Application of Antifreeze Protein for Food Preservation: Effect of Type III Antifreeze Protein for Preservation of Gel-forming of Frozen and Chilled Actomyosin

WARAPORN BOONSUPHTIP AND TUNG-CHING LEE

ABSTRACT: Type III antifreeze protein (AFP) remarkably preserved Ca\textsuperscript{2+} ATPase activity of actomyosin during frozen and chilled storage. Under frozen conditions, AFP helped to retain the Ca\textsuperscript{2+} ATPase activity of actomyosin much higher than that of conventional cryoprotectants (sucrose-sorbitol mixture). The Ca\textsuperscript{2+} ATPase activity increased with increasing AFP concentration and reached a maximum at 50g/L AFP. After 3-d chilled storage, the Ca\textsuperscript{2+} ATPase activity of control and sucrose-sorbitol samples had lost 80% and 50%, respectively, while the AFP samples remained unchanged. A Type III AFP mechanism based on freezing temperature depression (more unfrozen water) and inhibition of ice recrystallization that protects against the freezing of muscle proteins in chilled or frozen conditions is proposed.

Keywords: antifreeze protein (AFP), actomyosin, gel forming, Ca\textsuperscript{2+} ATPase activity, surimi, surimi product, frozen and chilled storage

Introduction

By their structural characteristics, Antifreeze Proteins (AFPs) are classified into four types: AFP type I, II, and III, and antifreeze glycoprotein (AFGP). Their properties include depressing freezing temperature without affecting the melting temperature (so-called thermal hysteresis), modulating ice crystal morphology, inhibiting ice crystal growth (recrystallization), enhancing cellular integrity, and reducing microbial growth (Hew and Yang 1992; Feeney and Yeh 1993; Griffith and Ewart 1995; DeLuca and others 1996; Chapsky and Rubinsky 1997; Harding and others 1999). The first 3 properties are associated with the interactions between AFP, water, and ice. The rest of the properties occur at temperatures a little above the freezing point of a system (for example, chilled systems).

AFPs have been shown to be useful in several systems, such as living cells (Rubinsky and others 1994; Chao and others 1996) or tissue and organs (Rubinsky and others 1994), in chilled and frozen storage. Only a small number of uses in foodstuffs have been reported. All of them involved frozen storage. However, according to their properties, AFPs are likely to be good preservatives, not only for frozen food but also for chilled food. To date, the only potential application of AFPs that has been published is the maintenance of small ice crystals as the most defined properties of AFPs (Griffith and Ewart 1995). In addition, studies of AFP application seem to be limited to liquid systems such as frozen dairy or ice cream products (Fletcher and others 1997; Lillford and others 1998; Clemmings and others 2000; Darling and Hoddle 2001), because AFPs are easily dispersed in such food matrices through simple mixing. Only one study has employed bovine meat. Small pieces of bovine meat were soaked in a concentrated AFP solution and drained to dry (Payne and others 1994). AFP was shown to help maintain the ice crystal size in the frozen meat; however, a problem was that the soaking time of the meat in the AFP solution was so long (2 wk) that the sample deteriorated. To avoid this problem, Feeney and Yeh (1999) suggested that lambs be injected with AFP before slaughter. With this approach, the frozen lamb meat had less drip loss and smaller ice crystals. Other AFP applications in foodstuffs should be explored with investigation of underlying mechanisms. This study focused on investigation of feasibility of AFP to preserve surimi (fish-gel) and surimi products by monitoring Ca\textsuperscript{2+} ATPase activity of actomyosin as a model study. These products offer a food matrix in which AFP can be uniformly incorporated by mixing and are, therefore, a feasible application. The investigation included chilled and frozen storage conditions.

The quality of surimi and surimi products is subject to the gel-forming ability of the constituent muscle proteins (Niwa 1992; Ohshima and others 1993; An and others 1996). To obtain a good quality product, one has to start immediately after the fish are slaughtered. Normally, before and during processing the fish is kept at a low temperature to minimize auto-degradation (Pearson 1986) and microbial spoilage (Lee 1992). The muscle proteins undergo proteolytic degradation with storage. For example, gel strength (compressive force) of red hake (Urophycis chuss) was reduced to 46% after being stored on ice for 3 d, and to 63% when stored in chilled seawater (Lee 1986). The gelling properties of squawfish (northern pikeminnow or Psocochelius), which was stored on ice for 24 h, was reduced by about 15% of the original values (Lin and Morrissey 1994). Unfortunately, no food preservative can effectively inhibit this muscle protein deterioration under chilled conditions.

