Availability of Partially Milled Rice as a Daily Source of γ -Aminobutyric Acid

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We investigated the use of a high-performance liquid chromatography (HPLC) with *o*-phthalaldehyde fluorescence detection to simultaneously quantify γ -Aminobutyric acid (GABA), glutamate and alanine. Using this method, the column (COSMOSIL 5C₁₈-MS-II, 4.6 mm i.d. $\times$ 15 cm) was eluted using a linear gradient of acetonitrile and 5 mM citrate buffer (pH 6.0). The GABA contents of both milled and cooked rice samples were measured, with a gradual decrease observed as the rice was milled in raw rice. However, GABA content was markedly diminished due to washing in rice milled for a longer period. In less milled rice, the GABA and glutamate contents increased when the rice was cooked without washing. As a result, the GABA content of cooked under-milled rice was twice that of cooked well-milled rice available in the market, and three times greater for under-milled cooked rice without washing. These findings revealed that partially milled rice could be utilized as a daily source of GABA.

Keywords: γ -aminobutyric acid, HPLC with a fluorescence detector, under-milled rice, cooked rice

Introduction

GABA is the most widespread inhibitory neurotransmitter (Bronstein and Cummings, 2001). GABA is expected to have a hypotensive effect on blood pressure and to be involved in alleviating mental stress. It has been shown that the GABA in rice germ could allay anxiety during menopause and in the elderly (Okada *et al.*, 2000). Subjects with high normal blood pressure were given fermented milk containing GABA for 12 weeks, and it was demonstrated that their blood pressure was significantly lowered by this treatment (Kajimoto *et al.*, 2004). Recently, in Japan, some foods supplemented with GABA have gained popularity. However, GABA is naturally synthesized by the action of a decarboxylase on glutamate, which is reversibly transformed into alanine by a transaminase. Rice germ and bran contain glutamate decarboxylase. Incubation with glutamate resulted in the immediate production of GABA (Ohtsubo *et al.*, 2000). Determination of the GABA content of brown rice, germinated brown rice, and germinated unhulled rice revealed that the GABA content of the germinated types was higher than that of brown rice (Ohisa *et al.*, 2003). Germinated brown rice, or *hatsuga-genmai*, has recently been introduced onto the market. It can be cooked well with well-milled rice and has a GABA content that is several times higher than that of well-milled rice. Thus, it is expected that a daily diet of cooked rice combined with 10% *hatsuga-genmai* can effectively increase the GABA intake. Conversely, brown rice also contains more GABA than well-milled rice but it needs to be soaked in water for more than 2–3 hours before boiling, and its palatability is low, which means that it is general-

ly avoided. However, partially-milled brown rice can absorb water over a short period of time and tastes as good as well-milled rice. In 2005, we reported that the GABA content of rice samples could be measured by HPLC with fluorescence detection and that the GABA content of rice milled for a shorter period was higher than that of types that had been milled for longer. To simultaneously quantify GABA, glutamate, and alanine, we analyzed the rice samples using this HPLC method using *o*-phthalaldehyde fluorescence instead of fluorescamine. We found that partially milled rice could be utilized as a daily source of GABA.

Materials and Methods

Rice samples Brown rice (*Oryza sativa* L.; 450 g; *Hino-hikari* from Nara Prefecture grown in 2004) was milled for 1.5, 2, 3, and 4.5 min using a milling machine (Tiger Co. RSD-A), and labeled as under-milled, well-milled 1, well-milled 2, and over-milled rice, respectively. Rice samples were stored at 4°C until use.

Chemicals *o*-Phthalaldehyde (OPA) and 2-mercaptoethanol were purchased from Wako Co. All other chemicals were of analytical grade. The OPA reagent used for precolumn derivatization of HPLC was prepared according to the method of Goto *et al.* (1993). Twenty milligrams of OPA was dissolved in 3 ml acetonitrile, and 7 ml 0.1 M borate buffer (pH 9.0) was added. This was kept under cold storage conditions overnight and filtered through a 0.45 μ m $\times$ 20 mm i.d. RC-membrane filter (Sartorius AG).

