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Relationship between breaking load and protein composition of acidic heat-induced gels prepared from the acidic precipitate of soy flour aqueous dispersions

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ABSTRACT

As one of the constituent materials of plant-based meat, a heat-induced gel was prepared from the aqueous paste (protein-to-water ratio: 0.30–0.35) of an acidic precipitate from soy flour called AP-SF. Among the broader pH range of 4.5–7.5, heat-induced gels prepared from the AP-SF paste adjusted to a pH range of 5.0–5.5 exhibited the similar breaking load (617 gf) and displacement (5.22 mm), to that of heat-induced gels prepared from minced beef, breaking load (667 gf) and displacement (6.45 mm). The results of two experiments, that is, the addition of fractionated protein components (7S, 11S, polar lipid-associated protein (PLAP), and oil body-associated protein (OBAP)) to the original AP-SF gels and the examination of a correlation between the breaking load of AP-SF gels and the protein composition of soybean cultivars used for the AP-SF preparation suggested that 7S globulin has an ability to effectively increased the breaking load of AP-SF gels. In globulins that contribute to gelation, the correlation between the content of 7S and 11S and the breaking load is 0.75 and 0.50, respectively, and the correlation coefficient of 7S is much higher than that of 11S. Analysis using dithiothreitol (DTT)-free SDS-PAGE confirmed that homodimers of the α′ and α subunits of 7S globulin play a dominant role in the heat-induced gelation via the formation of intermolecular disulfide bonds with themselves or other protein components.

1. Introduction

In the preparation of plant-based meat (PBM), textured vegetable protein (TVP) produced from defatted soybeans using an extruder is commonly used as the major ingredient (Zhang et al., 2021). However, it is widely accepted that TVP produced by an extruder has an odor problem (Yang et al., 2023). Because the texturing at high temperature ≥130 °C in the extrusion process (Noguchi, 2017; Nakano, 2021) caused an undesirable flavor, washing step is crucial for the application of plant-based meat to commercial food products. If TVP can be produced at a lower temperature (≤ 130 °C), the undesirable odors can be suppressed. Therefore, preparation methods that do not require an extruder and adopt the low-temperature heating condition should be developed to diversify the quality of plant-based meat.

In addition to the milder preparation methods, the use of soybean flour instead of defatted soybeans as a raw material offers several expected advantages. It eliminates the need for organic solvents used in the defatting process and simplifies the production process. However, such raw material contains oil, which relatively lowers the solid protein content contributing to the texturization of TVP as compared with defatted soybeans. Therefore, it is necessary to select soybean cultivars with a high protein content as starting materials to realize the high

Abbreviations: APP, acid-precipitated proteins; LP, lipophilic proteins; OBAP, oil body-associated protein; PLAP, polar lipid-associated protein; AP-SF, acid-precipitate of soybean flour; APEP, acid-precipitated extracted proteins; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DDT, dithiothreitol; TVP, textured vegetable protein.

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quality PBM.

In this context, we attempted to prepare a heat-induced gel using soybean flour instead of defatted soybean flour as the raw material (Sugiyama et al., 2022). The traditional soybean food "tofu" is prepared from soybean by the water extraction and heating of extracted soymilk to induce the gelation, giving us hints for the new methods. However, to prepare the gel suitable to the ingredient for PBM, the relative protein content in the water should be significantly higher than that in tofu, or at least as much as in meat. Since the main protein in soybeans is insolubilized and aggregated at a pH near the isoelectric point it can be collected as a precipitate. Globulin (7S + 11S) and lipophilic protein (LP), account for approximately 90% of the nitrogen compounds contained in soybeans, are present in the precipitate (Samoto et al., 2007; Sugiyama et al., 2022). Based on this finding, a paste was prepared from the acidic precipitate of an aqueous dispersion of soybean flour and called AP-SF (Sugiyama et al., 2024). AP-SF was redispersed in water and adjusted pH, and the heat-induced gelation of the resultant paste was tested at several conditions. The obtained gel was referred to as AP-SF gel and shown to have textural properties suitable for the ingredient for PBM. In addition to proteins, AP-SF contains dietary fiber and fat. On the other hand, the typical TVP and defatted soybeans contain dietary fiber but not lipids. As described above, since oil reduces the solid concentration of the protein, TVP has an advantage in protein content contributing the texturization over AP-SF. However, in the preparation process of AP-SF, soy whey components, i.e., sugars, minerals, amino acids, peptides, and some proteins that are considered to have no advantage in gelation are removed as supernatants when the AP-SF is collected by centrifugation. Therefore, it is considered that our new method using AP-SF satisfies the criteria for protein concentration and quality necessary for heat-induced gelation.

The proteins in AP-SF are insolubilized and aggregated at pH conditions near isoelectric point but can coagulate further with heating to form gels. Therefore, the pH of the AP-SF paste should not be limited to the pH range in which soy proteins show high solubility. Although it has been previously reported that AP-SF gels can be prepared in the neutral pH range (Sugiyama et al., 2024), the relationship between the pH and salt concentration of the AP-SF paste, as well as the quality of AP-SF gels, requires further investigation. One of the key factors in heating-induced gels is the amount of protein and water contained in the AP-SF paste. This is because fats, oils, and fibers present in the AP-SF paste do not significantly contribute to the gelation process unlike proteins. In this context, the protein-to-water content ratio in the AP-SF paste was calculated as the P/W value. The P/W value in this study was approximately 0.30–0.35, which is the same as that of meat. This value for tofu was approximately 0.08 (Food Composition Database). As the P/W value of AP-SF is significantly higher than that of tofu, the optimal heating conditions and gelation mechanisms may differ from those of tofu. In this study, we prepared AP-SF gels under various heating conditions and compare their breaking loads. In order to know whether textural properties of AP-SF gels satisfy the criterion for the ingredient for PBM, a heat-induced gel was prepared from minced beef (Round meat mainly) and the stress–strain curve of the gel was then compared with that of the AP-SF gel.

In addition to the protein content, the composition of soy major protein which differs according to soybean cultivar, should affect gelling ability (Tezuka et al., 2000; James and Yang, 2016). To know how greatly each component of soy protein contributes to the gelling ability of AP-SF is crucial for the future selection of soybean cultivars suitable for the production of the ingredient for PBM. Therefore, we investigated the effects of not only 7S and 11S globulins but also other protein components, such as LP, and its constituents (OBAP and PLAP) (Sugiyama et al., 2022), on the breaking load of AP-SF gels mainly in the acidic pH range, by performing the two experiments, that is, the addition of fractionated protein components to the original AP-SF gel and the examination of a correlation between the breaking load of AP-SF gels and the protein composition of soybean cultivars used for AP-SF preparation as raw materials. In addition, the formation of disulfide bonds between protein molecules upon heating was investigated as a mechanism for increasing the breaking load of the AP-SF gels.

