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## TEXTURAL AND MICROSTRUCTURAL PROPERTIES OF FROZEN FISH MINCE AS AFFECTED BY THE ADDITION OF NONFISH PROTEINS AND SORBITOL

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### Abstract

Changes in textural and microstructural properties of washed and unwashed frozen fish mince were studied as affected by the addition of nonfish proteins (soy protein isolate, milk protein isolate, egg white, and wheat gluten at 2, 4 or 6%) and 6% crystalline sorbitol. Soy and milk proteins and sorbitol reduced the hardness of frozen fish mince, while egg white and wheat gluten made the texture firmer without rubberiness developing after frozen storage. All nonfish proteins and sorbitol stabilized the myofibrillar organization by reducing freeze-induced contraction of myofibrils.

The mechanisms of reducing texture hardening appear to be different between sorbitol and nonfish proteins. Water binding properties and dispersibility made the difference among nonfish proteins in reducing freeze-contraction of myofibrils. Nonfish proteins not only reduced texture hardening during frozen storage, but also modified texture during cooking as they underwent thermal gelation specific to each protein used.

### Introduction

Fish mince offers flexibility in product formulation and texture modification. Fish mince blocks can be produced from species not suitable for fish fillets (Burgin *et al.*, 1985). Several commercial products such as cakes, fish sticks and fish patties have been developed from fish mince. However, fish mince blocks are more susceptible than fillet blocks to deterioration during frozen storage. Breakdown of tissue structure accelerates changes in texture, water holding capacity, flavor, and color (Rodger *et al.*, 1980; Arocha and Toledo, 1982).

Fish mince prepared from the gadoid white fish (i.e., cod, haddock, hake, pollock, cusk, whiting) develops hardness more rapidly than that prepared from flat fish (Sikorski *et al.*, 1976; Laird *et al.*, 1980; Svensson, 1980). Textural hardening of fish during frozen storage is attributed to a variety of mechanisms, including damage due to ice crystal formation (Dyer and Dingle, 1961; Love, 1968), lipid-protein interactions (Karel, 1973; Karel *et al.*, 1975), aggregation-denaturation of myofibrillar proteins (Sikorski *et al.*, 1976; Matsumoto, 1980) and formaldehyde-protein interactions (Childs, 1973; Castell and Smith, 1973; Castell *et al.*, 1973; Rodger and Hastings, 1984).

The functional properties of frozen fish mince can be preserved by washing and then immediately blending with cryo-protectants to control the denaturation of fish muscle protein (Miyauchi *et al.*, 1975; Noguchi and Matsumoto, 1975; Noguchi *et al.*, 1976; Park and Lanier, 1987). Various ingredients are also used to improve water binding ability and texture of fish mince during frozen storage (Miyauchi *et al.*, 1975; Patashnik *et al.*, 1976; Rodger *et al.*, 1980; Da Ponte *et al.*, 1985).

Removal of water-soluble sarcoplasmic proteins appears to facilitate freeze-induced contraction of myofibrils, leading to textural hardening of washed fish mince. Water soluble proteins may act as a blocking agents by filling the sarcoplasmic space between myofibrils, thus preventing extensive cross-linking between myofibrils (Yoon *et al.*, 1991). Similarly, in a surimi gel system, the addition of water-soluble nonfish proteins reduced gel strength (Lee and Kim, 1986; Chung

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and Lee, 1990). This was believed to be due to interference with gel formation through retardation of cross-linking of actomyosin (Okada, 1964; Shimizu and Nishioka, 1974). Therefore, it is probable that the addition of water-soluble nonfish proteins prevent textural hardening during frozen storage by acting similarly as water soluble sarcoplasmic proteins.

The objectives of the present study were to investigate the effects of added nonfish proteins on the textural properties, microstructure and sensory acceptability of washed and unwashed frozen fish mince, and the underlying mechanisms of texture modification by water-soluble nonfish proteins in fish mince during frozen storage.

### Materials and Methods

Water soluble soy protein isolate, milk protein isolate, dried egg white, and wheat gluten were used as texture modifying ingredients. Soy protein isolate (S-970, Lot No. 605621) was obtained from the Grain Processing Corporation, Muscatine, Iowa; milk protein isolate (1230, Ref. No. 5995) from New Zealand Milk Products, Petaluma, California; dry egg white (standard grade) from Monark Egg Corp., Kansas City, Missouri; and spray dried wheat gluten from Midwest Grain Products Inc., Atchison, Kansas. Crystalline sorbitol was obtained from ICI Americas, Wilmington, Delaware.

