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Co-expression of Gamma-Aminobutyrate Aminotransferase and Succinic Semialdehyde Dehydrogenase Genes for the Enzymatic Analysis of Gamma-Aminobutyric Acid in *Escherichia Coli*

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Abstract Gamma-aminobutyric acid (GABA) aminotransferase (*gabT*) and succinic semialdehyde dehydrogenase (*gabD*) genes from *Pseudomonas fluorescens* KCCM 12537 were cloned into a single pETDuet-1 vector and co-expressed in *Escherichia coli* BL21(DE3) simultaneously. The mixture of both enzymes, called GABase, is the key enzyme for the enzymatic analysis of GABA. The molecular mass of the GABA aminotransferase and succinic semialdehyde dehydrogenase were determined to be 52.8 and 46.7 kDa following computations performed with the pI/Mw program, respectively. The GABase activity between pH 6.0 and 9.0 for 24 h at 4°C remained over 75%, but under pH 6.0 decreased rapidly. The GABase activity between 25 and 35°C by the treatment at pH 8.6 for 30 min remained over 80%, but over 35°C decreased rapidly. When the activity against GABA was defined as 100%, the purified GABase activity against 5-aminovaleric acid having a similar structure to GABA showed 47.7% and GABase activity against β-alanine, ε-amino-n-caproic acid, L-ornithine, L-lysine, and L-aspartic acid showed between 0.3 to 2.3%. The GABA content was analyzed with this co-expressed GABase, compared with the other GABase which was available commercially. As a result, the content of GABA extracted from brown rice, dark brown rice, and black rice were 26.4±3.5, 40.5±4.7 and 94.7±9.3 μg/g, which were similar data of other GABase in the error ranges.

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Introduction

Gamma-aminobutyric acid (GABA) is known as a nonprotein amino acid produced by the α-decarboxylation of L-glutamic acid in a reaction catalyzed by glutamate decarboxylase (EC 4.1.1.15) (Jakoby and Scoott, 1959). It is known that GABA is involved in the regulation of neurological disorder like seizures, Parkinson's disease, Huntington's disease and Alzheimer's disease (Stanton, 1963). It is also involved in physiological functions such as neurotransmission, induction of hypotensive effects, diuretic effects, and tranquilizer effects in nonneural tissues (Omori et al., 1987; Oh and Choi, 2000). In addition, GABA induces insulin secretion strongly from the pancreas (Adeghate and Ponery, 2002) and prevents diabetic conditions (Hagiwara et al., 2004).

GABA is metabolized to succinic acid via succinic semialdehyde by two catabolic enzymes GABA aminotransferase (E.C.2.6.1.19) and succinic semialdehyde dehydrogenase (E.C.1.2.1.16). Jakoby and Scott (1959) have described a system for the enzymatic analysis of GABA with these two enzymes from *Pseudomonas fluorescens*. Therefore a mixture of GABA aminotransferase and succinic semialdehyde dehydrogenase allows for a spectrophotometric assay of GABA by the detection of change of absorbance due to NADPH production.

A quantitative analysis of GABA is possible with amino acid analyzer, a thin layer chromatography (TLC) or an enzymatic analysis using the mixture of GABA aminotransferase and succinic semialdehyde dehydrogenase, called as GABase. But the analysis with the amino acid analyzer is tedious and time-consuming and a fixed quantity of GABA cannot be determined by TLC. Also, a commercially available mixture of GABA

aminotransferase and succinic semialdehyde dehydrogenase called GABase (Sigma-Aldrich, USA) is somewhat expensive for the analysis of the plenitude of samples.

In this study, the GABA aminotransferase (*gabT*) and succinic semialdehyde dehydrogenase (*gabD*) gene from *P. fluorescens* KCCM 12537 was cloned into the single pETDuet-1 vector and investigated for the characterization of both enzymes co-expressed simultaneously. Also in order to verify the application of co-expressed both enzymes for the analysis of GABA, the content of GABA was measured with various samples for enzymatic analysis with co-expressed GABase compared to a commercial GABase purchased from Sigma-Aldrich (St. Louis, USA).

Materials and Methods

Bacterial Strains, Plasmids, and Culture Media. *P. fluorescens* KCCM 12537 was obtained from the Korean Culture Center of Microorganisms (Seoul, Korea) and used as the source of genomic DNA. *Escherichia coli* DH5α and *E. coli* BL21(DE3) were used as cloning and expression hosts. These strains were grown in LB medium and the medium was supplemented with ampicillin (100 μg/mL), when required. Plasmid pGEM-T Easy (Promega, USA) was used for cloning of PCR products. Plasmid pETDuet-1 (Novagen, USA) was used as an expression vector to construct *gabT* and *gabD* genes containing a six his-tag at their carboxy terminal.

