On the Solubility of Cod Muscle Proteins in Water

Gudmundur Stefansson and Herbert O. Hultin*

Massachusetts Agricultural Experiment Station, Department of Food Science, University of Massachusetts/Amherst, Marine Foods Laboratory, Marine Station, Gloucester, Massachusetts 01930

Cod muscle myofibrillar proteins were shown to be soluble in water at neutral pH if the final ionic strength was approximately 0.0003 or less. This solubility was sensitive to ionic strength and was reversible. At an ionic strength of 0.001 about 15% of the proteins were soluble compared to more than 90% at an ionic strength of 0.0002. SDS–PAGE of muscle and soluble proteins demonstrated that all the major myofibrillar proteins were soluble at an ionic strength of 0.0002. Increasing sodium chloride concentration or decreasing pH to 5.5 reduced the solubility of the myofibrillar proteins selectively. Our results suggest that extraction and re-precipitation of myofibrillar proteins at low ionic strength may be useful for separation of the different myofibrillar proteins.

Keywords: *Muscle proteins; protein solubility; solubility of muscle proteins; cod muscle proteins*

INTRODUCTION

The solubility of muscle proteins has been the subject of much research. Isolation of individual myofibrillar proteins necessitates first putting them into solution. Change in solubility under a variety of extracting conditions has been taken as a measure of change in protein conformation, i.e., denaturation, and has been used as an indicator of the quality change that a stored muscle food has undergone. In addition, many functional properties of muscle food proteins have been related to the solubilization of the protein in salt solutions. For example, the generally accepted hypothesis explaining the formation of fish gels has been that a high concentration of sodium chloride is required to solubilize the myofibrillar proteins (Suzuki, 1981; Lee, 1984, 1986; Shimizu, 1985), particularly myosin and actomyosin, which can then gel upon heating as the proteins denature, interact, and aggregate (Hermansson *et al.*, 1986; Wicker *et al.*, 1989; Niwa *et al.*, 1989; Xiong and Brekke, 1990).

We had previously shown that good gels could be prepared from the washed minced muscle tissue of some species of fish without the addition of the 2–3% sodium chloride thought to be required (Hennigar *et al.*, 1988, 1989; Vareltzis *et al.*, 1989). If the hypothesis above is correct, some of the myofibrillar proteins should be soluble in solutions of low salt content. After washing the minced muscle tissue of five species of white-fleshed fish, twice with water and a third time with 0.15% sodium chloride solution, a considerable proportion of the remaining protein was soluble when extracted with 20 volumes of water; the amount of protein extracted was 20–36% of the protein that was extracted with a buffered 1 M lithium chloride solution, a good protein extractant (Kelleher and Hultin, 1991). This indicated that the extracted proteins were most likely myofibrillar (Wu *et al.*, 1991). The salt concentration present in these low salt fish gels was estimated to be approximately 0.15% (26 mM) which was the level of salt added as an aid in the de-watering process. When these washed minces were extracted in water, the salt concentration of the extracting solution was estimated to be approximately 1.0–1.5 mM. In this report, we show that when the ionic strength is lowered further, essentially all of the myofibrillar proteins of cod muscle become soluble; some characteristics of the process are presented and discussed.

* Address correspondence to this author at U. Mass. Marine Station, P.O. Box 7128 Lanesville, Gloucester, MA 01930 [(508) 281-1930].

MATERIALS AND METHODS

Material. Fresh gutted whole cod (*Gadus morhua*) was obtained from a local fish dealer and transported on ice to the laboratory. The fish ranged from 4 to 48 h post mortem with an average of about 24 h. Sodium chloride, sodium iodide, potassium chloride, lithium chloride, magnesium chloride, calcium chloride, glycerol, Coomassie Brilliant Blue R-250, and ethylendiaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Co. (St. Louis, MO). Acrylamide/bis (37.5:1) premixed, tris(hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), ammonium persulfate, 2-mercaptoethanol, glycine, *N,N,N,N*-tetramethylethylenediamine (TEMED), bromophenol blue, dithiothreitol (DTT), and SDS–PAGE molecular weight standards (low and high) were purchased from Bio-Rad Laboratories (Richmond, CA). Precast polyacrylamide gels (3–27%) were from Integrated Separation Systems (Natick, MA). Coomassie protein assay reagent was obtained from Pierce Chemical Co. (Rockford, IL). Methanol, acetic acid, hydrochloric acid, and sodium hydroxide were from Fisher Scientific (Pittsburgh, PA). All reagents used for electrophoresis were of electrophoresis purity, and all other reagents were of ACS grade.

Preparation of Washed Muscle. The muscle from fresh cod (*G. morhua*) was ground (5 mm diameter plate) and washed three times with cold (4–6 °C) deionized, distilled water using in each wash 3 volumes of water per original weight of fish. In each washing step the mince and water were allowed to sit for 13 min after an initial stirring of 2 min. In the first and second washing steps the sediment was recovered by filtration through a fiberglass screen and the wash water discarded. After the third and final wash, the slurry was centrifuged at 30 000*g* for 30 min and the wash water discarded. The pH ranges of the washings were 6.7–7.0, 6.9–7.2, and 7.1–7.4 in the first, second, and third washes, respectively.

Solubility Measurements. Solubility was tested by mixing approximately 4.0 g of washed muscle and 200 mL of cold (4–6 °C) deionized, distilled water in a Waring blender (single speed) for 45 s in a glass cup. Approximately 50 g of the suspension was placed in 60 mL tubes and centrifuged for 20 min at 37000*g* at 4–6 °C. Samples for protein determinations were taken from the homogenate before centrifugation for measurement of total protein and from the supernate after centrifugation for measurement of soluble protein. The su-

0021-8561/94/1442-2656$04.50/0

Table 1. Changes in Water, Salt, and Protein during Washing and Extraction of Minced Cod Muscle

	mince						
	wt (g)	water (g)	protein (g)	equiv NaCl (mM)	water added (mL)	dilution	protein recovery[a] (%)
initial muscle	100	81.9	17.9	145			
					300	4.6	
first wash	147	131	15.4	35.3			96.6
					300	3.3	
second wash	180	166	14.4	14.3			96.8
					300	2.8	
third wash	176	163	14.1	7.9			100
			total dilution and protein recovery in washing steps:			42.5	95.5
extraction	4.0	3.7	0.3	0.17	200	55	
				total dilution in washing and extraction:		2338	

[a] Protein recovery refers to the protein recovered in the wash water and washed sediment compared to the initial protein before the washing step.

pernate consisted of two portions, each of which comprised about one half of the total. The upper part was clear while the lower half had a very slight turbidity seen on close examination. The supernate samples were taken by drawing 1 mL from the top clear layer. In the clear layer, there was no protein concentration gradient. Protein solubility was defined as the fraction of the total protein remaining soluble after centrifugation.

