Purification and Characterization of Myoglobin from Harp Seal (*Phoca groenlandica*)

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The purification and characterization of seal meat myoglobin after aqueous extraction of hemoproteins from muscle tissues was investigated. Of the 59.36 mg/g total hemoproteins in seal meat, only 26.9% of its myoglobin content was recovered by the commonly used buffer precipitation method. Similar observations were made for the content of myoglobin in other muscle tissues. Thus, quantification of myoglobin by the phosphate precipitation method is inaccurate. The amino acid composition of harp seal myoglobin was similar to those of gray and harbor seals as well as that of sperm whale.

INTRODUCTION

Myoglobin is a major hemoprotein of muscle foods and is the principal oxygen reservoir in the muscle tissues of live animals. In meats, it serves nutritionally as a source of iron but also acts as a catalyst for autoxidation of lipids (Shahidi and Hong, 1991). Thus, quantification of myoglobin is of interest to researchers. All methods of myoglobin determination involve the extraction of hemoprotein pigments from the tissue followed by separation from other proteins, including hemoglobin. Various procedures for the extraction of heme pigments from muscles have been developed. Hornsey (1956) used an acidified 80% acetone solution to convert both hemoglobin and myoglobin pigments to acid hematin. Water was used as an extractant of hemoproteins by Poel (1949), Ginger and Schweigert (1954), and Rickansrud and Henrickson (1967). Examination of different buffers as extractants of hemoprotein pigments was reviewed by Warris (1979). For determination of tissue myoglobin content, the methods of Ginger and Schweigert (1954) and Rickansrud and Henrickson (1967) are usually employed. In these procedures, separation of heme compounds from other proteins is achieved by precipitation of non-heme proteins with a saturated lead acetate solution. To the filtrate obtained is added a buffer consisting of mono- and dibasic phosphates so that the total phosphate concentration in the mixture reaches 3 M and the pH is 6.6. Then the hemoglobin fraction preferentially precipitates, leaving myoglobin in the supernatant.

Chromatographic separation of hemoglobin and myoglobin mixtures was first reported by Berman and Kench (1963) using human pigments and by Awad et al. (1963) using human hemoglobin and whale myoglobin. Warris (1976) also used a Sephadex stationary phase for separation of hemoproteins from each other. The relative quantities of these pigments were then determined after their conversion to corresponding cyanomet derivatives.

The purpose of this investigation was to determine the characteristics and concentration of myoglobin in seal muscles and to examine the possibility of its extraction from tissue samples. The validity of procedures available in the literature for myoglobin determination in myoglobin-rich sources of muscle foods was also re-examined.

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MATERIALS AND METHODS

Materials. Bled, skinned, and eviscerated seal carcasses, after storage for up to 3 days in ice, were washed with a stream of cold water (10 °C) for about 15 s to remove any blood remaining on the body frames. After most of the subcutaneous fat was trimmed, the carcasses were deboned using a POSS deboner (Model PDE 500, POSS Limited, Toronto, ON). Small portions of mechanically separated seal meat (MSSM) were vacuum packed and kept frozen at −20 °C until use.

The MSSM was washed once, twice, or three times with water at 5 °C for 10 min with manual stirring at a water to meat ratio of 3:1 (v/w). The washed meat was then filtered through three layers of cheesecloth.

Commercial beef and pork meat samples were bought from a local supermarket and were ground in Braun meat mincer (Model KGZ3, Braun AG, Frankfurt, Germany), vacuum packed in plastic bags, and stored at −20 °C until use.

The seal myoglobin for amino acid analysis was prepared according to the method of Ginger et al. (1954). After removal of hemoglobin and other proteins precipitated by a 3 M phosphate buffer at pH 6.6, the supernatant was dialyzed three times against distilled water and freeze-dried. The purity of myoglobin was monitored by polyacrylamide gel electrophoresis.

Analyses. Crude proteins content was calculated from total nitrogen (N) determined according to the AOAC (1990) method (i.e., N × 6.25). Total hemoprotein pigments were determined after three extractions of meat samples with acidified 80% acetone according to the method of Hornsey (1956). After 1 h, the absorbance of the extract was read at 640 nm using a Beckman DU-8 spectrophotometer. Total hemoprotein pigments were calculated using the equation

$$\text{total hemoprotein pigments (mg/g of sample)} = \frac{\text{hemin content (mg/g of sample)}\ (\mathrm{MW}_{\text{myoglobin}})}{\mathrm{MW}_{\text{hemin}}}$$

Total hemin, expressed as micrograms per gram of sample, was calculated by multiplying the absorbance of the acetone extract at 640 nm by Hornsey's factor of 680 and consideration of sample size, if different from 10 g.