Frozen storage is required to further prolong the shelflife of the finished products. However, it initiates another problem: freeze-induced denaturation of the muscle proteins (Matsumoto and Noguchi 1992). This denaturation can be prevented to some extent by cryoprotectants. Cryoprotectants used commercially are sucrose and sorbitol (4% each) (MacDonald and Lanier 1994; Carvajal and others 1998). However, these sugars result in sweetness which is undesirable for some types of food products and for some...
consumers (for example, consumers who hate sweetness and/or have health concerns such as diabetes). At present, a substitute preservative without this drawback is not commercially available. AFPs may be an answer.

The objective of this study is to investigate the potential usefulness of a Type III AFP in preservation of the gel-forming properties of fish muscle under frozen and chilled conditions. Fish muscle (Arai and others 1973) was selected as a model system of gel-forming products because it is thermally less stable than other gel-forming muscle proteins such as beef, rabbit, and chicken (Connell 1961). It more rapidly deteriorates under chilled and frozen conditions. Ca2+ ATPase activity was used to evaluate the gel-forming capacity of actomyosin which was normally used as an indicator of the quality of the muscle proteins (MacDonald and Lanier 1994; Carvajal and others 1999). The investigation of the chilling preservation ability of AFP for food is a novel application. No finding on this subject has ever been reported, and only a few publications have introduced unique functionalities of AFP for nonfood matters. For example, AFP was demonstrated to improve the viability of cell suspensions, tissues, and whole organs during chilled storage (Rubinsky and others 1994). Interaction between AFP and the cell membrane is suggested to be a protective mechanism.

Materials and Methods

The purified antifreeze protein (Yang and others 1998) was donated by Professor Daniel S.-C. Yang (Dept. of Biochemistry, McMaster Univ., Hamilton, Ontario, Canada L8N 3Z5 and Ice Biotech, Inc.). It is classified as a Type III antifreeze protein. Its molecular weight is approximately 8 kDa. Live tilapia hybrids obtained from a local retail store (New Brunswick, N.J.) were used as a source of actomyosin. All reagents were reagent grade.

Actomyosin (AM) preparation

The fish were gently removed from a tank, immediately sacrificed, and packed on ice. AM extract was prepared within 1 hr while the flesh was in the prerigor state (Korhonen and others 1990). The method of AM preparation was modified from MacDonald and Lanier (1994). 10-g sliced flesh kept on ice was homogenized in 100 mL chilled 0.6M KCl and 25mM Tris-Maleate (pH 7.0) using a tissue homogenizer (Polytron, Type PT10203500, Kinemetch GmbH, Switzerland) at speed Nr 4, using 25-s on and off periods for a total of 5 min. To prevent any deterioration from the excessive heating, the homogenizer blade was chilled and homogenization was conducted on ice. The extract was centrifuged at 5000 × g for 30 min at 4 °C and held at 0 °C. AM in the supernatant was precipitated with three volumes of chilled distilled water for 1 h and collected by centrifugation (5000 × g for 20 min). The AM pellet was redissolved in two volumes of chilled 0.825M KCl and 34.5mM Tris-Maleate (pH 7.0) for 1 h and centrifuged (5000 × g for 20 min) to remove nondissolved substances from the AM solution. The cryoprotectant or the AFP was added to this final AM extract suspension, stirred by a stirring bar for 1 h and then subjected to a storage temperature condition. Two different ranges of AFP concentrations were investigated. One was a high concentration range, 10 to 100 g/L AM extract, and the other was a low concentration range, 0.05 to 0.3 mg/L AM extract. A mixture of 50 g/L sucrose-sorbitol (1:1 by wt) was used to represent 8% sucrose-sorbitol of fish flesh. 10-g flesh provided 5.3-mL AM pellet. After 2 volumes of the KCl and Tris-Maleate solution were added, the AM extract contained 10-g fish flesh in 16 mL AM extract. 0.8 g of the conventional cryoprotectants was added to 16 mL of the AM extract (10-g fish flesh) to obtain 8% cryoprotectants in the fish flesh. This gave the concentration of 50-g Cryoprotectants/ L AM extract. This concentration of proteins in the AM extract was about 5800 to 6400 mg Protein/ 1 AM extract. The control sample was an AM extract containing no cryoprotectants. Ca2+ ATPase activity and protein concentration of the extracts were measured.

