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Hemoglobin-mediated lipid oxidation of protein isolates obtained from cod and haddock white muscle as affected by citric acid, calcium chloride and pH

Patroklos Vareltzis [b], Herbert O. Hultin [a,*], Wesley R. Autio [c]

[a] Massachusetts Agricultural Experimental Station, Department of Food Science, University of Massachusetts/Amherst, Marine Station, Box 7128, Gloucester, MA 01930, USA
[b] Department of Chemistry and Bioscience – Food Science, Chalmers University of Technology, Box 5401, S-40229 Göteborg, Sweden
[c] Department of Plant and Soil Sciences, University of Massachusetts/Amherst, Marine Station, Box 7128, Gloucester, MA 01930, USA

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Abstract

Acid or alkali solubilization followed by isoelectric precipitation can be used to isolate proteins with good functional properties from muscle tissue. Both acid (pH 3.0) and alkali (pH 10.5) treatment of the muscle decreased lipid oxidation catalyzed by hemoglobin. Citric acid and calcium chloride improved the oxidative stability of both acid- and alkali-solubilized muscle protein isolates when added to the homogenized muscle before separating the membrane or directly to the isolated membranes in the assay. Citric acid may have functioned in part by lowering the pH and destroying preformed peroxides. Exposing the muscle and the hemoglobin together at pH 3 promoted lipid oxidation, while addition of citric acid/calcium chloride or press juice to washed cod prior to solubilization inhibited lipid oxidation even when the tissue and hemoglobin were acidified together.

Key words: Lipid oxidation; Cod proteins; Haddock proteins; Hemoglobin; Citric acid; Calcium chloride; Acid treatment; Alkali treatment

1. Introduction

The major factors involved in lipid oxidation of muscle tissues are the substrate, the pro-oxidants and the anti-oxidants of the system. A main substrate for lipid oxidation is the membrane phospholipids because they are more susceptible to oxidation than the triacylglycerols (Pikul, Leszczynski, & Kummerow, 1984). This might be due to the high degree of unsaturation and large surface area of membranes per unit weight of lipid (Hultin, 1995). Hemoproteins, like hemoglobin and myoglobin, are believed to be the main pro-oxidants in muscle foods (Chan, Faustman, Yin, & Decker, 1997; Koizumi, Wada, & Oshima, 1987; Richards, Kelleher, & Hultin, 1998). Various pathways by which hemoglobin can promote lipid oxidation have been proposed including pseudo lipoxygenase-like activities (Everse & Hsia, 1997).

There is little doubt that the pH is an important variable in lipid oxidation. Predicting however, the effect of pH is very difficult due to the complexity of the catalysts involved and the reactions which they stimulate. A method using solubilization by acid or base to separate proteins from muscle tissue with good functional properties exposes the tissue to unusually low or high pH (Hultin & Kelleher, 1999; Hultin & Kelleher, 2000). The process involves extracting proteins using a low (~3) or a high (~10.5) pH to solubilize the muscle proteins and recovering the solubilized proteins by centrifugation after isoelectric precipitation (pH ~5.5). Acid and/or alkali solubilization provides the possibility of separating membranes from

* Corresponding author. Tel.: +1 978 281 1930; fax: +1 978 281 2618.
E-mail addresses: pkv1977@yahoo.com (P. Vareltzis), marinest@foodsci.umass.edu (H.O. Hultin).

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solubilized proteins by centrifugation or filtration. Hultin and Kelleher (2000) reported 37% and 51% of the phospholipids were removed from solubilized chicken breast muscle and chicken thigh/leg muscle, respectively, by centrifugation at 15,000×*g* for 30 min at pH 2.8. In a study with herring, Undeland, Kelleher, and Hultin (2002) found that only about 20–30% of the phospholipids were removed from herring light muscle by a combination of acid solubilization and 18,000×*g* centrifugation for 20 min if the holding time of the acidified muscle homogenate on ice prior to centrifugation did not exceed 65 min. No phospholipids were removed under the same experimental conditions when the holding time was 125 min.

It should be noted, that high centrifugal forces, such as 15,000 and 18,000×*g* used in the research work above, are not practical for industrial applications. Liang and Hultin (2005a) showed that calcium chloride, and to a lesser degree magnesium chloride, aided in the membrane removal from muscle homogenates solubilized at pH 3 in the presence of citric acid or malic acid but not lactic acid after centrifugation at 4000×*g* for 15 min. Citric acid at 5 mM and calcium chloride at 10 mM final concentrations when added before solubilizing the muscle homogenates showed a removal of phospholipid from homogenates solubilized at pH 3 up to 80%, while at the same time recovering a high percentage of protein. Since membrane phospholipids are highly susceptible to lipid oxidation, treatment with citric acid and calcium chloride might reduce lipid oxidation in protein isolates prepared by acid and/or alkali solubilization by effectively removing phospholipid.

The large pH shifts that occur during acid or alkali solubilization can have an impact on both the pro-oxidative properties of hemoglobin and the susceptibility of the muscle to lipid oxidation. It was shown that after treatment at pH 3, washed cod muscle became slightly more susceptible to lipid oxidation, while alkaline treatment slightly protected the muscle from trout hemoglobin-mediated lipid oxidation (Kristinsson & Hultin, 2004). The same authors demonstrated that exposure of trout hemoglobin to low pH increased its pro-oxidative properties. A longer unfolding time and a lower pH led to a less refolded hemoglobin with increased pro-oxidative activity. Autoxidation of hemoglobins was found to occur around pH 7.0 and below but much less at pH 8.0 (Harrington, 1986). The autoxidation of hemoglobin will produce $O_2^{\cdot-}$ and methemoglobin. Recent findings indicate that high oxidation state myoglobin species, i.e., perferryl- and ferryl-myoglobin, are produced in vivo and are major pro-oxidative candidates in muscle based foods (Carlsen, Moller, & Skibsted, 2005). Low pH favors the protonation of the ferryl species, which exhibit great instability and can be considered as possessing a radical-like nature. Therefore low pH leads to an enhanced rate of lipid peroxide consumption by metmyoglobin (Reeder & Wilson, 2001). Hemoglobin subunits were found to autoxidize more rapidly than tetramers (Griffon et al., 1998), and Dumoulin, Manning, Jenkins, Winslow, and Manning (1997) showed that subunit formation increases an order of magnitude for each unit of pH reduction.

