Biosci. Biotechnol. Biochem., **73** (6), 1257–1261, 2009

PFG-NMR Study for Evaluating Freezing Damage to Onion Tissue

Hiroko ANDO,[1] Mika FUKUOKA,[2] Osato MIYAWAKI,[3] Manabu WATANABE,[2] and Toru SUZUKI[2,†]

[1]*Department of Biological and Environmental Engineering, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*
[2]*Department of Food Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-847, Japan*
[3]*Department of Food Science, Ishikawa Prefectural University, 1-308 Suematsu, Nonoichi-Machi, Ishikawa 921-8836, Japan*

Received September 30, 2008; Accepted January 29, 2009; Online Publication, June 7, 2009
[doi:10.1271/bbb.80681]

We assessed the damage to onion tissue due to freeze-thawing as the water permeability determined by using PFG-NMR and light microscopy. The water diffusion in fresh onion tissue was restricted due to cellular barriers, and the estimated water permeability was 6.99×10^{-6} m/s. The water diffusion became considerably less restricted after freeze-thawing; the convergent value for the restricted diffusion coefficient increased and the water permeability significantly increased to 2.85×10^{-5} m/s. While NMR could detect a distinct change in the diffusion behavior of water molecules in freeze-thawed tissue, light microscopy revealed no significant tissue damage. These results suggest that freeze-thawing damaged the vegetable tissues primarily through destruction of the cell membrane rather than the cell wall.

Key words: diffusion coefficient; water permeability; freezing damage; cell membrane

Most vegetables soften after being frozen and then thawed. Destructive changes in the cell wall induced by volume expansion as liquid water transforms into ice has been considered for several decades to be responsible for this effect.[1,2] However, others have postulated that changes in the permeability of the cell membrane to water comprise the mechanism of plant tissue softening after freeze-thawing.[3–7] Indeed, damage to vegetable tissue due to freezing has been evaluated by electrical conductivity.[8] This hypothesis assumes that the cell "membrane" of plant cells has very low water permeability and that plant cells become swollen due to high turgor pressure, whereas the structure of a normal plant cell "wall" is a loose network. Therefore, fresh vegetables appear crispy and juicy. When the freeze-thawing process damages the "membrane," the water permeability increases, thus reducing the turgor pressure of the cells, which softens the vegetable tissues. According to this latter hypothesis, tissue softening after freeze-thawing should be solely attributed to the damaged cell membrane rather than to the cell wall. However, this hypothesis has not been confirmed, because the permeability of the cell membrane after freeze-thawing could not be demonstrated.

The membrane permeability of various types of individual cells has been measured by such methods as microscopic observation of changes in the cell volume under high osmotic pressure[9] and two-laminar flow method.[10] However, most of these measurements have been applied to isolated protoplasts without a cell wall. The properties of the cell membrane in a tissue matrix might not always be identical to that of individually suspended protoplasts. We therefore measured the permeability of the cell membrane *in situ* after freeze-thawing by using the pulse field gradient nuclear magnetic resonance (PFG-NMR) technique.

The PFG-NMR technique, which is basically a modified simple spin-echo experiment for measuring spin-spin relaxation time T_2, has been used to study the translational diffusion of water molecules in a material. "Restricted diffusion" can also be investigated as an alternative application of this technique.[11] When the self-diffusion of water molecules in a small-compartment system such as a cell is measured by PFG-NMR, the diffusion coefficient depends on the diffusion time and usually decays over time. This phenomenon is referred to as restricted diffusion and it can be explained as follows: Since water molecules cannot diffuse freely over a distance longer than the size of the compartment, the average diffusion distance does not always increase with the diffusion time. Therefore, the diffusion coefficient apparently starts to decrease when the water molecules diffuse sufficiently to fill the compartment. Furthermore, as the diffusion time becomes longer, total averaging of intra-cell diffusion is achieved due to coherence and/or restricting the permeability of the cell membrane;[12] that is, like hindered diffusion. Such diffusion time dependence on the diffusion coefficient is used to mathematically describe with a model that tissue is constituted by cells connected with a semi-permeable membrane. In this model, cell size a and permeability P are incorporated as adjustable parameters. This model has been used by several investigators[11–15] to simultaneously determine both these adjust-

