CHAPTER 4

EFFECT OF AN ALKALINE TREATMENT AND FISH MUSCLE COMPONENTS ON EXTRACTABILITY/SOLUBILITY OF HEMOGLOBIN

4.1. Introduction

The alkaline solubilization process, a novel processing for making surimi, has been recently developed at the University of Massachusetts Marine Station (Hultin and Kelleher, 1999,2001, 2002). With this process, fish homogenate was brought to pH of 10.8 in order to solubilize muscle protein. Insoluble components at this pH are separated by centrifugation. The supernatant is then readjusted its pH to isoelectric point of muscle protein for precipitation. Many advantages of producing surimi with this novel process comparing with the conventional process include higher protein recovery and better gelling property of the protein isolate (Hultin and Kelleher, 2002). This process has been paid increasing intention to produce the surimi from low-value fish especially fatty and dark-flesh fish (Hultin and Kelleher, 2000). With high content of heme proteins and their poor extractability, these proteins exhibit the detrimental effects on quality of post mortem fish and its products (Richards and Hultin, 2000; Chen et al., 1996; Hultin and Kelleher, 2000). Due to their high water solubility, low extractability of these soluble proteins would imply their molecular modification and/or the existence of an interaction with the insoluble components of fish muscle (Hultin et al., 1995). These changes possibly occur between capture and processing. Recently, Kristinsson and Hultin (2004) reported that no conformational changes in trout hemoglobin was observed after an alkaline treatment.

Therefore, the alkaline solubilization process is considered to be an appropriate technology for recovery high functional proteins of fatty and dark-flesh fish with minimal disturbance on the heme proteins. Nonetheless, the postmortem decrease in pH of fish muscle was associated with the lowered extractability of the heme proteins (Thongraung et al., 2005). Additionally, the alkaline pH of washing media was shown to increase hemoglobin extractability (Jiang et al., 1998; Chen et al., 1996; Thongraung et al., 2005). Fish muscle components affected the extractability of heme proteins differently. Sarcoplasmic reticulum caused the decrease in extractability, while the soluble components impeded the binding of hemoglobin to the muscle protein (Thongraung et al., 2005). These phenomena might be influenced by alkaline solubilization process. Thus, the objective of the present study was to investigate the effect of alkaline solubilization process and some fish muscle components on the extractability of cod hemoglobin from the muscle.

4.2. Materials

4.2.1 Materials

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

4.2.2 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, New Jersey, USA). Other chemicals of analytical grade were procured from Sigma Chemical (St. Louis, MO, USA)

4.3 Methods

4.3.1 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 units/ml) (Richards and 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. Whole herring in rigor-state was used to withdraw its blood from caudal vein of the fish.

Hemolysate was prepared immediately after the blood was drawn according to the method of Fyhn et al. (1979) as modified by Richards 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 700g 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 700g. 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 28,000g 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.

4.3.2 Determination of soluble/extractable heme protein concentration

Heme protein content was measured according to the method of Brown (1961) as modified by Richards 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 nm 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. Instead of absolute amount of soluble hemoglobin, a relative content of the protein was used in some experiments that was performed by comparison absorbance at 412 nm of the samples/supernatants having pH 7.0-10.8.

4.3.3 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 deionized 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 10,000g for 15 min at 10°C (Sorval RC-5B, refrigerated superspeed centrifuge, Dupont Instrument, Wilmington, Delaware, USA). The washed mince was kept on ice or in the refrigerator and used within two days.

4.3.4 Isolation of sarcoplasmic reticulum (SR)

Cod and herring sarcoplasmic reticulum were isolated from light muscle of the fishes as described by Borhan et al. (1984). The minces were 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 5 for 40 sec. The homogenates were adjusted to pH 7.4 with 1 M Tris-HCl buffer (pH 8.4) and centrifuged at 1,350g for 10 min using a Beckman L5-6513 centrifuge at 5°C, followed by the centrifugation at 15,000g for 20 min. The supernatants were 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 130,000g 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 130,000g 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 3 weeks.

4.3.5 Myosin preparation

Cod myosin was prepared from fresh cod light muscle. A modified method of Martone et al. (1986) as described by Park and Lanier (1989) was employed. Myosin sample was stored in 50% glycerol at -80°C and used within 2 months.

