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Property changes of frozen soybean curd during frozen storage in "Kori-tofu" manufacturing process

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ABSTRACT

It is generally accepted that the microstructure of frozen food changes even in freeze-storage due to the recrystallization of ice crystals. Kori-tofu is a traditional processed food in Japan, which is made from soybean curds availing refrigeration techniques to add an elastic texture. The elastic texture of soy bean curds which is Kori-tofu materials occurs during frozen-storage under subzero temperature, however, these mechanism have not clear. In this study, aiming to discuss the mechanism of the hard texture arising in soy bean curds during the freeze storage periods, characteristics changes in soy bean curds during frozen storage period such as texture, water holding capacity, ice crystal and frozen concentrated phase were investigated with time. In addition, how a character of soy protein changes was examined. The result indicated that the change in the balance of hydrophilic and hydrophobic region induced by dehydration with ice recrystallization at first stage of storage plays an important role in forming the new protein-protein interaction especially for amphipathic protein as α and α′subunit of β-conglycinin.

1. Introduction

Food refrigeration techniques are widely used as food preserving methods because they enable the extension of food shelf-life. However, physical and chemical changes always occur through food refrigeration processes; freezing process, storage process, and thawing process. It is generally accepted that the physical and the chemical changes in food refrigeration process were caused by the formation of ice crystals and by subsequent dehydration of the food matrix (Fennema, 1973).

It was well-known that most of the frozen-thawed food gels showed different microstructure, lower water holding capacity, and a different texture from unfrozen one (Fuchigami & Teramoto, 2003).

In Japan, Kori-tofu is one of the traditional processed foods manufactured from soybean curd availing refrigeration techniques. Kori-tofu has a characteristic hard texture, which is completely different from unfrozen tofu. In the manufacture of Kori-tofu, unfrozen soy bean curds, which is the Kori-tofu materials are frozen and subsequently settled in −2 °C static air for approximately 3 weeks. It is understood that empirically the 3 weeks storage is necessary to add the characteristic hard texture to soy bean curds materials of Kori-tofu. Besides, it is desired to shorten the period of the frozen-storage process for improving the production efficiency, while keeping the "Kori-tofu characters "intact.

Although a few studies are available to consider the mechanism of texture changing of soybean curds under frozen condition, it is yet to be understood in detail. It was reported that soybean curds became harder, springer, gummier and more cohesive after freeze-thawed depending on freezing time. (Xu, Tao, & Shivkumar, 2016). They estimated from the decreases of binding water in soy bean curds by freeze-thawing that the harder texture of freeze-thawed soy bean curds was led by the replacement of water-protein interaction to new protein-protein interaction.

Previous studies indicated that an intramolecular and/or an intermolecular disulfide bond formed by the oxidation of cysteine residues of soy protein mainly contributed to new protein-protein interaction during the freezing and frozen storage in defatted soy protein isolated (SPI) (Hashizume, Kakiuchi, Koyama, & Watanabe, 1971). Besides, other pervious study mentioned that not only covalent bonds like disulfide bond but also non-covalent bonds like hydrogen bond play an important role in new protein-protein interaction of SPI during the freezing and the frozen storage process. (Chen et al., 2016).

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These previous studies showed different conclusion and revealed only the degradation behavior of "soy proteins" during the frozen storage process and not of the "soy bean curds", since the previous studies used defatted SPI. A backbone of soy bean curds were mainly consisted oil globules as well as soy protein. When soy bean curd is aggregated from heated soy milk, the oil globules first adsorbs the particle soy protein in soy milk that is consisted basic subunit of glycinin (11s) and β subunit of β-conglycinin (7s), subsequently the aggregate of lipid surrounded by soy protein are linked to each other by absorbing α and α' subunit of β-conglycinin (7s) and acid subunit of glycinin (11s), to form the gel structure (Guo et al., 2002).

Therefore it is not clear how protein-protein interaction changes in soy bean curds during the frozen storage period and why these changes shows time dependency of storage period, even though it is also assumed that the changing of protein-protein interaction during the storage process caused by the regrowth of ice crystals and dehydration of soy bean curds matrix.

In this study, aiming to discuss the mechanism of the hard texture arising in soy bean curds during the freeze storage periods, characteristics changes in soy bean curds during frozen storage period such as texture, water holding capacity, ice crystal and frozen concentrated phase were investigated with time. In addition, to elucidate the changing of protein-protein interaction with storage period in the frozen concentrated phase, the relative rate of the disulfide bond, the hydrophobic or hydrophilic region at the surface of the frozen storage soy bean curds, and the protein composition in storage or no storage soy bean curds were investigated.

2. Materials and Methods

2.1. Materials

Soymilk and fresh tofu materials were prepared in the plant of Asahimatsu Foods Co., Ltd. as mentioned below. Soybeans were soaked in water for 8 h at 15 °C, and then, ground into a homogenate with fresh water using a blender (M-5A; NARA Machinery Co., Ltd., Tokyo, Japan) at 3300 rpm. The homogenate was heated to >98 °C for 3 min and subsequently separated into tofu refuse and soy milk using a press equipped with a screen mesh (Twin Meister; Yanagiya Machinery Co., Ltd., Yamaguchi, Japan). The soy milk was coagulated by adding calcium chloride solution at a concentration of 13 mM, and then, the curd was pressed to remove the whey.

Fresh soy bean curds were cut into 35 mm × 70 mm x 15 mm and frozen for 3 h by the −9 °C Air blast freezer (Fukusima Industry, Blast chiller/Shock freezer, QXF-006SF5). As shown in Fig. 1, one side of the soy bean curds were cooled by a blast of cold air, and another side were cooled by a cold metallic plate. After 180 min freezing, frozen fresh soy bean curds was settled at −2 °C for a maximum of 21 days for the freeze storage process.

