Interaction of Sucrose and Zinc for Cryoprotection of Surimi

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Stabilization of fish myofibrillar proteins by low molecular weight carbohydrates and polyols during frozen storage forms the basis of the surimi process. Studies have shown that certain divalent cations, notably zinc, may enhance the effectiveness of cryoprotective solutes for stabilizing labile enzymes. This study investigated the interaction of sucrose and zinc for cryoprotection of fish actomyosin (AM). Initial freeze−thaw studies, using a model actomyosin system, showed addition of $ZnSO_4$ generally destabilized AM with a loss of Ca^{2+}ATPase activity. This was confirmed by making surimi and comparing denaturation (Ca^{2+}ATPase, salt-soluble protein) and loss of gel-forming ability before and after freeze−thaw treatment. In addition, the surimi studies demonstrated the dual effect of sucrose, that of stabilizing the AM proteins while depressing the gelation properties of the surimi. Prior to freezing, $ZnSO_4$ reversed the effects of sucrose somewhat and improved gelation properties when high levels of sucrose were present.

Keywords: *Fish; protein; gel(ation); cation; freezing; stability*

INTRODUCTION

Stabilization of fish myofibrillar proteins using low molecular weight carbohydrates, such as sucrose and sorbitol, forms the basis of the surimi process whereby highly labile fish myofibrillar proteins are stored in a frozen form suitable for later manufacture into gel-based foods. The principal functional property of interest is the ability of the stabilized actomyosin to form gels in mild salt solution. However, there is a close correlation between the ability of actomyosin to form cohesive gels and its Ca^{2+}ATPase activity (Kawashima et al., 1973; Fukuda et al., 1984). Following the loss of Ca^{2+}ATPase activity of actomyosin solutions has provided the basis of model systems for studying the effects of cryoprotectants (Noguchi and Matsumoto, 1970; Noguchi, 1974).

Recently, in studies using labile enzymes such as phosphofructokinase, a remarkable synergism between cryoprotectant solutes (e.g. sucrose) and certain divalent cations, notably zinc, has been shown to exist (Carpenter et al., 1986, 1987; Hazen et al., 1988). In the presence of just 0.6 mM $ZnSO_4$, the concentration of cryoprotectant needed to protect phosphofructokinase during freezing and thawing could be greatly lowered (e.g. from 500 to 50 mM). As this synergism seems, thus far, to depend neither upon the protein nor the cryoprotectant solute involved (Hazen et al., 1988), the use of zinc ions could provide means for lowering the quantity of cryoprotectants needed to achieve stability of fish myofibrillar proteins in frozen storage. If successful, the cost of cryoprotectant could be decreased as could the level of sweetness compared to current cryoprotectant levels.

Generally, compounds that stabilize proteins in solution are also effective cryoprotectants (Carpenter and Crowe, 1988). Sugars and polyols increase the stability of proteins in solution by stabilizing intramolecular hydrophobic interactions (Back et al., 1979). They also increase preferential hydration of the protein and concomitantly the free energy of cavity formation, making it thermodynamically less favorable for the protein to unfold (denature) and increase its contact surface area with the solvent (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982). The extent of stabilization depends on both the concentration and stereochemistry of the sugar or polyol since the spacing and orientation of the hydroxyl groups determine how these compounds interact with water (Franks et al., 1972; Tait et al., 1972; Uedeira and Uedeira, 1980).

The effects of salts on myosin ATPase activity and structure generally follow the Hofmeister series for most ions studied (Warren et al., 1966; Tonomura et al., 1961; Seidel, 1969; Stafford, 1985). Thus, a salt's ability to stabilize proteins is a balance between its ability to increase the solution surface tension and its binding to anionic sites and peptide bonds on the protein (Arakawa et al., 1990). Increasing surface tension tends to stabilize intramolecular hydrophobic interactions, while binding of ions may stabilize or destabilize depending on the specific interactions between protein and ions (Arakawa and Timasheff, 1984; Arakawa et al., 1990). The ability of a solute to stabilize a protein in solution has been linked to its molal surface tension increment (Melander and Horvarth, 1977; Creighton, 1984). Zinc sulfate, which has a relatively high molal surface tension increment of 2.27×10^3 dyn·g/cm·mol, would therefore be expected to be a good protein stabilizer (Melander and Horvarth, 1977).

Castell et al. (1970) studied the effect of transition metal ions on muscle protein denaturation from various fish species, as measured by extractable protein nitrogen. Over the concentration range 10−50 ppm (ca. 150−750 μM) Zn^{2+} ions had relatively little effect, although the effects of the metal ions differed with muscle from different species. Okada (1963) reported that the low-temperature setting phenomenon in surimi depended on the Hofmeister series in a concentration-dependent manner and that salting-out ions such as sulfate generally inhibit gel formation.

