Solubility of Chicken Breast Muscle Proteins in Solutions of Low Ionic Strength

Ganesan Krishnamurthy,† Hsin-Sui Chang, Herbert O. Hultin,* Yuming Feng, Subramanian Srinivasan,‡ and Stephen D. Kelleher

Massachusetts Agricultural Experiment Station, Department of Food Science, University of Massachusetts, Amherst, Massachusetts 01003

Essentially all of the proteins of chicken breast muscle were soluble in sodium chloride solutions of physiological ionic strength or less and neutral pH. However, there was a critical order of treatment necessary to accomplish this. After removal of those proteins solubilized by homogenizing the tissue 1:10 (w/v) in water, it was necessary to solubilize a fraction of the remaining proteins in moderate concentrations (25−150 mM) of sodium chloride at neutral pH before the remaining proteins could be solubilized in water. Solubilization of chicken breast muscle proteins in water could be prevented or reversed by salt solutions of low concentration, suggesting that most of the solubility was not directly caused by proteolysis. SDS−polyacrylamide gel electrophoresis indicated that removal of specific peptides by moderate concentrations of sodium chloride at neutral pH was correlated with removal of the restriction on the water solubility of the remaining proteins.

Keywords: *Protein solubility; solubility of muscle proteins; chicken muscle protein solubility*

INTRODUCTION

The interaction of muscle proteins with water or among themselves is an important factor in the functional properties of meat products. Some of the factors that modify these interactions include pH, salt, and protein concentrations. Of these, the use of sodium chloride has been the most important for modifying the functional properties of comminuted muscle foods. Many of the desirable features of muscle foods have been attributed to solubilization of the myofibrillar proteins at high concentrations of sodium chloride (>0.3 M) (Hultin *et al.*, 1995). We have recently shown that essentially all of the proteins of cod muscle are soluble after washing in water and extracting in a solution of very low ionic strength (0.3 mM or less) (Stefansson and Hultin, 1994).

It was of interest to determine whether the muscle proteins of homeothermic species could also be solubilized at low ionic strength. Studies conducted several years ago to prepare sarcolemma from chicken breast muscle suggested that the myofibrillar proteins were soluble in solutions of low ionic strength, although careful evaluation of protein solubility was not done. Treatment of cell segments of chicken breast muscle with calcium chloride (0.5−50 mM) followed by washes with buffered sodium chloride solutions (25 mM) caused the material inside the muscle cell segments to be removed on dilution in slightly alkaline solutions of low ionic strength (Westort and Hultin, 1966; Stanley and Hultin, 1968). The purpose of the experiments reported here was to evaluate the solubility of chicken breast muscle proteins in solutions of low ionic strength.

* Address correspondence to this author at the University of Massachusetts Marine Station, P.O. Box 7128, Gloucester, MA 01930 [fax (508) 281-2618].

† Present address: Cains Foods, Inc., South Deerfield, MA 01373.

‡ Present address: Department of Animal Sciences, University of Kentucky, Lexington, KY 40546-0215.

MATERIALS AND METHODS

Materials. Adult female hens were obtained from the Department of Veterinary and Animal Sciences at the University of Massachusetts/Amherst. Mixed breeds were used. The birds were sacrificed by carbon dioxide asphyxiation. Immediately thereafter the skin around the breast muscles was cut open and the breast muscles removed. They were then wrapped in plastic film, immediately placed on ice, and removed to our laboratory where processing began. Connective tissue was removed to the extent possible, and the remaining muscle was cut into cubes of approximately 1−1.5 cm prior to homogenization. This procedure generally took about 45 min.

Extractions. The comminuted muscle was homogenized with cold extracting solution at a 1:10 (w/v) ratio in a commercial Waring Blendor for 60 s. The extracting solution was either water or salt solutions with or without pH adjustment as indicated below. The blender and containers were refrigerated first, and the sample was kept cold (3−7 °C) at all times during the experiments. Sequential washes (extractions) were carried out in a similar way.

Solubility Determination. After each homogenization, the samples were centrifuged at 18000*g* for 20 min at 4 °C (Sorvall Superspeed RC2-B centrifuge, DuPont Medical Products, Wilmington, DE; GSA rotor; 10 000 rpm). Samples were taken for protein determination before and after centrifugation, and percent solubility was calculated by taking the ratio of the protein in solution after centrifugation divided by the total protein in the suspension before centrifugation. Although samples were treated with 10 volumes of wash (extracting) solution, the final sediment was extracted with 50 volumes of water. This was done to reduce the ionic strength sufficiently to solubilize the proteins of this fraction.

Analyses. Protein was measured according to the Lowry procedure (Lowry *et al.*, 1951). If the sample contained insoluble protein, it was generally dissolved in 0.1 N sodium hydroxide to dissolve it before measurement. pH was determined with a glass electrode and pH meter (Model 611, Orion Research, Boston, MA). Salt concentration was estimated with a conductivity meter (Radiometer CDM 83). A standard curve of the conductivity reading versus salt concentration was obtained over the range 0−2 mM sodium chloride. Salt concentration was measured on solutions with low concentrations since variations under these conditions can greatly affect protein solubility (Stefansson and Hultin, 1994). Calcium was

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determined by atomic absorption spectroscopy; approximately 2 g of protein was predigested in 30 mL of concentrated HNO_3 (Ultrex II grade, J. T. Baker Inc., Phillipsburg, NJ) at 425 °C for 12 min. Cooled aliquots of the digested material were diluted with water (Ultrex II grade, J. T. Baker Inc.) to give a final concentration of 0.2% nitric acid. Lanthanum (Instra-analyzed grade, J. T. Baker Inc.), 0.1%, was used as a matrix modifier. Calcium concentrations were determined using a Perkin-Elmer (Norwalk, CT) 3030B atomic absorption spectrometer equipped with a calcium hollow cathode lamp. Elevated temperatures needed for element detection were obtained using a HGA-400 graphite furnace. Samples were atomized off the wall of a pyrolytically coated graphite tube. Calcium was determined at 422.7 nm with a slit width of 0.7 nm. Pyrolysis was performed at 1100 °C for 20 s with a 10 s ramp time. Atomization occurred at 2600 °C for 5 s with a 2 s ramp time. Argon flow was not disrupted during analytical detection. Data were analyzed using the peak area integration mode of the instrument software program.

