Accumulation of \( \gamma \)-Aminobutyric Acid in Rice Germ Using Protease

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The accumulation of \( \gamma \)-aminobutyric acid (GABA) in the rice germ by using protease was investigated. After the proteolytic hydrolysis of germ protein by trypsin, the amount of GABA reached about 2.26 g per 100 g of germ. This demonstrates that the GABA yield could be significantly increased by protease. Our method is efficient and safe for manufacturing health food enriched with GABA.

Key words: \( \gamma \)-aminobutyric acid; rice germ; glutamate decarboxylase; accumulation; trypsin

\( \gamma \)-Aminobutyric acid (GABA) is an important ubiquitous non-protein amino acid in both prokaryotic and eukaryotic organisms which is produced by glutamate decarboxylase (GAD; EC 4.1.1.15) from glutamic acid (Glu). It is a representative depressive neurotransmitter in the sympathetic nervous system, and has been proved to be effective for lowering the blood pressure of experimental animals and humans. Hypertension is an underlying disease that causes cardiovascular and cerebrovascular disorders. The number of hypertensives is increasing quickly around the world, making the prevention and management of hypertension very important. Besides improving hypertension, GABA-enriched food could also be used as a dietary supplement and/or nutraceutical to help treat sleeplessness, depression and autonomic disorders; chronic alcohol-related symptoms; and to stimulate immune cells. For these reasons, attempts to apply GABA to functional foods to prevent some diseases have been reported: GABA-rich green tea produced by an anaerobic or cycling treatment involving the anaerobic and aerobic incubation of tea leaves or shoots; GABA accumulation in rice germ by soaking in water; GABA enrichment of brown rice by a high-pressure treatment and germination; increase levels of GABA in germinated wheat by endogenous enzymes; GABA-enriched tempeh-like fermented soybean; and GABA-enriched dairy products and GABA accumulation in red-mold rice fermented with the Monascus fungus.

GABA has been produced for these methods from endogenous Glu by endogenous GAD or GAD from microbial organisms. Apart from fermentation by a few highly productive strains, the GABA content of these GABA-enriched foods is comparatively low (<400–600 mg/100 g of dry weight or <50 mM), and these foods cannot be used directly with much effect. Although the activities of protease and peptidase in rice are different between cultivars, in general, the activity of protease in rice roots is about 2–7 U per mg of the enzyme protein, and much lower than that of peptidase. Limited by the endogenous protease, the low quantity of endogenous Glu restricts the GABA accumulation level. Ohtsubo has developed an enzymatic production method for GABA in rice germ by adding exogenous Glu, and a up to 29.0 g/100 g of germ GABA content. This implies that rice germ is a good material for GABA accumulation because of its high activity for GAD. However, this method results in a large quantity of exogenous Glu remaining in the final products. It is not suitable because the use of a large amount of Glu is expensive.

We therefore attempted to establish a simple and effective technique for producing a high GABA content in rice germ without adding exogenous Glu. We selected trypsin as a protease to hydrolyze the germ protein and produce Glu for GABA accumulation.

Material and Methods

Materials. Rice germ was generously provided by Shanghai Store & Transport of Grain Co., Ltd., and stored at \(-4 \, ^{\circ}C\) before use. The purity of the germ was about 66%. Proteases were purchased from Nowzyme China. All other chemicals were of analytical grade.

Hydrolysis of the rice germ protein by protease. The reaction was performed in 150 ml of a 50 mM phosphate

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Abbreviations: GABA, \( \gamma \)-aminobutyric acid; Glu, glutamic acid; GAD, glutamate decarboxylase; TCA, trichloroacetic acid

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buffer with 10 g of rice germ. 200 U of protease was added (1 U of protease activity is defined as that to produce 1 µg of tyrosin per minute). The mixture was incubated for 6 hrs at the optimum pH and temperature for each protease: pH 8.0 and 45 °C for trypsin, pH 8.0 and 45 °C for protamex, pH 7.0 and 45 °C for neutease, and pH 7.0 and 45 °C for alcalase. The reaction was terminated by adding 32% (w/v) trichloroacetic acid (TCA) to a final concentration of 8%. The Glu produced was assayed as described later. The optimum type of protease and conditions for hydrolysis were chosen according to the amount of Glu produced. The pH change during proteolytic hydrolysis under the optimum conditions was also determined.

