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Original article

Effect of incubation time, buffer type and concentration on gamma-aminobutyric acid (GABA) production using Khao Dawk Mali 105 rice bran

Apinya Eamarjharn, Chockchai Theerakulkait,* Saipin Thanachasai

Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand

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ABSTRACT

Rice bran of Khao Dawk Mali 105 (KDML105) variety was selected for production of gamma-aminobutyric acid (GABA). The effect of incubation time on GABA production was studied and the maximum GABA was produced after 6 h of incubation. Different types of 50 mM buffers (containing 0.2% glutamic acid) consisting of Tris, citric acid, boric acid and phosphate buffer (pH 5.6) were used to stabilize the pH of the reaction system. The highest GABA content (5.05 mg/g of bran) was found in the phosphate buffer system. Therefore, the effect of phosphate buffer concentrations (0–200 mM) on GABA production was investigated. The results showed that rice bran with phosphate buffer at a concentration of 80 mM at pH 5.6 with a rice bran to phosphate buffer at a ratio of 1–8 (weight per volume) produced the highest GABA content ($p \leq 0.05$). GABA production was increased about 2.7 times in the phosphate buffer system compared with the control and about 11 times compared to the initial GABA content (0.58 mg/g of bran) in the rice bran. The results indicated that incubation time, buffer type and concentration significantly affect GABA production using rice bran.

Introduction

Rice (*Oryza sativa* L.) is one of the most important crops and is grown in almost every part of Thailand (Zeigler and Barclay, 2008; Jannoey et al., 2010). Rice bran is the main by-product produced during milling of whole rice grain and rice bran is a rich source of glutamate decarboxylase (L-glutamate-1-carboxylyase, EC 4.1.1.15, GAD) (Wang et al., 2010). It also contains high levels of glutamic acid (Juliano, 1985). In rice bran, glutamate decarboxylase enzyme (GAD) is naturally present in the cytosol of the cell and plays an important role in GABA production; GAD is a pyridoxal 5'-phosphate (PLP) dependent enzyme, which catalyzes the irreversible alpha-decarboxylation of L-glutamic acid (Glu) to produce gamma-aminobutyric acid (GABA) and carbon dioxide. GAD also naturally located in the cytosol of rice bran cell (Zhang et al., 2007). GABA is widely distributed in nature and plays an important role in the central nervous system as a neurotransmitter and lowers blood pressure in the human brain (Kimura et al., 2002; Wang et al., 2010). GABA also plays an important role in nitrogen storage, plant growth, glutamic acid utilization and in the plant's defense system against phytophagous insects (Bown and Shelp, 1997).

Control of the GABA and GAD levels in the brain was found to prevent many neurological disorders such as seizures, Parkinson's disease, stiff-man syndrome, and schizophrenia (Bao et al., 1995; Adeghate and Ponery, 2002). Furthermore, consumption of GABA-enriched foods can inhibit cancer cell proliferation and improve memory and the learning abilities (Oh and Oh, 2004; Dhakal et al., 2012). Therefore, GABA has been classified as a bioactive component in foods and pharmaceuticals. The health benefits of GABA have resulted in it becoming of keen interest to researchers in their work to develop functional foods containing high levels of accumulated GABA. Methods to increase GABA concentrations in rice have been studied by various researchers.

Ohtsubo et al. (2000) used rice germ as an enzyme (GAD) source. They produced 290 mg/g of germ of GABA by adding exogenous Glu. Zhang et al. (2006) hydrolyzed germ protein with the addition of trypsin and produced Glu for GABA accumulation. They produced GABA at a rate of 22.6 mg/g of rice germ. GABA production by Zhang et al. (2006) was much lower than the GABA

* Corresponding author.
E-mail address: fagicct@ku.ac.th (C. Theerakulkait).

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produced by Ohtsubo et al. (2000). This indicates that the GABA production level was lowered by the endogenous Glu naturally present in the rice germ and this was not sufficient for high GABA production. Karladee and Suriyong (2012) produced GABA at a level of 0.23 mg/g of grain by soaking brown rice for 24 h at 40 °C. Similarly, Jannoey et al. (2010) produced GABA at a level of 2.45 mg/g of grain by soaking germinated brown rice at a controlled temperature of 30 °C for 72 h. GABA has been produced in many other cereals by different methods. Nogata and Nagamine (2009) produced GABA from wheat bran by soaking in sodium phosphate buffer and obtained a GABA content of 1.48 mg/g of wheat bran under optimum soaking conditions (pH 5.5, 24 h, 40 °C). Iimure et al. (2009) developed a simple method of GABA production from barley bran supplemented with glutamate and found that the optimal reaction conditions for GABA production were sodium glutamate (10 mM), barley bran (150 mg:100 mL), reaction time (6 h) and reaction temperature (20 °C). As a result, 11 mM GABA could be produced using this method.