Dialysis. Dialysis was carried out in tubing (Spectrum Medical Industries, Inc., Los Angeles, CA), 32 mm in diameter, with a molecular weight cutoff of 6000−8000 against deionized water at a ratio of 1:50 (v/v). The dialysis tubing was boiled in deionized, distilled water for 2 min; the water was then replaced with deionized, distilled water at room temperature (22−25 °C) and stored at 4 °C until used. The water was changed a total of four times every 6 h during dialysis. After dialysis, the mixture was centrifuged at 18000*g* for 20 min to separate soluble from insoluble proteins. All operations were performed at approximately 4 °C.

Electrophoresis. Electrophoretic analyses were carried out as previously described (Stefansson and Hultin, 1994) using precast gels of a 3−27% linear polyacrylamide gradient (ISS, Natick, MA). Molecular masses were estimated from markers (Bio-Rad) and consisted of rabbit myosin heavy chain (200 kDa), *Escherichia coli* β-galatosidase (116.25 kDa), rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white albumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa). Destained gels were scanned using a Hoefer GS 300 scanning densitometer (San Francisco, CA); equal dye binding by the various muscle proteins was assumed.

Replications. All experiments were repeated at least twice. There was some variation between experiments even though the nature of the effects of various treatments was consistent. Typical experiments are reported. The only exception to the consistency in behavior were the samples that were extracted with 150 mM NaCl without pH adjustment before final extraction in water (control in Table 7). Solubility of the proteins in the final washed mince in these samples varied from 0.7 to 10.3%. The example given is the same one as used to illustrate the electrophoresis patterns.

All analytical measurements were done in duplicate.

RESULTS

The procedure developed for preparation of sarcolemma (Westort and Hultin, 1966) was evaluated as to its efficacy in solubilizing chicken breast muscle protein. Ninety-five percent of the proteins of washed, minced chicken breast muscle were soluble when the sodium chloride washes were buffered with histidine (Table 1). When the washes were done in sodium chloride solution without histidine buffer and pH adjustment, protein solubility was reduced (Table 1); the solubility in this case was also highly variable. The results indicate that most of the chicken breast muscle proteins are soluble in low ionic strength solutions and that histidine and/or pH adjustment increased solubilization. When minced cod muscle is washed with water to an ionic strength lower than 0.3 mM, protein solubilization greater than 90% is achieved (Stefansson and Hultin, 1994). When minced chicken breast muscle was treated this way, solubilization was less than 2%.

Table 1. Solubility of the Proteins of Washed, Minced Chicken Breast Muscle[a] in Water

washing solution[b]	% soluble protein
NaCl + histidine at pH 7.4	95.0 ± 4.2[c] (9)
NaCl only	29.2 ± 19.3 (6)

[a] Chicken breast muscle was homogenized in 10 volumes of 50 mM $CaCl_2$ and washed four times in 10 volumes of NaCl solution before extraction into 50 volumes of deionized, distilled water. [b] The first three washes contained 25 mM NaCl and 5 mM histidine adjusted to pH 7.4 with tris(hydroxymethyl)aminomethane and once with 2.5 mM NaCl and the same histidine. The washing solutions of the "NaCl only" samples contained no histidine and did not have any pH adjustment of the wash solutions. [c] The numbers are averages with standard deviations. Numbers in parentheses are the number of experiments conducted.

Table 2. Ranges of pH Values in the Treatment Steps

treatment	wash in NaCl plus histidine[a]	wash in NaCl only[a]
homogenization in $CaCl_2$	5.6−5.8	5.6−5.8
first wash	6.5−6.7	6.1−6.3
second wash	7.0−7.2	6.5−6.7
third wash	7.3−7.5	6.7−6.9
fourth wash	7.4−7.6	7.1−7.3
extraction with water		

[a] Conditions were as described in Table 1. The pH values indicated are those of the homogenized muscle suspensions. The pH of the muscle homogenized in water was 6.3−6.5.

Table 3. Effect of Post-Mortem Age on the Solubility of the Proteins of Washed, Minced Muscle in Water

h post-mortem	% soluble protein[a]
0.75	95.0 ± 4.2 (9)
4	91.7 ± 5.7 (3)
24	96.9 ± 0.7 (2)
72	95.7 ± 1.7 (2)

[a] Average values ± SD; numbers in parentheses are replicates.

Table 2 shows the ranges of pH values observed in several experiments in the various treatment steps when the samples were washed in sodium chloride solution adjusted with histidine to pH 7.4 or washed only in the sodium chloride solutions. It should be emphasized that it was not the suspensions that were adjusted to pH 7.4 but only the wash solutions. The first wash solutions had low pH values which were undoubtedly due to acid from the muscle tissue. By the third wash the pH was approximately that of the added solution. The important point to notice is that throughout the washes the pH of the suspensions that contained histidine were higher by 0.4−0.6 pH unit compared to the samples not containing histidine. The same solubility results were obtained when *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES) was used instead of histidine. Thus, it is likely that the effect was due to pH and not a specific effect of histidine.