Zinc ions at concentrtions of about 50 μM are also potentiators of muscle contraction and increase the maximum tetanic force along the ascending limb of the length−tension relationship during contraction (Lopez et al., 1981). The mechanism is thought to be related

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to the enhanced release of calcium during excitation−contraction coupling (Taylor et al., 1982).

The objective of this study was to investigate the protective effect and interaction of sucrose and zinc on fish actomyosin (AM) after freezing and thawing.

MATERIALS AND METHODS

Model AM System. *AM Preparation*. Actomyosin was extracted from freshly sacrificed tilapia hybrid (*Tilapia nilotica* × *Tilapia aurea*) by blending 7.5 g of flesh in 75 mL of chilled 0.6 M KCl (pH 7.0) using a Sorvall Omnimixer (DuPont Instruments, Newton, CT). Excessive heating during extraction was avoided by placing the blender cup in ice and blending for 20 s followed by a 20 s interval for a total extraction time of 4 min. The extract was centrifuged at 5000*g* for 30 min (4 °C). Actomyosin was precipitated by diluting with 3 volumes of chilled distilled water and collected by centrifuging at 5000*g* for 20 min (4 °C). The AM was then dissolved by gentle stirring of an equal volume of chilled 1.2 M KCl (pH 7.0) for 30 min (0 °C). The AM solution was centrifuged (5000*g*, 20 min, 4 °C) for a final time to remove insoluble protein. The supernatant was used in the following experiments.

Ca^{2+}ATPase Activity. To a 1 mL AM solution (2−3 mg/mL, pH 7.0, 0.6 M KCl) were added 0.5 mL of 0.5 M Tris−maleate buffer (pH 7.0), 0.5 mL of 0.1 M $CaCl_2$, 7.5 mL of deionized water, and 0.5 mL of 20 mM ATP solution (pH 7.0) and incubated for 3 min at 25 °C. The reaction was stopped by addition of 5 mL of chilled 15% trichloroacetic acid. The concentration of inorganic phosphate released during incubation was measured according to the Fiske and Subbarow (1925) method as modified by Arai (1974). The Ca^{2+}ATPase specific activity was defined as micromoles of inorganic phosphate liberated per milligram of protein [μM P_i (mg of protein)$^{-1}$ min^{-1}] at 25 °C.

Protein Assay. Protein concentrations were measured according to the biuret method (Gornall et al., 1949) using bovine serum albumin as standard.

Effect of Solute Concentration on Freeze−Thaw Stability of AM. To investigate the effect on AM stability during freezing and thawing, samples were frozen and thawed under time/temperature conditions that produced significant AM denaturation when no sucrose was present. Actomyosin solutions (2.5 mg/mL, pH 7.0, 10 mm i.d. polyethylene tubes) with various solute concentrations were frozen in liquid nitrogen (−196 °C) for 3 min. The frozen tubes were held in a bath at −5 °C for 60 min and then thawed in a water bath for 5 min at 25 °C. Tubes of unfrozen and thawed AM were stored (less than 30 min) in ice−water until measurement of Ca^{2+}ATPase activity. A check was done to ensure there was no loss in activity during storage of the solution in ice prior to activity measurement. The activity was used to compare unfrozen to frozen−thawed samples with the unfrozen control equal to 100%.

Preliminary freeze−thaw experiments were done using 0−0.6 M sucrose and also 0.3 M sucrose with various concentrations of zinc sulfate (0−1.0 M). Then response surface models were developed using a 6 × 6 latin square design with $ZnSO_4$ at six levels and sucrose at six levels. This design was repeated for two ranges of $ZnSO_4$ concentration (0−0.4 and 0−0.04 mM) with the sucrose concentration range the same for both sets (0−0.2 M).

Surimi System. To verify the results from the model AM experiments, sucrose and $ZnSO_4$ were added at various levels to washed mince. These mince mixtures were then either made into heat-set gels or repeatedly frozen and thawed and then made into gels to test freeze−thaw stability of the muscle proteins. Salt-soluble protein and Ca^{2+}ATPase activity of unfrozen and frozen−thawed minces were also compared.

Freshly caught Atlantic croaker (*Micropogan undulatas*) were processed as described by Park et al. (1990). Briefly, the fish were filleted, deboned, washed twice in chilled water, and then dewatered to give washed mince used in the following experiment. Final washed mince moisture as measured by oven-drying (105 °C for 18 h) was 82.8% (SD = 0.05%).

