD[**], in *Saccharomyces cerevisiae* was expressed in *S. cerevisiae* and *E. coli*. *S. cerevisiae* expression system gave specific activity of 60 nmol/min/mg, which was 9-fold higher than the value obtained from the parental strain. *S. cerevisiae* system expressing C-terminal His-tag GAD (GAD-His_6) gave the specific activity of 27 nmol/min/mg. SDS-PAGE results of the crude extract followed by Western blot analysis with the use of antibody against His-tag suggested that proteolysis took place. Because of this susceptibility to proteolysis, an expression of GAD-His_6 in *E. coli* was attempted, where GAD-His_6 was found in both soluble and insoluble fractions. The soluble GAD-His_6 in the crude extract exhibited an enzyme activity of 10 nmol/min/mg with a major band at 69 kDa. The expression systems can be a useful tool to study structure-function relationship of GAD, *i.e.*, preparation of antibody.

Key words: GABA, GAD, yeast, expression

γ-Aminobutyrate (GABA) is an amino acid found in animals, plants, and microorganisms. In higher animals, GABA functions as a major inhibitory neurotransmitter. In addition, GABA is reported to participate various physiological activities such as reduction of high blood pressure, diuretic effect and induction of growth factors [1-3]. Because of beneficial physiological roles, various commercial foods that contain GABA as an ingredient have been developed, where the fermentation is the main tool for the GABA production. Among the microorganisms, yeast is the most popular microorganism used for production of foods containing GABA, thus it is an ideal microorganism to investigate GABA-production mechanism.

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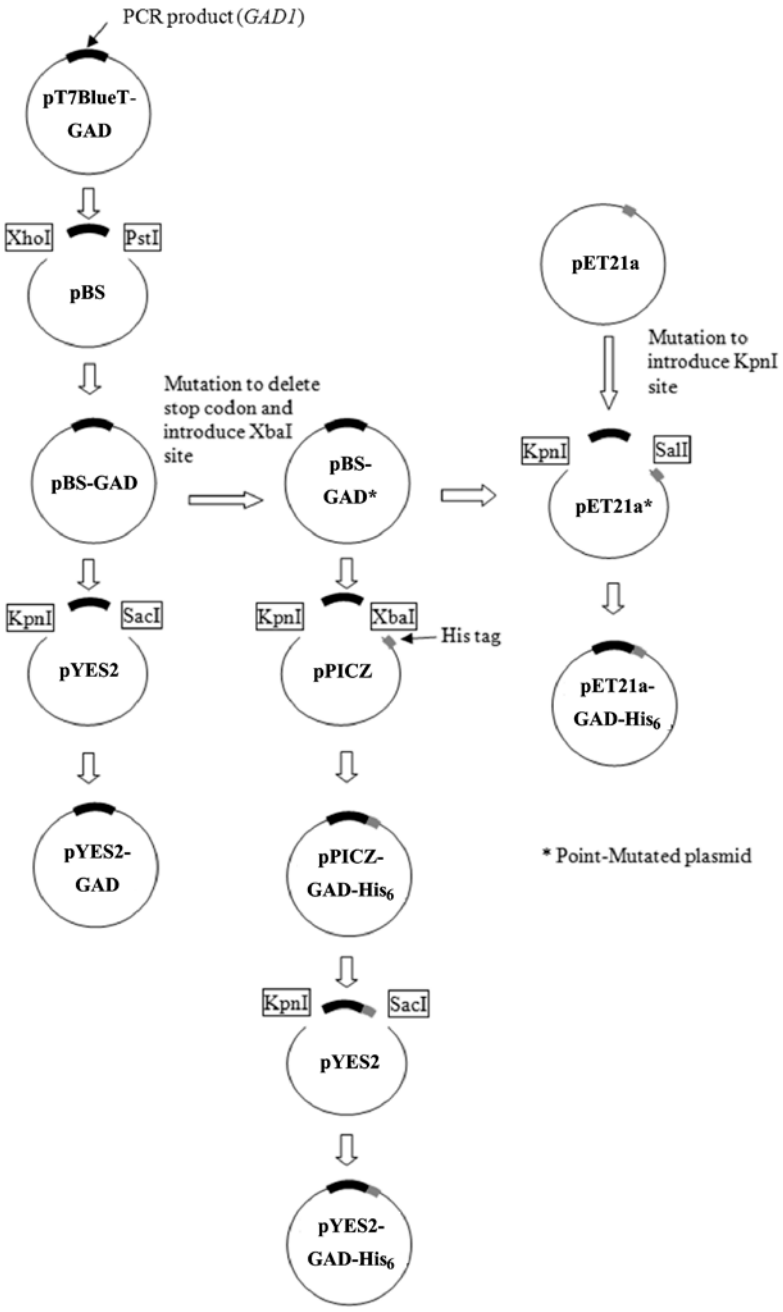
Abbreviations: GABA, γ-amino butyric acid; GAD, glutamate decarboxylase; TTBS, Tris-buffered saline with Tween-20
[**]Protein product of *GAD1* should be called Gad1p; however, because of the common name for glutamate decarboxylase, the expressed protein is called GAD in this text.

