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Important role of starch in the freeze-thaw damage of Nama-An particles prepared from adzuki beans (*Vigna angularis*)

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Abstract

In order to elucidate the mechanism of freezing damage of adzuki Nama-An (non-sugar bean jam), changes in the tissue structure of Nama-An and degree of damage according to different freeze-speeds and thawing conditions were investigated in this study. After Nama-An was frozen at −20 °C for 3 months, the An particle size shrank from 67.6 to 51.6 μm. Although the particle size was restored to 60.4 μm after being thawed at 20 °C, the An particles were not completely recovered, and many hollows were formed on the surface of the particles. Also, when An was stored at −20 °C, some spaces and voids were formed inside the particles and starch granules because starch granules might retrogradate and be dehydrated. The soluble carbohydrate extracted from the particles was in low level both in thawing and non-thawing conditions, although it tended to increase with freezing storage periods. The starch of raw beans had a lower swelling power and retrogradated easily, depending upon its structures and the ratio of amylose to amylopectin. The findings suggest that freezing damage of adzuki Nama-An may be mainly due to the major constituent, starch.

Keywords: Nama-An; Freeze-than damage; Starch

1. Introduction

An is used habitually in Japan as a traditional food with a unique taste. The quality of An is dependent on the characteristics of the raw beans (place of production, cultivation conditions, storage conditions) and the method of processing. All Nama-An exist as capsule-like ellipsoid particles that mainly consist of a cell wall-like layer of the solidified protein coagulated by heat and the content of the gelatinized starch (Fujimura & Kugimiya, 1993; Kobayashi, Michikawa, & Watanabe, 1992; Michikawa, Kobayashi, & Watanabe, 1992). Because the water content in Nama-An is high, 62–68%, the quality easily deteriorates (Michikawa et al., 1992). The limitation of preservation is 4 days even at 1–2 °C due to growth of a bacillus. Therefore, it is thought that refrigeration is a desirable method for the long preservation of Nama-An. But the Neri-An (sweet bean jam) made from frozen Nama-An with the usual freeze method is said to taste bad. Thus product value of the Neri-An falls.

Freezing damage of food (the animals and plants bodies) is classified roughly into changes of tissue structure (the mechanical damage, cell destruction, the gas expansion) and colloidal structure (release of free and bound waters, dehydration damage, salting out, protein denaturation, molecule density of protein). However, there has been no study about the freezing damage of Nama-An as a processed food. In order to elucidate the mechanism of freezing damage of Nama-An, the tissue structure and damage degree of An particles according to different freezing speeds (liquid nitrogen and −20 °C) and different thawing conditions (non-thawing and thawing at 20 or 100 °C) were investigated in this study. Also, the property of starch (over 70% on dry basis), the major component of adzuki beans as a raw material (Michikawa et al., 1992), was examined.

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2. Experimental

2.1. Materials

Mature adzuki beans (*Vigna angularis*) grown in Tenjin, China, in 1998 were used. Nama-An was provided by Hashimoto Food Industry Co., Ltd, Japan using the adzuki beans. Non-frozen Nama-An was used as normal Nama-An. Frozen Nama-An was prepared by packing 5 kg of Nama-An in polyvinyl bags spread to 10 cm thickness and stored for 3 months at −20 °C. Thawed Nama-An was prepared by thawing the frozen Nama-An at 20 °C for 12 h. All chemicals were purchased from commercial suppliers.

2.2. Property of Nama-An particle

Normal Nama-An and thawed Nama-An were freeze-dried with a freeze dryer (Iwaki Glass Co., Ltd, FRD-80 Type) after being frozen by liquid nitrogen. Frozen Nama-An was freeze-dried directly with the freeze dryer. The shape and size of freeze-dried Nama-An particles were observed by scanning electron microscopy (SEM) (Nippon Datam, JSM-5400 LV) at an accelerating voltage of 10–30 kV. Nama-An particle diameter was estimated by averaging the dimensions of 100 random particles from three SEM micrograms for each sample (Takeda, Takeda, Mizukami, & Hanasuiro, 1999). The moisture content of Nama-An was determined by the AOAC method.

