



Effects of a High-Pressure Treatment on Bovine Gamma Globulin and Its Reduction in Allergenicity

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The effects of a high-pressure treatment on the IgE-specific binding activity and structural changes to bovine gamma globulin (BGG), a beef allergen, were investigated. The allergenicity of pressure-treated BGG was also evaluated. We found that the IgE-specific binding activity and allergenicity of BGG were decreased by the high-pressure treatment. Almost no significant change in secondary structure was apparent for pressurized BGG, but a change in the tertiary structure was detected. The decreased IgE-specific binding activity and allergenicity of pressurized BGG were probably due to changes in the tertiary structure caused by pressurization. The results of this study suggest that a high-pressure treatment would be an effective food-processing technique to reduce the allergenicity of BGG.

Key words: high pressure; bovine gamma globulin (BGG); food allergy; IgE-specific binding activity

Food allergies have become increasingly widespread in recent years and are now a serious social problem. Food allergies typically appear in infants or children, and are often outgrown.¹⁾ However, children diagnosed as having food allergies after 3 years of age have been found to be less likely to outgrow the allergy.²⁾ Food allergies are now prevalent in both children and adults. Although it is desirable to avoid eating foods that cause allergic reactions, such foods, including milk, eggs, and meat, are often nutritious and indispensable for the growth of children. Avoiding these foods can therefore lead to other health concerns.

Several research groups have exploited new techniques to eliminate food allergenicity, and have developed low-allergenic food products.³⁻⁹⁾ For example, Watanabe *et al.*^{10,11)} have succeeded in eliminating an

allergenic component from rice by an enzymatic treatment, and Besler *et al.*¹²⁾ have eliminated allergenic elements from bovine milk by combining heat and enzymatic treatments with ultrafiltration. The hypoallergenic rice and milk are now produced commercially and can be supplied to allergic patients. Although enzymatic treatments have generally been used for eliminating food allergenicity, such a treatment results in a degradation of the processing food characteristics, as well as a deterioration in the taste; for example, the development of bitterness or an unpleasant odor. Moreover, the application of an enzymatic treatment to foods containing a high level of protein, such as meat, is not practicable.

Meat is an important food because of its high nutritional value and functional properties. Although meat allergy is considered to be a rare pathological problem, recent reports have demonstrated that meat allergy can be a serious problem, particularly in children, that strikes unexpectedly. A previous study has identified bovine serum albumin (BSA) and bovine gamma globulin (BGG) to be the major beef allergens.¹³⁾ Werfel *et al.*¹⁴⁾ have reported that the allergenicity of BSA and BGG could be reduced by sufficient heating. On the other hand, Fiocchi *et al.*¹⁵⁾ have reported that, in some cases, heated BSA caused an allergic reaction. There have also been reports that beef allergy showed high cross-reactivity with other meat allergies.^{16,17)} Beef allergy patients also show allergic symptoms against bovine milk,¹⁸⁾ as BSA and BGG are present in both beef and milk as allergens. Lebenthal *et al.*¹⁹⁾ have reported that 18% of bovine milk allergy patients reacted against BSA and 27% of bovine milk allergy patients reacted against BGG. Therefore, reducing BSA and BGG allergenicity is very important for both beef allergy and bovine milk allergy patients.

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Abbreviations: BGG, bovine gamma globulin; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PBST, Tween20 in phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; CD, circular dichroism; ANSA, 8-anilino-1-naphthalene-sulphonic acid; cPA, *cis*-parinaric acid

A high-pressure treatment is considered to be a useful food-processing technique for developing new food products. Such a treatment affects the structure of proteins resulting in the changes in the food characteristics. The efficiency of this treatment for meat has been reported by several research groups.^{20–22)} However, there is currently little data on the effects of a high-pressure treatment on food allergenicity. In several previous papers, the effects of high pressure on the antigenicity of a beef extracts,²³⁾ BSA (beef allergen)²⁴⁾ and ovomucoid (chicken egg white allergen)²⁵⁾ have been discussed from the point of view that structural changes in the allergens induced by pressure may lead to the reduction or elimination of antigenicity. The high-pressure-induced reduction of the allergenicity of Mal d 1, the major allergen in apples, has also been reported from the same point of view.²⁶⁾ In addition to this, Kato *et al.* have reported that removing the allergen from pressurized rice became easier than from untreated rice.²⁷⁾

We report in the present paper the effects of high pressure on the IgE-specific binding activity and structure of BGG, with the aim of evaluating the relationship between the allergenicity and structure of BGG.