GABA is synthesized by glutamate decarboxylase (GAD, E.C. 4.1.1.15) that catalyzes decarboxylation reaction of L-glutamate [4]. GAD is found in various species with different properties [5]. GAD found in *Escherichia coli* has two isoforms, GAD_A and GAD_B, both of which have the hexameric form and are active in the acidic conditions. When decarboxylation reaction is in progress, GAD utilizes one molecule of proton (H^+) together with L-Glu to give one molecule of GABA. This suggests that GAD acts to neutralize the acidified intracellular pH of the cell that has been exposed to the acidic environment. Hence, the property of acid resistance of *E. coli* may



Scheme I. Schematic representation of plasmid designs

owe to the decarboxylation reaction [6, 7]. Several forms of GAD, GAD1 to 5, are found in plant spieces and they have Ca^{2+}/calmodulin binding domain at the C-terminal. The role of calmodulin binding domain has been extensively studied and thought to be related to the plant growth [8]. The plant GADs are reported to be dimeric or hexameric enzymes [9, 10]. Mammals have two distinct genes for GAD that produce two isoforms, GAD67 and GAD65. In brain and nerve cells, while both isoforms are expressed within the same cells, very little information on the specific role(s) for each of the isoforms is available. Previous studies using knock-out mouse have given some clues for the individual roles of isoforms, in which GAD67 knock-out mice being born with cleft palate died within a day after the birth whereas GAD65 knock-out mice survived with an increased tendency having seizures [11, 12]. Despite of these clearly different functions for mammalian GAD isoforms, their enzymatic function in producing GABA from L-glutamate appears to be the same.

Saccharomyces cerevisiae, a common yeast employed for various food productions, expresses GAD protein that is encoded by *GAD1* gene. The role of GAD in yeast is not clearly understood; however, a report suggests that GAD is involved in the resistance for oxidative stress [13]. As shown in the recently available genome database of *S. cerevisiae*, an open reading flame, YMR250w, has been assigned for *GAD1* which is on chromosome 13. With this information, we have cloned *GAD1* cDNA and incorporated into expression vectors. After the transformation of either *S. cerevisiae* or *E. coli*, the expression profiles of the host cells were characterized and the biochemical study on the obtained GAD was carried out.

MATERIALS AND METHODS

Plasmids and host strains - Plasmid pYES2 was obtained from Invitrogen (Carlsbad, CA). The pYES2 incorporated with *GAD1* (pYES2-GAD) was used to transform GAD knockout yeast (clone ID 20835) obtained from Open Biosystem (Huntsville, AL). The GAD knockout yeast was created from strain BY4743 (*MATa/α his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, MET15/ met15Δ0, lys2Δ0/LYS2, ura3Δ0/ura3Δ0*) by the *Saccharomyces* genome deletion project (Open Biosystem). Plasmid pET21a purchased from Novagen (Madison, WI) was used for the expression of the His-tag GAD in *E. coli* strain BL21(DE3). Typical plasmid construction was summarized in Scheme I.