Measurement of the GAD activity. The reaction mixture consisted 2.0 ml of 50 mM sodium phosphate at pH 5.6, 1 ml of 100 mM L-glutamate, and 0.2 g of rice germ. The reaction solution was incubated at 40 °C for 60 min, and the reaction terminated by the addition of 1 ml of 32% (w/v) TCA. The GABA production was assayed as described later. One unit of GAD activity is defined as the release of 1 µmol of GABA produced from glutamate per 30 min at 40 °C. The GAD activity of rice germ is defined as units of GAD activity per gram of germ.

GABA accumulation in rice germ. Rice germ was hydrolyzed by a protease at the optimum conditions obtained from the experiments just described. The reaction mixture was incubated at 90 °C for 15 min to inactivate the enzyme, cooled to room temperature and centrifuged to obtain the supernatant. After adding 0.5–4 g of rice germ to 100 ml of the supernatant, the mixture was incubated at 40 °C for 8 hrs to accumulate GABA. The supernatant obtained from centrifugation was heated to 90 °C for 15 min to inactivate the enzyme, 1 ml of 32% TCA was added to 3 ml of the supernatant, and the GABA production was assayed as described later.

Assay of Glu, GABA and other amino acids. A 1-ml amount of the reaction mixture with 8% (w/v) of TCA was passed through a 0.45-µm membrane filter (Whatman, USA). The filtrate was analyzed with an L-8800 high-speed amino acid analyzer (Hitachi, Japan) to determine Glu, GABA and other amino acids.

Results and Discussion

Effect of the different proteases on Glu production

Figure 1 shows the Glu production with the different proteases. The Glu production was correlated with the type of protease, and reached a maximum level with trypsin. Trypsin is a pancreatic serine protease with substrate specificity based upon positively charged lysine and arginine side chains.23) The contents of arginine and lysine in the rice germ protein were relatively high compared with the other amino acids (Fig. 2). Consequently, the proteolytic hydrolysis by trypsin was relatively complete, and the Glu production was greater than that hydrolyzed by the other proteases. It is well known that the products of proteolytic hydrolysis by trypsin are mainly peptides with few amino acids, so it seems to need the cooperation of peptidase to obtain more amino acids. However, our study showed that the Glu production was markedly increased only by the trypsin digestion, so this phenomenon might be attributed to the high activities of endogenous peptidase. The total activities of amino-peptidase and carboxypeptidase in the rice root of some cultivars were 8 times that of protease.21) When we used peptidase combined with trypsin, less than 10% of the additional Glu could be produced (data not shown). However, the peptidase is expensive and it would make the final product cost high. By combining with the endogenous peptidase, trypsin digestion represents a more effective and lower cost method to generate a high amount of Glu.

Effect of trypsin on Glu production

We selected trypsin as the protease for germ proteolytic hydrolysis to produce Glu, and examined the optimal hydrolysis conditions for the ratio of germ to buffer, added amount of trypsin, temperature, and reaction time. Figure 3 shows that the optimal ratio of the germ to buffer was 1:14, and the optimal temperature and incubation time were 40 °C and 7 hrs. The optimal added amount of trypsin was 80 U/g of germ protein (Fig. 4).

The content of Glu in rice germ is relatively high among the different grain fractions, in addition to its high GAD activity, making rice germ a good material
for GABA accumulation. The total content of Glu in 100 g of rice germ was 3.87 g (Fig. 2), among which 0.11 g of Glu exists as a free amino acid; so, almost all Glu exists in the germ protein. Under the optimal hydrolysis conditions, about 85% of Glu was hydrolyzed to a free amino acid, and the amount of Glu in the reaction mixture reached 2.35 mg/ml. This resulted in a higher amount of Glu for GABA accumulation.

