Biosci. Biotechnol. Biochem., **67** (5), 1018–1023, 2003

The Production of a New *Tempeh*-like Fermented Soybean Containing a High Level of γ-Aminobutyric Acid by Anaerobic Incubation with *Rhizopus*

Hideyuki AOKI,[1,†] Ichiyo UDA,[1] Keiko TAGAMI,[1] Yuji FURUYA,[1] Yasushi ENDO,[2] and Kenshiro FUJIMOTO[2]

[1]*Research Laboratory, Ikeda Tohka Industries Co., Ltd., 95-7 Minooki-Cho, Fukuyama, Hiroshima 721-0956, Japan*
[2]*Department of Science of Biological Function, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Sendai 981-8555, Japan*

Received November 1, 2002; Accepted February 14, 2003

A cultivation procedure for the preparation of a new *tempeh*-like fermented soybean containing a high level of γ-aminobutyric acid was developed. Steamed soybeans were incubated aerobically with *Rhizopus microsporus* var. *oligosporus* IFO 8631 for 20 h, and then anaerobically incubated for 5 h by replacement of the atmosphere with nitrogen. The GABA content in the aerobically fermented soybeans was about 30 mg per 100 g dry fermented soybeans, while the anaerobically cultivation was about 370 mg/100 g dry fermented soybeans. The incubation with several strains of *Rhizopus* species showed that all of *R. microsporus* var. *oligosporus* and *R. oryzae* examined accumulated GABA in the anaerobically fermented soybeans. In particular, *R. microsporus* var. *oligosporus* IFO 32002 and IFO 32003 showed the highest content of GABA (1,740 mg/100 g dry fermented soybeans and 1,500 mg/100 g dry fermented soybeans, respectively). Moreover, the free protein amino acids increased greatly in the fermented soybeans during the anaerobic cultivation.

Key words: γ-aminobutyric acid; fermented soybean; GABA; *Rhizopus*; *tempeh*

γ-Aminobutyric acid (GABA) is an important compound of the free amino acid pool in both prokaryotic and eukaryotic organisms and it is also a representative depressive neurotransmitter in the sympathetic nervous system.[1,2] Moreover, it has been reported that GABA retarded the elevation of systolic blood pressure[3] and improved discrimination learning in rats.[4] In Japan, highly purified GABA is used as medication for amelioration of the brain bloodstream.

It is known that GABA is found in wide varieties of plant foods such as tea leaves[5,6] and rice germ,[7–9] although their levels are about 290 to 550 mg/100 g of dry weight. Various fermented foods, such as Korean *kimchi*, yogurt,[10] and red-mold rice,[11,12] have also been known to contain a considerable amount of GABA. Furthermore, feeding of GABA-enriched foods such as gabaron tea,[13] red-mold rice[14] and *Chlorella*[15] were reported to depress the elevation of the systolic blood pressure in spontaneously hypertensive rats (SHR).

GABA is known to be biosynthesized in plants and microorganisms *via* decarboxylation of glutamic acid by glutamate decarboxylase, which is induced by various stresses.[12,16] Soy protein is very rich in glutamic acid and it has been reported that *tempeh*,[17] which is a traditional non-salted soybean fermented by *Rhizopus* spp. in Indonesia, contains greater amounts of peptides and free amino acids than the unfermented soybean.[18,19] This is the reason the hydrolytic breakdown of soybean proteins takes place during fermentation. Therefore, the content of free glutamic acid in *tempeh* has been suggested to be greater than that in the unfermented soybean. Moreover, it is expected that GABA is produced by the decarboxylation of glutamic acid by glutamate decarboxylase with *Rhizopus*.

In this study, a new procedure for the preparation of a GABA-enriched *tempeh*-like fermented soybean by incubation with *Rhizopus*, partly under anaerobic conditions, is reported.