2.3. Internal structure of Nama-An particle

Soaked beans were prepared by soaking adzuki beans (about 200 g) in 400 ml of water at 10 °C for 24 h. Half of the soaked beans (100 g when dry) were cooked in boiling water for 1 h according to the processing method of Nama-An, to prepare cooked beans. The soaked beans and one part of the cooked beans were freeze-dried by the freeze dryer after being frozen by liquid nitrogen. The remainder of the cooked beans was cut into 2–3 mm fragments. One part of the fragments was embedded with the usual paraffin embedding method at room temperature without stain. The remainder of the fragments was stored at −20 °C for 3 months. One part of the frozen fragments was fixed with Carnoy fluid (ethanol: chloroform: acetic acid = 6:2:1) at −20 °C, and freeze-substituted for 24 h. And then, they were washed for five to six times by one time of every day in 99.5% of ethanol at −20 °C. Finally, they were routinely embedded in the paraffin wax (Bevilacqua & Zaritzky, 1982). The remainder of frozen fragments was embedded with the usual paraffin embedding method after being thawed at 20 °C for 2 h or in boiling water for 10 min. Frozen Nama-An was also embedded with the usual paraffin method after being thawed in boiling water for 10 min. The crossed and cut sections of the embedded samples were observed with scanning electron microscopy (Nippon Datam, JSM-5400 LV) at an accelerating voltage of 10–30 kV.

2.4. Chemical analysis of damage degree of freeze

Normal Nama-An (100 g) was packed in a vinyl bag, spread to 5 mm thickness, and then stored at −20 °C (Sanyo Medical Freezer MDF-332) for 6 months. The soluble carbohydrate (starch mainly) from the Nama-An was measured by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) every 4 weeks during freezing storage. Samples (exactly 10 g) non-thawed and thawed at 20 °C for 2 h were boiled in 100 ml of boiling water for 30 min, then filtered with a glass filter (G-5) and washed three times in 100 ml of water. The solution was diluted to 500 ml with water, and used as a sample for the measurement of soluble carbohydrate.

2.5. Analysis of properties of adzuki starch

The starch granules were isolated by the alkali method as described previously (Tang, Ando, Watanabe, Takeda, & Mitsunaga, 2000). Adzuki beans were soaked in five-fold weight of 0.1% NaOH solution at 5 °C for 48 h, then it was homogenized by a homogenizer (Nissei AM-8, Japan). The homogenized mixture was screened through a 125 mesh metal screen. The residue was washed with 0.1% NaOH solution for three times. The suspension was adjusted to pH 7.0 with 0.5 M HCl solution. After three treatments with 1-pentanol to remove protein, the suspension was screened through a 250 mesh metal screen. The starch granules were collected on glass-filter, washed with ethanol and diethyl ether, and dried in a vacuum desiccator.

The size of prepared starch granules was examined by an analyzer (Horiba, Ltd LA-700, Japan) (Tang, Watanabe, & Mitsunaga, 2002a).

Swelling power of starch was evaluated by the method reported previously (Tang, Watanabe, & Mitsunaga, 2002b). Starch (0.1 g) was weighed in glass tubes with coated screw caps to which 5 ml of a 0.1% $AgNO_3$ solution was added. The tubes were placed in a shaking water bath at 70 °C for 10 min and then transferred into a boiling water bath. After gelatinizing perfectly, the tubes were cooled in cold water (20 °C) for 5 min and centrifuged at 1700*g* for 4 min. The supernatant was removed carefully and swelling power was determined as sediment weight (g/g).

Differential scanning calorimetry (DSC) was performed with a starch-to-water ratio of 5 mg to 20 μl. The samples were heated from 25 to 100 °C at a heating rate of 5 °C/min (Rigaku, Ltd DSC-8240D). (Tang et al., 2000).

