Role of Calcium and Magnesium Ions in the Hardening of Pressure-Treated Root Vegetables

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We investigated the effect of calcium and magnesium ions on the hardening of Japanese radish by the pressure treatment at 400 MPa. The pectins were separated into the three components. Water-soluble pectin (WP) and hydrochloric acid-soluble pectin (HP) were decreased by the pressure treatment, whereas hexametaphosphate-soluble pectin (PP), which is a metal binding type pectin, was increased. The degree of esterification of WP was decreased by pressurization. Variations with time after pressure release of all pectin fractions and the concentrations of calcium and magnesium ions contained in pectin fractions were small. We studied the hardness of the pressure treated samples by soaking them with or without EGTA and EDTA and the effect of the order reversal of the soaking and the standing. It was found that the magnesium ions had a great influence on the hardness of both non-treated and pressure-treated uncooked samples and that calcium ions affected the hardness of pressure-treated cooked samples. The suppressing effect of the calcium ions on the softening during cooking suggested that the interactions between such components as proteins and hemicellulose were promoted by the calcium ions. The mass transfer processes followed by the collapse of the cell membranes by the pressure treatment are considered to strengthen these interactions.

Keywords: *Pressure treatment; pectin; calcium ion; magnesium ion; hardening*

INTRODUCTION

It has been reported that vegetables, fruits, and beans are hardened by preheating (Bartolome et al., 1972; Fuchigami, 1986; LaBelle et al., 1971; Manabe, 1980a), drying (Fuchigami, 1986), addition of salts (LaBelle, 1971), and storage (Jones et al., 1983; Liu et al., 1992a) and that their softening during cooking after these pretreatments is suppressed. In our previous papers (Yamamoto et al., 1992; Kasai et al., 1995), we found that such root vegetables as Japanese radish and carrots were hardened by the application of a high pressure of several hundreds MPa and that the hardening continued even after pressure release. The cell membranes were destroyed by the pressure treatment, and the decrease in the degree of esterification of pectins followed. Mechanism of the pressure-induced hardening could not be explained only in terms of the decrease of the degree of esterification of pectins, and some other factors were suggested to contribute. In this work, we focus on the role of metal ions such as calcium and magnesium in controlling the vegetable hardening.

Some important mechanisms have been proposed for the hardening of foods (Bartolome et al., 1972; Jones et al., 1983; Liu et al., 1992a–c; Manabe, 1980b; McFeeter et al., 1985; Richardson et al., 1991). In the case of preheating of potatoes, it was considered that the destruction of the cell membranes above 50 °C allowed intracellular ions to contact the cell wall materials and that it caused the enzymic demethylation of pectins followed by the formation of bridge bonds with divalent metal ions (Bartolome et al., 1972). On the other hand, it was reported that the decrease in the degree of esterification of pectins in preheated chestnuts was the main cause of the hardening and that the bridge bonds between metal ions and pectins were not recognized (Manabe, 1980b). An increase in cation-uptake capacity during the “hard-to-cook” development of cowpeas resulted from a loss of membrane integrity, leading to a passive flux of cations into cells (Liu et al., 1992b). Thus the role of the divalent ions in the tissue components is not well understood as yet.

In contrast to such treatments as heat and storage, pressure treatment without heat can mechanically damage the cell membranes in a very short time. This phenomenon suggests that pressure treatment could be an interesting new procedure for investigating the hardening mechanism for the purpose of controlling vegetable hardness. In this study, to clarify the hardening mechanism by the pressure treatment, we examined the relationship between the pressure-induced hardening and the concentrations of the calcium and magnesium ions in pectin fractions. By removing the divalent metal ions from the sample using chelating agents after pressure release, the effect of these metal ions on the pressure-induced hardening was investigated.

MATERIALS AND METHODS

Sample Preparation. Japanese radish used in this study was bought in a retail store on the day of experiment. The sample using the middle portion of the Japanese radish was cut into segments of 1 cm × 1 cm × 1 cm by means of a die. The sample preparation was done using the method reported previously (Yamamoto et al., 1992).