Zinc sulfate (0, 0.6 mM) and sucrose (0, 0.1, 0.3 M) were added to the mince by chopping in a vertical cutter/mixer (Stephan Machinery, Columbus, OH) for 1 min, packed into plastic bags, and held for 12 h at 0 °C. Samples were then portioned; half was used for protein extraction (salt-soluble protein and AM Ca^{2+}ATPase activity) and to make gels. The remainder of each mixture was then packed into plastic bags (2 cm depth), vacuum sealed, and subjected to repeated (five times) freeze−thaw cycles during which each sample was frozen at −35 °C for 16 h and then tempered at 5 °C for 8 h. After the final freeze−thaw treatment, the samples were tempered (30 min, 20 °C), salt-soluble protein and AM Ca^{2+}-ATPase activity were measured, and the remainder was made into heat-set gels.

The protein content of the supernatant after extraction and the first centrifugation step as described for the AM extraction above was measured as the protein soluble in 0.6 M KCl and is reported as milligrams of soluble protein per gram of flesh.

Actomyosin was extracted from mince mixtures and the Ca^{2+}ATPase activity was measured as described for the model system.

Gel Preparation and Rheological Properties. To measure the effect of solute level and freeze−thaw treatment on the ability of the myofibrillar proteins to form firm and cohesive heat-set gels, minces were first comminuted in a vertical cutter/mixer. After 1 min of chopping, 2.0% (w/w) salt based on mince weight was added with ice−water to give a final moisture of 82% (w/w). Chopping was continued under vacuum to give a final comminution time of 6 min. The final paste temperature was less than 15 °C. Pastes were placed in a plastic bag and vacuum sealed to remove air pockets. This bag was then placed in a laboratory sausage stuffer, the paste was stuffed into stainless steel tubes (length, 17.75 cm; i.d., 1.87 cm), and sealed for heat treatment. The tubes were heated in a water bath at either (1) 90 °C for 15 min or (2) 25 °C for 2 h followed by 90 °C for 15 min. The tubes were cooled in ice−water.

Gels were removed from the stainless steel tubes and warmed to room temperature (24 ± 1 °C) prior to shaping and testing to fracture in the torsional mode as described by Kim et al. (1986). Briefly, disposable disks were glued to each end of the cylindrical samples (2.87 cm length, 1.86 cm end diameter) with cyanoacrylate glue (Krazy Glue Inc.) for the purpose of gripping the samples in the subsequent grinding and testing steps. Capstan-shaped samples were prepared for torsion testing by grinding the cylindrical cooked gels on a lathe-type apparatus (Gel Consultants, Inc., Raleigh, NC) to a minimum diameter at the center.

The capstan-shaped specimens were twisted at 2.5 rpm to the point of structural failure on a Torsion Gelometer apparatus (Gel Consultants). For each treatment, at least eight specimens from each preparation were tested, with a standard deviation on individual measurements of about 10%. Shear stress and shear strain at failure were calculated from torque and angular displacement using the equations of Hamann (1991).

Statistical Analyses. Surface response models were developed using the General Linear Models (GLM) procedure of SAS (1982). Differences between means were tested for statistical significance using Student's test.

RESULTS AND DISCUSSION

Model AM Studies. *Effect of Sucrose Concentration*. Actomyosin freeze−thaw stability was enhanced as sucrose concentration increased (Figure 1). In this instance a linear relation ($p < 0.001$) was demonstrated with an increase in recovered activity of 56%/mol of sucrose. We have found in other studies (MacDonald and Lanier, unpublished data) that for less stable AM from other fish, the relationship is not necessarily linear since there is greater denaturation when no cryoprotectant is present and low levels of sucrose (less than 0.3 M) have a much greater effect percentagewise than shown by this data.

Increasing sucrose concentration depressed unfrozen AM Ca^{2+}ATPase activity ($p < 0.05$) at a rate of about 21.5%/mol of sucrose (Figure 1). This can be explained