Richards and Hultin (2000) showed that there was rapid lipid oxidation of washed cod muscle at pH 3.5 catalyzed by trout hemoglobin, while there was a considerable lag phase and a slower rate of oxidation at pH 7.8. However, in cod microsomes the rate of hemoglobin-mediated lipid oxidation increased in the order pH 6.8 ≫ pH 7.6 > pH 8.4 > pH 6 > pH 4.5 > pH 3.5 (Pazos, Medina, & Hultin, 2005). In washed cod muscle a decrease in pH from 7.6–6.4 decreased the lag phase and increased the rate of lipid oxidation. A further decrease in pH to 3.5 decreased the lag phase and increased the rate further. The authors postulated that structural changes of the isolated microsomal membranes could be the reason for the unexpected slow lipid oxidation in microsomes at pH 6.0 and below.

This research work studied primarily (a) the effect of citric acid and calcium chloride on lipid oxidation of cod muscle *in situ* at pH 3 and (b) the effect of acid or alkali solubilization on the susceptibility of cod muscle to hemoglobin-mediated lipid oxidation. Model systems comprising washed or unwashed minced cod muscle were used. Cod (*Gadus morhua*) muscle was chosen as it primarily contains membrane lipids with almost no triacylglycerols (Lochmann, Maillet, Frank, & Taggart, 1995). The effect of the different treatments on lipid oxidation was evaluated by chemical analyses.

2. Materials and methods

2.1. Materials

Fillets of Atlantic cod (*Gadus morhua*), and haddock (*Melanogrammus aeglefinus*) were purchased from a local fish distributor (J.B. Wright, Gloucester, MA) and transported to the laboratory on ice. Chemicals were purchased from Sigma Chemical Co., St. Louis, MO. All reagents were of ACS grade and all solvents were of HPLC grade.

2.2. Methods

2.2.1. Cod mince and washed cod or haddock preparation

Cod or haddock muscle fillets were trimmed from skin and red muscle. The white muscle was then minced using a Kitchen Aid ultra power grinder (model KSM90, St. Joseph MI) through a 5 mm perforated plate. Haddock was used when quality cod fish was not available. Raghavan and Hultin (2005) showed that haddock and cod exhibit similar hemoglobin-mediated lipid oxidation behavior.

The minced muscle tissue was washed twice in cold (8 °C) distilled, deionized water at 1:5 mince to water ratio (w/w) by stirring with a plastic rod for 2 min, allowing the mixture to sit for 15 min on ice and then dewatering on a fiberglass screen. In the second washing step, 50 mM NaCl (final concentration) was added in the wash water. The resulting paste was used as the washed cod and had a mois-

ture content of ~90%. The pH of the washed muscle was adjusted to the desired level using 2 N or 0.4 N NaOH or HCl.

2.2.2. Preparation of hemolysate

A modified method of Fyhn et al. (1979) was used for preparing hemolysate from cod frames (obtained after filleting whole cod). Cod frames were severed at the tail section. Blood was collected from the severed vein using a glass micro-pipette and transferred into heparin solution (containing 30 units of heparin per mL and 150 mM NaCl). Heparinized blood was washed with four volumes of ice cold 1.7% NaCl in 1 mM Tris buffer, pH 8.0. Centrifugation was done at 1000×*g* for 10 min at 4 °C using a tabletop clinical centrifuge (IEC, Needham Heights, MA). Plasma was removed and discarded. The red cells obtained were washed three times with 10 volumes of the above buffer followed by centrifugation at 1000×*g*. Cells were then lysed in three volumes of ice cold 1 mM Tris buffer, pH 8.0 for 1 h. One-tenth volume of 1 M NaCl was then added to aid stromal removal before centrifugation at 30,000×*g* for 15 min at 4 °C in a Beckmann Ultracentrifuge model L5-65B (Beckmann Instruments Inc., Palo Alto, CA). The supernatant was used as the hemolysate and was stored in a freezer at −80 °C until used.

2.2.3. Quantifying hemoglobin levels

The method of Brown (1961) was used to quantify the hemoglobin concentration in the hemolysate. The hemolysate was mixed in ice cold 50 mM Tris, pH 8.0 for dilution (1:100). Around 1 g of sodium hydrosulfite was added and mixed. Carbon monoxide (Matheson Gas, MA) was bubbled into the samples for 10 s. The samples were then scanned between 440 nm and 360 nm against a blank that contained only the buffer using a model U-3110 double beam spectrophotometer (Hitachi Instruments, Inc., San Jose, CA). The peak at 420 nm was recorded. Standard curves were plotted using bovine hemoglobin standard.

2.2.4. Addition of hemolysate

A volume of cod hemolysate sufficient to provide hemoglobin at the stated concentration was added to washed cod or haddock muscle and was expressed as μmol hemoglobin per kg of sample. Samples contained 200 ppm streptomycin sulfate to inhibit microbial growth. After the addition of hemolysate and streptomycin sulfate, samples were stirred by hand for ~2 min using a stainless steel spatula. The pH of samples was checked before and after the addition of the hemolysate. The samples were adjusted to 90% moisture content and were stored on ice for up to 15 days.

2.2.5. Preparation of press juice

Minced cod muscle was packed in 200 mL polypropylene centrifuge bottles and centrifuged at 22,000×*g* for 15 h at 4 °C (Beckman L8-M ultracentrifuge with a type 19 Ti rotor, Beckman Coulter Inc., Fullerton, California). The press juice obtained was filtered through four layers of cheesecloth and used immediately or after short-term storage at −80 °C.

2.2.6. Preparation of protein isolates

Washed or unwashed cod mince was homogenized in six volumes of cold distilled deionized water. The pH of the homogenate was brought down to 3 (acid solubilization) or to 10.8 (alkali solubilization). Then the pH of the homogenate was adjusted to 5.5 to precipitate the proteins without any removal of insoluble material, followed by centrifugation at 10,800×*g* for 20 min. The resultant sediment, called protein isolate, was adjusted to pH 7.4 and to a moisture content of 90%. Streptomycin sulfate (200 ppm) was added to the samples to prevent microbial spoilage. An appropriate volume of hemoglobin (Hb) was then added to a final concentration of 6 μmol per kg of tissue. The pH of the samples was checked before and after the addition of Hb and adjusted to the desired value if necessary. The samples were stored in 250 mL Erlenmeyer flasks and stored on ice (~4 °C).

2.2.7. Effect of citric acid, calcium chloride and HCl on the susceptibility of cod muscle to lipid oxidation

Citric acid and calcium chloride at final concentrations of 5 mM and 10 mM, respectively, were used during the preparation of the washed cod samples. The addition took place at different steps in the process, followed by 1 h incubation. When unwashed cod was used for the experiment, cod muscle was minced and homogenized in six volumes of cold (8 °C) distilled, deionized water. Citric acid and calcium chloride were added to the homogenate.