Cloning and expression of GAD in S. cerevisiae - The sequence of *GAD1* was obtained from *Saccharomyces* genome database (http://www.yeastgenome.org/). GAD cDNA was amplified by PCR with primer pairs, 5' GAA GTA TCT CGA GTA ACA GTG ATG CTC 3' and 5' TTA TAG GCG CTA GCC CTT TCA CC 3'. The PCR product was cloned into pT7BlueT vector purchased from Novagen and inserted into the *Xho*I-*Pst*I site of pBluescript to form pBS-GAD. Then, the pBS-GAD was digested with *Kpn*I-*Sac*I and inserted into the same site of pYES2 to form pYES2-GAD. In order to accommodate C-terminal His-tag, the cDNA of GAD in pBS-GAD was mutated to replace the stop codon with *Xba*I site by using Quick Change mutagenesis kit (Stratagene, Cedar Creek, Texas). The primer pairs for the mutation were 5' GGA TGA CCT TTT CAT CTA GAC ATG TTC CTC 3' (*Xba*I restriction site) and 5' GAG GAA CAT GTC TAG ATG AAA AGG TCA TCC 3' (*Xba*I restriction site). Then, the pPICZaB plasmid (Invitrogen) was digested with *Kpn*I and *Xba*I and the *Kpn*I-*Xba*I

segment was inserted into this plasmid to create pPICZ-GAD-His_6.

The pPICZ-GAD-His_6 was digested and inserted into *Kpn*I-*Sac*I site of pYES2 to obtain pYES2-GAD-His_6. Restriction digestion and partial sequencing of the plasmid was carried out in order to confirm the successful incorporation of the insert DNA. Then, the plasmids were introduced into the *GAD1* knock out yeast by means of electroporation (BioRad, Richmond, CA). The transformants on the plates containing minimal synthetic medium without uracil (SC-U) were selected for the uracil prototrophy. *S. cerevisiae* carrying pYES2-GAD or pYES2-GAD-His_6 were grown in a medium, containing yeast extract, peptone and glucose (YPD), at 28 °C in a shaking incubator rotating at 200 rpm for overnight. The cells were centrifuged and resuspended into galactose-containing medium (YPG) for the target protein induction. After the culture flasks being shaken for several hours at 28 °C, the cell pellets were collected by centrifugation and resuspended in the lysis buffer containing 1 mM 2-aminoethylisothiouronium bromide hydrobromide, 0.5 mM pyridoxal 5'-phosphate (PLP), 0.1 mM dithiothreitol, 60 mM potassium phosphate buffer (pH 7.1) and the protease inhibitor cocktail (Roche). The cells suspended in the lysis buffer was disrupted with a beads-beater (Biospec Products, Kyoto, Japan) for 1 min and cooled for 2 min, which was repeated 7 times. During the cell lysis, the temperature was maintained not to exceed 10 °C by surrounding the container with ice water. The lysate was centrifuged at 10,000 x g for 20 min at 4 °C and the supernatant was used as a crude enzyme extract.

Cloning, expression and purification of GAD in E. coli - Prior to the insertion of *GAD1* cDNA into pET21a vector, *Kpn*I site was introduced into the vector by using Quick Change mutagenesis kit (Stratagene). As a result, pET21a vector should have *Kpn*I and *Sal*I sites to accommodate the insert DNA. The primer pair sequences for *Kpn*I site were 5' GGT GGA CAG CAA AGG TAC CGC GGA TCC G 3' (*Kpn*I restriction site) and 5' CGG ATC CGC GGT ACC TTT GCT GTC CAC C 3' (*Kpn*I restriction site). Meanwhile a pBS-GAD plasmid, in which the stop codon for GAD ORF was removed, was digested with *Kpn*I and *Sal*I to obtain the insert DNA. Both pBS-GAD and pET21a were ligated together to construct pET21a-GAD-His_6, which has His-tag at its C-terminus region. The recombinant plasmid was confirmed by the restriction digestion mappings and partial sequencing. The plasmid was introduced into *E. coli* BL21(DE3) strain by the calcium chloride method.