The effect of different ratios of mince to extracting solution (200 mL of water) was tested using 10, 5, 4, 2.5, and 1.25 g of mince. The effect of homogenization time (30, 45, 60, and 120 s) and centrifugal force (10000*g*, 18000*g*, 37000*g*, 47000*g*, and 145000*g* for 20 min) was tested using 4 g of washed mince extracted with 200 mL of water. Solubility was routinely defined as the percentage of protein remaining in the supernate after centrifugation for 20 min at 37000*g*. The effect of added salts on the solubility was tested as above by using solutions of various ionic strengths in place of deionized, distilled water (extraction), or by adding salt to the deionized, distilled water extract (precipitation). This was done by adding an appropriate volume of salt solution with ionic strength of 1.0. The effect of pH was tested by adjusting the pH of the deionized, distilled water extract with 0.2–1.0 N solutions of HCl or NaOH before centrifugation.

Protein Measurements. The Bradford dye binding method (Bradford, 1976) was used for protein determination using commercially available Coomassie Brilliant Blue G-250 reagent solution (Anon., 1991) with BSA as a standard.

Conductivity Measurements. The ionic strength of protein solutions was estimated by measuring conductivity of appropriately diluted solutions. A YSI conductivity meter, Model 35, equipped with a YSI conductivity cell having a cell constant of 1 was used (YSI, Inc., Yellow Springs, OH). Ionic strength was estimated by comparing the conductivity readings of the protein solutions to a standard curve prepared with sodium chloride.

Electrophoresis. Electrophoretic analyses were carried out on polyacrylamide slab gels using a Hoefer SE 600 unit (Hoefer Scientific Instruments, San Francisco, CA). The gels were obtained precast (ISS, Natick, MA) and were composed of a 3–27% linear polyacrylamide gradient (acrylamide/bis-(acrylamide) = 37.5/1), 0.375 M Tris-HCl (pH 8.8), 0.1% SDS, 0.06% ammonium persulfate, and 0.075% TEMED. The stacking gels, composed of 2.43% acrylamide, 0.065% bis-(acrylamide), 0.1% SDS, 10% glycerol, 0.125 M Tris-HCl (pH 6.8), 0.05% ammonium persulfate, and 0.05% TEMED, were made in the laboratory. The composition of the reservoir buffer was as described by Laemmli (1970) with the addition of 5 mM mercaptoethanol to the upper reservoir buffer (Fritz *et al.*, 1989). Samples of soluble proteins were prepared by mixing in a 3/1 ratio (sample/buffer) (Wang, 1982). Samples of whole muscle or washed mince were dispersed by homogenization in cold deionized, distilled water with a Teflon pestle in a glass homogenization tube; the ratio of water to sample was adjusted to give a protein concentration of 1–2 mg/mL. The samples were heated for 2 min at 100 °C (Wang, 1982) before application to the gels. All gels were run at temperatures below 10 °C using a constant current of 30 mA per gel. The gels were stained with Coomassie Brilliant Blue R-250 and destained according to Hames (1982). Molecular masses were estimated from markers (Bio-Rad) ranging from 200 kDa to 14.4 kDa. The molecular markers were rabbit myosin heavy chain (200 kDa), *E. coli* β-galactosidase (116.25 kDa), rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa). The two protein bands having molecular masses higher than 200 kDa were tentatively identified as titin and nebulin by using fresh chicken breast muscle as a standard. The two high molecular mass components from cod showed the same migration distance as the components of the chicken breast muscle previously identified as titin and nebulin with reported molecular masses of 2100–2800 kDa for titin and 600–800 kDa for nebulin (Locker and Wild, 1986; Hu *et al.*, 1986; Hainfeld *et al.*, 1988; Kurzban and Wang, 1988). Destained gels were scanned using a Hoefer GS 300 scanning densitometer (San Francisco, CA) in transmittance mode at 580 nm and a scanning speed of 6.7 cm/min. Quantification was done by integrating individual peaks and calculating the percent of total area. This fraction of the total area was taken to be the same as the fraction of the total protein loaded on the gel. The amount of each component was calculated by multiplying the fraction of the total area by the total amount of protein put on the gel. Equal dye-binding by the various muscle proteins was assumed.

Statistical Analysis. All measurements were done in duplicate and results are means from two separate experiments. Statistical analysis for difference of means was done using two sample *t*-tests (Kvanli, 1988).

RESULTS

Conditions for Solubilization of Proteins in Water. In evaluating the solubility properties of washed cod mince proteins, a procedure was used for preparation of the washed minced muscle that was similar to that used in commercial practice for preparation of surimi. The final dewatering, however, was done in the absence of added salt so as to achieve the necessary low ionic strength when the material was extracted with 50 volumes of water. To dewater, the retentate from the final filtration was centrifuged at 30000*g* (maximum) for 30 min at 4 °C. The moisture, protein, and equivalent salt concentrations at each step of the process are shown in Table 1. As the minced fish muscle was washed, it increased in weight due to an increase in the water uptake ability of the muscle proteins. At the same time, there was a decrease in the ionic strength

Table 2. Effect of the Ratio of Washed Minced Cod Muscle: Deionized, Distilled Water and Homogenization Time on Protein Solubility

mince:water[a] (w:v)	solubility (%)	homogenization time (s)	solubility (%)
1:20	gel	30	87.7 ± 2.7
1:40	80.2 ± 2.7	45	98.3 ± 3.2
1:50	96.2 ± 3.0	60	94.9 ± 2.8
1:80	96.2 ± 2.9	120	81.2 ± 2.7
1:160	97.0 ± 3.1		

[a] The mince:water ratio refers to the ratio of the weight of the washed mince to the volume of deionized distilled water used in the extraction step.

expressed as equivalent millimolar sodium chloride. The final extraction was performed with 50:1 water/fish (v/w) to lower the ionic strength sufficiently for solubilization to occur. It was difficult to achieve this dilution gradually. With successive low volume washes, the minced muscle tissue became swollen and was difficult to dewater. Therefore, before excessive swelling occurred which prevented dispersion, it was necessary to extract into a large volume of water.

Washing is a dilution process. The theoretical extent of dilution at each wash can be calculated by determining the moisture content of the washed minced muscle and the amount of exogenous water that is used. Dilution at each washing stage as well as the extraction step is shown for a typical sample in Table 1. In theory, any water-soluble component of the minced muscle should be reduced in concentration by the dilution indicated at each step. The content of any water-soluble component on a gram of water basis can be followed throughout the procedure to see if this theoretical dilution is achieved. If it is not, it indicates that there was not a free exchange of the component between the tissue and the wash water during the process. We compared theoretical dilution with actual dilution by measuring conductivity to get an estimate of the salt content. The salt content in the final extract was 2.7-fold greater than the theoretical value indicating that prior to final homogenization and extraction, the ions in the muscle tissue were not completely exchangeable with the added wash water. The discrepancy between the actual salt content and that which should be achieved theoretically was eliminated when the minced tissue was homogenized. Thus, the amount of time of exposure of the muscle cell segments to the wash water was not sufficient to allow equilibration to be established between the salt in the segments and in the wash water. Presumably, other water-soluble components would be at least as restricted. When extraction of the final washed mince was made in 20 volumes of cold deionized distilled water, the samples gelled; at a ratio of 1:40 considerable solubility was observed while a maximum was reached at 1:50 which did not improve with the use of greater volumes of water (Table 2). Homogenization times of 45 and 60 s gave maximal extractability of the proteins; longer or shorter times decreased the protein solubility (Table 2). It is likely that extraction was less at the shorter time period due to insufficient breakage of the cells. The decrease in extractability seen in Table 2 at 120 s could have been caused by denaturation of the muscle proteins due to this greater time of exposure to the shear force and/or a high temperature reached because of the prolonged blending. Centrifugal forces up to 47000*g* (maximum) for 20 min did not cause greater removal of the proteins from solution as compared to those removed at 10000*g* under the same conditions. However, at a high centrifugal force (144000*g*), only one-third of the protein remained in the supernatant fraction.