To determine the individual contributions of citric acid, calcium chloride and HCl to the oxidative stability of washed cod, 5 mM citric acid, 10 mM calcium chloride or 2 N HCl were added separately to minced cod samples in the first step of washing, followed by 1 h of incubation at pH 5.2 (i.e. the pH reached when 5 mM citric acid was added to the sample). Then the pH was adjusted to 7.4 and moisture content to 90%. Streptomycin sulfate (200 ppm) and cod hemoglobin at a final concentration of 6 μmol/kg of tissue were stirred in and oxidation was followed by TBARS and/or peroxide value. All samples were kept on ice throughout the duration of the experiments.

2.2.8. Determination of lipid hydroperoxides

Approximately 1 g of sample was added to 10 mL chloroform:methanol (2:1, v/v) mixture, containing 500 ppm BHT in order to prevent any further peroxidation during the preparation of the samples. The chloroform used must have ethanol as its preservative since other preservatives caused high blank readings. The mixture was homogenized in a 16 × 125 mm disposable glass tube with a Bio homogenizer (M133/1281-0, Bio Spec Products Inc., Bartlesville, OK) at high speed for 30 s. The glass tubes were then centrifuged at approximately 2500×*g* for 5 min using a

tabletop centrifuge (Tabletop Clinical Centrifuge, IEC, Needham Heights, MA). Three mL of 0.5% NaCl were added to the test tubes, which were then vortexed at a moderate speed for 15 s and centrifuged for 5 min in the tabletop centrifuge at ~2500×*g* to separate the sample into two phases. Five mL of the lower phase were collected and transferred to a 16 × 125 mm glass tube using a 5 cc Luer-LOK syringe (Becton-Dickinson and Co, Franklin Lakes, NJ). Five mL of ice cold chloroform:methanol (2:1, v/v) mixture were added to the samples to make a final volume of 10 mL. Ammonium thiocyanate solution was prepared by adding 30 g of the reagent to 100 ml of water. To prepare the ferrous chloride solution, 0.4 g barium chloride was dissolved in 50 ml of water. This solution was added to an equal volume of ferrous sulfate solution which was prepared by dissolving 0.5 g ferrous sulfate in 50 ml of water. The mixture was centrifuged for 5 min at ~2000×*g* and the clear supernatant was used as the ferrous chloride solution (Shantha & Decker, 1994). Twenty-five μL of each reagent were added to the samples followed by vortexing for 3 s. Samples were incubated for 5 min at room temperature. Then absorbance was measured at 500 nm. A standard curve was prepared using cumene hydroperoxide. The lipid hydroperoxides are expressed as μmol per g of lipid.

2.2.9. Thiobarbituric acid-reactive substances

A modified method of Lemon (1975) was used for measuring thiobarbituric acid-reactive substances (TBARS). A sample (1.5 g) was homogenized with 5 mL of trichloroacetic acid extraction solution (0.1% propyl gallate and 7.5% trichloroacetic acid prepared in double-distilled water) using a Bio homogenizer (M133/1281-0, Bio Spec Products Inc., Bartlesville, OK) at high speed for 1 min. The homogenized sample was centrifuged in the tabletop clinical centrifuge and the supernatant was collected. Two mL of the supernatant was mixed with 2 mL of thiobarbituric acid solution (0.02 M thiobarbituric acid in double-distilled water) and heated for 40 min in a boiling waterbath. Absorbance readings were measured against a blank at 530 nm. Tetra-ethoxy propane was used for plotting the standard curve.

2.2.10. Statistical analysis

Data was analyzed by ANOVA using SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Differences between treatment means at the 5% level were determined using the Duncan Multiple Range Test. Each experiment was done at least twice ($n = 2$) and for each data point the average of at least two readings was taken.

3. Results

3.1. Hemoglobin-mediated lipid oxidation of washed or unwashed minced cod muscle in the presence or absence of citric acid and calcium chloride

Unwashed minced cod had a greater oxidative stability to hemoglobin-catalyzed lipid oxidation compared to the washed mince (Fig. 1). This could be due to the removal of the potent anti-oxidative system found in the aqueous fraction of cod muscle (Undeland, Hultin, & Richards, 2003). Incubation of the minced cod with 5 mM citric acid and 10 mM calcium chloride for 30 min in the washed sample completely prevented lipid oxidation after adjusting the pH to 7.4 over the 6 days of the experiment.

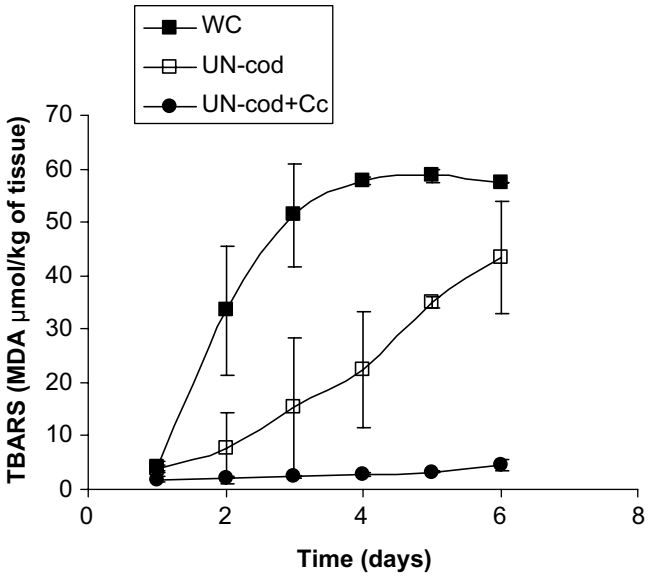

Fig. 1. Lipid oxidation of minced cod with or without citric acid and calcium chloride ($n = 2$). WC: washed minced cod. UN-cod: minced cod. UN-cod + Cc: minced cod incubated with 5 mM citric acid and 10 mM calcium chloride for 30 min before adjusting the pH to 7.4 and adding hemoglobin at a concentration of 6 μmol/kg of tissue; MC was 90% and pH of all samples 7.4.