E. coli transformant having pET21a-GAD-His_6 was grown at 37 °C for overnight in LB medium containing 50 μg/ml ampicillin. This overnight culture was diluted in the fresh LB medium with 50 μg/ml ampicillin to make initial OD at 0.05 and was grown at 37 °C until OD at 600 nm reached 0.6. The GAD protein expression was induced by adding 0.1 or 1mM isopropyl-β-D-thiogalactoside (IPTG) and then continued shaking the culture flasks at 18, 28, or 37 °C. After the centrifugation, the cell pellet was collected and suspended with an equal amount of PBS, constituted with 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 8 g/l NaCl and 1.15 g/l Na_2HPO_4. The suspension was treated with ultrasonication to disrupt the cells, and centrifuged at 10,000 x g for 20 min at 4 °C to obtain a crude enzyme extract. The insoluble fraction was solubilized in a denaturing buffer containing 1% SDS, 50 mM sodium phosphate, pH 8.0, and 0.3 M NaCl, and was applied to a resin of nickel based- (GE Healthcare, Buckinghamshire, UK) and cobalt based- (Clontech, Mountain

View, CA) immobilized metal affinity chromatography for protein purification according to the manufacturer's instructions.

Enzyme activity assay - The crude enzyme extract was applied to a desalting column to remove small molecular weight impurities that affect fluorescent detection of o-phthalaldehyde (OPA)-derivatized GABA. At the same time, the buffer in the crude enzyme extract was replaced with 50 mM potassium phosphate, pH 7.0. GAD activity was estimated by measuring the amount of GABA produced during the assay under the condition as described previously [14]. The assay mixture, contained 400 µl enzyme solution, 20 µl of 1 M L-glutamic acid neutralized to pH 7 with NaOH, 20 µl of 10 mM PLP neutralized to pH 7 with NaOH, 160 µl of 1 M sodium acetate buffer, pH 4.6, and 400 µl H_2O, was incubated at 37 °C for 30 minutes and the reaction was terminated by adding 50 µl of 60% perchloric acid (PCA). As a control, the same mixture was prepared but PCA was added at time 0. After the termination, aliquots of the mixture were applied on an HPLC (Shimadzu, Kyoto, Japan) equipped with the post-column derivatization system that delivered OPA. A CX Pak column (Shodex, Tokyo, Japan) packed with strong cation exchange resin was used to separate GABA and the abundant amount of L-glutamate by eluting with 0.2 N sodium citrate buffer, pH 5.12, as described by Spackman, *et al.* [15, 16] at a flow rate of 0.3 ml/min. The eluted solution of the CX Pak column was mixed with OPA solution and the derivatized amino acids were monitored by a fluorescence detector (Shimadzu) set for excitation at 355 nm and emission at 440 nm. The amount of GABA produced was calculated by comparing the peak area of the examining solution with that of the standard GABA solution of known concentration. The protein concentration in the enzyme solution was estimated by using BioRad protein assay.

SDS-PAGE and Western blotting - During the purification process, protein purity was evaluated by carrying out SDS-PAGE and Western blot analyses [17]. SDS-PAGE gel was electrically blotted to a PVDF membrane on a BioRad semi-dry blotter for 1 hr with the use of CAPS buffer, pH 11. The membrane was treated with skim milk for 1 hr, washed with TTBS buffer (20 mM Tris, 154 mM NaCl, 0.05% Tween-20, pH 7.4), and incubated with horseradish peroxidase-conjugated mouse

Table 1. Summary of activity for recombinant GAD expressed

	wet cell (g/L)	soluble protein (mg/g wet cell)	specific activity (nmol/mg/min)
S. cerevisiae BY4743 (Parental strain)	16	12	7
GAD Knockout *S. cerevisiae* (BY4743gad$^-$)	18	17	nd[1]
BY4743gad$^-$ transformed with pYES2-GAD	8	4.4	60
BY4743gad$^-$ transformed with pYES2-GAD-His_6	5	1.7	27
E. coli BL21(DE3)	9	7.1	nd
E. coli BL21(DE3) transformed with pET21a-GAD-His_6	6	25	10

[1]nd: Activity was not detected.

monoclonal antibody against a His-tag and the immuno-positive bands were visualized with ECL Western blotting detection reagent (GE Healthcare) according to the manufacturer's instructions. After the treatment with ECL reagents, the membrane was covered with plastic bag, heat sealed, and placed on a Polaroid film (Type 667) in a cassette (Invitrogen) to expose protein bands.