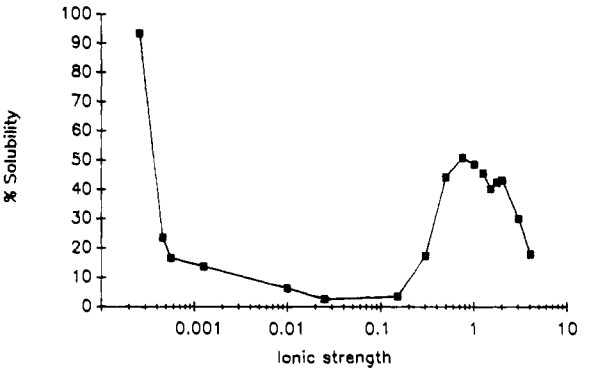

Figure 1. Effect of ionic strength on solubility of myofibrillar proteins from cod muscle. Solubility is expressed relative to total protein in the washed muscle.

Table 3. Solubility of Proteins from Washed Cod Muscle after Extraction or Reprecipitation in the Presence of Sodium Chloride[a]

added NaCl (ionic strength)	solubility (%)	
	extraction[b]	precipitation[c]
0	96.5 ± 3.0[a]	96.5 ± 3.0[a]
0.0002	23.0 ± 2.0[a]	24.7 ± 2.0[a]
0.0004	17.5 ± 1.0[a]	20.7 ± 1.0[b]
0.001	15.0 ± 1.0[a]	15.9 ± 1.0[a]
0.01	6.4 ± 1.0[a]	6.9 ± 1.0[a]

[a] Values in the same row having the same letter are not significantly different ($p < 0.05$). [b] In the extraction experiments, the procedure for determining solubility was as described under Materials and Methods. [c] In the precipitation experiments, the proteins were first solubilized; NaCl was then added to the solution to give the indicated ionic strength before centrifugation.

Effect of Salt on Protein Solubility. When the ionic strength of the extracting solution was decreased sufficiently, essentially all the proteins of washed, minced cod muscle were soluble. The solubility observed in water decreased rapidly with small increases in ionic strength and reached a minimum in the range of 0.025 to 0.15. At ionic strengths greater than this, the traditional salting-in effect was seen which reached a maximum at an ionic strength of approximately 0.75; protein solubility then decreased at higher salt concentrations (Figure 1). In these studies, solubility at very low ionic strengths was always much greater than that at high ionic strengths. It is possible that the low protein solubility at high salt concentrations is due to denaturation of the fish proteins under these latter conditions (Dyer *et al.*,1950; Kelleher and Hultin, 1991). We did not take any special precautions such as eliminating foaming and keeping the temperature below 5 °C to protect the fish muscle proteins from denaturation by sodium chloride in these experiments as recommended by Woyewoda *et al.* (1986).

A comparison was made between the effect of sodium chloride in preventing solubilization of proteins during the extraction step and reprecipitating the muscle proteins once they had been solubilized. In the first case, the salt was added in the extracting solution. In the second case, the proteins were first solubilized and the appropriate amount of salt added prior to centrifugation to separate the soluble and insoluble proteins. Similar solubilities were obtained as a function of sodium chloride concentration under these conditions (Table 3) indicating that the process of solubilization is entirely reversible.

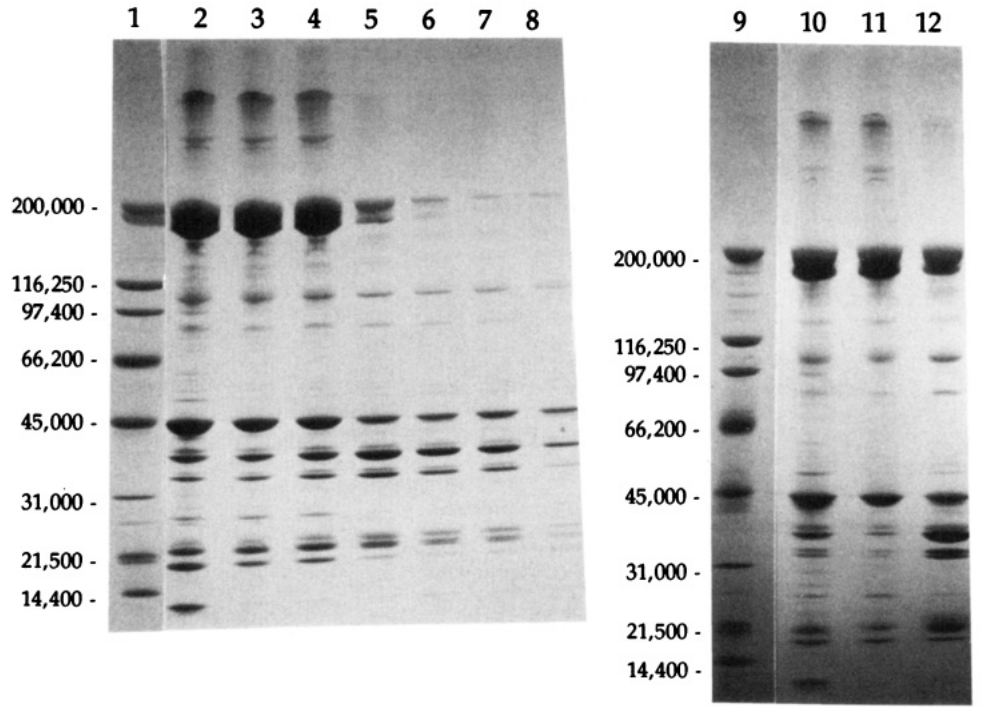

Figure 2. Cod muscle proteins analyzed by SDS gel electrophoresis. Lanes 1–4 represent molecular weight markers (1), ground cod muscle (2), cod muscle washed three times in water (3), and soluble proteins after extraction with deionized, distilled water (4); these samples were loaded on the gel on an equal protein basis (35 μg). Lanes 5–8 represent soluble proteins (supernate) after extraction of the washed mince with solutions containing 0.2–10 mM added sodium chloride. These samples were all loaded onto the gel at the same volume (30 μL) that was used for the sample in lane 4. Thus, they contained decreasing protein contents representing the decrease in solubility with increasing concentration of salt: (lane 5) 0.2 mM added NaCl; (lane 6) 0.4 mM added NaCl; (lane 7) 1.0 mM added NaCl; (lane 8) 10.0 mM added NaCl. Lanes 11 and 12 are soluble and insoluble fractions after reprecipitation of solubilized proteins from washed cod mince by addition of 0.2 mM NaCl; molecular weight markers (lane 9); muscle 15 μg (lane 10); insoluble fraction 15 μg (lane 11); soluble fraction 15 μg (lane 12).