Materials and Methods

Microorganisms. The *Rhizopus microsporus* var. *oligosporus* IFO 8631(=NRRL 2710), has been recommended for *tempeh* production and commercially used.[17] Beside this strain, we used 8 strains of fungi belonging to the genus *Rhizopus*: *R. microsporus* var. *oligosporus* IFO 31987, IFO 32002, IFO 32003, *R. oryzae* IFO 4705, IFO 4770, IFO 5438, and IFO 9364. All of the fungi used were chosen from

† To whom correspondence should be addressed. E-mail: aoki-hy@mbb.nifty.ne.jp
Abbreviations: GABA, γ-aminobutyric acid; SHR, spontaneously hypertensive rats; TCA, tricarboxylic acid

the culture collection of Ikeda Food Res. Co. (Fukuyama, Hiroshima, Japan).

Fermentation of soybeans. The fermented soybeans were prepared on the basis of the procedure for *tempeh* preparation as follows. The dehulled soybeans were soaked in 0.2% acetic acid at room temperature overnight. The soaked soybeans were steamed at 121°C for 5 min. Then 200 g of the steamed beans were inoculated with 1 ml of a suspension of spores of *R. microsporus* var. *oligosporus* IFO 8631 (about 1×10^7/ml). The inoculated beans were spread on a polyethylene sheet (20×30 cm) with small pinholes and incubated at 37°C under aerobic conditions. After the aerobic cultivation, the fermented soybeans were incubated at 37°C in an anaerobic vessel which was charged with an inert gas supplied from a commercial gas bomb for 1 min to decrease the oxygen level to less than 1%. The oxygen concentration in the vessel was monitored by an oxygen meter model RO-102 (Iijima Electronics Co., Gamagori, Aichi, Japan). The cultivation times varied and are shown in the Results. All samples were lyophilized and used for the GABA analysis. The growth of fungi in the fermented soybean was followed by the nucleic acid assay described by Ouchi *et al.*[20] The results of the fermentation are averaged based on at least two experiments.

Cultivation conditions for enrichment of GABA in fermented soybeans. The following factors on the cultivation conditions were assessed using *R. microsporus* var. *oligosporus* IFO 8631 against the accumulation of GABA in the fermented soybeans: length of aerobic and anaerobic cultivations and the composition of atmospheric gases for the anaerobic cultivation. The compositions of the solid media and the cultivation conditions are given in the legends of the figures and table.

Amino acid analysis. The free amino acids in the fermented soybeans were measured by the method of Saikusa *et al.*[8] using an automatic amino acid analyzer (L-8500, Hitachi, Tokyo) with a column (4.6×60 mm) filled with an ion-exchange resin (P/N 855-3501, Mitsubishi Chem., Tokyo).

The ethanol content in fermented soybeans. The ethanol contents in the fermented soybeans after aerobic cultivation for 20 h and that after the successive anaerobic cultivation for 20 h were measured by an enzymatic assay kit (F-Kits Ethanol, R-Biopharm GmbH, Darmstadt, Germany).

Results

Effects of cultivation time and conditions

The course of growth of *R. microsporus* var. *oligosporus* IFO 8631 and the GABA content in the fermented soybeans under the aerobic and anaerobic conditions are shown in Fig. 1. The cells grew almost linearly for the initial 30 h of cultivation under aerobic conditions and then the growth gradually slowed down, while the GABA content in fermented soybeans remained within a low level (less than 40 mg per 100 g dry fermented soybean) during 50 h of the aerobic cultivation. When the atmospheric gas was replaced with nitrogen after 10 to 50 h of the aerobic cultivation and the soybeans were then incubated anaerobically for 5 h, the GABA content increased greatly to a maximum 370 mg/100 g dry fermented soybeans. This value was about 12-fold higher than those obtained through the aerobic cultivation. The greatest GABA accumulation was observed in the anaerobically cultivated soybeans, in which *Rhizopus* was at the mid-logarithmic phase during the aerobic cultivation.

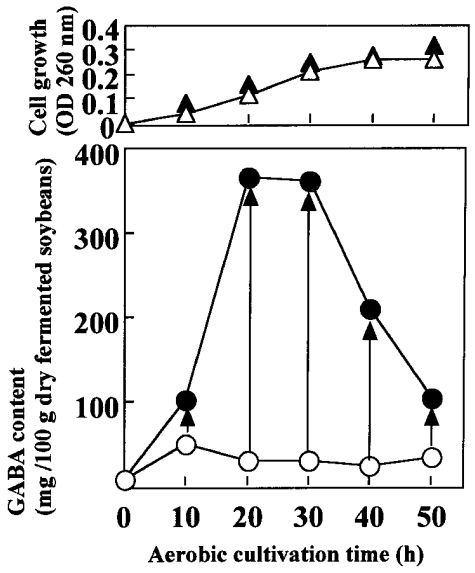

Fig. 1. Growth of *Rhizopus* and GABA Content in the Fermented Soybeans during the Aerobic and Anaerobic Cultivation.

