Pacific Hake (*Merluccius Productus*) Hydrolysates as Cryoprotective Agents in Frozen Pacific Cod Fillet Mince

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ABSTRACT: Fish protein hydrolysates produced by proteolysis of Pacific hake (*Merluccius productus*) with Alcalase® (FPH-A) or Flavourzyme® (FPH-F) were investigated as a potential alternative to the 1 : 1 blend of sucrose–sorbitol (SuSo) commonly used for cryoprotection of frozen fish mince. The physicochemical properties of cod mince samples in the absence (control) or presence of 8% FPH-A, FPH-F, or SuSo were evaluated before and after 6 freeze–thaw cycles, with differences noted at the 5% significance level. Freeze–thawing of control sample increased expressible moisture (from 22% to 33%) and cook loss (from 3% to 16%). These poor water retention properties were improved in samples containing FPH or SuSo. Differential scanning calorimetry showed higher proportion of unfrozen water in freeze–thawed samples containing FPH-F or FPH-A (0.36 g/g) compared to SuSo (0.33 g/g) and control (0.24 g/g) samples. Textural analysis of cooked mince from unfrozen samples indicated greater hardness for FPH than SuSo and control samples, while freeze–thawing resulted in decreased hardness for FPH and SuSo samples. Content and surface hydrophobicity of extractable natural actomyosin (NAM) were maintained after freeze–thawing of samples containing FPH-F or SuSo, compared to 50% decrease in extractable NAM and a significant increase in surface hydrophobicity for the control. The presence of oligopeptides in both hydrolysates and the high levels of free amino acids including Asp, Glu, Arg, and Lys in FPH-F might be responsible for their cryoprotective action. This study provides strong evidence to support development of FPH as a new generation cryoprotectant to maintain quality of frozen fish.

Keywords: amino acids, cod mince, cryoprotectant, fish protein hydrolysate, freeze–thaw

Introduction

Freezing and frozen storage have been widely used to slow down microbial and enzymatic deterioration of fish. However, studies have shown that prolonged frozen storage and/or repeated freeze–thawing abuse may impart changes in the texture and functional properties of the muscle proteins leading to an end in the shelf life of the frozen fish (Verma and others 1995; Benjakul and others 2005; Benjakul and Sutthipan 2009). Therefore, over the years, many investigations have attempted to look for suitable ingredients to stabilize fish muscle proteins during freezing or long-term frozen storage.

Cryoprotectants may prevent protein denaturation during frozen storage by increasing the surface tension of water as well as the amount of bound water, which will prevent ice crystal growth and migration of water molecules from the protein, thus stabilizing the protein in its native form during frozen storage (Carpenter and Crowe 1988). A 1 : 1 blend of sucrose and sorbitol is commonly used as a cryoprotectant for fish products, but several studies have also shown cryoprotective potential of polydextrose, lactitol, glucose syrup (Herrera and Mackie 2004), as well as trehalose and sodium lactate (Zhou and others 2006). Nevertheless, products containing these carbohydrate-based cryoprotectants would not be acceptable by consumers suffering from diabetes; furthermore, their tendency to impart a sweet taste to the final product might also not be desirable in some cases (Sych and others 1990a; Yoon and Lee 1990).

The application of protein hydrolysates has attracted much attention in the past decade mainly due to their high nutritive value and improved functionalities arising from the production of short peptides and free amino acids during hydrolysis (Clemente 2000). The potential cryopreventive property of protein hydrolysates has recently started to draw researchers' interest. Damodaran (2007) demonstrated the ability of gelatin hydrolysate to inhibit ice crystal growth in ice cream mix. Optimization of the gelatin hydrolysis conditions and molecular weight characterization of the peptide cryoprotectants indicated that the most active fraction consisted of cationic peptides in the range of 700 to 1400 Da (Wang and others 2009). Protein hydrolysates produced from fish scrap (Khan and others 2003), squid (Hossain and others 2004), shrimp chitin (Somjit and others 2005), and shrimp head (Ruttanapornvareesakul and others 2006) have all been shown to display cryoprotective ability through increasing the proportion of unfrozen water and decreasing the loss of Ca-ATPase activity in lizardfish surimi and myofibrillar protein extracts. In contrast, Sych and others (1990b, 1991) reported poor cryoprotection by 2 commercial hydrolysates ("APSL," a fish protein hydrolysate [FPH] and "Lactamine AA," a casein hydrolysate) on denaturation of cod surimi proteins during frozen storage as assessed by salt extractable protein content and differential scanning calorimetry (DSC). To our knowledge, no studies thus far have investigated the cryoprotective effects of protein hydrolysates in fish mince.

Pacific hake (*Merluccius productus*), also referred to as Pacific whiting, is an under-utilized fish species due to its susceptibility

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to autolytic degradation of muscle tissue leading to decreased protein functionality and loss of texture (Mazorra-Manzano and others 2008). Recently, Pacheco-Aguilar and others (2008) performed a study on the functionality of Pacific whiting muscle protein hydrolysates and found excellent peptide solubility, good emulsifying capacity, and foam stabilizing ability. However, the possible cryoprotective ability of these hydrolysates was not investigated.

