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Mercury Distribution and Lipid Oxidation in Fish Muscle: Effects of Washing and Isoelectric Protein Precipitation

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ABSTRACT: Nearly all the mercury (Hg) in whole muscle from whitefish (*Coregonus clupeaformis*) and walleye (*Sander vitreus*) was present as methyl mercury (MeHg). The Hg content in whole muscle from whitefish and walleye was 0.04−0.09 and 0.14−0.81 ppm, respectively. The myofibril fraction contained approximately three-fourths of the Hg in whitefish and walleye whole muscle. The sarcoplasmic protein fraction (e.g., press juice) was the next most abundant source of Hg. Isolated myosin, triacylglycerols, and cellular membranes contained the least Hg. Protein isolates prepared by pH shifting in the presence of citric acid did not decrease Hg levels. Addition of cysteine during washing decreased the Hg content in washed muscle probably through the interaction of the sulfhydryl group in cysteine with MeHg. Primary and secondary lipid oxidation products were lower during 2 °C storage in isolates prepared by pH shifting compared to those of washed or unwashed mince from whole muscle. This was attributed to removing some of the cellular membranes by pH shifting. Washing the mince accelerated lipid peroxide formation but decreased secondary lipid oxidation products compared to that of the unwashed mince. This suggested that there was a lipid hydroperoxide generating system that was active upon dilution of aqueous antioxidants and pro-oxidants.

KEYWORDS: oil, methyl mercury, rancidity, surimi, fish, contaminants

INTRODUCTION

Fish can be an important part of a healthy diet providing high quality protein and elevated levels of long chain omega-3 fatty acids. Long chain omega-3 fatty acids are particularly noted for providing cardiovascular benefits.[1] Eating fish products containing elevated levels of mercury may be neurodegenerative particularly in the absence of dietary selenium.[2] The distribution of mercury in fish muscle has received little attention. Mason et al.[3] stated that the predominance of methyl mercury in fish resides in the protein rather than fat tissue. This does not distinguish between sarcoplasmic proteins in the aqueous phase, myofibrillar proteins, and proteins associated with cellular membranes. A ternary complex of selenium, mercury, and selenoprotein P in the plasma fraction of blood has been described.[4] Neutral and negatively charged phospholipids in cellular membranes also have the potential to interact with methyl mercury by hydrophobic and electrostatic interactions.

Acid- or alkaline-sedimentation of cellular membranes with subsequent isoelectric precipitation (e.g., pH shifting) is done to prepare isolates from fish muscle that are rich in protein and mostly devoid of cellular membranes.[5] Thus, this pH shifting process has the potential to decrease mercury levels in the protein isolates in the event that substantial quantities of mercury associate with the membrane fraction.

Mercury distribution and lipid oxidation were jointly investigated because the procedures described herein have the potential to affect both lipid stability and final mercury content. Lake whitefish was chosen for this work because of their high economic value and the fact that this species is known to be particularly sensitive to lipid oxidation.[6] Thus, our work explored the ability of pH shifting to inhibit lipid oxidation in lake whitefish and affect mercury content. Walleye, like other top predator picivorous fish, are one of the highest of all freshwater species in accumulating mercury. Walleye are one of the most sought after sport fish of all freshwater fish, which makes them especially important in terms of human exposure. Lake whitefish was the most harvested and valued fish in the Great Lakes in 2000, while walleye was the third most valued.[7]

The main objective of this work was to examine partitioning of mercury between different phases of fish muscle including cellular membranes, triacylglycerols, myofibrillar proteins, and press juice that contains sarcoplasmic proteins. Another objective was to determine the oxidative stability of minced muscle compared to that of washed mince and isolates derived from pH shifting.

MATERIALS AND METHODS

Materials. Whitefish (catch A) were obtained from Larson's Reef off Sturgeon Bay, WI; the fillets were 20−25 cm in length. Whitefish (catch B) were obtained from Lake Michigan near Sheboygan, WI; fish lengths were 47−50 cm. Whitefish (catch C) were obtained from the Northern portion of Lake Michigan; fish lengths were 48−50 cm. Whitefish (catch D) were obtained from Lake Superior; fish lengths were 40−48 cm. Walleye (catch E) were obtained from Lake Michigan near Sturgeon Bay, WI;

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fish lengths were 56−71 cm. Walleye (catch G) were obtained from the northern portion of Lake Erie. Walleye (catch H) were obtained from Little Sturgeon Bay in Lake Michigan; fish lengths were 61−74 cm. Fish were obtained with the assistance of the Wisconsin Department of Natural Resources and personal contacts. All fish were transported at refrigerated temperatures and were obtained within 24−48 h of capture.

Bacterial proteinase (type XXIV), ammonium thiocyanate, iron(II) sulfate, barium chloride, 2-thiobarbituric acid, sodium ascorbate, sodium tartrate, Folin−Ciocalteu phenol reagent, bovine serum albumin, phenylmethysulfonylfluoride (PMSF), NaN_3, $Mg(CH_3COO)_2$, Tris-maleate, ethylene glycol-bis (β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetra acetic acid(EGTA), β-mercaptoethanol (β-MCE), Trition-X 100, and HEPES were obtained from Sigma Chemical A/S (St. Louis, MO). Chloroform, methanol, trichloroacetic acid, acrylamide, bis-acrylamide, tris [hydroxymethyl] aminomethane, sodium dodecyl sulfate, urea, and thiourea were obtained from Fisher Scientific (Fairlawn, NJ). Tetramethylethylenediamine (TEMED) and prestained broad-range SDS−PAGE standard were obtained (Biorad, Inc., Hercules, CA). All other chemicals and reagents were of analytical grade.