SDS–PAGE patterns of some of these treatments are shown in Figure 2. Lane 2 represents the proteins of whole cod muscle, while lane 3 shows the proteins present in the washed, minced tissue. The major proteins present in the washed mince were also in the whole muscle although there were some that were removed by the washings. Lane 4 shows the pattern obtained from the muscle proteins that were solubilized in water. The pattern in lane 4 is the same as that of the washed minced muscle tissue (lane 3) indicating that all of the proteins of the washed mince muscle were solubilized under these conditions.

Lanes 5–8 are the proteins extracted into solutions of increasing salt concentrations from 0.2 to 10 mM added sodium chloride. A sharp decrease in extractability is shown, and there is a selectivity in the solubilization of the minced muscle proteins with increasing salt concentration. Lane 10 shows the pattern of the whole muscle again and is compared to the patterns obtained from the insoluble and soluble fractions in lanes 11 and 12, respectively, after previously solubilized proteins from washed cod mince were reprecipitated by the addition of 0.2 mM sodium chloride. Comparison of lanes 11 and 12 shows that there was selectivity in the reprecipitation of proteins on adding salt. The high molecular weight components thought to be titin and nebulin were recovered completely and apparently intact by this procedure. These proteins are difficult to solubilize and separate at high salt concentrations (Wang, 1982; Nave *et al.*, 1990).

A quantitative estimate of the individual proteins solubilized at various equivalent concentrations of sodium chloride was made (Table 4). The individual fractions have been compared to the control sample extracted in water (ionic strength equal to approximately 0.0002) as 100%. The two high molecular weight proteins showed no solubility even at the lowest concentration of added salt. These two high molecular mass proteins were tentatively identified as titin and nebulin by similarity in size to titin and nebulin from chicken breast muscle (Locker and Wild, 1986; Hu *et al.*, 1986). The solubility of myosin was also very sensitive to low concentrations of salt as was the 25 kDa component. On the other hand, actin (43 kDa) and tropomyosin (35.5 kDa) retained considerable solubility at an added salt concentration of 1 mM.

A quantitative estimate was made of the proteins removed from the minced cod muscle at each wash (Table 5). Those proteins that had been shown previously to be sensitive to precipitation by very low concentrations of salt were, for the most part, little removed during the washing process, e.g., see data for titin, nebulin, myosin HC, C-protein, and the 25 kDa peptide. This is most likely due to the fact that the salt concentration was not lowered sufficiently until the final extraction in water to render these proteins soluble.

The effect of several different chloride salts, and one iodide salt, on solubility of proteins from washed minced cod muscle was determined (Table 6). The general patterns of solubility were similar. The major effect of different cations was with magnesium and calcium chlorides which at the higher concentrations reduced the solubility of the muscle proteins. It is possible that this effect was related to pH. In the unbuffered systems which comprised the final washed sediment, small changes in proton concentrations could cause very large changes in pH. Magnesium and calcium chlorides reduced the pH when added at an ionic strength of 0.01

Table 4. Effect of Sodium Chloride on Solubility of Different Myofibrillar Components from Washed Minced Cod Muscle

approx molec mass (kDa)	component[a]	peptides remaining soluble (%) at final equiv NaCl of 0.4 mM	0.6 mM	1.1 mM	10 mM
2800	titain	<4			
600	nebulin	<4			
200	myosin HC	13.0 ± 4.0	3.1 ± 1.0	0.7 ± 0.2	1.3 ± 0.4
155		9.3 ± 3.1	8.4 ± 1.2	6.2 ± 2.1	
130	C-protein	17.6 ± 10.3	9.2 ± 1.0		
105		30.4 ± 7.8	24.7 ± 5.5	17.8 ± 5.2	15.2 ± 4.0
83		32.0 ± 2.1	29.6 ± 8.9	21.3 ± 13.1	
43	actin	29.8 ± 5.7	27.3 ± 5.0	24.7 ± 5.2	16.3 ± 3.4
37		77.0 ± 11.4	40.6 ± 6.5	40.0 ± 6.5	
35.5	tropomyosin	122.9 ± 27.1	98.3 ± 20.6	79.3 ± 20.0	29.3 ± 7.3
33		91.4 ± 10.8	81.2 ± 26.6	60.9 ± 7.2	13.7 ± 6.3
32		136.7 ± 12.1	96.2 ± 18.0	76.0 ± 26.6	9.1 ± 6.3
25	myosin LCl	<4%			
19.5	myosin LC2	50.8 ± 8.5	32.6 ± 4.5	27.2 ± 5.5	9.1 ± 3.1
18	myosin LC3	19.5 ± 2.0	9.7 ± 1.0	8.8 ± 1.1	8.8 ± 1.1

[a] Tentative identification. [b] Percentage solubility is compared to the peptides obtained by extraction of washed minced muscle in water taken as 100%. Percentages greater than 100% indicate the protein is more soluble in that salt concentration than in water.

Table 5. Proteins Removed from Minced Cod Muscle by Three Washes in Water (w:v/1:3)

molec mass (kDa)	component[a]	muscle (mg)	first wash (mg)	second wash (mg)	third wash (mg)	% removed
2800	titin	579 ± 44				0
600	nebulin	205 ± 37				0
200	myosin HC	2707 ± 144				0
155		212 ± 18	6 ± 1	7 ± 1	5 ± 1	9 ± 1
130	C-protein	84 ± 9				0
105		274 ± 36	11 ± 1	6 ± 1	10 ± 2	10 ± 1
95		60 ± 16	30 ± 4	9 ± 1	4 ± 1	72 ± 20
83		59 ± 6	13 ± 1	3 ± 1	1 ± 1	29 ± 3
60		30 ± 4	17 ± 1	5 ± 1	2 ± 1	80 ± 11
57		23 ± 6	9 ± 1	3 ± 1	2 ± 1	61 ± 16
53.5		25 ± 7	7 ± 1	2 ± 1	1 ± 1	40 ± 12
51		75 ± 11	36 ± 3	10 ± 1	4 ± 1	67 ± 11
43	actin	1610 ± 133	332 ± 32	102 ± 12	45 ± 2	30 ± 3
37		134 ± 41	70 ± 4	5 ± 1	2 ± 1	57 ± 18
35.5	tropomyosin	457 ± 34	14 ± 1	9 ± 1	26 ± 7	11 ± 2
33		81 ± 30			3 ± 2	4 ± 3
32		128 ± 9			9 ± 3	7 ± 2
29		47 ± 4	14 ± 1	4 ± 1	2 ± 1	43 ± 4
26.5		42 ± 4	16 ± 5	4 ± 1	2 ± 1	52 ± 13
25	myosin LC1	81 ± 7				0
23		19 ± 1	17 ± 2	3 ± 2	1 ± 1	105 ± 17
19.5	myosin LC2	366 ± 32			11 ± 4	3 ± 1
18	myosin LC3	420 ± 4	146 ± 24	46 ± 12	18 ± 1	50 ± 6
13		291 ± 32	210 ± 31	67 ± 20	23 ± 1	103 ± 17
total		8011 ± 220	946 ± 51	287 ± 27	158 ± 10	
% removed			11.8 ± 0.7	3.6 ± 0.4	2.0 ± 0.1	17.4 ± 0.8