Therefore, the objective of this study was to examine the potential application of FPHs produced from Pacific hake, compared to sucrose–sorbitol (SuSo), as cryoprotective agents in frozen Pacific cod fillet mince.

Materials and Methods

Materials

Sucrose and D-sorbitol were purchased from Fisher Scientific (Ontario, Canada) and Sigma-Aldrich (Ontario, Canada), respectively, while 6-propionyl-2-dimethyl-aminonaphthalene (PRODAN) was purchased from Invitrogen Molecular Probes (Oreg., U.S.A.). Novozyme® commercial proteolytic enzyme preparations (liquid Alcalase, 2.4 AU/g, and liquid Flavourzyme, 500 LAPU/g) were donated by Brenntag Canada Inc. (BC, Canada).

FPHs were prepared from Pacific hake using Alcalase (FPH-A) or Flavourzyme (FPH-F) as described by Ho (2009). Briefly, a slurry of minced whole Pacific hake fish in water (1 : 2 w/v) was heated to 50 °C with constant stirring, after which 3% (v/w protein) of either Alcalase or Flavourzyme enzyme preparation was added. After incubation for 1 h, the hydrolysates were sieved to remove insoluble matter, and then centrifuged at 2800 × *g* for 15 min. The resulting supernatant was filtered with a double-layered filtered net, pasteurized (80 °C, 15 min), the fat removed and freeze-dried to yield the FPH-A and FPH-F used in this study.

Cod mince sample preparation. Pacific cod (*Gadus macrocephalus*) fillets, purchased from a local supermarket (Safeway, BC, Canada), were gently rinsed with tap water and then minced in a walk-in cold room (4 °C) using a Beem-Gigant Grinder Model Type F5-10 (Beem California Corp., Calif., U.S.A.) with a 4-mm screen. The mince was separated into approximately 225 g portions, to which one of the following was added: 8% SuSo in a 1 : 1 ratio, 8% FPH-A, 8% FPH-F, or no cryoprotectants (control). Individual samples were mixed thoroughly for 1 min using a KitchenAid bowl mixer (The Hobart MFG. Co., Ohio, U.S.A.) set on low speed (setting of "1"). Each mince sample was further divided into 2 portions (approximately 110 g) for the unfrozen and freeze–thaw treatments, and packaged in 0.1-mm thick 6″ × 12″ polyethylene bags, which were then heat sealed. Unfrozen samples were analyzed on the same day, while the others were subjected to 6 freeze–thaw cycles (18-h freezing at −25 ± 2 °C and 6-h thawing at 4 ± 2 °C for each cycle) before analysis.

Moisture and crude protein analysis. The mince samples were placed in aluminium pans and dried in a VWR® Vacuum Oven model 1430 (VWR Scientific Products, Ont., Canada) at 70 °C and 762 mmHg overnight (Ruiz 2001). The moisture content was determined as the weight difference before and after drying, averaged among 3 replicates. The dried samples were ground together and the total crude protein was determined by 6.25 × the total nitrogen content analyzed in duplicate using the Leco FP-428 instrument (Leco Instruments Ltd., Mich., U.S.A.) (Rhee 2001).

pH measurement. The pH of the mince samples was measured according to Sultanbawa and Li-Chan (1998). Duplicate mince samples were prepared as a 10% (w/v) solution in ddH_2O and homogenized using a Sorvall Omni mixer (Ivan Sorvall Inc., Conn., U.S.A.) at 1800 rpm for 1 min, after which the pH was immediately determined using a Fisher Accumet model 620 pH meter (Fisher Scientific Co., Pa., U.S.A.) at ambient temperature.

Expressible moisture (EM). EM was determined in triplicate according to the method by Gómez-Guillen and others (1996). Mince samples (1.5 g) were placed in a centrifuge tube containing a preweighed Gilson Pipetman pipet filter (Mandel Scientific Co. Ltd., Ont., Canada). The sample with filter was centrifuged at 4340 × *g* for 10 min at ambient temperature using a Sorvall centrifuge model RC 5B plus (Sorvall Instruments Dupont, Conn., U.S.A.). EM was expressed as percent weight gain on the filter over wet mince weight.