2. Materials and methods

2.1. Materials

Of the 13 soybean cultivars studied, eight were Japanese, and five were from outside Japan. The Japanese cultivars, all harvested in 2018, included Enrei, Fukuyutaka, Ootsuru, Tachinagaha, Satonohohoemi, and Hayahikari purchased from Kitao Kichisaburo Co., Ltd. (Kyoto, Japan); Yukihomare purchased from Yamato Shouten LPC. (Tochigi, Japan); and Ryuhou purchased from Hoshiroku LLC. (Niigata, Japan). Fukuyutaka and Enrei are popular for tofu processing. The five foreign soybean cultivars were procured from Fuji Oil Co., Ltd. (Osaka, Japan), and for one of these cultivars, samples from two different harvest years were included in the study. All chemicals and reagents were of analytical grade and were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan), and Junsei Chemical Co., Ltd. (Tokyo, Japan).

2.2. Preparation of soybean flour and measurement of solid content

The hull and hypocotyl of the soybeans were manually removed using tweezers. The dehulled soybeans were crushed using a micro-grinder (West LLC., Nagaoka, Japan). The diameters of flour particles were in the range of 90–350 μm (median: 120 μm). The solid content of the soybean flour was calculated from the weight change after drying at 105 °C for 5 h.

2.3. Preparation of heat-induced AP-SF gel

Fig. 1 shows a schematic of the preparation of the heat-induced AP-SF gel. Soybean flour (5.05 g) and deionized water (52.8 g) were added to a 50 mL conical tube, dispersed with a spatula, and adjusted to pH 7.2–7.5 using 1 M NaOH. After preheating the dispersion for 30 min at different temperatures (95, 75, 65, 55, and 25 °C), it was cooled to room temperature (20–25 °C) using water. Next, the dispersion was adjusted to a pH range of 4.5–4.7 using 1 M HCl to induce isoelectric precipitation of the main proteins. The mixture was centrifuged at 3000 *g* for 5 min, and the supernatant (soy whey) was removed. The precipitate was dehydrated with absorbent paper until it weighted less than 8 g. The dehydrated precipitate was recollected in a 50 mL conical tube, and each suitable amount of 1M NaOH was added to adjust the pH of the AP-SF paste to the following several pH ranges; I (4.5–5.0), II (5.0–5.5), III (5.5–6.0), IV (6.0–6.5), and V (7.0–7.5). To adjust the salt concentration, salt was added to the AP-SF paste, followed by the addition of deionized water were until the final weight was 10 g (protein-to-water ratio: 0.30–0.35). Subsequently, the paste was kneaded uniformly using a spatula. After covering and allowing it to stand at room temperature for 1 h, the sample was kneaded uniformly using a spatula. The obtained paste was centrifuged in a conical tube at 1400 *g* for 2 min for defoaming. If water separation occurred owing to the acidity of the paste, it was kneaded again and defoamed using centrifugation. To minimize the expansion of the air in the tube owing to heating, a wooden filling rod (Φ25 mm × 87 mm) was placed in the conical tube after defoaming. The tube lid was then lightly pressed down onto the surface of the paste. The conical tube was heated in an oil bath (BO400; Yamato Scientific Co., Ltd., Tokyo, Japan) at temperatures of 105, 110, 115, 120, 125 or 130 °C for gelation. These are the temperature ranges in which a high breaking load was obtained in the previous report (Sugiyama et al., 2024). The gel was cooled in water to room temperature and finally kept in a refrigerator for more than 30 min at 4–8 °C.

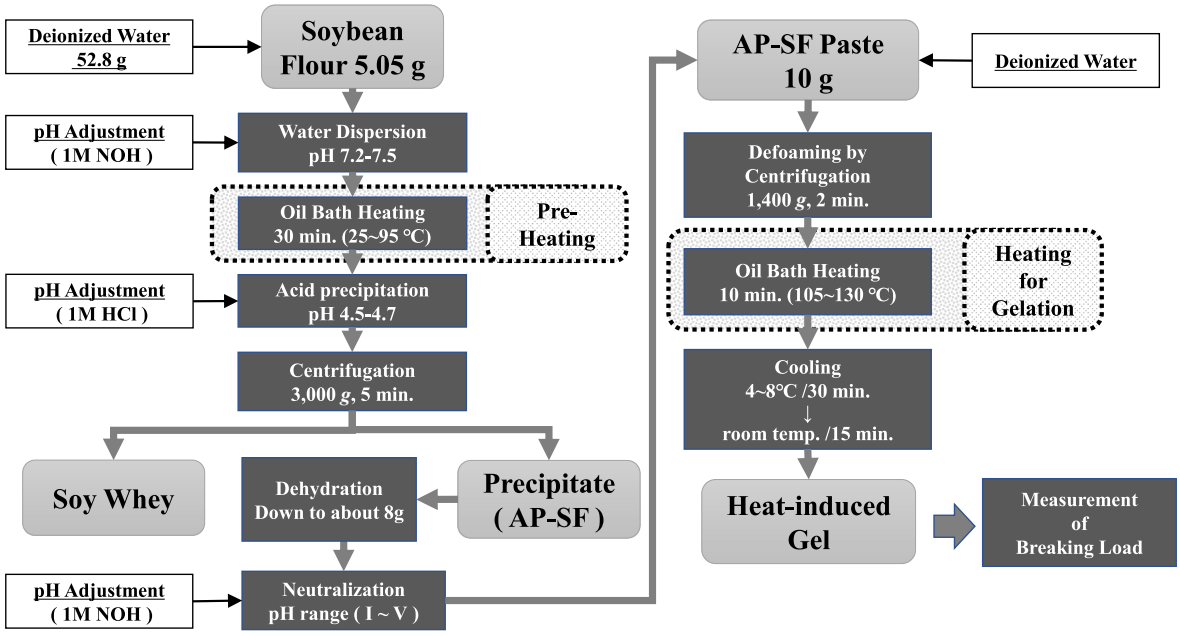

Fig. 1. Flow diagram for the preparation of heat-induced gels (AP-SF) and breaking load measurements.

2.4. Preparation of heat-induced gel for beef mince

Mince purchased from a grocery store was used to prepare the heat-induced gel for beef mince (Round meat mainly). The pH and P/W of the minced beef were 5.9 and 0.35, respectively. Ten grams of minced beef, matching the weight of the AP-SF gel, was placed in a 50 mL conical tube and uniformly kneaded with the addition of 1% NaCl by weight of the mince. The mixture was then defoamed by centrifugation (1400 g, 2 min). A wooden filling rod (φ25 mm × 87 mm) was inserted into the conical tube, and the tube was then sealed with its lid. Because the appropriate heating temperature range for meat is different from that of AP-SF, the mixture was heated in an oil bath at 80 °C for 30min. Subsequently, the mixture was cooled with water to 20 °C and further cooled in a refrigerator at 4–8 °C for 30 min.