#### Preparation of minced fish patty

Red hake (*Urophycis chuss*) was chosen because of its availability and great susceptibility to texture toughening during frozen storage (Dyer and Hiltz, 1974; Owusu-Ansah and Hultin, 1986). Fresh (one day old) fish were filleted and minced through 5 mm diameter perforations using a Baader deboner (Model 694, Baader North America, New Bedford, Massachusetts). Half of the fish mince (20 kg batch) was washed twice for 5 minutes each, using four parts soft chilled water ( $\text{Ca}^{++}$ ,  $\text{Mg}^{++} < 5$  ppm;  $\text{Na}^+$  80 ppm;  $10^\circ\text{C}$ ) to one part fish (by weight), where  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  were measured by an atomic absorption flame photometer and  $\text{Na}^+$  by an ion-selective electrode. Soft water was generated by passing tap water through a water softener (Model MM-24S, Marlo Inc., Racine, Wisconsin); after each washing, the wash water was drained through two layers of cheesecloth (50 grade, 20 mesh) and the resulting slurry was pressed to remove excess water at 1500 psi for 5 minutes using a Carver laboratory press (Carver Inc., Menomonee Falls, Wisconsin). It should be pointed out that two washings at a 3:1 water to meat ratio resulted in removal of 77.2% of the sarcoplasmic proteins with 90.7% of the myofibrillar proteins remaining, relative to 21% sarcoplasmic proteins and 76% myofibrillar proteins before washing (Lee, 1991). The other half was not washed.

Respective nonfish protein (soy protein isolate, milk protein isolate, dried egg white, or wheat gluten) at the 2, 4 or 6% level or 6% sorbitol was mixed with the washed and unwashed fish mince (based on the meat weight) at setting 3 (slow speed) for 2 minutes in a

Hobart Kitchen Aid bowl mixer (Model K 5-A). Cold water ( $2^\circ\text{C}$ ) was added to equalize the moisture content at 84% throughout the samples. Fish patties were formed by packing approximately 40 g of mince into aluminum dishes (6.0 cm diameter, 1.5 cm height). Patties with aluminum dishes for each group were then vacuum-packed in nylon bags (McKenna & Assoc., Weymouth, Massachusetts) and subjected to 3 freeze-thaw cycles for the determination of their freeze-thaw stability. Three freeze-thaw cycles were employed to accelerate freeze-induced physical changes. Each freeze-thaw cycle involved storage at  $-20^\circ\text{C}$  for 15 days and thawing at  $4 \pm 0.5^\circ\text{C}$  for 1 day.

#### Evaluation of texture

To measure the hardness of fish mince patties after frozen storage, the fish patties were completely thawed at  $4^\circ\text{C}$ , steam-cooked at  $90^\circ\text{C}$  and atmospheric pressure for 20 minutes and cooled in a refrigerator overnight while they were in the bag. The term "hardness" (Szczeniak *et al.*, 1963; Cardello *et al.*, 1982) was chosen over "toughness" (Jowitt, 1974) since hardness more closely reflects the texture changes in frozen fish mince while toughening is more appropriate for frozen filets. Texture was evaluated using an Instron testing machine (Model 1122, Instron Corp., Canton, Massachusetts) after the patties were equilibrated to room temperature and removed from the aluminum dishes.

Shear force was measured using the first peak as an index of hardness by shearing the sample perpendicularly with a flat rectangular blade (1 mm thick x 50 mm wide x 50 mm long). For the measurement of expressible moisture, each patty was compressed to 90% using a compression head (10 cm-diameter). The amount of moisture expressed upon compression (90%) was measured by collecting the fluid on filter papers (Whatman No. 4) and weighing the filter papers before and after collection. All measurements were done at 50 mm/min cross-head speed and 100 mm/min chart speed. Expressible moisture was calculated as a percentage of original sample moisture content.

Moisture was determined by drying the sample in an oven at  $110^\circ\text{C}$  for 18 hours. Drip loss on thawing at  $4^\circ\text{C}$  for 1 day and cooking loss on heating at  $90^\circ\text{C}$  and atmospheric pressure for 20 minutes were calculated by measuring weight losses and expressed in percentage of the original sample weight. All analyses were performed on six replicates for each sample.

#### Sensory evaluation

Sensory evaluation of texture was conducted by a panel of 7 people from the Department who had previously participated in the evaluation of similar products. After 3 freeze-thaw cycles, the fish patties in the bag were fully thawed at  $4^\circ\text{C}$ , steam-cooked at  $90^\circ\text{C}$  and atmospheric pressure for 20 minutes, and refrigerated ( $4^\circ\text{C}$ ) overnight before being evaluated by sensory panelists. Fish patty samples were cut into lots of small pieces (9 from each patty), numerically coded and randomly served as cold (approximately  $10^\circ\text{C}$ ) to panelists.