Isolation of Cellular Membrane Fraction. Isolated membranes from whitefish and walleye muscle were prepared mostly as described previously.[8,9] Filets were minced with a Kitchen-Aid model KSM 90WW household mixer (St. Joseph, MI) equipped with a grinding apparatus (5 mm plate diameter). Proteinase XXIV (0.5%, w/w) was added to the mince, hand-mixed for 2 min, and incubated for 60 min at 4 °C. Four volumes of HEPES (0.1 M, pH 7.5) were added to the muscle. The mixture was then homogenized for 1 min at speed 3 using a model PT 10-35 Polytron homogenizer (Kinmatica AG, Littau, Switzland). The homogenized mixture was centrifuged at 10,000*g* for 20 min at 5−10 °C using a Beckman L8-M Ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). The supernatant was centrifuged at 130,000*g* for 30 min. The pellet was resuspended in chilled 30 mL of 0.6 M KCl using the Potter-Elvehjem tissue grinder and centrifuged at 130,000*g* for 30 min. The resulting pellet was analyzed for mercury, protein, and phosphorus content.

Isolation of Triacylglycerols (TAG). Mince (100 g) was centrifuged at 104,000*g* for 30 min at 5−10 °C. The supernatant containing oil and aqueous phase was added to a separatory funnel, and 10 vol of hexanes was added, shaken vigorously, and stored overnight at 4 °C. The upper hexane layer was evaporated in a 500 mL flask by a vacuum rotary evaporator in a 50 °C water bath (Brinkman Instruments, Inc., Westbury, NY). TAG was collected and stored at −20 °C for Hg analysis.

Isolation of Press Juice Fractions. Approximately 50 g of mince was packed in a 250 mL centrifuge tube and centrifuged at 16,300*g* using a Sorvall RC5C centrifuge (Kendro Laboratory Products, Newtown, CT) for 1 h at 4 °C. After centrifugation, an aqueous phase, oil phase (top of tube), and sediment were obtained. The supernatant phase was added to a 50 mL polycarbonate centrifuge tube and centrifuged at 10,000*g* for 10 min at 5−10 °C. The aqueous phase was carefully removed with a glass pipet and designated as press juice.

Myofibril Preparation. Myofibrils were prepared with modifications according to a previous procedure.[10] Ten grams of mince was homogenized with a polytron using 80 mL of buffer containing 1% Triton-X 100 (peroxide and carbonyl free), 50 mM NaCl, and 10 mM HEPES (pH 6.8) for 1 min. The mixture was centrifuged at 10,000*g* for 20 min (5−10 °C). The supernatants were discarded, and the sediments were homogenized again in the buffer and centrifuged. The supernatants were discarded, and the sediments were centrifuged again. The supernatants were discarded, and the resulting sediments were designated as myofibrils. Phosphorus determination was done to ensure that cellular membranes were mostly removed compared to that of a minced whole muscle.

Myosin Preparation. Myosin was prepared with modifications according to previous procedures.[11,12] Solution A contained 0.10 M KCl, 1 mM phenylmethysulfonylfluoride (PMSF), 0.02% NaN_3, and 20 mM Tris-HCl (pH 7.5). Solution B contained 0.45 M KCl, 5 mM 0.2 M $Mg(CH_3COO)_2$, 20 mM Tris-Maleate buffer at pH 6.8, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and β-mercaptoethanol (β-MCE). In some cases, EGTA and MCE were removed in the isolation procedure. Solution C contained 0.50 M KCl, 20 mM Tris-HCl buffer at pH 7.5, and 5 mM β-MCE. Fifteen grams of mince was mixed with 10 vol of Solution A using a spatula at 4 °C for 30 min. The mixture was centrifuged at 1,000*g* for 10 min at 4 °C. The supernatant was discarded. The pellet was homogenized for 1 min in 5 vol solution B (75 mL), and ATP was added to a final concentration of 10 mM and kept at 4 °C for 1 h. The homogenate was centrifuged at 10,000*g* for 15 min at 4 °C. The pellet was discarded. The supernatant was diluted with 5 vol (75 mL) of 1 mM $NaHCO_3$, kept at 4 °C for 15 min, and centrifuged at 12,000*g* for 10 min. The supernatant was discarded. The pellet was suspended in 2.5 vol solution C, homogenized in a Potter-Elvehjem glass tissue with pestle, and kept at 4 °C for 10 min. The homogenate was diluted with 2.5 vol of 1 mM $NaHCO_3$. $MgCl_2$ was added to a final concentration of 10 mM and kept at 4 °C overnight. The mixture was centrifuged at 20,000*g* for 15 min. The supernatant was discarded. The pellets were analyzed for moisture, mercury, protein, and phosphorus content.