Pressure, Chelating, and Cooking Treatments. *(a) Pressure Treatment.* About 40 pieces of samples (ca. 40 g) were put into a polyethylene capsule with 0.01 mm thickness walls and packed with a vacuum sealer (SQ-202, Sharp Co., Ltd.). The samples were treated at 400 MPa for 10 min at room temperature using a high pressure equipment (R7K-3-10-5, Yamamoto Suiatsu Co., Ltd.). The time required to reach the

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Table 1. Variations of Pectin Fractions of Pressurized Japanese Radish with Standing Time (mg/100 g of tissue)[a]

		pressurized				
		standing time (h)				
pectin fraction	non-treated	0	2	7	12	24
WP[b]	56.3 ± 1.0[a]	36.1 ± 1.8[b]	32.7 ± 0.6[b]	33.9 ± 1.2[b]	27.1 ± 0.7[c]	28.6 ± 0.5[c]
PP[b]	141.3 ± 2.6[a]	157.5 ± 3.3[b]	163.3 ± 3.8[bc]	166.5 ± 1.5[c]	163.5 ± 3.2[c]	171.1 ± 3.4[d]
HP[b]	95.0 ± 2.6[a]	79.0 ± 0.9[b]	78.4 ± 1.5[b]	78.4 ± 1.5[bc]	88.7 ± 2.3[ac]	83.4 ± 6.2[bc]

[a] Samples were pressurized at 400 MPa for 10 min and left at atmospheric pressure after pressure release. [b] WP, water-soluble pectin; PP, hexametaphosphate-soluble pectin; HP, hydrochloric acid-soluble pectin. The means marked by different roman letters in the same row are significantly different ($p < 0.05$).

prescribed pressure was about 1.3 min, and the pressure was released to 0.1 MPa in about 0.3 min.

(b) Standing after Pressure Treatment. After the pressure release, the samples taken out from the package were left on the testing sieve of 0.71 mm aperture (Tokyo Screen Co., Ltd.) and wrapped loosely with a polyvinylidene chloride film (Saran Wrap, Asahi Kasei Co., Ltd.). They were left under 60% humidity for 0−24 h at 20 °C. The zero time here means that there is no standing time after the sample pressurization at 400 MPa for 10 min.

(c) Soaking Treatments. Samples a and b were either soaked in deionized water, chelating agents in deionized water, 0.07 M EDTA (ethylenedinitrilotriacetic acid, Tokyo Kasei Co., Ltd.), or 0.07 M EGTA (ethylenedioxybis-*N,N,N,N*-tetraacetic acid, Kantou Kagaku Co., Ltd.) for 6 h. Solutions of EGTA and EDTA were adjusted to pH 7.0 with 2 M NaOH (Liu et al., 1992a). The weight ratio of the sample to the soaking solution was 1:6. Soaking was carried out at 20 °C and 60% humidity. Sample a was left for 12 h after soaking treatment under atmospheric pressure, and sample b was soaked after 12 h standing.

(d) Cooking Treatment. About 40 g of samples b and c was cooked in 7 L of distilled water in a water bath (Yamato Science Co., Ltd.) at 99.5 °C for 5 min. After cooking, the samples were cooled down in flowing water of about 15 °C for 30 s and wiped with filter paper.

Measurement of Hardness. The hardness of samples c and d was measured by a texturometer (GTX-2, Zenken Co., Ltd.) under the following conditions: The plunger used was a V-type, the clearance was 1 mm, and the bite speed was 6 times/min. The average hardness (*N*) was obtained from the measurements of seven to ten samples.