Myoglobin content in the pooled mixture obtained after three homogenizations of 5 g of meat sample with water using a Polytron and purification according to the method of Rickansrud and Henrickson (1967) by lead acetate and then by phosphate buffer at pH 6.6 was determined as its cyanoprotein derivative. One milliliter of a 134.2 mM potassium ferricyanide and 1 mL of a 17.6 mM solution of potassium cyanide were added to the extract, and the absorbance of the final mixture was read at 540 nm. The myoglobin content was then calculated according to the equation

$$\text{myoglobin (mg/g of sample)} = A_{540\ \mathrm{nm}}(\mathrm{MW}_{\text{myoglobin}})Vf/EW$$

where V is the volume of final diluted extract in liters, W is the

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weight of meat sample in grams, E is the extinction coefficient of myoglobin in millimoles per liter, and f = (20 mL of extract + 1 mL of $K_3Fe(CN)_{6\ soln}$ + 1 mL of KCN_{soln})/20 mL of extract = 1.1.

Myoglobin content was also determined according to the method of Warris (1976). Pigments extracted by 0.04 M phosphate buffer at pH 6.8 were converted to their more stable cyanomet forms by addition of a couple of crystals of $K_3Fe(CN)_6$ and KCN, freeze-dried, redissolved in 1 mL of water, and dialyzed against 0.5 M NaCl solution to precipitate other sarcoplasmic proteins. Hemoglobin and myoglobin were separated from each other on a 30 cm × 1 cm i.d. column packed with Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden). The eluent buffer was 0.1 M phosphates at pH 6.8 containing 0.1 M NaCl.

The absorbance of hemoglobin fraction was read at 420 nm and that of the myoglobin at 540 nm. The amount of myoglobin was calculated as follows:

$$\text{myoglobin fraction in extract} = \frac{A_{540\ nm}}{A_{420\ nm}(0.11 + A_{540\ nm})}$$

myoglobin (mg/g of sample) = total hemoprotein pigments (mg/g of sample) × myoglobin fraction in extract

Hemoglobin content was calculated as the difference between the total content of hemoproteins and myoglobin.

SDS–polyacrylamide gel electrophoresis was carried out using a Laemmli (1970) buffer system and a Bio-Rad Mini-Protean II apparatus. All gels consisted of a 5% (w/v) stacking component and a 15% (w/v) separating component. Samples were prepared for electrophoretic examination in the following way: Lyophilized proteins were dissolved into Laemmli sample buffer to a concentration of 1 mg/mL and boiled for 2–3 min. Meats (unextracted and extracted), sample washings, and precipitated proteins were homogenized in Laemmli sample buffer lacking SDS, for 15–20 s, with a Polytron homogenizer (Brinkman Instruments, Rexdale, ON). The detergent was omitted to avoid excessive frothing and was added prior to boiling. All samples were applied to the gels via a 50-μL Hamilton syringe. The electropherograms were stained in a shaking bath containing 0.2% (w/v) Coomassie Brilliant Blue R-250 (ICN Biochemicals Canada Ltd., Mississauga, ON) in 50% (v/v) ethanol/10% (v/v) acetic acid and destained in 10% (v/v) ethanol/5% (v/v) acetic acid.

The individual amino acids in freeze-dried samples were determined (in triplicate) after their digestion in 6 N HCl at 110 °C according to the method of Blackburn (1968). The HCl solution was then removed under vacuum, and dried samples were reconstituted using a lithium citrate buffer at pH 2.2. The amino acid composition of the hydrolysate was determined using a Beckman 121 MB amino acid analyzer (Beckman Instruments Inc., Palo Alto, CA). Cysteine and methionine were determined by performic acid oxidation prior to their digestion in 6 N HCl and were measured as cysteic acid and methionine sulfone, respectively (Blackburn, 1968). Analysis of tryptophan was carried out by hydrolysis of the sample under vacuum in 3 M mercaptoethanesulfonic acid at 110 °C, as described by Penke et al. (1974).

Statistical Analysis. Analysis of variance and Tukey's Studentized range test (Snedecor and Cochran, 1980) were used to determine differences in mean values based on the data collected from four replicates of each measurement. Significance was determined at 95% probability.