A part of the freeze storage soy bean curds, for selected periods of storage time, were freeze-dried from −40 °C to +5 °C at approximately 5 Pa using freeze-dryer to sublimit the ice crystals.

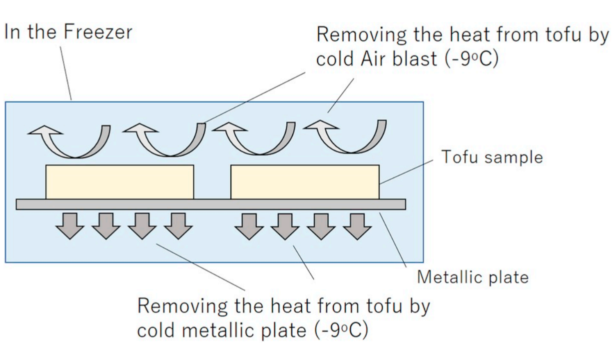

Fig. 1. The illustration of freezing system for Kori-tofu materials.

2.2. Texture analysis

The 0–14-day storage soy bean curd was thawed in iced water for 3 h breaking stress and breaking strain of it was measured by indentation test using the texture analyzer (Yamaden, Creep meter, RE2-33005B), with the columnar like plunger (ϕ3 mm) at 1.0 mm/s indentation speed. This test were repeated five times.

2.3. Water holding capacity

The 0–21 day storage soy bean curd was thawed in iced water for 3 h. Water holding capacity of it was measured as follows: the thawed soy bean curds was dehydrated in the Dehydrator (C14L SS, Somela) at 3000 rpm for 30 s. The remaining moisture content of the dehydrated soy bean curds was measured at 105 °C, and at constant weight. The moisture content indicates the water-holding capacity of the freeze storage samples. This test were repeated three times.

2.4. Observation of ice crystals in frozen storage soy bean curds by X-ray Micro-Computed Tomography with synchrotron radiation

For the *in situ* observation of ice crystals and the frozen concentrated phase in frozen storage soy bean curds, the X-ray Computed Tomography with synchrotron radiation was performed in accordance with developed method and using almost same condition in previous study (Sato, Kajiwara, & Sano, 2016) at BL19B2 beam line in SPring-8. In addition, frozen storage soy bean curds which were freeze-dried after aging were also scanned.

The CT imaging was performed using monochromatic X-rays of 12.4 keV energy. The charge-coupled device camera (C4880–41S; Hamamatsu Photonics K.K., Japan) and micro fluorescence imaging unit (AA40; Hamamatsu Photonics K.K.) were used as the sample detector. The sample stage was placed between the X-ray source and the sample detector, which was cooled by liquid nitrogen vapor at approximately −30 °C during the experiment. The distance between the sample and X-ray detector was set to 30 mm. The completely frozen sample cut into small pieces (approximately 5 mm × 5 mm x 10 mm) was subsequently set on the CT sample stage. The samples were scanned by rotating 180° at 0.49° per one step, and it were scanned ten times at every step; subsequently 10 transmission images were averaged to one transmission image with filtered back projection. The exposure time for each image acquisition was 250 msec? Size of pixels in the image data was 2.91 × 2.91 $\mu m^{2.}$

To identify the changing behavior of the 3D structural appearance of the freeze storage soy bean curds and to calculate the structure volume and the surface area of it, 600 pixels of ROI in 150 slices of tomograms of soy bean curds were converted to binary images, and subsequently 3D images of it were reconstructed and subsequently the structure volume and surface area in the 3D images of soy bean curds were calculated using the Image J (Rasband, 1997-2018, U. S. National Institutes of Health).

2.5. Disulfide bonds of soy protein in the frozen storage soy bean curds

20 mg of the freeze-dried soy bean curds powder was added to 1 mL of 1% SDS in 50 mM Tris-Cl (pH 6.8) with/without 1% of 2-ME. The mixture was incubated at 37 °C for 12 h, and then, centrifuged at 8000×*g* for 10 min. Protein concentration of the supernatant was measured by using the RC DC protein assay kit (Bio-Rad) based on the Lowry method. The disulfide bond ratio of soy protein was defined as follows;

The disulfide bond content rate of soy protein (%) = ([B] - [A])/[B]. where [A] is the protein concentration of the reagent without 2-ME, and [B] is the protein concentration of the reagent with 2-ME. It was generally accepted that the disulfide bond in protein were reduced to SH group when the 2-ME is added to the suspension containing protein. Therefore, it can be regarded as the difference in protein dissolubility to the buffered SDS solution with and without 2-ME as the relative rate of the disulfide bond in freeze storage soy bean curds. This test were repeated five times.

2.6. Water sorption isotherms

To estimate the hydrophobic or hydrophilic region at the surface, water sorption isotherms of the soy bean curd were measured as follows; freeze-dried powder of the freeze storage soy bean curds, for selected periods of time (0, 7, 14 days) was put through a sieve with 300 μm pore size to obtain a uniform granular diameter in order to cancel the effect of ice crystal marks on water sorption behavior. Furthermore, as a control, unfrozen and non-storage soy bean curd were frozen quite rapidly with liquid nitrogen and subsequently freeze-dried and powdered as above, with artifact minimizing. The powdered soy bean curd was placed in a desiccator with phosphorus pentoxide and heated for 3 h at 80 °C to gain the bone-dried soy bean curd powder. On the other hand, six saturated salt solutions of NaOH, CH_3COOK, $MgCl_2.6H_2O$, $Mg(NO3)_2.6H_2O$, NaCl, and KCl were placed in six each micro desiccators and kept at 25 °C for 1 day in order to increase the relative humidity from 8.2% to 84%. Subsequently, 12 g of bone-dried tofu powder were weighted precisely (W_o) in the grove box packed with dry nitrogen gas. To absorb the water on soy bean curd powder, the bone-dried soy bean curd powder was replaced in each micro desiccator and kept at 25 °C for a maximum of 7 days. During the absorption period, soy bean curd powder was re-weighted precisely in the grove box purged with dry nitrogen gas every two days. This test used four sample at same Aw. The absorption was considered to be complete when the soy bean curd powder showed a constant mass (W_c). After water absorption, the water content of each tofu powder (v) was calculated by the following:

The water content of each soy bean curd powder (v, %) = ((W_c-W_o)/W_o) x 100.