DNA Manipulation. Chromosomal DNA isolation, transformation and standard DNA manipulations were carried out as described by Sambrook et al. (1989). Plasmid DNA and PCR products were purified using the plasmid DNA preparation kit and PCR purification kit (Bioneer, Korea). All used enzymes were purchased from Takara (Takara, Shiga, Japan) and used oligonucleotides for PCR primer were obtained from Bioneer Co. (Bioneer, Korea).

PCR Amplification, Cloning and Sequencing. The *gabT* and *gabD* genes were amplified from *P. fluorescens* KCCM 12537 by PCR using the forward and reverse primers designed on the basis of the sequence of *gabT* and *gabD* genes from *P. fluorescens* Pf-5 complete genome sequence (GenBank accession no, CP000076). In order to amplify the *gabT* gene, two primers were designed: (sense) 5'-ATGAGCAAGACCAACGCATCTTTG-3' and (antisense) 5'-GATGATGGAGTTCAGCGAAGCACTCTTCGATGAT-3'. Two primers were designed to amplify the *gabD* gene: (sense) 5'-ATGCAGCTCAAAGACGCC-3' and (antisense) 5'-TGATGA TGATGATGGATACCCAGGCAGAGGTA-3'. PCR conditions were an initial denature step at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 50°C for 40 s, 72°C for 60 s, and a final extension step at 72°C for 5 min. The PCR products were cloned into pGEM-T Easy vector and transformed into *E. coli* DH5α. The nucleotide sequence analysis of the insert was entrusted to Bioneer Co. Homology searches in the GenBank database were carried out by using the BLAST program.

Expression and Purification of the Recombinant GABase. For expression and purification of the GABA aminotransferase and succinic semialdehyde dehydrogenase simultaneously in *E. coli* BL21(DE3), the structural region of *gabT* and *gabD* genes having six his-tag was amplified. As the first step, *gabT* gene was amplified using the *gabT* gene cloned in pGEM-T Easy vector as a template. The sense primer (5'-CGGGATCCATGAGCAAGAC CAACGCAT-3') was synthesized to contain *Bam*HI restriction site and the antisense primer (5'-GCCGAGCTCTCAATGATGAT GATGATGATGGAGTTCAGCGAA-3') was synthesized to contain *Sac*I restriction site behind six histidine codons to facilitate cloning in-frame into the pETDuet-1 expression vector. PCR was performed for 30 cycles consisting of 95°C for 30 s, 50°C for 1 min, and 72°C for 1.5 min. The amplified DNA fragment was digested with *Bam*HI and *Sac*I, and cloned into the corresponding multiple cloning site 1 of pETDuet-1 expression vector. As the second step, the *gabD* gene was amplified using the *gabD* gene cloned in pGEM-T Easy vector as a template. The sense primer (5'-ATACATATGCAGCTCAAAGACGCCCAG-3') was synthesized to contain a *Nde*I restriction site and the antisense primer (5'-CCGCTCGAGTCAATGATGATGATGATGGAGTTCAGC GAA-3') was synthesized to contain a *Xho*I restriction site behind six histidine codons to facilitate cloning in-frame into the pETDuet-1 expression vector. PCR was performed as described above. The amplified DNA fragment was digested with *Nde*I and *Xho*I, and then cloned into the corresponding multiple cloning site 2 of pETDuet-1 plasmid containing the above *gabT* gene, named pETDuet-GABase (Fig. 1). The recombinant plasmid, pETDuet-GABase having *gabT* and *gabD* genes simultaneously, was transformed into *E. coli* BL21 (DE3). The nucleotide sequence of the insert was confirmed by entrusting to Bioneer Co.

The his-tagged GABA aminotransferase and succinic semialdehyde dehydrogenase protein were purified from *E. coli* BL21(DE3) having pETDuet-GABase. Transformants were grown at 37°C in LB medium supplemented with ampicillin (100 μg/mL), for 2 h with shaking. Isopropyl-1-thio-β-D-galactoside (IPTG) was added to a final concentration of 1 mM and the cultures were further incubated for 16 h at 30°C with shaking. The cells were harvested by centrifugation, washed, and resuspended in 100 mM sodium phosphate buffer (pH 7.0). The resuspended cells were disrupted by sonication and the lysate was centrifuged at 15,000 × g for 30 min. The supernatant which contained GABA aminotransferase and succinic semialdehyde dehydrogenase was used as a crude enzyme for the purification of GABase. Affinity chromatography was carried out using Ni-NTA column (Qiagen, USA) according to the supplier's protocol. The purified GABase with six his-tag was used for analysis of enzymatic properties. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was accomplished on a 10% running gel and protein bands were visualized by staining with Coomassie Brilliant Blue R250 (Sigma-Aldrich, USA).