Cooking method The cooking method used in this experiment was in accordance with that reported in a previous paper (Iwaki *et al.*, 2005, in Japanese). Rice (462 g) was washed thrice with 700 ml water, drained, and water was again added to give a total weight of 1132 g.

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It was cooked in a rice cooker (Tiger Co. IH rice cooker JKA-A100HU, Japan) under conditions similar to those used for *hakumai-futsudaki* and was labeled as washed and cooked rice. Rice that was cooked without washing was labeled “non-washed” and “cooked” rice. After cooking, a sample of cooked rice was immediately collected from the central portion of the cooked rice.

Measurement of the moisture content Before cooking, the rice was ground into pieces using a mill (Iwatani Co. IFM-700G, Japan). Subsequently, 5 g was weighed, placed within an aluminum foil receptacle, and dried at 135°C for 3 h to measure the moisture content. A similar procedure was followed for the cooked rice sample, except that 10 g of cooked rice was weighed into an aluminum foil vessel and wrapped within the aluminum foil. The aluminum foil was then unfolded to dry at 135°C for 3 h.

Analyses of several rice characteristics The weight of 1000 rice grains was estimated using the method employed by the National Food Research Institute (Ministry of Agriculture and Forestry, 1969). The color of the surface of the rice grains was measured using a spectrophotometer (Nippondenshoku Co. SE 2000, Japan), as reported previously. Rice grains were placed within a transparent acrylic cylinder (31 mm diameter, 13 mm height) to measure the reflected light (Lab value) when the gauge diameter was 30 mm.

Extract preparation Rice powder (5 g) was homogenized (15000 rpm $\times$ 2 min) with 25 ml of 75% ethanol in a homogenizer (IKA Works T25 Basic 81), and the homogenate was filtered through a No. 5B filter paper. The residue was extracted thrice with 25 ml of 75% ethanol, and the volume of the collected filtrate was made up to 100 ml with 75% ethanol. In the case of cooked rice, approximately 18 ml of 99.5% ethanol was added to a 10 g sample, which is equivalent to 5 g of raw rice (Iwaki *et al.*, 2005). The other procedures were the same as those performed in the case of rice powder.

Analysis of GABA content The HPLC conditions were the same as those reported by Goto *et al.* (1993). The

sample was prepared by mixing 20 μ l of the extract solution, 200 μ l of the OPA reagent, and 200 μ l of 2-mercaptoethanol solution [50 μ l of 2-mercaptoethanol in 0.1 M borate buffer (pH 9.0)] in a vial; 10 μ l of the mixture was then immediately injected into the HPLC column (COSMOSIL 5C₁₈-MS-II, 4.6 mm i.d. $\times$ 15 cm). The method used a linear acetonitrile gradient (solution A) and 5 mM citrate buffer (pH 6.0) (solution B). This operation was performed mechanically (Shimadzu Co. LC-10AD with RF-10A, Japan). The mobile phase comprised 15% solution A and 85% solution B at 0 min, 16.5% solution A and 83.5% solution B at 15 min, 30% solution A and 70% solution B from 35 to 40 min, and 15% solution A and 85% solution B for 10 min. The flow rate and column temperature were set to 0.8 ml/min and 40°C, respectively. The fluorescence detector was set at Ex. 340 nm and Em. 450 nm.

Results and Discussion

Elution pattern of the rice extract In order to investigate the effect of concentration and pH of the citrate buffer on peak height, the concentration and pH were adjusted to 5 to 20 mM and 5.0 to 9.0, respectively. The peaks were most pronounced when the concentration of citrate buffer was 5 mM; however, the peaks were independent of the pH. Based on these findings, 5 mM citrate buffer (pH 6.0) was used as the mobile phase. The elution patterns of standard GABA (1 μ g/ml) and the under-milled rice extract are shown in Fig. 1. The major peaks from the under-milled rice extract were confirmed to correspond to L-glutamate (7.27 min), L-asparagine (11.74 min), L-arginine (13.59 min), L-alanine (29.72 min), GABA (31.97 min), and L-tyrosine (33.51 min). The contents of the other amino acids were comparatively lower. These results agreed with previously reported results on the composition of amino acids in cooked rice (*Oryza sativa* L., *Koshihikari*, harvested from Kyoto Prefecture in 2003) that had been obtained using an amino acid analyzer (Hitachi Co. L-8500) (Iwaki, *unpublished*). L-Glutamate and L-alanine are closely related to GABA biosynthesis

Fig. 1. Chromatograms of GABA standard (1 μ g/ml) (a) and under-milled rice extract (b).