Soaking or incubating the rice or cereal bran is an effective method for GABA production at control temperatures. However, the incubation time must be optimized because GABA production is adversely affected when the incubation time exceeds the optimum (Komatsuzaki et al., 2007). In a previous experiment on rice bran, the current authors compared the GABA content when external glutamic acid was added and when it was not (data not shown). GABA content with glutamic acid addition was higher than when no glutamic acid was added. Therefore, it was decided to add glutamic acid to increase the substrate of the alpha-decarboxylation reaction and produce more GABA. Moreover, the effect of buffer type and concentration on GABA production using rice bran has not been previously investigated. Therefore, the objective of this research was to investigate the effect of the incubation time on GABA production and to produce a higher GABA content from rice bran by selecting the proper buffer type and concentration at a stabilized pH during incubation.

Materials and methods

Materials and chemicals

Rice bran was prepared from milling rice grain from different cultivars of Thailand namely, *O. sativa* L. cv. Khao Dawk Mali 105 (KDML105), Supanburi 1 (SP1), Chainat 1 (CN1), Phitsanulok 2 (PS 2) and Pathumthani 1 (PT1). These varieties were selected because of their high production levels in the country. The rice bran was passed through a 30-mesh screen and stored at −4 °C until use. Prior to incubation, the rice bran was ground to break cells so the GAD could be released from the cells and react with glutamic acid (the substrate of GABA synthesis) that was added to the buffer solution for GABA synthesis.

A standard of gamma-aminobutyric acid (purity 99%) was purchased from Sigma Aldrich Chemicals (MO, USA). Sodium acetate trihydrate, sodium bicarbonate and L-Glutamic acid (99% purity) were purchased from Sigma Aldrich Chemicals (Japan). 4-Dimethyl-aminoazobenzene-4-sulfonyl chloride (DABSYL-Cl) analytical grade was purchased from Sigma Aldrich Chemicals (Switzerland), and acetonitrile (HPLC grade) was obtained from Mallinckrodt chemicals (MO, USA).

Effect of incubation time on gamma-aminobutyric acid production

This method has been modified from Iimure et al. (2009). Glutamic acid solution (0.2% weight per volume; w/v) was added to rice bran at a rice bran to glutamic acid solution ratio of 1–8 (w/v). The mixture was then incubated at 40 °C to accumulate GABA and centrifuged. The supernatant obtained from centrifugation was assayed for its GABA content.

Effect of buffer type on gamma-aminobutyric acid production

This method has been modified from Iimure et al. (2009). Different types of buffers (containing 0.2% glutamic acid) consisting of 50 mM of Tris, citric acid, boric acid and phosphate buffers (pH 5.6) were added to rice bran with the rice bran to buffer at a ratio of 1–8 (w/v). The mixture was incubated at 40 °C for 6 h to accumulate GABA and centrifuged. The supernatant obtained from centrifugation was assayed its GABA content.

Effect of buffer concentration on gamma-aminobutyric acid production

This method has been modified from Iimure et al. (2009). Proper buffer (containing 0.2% glutamic acid) pH 5.6 was added to rice bran with the rice bran to buffer at a ratio of 1–8 (w/v). The concentrations of buffer were varied from 0 to 100 mM. Then, the mixture was incubated at 40 °C for 6 h to accumulate GABA. The mixture was centrifuged and the supernatant obtained from centrifugation was assayed for its GABA content.