Steamed soybeans were incubated with *R. microsporus* var. *oligosporus* IFO 8631 at 37°C for various times under aerobic conditions. After aerobic cultivation, the soybeans were incubated at 37°C for 5 h under anaerobic conditions. Symbols: ○, GABA contents in the fermented soybeans after an aerobic cultivation; ●, After aerobic cultivation, GABA contents in the fermented soybeans under anaerobic conditions; △, Cell growth (OD 260 nm) after an aerobic cultivation; ▲, After aerobic cultivation, Cell growth (OD 260 nm) under anaerobic conditions.

Effects of anaerobic cultivation time

The changes of GABA content during the prolonged anaerobic cultivation were examined. As shown in Fig. 2, the GABA content in the fermented soybeans increased with the progress of the anaerobic incubation. It reached a maximum of approximately 600 mg/100 g dry fermented soybeans in fermented soybeans at 37°C after 20 h.

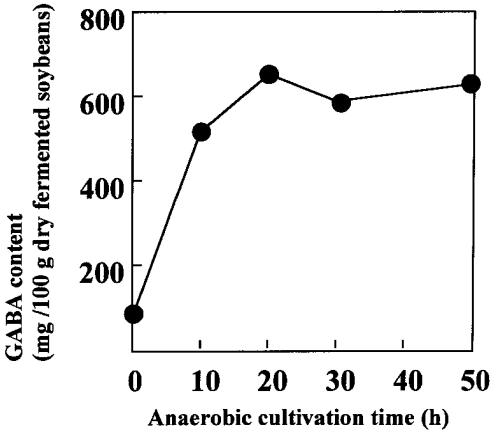

Fig. 2. Effects of Anaerobic Cultivation Time on the Accumulation of GABA in the Fermented Soybeans.

Steamed soybeans were incubated with *R. microsporus* var. *oligosporus* IFO 8631 at 37°C for 20 h under aerobic conditions and then incubated at 37°C for various times under anaerobic conditions.

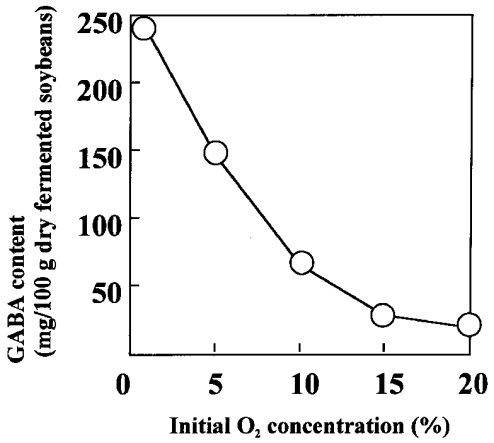

Fig. 3. Effects of the Initial Oxygen Concentration on the Accumulation of GABA in Fermented Soybeans with *Rhizopus*.

Steamed soybeans were incubated with *R. microsporus* var. *oligosporus* IFO 8631 at 37°C for 20 h under aerobic conditions. After the aerobic cultivation, the atmosphere of the fermentation vessels were replaced with nitrogen to various levels of oxygen concentration and then the vessels were sealed. The soybeans were successively incubated at 37°C for 5 h.

Effects of replacement of air with inert gases on GABA accumulation in soybeans

After the aerobic incubation for 20 h, the atmosphere was replaced with nitrogen at a different extent and incubated for 5 h. As shown in Fig. 3, the GABA content in the fermented soybeans increased with the decrease of the initial oxygen concentration. Under the more anaerobic conditions, *Rhizopus* accumulated a greater amount of GABA in soybeans.