Materials and Methods

Serum. Sera from patients with food allergies were provided by Yoshida Hospital (Niigata, Japan). Informed consent was obtained before blood was drawn, and the experimental protocol was carried out in accordance with the guide prepared by Niigata University.

High-pressure treatment. Pressurization was carried out as described by Homma *et al.*²⁸⁾ BGG (Sigma, St. Louis, MO, USA) solutions sealed in polyethylene bags were pressurized to 100, 200, 300, 400, 500, and 600 MPa at 5–7 °C for 5 min with an isostatic processor (Nikkiso Co., Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA). In order to evaluate the IgE-binding activity of sera from patients with food allergies, ELISA was performed in accordance with the method of Han *et al.*¹³⁾ with some modifications. Fifty microliters per well of a BGG solution (5 µg/ml in a 50 mM sodium carbonate buffer at pH 9.6) was added to a 96-well ELISA plate. The plate was incubated at 30 °C for 2 h, washed five times with phosphate-buffered saline (PBS, 137 mM NaCl, 8 mM Na₂HPO₄·12H₂O, 2.7 mM KCl and 1.5 mM KH₂PO₄ at pH 7.4), blocked at 30 °C for 1 h with a 2% gelatin-PBS solution, and then washed five times with 0.05% Tween20 in phosphate-buffered saline (PBST, pH 7.4). Fifty microliters of serum (diluted 20-fold in PBST) was then added to the plate which was incubated at 30 °C for 1 h. The plate was washed five times with PBST, and then the second antibody (an anti-human IgE alkaline phosphatase conjugate; Biosource, San Jose, CA, USA) was added. The plate was again washed five times with PBST. One hundred microliters of *p*-nitrophenyl phosphate (10 g in a 10% diethanolamine buffer at pH 9.8, 10 ml) was added, and after the plate had been incubated at 30 °C for 60 min, the absorbance was measured by a microplate reader with a 405-nm filter.

Dot-blotting. Dot-blotting was modified from the method of Morita *et al.*²⁹⁾ BGG was dissolved in PBS at a concentration of 1 mg/ml. BGG (2 µg/spot) was blotted on to nitrocellulose sheets (Atto, Tokyo, Japan), and these were blocked for 2 h at 37 °C in a buffer containing 0.1% human serum albumin and 0.1% Tween20 in PBS. The blocked sheets were rinsed with PBST, and were then incubated overnight in PBST containing serum (diluted 1:40 vol./vol. for the serum from patient No. 5 and 1:20 vol./vol. for the sera from patients No. 64 and No. 82) at 4 °C. The sheets were then washed with PBST. Subsequently, the sheets were incubated in PBST containing the second antibody, a 1:2500 dilution of HRP-conjugated goat anti-human

IgE (Biosource, San Jose, CA, USA) for 1 h at 37 °C. After washing with PBST, the proteins on the sheets were visualized by chemiluminescence with an ECL Plus kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Cells and cell culture. Human basophilic KU812F cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and were maintained in an RPMI-1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, 100 mg/ml of streptomycin, 12.5 mM NaHCO₃ and 15 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES). The cells were cultured at 37 °C in a humidified atmosphere containing less than 5% CO₂.

Measurement of the β -hexosaminidase release. Mast cells and basophils appear to play an important role in allergic reactions as effector cells in IgE-dependent immune responses. They express Fc ϵ RI, a high-affinity IgE receptor, on the cell surface and contain cytoplasmic granules that hold various mediators including histamine. These mediators are released from basophils and mast cells stimulated with multivalent antigens. KU812F human pre-basophilic cells originating from a patient with chronic myelogenous leukemia have been shown to bind IgE with high affinity, and to release such mediators as histamine upon stimulation with an antigen. β -Hexosaminidase activity was measured as a mediator in this study, because this enzyme is stored within secretory vesicles and is a marker of mast cells or basophil degranulation.