Determination of gamma-aminobutyric acid content

This method was modified from Varanyanond et al. (2005). One-fifth to one-half a gram of rice bran was weighed or one-fifth to one-half a milliliter of rice bran solution (supernatant) was pipetted into a plastic tube with 1.8 mL of deionized water and 200 μL of added 3% sulfosalicylic acid. The sample solution was shaken at room temperature for 1.5 h, then centrifuged at 4500 × *g* for 10 min a sample of 50 μL of supernatant was pipetted and added to 50 μL of 100 mM $NaHCO_3$ and 50 μL of 4 mM DABSYL-Cl acetonitrile solution. The reaction was performed at 70 °C for 20 min. After derivatization, 250 μL of absolute ethanol and 250 μL of 25 mM phosphate buffer (pH 6.8) were added. The sample was filtered into a vial and 5 μL of sample was injected into a high performance liquid chromatography (HPLC) system (Series 1100; Hewlett Packard; CA, USA) with a Supelcosil LC-DABS column, 4.6 × 150 mm, 2 μm (Supelco; PA, USA). The HPLC was equipped with an ultraviolet–Vis photodiode array detector set at 465 nm wavelength. The mobile phase was 25 mM acetate buffer and acetonitrile (80:20 volume per volume; v/v) adjusted at a flow rate of 1.0 mL/min at 35 °C. GABA was used as a calibration standard.

Statistical analysis

All the data were statistically analyzed using a completely randomized design by ANOVA with a least significant difference test at the 95% confidence level.

Results and discussion

Gamma-aminobutyric acid contents of rice bran

The GABA contents of the five different cultivars of rice bran are shown in Fig. 1. The initial GABA content of KDML105 (0.58 mg/g of bran) was not significantly different from those of PSL2 (0.48 mg/g of bran), PTT1 (0.52 mg/g of bran) and SP1 (0.44 mg/g of bran). However, production using KDML105 was the highest among the various rice cultivars. It was reported that 2 million t of KDML105 were exported in 2012 which is about 28% of total exported Thai rice and in addition, approximately 2 × 10^6 t of KDML105 rice bran per year were produced as a major by-product from the rice milling

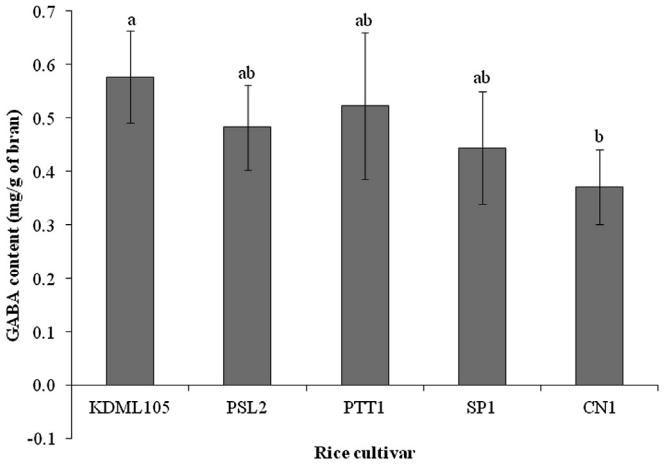

Fig. 1. Initial gamma-aminobutyric acid content of five different cultivars of rice bran. Means (N = 3) followed by the different letters are significantly different ($p \leq 0.05$). Error bars indicate ± SD.

industry (Office of Agricultural Economics, 2013). Due to its abundance, KDML105 variety rice bran was selected for further study of GABA production.

Effect of incubation time on gamma-aminobutyric acid production

The concentration of GABA increased with the incubation time and reached a maximum at 6 h (Fig. 2). However, when the incubation time was increased to 10 h, GABA production decreased. During the incubation, the pH of the mixture increased with the incubation time (Fig. 3) because of the GAD activity. This induced the removal of protons by catalyzing alpha-decarboxylation of glutamic acid.

The increase in GABA production might have been due to an increase in pH from its initial value (pH 4.8) to its optimum for alpha-decarboxylation in the system incubated for 6 h at pH 5.5. The decrease in GABA production after 6 h might have been because of an increase in the pH during the alpha-decarboxylation that continued until the acidity of the mixture increased to pH 8.0. This is the optimal pH for GABA transaminase (EC 2.6.1.19) and it catalyzed the reversible conversion of GABA to succinic semialdehyde (Bouché and Fromm, 2004) and hence the GABA concentration slightly decreased after 6 h.

During incubation, the increase in the pH was because GABA was synthesized primarily through H^+ consuming alpha-decarboxylation of L-glutamate in a reaction (L-glutamate + H^+ → GABA + CO_2) catalyzed by glutamate decarboxylase (GAD; EC 4.1.1.15) (Bown and Shelp, 1997). The increase in pH might have been due to using H^+ in this irreversible reaction. Even carbon dioxide from the GABA synthesized reaction can react with water ($H_2O + CO_2 \rightleftharpoons H_2CO_3$) to produce carbonic acid and carbonic acid can decrease the pH of the system; however, this reaction is reversible and might not have decreased the pH (Harned and Davis, 1943).