Next, the effects of the types of inert gases on the GABA accumulation were examined. Air was replaced with three types of inert gases, *i.e.*, carbon dioxide, nitrogen, or argon to reduce the oxygen concentration to less than 1%. As a result, the three gases similarly promoted the accumulation of GABA, as shown in Fig. 4. The nitrogen gas was the most favorable to the GABA accumulation of all.

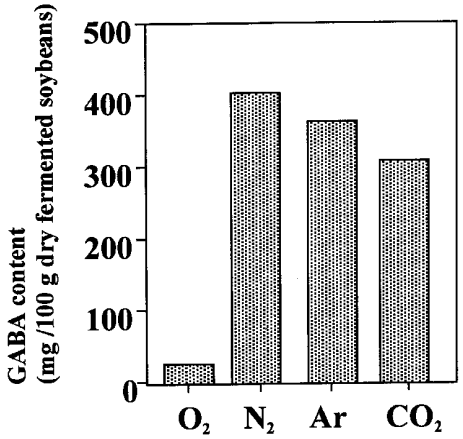

Fig. 4. Effects of the Replacement of the Atmosphere with Inert Gases on the Accumulation of GABA in the Fermented Soybeans.

Steamed soybeans were incubated with *R. microsporus* var. *oligosporus* IFO 8631 at 37°C for 20 h under aerobic conditions and then the atmosphere was replaced with some inert gases or was left open (indication of O_2) and incubated anaerobically at 37°C for 5 h.

Table 1. The Production of GABA-Enriched Fermented Soybeans by Several Strains of *Rhizopus*

Strains	GABA content (mg/100 g dry fermented soybeans)
Rhizopus microsporus var. *oligosporus* IFO 8631	720
Rhizopus microsporus var. *oligosporus* IFO 31987	810
Rhizopus microsporus var. *oligosporus* IFO 32002	1,740
Rhizopus microsporus var. *oligosporus* IFO 32003	1,500
Rhizopus oryzae IFO 4705	770
Rhizopus oryzae IFO 4770	510
Rhizopus oryzae IFO 5438	770
Rhizopus oryzae IFO 5780	420
Rhizopus oryzae IFO 9364	620

Variou strains incubated at 30°C for 20–22 h under aerobic conditions. After aerobic cultivation, fermented soybeans were incubated at 30°C for 20 h under anaerobic conditions.

Production of GABA by several Rhizopus strains

The GABA production by several strains of *Rhizopus* species were compared under the best conditions for the GABA accumulation by *R. microsporus* var. *oligosporus* IFO 8631. As shown in Table 1, all of *R. microsporus* var. *oligosporus* and *R. oryzae* considerably accumulated GABA in the fermented soybeans. In particular, *R. microsporus* var. *oligosporus* IFO 32002 and IFO 32003 showed the higher yield of GABA in the fermented soybeans (1,740 mg and

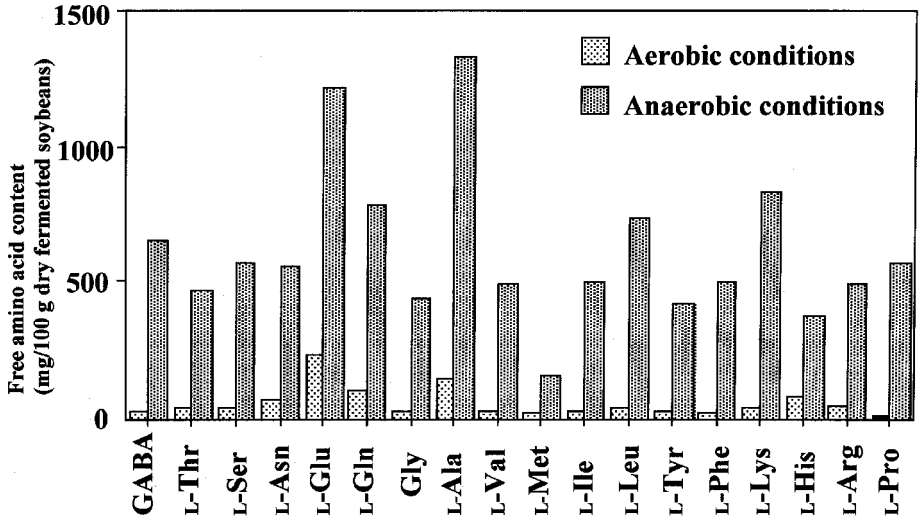

Fig. 5. The Changes of Free Amino Acid Contents during the Anaerobic Incubation in the Fermented Soybeans.
Steamed soybeans were incubated with *R. microsporus* var. *oligosporus* IFO 8631 at 37°C for 40 h under aerobic conditions. After the aerobic cultivation at 37°C for 20 h, the soybeans were successively incubated anaerobically at 37°C for 20 h.