Fractionation of amylose and amylopectin was carried out by the procedure of Takeda, Hizukuri, and Juliano (1986). Iodine absorption spectra were measured by the method of Takeda, Takeda, and Hizukuri (1983), and the amylose content was calculated from the blue value (BV) with the following equation, in which amylose content was equal to: [BV(starch − amylopectin)/BV(amylose − amylopectin)] × 100. The number-average degrees of polymerization (DPn) by the modified Park–Johnson method (Hizukuri, Takeda, Yasuda, & Suzuki, 1981), and average chain length (CL) were also determined by the same method after isoamylolysis. The average number of chains per molecule (NC) was the value of ((DPn/CL) − 1). Isoamylolysis of starch was performed by the method reported previously (Tang, Ando, Watanabe, Takeda, & Mitsunaga, 2001).

3. Results

3.1. Properties of Nama-An particles

The shape and size of Nama-An particles prepared by the freeze-dry method were observed with SEM (Fig. 1). Frozen Nama-An ((c) and (d)) clearly shrank in size, compared with normal Nama-An ((a) and (b)). The thawed Nama-An ((e) and (f)) seemed to have been restored to the oval of normal Nama-An particle, but some dents were observed on the surfaces of particles. The particle size was estimated by averaging the dimensions of 100 particles at random from three SEM micrograms for the Nama-An particles (Table 1). The average sizes of the smallest 30 particles (S_{30}) among 100 particles at random were 44.2 μm for normal Nama-An,