RESULTS AND DISCUSSION

The contents of myoglobin in mechanically separated seal meat (MSSM), beef, and light and dark pork were 13.20, 2.50, 0.46, and 1.09 mg/g of sample, respectively, (Table I) as determined by the method described by Ginger and Schweigert (1954) and Rickansrud and Henrickson (1967). Results for beef and pork are close to those reported by Rickansrud and Henrickson (1967) and Ginger and Schweigert (1954): 2.40–3.64 mg/g for beef and 0.61–0.98 mg/g for pork. However, the amount of myoglobin in MSSM determined by separation on Sephadex G-75, according to the method of Warris (1976), was 49.04 mg/g, about 3.7 times more than that determined using the method of Ginger and Schweigert (1954) and Rickansrud and Henrickson (1967). The same trend was observed in other samples of washed MSSM and in beef and pork (Table I). These differences probably arise from incomplete extraction of the pigments with water, under the conditions given by Rickansrud and Henrickson (1967), and possible loss of myoglobin during its purification. Extracted residues obtained using acidified acetone were colorless, while the residues after extraction with water or buffers were brown-gray. This suggests that complete removal of the pigments from muscle tissues was not achieved as reflected by the electrophoretic analysis of the residues after water extraction of the pigments. A relatively large proportion of myoglobin and hemoglobin, possibly together with other proteins of similar molecular weight, remained unextracted in MSSM (Figure 1). The band for myoglobin was also observed in proteins precipitated by a saturated lead acetate solution as well as in the hemoglobin residue separated at pH 6.6 by a 3 M phosphate buffer. The 3 M phosphate buffer precipitated not only hemoglobin but also a considerable proportion of myoglobin and a number of components with molecular weights higher than those of myoglobin and hemoglobin. Therefore, use of lower concentrations of buffers for precipitation of hemoglobin was investigated. Results given in Table II indicate that decreasing the buffer concentration from 3 to 1 M enhanced the hemoprotein content of the supernatant by about 42%. The amount of hemoproteins was linearly related to the phosphate buffer concentration; the correlation coefficient was r = 0.994. In addition, the myoglobin band became more intense when smaller concentrations of buffer were employed and also some hemoglobin remained in the solution (results not shown).

Table I. Total Hemoprotein, Myoglobin, and Hemoglobin Content of Muscle Foods (mg/g of Sample)[a]

sample	total hemoproteins[b]	myoglobin: separated on Sephadex[c]	myoglobin: extracted and precipitated[d]
unwashed MSSM	59.36 ± 1.41	49.04 ± 0.24	13.20 ± 0.56
washed MSSM			
1 × H_2O	29.97 ± 0.47	23.93 ± 0.62	6.56 ± 0.11
2 × H_2O	20.70 ± 0.08	16.32 ± 0.12	1.95 ± 0.02
3 × H_2O	19.30 ± 0.31	15.03 ± 0.32	0.66 ± 0.09
beef, longissimus dorsi	4.46 ± 0.23	4.13 ± 0.21	2.50 ± 0.20
pork, light biceps femoris	1.93 ± 0.19	1.66 ± 0.05	0.46 ± 0.03
pork, dark biceps femoris	3.64 ± 0.11	3.20 ± 0.02	1.09 ± 0.07

[a] Results are mean values of six to eight replicates ± standard deviation. [b] According to Hornsey's (1956) method. [c] According to Warris (1976). Hemoglobin may be obtained by difference. [d] According to Ginger and Schweigert (1954) and Rickansrud and Henrickson (1967).

Table III summarizes the analyzable content of myoglobin in water extracts as a function of the original concentration of hemoproteins in the assay solution. Importantly, after dilution of seal hemoproteins from their initial concentration of 5.67 mg/mL to 0.34 mg/mL, the proportion of assayable myoglobin increased from 26.5% of the assayable amount determined according to the method of Warris (1976) to 39.7% (Table III).

As an extension of this work, the amino acid composition of seal myoglobin was determined as summarized in Table IV. These results were very close to those for other seal