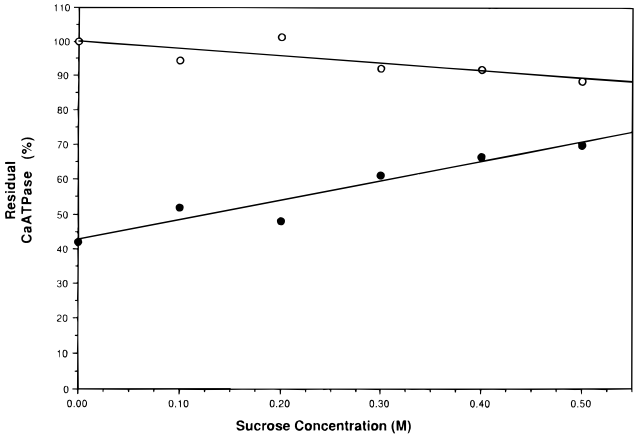

Figure 1. Effect of sucrose concentration on freeze−thaw stability of tilapia actomyosin (2.5 mg/mL, pH 7.0, 0.6 M KCl): unfrozen (○; $y = 100.0 - 21.5$ sucrose concentration, $R^2 = 0.632$) and frozen−thawed (●; $y = 42.4 + 56.3$ sucrose concentration, $R^2 = 0.911$).

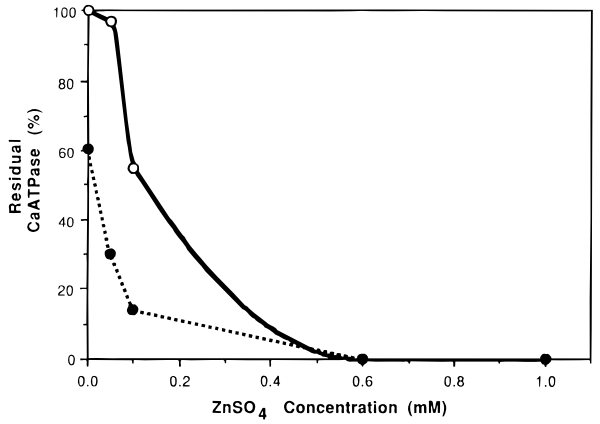

Figure 2. Effect of increasing $ZnSO_4$ concentration on freeze−thaw stability of tilapia actomysosin (2.5 mg/mL, pH 7.0, 0.6 M KCl) with 0.3 M sucrose added: unfrozen (○) and frozen−thawed (●).

by the structuring effect of sucrose on water (Franks et al., 1972; Tait et al., 1972), which would reduce the mobility of water molecules immediately surrounding the protein and consequently slow the rate of ATP hydrolysis. Protein flexibility would also be decreased due to compression of the solvent cavity containing the protein, further reducing enzyme activity. Tonomura et al. (1961), using dioxane, which like sucrose is a structure-making solute, found that myosin size and shape, as measured by intrinsic viscosity, were greatly influenced by dioxane concentration and that there was a close correlation between intrinsic viscosity and Ca^{2+}ATPase activity.

Increasing the $ZnSO_4$ concentration in the presence of 0.3 M sucrose resulted in a steady decline in Ca^{2+}-ATPase activity, both before freezing and after freeze−thaw treatment (Figure 2).

Surface Response Models. Since maximum stabilization of phosphofructokinase, with $ZnSO_4$ added, had been observed at concentrations of less than 50 mM sucrose (Carpenter et al., 1986), we tested for possible interactions between zinc and sucrose over a wide range of concentrations. From these results we developed surface response models. Initially, $ZnSO_4$ levels (0−0.4 mM) were in the range previously shown to be synergistic with sucrose for a number of proteins (Carpenter et al., 1986). Both before and after freeze−thawing, a dramatic decline in Ca^{2+}ATPase activity occurred with increasing $ZnSO_4$ at levels up to 0.4 mM, regardless of

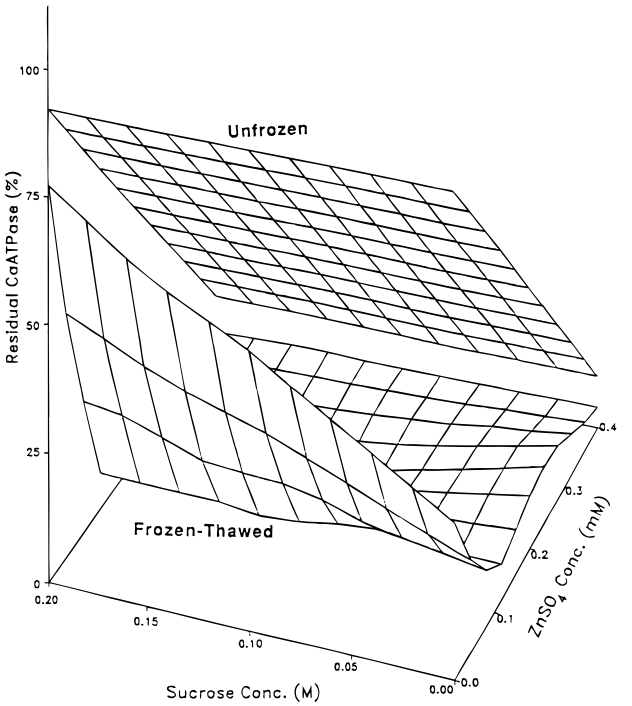

Figure 3. Surface response models for interaction of sucrose and $ZnSO_4$ (0−0.4 mM) on tilapia actomyosin (2.5 mg/mL, pH 7.0, 0.6 M KCl) before and after freeze−thaw treatment.

