Solubility of Beef and Chicken Myofibrillar Proteins in Low Ionic Strength Media

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Myofibrillar proteins were extracted and solubilized at ionic strengths not exceeding 30 mM. This was accomplished by treating homogenized muscle with a succession of washes in dilute salt solution (25 mM NaCl, 2.5 mM histidine, pH 7.4). It was possible to achieve up to 36% solubility of total protein from various beef and chicken muscles. These values compared favorably with published results for extractions using high salt concentrations (0.6 M). Electrophoretic separation of extracts demonstrated that all myofibrillar species were present. A strong positive relationship ($r = 0.95$) was found between protein yield in the initial homogenate and literature values for percentage of white (type IIb) fibers in the muscle. Swelling of muscle proteins was associated with a higher level of protein solubilization.

INTRODUCTION

Much of the foundation for our current knowledge of protein extraction and subsequent solubilization from the vertebrate muscle myofibril comes from work done by Hungarian researchers in the 1940s. Using rabbit muscle, these workers reported the first successful isolation and purification of myosin and actin. In both cases, the proteins were defined in terms of the extracting solution. Myosin was taken as the protein fraction that precipitated at pH 7.0 when the solution was diluted to 0.1 M KCl after extraction of the muscle proteins by 0.6 M KCl solution at pH 8.5–9.0 (Banga and Szent-Györgyi, 1942). Actin was isolated by isoelectrically precipitating a water extract of an acetone powder prepared from muscle initially extracted as above (Straub, 1942). Later, it was recognized that the original myosin fraction was, in fact, actomyosin, a mixture of actin and myosin. To separate myosin from actomyosin, Szent-Györgyi (1943a) prepared actomyosin using 0.3 M KCl at pH 6.5 and diluted this with water; actomyosin was precipitated, leaving behind a solution of myosin that was precipitated by further dilution. It is important to note that this myosin preparation would swell when suspended in water and, on further dilution, became totally water soluble. Addition of small concentrations of salts precipitated the protein; 0.005–0.01 M KCl caused "almost quantitative precipitation", and even 0.0015 M KCl resulted in precipitation (Szent-Györgyi, 1943a). Szent-Györgyi concluded that while myosin is water soluble, actomyosin only swells in the presence of this solvent (Szent-Györgyi, 1943b).

On the basis of these and subsequent studies, it is now common practice to begin an extraction of muscle by the use of a buffered 0.6 M KCl solution to solubilize the major proteins. Extraction of muscle proteins is necessary for subsequent separation, purification, and isolation operations required in biochemical studies and is also the first step in preparing these proteins for use as functional ingredients such as binders and emulsifiers and for thermal gel formation in various foods. Reducing the salt content of muscle protein preparations would allow the determination of their properties under more physiological conditions. For example, it is known that salts, in particular KCl and NaCl, influence muscle characteristics including thermal properties (Barbut and Findlay, 1991), water-binding capacity (Richardson and Jones, 1987; Bernthal *et al.*, 1991), heat-induced gelation (Careche *et al.*, 1991; Nuckles *et al.*, 1991), ability to bind restructured meat products (Kenney *et al.*, 1992), and emulsification properties (Gillett *et al.*, 1977). Few, if any, of these studies have been performed using low, *i.e.*, ≤30 mM, ionic strengths. Lowering the salt content of edible muscle protein foods would also reduce the level of an undesirable nutritional component.

Our laboratories have previously developed procedures for preparing sarcolemmae from muscle fibers that are based on extraction of the cell contents into water (Westort and Hultin, 1966; Stanley and Hultin, 1968; Stanley, 1970, 1983). More recently, Wu *et al.* (1991) showed that about 20–36% of the proteins extractable with 1.0 M LiCl from low-fat white-fleshed fish muscle could be extracted in salt solutions of approximately 1 mM NaCl. It was the purpose of this investigation to determine if these procedures could be modified to yield large quantities of myofibrillar proteins soluble at low ionic strength from mammalian and avian muscle tissue.