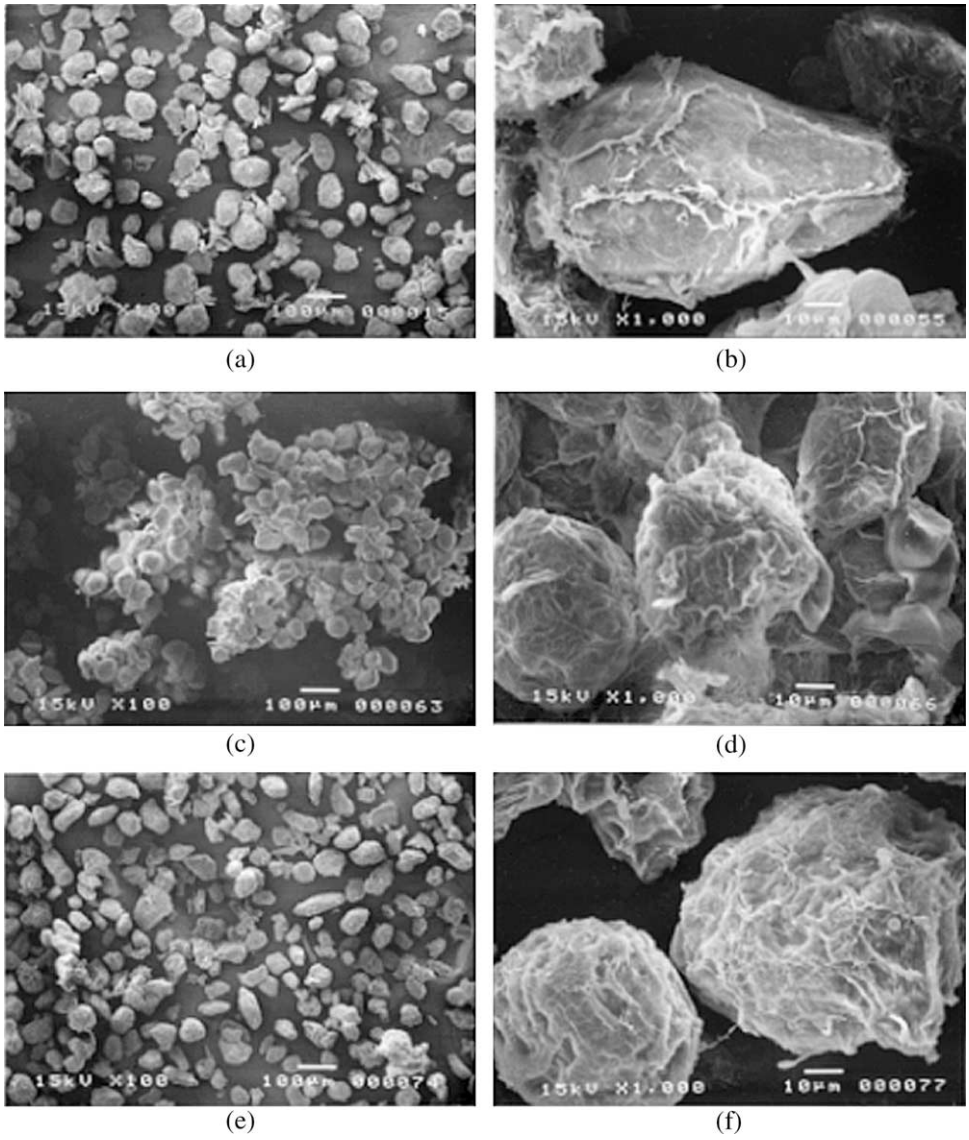

Fig. 1. Shape and size of Nama-An particles by SEM: (a) and (b) were normal Nama-An (without freezing storage), which was freeze-dried with liquid nitrogen as control; (c) and (d) were frozen Nama-An (−20 °C for 3 months), which was freeze-dried directly; (e) and (f) were thaw Nama-An (thawed at 20 °C for 2 h after storing at −20 °C for 3 months), which was freeze-dried with liquid nitrogen.

Table 1
Diameter of Nama-An particles

Sample	S_{30} (μm)[a]	L_{30} (μm)[b]	AD_{100} (μm)[c]	Moisture (%)[d]
Normal An[e]	44.2±7.5	91.1±12.0	67.6±20.1a	64.1±1.0
Freeze An[f]	35.9±6.3	67.8±7.5	51.6±13.8b	65.0±1.2
Thawed An[g]	38.6±8.2	83.5±10.0	60.4±19.2c	64.3±0.9

[a] Average dimensions±SD of the smallest 30 particles in 100 random particles estimated.
[b] Average dimensions±SD of the largest 30 particles in 100 random particles estimated.
[c] Average dimensions±SD of 100 random particles estimated. Values followed by different letters are significantly different ($p<0.01$).
[d] Determined by the AOAC method.
[e] Nama-An was freeze-dried by liquid nitrogen without freeze-storage as control.
[f] Nama-An was freeze-dried directly after freeze-storage at −20 °C for 3 months.
[g] Nama-An was thawed at 20 °C after freeze-storage at −20 °C for 3 months, and then freeze-dried by liquid nitrogen.

35.9 μm for frozen Nama-An, and 38.6 μm for thawded Nama-An, whereas the largest 30 particles (L_{30}) were 91.1, 67.8 and 83.5 μm, respectively. Average dimensions of 100 particles at random (AD_{100}) were 67.6 μm for normal Nama-An, 51.6 μm for frozen Nama-An, and 60.4 μm for thawed Nama-An. Nama-An particles shrank significantly after being frozen. However, the moisture content were 64.1% in normal Nama-An, 65.0% in frozen Nama-An, and 64.3% in thaw Nama-An. The moisture content in An did not change before and after being frozen.

3.2. Internal structure of cooked bean and Nama-An particles

From scanning electron micrographs of the soaked beans (Fig. 2(a)), there were at least seven starch granules in a cell. Five to six Nama-An particles were formed in two or three cells from SEM of the cooked beans (Fig. 2(b)). It was thus supposed that one Nama-An particle contained about three starch granules. The estimate was supported also by images of the internal structure of Nama-An particles from cut sections of the cooked bean (Fig. 2(c)). The cell wall-like layer of Nama-An particles was around 3 μm thick. Starch granules in the Nama-An particles were obviously swollen, compared with those in the soaked beans. But the shape of starch granules was unchanged. Internal structures of frozen Nama-An particles with SEM are given in Fig. 3. Compared with Fig. 2(c) as a control, the internal structure of the An particles in the cooked beans with the freeze substitution (a) had collapsed. Starch granules in An particles shrank, became uniform, and had cavities in the center. The An particles of beans after thawing at 20 °C (c) were broken more than those of beans with the freeze substitution (a). The An particles of beans after thawing in boiling water (e) seemed to have been restored to the control state (Fig. 2(c)), but the shape of starch granules inside the An particles was changed. The changes of the starch granules were similar among (c), (e) and frozen Nama-An after thawing in boil water (g). Under higher magnification SEM, the particle-like profile was only observed in the organization of starch granules for the freeze substitution (b) and thawing at 20 °C (d), whereas