Measurement of Pectins. Pectin was measured by the carbazole method (McComb et al., 1952). Alcohol-insoluble solid (AIS) was obtained using 40 g of the samples. AIS of 0.4 g was fractionated into three portions, water-soluble pectin (WP), 4% hexametaphosphate-soluble pectin (PP), and 1 M hydrochloric acid-soluble pectin (HP). The pectin content in each portion was determined by a spectrophotometer (UV-2000, Shimadzu Co., Ltd.) at 520 nm. A calibration curve was made with anhydride galacturonic acid (15−60 μg/mL). The content of pectin was calculated in the form of anhydride galacturonic acid.

Measurement of the Degree of Esterification of Pectin Fractions. The degree of esterification of each pectin fraction was measured by gas chromatography in the previous paper (Yamamoto, 1992).

Measurement of Metal Ions. The calcium and magnesium ions contents of the each pectin portion were determined by atomic absorption spectrophotometry (AA-660, Shimadzu Co., Ltd.). The pectin solutions were diluted by 1% hydrochloric acid. Lanthanum chloride was added to suppress such anionic interference as phosphoric ions; the final concentration was 1000 ppm. Wavelengths used were 442.7 and 285.2 nm for the calcium and magnesium ions, respectively.

Measurement of Different Forms of Calcium Ions. Minamide's method (1986) was applied to fractionate the calcium ions. Deionized distilled water was added to a 20 g sample. After the samples were homogenized, the calcium ions were extracted using deionized water, 1 M sodium chloride, 2% acetic acid, and 5% hydrochloric acid. The calcium content of each fraction was determined by the method mentioned above.

Statistical Analysis. The data sets were subjected to the analysis of variance (STATISTICA, THREE'S Company, Inc.) to determine least significant differences (LSD) among non-treated and pressurized samples. All expreriments were repeated three or four times.

RESULTS AND DISCUSSION

Variations of Pectin Fractions with Time after Pressure Release. First of all, it is important to analyze pectins for a better understanding of the mechanism of the hardening of vegetables. It is well-known that the pectin fraction bound to metal ions play a role in controlling the tissue strength. Thus we have fractionated pectins into the water-soluble pectin (WP; free), the hexametaphosphate-soluble pectin (PP; bridged by divalent metal ions), and the hydrochloric acid-soluble pectin (HP; combined to cell wall components as cellulose). We have examined how the time dependence of the pectin fractions is related with the hardening of the pressurized sample with standing time.

As seen in Table 1, each pectin fraction shows the largest change just after the pressurization. WP and HP are decreased by the application of pressure up to 400 MPa for 10 min, whereas PP is increased. The decrease in the sum of WP and HP in excess of the increase in PP is due to the effusion of the pectins by pressurization. Although the increase in PP corresponds to the hardening mechanism known, its variation with time after pressure release exhibits a small change. Such a small change corresponds to the marked increase in the hardness with standing time (Kasai et al., 1995). However, we cannot see any quantitative relation between pectin fractions and the hardness here. The variations of WP and HP with standing time are also small.

The pressure-induced increase in PP suggests that the deesterification of pectins occurs. To confirm this, we have analyzed the degree of the esterification of the pectin fractions. The results are summarized in Table 2. The esterification degree of WP is significantly decreased by the application of high pressure. The slight decrease continues until 12 h after pressure release. The esterification degrees of PP and HP are changed neither by pressurization nor by standing. The decrease in the degree of esterification of WP causes the suppression of the β-elimination (Albersheim et al., 1960) and contributes to the hardening by the pressure treatment. However, the relatively weak time dependence of the esterification degrees of pectins after pressure release does not correspond to the increase in the hardness with standing time. The reason for this is unknown at present time. In the next step, therefore, we need to study how the concentrations of the divalent metal ions contained in the pectin fractions vary with pressurization and standing.