Effect of buffer type on gamma-aminobutyric acid production

The effect of different types of buffer on GABA production using KDML105 rice bran was investigated. The results are shown in Fig. 4. After incubation for 6 h at 40 °C, the highest GABA content ($p \leq 0.05$) was found in a reaction mixture using 50 mM phosphate buffer (6.13 mM or 5.05 mg/g of bran), pH 5.6 followed by Tris buffer (5.21 mM or 4.30 mg/g of bran), boric acid buffer (4.68 mM or

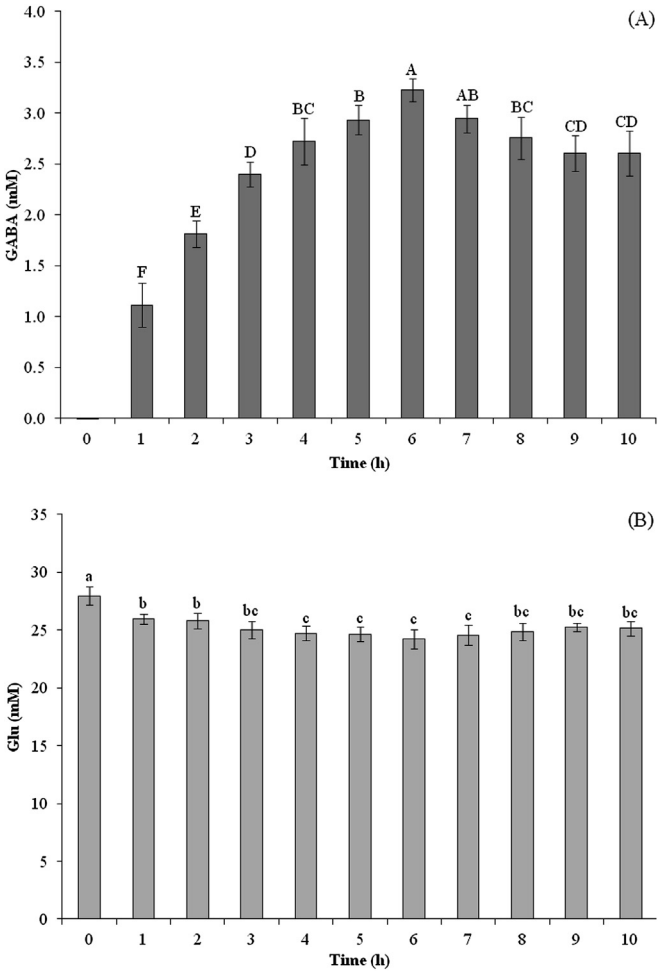

Fig. 2. Effect of incubation time on: (A) gamma-aminobutyric acid (GABA) content; (B) Glutamic acid content during GABA production. The reaction mixture consisted of rice bran to 0.2% Glu solution in a ratio of 1–8 (w/v); the mixture was incubated at 40 °C to accumulate GABA. Means (N = 3) followed by the different letters are significantly different ($p \leq 0.05$). Error bars indicate ± SD.

3.86 mg/g of bran) and citric acid buffer (4.05 mM or 3.34 mg/g of bran). However, GABA production in the reaction mixture using phosphate buffer decreased after 6 h. The control (no buffer system) produced GABA at a level of only 2.66 mg/g of bran.

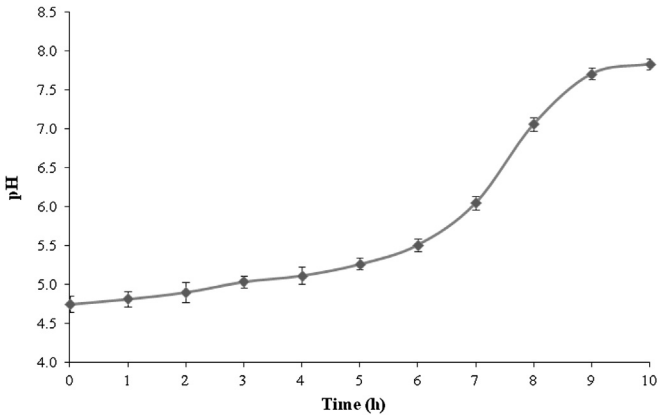

Fig. 3. Increase of pH during incubation as a function of time. The reaction mixture consisted of rice bran to 0.2% Glu solution in a ratio of 1–8 (w/v). The mixture was incubated at 40 °C to accumulate gamma-aminobutyric acid. Error bars indicate ± SD.

