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Effect of pH, ADP and muscle soluble components on cod hemoglobin characteristics and extractability

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Abstract

The effect of pH, ADP and the soluble components of the muscle on the extractability of cod hemoglobin was studied. A marked decrease in hemoglobin extractability from washed and unwashed mince was observed at pH 6.0. This coincided with the binding of the hemoglobin to cod sarcoplasmic reticulum at pH 6.0. After 10 min of incubation at pH 6.0, most non-extractable hemoglobin could be extracted at pH 7.0 or higher. The soluble fraction of cod muscle increased hemoglobin extractability and decreased the hemoglobin bound to the sarcoplasmic reticulum. A decrease in hemoglobin extractability in unwashed mince with added ADP at pH 7.0 occurred with an increased amount of deoxyhemoglobin. The results suggested that medium pH and deoxygenation of hemoglobin were related to the decrease in hemoglobin extractability at pH 6.0.

Keywords: Hemoglobin; Extractability of hemoglobin; Sarcoplasmic reticulum; Binding of hemoglobin

1. Introduction

Color is an important parameter determining the quality of surimi. Washing conditions in conventional surimi processing, such as washing time, number of washing cycles, and water quality, have been modified in order to remove more muscle pigments and to improve surimi whiteness (Chen & Lao, 1997; Lin & Park, 1996). Nevertheless, little success has been reported (Nishioka, Tokunaga, Fujiwara, & Yoshioka, 1990). Washing mechanically deboned chicken meat with 0.5% sodium bicarbonate or 40 mM phosphate buffer at pH 8.0 was effective for removal of meat pigments (Yang & Froning, 1992). Chen, Chiu, and Huang (1997) reported that washing with 0.5% sodium bicarbonate did not improve the color of horse mackerel mince. Similar results have also been reported for mackerel (Jiang, Ho, Jiang, Lo, & Chen, 1998). However, Chen, Chow, and Ochiai (1996) found that washing milkfish mince at alkaline pH either by using phosphate buffer or sodium bicarbonate removed fish pigments efficiently.

The pigments primarily responsible for the color of the dark muscle of fish are hemoglobin and myoglobin (Bone, 1978). These proteins are highly water-soluble. Thus, low extractability of the soluble proteins would imply their molecular modification and/or the existence of an interaction with the insoluble components of fish muscle (Hultin, Feng, & Stanley, 1995). These changes possibly occur between capture and processing. The myoglobin of milkfish and herring was less soluble after iced storage than in the freshly caught state (Chen et al., 1996). Autoxidation of tuna myoglobin was coincidental with the decrease in its solubility (Chow, Ochiai, Watabe, & Hashimoto, 1987). Richard and Hultin (2000) re-

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ported a sharp decrease in oxygenation of trout hemoglobin when the pH was lowered from 7.5 to 6.0 with an increase in lipid oxidation rate in washed cod mince. Thus, the decrease in pH of fish muscle during postmortem handling or storage may favor modification of the proteins especially hemoglobin.

Hemoglobin binds to membrane components readily (Salhany, Sloan, & Cordes, 1990; Walder et al., 1984). Marva and Hebbel (1994) proposed that an interaction between hemoglobin and phosphatidylserine liposomes, as a membrane model system, involves an initial and reversible electrostatic interaction. Binding to lipid exaggerates the instability of hemoglobin (Andreyuk & Kisel, 1999). After initial electrostatic interaction, myoglobin may partially unfold to semirandom coils at the membrane/water interface, which would permit the nonpolar residues to contact the apolar region of the bilayer (Bergers et al., 1993). Therefore, many factors can affect the extractability of hemoglobin from fish muscles, which can affect the color of washed mince.

The objective of the present study was to investigate the effect of pH on the extractability and binding of cod hemoglobin to cod sarcoplasmic reticulum.

2. Materials and methods

2.1. Materials

Fresh Atlantic cod (*Gadus morhua*) fillets or whole fish, was obtained from J.B. Wright Fish Co. (Gloucester, MA). Carbon monoxide and nitrogen gas were supplied by Matheson Gas (Gloucester, MA).

2.2. Reagents

Chloroform, methanol, sodium dithionite, sodium chloride, sodium hydroxide, sodium phosphate (monobasic and dibasic), and sodium potassium tartrate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Other chemicals of analytical grade were procured from Sigma Chemical (St. Louis, MO, USA)

2.3. Methods

2.3.1. Hemolysate preparation

Cod frame obtained after filleting rigor-state fish was used to collect cod blood. To open its caudal vein, the tail of the fish frame was cut off. Fish blood was drawn from the opened caudal vein by using a transfer glass pipet rinsed with salined sodium heparin solution (30 U/ml) (Richard & Hultin, 2000). Pressure was applied to the fish frame to facilitate blood flow and several cuttings were used to increase yield. The blood was transferred into a glass test tube containing 0.5 ml of the cold heparin solution.

