Production of γ -Aminobutyric Acid in Pumpkin Tissue Treated with Freeze-thaw Infusion of L-Glutamic Acid Monosodium Salt

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Received December 12, 2012; Accepted March 13, 2013

We studied a method for producing high concentrations of γ -aminobutyric acid (GABA) in pumpkin tissue using freeze-thaw infusion. We introduced L-glutamic acid monosodium salt (MSG) into pumpkins by freeze thaw infusion, and observed that high concentrations of GABA were produced due to the action of the glutamate decarboxylase present in the pumpkins. For an enzyme reaction at 3°C, the amount of GABA peaked when the MSG concentration was 1% (w/w) and enzyme reaction time was about 48 h. When a softening enzyme (Macerozyme 2A) was added together with MSG and infusion was performed, a soft pumpkin that contained GABA at high concentrations was obtained. By freeze-thawing with MSG, it is now possible to manufacture functional foods that have two added values, i.e., in addition to enrichment of GABA, their hardness can also be adjusted.

Keywords: freeze-thaw infusion, γ -aminobutyric acid, pumpkin, enzyme reaction

Introduction

Freeze-thawing is a method used to immediately introduce a target substance into food, and is widely employed in the manufacture of functional foods in nursing and medical care. For example, by introducing plant tissue-degrading enzymes such as pectinase, the hardness of food materials can be adjusted while retaining their shape, and this is useful for the manufacture of foods for swallowing disorders from the viewpoint of improving the quality of life (QOL) of patients with dysphagia (Sakamoto *et al.*, 2004; 2005; 2006; Shibata *et al.*, 2006; Nakatsu *et al.*, 2010). In efforts to manufacture functional foods, research has also been carried out to infuse soy beans with a protease to generate blood pressure-suppressing peptides (Kajihara *et al.*, 2011), to infuse potatoes with amylase so that they contain large amounts of oligosaccharides (Shibata *et al.*, 2010), and to infuse oils and fats in which enzymes and β -carotene have been dissolved (Watanabe *et al.*, 2011). Much of the research involving application of freeze-thawing to date was aimed at altering the

molecules of food item components to give the food added functions, by infusing added enzymes into the food. In this research, we focused instead on the enzymes originally present in foods, and in order to add new functions by introducing a substrate from outside, we targeted γ -aminobutyric acid (GABA). As this procedure can add functionality by using substances less expensive than enzymes, it is industrially advantageous.

GABA is widely distributed in nature, and is found in animals, plants and microbes. It is an inhibitory neurotransmitter that is present in large amounts in the cerebellum, spinal-cord and cerebrum of mammals (Yoshikawa and Sumida, 2009), and is receiving attention as a functional ingredient due to its physiological actions, such as ameliorating hypertension (Kajimoto *et al.*, 2004; Kayahara *et al.*, 2001; Matsubara *et al.*, 2002; Yamamoto, 2006) and calming nerves (Horie *et al.*, 2003; Sonoda, 2001).

In the food industry, GABA is added to lactic acid beverages, powdered cold drinks and candy for the purpose of improving hypertension, and has been approved as a food for specified health uses in Japan¹⁾. In addition, agricultural products or food items enriched with GABA have also been

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developed. Here, the aim was to accumulate GABA in agricultural products using a reaction where glutamic acid is converted to GABA by the action of glutamic acid decarboxylase (Matumoto *et al.*, 1997; Ohno *et al.*, 2007). The authors, noting that some vegetables have a high glutamic acid decarboxylase activity, and the fact that glutamic acid, which is a substrate of glutamic acid decarboxylase, is widely employed to enhance taste in cooking, studied a method to produce GABA at high concentrations in food using freeze-thaw infusion.

Materials and Methods

Preparation of experimental material and extracts

Japanese pumpkins purchased at stores in Hiroshima city from May to August, 2012 were used as raw materials for this experiment. On the day when the starting materials were purchased, the pumpkin was cut to approximately 20 mm square, and frozen to -20°C using a blast chiller (QXF-006SF5; Fukushima Co. Industries, Osaka, Japan) to provide samples.