3.2. Separate effect of citric acid, calcium chloride and HCl on washed cod/haddock oxidation

The next experiment was to determine the individual roles of citric acid and calcium chloride in the oxidation of washed, minced haddock muscle. Washed flesh was used to eliminate the effect of soluble anti-oxidative components. Citric acid at a final concentration of 5 mM or calcium chloride at a final concentration of 10 mM were added in the first step of washing and incubated for 1 h. The samples were then adjusted to 7.4. The oxidative stability of these samples was compared to a muscle sample washed with water and to a sample that had both citric acid and calcium chloride in the washing solution at the above concentrations. The addition of citric acid and/or calcium chloride in the first washing step decreased the pH of the washed minced fish (Table 1). The citric acid lowered the pH more than did the calcium chloride. As is evident from both the TBARS and peroxide values (Fig. 2a and b, respectively), samples treated with both citric acid and calcium chloride exhibited good oxidative stability compared to the washed haddock sample. Citric acid alone also provided good protection against lipid oxidation. Calcium

Table 1
pH of samples during incubation with citric acid, calcium chloride or both in the first washing step ($n = 2$; ±SE)

Sample	pH
WC, citric	5.09 ± 0.08
WC, calcium	6.49 ± 0.01
WC, Cc	4.85 ± 0.08

WC, citric: washed haddock with added citric acid in the first wash (5 mM).
WC, calcium: washed haddock with added calcium chloride in the first wash (10 mM).
WC, Cc: washed haddock with added citric acid (5 mM) and calcium chloride (10 mM) in the first wash.
The initial pH of washed, minced cod was 6.95 ± 0.20.

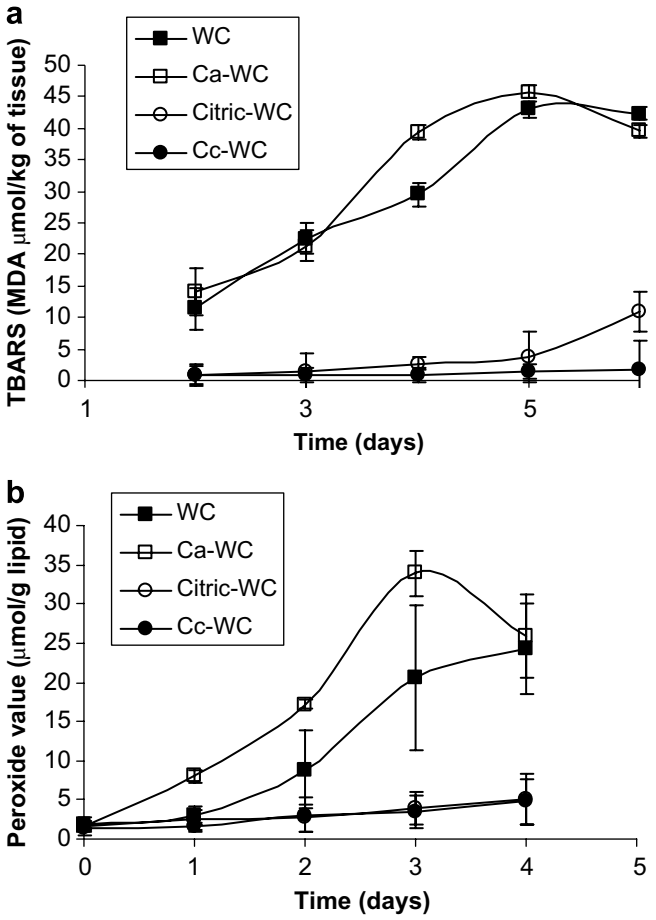

Fig. 2. Effect of citric acid, calcium chloride or both on the oxidation of washed haddock (a) TBARS and (b) peroxide value ($n = 2$). WC: washed haddock. Ca-WC: washed haddock with calcium chloride added in the first wash (10 mM). Citric-WC: washed haddock with citric acid added in the first wash (5 mM). All samples were incubated during the first washing step for 1 h; Hb was added to all samples after washing at 6 μmol/kg of sample. Final pH 7.4.

chloride alone did not give protection against oxidation when measured by TBARS and significantly increased peroxide values.

The protection that citric acid offered against lipid oxidation might be attributed either to the low pH that is achieved when it is added to the muscle or to some other more specific effect, e.g., breakdown of peroxides. Treatment with citric acid was compared to treatment with HCl at the same pH. Citric acid at a final concentration of 5 mM or HCl, sufficient to lower the pH of the sample to the same value as citric acid did, were added to washed haddock samples. The addition of citric acid and HCl took place in the first washing step and the samples were incubated for 1 h. Both the citric acid and HCl treatments significantly ($p < 0.05$) protected the muscle from lipid oxidation compared to the control (i.e., no treatment), as measured by TBARS (Fig. 3). Citric acid showed somewhat more inhibitory effect than the HCl, but only on the fourth and last day of the experiment was the effect significantly better ($p < 0.05$). The results indicate that the protective effect was mainly due to the pH and not the specific acid used.

To determine whether citric acid or low pH have an effect on the breakdown of peroxides, oxidized washed cod samples were used. Hemoglobin (6 μmol/kg of tissue) was added to washed cod samples and after 2–3 days the peroxide value of the oxidized sample was measured. Then citric acid at a final concentration of 5 mM or an amount of 0.4 N HCl sufficient to lower the pH to the same value as the citric acid (~5.0–5.3), was stirred in. The pH was adjusted back to 7.4 and the new peroxide value was determined (Table 2). The results indicated that both citric acid and HCl were able to break down some of the formed peroxides. The decrease in peroxide value after the treatment with citric acid ranged from ~30% to ~50%. A longer exposure to the low pH might have destroyed more of the peroxides.

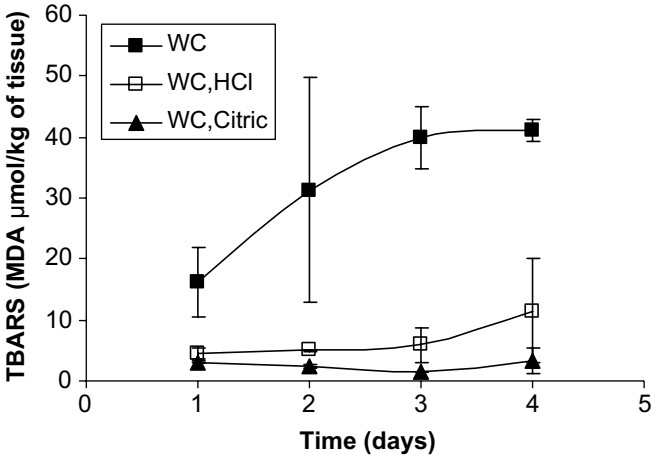

Fig. 3. Effect of HCl and citric acid on washed haddock oxidation; TBARS ($n = 2$). WC: washed haddock. WC, citric: washed haddock with added citric acid in the first wash (5 mM); pH of first wash was 5.25. WC, HCl: hydrochloric acid used in the first wash to lower the pH to 5.25. Oxidation assays ran at pH 7.4; Hb had a concentration of 6 μmol/kg of tissue.