GABase Assay. GABase activity was assayed by the method described by Jakoby (1962). A mixture of GABA aminotransferase and succinic semialdehyde dehydrogenase (GABase) allows for a spectrophotometric assay of GABA by the detection of change of absorbance due to NADPH production. Therefore GABase

activity was measured spectrophotometrically by the increase of NADPH (millimolar extinction coefficient, ε =6.22 mM^{-1} cm^{-1}) at 340 nm. The reaction mixture contained 2.3 mL of 0.3 M Tris-HCl buffer (pH 8.6), 0.1 mL of 100 mM β-mercaptoethanol, 0.15 mL of 100 mM α-ketoglutaric acid, 0.15 mL of 25 mM β-$NADP^+$ and 0.3 mL of 60 mM GABA. The reaction was started by adding 20 μL of enzyme solution. Absorbance at 340 nm was read after incubation of 60 s and the activity was calculated as described by Jakoby (1962).

pH and Temperature Stability of GABase. The GABase was incubated to determine pH stability at different pH values by using 100 mM citrate phosphate buffer for the range from pH 4.0 to 6.0, 100 mM sodium phosphate buffer for the range from pH 6.0 to 8.0, and 100 mM Tris-HCl buffer for the range from pH 8.0 to 10.0 for 24 h at 4°C. After incubation, the pH was adjusted to pH 8.6 with 0.3 M Tris-HCl buffer. To determine thermostability of GABase, The purified enzyme was incubated in various temperatures (25–60°C) at pH 8.6 for 30 min. The remaining GABase activity was measured as described above.

Preparation of Standard Calibration Curve Using GABA for Quantitative Analysis. For the preparation of standard calibration curve, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg of GABA was dissolved in 1 mL of the deionized water and used as substrate for the standard calibration curve. The reaction mixture contained 2.3 mL of 0.3 M Tris-HCl buffer (pH 8.6), 0.1 mL of 100 mM β-mercaptoethanol, 0.15 mL of 100 mM α-ketoglutaric acid, 0.15 mL of 25 mM β-$NADP^+$and 0.3 mL of each concentration of GABA for standard calibration curve. The reaction was started by adding 20 μL (0.04 unit) of the enzyme solution.

Extraction of GABA from the Brown Rice, Dark Brown Rice, and Black Rice. GABA was extracted according to the method described by Oh and Choi (2000). For the extraction of GABA, the brown rice (Juan-byeo rice, National Institute of Crop Science, R.D.A, Korea), dark brown rice (Noreunjachal, College of Natural Resources, Yeungnam Univerity, Korea), and black rice (Josaeng-heukchal, National Institute of Crop Science, R.D.A, Korea) were frozen with liquid nitrogen and pulverized in a mortar. Twenty grams of the pulverized rice were mixed vigorously with 80 mL of a solvent (methanol: chloroform: water; 12:5:3) for 1 min and centrifuged 15,000 × g for 30 min. After the collection of supernatant, the pellet was mixed vigorously with 80 mL of a solvent (chloroform : water; 2:1) for 1 min and centrifuged 15,000 × g for 30 min. The two supernatants were combined and concentrated in rotary vacuum evaporation (Hahnshin Scientific Co., Korea) and then dissolved with 20 mL of the deionized water. The concentrated samples were centrifuged 15,000 × g for 30 min and the supernatant was filtered with 0.22 μm syringe filter (Millipore, USA).