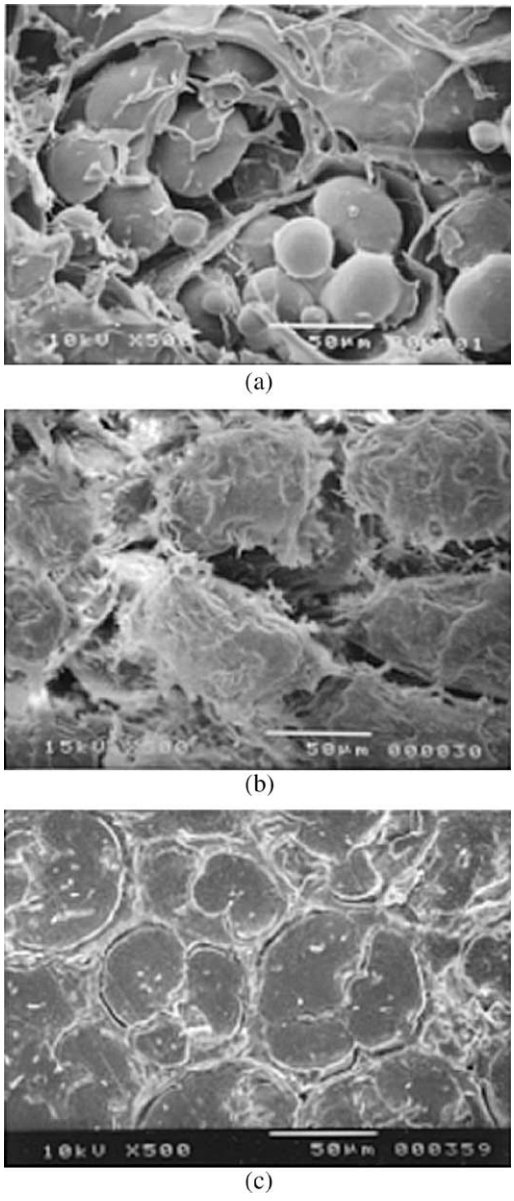

Fig. 2. Scanning electron micrographs of soaked and cooked beans: (a) and (b) were freeze-dried with liquid nitrogen, (a) was a section of soaked bean, and (b) a section of bean boiled for 30 min, (c) was a histological cut of cooked bean prepared by the usual paraffin embedding method.

