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Optimal Conditions for the Distribution of Cryoprotectant into the Intact Fish Muscle of *Oncorhynchus mykiss* during Freeze/Thaw Cycling

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Conditions for sufficient and rapid distribution of a cryoprotectant (sorbitol solution) into intact fish muscle (*Oncorhynchus mykiss*) were studied as changing in the residual Ca^{2+} ATPase activity during freeze/thaw cycling. Chunks of the fish muscle were immersed in 4 concentrations of sorbitol solutions (20%, 30%, 45%, and 60%) by a shaker mechanism at 5℃. Whole immersion samples (W) showed a higher value of the residual Ca^{2+} ATPase activity than those in the untreated controls (C), except in the treated controls (TC), while less effect of immersion concentration could be found. Comparing the extent of penetration of sorbitol into the surface layer to inner layer of immersed fish chunks, outer portion samples achieved excellent cryoprotection with 100% of the residual ATPase activity values or more. For the inner portion samples, 30% and 45% sorbitol solution treatments indicated ahigher ATPase activity than 60% treatment. At high concentrations, mass transfer rates during osmotic dehydration might berapid and it causes faster surface drying by dewatering at surface solute layer. Periodically immersed and relaxed samples, W (5-3-1), led to good cryoprotection effect: W (5-3-1) indicated high residual Ca^{2+} ATPase activity values and the residual ATPase activity values excess 100% in immersion of 30% and 45% sorbitol solutions.

Key words: Cryoprotectant, Intact trout fish meat, Sorbitol, ATPase activity, *Oncorhynchus mykiss*

Introduction

Freezing process has been employed for the purpose of keeping the preservation of muscle protein and preventing textural weakening of fish muscle (Matsumoto and Noguchi, 1992). Quality of fish muscle, however, might be deteriorated or changed by freezing process. That is, the denaturation of myofibrillar protein in the fish muscle might occur during frozen process (Matsumoto, 1979; Matsumoto and Noguchi, 1992). Carvajal et al. (1999) found that the infusion of intact fish muscle with cryoprotectants stabilized their proteins during frozen storage. Fish muscle (myofibrillar) proteins are known to be more labile to denaturation than the contractile proteins of homeotherms commonly converted to meat, including beef, pork, and poultry (Connell, 1961; Hashimoto et al., 1982). Hence, the denaturation of fish protein easily occurs during frozen storage. Muscle protein aggregation, textural changes, and loss of gelling and water-holding functionality in fish are known to occur during frozen storage (Haard, 1992; Niwa, 1992).

Compounds that stabilize proteins in solution showed effective cryoprotection effect (Carpenter and Crowe, 1988). Sugar and polyols increase the stability of proteins in solution, which is due to stabilizing intramolecular hydrophobic interaction (Back et al., 1979). These compounds also increase preferential hydration of the protein and concomitantly the free energy of cavity formation, hence, thermodynamically it could not be favorable for protein denaturation and its contact surface area with the solvent increase by a solute exclusion mechanism (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982). Both the concentration and stereochemistry of the sugar or polyol play an important role to determine the extent of stabilization, since the interactions of the compounds with water are determined by the spacing and

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orientation of the hydroxyl groups (Franks et al., 1972; Tait et al., 1972; Uedeira, 1980).

Sucrose and sorbitol are used as principal cryoprotectants during freezing or frozen storage process because of low cost, availability, and low tendency to cause Maillard browning in the typical white Japanese kamaboko (surimi gel) (MacDonard and Lanier, 1994). However it could be difficult for the cryoprotectants to achieve within tissue of intact fish muscle, comparing to minced muscle. The mechanism of mass transfer for the samples immersed into sorbitol solution can be hypothesis as follows: (1) At first, sorbitol solution is diffused into the fish chunk by the difference of the sorbitol concentration, thereby increasing the osmotic pressure at surface. (2) Osmotic pressure at surface depending on the concentration at the surface reached a critical value, so that the surface structures are ruptured and shrunk due to water loss. (3) At high concentrations, mass transfer rates during osmotic dehydration are rapid, and it causes faster surface drying by dewatering at surface solute layer.