EXPERIMENTAL PROCEDURES

Materials. Samples (*ca.* 1 kg) of beef muscles (sternomandibularis, semimembranosus and soleus from 12-month-old Hereford cross-breed steers) were obtained at the University of Guelph abattoir after having been removed from the carcass *ca.* 30 min post-mortem. Whole chicken muscles (pectoralis major and iliotibialis from 10-week-old White Rock birds) were obtained from 24-h post-mortem carcasses purchased at a local poultry purveyor. Except for the material used after slaughter, all samples were stored at 0–5 °C in sealed, vacuum-packaged plastic bags until required. Beef samples were extracted at 2–3-h post-mortem and following 1 and 6 days total storage. Three beef animals were used; in the case of the last animal duplicate extractions were performed. Chicken samples were extracted 1 day post-mortem and following 6 days total storage; six birds were used.

Methods. *Extraction.* Protein extraction was carried out using a method based on that of Westort and Hultin (1966) for sarcolemmae preparation. Coarsely chopped muscle (20.0 g) was homogenized in 200 mL of 50 mM $CaCl_2$ using a commercial Waring Blendor. Samples were homogenized using a prechilled glass vessel and two 30-s bursts separated by a 10-s waiting period. The homogenized slurry was filtered through four layers of cheesecloth, a 50-g aliquot centrifuged in a Beckman L7-70M

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Table 1. Extraction and Solubilization Data for Muscle Tissue (Mean ± SD)

	pectoralis	iliotibialis	sternomandibularis	semimembranosus	soleus
total protein (g)[a]	4.60 ± 0.10	3.89 ± 0.19	4.13 ± 0.19	4.42 ± 0.05	4.59 ± 0.34
1–2 h post-mortem					
homogenate yield (%)[b]			41.1 ± 4.2	54.1 ± 6.4	36.2 ± 3.6
homogenate pH[c]			6.25 ± 0.10	6.24 ± 0.12	6.32 ± 0.03
final pellet yield (%)[d]			21.7 ± 6.0	27.9 ± 6.3	20.2 ± 4.7
solubility—final pellet (%)[e]			33.5 ± 12.6	75.4 ± 10.9	58.8 ± 32.8
solution pH[f]			7.26 ± 0.07	7.25 ± 0.05	7.20 ± 0.09
solution conductivity (mM)[g]			0.342 ± 0.03	0.268 ± 0.02	0.268 ± 0.02
overall protein yield (%)[h]			6.9 ± 1.7	20.6 ± 4.0	10.6 ± 4.6
1 day post-mortem					
homogenate yield (%)	77.6 ± 2.5	52.5 ± 5.4	45.4 ± 1.1	62.4 ± 3.9	37.1 ± 5.5
homogenate pH	5.44 ± 0.17	6.01 ± 0.04	5.35 ± 0.13	5.19 ± 0.13	5.65 ± 0.06
final pellet yield (%)	36.3 ± 5.0	46.5 ± 8.4	26.2 ± 3.0	38.4 ± 7.3	23.5 ± 1.5
solubility—final pellet (%)	89.7 ± 4.3	49.8 ± 30.0	27.6 ± 3.2	46.9 ± 7.3	62.7 ± 22.0
solution pH	7.35 ± 0.10	7.07 ± 0.15	7.30 ± 0.11	7.20 ± 0.08	7.29 ± 0.25
solution conductivity (mM)	0.178 ± 0.02	0.217 ± 0.01	0.275 ± 0.04	0.195 ± 0.02	0.180 ± 0.02
overall protein yield (%)	32.5 ± 4.6	21.7 ± 12.0	7.3 ± 1.3	17.6 ± 2.0	14.5 ± 4.5
6 days post-mortem					
homogenate yield (%)	70.2 ± 1.0	54.4 ± 5.2	51.1 ± 5.7	67.9 ± 5.4	36.4 ± 6.5
homogenate pH	5.52 ± 0.02	6.15 ± 0.22	5.23 ± 0.09	5.17 ± 0.04	5.31 ± 0.11
final pellet yield (%)	35.9 ± 5.7	37.4 ± 4.2	36.6 ± 2.8	40.3 ± 2.2	20.6 ± 8.1
solubility—final pellet (%)	84.4 ± 7.1	72.1 ± 19.2	76.6 ± 19.6	88.8 ± 6.4	64.7 ± 21.1
solution pH	7.27 ± 0.11	7.06 ± 0.22	7.59 ± 0.34	7.36 ± 0.19	7.20 ± 0.13
solution conductivity (mM)	0.145 ± 0.01	0.192 ± 0.02	0.162 ± 0.01	0.152 ± 0.01	0.135 ± 0.02
overall protein yield (%)	29.9 ± 2.4	26.5 ± 5.9	27.5 ± 5.6	35.8 ± 2.8	12.0 ± 3.7