Sensory evaluation of deep-fried patties was also conducted. Frozen patties were battered and breaded with the Golden Dip "Fish and Chip" batter and breading (Golden Dip, St. Louis, Missouri) and deep fried in Mazola Corn oil at 190°C for 5 minutes. Fried patties were immediately presented whole to panelists and tasted while hot. Four textural characteristics (firmness, chewiness, rubberiness and moistness) were evaluated for their intensity and desirability, as well as overall desirability (relative to the control without added proteins) using a 9-point scale (1 : least, 5 : moderate, 9 : greatest).

#### Determination of bound water capacity of nonfish proteins

The amount of unfreezable water at -30°C (ionically bound) in the sample was measured, according to the procedure described by Chung and Lee (1991), as the bound water capacity (BWC) of nonfish proteins and sorbitol using a differential scanning calorimeter DSC-2 (Perkin-Elmer Corp., Norwalk, Connecticut). Cooling to -30°C was decided based on the incipient melting temperatures ranging from -13.3 to -17.8°C which were reported by Roos (1986). The results from this measurement were compared with those from the water binding measurement, based on a centrifugation method (Chung and Lee, 1990).

To convert the endothermic peak area to the quantity of freezable water, a standard curve of peak area versus moisture content (1-8 mg) was constructed using deionized water, which is all freezable. The amount of freezable water in the samples was detected by the fusion endotherm peak and calculated in reference to peaks obtained with known quantities of freezable water. The intercept of the calibration curve (Y axis: g of total water / g of dry sample; X axis: g of freezable water / g of dry sample) was used as the amount of unfreezable water (bound water capacity) (Simatos *et al.*, 1975).

#### Evaluation of structure

**Light microscopy:** The relationship of the dispersion pattern of nonfish proteins to the structure of frozen fish mince was studied after 3 freeze-thaw cycles using light microscopy following the procedure described by Lee (1985). An approximately 0.4 cm thick specimen of minced fish patty was quick frozen in liquid nitrogen and cut into 16 µm sections using a microtome cryostat (Damon/IEC division, Needham Heights, Massachusetts). The sections were mounted directly on microslides and allowed to dry at room temperature. The dried section was dipped in distilled water for 1 second, then dipped in 60% isopropyl alcohol for 2 seconds, stained for 20 minutes with hematoxylin and rinsed with tap water. After the section was dried, a small drop of warm glycerin jelly was applied and a cover glass placed. Color photomicrographs of the stained sections were taken at 60 x magnification.

**Electron microscopy:** Effects of nonfish proteins and sorbitol on the microstructure of fish mince during frozen storage were evaluated using transmission elec-

tron microscopy after freeze substitution (Martino and Zaritzky, 1986). To prepare specimens, a frozen patty was cut into several pieces (2 mm x 2 mm x 1 mm) with a razor, immediately immersed in 5 ml cold (-20°C) freeze-substitution fluid (methanol containing 1% OsO<sub>4</sub> and 0.025% glutaraldehyde), and fixed at -20°C for 2 days. Osmium tetroxide and glutaraldehyde were purchased from Stevens Metallurgical Corp., New York. Specimens were then transferred into 100% methanol (-20°C) (protein sequencing reagent grade, Sigma Chemical Co., St. Louis, Missouri): kept for 6 hours in a freezer (-20°C), overnight in a refrigerator, and for 2 hours at room temperature (20°C). The fixed specimens were washed, for 15 minutes each, consecutively in 2 changes each of methanol and propylene oxide (PO) (Ted Pella Inc., Tustin, California). To prepare control specimens, an unfrozen washed fish patty was cut into pieces (2 mm x 2 mm x 1 mm) and fixed with 1% OsO<sub>4</sub> and 0.025% glutaraldehyde in 0.1 M cacodylate buffer for 2 hours at 20°C. The fixed specimens were washed three times with 0.1 M cacodylate buffer for 1 hour and dehydrated by immersion in increased concentrations (35, 50, 70 and 95%) of ethanol for 20 minutes each. This was followed by washing for 15 minutes each, consecutively in 2 changes each of 100% ethanol and 50:50 ethanol:propylene oxide (PO) and 100% PO. All steps in the preparation of control specimens were done at room temperature (20°C).

Both fresh and frozen specimens were then transferred into a vial containing PO:Epon 812 (Polaron, Cambridge, Massachusetts) (50:50%) and agitated in a tissue rotator for 24 hours. The solution was then replaced with PO:Epon 812 (25:75%) and again agitated for 24 hours. Specimens were then transferred into 100% Epon 812 in an embedding dish and held at 60°C for 24 hours.

Ultrathin microtome sections (60-90 nm) were mounted on grids and stained with 5% uranyl acetate in ethanol at 60°C for 20 minutes. They were then stained with lead citrate for 5 minutes at room temperature (20°C) and rinsed with 0.02 N NaOH and distilled water. Specimens were examined with a JOEL 1200 EX transmission electron microscope (JEOL U.S.A. Inc., Peabody, Massachusetts) operated at 80 kV. Photomicrographs were taken at magnifications of 10,000 and 20,000 x.