KU812F cells (3.0×10^5 cells/sample) were washed twice in a Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH₂PO₄·2H₂O, 12 mM NaHCO₃, 56 mM D-glucose, and 10 mM HEPES at pH 7.2) and resuspended in an RPMI-1640 medium (1.0×10^6 cells/100 µl). To permit IgE binding to the high-affinity Fc ϵ RI IgE receptor on the surface of the KU812F cells, the cells were incubated at 37 °C for 2 h with serum (diluted 1:100) exhibiting IgE-binding activity to BGG. The cells were then washed twice and resuspended in a Tyrode buffer containing Ca²⁺ and Mg²⁺. The β -hexosaminidase release was measured after stimulating the serum-sensitized cells with pressurized BGG at 37 °C for 40 min. Total β -hexosaminidase release was determined by using cells treated with 0.1% Triton X-100. Stimulation was stopped by placing the cells on ice. After centrifugation, the amount of β -hexosaminidase activity released from the cells in the supernatant was measured according to the method of Shibata *et al.*³⁰⁾ and Matsuda *et al.*³¹⁾ with some modifications. Fifty microliters of supernatant and 50 µl of 1 mM 4-nitrophenyl *N*-acetyl- β -D-glucosaminide (in a 0.1 M sodium citrate buffer at pH 4.5) were mixed in a 96-well plate, and then incubated at 37 °C for 2 h. The reaction was stopped by adding 200 µl of a 0.1 M sodium bicarbonate buffer (pH 10.0), and the β -hexosaminidase activity in the supernatant was quantified by measuring the absorbance with a microplate reader having a 405-nm filter. The BGG-induced β -hexosaminidase release is expressed as a percentage of the total β -hexosaminidase release.

Measurement of the absorbance of the supernatant of the high-pressure-treated BGG solutions. Pressure-treated (0.1–600 MPa, 5 min) BGG solutions (1 mg/ml in PBS at pH 7.4) were centrifuged at $8,000 \times g$ for 30 min. The absorbance of the supernatant was measured at 280 nm with a U-3310 instrument (Hitachi High-Tech Co., Tokyo, Japan).

Determination of the secondary structure of BGG. Circular dichroism (CD) spectra for a pressurized BGG solution (0.2 mg/ml in a 20 mM Tris-HCl buffer at pH 7.4) were recorded with a J-725 spectropolarimeter (Jasco Co., Tokyo, Japan). The mean residual ellipticity $[\theta]$ is expressed in degrees-cm²-dmol⁻¹, and was calculated by using a molecular weight of 160,000 and 918 residues. The secondary structure of BGG was analyzed by using the program developed by Yang *et al.*³²⁾

Measurement of the fluorescence spectra and center of spectral mass of pressurized BGG. Red shift, which is the movement to the long wavelength side of a fluorescence peak, is one of the indices for change in the protein tertiary structure. When the unfolding of a protein is

Table 1. IgE Binding Activity to BGG in Sera from Patients with Food Allergies

Allergic patient's serum No.	ELISA value
5	0.786
64	0.252
82	0.236
27	0.009

monitored by changes in the intrinsic tyrosine and tryptophan fluorescence, an increase in pressure generally induces a decrease in the fluorescence intensity associated with a red shift. Such a red shift has been observed in many pressure-induced denaturation studies.^{33–37}

Fluorescence spectra were obtained with an F-2500 spectrofluorometer (Hitachi High-Tech Co., Tokyo, Japan) fitted with a high-pressure vessel and pump (Teramecs Co., Kyoto, Japan). BGG solutions (0.2 mg/ml in a 20 mM Tris-HCl buffer at pH 7.4) were subjected to pressures of between 0.1 and 400 MPa, and fluorescence spectra were measured after maintaining each pressure for 5 min. After a pressure of 400 MPa had been reached, the BGG sample was decompressed in stages. Fluorescence spectra between 300 and 450 nm were recorded for BGG during pressurization and depressurization, with excitation at 280 nm. Changes in the center of spectral mass ($\langle\nu\rangle$) were calculated in accordance with the method of Ruan *et al.*³⁸

$$\langle\nu\rangle = \sum \nu_i * F_i / \sum F_i$$

where ν_i is the wavenumber and F_i is the fluorescence intensity at ν_i .