† To whom correspondence should be addressed. Fax: +81-3-5463-0585; E-mail: toru@kaiyodai.ac.jp

Abbreviations: D, self-diffusion coefficient [m^2/s]; D_∞, self-diffusion coefficient at diffusion time $\rightarrow \infty$ [m^2/s]; D_0, self-diffusion coefficient at diffusion time $\rightarrow$ zero [m^2/s]; R, NMR echo amplitude; R_0, echo amplitude with zero field gradient; γ, gyromagnetic ratio [Hz/Gauss] (gyromagnetic ratio of proton, 4258 Hz/Gauss); g, magnitude of the field gradient pulse [Gauss/m]; Δ, interval between two field gradient pulses or diffusion time [ms]; δ, gradient pulse duration [ms]; P, water permeability [m/s]; a, distance between barriers corresponding to cell size [μm]

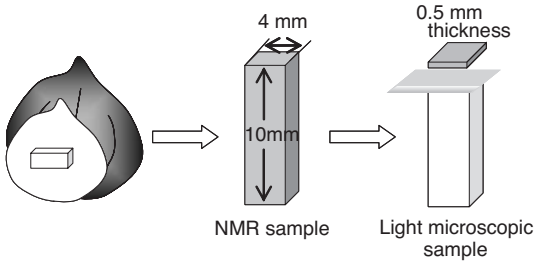

Fig. 1. Preparation of Test Sample for Light Microscopy and NMR Experiments.
The centre of the onion sample was cut to about a $4 \times 4 \times 10$ mm cube.

able parameters, cell size a and membrane permeability P in a biological tissue by fitting to the experimental results. For example, Anisimov *et al.*[12] measured the water permeability of the cell membrane in cotton fiber. However, absolute values for these parameters are difficult to determine from PGF-NMR data alone, since both of these parameters are characteristically alternative and adjustable in nature.

On the other hand, Merboldt *et al.*, 1987[16] have found by NMR-imaging that the diffusion coefficient of water molecules was reduced due to restricted diffusion in onion tissue. Moreover, Weerd *et al.*, 2002[17] have shown by NMR-imaging, using simulation with Fick's second law of diffusion, that the cell membrane permeability of maize tissue changed due to damage caused by such factors as the duration of preservation and freezing. However, it is difficult by the NMR-imaging method to detect the decay of the diffusion coefficient over a wide range of diffusion time from μsec to msec due to such technical problems as the unavailability of a high gradient, even when restricted diffusion actually occurs. NMR-imaging is therefore considered unsuitable for precisely estimating the water permeability in vegetable tissues.

We therefore evaluated in this study the damage to vegetable tissue caused by freeze-thawing by combining data for the water permeability calculated from PFG-NMR data and cell size information obtained from light microscopy. We also compared the effects of freeze-thawing with those of removing the cell membrane from vegetables by using chloroform vapour to understand the mechanism for cell tissue softening induced by freezing.

Materials and Methods

Sample preparation. Fresh onion was used in all experiments within 1 d of purchase from a local retail store. As shown in Fig. 1, the soft core of a fresh onion was cut into a $4 \times 4 \times 10$ mm cube after removing the surface skin layer. Three samples were prepared: fresh after cutting as is, after freeze-thawing, and after the chloroform vapour treatment.

