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ARTICLE

Generation of Free Amino Acids and γ-Aminobutyric Acid in Water-Soaked Soybean by High-Hydrostatic Pressure Processing

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The effects of high-hydrostatic pressure processing (HPP) on soybean cotyledon as a cellular biological material were investigated from the viewpoints of the cell structure and enzyme reaction system. Damage to cell structure was evaluated by measuring dielectric properties using the Cole−Cole arc, the radius of which decreased as pressure level increased. Results suggested that cell structure was damaged by HPP. The distribution of free amino acids was measured after HPP (200 MPa) of soybean soaked in water or sodium glutamate (Glu) solution. HPP resulted in high accumulation of free amino acids in water-soaked soybean, due to proteolysis. HPP of soybean in Glu solution caused higher accumulation of γ-aminobutyric acid, suggesting that both proteolysis and specific Glu metabolism were accelerated by HPP. We concluded that HPP partially degraded cell structure and accelerated biochemical reactions by allowing enzyme activities to remain. These events can be considered "high-pressure induced transformation" of soybean.

KEYWORDS: Soybean; high pressure; free amino acid; γ-aminobutyric acid; scatter plot

INTRODUCTION

High-hydrostatic pressure processing (HPP) can preserve small molecules such as vitamins and free amino acids in foods and agroproducts because of nonthermal treatment (*1*, *2*), and the formation of self-supporting soybean protein gels by HPP at or above 300 MPa has been reported (*3*, *4*). HPP significantly modifies structures of proteins and other macromolecules by affecting noncovalent bonds. On the other hand, HPP at 100−400 MPa induces various changes in agroproducts (*5*, *6*) by damaging internal cell structures and membranes (*7*, *8*), leading to mass transfer of materials within cells (*9*, *10*). Since certain enzymes are still active even at 600 MPa, several biochemical reactions can often occur after HPP (*11*, *12*). Therefore, if HPP would confer suitable conditions for a certain biochemical reaction to the inside of an agroproduct, the agroproduct could behave as a bioreactor. The biochemical reactions related to proteins and peptides are predicted to change the free amino acid composition in pressurized biological materials.

Soybean is rich in nutrients and other bioactive substances, and the effects of soybean components on human physiology have been studied. Certain soybean components have been associated with the reduction of maladies such as certain cancers (*13*, *14*) and heart disease (*15*). In addition, the role of soybean in the reduction of cholesterol and in the prevention of obesity has been established (*16*, *17*). Soybean contains a high concentration of protein. Soybean foods such as natto, miso and soy sauce have been processed traditionally to increase nutritional values by hydrolysis of soybean protein with protease (*18*−*20*). Degradation of soybean protein in fermented soybean resulted in changes of amino acid distribution and increased its nutritional ingredients (*21*). Unfortunately, fermented soybean has unique sensory characteristics, which are not widely acceptable. Environmental stresses, such as salt stress, low temperature and drought stress, also induced changes in amino acid distribution of soybean without addition of protease (*22*−*24*). In these reports, γ-aminobutyric acid (GABA), a non-protein amino acid, also increased. Salt stress on soybean roots strongly promoted the activity of diamine oxidases to stimulate polyamine degradation (*23*). After a longer stress period, soybean plants were able to adjust their amino acid metabolism to the unfavorable conditions (*24*). Some researchers investigated that GABA accumulation was caused by environmental stress-induced proteolysis, but these reports were mainly about soybean plants, not soybean cotyledon. The effect of germination and breeding on chemical composition of soybean cotyledon was reported (*25*, *26*). GABA content increased in the early germination stage of soybean. Soybean cotyledon without germination, which is enriched in nutritional ingredients, is desired as a raw material for value-added soybean food.

High pressure is a kind of stress on plant tissues. Therefore, application of HPP on soybean is expected to lead to proteolysis, which will result in a novel technique for increasing amino acid

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content. In this study, we examine the high-pressure induced changes in free amino acid distribution in pressurized soybean, and investigate the effect of HPP on the reaction related to amino acid metabolism.

MATERIALS AND METHODS

Preparation of Soybean Grains. Soybean (*Glycine max* L.) seeds were purchased at a local supermarket in Niigata, Japan. Seeds suspended in distilled water or sodium glutamate solution (0.05 g/mL) were vacuum-packed in a polyethylene pouch (Eiken Kizai, Tokyo, Japan) using a vacuum sealer (FCB270, Fuji Impulse, Osaka, Japan); sodium azide (0.2 w/v %) was added to prevent microbial growth. Samples were allowed to imbibe and swell at 25 °C for 22 h.