2.5. Measuring the breaking load of the heat-induced gel

The refrigerated gel in the conical tube was allowed to stand at room temperature for 15–30 min and was then removed from the tube. The cylindrical gel was cut to a thickness of approximately 15 mm, and its breaking load was measured using a creep meter (RHEONER II RE2-3305C, YAMADEN Co., Ltd., Tokyo, Japan). The measurement conditions were as follows: storage pitch, 0.03 s; measurement distortion factor, 80%; and measurement speed, 1 mm/s. The reported breaking load represents the average value from three measurements.

2.6. Measurements of pH and moisture in the heat-induced gels of AP-SF or minced beef

An HI981038 bread and dough pH tester (Hanna Instruments Japan Co., Ltd., Chiba, Japan) was used to measure gel pH. To determine the water content, 3 g of the gel was placed in an aluminum cup and heated in a dry oven (DV 400, Yamato Scientific Co., Ltd., Tokyo Japan) at 105 °C for 5 h using the dry weight method.

2.7. Measurement of protein concentration in the heat-induced gel of AP-SF or minced beef

To determine the protein concentration in the gels, 1 g of gel was added to 9 g of NaOH (0.1 M) and dispersed for 5 min using an ultrasonic homogenizer (SFX150HH, BRANSON, Emerson Japan Ltd., Tokyo, Japan) to solubilize soy proteins with strong alkali condition. Furthermore, 1.5 mL of the dispersion was placed in a microtube and centrifuged (Allegra X-30R, Beckman Coulter, Inc., CA USA) at 28,670 *g* for 1 min. To avoid fiber in the lower layer and oil content in the upper layer, 20 μL of the middle layer was collected into a microtube, diluted 10-fold with deionized water, and stirred with a touch mixer. Subsequently, 20 μL of the solution was diluted 10-fold with deionized water and used as a sample solution for protein measurements. Protein quantification was performed using the Lowry method using a Modified Lowry assay kit (Thermo Fisher Scientific K. K., Tokyo Japan). One milliliter of a modified Lowry reagent was added to a microtube containing 200 μL of the sample solution for protein measurements. The mixture was then stirred with a touch mixer and allowed to stand at 20 °C for 10 min. Absorbance at was measured at 750 nm using a microplate absorbance reader (iMark, Bio-Rad Laboratories, Inc., CA, USA). A standard curve for AP-SF was prepared using commercially available soy protein isolate (SPI) (Fuji Oil Co., Ltd., Osaka, Japan) with known protein content. A standard curve for the minced beef was prepared using BSA. The protein-to-water ratio in the gel was expressed as P/W.

2.8. Preparation of OBAP, PLAP, 7S, 11S, and acid-precipitated extracted protein (APEP)

Protein fractionation and quantification were performed by preparing four types of acidic precipitated proteins (OBAP, PLAP, 7S, and 11S) as well as acid-precipitated extracted proteins (APEP) for the control group, following a previously reported method (Sugiyama et al., 2024). The fractionation status of each protein fraction is shown in the SDS-PAGE of the previous report (Sugiyama et al., 2024). Separated protein fraction were used for the addition experiments in the next section.

2.9. Adding different protein fractions to AP-SF

The protein fraction powder (OBAP, PLAP, 7S, 11S, or APEP) was dispersed in deionized water to obtain a protein concentration of 1%, and the protein dispersion was treated for 1 min using an ultrasonic homogenizer (SFX150HH, BRANSON, WAKENYAKU Co., Ltd., Tokyo, Japan). AP-SF was prepared using 5.05 g of soybean flour, 30.1 g of

deionized water, and 22.7 g of this solution (instead of 52.8 g deionized water, as described in method Section 2.3). To study the possible synergy between the two protein fractions, a 1% protein solution of each fraction (11.35 g) was used. Replacing water with these protein solutions introduced an addition 10% protein into the soybean flour. These dispersions containing 10% top-up protein content were used to prepare the heat-induced AP-SF gel samples. The water content of the AP-SF paste was further adjusted to consider the solid and protein contents, ensuring that the P/W ratio in the gel matched that of the control gel containing APEP. The breaking load of each gel sample was measured according to the method described in section 2.4. Furthermore, when the protein concentration in the AP-SF gel was increased by 20%, a 2% fractional protein solution was prepared and added in the same way as the 1% solution.

2.10. Correlation between the gel breaking load and the content of four protein fractions in each soybean cultivar

We recently reported the contents of OBAP, PLAP, 7S, and 11S in 15 soybean samples from 13 cultivars (Sugiyama et al., 2023). In this study, we used the same soybean samples to prepare heat-induced AP-SF gels in the 5.0–5.5 pH range and measured their breaking loads. The soybean flour dispersion was preheated at 55 °C and further heated at 120 °C for gelation. We calculated the correlation coefficient between the gel-breaking load and the content of each protein fraction, as well as each combined fraction, including LP (OBAP + PLAP), globulins (11S + 7S), and acid-precipitated proteins (LP + globulins).

2.11. Detection of intermolecular disulfide bonds using DTT-free SDS-PAGE

SDS-PAGE was performed using the Laemmli buffer system (Laemmli, 1970) with a 12.5% gel and an electrophoresis kit (Atto Corporation, Tokyo, Japan). Various AP-SF gels (1 g) heated at different temperatures for gelation were dispersed in 9 g of 1% SDS solution. Additionally, the mixture was sonicated for 5 min using an ultrasonic homogenizer (SFX150HH). The obtained dispersion was neutralized by adding 100 μL of 1 M NaOH. Subsequently, the dispersion was diluted 5-fold using deionized water to obtain a 0.4% protein solution. The solution was further diluted 4-fold using the reducing agent DTT-free SDS-PAGE sample buffer and allowed to stand at room temperature overnight. To each well, 10 μL of the solution was applied.

To increase the distance between each band of high-molecular-weight soy proteins, the electrophoresis time was set to 85 min instead of the usual 25 min at a constant voltage.

2.12. Quantification of various subunits of globulins using densitometry

Electrophoresis patterns of the CBB (Coomassie brilliant blue) -stained proteins were displayed on a scanner (GT-X980; EPSON Corporation, Nagano, Japan). After converting the image of the electrophoresis gel to 16 bits using CSAnalyser4 software (manufactured by ATTO Co., Ltd. Tokyo, Japan), the lane width was unified, and the separated soybean protein components were quantitatively analyzed.

When various bands of proteins contained in the AP-SF gels were expanded using the reducing agent (DTT)-free SDS-PAGE, CBB staining of the bands was reduced due to polymerization caused by the formation of disulfide bonds between protein molecules upon heating. In other words, the subunits not involved in polymerization upon heating should not show a decrease in CBB staining. The integrated value calculated from the absorbance of each band was determined using densitometry. The change in the integral value of the absorbance of each subunit upon heating in the AP-SF paste was expressed as the relative ratio (%) to that of the unheated sample.