Light microscopic observation. The onion tissue blocks were cut into 0.5-mm-thick slices (Fig. 1), each was each placed on a glass slide and then immediately observed at room temperature without staining with a BX51 microscope (Olympus Optical Co., Tokyo, Japan) equipped with an LK-600PMS cold stage (Japan High Tech Co., Fukuoka, Japan), before the image was recorded. The specimen was then cooled on the stage to $-20\,^{\circ}C$ at a rate of $-5\,^{\circ}C$/min,

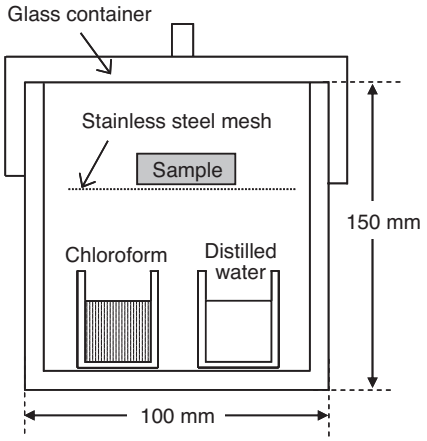

Fig. 2. Glass Container Used for Preparing of Cell-Membrane-Free Onion Tissue with Chloroform Vapour.
The onion sample tissue was placed in the glass container saturated with chloroform vapour for 3 h at 25 °C.

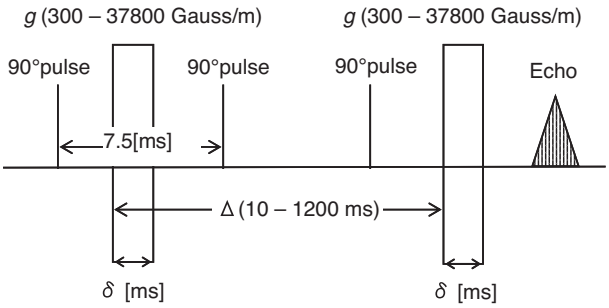

Fig. 3. NMR Pulse Sequence for Diffusion Measurements by the Stimulated Echo Method.

before being immediately thawed to room temperature and observed again under the microscope. This image was also recorded. A chloroform vapour treatment was also carried out to remove the cell membrane from the tissue.[18] A 0.5-mm-thick sliced onion tissue sample on a glass side was put into a glass container, in which a 50-ml beaker containing with 30 ml of chloroform and another 50-ml beaker containing 30 ml of deionized water had been placed to saturate the container, and kept for 3 h at room temperature as shown in Fig. 2. The specimen, *i.e.*, the cell-membrane-free tissue, was stained with 1.0% Safranin (Wako Pure Chemical Industries, Osaka, Japan) and then observed by the same light microscopic system.

NMR measurement. A block of fresh tissue was wrapped in polypropylene film before NMR measurements. A freeze-thawed sample for the NMR analysis was also prepared by placing a second block in a commercial freezer at $-18\,^{\circ}C$ before thawing at room temperature, and a third sample, the cell-membrane-free tissue, was exposed to saturated chloroform vapour for 3 h as already described. We also measured a drip solution from the freeze-thawed onion tissue as a reference material for deterministic the restricted diffusion. The drip solution was obtained from cut tissue placed in several centrifuge tubes (10 ml, $\phi 16.2 \times 82.5$ mm; Tomy Seiko Co., Tokyo, Japan) which were centrifuged at 100,443 *g* and 0 °C for 30 min. The supernatant was retained as the drip sample.

Each sample was placed in an NMR tube (5 mm internal diameter), and subjected to Bruker AM200WB NMR spectrometry with a 4.7 Tesla magnet equipped a pulse field gradient accessory. The experiments were conducted with stimulated echo pulse sequence with the magnitude of the field gradient pulse g varying from 300 to 37800 Gauss/m at selected diffusion time Δ (Fig. 3). All experiments were carried out at $20 \pm 2\,^{\circ}C$.

