

## CHAPTER 5

### REMOVAL OF HEME PROTEINS AND COLOR OF HERRING WASHED MINCE/PROTEIN ISOLATE BY WASHING OR THE ALKALINE SOLUBILIZATION PROCESS

#### *5.1 Introduction*

Fatty and dark-flesh fish species currently make up 40-50% of the total fish caught in the world and most of them are still considered as underutilized fish (Gunning, 1997; Okada, 1980). Main characteristics of those fish, which limit their use, are high lipid content, less stable proteins, high concentration of heme proteins, low ultimate pH, and high proteolytic activity (Whittle et al., 1990). Removal of water soluble substances by appropriate washing is prerequisite for surimi production from those species (Okada, 1980).

Color is an important parameter determining the quality of surimi. Hemoglobin and myoglobin are primary pigments of the dark muscle of fish (Bone, 1978). These proteins are highly water-soluble. However, loss in extractability of the heme proteins in post mortem fish was reported (Chen et al., 1996; Chow, 1991). Several washing conditions including washing time, number of washing cycles, and water quality, have been modified as well as new washing strategies have been developed in order to remove more muscle pigments (Barrero and Bello, 2000; Chen and Lao, 1997; Jiang et al., 1998; Lin and Park, 1996). Nevertheless, little success has been reported (Nishioka et al., 1990). Recently, a new approach for obtaining functional protein from dark-flesh fish has been developed at the University of Massachusetts Marine Station (Hultin and Kelleher, 1999, 2001, 2002).

The process minimizes the drawback of surimi production from the pelagic fish, compared with the standard process. The color of the protein prepared by the new process was much better than the color obtained by the standard procedure as indicated by the lowering of “b” value (Kelleher et al., 1994).

The objective of the present study was to investigate the effect of washing and the alkaline solubilization process on the extractability of herring proteins and color values of the washed mince/protein isolate.

## **5.2. Materials**

### **5.2.1 Materials**

Atlantic herring (*Clupea harengus*) was supplied by the Cape Seafoods Inc (Gloucester, MA). Carbon monoxide gas was supplied by Matheson Gas (Gloucester, MA).

### **5.2.2 Reagents**

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)

## **5.3 Methods**

### **5.3.1 Determination of extractable heme protein concentration**

Heme protein content was measured according to the method of Brown, (1961) as modified by Richards and Hultin, (2000). A muscle extract 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.

### **5.3.2 Effect of iced storage on extractability of herring heme proteins**

Fresh herring was headed and eviscerated before storage on ice with a fish/ice ratio of 1:2 in the insulated box which was kept in chilled room (10-13°C) for 7 days. Three fishes were randomly taken at day 0, 1, 3, 5, and 7 for extractable heme proteins assessment. Their light and whole muscles were removed manually. Heme proteins were extracted from both muscles by homogenizing the muscles with 10 volumes of water with a Polytron homogenizer at speed No. 5 for 40 sec (PT 10-35, Kinematica AG, Luzern, Switzerland). The homogenates were then centrifuged at 46,500xg at 4°C for 15 min (Beckman Ultracentrifuge model L5-65B, Beckman Instruments Inc., Palo Alto, CA). The extractable heme protein in the supernatant was quantified.

### **5.3.3 Effect of pH and salt at extraction on extractability**

Herring light muscle was manually separated from fresh herring. Heme proteins were extracted from light muscle of fresh herring by homogenizing the muscles with 10 volumes of water with a Polytron homogenizer at speed No. 5 for 40 sec (PT 10-35, Kinematica AG, Luzern, Switzerland). NaCl (0, 20, 150 mM) was added to the homogenate and the samples were adjusted to pH 7.0 or 8.0 with 1 N NaOH. The mixtures were then centrifuged at 46,500xg at 4°C for 15 min (Beckman Ultracentrifuge model L5-65B, Beckman Instruments Inc., Palo Alto, CA). The extractable heme protein in the supernatant was quantified.