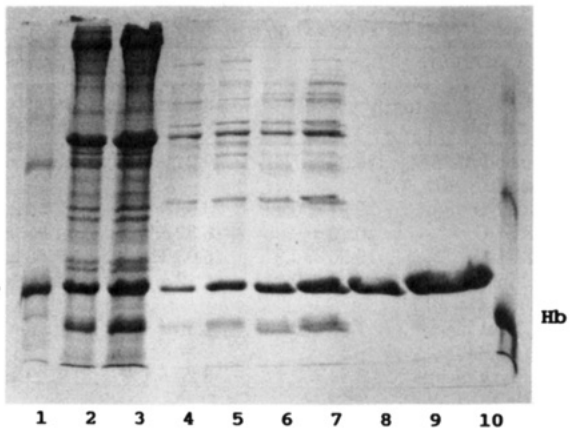

Figure 1. SDS–polyacrylamide gel electrophoresis of (1) horse myoglobin (Sigma, St. Louis, MO), (2 and 3) meat residue after extraction of pigments according to the method of Rickansrud and Henrickson (1967), (4 and 5) proteins precipitated by lead acetate, (6 and 7) proteins precipitated by 3 M phosphate buffer, (8 and 9) myoglobin extract according to the method of Ginger et al. (1954), and (10) human hemoglobin (Sigma). Samples were prepared for electrophoresis as outlined under Materials and Methods. The loadings were as follows: (lane 1) 5 μg of horse myoglobin; (lanes 2 and 3) 5 and 10 μL, respectively, of a 1:10 (w/v) extract; (lanes 4 and 5) 5 and 10 μL, respectively, of a 1:2 (w/v) extract; (lanes 6 and 7) 5 and 10 μL, respectively, of a 1:5 (w/v) extract; (lanes 8 and 9) 5 and 10 μg of myoglobin extract, respectively; (lane 10) 5 μg of human hemoglobin.

Table II. Influence of Phosphate Buffer Concentration on the Amount of Hemoproteins from MSSM Not Precipitated at pH 6.6[a]

phosphate buffer concn (X), M	hemoproteins in supernatant (Y), mg/g of sample
3.0	13.45 ± 0.04
2.5	15.20 ± 0.06
2.0	15.95 ± 0.07
1.0	19.09 ± 0.04

[a] Results are mean values of eight replicates ± standard deviation. The content of hemoproteins determined (Y) is related to the buffer concentration (X) by $Y = 21.75 - 2.74X$, $r = 0.994$.

Table III. Influence of Hemoprotein Concentration of MSSM on Its Aliquot Not Precipitated by a 3 M Phosphate Buffer at pH 6.6[a]

concn of hemoproteins,[b] mg/mL of extract	myoglobin, mg/g of sample
5.67	13.02 ± 0.04
2.83	15.71 ± 0.01
1.20	16.00 ± 0.10
0.34	19.45 ± 0.00

[a] Results are mean values of eight replicates ± standard deviation. [b] The original extraction solution contained 5.67 mg of hemoproteins/mL of solution. This solution was then diluted to obtain other solutions. [c] Total myoglobin determined according to the method of Warris (1976) by separation on Sephadex G-75 was 49.04 ± 0.24 mg/g of sample.

species such as gray or harbor seal myoglobin as they were calculated from protein sequence data reported by Bradshaw and Gurd (1969). Furthermore, only small differences in amino acid composition between harp seal myoglobin and that from sperm whale were noticed (Bodwell et al., 1971). Seal myoglobin, similar to myoglobin from other sea mammals, did not contain cysteine (Bannister and Bannister, 1976; Bodwell et al., 1971). However, the presence of cysteine has been reported in myoglobin from both tuna (Balestrieri et al., 1978) and horse (Bodwell et al., 1971).

As compared to dolphin fish (*Coryphanea pippurus* L.) and bluefin tuna (*Tunnus thynnus*), seal myoglobin contained less aspartic acid equivalent (Asp plus Asn) and more histidine (Table IV). This is due to genetic differences as noted by various authors (Bannister and Bannister, 1976; Hirs and Olcott, 1964; Edmundson and Hirs, 1962). Generally, mammalian and fish myoglobins have a higher content of histidine and a lower amount of aspartic acid equivalent as compared to that of seal myoglobin. Data given in Table V show that seal and sperm whale myoglobins contained less aliphatic and more basic amino acids than myoglobins from bluefin tuna and dolphin fish. The smaller content of histidine in fish myoglobin is responsible for the latter observation.