4.3.6 Effect of pH on solubility and extractability of cod hemoglobin.

Cod hemoglobin was diluted with 20 mM $NaHCO_3$ (pH 7.0) to obtain 1 μM heme protein. Cod mince (pH ~7.2) containing ~10 μmole heme protein/kg was then prepared. To prepare the homogenate, the mince was homogenized by Polytron homogenizer at speed No. 5 for 40 sec (PT 10-35, Kinematica AG, Luzern, Switzerland) with 9 volumes of deionized water. The heme protein solution and the homogenate were subjected to various pH treatments either by adjusting directly to pH 6.0 or 5.5 (7->6.0 or 5.5) or by first adjusting pH to 10.8, followed by centrifugation at 104,630xg for 15 min and then readjusting their supernatant to pH 7.0, 6.0 or 5.5 (7->10.8->7, 6 or 5.5). All pH adjustment was done using either 1 N NaOH or 1 N HCl. At the final pH values, the samples were re-centrifuged under the same condition. Solubility of heme protein of the hemolysate and its extractability from homogenate samples were quantified from soluble heme protein in the supernatants.

For another experiment, cod homogenate was prepared from cod mince without added cod hemoglobin. After pH adjustment to 10.8 with 1 N NaOH, the predetermined volume of cod hemoglobin was added into either the homogenate before the centrifugation or the supernatant obtained after the centrifugation. The hemoglobin-added homogenate was then centrifuged under the same condition. The supernatants were subsequently adjusted to pH 7.0, 6.0 or 5.5 with 1 N HCl and re-centrifuged under the identical condition. Concentration of heme protein remained in the supernatants was then quantified.

4.3.7 Effect of pH on binding of hemoglobin to SR

The cod hemoglobin (7.4×10^{-2} μM heme protein) in 20 mM $NaHCO_3$ (pH 7, 8, 9, 10, and 10.8) suspended with or without SR (0.14 mg protein/ml) was incubated in an ice bath for 2 or 30 min. The samples were then centrifuged at 186,000xg for 15 min at 10°C.

The SR suspension (0.14 mg protein/ml), and the cod hemoglobin solution ($7.4x10^{-2}$ μM in 20 mM $NaHCO_3$) were incubated at pH 10.8 for 30 min. These samples were subsequently readjusted to pH 7.0 and added with an untreated SR or an untreated cod hemoglobin into the hemoglobin solution and the SR suspension, respectively. The samples were then centrifuged as previously described.

To study the effect of SR or hemoglobin source on their binding, 0.8 μM cod or herring hemoglobin in 20 mM $NaHCO_3$ buffer (pH 10.8) was added with SR (0.14 mg protein/ml) from either cod or herring. The mixtures were incubated in an ice bath for 30 min, then adjusted to either pH 7.0, 6.0, or 5.5 and subsequently centrifuged at 186,000xg for 15 min.

The supernatants after various treatments were collected and used for assessment of the soluble heme protein concentration. The controls were the heme protein solutions without the added SR but receiving identical treatment. Decrease in the relative soluble heme protein concentration due to the existence of SR was regarded as heme protein bound to SR.

4.3.8 pH measurement

The pH values of 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, New Jersey, USA) equipped with a thermocouple to compensate for temperature**.**

4.3.9 Protein analysis

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

4.3.10 Statistical analyses

The experiments were conducted at least twice. Data were subjected to Analysis of Variance (ANOVA) and mean comparisons were performed using Duncan New Multiple range test. Statistical analyses were carried out using the SAS statistical software (SAS, 1996).

4.4 Results and discussion

4.4.1 Effect of pH on solubility and extractability of cod hemoglobin.

Effect of pH treatments on solubility of hemoglobin in 20 mM sodium bicarbonate buffer and on the extractability of hemoglobin in cod homogenate is shown in Table 4-1. The increased loss of soluble or extractable hemoglobin was observed with a decrease in the final pH from 7.0 to 6.0 or 5.5, regardless of how these final pH values were reached. Increased loss of soluble/extractable hemoglobin when pH was adjusted from the neutral value to acidic value was in accordance with the results of the previous chapter. However, the samples whose pH were initially adjusted to pH 10.8 showed a greater loss of soluble and extractable hemoglobin than those of the samples whose pH were brought directly to those identical final pH values. This result suggested that the exposure of hemoglobin to high pH enhanced its precipitation at final pHs.