[a] Tentative identification.

Table 6. Effectiveness of Different Salts on Precipitating Solubilized Proteins of Washed Cod Mince[a]

salt	solubility (%) at added ionic strength of 0.0001	0.0002	0.001	0.01
NaCl	32.0 ± 3.0[a]	25.3 ± 4.0[a]	21.8 ± 3.0[a]	13.6 ± 1.0[a]
KCl	30.7 ± 1.0[a]	26.0 ± 1.0[a]	19.8 ± 1.0[a]	13.2 ± 1.0[a]
LiCl	31.8 ± 1.0[a]	32.1 ± 2.0[b]	21.4 ± 2.0[a]	13.6 ± 1.0[a]
NaI	32.0 ± 3.0[a]	25.9 ± 2.0[a]	19.8 ± 3.0[a]	12.9 ± 1.0[a]
$MgCl_2$	29.2 ± 1.0[a]	24.4 ± 1.0[a]	16.7 ± 1.0[b]	4.8 ± 1.0[b]
$CaCl_2$	30.4 ± 3.0[a]	26.4 ± 1.0[a]	16.3 ± 1.0[b]	4.1 ± 1.0[b]

[a] Values in the same column having the same letter are not significantly different ($p < 0.05$).

by about 0.2 unit, roughly 6.5 compared to 6.7. This difference in pH was possibly caused by the strong interactions between the muscle protein side groups and the divalent cations releasing more protons than did the monovalent cations. This decrease in pH would probably be sufficient to cause the differences in solubility observed (see Figure 3).

Effect of pH on Protein Solubility. The effect of pH on the removal of the minced muscle proteins from solution after prior solubilization into water is shown in Figure 3. In these experiments pH was reduced by the addition of hydrochloric acid. Presumably, the protons from the acid displaced bound cations as the concentration of the acid was increased. This led to an increase in ionic strength (measured by conductivity and expressed as sodium chloride concentration) as the pH was lowered. At a pH of 4.3, over 80% of the muscle proteins remained soluble even though the ionic strength approached an equivalent sodium chloride concentration of 0.7 mM, a concentration which would lead to less than 20% solubility at neutral pH. The decrease in solubility as the pH was lowered from 7 to 6.5 was large, and gradually leveled off as the pH was decreased from 6.5

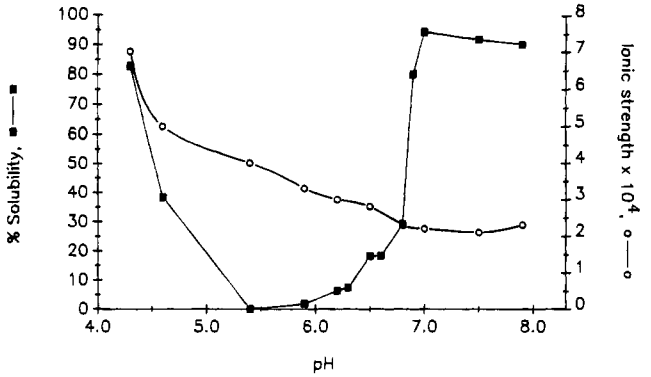

Figure 3. Effect of pH on solubility of myofibrillar proteins from cod muscle: solubility (■); ionic strength (○). Solubility is related to the total protein in the washed muscle.

Table 7. Effect of pH on Precipitation of Different Myofibrillar Components Extracted from Washed Minced Cod Muscle

		peptides remaining soluble[b] (%) at			
approx molec mass (kDa)	component[a]	pH 7.04	pH 6.90	pH 6.83	pH 6.52
2800	titin	100	75	22	0
600	nebulin	100	62	0	0
200	myosin HC	100	90	42	18
155		100	76	23	11
130	C-protein	100	68	14	0
105		100	85	27	12
95					
83		100	69	32	23
43	actin	100	76	29	24
37		100	80	51	27
35.5	tropomyosin	100	92	84	68
33		100	83	65	29
32		100	85	78	50
25	myosin LC1	100	68	0	0
24					
21		100	97	85	76
19.5	myosin LC2	100	87	49	37
18	myosin LC3	100	82	33	23

[a] Tentative identification. [b] Percentage solubility is compared to the peptides obtained by extraction of washed minced muscle in water (pH 7.04) taken as 100%.

to 5.5. At this latter pH, there was no measurable solubility.

A quantitative assessment of the individual peptides that were removed from solution as the pH was reduced from 7 to 6.5 was made and expressed in terms of the quantity of the soluble components taken as 100% at pH 7 (Table 7). The data in this table represent a single experiment and are used to show that there is a selective solubilization of different proteins at different pH values. This selectivity has some similarity to that shown in Table 4 for the effect of salt, but there are some differences as well. Experimental data obtained by precipitation of the proteins solubilized by water extraction on reducing the pH were very difficult to replicate exactly. A sharp decrease in solubility was consistently seen as the pH was lowered from neutrality or slightly above to a lower pH, but the point at which this happened varied from preparation to preparation. The reason for this is not known. However, in the unbuffered muscle system small changes in either ionic strength and/or pH had very marked effects on percent soluble proteins. It was difficult to control these conditions due to the difficulty in reproducing washing conditions and swelling of the minced muscle.

Effect of Post-Mortem Age on Protein Solubility. The post-mortem age of the cod muscle used in the studies described above generally varied between 4 and 48 h with an average of about 24 h. We were interested in what the effect of longer post mortem times on solubility would be. Percent solubility as a function of time post mortem for a period of up to approximately 9 days is given in Table 8. The samples were from a batch of cod that were approximately 24 h post-mortem when received. The 0 h sample was obtained within 10 min from a fish that was brought live to the laboratory. Protein solubility was high in all samples. Solubility of the proteins of muscle tissue removed from the fish and washed within 10 min of death appeared to be somewhat lower than the others. However, insufficient samples were done under these conditions to determine if there was an actual difference. Samples of live cod are difficult and expensive to obtain. The electrophoretic pattern of the soluble proteins from the 0 h sample is the same as that ordinarily observed with the exception that the titin band was a little less smudged, although it still existed as a doublet (Small *et al.*, 1992). The effect of increasing concentrations of sodium chloride on protein solubility of the same samples was also determined (Table 8). There were modest increases in protein solubility from aged muscle at these low salt concentrations over the period from 30 to 216 h. An increase in protein solubility in low ionic strength medium of chicken and beef myofibrillar proteins with post mortem storage time has also been observed (Stanley *et al.*, 1993).