Measurement of the surface hydrophobicity of BGG. The surface hydrophobicity of the pressurized BGG solutions was measured in accordance with the method of Boyer *et al.*³⁹ Either 10 μ l of 5 mM 8-anilino-1-naphthalene-sulphonic acid (ANSA; Sigma, St. Louis, MO, USA) or 15 μ l of 1 mM *cis*-parinaric acid (cPA; Sigma, St. Louis, MO, USA) was added to 3 ml of the BGG solution (0.05 mg/ml in a 20 mM Tris-HCl buffer at pH 7.4). The fluorescence intensity of the ANSA-protein conjugates was then measured at 475 nm, with excitation at 380 nm. The excitation and emission wavelengths were 325 nm and 410 nm, respectively, for the cPA-protein conjugates.

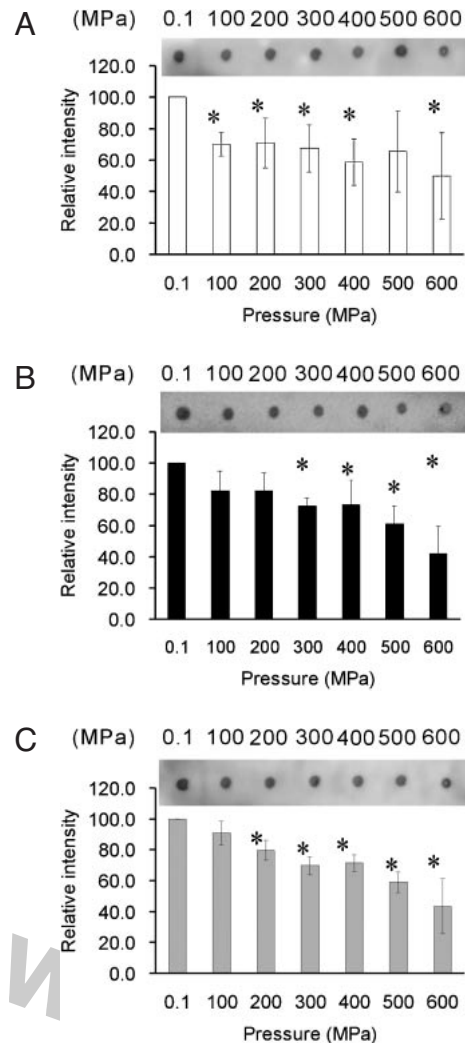
Results and Discussion

Effect of the high-pressure treatment on the IgE-binding activity against BGG

The IgE-binding activity against BGG in sera from patients with food allergies was evaluated by ELISA (Table 1). Sera from patients No. 5, No. 64, and No. 82, which showed high ELISA values in comparison to the sera from patient No. 27 who did not have beef and bovine milk allergy, were used in this study.

The IgE-specific binding activities of pressurized and unpressurized BGG in the sera from patients allergic to BGG, as assessed by dot-blotting, are shown in Fig. 1. The IgE-specific binding activities of BGG decreased significantly at 100–400 MPa and 600 MPa in patient No. 5 (Fig. 1A), at up to 300 MPa in patient No. 64 (Fig. 1B) and at up to 200 MPa in patient No. 82 (Fig. 1C). These results indicate that the high-pressure treatment influenced the reduction of IgE-specific binding activity.

Previous studies have shown BGG to be a major beef allergen and that BGG is present in beef extracts and bovine milk. Han *et al.*¹³ have reported that a high-pressure treatment did not affect the IgE-specific binding activity of BGG to sera. The difference between their results and the present results appears to have been due to the different preparations used. Beef extracts were used in the former work, and purified BGG was used in the latter. It is therefore possible that beef proteins other

**Fig. 1.** Dot-Blotting of the IgE-Binding Activities to BGG in Sera from Patients with Beef Allergy after a High-Pressure Treatment.