Hemolysate was prepared immediately after the blood was drawn according to the method of Fyhn et al. (1979) as modified by Richard and Hultin (2000). Four volumes of cold 1 mM Tris–HCl buffer containing 1.7% NaCl, pH 8.0, were added to the heparinized blood. Centrifugation was done at 700*g* for 10 min at 4 °C using a tabletop clinical centrifuge (IEC, Needham Heights, MA). Plasma was then removed and the red blood cells were washed by suspending them in 10 volumes of 1 mM Tris–HCl buffer, pH 8.0 and centrifuging at 700*g*. The washing was conducted three times. The red blood cells were lysed in 3 volumes of 1 mM Tris–HCl buffer, pH 8.0, for 1 h. One-tenth volume of 1 M NaCl was then added to aid in stroma removal before centrifugation at 28,000*g* for 15 min at 4 °C in a Beckman Ultracentrifuge model L5-65B (Beckman Instruments Inc., Palo Alto, CA). The supernatant obtained was the hemolysate and is referred to as ''cod hemoglobin''. It was kept at −80 °C and thawed just before use.

2.3.2. Determination of soluble/extractable heme protein concentration

Heme protein content was measured according to the method of Brown (1961) as modified by Richard and Hultin (2000). The diluted hemoglobin in 50 mM phosphate buffer, pH 8.6, was bubbled with carbon monoxide gas for 30 s before mixing with 1 mg of sodium dithionite. The sample was subsequently scanned from 440 to 370 against a blank containing only the buffer using a model U-3110 double-beam spectrophotometer (Hitachi Instruments, Inc., San Jose, CA). The difference between absorbance at the peak and valley was recorded. Heme protein concentration was calculated using bovine hemoglobin as a standard.

2.3.3. Cod mince and washed cod mince preparation

The light muscle of cod was minced in a Kitchen Aid mincer (Kitchen Aid Inc., St. Joseph, MI) with orifices of 4.7-mm diameter. Minced fish was washed three times with deionised water using a water to mince ratio of 3:1 (v/w). During washing, the mince slurry was occasionally stirred with a plastic rod and allowed to stand for 10 min and then drained on cheesecloth. A solution of NaCl (0.3% w/v) was used in the third wash to aid in dewatering. Excess moisture of the washed mince was removed by centrifugation at 10,000*g* for 15 min at 10 °C (Sorval RC-5B, refrigerated superspeed centrifuge, Dupont Instrument, Wilmington, DE, USA). The washed mince was kept on ice or in the refrigerator and used within two days.

2.3.4. Soluble fraction of fish muscle and its components

A soluble fraction of fish muscle was separated from minced cod light muscle. The minced muscle was packed into centrifuge tubes and centrifuged at 4 °C

for 16 h using a Beckman Ultracentrifuge model L5-65B with a fixed angle rotor type 19 (Beckman Instruments Inc., Palo Alto, CA). The centrifugation force was applied as follows; firstly at 14,900*g* for 1 h, then at 21,500*g* for 2 h, and finally at 38,200*g* for 13 h. This procedure provided a soluble fraction of the fish muscle of about 32% of the initial mince weight. The liquid is referred to as "press juice". The press juice was gently withdrawn from the centrifuge tube with a disposable transfer pipet, kept in an ice bath and used within three days.

The press juice was further separated into two fractions by using ultrafiltration or dialysis. The molecular weight cut-off of the membrane used in dialysis was 1000 daltons. Press juice (40 ml) was dialysed for 6 h with three changes of the dialysis buffer (4 L, 50 mM phosphate buffer solution, pH 7.0). The liquid remaining in the dialysis tubes was collected and referred to as the "high molecular weight fraction of press juice". All operations were performed in a cold room.

A low molecular weight fraction of press juice was obtained using an ultrafiltration technique. The filtration was performed in the Millipore stirrer cell (Milipore Corporation, Bedford, MA) under nitrogen gas (50 psi, Matheson Gas, Gloucester, MA). The filtrate fraction was collected and referred to as the "low molecular weight fraction of press juice". All operations were conducted in a cold room. Both fractions were used within three days after preparation.

2.3.5. Isolation of sarcoplasmic reticulum (SR)

Sarcoplasmic reticulum was isolated from light muscle of cod as described by Borhan, Shewfelt, and Hultin (1984). The mince was homogenised with chilled 1 mM HEPES-Cl buffer (pH 7.4) containing 0.3 M sucrose with a mince/buffer ratio of 1:3 (w/v) using a Polytron homogeniser (PT 10-35, Kinematica AG, Luzern, Switzerland) at speed No. 5 for 40 s. The homogenate was adjusted to pH 7.4 with 1 M Tris–HCl buffer (pH 8.4) and centrifuged at 1350*g* for 10 min using a Beckman L5-6513 ultracentrifuge at 5 °C, followed by the centrifugation at 15,000*g* for 20 min. The supernatant was filtered through four layers of cheesecloth and placed on a sucrose gradient (supernatant:20% w/w sucrose:45% w/w sucrose; 50:9:3 ml). A type 45-rotor was used to centrifuge the sucrose gradient at 130,000*g* for 90 min. The upper layers were removed by aspiration, and the protein collected at the interface of the 20% and 45% sucrose layers was suspended in 5 mM histidine-buffer containing 0.6 M KCl (pH 7.3). The suspension was centrifuged at 130,000*g* for 60 min. The resultant sediment was suspended in the same buffer and recentrifuged similarly. The supernatant was decanted and the sediment was resuspended in this same buffer. It was kept at −80 °C and used within three weeks.