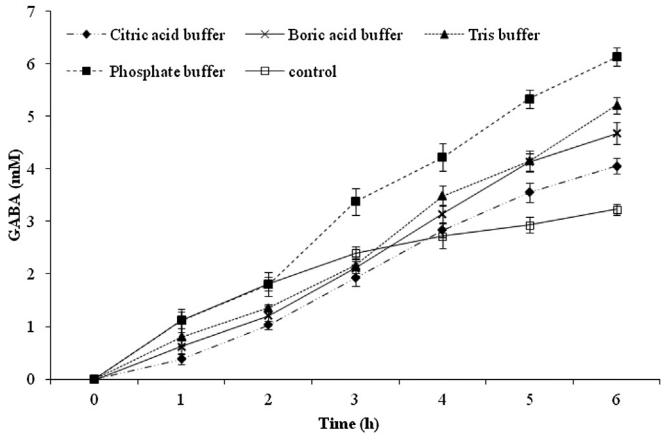

Fig. 4. Effect of buffer type on gamma-aminobutyric acid (GABA) production. The reaction mixture consisted of KDML105 rice bran to 50 mM buffer (pH 5.6) containing 0.2% Glu in a ratio of 1–8 (w/v). The mixture was incubated at 40 °C for 6 h to accumulate GABA. Error bars indicate ± SD.

Phosphate buffer was better than the other types of buffer at the same concentration (50 mM). This might have been due to free phosphate ions in the buffer system which could have enhanced the formation of a Schiff base by serving as both a proton donor and acceptor (Huang et al., 2001). Schiff base has been known as an intermediate in the irreversible alpha-decarboxylation reaction of Glu to produce GABA (Toney, 2005). Therefore, phosphate buffer was chosen for further study on the effect of the buffer concentration on GABA production.

Although Tris and boric acid buffers have slightly alkaline buffering capacity, the results from the current study showed that these buffer types were able to stabilize the pH of the mixture. The pH was only slightly changed at the end of incubation and was not significantly different from the initial pH of system before incubation (Table 1).

Citric acid buffer, which has a working range that included the pH values observed in the current study, produced the lowest levels of GABA. This was in agreement with Fonda (1972) who reported that various carboxylic acids were demonstrated to be substrate-competitive inhibitors of GAD.

Effect of buffer concentration on gamma-aminobutyric acid production

The effects of phosphate buffer concentration at pH 5.6 upon GABA production from KDML105 rice bran are shown in Fig. 5 and show that increasing the phosphate buffer concentration increased the GABA production. Maximal GABA production was found with 80 mM phosphate buffer. GABA was produced at 6.36 mM (7.71 mg/g of bran). However, when the concentration increased to more

Table 1
Changes in the pH of various buffers before and after incubation.

Buffer type	pH	
	Before incubation	After incubation
Citric acid buffer	5.60 ± 0.00^{A}	5.60 ± 0.00^{A}
Phosphate buffer	5.60 ± 0.00^{A}	5.60 ± 0.00^{A}
Boric acid buffer	5.60 ± 0.00^{A}	5.61 ± 0.01^{A}
Tris buffer	5.60 ± 0.00^{A}	5.63 ± 0.02^{A}
Control	4.73 ± 0.09^{A}	5.51 ± 0.08^{B}

Note: The reaction mixture consisted of KDML105 rice bran to 50 mM buffer (pH 5.6) contained 0.2% Glu in the ratio of 1–8 (w/v), the mixture was incubated at 40 °C for 6 h to accumulate GABA. Means (N = 3) followed by the different upper case letters in the same row are significantly different ($p \leq 0.05$). Values are mean ± SD.