Relative intensity is expressed as a percentage of the value at 0.1 MPa. A, patient No. 5; B, patient No. 64; C, patient No. 82. Each value is the mean $\pm$ standard deviation ($n = 4$). * $p < 0.05$.

than BGG in the beef extracts influenced the IgE-specific binding activity of BGG and that individual differences in the IgE-binding activities in sera from several patients caused the differences in experimental results.

Effects of the high-pressure treatment on the β -hexosaminidase release activity

Pressurized and unpressurized BGG-induced β -hexosaminidase release from KU812F human basophilic cells sensitized by using sera from allergic patients is shown in Fig. 2. β -Hexosaminidase release was significantly inhibited by the high-pressure treatment of BGG at above 300 MPa for patient No. 5, and at above 100 MPa for patients No. 64 and No. 82, and was profoundly inhibited by the high-pressure treatment of BGG at 400 MPa for all the patients. These results suggest that the allergenicity of BGG was lower after the high-pressure treatment. Nogami *et al.*²⁴ have reported that histamine release was significantly inhibited by a high-pressure treatment of BSA at above 300 MPa, and was markedly inhibited at above 500 MPa. Thus, allergic reduction by a high-pressure treatment above 400 MPa

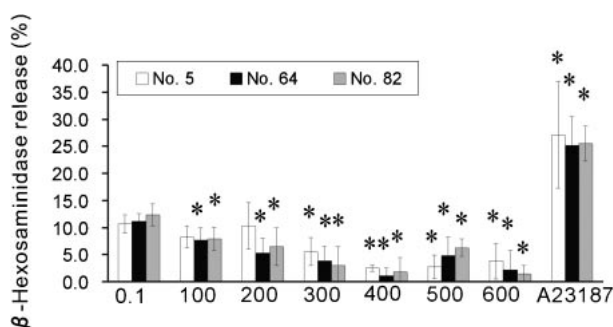


Fig. 2. Effect of a High-Pressure Treatment on the β -Hexosaminidase Release from KU812F Human Basophilic Cells Sensitized with Sera from Allergic Patients.

Each value is the mean $\pm$ standard deviation ($n = 4$). * $p < 0.05$.

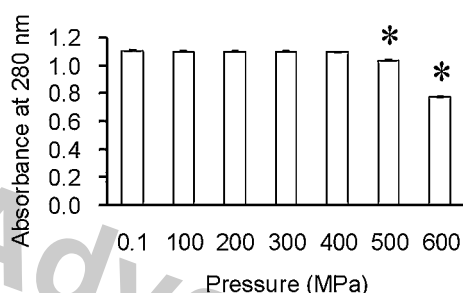


Fig. 3. Absorbance of the Supernatant from Pressure-Treated BGG. Each value is the mean $\pm$ standard deviation ($n = 6$). * $p < 0.05$.

may be effective in foods containing BSA and BGG. However, Han *et al.*¹³⁾ have reported that the β -hexosaminidase release was significantly inhibited by a high-pressure treatment of a beef extract at 600 MPa, while no significance was apparent at 400 MPa. These differences in β -hexosaminidase release may have been caused by the fact that they used beef extracts, and there may have been interactions between BGG and other proteins in the beef extracts.

Nonetheless, these data have confirmed that the high-pressure treatment could inhibit the release of mediators. Based on the present and previous results, a high-pressure treatment of foods has the possibility of reducing allergenicity.

Effects of pressure-induced coagulation on the allergenicity of BGG

It is well known that a high-pressure treatment causes the coagulation or gelation of proteins. The pressure-induced coagulation of allergen proteins may result in masking epitopes of the allergen proteins which may induce a decrease in the IgE-specific binding activity and reduction of the allergenicity. We therefore investigated in this study the relationship between the pressure-induced coagulation of BGG and the decrease of IgE-specific binding activity and reduction of the allergenicity of BGG.