2.3.6. Effect of pH and ADP on deoxygenation of cod hemoglobin

The cod hemoglobin (6.5 μM) in 50 mM phosphate buffer at pH 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 was scanned from 630 to 500 nm using a double-beam spectrophotometer model U-3110 (Hitachi Instruments, Inc., San Jose, CA) against phosphate buffer. The difference between the absorbance at the peak (574 ± 1 nm) and the valley (559 ± 1 nm) was used for calculation of the relative oxygenation of the hemoglobin. Oxygenation was assigned a value of 100% at pH 8.0. Effect of ADP on hemoglobin deoxygenation was assessed by using cod hemoglobin (6.5 μM) in 50 mM phosphate buffer at pH 7.0 with and without 1 mM ADP.

2.3.7. Extractability of cod hemoglobin from washed and unwashed cod mince at pH 6.0 and 7.2

Washed and unwashed cod mince were adjusted to pH 6.0 or 7.2 with 1 N NaOH or 1 N HCl. Cod hemolysate was then added into the pH-adjusted minces to obtain 5.8 μmol hemoglobin/200 g dry weight of mince. The pH of the minces was rechecked just after addition of hemolysate. If necessary, the mince was adjusted to 88% moisture with deionised water before hemoglobin addition After incubation on ice for 10 min, the heme protein was extracted by homogenisation with 10 volumes of deionised water using a Polytron homogenizer at speed No. 5 for 40 s (PT 10-35, Kinematica AG, Luzern, Switzerland) followed by centrifugation at 46,500*g* at 4 °C for 15 min. Extractable hemoglobin in the supernatant was quantified by measuring the amount of soluble heme protein in the supernatants. The percentage of extractable hemoglobin was calculated based on the total added heme protein (plus endogenous extractable heme proteins in the case of the unwashed mince).

2.3.8. Effect of extraction pH and salt on extractability of cod hemoglobin from unwashed mince originally at pH 6.0

The unwashed mince to which hemoglobin was added originally at pH 6.0 was prepared as previously described. After incubation on ice for 10 min, the mince containing hemoglobin was homogenized with 10 volumes of deionised water as previously described. The effect of extraction pH at 7.0, 7.5, or 8.0 was investigated by instantly adjusting the pH of the homogenates obtained with 1 N NaOH. The effect of salt at different concentrations (0, 23 and 139 mM) on hemoglobin extractability was studied at pH 6.0 and 7.0. Salt was added into the homogenates and the pH was immediately corrected to 6.0 or 7.0 with 1 N NaOH. The supernatants were obtained by centrifugation as previously described in Section 2.3.7 and used for quantifying extractable hemoglobin. Soluble protein co-extracted in the supernatants was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

Per cent extractable hemoglobin was calculated base on the amount of hemoglobin added.

2.3.9. Effect of cod muscle soluble fraction on hemoglobin extractability

Whole press juice or its fractions were separately added into the washed cod minces (pH 6.0) to account for 35% (v/v) of the final moisture of the minces, which was 85% moisture. Predetermined volumes of cod hemolysate were subsequently added into the minces to give a concentration of 5.8 μmol heme protein/200 g dry weight muscle. The hemoglobin was then extracted with 10 volumes of deionised water as previously described after incubation in an ice bath for 10 min. Per cent extractable hemoglobin was calculated.

2.3.10. Binding of hemoglobin to sarcoplasmic reticulum

Cod hemoglobin was added to a suspension of cod SR (0.14 mg protein/ml) in 10 mM phosphate buffer at pH 6.0 or 7.0 containing NaCl at different concentrations to have a final heme protein concentration of 0.7 μM. NaCl concentrations were 0, 30, 80, 130, 180 or 230 mM. The pH of the mixtures was checked and corrected with small amounts of 1 N HCl or 1 N NaOH if necessary. The mixtures were incubated in an ice bath for 30 min. Insoluble material was then removed by centrifugation at 186,000*g* for 15 min at 4 °C using a Beckman L8-55M ultracentrifuge. Soluble hemoglobin in the supernatant was quantified and per cent soluble hemoglobin was calculated.

The effect of press juice and its low and high molecular weight fractions on binding of hemoglobin with SR was also investigated. The press juice or its fractions were added into the mixture of cod hemoglobin (0.7 μM) and cod SR (0.14 mg protein/ml) in 20 mM phosphate buffer at pH 6.0 to a final concentration of 35% (v/v). Incubation and centrifugation of the samples were carried out as previously mentioned. Per cent soluble hemoglobin was calculated.

2.3.11. Effect of ADP on hemoglobin extractability

The effect of ADP on hemoglobin extractability at pH 7.0 at concentrations of 0, 1 and 8 mM was tested. ADP solution at pH 7.0 was added into the washed mince to give a final concentration of 1 or 8 mM ADP. Thereafter the cod hemoglobin was mixed into the ADP containing washed mince to have 5.8 μmol heme protein/200 g dry weight. For one sample, the ADP solutions at pH 7.0 were separately mixed into the washed mince and the hemoglobin stock solution to give a final concentration of 1 mM ADP. After holding for 1 min in an ice bath, the ADP-hemoglobin solution was added to the mince to which ADP had been previously added to obtain a mince having 5.8 μmol heme protein/200 g dry weight and 1 mM ADP. The samples were incubated in a refrigerator for 0, 6 and 12 h before assessment for extractability of hemoglobin as previously described.