Preparation of Protein Isolates by pH Shifting. Protein isolates (PI) from mince were prepared according to previous procedures.[13,14] Six volumes of ice-cold distilled−deionized water was added to the mince (50−100 g). The mixture was homogenized for 1 min at speed 3 by a model PT 10-35 Polytron homogenizer. Sodium citrate was added to 5 mM in the homogenate and incubated at 4 °C for 1 h. $CaCl_2$ was added to the homogenate to achieve 10 mM. pH was adjusted to pH 2.8 using 2 N HCl (acid solubilization) or to pH 11 by 2 N NaOH (alkaline solubilization). The solubilized homogenate was centrifuged at 8000*g* for 20 min at 4 °C. The sediment was discarded. The floating top layer was separated from the supernatant by filtering through three layers of cheesecloth (American Fiber and Finishing, Inc., Albemarle, NC). The acidic supernatant fraction was adjusted to pH 5.8 with 2 N NaOH. The basic supernatant fraction was adjusted to pH 5.2 with 2 N HCl. Centrifugation was then done once or twice at 8000*g* for 20 min. The sediment layer comprised the protein isolate. The protein isolates were collected and analyzed for moisture, protein, total lipids, and mercury content.

Preparation of Traditional Surimi in Raw State. Mince was mixed with 4 parts water (4 °C) and held for 15 min after 3 min of stirring.[15] The washed mince was then strained using 3 layers of cheesecloth. The washing and filtering process was repeated once with water and once with the last wash including 0.2% NaCl in the wash water. The sediment was centrifuged at 8000*g* for 20 min at 4 °C and collected.

Thermal Processing of Surimi. Surimi gels were prepared as described previously.[16] In brief, the moisture of the raw surimi was adjusted to 80% and chopped with 2% NaCl, 0.3% sodium tripolyphosphate, and 0.4% sodium bicarbonate in a FP1510 PowerPro II food processor (Black and Decker, Hunt Valley, MD) for 4−5 min at 4 °C. The samples were then adjusted to pH 7.1 while chopping using 1 N NaOH or 1 N HCl. The paste was stuffed into a 2 cm synthetic casing (Viscase, Loudon, TN) and then cooked at 90 °C for 30 min. After cooking, the gels was cooled in an ice water bath for 15 min and stored at 4 °C for 48 h prior to mercury analysis.

Preparation of Surimi Treated with Cysteine. Ground muscle (1 part) was mixed with (4 parts) cold (4 °C) 0.5% cysteine solution and held on ice for 15 min after 3 min of stirring. The mixture was then filtered with 3 layers of cheesecloth. The washing and filtering processes were repeated with cold 0.5% cysteine solution. The last washing and filtering process was done with cold 0.2% NaCl in the wash water. The sediment was centrifuged at 8000*g* for 20 min and stored at −20 °C.

Table 1. Methyl Mercury (MeHg) and Total Mercury Concentrations in Whole Muscle and Protein Isolates from Whitefish and Walleye[a]

sample	batch of fish	methyl mercury (ng/g dry weight)	total mercury (ng/g dry weight)
whitefish whole muscle	C	142.1 ± 6.3	143.1 ± 6.1
whitefish PI (alkaline)	C	200.5 ± 15.7	233.2 ± 10.3
whitefish PI (acid)	C	210.7 ± 18.4	206.5 ± 2.6
walleye whole muscle	H	3,599.4 ± 48	3,788 ± 73

[a] PI: protein isolate. Means and standard deviations are reported.

Total Mercury and Methyl Mercury Determinations. Mercury content in tissues and isolates was determined at the United States Geological Survey (USGS) Wisconsin Water Science Center facility (Middleton, WI). Samples were first digested in a Teflon bomb containing 7 mL of a 5/2 mixture of concentrated nitric and sulfuric acids, respectively. The bombs were sealed (screw top bombs) and held in an oven at 125 °C for two hours. The samples were brought up to 30 mL of total volume with a 5% bromine monochloride solution to ensure complete oxidation and heated at 50 °C in an oven overnight. Samples were then analyzed according USEPA method 1631 with an automated purge and trap flow injection system that incorporates gold traps and a cold vapor atomic fluorescence spectrometer (Tekran model 2600).

SDS−PAGE Electrophoresis. SDS−PAGE was done as described previously.[17] Protein standards were myosin (215 kDa), phosphoylase B (100 kDa), bovine serum albumin (84 kDa), ovalbumin (60 kDa), carbonic anhydrase (39 kDa), trypsin inhibitor (28 kDa), and lysosyme (18.3 kDa). A modified Lowry's method was used to determine protein content.[18]

Drying of Tissues and Moisture Determination. Aluminum weighing boats were predried in a laboratory oven at 105 °C for 20 to 30 min. Fish tissue (around 1 g) was dried in a laboratory oven at 105 °C overnight (about 17 h). The dried tissue in the aluminum weigh boats were placed in desiccators for half an hour at room temperature and were then weighed. Moisture content was calculated. Dried fish tissue was put into glass vials (5 mL capacity) and stored in desiccators prior to Hg analysis.

Storage Studies. Minced muscle, traditional surimi, acid protein isolates, and alkaline protein isolates (20−25 g) were packaged in O_2-permeable, 10 × 15 cm, low density polyethylene bags (Nalgene, Rochester, NY) and stored at 2 °C. The tissue was spread thin (3−5 mm) to ensure access to oxygen. pH of the surimi and protein isolates were adjusted to the pH of the whole muscle. Whitefish muscle was at pH 6.5, and walleye muscle was at pH 6.6. The moisture content of whitefish mince was 77.8%. The moisture of the surimi and protein isolates prepared from whitefish was 80.9%. The moisture content of the walleye mince was 79.0%. The moisture of the surimi and protein isolates prepared from walleye were 82.5%.