Most of the studies described in this paper used prerigor muscle, obtained approximately 45 min after the hen was slaughtered. When longer times post-mortem were evaluated, there was little change in protein solubility (Table 3).

The effect of salt was the same in preventing solubilization or precipitating previously solubilized proteins (Table 4) as had been previously observed for cod muscle proteins (Stefansson and Hultin, 1994). The proteins of the washed minced muscle were 97% soluble in water, and the dilutions used gave a final salt concentration of approximately 0.16 mM. When 0.3 mM added sodium chloride was used to extract the proteins, the sodium

Table 4. Inhibition and Reversal of Solubilization of Washed, Minced Muscle[a] Proteins by Salt

extraction medium	% soluble protein	NaCl in supernate, mM
water	97 ± 2.6	0.16
0.3 mM NaCl	58 ± 2.5	0.50
water, adjusted to salt concentration of "0.3 mM" sample after extraction	55 ± 3.3	0.50
0.6 mM NaCl	9 ± 2.0	0.85
water, adjusted to salt concentration of "0.6 mM" sample after extraction	6 ± 2.2	0.85

[a] The samples were prepared as described in Table 1 except that the original homogenization was done in water (see Table 6 and discussion thereof).

chloride content in the muscle suspension was 0.50 mM. This solubilized 58% of the proteins. Fifty-five percent of the proteins remained soluble after the proteins were solubilized in water and the mixture was adjusted to the same salt concentration by addition of sodium chloride. When the samples were exposed to 0.85 mM sodium chloride, comparable values of soluble proteins by inhibition and reprecipitation were 9 and 6%, respectively.

It was previously suggested that washing with buffered sodium chloride solution was needed to remove calcium ions from actomyosin to allow extraction of myofibrillar proteins from chicken breast muscle segments in low ionic strength solution (Stanley and Hultin, 1968). To test whether buffered sodium chloride solution did remove calcium, washed mince was prepared for protein solubilization in the usual way, i.e., homogenization in calcium chloride and four washes in sodium chloride−histidine solution. As expected, the proteins of the washed mince were mostly soluble when extracted in 50 volumes of water, i.e., 90% (Table 5). If an aliquot of the original washed mince that was 90% soluble in water was treated with 10 volumes of 50 mM calcium chloride solution and rewashed four times with sodium chloride−histidine buffer in the standard way, the proteins remained mostly soluble, i.e., 94% (Table 5). However, when an aliquot of the original washed mince was treated with 50 mM calcium chloride and washed four times with the same volume of water, most of the proteins were insoluble, i.e., only 10% were soluble (Table 5). Since muscle tissue treated in the usual way allowed the proteins to be solubilized on extraction with a large volume of water, it can be assumed that whatever other role calcium chloride may play, it can inhibit the solubilization of the proteins if it is not removed. The solubility results indicated that the sodium chloride−histidine solution removed enough of the calcium chloride to render the proteins soluble. In this experiment calcium decreased from 827 mg/g of protein in the sample that was washed with water to 492 mg/g of protein for the sample that was washed with the sodium chloride−histidine solution.

Since it was necessary to remove excess calcium ions before the chicken breast muscle proteins became soluble in water, it was of interest to determine whether the presence of calcium chloride in the homogenizing medium was necessary for solubilization of chicken muscle proteins. Chicken breast muscle was homogenized in either 50 mM $CaCl_2$, 150 mM NaCl, or water; these homogenization treatments were followed by the standard four washes in histidine-buffered salt solutions prior to extraction in 50 volumes of water. The amount of protein removed at each step was determined as well as the pH in each of the wash suspensions (Table 6). High total solubilities were achieved in all three samples (95−98%). The patterns of the solubilities, however, were different between the samples homogenized in water and those homogenized in the two salt solutions which were used at equal (and physiological) ionic strength. The amount of protein removed in the supernate of the centrifuged homogenate ranged between 45 and 50% when done in salt solutions of approximately physiological ionic strength, while it was about 25% when the homogenization was carried out in water. The difference in protein extracted at the first homogenization step between those samples homogenized in salt and that in water was made up for primarily in two of the subsequent steps. One of these was the first wash in histidine-buffered 25 mM NaCl. The final extraction into 50 volumes of water also removed considerably more protein in the sample homogenized in water than those that were originally homogenized in salt. The net effect was that the total protein solubilities were approximately the same for all of the samples.

When chicken breast muscle was homogenized in water and washed extensively in water, the solubility of the proteins was low. The total protein extracted was usually under 30%, most of it being "sarcoplasmic" proteins. The high solubility shown after homogenization in water followed by washes in the sodium chloride−histidine solutions (Table 6) indicates that salt and pH adjustment may be necessary to achieve high solubilization of the muscle proteins. Since homogenization with water does remove a considerable portion of the soluble or sarcoplasmic proteins, it is advantageous to remove these proteins before one studies the effect of salt and pH on the solubility of the myofibrillar proteins. The sarcoplasmic proteins were removed by homogenizing chicken breast muscle in 10 volumes of deionized distilled water, centrifuging, resuspending the sediment in the same volume of water, and centrifuging again. The second sediment is hereafter referred to as the "twice-washed homogenized mince" and was used to evaluate the effects of extracting solutions on myofibrillar protein solubility.