sucrose concentration (Figure 3). Higher levels of sucrose gave greater AM stability at low levels of $ZnSO_4$ addition. The models developed were as follows:

unfrozen

$$\text{ATPase activity \%} = 91.6 - 2020.2\ ZnSO_4\ (\text{mM})$$

$$R^2 = 0.8489\ (p < 0.0001),\ \text{MSE} = 149.1$$

frozen−thawed

$$\text{ATPase activity \%} = 29.2 + 240.1\ \text{sucrose (M)} - 3812.7\ ZnSO_4\ (\text{mM}) - 24714.3\ \text{sucrose} \times ZnSO_4$$

$$R^2 = 0.7805\ (p < 0.0001),\ \text{MSE} = 94.7$$

To be certain that $ZnSO_4$ addition was not synergistic with sucrose at very low concentrations, even lower levels of $ZnSO_4$ were tested in combination with sucrose. These low levels of $ZnSO_4$ had little effect on Ca^{2+}-ATPase activity prior to freezing and after freezing−thawing at low sucrose levels (Figure 4). An antagonistic effect is evident at higher sucrose levels. The models developed were as follows:

unfrozen

$$\text{ATPase activity \%} = 98.7 - 229.3\ \text{sucrose (M)} - 77.0\ ZnSO_4\ (\text{mM}) + 3105.5\ \text{sucrose} \times \text{sucrose} - 10559.6\ \text{sucrose} \times \text{sucrose} \times \text{sucrose}$$

$$R^2 = 0.1898\ (p < 0.151),\ \text{MSE} = 25.7$$

frozen−thawed

$$\text{ATPase activity \%} = 43.4 + 315.0\ \text{sucrose (M)} - 238.6\ ZnSO_4\ (\text{mM}) - 823.1\ \text{sucrose} \times \text{sucrose} - 3089.9\ \text{sucrose} \times ZnSO_4$$

$$R^2 = 0.7521\ (p < 0.0001),\ \text{MSE} = 44.0$$

Overall, these model actomyosin studies show that for tilapia AM there is no $ZnSO_4$ interaction with

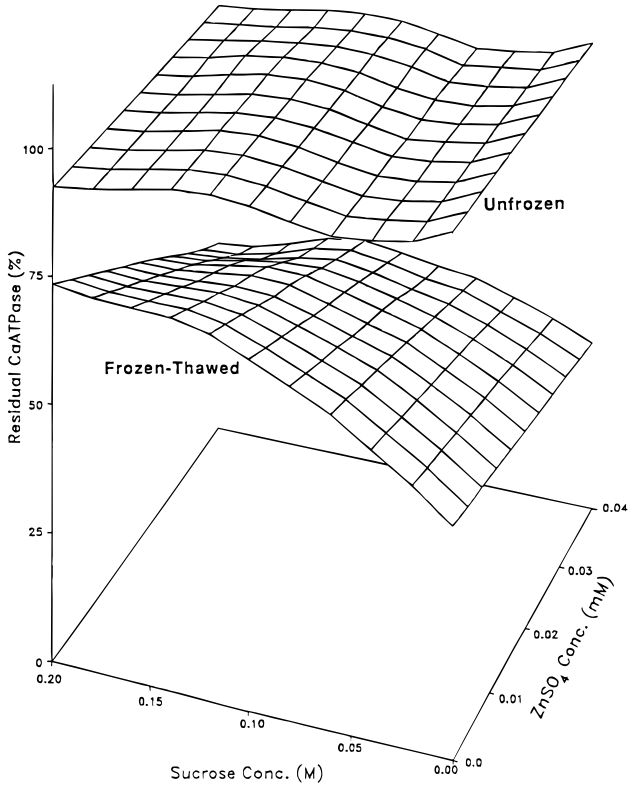

Figure 4. Surface response models for interaction of sucrose and $ZnSO_4$ (0−0.04 mM) on tilapia actomyosin (2.5 mg/mL, pH 7.0, 0.6 M KCl) before and after freeze−thaw treatment.