Lipid Assessment and Lipid Oxidation Indicators. Total lipid content was analyzed as described previously.[19] The phosphorus contents were determined as described previously.[20] Lipid peroxides were determined according to the method of Shantha and Decker[21] as modified by Undeland et. al.[22] Lipids were extracted with chloroform/methanol, which was followed by the addition of ferrous chloride and thiocyanate, which produced a colored complex upon reaction with peroxides. Thiobarbituric acid reactive substances (TBARS) were determined as described previously except that 1 g of sample was extracted with 6 mL of trichloroacetic acid (TCA).[23] Lipid oxidation products, mostly aldedhydes, were extracted with TCA, filtered, and reacted with TBA, which produced a colored complex.

Statistical Evaluations. A MIXED procedure of the SAS system was used to analyze data from the storage studies.[24] Means were separated using the p-diff test. The MIXED procedure was also used to analyze multiple treatments in which the measurements did not include a storage component. At least three preparations of each treatment were examined and used as the source of replication for the statistical procedures.

Table 2. Hg Levels in Different Isolates from Whitefish Whole Muscle[a]

	batch of fish	ng/g dry weight	μg/100 g muscle (wet weight)	% Hg in the fraction
whole muscle	A	437.3 ± 8.1	9.7	
triacylglycerols		243.7 ± 73.9	0.7[b]	7.9
membrane		283.3 ± 10.3	0.9[c]	7.5
myosin		70.1 ± 3.0	0.4[d]	3.6
whole muscle	B	408.0 ± 21.5	8.4	
membrane		251.4 ± 11.3	0.7	8.3
whole muscle	C	143.1 ± 6.1	3.8	
myofibril		239.1 ± 11.4	2.7[e]	71.1
membrane			0.20	5.3
press juice		97.8 ± 8.1	0.49[f]	12.8

[a] The total lipid content in the whole muscle from batches A, B, and C was 3.19 ± 0.35, 3.54 ± 0.15, and 8.17 ± 0.75, respectively. The phospholipid content in the whole muscle from batches A, B, and C was 0.52 ± 0.02, 0.57 ± 0.02, and 0.62 ± 0.06, respectively. Means and standard deviations are reported. [b] Triacylglycerol content in the whole muscle was obtained by subtracting the phospholipid content from the total lipid content. [c] Membrane content in the whole muscle was obtained by weighing the dried membrane isolate gravimetrically and then accounting for the fraction of the membrane that was not obtained in the isolate. Approximately 30% of the total membrane was isolated based on the phospholipid contents in a given mass of whole muscle and in the membrane isolate obtained from the same mass of whole muscle. [d] An approximation of 5% myosin in whole muscle was used.[25] [e] An approximation of 11.5% myofibril in whole muscle was used.[25] [f] An approximation of 5% sarcoplasmic proteins in the whole muscle was used.[26] Thus, 100 g of whole muscle (wet weight basis) will contain approximately 5 g of dry matter from press juice.

RESULTS

Levels of Methyl Mercury and Total Mercury in Muscle and Protein Isolates. Methyl mercury (MeHg) and total mercury levels (that include inorganic and organic forms) were determined in whole muscle from whitefish and walleye as well as protein isolates from whitefish using the acid and alkaline pH shifting procedures. The % MeHg was calculated as nanograms of MeHg/nanograms of total Hg × 100. MeHg comprised 99%, 86%, 102%, and 95% of the total mercury based on the mean values from three separate preparations for each type of sample (Table 1). Mercury levels were determined in dried samples. Moisture contents were determined before drying so that the mercury content could also be reported on a wet weight basis in the muscle and protein isolates.

Levels of Mercury in Muscle and Muscle Fractions. Hg levels were determined in whole muscle from whitefish and compared to levels in isolates that included triacylglycerols, cellular membranes, myosin, myofibrillar protein preparations, and press juice, which contained mostly sarcoplasmic proteins. The Hg level in whole muscle from four catches of whitefish varied from 0.04 to 0.10 μg/g (wet weight basis) (Tables 2 and 4). The triacylglycerol, cellular membranes, and myosin isolates

Table 3. Hg in Different Isolates from Walleye Whole Muscle[a]

	batch of fish	ng/g dry weight	μg/100 g muscle (wet weight)	% Hg in the fraction
whole muscle	E	2892.4 ± 114.9	58.4	
triacylglycerols		62.3 ± 64.1	0.1[b]	0.2
membrane		672.6 ± 29.7	1.1[c]	1.9
myosin		599.9 ± 74.9	3.0[d]	5.1
myofibril		3848.2 ± 43	44.3[e]	76.0
whole muscle	G	702.0 ± 52.0	14.3	
membrane			0.2	1.4
press juice		1045.2 ± 39.2	5.2[f]	36.4

[a] The total lipid content in the whole muscle from batches E and G was 2.34 ± 0.09 and 2.15 ± 0.01, respectively. Means and standard deviations are reported. [b] Triacylglycerol content in the whole muscle was obtained by subtracting the phospholipid content from the total lipid content. [c] Membrane content in the whole muscle was obtained by weighing the dried membrane isolate gravimetrically and then accounting for the fraction of the membrane that was not obtained in the isolate. Approximately 30% of the total membrane was isolated based on the phospholipid contents in a given mass of whole muscle and in the membrane isolate obtained from the same mass of whole muscle. [d] An approximation of 5% myosin in whole muscle was used.[25] [e] An approximation of 11.5% myofibril in whole muscle was used.[25] [f] An approximation of 5% sarcoplasmic proteins in the whole muscle was used.[26] Thus, 100 g of whole muscle (wet weight basis) will contain approximately 5 g of dry matter from press juice.