Results and Discussion

PCR Cloning and Amino Acid Sequence Analysis. For the cloning of *gabT and gabD* genes of *P. fluorescens* KCCM 12537, *gabT* (1,278 bp) and *gabD* (1,443 bp) structural genes having start codon and stop codon were amplified by the primers designed on the basis of sequence of *gabT* and *gabD* genes from *P. fluorescens* Pf-5 complete genome sequence (GenBank accession no, CP000076). The *gabT* and *gabD* genes were analyzed their sequence. The deduced amino acid sequence of *gabT* and *gabD* genes were used to search for the homologous sequences in the BLAST database. The amino acid sequence of GABA amino-transferase of *P. fluorescens* KCCM 12537 was homologus at 99% with *P. fluorescens* SBW25 (GenBank accession no. CAY46464). The amino acid sequence of GABA aminotransferase from *P. fluorescens* KCCM 12537 also had high sequence similarity with *P. fluorescens* pf-5 (96% identity, GenBank accession no. AAY95596) and *P. fluorescens* pf0-1 (95% identity, GenBank accession no.ABA71931) (data not shown). The amino acid sequence of succinic semialdehyde dehydrogenase of *P. fluorescence* KCCM 12537 was homologus at 98% with *P. fluorescens* SBW25 (GenBank accession no. CAY46464). The amino acid sequence of succinic semialdehyde dehydrogenase of *P. fluorescence* KCCM 12537 also had a high sequence similarity with *P. fluorescens* pf-5 (97% identity, GenBank accession no. AAY95596) and *P. fluorescens* pf0-1 (95% identity, GenBank accession no.ABA71931) (data not shown).

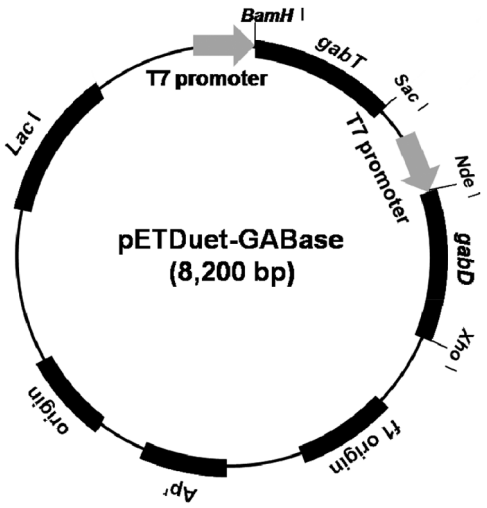

Fig. 1 Physical map of pETDuet-GABase. The *gabT* (1,278 bp) and *gabD* (1,443 bp) genes were inserted into multiple cloning site 1 and 2 in single pETDuet-1 vector (Novagen, USA), respectively.

Co-expression and Purification of the Recombinant GABase. For the co-expression of GABA aminotransferase and succinic semialdehyde dehydrogenase in the single plasmid pETDuet-1, the *gabT* and *gabD* genes were inserted into multi-cloning site 1 and 2 of pETDuet-1, respectively (Fig. 1). To facilitate purification by affinity chromatography, a six his-tag was added to each carboxyl terminal of both enzymes. The purified proteins gave two bands with a molecular mass of approximately 52 kDa and 46 kDa on the SDS-PAGE corresponding to GABA aminotransferase and succinic semialdehyde dehydrogenase, respectively (Fig. 2). When the molecular mass was calculated with Compute pI/Mw

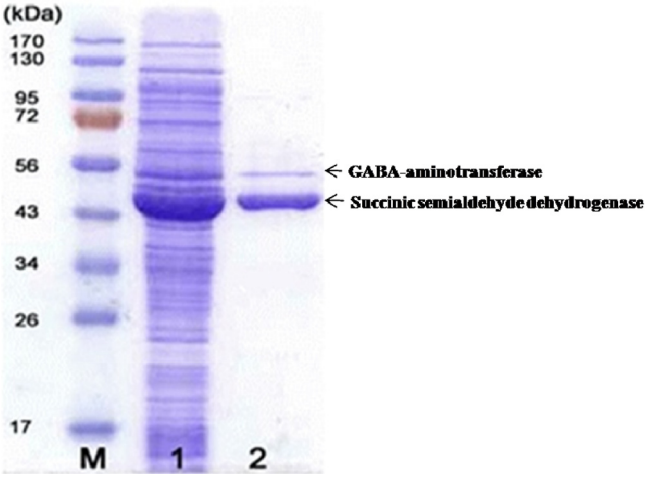

Fig. 2 SDS-PAGE profile of co-expressed GABase from pETDuet-GABase/BL21(DE3). M, molecular size marker; 1, crude extract; 2, purified GABase (GABA aminotransferase and succinic semialdehyde dehydrogenase) from pETDuet-GABase/BL21(DE3).