Unfrozen water determined using DSC. DSC measurements were conducted in triplicate using a MC-DSC model 4207 (Calorimetry Science Corp., Del., U.S.A.). Mince samples (approximately 0.05 g, equivalent mass of water in all samples) were placed in Hastelloy-C ampoules, which were then sealed and accurately weighed. An empty cell was used as the reference. The following cooling and heating profiles were applied to the samples: (1) cooling from 20 to 5 °C at a rate of 1 °C/min and equilibrate for 600 s; (2) cooling from 5 to −10 °C at a rate of 0.1 °C/min and equilibrate for 1800 s; (3) cooling from −10 to −20 °C at a rate of 1 °C/min and equilibrate for 600 s; (4) heating from −20 to −10 °C at a rate of 1 °C/min and equilibrate for 1800 s; (5) heating from −10 to 5 °C at a rate of 0.1 °C/min and equilibrate for 600 s; and (6) heating from 5 to 25 °C at a rate of 1 °C/min and equilibrate for 600 s. The resulting thermograms of heat capacity as a function of temperature showed an endothermic peak of melting for each sample. The endothermic peak was integrated from −10 °C to the temperature where the baseline was reestablished to measure the heat of fusion (ΔH_A) necessary to melt the ice, which corresponded to the free or frozen water in each sample. The antifreeze activity (AF) of the different cryoprotectants was determined using an equation modified from Mitsuiki and others (1998), and defined as the grams of unfrozen water per gram of total water in the sample, calculated according to Eq. (1):

$$AF = 1 - [\Delta H_A / \Delta H_{water}] \quad (1)$$

where ΔH_A = peak area of sample (J)/mass of water in sample (g), and ΔH_{water} = heat of fusion of pure water. The value for ΔH_{water} measured in this study was 336 J/g, which is in close agreement to the reported value of 333.271 J/g (Khan and others 2003), and the mass of water in each sample was determined from the moisture analysis.

Cook loss (CL). CL of samples was determined according to Honikel (1998) with some modifications. A total of 6 replicates (approximately 4 g) from each mince sample were placed inside previously weighed plastic cylindrical molds (2.5-cm high and 1.3-cm in diameter). The molds were wrapped with all purpose plastic food wrap (AEP Canada Inc., Ont., Canada), placed inside a polyethylene bag and cooked in a water bath (PB-2800 Boekel Scientific, Pa., U.S.A.) at 75 °C for 15 min. Molds were then inverted over paper towels and allowed to equilibrate for 10 min at room temperature before weighing. The difference in weight of the mold and mince before and after cooking was measured to determine CL, which was expressed as a percentage of the original wet mince weight.

Texture analysis (TA). The cooked mince samples after CL analysis were used for TA. Samples were carefully removed from the mold and cut cross-sectionally into 2 equal halves, with the cut surface used for TA. Texture profile analysis was performed on a TA-XT2 texture analyzer (Texture Technologies Corp., N.Y., U.S.A.) using a 5-mm diameter stainless steel probe. The probe was set at a test speed of 1 mm/s and penetration distance of 7 mm with a contact force of 5 g. Textural hardness (in gram) of the sample was

measured using the puncture test where hardness was identified as the maximal force required for the 1st deformation (peak 1) after a 7 mm penetration distance (Lian and others 2000). A total of 12 replicates were analyzed for each sample and data that fell outside the range between (Q1 − 1.5 × IQR) and (Q3 + 1.5 × IQR) were considered outliers, where IQR is the interquartile range, and Q1 and Q3 are the 1st and 3rd quadrants (Moore and McCabe 2003).

Natural actomyosin (NAM) extraction. NAM was extracted from mince samples by modification of the protocols described by Sultanbawa and Li-Chan (2001) and Wang and others (2003). Mince samples (approximately 20 g) were homogenized with 100 mL of 0.02 M sodium phosphate buffer (pH 7) in 0.05 M NaCl (Buffer A) for 1 min using a Waring™ commercial blender (Waring Products Div. Dynamics Corp. of America, Conn., U.S.A.). The homogenate was centrifuged at 10000 × *g* for 5 min at 4 °C, after which the supernatant was discarded; the pellet was again homogenized with 100 mL buffer A and centrifuged. The pellet was then homogenized with 200 mL of 0.02 M sodium phosphate buffer (pH 7) in 0.6 M NaCl (Buffer B) and centrifuged at 10000 × *g* for 10 min at 4 °C. The supernatant was filtered through double layer cotton gauze (grade 40) into a beaker with 1200 mL of 0.02 M sodium phosphate buffer (pH 7), and kept at 4 °C for 30 min with constant stirring, prior to centrifugation at 10000 × *g* for 10 min at 4 °C. The pellets were collected and homogenized with 100 mL of buffer A, blended, and centrifuged; the resulting viscous pellet was referred to as NAM. The protein concentration of the extracted NAM was determined by the bicinchoninic acid protein assay according to manufacturer's protocol using bovine serum albumin as a standard (Thermo Scientific–Pierce, Ont., Canada).