2.3.12. Moisture determination

Moisture content was quantified by using a Cenco rapid moisture determination balance (CSC Scientific Co., Inc., Fairfax, VA) equipped with an infrared lamp.

2.3.13. pH measurement

The pH values of the samples were obtained by homogenizing 1 g of sample with 10 ml of deionised water and determining pH using an Accumet pH/conductivity meter model 20 (Fisher Scientific, Fair Lawn, NJ, USA) equipped with a thermocouple to compensate for temperature.

2.3.14. Protein analysis

Protein content was measured according to Lowry et al. (1951).

2.3.15. Statistical analyses

Data were subjected to analysis of varince (ANOVA) and mean comparisons were performed using Duncan New Multiple range test. Statistical analyses were carried out using the SAS statistical software (SAS, 1996).

3. Results and discussion

3.1. Effect of pH and ADP on deoxygenation of cod hemoglobin

The absorbance spectra (350–630 nm) of cod hemoglobin (Fig. 1) possessed the general characteristics of hemoglobin from other animals reported elsewhere. An absorbance peak in the Soret region of 412 nm was observed. Absorbance peaks at 574 and 540 nm are indicative of the characteristic of oxyhemoglobin. A

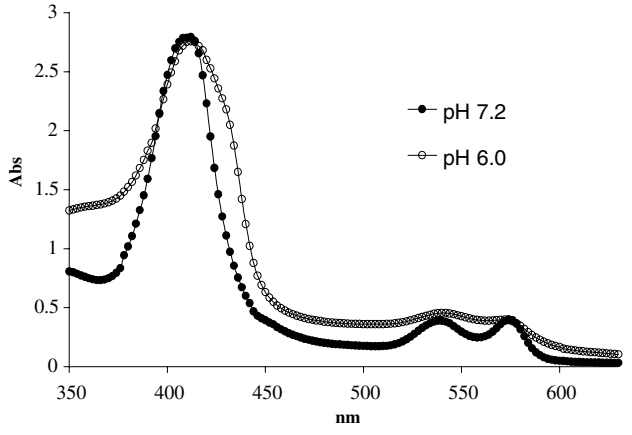

Fig. 1. Typical visible light absorbance spectrum of cod hemoglobin (10 μmol) in 50 mM phosphate buffer at pH 6.0 or 7.2.

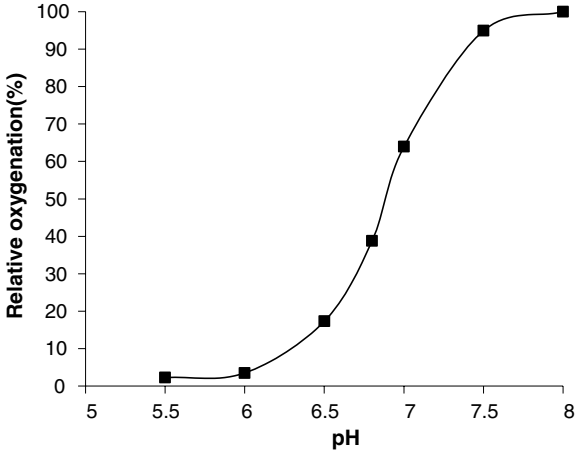

Fig. 2. Relative oxygenation of cod hemoglobin at various pH values. Cod hemoglobin (6.5 μM) in 50 mM sodium phosphate buffer at pH 5.5–8.0. The solutions were incubated in an ice bath for 10 min after dilution and scanned for their absorbance spectrum. Relative oxygenation was related to the difference between the peak (~574 nm) and the valley (~559 nm) of the spectra. The relative oxygenation of the solution at pH 8.0 was taken as 100%.

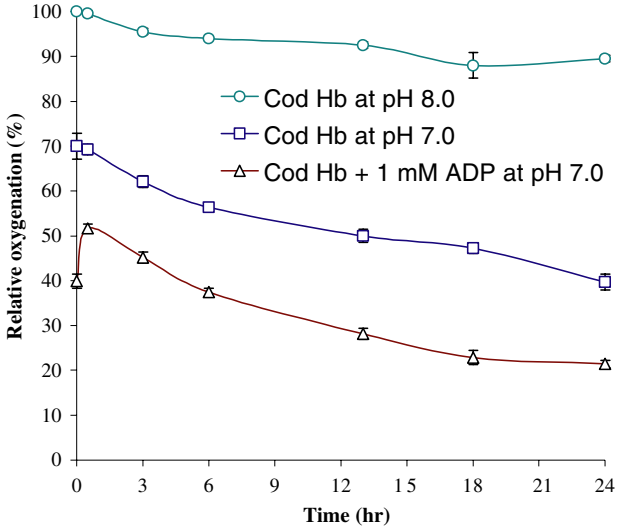

Fig. 3. Effect of ADP and pH 7.0 on relative oxygenation of cod hemoglobin. The samples were cod hemoglobin (6.5 μM) in 50 mM phosphate buffer at pH 7.0 with and without 1 mM ADP and the solution at pH 8.0 without added ADP. The solutions were incubated in an ice bath and scanned for their absorbance spectrum at an appropriate duration. Relative oxygenation was calculated as described in Fig. 2. Relative oxygenation of the solution at pH 8.0 at 40 s after mixing was taken as 100%. Bars indicate SD of duplicate experiments.