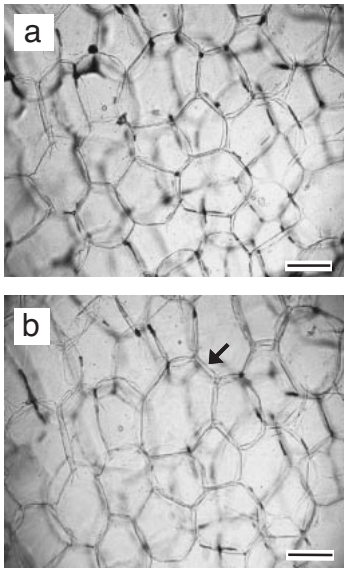

Fig. 4. Microscopy Images of Onion Tissue Samples before and after Freezing.

The change of the cell wall structure (indicated by the arrow) was hardly apparent after freeze-thawing by when viewed by light microscopy. a, fresh tissue (untreated); b, frozen-thawed tissue. Scale bar, 100 μm.

Echo amplitude R obtained in this pulse sequence with field gradient pulse g can be expressed by:

$$\ln(R) = \ln(R_0) - D(\gamma g\delta)^2(\Delta - \delta/3) \quad (1)$$

where R_0 is the echo amplitude with zero field gradient. Self-diffusion coefficient D was obtained from the slope of a linear ln(R) *vs.* $(\gamma g\delta)^2(\Delta - \delta/3)$ plot. In the case of restricted diffusion, the diffusion coefficient obtained from the slope changed with diffusion time Δ.

Results and Discussion

Comparison of tissues before and after freeze-thawing by using light microscopy

Although the onion tissue sample after freeze-thawing showed slight broadening of the membrane-cell wall interface as shown by the arrow in Fig. 4, no significant destruction of the morphology of the cell wall construction could be recognized after freeze-thawing. In other words, the cell size did not apparently change due to freeze-thawing. There exists a real barrier in appearance, even if it may have lost the resistance characteristics against water permeability. It is however known that the fusion of some vegetable membranes after freeze-thawing could be observed by electron microscopy[19,20] at far higher resolution than that of light microscopy. We therefore assumed the cell size before and after freeze-thawing to be *ca.* 150 μm as estimated from the images by light microscopy.

Chloroform dissolves phospholipids in the cell membrane and thus the cell membrane can be selectively removed from various tissues.[16] This has become the standard technique for removing the cell membrane from animal tissues.[21] The application of this technique has resulted in significant changes in the rheological characteristics of carrot tissues.[22] However, our light microscopy findings revealed minimal changes in onion tissues after exposure to chloroform (Fig. 5).

Safranin stained the nuclei more clearly in those tissues stripped of their cell membrane than in fresh tissue as shown by the arrow in Fig. 5b. The structure of cotton cell walls is loose enough to allow large molecules such as safranin to permeate.[23] However, the intact membrane in untreated cells probably acts as a barrier to penetration by large molecules, and thus the dye could not stain the nuclei *in situ* before exposure to chloroform. The finding that safranin stained the cell nuclei after exposure to chloroform indicates that the cell membrane had been selectively eliminated from the onion tissue. Moreover, the cells exposed to chloroform appeared to retain their compartmental structure despite the absence of a membrane.

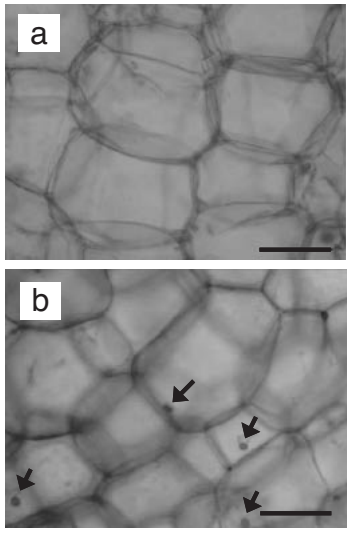

Fig. 5. Microscopic Images of Onion Tissue Samples before and after Chloroform Exposure.

The cell structure was stained with 1.0% Safranin. The cell nucleus after the chloroform exposure treatment was well stained (indicated by an arrow). a, fresh tissue (untreated); b, after chloroform exposure. Scale bar, 100 μm.