Table 1. Some characteristics and GABA content of milled rice.

Sample	Milling yield(%)	Weight of 1000 grain (g)	Remain of germ (%)	Content of water (%)	Color of grain surface			Content of GABA ($\mu\text{g/g}\cdot\text{dry}$)
					L value	a value	b value	
under-milled	94.0	20.27 $\pm$ 0.15 ^a	39.59 $\pm$ 5.59 ^a	15.89 $\pm$ 0.10	65.38 $\pm$ 0.41 ^a	-0.22 $\pm$ 0.11	19.52 $\pm$ 0.34	27.71 $\pm$ 1.40 ^a
well-milled 1	92.4	19.73 $\pm$ 0.01 ^b	12.20 $\pm$ 0.93 ^b	16.06 $\pm$ 0.12	66.92 $\pm$ 0.51 ^b	-1.36 $\pm$ 0.07	17.70 $\pm$ 0.18	22.48 $\pm$ 1.16 ^b
well-milled 2	91.3	19.56 $\pm$ 0.13 ^b	6.97 $\pm$ 0.60 ^c	15.86 $\pm$ 0.12	69.25 $\pm$ 0.67 ^c	-1.76 $\pm$ 0.04	16.88 $\pm$ 0.36	20.87 $\pm$ 2.55 ^{bc}
over-milled	88.8	19.26 $\pm$ 0.01 ^b	1.80 $\pm$ 0.49 ^d	15.72 $\pm$ 0.05	71.22 $\pm$ 0.36 ^d	-2.27 $\pm$ 0.04	14.78 $\pm$ 0.10	17.64 $\pm$ 1.47 ^c

Each value represents the mean $\pm$ S.D. calculated from 3 determinations.

Means in a column without a common letter differ, $P < 0.05$.

and were measured with GABA in order to plot the following calibration curves: L-glutamate ($y = 676475x + 29411$, $R^2 = 0.9992$), L-alanine ($y = 759652x + 57676$, $R^2 = 0.9989$), and GABA ($y = 1218500x + 19236$, $R^2 = 0.9994$). The limits of detection for GABA and the other amino acids were $0.05 \mu\text{g/ml}$ and $0.1 \mu\text{g/ml}$, respectively; whereas, the detection limits of were $0.02 \mu\text{g/ml}$ and $0.05 \mu\text{g/ml}$, respectively.

Some characteristics and the GABA content of rice samples A few characteristics and the GABA content of four rice samples are shown in Table 1. In a previous paper, the milled rice samples were over-milled due to the use of a large milling machine. However, a smaller milling machine was used in this study and the milling yields of the four samples were 94.0%, 92.4%, 91.3%, and 88.8%, respectively. In general, the milling yield of well-milled rice derived from brown rice was approximately 92%. Further, the samples were labeled as being “under-milled”, “well-milled 1”, “well-milled 2”, and “over-milled” rice, while the identically labeled samples reported in the previous paper were labeled as “more milled”.

The weight of 1000 grains was observed to decrease in proportion to the milling yield. The residual amounts of the germs present in all the samples were significantly different. The color of the grain surface indicated that the L-value increased in proportion to the milling yield, whereas the a- and the b-values decreased. The color of rice gradually became brighter and tinged with green. However, it changed to pale yellow in proportion to the milling yield. The GABA content was significantly higher in under-milled than in well-milled 1 rice, and it was also higher in well-milled 1 than in over-milled rice. There was no significant difference between well-milled 1 and well-milled 2 rice. The relationship between GABA content and the weight of 1000 grains is shown in Fig. 2 along with additional results from a previous paper. Although the harvest year of the two samples was differed, the GABA content was in direct proportion to the weight of 1000 grains. This showed that the GABA content gradually decreased as the rice was milled.