DISCUSSION

The need for sodium chloride in a concentration range of 0.3–0.6 M to solubilize myofibrillar proteins is widely quoted (Hultin, 1985; Bodwell and McClain, 1978). The classic procedure for the solubilization of myofibrillar proteins has been to dissolve them in a salt solution varying from roughly 0.4 to 0.6 M at neutral or slightly acid or alkaline pH with or without compounds such as ATP and magnesium salts. The solubilization is thought to occur in two steps; first, there is depolymerization of the thick filaments followed by dissociation of actin from myosin (Parsons and Knight, 1990). These authors showed that at high salt concentrations thick filaments from different muscle fibers may have different solubilities. The difference is likely due to the different isoforms of myosin which are specific to the function of the muscle. Martinez *et al.* (1990) found four isoforms of myosin in the muscle of Atlantic cod.

The salting-in phenomenon has been ascribed to nonspecific electrostatic interactions between charged proteins and the ionic environment. This leads to a net decrease in the activity coefficient of the protein and gives a net solubilization (von Hippel and Schleich, 1969). This process is generally independent of ion type. Salting-out, on the other hand, is strongly influenced by ion type. It occurs at higher salt concentrations and is generally ascribed to the loss of a stable hydrophilic surface, causing the exposed hydrophobic areas of proteins to interact, inducing aggregation and precipitation. Salts that increase the solubility of proteins also tend to denature them (see, for example, von Hippel and Schleich, 1969; Arakawa *et al.*, 1990a,b). There have been many fewer studies on the effects of low salt concentrations on proteins. A variety of effects of low salt concentrations related to enzyme inhibition and salt-binding have been discussed by Jencks (1969), who indicated that effects on rates of reactions of charged

Table 8. Effect of Post-Mortem Time on Solubility of Washed Minced Cod Muscle Proteins[a]

time post-mortem (h)	muscle pH	equiv final NaCl[b] (mM)	solubility (%) at added NaCl concn of			
			0 mM	0.5 mM	1.0 mM	10.0 mM
0[c]	7.1 ± 0.2	0.19 ± 0.02	87.8 ± 6.0[a]			
30	6.8 ± 0.2	0.16 ± 0.02	94.5 ± 4.0[a,b]	27.3 ± 0.8[a]	24.2 ± 0.6[a]	12.1 ± 1.0[a]
74	6.9 ± 0.2	0.14 ± 0.02	97.7 ± 2.0[a,b]	26.3 ± 1.8[a]	22.9 ± 0.7[b]	14.8 ± 1.9[a,b]
146	7.1 ± 0.2	0.22 ± 0.02	99.0 ± 1.0[b]	30.9 ± 1.6[b]	24.5 ± 0.8[a]	15.4 ± 1.3[b]
216	7.4 ± 0.2	0.22 ± 0.02	93.7 ± 2.0[a]	32.5 ± 0.4[b]	25.1 ± 0.5[a]	20.1 ± 0.8[d]

[a] Values in the same column having the same letter are not significantly different ($p < 0.05$). [b] Concentration when no salt is added, estimated by conductivity. [c] Muscle ground and washed within 10 min of death for the 0 time sample. Other samples were from a different batch of cod obtained at about 24 h post mortem.

substrates can usually be accounted for by the Debye–Hückel theory at low salt concentrations, i.e., it is an ionic strength effect.

In spite of the prevailing belief of the requirement of relatively high salt concentrations for solubilization of myofibrillar proteins, sporadic reports have appeared in the literature indicating that some of the myofibrillar proteins are soluble in water. As early as 1947, Albert Szent-Györgyi showed that rabbit myosin was soluble in water and insoluble in very low concentrations of salt, although he indicated that salt was needed to extract the contractile proteins from the muscle (Szent-Györgyi, 1947). Matsumoto (1959) reported on the water solubility of an actomyosin-like protein obtained by aqueous extraction of squid mantle, and Stanley (1970) found that the proteins of horseshoe crab muscle cells could be solubilized in 1 mM Tris solution at pH 7.5 by extraction with a large volume; he suggested that four factors were involved in the solubility of horseshoe crab actomyosin: degree of purity, ionic strength, concentration of protein, and the presence of low molecular weight substances such as calcium and magnesium ions and ATP. Wilkinson *et al.* (1972) solubilized the inhibitory-factor proteins (troponin and tropomyosin) from muscle by exhaustive dialysis against water. Trevino *et al.* (1990) observed about 55% protein solubility from sardine surimi when extracted with water at pH 9. In all cases where myofibrillar protein solubility was observed, it can be supposed that the ionic strength was reduced to a low value by the extent of washing or dialysis involved.

In this paper we show that essentially all of the proteins of cod muscle became soluble if the ionic strength was sufficiently reduced. A possible explanation for the phenomenon that is consistent with the data of the effects of ionic strength and pH is that the myofibrillar proteins have negative charges at neutral pH. In water, or solutions of very low ionic strength, the repulsive forces from these negatively charged side chains are sufficient to drive the individual protein molecules apart when sufficient water is made available. Conditions that reduce the repulsive forces allow the proteins to associate, most probably driven by hydrophobic interactions. The proteins can then be removed from solution by an appropriate centrifugal force. Reduction of the repulsive forces can be achieved by lowering the pH to values near the isoelectric point(s) of the proteins. Increasing salt concentrations accomplish the same result by shielding the negative charges by the cationic component of the salt. Solubilization of the myofibrillar proteins by decreasing the pH to below the isoelectric points of the proteins would reinstitute a repulsive force between the molecules due to overall positive charges. This theory would predict a close association between the effects of salt and pH.

Solubilization of cod muscle myofibrillar proteins was not caused by extensive hydrolysis. This was shown by several facts. First, very low concentrations of sodium chloride applied just at the extraction step inhibited the solubilization. It is very unlikely that this level of sodium chloride added during the extraction step, inhibited a proteolytic activity which caused solubilization. Further, the proteins once solubilized in water by extraction into deionized, distilled water could be reprecipitated at salt concentrations exactly analogous to those that would prevent solubilization initially. Finally, there appeared to be no apparent loss of peptides before and after extraction into water as seen by the patterns formed on SDS–polyacrylamide gel electrophoresis. We cannot rule out the possibility that some low hydrolytic activity against critical proteins may pave the way for solubilization of the rest of the proteins.