### **5.3.4 Effect of conventional washing process and alkaline solubilization process on heme proteins removal**

Conventional washing process was performed to extract heme proteins by three washing cycles. The whole muscle of herring was minced in a Kitchen Aid (Kitchen Aid Inc., St. Joseph, MI) mincer (diameter 4.7 mm). The mince was washed for 10 min with 3 volumes of water. A gentle stirring of 10 sec was conducted for each 3 min standing. Water was drained off. Washing was repeated for another two consecutive washes. NaCl solution (0.2%) was used for the last wash. Drained water of each wash was collected and

centrifuged at 10,000xg at 10°C for 30 min (Sorval RT8000 refrigerated centrifuge) and its supernatant was used for analysis.

For the alkaline solubilization process, the herring mince was homogenized with 9 volumes of water. The homogenate was adjusted to pH 10.8 with 1 N NaOH and centrifuged at 10,000xg at 10°C for 30 min (Sorval RT8000 refrigerated centrifuge). The obtained supernatant was then readjusted to pH 6.0 with 1 N HCl and centrifuged under the previous condition.

For control sample, the mince was homogenized with 9 volumes of chilled-deionized water with a Polytron homogenizer at speed No. 5 for 40 sec (PT 10-35, Kinematica AG, Luzern, Switzerland). pH of the homogenate was instantly checked and corrected to 7 with 1N NaOH. Insoluble fish component was then collected by centrifugation at 10,000xg at 10°C for 30 min (Sorval RT8000 refrigerated centrifuge).

The supernatants of all samples were determined for extractable heme proteins and total extractable protein. The washed mince or protein isolate was used for color measurement.

### **5.3.5 Effect of pre-washing on amount of heme proteins removal by the alkaline solubilization process and color of protein isolate.**

Herring mince was separated into 2 groups: mince and washed mince. To prepare washed mince, the mince was washed for 1 min with one volume of cold-deionized water. Water was drained off on screen for 1 min. The washing process was repeated twice. The drained water was collected and centrifuged at 10,000xg at 10°C for 30 min (Sorval RT8000 refrigerated centrifuge). The washed mince and mince were subjected to the alkaline solubilization process as described in the previous section. The protein isolate obtained at pH 6.0 were readjusted to pH 7.0 with powder of NaHCO<sub>3</sub>. Extractable protein content in the supernatants was measured. Color measurement of cooked and raw samples was carried out.

### **5.3.6 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, New Jersey, USA) equipped with a thermocouple to compensate for temperature.

### **5.3.7 Protein analysis**

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

### **5.3.8 Color measurement**

The samples were chopped with 3% salt for 5 min and heated in a water bath at 95°C for 20 min. Color measurement of either cooked or uncooked samples was performed after the sample equilibration at room temperature. A CIE Lab scale was used to measure color values of raw and cooked samples by using a Hunter LabScan II colorimeter (Hunter Associates Laboratories, Reston, VA). Raw and cooked samples were filled into a disposable polystyrene culture dish (60\*15mm, Fisherbrand) before the color measurement. The port size was about 6 mm. The instrument was standardized using a black tile and white tile with the following values: x, 78.62; y, 83.49; and z, 87.78. Whiteness value was calculated using the following equation. (Tseo et al., 1983)

$$\text{Whiteness} = 100 - [(100-L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

### **5.3.9 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).

## ***5.4 Results and discussion***

### **5.4.1 Effect of iced storage on extractability of herring heme proteins**

Initial amount of extractable heme proteins of herring whole muscle was about two times higher than that of light muscle (Figure 5-1), owing to the incorporation of dark muscle in whole muscle sample. Dark color of the washed mince suggested that certain amount of herring heme proteins was initially non-extractable. Insoluble pigments and cell

structures such as mitochondria which contain cytochromes are responsible for low whiteness of washed mince/surimi (Park, 1995; Chen et al., 1997).