In an experiment to study the relationship between L-glutamic acid monosodium salt (MSG; Nacalai Tesque, Inc., Kyoto, Japan) and GABA production, in the freeze-thaw infusion group, MSG was added to the samples at a predetermined concentration, and enclosed in special vacuum packaging film. After sealing these with a vacuum packaging machine (V-380G; Tosei Corporation, Shizuoka, Japan) under 70% vacuum for 30 s, they were thawed in a steam convection oven (CS2-100; Comet Kato Co., Aichi, Japan) at 40°C for 10 min. After thawing, and packing with a vacuum packaging machine under 95% vacuum for 90 s, an enzyme reaction was carried out in a refrigerator (HR-75S; Hoshizaki Electric Co., Ltd., Aichi, Japan) at 3°C for a predetermined time. After the enzyme reaction, the sample wherein the enzyme had been deactivated in a steam convection oven at 90°C for 15 min was pulverized, and filtered through 5C filter paper to obtain a pumpkin extract. On the other hand, in the group not subjected to freeze-thaw infusion (control group), the sample was subjected to identical processing as that of the freeze-thaw infusion group, except that vacuum packaging was not performed after thawing.

Separately, in an experiment to study the effects of plant tissue-degrading enzymes, the enzyme agent was Macerozyme 2A (Yakult Pharmaceutical Industry Co., Tokyo, Japan), and an aqueous extract was obtained through an identical procedure as above. Finally, in the freeze-thaw infusion and control groups, Macerozyme 2A was added to a predetermined concentration, and GABA production was measured in the presence and absence of 1% (w/w) MSG.

Quantization of GABA The pumpkin extract obtained

in 1 was diluted 3 times and used as a sample. This sample was mixed with 10 μL of 1 M Tris-HCl (pH 8.9), 0.1 M d-ketoglutaric acid, 20 mM β -mercaptoethanol and 5 mM NADP⁺ (Nacalai Tesque) and 40 μL of distilled water, and to this, 10 μL of 2.5 U/mL GABase from *Pseudomonas fluorescens* (GABase; Sigma-Aldrich, St. Louis, MO, USA) was added to prepare 100 μL of enzyme reaction solution. After allowing this enzyme reaction solution to react for 40 min in an incubator (MIR-154; Sanyo, Osaka, Japan) set to 27°C , 400 μL distilled-water was added, and the absorbance at 340 nm was measured. As a blank, the same solution was prepared by adding 10 μL of distilled water instead of 2.5 U/mL GABase. GABA contents were calculated from a calibration curve prepared using the value obtained by subtracting the value for the blank from the absorbance of the enzyme reaction solution, and a GABA standard product (Sigma-Aldrich). GABA contents (mg) per 100 g of sample are expressed as average values $\pm$ standard deviation, taking the extraction efficiency of the pumpkin extract used for this experiment as 100%.

Measurement of hardness The sample was kept at $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and placed on the sample stand of a creep meter (Reona RE-3305; Yamaden, Tokyo, Japan), and the maximum stress (N/m^2) obtained using a resin plunger of diameter 3 mm with a plunger speed of 10 mm/s and clearance of 30% was taken as the hardness.

Statistical processing Using a dispersion analysis or *t*-test, a difference was considered to be significant at $p < 0.05$.

Results and Discussion

Effects of MSG concentration on GABA production in freeze-thaw infusion-treated pumpkin The MSG concentrations in the infusion solution were set respectively at 0.2%, 0.5%, 1%, 2% and 3% (w/w), and GABA production in the freeze-thaw infusion group and control group was compared after infusion and enzyme reaction at 3°C for 48 h (Fig. 1). Whereas in the control group, GABA production did not significantly change, even with increasing MSG concentration, in the freeze-thaw infusion group, GABA production increased with MSG concentration up to an MSG concentration of 1% (w/w), when it was 475 ± 190 mg/100 g. However, when more MSG was added, further increases in GABA production were not observed. At this time, although a significant difference was not seen between the freeze-thaw infusion group and the control group at each processing concentration of MSG, significant differences were seen between the freeze-thaw infusion group at MSG concentrations of 0.5% (w/w), 1% (w/w) and 2% (w/w), and the control group at an MSG concentration of 0% (w/w) by *t*-test ($p < 0.05$). We believe that there were indi-

Fig. 1. Effects of MSG concentration on GABA production in the freeze-thaw infusion-treated pumpkin.