Surface hydrophobicity of NAM. Protein surface hydrophobicity (S_o) of the extracted NAM was determined in triplicate using the fluorescent probe propionyl-2-dimethylaminonaphthalene (PRODAN) by modification of the method described by Alizadeh-Pasdar and Li-Chan (2000). The concentration of PRODAN stock solution (0.032% [w/v] in spectral grade methanol) was determined from its absorbance at 360 nm and assuming the molar absorption coefficient of $1.8 \times 10^4\ M^{-1}cm^{-1}$. A dilution series of NAM in buffer B was prepared with final protein concentrations of 0, 0.050, 0.100, 0.150, 0.200, and 0.225 mg/mL. To 4 mL of each NAM solution was added 10 μL of PRODAN stock solution. After mixing by inversion and incubation in the dark for 15 min, relative fluorescence intensity (RFI) was measured on a Shimadzu RF-5301 spectrofluorometer (Shimadzu Corp., Kyoto, Japan) with excitation and emission slit widths set at 5 and 3 nm, respectively, excitation wavelength of 365 nm, and emission scans from 400 to 650 nm. The fluorescence spectrum acquisition process was performed in the dark due to the light sensitive nature of PRODAN. For each concentration of NAM, the net RFI value at 442 nm was calculated as the difference between the measured RFI of sample with PRODAN and the corresponding sample blank (NAM with no PRODAN), and then standardized to the RFI at 518 nm of the PRODAN blank (PRODAN in buffer only). The standardized net RFI values were plotted against percent protein concentrations to yield the slope, representing S_o.

Amino acid analysis of FPH. Freeze-dried hydrolysates FPH-A and FPH-F were sent to the Advanced Protein Technology Centre (APTC) at the Hospital for Sick Children in (Toronto, Ont., Canada) for amino acid analysis (http://www.sickkids.ca/Research/APTC/Amino-Acid-Analysis/index.html). Total amino acid contents were determined by hydrolysis of samples with 6 N HCl and 1% phenol at 110 °C for 24 h, precolumn derivatization using phenylisothiocyanate, followed by reverse phase high-performance liquid chromatography (HPLC). Free amino acid contents were determined by analysis without acid hydrolysis.

Statistical analysis. Analysis of variance general linear model (ANOVA-GLM) from the Minitab® 15 statistical software (Minitab Inc., Pa., U.S.A.) was used to determine whether the ingredients and freeze–thaw treatment had a significant effect on the mince samples. The differences among samples and between unfrozen and freeze–thaw treatments were analyzed by Tukey's test. Statistical significance was based on $\alpha = 0.05$ unless otherwise noted.

Results and Discussion

Moisture contents, crude protein contents, and pH

Moisture content of mince sample without the addition of ingredients was 81%, compared to 75% for samples with the addition of SuSo or FPH. The crude protein content, on a dry basis, was lower for SuSo (66%) than the control (96%), FPH-A (93%), and FPH-F (92%) samples. The incorporation of SuSo diluted the amount of crude protein in the mince sample whereas FPH, mainly consisting of free amino acids and peptides, maintained the overall crude protein content, which was determined by 6.25 × total nitrogen content.

The pH of the unfrozen samples was in the range of 7.20 to 7.32. After 6 freeze–thaw cycles, significant changes in pH were observed for all samples. Freeze–thawed control sample displayed a significant decrease in pH to 6.97 while freeze–thawed samples with SuSo, FPH-A, and FPH-F had pH of 7.55, 7.48, and 7.72, respectively. The pH of ling cod surimi was also reported to be significantly lower after frozen storage (Sultanbawa and Li-Chan 1998), and the decrease in pH observed in crab muscle after frozen storage was attributed to the formation of fatty acids by enzymatic hydrolysis of neutral fats and phospholipids (Benjakul and Sutthipan 2009). Nevertheless, the increase in pH observed in the present study after frozen storage of cod mince samples containing SuSo or FPH has not previously been reported.

Expressible moisture (EM). Unfrozen control sample had significantly higher EM (22%) than SuSo sample (15%), which was also significantly higher than samples with FPH-A (5%) and FPH-F (4%) (Figure 1). This indicates that the addition of FPH resulted in a positive effect on binding the water in the unfrozen mince samples.

After 6 freeze–thaw cycles, SuSo, FPH-A, and FPH-F samples did not incur a significant change in EM, in contrast to the 50% increase

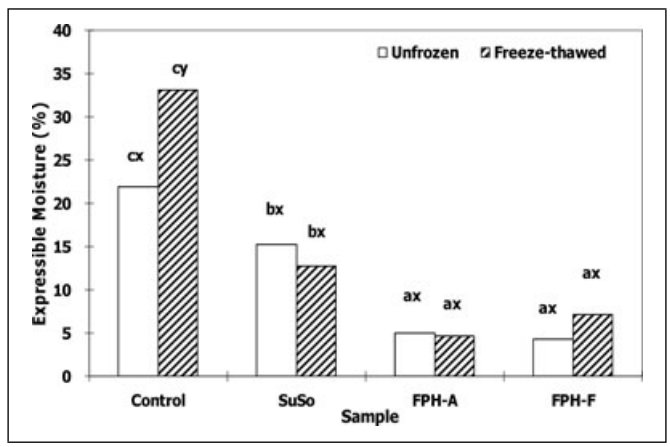

Figure 1 — EM (%, w/w) of control (with no added cryoprotectant), SuSo (1 : 1 SuSo blend), and FPH (FPH-A and FPH-F) samples before and after freeze–thaw treatment. Bars represent mean values from 3 replicates. Significant differences ($P < 0.05$) detected between samples within each treatment are denoted by letters (a, b, c) and between unfrozen and freeze–thaw treatments within each sample are denoted by x, y.