[a] Grams of total protein per 20.0 g of muscle sample. [b] Percent yield of protein in homogenate. [c] pH of homogenate. [d] Percent yield of protein in final pellet. [e] Percent protein solubilization of final pellet. [f] pH of solution. [g] Conductivity of solution expressed as mM NaCl. [h] Yield of initial protein in final solution.

ultracentrifuge (18000*g*, 4 °C, 20 min), and the residue retained. This sediment was washed first in 50 mL of 25 mM NaCl/2.5 mM histidine that had been previously adjusted to pH 7.4 with dropwise addition of 0.1 M Tris. A small volume of the wash solution was initially added to the sediment that was then mixed/resuspended with a spatula prior to addition of the rest of the wash solution. The resuspended residue was then centrifuged as before. This washing step was repeated a further two times (total of three washes). A fourth washing step was carried out using 50 mL of 2.5 mM NaCl/2.5 mM histidine (pH 7.4, as before), the slurry being centrifuged as before.

Solubilization. A 4.0-g aliquot of the pellet from the fourth wash was then blended using a prechilled Waring Blendor with 200 mL of distilled deionized water (4–7 °C) for 10 s and filtered through four layers of cheesecloth. The filtrate was centrifuged as previously described and the resulting supernatant analyzed for soluble protein content. Soluble protein was defined as that remaining in the supernatant following centrifugation at 18000*g* for 20 min at 4 °C.

Analysis. Protein determinations were performed using the Kjeldahl procedure (N × 6.25) in duplicate for intact muscle and homogenates and the biuret method in triplicate for final filtrates and extracts. Biuret samples were incubated at 38 °C for 15 min prior to measurement of absorbency at 540 nm; readings were standardized against bovine serum albumin (Sigma, 96–99% purity). Sample pH was measured for the initial homogenate and the supernatant of the final extracting step using a temperature-compensated probe. Conductivities were taken using a Radiometer (Copenhagen) CDM3 conductivity meter at 4–7 °C. These readings were standardized against known NaCl concentrations and expressed as millimolar.

Proteins of the final filtrate and the resulting supernatant from one extraction of all treatments were separated by SDS–PAGE using a Bio-Rad Mini-protein II electrophoresis system (Bio-Rad, Mississauga, ON) as directed by the manufacturer and following the procedure of Laemmli (1970). A 12% polyacrylamide gel was loaded with 20-μL samples containing 0.4 mg of protein or 5 μL of Bio-Rad protein standards ranging in molecular weight from 200 to 14 400 in denaturing buffer. Protein samples were prepared by heating the muscle extracts in 2% sodium dodecyl sulfate/2% 2-mercaptoethanol denaturing buffer (sample/buffer ratio of 1:4 v/v) at 100 °C for 5 min. Separation was carried out for 45 min at 200 V. Gels were then stained with Coomassie R-250 (Bio-Rad).