Storage conditions

Frozen condition. AM extracts were stored at −20 °C for one week and then subjected to 3, 7, or 10 temperature-fluctuation cycles. Temperature-fluctuation cycles were applied to accelerate the freeze-induced denaturation process of actomyosin (Kim and others 1986; Watanabe and others 1988; Benjakul and Bauer 2000). A single cycle consisted of holding the extract at −20 °C for 16 h and then at −12 °C for 8 h. Usually, frozen surimi products are stored at a temperature lower than −25 °C by manufacturers and at −15 °C to −18 °C by consumers. The temperatures applied here were higher to introduce a condition of severe temperature abuse.

Chilled condition. The extracts were stored at 6 °C for 3 d.

Protein assay

Equivalent protein concentration of the extract was measured using the Protein Assay Kit 11 (Bio–Rad Laboratories Inc., Hercules, Calif., U.S.A.) based on the Bradford dye-binding technique (Bradford 1976). Bovine serum albumin (BSA) was used as a known protein standard.

Ca2+ ATPase activity

The measurement of Ca2+ ATPase activity was modified from the method of MacDonald and Lanier (1994). The AM extract was diluted with 0.6M KCl, pH 7.0 to obtain the final protein concentration of 1.5–3 mg/mL. 0.5–mL of this diluted extract was mixed well with 0.25 mL 0.5M Tris-Maleate buffer (pH 7.0), 0.25 mL 0.1M CaCl2, and 3.75 mL deionized water. To trigger the enzymatic reaction, 0.25 mL 20 mM Na-ATP (pH 7.0) was added to the mixture. The reaction was conducted at 25 °C for 3 min and then immediately stopped by adding 2.5 mL chilled 15% trichloroacetic acid. The sample was then kept cold on ice. The precipitated proteins were removed by centrifugation at 10000 × g for 5 min. The concentration of inorganic phosphate in the sample was measured by the method of Chen and others (1956). 0.01 μM inorganic phosphate results in an absorbance of 0.260 at 820 rim. Ca2+ ATPase specific activity was defined as μM pi/mg protein/min at 25 °C. M is a molarity applied to solution. To compare activities, the control before freezing was set at 100%.

Statistical analysis

Regression analysis was performed using the Statistical Analysis System (SAS 1997) to reveal the concentration effects of the AFP on the Ca2+ ATPase activity. One-way ANOVA analysis of variance and Least Significant Difference (LSD) were performed for comparisons among means.

Results and Discussion

Changes in Ca2+ ATPase activity during frozen storage

When subjected to 3 to 10 temperature-fluctuation cycles, the frozen actomyosin extracts containing the AFP at all the examined concentrations, 10, 50, and 100 g/L, retained their Ca2+ ATPase activity much higher than the controls (Figure 1). For the most extreme denaturation condition of 10 cycles, 10-g/L AFP preserved the enzyme activity approximately 2 times higher than the control. The high activity of the enzyme is assumed to result from the actomyosin which has high gel-forming ability.

This assumption has been commonly accepted in muscle-protein-protection studies of cryoprotectants such as sorbitol (Konno
and others 1997), maltodextrins (Carvajal and others 1999), sodium glutamate (Noguchi and Matsumoto 1970; Akahane and others 1981), sodium lactate (MacDonald and Zanier 1994), and amino acids (Noguchi and Matsumoto 1975). Therefore, our result is evidence that the AFP helped to protect the gel-forming properties of the frozen muscle proteins. This is the first finding of this functionality in AFPs for frozen muscle preservation.

It should be noted that the effect of AFP concentration on the enzyme activity retention of the extracts before (kept on ice for 1 h) and after freezing was different (Figure 1). The higher concentrations of the ECINs helped increase the Ca^{2+} ATPase activity of unfrozen fish muscle. After freezing, the fish muscle lost the Ca^{2+} ATPase activity faster when the AFP concentration increased. Probably, the Type III AFP interacts differently with unfrozen and frozen AM. In addition, this concentration effect of the AFP on unfrozen fish muscle is different from those of spherical dextrose and hydroxypropyl starch hydrolyzate (up to 15% concentration) (Rogols 1995), and sucrose and sodium lactate (up to 20% concentration) (MacDonald and Lani er 1994). These four cryoprotectants decreased the enzyme activity as their concentration increased. This different concentration effect on the enzymatic activity between the Type III AFP and the cryoprotectants supports that their protective mechanisms may be different.