The experimental data were fitted to the Brunauer-Emmett-Teller equation (B.E.T. equation) to measure the moisture content of the monolayer (v_m).

2.7. SDS-PAGE

SDS-PAGE was performed according to Laemmli method with 5% stacking gel and 12% running gel. 10 mg of the freeze-dried soy bean curd powder was added to 1 mL of 8M Urea solution with/without 1% of 2-ME. The mixture was incubated at 25 °C for 15min, and then, centrifuged at 10,000×g for 10 min. The supernatant were diluted with 8M Urea in order to maintain protein content consistency. The diluted supernatant were mixed with premixed Laemmli sample buffer (Bio rad) at the rate of 3:1. After that, mixture were heated at 100 °C for 5min and used as electrophoresis sample.

Each sample was put into a sample well in the stacking gel and electrophoresed. The electrophoresis gels were dyed with the Coomassie Brilliant Blue (CBB) stain solution over night, and subsequently removed surplus color by using distilled water for 1 h. This test repeated three times.

3. Result and discussion

The breaking stress and the breaking strain of frozen and 0–14 days storage soy bean curds after thawing are shown in Fig. 2. In order to compare the breaking stress or the breaking strain between the different ice characteristics, partly owing to the difference in the local freezing

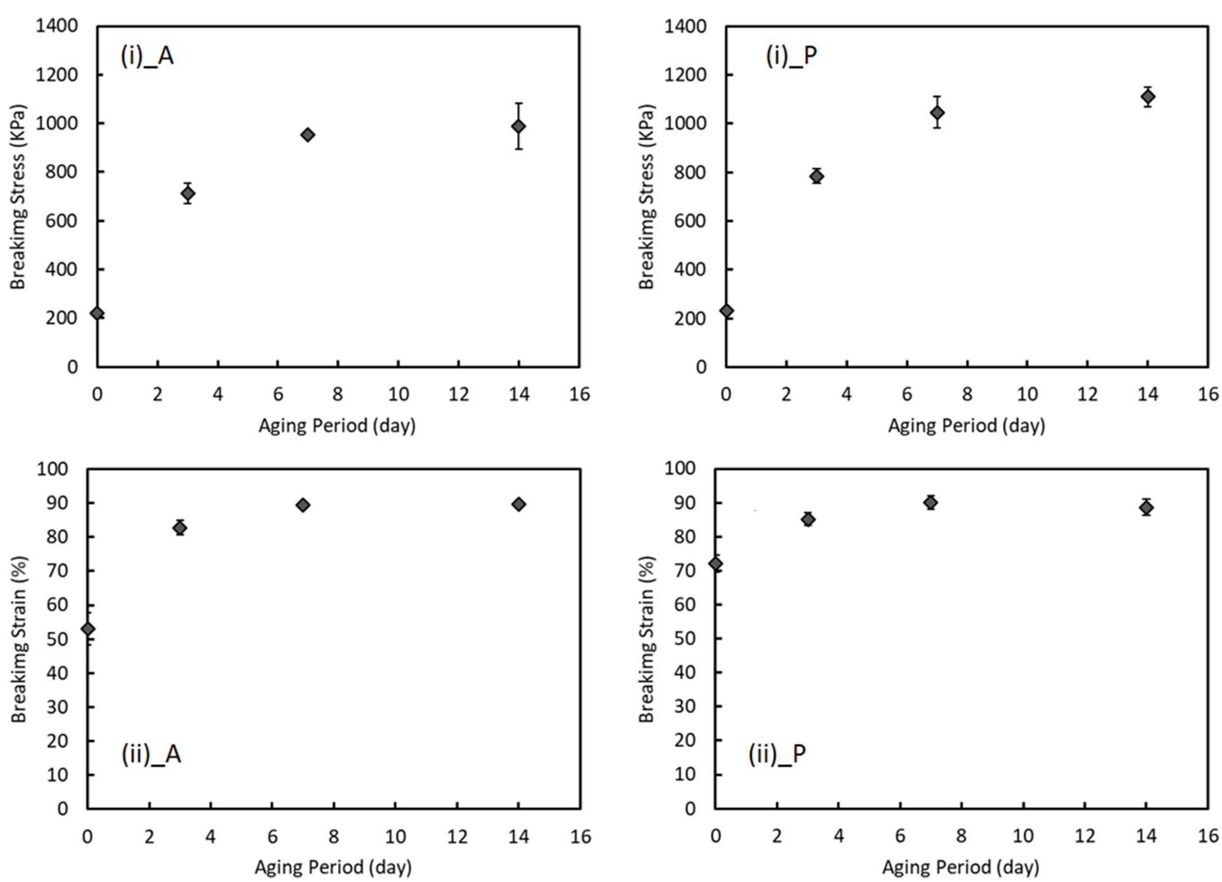

Fig. 2. The breaking stress (i) and the breaking strain (ii) of the freeze storage soybean curds after thawing was evaluated by the penetrate test, where the plunger was penetrated to the surface of soybean curds which is adjacent to the air (A), and to the surface of soybean curds which is adjacent to the metallic plate (P).

rate, the breaking stress or the breaking strain were evaluated by penetrating the plunger at places on the soy bean curds, which are adjacent to the air blast side and to the metallic plate side.

As mentioned in Materials and Methods, one side of the fresh soy bean curds materials was cooled by a blast of cold air (air blast side), which freeze slower than the other side cooled by a cold metallic plate (metallic plate side).