A comparison was made of the effectiveness of calcium chloride vs sodium chloride with or without buffering with sodium carbonate on the solubility of the proteins of twice-washed homogenized mince (Figure 1). Protein extractability increased with increasing ionic strength up to a concentration of 1 M. The results with calcium chloride are interesting since other data (Table 5) indicated that calcium ions inhibited solubilization of proteins when a final washed sediment was extracted with deionized distilled water. At low ionic strengths the effectiveness of calcium chloride solution as an extractant was similar to that of the sodium chloride solution adjusted to neutral pH with sodium carbonate.

Table 5. Inhibition of Solubilization of Washed Muscle Mince Proteins by Calcium Ions

sample	% soluble proteins	pH of final supernate	NaCl concentrations of final supernate, mM
washed mince prepared for protein solubilization	90	7.2	0.18
washed mince treated with 50 mM $CaCl_2$ and rewashed four times with NaCl/histidine	94	7.3	0.14
washed mince treated with 50 mM $CaCl_2$ and washed four times with water	10	7.0	0.04

Table 6. Comparison of Muscle Samples[a] Homogenized in $CaCl_2$, NaCl, or Water

sample	homogenized in 50 mM $CaCl_2$	150 mM NaCl	water
pH			
homogenate	6.25	6.35	6.00
wash 1	6.86	6.63	6.32
wash 2	7.18	6.96	6.72
wash 3	7.24	7.03	6.79
wash 4	7.66	7.38	7.49
final extract	6.66	6.86	6.64
% soluble protein			
homogenization	46.7	48.8	24.1
wash 1	4.3	5.2	16.4
wash 2	6.6	6.3	8.7
wash 3	0.2	0.2	0.5
wash 4	0.3	0.4	0.4
final extract in water	38.2	34.3	47.7
total protein extracted	96.3	95.2	97.8
final extract as % of final washed mince[b]	91.1	87.9	95.8

[a] The samples were prepared according to the procedure described in Table 1 by varying the homogenizing media as indicated. Histidine-buffered salt solutions were used in the washes. [b] These data represent the amount of protein that was extracted from the sediment after it had been washed four times expressed as a percentage of the protein in that sediment.

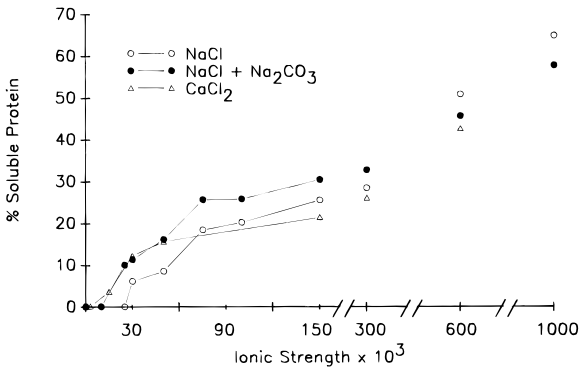

Figure 1. Solubility of proteins by $CaCl_2$ and NaCl from twice-washed homogenized mince as a function of ionic strength. When the NaCl was adjusted to neutrality, the sodium carbonate contributed about 1−2 mM Na^+ to the extracting solution.

There was a relatively constant difference in the amount of protein extracted by the sodium chloride solutions with or without pH adjustment at ionic strengths from 25 to 150 mM. Greater extraction was obtained with sodium chloride solutions at the higher pH.

The data in Figure 1 suggested that an additional amount of protein was removed from the twice-washed homogenized mince when the wash solution containing 150 mM sodium chloride was adjusted to neutral pH with sodium carbonate. The next experiment was designed to test whether the removal of this additional protein affected protein solubilization. The experimental protocol is shown in Figure 2. After preparation of the twice-washed homogenized mince according to the usual technique, it was extracted first with 150 mM sodium chloride solution (W_2). The sediment was then washed with water. One portion of this sediment was then washed with a solution of 2.5 mM sodium chloride. The purpose of the last two washes was to reduce the ionic strength of the water in the sediment. The use of 2.5 mM sodium chloride in the second wash was to prevent excessive swelling. The sediment was then extracted into 50 volumes of water.

A portion of the washed minced muscle was taken

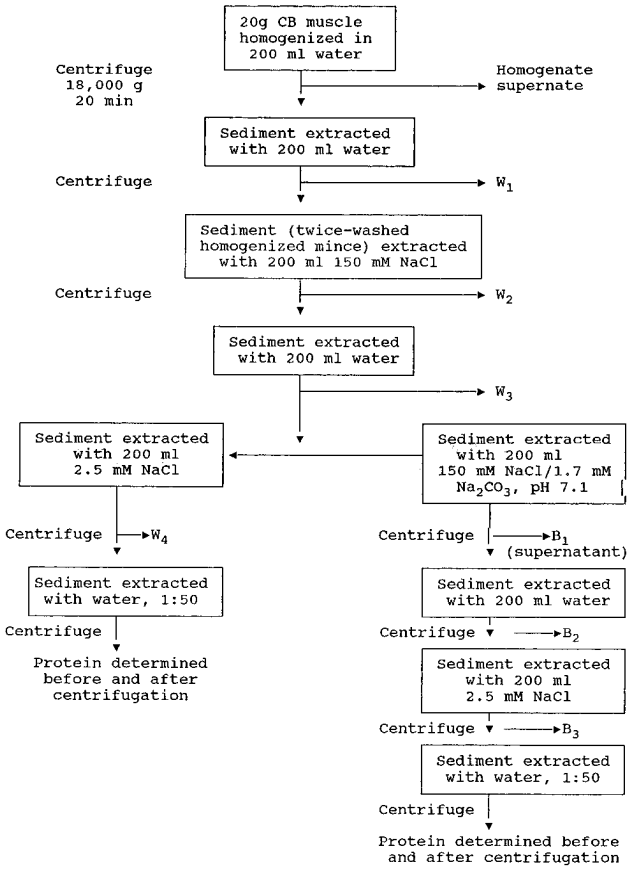

Figure 2. Protocol for assessing requirements for conferring water solubility on chicken breast muscle myofibrillar proteins.

after extraction of the twice-washed homogenized mince with 150 mM sodium chloride solution and the first wash with water. It was then extracted with a solution of 150 mM sodium chloride adjusted to neutrality with sodium carbonate (B_1). This generally required around 1.5−2.0 mM sodium carbonate. The purpose of this extraction was to determine whether additional proteins were extracted when the same sodium chloride solution used in the earlier extraction (W_2) was adjusted to a higher pH. The sediment from this centrifugation was then, as before, washed with water followed by a wash in 2.5 mM sodium chloride before final extraction in 50 volumes of water.