In some cases, the Ca^{2+} ATPase activity retention exceeded 100% (Figure 1). Activity retention is defined as the ratio of Ca^{2+} ATPase activity of frozen sample to that of the unfrozen control sample. When the activity retention is higher than 100%, the frozen actomyosin samples after thawing are in a more suitable configuration for the enzyme reaction than the unfrozen control (Carvajal and others 1999). This excessive enzyme activity phenomenon was also reported with several cryoprotectants (Noguchi and Matsumoto 1970; Akahane 1982; Reid and others 1986; MacDonald and Lani er 1994; Carvajal and others 1999). The studies suggested that it involves an initial unfolding of muscle proteins which is later followed by aggregation (Carvajal and others 1999). The unfolding possibly facilitates the myosin head for both binding ATP and ATP hydrolysis (Momet and others 1989). If this is the case, the AFP seems to have higher facilitating ability than the sucrose-sorbitol mixture (typical cryoprotectant) as shown by the higher enzyme activity retention in Figure 1.

Compared to the sucrose-sorbitol mixture, the AFP was more effective at actomyosin cryoprotectant (Figure 1). The AM extracts containing only 10 g/L AFP retained as much activity as those containing a 50g/L sucrose:sorbitol (1:1 by wt) mixture under all temperature treatments ($p < 0.05$). At 50 g/L AFP, the retention of Ca^{2+} ATPase activity was approximately 1.2 times greater. On a molar basis, the AFP is about $10^6$ to $10^7$ times more effective than the sucrose: sorbitol mixture (Table 1). This illustrates that a molecule of AFP is considerably more powerful than sorbitol-sucrose.

Table 1—Comparison of Ca^{2+} ATPase activity on a molar basis. The extracts contained 50 g/L sucrose:sorbitol (1:1 by wt) (Suc:Sor), or 10, 50, or 100 g/L AFP (F 10, F 50, or F 100, respectively).

<table>
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<tr>
<th>Treatment (Cycle)</th>
<th>Suc:Sor</th>
<th>F 10</th>
<th>F 50</th>
<th>F 100</th>
</tr>
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<tr>
<td>3</td>
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<td>5.24 x 10^{-2}</td>
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<td>7</td>
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<tr>
<td>10</td>
<td>5.29 x 10^{-2}</td>
<td>5.76 x 10^{-2}</td>
<td>1.29 x 10^{-2}</td>
<td>6.91 x 10^{-3}</td>
</tr>
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Table 1—Comparison of Ca^{2+} ATPase activity on a molar basis. The extracts contained 50 g/L sucrose:sorbitol (1:1 by wt) (Suc:Sor), or 10, 50, or 100 g/L AFP (F 10, F 50, or F 100, respectively).

Note: The unfrozen control was set as 100% Ca^{2+} ATPase activity.

Ca^{2+} ATPase activity retention $= \frac{Ca^{2+} ATPase activity of the unfrozen sample \times 100}{Ca^{2+} ATPase activity of a sample}$.

FIGURE 1—Effect of the Type III AFP on the Ca^{2+} ATPase activity of the AM extracts compared to that of the conventional cryoprotectants during frozen storage of several degrees of temperature abuse (3, 7, and 10 cycles of temperature fluctuation). Zero-cycle temperature fluctuation represented the AM extract before being frozen. The extracts contained no cryoprotectant (Control), 50 g/L Sucrose: sorbitol (1:1 by wt) (Suc:Sor), or 10, 50, or 100 g/L AFP (F 10, F 50, or F 100, respectively).

FIGURE 2—Effect of the Type III AFP concentration on Ca^{2+} ATPase activity during frozen storage. Several temperature-fluctuation cycles were applied to introduce different severity of storage temperature abuse.
the frozen domain (Carvajal and others 1998). It remains in the unfrozen water domain due to a higher cohesive force between the cryoprotectant and water than between the cryoprotectant and other constituents. The presence of the cryoprotectant in the unfrozen domain favors thermodynamic enhancement of protein stabilization. The denatured or unfolded stage of the proteins is suppressed in order to minimize protein-solvent surface contact, which further increases free energy of the system (Arakawa and Timashoff 1982). This phenomenon may not occur in the case of AFPs. It is well recognized that AFPs generally depress the freezing point of aqueous systems. A proposed mechanism of this freezing point depression involves the absorption of the AFPs on ice crystals in a manner similar to the enzyme-substrate adsorption (Burcham and others 1986). This mechanism suggests that, in the actomyosin extracts, AFPs would not entirely disperse freely in the unfrozen water domain, but would rather attach (at least partially) to ice crystals. As a result, their presence may interfere with the typical arrangement of both water and ice molecules. It is also likely that it may affect the typical interactions among water and the neighboring components. However, their interference in the unfrozen and frozen domains should be in such a way as to support the stability of the muscle proteins as suggested by the high retention of the Ca$^{2+}$ ATPase activity.