High-Hydrostatic Pressure Treatment and Storage. Vacuum-packed samples were soaked in distilled water (hydraulic fluid) in the stainless-steel vessel of a high-hydrostatic pressure apparatus that had a maximum pressure of 686 MPa (WIP, Kobe Steel, Kobe, Japan). The inner diameter of the high-pressure vessel was 60 mm, and the depth was 180 mm. The piston moved into the vessel and was adjusted to pressurize samples at 200 or 400 MPa for 10 min at 20 °C. The pressure increase rate was 3.0 to 3.7 MPa/s; decompression was completed within 40 s. The temperature of distilled water in the high-pressure vessel changed within 2 °C during pressure treatment. After high-pressure treatment, seeds were washed with water, wiped with paper towels, vacuum-packed in polyethylene pouches, and stored for 4 days at 25 °C.

Dielectric Measurement. Dielectric properties of untreated and pressurized soybeans were measured immediately after pressure release. Pressurized and control soybean seeds were removed from pouches, each 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 Ti−Pt electrodes (Tanaka Kikinzoku Kogyo, Tokyo, Japan), each 1.0 mm in outer diameter with 5.0 mm interelectrode distance from the equatorial plane. Dielectric properties of cotyledons were measured at frequencies between 100 Hz and 5 MHz using an LCR meter (3532-50, Hioki, Nagano, Japan) with a fixture tool (9252, Hioki, Nagano, Japan). For each cotyledon, resistance was plotted against reactance, giving rise to a Cole−Cole plot (*27*), which reflects the integrity of the cellular structure (*28*). The Cole−Cole plot shows the characteristic shape as half round or partial arc. The circle equation can be described as follows (*29*):

$$(R - a)^2 + (X - b)^2 = r^2 \quad (1)$$

where a and b are parameters to be optimized and r is the radius of the Cole−Cole arc. This equation is transformed into a linear equation form as

$$Y = aX_1 + bX_2 + c \quad (2)$$

$$c = r^2 - a^2 - b^2 \quad (3)$$

The optimal parameters eq **2** were obtained by a least-squares method with analytical tool of Excel (Excel2000, Microsoft, WA), and the radius of the Cole−Cole arc, r, was obtained by eq **4**.

$$r = (a^2 + b^2 + c^2)^{0.5} \quad (4)$$

GC-FID Analysis of Amino Acids. Soybean seeds (2 g) were ground with 18 mL of distilled water in a mortar and centrifuged at 20400*g* for 3 min at 4 °C. Free amino acids in the supernatant were measured with GC-FID (GC-14A, Shimadzu, Kyoto, Japan), utilizing the free amino acids analysis kit "EZ:faast" from Phenomenex (Torrance, CA) (*30*, *31*). This kit detects up to 50 amino acids and related compounds with no interference by the presence of proteins. The procedure involves a solid phase extraction, followed by rapid derivatization conducted in the aqueous phase at room temperature. The carrier gas was helium. The oven temperature program was as follows: initial temperature 110 °C, followed by a linear ramp of 24 °C/min up to 320 °C and held for 5 min. The temperature of the injection port was 250 °C, while the detector temperature was kept at 320 °C. Under these conditions a 2 μL sample was injected in split mode.

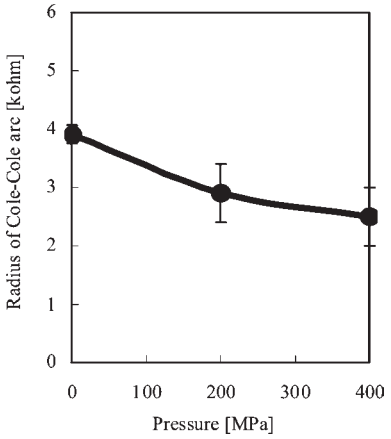

Figure 1. Effect of high-pressure treatment on radius of Cole−Cole arc of soybeans.