Variations of Divalent Metal Ions Contained in Pectin Fractions with Standing Time. Pectins are

Table 2. Variation of the Degree of Esterification in Each Pectin Fraction of Pressurized Japanese Radish with Standing Time[a]

pectin fraction	non-treated	pressurized, standing time (h): 0	2	7	12	24
WP[b]	50.2 ± 5.5[a]	33.6 ± 5.5[b]	26.9 ± 2.9[bc]	25.7 ± 2.8[c]	23.2 ± 4.5[c]	21.6 ± 1.5[c]
PP[b]	9.8 ± 4.4[a]	8.9 ± 2.0[a]	7.8 ± 0.5[a]	7.3 ± 2.4[a]	7.4 ± 2.3[a]	6.9 ± 1.0[a]
HP[b]	9.0 ± 1.6[a]	9.9 ± 1.1[a]	10.0 ± 2.8[a]	11.7 ± 3.9[a]	9.7 ± 3.5[a]	9.5 ± 1.0[a]

[a] Pressurized samples and pectin fractions are the same as in Table 1. [b] The same meaning as in Table 1.

Table 3. Variations of the Concentrations of Calcium and Magnesium Ions in Each Pectin Fraction of Pressurized Japanese Radish with Standing Time (mg/g of Each Pectin Fraction)[a]

	non-treated	pressurized, standing time (h): 0	2	7	12	24
calcium ion						
in WP[b]	43 ± 9.5[a]	46.3 ± 4.8[a]	31.8 ± 5.6[b]	21.1 ± 2.3[c]	30.5 ± 1.5[b]	30.6 ± 1.2[b]
in PP[b]	18.9 ± 1.1[a]	17.3 ± 1.0[b]	15.4 ± 0.7[c]	16.1 ± 0.5[bcd]	17.2 ± 0.5[bd]	15.8 ± 0.5[cd]
in HP[b]	5.8 ± 0.8[a]	6.4 ± 0.8[a]	6.2 ± 0.5[a]	6.1 ± 0.7[a]	7.6 ± 0.4[b]	6.5 ± 0.3[a]
magnesium ion						
in WP[b]	9.4 ± 0.7[a]	12.9 ± 1.0[b]	10.0 ± 1.6[a]	7.4 ± 0.2[c]	11.6 ± 0.7[b]	11.5 ± 0.2[b]
in PP[b]	1.0 ± 0.1[a]	1.2 ± 0.1[a]	1.2 ± 0.1[a]	1.1 ± 0.1[a]	1.2 ± 0.02[a]	1.1 ± 0.04[a]
in HP[b]	6.7 ± 0.1[ab]	7.1 ± 0.2[c]	6.4 ± 0.3[a]	6.8 ± 0.1[bc]	7.7 ± 0.1[d]	7.6 ± 0.2[d]

[a] Pressurized samples and pectin fractions are the same as in Table 1. [b] The same meaning as in Table 1.

Table 4. Variations of Various Forms of Calcium Ions of Pressurized Japanese Radish with Standing Time (mg/100 g of tissue)[a]

form	non-treated	pressurized, standing time (h): 0	2	7	12	24
effusion[b]	0[a]	1.0 ± 0.3[b]	0.9 ± 0.2[b]	1.0 ± 0.1[b]	0.9 ± 0.1[b]	0.9 ± 0.2[b]
water-soluble[b]	5.5 ± 0.6[a]	4.3 ± 0.1[b]	4.1 ± 0.1[b]	4.1 ± 0.3[b]	3.5 ± 0.3[c]	3.5 ± 0.4[c]
sodium chloride-soluble[b]	5.2 ± 0.3[a]	5.7 ± 0.1[ab]	5.6 ± 0.1[ab]	5.9 ± 0.1[abc]	6.1 ± 0.2[bc]	6.3 ± 0.2[c]
acetic acid-soluble[b]	3.1 ± 0.3[a]	3.1 ± 0.3[a]	3.0 ± 0.1[a]	3.2 ± 0.1[a]	3.1 ± 0.3[a]	3.0 ± 0.3[a]
hydrochloric acid-soluble[b]	1.6 ± 0.1[a]	1.7 ± 0.2[a]	1.8 ± 0.2[a]	1.8 ± 0.1[a]	1.8 ± 0.2[a]	1.7 ± 0.2[a]

[a] Pressurized samples are the same as in Table 1. [b] The same meaning as in Table 1.

considered to increase the tissue strength by forming bridge bonds to metal ions (Bartolome et al., 1972). To confirm this, we have analyzed the amounts of calcium and magnesium ions that are the main divalent ions of Japanese radish in the pectin fractions and those contained in the whole tissue.