The need to reduce the ionic strength of the extracting solution to a value low enough to permit solubilization of the cod muscle proteins is clear. The point not answered by the data presented in this paper is whether any of the proteins that are removed during the washing steps must be removed for the myofibrillar proteins to become soluble. The point is mentioned because we have observed that in two other types of muscle, chicken breast muscle and the ordinary muscle of Atlantic mackerel, removal of specific proteins is required for all of the myofibrillar proteins to be solubilized (unpublished observations). Comparable proteins were removed in the washes of the minced cod muscle, e.g., the polypeptide with molecular mass of approximately 95 kDa. Those proteins which were easily precipitated by very low concentrations of sodium chloride were not extracted from the tissue during the washing procedure.

The total amount of protein removed in the washing steps was lower than would be expected from the expected content of sarcoplasmic proteins reported in fish muscle tissue (Nakagawa *et al.*, 1988). There are probably at least two reasons for this. The muscle tissue was not homogenized until the final extraction step and thus consisted of some relatively large cell segments. The washing times were not sufficiently long to allow for complete equilibration between the washing solution and the water-soluble components within these segments as noted by the discrepancy between the actual salt concentration found in the tissue and that predicted based on dilution with the wash water. It would be expected that the diffusion rate of the soluble proteins out of the muscle cell segments would be no greater than that of the salts and, therefore, would not reach equilibrium with the wash water. The other factor is that as the washes progressed, the ionic strength was lowered. Many of the glycolytic proteins are bound to subcellular structures at low ionic strengths (Kurganov, 1985; Morton *et al.*, 1988) and would not have been extracted in the washes. The experiments mimicked the process for producing surimi.

Results of selective extractability at increasing concentrations of sodium chloride showed that the myofibrillar proteins behaved independently. That is, the solubility of the individual proteins appeared to depend only on the concentration of salt and the pH of the extracting solution. It is not possible to say at this time with any certainty that the proteins are extracted as individual entities. It is difficult to describe the process of solubilization with myofibrillar proteins, because in addition to protein–protein and protein–solvent interactions, there are most likely conformational changes and polymerization–depolymerization shifts. The forces that determine whether protein molecules are stable in solution are also those that are involved in polymerization and depolymerization of the proteins (filament or monomer formation). Associations of these proteins in solutions of low ionic strength and the possibility of reformation of any structures on reprecipitation of the proteins are questions that deserve further research. There is, perhaps, a suggestion that some proteins do form complexes at low ionic strength. Most of the myosin heavy chain was removed by the addition of 0.2 mM sodium chloride giving a final ionic strength equivalent to approximately 0.4 mM. Under these conditions, there was a large loss in soluble actin content, about 70% of the total. Further precipitation of actin with increasing salt concentrations was much less. It is possible that precipitation of the myosin removed some of the initial actin because it formed a complex with the actin either in the monomeric or polymeric form. Approximately 3 mol of actin was removed with every mole of myosin under our conditions as determined by our method of peptide estimation on the electropherograms.

Understanding the solubility characteristics of muscle myofibrillar proteins is important since solubility may be related to functional properties. It has been widely suggested, for example, that solubilization of myosin and actomyosin is necessary for gel formation (Suzuki, 1981; Lee, 1984, 1986). Thus, an understanding of the one may lead to a better understanding of the other with the potential for better process control. The selective inhibition of solubilization and the selective precipitation of the muscle proteins as a function of salt concentration could be the basis for isolation and fractionation of myofibrillar proteins. Solubilization of myofibrillar proteins was higher at low salt concentrations than it was at high. Preliminary results also indicated that the proteins were much more stable in the absence of high concentrations of sodium. Some of the myofibrillar proteins such as titin and nebulin have been reported to be difficult to extract and are unstable at high salt concentrations (Wang, 1982; Nave *et al.*, 1990). Our results indicated they are reversibly soluble in water. Fractionation may also be a way to improve the functional properties of the muscle proteins. For example, both C-protein (Yamamoto *et al.*, 1987) and tropomyosin (Sano *et al.*, 1989) interfere with the process of gelation. Selective removal of these proteins might serve as the basis for improving gelation characteristics of the remaining proteins. Further advantages will become apparent as more information on the mechanism of solubilization and the properties of the proteins at reduced salt concentrations becomes available.

ACKNOWLEDGMENT

This work was supported in part by the Massachusetts Agricultural Experiment Station and by the MIT Sea Grant College Program (Project RB-32) under Federal Grant NA90AA-D-SG424 from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce.

LITERATURE CITED

Anon. "Instruction for Coomassie protein assay"; Pierce Chemical Co., Rockford, IL, 1991.

Arakawa, T.; Bhat, R; Timasheff, S. N. Preferential interactions determine protein solubility in three-components solutions: the $MgCl_2$ system. *Biochemistry* **1990a**, *29*, 1914–1923.

Arakawa, T.; Bhat, R.; Timasheff, S. N. Why preferential hydration does not always stabilize the native structure of globular proteins. *Biochemistry* **1990b**, *29*, 1924–1931.

Bodwell, C. E.; McClain, P. E. Chemistry of animal tissues, proteins. In *The Science of Meat and Meat Products*; Price, J. F., Schweigert, B. S., Eds.; Food and Nutrition Press: Westport, CT, 1978; pp 78–133.

Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

Dyer, W. J.; French, H. V.; Snow, J. M. Proteins in fish muscle. Extraction of protein fractions in fish flesh. *J. Fish. Res. Bd. Can.* **1950**, *7*, 585–593.

Fritz, J. D.; Swartz, D. R.; Greaser, M. L. Factors affecting polyacrylamide gel electrophoresis and electroblotting of high-molecular-weight myofibrillar proteins. *Anal. Biochem.* **1989**, *180*, 205–210.

Hainfeld, J. F.; Wall, J. S.; Wang, K. Titin: Quantitative mass measurements by scanning transmission electron microscopy and structural implications for the sarcomere matrix of skeletal muscle. *FEBS Lett.* **1988**, *234*, 145–148.

Hames, B. D. An introduction to polyacrylamide gel electrophoresis. In *Gel Electrophoresis of Proteins: A Practical Approach*; Hames, B. D., Rickwood, D., Eds.; IRL Press: Oxford, U.K., 1982; pp 1–91.

Hennigar, C. J.; Buck, E. M.; Hultin, H. O.; Peleg, M.; Vareltzis, K. The effect of washing and sodium chloride on mechanical properties of fish muscle gels. *J. Food Sci.* **1988**, *53*, 963–964.

Hennigar, C. J.; Buck, E. M.; Hultin, H. O.; Peleg, M.; Vareltzis, K. Mechanical properties of fish and beef gels prepared with and without washing and sodium chloride. *J. Food Qual.* **1989**, *12*, 155–166.

Hermansson, A.-M.; Harbitz, O.; Langton, M. Formation of two types of gels from bovine myosin. *J. Sci. Food Agric.* **1986**, *37*, 69–84.