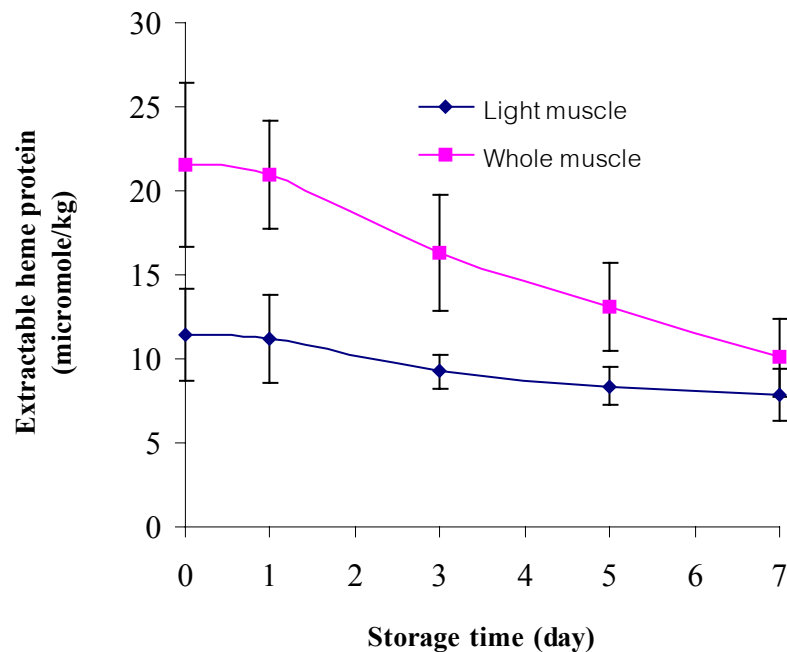


Figure 5-1 Extractable heme protein content of herring light and whole muscle during iced storage.

Fresh herring was headed and its viscera was removed before storage on ice for 7 days. Heme protein was extracted from light and whole muscle of herring by homogenizing the muscles with 10 volumes of water and centrifuging at 46,500xg at 4°C for 15 min.

The extractable heme proteins in both muscles decreased gradually over an ice-storage with a higher degree on that of whole muscle ( $p \leq 0.05$ ) (Figure 5-1). Loss of the extractable herring heme proteins was also found during frozen storage (Appendix: Table 9-4). The results of Figure 5-1 suggest a greater loss of extractable heme protein in dark muscle, compared with whole muscle. Drastic loss of extractable heme pigments in dark muscle of pelagic fish during postmortem storage was well documented (Chen et al., 1996; Chen et al., 1997; Chen and Lao, 1997). Partial removal of the dark muscle before surimi processing was therefore proposed to improve surimi whiteness (Sonu, 1986). Both

hemoglobin and myoglobin are more concentrated in the dark muscle than in the light to meet energy need of oxidative metabolism (Mathieu-Costello, 1993). The limited loss of extractable heme proteins in the light muscle suggested the restricted alteration of hemoglobin, the main heme proteins in the muscle, which is normally compartmentalized in erythrocytes confined in an intact capillary system. This fact puts forward the assumption that the radical loss in the heme proteins of whole muscle or, precisely, dark muscle was possibly due to the changes of myoglobin. It is important to note that pH of herring was around 7.0, a typical physiological pH value of fresh herring normally used. It is notably different from a lower value reported for other pelagic fishes (Pacheco-Aguilar et al., 2000; Bennour et al., 1991). This suggested that changes of heme proteins resulting in loss of their extractability also occurred without the exposure to low pH of post mortem fish.

There are several possible changes of heme proteins, especially deoxygenation and autoxidation, during an iced storage and extraction that might have the significant contribution to the loss of extractability of heme proteins. It was found that the absorbance spectrum of the muscle extracts changed from the spectrum of oxy-heme protein to resemble that of deoxy-heme proteins upon increasing storage (data not shown). The stimulation of cod hemoglobin deoxygenation at pH 7.0 by addition of ADP increased amount of non-extractable hemoglobin (Thongraung et al., 2005). Normally, autoxidation of heme proteins is inevitably in nature (Everse and Hsia, 1997; DeYoung et al., 1994) and the change occurred in tuna myoglobin was coincidental with the decrease in its solubility (Chow et al., 1987).