DTT-free SDS-PAGE was used to separate the proteins contained in the 13 soybean cultivars with molecular masses. The CBB staining ratios of monomers, dimers of α′ and α subunits, and β subunits were calculated, and the content of each subunit in soybean was determined by multiplying these ratios by the 7S globulin content.

2.13. Statistical analysis

The error bars indicate the standard deviation. The correlation between the protein content in each fraction and the breaking load values of AP-SF gels was analyzed using SSPS ver 2.8 (IBM, Armonk, NY, USA), and p-values were calculated to assess statistical significance. The one-way ANOVA and Tukey method were used for detecting significant differences between groups.

3. Results and discussion

3.1. Effects of salt concentration at acidic pH on the breaking load of AP-SF gels

It is generally accepted that pH and ionic strength (salt concentration) affect the gelation behavior of proteins (Doi and Kitabatake, 1989). At pH regions far from isoelectric point, protein molecules regularly associate to form thin strands resulting in the formation of firm gel with well-developed network structure. On the other hand, at pH regions near isoelectric point, precipitates or soft gels consisting pf randomly aggregated particulates are normally produced. Ionic strength affects such gelation behavior of protein molecules by changing the attractive and repulsive electric forces. The gel prepared in this study exhibited a P/W of 0.30–0.35, and the protein content relative to the weight of the gel was approximately 20%. Even in a state of aggregation owing to the acidic pH near the isoelectric point, the heat-induced AP-SF gel may become a suitable gel material for plant-based meats owing to the high protein concentration of the AP-SF paste. In addition, as reported for the fractionation method of protein composition of major globulins (Sugiyama et al., 2022), solubility increases with increasing ionic strength, even near the isoelectric point. Therefore, the effects of pH including region near the isoelectric point and ionic strength on AP-SF gels should be investigated.

The effects of the pH and salt concentration on the breaking load of the AP-SF gel are shown in Fig. 2. The AP-SF gels were prepared at three acidic pH ranges: I (4.5–5.0), II (5.0–5.5), and III (5.5–6.0). The salt concentration in the AP-SF gel was adjusted by adding NaCl. Following the method described in a previous report (Sugiyama et al., 2024),

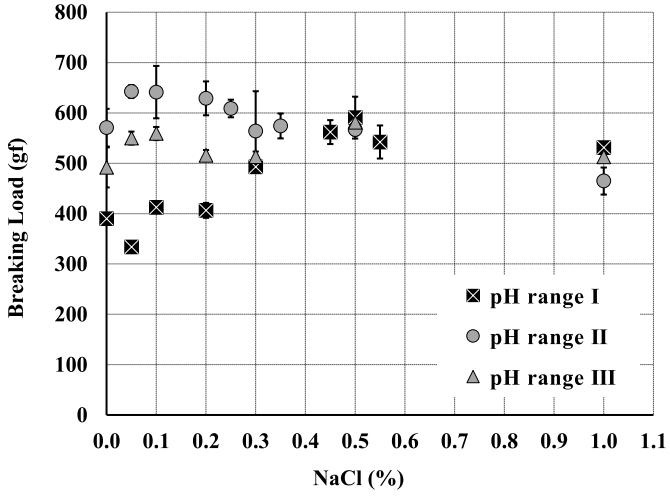

Fig. 2. Effect of salt concentration on the breaking load of AP-SF gels at three acid pH ranges.
AP-SF gels were prepared at three pH ranges: I, 4.5–5.0; II, 5.0–5.5; and III, 5.5–6.0. NaCl was added to the AP-SF paste at different concentration.

soybean flour water dispersion was preheated at 55 °C for 30 min, and the AP-SF paste was heated at 115 °C for 10 min for gelation. In the AP-SF paste with a salt concentration of less than 0.4% (w/w), the pH values affected the breaking load of the AP-SF gel. That is, the breaking load increased in the following order of pH ranges: I, III, and II. When the salt concentration was approximately 0.1–0.3%, the delicate electrostatic balance between protein molecules seems to be changed by an increase of salt concentration thereby influencing the breaking load.

However, when the salt concentration exceeded 0.4%, a breaking load value of approximately 550 gf was observed constantly, regardless the AP-SF gel pH. This may be attributed to the enhancement of the solubility of the major globulin with an increase in ionic strength. Based on these results, the subsequent experiments were conducted under the conditions that maximized the breaking load of AP-SF gel, this is, the pH range II (pH 5.0–5.5) and 0.1%, respectively.

3.2. Effect of heating conditions on breaking load

The effect of heating conditions on the breaking load of AP-SF gels, with pH and NaCl concentration adjusted to 5.0–5.5 and 0.1%, respectively, was investigated. Similar to tofu, two-step heating (Nakamura et al., 1984; Liu et al., 2004) was adopted as the heating method. AP-SF gels prepared by preheating at different temperatures (25, 55, 75, and 95 °C) and subsequent heating at different gelation temperatures (105, 110, 115, 120, 125, and 130 °C). The breaking load of obtained AP-SF gels were shown in Fig. 3.

When the AP-SF gel was preheated at 25 °C or 55 °C, its breaking load increased significantly with the increase in the subsequent heating temperature for gelation, exhibiting a maximum value of 638.3–690.0 gf at 120–130 °C. On the other hand, when the preheating temperature was 75 °C or 95 °C, no significant changes in the breaking load were observed despite increasing the heating temperature for gelation. Given the fact that the denaturation temperatures of the 11S and 7S globulins are approximately 90 °C and 70 °C (Nagano et al., 1992; Zhang et al., 2004; Sirison et al., 2017), respectively, the results suggest that both globulins should remain un-denatured state during preheating to maximize the breaking load. We have already reported (Sugiyama et al., 2024) that when the AP-SF gels are prepared at the neutral pH range of 7.0–7.5, no significant changes in the breaking load were observed at a preheating temperature of 95 °C; however, the breaking load increased at 75 °C. Comparison of our new and previous results indicates that the effect of thermal denaturation of 7S globulin on the breaking load by preheating did not appear in the neutral range but appeared in the acidic range. The reason for these different results is unclear, but it is presumed that when 7S globulin is denatured by heating, subtle changes in solubility are more likely to occur in acidic pH ranges compared to neutral ranges.

The breaking load of the acidic AP-SF gel tends to be slightly higher when preheated at 55 °C than at 25 °C. This difference might be attributed to the thermal denaturation of non-globulin proteins or variations in protein extraction from soybean flour (notice that the preheating was carried out for “water dispersion” in Fig. 1), although the exact cause remains unclear. Based on these results, the preheating and gelation temperatures in the subsequent experiments for preparing acidic AP-SF gels were set to 55 °C and 120 °C, respectively.