GABA production was measured between the freeze-thaw infusion group (closed column) and control group (open column). The reaction was conducted at 3°C for 48 h. The values represent the mean $\pm$ S.D. (n = 4). The significance of differences (*: $p < 0.05$) was determined with *t*-test.

vidual differences between the pumpkin samples. Ohno *et al.* (2003) examined GABA production in frozen or refrigerated squeezed residues of satsuma mandarins. They showed that the frozen sample maintained GABA production at more than 85% before storage, even after six months; on the other hand, slight GABA production was observed after storage for 14 days or more at the refrigeration temperature. In this experiment, the storage conditions in the distribution process for experimental samples were unidentified. Thus, differences in these conditions could have increased the differences in GABA production. However, when the GABA production obtained with various concentrations of MSG was compared between the freeze-thaw infusion group and the control group by dispersion analysis, the amount was found to be significantly higher in the freeze-thaw infusion group. These results demonstrate that when MSG was introduced into pumpkin by freeze-thaw infusion, GABA was produced due to the action of glutamic acid decarboxylase present in the pumpkin. Moreover, GABA production amount peaked when MSG concentration was 1% (w/w). Although it is not clear whether there is any enzyme inhibition in this reaction, enzyme reactions in freeze-thaw infusion are different from such reactions in enzyme solutions; as there is no stirring, the frequency of collisions between the substrate and the enzyme is limited. Moreover, in freeze-thaw infusion, most of the

MSG is conjectured to accumulate in the intercellular space. Therefore, in order to increase the amount of GABA production, methods are required to introduce MSG into cells, such as gentle heating to the extent that glutamic acid decarboxylase is not deactivated. In addition, in this experiment, the GABA production in the control group before treatment was 115 ± 31 mg/100 g, and this value was higher than the 9.7 mg/100 g previously reported with fresh pumpkins (Matumoto *et al.*, 1997). The main reason for this was considered to be the effect of freeze treatment of the sample. Specifically, Matumoto *et al.* (1998) reported that since the reaction between glutamic acid and glutamic acid decarboxylase is promoted due to partial damage of tissues caused by freezing, the GABA production, which was 9.7 mg/100 g before freezing, increased to 207 mg/100 g. In our experiment, we also used a sample wherein pumpkin had previously been frozen at -20°C . This suggests that a reaction occurred between the glutamic acid originally present in the pumpkin and glutamic acid decarboxylase; thus, a high GABA content was observed in the control group with 0% MSG. Freeze-thaw infusion uses the principle whereby the slackness of tissue due to freezing markedly increases the decompression infusion effect, so freezing is essential. As GABA production is influenced by processing conditions such as freezing and MSG infusion, optimization of these conditions is required in the future.

Effects of MSG on time course of GABA production in freeze-thaw infusion-treated pumpkin As shown in Fig. 1, when pumpkin was subjected to freeze-thaw infusion and MSG was added, GABA production increased. To examine this point in more detail, the time-dependent changes in GABA production were measured between the freeze-thaw infusion group and the control group when 1% (w/w) MSG was added (Fig. 2). As a result, in the control group, a slight time-dependent increase in GABA production was seen in the control group due to the reaction of intrinsic glutamic acid and glutamic acid decarboxylase. In the freeze-thaw infusion group, GABA production increased with elapsed time, and although it was 232 ± 74 mg/100 g at the beginning of the experiment, it increased to 583 ± 114 mg/100 g after 48 h of freeze-thawing, which is an increase of approximately 2.5 fold. At this time, however, a significant difference was not seen between the freeze-thaw infusion group and the control group for each reaction time. On the other hand, a significant difference was seen between the freeze-thaw infusion group after 24 h and 48 h of freeze-thawing, and the control group after 0 h with *t*-test ($p < 0.05$). This result was likely due to reasons explained in Fig. 1. In addition, comparing the GABA production shown in Fig. 2 between the freeze-thaw infusion group and the control group by dispersion analysis,