large difference between absorbance at the peak (574 nm) and at the valley between 574 and 540 observed at pH 7.2 suggested that oxyhemoglobin was the dominant form at this pH (Pelster & Weber, 1991). A decrease in this difference was observed when the pH was 6.0, suggesting deoxygenation of the hemoglobin. A blue shift in the Soret peak (~4 nm) and the existence of a shoulder at ~450 nm were typical characteristics of the absorbance spectra of cod hemoglobin at pH 6.0 (Fig. 1).

The relative oxygenation of cod hemoglobin decreased with decreasing pH from 8.0 to 5.5 in a sigmoidal fashion (Fig. 2). This result was in agreement with Richard and Hultin (2000) who reported that deoxygenation of trout hemoglobin occurred when the pH decreased from 8.0 to 6.0. The results suggest that cod oxyhemoglobin released oxygen readily at pH values below 7.6. It is a typical characteristic of the Bohr effect of fish hemoglobin and is a property of the anodal hemoglobins. It has been suggested that this loss of ability to bind oxygen is related to transport and off-loading oxygen in the cell (Jensen, Fago, & Weber, 1998). At pH 6.0, less than 5% of cod hemoglobin was in the oxy form, whereas oxyhemoglobin was dominant at pH 8.0.

The relative oxygenation of cod hemoglobin decreased continuously with increasing storage time at both pH 8.0 and 7.0. However, the relative oxygenation of cod hemoglobin at pH 8.0 was much greater than that observed at pH 7.0 (Fig. 3). Less relative oxygenation of the hemoglobin was observed at pH 7.0 in the presence of 1 mM ADP than in its absence, but the rates of decrease were similar. At the same concentration used, ADP decreases oxygenation of trout hemoglobin less than ATP does (Richard & Hultin, 2000). It is believed that ADP binds at the entrance to the central cavity between the two β chains in the T form of hemoglobin as does ATP. Organic phosphates are known to be potent allosteric effectors of hemoglobins in fish and decrease their oxygen affinity (Jensen et al., 1998). At pH 8.0, ADP had no significant effect on oxygenation of cod hemoglobin (data not shown).

3.2. Effect of pH and salt on extractability of cod hemoglobin

The endogenous extractable heme protein of cod light muscle was around 0.54 μmol/kg. Although an extract of the washed mince showed no detectable soluble heme proteins, the possible existence of non-extractable heme proteins in the washed mince was possible. The amount of hemoglobin added to the samples used in this study is roughly equivalent to the amount of hemoglobin found in the light muscle of trout (Gingerich, Pityer, & Rach, 1990; Richard & Hultin, 2000). This added hemoglobin was taken as the initial total extractable hemoglobin.

Cod hemoglobin extractability with water from mince and washed mince at pH 6.0 and 7.2 is shown in Table 1. Exposure of hemoglobin to pH 6.0 decreased its extractability drastically in both washed and unwashed mince compared to pH 7. Since we did not observe significant loss of solubility of hemoglobin at pH 6.0 (data not shown), it is likely that this loss of extractability was due to interaction of the hemoglobin with structural components of the muscle such as the myofibrillar

Table 1
Per cent extractability of hemoglobin of washed and unwashed cod mince at pH 6.0 and 7.2

pH	Washed mince	Unwashed mince
6.0	9.8 ± 2.4by[a,b]	45.6 ± 3.9bx
7.2	78.9 ± 3.6ax	83.9 ± 4.3ax

Washed and unwashed cod mince were adjusted to pH 6.0 or 7.2 with 1 N NaOH or 1 N HCl then mixed well with cod hemoglobin (3.48 μmol heme protein/kg mince, 88% moisture content). The hemoglobin was extracted with 10 volumes of deionized water after incubation on ice for 10 min. Supernatants were obtained by centrifugation at 46,500*g* at 4 °C for 15 min. The per cent extractable hemoglobin was quantified by measuring the amount of soluble hemoglobin in the supernatants (by conversion to carbon monoxyhemoglobin) and calculated based on total added hemoglobin.

[a] Means ± SD from triplicate experiments.

[b] The means followed by different letters in the same row (xyz) or column (abc) are significantly different ($P > 0.05$).

proteins or cellular membranes. The observed spectra at pH 6.0 (Fig. 1) suggest conformational changes which may involve the exposure of more hydrophobic surfaces for these interactions. Higher extractability was observed in unwashed mince at both pHs tested. There was a marked decrease in hemoglobin extractability in washed mince compared to unwashed mince, particularly at pH 6.0. The result suggests that components in unwashed mince might interfere with the interaction between hemoglobin and muscle components.