The principal functional property of fish muscle is the ability of stabilized actomyosin to form cohesive gels in mild salt solution. The ability of actomyosin to form cohesive gels is correlated to its Ca^{2+} ATPase activity (Kawashima et al., 1973; Fukuda et al., 1984). Stabilization of myofibrillar protein by cryoprotectants (low MW carbohydrates) is based on manufacture of stabilized fish or surimi (MacDonard et al., 1990; Okada, 1992). The loss of Ca^{2+} ATPase activity of fish actomyosin on mechanism for stabilization of fish myofibrillar proteins by cryoprotectants has been studied (MacDonald et al., 1994; 1996; Noguchi and Matsumoto, 1970; Noguchi, 1974).

The objective of this study was to optimize conditions for sufficient and rapid distribution of a cryoprotectant, sorbitol solution, into intact muscle of rainbow trout (*Oncorhynchus mykiss*). The cryoprotection effect was explained as changing in the residual Ca^{2+} ATPase activity during freeze/thaw cycling.

Materials and Methods

Materials

Fillets of North Carolina rainbow trout (*Oncorhynchus mykiss*) were cut into rectangular chunks (dimensions 3×2×1 cm). Liquid sorbitol solution (70% w/w), which is supplied by Roquette America by Roquette America (Keokuk, IA), and deionized water were used to adjust to 4 kinds of sorbitol solutions (20%, 30%, 45%, and 60% sorbitol w/v). All other chemicals were purchased from Sigma (St. Louis, MO).

Incorporation by immersion

Fish meat chunks were immersed (infused) in sorbitol solutions at a weight ratio of 10:1 cryoprotectant solution to avoid significant dilution of the medium during the diffusion period. The chunks and sorbitol solution were in plastic beakers, and hardware cloth (0.64 cm mesh) was placed on top of the fish chunks to ensure complete immersion. The solution was agitated during the immersion time by shaker mechanism at 5℃.

Cryoprotection of meat chunks by immersion/diffusion of solute

Samples immersed in each sorbitol solutions were individually packaged in Fisher brand polypropylene sampling bags and allowed to equilibrate in a refrigerator before first freezing, followed by 9 freeze/thaw (F/T) cycles: one cycle is still air frozen for at least 8 hrs at -20℃ and then thawed at 5℃ for 12 hrs to accelerate freeze-related degradation. The samples were assayed for ATPase activity after 1 F/T cycle and 9 F/T cycles. For most treatments except I/O samples, two pieces were used to constitute one sample for ATPase activity.

Treatments of fish meat chunk samples

Fish meat chunks of rectangular were immersed in one of 4 kinds of sorbitol solutions (20%, 30%, 45%, and 60%) for 15 min at 5℃. Fish meat chunks were subjected to one of 6 treatments for each sorbitol solutions as follows: (1) C, no treatment control; (2) TC, treatment control (5% dry sorbitol was added into minced fish meat chunks); (3) W, whole immersion sample; (4) I/O, Inner/Outer portions samples (three pieces of immersed chunks were sectioned using a meat slicer into inner and outer (the upper and lower 1/4 of chunk thickness); (5) W (5-3-1), the other whole immersion sample (Fish meat chunks were immersed for 5 min and withdrawn out of sorbitol solution for 10 min, immersed again for 3 min and withdrawn for 10 min followed by immersion for 1 min); (6) TC (imm), immersion treatment control (The minced fish meat mixed with 5% dry sorbitol, was immersed in each sorbitol solution).