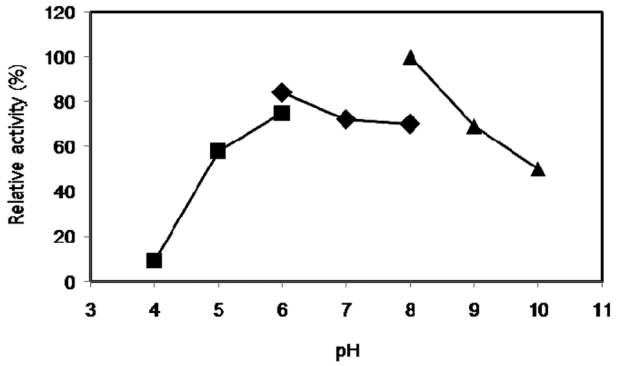

Fig. 3 pH stability of co-expressed GABase from pETDuet-GABase/BL21(DE3). ■-■, 100 mM citrate phosphate buffer; ◆-◆, 100 mM sodium phosphate buffer; ▲-▲, 100 mMTris-HCl buffer. The purified enzyme was incubated at 4°C for 24 h in each buffer. After the enzyme solution was adjusted to pH 8.6, the remaining activity was measured under the standard conditions.

program of Expasy (http://www.expasy.ch/cgi-bin/pi_tool) using the amino acid sequence, the molecular mass of GABA aminotransferase and succinic semialdehyde dehydrogenase was 52.8 and 46.7 kDa, respectively. Therefore the molecular mass of the purified GABA aminotransferase and succinic semialdehyde dehydrogenase on SDS-PAGE was almost matched with the calculated molecular mass by Compute pI/Mw program.

pH and Temperature Stability of GABase. The GABase was incubated to determine pH stability at different pH for 24 h at 4°C. After incubation, the pH was adjusted to pH 8.6 with 3.0 M Tris-HCl buffer. As shown in Fig. 3, the GABase activity between pH 6.0 and 9.0 remained over 75%, but under the pH 6.0 rapidly decreased. After the purified enzyme was incubated in various temperatures between 25 and 60°C at pH 8.6 for 30 min, the remaining activity was assayed. The GABase activity between 25 and 35°C remained over 80%, but over 35°C rapidly decreased (Fig. 4).

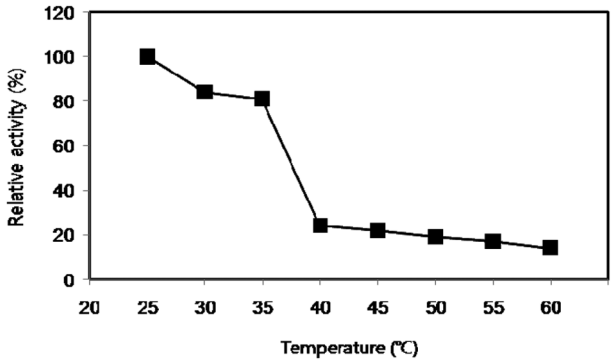

Fig. 4 Thermal stability of co-expressed GABase from pETDuet-GABase/BL21(DE3). The purified enzyme was incubated at pH 8.6 for 30 min at the indicated temperature. The remaining activity was measured at standard conditions.

Table 1. Substrate specificity of GABase from pETDuet-GABase/BL21(DE3)

Substrate	Structural formula	Relative activity (%)
β-Alanine	$NH_2CH_2CH_2COOH$	1.6
GABA	$NH_2CH_2CH_2CH_2COOH$	100.0
5-Aminovaleric acid	$NH_2(CH_2)_4COOH$	47.7
ε-Amino-n-caproic acid	$NH_2(CH_2)_5COOH$	2.3
Ornithine	$NH_2(CH_2)_3CHNH_2COOH$	0.3
L-Lysine	$NH_2(CH_2)_4CHNH_2COOH$	1.1
L-Aspartic acid	$COOHCH_2CHNH_2COOH$	2.0

The activity against GABA was defined as 100%.

Jakoby (1962) has described that the succinic semialdehyde dehydrogenase of *P. fluorescens* (ATCC 13430) displays maximal activity at pH 8.5 and is most stable at pH 6.5 to 7.1 and GABase stored at pH 7.0 in 5 mM mercaptoethanol-0.05M phosphate buffer at 2°C lost 10% of their activity per week. Also the optimum activity of GABA aminotransferase is obtained between pH 8.5 and 9.0 (1962).

Substrate Specificity. For the investigation of substrate specificity, the GABase activity was assayed against β-alanine, 5-aminovaleric acid, ε-amino-n-caproic acid, L-ornithine, L-lysine, and L-aspartic acid which were similar to the structure of GABA. When the activity against GABA was defined as 100%, the purified GABase activity against 5-aminovaleric acid showed 47.7%. The purified GABase activity against the rest of substrates showed between 0.3 to 2.3% of the activity against GABA (Table 1).