3.3. Comparison of the stress–strain curves between the heat-induced gels prepared from minced meat and the AP-SF gels

The stress–strain curve indicating the breaking load and deformation value at the break point was measured for the heat-induced gel of minced beef and AP-SF gels. AP-SF gels were prepared under conditions of 0.1% (w/w) NaCl and at different pH ranges: I (pH 4.5–5.0), II (pH 5.0–5.5), IV (pH 6.0–6.5) and V (pH 7.0–7.5). The results are shown in Fig. 4.

The pH conditions of the AP-SF gel exhibiting the strain value similar to that of the minced beef gels were within pH ranges I and II. In contrast, the strain values of the AP-SF gels prepared at the pH ranges IV and V were higher than that of the minced beef gel. The breaking load of the AP-SF gel prepared at pH range I was the lowest, whereas those of the other gels were similar. In spite of the difference in the break point, the stress–strain curve of the AP-SF gel in pH range II was similar to that of the minced beef gel. These results suggest that even in the pH range, where proteins are nearly insoluble and aggregated, AP-SF paste with a high P/W value may contribute to a new texture as a constituent material for plant-based meats.

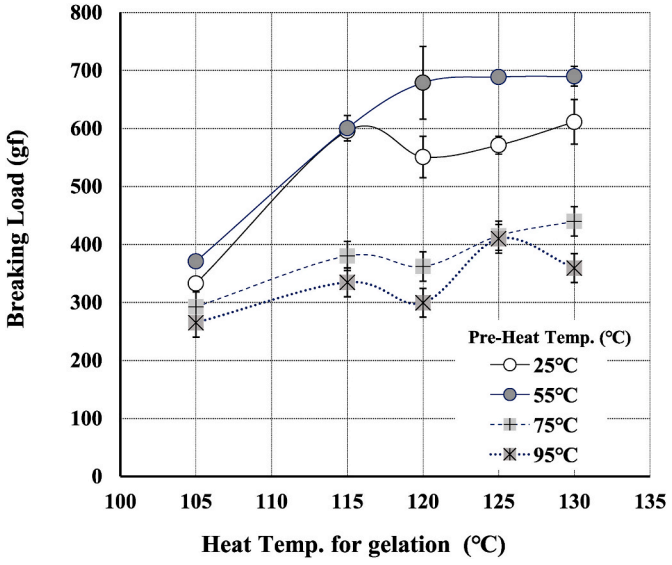

Fig. 3. Effect of temperatures used in two-step heating (preheating and gelation) on the breaking load of the heat-induced AP-SF gels. The lines represent different pre-heating temperatures. AP-SF gels were prepared at pH range II (5.0–5.5), by adding 0.1% salt to 10 g of the AP-SF paste.

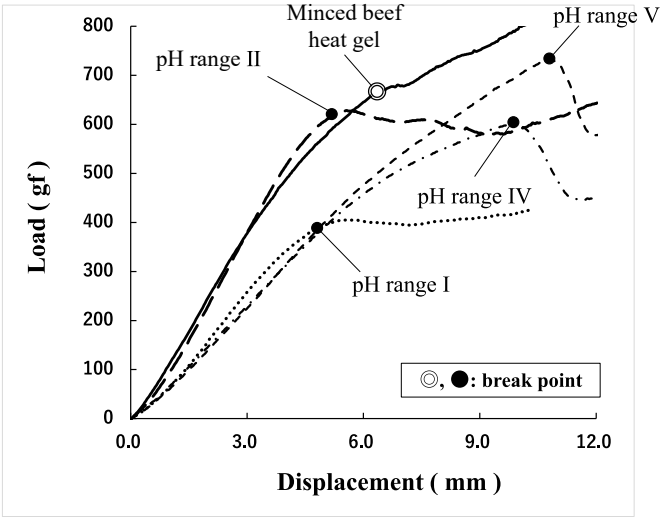

Fig. 4. Stress–strain curves of heat-induced gels prepared from minced beef and AP-SF. AP-SF gels were prepared at the following pH ranges: I, 4.5–5.0; II, 5.0–5.5; IV, 6.0–6.5; and V, 7.0–7.5. The symbol ● indicates the breaking point of AP-SF gels, and ◎ indicates the breaking point of minced beef gel.

3.4. Effect of the protein composition in soybeans on the breaking load of acidic AP-SF gels

To investigate the contribution of protein components to the textural properties of AP-SF gels, each component was added to AP-SF gels and changes in breaking load were measured. Acidic AP-SF gels in pH range II were selected as a base gel because this gel was found to mimic well the textural behavior of the heat-induced gels prepared from minced meat in the previous section.

Before the gelation, the protein fractions OBAP, PLAP, 7S, and 11S were added to increase the protein concentration by 10% to the base AP-SF paste. Furthermore, to test the synergy effects between the two components, the two protein fractions were respectively added to increase the protein content of AP-SF by 5% (total 10%). In addition, APEP, an acidic precipitated protein extracted from soybean flour, was used as a non-fractionated protein control. Acidic AP-SF gels with APEP were prepared to increase the protein concentration by 10%. The breaking loads of the acidic AP-SF gels with different protein fractions are shown in Fig. 5 as relative values, with the average breaking load set to 100.

The average breaking load of acidic AP-SF gels with added APEP was 772 gf, set as 100% for comparison. Relative to this, the breaking loads of gels with added OBAP, PLAP, 11S, 7S, OBAP +11S, OBAP + 7S, and 7S + 11S were 88.7%, 90.7%, 96.0%, 113.3%, 100.6%, 100.3%, and 109.9%, respectively. These results indicate that adding 7S globulin and the combination of 7S and 11S globulins significantly increased the breaking load of the acidic AP-SF gel. Statistical analysis revealed significant differences ($p < 0.05$) in breaking load values between gels containing 7S or 11S + 7S and gels containing OBAP or PLAP. A previous study (Sugiyama et al., 2024) reported that only the AP-SF gel with 7S + 11S in the neutral pH range of 7.0–7.5 exhibited a high breaking load, indicating a remarkable synergistic effect between 7S and 11S globulins. However, the result differs between the acidic and neutral pH ranges, and the effect of adding 7S alone becomes significant with an increase in the breaking load of the acidic AP-SF gel.

To confirm the contribution of 7S and 7S + 11S protein fractions to the increase of breaking load, these globulins were more added to increase the protein content of the AP-SF by 20%. To investigate the synergy between the two components, the two protein fractions were added to increase the protein content by 10%. In addition to the AP-SF gels formed at the pH range II, gel prepared at the pH range IV and V were also used in this experiment. The results are shown in Fig. 6. Adding 7S globulin significantly increased the breaking load of the AP-SF gels in the pH range of 5.0–5.5. This increase was notably greater compared to gels with 11S globulin or a combination of 7S and 11S globulins. On the other hand, although the breaking load of the AP-SF gel in the pH range 7.0–7.5 increased due to the addition of 7S and 11S, no significant difference was observed among 7S, 11S and 7S + 11S addition. The result is in disagreement with the result of Sugiyama et al. (2024), showing the synergistic effects of 7S and 11S as described above. Such difference may be the experimental condition employing the substantially higher P/W than that used in a previous report (Sugiyama et al., 2024).