Table 4. Hg in Whole Muscle, Surimi, and Protein Isolates from Whitefish Using the Acid and Alkaline Process[a]

treatment	batch of fish	ng/g dry weight
whole muscle	A	437.3 ± 8.1 a
cooked surimi		357.6 ± 1.4 b
whole muscle	C	143.1 ± 6.1 d
PI (acid)		209.0 ± 6.5 b
PI (acid) + EDTA		182.9 ± 5.9 c
PI (alkaline)		239.5 ± 6.4 a
whole muscle	D	287.2 ± 11.4 b
uncooked surimi		393.0 ± 23.8 a
uncooked surimi + cysteine		224.1 ± 11.0 c

[a] PI (acid): protein isolated by acid, pH 2.8. PI (alkaline): protein isolated by base, pH 11.0. Same letters in a column (within a batch of fish) indicate no significant difference. Means and standard deviations are reported

contained less than 10% of the Hg determined in the whole muscle. The myofibrillar protein preparation contained around 71%, while the press juice fraction contained 13% of the Hg determined in the whole muscle (Table 2).

Hg levels were also determined in whole muscle from walleye and compared to levels in the isolates. The Hg level in whole muscle from three catches of walleye varied from 0.14 to 0.81 μg/g (wet weight basis) (Tables 3 and 5). The triacylglycerol, cellular membranes, and myosin isolates contained less than 10% of the MeHg determined in the whole muscle. The myofibrillar protein preparation contained around 76% of the Hg in the whole muscle when Batch E was examined. Press juice contained 36% of the Hg determined in the whole muscle when Batch G was examined (Table 3).

Table 5. Hg in Whole Muscle, Surimi and Protein Isolates from Walleye Using the Acid and Alkaline Process[a]

treatment	batch of fish	ng/g dry weight
whole muscle	E	2892 ± 115 ab
PI (acid)		2140 ± 601 b
cooked surimi		3577 ± 106 a
whole muscle	G	702.0 ± 52.0 c
PI (acid)		862.3 ± 39.9 b
PI (acid) + EDTA		881.9 ± 56.8 b
PI (alkaline)		1011 ± 100 a
whole muscle	H	3788 ± 73 b
uncooked surimi		4766 ± 110 a
uncooked surimi + cysteine		1859 ± 118 c

[a] PI (acid): protein isolated by acid, pH 2.8. PI (alkaline): protein isolated by base, pH 11.0. Same letters in a column (within a batch of fish) indicate no significant difference. Means and standard deviations are reported.

Levels of Mercury in Muscle Compared to Washed Muscle and Protein Isolates. Hg was determined in extensively washed muscle (e.g., surimi) from whitefish and walleye. A significant decrease in Hg (around 18%) was observed when cooked surimi was compared to raw, whole muscle of whitefish ($p < 0.05$) (Table 4). In walleye, there was no significant difference in Hg content when comparing cooked surimi to the raw, whole muscle (Table 5). A significant increase in Hg (25−37%) was observed when uncooked surimi was compared to that of the respective whole muscle from whitefish and walleye ($P < 0.05$) (Tables 4 and 5). The addition of 0.5% cysteine to the washing solution in uncooked surimi significantly decreased Hg in whitefish and walleye ($P < 0.05$) (Tables 4 and 5). The MeHg levels in cysteine-treated whitefish and walleye surimi were 43% and 61% less compared to those of the respective surimi preparations not containing added cysteine.

Protein isolates prepared by acid or alkaline pH shifting had significantly higher Hg values (22−67% higher) compared to those of whole muscles from whitefish and walleye ($P < 0.05$) (Tables 4 and 5). The addition of EDTA to the washing solutions had no effect on Hg levels in the protein isolates. Hg values were higher in protein isolates using alkaline pH shifting (14−31% higher) compared to acid pH shifting ($P < 0.05$) (Tables 4 and 5).

Lipid to Protein Ratios in Muscle Compared to Protein Isolates. The lipid to protein ratios in whole muscle, acid protein isolates, and alkaline protein isolates were determined in whitefish and walleye. This was done to determine the efficiency of removing cellular membranes and neutral lipids by pH shifting. The protein isolates contained significantly reduced lipid levels compared to that of the respective whole muscles ($P < 0.05$) (Table 6). There was as much as a 6-fold reduction in the lipid to protein ratio in the protein isolates based on the mean values, while the smallest reduction was 1.4-fold (Table 6).