As concentration of the AFP increased, the Ca$^{2+}$ ATPase activity retention also increased by reaching a plateau at about 50g/L (Figure 2). Once the concentration increased twice from 50 g/L to 100 g/L, the activity retentions were not significantly improved. This behavior is different from other cryoprotectants such as sucrose, spherical dextrose, hydroxypropyl starch, hydrolyzate, and lactate salt. The activity retention of the first three cryoprotectants increases linearly as their concentration increases (Rogols 1995). However, it may reach a plateau if higher concentrations of these cryoprotectants are examined. Sodium lactate presented maximum cryoprotection at about 6% concentration with 80% Ca$^{2+}$ ATPase activity recovered (MacDonald and Lanier 1994). A higher concentration of sodium lactate decreased the enzyme activity. This variety of concentration effects of the different cryoprotectants suggests different interactions between the cryoprotectants and actomyosin. The linearly increasing phenomenon of the concentration effect suggests an existence of a favorable interaction(s). The nonlinear phenomenon would involve more complicated interactions. Some promote and some damage the integrity of the muscle proteins. For example, sodium lactate induces the latter one more strongly than the former once the concentration of the cryoprotectant becomes relatively high.

Several studies have supported that freezing temperature depression increased and reached a plateau with an increase in AFP concentration (Devries and others 1970; Feeney and Yeh 1993; Grif-ffith and Ewart 1995). As shown in Figure 2, the same characteristic occurred with the cryoprotection of the enzyme. A further consideration is the relationship between the magnitude of the freezing temperature depression and the amount of unfrozen water in the frozen system, as it may aid an understanding of the cryoprotective mechanism. It is known that the amount of unfrozen water increases as the depression of freezing temperature increases (Heldman 1974; Mannonperuma and Singh 1989). This implies that the ability of AFP to depress the freezing temperature helps to increase the amount of unfrozen water in the frozen AM extracts. It is believed that unfrozen water prevents the frozen muscle proteins from dehydrating, which in turn causes aggregation (Matsumoto and Noguchi 1992). This suggests that the cryoprotective mechanism of AFP is associated with the prevention of muscle protein dehydration as a result of a concentration of unfrozen water. This also helps to explain why, on a molar basis, the AFP provided a much higher Ca$^{2+}$ ATPase activity retention than the conventional cryoprotectants (Table 1). In general, AFPs were 200 to 500 times more powerful than ideal solutes, on a molar basis, in depressing the freezing temperatures of solutions (Devries 1971). As a result, more unfrozen water is expected, leading to greater hydration of the muscle proteins.

The application of AFP at much lower concentrations was inves-
tigated in combination with a 3-cycle treatment. The results are shown in Figure 3. Compared to the control, the AFP in the 0.05 to 0.3 mg/L concentration range provided significant improvement in the levels of enzyme activity retention (p < 0.05). At such low concentrations, AFPs do not usually have significant effects on the freezing temperature depression of a system, but do effectively inhibit ice recrystallization (Griffith and Ewart 1995). Therefore, the improvement in the enzyme activity in this case could involve the AFP inhibition of ice recrystallization, preventing tissue damage from penetration by large ice crystals.

**Change in Ca²⁺ ATPase activity during chilled storage**

The ability of the AFP to retain Ca²⁺ ATPase activity at 6 °C is illustrated in Figure 4. The control AM extract lost 80% Ca²⁺ ATPase activity retention by the 3rd d of storage. The level of activity retention of the AM extract containing sucrose-sorbitol mixture was relatively a little different from that of the control after 3-d storage. Therefore, these cryoprotectants are not effective in protection of fish muscle protein deterioration. In contrast, AFP increased the levels of activity retention which remained constant for 3 d. This was an indicator that the AFP can efficiently preserve the Ca²⁺ ATPase activity during cryopreservation. J Experim Bio 199:207-1-7. 

**References**


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Authors Boonsupthip and Lee are with Dept. of Food Science and Center for Advanced Food Technology, Rutgers, The State Univ. of New Jersey, 65 Dudley Rd, New Brunswick, NJ 08901. Direct inquiries to author Lee (E-mail: lee@aesop.rutgers.edu).