Table 2
Peroxide values of oxidized washed cod samples before and after citric acid or HCl treatment ($n = 3$; ±SE)

Sample	Peroxide value before citric acid treatment (μmol/g lipid)	Peroxide value after citric acid treatment (μmol/g lipid)	Peroxide value after HCl treatment
1	55.5 ± 0.2	40.2 ± 0.1	
2	53.2 ± 0.2	27.3 ± 0.3	31.2 ± 0.3
3	50.1 ± 0.3	29.2 ± 0.1	32.1 ± 0.2

The peroxides values of oxidized washed cod samples (pH 7.4) were determined. Then citric acid (5 mM) or 2 N HCl were added to the samples (pH reached after citric acid or HCl addition was 5.2) and the pH was immediately readjusted to 7.4 by addition of 2 N NaOH. The new peroxide value was determined.

3.3. Role of citric acid and calcium chloride added at different processing steps

Citric acid and calcium chloride at final concentrations of 5 mM and 10 mM, respectively, were added to minced cod in different washing steps. In every case, the addition of citric acid and calcium chloride to the muscle was followed by incubation for 1 h prior to exposing the proteins to pH 3 and later neutralization. Thus, the citric acid and calcium chloride were added to the fish mince from which the protein isolate was prepared. Lipid oxidation was followed by TBARS. The results showed that when citric acid and calcium chloride were used in the first wash of the minced muscle, the oxidative stability of the muscle was improved at neutral pH after the washed cod was subjected to low pH (Fig. 4). Citric acid and calcium chloride in the first wash were more effective in preventing lipid oxidation later at neutral pH than acid treatment (HCl) alone (compare "PI" and "PI, Cc 1st wash"), but prior acid treatment alone also gave considerable protection from hemoglobin-mediated oxidation when assayed at neutral pH. Citric acid and calcium chloride inclusion in the second wash provided oxidation protection to a lesser extent than either treatment used in the first wash.

3.4. Effect of acid and alkali solubilization on hemoglobin-mediated lipid oxidation of a muscle protein isolate

In a typical procedure to isolate functional proteins from low value materials, homogenized muscle is treated at low ($\leqslant 3$) or high ($\geqslant 10.5$) pH to solubilize the proteins. Here, unwashed cod was subjected to pH 3 or 10.8 in the presence or absence of citric acid and calcium chloride (5 mM and 10 mM, respectively). After adjustment to pH 5.5 and centrifugation, the protein isolates were collected and the pH was brought to 7.4 and moisture content to 90%. Hemoglobin was then added (6 μmol/kg of tissue) to promote lipid oxidation. Both acid and alkali treatments inhibited lipid oxidation markedly, but citric acid and calcium chloride at either acid or alkali conditions had an additional effect (Fig. 5) essentially eliminating TBARS

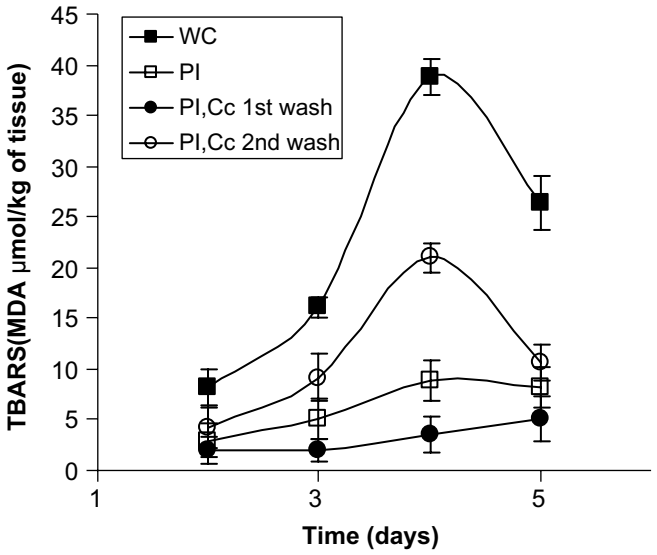

Fig. 4. Effect of adding citric acid and calcium chloride in different steps during the washing procedure ($n = 2$). WC: washed cod, no treatment. PI: protein isolate from acid solubilized washed cod. PI, Cc 1st wash: protein isolate from acid solubilized washed cod. Citric acid and calcium chloride added in the first step of washing followed by 1 h of incubation. PI, Cc 2nd wash: protein isolate from acid solubilized washed cod. Citric acid and calcium chloride added in the second step of washing followed by 1 h of incubation. Protein isolates were collected at pH 5.5 after centrifugation at 10,000×g of the solubilized washed cod muscle at pH 3. Final pH was adjusted to 7.4 and 6 μmol Hb/kg of sample were added to all samples at the end and moisture content adjusted to 90%.

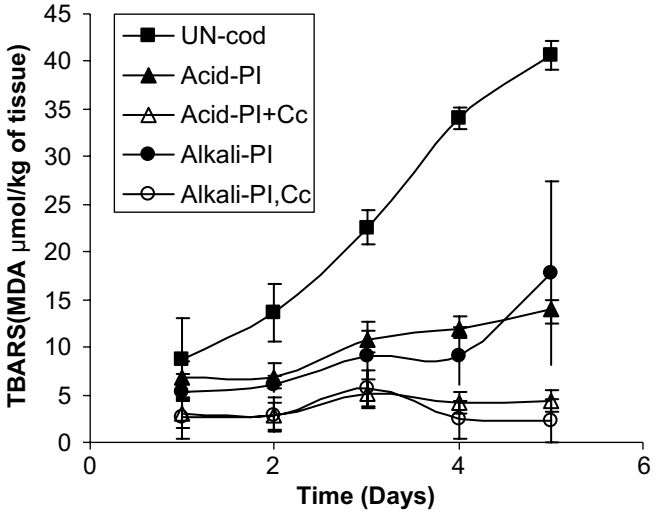

Fig. 5. Oxidation of protein isolate from acid (pH 3) or alkali (pH 10.8) solubilized unwashed cod with or without the addition of citric acid and calcium chloride in the homogenization step ($n = 2$). UN-cod: unwashed cod. Acid-PI: protein isolate from acid solubilized unwashed cod. Acid-PI + Cc: protein isolate from acid solubilized unwashed cod, where 5 mM citric acid and 10 mM calcium chloride were added in the homogenate. Alkali-PI: protein isolate from alkali solubilized unwashed cod. Alkali-PI + Cc: protein isolate from alkali solubilized unwashed cod, where 5 mM citric acid and 10mM calcium chloride were added in the homogenate. Protein isolates were adjusted to pH 7.4 and a moisture content of 90%. Hemoglobin was then added to the protein isolates at a final concentration of 6 μmol/kg of tissue.

formation in the protein isolate samples over the 5 days of storage.