in the control sample (Figure 1). The significant increase in EM in control minced cod after freeze–thaw abuse suggests protein structural changes leading to an inability to retain water. This was similarly observed after freeze–thawing of cod muscle fillet (Benjakul and Bauer 2000) and shrimp muscle (Sriket and others 2007), and during frozen storage of croaker, lizard fish, threadfin bream, and bigeye snapper (Benjakul and others 2003).

In this study, there was an eightfold difference in EM between FPH and control samples after freeze–thawing; moreover, freeze–thawed samples with FPH-A and FPH-F both had significantly lower EM than the freeze–thawed sample with SuSo (Figure 1). Effective protection by carbohydrate-based ingredients against moisture loss after frozen storage or freeze–thaw abuse is well documented (Yoon and Lee 1990; Sultanbawa and Li-Chan 1998; Jittinandana and others 2005) but the discovery of better water-holding capacity of FPH samples suggests that FPH could be considered as an alternative to the carbohydrate-based ingredients.

Unfrozen water determined using DSC. To provide further insight on the influence of FPH on water binding properties of the minced fish, the proportion of unfrozen water in the samples was investigated using DSC. For the unfrozen samples, the control showed the lowest proportion of unfrozen water (0.24 g/g), followed by the sample with SuSo (0.29 g/g), while both minced cod with FPH-A and FPH-F had the highest proportion of unfrozen water (0.34 g/g) (Table 1). After 6 freeze–thaw cycles, minced cod with SuSo showed significantly higher proportion of unfrozen water (0.33 g/g) than the unfrozen SuSo sample but the freeze–thawed SuSo sample still had significantly lower proportion of unfrozen water than the freeze–thawed FPH samples (0.36 g/g) (Table 1). The increase in the proportion of unfrozen water was reported upon addition of other cryoprotective ingredients such as fish-scrap protein hydrolysate (Khan and others 2003), chitin hydrolysate (Yamashita and others 2003; Somjit and others 2005), and squid protein hydrolysate (Hossain and others 2004). The significantly lower proportion of unfrozen water in the control sample could be attributed to the significantly lower solid content of the sample, which might have reduced its ability to bind water.

The higher proportion of unfrozen water observed in SuSo and FPH samples was also consistent with the data obtained from EM analysis. The higher amount of unfrozen water in SuSo and especially in the FPH samples contributed to the lower EM as the water was tightly bound. Conversely, the control, which contained less unfrozen water, displayed higher EM due to the availability of free water that was easily expressed out of the muscle system during freeze–thawing. It is worth noting that at the same (8%) level of incorporation to the minced cod, both FPH-A and FPH-F samples resulted in higher proportions of unfrozen water, as well as lower EM, than the SuSo blend.

Table 1 — The proportion of unfrozen water determined by DSC of control (with no added cryoprotectant), SuSo (1 : 1 SuSo blend), and FPH (FPH-A and FPH-F) samples before and after 6 freeze–thaw cycles.[A]

	Unfrozen water (g/g)	
	Unfrozen	Freeze–thawed
Control	0.24 a,x	0.24 a,x
SuSo	0.29 b,x	0.33 b,y
FPH-A	0.34 c,x	0.36 c,x
FPH-F	0.34 c,x	0.36 c,x

[A]Reported values are mean values from 3 replicates. Values bearing different letters (a, b, c and x, y) within each column and each row, respectively, are significantly different ($P < 0.05$).

Cook loss (CL). Maintaining the juiciness and yield of the cooked product is an important quality parameter that can be assessed by measuring the loss in weight after cooking. No significant differences were observed among unfrozen minced cod samples with or without the addition of cryoprotectants (Figure 2). However, after 6 freeze–thaw cycles, the sample containing FPH-A did not change in CL while control, SuSo and FPH-F samples displayed significant increases in CL. SuSo and FPH-F samples both had a twofold increase in CL, compared to an increase from 2.8% of the unfrozen control to 15.5% of freeze–thawed control minced cod (Figure 2). The increase in CL was also observed from the frozen storage of oil sardine mince (Verma and others 1995) and red hake (Lian and others 2000).

When the CL of the freeze–thawed SuSo, FPH-A, and FPH-F samples were compared to the unfrozen control, it was found that only freeze–thawed FPH-A minced cod remained similar in CL while both samples containing SuSo and FPH-F had higher CL. The difference in the level of CL could be attributed to compositional differences in the ingredients that impact on their effectiveness to act as cryoprotectants. The significant difference in CL observed for FPH-A and FPH-F hydrolysates prepared from the same batch of Pacific hake but using different proteolytic enzyme preparations was evident.

