Freeze–thaw–induced Structural Destruction and Generation of γ–aminobutyric Acid in Water–soaked Soybeans

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In this study, we investigated the effects of freezing and subsequent storage on γ–aminobutyric acid (GABA) accumulation in soybean (Glycine max) cotyledons. According to direct observations and an indirect analysis with Cole–Cole plots of soybeans, the membrane structure in freeze–thawed soybeans was partially destroyed and the degree of destruction increased with a decrease in the freezing temperature. GABA generation in freeze–thawed soybeans was accelerated during storage after thawing. The optimum pH for GABA generation reaction was investigated; the decrease in pH in stored soybeans was found to result in an increase in GABA generation. The higher GABA accumulation after freeze–thawing of soybeans may have been caused by the freeze–thaw–induced structural destruction, which improved substrate transfer and enzymatic reactions.

Keywords: freezing; γ–aminobutyric acid; soybean; glutamate decarboxylase; pH

1. Introduction

Freezing is a traditional technique used to store food for a long period of time. However, in some cases, freezing induces injury to food materials by inducing the destruction of cell membranes which, in effect, diminishes food quality. Many factors contribute to the destruction of cell membranes, one of which is mechanical stress due to volume changes after the formation of ice crystals [1]. Of note, cell destruction leads to a change in the rheological properties of food. In agricultural products, the rheological properties reflect the turgor pressure [2], which is very sensitive to the state of the plasma membrane in plant cell tissues. The plasma membrane is considered the primary site of freezing injury because of its weak structure [3–6]. Numerous studies have investigated the optimal freezing conditions to minimize ice crystal formation [7–10]. Also, some pre–freezing treatments have been suggested to limit ice crystal damage to plant cell structures during freezing [11]. Interestingly, the freezing process was also found to modify the compounds/molecules in plant cells; for example, the total phenols in broccoli (Brassica oleracea) [12] and celery (Apium graveolens) [13] increased after freezing, providing an antioxidant activity.

High hydrostatic pressure (HHP) is an innovative non–thermal food processing technology. The application of HHP also leads to the destruction of cell membranes, resulting in the acceleration of mass transfer [14–16]. Furthermore, under selected HHP conditions, specific compounds in plant–based foods were increased and modified [17–20]. Particularly in soybeans (Glycine max), the use of HHP and subsequent storage led to an enrichment of γ–aminobutyric acid (GABA) [21], which has been shown to have anticancer and anti–hypertensive properties [22] with taste improvement [23]. The HHP–mediated destruction of cells that consequently promoted cellular enzymatic reactions presumably accounted for the observed enrichment of a beneficial compound and properties in food. However, in spite of this positive effect of HHP to the food material, its application is very limited because HHP instruments are expensive and require a large space. Methods for enrichment of bioactive compounds in foods have been developed because of the increasing worldwide interests in human health and quality of life. One possible enrichment method is the food freezing process [24,25]. Thus, this study utilized freezing to enrich for beneficial bioactive compounds such as GABA as a potential alternative to the HHP method.

The objectives of this study were to investigate the

(Received 16 May. 2019: accepted 31 May. 2019)
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GABA enrichment condition by freezing and to investigate the mechanisms of freeze–thaw-induced enrichment of the GABA generation enzymatic reaction.

2. Materials and methods

2.1 Sample preparation

All reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and they were of analytical grade except for acetonitrile, which was high-pressure liquid chromatography (HPLC) grade.

Dried soybean seeds harvested in Hokkaido were purchased from a local market. Soybean seeds were soaked for 22 h at 20°C in distilled water containing 0.1% (w/v) sodium azide to prevent microbial contamination. The dimensions of the water–soaked soybeans were 15.9±0.6 mm in the long axis, 9.4±0.4 mm in the short axis (n=30). After soaking, the soybean seeds were wiped and vacuum-sealed in a polyethylene pouch (Mekkin Kensa Bag; Eiken kizai, Tokyo, Japan) with 10 g of seeds per pack arranged in a single layer. The vacuum-packed seeds were frozen using a freezing apparatus (MC711T; Espec, Osaka, Japan) or liquid-nitrogen in a Dewar vessel at -20°C, -80°C, or -180°C. The dimensions of the freezing apparatus and Dewar vessel were 400×230 mm in height, respectively.

The temperature history of the freezing apparatus/Dewar vessel and soybean samples were monitored with T-type thermocouples and a data logger (GL200A-UM-801, midi LOGGER; Graphtec, Yokohama, Japan) in preliminary experiments without vacuum seal. Thermocouples were fixed at the central part of the freezing chamber or Dewar vessel, and the ambient temperature inside the freezing chamber was regarded as the freezing temperature, which was different from the temperature inside the freezing chamber or Dewar vessel, and the ambient temperature was plotted against reactance to generate a Cole–Cole plot, which reflected the integrity of the cellular structure [28]. Radii of Cole–Cole plots were calculated using the method of Ohnishi and Miyawaki [29].