Ca^{2+} ATPase activity assay

Fish meat (7.5 g) was blended with 67.5 g chilled

0.6 M KCl (pH 7.0) using a Sorvall Omnimixer for 10 sec on/off intervals at speed 2 for a total time of 1 min. Excessive heating during extraction was avoided by placing the blender cup in ice. Ca^{2+} ATPase activity of sample was measured using a modified method of MacDonald et al. (1996). In a microcentrifuge tube, 850 μL of 33.8 mM Tris-maleate, 5.8 mM $CaCl_2$ buffer (pH 7.0), and 100 μL of fish solution were added and brought to 25℃ for 1 min. Then 50 μL of 20 mM ATP substrate solution (pH 7.0) was added, and the solution was lightly vortexed. Samples were incubated for 3 min in microcentrifuge tubes placed in a water bath of 25℃. The reaction was stopped by adding 500 μL chilled 15% trichloroacetic acid. Samples were microcentrifuged at 12,500×g for 10 min. in a Marathon Micro A Centrifuge (Fisher Scientific, Pittsburg, PA). The concentration of inorganic phosphate released during incubation was measured by the Fiskke and Subbarow (1925) using 0.25 mL of the sample solution; 0. 9 mL of 5.77 M ammonium molybdate in 1.43 N H_2SO_4 and 1.25 mL of Elon reagent (316 mM sodium bisulfite; 50 mM p-methyl-amino-phenol hemisulfate). The total ATPase activity was defined as micromoles inorganic phosphate liberated per g of fish per 3 min assay (μM Pi/g fish/3 min) using a standard curve made with potassium phosphate at 25℃. Phosphate determinations were made in quadruplicate.

Statistical analysis

Statistic analyses were carried out using the Statistical Analysis System (SAS Institute, 1999). Significant differences between treatment means were determined by using Duncan's multiple range tests. Significance of differences was defined at the $p<0.05$ level.

Results and Discussion

Treated and Untreated Controls

The residual Ca^{2+} ATPase activity after 9 freeze/thaw cycles for the untreated (C) and the treated control (TC) samples were compared. ATPase activity values at 1 freeze/thaw cycle can be used as initial values. Generally 8% sucrose or sorbitol, alone or mixture, to leached fish muscle (w/w), has been served as a cryoprotectant in manufacture of Alaska pollack surimi (MacDonald and Lanier, 1994). Since sucrose and sorbitol give a considerable sweetness, however, it is necessary to reduce sweetness during freezing process (Noguchi and Matsumoto, 1975; Park et al., 1988; Sych et al., 1991a, b). Therefore, as minimum sorbitol level to cryoprotect fish muscle, 5% dry sorbitol was added to meat chunks followed by thorough chopping of the meat to mix the sorbitol well. The mixed meat paste was produced as treated control (TC). The untreated control resulted in 76.42% of the residual Ca^{2+} ATPase activity. A level of 5% sorbitol mixed into the fish mince resulted in 100% of the residual Ca^{2+} ATPase values or more. The treated control showed better cryoprotection effect than the untreated control. This value well explains the role of sorbitol as a cryoprotectant, and thus this value can be used as the standard value for comparing the cryoprotection effect on sorbitol solution.

Immersed Samples

Whole immersion samples (W) showed a higher value of the residual Ca^{2+} ATPase activity than those in the untreated controls (C) except in treated controls (TC) (Fig. 1). For whole immersion samples, the residual Ca^{2+} ATPase activity was increased slightly with sorbitol concentrations. Less effect on immersion concentration, however, could be found. Minimum cryoprotection level was not achieved in any concentrations. Sorbitol solution uptake showed almost same value. Considering the mechanism of mass transfer, initially water might be diffused from the outer layer of the fish meat to osmotic medium,

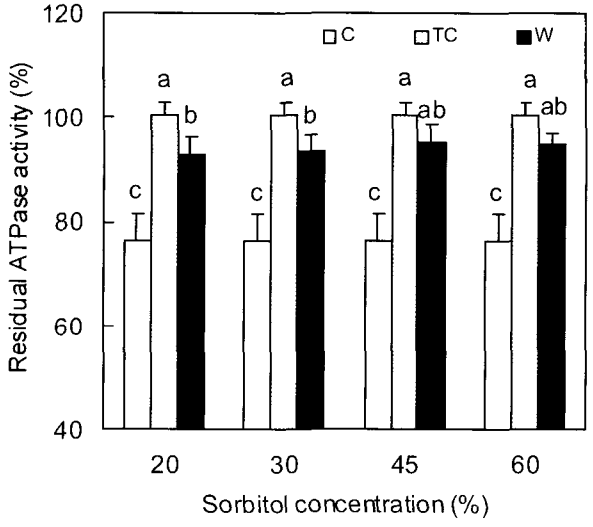

Fig. 1. Changes in the residual ATPase activity for whole immersion sample in the varied sorbitol concentrations. C: no treatment control, TC: treatment control (5% dry sorbitol was added into minced fish meat chunks), W: whole immersion sample. Different alphabetical letters indicate a significant difference ($p<0.05$).