Table IV. Amino Acid Composition (g/100 g of Myoglobin) of Harp Seal Myoglobin As Compared with That of Myoglobin from Other Sources[a]

amino acid	harp seal	gray and harbor seal[b]	sperm whale[c]	dolphin fish[d]	bluefin tuna[e]
Ala	6.54 ± 0.02	9.2	7.53	8.95	10.01
Arg	4.37 ± 0.07	3.3	3.57	1.92	1.93
Asp[f]	7.24 ± 0.04	7.2	5.31	13.87	10.31
Cys					0.68
Glu[g]	12.16 ± 0.01	11.2	13.99	11.07	9.01
Gly	4.41 ± 0.02	7.8	4.14	5.40	5.88
His	10.27 ± 0.03	8.5	9.25	5.10	5.32
Ile	5.10 ± 0.06	5.2	5.96	5.73	6.74
Leu	12.28 ± 0.02	12.4	11.80	11.66	13.08
Lys	13.59 ± 0.11	12.4	13.93	13.35	12.20
Met	1.67 ± 0.01	1.3	1.46	0.93	1.71
Phe	5.09 ± 0.01	4.6	5.00	5.55	5.45
Pro	2.28 ± 0.01	2.6	2.36	2.72	3.35
Ser	3.01 ± 0.03	4.6	3.18	2.31	2.20
Thr	3.05 ± 0.03	3.3	2.97	3.49	4.58
Trp	1.15 ± 0.01	1.3	1.99	1.07	1.14
Tyr	1.52 ± 0.04	1.3	2.74	2.02	1.98
Val	3.96 ± 0.03	3.9	4.69	4.73	5.40

[a] Results are mean values of three determinations ± standard deviation. [b] Bradshaw and Gurd (1969). [c] Bodwell et al. (1971). [d] Bannister et al. (1976). [e] Balestrieri et al. (1978). [f] Value includes asparagine. [g] Value includes glutamine.

SDS–polyacrylamide gel patterns of harp seal muscle were compared with muscles of other species (Figure 2). The major difference in the proteins of the various muscles was the very high content of myoglobin in seal as opposed to that in chicken, pork, and beef. In general, we have observed that the electrophoretic mobility of harp seal myoglobin on SDS gels is similar to that of myoglobin from other species. While Höyen and Thorson (1970) have discussed the presence of a secondary band in some species, this was not apparent in our studies. It should be pointed out, however, that we have employed an electrophoretic system different from that used by these authors. In addition, multiwelled slab gels were used, as opposed to their individual tube gels, to facilitate mobility comparisons. Using the Laemmli method alone, it was not possible to assess the entire protein content of the contractile apparatus of seal muscle and contrast it with that of other species. This is a question we are currently exploring with high-resolution two-dimensional gel electrophoresis in combination with established muscle protein isolation methods (Frederiksen and Cunningham, 1982).

In another set of studies we investigated the possibility of partial extraction of myoglobin from seal meat (Table VI). On a dry weight basis, the myoglobin content after one, two, or three washings with water at pH 6.2–6.5 decreased by about 16.4, 40.5, and 44.2% of its initial amount in MSSM. However, the amount of hemoglobin after three extractions with water decreased by about 24.6% (from 33.39 to 26.68 mg/g, on a dry basis). These observations were supported by polyacrylamide gel patterns provided in Figure 3. After each extraction with

Table V. Amino Acid Profile of Harp Seal Myoglobin, As Compared with Those from Other Sources, as Percent of Total Amount

amino acid group	harp seal	gray and harbor seal[a]	sperm whale[b]	dolphin fish[c]	bluefin tuna[d]
aliphatic (Ala + Gly + Ile + Leu + Val)	34.3	34.5	34.1	36.5	41.1
hydroxy (Ser + Thr)	6.1	7.9	6.2	5.8	6.8
acidic and their amides (Asn + Asp + Gln + Glu)	19.4	18.4	19.3	24.9	19.3
basic (Arg + His + Hyly + Lys)	27.2	24.2	26.8	20.4	19.5
aromatic, excluding histidine (Phe + Try + Tyr)	7.8	7.2	9.7	8.6	8.6
sulfur-containing (Cys + Met)	1.7	1.3	1.5	0.9	2.4
imino (Pro)	2.3	2.6	2.4	2.7	3.4

[a] Bradshaw and Gurd (1969). [b] Bodwell et al. (1971). [c] Bannister et al. (1976). [d] Balestrieri et al. (1978).