After freezing storage, vacuum-sealed soybeans were thawed in an ice bath for 1 h, removed from the pouches, and then stored at 20°C for another 1 h. After the soybean seeds were completely thawed, they were immediately analyzed and designated as 0-days storage. Other samples were stored in an incubator (BR-23FP; Taitec, Saitama, Japan) at 20°C for 3 days for subsequent analyses.

2.2 Microscopy

The internal structures of the soybean cells were observed by transmission electron microscopy (TEM; H-7500; Hitachi, Tokyo, Japan). Soybean cotyledons were fixed with 2%(w/w) osmium tetroxide in 0.05 M potassium-phosphate buffer [26]. For TEM, dehydration was performed in an acetone series and the cotyledons were embedded in Spurr’s resin. To achieve ultrathin sections for TEM, samples were cut with a diamond knife on an ultramicrotome and sections were stained with 2% (w/w) uranyl acetate for 10 min followed by lead citrate for 5 min [27].

2.3 Dielectric measurement

The dielectric properties of the soybeans were measured at 20°C. Briefly, soybean seeds were removed from the pouches after thawing and stored at 20°C for 1 h. Each thawed seed was cut in half along the long axis and the seed hull was peeled off. The cotyledon of the half-seed was inserted between two titanium-platinum electrodes, each with an outer diameter of 1.0 mm and a 5.0–mm inter-electrode distance from the equatorial plane [21]. The cotyledon dielectric properties were measured at frequencies between 100 Hz and 5 MHz using an inductance–capacitance–resistance meter (LCR HITESTER 3532–50; HIOKI E. Corporation, Nagano, Japan) with the electrodes at 20°C. For each cotyledon, resistance was plotted against reactance to generate a Cole–Cole plot, which reflected the integrity of the cellular structure [28]. Radii of Cole–Cole plots were calculated using the method of Ohnishi and Miyawaki [29].

2.4 pH of homogenized freeze–thawed soybeans milk

Ten grams of soybean seeds were homogenized with 30 mL of distilled water. One milliliter of obtained homogenized soymilk was poured onto the pH electrode (Laqua twin, Horiba, Kyoto, Japan) and soymilk pHs were measured at 20°C. The remaining soymilk was used in the following GABA analysis.

2.5 GABA analysis

GABA concentrations were determined as per previous methods [30]. Briefly, the remaining soymilk from the pH measurement was centrifuged at 15,000×g for 15 min. The supernatant was collected and filtered through a ultrafiltration unit (Amicon Ultra 0.5, 10 kDa cut off; Millipore, Billerica, MA, USA). The filtrate solution (100 μL) with 4-dimethylaminoazobenzene–4’-sulfonyl chlo-
ride (dabsyl chloride, 1 mg/mL, 100 μL) was analyzed for GABA concentration using HPLC. The HPLC system consisted of an ultra violet detector at 425 nm (SPD-20A; Shimadzu, Kyoto, Japan), a column oven at 30°C, and a C18 reversed–phase octadecyl–silica column (4.6 mm inner diameter×150 mm; Shiseido Co., Ltd., Tokyo, Japan). Preparation of glutamate decarboxylase (GAD) from soybean cotyledons and an in vitro assay of GAD activity at 40°C were also performed as per previous in vitro methods [30].

2.6 Statistical analysis
Average and standard deviation values were calculated using Microsoft Excel (Excel 2013; Japan Microsoft, Tokyo, Japan). All other statistical analyses were performed using statistical software (Excel–based multiple comparison; Kodansha, Tokyo, Japan). Significant differences were analyzed by Tukey–Kramer’s test at a significance level of \( p < 0.05 \).

3. Results and discussion

3.1 Freezing and thawing of soybeans
A typical freezing curve at \(-80^\circ C\) and thawing curve are shown in Fig. 1.

The freezing rates at different temperatures were calculated from the slopes of each freezing curve of the soybean core between \(-10^\circ C\) to \(0^\circ C\). The freezing rates were 0.0458°C/s, 0.170±0.0480°C/s, and 23.5±15.3°C/s at the freezing temperatures of \(-20^\circ C\), \(-80^\circ C\), and \(-180^\circ C\), respectively.