Characterization of Proteins in Muscle, Muscle Fractions, and Protein Isolates. SDS−PAGE was done to characterize the polypeptides present in whole muscle and the different isolates. Myosin isolates from whitefish and walleye showed a major band at around 200 kDa, indicative of myosin (lanes 4 and 6, Figure 1). The myofibril isolate (lane 5) was rich in myosin and had substantial band densities that were tentatively identified as actin and tropomyosin (marked by *).[25] Acid protein isolates from whitefish and walleye showed numerous high, intermediate, and

Table 6. Lipid/Protein Ratio in Whole Muscle from Whitefish and Walleye after the Acid or Alkaline Solubilization Process[a]

treatment	lipid/ protein ratio whitefish	lipid/ protein ratio walleye
minced whole muscle	0.32 ± 0.14 a	0.13 ± 0.01 a
PI (acid)	0.08 ± 0.02 b	0.08 ± 0.01 b
PI (acid) + EDTA	0.11 ± 0.01 b	0.09 ± 0.04 b
PI (alkaline)	0.05 ± 0.01 b	0.05 ± 0.00 c

[a] Same letters in a column indicate no significant difference. Means and standard deviations are reported.

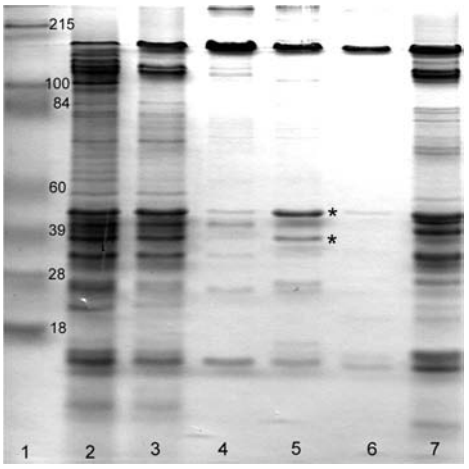

Figure 1. SDS−PAGE of proteins from whitefish and walleye; (lane 1) protein standards, (lane 2) acid protein isolate from whitefish, (lane 3) acid protein isolate with added EDTA from whitefish, (lane 4) myosin preparation from whitefish, (lane 5) myofibril preparation from whitefish, (lane 6) myosin preparation from walleye, and (lane 7) acid protein isolate from walleye. (*) Indicates actin (upper band) and tropomyosin (lower band) in lane 5.

low molecular weight polypeptides. Acid protein isolates from whitefish with added EDTA contained less polypeptide bands in the 100−200 kDa range compared to that of the acid protein isolates from whitefish without added EDTA (lanes 2 and 3, Figure 1).

SDS−PAGE of the polypeptides in press juice from whitefish and walleye are shown in Figure 2. Creatine kinase (84 kDa) is a dimeric protein that comprises around 5% of the sarcoplasmic proteins. The 42 kDa band in press juice likely contains monomeric creatine kinase. Glyceraldehyde-3-phosphate dehydrogenase (143 kDa) is a tetrameric protein that comprises around 12% of the sarcoplasmic proteins. The 35 kDa band from press juice likely contains monomeric glyceraldehyde-3-phosphate kinase. These and 16 additional sarcoplasmic proteins have been described.[26] The polypeptides pattern in press juice from whitefish and walleye were similar.

SDS−PAGE of the polypeptides in whole muscle, alkaline protein isolates, and surimi were examined. Whole muscle of walleye and whitefish contained a low molecular weight polypeptide that was not present in the protein isolates and surimi (data not shown). Overall, whole muscle and protein isolates were similar, while the surimi had fewer polypeptide bands in terms of quantity and density. This is consistent with the removal

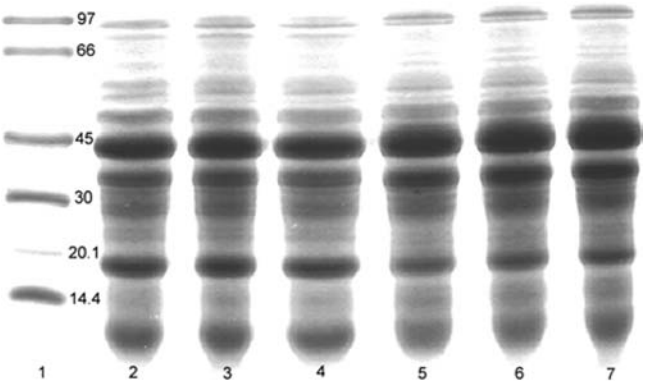

Figure 2. SDS−PAGE of press juice from whitefish and walleye; (lane 1) protein standards, (lanes 2−4) whitefish press juice, and (lanes 5−7) walleye press juice.

of sarcoplasmic proteins by extensive washing in surimi production, while preparing protein isolates by pH shifting can recover the sarcoplasmic proteins.[13] Washing with cysteine did not appear to alter the protein patterns.