In the previous experiments native, untreated hemoglobin was added to the samples at the end of the treatments to promote lipid oxidation. In any practical application of the protein isolation procedure, however, hemoglobin would be part of the tissue and would be exposed to the low pH at the same time as the muscle tissue. The effect of treating hemoglobin and the muscle together or separately at low pH was investigated. The results indicated that when hemoglobin was treated separately at low pH, somewhat less oxidation occurred when the treated hemoglobin was added to the protein isolates (washed cod, treated at pH 3.0, adjusted to pH 5.5 and centrifuged) compared to the results with untreated Hb (closed and open circles, Fig. 6). Furthermore, protein isolates were less susceptible to untreated hemoglobin-mediated lipid oxidation compared to washed (untreated) samples. On the other hand, it was noticed that when washed cod was brought to pH 3.0 in the presence of hemoglobin during acid solubilization, the lag phase of oxidation decreased (Figs. 6 and 7). Addition of citric acid and calcium chloride in the first washing step prior to subjecting the washed tissue and the hemoglobin to low pH together protected the muscle from oxidation even after 60 min incubation of the washed tissue with hemoglobin at pH 3 (Fig. 7).

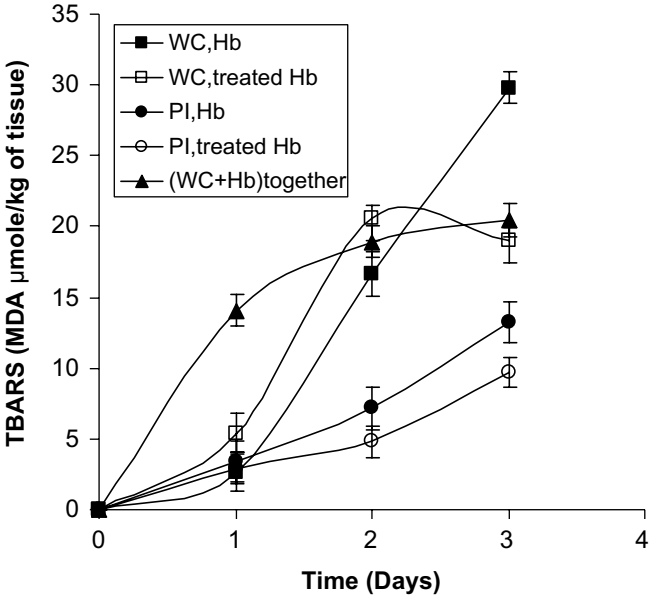

Fig. 6. Lipid oxidation of protein isolates collected from washed cod solubilized at pH 3 in the presence or absence of hemoglobin. WC + Hb: washed cod and untreated hemoglobin (6 μmol/kg of tissue). WC + Hb treated: hemoglobin (6 μmol/kg of tissue) was exposed to pH 3 for 20 s and readjusted to 7.4 before adding to washed cod. PI + Hb: washed cod exposed to pH 3, adjusted to 5.5 and centrifuged to collect the protein isolate; untreated Hb (6 μmol/kg of tissue) was added to the protein isolates. PI + treated Hb: washed cod exposed to pH 3, adjusted to 5.5 and centrifuged to collect the protein isolate; Hb (6 μmol/kg of tissue) exposed alone to pH 3 for 20 s and then readjusted to 7.4 was added to the protein isolates at pH 7.4. PI from (WC + Hb) together treated: washed cod and 11.3 μmol/kg of tissue of untreated hemoglobin were homogenized together in six volumes water. The pH of the homogenate was adjusted to 3 and then back to 5.5 for centrifugation to collect the protein isolate. The oxidation assays run at pH 7.4 and the MC of all samples was 90%.

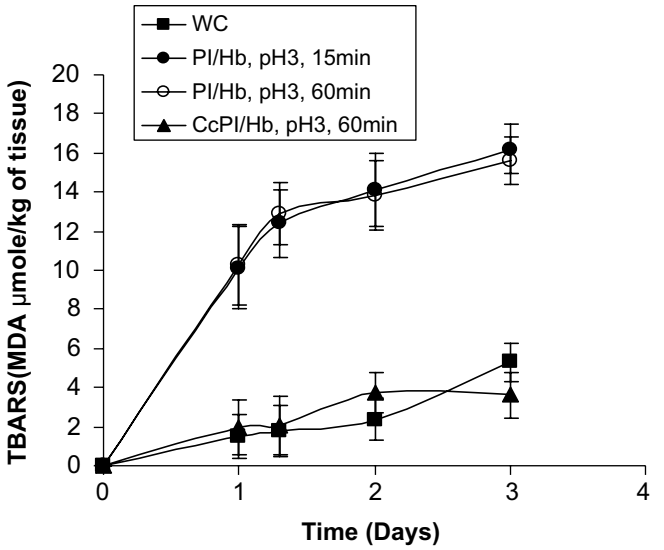

Fig. 7. Inhibition of oxidation of protein isolates by citric acid and calcium chloride after exposing washed cod and hemoglobin together to pH 3 (assays at pH 7.4; $n = 2$). WC: twice-washed mince, Hb 5.8 μmol/kg of tissue (■). PI/Hb, pH 3, 15 min: protein isolate from twice-washed cod, 11.3 μmol Hb added to the homogenate and 15 min incubation at pH 3 (●). PI/Hb, pH 3, 60 min: protein isolate from twice-washed cod, 11.3 μmol Hb added to the homogenate and 60 min incubation at pH 3 (○). CcPI/Hb, pH 3, 60 min: protein isolate from twice-washed cod, where citric acid and calcium chloride (5 mM and 10 mM, respectively) were added in the first wash. 11.3 μmol Hb/kg of tissue were added to the homogenate and incubation time at pH 3 was 60 min (▲).

3.5. Effect of the soluble fraction of the muscle on lipid oxidation

It was observed earlier (Fig. 1) that washed cod was more susceptible to hemoglobin-mediated lipid oxidation compared to unwashed cod. When the muscle is washed the soluble constituents of the muscle are largely removed. It has been demonstrated that cod press juice has anti-oxidative properties (Undeland et al., 2003). However, it is not known if the press juice (PJ) can retain these properties during acid treatment or if it can prevent lipid oxidation when muscle and hemoglobin are exposed to low pH at the same time. To investigate this, hemoglobin at a concentration of 11.3 μmol/kg of tissue and press juice (~7-fold dilution) were added to homogenized washed cod. The pH was adjusted to 3.0 and then to pH 5.5 with or without a 15 min incubation. The protein isolates were then collected and oxidation assays were run at pH 7.4. The protective effect of press juice against oxidation was profound, even for the 15 min incubation time at pH 3.0 (Fig. 8).