Fig. 2 shows the breaking stress (i) and the breaking strain (ii), evaluated by penetrating of the plunger on the surface of soy bean curds which is adjacent to the air blast side (A) and to the metallic plate side (P). As shown in Fig. 2 (i) _A and (i) _P, the breaking stress increased depending on the storage periods until 7 days of storage; subsequently, a nearly constant value was maintained until the end of the storage period. Additionally, the breaking strength value in Fig. 2 (i) _A and (i) _P showed almost same value at the same storage periods, even though the local freezing rate might be different depending on the local location in soy bean curds.

The changing behavior of breaking strain, as shown in Fig. 2 (ii) _A and (ii) _P also shows a notable increase until first 3 days of storage, which has been increasing slightly until 14 days of it. Comparing Fig. 2 (ii) _A and (ii) _P, the breaking strain of soy bean curds before storage (0 day) were different by the local position of soy bean curds, it showed similar value after 3–14 days storage in a different local position of soy bean curds. Previous study about texture changes of soy bean curds by freeze-thawing reported that slower freezing rate induced firmer soy bean curds, since intense ice crystal grows by slow freezing induce harder dehydrate from protein and change water-protein interaction to protein-protein interaction in back bone of soy bean curd (Xu et al., 2016).

Even though, the results of this study implied that the effect of the local freezing rate during the freezing process was not strong affected the firmness of soy bean curds after frozen storage. It is estimated that the dehydration degree from back bone of soy bean curds depending on different ice crystal growth during freezing process are not stronger than the dehydration during the storage process. The changing behavior of the water holding capacity (WHC) during the storage is shown in Fig. 3. The WHC was valued as the water content after the free water was removed from soy bean curds by strong external force in this experiment. Therefore, the water holding capacity in this experiment represented the capacity of the protein itself to bind water in the freeze storage soy bean curds.

As shown in Fig. 3, the water holding capacity of the freeze storage soy bean curds decreased depending on the storage periods; it dropped sharply during the early period of the storage process (0 day–7 days). Subsequently it showed few decrease until 21 days. These results indicated that the early stage of storage from 0 day to 7 days have lost the water-protein interaction in the soy bean curds with time. From the result, the decrease in the WHC of soy bean curds remained same behavior of the losing softness of it. It was considered that losing water-protein interaction and subsequent forming new protein-protein interaction are related directly with the increasing firmness in soy bean curds during storage. In order to discuss the reason why water-protein interaction have lost during 0day–7day storage, ice crystals and frozen concentrated phase were observed by X-ray CT.

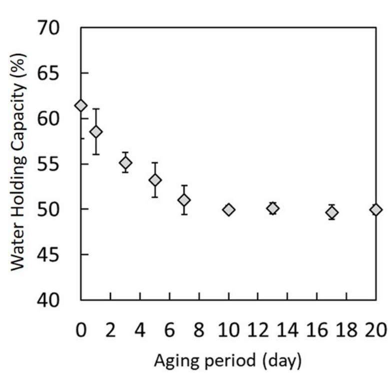

Fig. 3. The decreasing water-holding capacity during storage process.

Fig. 4.1 shows the tomograms of the soy bean curds in the frozen state at 0 day, 7 days, and 14 days of storage. In these tomograms, the darker part indicated a part with lower X-ray linear coefficient, i.e., lower electron density area, and the lighter part indicated a part with higher X-ray linear coefficient, i.e., higher electron density area.

In addition, in order to semi-quantify the shifting of the electron density in the frozen concentrated phase, the frequency distribution of the X-ray linear attenuation coefficient was drawn out from the tomograms as shown in Fig. 4.2. Fig. 4.2-i shows the frequency distribution of the X-ray linear attenuation coefficient at all range in the tomograms of the frozen storage soy bean curds, which is sliced near the air blast side surface. The enlarged figure of the right side of the peak shoulder of the frequency distribution in Fig. 4.2-i are shown in Fig. 4.2-ii, in which Y-axis was changed to semi-log in order to clarify the change in the frequency distribution at the shoulder part.

As seen in Fig. 4.1, the tomograms of soy bean curd mainly consisted of three different brightness areas, a dark gray area surrounding the rectangular shapes, a light gray area, and a white-gray area in rectangular shapes. It was considered that rectangular shapes surrounded by the dark gray area was the backbone of soy bean curd. Furthermore, a lighter gray area and a lightest-gray area in the rectangular shapes indicated ice crystals and frozen concentrated phase, respectively. From Fig. 4.1, it have not recognized that the changing of size or shapes of ice crystals during the storage process, especially 0 days–7 days. Besides, it was observed that the shoulder of the distribution was asymmetric at any storage period in Fig. 4.2-i, even though the frequency distribution at all ranges had a single peak top at around 2.5 cm^{-1} of X-ray linear attention coefficient, regardless of the storage periods. It was seen that the right side of the peak shoulder was broader than the left side. It was considered that the peak of the distribution in X-ray linear attention coefficient around 2.5 cm^{-1} indicated the existence of ice crystals and the broader peak shoulder in the right side indicated the existence of frozen concentrated phase of soy bean curds which mainly consisted of protein, since it was reported that the X-ray linear attention coefficient of ice crystals was calculated as 2.45 cm^{-1} in this experiment condition (Sato et al., 2016), and it of protein is considered as higher than it of ice crystals.

During the storage process, the height of the peak top was around 2.45 cm^{-1} of the X-ray linear attention coefficient (Fig. 4.2-i), which greatly decreased between 0 day and 7 days and decreased slightly between 7 days and 14 days. On the other hand, the right side peak shoulder (Fig. 4.2-ii) broadened between 0 day and 7 days, towards the higher side of the X-ray linear coefficient, but showed a nearly constant value between 7 days and 14 days.