RESULTS AND DISCUSSION

Damage to Cell Structure of Pressurized Soybean Seeds. The dielectric property of soybean cotyledons, measured by the Cole−Cole arc, indicated the integrity of the cell structure. The radius of the Cole−Cole arc decreased as hydrostatic pressure increased (**Figure 1**), indicating that the structure of soybean cells was damaged by HPP. Ueno and others (*32*) showed that the radius of the Cole−Cole arc of *Brassica rapa* decreased as pressure increased, suggesting that HPP damages cell structure in plant tissue. The Cole−Cole arc of potato tissue disappeared after a freeze−thaw cycle or osmotic dehydration treatment (*33*). Ohnishi and others (*34*) found that Cole−Cole arcs completely disappeared after physical treatments of many kinds of vegetables whereas the radius of the Cole−Cole arc of soybean after a freeze−thaw cycle was only partially reduced, a result similar to those found using HPP. Omi and others (*35*) reported large structural changes in cotyledon surface and epidermal cells after pressurization of seeds. It is proposed that HPP damages cellular membrane systems, accelerating mass transfer.

Distribution of Free Amino Acids in Water-Soaked and Pressurized Soybean. Partial damage of cell structure at 200−400 MPa would induce certain enzymatic reactions (*32*). Puppo and others (*36*) reported that treatment of soybean with HPP higher than 200 MPa caused protein aggregation and disrupted protein secondary structure. Retention of lipoxygenase activity decreased after multicycling of pressure (350 MPa) and thermal (40 °C) treatments (*11*). To avoid protein conformational changes and to retain enzymatic activity, we pressurized soybean at 200 MPa.

Concentrations of GABA and free amino acids during storage in water-soaked soybeans with or without HPP are shown in **Figure 2**. Immediately after HPP, there was little difference in free amino acid concentrations between unpressurized and pressurized samples: for example, for Ala, Lys, and GABA, the values were 0.72 and 0.53 μmol/g, 1.12 and 1.28 μmol/g, and 0.90 and 1.05 μmol/g, respectively. Concentrations of other amino acids did not significantly change. After 3 day storage, however, concentrations of most amino acids increased in pressurized soybeans after soaking in water compared with unpressurized ones. For Ala, Lys, and GABA, respectively, the values of unpressurized vs pressurized soybeans after 3 days were 1.15 and 1.98 μmol/g, 1.61 and 2.05 μmol/g, and 1.70 and 2.32 μmol/g. Thus, production of free amino acids in water-soaked soybean during storage was accelerated by HPP at 200 MPa.

Puppo and others (*36*) reported that a decrease of free sulfhydryl content and a total denaturation soy protein were observed at 400 and 600 MPa, but not observed at 200 MPa. This

suggested that the secondary structure of soy protein might be mostly maintained at 200 MPa, and enzymes for production of certain amino acids might retain activities at 200 MPa. Penas and others (*15*, *37*) reported an enhancement of enzymatic proteolysis of soybean whey by HPP. In addition, they reported that pressure levels of 200 and 300 MPa were the best pressure conditions for enhancement of proteolysis in the soybean whey-protease system. Activities of certain proteases, such as Alcalase, Neutrase, Corolase 7089 and Coronase PNL, were reportedly enhanced by HPP (*15*). They also pointed out that proteolysis was improved when proteins were structurally modified. Concentration of GABA in water-soaked rice germ increased during storage at room temperature (*38*). In our study, higher accumulation of free amino acids was promoted by HPP in water-soaked soybean.

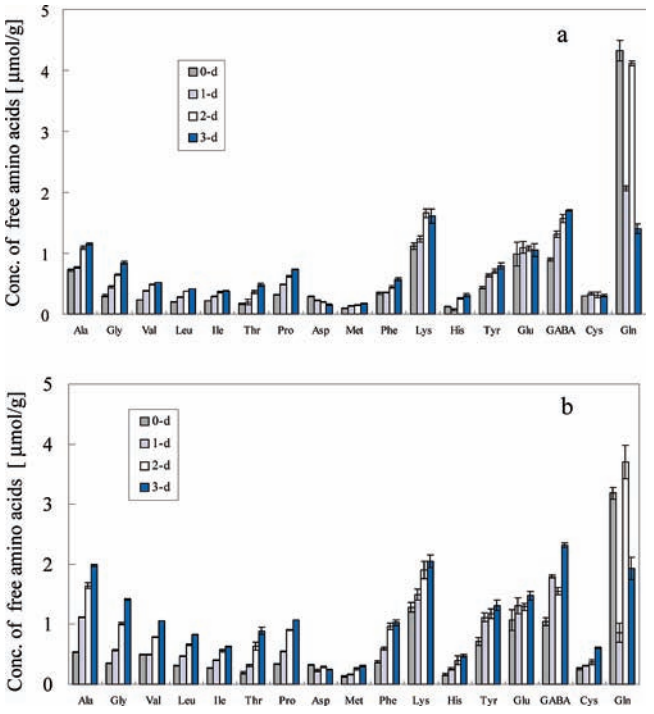

Figure 2. Free amino acid concentrations in unpressurized (**a**) and pressurized (**b**) soybeans after soaking in water.