Statistical analysis of the data was performed using the JMP statistical program for the Macintosh (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Protein Solubilization. Depending upon the experimental conditions, solubilization of proteins from the final pellet into water ranged from about 30 to 90% and overall yield of initial protein into the final solubilized extract ranged from about 7 to 36% (Table 1). This was achieved in a solution whose final ionic strength did not exceed 30 mM. As reported by Wu *et al.* (1991), ionic strengths above this level failed to produce high solubility of fish muscle proteins. It would seem likely that most of the ions contributing to ionic strength at the final extract stage were contributed by residual washing solution. Considering that skeletal muscle contains, on average, 50–60% of the protein fraction as contractile proteins (Forrest *et al.*, 1975; Lawrie, 1979), that the majority of the stroma protein would be expected to be physically removed in the homogenization and filtration steps, and that the majority of the sarcoplasmic proteins would be expected to be solubilized by washing, the data for overall protein yield from 6-day post-mortem semimembranosus beef muscle (Table 1) represent a best case recovery of about 65% of contractile proteins or 35% of the total protein. In contrast, extraction of beef, pork, or poultry muscle with high-salt solutions has been reported to yield 35–50% of the total protein, depending upon conditions (Sayre and Briskey, 1963; Regenstein and Rank Stamm, 1979; Li-Chan *et al.*, 1986; Richardson and Jones, 1987; Bernthal *et al.*, 1991) and would be expected to contain the sarcoplasmic proteins unless an initial water wash was used.

Protein Separation. It is important to determine the nature of the protein extract. Electrophoretic separation of the extracts (Figure 1) demonstrates that these proteins are of myofibrillar origin, with myosin and actin comprising the majority of the sample. The electrophoretic patterns are comparable to those reported for salt-extracted proteins of chicken (Li-Chan *et al.*, 1986; Xiong and Brekke, 1991) and beef (Paterson *et al.*, 1988; Kenney *et al.*, 1992; Kendall *et al.*, 1993).

Statistical Analysis. Analysis of variance of the beef extraction data (Table 2) indicates that the two most significant factors influencing the percent protein solu-

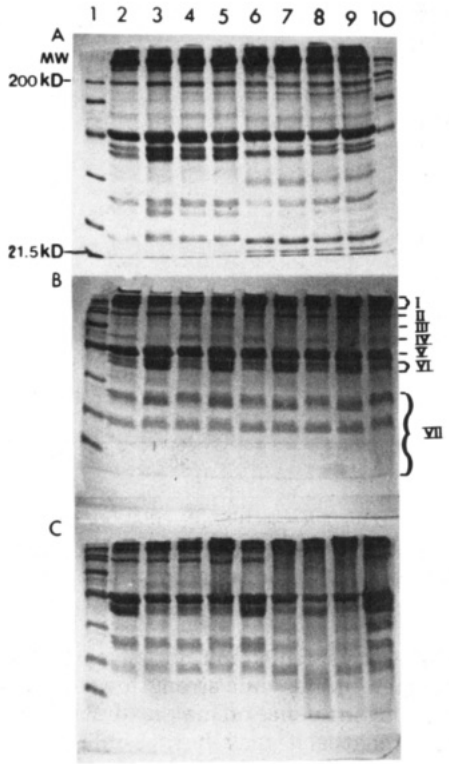

Figure 1. Electrophoretic patterns of chicken and beef muscle extracts and solutions. (A) Lane: 1, standards; 2, sternomandibularis (6 days post-mortem) whole extract; 3, same as lane 2 but centrifuged extract; 4, semimembranosus (6 days post-mortem) whole extract; 5, same as lane 4 but centrifuged extract; 6, pectoralis (6 days post-mortem) whole extract; 7, same as lane 6 but centrifuged extract; 8, iliotibialis (6 days post-mortem) whole extract; 9, same as lane 8 but centrifuged extract; 10, standards. (B) Lane: 1, standards; 2, sternomandibularis (1–2 h post-mortem) whole extract; 3, same as lane 2 but centrifuged extract; 4, sternomandibularis (1 day post-mortem) whole extract; 5, same as lane 4 but centrifuged extract; 6, semimembranosus (1–2 h post-mortem) whole extract; 7, same as lane 6 but centrifuged extract; 8, semimembranosus (1 day post-mortem) whole extract; 9, same as lane 8 but centrifuged extract; 10, soleus (1–2 h post-mortem) whole extract. (C) Lane: 1, standards; 2, soleus (1–2 h post-mortem) centrifuged extract; 3, soleus (1 day post-mortem) whole extract; 4, same as lane 3 but centrifuged extract; 5, soleus (6 days post-mortem) whole extract; 6, same as lane 5 but centrifuged extract; 7, pectoralis (1 day post-mortem) whole extract; 8, same as lane 7 but centrifuged extract; 9, iliotibialis (1 day post-mortem) whole extract; 10, same as lane 9 but centrifuged extract. Identification of bands: I, titin, nebulin; II, myosin heavy chains; III, c protein; IV, α actinin; V, actin; VI, troponin T, tropomyosin; VII, myosin light chains, troponin.