These phenomena indicated that the X-ray linear attenuation coefficient, which is calculated from the X-ray penetrating the frozen concentrated phase, has shifted higher between 0 day and 7 days of the storage process. It was estimated that the electron density of the frozen concentrated phase in the soy bean curds shifted higher during at first 7 days of storage, as same as the firmness increasing and the WHC decreasing of soy bean curd, were owing to dehydration from backbone of soy bean curds by recrystallization during storage.

Summarizing the results showed in Figs. 2–4, it is demonstrated that the dehydration of backbone in soy bean curds at first stage of storage (0day–7day) have destroyed water-protein interaction of soy bean protein, which can induce new protein-protein interaction, and result in the firmer texture of soy bean curds.

Then, in order to discuss a factor causing the dehydration of backbone in soy bean curds during 0day–7day storage, the changes of size and shapes in ice crystals were investigated by scanning of the freeze-dried soy bean curds which is specimen of ice crystals using X-ray CT. It is known that freeze-drying could sublimit the ice crystals with

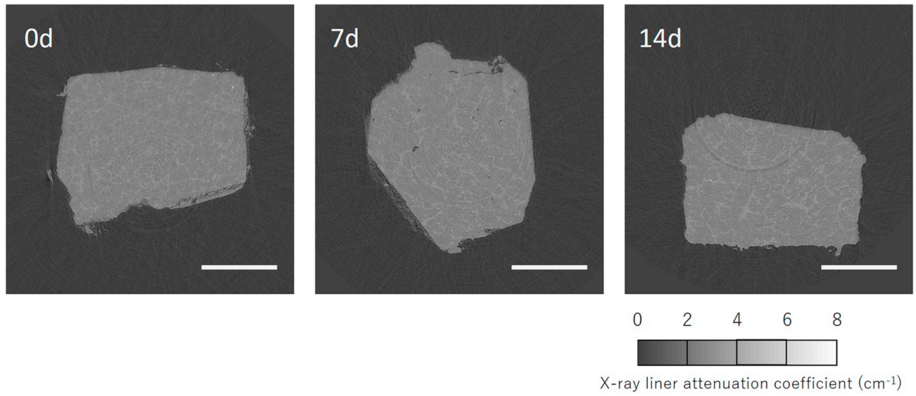

Fig. 4a. Tomograms of the freeze storage soybean curds scanned by synchrotron monochromatic X-ray CT after 0 day, 7 days, and 14 days of storage (0 d, 7 d, and 14 d), sliced at the materials surface, which is adjacent to the air blast side. Scale bar = 2 mm.

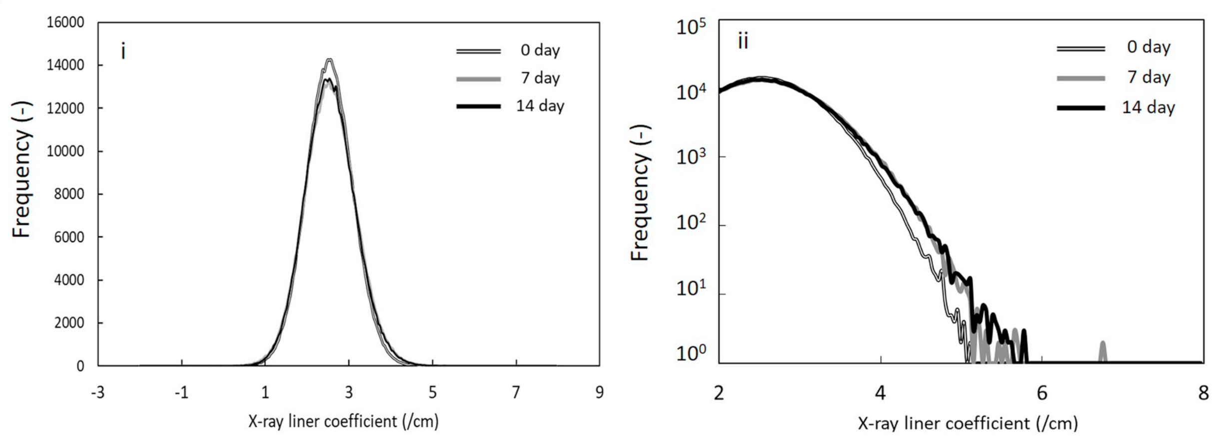

Fig. 4b. The frequency distribution of the X-ray linear attenuation coefficient of soy bean curds.

minimum changes to the frozen concentrated phase character; therefore, the freeze-dried frozen samples have been used as the specimen of ice crystals in frozen materials (Kobayashi, Kimizuka, Watanabe, & Suzuki, 2015). The pores represent the ice crystal structure and the solid structure represents the back bone of soy bean curd.

In addition, scanning of the freeze-dried soy bean curds can gain more determinate image of soy bean curd backbone and ice crystal marks, since the difference of the X-ray linear attenuation coefficient between the pore filled with air and the backbone of soy bean curds mainly consisted by protein were larger than the ice crystals and the backbone of soy bean curds.

Fig. 5.1 shows the tomograms of the freeze-dried soy bean curds that were frozen and storage from 0 to 14 days. In tomograms, the solid structure indicating the frozen concentrated phase having lower X-ray transmission coefficient, were presented as the lighter part and the pore structure having higher X-ray transmission coefficient were presented as the darker part. Furthermore, Fig. 5.2 shows the 3D structure of the freeze-dried soy bean curds, reconstructed from the 150 binary images of the tomograms.