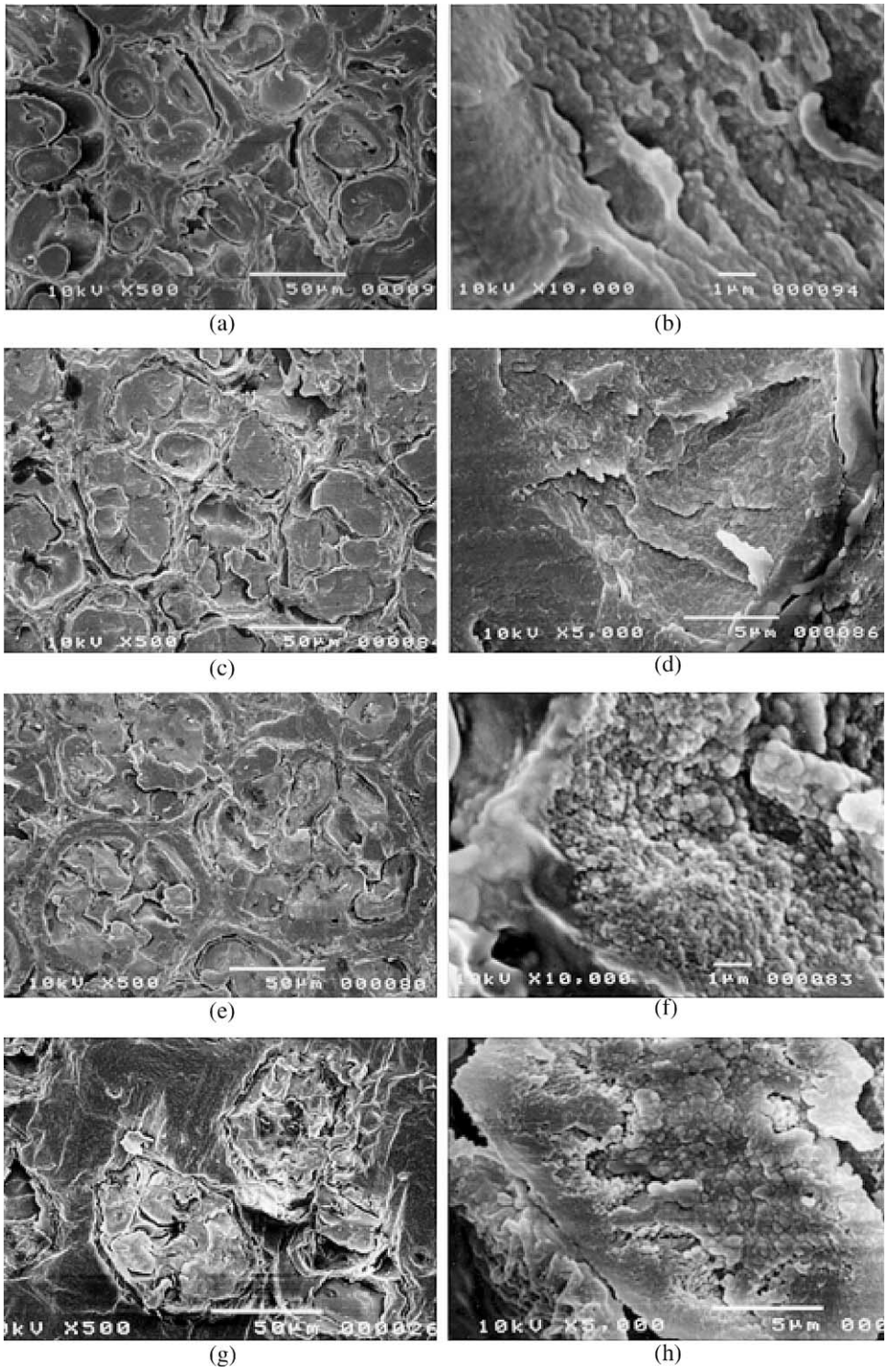

Fig. 3. Internal structure of cooked beans and frozen Nama-An particles on SEM. Cooked bean sample (a–f) and Nama-An (g and h) were stored at −20 °C for 3 months: (a) and (b) were prepared by the freeze substitution method followed by the usual paraffin embedding method, (c) and (d) were prepared by the usual paraffin embedding method after being thawed at 20 °C for 2 h, (e)–(h) were prepared by the usual paraffin embedding method after being thawed in boiling water for 10 min.

the particle-like structure had been formed in starch granules for frozen beans (f) and frozen Nama-An (h) after thawing in boiling water. The formation of the particle-like structure seemed to be promoted by thawing.

3.3. Chemical analysis of damage degree of freeze

Normal Nama-An was stored at −20 °C for 0–6 months. The change of soluble carbohydrate during freeze storage is