The decrease in hemoglobin extractability due to exposure to pH 6.0 for 10 min was restored in part if the extraction was performed at pH 7.0 or higher (Fig. 4). The hemoglobin extracted at pH 7.0 decreased

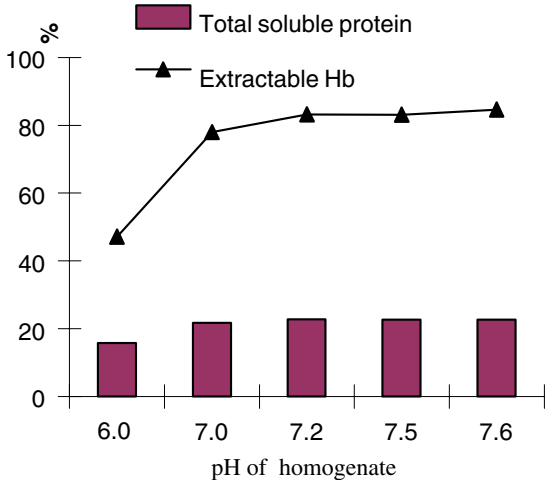

Fig. 4. Effect of extraction pH on percentage of extractable hemoglobin and protein of the unwashed mince originally at pH 6.0. The experiment was conducted in the same manner as described in Table 1 with the exception that the unwashed minces with added hemoglobin were held for 10 min at pH 6.0 before they were extracted at pH 6.0, 7.0, 7.5, or 8.0. Total protein (100% extractable protein was 175.4 ± 5.0 g protein/kg) and extractable protein of the samples were measured by the Lowry method. The initial total extractable hemoglobin of the samples was 3.48 μmol heme protein/kg (88% moisture content).

if the incubation at pH 6 was extended beyond 10 min. Within 24 h, there was no extractable hemoglobin (data not shown). Washing with alkaline solutions, especially $NaHCO_3$, to neutralize pelagic fish mince has been recommended to enhance functional properties of surimi (Shimizu, Toyohara, & Lanier, 1992). A basic wash also showed improvement of pigment removal of sardine (Barrero & Bello, 2000; Ramirez-Suarez, Pacheco-Aguilar, & Mazorra-Manzano, 2000) and milkfish (Chen et al., 1996) but not mackerel (Jiang et al., 1998). Hemoglobin is normally compartmentalized in erythrocytes in the living animal and blood is confined in an intact capillary system. General processing such as heading, filleting, and/or mincing will expose hemoglobin to muscle cell components. If the muscle tissue has a low pH this can induce changes in the hemoglobin and accelerate lipid oxidation (Richard & Hultin, 2000; Richard, Østdal, & Andersen, 2002). Our results from the model system suggest that hemoglobin will remain extractable by alkaline solutions only within a limited length of time.

Raising the extraction pH resulted in an increased loss of soluble protein (Fig. 4). Removal of heme pigment while maintaining functional muscle proteins is desirable for surimi processing. Loss of valuable proteins in this process occur either as small muscle particles or as soluble proteins. Extraction of hemoglobin leveled off slightly above pH 7, while extractable protein continuously increased with increasing extraction pH. Therefore, extraction of hemoglobin should be performed at pH 7.0.

The use of salt improved extractability of hemoglobin when the extraction was conducted at pH 6.0 but had little or no effect on hemoglobin extraction at pH 7.0 (Table 2). This use of sodium chloride at 139 mM caused a small increased loss of soluble protein and stimulated hemoglobin autoxidation as observed by fading of sample redness.

3.3. Effect of pH and salt on binding of cod hemoglobin to SR

Fig. 5 shows the effect of salt on the sedimentability of hemoglobin in the presence and absence of sarcoplasmic reticulum. There was a trend, although there was no significant difference, for the hemoglobin to retain its solubility a little less well at pH 6.0 compared to pH 7.0 in the absence of the membrane. This effect of pH was independent of salt concentration. In the presence of the sarcoplasmic reticulum, there was a considerable decrease in the percentage of soluble hemoglobin at pH 6.0, especially in the absence of added sodium chloride. Since the addition of salt retained the hemoglobin in the soluble phase, it is probable that the hemoglobin interacted with the sarcoplasmic reticulum through electrostatic interactions which are sensitive to ionic strength.

Table 2
Per cent extractable hemoglobin and soluble protein of the unwashed cod mince originally at pH 6.0 after extraction at pH 6.0 or 7.0 with or without added salt

Added NaCl (mM)	Extraction at pH 6.0		Extraction at pH 7.0	
	Soluble Hb (%)	Soluble protein (%)	Soluble Hb (%)	Soluble protein (%)
No added salt	56.1 ± 4.3by[a,b]	9.1 ± 0.3by	82.1 ± 4.0ax	12.6 ± 1.9bx
23	78.3 ± 4.9ay	10.8 ± 0.2bx	87.1 ± 3.1ax	12.6 ± 1.9bx
139	86.6 ± 8.5ax	15.0 ± 0.2ax	87.6 ± 7.3ax	16.4 ± 1.2ax

The experiment was conducted in the same manner as described in Table 1 with the exceptions that the minces at pH 6.0 were extracted at pH 6.0 or 7.0 either with or without added salt. NaCl (1 M) was added to the homogenates at both pH values to final concentrations of 23 or 139 mM NaCl. NaOH (1 N) was used for adjusting the homogenate pH. Total protein (175.4 ± 5.0 g protein/kg) and soluble protein were measured by the Lowry method.