#### **5.4.2 Effect of pH and salt at extraction on extractability of herring heme proteins.**

Effect of pH and salt on amount of extractable heme proteins of herring whole muscle is shown in Table 5-1. Extraction at both pH values (7.0 and 8.0) showed no different effect on extractability of herring heme proteins regardless of amount of added salt. Although, alkaline washing was generally recommended for increasing removal of heme pigments of pelagic surimi (Chen et al., 1996; Chen and Lao, 1997). The solution of alkaline phosphate or bicarbonate buffers at various concentrations successfully enhanced

pigment removal from red meats and fish mince (Barrero and Bello, 2000; Hernandez et al., 1986; Dawson et al., 1988; Chen et al., 1996; Roussel and Cheftel, 1988). However, alkaline washing showed no effect on the color improvement of pelagic surimi (Jiang et al., 1998). Addition of salt had no beneficial effect on extractability of heme proteins. Moreover at high salt concentration, lower extractability was noticeable.

All conditions used had no effect on extraction of heme proteins, suggesting that the conditions favored extractability of proteins could not apply to increase extractable heme proteins of the fish. The rupture of an electrostatic interaction either by increasing of pH or ionic strength of medium used was not associated with the release of bound heme proteins. Together with the observation that decrease of extractable heme proteins in ice-stored herring without a noticeable change in pH reported in the previous section, a limited role of pH on loss of heme protein extractability was likely. Precise mechanism responsible for the loss of heme proteins extractability needs to be further investigated. Although, myoglobin has been postulated to possess specific electrostatic and hydrophobic binding sites for fatty acids (Yackzan and Wingo, 1982), partial unfolding of the tertiary structure of globular protein is a prerequisite (Hanssense et al., 1985).

Table 5-1 Extractable heme protein content of herring whole muscle at pH 7.0 and 8.0 with and without NaCl.

pH at extraction	NaCl content at extraction (mM)	Extractable heme protein ( $\mu\text{mole/kg}$ )
7.0	0	$19.5 \pm 1.0$ <sup>a,b</sup>
	20	$19.5 \pm 0.7$ a
	150	$16.3 \pm 1.2$ b
8.0	0	$20.2 \pm 1.1$ a
	20	$19.7 \pm 0.6$ a
	150	$16.6 \pm 1.3$ b

Heme protein was extracted from whole muscle of herring by homogenization the muscles with 10 volumes of water. NaCl was added to the homogenate and adjusted to pH 7.0 or 8.0 with 1 NaOH. They were then centrifuged at 46,500xg at 4°C for 15 min. The extractable heme protein in the supernatant was quantified.

<sup>a</sup>Mean  $\pm$  SD of four experiments.

<sup>b</sup>The means followed by different letters are significantly different ( $P \leq 0.05$ ).



### **5.4.3 Effect of the conventional washing process and the alkaline solubilization process on removal of herring heme proteins**

Homogenisation of the mince with water provided the highest amount of extractable heme proteins (Figure 5-2). Obviously, leaching was effective with very fine fish muscle particle. Similar washing condition was also existed in the alkaline solubilization since homogenisation is the prerequisite step of the alkaline solubilization process. The process showed the lower removal of extractable heme protein. The difference in amount of removable heme proteins by these two methods might due to the loss of the soluble heme protein by co-precipitation with insoluble fish muscle component at pH 10.8 and/or with protein isolate at a final pH. The first case is desirable since the proteins will be discarded with insoluble components. Residue of heme proteins in the protein isolate would deteriorate its quality, therefore the latter has to be minimized. Our previous work revealed that exposure of hemoglobin at pH 10.8 enhanced its binding to muscle component both at pH 10.8 and a final pH (Thongraung et al., 2005). As expected, removal of heme protein from fish mince by three washes showed the least efficiency with total extractable heme protein of 8.3  $\mu\text{mole}$  heme protein/kg of fish mince. Size of the fish mince particle was essentially responsible for the extraction.

Each extraction methods caused the loss of soluble protein differently ( $p < 0.05$ ) (Table 5-2). Increase in removal of heme protein by washing a very fine fish muscle particle paid off the highest loss of soluble protein. Of this study, centrifugation at 10,000xg was used to recovery a fine solid particle after the wash. Therefore the loss would be presented almost exclusively by soluble protein rather than by a fine muscle particle. The increasing in protein recovery obtained by the alkaline process is the most outstanding advantages of the process. It is due to co-precipitation of soluble muscle proteins with muscle protein at final pH (Hultin and Kelleher, 2002).