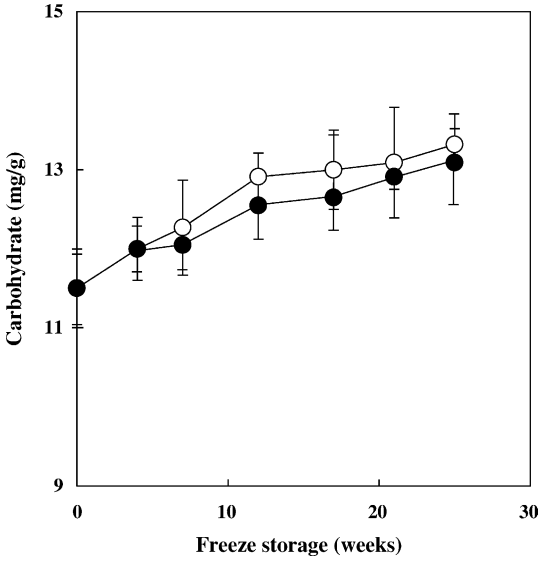

Fig. 4. Determination of soluble carbohydrate from the Nama-An particles. The Nama-An samples were stored at −20 °C for 0–6 months. (○) Extracts with boiling water after being thawed at 20 °C for 2 h, (●) extracts with boiling water without thawing.

given in Fig. 4. The soluble carbohydrate from Nama-An was 11.5–13.3 mg/g for boiling water extract after thawing at 20 °C for 2 h, and 11.5–13.1 mg/g for boiling water extract without thawing. The two treatments were not significantly different in soluble carbohydrate in which both increased with freeze periods. However, the levels of soluble carbohydrate were low.

3.4. Properties of adzuki starch

Properties of adzuki starch are shown in Table 2. The size distribution of the starch granules ranged from 15.2 to 67.5 μm according to a particle size analyzer (Horiba, Ltd LA-700, Japan). The median size was around 32.0 μm, and similar to that reported previously (Tang et al., 2002a). The swelling power of starch granules was 12.3 g/g, and smaller than those reported previously (Sasaki & Matsuki, 1998; Tang et al., 2002b; Vasanthan & Bhatty, 1996). For gelatinization of starch granules, T_o, T_p, T_f and ΔH were 62.0, 69.8, 80.1 °C and 12.8 J/g, respectively. The values were similar to those of adzuki starch reported previously (Tang et al., 2002a), but had a higher gelatinization temperature and enthalpy change than starch from other plant seeds. The λ_{max} and BV were 608 nm and 0.493 for non-granular starch, 657 nm and 1.371 for amylose, and 569 nm and 0.241 for amylopectin, respectively. The amylose content was 22.3% as estimated by the BV of starch, amylose and amylopectin. DPn, CL and NC were 2200, 1800 residues and 0.2 chains for amylose, and 4550, 24 residues and 189 chains for amylopectin, respectively. Amylose molecules were large and almost not branched, whereas amylopectin molecules were small and had long side-chains. The results agreed with our previous report (Tang et al., 2002a). The properties of adzuki starch such as swelling and gelatinization were supported by its molecular structure.

Table 2
Properties of adzuki starches[a]

Granular starch[b]	
Distribution of size (μm)	67.5–15.2
Median size (μm)	32.0±2.0
Swelling power (g/g)	12.3±0.2
Gelatinization[c]	
T_o (°C)	62.0±0.6
T_p (°C)	69.7±0.3
T_f (°C)	80.1±0.5
ΔH (J/g)	12.8±0.2
Non-granule starch[d]	
Amylose content (%)[e]	22.3±2.1
λ_{max} (nm)[f]	608±2
BV[g]	0.493±0.005
Amylose	
λ_{max} (nm)	657±2
BV	1.371±0.053
DPn[h]	2200±110
CL[i]	1800±60
NC[j]	0.2±0.1
Amylopectin	
λ_{max} (nm)	569±2
BV	0.241±0.003
DPn	4550±460
CL	24±2
NC	189±22

[a] Values are the mean±SD of three at least separate measurements.
[b] Starch prepared by the modified alkali method (Tang et al., 2000).
[c] T_o, Onset; T_p, peak; T_f, final temperature; ΔH, enthalpy change by DSC.
[d] Defatted starch.
[e] Amylose content (%)=[(BV(starch)−BV(amylopectin))/(BV(amylose)−BV(amylopectin))] ×100.
[f] Maximum absorption wavelength.
[g] Blue value at 680 nm.
[h] Number-average degrees of polymerization.
[i] Average chain-length.
[j] Average number of chains per molecule.