Texture analysis. After cooking, the unfrozen samples containing FPH, especially FPH-A, showed significantly higher textural hardness than unfrozen control and SuSo samples (Figure 3). It was expected that unfrozen mince samples with the addition of ingredients would result in a harder texture, owing to their lower moisture content. Nevertheless, despite having lower moisture content, the unfrozen SuSo sample maintained a similar textural hardness to the unfrozen control, suggesting the ability of SuSo in retaining the original texture of the mince. After 6 freeze–thaw cycles, mince containing SuSo showed a significant decrease in hardness, and was softer than the freeze–thawed control sample (Figure 3). Textural hardness of freeze–thawed FPH samples also decreased significantly, and there was no longer any significant difference between freeze–thawed control and FPH samples (Figure 3). In fact,

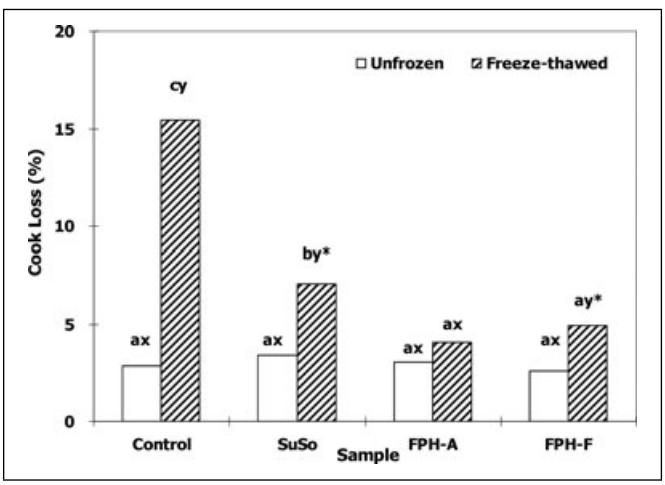

Figure 2 — CL (%, w/w) of control (with no added cryoprotectant), SuSo (1 : 1 SuSo blend), and FPH (FPH-A and FPH-F) samples before and after freeze–thaw treatment. Bars represent mean values from 6 replicates. Significant differences ($P < 0.05$) detected between samples within each treatment are denoted by letters (a, b, c) and between unfrozen and freeze–thaw treatments within each sample are denoted by x, y. Freeze–thawed samples with significantly different CL ($P < 0.05$) from the unfrozen control are denoted by an asterisk.

when the freeze–thawed samples were compared to the unfrozen control minced cod, which should reflect the original quality of the mince prefreezing, it was interesting to note that the SuSo sample had a significantly softer texture and the sample with FPH-A had a harder texture, whereas the sample with FPH-F maintained a similar texture to the unfrozen control sample (Figure 3). The softer texture of cooked products incurred by the addition of 8% SuSo has been previously reported (Jittinandana and others 2005). The initial textural hardening observed in the FPH samples could arise from interactions between amino acids and peptides in FPH with cod proteins to yield a stronger gel network in the cooked mince, similar to the reported action of the casein hydrolysate Lactamine AA in preventing loss of gel cohesiveness of cod surimi during frozen storage (Sych and others 1991). Although the mechanism by which FPH induced a textural softening effect post freeze–thaw treatment is not clear, the similarity in textural hardness in freeze–thawed FPH-F and unfrozen control samples indicates the potential advantage of using FPH-F to maintain quality of frozen fish mince.

NAM extractability and surface hydrophobicity. Changes in extractability and surface hydrophobicity of NAM can be a useful tool to evaluate the extent to which fish protein denaturation and aggregation may have occurred during frozen storage or as a result of temperature abuse situations such as freeze–thawing cycles (del Mazo and others 1999; Lian and others 2000). During frozen storage, actin and myosin become gradually less extractable in salt solutions, forming high molecular weight protein aggregates (Careche and Li-Chan 1997; Careche and others 1999).

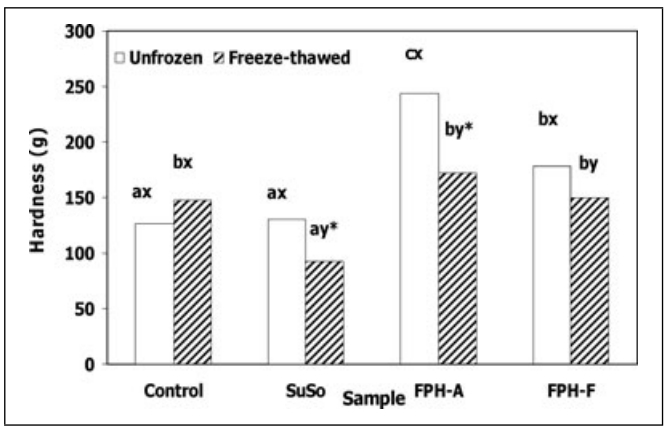

Figure 3—Textural hardness (g) after cooking of control (with no added cryoprotectant), SuSo (1 : 1 SuSo blend), and FPH (FPH-A and FPH-F) samples before and after freeze–thaw treatment. Bars represent mean values from 12 replicates. Significant differences ($P < 0.05$) detected between samples within each treatment are denoted by letters (a, b, c) and between unfrozen and freeze–thaw treatments within each sample are denoted by x, y. Freeze–thawed samples with significantly different textural hardness ($P < 0.05$) from the unfrozen control are denoted by an asterisk.