During the 14 days of storage, it was seen that a part of the small

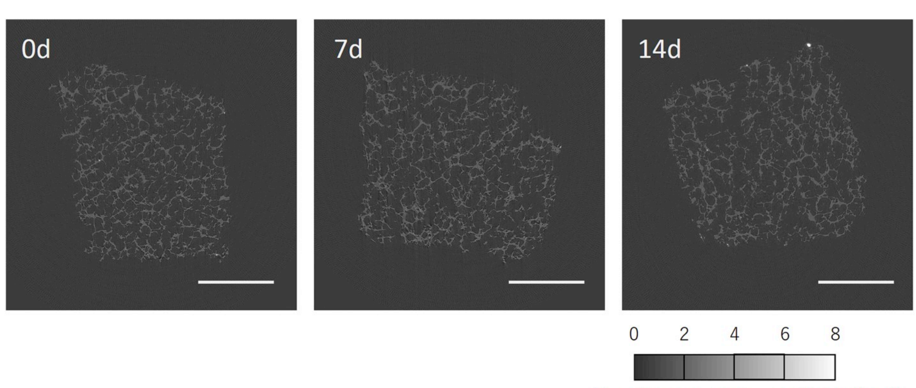

Fig. 5a. Tomograms of freeze-dried soybean curds scanned by synchrotron monochromatic X-ray CT at 0 day, 7 days, and 14 days of storage (0d, 7d, and 14d). Scale bar = 2 mm.

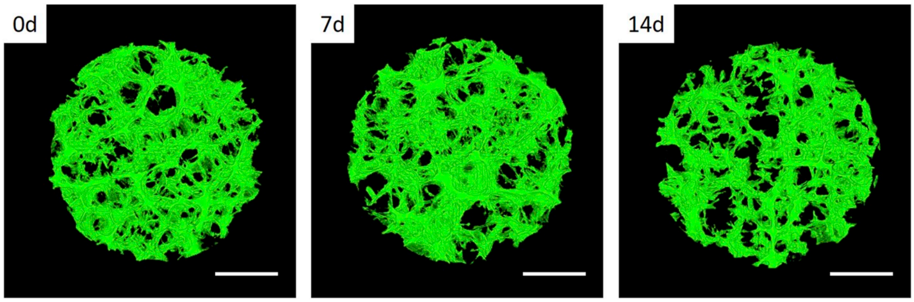

Fig. 5b. The 3D structure of freeze-dried soybean curds at 0 day, 7 days, and 14 days of storage (0 d, 7 d, and 14 d). Scale bar = 0.5 mm.

pores disappeared and the large pores increased by the ripening of the ice crystals. In addition, a closed pore was split opened and coalesced with another pore nearby. Fig. 5.2 revealed that the structural changes mentioned above occurred in the whole samples and especially from 7 days to 14 days of storage.

Table 1 shows the 3D characteristics of soy bean curds, calculated from 150 binary images of the tomograms. It was revealed that the structure-volume of the soy bean curds did not change during the 14 days of the storage process. Besides, the surface area of the soy bean curds notably decreased after 14 days of aging. These phenomena were caused by ice recrystallization.

The surface area of the soy bean curds showed constant value from 0 day to 7 days, and then, dramatically decreased from 7 days to 14 days.

In other words, the physical destruction of the soy bean curds backbone owing to the ice crystal regrowth have showed not same trends of the increasing of electron density of the frozen concentrated phase in the freeze storage soy bean curds.

It is considered that the ice recrystallization during storage periods induces two different changes that have a different time dependency, which is the dehydration from and the destruction in the soybean curds backbone.

The former occurring at the early stage of storage affects the increasing firmness in soybean curds during storage. This is the reason why firmness of soy bean curds increased during 0–7day storage.

In order to illustrate new protein-protein interaction, which is formed in the soybean curd backbone instead of water-protein interaction during storage, the relative rate of the disulfide bond and the hydrophobic region at the surface of the soybean curds were examined.

The relative rate of the disulfide bond in the frozen storage soy bean curds is shown in Fig. 6. As shown Fig. 6, the relative rate of the disulfide bond in the unfrozen soy bean curds and frozen and no storage soy bean curd was approximately 0.25 and 0.5, respectively. It was observed that the disulfide bond ratio of the soy bean curds was increased considerably by the freezing process. On the other hand, increase in disulfide bonds during the storage process was smaller compared to the freezing process, even though the disulfide bond ratio slightly increased during the storage process depending on the its period. Moreover, the increasing rate of disulfide bond content from 0day to 7day showed similar as from 7day to 14day.

Table 1
The 3D structure of soybean curds backbone at 0 day, 7 days, and 14 days of storage (0 d, 7 d, and 14 d).

	Storage period		
	0 day	7 day	14 day
The Structure Volume of tofu backbone (mm^3)	0.185 ± 0.005	0.178 ± 0.001	0.182 ± 0.017
The Percentage of Structure volume of tofu backbone (%)	17.5 ± 0.50	16.9 ± 0.10	17.3 ± 1.63
The surface area of tofu backbone (mm^2)	33.0 ± 0.49	32.4 ± 1.13	26.4 ± 1.63
The specific surface area of tofu backbone (mm)	179 ± 7.26	182 ± 5.28	146 ± 6.85

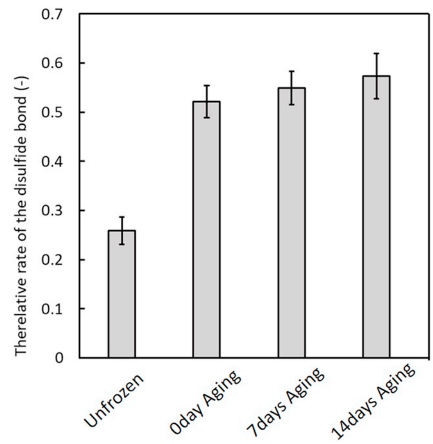

Fig. 6. The relative rate of the disulfide bonds in the freeze storage soybean curds.