However, the similar experiment done by addition of hemoglobin into the homogenate either before or after the centrifugation at pH 10.8 revealed that the loss of the soluble hemoglobin could occur at pH 10.8 (Appendix: Table 9-2). It was estimated that the centrifugation removed about 20% of total added hemoglobin. It is the most optimism to precipitate heme protein at this stage since it decreased the heme protein residue in the protein isolate. In contrast, the insoluble hemoglobin occurred at the final pH would remain in the protein isolate. Loss of hemoglobin solubility at pH 10.8 suggested that interaction of hemoglobin with muscle components could take place at pH 10.8.

Non-significant change on visible absorption spectrum of the hemoglobin solution at pH 10.8 was found within time scale of this experiment. Nonetheless, the significant and gradual reduction of the Soret peak of cod hemoglobin was noted after incubation for 2 h during incubation at pH 10.8 in refrigerator (data not shown). The disappearance of the Soret band is known to occur if the conjugation of the porphyrin ring is interrupted for some reason (Falk, 1964).

The percent extractable hemoglobin of the homogenate samples was relatively low in comparison with that of the buffer samples receiving the same treatments (Table 4-1). It

suggests the possible interaction between hemoglobin and fish insoluble muscle components.

Table 4-1 Relative soluble hemoglobin in buffer system and relative extractable hemoglobin in cod homogenate after various pH treatments.

pH treatment	Relative soluble/extractable hemoglobin (%)	
	Hemoglobin solution	Cod homogenate
7.0	100.0	100.0
7.0-> 6.0	81.7 ± 4.1a	58.5 ± 1.7a
7.0-> 5.5	65.7 ± 2.1b	48.4 ± 0.1c
7.0->10.8->7.0	76.6 ± 5.5a	52.8 ± 3.5b
7.0->10.8->6.0	61.3 ± 5.6b	41.0 ± 5.1d
7.0->10.8->5.5	54.3 ± 4.5c	36.5 ± 2.8d

The solution was 1 μM hemoglobin in 20 mM $NaHCO_3$, pH 7.0. The cod homogenate (pH 7.0-7.15) was prepared by homogenizing the cod mince containing 10 μmole heme protein/kg of mince, with 9 volumes of deionized water. Both samples received various pH treatments either by adjusting directly to the acid values (6.0 or 5.5) or by first adjusting to 10.8, followed by centrifugation at 104,630xg for 15 min and then readjusting their supernatant pH to 7.0, 6.0 or 5.5. The samples were again centrifuged at these pH values. The relative soluble or extractable hemoglobin in the supernatants was quantified by measuring their absorbance value at 412 nm (A_{412}) at pH 7.0. The A_{412} of the buffer sample (0.312±0.011) at pH 7.0 and A_{412} of the cod homogenate sample (0.411±0.022) without receiving pH treatment and after centrifugation were 100% soluble hemoglobin and extractable hemoglobin, respectively.

[a]Mean ± SD from triplicate experiments.

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

4.4.2 Effect of pH on binding of hemoglobin to sarcoplasmic reticulum (SR).

Incubation of cod hemoglobin solutions at pH range of 7.0 to 10.8 for 30 min showed no significant effect on the protein solubility (p>0.05). However, in the presence of SR, the amount of insoluble hemoglobin increased progressively as pH of the solutions increased from 7.0 to 10.8 (Figure 4-1). This result together with a noticeable reddish-brown of the SR pellet was evident that binding of the hemoglobin to SR was primary reason for the loss of the hemoglobin solubility. Insoluble hemoglobin increased significantly when the incubation time increased from 2 to 30 min only when pHs of the solution were 10.0 and 10.8. This suggested that the exposure of hemoglobin to these alkaline pHs with a sufficient time was crucial for the binding.