#### Statistical analysis

The data were analyzed using the Statistical Analysis System Package (SAS, 1985). Duncan's multiple range test was used to determine the significance of differences between treatments.

### Results and Discussion

#### Effects of ingredients on texture and water binding ability

As seen in Fig. 1, addition of soy and milk proteins significantly reduced freeze-induced hardness of

washed fish mince ( $P < 0.001$ ). As the level of protein was increased, the effect became more pronounced. The gluten-containing mince remained firm without a significant difference over the levels tested (Fig. 1). Fig. 2 shows the effects of various nonfish proteins and sorbitol at a 6% level on the water binding ability of washed and unwashed fish mince after 3 freeze-thaw cycles. Addition of soy protein significantly ( $P < 0.0001$ ) reduced the expressible moisture and cooking loss in both washed and unwashed fish mince, while milk protein and egg white significantly ( $P < 0.001$ ) reduced the expressible moisture and cooking loss in unwashed fish mince only. Sorbitol, on the other hand, did not reduce expressible moisture as well as cooking loss in both washing and unwashed mince. A similar observation was reported by Yoon and Lee (1990). The apparent reason is that sorbitol does not bind the water during cooking, unlike nonfish proteins, which are able to bind water during thermal gelation. Drip loss was reduced by all nonfish proteins and sorbitol in washed fish mince, while only soy and milk proteins reduced drip loss in unwashed fish mince. The different effects of nonfish proteins on water binding abilities during freezing and cooking may be attributed to the differences in their physicochemical properties which were reported to have effects on the water binding ability of nonfish protein-containing surimi gels (Chung and Lee, 1990).

The DSC data indicate that the bound water capacity (BWC) differed from one protein to another. Wheat gluten showed the lowest BWC of 0.22 g water/g sample, while milk protein had the highest BWC of 0.45 g water / g sample. Although the bound water capacity of soy protein (0.30 g water/g sample) was not greater than that of milk protein and egg white (0.42 g water / g sample), soy protein-containing mince showed the greatest water binding ability (WBA) with the least drip loss, cooking loss and expressible moisture (Fig. 2). This means that the DSC-measured BWC of the protein does not appear to be a good indicator of the WBA of the protein-containing fish mince.

In a previous study (Chung and Lee, 1990), when primarily interstitial free water was measured by a centrifugation method, soy protein showed the highest water binding value, the next was egg white, followed by wheat gluten and milk protein. It was found that the WBA of nonfish proteins based on the interstitial free water (physically bound water) correlated better with the surimi gel strength than the DSC-measured bound water. It should be noted that the amount of unfreezeable bound water is relatively smaller than the interstitially bound water (freezeable). The ice crystal formation responsible for expressible water and freeze drip is a function of the available freezeable water. Therefore, the amount of expressible water and freeze drip in the frozen fish mince is determined primarily by the amount of freezeable water present, which is in turn affected by the water binding of added proteins.

Fig. 3 shows the effect of nonfish proteins and sorbitol at the 6% level on the shear force of the mince

patties. Before 3 freeze-thaw cycles, the addition of sorbitol and all nonfish proteins except egg white significantly reduced firmness in washed fish mince, while only soy and milk proteins significantly reduced the firmness in unwashed fish mince ( $P < 0.001$ ). After 3 freeze-thaw cycles, the addition of soy and milk proteins resulted in a significantly softer texture in both washed and unwashed mince patty ( $P < 0.001$ ). When egg white and gluten were added, on the other hand, the texture was firm, but less firm than the control. Sorbitol showed a moderate ability to produce a soft texture in both washed and unwashed mince patty. Egg white gave greater texture firming in washed fish mince than in unwashed fish mince. The present results suggest no clear pattern with added ingredients on the texture of the fish mince between washed and unwashed fish mince.

Washed mince patties had a greater shear force than unwashed ones without ingredients (Fig. 3) suggesting that water soluble proteins have a texture softening effect. This effect was explained by a greater degree of freeze contraction of myofibrils in the absence of water soluble proteins during frozen storage (Yoon, *et al.*, 1991). In this report they postulated that water soluble sarcoplasmic proteins were involved in blocking the cross-linking of muscle fibrils, thus reducing contraction of muscle fiber units.

The pattern of texture changes in frozen fish mince did not follow that observed with expressible moisture and freeze drip. Texture softening or firming of fish mince by adding the nonfish proteins or sorbitol was not always related to the WBA of added ingredients. This suggests that some mechanism other than water binding is implicated in the role of nonfish proteins in texture changes of frozen fish mince.