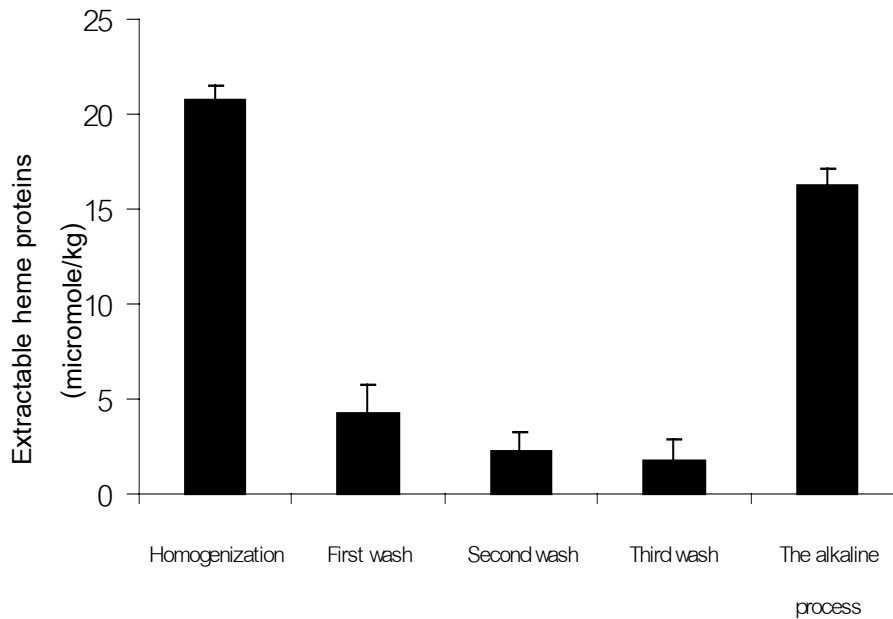


Figure 5-2 Amount of heme proteins in herring whole muscle removed by different methods.

**Homogenization:** Herring whole muscle was homogenized with 9 volumes of water at pH 7.0. Insoluble fish component was removed by centrifugation at 10,000xg at 10°C for 30 min. The extractable heme protein in the supernatant was quantified.

**The conventional washing process:** Herring whole muscle mince was washed for 10 min with 3 volumes of water. Water was drained off and washing was repeated for another two consecutive washes. Extractable heme protein in water after the washing was quantified.

**The alkaline solubilization process:** Herring whole muscle was homogenized with 9 volumes of water. The homogenate was adjusted to pH 10.8 with 1 N NaOH and centrifuged at 10,000xg at 10°C for 30 min. The obtained supernatant was then readjusted to pH 6.0 with 1 N HCl and centrifuged at 10,000xg at 10°C for 30 min. Extractable hemoglobin in the supernatant was quantified.

Mean  $\pm$  SD of triplicate experiments.

Table 5-2 Soluble protein of herring whole muscle removed by different washing protocols.

Treatment	Soluble protein <sup>a</sup> (g protein/ 100 g mince)
Homogenisation with 9 volumes of water <sup>b</sup>	3.5 $\pm$ 0.4 <sup>a,c,d</sup>
First wash with 3 volumes of water <sup>b</sup>	1.1 $\pm$ 0.1 <sup>b</sup>
Second wash with 3 volumes of water <sup>b</sup>	0.4 $\pm$ 0.0 <sup>c</sup>
Third wash with 3 volumes of 0.2% NaCl solution <sup>b</sup>	0.3 $\pm$ 0.0 <sup>c</sup>
Alkaline solubilization of whole muscle mince <sup>b</sup>	1.5 $\pm$ 0.2 <sup>b</sup>

<sup>a</sup>Soluble protein was quantified by the Lowry method. To remove a fine muscle particle, the drained water obtained after the washing was centrifuged at 10,000xg at 10°C for 30 min before the analysis. Total protein of herring was 18.4  $\pm$  0.5 g protein/ 100 g mince.