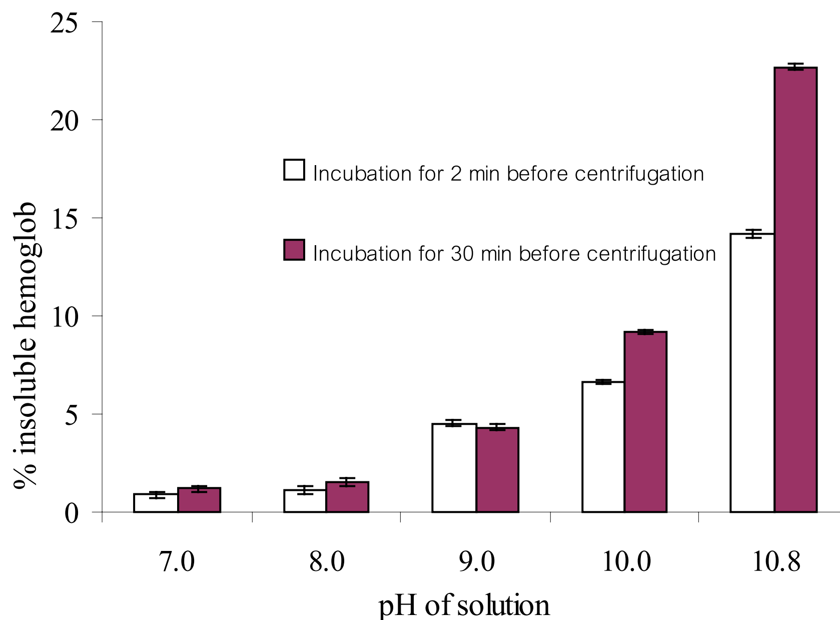

Figure 4-1 Percent insoluble hemoglobin after incubation with SR at various pH values.

The solutions of $7.4x10^{-2}$ μM hemoglobin in 20 mM $NaHCO_3$ (pH 7-10.8) were suspended with SR (0.14 mg protein/ml). The controls were the hemoglobin solutions without SR at identical pH. The solutions were incubated in an ice bath for 2 or 30 min before centrifugation at 186,000xg for 15 min. The percent soluble hemoglobin in supernatant was quantified and hemoglobin bound to SR was calculated based on the difference between the soluble hemoglobin remaining in the supernatants of the solutions with and without SR.

When either hemoglobin or SR was incubated at pH 10.8 separately, readjusted to pH 7.0 and added to the untreated components; either SR or hemoglobin at pH 7.0, different amount of soluble hemoglobin was observed (Table 4-2). The result revealed that alkalinizing the hemoglobin and readjusting pH to 7.0 made it more potent to bind to the untreated SR, compared to the untreated hemoglobin. Relative percent soluble hemoglobin of the treated hemoglobin and the untreated SR mixture at pH 7.0 was comparable with that of the suspension of both components that had undergone an alkaline treatment together. The result suggested that the change in cod hemoglobin molecule might be responsible for its binding to membrane. On the other hand, SR subjected to an alkaline treatment did not cause the loss of the soluble-untreated hemoglobin at pH 7.0.

Table 4-2 The relative percent soluble hemoglobin after various treatments

Treatment	Soluble hemoglobin (%)[a]
Hb (7.0, 30min)[b] ->C	97.5 ± 0.8a[d,e]
Hb+SR (7.0, 30min)[b] ->C	97.1 + 0.6a
Hb ->10.8 (30min)[b]->7->C	93.1 + 2.3b
Hb+SR ->10.8 (30 min)[b]->7->C	79.2 + 6.7c
SR-> 10.8 (30min)[c]->7+Hb->C	97.5 + 0.5a
Hb-> 10.8 (30min)[c]->7+SR->C	79.7 + 0.9c

[a]The relative soluble hemoglobin was quantified based on that found in the untreated hemoglobin. A_{412} of hemoglobin solution (0.246 ± 0.006) at pH 7.0 before the treatments was 100% soluble hemoglobin. Insoluble components of all samples were removed at pH 7.0 by centrifugation (C) at 186,000xg for 15 min.
[b]The samples of hemoglobin solutions (7.4×10^{-2} μM in 20 mM $NaHCO_3$) at pH 7.0 or 10.8 were incubated in an ice bath for 30 min with or without SR (0.14 mg protein/ml).
[c]Only hemoglobin or SR was incubated at pH 10.8 for 30 min, subsequently readjusted to pH 7.0 and then added with untreated SR or hemoglobin, respectively.
[d]Mean + SD from triplicate experiments.
[e]The means followed by different letters in the same column (abc) are significantly different ($P<0.05$).

pH is known to influence the stability of a protein by altering its net charge, and many proteins undergo denaturation at extreme pH because of the destabilizing repulsive interactions between the same charges. Alternatively, if a titratable group is buried as an un-ionized form in the interior of the protein and can be titrated only upon unfolding, the unfolded state will be favored upon titration of the group by changing the pH. Most amino acids have their pK_a for amino group between pH 9 to 10. However, there are, at least, 4 amino residues whose their amino group or side chain have pK equaled or closed to 10.8. Those include proline (10.6), cysteine (10.8), side chain-amino group of lysine (10.8), and tyrosine (10.1) (Tanford, 1968; Tanford, 1962). Ionization of these groups might associate with the aggregation of hemoglobin with SR. The ionization of proline is particularly interesting since it lies at the NH_2-terminal end of the several α-helices of both myoglobin and hemoglobin (Dickerson and Geis, 1983). It contributes to the peptide backbone rigidity by preventing the rotation around its α-carbon. The regions between the helices may act as hinges between the helices and affect the conformational flexibility of proteins (Perutz, 1990). Ionization of proline at high pH may provide a greater degree of freedom in the α-carbon-nitrogen bond and could therefore allow greater movement of the helices.