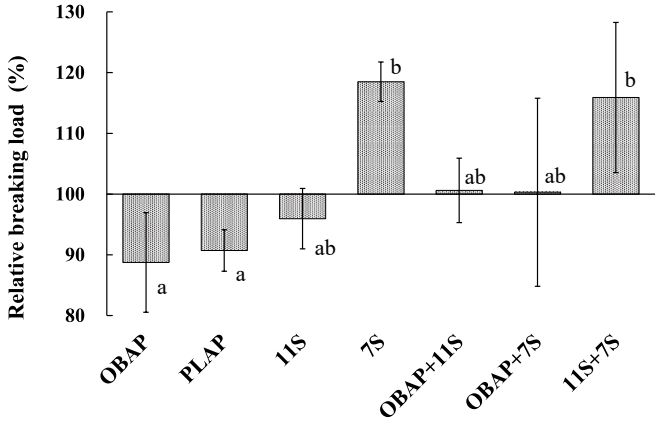

Fig. 5. Effect of the addition of one or two protein fractions (APEP as control, OBAP, PLAP, 11S, and 7S) on the breaking load of the AP-SF gels. The breaking load of the AP-SF gel, for which APEP was used as a control, was set to 100, and the relative value of each AP-SF gel was determined. The change in the breaking load due to the addition of various proteins was expressed as a relative value. Error bars represent experimental values (means ± SD, n = 3). a, b: Significant differences between groups ($p < 0.05$). Abbreviations: APEP, acid-precipitated extracted protein; AP-SF, acid precipitate of soybean flour; OBAP, oil body-associated protein; PLAP, polar lipid-associated protein.

3.5. Correlation between protein compositions in raw soybeans and the breaking load of AP-SF gels

As mentioned previously, the key protein affecting the breaking load of the acidic AP-SF gel is 7S globulin. To investigate if this finding is also supported by raw material selection, we evaluated the correlation between the various protein contents in 13 soybean cultivars and the breaking load of AP-SF gels prepared from these soybeans. The content of various protein compositions in those soybeans has already been reported (Sugiyama et al., 2024). Results are presented in Fig. 7. The breaking load of the acidic AP-SF gel strongly correlated with the APP content and the 7S globulin content, followed by the combined 7S and 11Sglobulin content. The strong positive correlation could not be observed between the breaking load and the 11S globulin content. In contrast, the LP (OBAP + PLAP), OBAP, and PLAP contents did not correlate with the breaking load of the acidic AP-SF gel. These results support the finding of the previous section that 7S globulin is a key protein that increases the breaking load of acidic AP-SF gels. Therefore, we can say that the globulin content, especially 7S globulin content, as well as the total protein content of soybeans, should be considered for selecting the soybean cultivar suitable to the production of the acidic AP-SF gel with a high breaking load beneficial for the plant-based meat.

The relationship between breaking load and protein composition in tofu has been explored with regard to the content of 7S and 11S globulins, as well as 7 S/11S ratio (Cai and Chang, 1999; Stanojevic et al., 2011). In this case, the protein composition and concentration of soy-milk may depend on the extraction conditions of raw soybeans (Poysa and Woodrow, 2002). These uncertain factors may interfere with the accurate determination of the relationship between the gel-forming

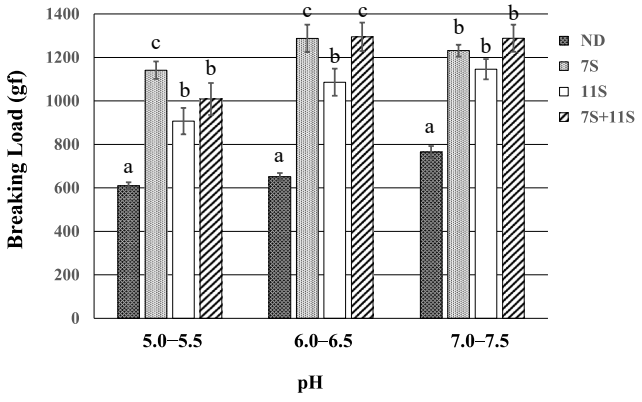

Fig. 6. Effect of the addition of one or two protein fractions (7S and 11S) on the breaking load of the AP-SF gels. The effect of globulin addition to AP-SF gels prepared in each pH region is shown. Error bars represent experimental values (means ± SD, n = 3). ND: no globulins added. a, b, and c: Significant differences between the same pH range group ($p < 0.05$).

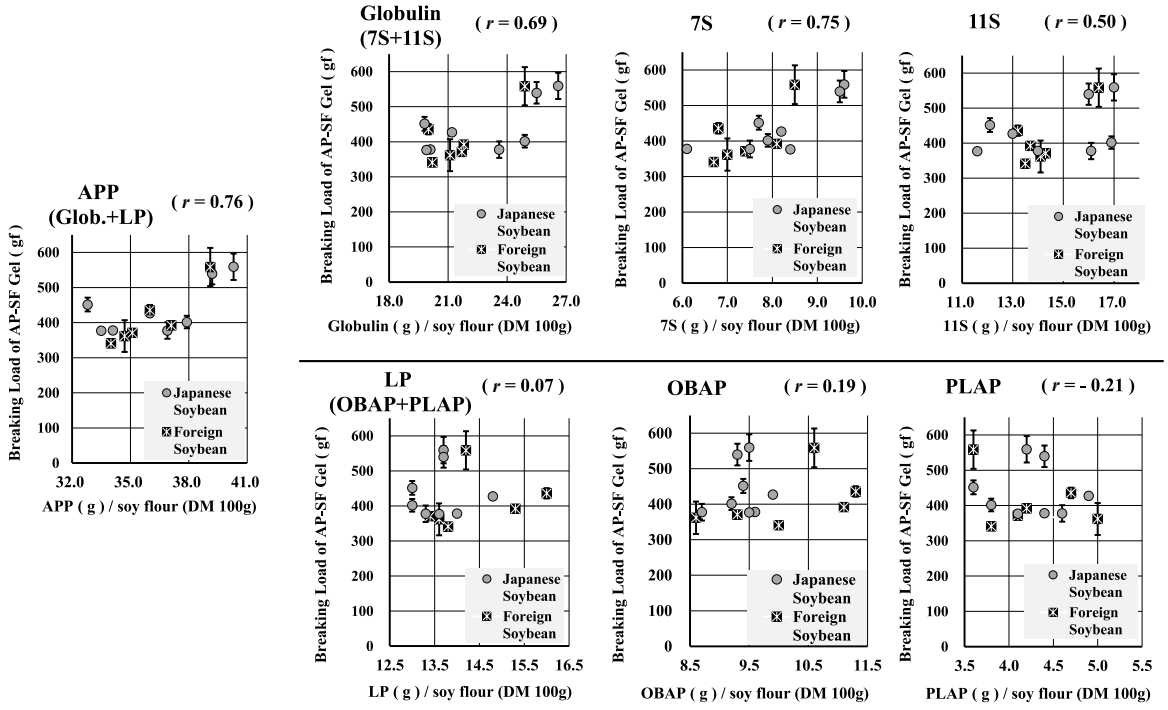

Fig. 7. Correlation between each fractionated protein content in soybean flour and the breaking load of the AP-SF gels. (r =): correlation coefficient of breaking load for each fractionated protein content in 14 soybean flour samples.