As shown in Table 3, calcium and magnesium ions in the pectin fractions are changed little by pressurization or standing time except for the decrease in the calcium concentration in WP. The invariance of the concentration of both ions despite the pressure-induced increase in PP shown in Table 1 implies that the amount of these ions in PP is increased by pressurization. For a similar reason, the amount of calcium ions in WP is concluded to be decreased by pressurization. This can be explained by considering that the binding of calcium ions to WP is replaced by that to some other components. The contents of calcium and magnesium ions in the whole tissue (ca. 15 and 5 mg %, respectively) show a slight decrease by pressurization due to effusion, but they don't change during standing after pressure release; data are not shown.

The hardening induced by the heat treatment of potatoes (Bartolome et al., 1972) and the storage of beans (Jones et al., 1983) has been interpreted in view of the increase in the amount of calcium and magnesium ions in the crude cell walls. These observations suggest that divalent metal ions are bound not only to pectins but also to some other components.

Time Dependence of Different Forms of Calcium Ions in the Tissue. It is conceivable that the existing form of calcium ions is changed because their concentration in WP is decreased with time after pressure release (Table 3). Calcium ions are separated into the following forms: the water-soluble calcium (mainly water soluble organic acid salts and calcium ion), 1 M NaCl-soluble calcium (calcium pectate, calcium-bound protein, and calcium carbonate), 2% acetic acid-soluble calcium (calcium phosphate), and 5% hydrochloric acid-soluble calcium (calcium oxalate). To see whether they are related to the hardening of pressurized Japanese radish or not, the variation of each form of calcium ions with standing time has been examined.

As shown in Table 4, in general, the variation of each form of calcium ions with pressurization and standing is relatively small in view of the experimental error. Although the standing time dependence of most of the calcium forms is quite small, the water-soluble form shows a detectable decrease after 12 h standing. This suggests that some of free calcium ions are gradually transformed into bound forms.

In the present work, we have observed that the changes in pectins (increase in PP and decrease in the degree of the esterification) occur by the pressure treatment, but their variations with time after pressure release are relatively small. The mass transfer processes followed by the collapse of the cell membranes by pressure treatment are considered to cause the hardening during standing by changing the interactions among the tissue components.

Effect of Calcium and Magnesium Ions on the Hardness of the Pressurized Samples. We have

Table 5. Effects of Calcium and Magnesium Ions on the Hardness of Pressurized Japanese Radish

treatments[a]				hardness (*N*)	
sample	pressurizing			before cooking[b]	after cooking[b]
A	no	water	–	25.2 ± 0.9^{a}	12.6 ± 2.2^{a}
B[c]	no	water	–	36.4 ± 2.2^{b}	19.5 ± 0.4^{b}
C[c]	no	EGTA	–	31.3 ± 0.7^{c}	10.4 ± 1.1^{a}
D[c]	no	EDTA	–	0.9 ± 0.3^{d}	nm[d]
E	yes	standing	water	43.4 ± 1.9^{e}	40.9 ± 0.6^{c}
F	yes	standing	EGTA	38.7 ± 2.1^{b}	20.4 ± 4.3^{b}
G	yes	standing	EDTA	2.3 ± 0.4^{d}	nm[d]
H	yes	water	standing	43.1 ± 1.3^{e}	35.8 ± 0.3^{d}
I	yes	EGTA	standing	39.3 ± 0.3^{b}	11.7 ± 1.8^{a}
J	yes	EDTA	standing	0.7 ± 0.2^{d}	nm[d]