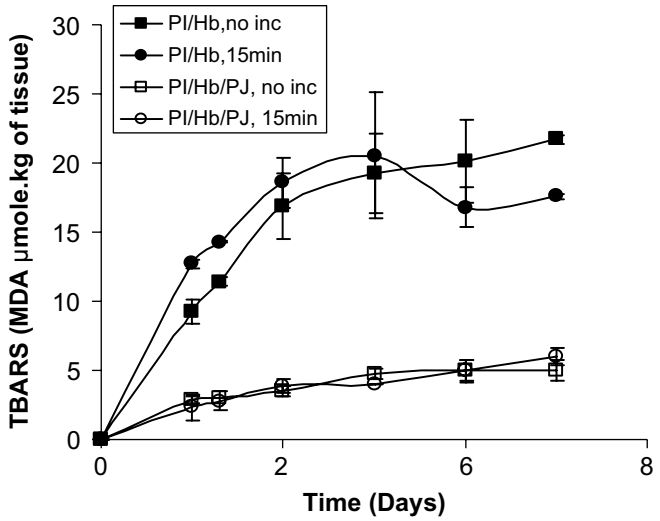

Fig. 8. Inhibition of oxidation of protein isolates by exposure of cod and hemoglobin together to cod press juice at pH 3 (assays at pH 7.4; $n = 2$). PI, no inc: protein isolate from washed cod and 11.3 μmol Hb that have been exposed together to pH 3 and immediately brought to pH 5.5 and centrifuged (■). PI, 15 min inc: control sample: protein isolate from washed cod and 11.3 μmol Hb that have been exposed together to pH 3 for 15 min and then brought to pH 5.5 and centrifuged (●). PJ-PI, no inc: protein isolate from washed cod, press juice (~7-fold dilution) and 11.3 μmol Hb/kg of tissue that have been exposed together to pH 3 and immediately brought to pH 5.5 and centrifuged (□). PJ-PI, 15 min inc: protein isolate from washed cod, press juice (~7-fold dilution) and 11.3 μmol Hb/kg of tissue that have been exposed together to pH 3 for 15 min and then brought to pH 5.5 and centrifuged (○).

4. Discussion

Liang and Hultin (2005a) showed that the isolated membranes from cod muscle could be sedimented at a low centrifugal force if they were exposed to a pH of about 5 or below. They showed that calcium chloride and an organic acid like citric added before solubilizing the muscle homogenates allowed up to 80% removal of phospholipid from homogenates solubilized at pH 3.0 in nine volumes of water and centrifuged at 4000×*g* for 15 min (Liang & Hultin, 2005a). It was suggested that citric acid and calcium chloride aided in the breaking of the linkages between membranes and cytoskeleton proteins. Citric acid might play a role as a binding agent to the basic amino acid residues of cytoskeletal proteins competing with the acidic phospholipids of membranes (Liepina, Czaplewski, Janmey, & Liwo, 2003; Haleva, Ben-Tal, & Diamant, 2004). Subsequently, the free membranes might aggregate due to the low pH and be removed by centrifugation at pH 3 (Liang & Hultin, 2005b). In addition to that, Pazos et al. (2005) showed that isolated cod membranes at pH 3 are less susceptible to hemoglobin-mediated lipid oxidation at pH 3 than at pH values around neutrality. Thus, it was thought possible that treatment with citric acid and calcium chloride might release membrane from the cytoskeletal proteins *in situ*. Subsequent lowering of the pH below 5 could then encourage aggregation of the membranes released from the cytoskeleton, rendering them less susceptible to lipid oxidation.

Citric acid and calcium chloride were most effective in preventing lipid oxidation of acid-treated washed muscle (i.e., protein isolates), only when they were added in the first washing step (Fig. 4) or directly to the mince (Fig. 1). The fact that citric acid and calcium chloride were not as effective when added in any other step of the process suggests that some of the soluble components of the muscle that are removed by the washing procedure, could be interacting with and/or aiding citric acid and calcium chloride in preventing lipid oxidation in the protein isolates even after these soluble components were removed. These soluble components themselves were good anti-oxidants as evidenced by the greater stability of the unwashed compared to the washed muscle.

Acid treatment of the muscle without added citric acid and/or calcium chloride, enhanced the oxidative stability of protein isolates from washed or unwashed cod. In contrast to these results, Kristinsson and Hultin (2004) showed that acidified washed cod muscle oxidized faster than untreated muscle. In their experiments, the pH of washed cod was brought down to 3.0 and then adjusted to 7.0. Hence, exposure time to pH 5.5 of the solubilized muscle was minimized and no centrifugation took place at pH 5.5, as it does during the protein isolate procedure. During the process followed in this study, it is possible that the low pH during the solubilization procedure caused *in situ* aggregation of the membranes, which then precipitated together with the muscle proteins after centrifugation at pH 5.5 at 10,000×*g*.

In an effort to explain and determine the individual contribution of each treatment (pH, citric acid, calcium chloride) to the overall improvement of the oxidative stability of cod muscle, their separate effects were studied. It was shown that citric acid and low pH treatment improved the oxidative stability of the muscle, while calcium chloride alone did not. The antioxidant capacity of citric acid is generally attributed to its ability to chelate metal ions, such as iron and copper, by forming bonds between the metal and the carboxyl or hydroxyl groups of the citric acid molecule (Decker, 2002; Hras, Hadolin, Knez, & Bauman, 2000). Iron concentration in cod muscle is low, with an average value of 6 ppm (Waterman, 2001). "Free" catalytic iron in the washed cod model system might be generated by the destruction of the heme and release of iron. However, it has been shown that low molecular weight iron added at a concentration of 23.2 μM (which corresponds to the iron concentration from 5.8 μM hemoglobin) did not induce oxidation of washed minced cod lipids while hemoglobin at this concentration was very pro-oxidative (Richards & Hultin, 2000). Undeland et al. (2003) demonstrated the inability of phosphates to inhibit hemoglobin-catalyzed lipid oxidation of washed cod muscle and suggested that the "free" iron has a negligible effect on the oxidation of this system.