Hu, D. H.; Kimura, S.; Maruyama, K. Sodium dodecyl sulfate gel electrophoresis studies of connectin-like high molecular weight proteins of various types of vertebrate and invertebrate muscles. *J. Biochem.* **1986**, *99*, 1485–1492.

Hultin, H. O. Characteristics of muscle tissue. In *Food Chemistry;* Fennema, O. R., Ed.; Dekker: New York, 1985; pp 725–789.

Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, 1969.

Kelleher, S. D.; Hultin, H. O. Lithium chloride as a preferred extractant of fish muscle proteins. *J. Food Sci.* **1991**, *56*, 315–317.

Kurganov, B. I. Control of enzyme activity in reversibly adsorptive enzyme systems. In *Organized Multienzyme Systems: Catalytic Properties*; Welch, G. R., Ed.; Academic: New York, 1985; pp 241–270.

Kurzban, G. P.; Wang, K. Giant polypeptides of skeletal muscle titin: sedimentation equilibrium in guanidine hydrochloride. *Biochem. Biophys. Res. Commun.* **1988**, *150*, 1155–1161.

Kvanli, A. H. *Statistics. A Computer Integrated Approach;* West Publishing: San Francisco, 1988.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophase T4. *Nature* **1970**, *227*, 680–685.

Lee, C. M. Surimi processing technology. *Food Technol.* **1984**, *38*, 69–80.

Lee, C. M. Surimi manufacturing and fabrication of surimi-based products. *Food Technol.* **1986**, *40*, 115–124.

Locker, R. H.; Wild, D. J. C. A comparative study of high molecular weight proteins in various types of muscle across the animal kingdom. *J. Biochem.* **1986**, *99*, 1473–1484.

Martinez, I.; Ofstad, R.; Olsen, R. L. Electrophoretic study of myosin isoforms in white muscles of some teleost fishes. *Comp. Biochem. Physiol.* **1990**, *96B*, 221–227.

Matsumoto, J. J. Identity of M-actomyosin from aqueous extract of the squid muscle with the actomyosin-like protein from salt extract. *Bull. Jpn. Soc. Sci. Fish.* **1959**, *25*, 38–43.

Morton, D. J.; Weidemann, J. F.; Clarke, F. M. Enzyme binding in muscle. In *Developments in Meat Science–4*; Lawrie, R., Ed.; Elsevier Applied Science: London, 1988; 37–61.

Nakagawa, T.; Watabe, S.; Hashimoto, K. Electrophortic analysis of sarcoplasmic proteins from fish muscle on polyacrylamide gels. *Nippon Suisan Gakkaishi* **1988**, *54*, 993–998.

Nave, R.; Fürst, D. O.; Weber, K. Interaction of α-actinin and nebulin in vitro. Support for the existence of a fourth filament system in skeletal muscle. *FEBS Lett.* **1990**, *269*, 163–166.

Niwa, E.; Yamada, H.; Kanoh, S.; Nakayama, T. Thermal behavior of actomyosin subunits during setting of salted fish flesh sol. *Nippon Suisan Gakkaishi* **1989**, *55*, 1997–2000.

Parsons, N.; Knight, P. Origin of variable extraction of myosin from myofibrils treated with salt and pyrophosphate. *J. Sci. Food Agric.* **1990**, *51*, 71–90.

Sano, T.; Noguchi, S. F.; Tsuchiya, T.; Matsumoto, J. J. Contribution of tropomyosin to fish muscle gel characteristics. *J. Food Sci.* **1989**, *54*, 258–268.

Shimizu, Y. Biochemical and functional properties of material fish. In *Proceedings of the International Symposium on Engineering Seafood Including Surimi*; Martin, R. E., Collette, R. L., Eds.; National Fisheries Institute: Washington, DC, 1985; pp 148–167.

Small, J. V.; Fürst, D. O.; Thornell, L.-E. The cytoskeletal lattice of muscle cells. *Eur. J. Biochem.* **1992**, *208*, 559–572.

Stanley, D. W. Actomyosin solubility and skeletal muscle cell emptying of horseshoe crab. *Comp. Biochem. Physiol.* **1970**, *36*, 279–284.

Stanley, D. W.; Stone, A. P.; Hultin, H. O. Solubility of vertebrate muscle myofibrillar proteins in low ionic strength media. *J. Agric. Food Chem.* **1994**, *42*, 863–867.

Suzuki, T. *Fish and Krill Protein: Processing Technology;* Applied Science Publishers: London, 1981.

Szent-Györgyi, A. *Chemistry of Muscular Contraction;* Academic: New York, 1947.

Trevino, B.; Moreno, V.; Morrissey, M. Functional properties of sardine surimi related to pH, ionic strength and temperature. In *Advances in Fish Technology and Biotechnology for Increased Profitability*; Voigt, M. N., Botta, J. R., Eds.; Technomic Publishing: Lancaster, PA, 1990; pp 413–422.

Vareltzis, K.; Buck, E. M.; Hultin, H. O.; Laus, M. J. Fish gel formation without added salt: improvement via mixed species. *J. Food Process. Preserv.* **1989**, *13*, 107–121.

von Hippel, P. H.; Schleich, T. The effects of neutral salts on the structure and conformational stability of macromolecules in solution. In *Structure and Stability of Biological Macromolecules*; Timasheff, S. N., Fasman, G. D., Eds.; Dekker: New York, 1969; pp 417–574.

Wang, K. Purification of titin and nebulin. *Methods Enzymol.* **1982**, *85*, 269–274.

Wicker, L.; Lanier, T. C.; Knopp, J. A.; Hamann, D. D. Influence of various salts on heat-induced ANS fluorescence and gel rigidity development of Tilapia (*Serotherodon aureus*) myosin. *J. Agric. Food Chem.* **1989**, *37*, 18–22.

Wilkinson, J. M.; Perry, S. V.; Cole, H. A.; Trayer, I. P. The regulatory proteins of the myofibril. Separation and biological activity of the components of inhibitory factor preparations. *Biochem. J.* **1972**, *127*, 215–228.

Woyewoda, A. D.; Shaw, S. J.; Ke, P. J.; Burns, B. G. Recommended laboratory methods for assessment of fish quality. *Can. Tech. Rep. Fish. Aquat. Sci.* **1986**, *No. 1448*, 73–76.

Wu, Y.-J.; Atallah, M. T.; Hultin, H. O. The proteins of washed, minced fish muscle have significant solubility in water. *J. Food Biochem.* **1991**, *15*, 209–218.

Xiong, Y. L.; Brekke, C. J. Physicochemical and gelation properties of pre- and postrigor chicken salt-soluble proteins. *J. Food Sci.* **1990**, *55*, 1544–1548.

Yamamoto, K.; Samejima, K.; Yasui, T. The structure of myosin filaments and the properties of heat-induced gel in the presence and absence of C-protein. *Agric. Biol. Chem.* **1987**, *51*, 197–203.

Received for review December 1, 1993. Revised manuscript received September 14, 1994. Accepted September 28, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1994.