1,500 mg/100 g dry fermented soybeans, respectively) than other strains.

The content of free amino acids in fermented soybeans after aerobic and anaerobic cultivation

The free amino acids in the soybeans fermented with *R. microsporus* var. *oligosporus* IFO 8631 aerobically for 40 h or aerobically for 20 h and successively anaerobically for 20 h are shown in Fig. 5. The sum of the free amino acids in the aerobically fermented soybeans was about 1% at the dry base, while that in the anaerobically fermented was about 11%. All free amino acids, particularly GABA and alanine, increased with anaerobic cultivation.

The ethanol content in fermented soybeans after aerobic and anaerobic cultivation

The ethanol contents in the fermented soybeans after aerobic cultivation for 20 h was 8.4 mg/100 g wet fermented soybeans, while that after the successive anaerobic cultivation for 20 h was 83.1 mg/100 g.

Discussion

In plants, GABA has been reported to be synthesized from glutamic acid by decarboxylation with glutamate decarboxylase, which has been reported to be activated by stresses such as low temperature or low atmospheric oxygen. In general, GABA is metabolized to succinic acid, however, stress blocks the metabolism of GABA to succinic acid in plants.[16] As a result, GABA accumulates in the stressed plants. The anaerobic incubation has been successfully used for the enrichment of GABA in tea leaves,[5,6] *Chlorella*,[15,21] and radish leaves.[22] In these studies, it was found that the anaerobic cultivation of soybeans after the aerobic cultivation by *R. microsporus* var. *oligosporus* drastically promoted the GABA accumulation. The GABA content in the anaerobically fermented soybeans with several types of *R. microsporus* var. *oligosporus* reached between 700 and 1,700 mg per 100 g dry fermented soybeans, and these values are much higher than those in red-mold rice,[12] gabaron tea,[5] or soaked rice germ,[7] which have been reported to be approximately 50, 290, and 550 mg in 100 g of dry foods.

The GABA content in the fermented soybeans increased with a decrease in the initial oxygen concentration and an increase of the anaerobic incubation time. These results suggest that more strictly anaerobic conditions favor with the accumulation of GABA in the fermented soybeans. In plants, stress initiates a signal-transduction pathway, in which increased cytosollic Ca(II) activates Ca(II)/calmodulin-dependent glutamate decarboxylase activity and GABA synthesis.[16] Furthermore, the anaerobic incubation of tea leaves and red-mold rice has been reported to induce cytosollic acidification,[6,12,16] which shifts the intracellular pH to the optimum pH of glutamate decarboxylase activity. During the aerobic fermentation of soybeans with *Rhizopus*, it is known that the pH of the fermented soybeans turned alkaline, probably due to the generation of ammonia by the deamination of amino acids.[19] However, the pH of the fermented soybeans after the anaerobic cultivation remained at about six in this study. This pH is optimum for the glutamate decarboxylase of *Rhizopus* (data not shown). These results suggest that the acidification caused by anaerobic fermentation may contribute to the accumulation of GABA in the fermented soybeans as well as tea leaves and red-mold rice.

Among the soybeans aerobically cultivated with *Rhizopus* for various lengths of time, the ones in which *Rhizopus* were in the mid-logarithmic phase stage, i.e., cultivated for 20 to 30 h, accumulated GABA most extensively after the anaerobic incubation. On the other hand, the fermented soybeans at stationary phase, for example the soybeans fermented for 50 h, accumulated only a small amount of GABA after anaerobic cultivation, although the glutamic acid content in the 50 h cultivated soybeans was greater than those in the 20 or 30 h cultivated ones (data not shown). The higher metabolic activities in *Rhizopus* with the mid-logarithmic phase may be responsible for the efficient accumulation of GABA.