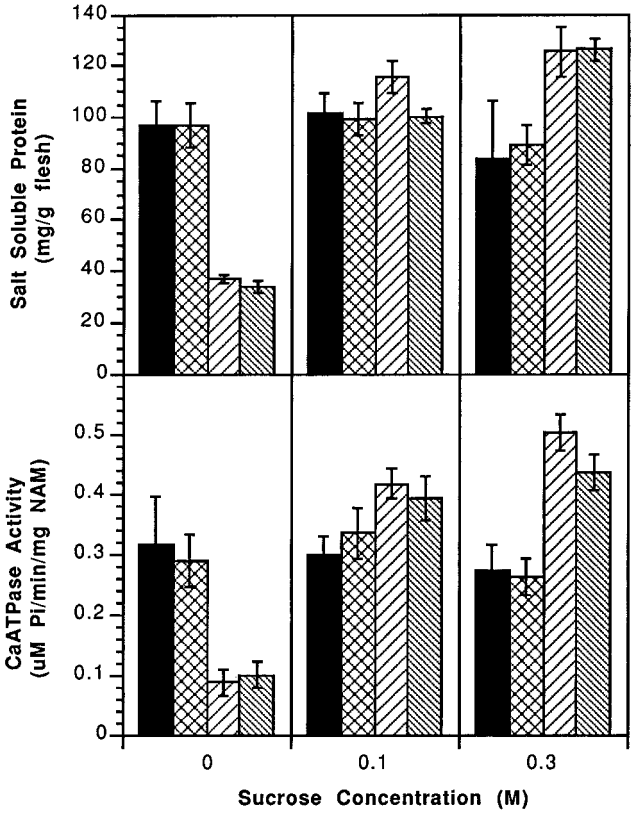

Figure 5. Effect of sucrose concentration, $ZnSO_4$ addition (0, 0.6 mM), and freeze−thaw treatment on salt-soluble protein extractability and Ca^{2+}ATPase activity of washed croaker mince: unfrozen [(solid bar) no Zn^{2+}; (cross-hatched bar) Zn^{2+} added] and frozen−thawed [(slashed bar) no Zn^{2+}; (back-slashed bar) Zn^{2+} added]. Bars show the 95% confidence interval.

sucrose to confer improved freeze−thaw stability. In fact, $ZnSO_4$ destabilized the AM even at very low concentrations.

Surimi System. To check the results of our model studies, we made surimi from fresh croaker (*M. undulatas*) and tested three levels of sucrose and two levels of $ZnSO_4$. After the additives were mixed in, samples were tested prior to freezing and after repeated freeze−thaw cycles. To assess protein denaturation and loss of functionality, we measured salt-soluble protein, AM Ca^{2+}ATPase activity, and rheological properties of gels made from the surimi.

Effect of Sucrose and $ZnSO_4$ on Salt-Soluble Protein Extractability (SSP) and Ca^{2+}ATPase Activity. After freeze−thaw treatment, there was a dramatic decrease in SSP and Ca^{2+}ATPase activity when no sucrose was present (Figure 5). Addition of 0.1 M sucrose increased both SSP and Ca^{2+}ATPase activity to levels close to the level prior to freezing, and addition of 0.3 M sucrose increased these levels further. Addition of 0.6 mM $ZnSO_4$ had little effect on either parameter prior to freezing but decreased the measurements in some frozen−thawed samples.

Interestingly, a close relationship between Ca^{2+}-ATPase activity and salt-soluble protein extractability was noted (Figure 6, $p < 0.001$) and showed that the reduction in Ca^{2+}ATPase activity effected by $ZnSO_4$ addition was due to protein denaturation and not enzyme inhibition by salt binding.

Effect of Sucrose and $ZnSO_4$ on Heat Gelation Ability of Washed Croaker Minces. Effects of $ZnSO_4$ addition were similar regardless of cooking schedule, although stress values were higher for gels cooked according to the two-step (25 °C/90 °C) schedule (Figures 7 and 8). For gels made from unfrozen mince, high sucrose concentration depressed stress values considerably ($p < 0.001$) and strain slightly. Addition of $ZnSO_4$ reversed

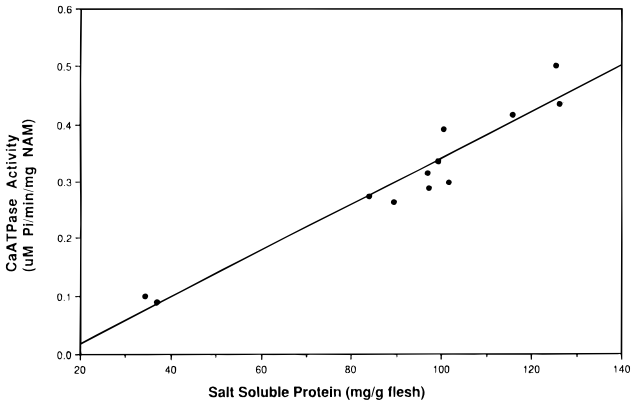

Figure 6. Relationship between Ca^{2+}ATPase and salt-soluble extractability for croaker minces ($y = -0.064 + 0.004x$; $R^2 = 0.928$).

the effect of sucrose and enhanced gel-forming ability, particularly for stress at the 0.3 M sucrose level.