thereby increasing the osmotic pressure at surface layer of the meat. As the osmotic pressure reached a critical value, the cell membranes are ruptured and shrunk due to water loss. Shrinkage causes loss of contact between cell membranes and cell well (Rastogi et al., 2000). The rupture of the surface structure might impede further mass transfer of sorbitol into the fish meat. The rupture of the surface structure could be promoted in the increased concentrations.

Inner and Outer Portion Samples

Under the consideringthe extent of penetration of sorbitol into the surface layer, Ca^{2+} ATPase activity remaining in outer layer and inner layer of immersed fish chunks were compared (Fig. 2). Salvatori et al. (1998) observed the structural changing of the inner surface of apple slices immersed in sucrose solution (65% w/w) from the interface of apple slices to the center plane. The result showed that, whereas inside of apple cell walls were completely convex, near the interface they appeared full of cavities due to shrinking and deformation to 2.5 mm depth from the interface. In the similar research (Bolin et al. 1983), the apple slices immersed in concentrated sucrose solution showed the rapid formation of a concentrated sucrose surface layer under the less than 2 mm depth. Here, the immersed fish meat chunks were separated

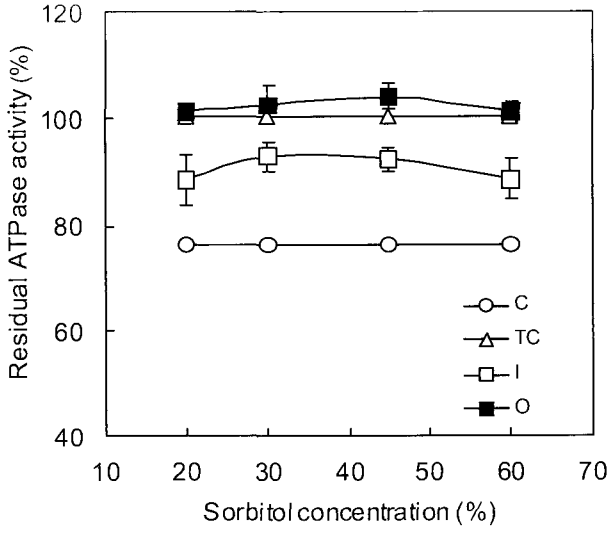

Fig. 2. Changes in the residual ATPase activity in inner and inner layers of fish meat chunks immersed in the varied sorbitol concentrations; ○: C, △: TC, □: I, ■: O. C: no treatment control, TC: treatment control (5% dry sorbitol was added into minced fish meatchunks), I/O: Inner/Outer portion samples of fish meat chunks.

according to the extent of penetration of sorbitol into the surface layer of fish muscle. Therefore, the three pieces of immersed chunks were sectioned to the upper and the lower 1/4 of chunk thickness (about 2.5 mm depth) into the inner and the outer portions. As we expected, the outer portion samples of the fish meat chunk showed a higher value of the residual ATPase activity than the inner portion samples. For the outer portion samples, achieving excellent cryoprotection with 100% of the residual ATPase activity values or more, which indicate an adequate cryoprotection, was mostly formed in any concentrations. Although we expected that no differences was seen between the untreated chunk samples (C) and the inner portion samples of the fish meat chunk (I), the inner portion samples showed slightly higher values of the residual ATPase activity than the untreated samples. However, good cryoprotection was not achieved in all inner portion samples. The diffusion of sorbitol into fish meat chunks might not be enough at the bottom in the beaker due to hardware cloth placed on the top of the chunks. The diffusion of sorbitol into fish meat chunks penetrated into deeper than 2.5 mm, but not completely. For the inner portion samples, 30% and 45% sorbitol solution treatments indicated a higher ATPase activity than 60% treatment.