The remarkable increase of ethanol in the anaerobically cultivated soybeans suggests that TCA cycle was blocked and compensately alcoholic fermentation was induced. It is assumed that succinic semialdehyde dehydrogenase, which is the key enzyme for the metabolism of succinic semialdehyde, is inhibited by this serious stress. The accumulation of succinic semialdehyde in turn blocks GABA aminotransferase, which catalyzes the conversion of GABA to succinic semialdehyde.[16]

The anaerobic cultivation induced the accumulation of not only GABA, but also other amino acids such as alanine, as shown in Fig. 5. The accumulation of alanine as well as GABA was already reported in plants during anaerobic incubation.[6,22] On the other hand, it is known that when an aerobe such as mold or bacteria meet with steep changes from the aerobic to anaerobic conditions, autolysis occurs on most of the proteins and nucleic acids in cells to degrade them. A similar mechanism was suggested in the fermented soybeans with *R. microsporus* var. *oligosporus* aerobically cultivated at first and then anaerobic cultivated. The cellular autolysis of mycelia that leak proteases may also contribute to increase the free amino acids in the fermented soybeans.

These combined results showed that the anaerobic cultivation of pre-aerobically fermented soybeans with *Rhizopus* is a suitable procedure for producing the GABA-enriched *tempeh*-like fermented soybeans.

The newly developed *tempeh*-like fermented soybeans with anaerobically cultivation is rich not only in GABA, but also alanine and other free amino acids, as shown in Fig. 5. The oligopeptides soluble in 70% ethanol was also produced in soybeans during anaerobic cultivation (data not shown). Recently, the nutritional effects of dietary free amino acids[23,24] and peptides[25] have been attracted much attention. Free amino acids and oligopeptides in foods are expected that not only improve the umami taste, but also have some nutritional advantages: (1) rapid absorption,[23,25] (2) muscle protein maintenance,[23] (3) antioxygenic activity,[24] and so on.

As the newly developed and anaerobically cultivated soybean is rich not only in GABA but also various free amino acids and peptides, the antihypertensive and some other functions will be expected. The expected nutritional effects of the anaerobically fermented soybeans are now being assessed. Sensory tests showed that the flavor of the new type of the fermented soybeans was similar to and better than the conventional *tempeh*.

Acknowledgment

We are grateful to Ms. Yoshiko Sugimoto for her outstanding technical assistance.

References

1) Ohkuma, S., Katsura, M., and Hirouchi, M., GABA and GABA receptors. *Shinkei Seishin-yaku "Neurotransmitter"* (in Japanese), **19**, 167–180 (1997).
2) Nishikawa, T., GABA system. *Clinical Neuroscience* (in Japanese), **14**, 404–407 (1996).
3) Vemulapalli, S., and Barletta, M., The role of the sympathetic nervous system in the cardiovascular effects of systemically administered γ-aminobutyric acid. *Arch. Int. Pharmacodyn.*, **267**, 46–58 (1984).
4) Ishikawa, K., and Saito, S., Effect of intraventricular γ-aminobutyric acid (GABA) on discrimination learning in rats. *Psychopharmacology*, **56**, 127–132 (1978).
5) Tsushida, T., Murai, T., Omori, M., and Okamoto, J., Production of a new type tea containing a high level of γ-aminobutyric acid. *Nippon Nōgeikagaku Kaishi* (in Japanese), **61**, 817–822 (1987).
6) Tsushida, T., Anaerobic metabolism of tea and its application. *Kagaku to Seibutsu* (in Japanese), **25**, 699–701 (1987).
7) Saikusa, T., Horino, T., and Mori, Y., Accumulation of γ-aminobutyric acid (gaba) in the rice germ during water soaking. *Biosci. Biotechnol. Biochem.*, **58**, 2291–2292 (1994).
8) Saikusa, T., Horino, T., and Mori, Y., Distribution of free amino acids in the rice kernel and kernel fractions and the effect of water soaking on the distribution. *J. Agric. Food Chem.*, **42**, 1122–1125 (1994).
9) Saikusa, T., Okada, T., Murai, H., Omori, M., Mori, Y., Horino, T., Itou, M., and Onoda, A., The effect of defatting with organic solvent on accumulation of 4-aminobutyric acid (GABA) in the rice germ. *Nippon Shoukuhin Kagaku Kogaku Kaishi* (in Japanese), **48**, 196–201 (2001).
10) Hayakawa, K., Ueno, Y., Kawamura, S., Taniguchi, R., and Oda, K., Production of γ-aminobutyric acid by lactic acid bacteria. *Seibutsu Kougaku* (in Japanese), **75**, 239–244 (1997).
11) Kono, I., and Himeno, K., Changes in γ-aminobutyric acid content during *beni-koji* making. *Biosci. Biotechnol. Biochem.*, **64**, 617–619 (2000).
12) Kono, I., and Himeno, K., Accumulation of γ-aminobutyric acid in beni-koji after anaerobic incubation. *J. Brew. Soc. Japan*, **97**, 785–790 (2002).
13) Omori, M., Yano, T., Okamoto, J., Tsushida, T., Murai, T., and Higuchi, M., Effect of anaerobically