Distribution of Free Amino Acids in Glu-Soaked Pressurized Soybean. Concentrations of GABA and free amino acids during storage in Glu-soaked soybean with or without HPP are shown in **Figure 3**. Concentrations of Ala and Gly, in Glu-soaked soybean immediately after soaking and 3 days of storage, were 1.37 and 4.23 μmol/g and 0.31 and 1.09 μmol/g, respectively (**Figure 3a**). Concentrations of several other free amino acids, Val, Leu, Ile, Thr, Pro, Asp, Met, Phe, Lys, His, Tyr, GABA and Cys, in Glu-soaked soybean also increased during storage. In contrast, the concentrations of Glu and Gln in Glu-soaked soybean decreased from 15.8 to 10.7 μmol/g and from 12.8 to 4.52 μmol/g, respectively, during storage (**Figure 3a**). Free amino acid concentrations in Glu-soaked soybeans after HPP were also analyzed (**Figure 3b**). Concentrations of Ala and Gly in Glu-soaked and pressurized soybean immediately after and 3 days after soaking were 1.36 and 6.00 μmol/g and 0.35 and 1.62 μmol/g, respectively (**Figure 3b**). Concentrations of other free amino acids in Glu-soaked and pressurized soybean were also higher than those in Glu-soaked but not pressurized soybean (**Figure 3b**). Concentrations of Ala, Gln and Glu in Glu-soaked soybeans increased significantly by addition of Glu, with or without HPP, whereas HPP caused an increase in GABA concentration in Glu-soaked soybean.

HPP Effect on GABA Concentration. The time course of GABA concentration in water-soaked or Glu-soaked soybeans is shown in **Figure 4**. HPP caused a slight increase in GABA concentration in water-soaked samples, but would be predicted to cause a greater increase in Glu-soaked samples, since Glu is a precursor of GABA. The highest GABA concentration was 4.20 μmol/g (12.35 μmol/g dry basis, d.b.) in Glu-soaked pressurized soybean at 2 days, while that in water-soaked at 0 day was 0.90 μmol/g. GABA concentration eventually increased 5-fold during storage.

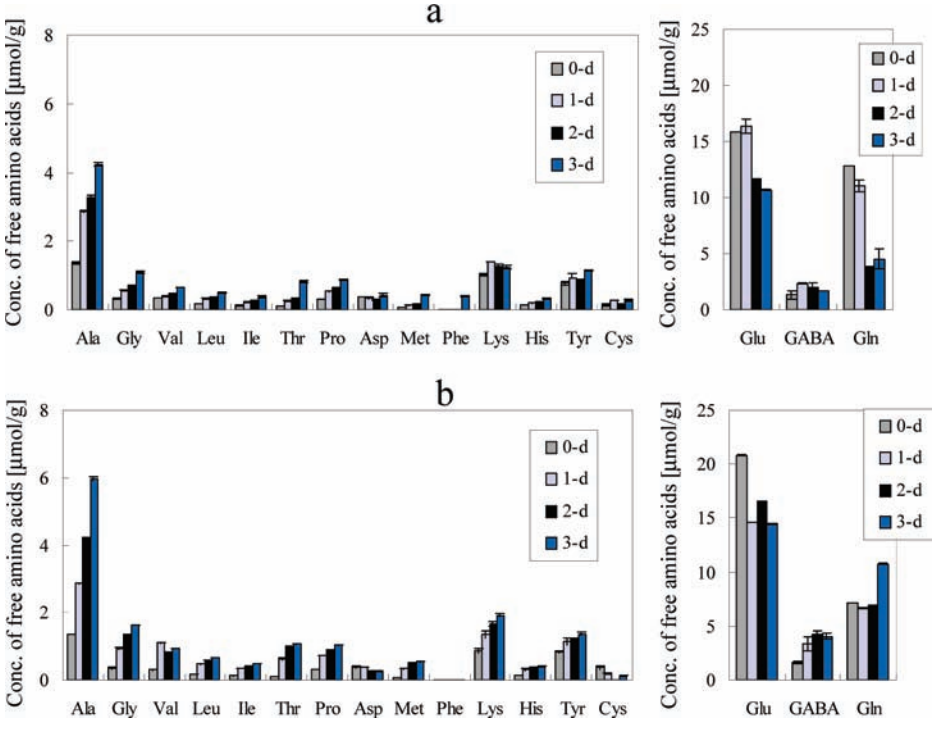

Figure 3. Free amino acid concentrations in unpressurized (**a**) and pressurized (**b**) soybeans after soaking in sodium glutamate solution.