Then, in order to investigate the changes in the hydrophobic or hydrophilic region at the surface of the soy bean curds, which is related to the hydrophobic interaction or hydrogen bonds during the storage process, the water sorption isotherms of the powdered backbone of soy bean curds were evaluated. Subsequently, the amount of monomolecular layer adsorption of water at the surface of the powder was obtained by the Brunauer-Emmett-Teller equation using the data of the water sorption isotherms. In this experiment, to cancel the effect of the difference of specific surface area caused by the ice crystal marks on the moisture adsorption ability of the samples, powdered backbone of soy bean curd of uniform particle size were unified 300 μm below.

The water sorption isotherms are shown in Fig. 7.1. It was revealed that the moisture sorption isotherm of the freeze and storage samples

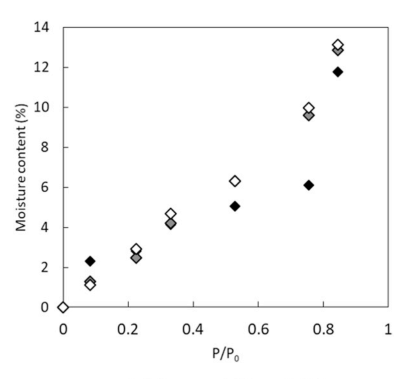

Fig. 7a. Water adsorption isotherms of control (unfrozen) and 0-day or 14-day storage soybean curds at 25 °C.

were considerably different from the control sample (unfrozen sample). Although the sorption isotherm of the freeze storage samples and of the control sample showed sigmoid curves, like a Type II isotherms, sorption isotherms of the freeze storage samples showed more moderate curves than compared to the control sample, which showed form like a Type III isotherms classified by Research of the past (Brunauer, Deming, Deming, & Troller, 1940). In more details, the freeze storage samples showed lower moisture content than the control sample below 0.2 water activity (Aw) called as Region A, besides it showed a higher moisture content than the control sample at 0.2 to 0.6 water activity (Aw) and at 0.6 to 1.0 water activity (Aw) called as Region B and Region C. It was accepted that the water absorption at Region A is a result of the monomolecular layer adsorption, the water absorption at Region B is a result of multilayer adsorption by capillary condensation, and the water absorption at Region C present the water having a similar property like bulk water. Namely, the result in difference of the water sorption isotherms indicated that the changes of intermolecular water-soybean protein powder interaction of the monomolecular layer adsorbing water or the water-water interaction of the multilayer adsorbing water in freeze storage soybean curds. It was also reported that isotherm of many kinds of foods like Starch gels, Corn, Potato showed Type II isotherms, besides isotherm of some sugar rich foods like pineapples, apples, and Sucrose-Starch showed Type III isotherms (Al-muhtaseb, Mcminn, & Magee, 2002). Therefore, these results implied that the freezing processes cause changes in the characteristics of the soy bean protein to hydrophilicity.

Furthermore, moisture adsorption quantity of the 14 days storage soybean curds material at the any water activity (Aw) was higher than the 0 day storage soybean curds, even though they showed the same form of isotherms. These results indicated that the storage processes also changes in the surface characteristics of the soybean protein to hydrophilicity, even though it was estimated mechanism of changing in surface characteristics to hydrophilicity were different since from of isotherms in the control sample and the frozen - 0day or 14day storage sample have completely been different.

The plot of Brunauer-Emmett-Teller equation was constructed from the data shown in Fig. 7.1, within the range of below 0.35 of Aw as shown in Fig. 7.2, in order to quantify the hydrophilic regions in the surface of the soybean protein. They showed a straight line, therefore, the water amount of the monomolecular layer adsorption and the surface area adsorbed by water were calculated using B.E.T. plot and the results are shown in Table 2. Both the moisture content of the monolayer (g/g) and the specific surface area which sorb the monolayer adsorption water (m^2/g) increased by both freezing and storage processes. The result in Table 2 showed that approximately 1.46 times of the hydrophilic surface area that adsorbed water was increased in the storage process.

Fig. 8 show the SDS-PAGE patterns of the freeze and no storage or 18 day storage soy bean curds, with or without 2-ME in sample buffer. It could be seen the band of α and α′subunit of β-conglycinin, acidic subunit of glycine, and β subunit of β-conglycinin at all lane. There was no difference of band pattern between the no storage sample with 2-ME and the 18 day storage sample with 2-ME (line2 and line3). On the other hands, the band pattern of α and α′subunit of β-conglycinin and acidic subunit from 18 day storage sample without 2-ME (line 4) get thinner, comparing with no storage sample without 2-ME (line5). These result indicated that α and α′subunit and acidic subunit were involved in formation of protein-protein interaction induced by the storage after freezing.

Previous study reported that soy milk which is suspension of hydrocolloids was gelatinized by freezing and thawing process (Simoyamada, Tomatsu, & Watanabe, 1999), and glycinin (11S) of soy protein participate in aggregation on the soy milk by freezing process (Morita & Shimoyamada, 2013). However, the result in this study showed β-conglycinin (7S) play important role in aggregation on the freeze storage soybean curds.

Table 2
The moisture content of the monolayer, and the specific surface area calculated from the B.E.T plot of water adsorption isotherms of soybean curds.

	Unfrozen	0day	14day
The moisture content of the monolayer (g/g)	0.0288	0.0392	0.0573
The specific surface area (m^2/g)	120.4	163.9	239.6

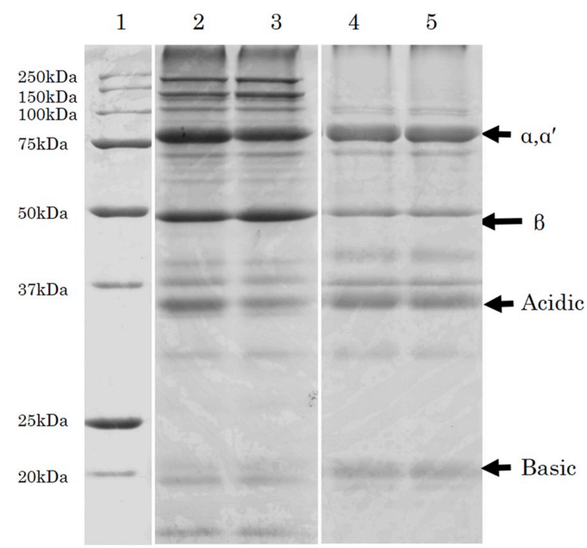

Fig. 8. SDS-PAGE profiles of the freeze and no storage or18 day storage soybean curds, Line1: Standard protein maker; Line2; the freeze and no storage soybean curds with 2-ME; Line3; the freeze and 18 day storage soybean curds with 2-ME; Line4; the freeze and no storage soybean curds without 2-ME; the freeze and 18 day storage soybean curds without 2-ME.