3.2 Microscopic observations
The internal structures of these soybean cotyledons were directly observed by TEM (Fig. 2). Untreated soybean cotyledons showed a uniform square shape or circular cells in the palisade tissue, and there were smaller cells in the spongy tissue. These cells were filled with larger protein bodies (P) and smaller lipid bodies (L), filling between protein bodies (Fig. 2A). However, freeze–thawed soybeans possessed partially destroyed membrane structures and the protein bodies appeared to be ruptured (Fig. 2B–D). The freeze–thaw process mainly destroyed the membrane structures of the protein bodies in soybean cotyledons with a minimal destruction of the lipid bodies. The presence of partially destroyed protein bodies and lipid bodies even after freeze–thawing indicated the preservation of some membrane structures.

3.3 Dielectric properties
In general, the Cole–Cole arc indicated the existence of membrane structures in cellular biological materials, showing turgor pressure [5,6]. The mechanically damaged materials resulted in smaller Cole–Cole arc than the intact cells [21,29]. The dielectric properties of the soybean cotyledons, as measured by the Cole–Cole arc, indicated the integrity of the cell structure. Untreated soybean cotyledons without a freeze–thaw treatment were expected to have intact membrane structures, and thus regular Cole–Cole arcs. Cole–Cole arcs from

![Typical freezing (A) and thawing (B) curves of a soybean as a preliminary freezing test. A soybean without vacuum-sealing were frozen for at \(-80^\circ C\) for 1 h, and then transferred to an ice bath at \(0^\circ C\) for 1 h. Finally, soybean was transferred from an ice bath and stored at \(25^\circ C\) for 1 h.](image-url)
untreated soybean cotyledons were significantly larger than those of freeze–thawed soybeans (Fig. 3) [31]. The frequency range used for the calculation for Cole–Cole radii was around 10 kHz to 5 MHz. To note, the radii of the Cole-Cole arcs after freezing became smaller as the freezing temperature decreased (Fig. 4). This indicated that the soybean cell structure was damaged by the freeze–thaw cycles. However, the Cole–Cole arc was still observed in cotyledons frozen at −180°C.

The Cole–Cole arc of potato (Solanum tuberosum) [32], carrot (Daucus carota var. sativus), and broccoli tissue [29] disappeared after the freeze–thaw process. The Cole-Cole arcs of osmotically dehydrated plant cells after freeze–thaw rehydration did not disappear; however, they became smaller than those after dehydration–rehydration. Furthermore, the Cole–Cole arc of pressurized soybeans between 50 to 400 MPa did not disappear and they became smaller than those of intact soybean cells [21]. The radius of Cole–Cole arc in freeze–thawed soybeans at all temperatures observed in our study was because of the relatively rigid structure of the cotyledon. Due to its structure, the Cole–Cole arc did not disappear compared with that of other vegetables that lack this rigid structure. In fact, intact lipid bodies were still observed even after freeze–thawing (Fig. 2 B–D), which led to the existence of the Cole–Cole arc.

In this study, the faster freezing rate at the lower freezing temperature led to more damage to the internal structures of the soybeans. These results were not common in previously reported plant cell–based food materials. However, Vertucci [33] also reported a similar phenomenon in the increased rate of electrolyte leakage from soybeans at a lower freezing temperature, which was an index of cellular destruction. Vertucci [33] investigated the relationship between thermal transitions and freezing injury in soybean seeds by differential scanning calorimetry, which showed several endothermic peaks above −40°C and a devitrification event at about −70°C. Lipid transitions may be involved in the devastating effects on seed viability. It is speculated that triglycerides
interact with water and that this interaction can trigger sensitivity to freezing damage. Our freezing temperatures at -80°C and -180°C were below these thermal transition temperatures of soybean compounds; hence, our results could be explained by a thermal transition of compounds in soybeans.

Another reason why the faster freezing rate led to more damage to the internal structures of soybeans was the existence of osmotic pressure-assisted destruction by the freeze concentration of soluble compounds. Osmotic pressure (Π, MPa) was expressed by the following equation [34]:

$$\Pi = \left(\frac{-RT}{V_w}\right) \times \ln Aw$$  \hspace{1cm} (1)

where, R is the gas constant, T (K) is the freezing point of water, Vw (=18.018 cm³/mol) is the molar volume of water, and Aw is the water activity. The estimated water activities were 0.822, 0.458, and 0.324 at -20°C, -80°C, and -180°C, respectively [34]. The calculated osmotic pressure for the freeze-concentrated solution was 27, 107, and 154 MPa at -20°C, -80°C, and -180°C, respectively. Based on this calculation, the faster freezing rates at a lower freezing temperature resulted in a higher osmotic pressure. This osmotic pressure would result in the partial destruction of the internal soybean structures. In fact, the high hydrostatic pressure—treated soybean cotyledons showed smaller Cole–Cole arcs than the untreated ones [21]. Additionally, the Cole–Cole arcs of high hydrostatic pressure—treated onion drastically decreased between 100 to 200 MPa, not up to 50 MPa [35]. Therefore, the serious destructions of soybean cotyledon cells at -80°C and -180°C, as shown in Figs 2 and 3, would be caused by the combination of ice crystal formation and osmotic pressure by the freeze concentration. Then, the diffusion of compounds such as GABA in these injured soybeans would be accelerated following the freezing–induced structural destruction.