RESULTS AND DISCUSSION

Expression of recombinant GAD and His-tag GAD in Saccharomyces cerevisiae - A *S. cerevisiae* strain was transformed with an expression plasmid, pYES2-GAD, and cultured in YPD medium. The recombinant GAD protein production was initiated by replacing YPD medium with YPG that contained galactose as a carbon source. After cells were harvested, lysed, and centrifuged, the soluble fraction was collected and its aliquots were measured for GAD activity. Specific activity of about 60 nmol/min/mg was obtained from the sample of 4 h induction in YPG at 28°C. This value is about 9-fold greater than that obtained from the parental strain, BY4743, which lacks plasmid (Table 1). When both cell lysates from the recombinant and original strains were subjected for SDS-PAGE analysis stained with Coomassie Brilliant Blue R-250 (CBB), they exhibited nearly identical band patterns at around 66 kDa as expected for GAD protein, however, the staining of the 66 kDa band for the recombinant strain was weak and the expression level of GAD protein was not extensive enough for the biochemical studies.

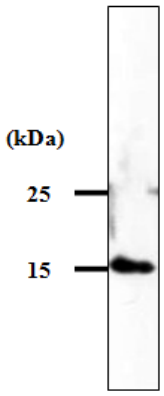


Fig. 1. Western blot of insoluble fraction of *S. cerevisiae* transformed with pYES2-GAD-His_6.

Antibody used was horseradish peroxidase-conjugated mouse monoclonal antibody raised against His-tag.

It has been desired to have antibody against yeast GAD in order to carry out detailed biochemical studies, however, antibody is not available. Hence, at the moment, it is not possible to carry out the Western blot study. When the sequences between yeast and mammalian GADs were compared, there were some homologies that suggested some possibilities of cross-reaction of available GAD antibodies. Hence, we have tried using antibodies against mammalian GADs; but we were unable to detect any band with these antibodies. Because weak expression of GAD in the yeast expression system and lack of the immuno-detection method have discouraged us to further characterizing the above described system.

Thus, we have constructed a pYES2-GAD-His_6 by inserting His-tag segment of pPICZaB into pYES2-GAD. pYES2-GAD-His_6 plasmid was introduced into the competent cell of *S. cerevisiae*. The colonies carrying pYES2-GAD-His_6 plasmid were isolated and subjected for His-tag GAD protein production by the induction with galactose. The optimal expression of His-tag GAD protein was achieved by 6 h induction in YPG at 28 °C with specific activity of 27 nmol/min/mg, which is about a half of the activity obtained from GAD expressed in pYES2-GAD. The expression