bilization of the final pellet ($P \leq 0.01$) are the total protein level in the homogenate and the pH of the homogenate. In this two-step process of protein extraction and solubilization, efficiency in the first phase is necessary for subsequent success during final solubilization. Less significant ($P \leq 0.05$) are post-mortem age, differences among animals, and, marginally, conductivity of the final solution. Overall percentage yield of protein in the final solution is affected most significantly ($P \leq 0.01$) by post-mortem age, protein level in the homogenate, and homogenate pH. Of less significance ($P \leq 0.05$) are differences among animals and pH of the solution. Values for R^2 indicate that 68% (solubilization of final pellet) and 83% (overall yield) of the total variation in results can be explained by the model used. Similar trends were seen for the chicken extraction data, but significance for these effects was not attained, due most likely to fewer data points and large variability.

From correlation analysis (data not shown) it was found that the two factors of protein level in the homogenate and pH of the homogenate, both of high significance in determining solubility and overall yield of protein, were not closely related ($r = -0.39$, NS). This suggests that these factors are independent and at least two underlying physiological parameters are important in these processes.

Table 2. Analysis of Variance for Beef Muscle Tissue Extraction and Solubilization Data

source	df	F	prob
Parameter: Percent Solubilization of Final Pellet ($R^2 = 0.68$)			
muscle	2	0.4799	0.4944
animal	2	5.7192	0.0240
post-mortem age	2	5.9734	0.0213
% homogenate yield	1	9.4247	0.0048
homogenate pH	1	9.5548	0.0046
% final pellet yield	1	2.9610	0.0967
solution pH	1	2.2529	0.1450
solution conductivity	1	4.2039	0.0502
Parameter: Percent Overall Yield ($R^2 = 0.83$)			
muscle	2	0.4828	0.4931
animal	2	5.0914	0.0323
post-mortem age	2	10.5583	0.0031
% homogenate yield	1	10.4507	0.0032
homogenate pH	1	7.9442	0.0089
% final pellet yield	1	2.8601	0.1023
solution pH	1	4.6722	0.0397
solution conductivity	1	1.8731	0.1824

Table 3. Literature Values for Fiber Types in Experimental Muscles (Percent)

	I	IIa	IIb
beef			
sternomandibularis[a]	53	47	0
semimembranosus[a]	20	42	38
soleus[a]	97	0	3
chicken			
pectoralis[b]	8	20	72
iliotibialis[c]	27	54	19

[a] Totland and Kryvi (1991). [b] Kaiser and George (1973). [c] Suzuki *et al.* (1985).

Mechanisms. What physiological parameters can influence protein level in the homogenate? It is to be expected that, since no relationship was found between the initial protein level of the muscle tissue and any of the parameters measured in this study, efficiency of the homogenization step would be of importance. This, in turn, would reflect the type or state of the muscle. Vertebrate skeletal muscles can be differentiated into two principal fiber types (with subtypes) on the basis of their morphological and biochemical properties (Swatland, 1984). These types are the red, oxidative, slow, tonic type, characterized by a small diameter, high mitochondrial content, and high levels of myoglobin, fat, and oxidative enzymes, and the white, glycolytic, fast twitch type, characterized by a larger diameter, lack of myoglobin, and low levels of fat, mitochondria, and oxidative enzymes.