### 3.4 Measurement of soybean pH during storage

Prior to soybean soaking, sodium azide was added to soaking distilled water; therefore, the physicochemical changes during storage were not oriented by microbial reaction in this study.

The soybean samples were stored at 25°C for 3 days after the freeze–thaw treatment, and their chemical characteristics were analyzed based on enzymatic reactions. The soybean pH values were measured over the 3 days (Table 1). The pH values of untreated soybeans increased at earlier time points in the storage period compared to those of treated soybeans, but these values significantly decreased after 3 days of storage (p<0.05) (Table 1). Soybean vacuoles formed within a couple of days after water uptake [36]. Vacuoles contain acids, and the eruption of vacuoles decreases the pH [37]. The pH values of soybeans frozen at -80°C and -180°C was lower than those of untreated soybeans and soybeans frozen at -20°C. This is due to the high destruction of cellular structures at -80°C and -180°C, wherein the internal components poured out.

Kasai [36,37] reported that heating vegetables resulted in a reduced pH. Heating particularly affected the demethylation of pectin and elicited the release of pectin esterase, causing a lower pH. Thus, the observed decrease in the pH of freeze–thawed soybeans was probably due to a combination of simultaneous events such as the release of acids and specific enzymes.

### 3.5 GABA concentrations

The time course of GABA concentrations during storage is shown in Table 2. The initial GABA concentration of untreated soybeans was 6.17±1.22 μmol/g-soybean. During storage, the GABA concentrations of untreated soybeans increased up to 10.1±1.18 μmol/g-soybean at 2 days of storage. By contrast, the GABA concentrations of freeze–thawed soybeans after 1 day of storage increased significantly, while that of untreated soybean retained the similar value even after 1 day of storage. The changes of GABA concentrations in freeze–thawed soybeans during 1 day of storage were 2.42 μmol/g-soybean at -20°C, 3.93 μmol/g-soybean at -80°C, and 2.11

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Table 1  Time course of pH values in soybean cotyledons during storage after freeze–thaw treatment.

| Storage period [d] | 0      | 1      | 2      | 3      |
|--------------------|--------|--------|--------|--------|
| Untreated          | 6.38±0.04a | 6.54±0.05b | 6.58±0.04b | 6.22±0.04c |
| -20°C              | 6.36±0.05ab | 6.48±0.13ac | 6.54±0.09c | 6.28±0.04b |
| -80°C              | 6.26±0.05a | 6.38±0.07bc | 6.36±0.05d | 6.00±0.00d |
| -180°C             | 6.32±0.08a | 6.32±0.04a | 6.34±0.05b | 6.02±0.04b |

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μmol/g-soybean at -80℃, respectively. The highest GABA concentration in this study was found in freeze-thawed soybeans at -180℃ (11.6±0.84 μmol/g-soybean) at 3 days of storage.

The relative GABA concentrations were calculated as a concentration ratio between GABA concentration during storage and that of untreated soybeans at 0 day of storage.

Compared with the initial GABA concentration of untreated soybeans, the relative GABA concentrations of freeze-thawed soybeans frozen at -80℃ and -180℃ were higher with maximum values of 1.65 and 1.89, respectively (Table 3). The GABA production rates were estimated from the slopes of GABA concentrations during the storage period from 0 to 2 days. The GABA production rate of untreated soybeans was 1.97 μmol/g-soybean·d, while those of freeze-thawed soybeans were 2.25 μmol/g-soybean·d at -20℃, 1.87 μmol/g-soybean·d at -80℃, and 1.61 μmol/g-soybean·d at -180℃. Additionally, the GABA production rate during 3 days of storage were 1.21 μmol/g-soybean·d in untreated, 1.44 μmol/g-soybean·d at -20℃, 1.24 μmol/g-soybean·d at -80℃, and 1.10 μmol/g-soybean·d at -180℃, respectively.