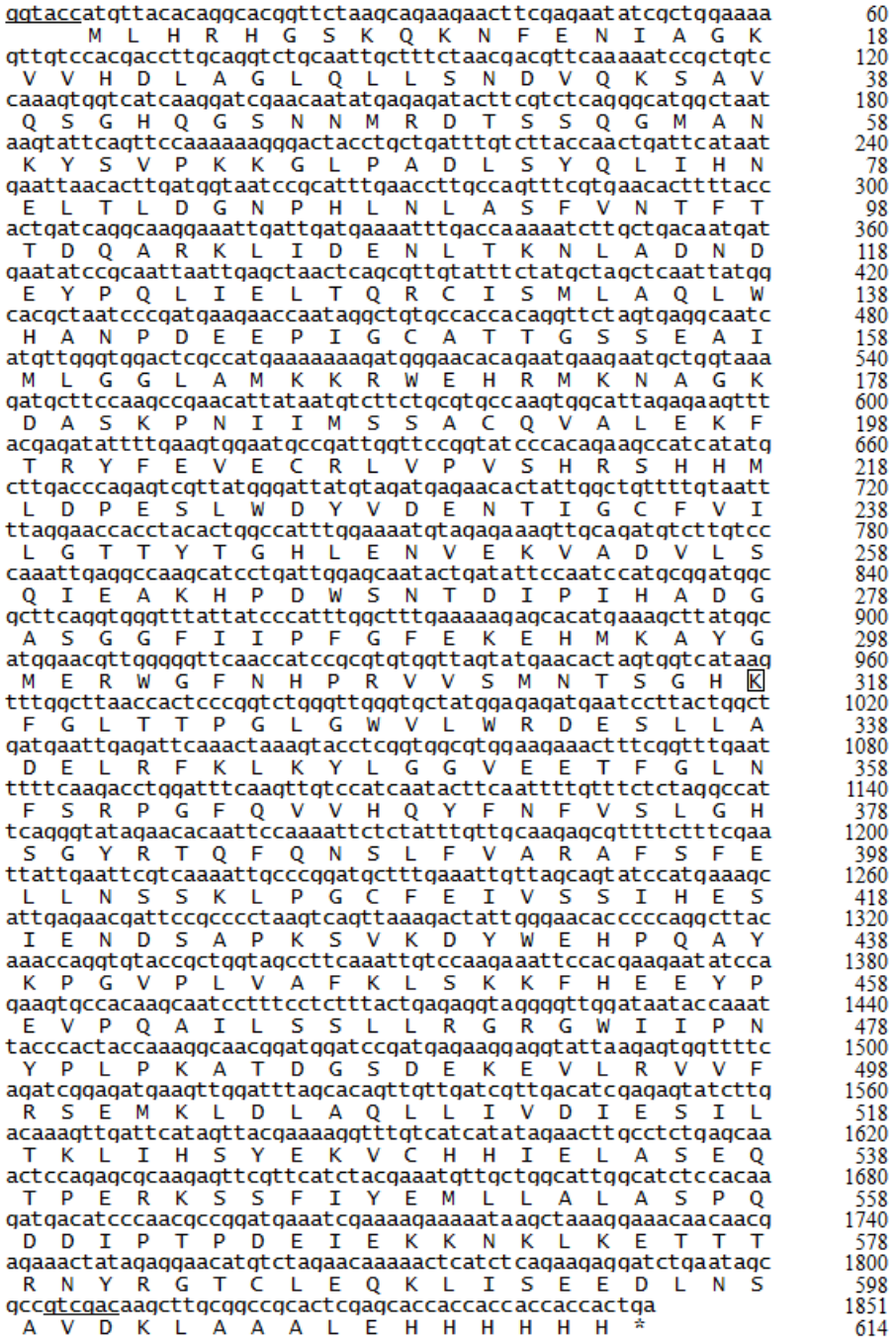


Fig. 2. Nucleotide and deduced amino acid sequence of *GAD1*

Nucleotide sequence and deduced amino acid sequence are numbered on the right. Boxed amino acid residues (Lys318) is marked as a PLP bind site. Restriction enzyme sites, *Kpn*I and *Sal*I, are shown as underlined.

of His-tag GAD was not detectable in SDS-PAGE gel stained with CBB (data not shown). By using antibody against His-tag, Western blot analysis gave a band at 15 kDa instead of 69 kDa, which suggests proteolysis may have occurred inside of the yeast cell (Fig. 1). There were no other immuno-positive bands observed on the