Results of these extractions are shown in Table 7. Approximately 21% of the total protein was removed in the supernatant fraction after homogenization in water (1:10) and 15.5% by extraction with 150 mM sodium chloride. When a second extraction was done with 150 mM sodium chloride adjusted to neutral pH with sodium carbonate, a further 9% protein was extracted. This value is similar to the difference observed between the amount of protein extracted with the adjusted and nonadjusted sodium chloride solutions shown in Figure 1. When the final sediment of this fraction was extracted with 50 volumes of distilled deionized water, some 98% of the protein was extracted. This gave a total extraction of the initial protein of almost 99%.

In contrast, when the sample from step 2 in Table 7 was reduced in ionic strength by two washes and then directly extracted with 50 volumes of deionized distilled water, only 1.2% of the proteins of the final sediment became soluble. Overall, only some 40% of the total muscle proteins were extracted. These results suggest that the extra proteins removed by adjusting the 150 mM sodium chloride extracting solution to neutral pH

Table 7. Sequential Extraction of Water-Washed Mince with Salt and Salt with pH Adjustment

treatment step	pH	ionic strength[a]	% soluble protein[b]
1. homogenization and wash in water			
homogenate (1:10)	5.83	7.0	21.3
water wash (1:10), W_1	5.81	0.7	0.3
2. extraction with 150 mM NaCl (1:10), W_2	5.51	72.0	15.5
3. wash with water (1:10), W_3	5.45	6.2	1.8
4. extraction with 150 mM NaCl/Na_2CO_3 to neutral pH (1:10), B_1	7.08	81.8	9.0
5. wash with			
water (1:10), B_2	6.91	8.8	2.3
2.5 mM NaCl (1:10), B_3	7.02	2.7	2.3
6. extraction with water (1:50)	7.10	0.09	46.7
% total protein extracted	99.2		
% protein extracted from final washed mince*	98.1		
control[c]			
sample from step 3 above washed with 2.5 mM NaCl (1:10)	5.85	2.6	0
extraction with water (1:50)	6.19	0.27	1.2

[a] Expressed as equivalent concentration of NaCl, mM. [b] Percent expressed on the basis of the original protein except where indicated by *. [c] Control sample was not subjected to the second wash with 150 mM NaCl adjusted to neutral pH with Na_2CO_3.

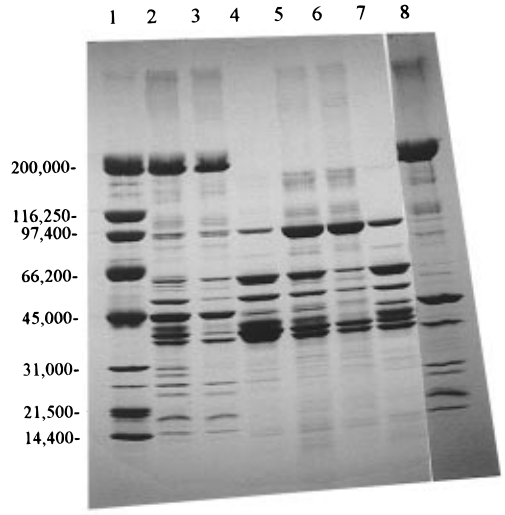

Figure 3. Chicken breast muscle proteins at various stages of extraction analyzed by SDS−polyacrylamide gel electrophoresis: lane 1, molecular weight markers; lane 2, ground chicken breast muscle; lane 3, twice-washed homogenized mince; lane 4, proteins extracted from twice-washed homogenized mince with 150 mM NaCl (W_2); lane 5, proteins extracted from sediment by 150 mM NaCl adjusted to pH 7.1 with Na_2CO_3 after previous extractions with unadjusted 150 mM NaCl and water to reduce salt content (B_1); lane 6, insoluble proteins from B_1 after dialysis; lane 7, soluble proteins from B_1 after dialysis; lane 8, proteins extracted from the final sediment in 50 volumes of water, i.e., the "water-soluble" myofibrillar proteins.

with sodium carbonate were critical in allowing solubilization of the remaining proteins in water after sufficient dilution of the added salt. The final ionic strength was 0.09 mM.

The peptides removed at the various extraction steps were evaluated by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) (Figure 3). Lane 1 contained the molecular weight markers, lane 2, whole chicken breast muscle, and lane 3, the twice-washed homogenized mince. Some proteins were removed from the whole muscle protein compared to the twice-washed homogenized mince; presumably these were "sarcoplasmic" proteins. The proteins extracted from the twice-washed homogenized mince by 150 mM sodium chloride solution are shown in lane 4 (W_2 in Figure 2). The peptides in lane 5 are those that were removed by the sodium chloride−sodium bicarbonate solution after previous extraction with just the sodium chloride solution (B_1 from Figure 2). Some six or seven proteins were removed at the higher pH that were not removed or were removed to a lesser extent with just the 150 mM sodium chloride solution. Most of these are of relatively high molecular mass, >66 kDa. The approximate molecular masses of the proteins are as follows (possible identifications in parentheses): 151 kDa (X-protein; Starr and Offer, 1982); 137 kDa (C-protein; Starr and Offer, 1982); 109 kDa; 105 kDa; 95 kDa (α-actinin; Nave *et al.*, 1990); 84 kDa (amorphin; Chowrashi and Pepe, 1982).