After freeze−thawing, there was a dramatic decrease in both stress and strain which improved with increasing sucrose concentration. Zinc sulfate addition increased slightly the heat-gelling ability at low sucrose concentration but depressed gel-forming ability (particularly stress values) at higher sucrose concentrations.

Contrary to the work of Hazen et al. (1988) and Carpenter et al. (1987), addition of $ZnSO_4$ did not interact synergistically with sucrose to cryoprotect fish actomyosin during freeze−thawing. Rather, $ZnSO_4$ ions were antagonistic and this effect was seen even at very low concentrations of $ZnSO_4$. Possibly Zn^{2+} ions bind to AM, destabilizing the protein structure, since at pH

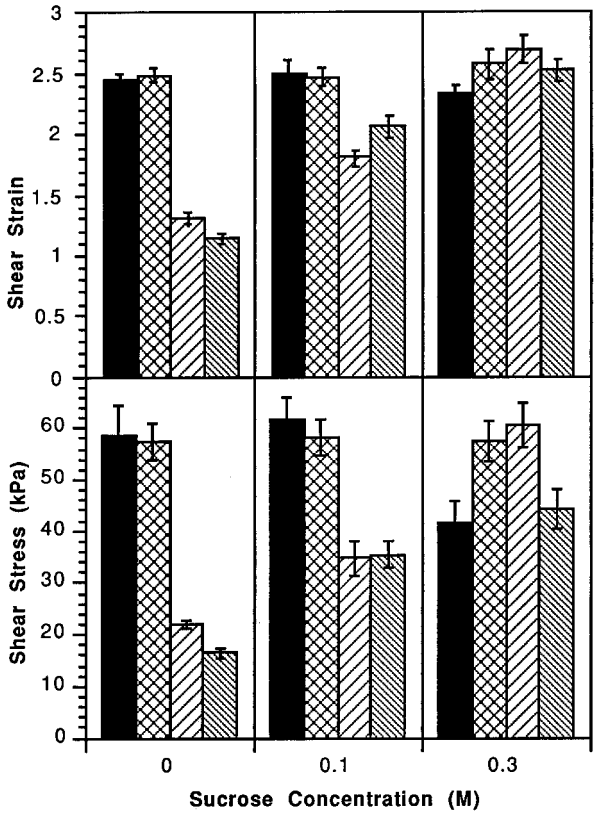

Figure 7. Effect of sucrose concentration, $ZnSO_4$ addition (0, 0.6 mM), and freeze−thaw treatment on gels (90 °C/15 min) made from leached croaker mince: unfrozen [(solid bar) no Zn^{2+}; (cross-hatched bar) Zn^{2+} added] and frozen−thawed [(slashed bar) no Zn^{2+}; (backslashed bar) Zn^{2+} added]. Bars show the 95% confidence interval.