It is possible to quantitate the content of fiber types using histochemical techniques. Although several, some contradictory, nomenclatures have been used, the one that seems to be most accepted denotes "red" fibers as type I, "white" fibers as type IIb, and those containing both aerobic and anaerobic energy systems as type IIa. Literature values of distributions for the muscles used in this study are given in Table 3. If these values for white fibers (IIb) in beef and chicken are plotted against the protein yield in the homogenate averaged over all time periods, a positive linear relationship is obtained with a coefficient of determination of 91.2% (Figure 2). This indicates that much more protein is extracted from muscles with a higher content of white fibers when using the procedure described. Other authors also have noted that protein extractability varies with muscle type. For example, Richardson and

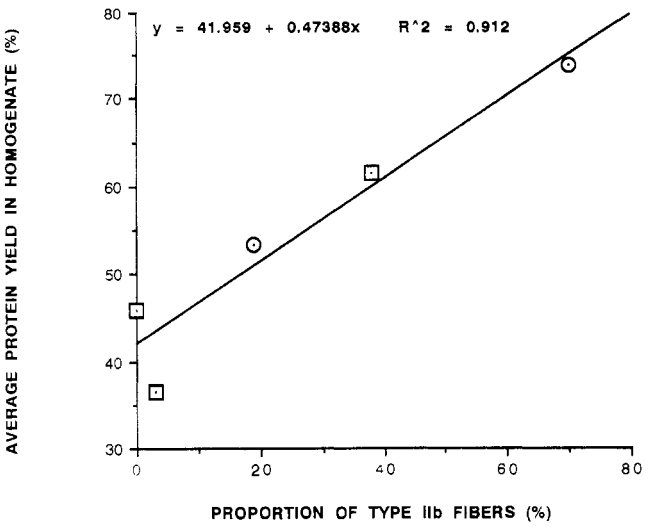

Figure 2. Protein yield in the homogenate averaged over post-mortem time as influenced by proportion of type IIb fibers: (○) chicken; (□) beef.

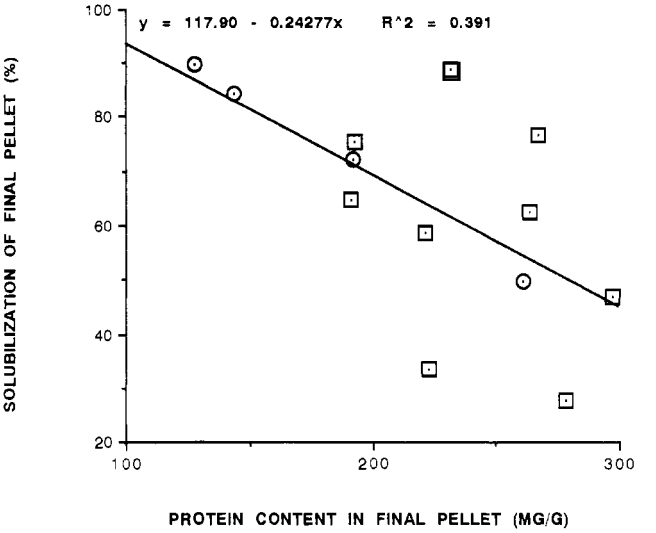

Figure 3. Percentage protein solubilization of the final pellet as influenced by protein content of the pellet: (○) chicken; (□) beef.

Jones (1987) found that turkey breast yielded more salt-extractable protein than thigh muscle. Totland and Kryvi (1991) report high intramuscular variations in fiber type distribution of a large number (106) of bovine animals that were alike with respect to age, gender, breed, nutrition, level of activity, and slaughter procedures. This could contribute to the differences in extractability within treatments seen in Table 1.