The amount of NAM extracted from the unfrozen mince samples varied significantly, with control, FPH-F, SuSo, and FPH-A samples containing 0.81, 0.73, 0.66, and 0.60 g extractable NAM/g protein in the fish mince, respectively, after taking into account the potential dilution effect by the addition of ingredients (Table 2). The variation in extractable NAM content may be related to changes in muscle protein structure as reflected by the higher surface hydrophobicity of NAM extracted from unfrozen samples with SuSo, FPH-A, and FPH-F (Table 2). Structural changes in NAM and surimi from ling cod in the presence of various polyol cryoprotectants have also been reported (Sultanbawa and Li-Chan 2001).

After 6 freeze–thaw cycles, the control minced cod showed > 50% loss in NAM extractability and >50% increase in surface hydrophobicity, suggesting freeze–denaturation and aggregation of proteins (Table 2). Even though the sample containing FPH-A also showed a significant decrease in extractable NAM after freeze–thawing (from 0.60 to 0.50 g/g), the decrease was noticeably less than that of the control sample (Table 2). In contrast to the increase in surface hydrophobicity observed in NAM extracted from control mince after freeze–thawing, there was a decrease in surface hydrophobicity in NAM from freeze–thawed FPH-A sample. On the other hand, samples with SuSo and FPH-F displayed a protective effect on muscle proteins, resulting in higher extractable NAM contents than the control sample after freeze–thawing, as well as the lack of any significant difference in surface hydrophobicity in NAM extracted from these samples before or after freeze–thaw treatment (Table 2).

A decrease in salt soluble proteins after frozen storage in the absence of added cryoprotective ingredients has been reported for many fish species including red hake mince (Lian and others 2000), farmed rainbow trout (Herrera and Mackie 2004), tilapia surimi (Zhou and others 2006), and ling cod surimi (Sultanbawa and Li-Chan 1998). The ability of a SuSo blend to minimize the loss of salt extractable proteins and changes in surface hydrophobicity upon frozen storage is also well documented (Sych and others 1990a; Sultanbawa and Li-Chan 1998; Herrera and Mackie 2004; Zhou and others 2006). However, the effectiveness of FPH-F hydrolysate for this purpose has not been reported before, and this characteristic, in combination with the previously mentioned water-holding capacity and the protection against CL, provides further support for the application of FPH as an alternative to SuSo as an ingredient for cryoprotection.

Amino acid composition of FPH

Table 3 shows the contents of free and total amino acids of the 2 FPHs used as potential cryoprotectants in this study. Overall, hydrolysates FPH-A and FPH-F contained similar amounts of total amino acids (Table 3). FPH-F was prepared by hydrolysis with Flavourzyme, an enzyme mixture containing exo- and endopeptidases, which resulted in almost 50% of its amino acid content being present in free form; on the contrary, FPH-A, produced using

Table 2—The extractability and surface hydrophobicity of NAM from control (with no added cryoprotectant), SuSo (1 : 1 SuSo blend), and FPH (FPH-A and FPH-F) samples before and after 6 freeze–thaw cycles.[A]

	Extractable NAM (g/g protein in mince)		Surface hydrophobicity ($\%^{-1}$)	
	Unfrozen	Freeze–thawed	Unfrozen	Freeze–thawed
Control	0.81 d,x	0.40 a,y	23 a,x	36 c,y
SuSo	0.66 b,x	0.72 c,y	33 b,x	30 ab,x
FPH-A	0.60 a,x	0.50 b,y	37 c,x	26 a,y
FPH-F	0.73 c,x	0.68 c,x	31 b,x	32 bc,x

[A]Reported values are mean values from triplicate analyses. Values of extractable NAM or surface hydrophobicity bearing different letters (a, b, c, d and x, y) within each column and each row, respectively, are significantly different ($P < 0.05$).

Alcalase that is an endoprotease, had only about 20% free amino acids (Table 3).