Fig. 2. Effects of MSG on the time course of GABA production in the freeze-thaw infusion-treated pumpkin.

GABA production was measured between the freeze-thaw infusion group (closed circle and solid line) and control group (closed square and dotted line). Both experiments were performed in the presence of 1% (w/w) MSG and the reaction temperature was 3°C. The values represent the mean $\pm$ S.D. ($n = 4$). The significance of differences (*: $p < 0.05$) was determined with *t*-test.

significantly higher production was seen in the freeze-thaw infusion group than in the control group. Thus these results suggest that freeze-thaw infusion increased GABA production in the presence of MSG. In the freeze-thaw infusion group, as the frequency of contact between the substrate, glutamic acid and glutamic acid decarboxylase is not thought to be very high, under enzyme reaction conditions at 3°C, it effectively reaches a maximum value in the reaction after about 48 h.

With regard to the effects of temperature and time on GABA production, Ohno *et al.* (2008) reported that when aubergine was used as a sample and 1% (w/w) MSG was added, the GABA production peaked after 2 h at 35°C, after 7 to 9 h at 10°C, and after 15 h at 5°C. Thus, if the experimental temperature is high, the activity of glutamic acid decarboxylase increases, and a peak in GABA production can be obtained in a short time. Moreover, if the enzyme reaction is performed at the optimal temperature for glutamic acid decarboxylase, although it is then possible to produce GABA in a shorter time, when the enzyme reaction takes place at 30°C or higher, the number of bacilli increases by one order of magnitude in about 1 h, depending on the type of bacteria. In particular, because an unheated agricultural product was used as the sample, the number of bacilli in the raw material stage due to primary contamination would probably be high,

Fig. 3. Effects of simultaneous addition of Macerozyme 2A and MSG on GABA production in the freeze-thaw infusion-treated pumpkin.

GABA production was measured between the freeze-thaw infusion group (closed column) and control group (open column) in the presence of 2% (w/w) Macerozyme 2A (left) or 2% (w/w) Macerozyme and 1% (w/w) MSG (right). The reaction was conducted at 3°C for 48 hours. The values represent the mean $\pm$ S.D. ($n = 5$). The significance of differences (*: $p < 0.05$) was determined with *t*-test.

and consequently, the risk of quality deterioration and health damage, for example due to putrefaction, also increases. Hence, in this experiment, with a view to actual application in food processing and cooking, and to prevent quality deterioration of food items due to temperature increases, we performed the experiment at 3°C with reference to the cook-chill storage temperature in modern cooking systems.

Effects of simultaneous addition of Macerozyme 2A and MSG on GABA production in freeze-thaw infusion-treated pumpkin From the results of the previous experiments, it was clear that freeze-thaw infusion of MSG increases GABA production. Freeze-thaw infusion is a technique whereby substances can be efficiently incorporated into food products, and as it is now possible to infuse degrading enzyme agents into the tissues of food items, and to adjust the hardness of food items through enzyme reactions, the technique is being increasingly used in foods for nursing care and dysphagia patients. As GABA is produced in plant foods even when their tissues are damaged by freezing, we investigated the effects of a plant tissue-degrading enzyme (Macerozyme 2A) on GABA production.