[a] Samples E, F, and G were left at atmospheric pressure after pressure release and then soaked. Samples H, I, and J were soaked after pressure release and then left at atmospheric pressure. Pressure treatment, 400 MPa for 10 min; chelating treatment, 20 °C for 6 h under atmospheric pressure; standing treatment, 20 °C for 12 h under atmospheric pressure; cooking, 99.5 °C for 5 min. Non-treated samples were only soaked. [b] Means marked by different roman letters in the same column are significantly different ($p < 0.05$). [c] Samples B, C, and D are heated at 99.5 °C for 45 s before the chelating to destroy the cell membranes. [d] nm: The hardness could not be measured due to the destruction of the tissue by cooking.

been unable to explain the pressure-induced hardening accompanying standing time dependence on the basis of chemical analyses mentioned above. It is important to examine whether calcium and magnesium ions play a key role in controlling the pressure-induced hardening. We can clarify this by removing these metal ions from the pressurized samples hardened enough. We measured the hardness of the pressurized samples soaked with or without chelating agents that bind with metal ions in the tissue. To know whether the divalent metal ions are necessary to pressure-induced hardening, we also reversed the order of standing and soaking. The chelating agents used are EGTA, which is a calcium ion specific chelator, and EDTA, which is a chelator for di- and multivalent ions. Samples soaked in deionized water in place of chelating agents were prepared as a reference before the chelating treatments.

Table 5 shows the hardness of the non-treated (A–D) and pressurized samples (E–J) before and after cooking at 99.5 °C for 5 min. The pressurized samples E–G were soaked after standing, and samples H–J were soaked just after pressurization and then left to stand. Non-treated samples B–D were cooked at 99.5 °C for 45 s to destroy the cell membrane for the diffusion of chelating agents. Sample B caused an avoidable heat-induced hardening only to a small extent in the high-temperature region (Kasai et al., 1994). Hence we can consider the difference between samples B–D and samples E–J is mainly due to pressure-induced hardening.

As shown in Table 5, before cooking, the hardness of samples C and F where calcium ions are removed decreased up to about 90% of samples B and E, respectively. On the other hand, the hardness of samples D and G where calcium and magnesium ions are removed decreased up to several percent of samples B and E. This indicates the strong influence of magnesium ions on the vegetable hardness. After cooking, the hardness of samples C and F is equal to half that of samples B and E. This result means that the effect of calcium ions on the hardness of the sample after cooking is larger than that before cooking. This suggests that the factors affecting the hardness before cooking are pectins and other cell wall components. However, the influence of pectins decreases drastically due to β-elimination after cooking. The calcium ions, therefore, seem to bring about some interactions with tissue components other than pectins. The hardness of sample F is significantly higher than that of sample C. It is speculated that the pressure-induced hardening is also caused by some factors other than the calcium ion binding. Furthermore, the hardness of samples D and G could not be measured due to the destruction by heat. This fact also shows the great influence of magnesium ions on the hardness.

Finally, we compare the effect of the order reversal of the chelating and the standing on the hardness. The degrees of hardness of samples E, H and F, I before cooking are almost the same. After cooking, however, samples E and F are significantly harder than samples H and I, respectively. The hardness of sample I where calcium ions are removed just after pressure treatment is about one-half that of sample F where calcium ions remain during standing. The difference in the hardness between samples F and I indicates that calcium ions play an important role in pressure-induced hardening. In other words, in the case of sample F, some interactions occur among tissue components not involving the calcium binding during standing after pressure release. Although sample I contains magnesium ions, no hardening occurs. This supports the occurrence of interactions among tissue components, such as hemicellulose and proteins, under the influence of the calcium ions.

Concerning the effect of the divalent metal ions on the pressure-induced hardening, we conclude that the magnesium ions mainly affect the hardness of uncooked samples and that the calcium ions affect that of cooked samples. It is also suggested that the interactions among the tissue components other than the bridge bonds between metal ions and pectins are related to the hardening under the influence of calcium ions. In this study, we have found that the role of divalent metal ions is more complex than we expected.

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