The absorbance of the supernatant of pressure-treated BGG is shown in Fig. 3. The absorbance decreased significantly under pressures up to 500 MPa, suggesting that BGG had coagulated. The results of this study demonstrate that the decrease of IgE-specific binding activity and reduction of the allergenicity of BGG under less than 400 MPa were not correlated with coagulation. Furthermore, the IgE-specific binding activity and

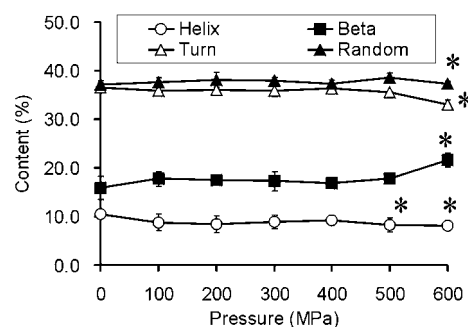


Fig. 4. Effect of a High-Pressure Treatment on the Secondary Structure of BGG.

Each value is the mean $\pm$ standard deviation ($n = 4$). * $p < 0.05$.

allergenicity of BGG under more than 500 MPa were not markedly different from the results under less than 400 MPa. These characteristics show that the decrease of IgE-specific binding activity and reduction of the allergenicity of BGG did not result from the pressure-induced coagulation of BGG.

Effect of the high-pressure treatment on the change in secondary structure of BGG

The change in secondary structure of BGG after pressurization is shown in Fig. 4. Except for the slight increase in β -structure after the treatment at 600 MPa, there was almost no significant change in secondary structure due to the high-pressure treatment. Scheibenzuber *et al.*²⁶⁾ have indicated that the high-pressure-induced decrease in the allergenicity of Mal d 1, which causes an allergic reaction to apples, might have been due to the decrease in α -helix and increase in β -sheet structures. Our results, however, do not suggest that the reduction of allergenicity in BGG was due to any change in the secondary structure of BGG.

Effect of the high-pressure treatment on the change in tertiary structure of BGG

Changes in the fluorescence spectra of BGG during and after the high-pressure treatment are shown in Fig. 5. A red shift was apparent, with a decrease in the intrinsic fluorescence intensity, thus suggesting a considerable increase in the polarity of the tyrosine and tryptophan environment accompanying the dissociation of subunits under high pressure.⁴⁰⁾ These results indicate that the environmental polarity of the tyrosine and tryptophan residues became stronger due to the pressure-induced change in the tertiary structure of the BGG molecule. As shown in Fig. 6, the center of the spectral mass ($\langle \nu \rangle$) values decreased gradually with increasing pressure. The $\langle \nu \rangle$ values during decompression were lower than those at the same pressures during compression, and the $\langle \nu \rangle$ values after release of the pressure did not return to the initial values. The $\langle \nu \rangle$ values therefore suggest that the high-pressure-induced structural changes in BGG were irreversible. In other words, hysteresis in the recovery of the fluorescence parameters was apparent during decompression.

Measurement of the surface protein hydrophobicity is one of the possible indices to detect a change in tertiary protein structure. Changes in the aromatic and aliphatic surface hydrophobicity of BGG measured by using ANSA and cPA are shown in Fig. 7. The surface

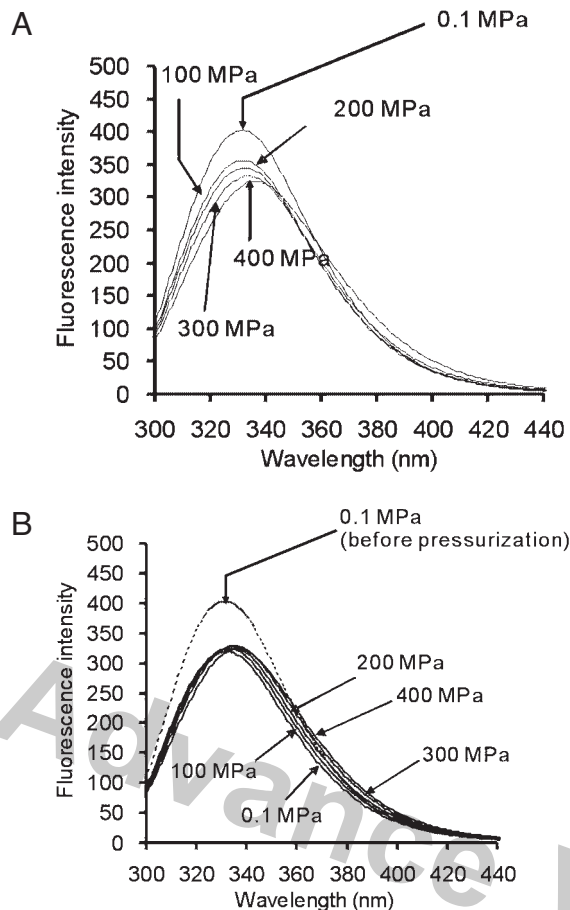


Fig. 5. Change in the Fluorescence Spectra of BGG under High Pressure.
A, compression; B, decompression.