#### Sensory evaluation

When fish patties were steam-cooked, the highest overall desirability score was given to washed patties containing sorbitol (Fig. 4). The next higher overall desirability scores were given to the fish patties containing egg white and gluten with comments that they were firmer and somewhat meatier but not rubbery compared to other patties. Panelists gave the lowest overall desirability score to the washed mince patty containing soy and milk proteins ("very soft texture") and to the control ("rubbery texture"). However, when fish patties were breaded and fried, the one containing egg white received the highest score followed by wheat gluten, sorbitol, soy protein, and milk protein. Steam-cooked fish patties always received higher firmness intensity score than deep-fried ones. The discrepancy in the result between steam-cooking and frying could be due to the differences in the heating rate, the cooking temperature, and the temperature of samples served. The steam-cooked patties were cooked in a nylon bag and refrigerated before being served, while fried ones were served warm. The shear force more closely correlated with firmness ( $r = 0.8671$ ,  $P < 0.001$ ) than with rubberiness ( $r = 0.7923$ ,  $P < 0.001$ ).

## Texture and microstructure of frozen fish mince

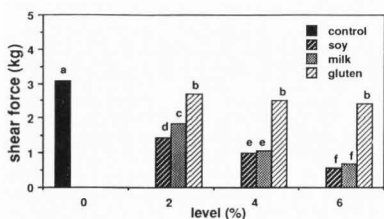


Fig. 1. Effect of nonfish proteins on the shear force of the washed fish mince after 3 freeze-thaw cycles. Samples tested were fish mince patties that had been cooked after freeze-thaw cycles. Different letters in the same group of bars are significantly different ( $P < 0.001$ ).

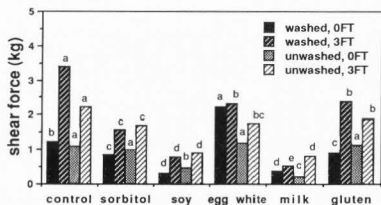


Fig. 3. Effect of nonfish proteins and sorbitol on the shear force of the washed and unwashed fish mince. Different letters in the same group of bars are significantly different ( $P < 0.001$ ). Tested at the 6% level. FT: freeze-thaw cycle.

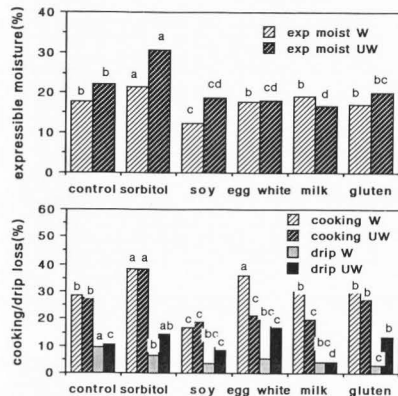


Fig. 2. Effect of nonfish proteins and sorbitol on the water binding ability of the washed and unwashed fish mince after 3 freeze-thaw cycles. Tested at the 6% level. W: washed, UW: unwashed.

### Light and electron microscopic observations

The effect of 6% nonfish proteins and sorbitol on the ultrastructure of washed fish mince after 7 weeks of storage at  $-20^{\circ}\text{C}$  is shown in Fig. 5. Without addition of nonfish proteins or sorbitol, myofibrils were distorted by ice crystals, resulting in a denser structure (Fig. 5b) compared to the myofibrillar structure of unfrozen fish mince (Fig. 5a). Sorbitol (Fig. 5c) and soy protein isolate (Fig. 5d) in washed fish mince suppressed intracellular ice crystals and stabilized the myofibrils during

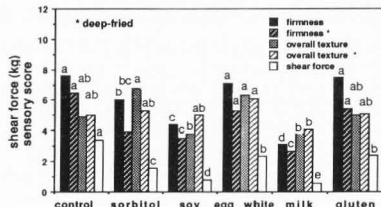
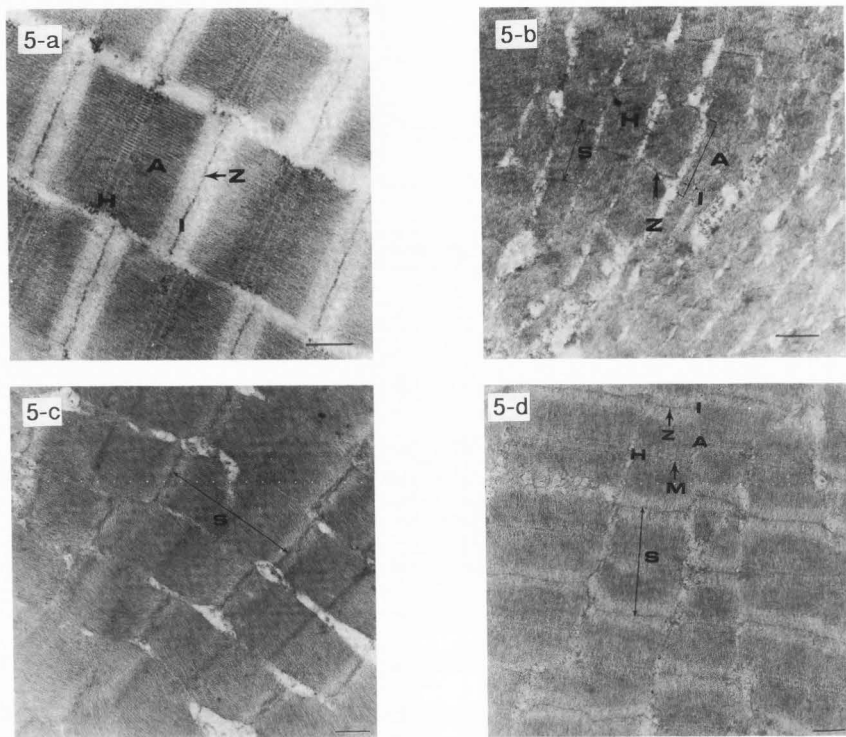