Lipid Oxidation in Muscle, Washed Muscle, and Protein Isolates. The progress of lipid oxidation in minced whole muscle from whitefish and walleye was compared to that in surimi and isolates prepared by pH shifting (acid and alkaline). All samples were adjusted to pH 6.5, and samples were stored at 2 °C. Minced whole muscle from whitefish was more susceptible to lipid oxidation compared to that of walleye based on lipid peroxide and thiobarbituric acid reactive substance (TBARS) values (Figure 3). Lipid peroxide formation was more rapid in surimi from walleye compared to that of the minced whole muscle ($P < 0.05$). Lipid peroxide formation was also more rapid in surimi from whitefish compared to that of the minced whole muscle when comparisons were made at the initial time point and day 2 of storage ($p < 0.05$). TBARS values were lower in surimi of whitefish and walleye compared to those of the respective minced whole muscle ($P < 0.05$). Lipid peroxides and TBARS values were lower in the isolates prepared by pH shifting compared to those of surimi ($P < 0.05$). There was no significant difference in progress of lipid oxidation based on TBARS and lipid peroxides when comparing isolates from acid pH shifting and alkaline pH shifting.

■ DISCUSSION

The ratio of methyl mercury (MeHg) to mercury that is not methylated should be determined when considering partitioning of the metal between different phases of muscle. Methylation will increase the nonpolar character of mercury, which can affect solubility of the metal in different phases of the muscle. Nearly all the mercury present in walleye and whitefish was present as MeHg (Table 1). Electrostatic interactions should also be considered because charge is associated with metals, free amino acids, amino acids of proteins, and head groups of phospholipids.

MeHg has the potential to associate with various fractions of the muscle including triacylglyerols (e.g., oil phase), cellular membranes that contain phospholipids and imbedded proteins, sarcoplasmic proteins of the aqueous phase, and myofibrillar proteins that are somewhat insoluble in muscle. Our results indicated that MeHg was mostly associated with the myofibrillar protein preparations (Tables 2 and 3). Lower but substantial

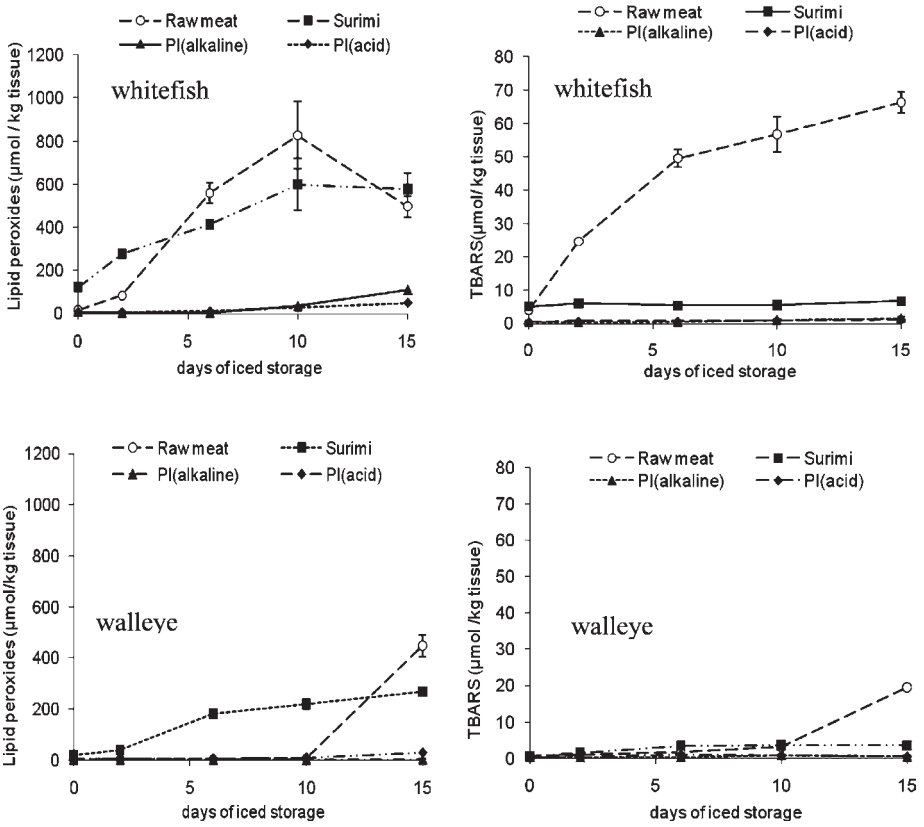

Figure 3. Lipid peroxide and thiobarbituric acid reactive substance (TBARS) values in whole muscle (minced), surimi, alkaline isolates, and acid isolates from whitefish and walleye during 2 °C storage. All samples were at pH 6.5 ± 0.1.

quantities of MeHg were found in the press juice phase that contains sarcoplasmic proteins, while little MeHg was found in the cellular membrane and the oil phase. Apparently, methylation of Hg does not decrease polarity enough to facilitate incorporation into lipids (e.g., phospholipids and triacylglycerols). Rather, polar and electrostatic interactions likely explain the association of MeHg with the myofibrillar protein fraction.