4. Discussion

The previous observations on Nama-An particles were focussed on the shape and size. In this study, we observed the inside of Nama-An particles clearly with SEM for the first time. Nama-An was an oval particle, and contained around three starch granules in each particle. The observations agreed with those of previous reports (Kobayashi et al., 1992; Michikawa et al., 1992). The shape of the starch granules was maintained in Nama-An particles, although it had become swollen and gelatinized. The formation mechanism of An particle can be considered to be as follows: The starch in the cell of the bean swells and other components (protein, pectin and fiber) dissolve in water by soaking. The other components denature by subsequent heating, form the cell wall-like layer, and the layer embeds

the starch granules before the starch granules begin to gelatinize, because adzuki starch has a low swelling power and high gelatinization temperature (Table 2). The starch granules in the layer gelatinize by successive heating. Thus, Nama-An particle are formed. The layer is around 3 μm thick (by SEM) and strong. Therefore, the starch granules in Nama-An particle do not collapse in shape and keep a stable structure.

Nama-An particles contained around 64% moisture. It is thought that most of the moisture is saved by the gelatinized starch in the Nama-An particles, because the particles contain 70% more starch (on dry basis) (Michikawa et al., 1992). All samples were sealed by polyvinyl bag in order to prevent loss or invasion of water during freezing storage. After freezing storage at −20 °C for 3 months, Nama-An particles decreased significantly in size although the moisture content did not change between before and after freezing storage. It can be presumed that the decrease in size may be due to release of a lot of water from Nama-An particles during the freezing storage, because a lot of ice occurs among the Nama-An particles (data not shown). The characteristic freezing of Nama-An particle resembled extracellular freezing of a plant. However, Nama-An particles are a denatured mixture with a capsulated structure, and are essentially different from a living plant cell. When frozen Nama-An was thawed with a calmer condition (at 20 °C), both shape and size could be recovered. However, many hollow were formed on the surface of Nama-An particles. This may be explained by the retrogradation of the starch in frozen Nama-An being unable to recover completely during the thawing. The retrogradation of the starch may differ from the cell wall-like layer with denatured protein in its ability to recover.

According to our observation with SEM, the internal structure of frozen Nama-An particles was consistent with there interpretations. Also, the thawing seemed to confuse the structure of Nama-An particle more than non-thawing. When we observed starch granules inside Nama-An particles under high magnification, an interesting thing was found about the structure of starch granule although it is not related with a purpose of this study: the structure of starch granules in Nama-An particles may hardly collapses, in addition to the shape of starch granules being maintained. In other words, starch granules just swell in Nama-An particles, rather than being gelatinized. Starch granules do not become paste of homogeneity. A particle-like structure was clearly observed, in particular in the Nama-An particles thawed with boiling water. One unproven hypothesis is that the starch granule is an aggregated organization of spherical structures termed 'blocklets' (Gallant, Bouchet, & Baldwin, 1997). Whether our finding can or not be used as evidence of this hypothesis needs to be examined further. However, we think that significant information may be provided by starch of the special state in Nama-An particles in order to elucidate structure of starch granule.

From the level of the soluble carbohydrate, the degree of freeze damage of Nama-An particles was low in both thawing and non-thawing, and both were similar in value of the level. The results supported the observations of SEM. Because ice crystallization might be mainly outside of Nama-An particles, little mechanical damage to the particles was caused by ice crystallization.

5. Conclusion

The present results indicated that starch plays the most important role in both the form and freezing damage of Nama-An particles. Also, the paraffin embedding using cooked beans was easy, and avoided structural damage of Nama-An particles in the preparation of histological preparations. The freezing damage of Nama-An particles may not be collapse of particles, but rather a transformation of particles, perhaps mainly due to the retrogradations of starch and the increase and release of free water in Nama-An particles at low temperature during slow freezing. It was difficult to completely restore these changes at least on structure and morphology. It is demonstrated that appropriate freezing and thawing conditions are important in order to maintain the quality of Nama-An in industrial production. In particular, thawing conditions should have the top priority in its industrial production.

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