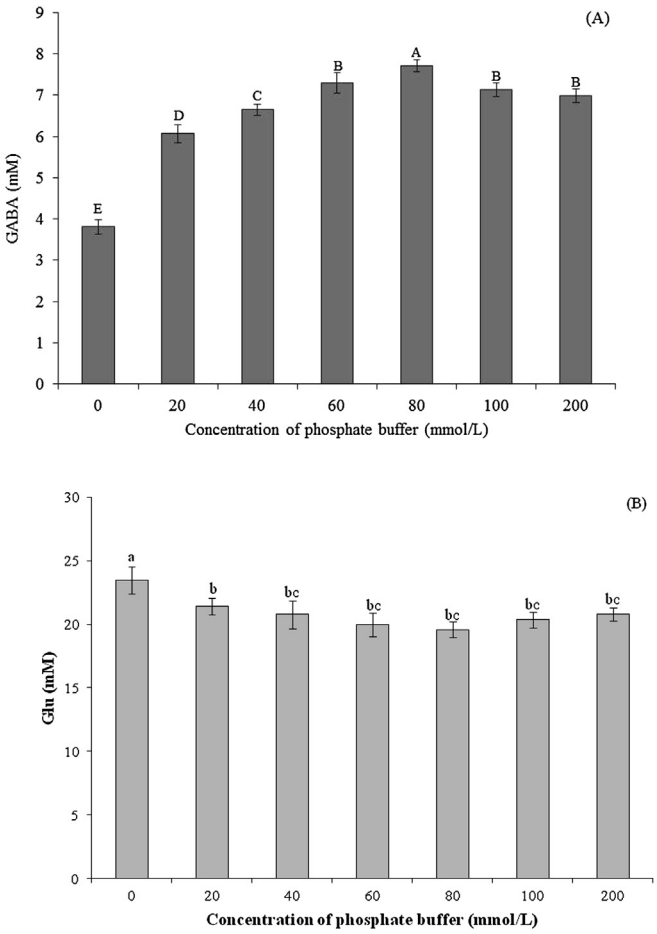

Fig. 5. Effect of buffer concentration: (A) gamma-aminobutyric acid (GABA) content; (B) glutamic acid content during GABA production. The reaction mixture consisted of KDML105 rice bran to phosphate buffer (pH 5.6) containing 0.2% Glu in a ratio of 1–8 (w/v), the mixture was incubated at 40 °C for 6 h to accumulate GABA. Means (N = 3) followed by different letters are significantly different ($p \leq 0.05$). Error bars indicate ± SD.

than 80 mM, the GABA production decreased slightly. This was possibly due to an increase in the ionic strength in the reaction system. A higher ionic strength might reduce the activity of the enzyme (Vincent and Blanchard, 1990).

The initial GABA content in the rice bran was only 0.58 mg/g of bran. The GABA produced in the current experiment was 7.71 mg/g of bran (11 times higher than the initial GABA content). The GABA produced in the current study was much higher in concentration than the GABA from germinated brown rice produced by Karladee and Suriyong (2012) and Jannoey et al. (2010) who reported 0.23 mg/g and 2.45 mg/g of grain, respectively.

The GABA produced in the current experiment was also higher in concentration than the GABA produced from wheat bran by Nogata and Nagamine (2009) which contained a GABA concentration of 1.48 mg/g of wheat bran. Production of GABA from rice bran in the current study was lower than that produced from rice germs by Ohtsubo et al. (2000) and Zhang et al. (2006). However, rice bran is a by-product from rice milling and much cheaper than rice germ. Therefore, rice bran is very useful and was selected for GABA production in the current research.

The highest initial GABA content was found in rice bran of the KDML105 cultivar (0.58 mg/g of bran) and for this reason, it was selected for this research. Phosphate buffer stabilized the pH in the reaction system. Using 80 mM phosphate buffer (pH 5.6) incubated

for 6 h produced the highest GABA concentration of 6.36 mM (7.71 mg/g of bran). The yield of GABA was about 11 times higher compared to the initial GABA content in the rice bran and about 2.7 times that of the un-buffered system (control). The results indicated that the incubation time, buffer type and concentration affected the GABA production from rice bran.

Conflict of Interest

No conflicts of interest influenced this research.