Fig. 4. Effect of nonfish proteins and sorbitol on the shear force (hardness) and the sensory scores of washed fish mince after 3 freeze-thaw cycles. Tested at the 6% level. Samples were either steam-cooked or deep fried; sensory score range from 1 to 9 where 1 = least, 5 = moderate, 9 = greatest. Firmness (intensity), overall texture (desirability).

frozen storage, while wheat gluten did not appear to show a stabilizing effect (Fig. 5e). Compared to soy protein, egg white and milk protein, its lower water binding ability and poor dispersibility (Fig. 6) may explain the reason for wheat gluten being less effective.

Interestingly, the size (area) of the sarcomeres was significantly different for each nonfish protein ( $P < 0.001$ ). The least size reduction was observed in fish mince containing sorbitol, followed by soy protein, egg white, milk protein, and wheat gluten (Table 1). Although the degree of freeze-contraction of myofibrils appeared to be similar, the fish mince containing egg white had a markedly firmer texture than that containing milk protein isolate. This suggests that nonfish proteins are involved in other structural changes besides reducing freeze contraction of muscle fibrils and that the texture changes were specific to the type of added nonfish protein.

Although the degree of textural softening by sorbitol and soy protein isolate appeared to be similar, the results of light and electron microscopic examinations suggest different mechanisms for their action.



**Fig. 5.** Electron micrographs of a longitudinal section of skeletal muscle of washed fish mince containing 6% sorbitol or nonfish proteins after 7 weeks of storage at  $-20^{\circ}\text{C}$ . **a:** control (washed unfrozen); **b:** control (washed-frozen); **c:** sorbitol; **d:** soy protein; **e:** wheat gluten. Bar = 500 nm. A: A band; H: H band; I: I band; Z: Z line; M: M line; S: Sarcomere.

**Fig. 6 (facing page, top).** Light micrographs of washed fish mince containing 6% nonfish proteins after 7 weeks of storage at  $-20^{\circ}\text{C}$ . **a:** soy protein; **b:** milk protein; **c:** egg white; **d:** wheat gluten. Bar =  $200\mu\text{m}$ . S: soy protein isolate; M: milk protein isolate; E: egg white; G: wheat gluten.

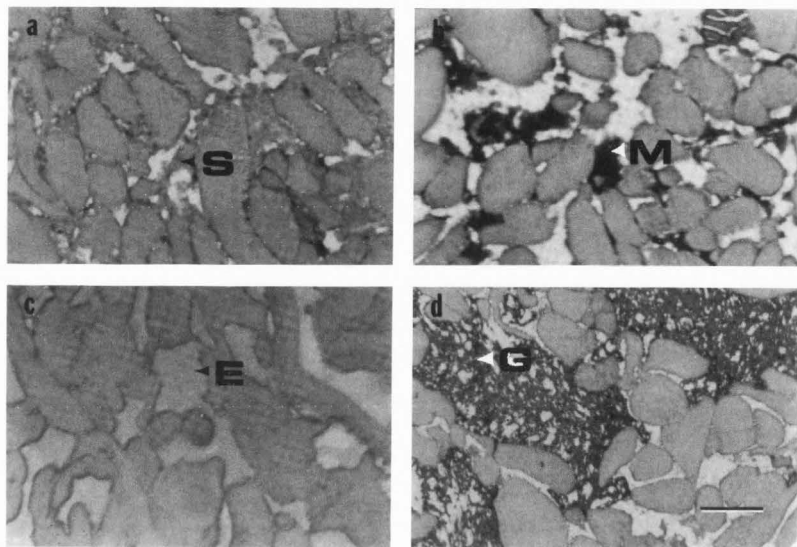


Table 1. Effect of added nonfish proteins and sorbitol on the size of the sarcomere during frozen storage<sup>1</sup>

sample	size of sarcomere <sup>2</sup> ( $\mu\text{m}^2$ )	% sarcomere reduction <sup>3</sup>
control <sup>4</sup>	$3.17 \pm 0.36$ a	0
control <sup>5</sup>	$0.41 \pm 0.02$ g	87.06
sorbitol	$1.96 \pm 0.15$ b	38.17
soy	$1.47 \pm 0.36$ c	53.63
egg white	$0.88 \pm 0.12$ d	72.24
milk	$0.73 \pm 0.08$ e	76.97
gluten	$0.55 \pm 0.09$ f	82.65