ability and protein composition in raw soybeans. However, in the AP-SF gels, approximately 90% soy protein, excluding whey protein, was contained in the AP-SF paste. The water content and P/W of the protein solutions can be adjusted to the desired level. Therefore, our new method using AP-SF may be more convenient for knowing the potential of soybean cultivar as raw materials for gel foods including PBM.

3.6. Intermolecular disulfide bonds of 7S globulins formed in the heating process of the acidic AP-SF paste

At acidic and neutral pH ranges, the breaking load of the AP-SF gel was significantly reduced by the addition of a reducing agent, such as sodium hyposulfite (data not shown). The formation of macromolecules by 7S globulins via disulfide bonds has rarely been reported. This is because the α′ and α subunits have only one SH group in the subunit. Disulfide bonds between 7S globulins and other proteins, such as 11S

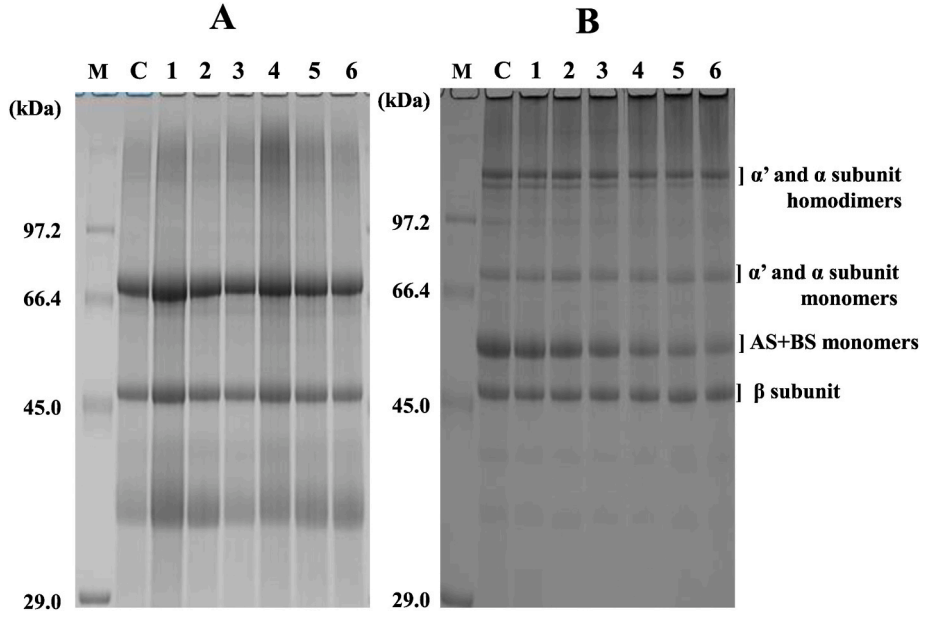

Fig. 8. Protein patterns in AP-SF paste heated at different temperatures, as analyzed by DTT-contained SDS-PAGE (left) and DTT-free SDS-PAGE (right). The AP-SF paste was heated for 10 min at each temperature and loaded with 10 ng of protein per well. Lanes: M: maker; C: not heated; 1: 105 °C; 2: 110 °C; 3: 115 °C; 4: 120 °C; 5: 125 °C; 6: 130 °C. AS: 11S globulin acidic subunit; BS: 11S globulin basic subunit.

globulins, may increase the breaking load of AP-SF. Therefore, the reducing agent, DTT-free SDS-PAGE, was carried out to separate the proteins in the acidic AP-SF gel without the disruption of disulfide bonds. Electrophoretic patterns, shown in Fig. 8 (B), revealed that the 7S globulin migrates as both monomers of the α′ and α subunits and as dimers linked by disulfide bonds (Samoto et al., 1996). On the other hand, shown in Fig. 8 (A), SDS-PAGE containing DTT, each band of all heated samples was completely restored, suggesting that polymerization via intermolecular S-S bonding is caused by heating.

When the protein within AP-SF became a polymer due to the formation of disulfide bonds during the heating process, it did not migrate to a predetermined band position. A decrease in the degree of CBB staining of each protein band was observed. The relative CBB staining values of the AP-SF protein during the heating process are shown in Fig. 9 (B). First, the polymerization of 11S globulin owing to the formation of new disulfide bonds during the heating process (Utusmi, 1989) was confirmed, and the amount of staining of the monomer band originated from the acidic and basic subunits was significantly reduced with increasing heating temperature. On the other hand, the amount of staining of 7S α′ and α subunits dimers decreased with increasing heating temperature. However, those of the monomers of the α′ and α subunits and β subunit did not decrease. The reason for such behavior of 7S dimers is unclear, but as shown in Fig. 10 depicting 7S state in AP-SF solution, Wadahama et al. (2012) suggested the possibility that the dimer takes on the three-dimensional structure of dimer, which enhances of dimers are difficult to be dispersed in water. In contrast, 7S subunit monomers, being well-dispersed in water, are less likely to interact with other proteins. Heating may cause the further interaction of these dimers with other dimers or 11S globulin subunits to form the polymer through disulfide bonds because of the compact packing in the aggregates of AP-SF solution, but 7S monomers outside the aggregates are possibly not involved in the interaction with other protein molecules.

In addition, we investigated the relationship between the content of each subunit (dimers, monomers and β subunit) of 7S globulin in raw soybeans and the breaking load of the acidic AP-SF gels to know the contribution of 7S subunits to gelation. The dimer content exhibited a significantly stronger correlation with the breaking load of the AP-SF gel than with the monomer content indicating the more involvement of dimer in the formation and strengthening of gel. The correlation coefficients for dimer, monomer, and β subunit content were 0.48, 0.20, and 0.42, respectively (data not shown). They are not statistically significant ($P > 0.05$), however there is a limited difference between the dimers and monomers of 7S globulin.

4. Conclusion

To prepare plant-based meat, we prepared an AP-SF gel from an acidic precipitate of a soybean flour aqueous dispersion, aiming to achieve a protein-to-water ratio (P/W) of 0.30–0.35, similar to that of meat. The detailed examinations of the AP-SF gels at the acidic pH range suggested that the optimal condition for increasing the breaking load of the gels are: a pH range of 5.0–5.5, NaCl salt concentration of 0.1%, and the gelation heating temperature of approximately 120 °C. Additionally, the preheating temperature should be sufficiently low to prevent denaturation of 11S and 7S globulins.