Acknowledgments

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References

Adeghate, E., Ponery, A.S. 2002. GABA in the endocrine pancreas: cellular localization and function in normal and diabetic rats. Tissue Cell. 34: 1–6.
Bao, J., Cheung, W.Y., Wu, J.Y. 1995. L-glutamate decarboxylase: Inhibition by phosphorylation and activation by dephosphorylation. J. Biol. Chem. 270: 6464–6467.
Bouché, N., Fromm, H. 2004. GABA in plants: just a metabolite? Trends Plant Sci. 9: 110–115.
Bown, A.W., Shelp, B.J. 1997. The metabolism and functions of γ- aminobutyric acid. Plant Physiol. 115: 1–5.
Dhakal, R., Bajpai, V.K., Baek, K. 2012. Production of GABA (γ-amino butyric acid) by microorganisms: a review. Braz. J. Microbiol. 1230–1241.
Fonda, M.L. 1972. Glutamate decarboxylase. Substrate specificity and inhibition by carboxylic acids. Biochemistry 11: 1304–1309.
Harned, H.S., Davis, R.D. 1943. The ionization constant of carbonic acid in water and the solubility of carbon dioxide in water and aqueous salt solutions from 0 to 50°C. Anal. Chem. 65: 2030–2037.
Huang, T.C., Chen, M.H., Ho, C.T. 2001. Effect of phosphate on stability of pyridoxal in presence of lysine. J. Agric. Food Chem. 49: 1559–1563.
Iimure, T., Kihara, M., Hirota, N., Zhou, T., Hayashi, K., Ito, K. 2009. A method for production of γ-amino butyric acid (GABA) using barley bran supplemented with glutamate. Food Res. Int. 42: 319–323.
Jannoey, P., Niamsup, H., Lumyong, S., Suzuki, T., Katayama, T., Chairote, G. 2010. Comparison of gamma-aminobutyric acid production in Thai rice grains. World J. Microbiol. Biotechnol. 26: 257–263.
Juliano, R.O. 1985. Rice: Chemistry and Technology, second ed. American Association of Cereal Chemists, St. Paul, MN, USA.
Karladee, D., Suriyong, S. 2010. Gamma-aminobutyric acid (GABA) content in different varieties of brown rice during germination. Sci. Asia 38: 13–17.
Kimura, M., Hayakawa, K., Sansawa, H. 2002. Involvement of γ-aminobutyric acid (GABA) B receptors in the hypotensive effect of systemically administered GABA in spontaneously hypertensive rats. Jpn. J. Pharmacol. 89: 388–394.
Komatsuzaki, N., Tsukahara, K., Toyoshima, H., Suzuki, T., Shimizu, N., Kimura, T. 2007. Effect of soaking and gaseous treatment on GABA content in germinated brown rice. J. Food Eng. 78: 556–560.
Nogata, Y., Nagamine, T. 2009. Production of free amino acids and γ-aminobutyric acid by autolysis reactions from wheat bran. J. Agric. Food Chem. 57: 1331–1336.
Office of Agricultural Economics 2013. Agricultural Export. Thailand, Bangkok. Available from: http://www.oae.go.th/oae_report/export_import/export_result.php [1 December 2013].
Oh, C.H., Oh, S.H. 2004. Effect of geminated brown rice extract with enhanced levels of GABA on cancer cell proliferation and apoptosis. J. Med. Food 7: 19–23.
Ohtsubo, S., Asano, S., Sato, K., Matsumoto, I. 2000. Enzymatic production of gamma-aminobutyric acid using rice (*Oryza sativa*) germ. Food Sci. Technol. Res. 6: 208–211.
Toney, M.D. 2005. Reaction specificity in pyridoxal phosphate enzymes. Arch. Biochem. Biophys. 433: 279–287.
Varanyanond, W., Tungtrakul, P., Surojanametakul, V., Watanasiritham, L., Luxang, W. 2005. Effects of water soaking on gamma-aminobutyric acid (GABA) in germ of different Thai rice varieties. Kasetsart J. Nat. Sci. 39: 411–415.
Vincent, S.S., Blanchard, J.S. 1990. Buffers: principles and practice. Chap. 6. In: Deutscher, M.P. (Ed.), Methods in Enzymology, Guide to Protein Purification, 182. Academic Press, Inc, San Diego, CA, USA, pp. 24–38.
Wang, L., Xu, D.X., Lv, Y.G., Zhang, H. 2010. Purification and biochemical characterization of a novel glutamate decarboxylase from rice bran. J. Sci. Food Agric. 90: 1027–1033.
Zhang, H., Yao, H., Chen, F. 2006. Accumulation of γ-aminobutyric acid in rice germ using protease. Biosci. Biotechnol. Biochem. 70: 1160–1165.
Zhang, H., Yao, H., Chen, F., Wang, X. 2007. Purification and characterization of glutamate decarboxylase from rice germ. Food Chem. 101: 1670–1676.
Zeigler, R.S., Barclay, A. 2008. The relevance of rice. Rice Sci. 1: 3–10.