<sup>b</sup>as describe in Figure 5-2.

<sup>c</sup>Mean  $\pm$  SD of triplicate experiments.

<sup>d</sup>The means followed by different letters (abc) are significantly different (P<0.05).

#### **5.4.5 Effect of pre-washing on removal of herring heme proteins by an alkaline solubilization process.**

Some extractable hemoglobin became unextractable after the alkaline solubilization process especially if the precipitation was performed at pH 5.5. Precipitation at pH 6.0 and decreasing the initial amount of heme protein in fish mince are strategy to minimize the residual heme protein in the protein isolate obtained. As shown in Figure 5-3, two quick washes removed about 8.3  $\mu$ mole heme proteins/kg. The heme proteins of about 9.6  $\mu$  mole/kg were further removed by the alkaline process. Therefore the process with two pre-washing steps removed heme proteins about 17.9  $\mu$ mole/kg. Total removable heme proteins of 19.4  $\mu$ mole/kg were found with the homogenisation process and heme proteins of 14.4  $\mu$ mole/kg were removed by the process without the pre-washing.

Even though the different procedures provided significant differences in heme protein removal, no differences in color values of washed mince and protein isolate were noticeable (Table 5-3). Raw protein isolate showed higher value in lightness and whiteness than raw washed mince. However, a marginal difference in color values might correlate with the difference in amount of heme pigments remained in both samples. However, no differences in lightness and whiteness of cooked samples. The color values of cooked samples were similar to those of surimi gel prepared from medium-grade frozen surimi of Alaska pollock ( $L^* \sim 78$ ,  $a^* \sim -4$ ,  $b^* \sim 3.8$ , and whiteness  $\sim 77$ ) reported by Park (1995). With respect to color values, the approach to reduce insoluble heme protein caused by the process by pre-washing is not sufficient to improve quality of the protein isolate. However, the alkaline process with pre-washing may be sufficient to improve color values of fish containing low initial non-extractable heme protein. The two washes totally caused a loss of 1.3 g soluble protein/100 g mince (Table 5-4). Subsequently, soluble protein in the pre-washed mince was further leached out after the alkaline process, resulting in total loss of 2.2 g protein/100 g mince. Without the pre-washing step, the loss of soluble protein was only 1.5 g soluble protein/100 g mince. Nonetheless, the process either with or without pre-washing yielded the less loss of soluble protein than extraction by homogenisation of fish mince (Table 5-2).

The development in painty odor of the herring protein isolate was noticeable after two days storage in refrigerator (data not shown). It was likely that pH treatment of the process might intensify pro-oxidative activity of heme protein. This change of heme protein however needs to be further investigated and may be crucial for the application of the alkaline process.

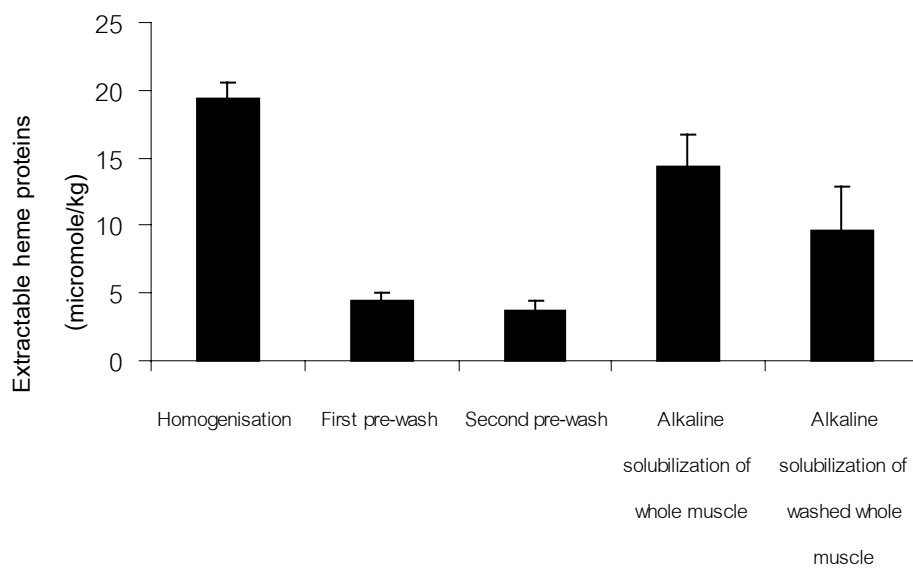


Figure 5-3 Extractable heme protein of herring whole muscle removed by pre-washing and the alkaline solubilization process.