<sup>1</sup> frozen for 7 weeks

<sup>2</sup> mean values of 10 myofibril units except control (n=6)

<sup>3</sup> reference to unfrozen control, 0 % = no reduction in the size of sarcomere

<sup>4</sup> unfrozen sample

<sup>5</sup> sample frozen for 7 weeks

Means followed by different letters are significantly different ( $P < 0.001$ ).

Light microscopy shows that all of the nonfish proteins appeared to remain in the interstitial spaces between muscle fibers (Fig. 6). This means that their primary influence must be limited to the extracellular fluid. The cryoprotective action of macromolecules, which cannot penetrate into the cell, has been explained by two mechanisms: 1) macromolecules form hydrogen bonds with water and this structural interaction increases the resistance of protein solutions to freezing (Lozina-Lozinskii, 1974), and 2) they form supersaturated solutions of very high viscosity, resistant to freezing (Meryman, 1974; Echlin *et al.*, 1977).

Contrary to nonfish proteins, sorbitol completely dissolves in the extracellular fluid and thus can interact with myofibrillar protein molecules (Lozina-Lozinskii, 1974) in such a way that it increases the hydration of the protein molecule necessary for protein stabilization (Carpenter and Crowe, 1988; Lee and Timasheff, 1982). They claimed that certain solutes such as sugar, polyols and amino acids promote a preferential hydration of protein with non-freezable water, which forms the basis of cryo-protection.

The transmission electron micrographs of frozen-stored washed fish mince show the reduced freeze-contraction of myofibrils when soy protein isolate, egg white and milk protein isolate were added. Such



reduced freeze-contraction after addition of nonfish proteins appears to be related to the proteins' water binding measured by centrifugation (Chung and Lee, 1990) and dispersibility (Chung and Lee, 1991). It is suggested that the good water binding and dispersibility of nonfish proteins reduced the amount of free water available for ice crystallization and allowed uniform ice crystal formation. This in turn prevented the formation of large ice crystals which caused freeze syneresis leading to freeze-contraction of muscle myofibrils during frozen storage as shown by the electron micrographs.

### Conclusion

The development of a hard and rubbery texture appears to be related to the freeze-induced contraction of the myofibrillar units. The removal of water from protein molecules during ice crystal formation may have promoted such freeze-contraction through cross-linking resulting from molecular proximity. The ability of nonfish proteins and sorbitol to prevent or minimize such textural hardening of fish mince during frozen storage is related to the reduced freeze-contraction of myofibrils through water binding and uniform dispersion. The effect of nonfish proteins on texture improvement and modification was highly specific to each protein. Electron microscopic observations showed that sorbitol and all nonfish proteins except wheat gluten were effective in ice crystal formation and stabilizing myofibrillar structure from freeze-contraction.

### Acknowledgements

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#### Discussion with Reviewers

**Reviewer 1:** The time used for isothermal freeze fixation appears to be far less than that which one would normally expect in light of the fact that the rate of fixative penetration is significantly reduced at low temperature.

**Authors:** The two-hour fixation time at  $-20^{\circ}\text{C}$  was found to be adequate (Yoon, 1990). According to Robards and Sleytr (1985), it is desirable to have a specimen in contact with the substitution liquid for the shortest time possible. Humble *et al.* (1983), and Humble and Muller (1984) indicated that methanol substitution and fixation by glutaraldehyde in methanol proceed extremely rapidly at temperatures above  $-30^{\circ}\text{C}$ , and that both should be completed within a few hours. The use of methanol and a relatively higher substitution temperature thus allowed a shorter fixation time in our study. The substitution temperature of  $-20^{\circ}\text{C}$  was chosen since all samples were frozen and stored at this temperature, and unlike unfrozen samples there was no need to provide a long fixation time at a ultra-low temperature to avoid ice recrystallization. Methanol was found to be compatible to the samples studied for the following reasons: 1) methanol remains liquid at  $-20^{\circ}\text{C}$  and does not require additives; 2) it removes ice in a few hours and thus reduces the duration of fixation; and 3) it causes the least damage to the proteinaceous structure among the organic solvents (Zalokar, 1966). Additional information on freeze-substitution and freeze-fixation techniques can be found in MacKenzie *et al.* (1975), Asquith and Reid (1980), and Hunt (1985).