Comparison of the free amino acid contents of the 2 hydrolysates clearly indicates that FPH-F contained higher contents of free Asp, Glu, Arg, and Lys, which have been reported to be cryoprotective (Jiang and others 1987; Carpenter and Crowe 1988; Heinz and others 1990; Chen and others 2005). This could in part explain the better cryoprotective ability of FPH-F, in comparison to FPH-A, as demonstrated in the present study. However, the majority of the free amino acids in both FPH-A and FPH-F appeared to be hydrophobic in nature. Since hydrophobic amino acids have been previously reported to be cryosensitizers (Heber and others 1971; Heinz and others 1990; Chen and others 2005), the presence of many hydrophobic amino acids in free form in the hydrolysates might therefore raise questions as to why they did not become cryosensitive and implies that there might be other contributors to the cryopreventive ability that have outweighed the cryosensitivity introduced by the hydrophobic side chains.

Aside from free amino acids, peptides constitute a dominant portion of the protein hydrolysates. The size of the peptides could be an important factor influencing the differences in their interactions with the cod proteins, and in ice crystal formation and growth in cod mince during frozen storage. Size exclusion chromatography on Sephadex G-25 indicated that FPH-F had a higher proportion of dipeptides or amino acids with molecular weight below 0.2 kDa, as well as polypeptides with size > 14 kDa, while FPH-A contained more peptides of intermediate size between 1 and 10 kDa (Ho 2009), in agreement with the amino acid results that FPH-F contained more free amino acids than FPH-A. In theory, since FPH-A contained higher proportions of oligopeptides than FPH-F, it should offer better cryoprotection as suggested by Damodaran (2007) that short-chain peptides may be important in protection against freezing by binding to ice nuclei via hydrogen bonding and thus inhibiting ice crystal growth. Nevertheless, results from the current study displayed stronger cryoprotection in FPH-F, suggesting that peptide composition might also be a critical factor in determining cryopreventive ability (Tyankova 1972). Up to 60% of the total amino acid content of both FPH-A and FPH-F was composed of charged or hydrophilic residues such as Asp, Glu, Ser, Gly, His, Arg, Thr, Pro, and Lys, which have been previously reported to offer effective protection against freezing (Heber and others 1971; Rudolph and Crowe 1985; Jiang and others 1987; Carpenter and Crowe 1988; Chen and others 2005). The presence of these amino acid residues in oligopeptides present in both FPH might therefore be contributing to cryoprotection of minced cod.

Therefore, results from this study points towards peptides likely being the major contributors to the cryopreventive ability of both FPH, although free amino acids such as Glu, Asp, Arg, and Lys could play a role in FPH-F. Further research should focus on examining the composition and characteristics of free amino acids and peptides in FPH that provide strongest cryoprotective activity. Furthermore, through understanding the structural requirements of amino acids and peptides to be effective cryoprotectants, an optimized process could be devised to produce FPH with ideal cryoprotective capacity.

Table 3 — Free and total amino acid composition of FPHs FPH-A and FPH-F.[A]

Amino acid	Amino acid content, g/100 g dry sample			
	FPH-A		FPH-F	
	Free[a]	Total[b]	Free[a]	Total[b]
Asp	0.20	5.13	1.24	4.59
Glu	0.43	7.93	1.39	7.28
Asn	0.19	0.19	0.28	0.28
Ser	0.41	2.23	0.97	2.02
Gln	0.38	0.38	0.86	0.86
Gly	0.13	3.13	0.44	3.04
His	0.11	1.01	0.10	0.61
Tau	0.32	0.35	0.36	0.42
Arg	0.79	3.47	1.76	3.23
Thr	0.38	2.30	0.93	1.99
Ala	0.73	3.29	1.39	3.11
Pro	0.08	2.18	0.09	1.95
Tyr	0.71	1.58	1.01	1.23
Val	0.49	2.47	1.37	2.36
Met	0.65	1.64	1.05	1.44
Cys	1.23	1.37	1.25	1.42
Ile	0.30	2.12	1.13	1.97
Leu	1.32	4.05	2.70	3.71
Phe	0.78	1.96	1.20	1.59
Trp	0.13	0.13	0.19	0.19
Lys	0.74	4.65	2.07	3.94
Total	10.50	51.56	21.78	47.22

[A]Data were adapted from Ho (2009).
[a]Free amino acid contents by analysis without hydrolysis.
[b]Total amino acid contents by analysis after acid hydrolysis.

Conclusions

The present study provides evidence to support development of FPH as a potential alternative to carbohydrate-based cryoprotective ingredients commonly used to stabilize frozen fish products. Minced cod samples containing either FPH-A or FPH-F have shown comparable or better cryoprotective properties than SuSo sample, likely linked to the presence of oligopeptides in both hydrolysates. Furthermore, the enhanced cryoprotection exhibited by FPH-F hydrolysate could also be attributed in part to its composition of free amino acids, especially the high contents of Asp, Glu, Arg, and Lys. Overall, the data in this study provide evidence that FPH could be utilized as an effective new generation of cryoprotectants for frozen storage of fish. Further research should be conducted to elucidate the influence of varying free amino acid and peptide composition on the effectiveness and mechanism of action of cryoprotection by FPH.

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