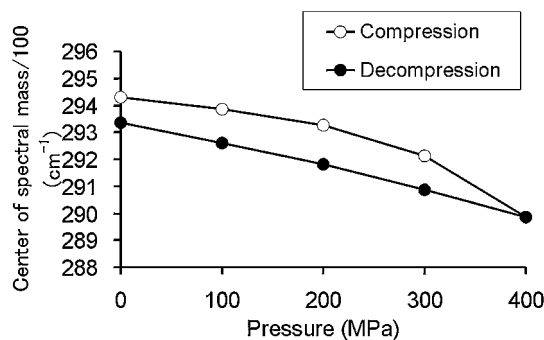


Fig. 6. Change in the Center of Spectral Mass of BGG under High Pressure.
Each value is the mean $\pm$ standard deviation ($n = 5$).

hydrophobicity of BGG increased significantly at pressures up to 500 MPa and then increased markedly when pressurized to 600 MPa. The results of this study demonstrate that high pressurization led to an irreversible change in the tertiary structure, as detected by measuring the surface hydrophobicity.

In the cases of other food allergen proteins, some researchers^{24,25} have reported that the tertiary structure change in allergen proteins affected the reduced allergenicity, and the present study agree with these results. Based on the results here, it is clear that pressure affected the structure of BGG, particularly the tertiary structure. Therefore, the lower IgE-specific binding activity and allergenicity of BGG after pressurization

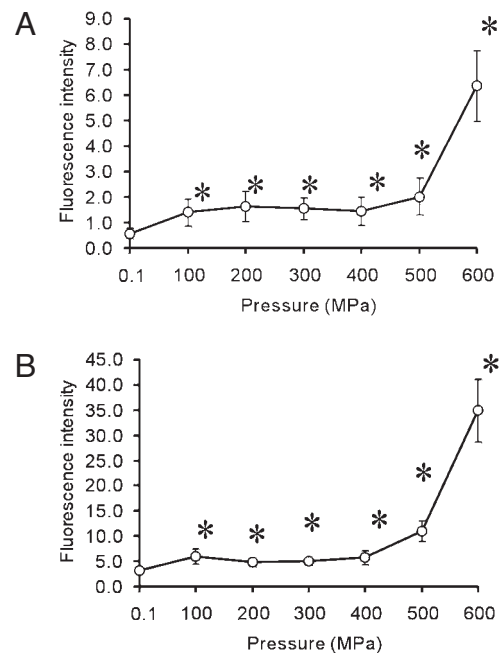


Fig. 7. Effect of a High-Pressure Treatment on the Aromatic (A) and Aliphatic (B) Surface Hydrophobicity of BGG.
Each value is the mean $\pm$ standard deviation ($n = 4$). * $p < 0.05$.

were probably due to a change in the tertiary structure. Furthermore, no epitopes of BGG have ever been demonstrated. Our studies suggest that the pressure-induced modification of the tertiary structure of BGG was confirmed in the parts which may include tyrosine and tryptophan residues. These results suggest that the epitopes of BGG may contain tyrosine and tryptophan residues.

The IgE-specific binding activity of BGG decreased in response to the high-pressure treatment. The BGG-induced β -hexosaminidase release from KU812F human basophilic cells sensitized with sera from allergic patients was also lower after the high-pressure treatment of BGG. Almost no significant change in the secondary structure was apparent in BGG due to the high-pressure treatment, but a change in the tertiary structure was seen. The decreases in IgE-specific binding activity and allergenicity of BGG were therefore probably due to this change in the tertiary structure. A high-pressure treatment may therefore be effective as a food processing technique in order to reduce the allergenicity of BGG without its elimination.

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