treated tea (gabaron tea) on blood pressure of spontaneously hypertensive rats. *Nippon Nōgeikagaku Kaishi* (in Japanese), **61**, 1449–1451 (1987).
14) Tsuji, K., Ichikawa, T., Tanabe, N., Abe, S., Tarui, S., and Nakagawa, Y., Antihypertensive activities of beni-koji extracts and γ-aminobutyric acid in spontaneously hypertensive rats. *Eiyogaku Zasshi* (in Japanese), **50**, 285–291 (1992).
15) Nakamura, T., Matsubayashi, T., Kamachi, K., Hasegawa, T., Ando, Y., and Omori, M., γ-Aminobutyric acid (GABA)-rich *Chlorella* depresses the elevation of blood pressure in spontaneously hypertensive rats (SHR). *Nippon Nōgeikagaku Kaishi* (in Japanese), **74**, 907–909 (2000).
16) Shelp, B. J., Bown, A. W., and McLean, M. D., Metabolism and functions of gamma-aminobutyric acid. *Trends Plant Sci.*, **4**, 446–452 (1999).
17) Hachmeister, K. A., and Fung, D. Y. C., Tempeh: a mold-modified indigenous fermented food made from soybeans and/or cereal grains. *Crit. Rev. Microbiol.*, **19**, 137–188 (1993).
18) Baumann, U., Bisping, B., and Rehm, H.J., Content and release of amino acids during the fermentation of tempe by several strains of *Rhizopus* spec. In ''Dechema Biotechnology Conference'', vol. 4, ed. Behrens, D., Wiley Europe, Weinheim, pp. 205–208 (1991).
19) Matsumoto, I., and Imai, S., Changes of chemical composition of tempe during fermentation. *Nippon Shokuhin Kogyo Gakkaishi* (in Japanese), **37**, 130–138 (1990).
20) Ouchi, K., Ishido, T., Sugama, S., and Nojiro, K., The ecophysiology of yeast in koji. *Nippon Jozo Kyokaishi* (in Japanese), **62**, 1029–1033 (1967).
21) Lane, T. R., and Stiller, M., Glutamic acid decarboxylation in Chlorella. *Plant Physiol.*, **45**, 558–562 (1970).
22) Streeter, J. G., and Thompson, J. F., Anaerobic accumulation of γ-aminobutyric acid and alanine in radish leaves (*Raphanus sativus* L.). *Plant Physiol.*, **49**, 572–578 (1972).
23) Kamiya, T., Biological functions and health benefits of amino acids. *Foods Food Ingredients J. Jpn.* (in Japanese), **206**, 33–44 (2002).
24) Hoppe, M. B., Jha, H. C., and Egge, H., Structure of antioxidant from fermented soybeans (tempeh). *J. Am. Oil Chem. Soc.*, **74**, 477–479 (1997).
25) Aoyama, N., Fukui, K., and Yamamoto, T., Effect of various forms of force-fed nitrogen sources on gastric transit times in rats. *Nippon Eiyo Shokuryo Gakkaishi* (in Japanese), **49**, 46–51 (1996).