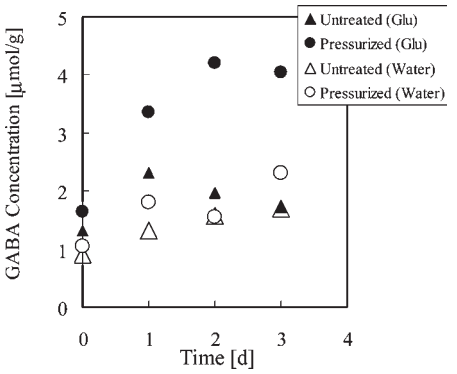

Figure 4. Effect of HPP on the time course of GABA concentration in soybeans after soaking in sodium glutamate solution or water.

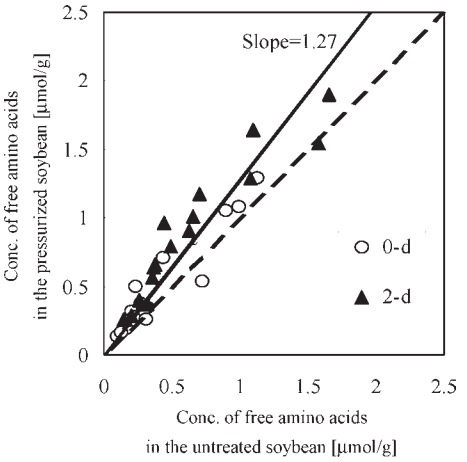

Figure 5. Scatter plot of free amino acids in untreated and pressurized soybeans soaked in water.

Typically, GABA levels in plant tissue are low, ranging from 0.03 to 2.00 μmol/g (*39*). Martinez-Villaluenga and others (*40*) reported that the GABA concentration in dry soybean seeds was 2.42 μmol/g d.b., while after 3 day germination it was 7.95 μmol/g d.b. GABA concentrations in mechanically or cold stimulated soybean leaves rise to 2 μmol/g (*22*). In our study, GABA concentration reached up to 12.35 μmol/g d.b. in Glu-soaked pressurized soybean at 2 days. Higher accumulation of GABA in soybean cotyledon was performed by the combination of precursor-soaking and HPP.

Evaluation of HPP Effect. The evidence indicates that HPP at 200 MPa causes partial disruption of cell structures, leaving certain enzymes still active. Some of these active enzymes would be expected to cause proteolysis in soybean pressurized at 200 MPa. In general, two types of physiological processes, proteolysis or amino acid metabolism, produce free amino acids. Proteolysis leads to increased amino acid concentration, while amino acid metabolism decreases concentration of certain amino acids.

To analyze the process by which free amino acids are generated in pressurized soybean, we made scatter plots of concentration values after HPP (*y*-axis) vs values without HPP (*x*-axis). If the slope of a line fitted to the data points is 1.0, it means amino acid concentrations are the same in both types of samples. In contrast, if the slope is greater than 1.0, it means HPP caused an increase in free amino acid concentration. Ueno and others (*41*) used scatter plots to evaluate high-pressure induced changes in flavonoids of onion, and successfully extracted certain components that were increased by HPP. In the present study, we used scatter plots to

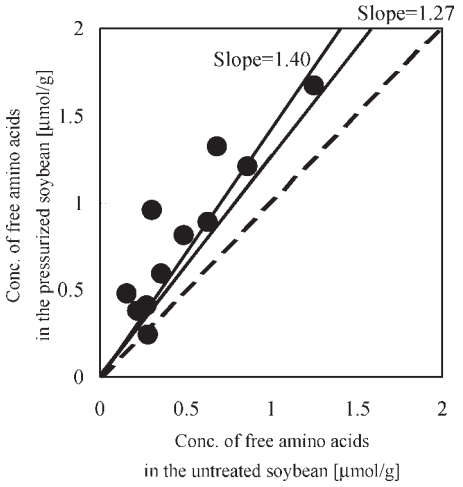

Figure 6. Scatter plot of free amino acids in untreated and pressurized soybeans 2 days after soaking in sodium glutamate solution.