Diffusion of water molecules

Figure 6 shows a typical plot of the NMR echo amplitude, ln(R) *vs.* $(\gamma g\delta)^2(\Delta - \delta/3)$, for a fresh onion tissue sample at fixed diffusion time Δ of 30 ms. The diffusion coefficient, D, of water molecules could be calculated from the slope of the straight line. The relationship between $\ln(R)$ and $(\gamma g\delta)^2(\Delta - \delta/3)$ was linear for all tested samples at all diffusion times >30 ms. Therefore, diffusion coefficients were obtained at various diffusion times.

When the self-diffusion of water molecules measured by PFG-NMR is applied to a compartment system such as cells, the diffusion coefficient usually decreases with increasing diffusion time Δ. Figure 7 shows that, while the diffusion coefficient of the fresh onion tissue sample also decreased with increasing diffusion time Δ, the change gradually decreased and approached a constant value. To more easily compare the results from each sample, the diffusion coefficient was normalized by dividing by diffusion coefficient D_0 at minimum diffusion time $\Delta = 10$ ms. At diffusion time Δ of 1200 ms, the diffusion coefficient approached a constant value (0.59×10^{-9} m^2/s). This diffusion behavior is evidence for restricted diffusion in which the water molecules cannot move freely due to a barrier such as the cell wall or membrane. In contrast, the diffusion coefficient of

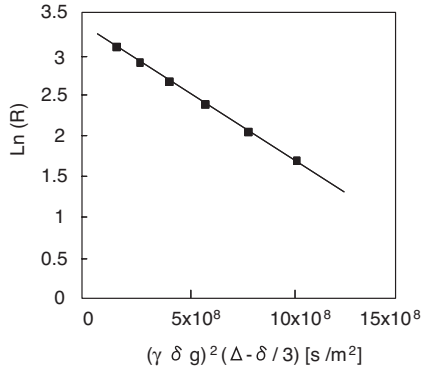

Fig. 6. Relationship between ln(R) *vs.* $(\gamma\delta g)^2(\Delta - \delta/3)$ in Fresh Onion Tissue When Diffusion Time Δ Was 30 ms.

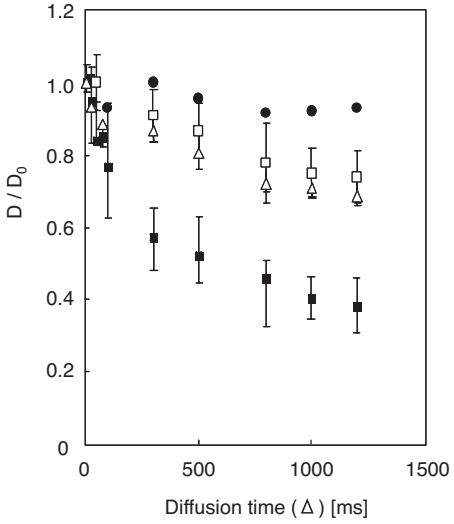

Fig. 7. Measured Diffusion Coefficients of Water in Fresh and Treated Onion Tissue Samples as Function of Diffusion Time.
■, fresh tissue (untreated) (n = 5); □, frozen-thawed tissue (n = 5); △, cell-membrane-free tissue (n = 5); ●, drip (n = 1). Error bars indicate the range of maximum to minimum.

the freeze-thawed sample only slightly decreased with diffusion time Δ, indicating less restricted diffusion of the water molecules. The result for cell-membrane-free tissue which has been treated by chloroform vapour was similar to that of the sample after freeze-thawing. The diffusion coefficient of the drip solution without a cell structure remained constant independently of the observation time, indicating unrestricted diffusion. These results suggest that diffusion was restricted due to the nature of the cell structure. Since freeze-thawing and chloroform exposure both destroyed the function of the cell membrane, water molecules could diffuse more freely into the inner and outer cells of the onion tissue. However, such diffusion was still weakly restricted, presumably by the cell wall, compared with that in the drip solution, in which diffusion was not restricted.