When the extract obtained by the pH-adjusted sodium chloride solution (B_1) was dialyzed against distilled water to a value less than 0.2 mM ionic strength, some of the proteins precipitated and some remained in solution (lanes 6 and 7, respectively). It is interesting that the proteins remaining in solution after dialysis had a peptide pattern similar to that obtained by the first extract in 150 mM sodium chloride (W_2). The peptides from the proteins which precipitated on dialysis (lane 6) contained most of the peptides that were additionally extracted by pH adjustment of the sodium chloride. The amount of the 95 kDa peptide extracted with the adjusted pH salt solution (B_1) was much higher than that extracted with the 150 mM sodium chloride solution without pH adjustment (W_2). Although some of the 97 kDa peptide from B_1 (extracted with pH adjustment) remained soluble after dialysis, a much greater amount was insoluble.

Some proteins showed no solubility in the 150 mM sodium chloride solutions with or without pH adjustment. These proteins were present in the final sediment that was extracted with water. These peptides (lane 8) included myosin heavy chain, a 27 kDa peptide, and three myosin light chains. Thus, chicken breast muscle myosin was soluble at low and high ionic strengths but not at moderate ionic strength. Although there was some actin extracted with moderate concentrations of sodium chloride, a large portion of it remained in the final sediment and was extracted with water (lane 8).

DISCUSSION

When the washed minced muscle of cod is washed three times and then extracted into water so as to reduce its ionic strength to less than 0.3 mM, the great majority of the proteins of the muscle are solubilized (Stefansson and Hultin, 1994). When a similar procedure is used with chicken breast muscle, the solubility is low and limited to 20−25% of the total protein, an amount usually associated with the sarcoplasmic fraction. More extensive washing has little effect. How-

ever, when a procedure developed for production of the sarcolemmal sheaths of muscle cells (Westort and Hultin, 1966) was used, solubilization of the chicken breast muscle proteins was almost complete. This procedure was based on that originally developed by McCollester (1962) and consisted of homogenization in 50 mM calcium chloride, followed by several washes in buffered 25 mM sodium chloride before extraction into 50 volumes of water. Results were then comparable to those observed with washed cod muscle.

An interesting difference was observed between cod and chicken breast muscle proteins concerning the centrifugal force required to remove the proteins from the clear upper phase of the centrifuge tube. In the case of cod muscle, centrifugal forces of up to 47000*g* for 20 min did not remove the proteins from solution (Stefansson and Hultin, 1994). When the "solubilized" proteins of chicken breast muscle were centrifuged at 30000*g* for 20 min, only 22% of the proteins remained in the clear upper layer of the centrifugate compared to 89% when centrifuged at 18000*g* for 20 min. Therefore, the lower centrifugal force was used to define solubility of the chicken breast muscle proteins. Centrifugal forces from 5000*g* to 41000*g* for 10−60 min have been used to define solubility of muscle proteins (Hultin *et al.*, 1995).

The solubilization of the final washed, minced muscle could be prevented and/or reversed by the addition of low concentrations of sodium chloride. This is similar to what was observed with the myofibrillar proteins of cod muscle (Stefansson and Hultin, 1994). These facts argue against extensive proteolysis of the myofibrillar proteins as an explanation for the high solubilization. It seems unlikely that low concentrations of sodium chloride added just before the final extraction in water could be inhibiting proteolytic enzymes. The reversibility of the solubilization likewise makes it extremely unlikely that a high degree of hydrolysis of the proteins is involved. The possibility of a small amount of proteolysis that is highly selective toward critical proteins cannot be ruled out.

The role of calcium ions in post-mortem muscle is complex. While ATP remains, calcium ions may trigger contraction by its interaction with the troponin−tropomyosin system (Ishikawa and Wakabayashi, 1994). Calcium ions bind to proteins such as α-actinin (Wenegieme *et al.*, 1994). Fragmentation of nebulin filaments is induced by binding of large amounts of calcium ions in chicken breast muscle myofibrils (Tatsumi and Takahashi, 1992). Takahashi and Hattori (1989) reported that a solution of 0.1 mM calcium ions produced a weakening of Z-disks that was nonenzymatic. Comissiong *et al.* (1971) demonstrated that polyvalent cations, including calcium, reduced the extractability of the contents of muscle cell segments, and it was proposed that this was caused by precipitation of the proteins by the polyvalent cations. Szent-Györgyi (1943a,b) reported that divalent cations precipitated both myosin and actomyosin. In the studies reported in this paper, we demonstrated that addition of calcium ions reduced solubility of chicken breast muscle proteins when extracted into water (Table 5). It was also shown that washing with a sodium chloride solution buffered with histidine could remove some of the calcium and restore the water solubility of the myofibrillar proteins.

While low concentrations of calcium ions had an inhibitory effect on the solubilization of chicken breast muscle myofibrillar proteins in water, solubilization of the proteins of twice-washed homogenized mince by calcium chloride was somewhat better than that by sodium chloride at ionic strengths of 50 mM and below and comparable over that range of ionic strength to sodium chloride solutions the pH of which had been increased to neutrality. It was only slightly less effective than sodium chloride at ionic strengths between 50 and 600 mM. We suggest that calcium ions form insoluble complexes of myosin and actomyosin such that they cannot be solubilized in water. However, at higher concentrations of calcium chloride, a selective and progressive salting-in of these proteins occurs.