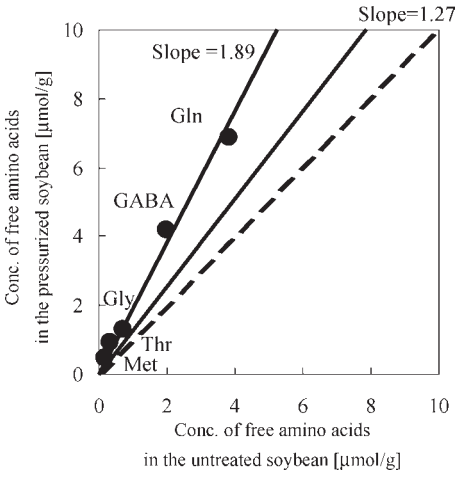

Figure 7. Scatter plot of GABA, Gln, Gly, Met and Thr in untreated and pressurized soybeans 2 days after soaking in sodium glutamate solution.

determine if HPP causes an increase in free amino acid concentration in soybean.

Scatter plots of values after water-soaking vs values after water-soaking and HPP are shown in **Figure 5**. After HPP, values for most free amino acid concentrations plotted linearly, giving a straight line with a slope of 1.27. This result indicates that HPP promotes proteolysis in pressurized soybean. If soybeans were Glu-soaked rather than water-soaked, free amino acid concentration values plotted linearly (except for GABA, Gln, Gly, Met and Thr) and the slope of the line was 1.40 (**Figure 6**). Comparison of the Glu-soaked value of 1.40 (**Figure 6**) with the water-soaked value of 1.27 (**Figure 5**) suggests that the increase in free amino acid concentration is related to metabolic pathways of amino acids. Scatter plots for GABA, Gln, Gly, Met and Thr in Glu-soaked and pressurized soybean at 2 days are shown in **Figure 7**. The slope of the plots for these amino acids is 1.89, significantly larger than 1.27, which is the slope of water-soaked and pressurized soybean. These results indicate that, for these five amino acids, Glu-soaking and pressurization causes even higher accumulations than for the set of free amino acids shown in **Figure 6**.

GABA was produced primarily by α-decarboxylation of Glu catalyzed by glutamate decarboxylase (GAD) (*39*, *42*). Saikusa and others (*38*) reported that GAD in rice germ might be responsible for antagonistic changes in GABA and Glu concentration.

The progressive increase of GABA in rice germ indicated that the amount of Glu was large enough for GAD activity to exceed that of the GABA-degrading enzymes. Wallace and others (*22*) indicated that GAD activity of soybean leaf considerably exceeded (>30-fold) that of GABA pyruvate transaminase. The second enzyme involved in the GABA shunt, GABA transaminase, catalyzes the reversible conversion of GABA to succinic semialdehyde using either pyruvate or α-ketoglutarate as amino acceptors (*39*). α-Ketoglutarate transaminase also catalyzes the reversible conversion of α-ketoglutarate to Ala. In our study, concentrations of Ala in Glu-soaked and pressurized soybean were relatively high. Higher accumulation of GABA in pressurized soybean would accelerate Ala synthesis catalyzed by α-ketoglutarate transaminase. Ala is also irreversibly converted to glycine by alanine-glyoxylate aminotransferase (*43*). Glu is reversibly converted to Gly by glutamate aminotransferase (*44*). Gly concentration in Glu-soaked and pressurized soybean would be caused to increase by syntheses from both Ala and Glu. Gly is reversibly converted to Thr by threonine aldolase (*45*). Therefore, when Gly concentration increases in Glu-soaked and pressurized soybean, Thr concentration would also increase. Consequently, metabolic pathways associated with Glu related components would be accelerated in Glu-soaked and pressurized soybean. Certain enzymes were still active at 200 MPa, which resulted in higher accumulation of free amino acids in Glu-soaked and pressurized soybean.

HPP at 200 MPa for 10 min was used to cause partial degradation of internal soybean cell structure. Cellular membrane systems were damaged in pressurized soybean. Thus, mass transfer in pressurized soybean was accelerated, compared with that in intact soybean, which promoted enzymatic reactions. HPP at 200 MPa caused an increase in free amino acids in water-soaked or Glu-soaked soybean during storage, which resulted in high accumulation of GABA. The combination of HPP with a presoak of soybean seeds is a novel technique for generation of functional compounds. We propose that high-pressure induced physicochemical cellular changes be called "high-pressure induced transformation (Hi-Pit)" of soybean. Application of "Hi-Pit" is suggested as a treatment for food processing.

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