Manuel and Ramos (2001) characterized one of the genes which encode an enzyme with 5-aminovalerate aminotransferase activity involved in lysine catabolism. According to their research, the 5-aminovaleric acid which is an intermediate of lysine catabolism pathway was converted to glutaric acid semialdehyde by 5-aminovalerate aminotransferase. The glutaric acid semialdehyde was converted to glutaric acid by glutaric acid semialdehyde

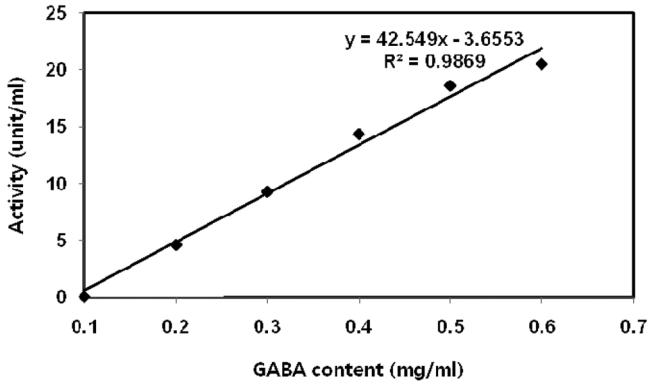

Fig. 5 GABA standard calibration curve. One unit of enzyme activity was defined as the amount of enzyme required to convert 1 μmol of GABA to succinic acid via succinic semialdehyde per min with a stoichiometric reduction of 1 μmol of $NADP^+$ at pH 8.6 at 25°C.

dehydrogenase, and used as a carbon source (Phillips, 1986). Because the recombinant GABase had 47.7% activity against 5-aminovaleric acid, compared to GABA, it was presumed that the purified GABA aminotransferase and succinic semialdehyde dehydrogenase might be different from the 5-aminovalerate aminotransferase and glutaric acid semialdehyde dehydrogenase, respectively.

Determination of the GABA Content from the Brown Rice, Dark Brown Rice, and Black Rice. The GABA standard calibration curve was prepared as described in Materials and Methods (Fig. 5). The GABA was extracted from the brown rice, dark brown rice, and black rice. The GABA content of the brown rice, dark brown rice, and black rice measured with the co-expressed GABase was 26.4±3.5, 40.5±4.7 and 94.7±9.3 μg/g, respectively. To verify the determination method using the co-expressed GABase, the GABA content of the brown rice, dark brown rice, and black rice was assayed with GABase (G7509) purchased from Sigma-Aldrich (USA). The GABA content of the brown rice, dark brown rice, and black rice measured with the GABase of Sigma-Aldrich was 25.4±3.1, 41.3±4.0 and 97.3±11.1 μg/g, respectively (Table 2). The GABA content of the rices determined by the co-expressed GABase in pETDuet-GABase were similar to those of Sigma-Aldrich in the error range.

Numerous assays for the measurement of GABA concentrations were used: the enzymatic GABase method (Baxter and Roberts, 1972), TLC with fluorescence detection (Seiler, 1981), and high performance liquid chromatography (HPLC), followed by electrochemical (Lindgren and Anden, 1985) or spectrofluorimetric detection (Griesmann et al., 1982). But the TLC method was inaccurate and the HPLC method was somewhat inconvenient because GABA has to be a derivatized by *O*-phthaladehyde or phenyl isothiocyanate for the UV detection. Also for the GABase to be available commercially is very expensive, therefore the recombinant GABase co-expressed in pETDuet-GABase/BL21(DE3) is useful to analyze the GABA with a low price. It is the first trial that the *gabT* and *gabD* genes are coexpressed in a single vector to produce the enzyme assay kit for the analysis of GABA.

Table 2. The content of GABA extracted from the brown rice, dark brown rice, and blackrice determined with GABase from pETDuet-GABase/BL21(DE3) and GABase from Sigma-Aldrich

	GABA content (μg/g)*	
	GABase (pETDuet-GABase)	GABase (Sigma-Aldrich)
Brown rice	26.4 ± 3.5	25.4 ± 3.1
Dark brown rice	40.5 ± 4.7	41.3 ± 4.0
Black rice	94.7 ± 9.3	97.3 ± 11.1

*Values are the mean ± standard deviation for three determinations.

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