GABA is mainly generated from glutamate as a substrate and the enzyme GAD that exists in the cytosol of soybean cells. After the freezing and thawing treatment, GAD in the cytosol could permeate into the membrane structure by a mass transfer acceleration based on cell destruction. Thus, GAD activity increased at lower temperatures and GABA concentrations rapidly increased as well.

Theoretically, enzymatic reactions inside cells are dependent on both diffusion and reaction. Freeze-thawed soybeans were partially destroyed. Thus, the mass transfer of glutamate was promoted. To further understand the mechanism of GABA enrichment, we investigated the optimal pH conditions for enzymatic GABA production.

### 3.6 Optimal pH of GAD

GABA is mainly generated from glutamate and catalyzed by GAD. There is literature on the optimal GAD pH in brown rice [38]; however, optimal GAD conditions in soybeans have rarely been reported. In this study, we investigated the optimal pH of crude GAD in soybeans at 40℃. The crude GAD obtained from untreated soybean showed a bell-shaped pH-dependent activity. The optimal pH of soybean GAD was around 5.6 (Fig. 5), and the GAD activity curve showed a higher activity in the pH range from 5.6 to 6.2. By contrast, it decreased drastically in other pH ranges.

From the pH time course of freeze-thawed soybeans, the pH values of soybeans frozen at ~80℃ and ~180℃ were lower than those of untreated soybeans. Soybeans with a lower pH showed a higher GABA concentration due to increased GAD activity during storage (Fig. 5). Another explanation for the rapid increase in the GABA concentration is that the destruction of cell membranes led to the elution of internal acidic components from the membrane structure. Thus, the internal cellular pH decreased and GAD activity of the soybean cotyledon

| Storage period [d] | 0   | 1   | 2   | 3   |
|-------------------|-----|-----|-----|-----|
| Untreated         | 1.0 | 0.96±0.10 a | 1.63±0.21b | 1.43±0.24b |
| -20℃             | 0.78±0.08a | 1.17±0.22b | 1.51±0.33c | 1.41±0.19c |
| -80℃             | 0.97±0.55a | 1.61±0.21b | 1.58±0.10b | 1.65±0.10b |
| -180℃            | 1.34±0.17a | 1.69±0.11b | 1.86±0.11c | 1.89±0.14c |
increased, thereby inducing an increase in GABA concentration. In fact, the pH of untreated soybean seeds was initially 6.38 while that of soybean seeds frozen at -180 °C was 6.02 after 3 days of storage. Additionally, the optimal pH for GAD activity in rice germs was 5.8 and the specific GAD activity at pH 6.0 was approximately twice that at pH 6.4 [38]. These reported results regarding the optimal pH for GAD activity support our findings.

At an earlier period of storage, the GABA generation reaction would be accelerated by a mass transfer improvement due to cellular destruction. Then, the decreasing pH at 3 days of storage led to an improved GAD activity, which resulted in a higher accumulation of GABA in soybeans.

4. Conclusions

In conclusion, the internal structure of soybeans such as protein bodies were partially destroyed by the freeze–thawing process investigated by dielectric and microscopic observation. The relative GABA concentrations of freeze–thawed soybeans at -80°C and -180°C were higher with maximum values of 1.65 and 1.89, respectively. The cell destruction led to glutamate diffusion and a lower pH (6.0–6.2), which provided optimal conditions for the enzymatic production of GABA in soybeans. In summary, we have developed a simple and optimal freeze–thawing process to produce GABA-enriched soybeans.

Acknowledgments

This study was supported by the JSPS KAKENHI (grant number 25350091) and the Salt Science Research Foundation (grant number 1323).

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凍結解凍処理による吸水大豆の組織破壊によるγ-アミノ酪酸生成

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細胞性食素材は凍結により膜構造が破壊され、食品の品質を劣化することがある。われわれは、高圧処理を施した吸水大豆を保存することにより、大豆中にγ-アミノ酪酸が高濃度に蓄積可能であることを明らかにしてきた。これは高圧処理による膜構造破壊が、大豆中の有機の酵素反応を促進したためと考えられている。本研究では、膜構造を破壊可能な凍結解凍処理によっても、大豆中にγ-アミノ酪酸が高濃度に蓄積可能であるかを実験的に明らかにすることを目的とした。凍結解凍処理を施した吸水大豆の内部構造を誘電特性計測および電子顕微鏡で評価し、保存後のγ-アミノ酪酸およびpHを測定した。その結果、高圧処理による大豆内部構造の破壊によって、γ-アミノ酪酸濃度が増加したことが示唆された。また、このγ-アミノ酪酸の増加は、大豆内のpHが低下しγ-アミノ酪酸生成酵素の至適pHに近づいたことにより、みかけの酵素活性が増加したことによるものと推察された。