At high concentrations, mass transfer rates and faster surface drying can be explained from the comparing the immersion treatment control (TCimm) to another sample (Fig. 3). The minced fish meat, added with 5% dry sorbitol, was immersed in each sorbitol solution as immersion treatment control (TCimm). It seems that the immersion treatment control samples (TCimm) follow the same propensity of treated control (TC) except 60% sorbitol treatment sample. Immersion of treatment control led to rather decrease the cryoprotection effect of sorbitol solution, comparing to the treatment control (TC). The highest decrease of cryoprotection effect was found in 60% sorbitol treatment, and the concentration dependence was not found in 20, 30, and 45% sorbitol solution treatments. Comparing to the whole immersion samples (W) (Fig. 4), TCimm samples indicated a lower value of the residual Ca^{2+} ATPase activity than the whole immersion samples (W) in 60% sorbitol solution treatment.

Periodical Immersing and Relaxing

It is necessary to slake mass transfer rate and relax diffusion of sorbitol solutions into fish meat chunks

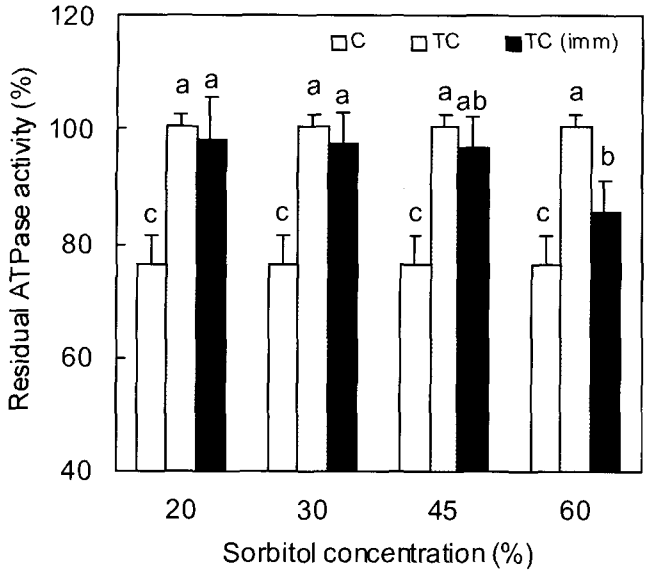

Fig. 3. Effects of sorbitol immersion treatment on treatment control samples. C: no treatment control, TC: treatment control, TCimm: immersion treatment control (the minced fish meat, 5% dry sorbitol was added into, was immersed in each sorbitol solution). Different alphabetical letters indicate a significant difference ($p<0.05$).

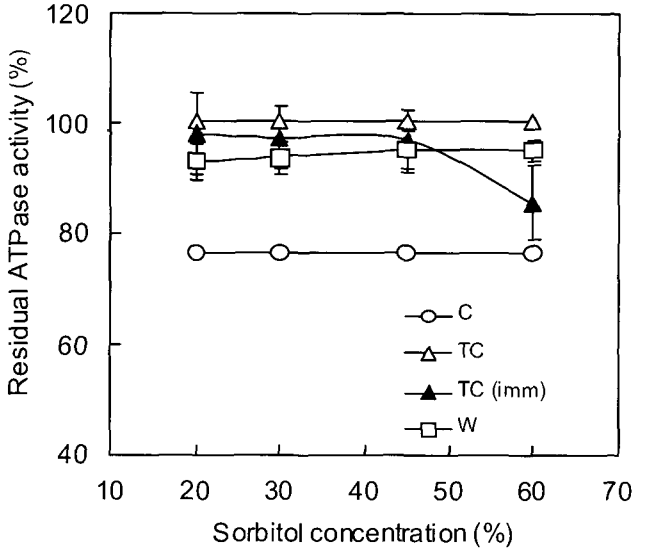

Fig. 4. Comparing the residual ATPase activity of the immersion treatment control samples to the whole immersion samples in the varied sorbitol cencentrations; ○: C, △: TC, ▲: TCimm, □: W, C: no treatment control, TC: treatment control, TCimm: immersion treatment control (the minced fish meat, added with 5% dry sorbitol, was immersed in each sorbitol solution), W: whole immersion sample.