One of the ways citric acid could protect the muscle from lipid oxidation might be that citric acid breaks down

preformed peroxides. The decrease in peroxide value of washed cod samples after treatment with citric acid ranged from ~30% to 50% with no incubation time (Table 2). It is possible to make an estimate of what this type of reduction might mean in terms of the development of sensory rancidity in the washed cod. A lipid content of 0.5% is 5 g of lipid per kg of tissue. At 50 mol of hydroperoxide per gram of lipid, this would be 250 mol of peroxide per kg of tissue. If we use a typical ratio of the conversion of peroxide into TBA-reactive substances as 6 mol of peroxide producing 1 mol of TBARS (McDonald & Hultin, 1987), this would give a total TBARS of approximately 40 mol per kg of sample. A reduction of 50% of the peroxides could lead to a like reduction in TBARS (40–20 mol). The value of 20 mol TBARS per kg of tissue is roughly the break point where we generally detect rancidity development (Jia, Kelleher, Hultin, Petillo, Maney et al., 1996). Thus the potential decrease of peroxides could possibly produce an acceptable product from one that was not acceptable. A further consideration is that the 30–50% of the peroxides that are destroyed by citric acid might be those that are more unstable and which could lead to a disproportionately high amount of breakdown products during later storage. There appeared to be a slight advantage of citric acid over hydrochloric acid with the muscle adjusted to the same pH, but the improvement with citric acid vs. hydrochloric acid was relatively small. A specific effect of citric acid vs. HCl needs confirmation.

Controlling lipid oxidation during protein isolate production from fatty fish using the acid solubilization process has been a challenge (Kristinsson & Liang, 2006; Undeland, Hall, Wendin, Gangby, & Rutgersson, 2005). The unexpected results reported in this paper showed that acid treatment of cod hemoglobin alone was mildly to strongly anti-oxidative, while the treatment of the washed cod at low pH was very strongly anti-oxidative. When the hemoglobin and the membrane suspension were treated at the low pH separately before running the assay at about neutral pH, the anti-oxidative effect was also observed. It was only when the hemoglobin and the cod were treated at an acid pH together that the system became strongly pro-oxidative. The necessity for the presence of both the substrate (membrane) and the catalyst at the same time indicates a specific interaction between them at the low pH. We suggest that the oxidation occurs by the release of the heme pigment from the hemoglobin which is followed by the uptake of pigment by the membrane (Grunwald & Richards, 2006; Richards, Dettmann, & Grunwald, 2005). We suggest that the release of the heme is more rapid than the aggregation of the membranes and thus the membrane has the opportunity to take the heme pigment up before it aggregates. When the muscle is treated at low pH before the addition of hemoglobin, the aggregation occurs and the aggregated membrane does not take up the released heme pigment. Free heme pigment is known to be a very potent pro-oxidant (Everse & Hsia, 1997).

We suggest further that the released heme pigment gets absorbed into the membrane lipids and rapidly brings about the destruction of any pre-formed lipid peroxides. The concentration of pre-formed peroxides which we observed in this study was of the order of about 250 mol of hydroperoxide per kg of muscle sample. This would have been sufficient as discussed above to bring about rapid oxidation of the tissue. It has been proposed that electrostatic interaction between hemoglobin and membranes is required for the initial binding of hemoglobin to liposomes and presumably membrane surfaces (Marva and Hebbel, 1994). Consistent with our proposal is the work of Richards et al. (2005) who demonstrated the release of the heme group from heme proteins was more related to lipid oxidation processes than the formation of the met heme (Fe^{+3}) compounds. In further work of that laboratory, Grunwald and Richards (2006) have shown that a myoglobin variant with a low affinity for heme is a potent promoter of lipid oxidation whereas another variant that bound heme tightly was a poor pro-oxidant. In addition, they also found that a myoglobin variant sensitive to heme degradation was a less efficient promoter of lipid oxidation than wild-type myoglobin. Thus they concluded that degradation of the heme ring and release of iron actually decreased the ability of myoglobin to promote lipid oxidation, an indication that it is the iron located in the heme pigment which is primarily responsible for oxidation. These workers used a model system of washed cod muscle similar to what was used in our experiments.

This mechanism to explain the reason why low pH applied to cod hemoglobin and washed, minced cod muscle separately is anti-oxidative while the low pH applied to these components when they are together is pro-oxidative could also explain a couple of other observations that were made. When the protein isolate is made by solubilizing the cod muscle proteins at pH 3, there is a dilution of approximately 8.5-fold. This would reduce the normal ionic strength of the tissue considerably which would make it easier for electrostatic interactions between the hemoglobin and membrane surfaces to occur. Adding the press juice at a 7-fold dilution would essentially double the ionic concentrations in the mixture and might have a negative effect on the interaction of the hemoglobin with the membranes. If this binding is prevented, it would be predicted that there should be less oxidation as was observed in the presence of the press juice.

It has been shown that aqueous extracts from some muscles inhibit hemoglobin-mediated oxidation of the cod muscle membrane lipids. Cod, mackerel, haddock, winter flounder, herring and chicken aqueous extracts were able to significantly extend the lag phase of painty odor development in washed cod model system or soybean phosphatidylcholine liposomes. At 2-, 3.2- and 6-fold dilution levels of cod press juice, the average lag phases were $\geqslant$8 days, 6.7 days, and 4.1 days, respectively (Undeland et al., 2003).

Another possibility is that the press juice hinders the binding of hemoglobin to membrane components, decreasing therefore hemoglobin's pro-oxidative activity.

Thongraung, Benjakul, and Hultin (2006) showed that cod press juice improved the extractability of hemoglobin added to minced cod and decreased the binding of soluble hemoglobin to sarcoplasmic reticulum. It was suggested that the ionic strength of the press juice could inhibit the electrostatic interactions between hemoglobin and membranes, which according to Marva and Hebbel (1994) are required for the initial binding of hemoglobin to liposomes. Previous research has excluded proteins or other biopolymers as candidates for the observed inhibition of lipid oxidation by the press juice, since the anti-oxidative properties were attributed to the <1 kDa fraction of the press juice, which retained its properties after heating and centrifugation. A few low molecular weight candidates, such as phosphates, spermine, TMAO and 2,3-DPG were also tested, but ruled out, since they were unable to prevent oxidation to the same degree as the press juice (Undeland et al., 2003). It was suggested that low molecular weight nucleotides and/or reducing agents might be involved in the inhibition.

A similar effect to the inhibition of lipid oxidation observed after treatment of the muscle tissue at low pH is observed at high pH. We have at present no explanation for this phenomenon. Muscle membranes at high pH do not aggregate in the absence of calcium ion, most likely due to their high negative surface charge. It is possible that such a high negative charge of both the membrane proteins and lipids could lead to their being expelled from the membrane, destroying the membrane structure. The cause of the oxidation effect of treating the muscle tissue at high pH needs further study.

5. General conclusions

Treating minced cod or haddock at either a low (3.0) or high (10.8) pH conferred resistance to lipid oxidation catalyzed by cod hemoglobin. Exposing the minced fish or the hemoglobin to a low pH separately was anti-oxidative while treating the fish and the hemoglobin at low pH together was pro-oxidative. Since this latter case represents actual processing conditions, solving the problem will involve taking into account the interaction of the fish membrane lipids (membranes) with the heme proteins at the low pH.

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