**Reviewer 1:** Is there any protease activity associated with this species of hake? Since the researchers have

investigated the effect of added proteins with different levels of sulfhydryl proteins; how do they eliminate the heat-induced effects of sulfhydryl-disulfide interchange on hardness of the patties? Since presumably gluten contains the greatest level of SH-proteins tested; does this imply that gluten increases hardness after cooking? Do soy and dairy proteins interfere with texture development?

**Authors:** Yes, this species (red hake) has protease activity, namely, cathepsin during cold storage and alkaline protease during heating at 60-70°C. No attempts were made to differentiate SH-protein effect of added proteins from the fish muscle protein in evaluating hardness of patties. Gluten-added fish patties showed a noticeable increase in hardness, while soy and milk proteins tended to soften the texture. This was explained by reduced freeze-contraction of muscle fibrils which is shown in the EM micrographs.

**Reviewer 1:** Would the addition of soy protein and wheat gluten impart an objectionable flavor when used at levels exceeding 2%?

**Authors:** We have not experienced this problem yet during sensory analysis in terms of any objectionable taste notes. However, we agree with you that one should take this matter into consideration in product formation.

**Reviewer 1:** Wouldn't it be rather obvious that the control sustains greater ice crystal damage as there is no dehydrated protein added.

**Authors:** The moisture content of all sample preparations were adjusted to that of the control by adding water. Although there is an equal amount of water in both control and protein-added sample, the actual amount of the free water available to ice crystal formation will differ between them. The differences among proteins depend upon their water-binding properties and dispersibility as discussed in the text.

**J.M. Regenstain:** Is the shear test an appropriate method for the patty?

**Authors:** We found the shear test using a rectangular blade responded with a single peak in the most discriminative manner to the texture changes due to freezing. We agree with you that a shear test using double blades is worth trying. However, we are concerned about interpreting multiple peaks generated from multi-blades which are used in a Kramer shear test although it will give a force-deformation response similar to a test using a single blade.

**J.M. Regenstain:** Why did authors use different fixation procedures for frozen and fresh control samples?

**Authors:** There is already a difference in physical properties because of one being frozen and the other not being frozen. The use of freeze substitution method is critical to frozen samples in preserving the original state of frozen tissue without subjecting the samples to thaw-

ing which would in turn result in alteration in structure.

**J.M. Regenstain:** Do you have any proof whether hardness was induced by freeze effect or freeze-thaw cycle effect?

**Authors:** Frozen storage and freeze-thaw cycle will not give the same identical effect, but the freeze-thaw cycle method can be used to accelerate freeze-induced physical or chemical changes without committing to a long-term storage.

**J.M. Regenstain:** Would drying at 110°C remove all "bound water"?

**Authors:** Yes, presumably so in the fish muscle tissue. However, reportedly, in some materials such as confectionery products it is difficult to remove all bound water because of the high degree of water binding property.

**Reviewer 3:** Were the fish used in the post-rigor state?

**Authors:** Yes, we believe so since it was at least one day old and was not stiff as seen in the fish in the rigor state.

**Reviewer 3:** I believe that the effect seen by milk and soy protein were mainly their interference with muscle protein interactions during heating (not frozen storage), while gluten and egg white gelled in the interstitial spaces to strengthen the patties.

**Authors:** The points are well taken, however, the conclusion we have drawn was based on the freeze contraction by measuring the size of sarcomeres where it is clear that soy exhibited the least shrinkage with a low shear force. The inconsistent relationship between the contraction of sarcomere units and the shear force of milk, in particular, suggests there are other causative factors than freeze contraction for texture outcome. On the other hand, soy proteins showed an equal or a greater gel strengthening effect compared to egg white (Chung and Lee, 1990), and yet the patties prepared from soy protein were markedly softer than those from egg white. This suggests there are other reasons for such differences than protein's interference or gelling ability.

**Reviewer 3:** It would have been helpful if mince frozen without the nonfish proteins has subsequently had the nonfish proteins added to it for comparison. Alternatively, the minces containing nonfish proteins could have been rewashed after frozen storage to remove them, and the functionality of the proteins evaluated on an equal basis without the added effect of the nonfish proteins' presence.

**Authors:** Your points are well taken. This suggestion will be considered in our next experiment. One concern we have is a disruption of patty's matrix that had been molded and set frozen, by breaking while adding nonfish proteins. Nevertheless, our original approach was to see if there is any correlation between structural change and texture due to frozen storage in fish muscle tissue.

**S.H. Cohen:** Why were the grids stained with uranyl acetate at 60°C and then with lead acetate at room temperature?

**Authors:** This staining procedure has been most commonly employed for TEM, and the use of 60°C was to hasten the staining process.

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