Others have observed solubilizing effects of calcium. Taylor and Etherington (1991) showed that 30 mM calcium chloride immediately solubilized C-protein and troponin I with small amounts of troponin T, tropomyosin, and a peptide of molecular mass 80 kDa from rabbit psoas myofibrils. Longer incubations with calcium chloride solubilized increasing amounts of troponin T, troponin I, and tropomyosin among others. Since protease inhibitors had no effect on these solubilizations, the authors concluded that solubilization of these proteins by calcium ions was due to salting-in rather than to proteolysis. Whipple *et al.* (1994) found no difference in the ability of buffered 0.1 M potassium chloride (pH 7.0) and 0.1 M potassium chloride plus 30 mM calcium chloride to solubilize several myofibrillar proteins from ovine myofibrils, although the solubilization was faster in the presence of the calcium salt. A protease inhibitor had no effect on protein solubility. M-protein, C-protein, α-actinin, actin, troponin-T, troponin-I, tropomyosin, and several peptides ranging in molecular mass from 22 to 72 kDa were solubilized. Myosin heavy chain was slightly solubilized.

Since it appeared to be necessary to remove calcium chloride to solubilize a portion of the myofibrillar proteins in water, it would be better not to add it. The data comparing protein solubilizations of the twice-washed homogenized mince suggested that sodium chloride at the same ionic strength would work as well. This was in fact shown to be the case. It was further demonstrated that an initial wash in water was sufficient as long as the minced tissue was later washed in sodium chloride at a sufficiently high concentration and with pH adjusted to neutrality. There appeared to be no specific role of histidine, so sodium carbonate was substituted. The homogenized chicken muscle was washed twice in water to remove sarcoplasmic proteins and make interpretation of the role of various myofibrillar proteins simpler. These preliminary washes may not be necessary to achieve solubilization.

The difference in proteins solubilized by sodium chloride solution compared to sodium chloride solution in which the pH was adjusted with sodium carbonate was not large and remained relatively constant over a wide range of sodium chloride concentrations (Figure 1). The difference of some 6−8% had a strong effect, however, on the water solubility of the myofibrillar proteins. The specific peptides that were removed with and without pH adjustment of the sodium chloride solution were examined to see if the restrictive ability on water solubility correlated with the removal of specific proteins. For this study, extraction at 0.15 M sodium chloride was chosen since this approximates physiological ionic strength.

The protocol to determine potential peptides involved with restricting the water solubility of the myofibrillar proteins was to sequentially extract the twice-washed homogenized mince, first with 150 mM sodium chloride

and then with 150 mM sodium chloride adjusted to neutrality with sodium carbonate. Wash steps were used between these extractions and also before the final extraction with water to adjust the ionic strengths to low values before the succeeding extraction. A control sample was extracted only with the 150 mM sodium chloride that was not adjusted to neutral pH. In all cases, the total solubility of the chicken breast muscle proteins extracted by the double salt wash procedure (Figure 2) was very high (≥99% in four trials). On the other hand, if the sample was not treated with the 25 mM sodium chloride adjusted to neutrality, the great bulk of the soluble protein occurred in the first wash (homogenization) and with the extraction at 150 mM sodium chloride; very little protein was extracted thereafter. There was variation in the amount of protein extracted in the final sediment when only the one wash in sodium chloride solution without pH adjustment was used. It varied from 0.7 to 10.3%. The pH of the washed muscle−water mixture was not controlled and varied from 6.2 to 6.8. The higher solubilities were observed at the higher pH values. A dependence of myofibrillar protein solubility on pH has been observed for cod (Stefansson and Hultin, 1994).

The quantitative removal of protein (Table 7) can be compared with the peptides removed by the various procedures (Figure 3). The homogenized whole muscle (lane 2) has a few more peptide bands than did the twice-washed homogenized mince (lane 3). Presumably these are part of the sarcoplasmic proteins. A comparison of the peptides first extracted with the non-pH-adjusted sodium chloride solution (lane 4) can be made with the peptides extracted by subsequent treatment with the same sodium chloride solution but with the pH raised to neutrality (lane 5). There were several proteins that were removed at the higher pH that were not removed at the lower pH or that were removed in much larger quantity at the higher pH than in the sodium chloride solution without pH adjustment.

When the extract obtained with the sodium chloride plus sodium carbonate (lane 5) was dialyzed against water until the ionic strength of the retentate was reduced to less than 0.2 mM, some proteins precipitated (lane 6) while some remained in solution (lane 7). These last "water-soluble" peptides were almost identical to the peptides that were removed by extraction with sodium chloride without pH adjustment. The proteins that were precipitated by this dialysis treatment contain most of the peptides that were not solubilized until the pH of the sodium chloride solution was raised. The peptide of approximately 95 kDa was present in both fractions but at a higher concentration in the insoluble fraction. The insoluble fraction also contained some of the proteins that had been solubilized without pH adjustment, but these were present at lower concentrations than they were in the water-soluble fraction.

Certain proteins must be removed to render the proteins of the final washed sediment soluble in water. It is tempting to speculate that the proteins responsible for preventing these proteins from being solubilized in water are those proteins that were removed preferentially by the sodium chloride solution that had been adjusted to neutral pH. These were also the principal proteins that became insoluble when the ionic strength of the extract was reduced to a low value. On the basis of estimates of molecular mass and relative abundance, X-protein, C-protein, α-actinin, and amorphin were tentatively identified. The first two are associated with the thick filaments (Starr and Offer, 1982) and the latter two with the Z-disk (Wang, 1985). Two other peptides with molecular masses of 109 and 105 kDa also belonged to this group. Further studies will be required to specifically identify the peptides in this fraction and to show that they are indeed restricting the water solubility of myosin and the other proteins in this fraction.