[a] Means ± SD from triplicate experiments.

[b] The means followed by different letters in the same row (xyz) or column (abc) are significantly different ($P > 0.05$).

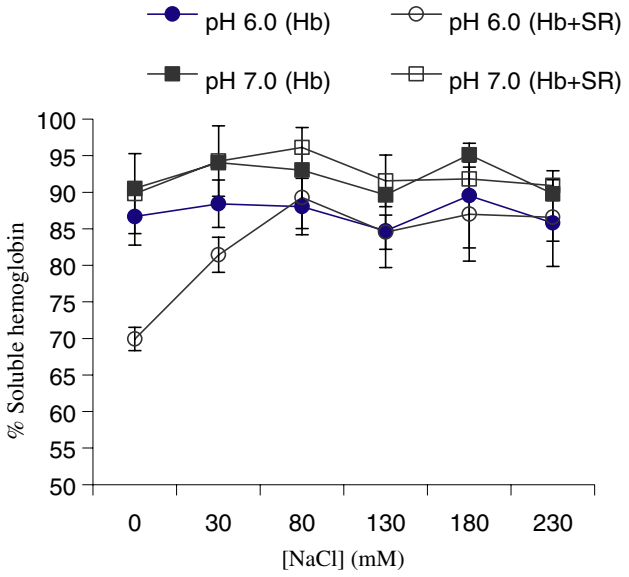

Fig. 5. Effect of pH and sodium chloride on per cent soluble hemoglobin after incubation with and without cod SR. The samples were 0.7 μM cod hemoglobin and NaCl in 10 mM phosphate buffer at pH 6.0 or 7.0 suspended with or without SR (0.14 mg protein/ml). Insoluble material was removed by centrifugation at 186,000*g* for 15 min after the samples were incubated in an ice bath for 30 min. The soluble hemoglobin was quantified based on the method described in Table 1. The per cent soluble hemoglobin was calculated based on soluble heme protein remaining from the total added hemoglobin in the supernatants after the centrifugation.

Since this interaction occurred only at pH 6 and not at pH 7, it suggests that there are changes in the charge either in the hemoglobin or in the membrane which reduce the electrostatic interactions at the higher pH.

3.4. Effect of the cod muscle soluble fraction on extractability and binding to SR of hemoglobin at pH 6.0

Addition of soluble muscle fractions to washed cod mince improved the extractability of hemoglobin added to the mince (Table 3). The binding of soluble hemoglobin to SR was also reduced in the presence of these fractions (Table 4). Both the high and low molecular weight fractions of the press juice decreased interactions in both systems, i.e., washed mince muscle and SR. However, the effective role of the high molecular weight fraction might be due to its ionic strength which was equal to that of the dialysate buffer used in preparation of this fraction. High extractability of hemoglobin at pH 6.0 was observed in unwashed mince compared with washed mince (Table 1). The preventive role of the muscle soluble fraction on the binding of hemoglobin to the minced muscle might be associated with its capability to retard modification of hemoglobin and/or to hinder the binding of hemoglobin to the muscle cell structural components.

3.5. Effect of ADP on extractability of hemoglobin at pH 7.0

The effect of ADP on extractability of cod hemoglobin at pH 7.1–7.2 is shown in Table 5. Although hemoglobin extractability of the samples decreased considerably with time of incubation, the presence of ADP up to 8 mM in the mince before hemoglobin addition had no significant effect on hemoglobin extractability, compared to that of the samples without added ADP. In contrast, there was a significant loss of hemoglobin extractability in samples where both hemoglobin and the washed mince were separately exposed to ADP. ADP stimulated deoxygenation of cod hemoglobin at pH 7.0 (Fig. 3). As a result, the added ADP increased the proportion of deoxyhemoglobin relative to oxyhemoglobin. Loss of hemoglobin extractability of this sample could occur from exposure of hydrophobic areas on the hemoglobin molecule due to the conformational changes brought about by deoxygenation and, perhaps, subsequent dissociation of the hemoglobin subunits. Relative oxygenation of hemoglobin alone with or without ADP was initially around 50% and 70%, respectively (Fig. 3). This corresponds to their initial extractability of around 80% and 95%, respectively, found here (Table 5). No effect of ADP (1 or 8 mM) on extractability pre-added into washed mince was observed, possibly

Table 3
Effect of press juice and its components on the percentage of extractable hemoglobin of washed cod mince at pH 6.0

Sample	% Press juice or press juice components in the sample	Extractable Hb (%)
Unwashed mince	63–71	56.6 ± 2.7a[a,b]
Washed mince	1[c]	14.1 ± 1.0d
Washed mince + Press juice (PJ)	35	46.4 ± 4.1b
Washed mince + HMW fraction of PJ	35	31.2 ± 3.5c
Washed mince + LMW fraction of PJ	35	34.6 ± 2.0c

The samples were of washed cod mince (pH 6.0) of which 35% of their total liquid was added press juice (PJ) or either high molecular (HMW) or low molecular weight (LMW) fractions of the press juice. The moisture content of the unwashed and the washed minces were adjusted to 85% with water. They were added with cod hemoglobin to contain 4.35 μmol heme protein/kg muscle. Hemoglobin was extracted with 10 volumes of deionized water after incubation in an ice bath for 10 min. The per cent extractable hemoglobin was calculated as described in Table 1.