Estimation of water permeation through the membrane

The permeability of the intercellular barrier was estimated from the relationship between diffusion coefficient D and diffusion time Δ, based on a one-dimensional structural model.[11] In this model, diffusion coefficient D_∞ for infinite diffusion time Δ, obeys Eq. 2,

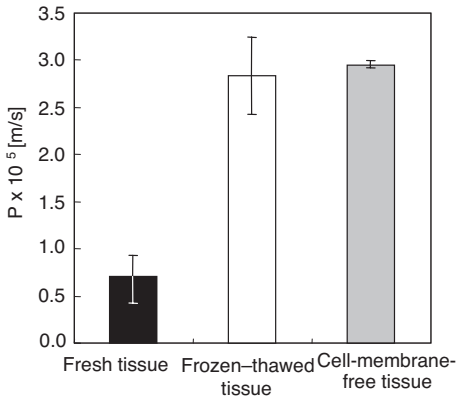

Fig. 8. Estimated Permeability of the Onion Cell Membrane *in Situ* in Fresh, Frozen-Thawed and Cell-Membrane-Free Tissues. Error bars indicate the range of maximum to minimum.

$$1/D_\infty = 1/D_0 + 1/(P \times a) \qquad (2)$$

where D_0 is the diffusion coefficient at $\Delta \rightarrow$ zero.

Many investigators have estimated the permeability of plant[12] and animal tissues[13,14] by using a model based on the assumption that the diffusion of water molecules between a cell compartment (barrier) such as the cell wall and membrane was one-dimensional. However, when the restriction to diffusion weakens, as in onion tissue after freeze-thawing or exposure to chloroform, the error in estimating the cell size becomes large, since heterogeneity in the cell size as a barrier to water diffusion increases. This considerably affects the accuracy of permeability measurements, since both parameters of cell size a and permeability P are characteristically alternative and adjustable in nature.

We therefore fixed cell size parameter a by using independently obtained microscopic data. This modified method allowed a more accurate estimate for the permeability of the water molecules.

Thus, the known parameter values for fresh onion tissue ($a = 150\,\mu m$ from the microscopic data; $D_\infty = 0.59 \times 10^{-9}\,m^2/s$ at $\Delta = 1200$ ms; $D_0 = 1.52 \times 10^{-9}\,m^2/s$ at $\Delta = 10$ ms from the NMR data) were introduced into Eq. 2, which resulting in estimated water permeability P being 6.99×10^{-6} m/s. This is similar to the reported permeability value of 1×10^{-5} m/s[12] for several fresh plant cells. The water permeability of the onion cell membrane is about 10^{-6} m/s according to a study using plasma cells.[24] This agreement between our data and the literature values demonstrates the validity of the assumption that the actual cell size from microscopic data should be equivalent to the compartment size on the model. In turn, it suggests that materials and subcompartments such as vacuoles inside the cell membrane or wall have negligible effect on the restricted diffusion phenomenon of water molecules in onion tissue compared with the resistance of the outer membrane.

On the other hand, the estimated water permeability, P, of onion tissue after freeze-thawing ($a = 150\,\mu m$; $D_\infty = 1.50 \times 10^{-9}\,m^2/s$; $D_0 = 1.11 \times 10^{-9}\,m^2/s$) was 2.85×10^{-5} m/s. This value was considerably larger in freeze-thawed than in fresh onion tissue. Moreover, the water permeability of the cell-membrane-free tissue sample after exposure to chloroform (2.96×10^{-5} m/s) was closer to that after freeze-thawing. Figure 8 shows

that the water permeability of freeze-thawed tissue was up to 10-fold higher than that of fresh tissue. This result demonstrates that freeze-thawing damaged the cell membrane of onion tissues. In other words, we quantified the cell damage in onion tissues due to freeze-thawing by measuring the change in water permeability of the cell membrane. Levitt *et al.*, 1936,[24] Oertli, 1976[9] and Sotome *et al.*, 2004[10] have also measured the water permeability of the cell membrane of protoplasts from vegetables and other plants, but they could not do so for cells *in situ*.