in order to achieve the rapid and sufficient penetration or diffusion of cryoprotectants into the intact fish muscle with minimal damage of the meat surface. Giving enough time for diffusion of sorbitol into the fish meat chunks, we might secure more unaffected special volume or surface area, since shrinkage at the outer surface was occurred in local but not overall. Here, the other whole immersion sample, W (5-3-1), was designed to relax the diffusion of sorbitol solutions. Fish meat chunks were immersed for 5 min and drain out of sorbitol solution for 10 min, immersed again for 3 min and drain for 10 min, followed by immersion for 1 min. Two pieces were used to constitute one sample for Ca^{2+} ATPase activity, and the sample was assayed for Ca^{2+} ATPase activity after 1 F/T cycle and 9 F/T cycles as the other immersed whole samples. Although the other whole treatment samples W (5-3-1) had a shorter total immersion time (9 min) than the W samples (15 min), the other whole treatment samples W (5-3-1) indicated a higher value of the residual Ca^{2+} ATPase activity. This might be caused by a high sorbitol concentration inside of fish chunks in whole range of sorbitol concentrations from 20% to 60% (Fig.

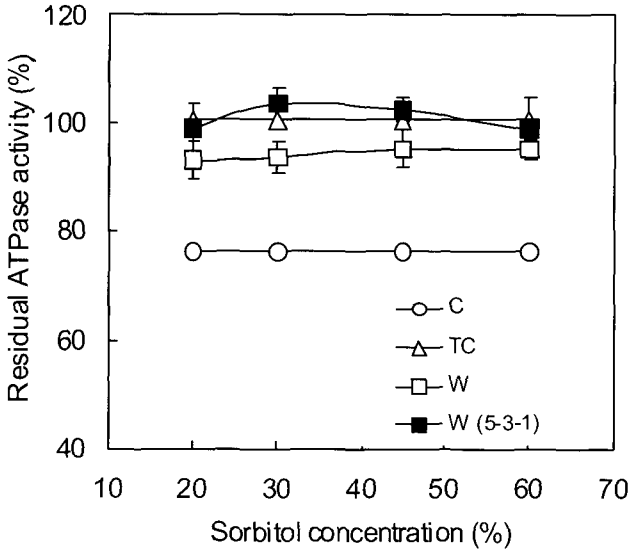

Fig. 5. Effects of sorbitol concentrations on periodically immersed and relaxed samples; ○: C, △: TC, □: W, ■: W(5-3-1). C: no treatment control, TC: treatment control, W: whole immersion sample, W(5-3-1): the other whole immersion sample (fish meat chunks were immersed for 5 min and were withdrawn out of sorbitol solution for 10 min, then those were immersed again for 3 min and withdrawn for 10 min, followed by immersion for 1 min).

5). For W (5-3-1) samples, the residual ATPase activity values excess 100% in immersion of 30% and 45% sorbitol solutions. W (5-3-1) treatment indicated better cryoprotection effect than whole immersion sample (W). This indicated that periodically immersing and relaxing treatment made a good cryoprotection effect.

We can hypothesize a possible mechanism to get a higher and more uniform sorbitol concentration for better cryoprotection effect. That is the process slaking osmotic pressure at surface and processing the mass transfer from the outer portion to inner portion in fish meat chunks without impeding by surface rupture as follows: (1) Immersing the sample into sorbitol solution before sorbitol concentration at the surface reaches a critical value. (2) Allowing the relaxation time for the mass transfer only from the outer portion to the inner portion induced by the difference of sorbitol concentration between the two portions by withdrawing the sample from the sorbitol solution, that is, cutting off the de-watering effect leading the rupture of the surface structure. Thereby the surface of the fish chunks is achieved a lower sorbitol concentration. (3) After getting the moderate sorbitiol concentration at the surface of the fish chunks, immersing the samples into the sorbitol solution again before the concentration at the surface reaches a critical value. (4) Repeating this process.

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