However, extreme pH do not always completely denature a protein but may lead to only partly folded states, commonly referred to as molten globules (Kristinsson and Hultin, 2003). The observation that high oxygen affinity of hemoglobin remained at pH 10.8 (data not shown) suggested that the protein is not fully unfolded. Existence of molten globule of hemoglobin is likely based on two facts. First, significant association of hemoglobin with SR occurred only at pH 10.8 at which the protein has mostly negative charge. The electrostatic aggregation of hemoglobin either with protein or lipid head groups of SR was unlikely due to the repulsive force of same charge (Szebeni et al., 1988). Hydrophobic interaction among new reactive binding sites of unfolded groups might instead mediate the aggregation. Second, a modification of hemoglobin at pH 10.8 favoring the binding seems to persist even though the medium was adjusted to neutral pH (Table 4-2). It seemed to be likely that refolding of partially unfolded hemoglobin might not be favored upon reduction of medium pH. This connects to the similar co-precipitation magnitude at pH 7.0 between the sample containing the alkaline-treated hemoglobin and untreated SR and the sample whose components co-existed at pH 10.8 (Table 4-2). Therefore binding of hemoglobin with membrane observed at the final pH of 7.0 (Table 4-1) possibly commenced when pH of the homogenate was 10.8 and was subsequently immense upon the reduction to pH lower than 7.0.

Effects of the final pH values and the presence of hemoglobin and SR of different fish species on hemoglobin solubility after alkaline solubilization were also investigated. As shown in Table 4-3, the percent soluble hemoglobin of both species decreased as the final pH decreased from 7.0 to 6.0 or 5.5. Without SR, cod hemoglobin was less soluble at pH 7.0 and 6.0 than herring hemoglobin. At pH 5.0, there was non-significant difference in solubility between these hemoglobins. The existence of SR from any species promoted further decrease in soluble hemoglobins and a higher decrease was observed with cod hemoglobin. From the result, the mixture of cod hemoglobin and herring SR had the lowest soluble hemoglobin. This finding implied that alteration of cod hemoglobin or herring SR induced by alkaline pH, if occurred, might flavor the binding.

Distribution profile of soluble herring and cod hemoglobin in the presence of SR after the alkaline treatment was different. A drastic decrease in soluble hemoglobin was found when the precipitation pHs were changed from 7.0 to 6.0 and from 6.0 to 5.5 for cod

and herring hemoglobin, respectively. It highlights the appropriate final pH to reduce co-precipitation of hemoglobin with protein isolate by the isoelectric precipitation.

Table 4-3 Effect of the final pH after the alkaline treatment on percent soluble hemoglobin of cod and herring with added SR either from cod or herring.

Hemoglobin	Sarcoplasmic reticulum	Soluble hemoglobin (%)		
		pH 7.0	pH 6.0	pH 5.5
Cod	Without SR	84.5 $\pm$ 3.0ax[a,b]	58.3 $\pm$ 8.3by	52.3 $\pm$ 1.5ay
	Cod	67.8 $\pm$ 2.3cx	35.3 $\pm$ 1.3cy	33.5 $\pm$ 3.0cy
	Herring	53.3 $\pm$ 1.1dx	23.7 $\pm$ 1.2dyy	20.9 $\pm$ 2.4dy
Herring	Without SR	91.0 $\pm$ 4.0ax	78.9 $\pm$ 3.1ay	55.2 $\pm$ 6.7az
	Cod	82.6 $\pm$ 4.8bx	63.0 $\pm$ 0.9by	42.1 $\pm$ 2.7bz
	Herring	82.9 $\pm$ 4.8bx	63.0 $\pm$ 2.4by	25.6 $\pm$ 4.5dz

The samples were 0.8 μM of the cod or herring hemoglobin in 20 mM $NaHCO_3$ buffer (pH 10.8) with added sarcoplasmic reticulum (0.14 mg protein/ml) from either cod or herring. The mixtures were incubated in an ice bath for 30 min then adjusted to pH 7.0, 6.0 or 5.5 and subsequently centrifuged at 186,000xg for 15 min. The soluble hemoglobin of the supernatants was quantified by measuring their absorbance values at 412 nm (A_{412}). The controls were the hemoglobin solutions without the added membrane but receiving identical treatment. The A_{412} of the cod and herring control samples before the treatment at pH 7.0 were 0.296 $\pm$ 0.003 and 0.268 $\pm$ 0.007, respectively. The relative percent soluble hemoglobin remaining after the treatments was measured and expressed relative to that of the corresponding controls.