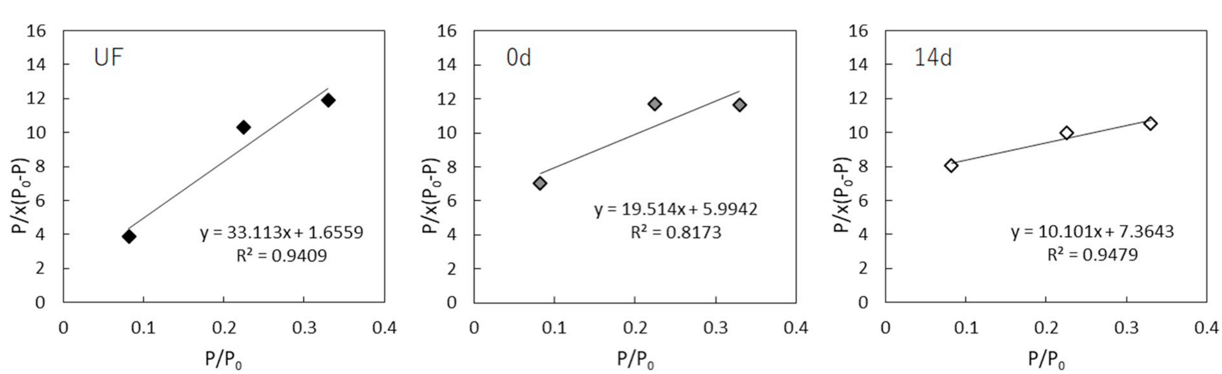

Fig. 7b. B.E.T plot of water adsorption isotherms of control (unfrozen) and 0 day or 14 day storage soybean curds.

Summarizing the result showed in Figs. 6–8, the protein-protein interaction induced during storage after freezing is estimated as follows. The backbone of soybean curds mainly consists of oil globules that have a triple protein layer. In addition, the outside layer mainly includes α and α′subunit of β-conglycinin which is a glycoprotein, and acidic subunit of glycinin which have the SH group (Chen & Ono, 2014). Therefore, it is considered that the increasing of disulfide bond shown in Fig. 6 were owing to formation of new disulfide bond between Acid subunit and other protein subunit. In addition, the increase in the hydrophilic area of the protein surface were induced by forming of new protein-protein interaction with α and α′subunit, since α and α′subunit have both hydrophilic and hydrophobic area. During storage, the balance of hydrophobicity and hydrophilicity in the whole backbone of soybean curd becomes unbalanced since the backbone is dehydrated and destroyed hydrogen bond between water and protein by the ice recrystallization. It is estimated that the changes of balance of hydrophobicity and hydrophilicity promote aggregation of amphiphilic protein such as α and α′subunit. It was also reported that the microstructure change of the egg yolk gel continues until 168 days at −20 °C and hydrophobic interaction (Au, Acevedo, Horner, & Wang, 2015), and it was also reported that the concentrated coffee extraction was gelatinized by freeze storage at −2.5 °C owing to the occurring hydrogen bonds in frozen concentrated phase (Sequera, Ruiz, Moreno, Quintanilla-carvajal, & Salcedo, 2019).

It is considered that sugar chain of α and α′subunit of β-conglycinin involved in increasing of hydrophilicity in freeze-dried soybean curds. It is assume the thawing process also change the balance in hydrophilicity and hydrophobicity of protein, since ice crystal that shows hydrophobicity melts and become water, and new protein-protein interaction as hydrogen bond and result in decrease of WHC. Even though, the increasing of disulfide bonds and the increasing in the hydrophilic surface area were measured in “freeze-dried” soy bean curds, therefore the changing of protein-protein interaction during thawing process was ignore in this study. It is necessary to discuss the effect of thawing process as future perspective.

4. Conclusion

In this study, aiming to discuss the mechanism of the hard texture arising in soy bean curds during the freeze storage periods, characteristics changes in soy bean curds during frozen storage period such as texture, water holding capacity, ice crystal and frozen concentrated phase were investigated with time. Furthermore, in order to illustrate new protein-protein interaction, which is formed in the soybean curd backbone instead of water-protein interaction during storage, the relative rate of the disulfide bond and the hydrophobic region at the surface of the soybean curds, and SDS-PAGEs were examined. The result demonstrated that the dehydration from backbone of soy bean curds have played important role for inducing a new protein-protein interaction and the firmer texture of soy bean curds at first stage of storage (0day–7day). Additionally it is indicated that the change in the balance of hydrophilic and hydrophobic region induced by dehydration with ice recrystallization plays an important role in forming the new protein-protein interaction especially for amphipathic protein as α and α′subunit of β-conglycinin.

CRediT authorship contribution statement

Rika Kobayashi: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Project administration, Writing - original draft, Writing - review & editing. **Takahiro Ishiguro:** Formal analysis, Funding acquisition, Investigation, Methodology, Project administration. **Ami Ozeki:** Formal analysis, Investigation. **Kiyoshi Kawai:** Formal analysis, Investigation. **Toru Suzuki:** Conceptualization, Project administration.

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