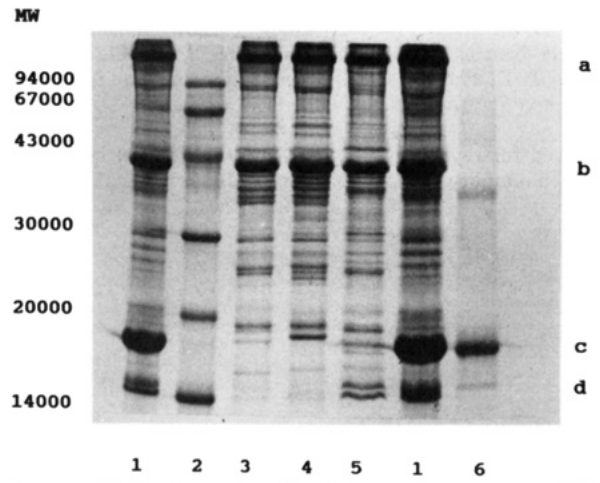

Figure 2. SDS–polyacrylamide gel electrophoresis of (1) MSSM, (2) molecular weight markers (14 000–94 000, Pharmacia, Baie D'urfe, PQ), (3) pork, (4) beef, (5) chicken, and (6) horse myoglobin (Sigma). The loadings were as follows: (sample 1) lower loading, 4 μL of a 1:10 (w/v) extract, and higher loading, 8 μL of same extract; (sample 2) 2.5 μg of molecular weight markers; (sample 3) 3 μL of a 1:10 (w/v) extract; (sample 4) 4 μL of a 1:10 (w/v) extract; (sample 5) 6 μL of a 1:10 (w/v) extract; (sample 6) 5 μg of horse myoglobin. Main protein bands are (a) myosin heavy chain, (b) actin, (c) myoglobin, and (d) hemoglobin.

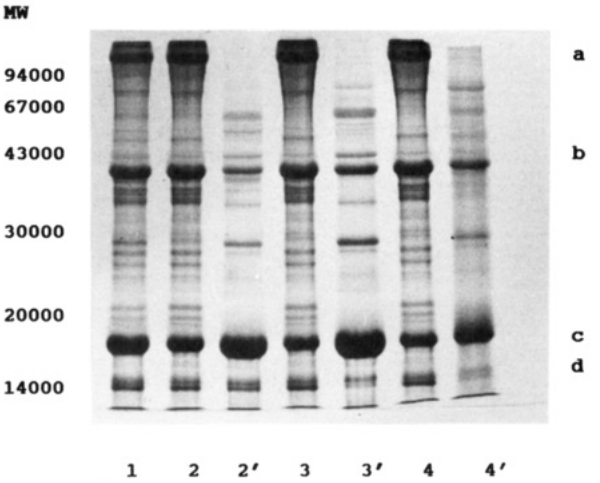

Figure 3. SDS–polyacrylamide gel electrophoresis of (1) unwashed MSSM, (2) residue and (2′) solution after first washing with water, (3) residue and (3′) solution after second washing with water, (4) residue and (4′) solution after third washing with water. The loadings were as follows: (sample 1) 4 μL of a 1:10 (w/v) extract; (samples 2–4) 4–6 μL of 1:5 (w/v) extracts; (sample 2′) 2 μL of a 1:1 (v/v) extract; (sample 3′) 5 μL of a 1:1 (v/v) extract; (sample 4′) 15 μL of a 1:1 (v/v) extract. Main protein bands are (a) myosin heavy chain, (b) actin, (c) myoglobin, and (d) hemoglobin.

Table VI. Effect of Washings with Water on Total Hemoproteins and Myoglobin Content of Mechanically Separated Seal Meat[a]

	total hemoproteins, mg/g		myoglobin, mg/g	
MSSM	in wet tissue	in proteins	in wet tissue	in proteins
unwashed	59.36 ± 1.41	255.75 ± 6.07	49.04 ± 0.24	211.29 ± 1.03
washed				
1 × H_2O	29.97 ± 0.47	215.61 ± 3.38	23.93 ± 0.62	172.15 ± 4.46
2 × H_2O	20.70 ± 0.08	154.47 ± 0.59	16.32 ± 0.12	121.79 ± 0.89
3 × H_2O	19.30 ± 0.31	145.77 ± 2.34	15.03 ± 0.32	113.51 ± 2.41

[a] Results are mean values of six to eight determinations ± standard deviation. Data in each column are significantly different ($P < 0.05$) from each other.

water, both myoglobin and hemoglobin were detected in the washed residue. Also, a number of other components were extracted along with these proteins, one of which had an electrophoretic mobility corresponding to actin. The presence of a relatively large amount of pigment in washed MSSM shows that the literature procedures for determination of total tissue heme pigments, which are based on their prior extraction with water (Rickansrud and Henrickson, 1967; Ginger et al., 1954), are inadequate for high-myoglobin muscle tissues such as those of seal. Furthermore, on the basis of the present results, we suggest that while the method of Rickansrud and Henrickson (1967) for preparation of reasonably pure myoglobin is quite useful, its value for quantification of myoglobin, especially in myoglobin-rich muscle tissues, is questionable.

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