Change in the pH value during proteolytic hydrolysis
During the course of proteolytic hydrolysis, the pH value of the reaction mixture decreased and reached 5.5–6.0 before the reaction was terminated. Saikusa et al.\textsuperscript{15} have shown that the optimum pH for GABA accumulation in the rice germ by water soaking was 5.8. The maximal activity of GAD in rice germ was observed at pH 5.6 in our research, which is the same as that for other kinds of plant,\textsuperscript{24–26} so the reaction supernatant could be used to accumulate GABA directly without adjusting the pH value.

GAD activity of rice germ
Saikusa et al.\textsuperscript{27} have shown the amount and pattern of GABA accumulation varied considerably with cultivar. This variation might have been due to the different GAD activity which could vary with the breed and purity of the rice germ. This means that the GAD activity of rice germ should be measured before use, the GAD activity of the germ we used being about 84 U/g. Maximal GAD activity was at pH 5.6 and 40 °C.

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**Fig. 2.** Amino Acids in Rice Germ.

One gram of rice germ was completely hydrolyzed by 1 N HCl, and the amino acids determined with an L-8800 high-speed amino acid analyzer.

**Fig. 3.** Effect of the Reaction Conditions on Glu Production.

The hydrolytic reactions were performed by the method described in the Material and Methods section by using trypsin, the other conditions being (a) 45 °C, pH 8.0, adding 200 U of trypsin, with different ratios of the germ to buffer for 6 hrs; (b) 45 °C, pH 8.0, 1:14 of the germ to buffer ratio, 200 U of trypsin, and at different temperature for 6 hrs; (c) 45 °C, pH 8.0, 1:14 of the germ to buffer ratio, with 200 U of trypsin for different times.
Factors influencing the GABA production

After 7 hrs of hydrolyzing, a little GABA (0.25 mg/ml) had been produced in the supernatant by the remaining GAD activity. To accumulate more GABA, the supernatant obtained from the germ proteolytic hydrolysis was added to raw germ, and incubated at 40°C for several hours. One of the factors affecting the GABA production was the germ-to-supernatant ratio. In the supernatant, not only the Glu produced by germ proteolytic hydrolysis was decarboxylated to GABA by endogenous GAD from the added germ, but the added germ also produced GABA by its endogenous protease and GAD. Consequently the more germ that was added, the more GABA that would be produced. The GABA production was increased with increasing germ-to-supernatant ratio and reached a plateau at the ratio of 4.0 g/100 ml (Fig. 5). However, the GABA production at a germ-to-supernatant ratio of 1.5 g/100 ml was 86.4% of that at the ratio of 4.0 g/100 ml, so there was not a marked increase between 1.5 g/100 ml and 4.0 g/100 ml. In order to obtain the greatest GABA yield per unit germ, we performed the batch GABA production at the ratio of 1.5 g/100 ml. The other influencing factor was the reaction time, the optimal time being 6 hrs.

After 6 hrs of the reaction, the mixture was centrifuged, and the resulting supernatant was heated to 90°C for 15 min to inactivate the enzyme. The main components and amino acids in this supernatant are shown in Tables 1 and 2.

As shown in Table 1, the amount of GABA produced under these conditions was 190 mg/100 ml, equivalent to about 2.26 g per 100 g of germ. Compared with 28 mg of GABA per 100 g of raw germ, the GABA content was increased by more than 80 times and was higher than that (300–400 mg/100 g of germ) by simple water soaking. The germ we used was prepared from round-shaped rice, and the purity of the germ was about 66% which was not particularly so high. The amount of GABA produced by our method could be improved by selecting a more appropriate rice cultivar or germ with higher purity.

We developed in this study an efficient and simple
method for GABA production by using rice germ without adding exogenous Glu. Compared with the soaking method, we used protease and increased the reaction time to 7 hrs, resulting in GABA production about 6 times that accumulated by simple water soaking. In addition, the solution containing a high GABA level prepared by our method was also enriched with other amino acids (Table 2). The method can therefore be directly applied to produce not only a kind of anti-hypertensive food, but also a kind of nutrient food. Rice production is about 400 million tons a year throughout the world. Rice germ is in the waste product from the rice-polishing process and can be inexpensively obtained. There are almost 4–8 million tons of rice germ produced per year in P. R. China. Our method would be cost-effective for producing health food enriched with GABA and valuable as a means for the effective utilization of agricultural waste.

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