The herring whole muscle mince was received a quick wash for 1 min with one volume of water. Water was drained off by screening for 1 min after washing. The first washed mince was re-washed with the same condition. The collected washed water was used to quantify extractable heme protein.

Table 5-3 Color values of washed herring whole muscle and protein isolate.

Color values <sup>1</sup>	Extraction with water <sup>a</sup>		Alkaline solubilization process <sup>a</sup>			
	Raw	Cooked	Without pre-washing step		With pre-washing step	
			Raw	Cooked	Raw	Cooked
L*	56.4 ± 1.6z <sup>c,d</sup>	71.8 ± 1.2x	60.1 ± 1.7z	71.6 ± 4.1x	65.7 ± 1.1y	75.4 ± 3.4x
a*	-1.1 ± 0.3y	-1.6 ± 0.3y	-3.1 ± 0.8x	-2.6 ± 1.5x	-3.0 ± 0.6x	-2.6 ± 0.9x
b*	10.0 ± 0.5x	10.9 ± 0.4x	8.6 ± 1.4x	10.6 ± 1.8x	9.0 ± 1.4x	9.4 ± 0.8x
Whiteness	55.2 ± 1.8z	69.7 ± 1.6x	59.1 ± 1.6y	69.5 ± 3.3x	64.4 ± 1.0y	73.7 ± 3.1x

Color values of raw and cooked samples were measured with Hunter colorimeter. The samples were adjusted to 85% moisture content with water and allowed to equilibrate at an ambient temperature for one h before the measurement.

<sup>a</sup>as described in Figure 5-2.

<sup>b</sup>Mean ± SD of triplicate experiments.

<sup>c</sup>The means in the same row followed by different letters (xyz) are significantly different (P<0.05).

Table 5-4 Soluble protein of herring whole muscle removed by different washing conditions.

Treatment	Extractable protein <sup>1</sup> (g protein/100g mince)
Extraction with 9 volumes of water <sup>a</sup>	3.5 ± 0.4 <sup>a,b,c</sup>
First pre-wash with one volumes of water <sup>a</sup>	0.7 ± 0.1c
Second pre-wash with one volumes of water <sup>a</sup>	0.6 ± 0.1c
Alkaline solubilization of whole muscle <sup>a</sup>	1.5 ± 0.2b
Alkaline solubilization of second-washed whole muscle <sup>a</sup>	0.9 ± 0.1c

Soluble protein was quantified by the Lowry method. The drained water obtained after the washing of the mince with one volume of water was centrifuged at 10,000xg at 10°C for 30 min before the measurement. Total protein of herring was 19.4 ± 0.9 g protein/ 100 g mince.

<sup>a</sup>as described in Table 5-3.

<sup>b</sup>Mean ± SD of triplicate experiments.

<sup>c</sup>The means followed by different letters (abc) are significantly different (P>0.05).

### 5.5 Conclusion

Dark color of washed herring suggested the presence of non-extractable heme proteins. Storage of the fish in ice caused reduction of heme protein extractability especially in whole muscle. The changes of myoglobin in herring dark muscle primarily contributed to the loss of heme protein extractability. Extraction at pH 7.0 or 8.0 and addition of NaCl failed to enhance extractability of the heme proteins. Extraction of the heme proteins either by homogenization with water, washing with water, or the alkaline solubilization process resulted in the removal of heme proteins differently. Compared with the conventional washing process, the alkaline solubilization process resulted in higher amount of extractable heme proteins. Pre-washing of herring mince before the alkaline solubilization process increased total extractable heme proteins. However, color values of the protein isolate obtained by different methods were not different (p>0.05).