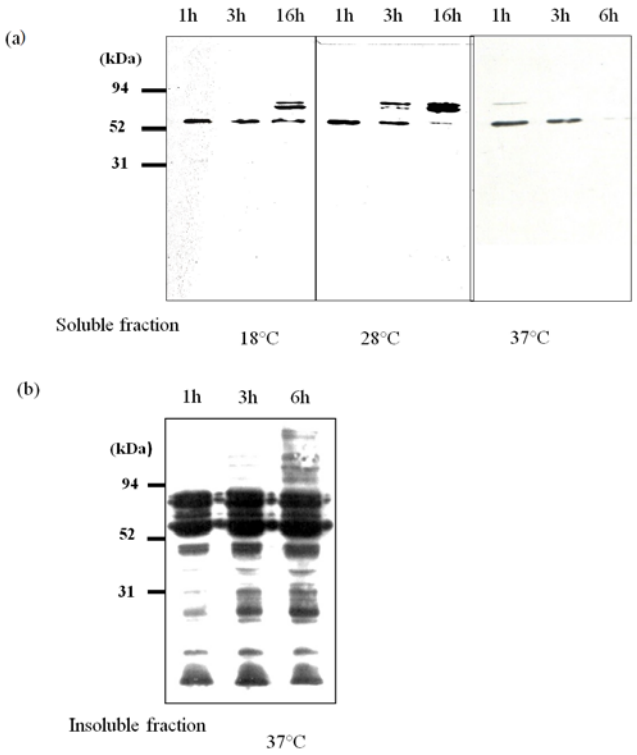


Fig. 3. Western blot analysis of *E. coli* transformed with pET21a-GAD-His_6

(a) Soluble fraction, induced at 18, 28 and 37 °C. (b) Insoluble fraction, induced at 37 °C. Antibody used was a horseradish peroxidase-conjugated mouse monoclonal antibody raised against His-tag.

Western blot analysis. By inspecting the amino acid sequence of GAD deduced from *GAD1* gene sequence, 15 kDa protein portion, including C-terminal end, should not have K318, the Lys residue at the active site, to form a Schiff base with PLP (Fig. 2). Therefore, the activity we have obtained must come from the remaining portion of GAD. However, under our experimental conditions, there was no way to detect it in the SDS-PAGE by CBB stain. Therefore, we have concluded that the yeast expression system should be redesigned or not suitable for GAD production.

Overexpression of His-tag GAD in Escherichia coli - An expression system using *E. coli* was constructed for His-tag GAD. As described in Materials and Methods, a plasmid pET21a-GAD-His_6 was created that contained the His-tag element at the C-terminal of GAD. The cell growth was carried out at 18°C and 16 h and it gave GAD activity of ~10 nmol/min/mg for the crude extract. After the induction carried out at 37°C with an addition of IPTG, cells, being harvested by centrifugation and disrupted by ultrasonication, showed no GAD activity, probably due to the misfolding. Under our experimental conditions, *E. coli* host cells did not give any residual GAD activity. As shown in Fig. 3, while GAD protein was detected in both soluble and insoluble fractions, most of GAD appeared in the insoluble fraction. When SDS-PAGE and Western blot analysis were carried out, several bands were

immuno-positive as observed for the expression in *S. cerevisiae* (data not shown), probably due to the proteolytic cleavage.

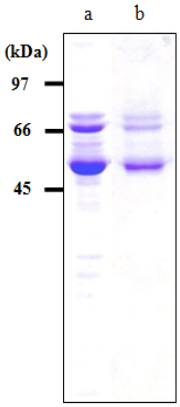


Fig. 4. SDS-PAGE analysis of insoluble fraction of *E. coli*.
(a) Insoluble fraction was dissolved in 1% SDS and applied. (b) Dissolved insoluble fraction was purified on His-tag column and the purified fraction was applied. For His-tag GAD expression, *E. coli* strain was grown and the protein expression was induced at 37 ℃ for 1 hour in the presence of 1mM IPTG. After SDS-PAGE, protein bands were visualized by staining the gels with CBB.

When the major band at 55 kDa was analyzed by protein sequencing analysis, the sequence was MKNAGKDASKPN that matched to Met-173-Asn184. This suggests that N-terminal 172 amino acids were absent for the 55 kDa protein.

Affinity purification on Ni-column was carried out for the soluble and insoluble fractions where the latter was solubilized by SDS. SDS-PAGE analysis for the insoluble fraction gave a protein band at around 66 kDa and at 55 kDa (Fig. 4). Amino acid sequencing of 66 kDa band confirmed that it is derived from GAD (data not shown). We have planned to use the affinity purified protein for the preparation of an antibody against GAD.

In this study, both yeast and *E. coli* expression systems were attempted for the production of GAD protein. The *E. coli* system was found to be more promising than the yeast system, due to the less proteolysis that yielded unwanted cleavage on the recombinant GAD. Solubilization of the inclusion body gave a suitable purity for raising the antibody. We will continue optimizing the expression system to provide GAD suitable for biochemical characterization.

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