[a]Mean $\pm$ SD from duplicate experiments.

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

4.4.3 Effect of myosin on solubility of hemoglobin.

Table 4-4 shows the percent soluble myosin and percent soluble hemoglobin with and without added myosin at various final pHs after the alkaline treatment. The lowest soluble myosin and hemoglobin were found at pH 5.5. Electrostatic aggregation between hemoglobin and myosin might contribute to this observation. pH 6.0 is the value that falls between pI of hemoglobin (somewhere between pH 7.0 and 6.0) (van Eijndhoven et al., 1995) and pI of myosin (5.5) (Ojima et al., 1997). In this pH range, hemoglobin having net positive charge should interact with myosin having net negative charge. It was also possible that the smaller molecule of hemoglobin might be co-precipitated with macromolecule like myosin. This was supported by an observation that drastic loss of myosin was occurred when pH decreased from 7.0 to 6.0. Similar result was found in the hemoglobin with added myosin. However, this result was contradicting to the assumption

that existence of myosin would render the precipitation of hemoglobin. Partial unfolding of myosin resulting in an increase of the available binding site is expected to occur at alkaline environment such as at pH 10.8 (Kristinsson and Hultin, 2003). This change could allow the partially unfold hemoglobin to readily bind to myosin, resulting in the co-precipitation of hemoglobin with myosin. In this experiment, the viscous myosin stock suspension was suspended into the buffer to get the myosin solution at pH 10.8 before hemoglobin addition. Thus, it is possible that partial unfolding and self-aggregation of myosin might occur upon dissolving into the buffer. This might result in no available binding site for the hemoglobin.

Table 4-4 Effect of pH on percent soluble myosin and percent soluble hemoglobin in presence and absence of myosin after alkaline treatment.

pH	Soluble myosin (%)	Soluble hemoglobin (%)	
		Without myosin	With myosin
7.0	29.0 ± 1.8a	85.3 ± 4.2ax[a,b]	79.6 ± 7.1ax
6.0	8.2 ± 0.8b	71.5 ± 5.3bx	61.9 ± 6.7by
5.5	4.7 ± 0.9c	61.2 ± 7.8cx	60.4 ± 7.0bx

The samples were the hemoglobin solutions (1.05 μM in 20 mM $NaHCO_3$) with or without 0.55 mg/ml myosin and the myosin solution (0.55 mg/ml). The samples were incubated in an ice bath at pH 10.8 for 30 min and adjusted to pH 7.0, 6.0 or 5.5 with 1 N HCl. The insoluble component was removed by centrifugation (Sorval RT8000 refrigerated centrifuge) at 2,250xg for 15 min. The total soluble protein of the myosin solution at pH 10.8 and after precipitation and centrifugation was quantified by the Lowry method. The relative soluble hemoglobin in the samples after the treatments was based on A_{412} of their supernatant obtained after readjusting their pH to 7.0. The A_{412} (0.285 ± 0.015) of the untreated hemoglobin at pH 7.0 was taken as 100% relative soluble hemoglobin.

[a]Mean ± SD from duplicate experiments.

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

4.5. Conclusion

pH treatment of the alkaline process decreased hemoglobin extractability. Exposure of hemoglobin at pH 10.8 before readjustment to pH 7.0, 6.0 or 5.5 enhanced the decrease in hemoglobin extractability compared with those of the samples whose pHs were brought directly to those identical final pH values. The results suggested that the hemoglobin could

be co-precipitated with the muscle components both at pH 10.8 and at final pH. Binding between hemoglobin and SR also occurred at alkaline pH. Alkalinizing hemoglobin and readjusting pH to 7.0 made it more potent to bind to SR. The presence of myosin increased precipitation of hemoglobin at pH 6.0 possibly by coprecipitation mediated by an electrostatic interaction.