We used here a novel combination of PFG-NMR and light microscopic data to estimate the cell membrane permeability in onion tissues. This new method should serve as a quantitative tool with which to compare degradation in vegetable tissue before and after freezing and help to increase understanding of the mechanism for damage induced by freezing.

In conclusion, we experimentally confirmed by observing the restricted diffusion of water molecules in onion tissue samples that freeze-thawing damaged the cell membrane rather than the cell wall. This insight provides a step towards understanding the mechanism for damage to vegetables caused by freezing. However, the correlation between damage to the membrane and the change in texture or drips after freeze-thawing remains to be resolved, and future studies should consider differences among various types of vegetables.

References

1) Brown MS, *J. Sci. Food Agric.*, **18**, 77–81 (1967).
2) Cano MP, "Freezing Effects on Food Quality," ed. Jeremiah LE, Marcel Dekker, New York, pp. 247–298 (1996).
3) Virgin H, *Physiol. Plant.*, **8**, 954–962 (1955).
4) Steponkus PL, *Ann. Rev. Plant Physiol.*, **35**, 543–584 (1984).
5) Wedd MS, Uemura M, and Steponkus PL, *Plant Physiol.*, **104**, 467–478 (1994).
6) Miyawaki O, *Cryobiol. Cryotechnol.*, **44**, 43–50 (1998).
7) Dejmek P and Miyawaki O, *Biosci. Biotechnol. Biochem.*, **66**, 1218–1223 (2002).
8) Ishikawa E, Miyawaki O, and Nakamura K, *Biosci. Biotechnol. Biochem.*, **61**, 1826–1830 (1997).
9) Oertli JJ, "Water and Plant Life," eds. Lange OL, Kappen ED, and Schulze ED, Springer-Verlag, Berlin, pp. 19–31 (1976).
10) Sotome I, Oshita S, Kawagoe T, Torii T, and Seo Y, *Trans. ASAE*, **4784**, 1207–1213 (2004).
11) Tanner JE, *J. Chem. Phys.*, **69**, 1748–1754 (1978).
12) Anisimov AV, Sorokina NY, and Dautova NR, *Magn. Reson. Imaging*, **16**, 565–568 (1998).
13) Meerwall VE and Ferguson RD, *J. Chem. Phys.*, **74**, 6956–6959 (1981).
14) Kinsey ST, Locke BR, Penke B, and Moerland TS, *NMR Biomed.*, **12**, 1–7 (1999).
15) Clark CA and Bihan DL, *Magn. Reson. Med.*, **44**, 852–859 (2000).
16) Merboldt KD, Hanicke W, and Frahm J, *Ber. Bunsenges. Phys. Chem.*, **91**, 1124–1126 (1987).
17) Weerd L, Claessens MMAE, Efde C, and As HV, *Plant Cell Environ.*, **25**, 1539–1549 (2002).
18) Ohnishi S and Miyawaki O, *Food Sci. Technol. Res.*, **11**, 52–58 (2005).
19) Uemura M, Tominaga Y, Kamata T, Nakagawara C, Kawamura Y, and Kojima K, *Cryobiol. Cryotechnol.*, **50**, 15–20 (2004).
20) Yamada T, Kuroda K, Jituyama Y, Takezawa D, Arakawa K, and Fujikawa S, *Planta*, **215**, 770–778 (2002).
21) Råbergh CMI and Lipsky MM, *Aquat. Toxicol.*, **37**, 169–182 (1997).
22) Ohnishi S, Fujii T, and Miyawaki O, *Food Sci. Technol. Res.*, **9**, 367–371 (2003).
23) Carpita N, Sabularse D, Montezinos D, and Delmer DP, *Science*, **205**, 1144–1147 (1979).
24) Levitt J, Scarth GW, and Gibbs RD, *Protoplasma*, **26**, 238–248 (1936).