What physiological parameter can influence the pH level of the homogenate and what is the role of pH in extractability? It would be expected that higher pH levels would be associated with greater extraction since the problems of protein denaturation and precipitation would be avoided. However, referring to Table 1, it may be seen that post-mortem aging reduces homogenate pH, as would be expected due to the accumulation of metabolic acids, but improves extractability. This increase in protein extractability with post-mortem aging results, perhaps, from cytoskeletal breakdown that allows greater penetration of the homogenizing and washing media of which pH is an indirect measure. Another factor that may deserve consideration is the post-mortem release of calcium from muscle membranes (Stanley, 1991). More importantly, the conductivity of the final solution is strongly related to the homogenate pH ($r = 0.82$, $P \leq 0.01$, 11 df). Thus, the higher the pH of the homogenate, the higher the final conductivity or ion content, and final conductivity is negatively related to the final extraction ($r = 0.57$, $P \leq 0.05$, 11 df). It was found to be important to keep the pH of the washing solutions above 7. At this pH muscle proteins are above the isoelectric point (IEP) and therefore carry a net negative charge and an electrostatic repulsive force exists between them. Increasing ionic strength below physiological values would be expected to reduce this repulsion and interfere with solubility.

An alternative to these hypotheses is based on the swelling observations of Szent-Györgyi (1943a,b). It is known that the water content of muscle fibers is influenced by ionic strength. This is an important effect since it is related to water-holding capacity, an important industrial parameter. Swelling is thought to be a function of the spaces within and between myofilaments that hold water (Offer and Trinick, 1983; Offer and Knight, 1988a,b). Muscle myofilaments are ordered in a hexagonal lattice, the dimensions of which are a function of sarcomere length, pH, ionic strength, ion type, and sarcolemmal and endomysial integrity (Wilding *et al.*, 1986). In aged muscle tissue, where the sarcolemma no longer acts as a semipermeable membrane, ionic strength would be expected to have a strong influence on matrix dimensions. At very low ionic strengths it may be possible that repulsive protein–protein interactions lead to swelling. The influence of the restraining endomysial sheath must also be considered. There is some evidence that certain collagen-based structures in muscle tissue break down during post-mortem aging (Stanley and Brown, 1973) and that the addition of collagenase to muscle fibers leads to increased swelling (Wilding *et al.*, 1986).

The swelling hypothesis may be useful in explaining the current data. Initial microscopic observations indicate that the homogenate mainly contains a dispersion of myofibrils. Thus, the effect of washing would be to remove ions so that these can swell or take up water. The higher the pH (the further away from the IEP) and the lower the ionic strength, the more swelling that occurs. Swelling of muscle proteins followed by solubilization in water parallels the observations of Szent-Györgyi (1943a). Any treatment that leads to internal disruptions (*e.g.*, post-mortem aging) could promote swelling, perhaps by decreasing contact regions among protein subunits through repulsive forces. In the current study it was possible to estimate swelling by measuring the protein content of the final pellet or sediment. The pellet had a swollen appearance and it is assumed that lower protein levels mean higher water levels and more swelling (Offer and Trinick, 1983). If the protein content of the pellet (milligrams per gram) is plotted against percentage solubility of the final pellet (Figure 3), a significant negative correlation is obtained ($r = -0.63$, $P \leq 0.05$), strongly suggesting that swelling is associated with a higher level of protein solubilization. It should be cautioned, however, that a higher protein content in the final pellet will result in a smaller dilution of salt when the pellet is solubilized with 50 volumes of solution. Also, it is interesting to note prior suggestions that variations in swelling between myofibrils could be a result of differences in response to ionic strength of different fiber types (Knight and Parsons, 1984; Richardson and Jones, 1987).

Summary. It has been possible to obtain relatively high levels of muscle protein solubility in an aqueous solution of low (<30 mM) ionic strength. Electrophoresis separation indicates the dominant proteins in the extract are those of the myofibril. Two seemingly independent factors have been identified thus far that influence significantly the degree of protein solubility obtained.

These are the amount of protein extracted into the homogenate and the pH of this homogenate. The first factor is a strong function of the white fiber content in the muscle, while the second is likely related to the presence of ions released from the tissue during the homogenization step. An alternative explanation rests on the swelling phenomenon.

While further experimentation is needed to ascertain in more detail the mechanism of the solubility procedure, the implications of these findings to such technological processes as water binding, emulsification, and gelling are also important reasons to continue these studies.

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