The proteins from the final sediment that were extracted in 50 volumes of water (lane 8) were for the most part proteins which were not extracted well in physiological concentrations of sodium chloride with or without pH adjustment. A major one of interest is the myosin heavy chain, which was not solubilized at all at moderate ionic strengths. The amount of protein in the actin band was also highest in the final water extract. In addition, there were four low molecular weight proteins that were essentially not solubilized until the final extraction into water. These could include the myosin light chains and/or one of the troponins, but this is not certain. There also appeared to be a considerable amount of titin that was not solubilized until extraction with water. Chicken muscle titin breaks down rapidly with time post-mortem (Tatsumi and Takahashi, 1992).

Our conclusion from this work is that essentially all of the proteins of chicken breast muscle are soluble at neutrality in solutions of sodium chloride at physiological concentrations or less. Achieving complete solubility requires a specific order of removal. Some proteins soluble at moderate ionic strengths and neutral pH must be removed before other proteins that are not soluble in low concentrations of salt can be solubilized in water. Presumably the proteins that are solubilized by moderate concentrations of salt restrict the water solubility of the other proteins. It would be of interest to determine which protein(s) are responsible for this. Ultimate proof of this capacity residing in any proteins would be demonstrated by the inhibition of solubilization by readdition of protein(s) previously removed.

Differences were observed in the solubilization characteristics of chicken breast muscle proteins compared to cod muscle proteins (Stefansson and Hultin, 1994). The possibility of solubility properties different from either chicken breast muscle or cod muscle in other muscle types must be considered. This may be of special interest with regard to red muscles since it has been shown that proteins of red muscle are generally less soluble than those of white muscle at both high (Wu, 1969) and low (Stanley *et al.*, 1994) salt concentrations. The observation that muscle proteins are soluble at physiological ionic strength or less and neutral pH may be useful in the isolation of individual proteins for research purposes or for fractionation of proteins for commercial use.

LITERATURE CITED

Chowrashi, P. K.; Pepe, F. A. The Z-band: 85,000-dalton amorphin and alpha-actinin and their relation to structure. *J. Cell Biol.* **1982**, *94*, 565−573.

Comissiong, E. A.; Stanley, D. W.; Hultin, H. O. Inhibition of emptying of skeletal muscle cell segments by adenine nucleotides and polyvalent cations. *J. Cell Biol.* **1971**, *49*, 430−437.

Hultin, H. O.; Feng, Y.; Stanley, D. W. A re-examination of muscle protein solubility. *J. Muscle Foods* **1995**, *6*, 91−107.

Ishikawa, T.; Wakabayashi, T. Calcium induced change in three-dimensional structure of thin filaments of rabbit skeletal muscle as revealed by cryo-electron microscopy. *Biochem. Biophys. Res. Commun.* **1994**, *203*, 951−958.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

McCollester, D. L. A method for isolating skeletal-muscle cell-membrane components. *Biochim. Biophys. Acta* **1962**, *57*, 427−437.

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.

Stanley, D. W.; Hultin, H. O. Mechanism of emptying of skeletal muscle cell segments. *J. Cell Biol.* **1968**, *39*, C1−C4.

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

Starr, R.; Offer, G. Preparation of C-protein, H-protein, X-protein and phosphofructokinase. *Methods Enzymol.* **1982**, *85*, 130−138.

Stefansson, G.; Hultin, H. O. On the solubility of cod muscle proteins in water. *J. Agric. Food Chem.* **1994**, *42*, 2656−2664.

Szent-Györgyi, A. The crystallization of myosin and some of its properties and reactions. *Stud. Inst. Med. Chem. Univ. Szeged* **1943a**, *3*, 76−85.

Szent-Györgyi, A. Observations on actomyosin. *Stud. Inst. Med. Chem. Univ. Szeged* **1943b**, *3*, 86−92.

Takahashi, K.; Hattori, A. α-Actinin is a component of the Z-filament, a structural backbone of skeletal muscle Z-disks. *J. Biochem.* **1989**, *105*, 529−536.

Tatsumi, R.; Takahashi, K. Calcium-induced fragmentation of skeletal muscle nebulin filaments. *J. Biochem.* **1992**, *112*, 775−779.

Taylor, M. A. J.; Etherington, D. J. The solubilization of myofibrillar proteins by calcium ions. *Meat Sci.* **1991**, *29*, 211−219.

Wang, K. Sarcomere-associated cytoskeletal lattices in striated muscle. In *Cell and Muscle Motility;* Shay, J. W., Ed.; Plenum Press: New York, 1985; pp 315−369.

Wenegieme, E.; Babitch, J. A.; Anjaparavanda, P. N. Cation binding to chicken gizzard α-actinin. *Biochim. Biophys. Acta* **1994**, *1205*, 308−316.

Westort, C.; Hultin, H. O. A procedure for the preparation of empty cell segments from skeletal muscle using solutions of low ionic strength. *Anal. Biochem.* **1966**, *16*, 314−319.

Whipple, G.; Koohmaraie, M.; Arbona, J. R. Calcium chloride *in vitro* effects on isolated myofibrillar proteins. *Meat Sci.* **1994**, *38*, 133−139.

Wu, C.-S. Comparative studies on myosins from breast and leg muscles of chicken. *Biochemistry* **1969**, *8*, 39−48.

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