[a] Means ± SD from triplicate experiments.

[b] The means followed by different letters in the same column (abcd) are significantly different ($P > 0.05$).

[c] The estimated amount of endogenous soluble fraction remaining after three washing cycles, each with three volumes of deionized water.

Table 4
Effect of press juice or its high and low molecular weight components on per cent soluble hemoglobin after incubation with SR at pH 6.0 for 30 min

Sample	Soluble Hb (%)
Hb + SR in buffer	51.00 ± 2.65[a]
Hb + SR + Press juice (PJ)	85.77 ± 6.61
Hb + SR + HMW fraction of PJ	83.70 ± 8.94
Hb + SR + LMW fraction of PJ	85.60 ± 6.00

The samples were 0.8 μM cod hemoglobin and SR (0.15 mg protein/ml) in 20 mM phosphate buffer solution at pH 6.0 with or without 35% (v/v) press juice or its components. Insoluble material was removed by centrifugation at 186,000*g* for 15 min after incubation in an ice bath for 30 min. Per cent soluble hemoglobin was calculated by dividing the amount determined after centrifugation by the total amount that was added.

[a] Means ± SD from triplicate experiments.

Table 5
Per cent extractable hemoglobin of washed cod mince with added ADP

Treatment	Incubation time in an ice bath		
	0 h	6 h	12 h
Washed mince (WM) + Hb[a]	94.6 ± 3.6ax[c,d]	83.5 ± 2.9ay	72.6 ± 1.7az
WM + 1 mM ADP-> + Hb	93.5 ± 2.7ax	78.5 ± 3.0ay	68.6 ± 4.7abz
WM + 8 mM ADP-> + Hb	89.5 ± 4.0ax	81.7 ± 2.8ay	69.5 ± 2.4abz
Hb + 1 mM ADP (1 min)-> + (WM + 1 mM ADP)[b]	79.5 ± 3.0bx	68.0 ± 2.5by	63.5 ± 3.3bz

Per cent extractable hemoglobin was quantified as described in Table 1.

[a] Cod hemoglobin (6 μmol/kg, moisture content 89%) was added to washed cod mince at pH 7.1–7.2 with and without ADP (1 or 8 mM).

[b] Washed cod mince at pH 7.1–7.2 incubated with 1 mM ADP for 3 min was mixed with cod hemoglobin (6 μmol/kg, moisture content 89%).

[c] Means ± SD from duplicate experiments.

[d] The means followed by different letters in the same row (xyz) or column (abc) are significantly different ($P > 0.05$).

because ADP was bound to muscle cell proteins and not available to interact with hemoglobin when the latter was added.

There are many possible situations leading to exposure of hemoglobin to high concentration of organic phosphates such as a rough handling of fish on board vessels and the use of pre-rigor or rigor fish for processing. Mincing of just-killed fish, for instance, could increase the concentration of ATP available for hemoglobin from 0.4 to 9.9 mM since the hemoglobin in the plasma would be mixed with the 'nucleotide-rich sarcoplasm (Richard & Hultin, 2000). ADP, like ATP, can induce the Bohr effect in fish hemoglobins, i.e., cause a rapid release of oxygen.

The results in Table 5 show that extractability of hemoglobin at pH 7.1–7.2 decreased with time of exposure. This suggests that the initial reversible electrostatic interaction is followed by an irreversible interaction, perhaps hydrophobic. Electrostatic interactions are affected by medium pH and ionic strength (Tanford, 1968). Both electrostatic and hydrophobic interactions were proposed to be involved in the binding of hemoglobin to phospholipids (Gorbenko, 1998). Hydrophobic reactivity of hemoglobin and myoglobin are likely due to externally accessible hydrophobic regions of the proteins (Tilton, Kuntz, & Petsko, 1984; Yackzan & Wingo, 1982). Dissociation of hemoglobin occurs at acid pH and/or dilution. Monomers of hemoglobin unfold read-

ily (Griffith & Kaltashov, 2003); such unfolding may expose hydrophobic groups on the protein surface. The muscle insoluble matrix or the SR might also induce conformational changes in hemoglobin, resulting in the exposure of hydrophobic portions of the molecule.

4. Conclusion

Exposure of cod hemoglobin to pH 6.0 decreased extractability of the hemoglobin drastically. Associations (probably electrostatic and hydrophobic) between hemoglobin and muscle insoluble components at pH 6.0 mediated the loss of its extractability. Membranes or muscle proteins may be responsible for this binding. The aqueous fraction of cod muscle increased extractable hemoglobin as well as inhibited the binding of hemoglobin to SR at pH 6.0. Loss of oxygen from hemoglobin correlated with low extractability.

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