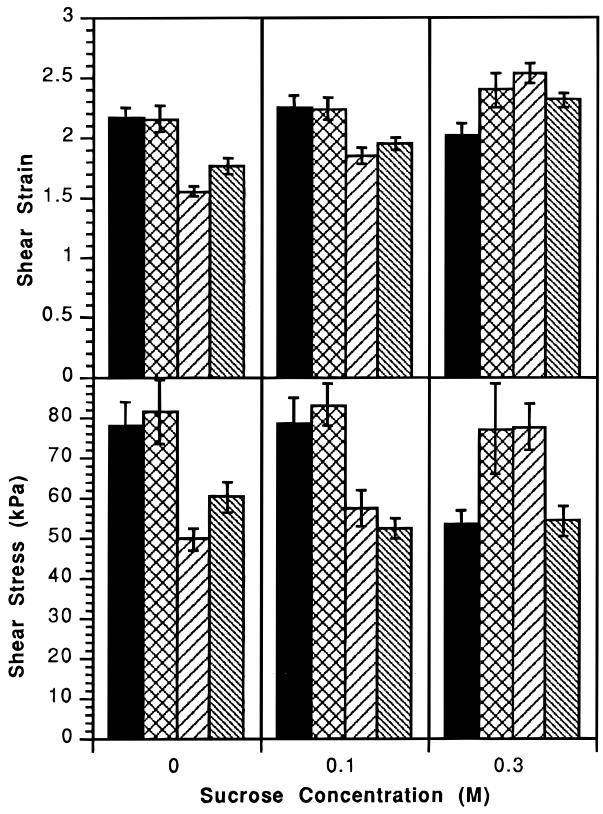

Figure 8. Effect of sucrose concentration, $ZnSO_4$ addition (0, 0.6 mM), and freeze−thaw treatment on heat-set gels (25 °C/2 h and 90 °C/15 min) made from leached croaker mince: unfrozen [(solid bar) no Zn^{2+}; (cross-hatched bar) Zn^{2+} added] and frozen−thawed [(slashed bar) no Zn^{2+}; (backslashed bar) Zn^{2+} added]. Bars show the 95% confidence interval.

7.0 there are probably sufficient negative charges on the AM to bind Zn^{2+} ions such that denaturation takes place. In addition, Klee (1988) has previously shown that Zn^{2+} ions can bind to free sulfhydryl groups and, as there are one or more −SH groups essential for myofibrillar ATPase activity, a decrease in enzyme activity would be expected. Further, considering that Atlantic croaker has 6.0 mol of available SH groups/10^5 g of protein (Liu et al., 1982), there appears to be ample −SH groups in myosin for the binding of Zn^{2+} to cause general destabilization of the native protein structure as suggested by the SSP data.

Interestingly, it appears that a small amount of denaturation can actually enhance SSP, Ca^{2+}ATPase activity, and gel formation in certain situations as shown by the data from samples containing a high sucrose concentration. An increase in myofibrillar Ca^{2+}-ATPase has been reported to precede subsequent denaturation and loss of enzyme activity in a number of other studies. The data of Reid et al. (1986) showed an initial increase in Ca^{2+}ATPase activity following fast freezing of rockfish fillets. In addition, Liu et al. (1982) reported an increase in Ca^{2+}ATPase and apparent viscosity as Atlantic croaker actomyosin was heated in the range 30−35 °C, apparently due to partial unfolding of the myosin. Above 35 °C, both enzyme activity and apparent viscosity decreased sharply. In studies on other fish species this temperature range correlated closely with a relative increase in surface hydrophobicity of these muscle proteins during heating (Niwa et al., 1981).

Recent research showing the need for flexibility in the myosin head for both binding ATP and ATP hydrolysis (Mornet et al., 1989) may help to explain why Ca^{2+}-

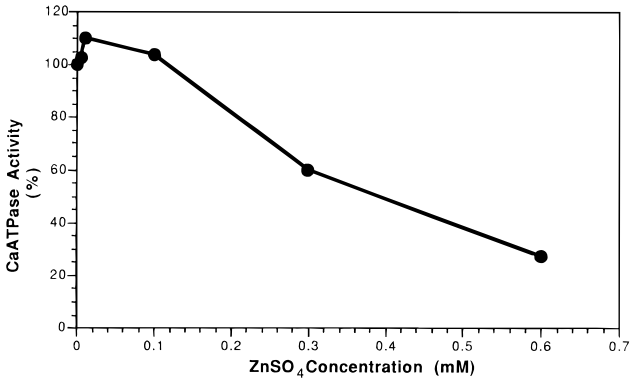

Figure 9. Effect of low concentrations of $ZnSO_4$ on croaker actomyosin Ca^{2+}ATPase activity.

ATPase activity decreased with increasing sucrose concentration and also why enzyme activity increased after freeze−thaw treatment compared to the unfrozen state when 0.3 M sucrose was added. For example, Cheung (1969) showed that maximal activation of myosin ATPase was gained by addition of a low concentration (<2 M) of urea. Increasing concentration (>3 M) resulted in rapid inactivation.

To test the hypothesis that slight denaturation would actually increase enzyme activity, we added $ZnSO_4$ to AM extracted from the croaker used in the surimi studies. Addition of very small amounts of $ZnSO_4$ actually did enhance Ca^{2+}ATPase activity (Figure 9), presumably by decreasing stability and increasing flexibility in the head region of the myosin heavy chain. Increasing $ZnSO_4$ concentration above 0.1 mM decreased enzyme activity by inducing denaturation and consequent aggregation in these samples. The com-

bined decrease in ATPase activity with increasing sucrose concentration and increase in activity with very low Zn^{2+} concentration supported previous work that showed a certain flexibility is necessary for ATP binding and hydrolysis by myosin.

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