Water and GABA content of cooked rice The water and GABA contents of cooked rice are shown in Table 2. Although there was no significant difference in the water content of cooked rice, the water content of washed, under-milled rice was slightly higher than that of the other samples. In washed and cooked rice samples, the GABA content was significantly higher in under-milled

Fig. 2. Correlation between GABA content and weight of 1000 grains of Hinohikari harvested in 2004 ($\diamond$) and 2003 in the previous paper ($\blacksquare$).

Table 2. Contents of water and GABA in cooked rice.

Sample	Washed		Non-washed	
	Water (%)	GABA ($\mu\text{g/g}\cdot\text{dry}$)	Water (%)	GABA ($\mu\text{g/g}\cdot\text{dry}$)
under-milled	63.02 $\pm$ 1.10	28.83 $\pm$ 0.10 ^a	61.42 $\pm$ 1.28	37.96 $\pm$ 0.68 ^a
well-milled 1	61.45 $\pm$ 0.23	20.97 $\pm$ 1.70 ^b	59.55 $\pm$ 0.90	27.15 $\pm$ 0.51 ^b
well-milled 2	60.22 $\pm$ 0.50	12.12 $\pm$ 0.18 ^c	59.36 $\pm$ 0.27	19.50 $\pm$ 0.79 ^c
over-milled	61.17 $\pm$ 0.79	11.30 $\pm$ 0.39 ^c	61.68 $\pm$ 0.61	17.52 $\pm$ 1.46 ^c

Each value represents the mean $\pm$ S.D. calculated from 3 determinations.

Means in a column without a common letter differ, $P < 0.01$.

than in well-milled 1 rice, and it was also higher in well-milled 1 than in well-milled 2 rice. The GABA content of under-milled rice was 2.6 times that of over-milled rice. A comparison of the GABA contents of washed and cooked rice with that of raw rice indicated that it was barely altered by washing and cooking in under-milled rice. Conversely, however, it was reduced to 58% and 64% in well-milled 2 and over-milled rice, respectively. On the other hand, the GABA content was considerably higher in non-washed and cooked rice than it was in washed and cooked rice. A comparison of the GABA content in non-washed and cooked rice with that of raw rice suggested that it was remained relatively constant in well-milled 2 and over-milled rice but increased markedly

Fig. 3. Contents of GABA, glutamate and alanine in raw rice and non-washed cooked rice.

in under-milled and well-milled 1 rice. This suggested that the GABA content decreased primarily due to washing in well-milled rice such as over-milled and well-milled 2 rice. However, in less well milled rice such as under-milled and well-milled 1 rice, the loss of GABA due to washing was either low or the production of GABA while cooking was equal to the amount lost. The contents of GABA, glutamate, and alanine in raw and non-washed cooked rice are shown in Fig. 3. Glutamate was converted to GABA by a glutamate decarboxylase, and alanine was produced by a transfer reaction of amine with pyruvate (Sanga T., 2002). In under-milled and well-milled 1 rice, the GABA and glutamate contents markedly increased, while that of alanine increased only slightly. This indicated that more glutamate was produced than was lost due to conversion to GABA by a glutamate decarboxylase in under-milled and well-milled 1 rice. In over-milled rice, the glutamate and alanine contents increased markedly but that of GABA changed only slightly. This indicated that the glutamate produced during cooking was converted to alanine.

In conclusion, the GABA content in raw rice gradually decreased in proportion to the milling yield. With regard to washed and cooked rice, the GABA content decreased primarily during cooking in more milled rice. The GABA content of non-washed less milled and cooked rice was significantly higher than that of the more milled varieties. As a result, the GABA content of cooked un-

der-milled rice was twice that of cooked well-milled rice available on the market, that of cooked under-milled rice without washing was three times higher still. These findings revealed that partially milled rice without washing could be utilized as a daily source of GABA.

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