Figure 3 shows the results when freeze-thaw infusions of Macerozyme 2A and MSG were performed simultaneously. When only 2% (w/w) Macerozyme 2A was infused, GABA production was 199 ± 101 mg/100 g for the freeze-thaw in-

fusion group, and 196 ± 88 mg/100 g for the control group. As there was no significant difference between the two (Fig. 3, left), it was clear that infusion of Macerozyme 2A had no effect on GABA production in the pumpkin. On the other hand, when 2% (w/w) Macerozyme 2A and 1% (w/w) MSG were infused simultaneously, GABA production increased to 426 ± 26 mg/100 g in the freeze-thaw infusion group and to 387 ± 12 mg/100 g in the control group; thus, GABA production in the freeze-thaw infusion group was significantly higher than in the control group (Fig. 3, right). We conducted simultaneous addition of Macerozyme 2A and MSG in this experiment from a practical standpoint. From the present data, we inferred that MSG is introduced into the cells before the action of Macerozyme 2A and the incorporation of MSG into the cells was only moderately enhanced.

Looking now at the changes in hardness of the pumpkin due to Macerozyme 2A, whereas the hardness without addition of Macerozyme was $45.4 \pm 20.6 \times 10^3$ N/m², when 2% (w/w) Macerozyme 2A and 1% (w/w) MSG were simultaneously infused, and the enzyme reaction was carried out for 48 h, it was $22.3 \pm 7.0 \times 10^3$ N/m²; thus, a remarkable softening effect was observed due to addition of Macerozyme 2A (data not shown). Freeze-thaw infusion can be performed on raw foods or heated foods, but most of the studies aimed at softening have attempted to control hardness using heated samples with the objective of destroying microorganisms and deactivating internal enzymes. The hardness of fresh pumpkin used in this experiment was $5.7 \pm 0.7 \times 10^5$ N/m², which is fairly hard, and infusion into its tissues is difficult, but judging from the softening due to Macerozyme 2A and GABA production results, we considered that the introduction of substances by freeze-thaw infusion into even fresh pumpkin is quite feasible.

In this study, it was found that the GABA production amount could be increased by adding MSG and performing freeze-thaw infusion using pumpkin as an experimental material. GABA is known to have various physiological effects. Regarding examples where consumption of foods containing GABA is effective for improving health, foods containing 10 – 20 mg of GABA lower blood pressure, and it has therefore been approved for use as a food for specified health uses in Japan¹⁾. In experiments on menopausal disorders and pre-senile mental disorders, 60% or more improvement has been reported with continuous consumption of drinks containing approximately 210 mg/day of GABA (Sonoda, 2001). Here, based on the results of this study, assuming that pumpkin enriched with GABA by using MSG is consumed in a meal by cooking with freeze-thaw infusion, if 50 g of stewed pumpkin is consumed on one occasion, the consumption of GABA would be 100 – 200 mg. This is what would be

consumed from a typical food item shown above, and it may be expected to have useful effects on a living organism. As MSG is widely employed as a flavor enhancer, a meal with a high GABA content could be provided by cooking with freeze-thaw infusion. With regard to the amount of MSG used, The Joint FAO/WHO Expert Committee on Food Additives (1988) considers that if it is used correctly as a food additive, there is no need to set an upper limit; thus, there are no restrictions on its use. Incidentally, the 1% (w/w) MSG used in this experiment would be equivalent to about 0.5 g of MSG used in cooking, and as this is a small amount, there are considered to be no health risks.

However, when using MSG in cooking, it is necessary to be vigilant about the sodium contents. MSG contains approximately 12% sodium, and assuming that it is necessary to restrict sodium intake from health considerations, it is possible to cook with less MSG, and the method is therefore effective for suppressing sodium intake. At the same time, regarding adjustment of hardness, with freeze-thaw infusion, softening could be obtained while retaining the shape of the pumpkin.

Taken together, the present results suggest that with freeze-thaw infusion of MSG, it is now become possible to manufacture functional foods with two added values, i.e., enrichment with GABA and adjustment of hardness. As it is possible to manufacture new functional foods using more economical methods, taking advantage of the features of freeze-thaw infusion, which can be used for various types of food processing while retaining the original shape of the food item, new applications of freeze-thaw infusion are expected to be developed in the future.

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