Myofibrillar proteins consist of myosin (49%), actin (26%), titin (14%), troponin (6%), and tropomyosin (6%).[25] Myosin isolates were mostly void of Hg (Tables 2 and 3). Myosin was prepared with and without EDTA as a reagent because the possibility of EDTA chelating MeHg was considered. MeHg levels in myosin isolates prepared with and without EDTA were similar. Thus, actin, titin, tropomyosin, and troponin are candidate proteins that potentially harbor most of the MeHg in whitefish and walleye muscle. Regarding the sarcoplasmic proteins, a ternary complex of selenium, mercury, and selenoprotein P in the plasma fraction of blood has been described.[4] Residual blood levels in muscle even after gutting and bleeding have been shown to be substantial.[27,28]

Protein isolates prepared by pH shifting increased the Hg levels in most cases compared to those of the whole muscle (Tables 4 and 5). Oil and cellular membrane contents are reduced by the pH shifting process, while the myofibrillar and sarcoplasmic proteins are retained.[13] The oil and membrane fractions were found to be low in Hg; therefore, an Hg reduction did not occur by pH shifting. The fractions that contain substantial amounts of Hg (myofibrils and sarcoplasmic proteins) were apparently concentrated to some degree when preparing the protein isolates, which would explain the elevated Hg levels.

Surimi processing did not reduce the Hg content in most cases (Tables 4 and 5). The repeated washing steps in surimi processing will remove a majority of the sarcoplasmic proteins and some of the oil phase.[29] This might be expected to cause a net decrease in Hg since the sarcoplasmic proteins contained significant levels of Hg. At the same time, there is some concentration of the myofibrillar proteins by the washing steps. This may explain why a majority of the time there was no reduction in Hg by preparing surimi, and on one occasion, there was a modest reduction in Hg.

Washing with cysteine did decrease Hg concentrations in the surimi prepared from whitefish and walleye (Tables 4 and 5). This approach has also decreased Hg levels in shark, halibut, hake, and mackerel.[30−32] The protein profiles of surimi based on electrophoresis with and without added cysteine were similar (data not shown). The pK_a of cysteine is near 8.5, and the pH of the washing solution was near pH 6.5. This indicates that around 1% of the sulfhydryl groups in the cysteine solution were unprotonated (e.g., negatively charged), which equates to 6 mM negatively charged cysteine. This represents an excess of negatively charged cysteine that can electrostatically interact with the positive charge on the methyl mercury present.

The techniques used to fractionate the muscle components have the potential of introducing errors in the Hg determination. Methyl Hg is quite stable unless it is subjected to strong chemical oxidation or significant heat.[33] We avoided strong oxidation conditions, and the effect of our heat drying protocol was assessed. Hg content was measured in both the wet tissue and dried tissue from whole muscle and isolates. Comparisons of nondried (e.g., wet) samples to that of dried samples yielded

similar Hg values when adjustments were made to account for the moisture difference (data not shown).

Minced whole muscle from whitefish was more susceptible to lipid oxidation compared to that of walleye. Heme pigment content, pH of the post mortem muscle, fatty acid composition, inhibitors of lipid oxidation in the aqueous phase, tocopherol levels in lipid phases, and depletion rates of tocopherols were noted as factors that explained variation in the oxidative stability of chicken, turkey, and duck muscle.[34]

The pH shifting process inhibited lipid oxidation more effectively compared to that of an extensive washing by the traditional surimi process. This can be attributed to removing cellular membranes by the acid and alkaline processes (Table 6). It is recognized that cellular membranes are the main lipid substrates in muscle lipid oxidation.[35]

Washing steps by the surimi process decreased the oxidative stability of whitefish and walleye muscle based on lipid peroxide values (Figure 3). Washing is generally thought of as a stabilizing procedure in which aqueous pro-oxidants are diluted. However, antioxidative and pro-oxidative components in the aqueous phase will be diluted by washing.[36] It may be that aqueous pro-oxidant(s) can function at reduced concentrations, while aqueous antioxidants are rendered ineffective by the dilution.[37] Pro-oxidants that are not diluted by washing, such as membrane bound components, should also be considered. Lipoxygenases are generally present in the cytosol but are dispatched to cellular membranes in order to convert polyunsaturated fatty acids into lipid hydroperoxide intermediates.[38] It would be of value to determine the mechanism by which lipid hydroperoxide formation occurs in washed muscle. Inhibiting this pathway would likely decrease off-flavor development during storage since lipid hydroperoxides are the precursors of the volatiles that comprise rancid and painty odors in stored fish muscle.

Washing steps increased the oxidative stability of whitefish and walleye muscle based on TBARS values. TBARS are secondary oxidation products that form from the decomposition of lipid hydroperoxides. Hemoglobin and myoglobin concentrations (and low molecular weight metal complexes) are lowered by washing, which can partly explain the lowered TBARS values due to washing. If heme pigments are not removed by washing, hemin dissociates from fish hemoglobins rapidly at postmortem pH.[39] Hemin is noted for decomposing lipid hydroperoxides to free radicals that can initiate lipid oxidation.[40] Heme proteins can also promote lipid oxidation by other mechanisms including ferryl heme protein species and ferryl heme.[41,42]

In conclusion, whitefish and walleye protein isolates were highly resistant to lipid oxidation during 2 °C storage. This was partly attributed to the removal of cellular membranes by the pH shifting process. Extensively washed muscle, without membrane removal, was highly susceptible to lipid peroxide formation. Secondary lipid oxidation products were lowered by washing but not to the level in the protein isolates. Hg was mostly associated with the myofibrillar fraction of the muscle, while less Hg was found in the other phases of the muscle. Washing the muscle with cysteine was effective at removing Hg although removal from protein isolates was not examined. In the cases when Hg contamination is substantial, future work should examine strategies to dissociate Hg from protein isolates so that isolates with high oxidative stability and low levels of mercury contamination can be achieved.

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