A comparison of the stress–strain curves showed that the heat-induced gel of minced beef more closely resembles the acidic AP-SF gel than the neutral AP-SF gel. To investigate the effect of the four protein compositions (7S, 11S, OBAP, and PLAP) on the breaking load of the acidic AP-SF gels, we added these protein fractions to AP-SF and found that 7S globulin was the most involved in the breaking load.

To confirm whether this result was supported in terms of soybean cultivar selection, we evaluated the correlation between the content of each protein in the 13 soybean cultivars and the breaking load of acidic AP-SF gels prepared from these soybeans. A strong correlation was observed between the breaking load of the acidic AP-SF gel and the protein compositions of APP, 7S globulin, and globulin (7S + 11S). Therefore, it was suggested that the 7S globulin and globulin contents, as well as the APP content are key factors in the selection of soybeans as the starting material.

In addition, the formation of AP-SF gels was strongly inhibited by the reducing agents. Therefore, the involvement of disulfide bonds can be considered a factor in the formation mechanism of the gel. We used reducing-agent-free SDS-PAGE to confirm the formation of intermolecular disulfide bonds during the heating process. Regarding the α′ and α subunits of the 7S globulin, it is suggested that the dimers rather than the monomers may be disulfide bonded to other proteins. Therefore, the abundance of these dimers may be important for increasing the breaking load through disulfide bonds between the molecules.

We demonstrated that AP-SF gel has a great potential as the raw material for PBM. However, in order to create the real make-link texture of AP-SF gel-based products, we should investigate interactions of AP-SF gel with other materials by applying the analysis of various textural

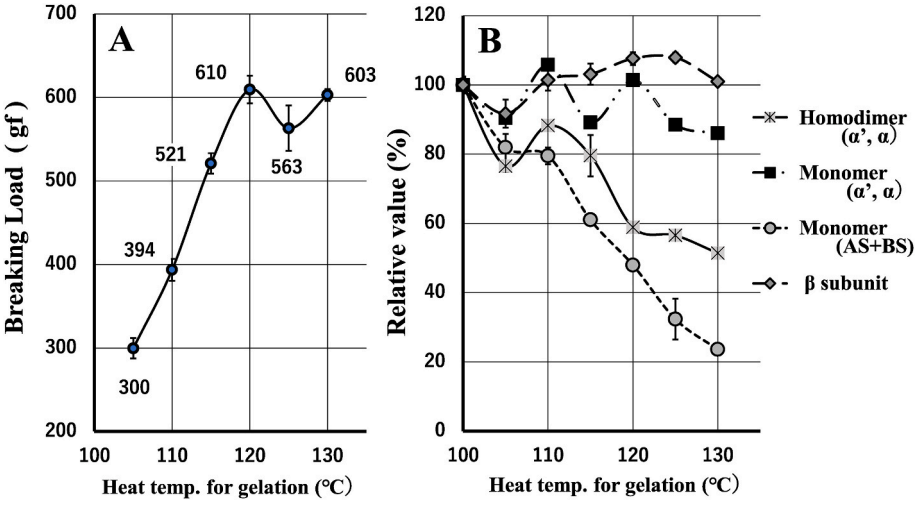

Fig. 9. Breaking load of the AP-SF gel (left) and relative CBB staining values of AP-SF proteins on DTT-free SDS-PAGE (right) when heated at each temperature. AP-SF was heated for 10 min at each temperature, and the breaking load was measured. Subsequently, the AP-SF gels were prepared in DTT-free SDS-PAGE sample buffer, loaded with 10 ng of protein per well, and subjected to electrophoresis. The relative CBB staining intensity of each globulin subunit was calculated based on the unheated AP-SF paste using densitometry, with the intensity set to 100%. AS: 11S globulin acidic subunit; BS: 11S globulin basic subunit.

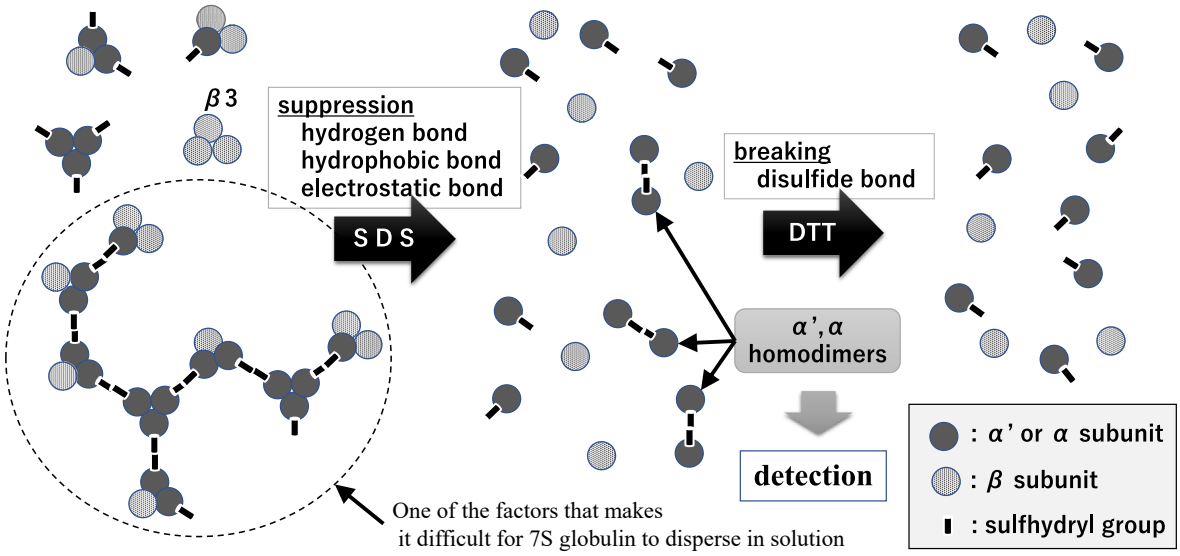

Fig. 10. State changes of 7S globulin owing to SDS and DDT reagents.

parameters other than breaking load.

CRediT authorship contribution statement

Masahiko Samoto: Conceptualization, Methodology, Writing – original draft, Visualization. **Ryo Okuzono:** Validation, Formal analysis, Investigation. **Mako Igarashi:** Validation, Formal analysis, Investigation. **Sayuri Sakurada:** Data curation. **Yasuki Matsumura:** Writing – review & editing, Visualization. **Akihiro Nakamura:** Data curation, Supervision, Project administration. **Masayuki Shibata:** Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Shibata masayuki reports was provided by fuji oil co. ltd. Shibata masayuki reports a relationship with fuji oil co., ltd that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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