# Functional Chicken Muscle Protein Isolates Prepared Using Low Ionic Strength, Acid Solubilization/Precipitation

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## Introduction

Full utilization of all the parts of poultry and fish is increasingly becoming an industry goal. Processes such as mechanically deboning are presently used to gain greater yields from whole chickens and fish. The resulting residue from mechanically deboned chicken backs and necks contains between 16-20% protein (Opiacha et al., 1994). If the meat fraction of the residue can be properly separated from the bone it can be utilized as human food. Solubilization of the muscle proteins using either high salt or alkaline conditions and low force centrifugation, has been used by researchers to fractionate the proteins from other components, such as bones (Kijowski and Niewiarowicz, 1985; McCurdy et al., 1986; Opiacha et al., 1994). Recently we (Hultin and Kelleher, 1999) used low pH, low ionic strength solubilization and high force centrifugation to separate fish proteins from head and gutted (bone-in) starting material. In contrast to other methods, the high g-force centrifugation was found to substantially reduce the membrane phospholipids while the proteins were recovered in high yields and retained their functionality, especially their ability to gel. Gandemer (1999) recently stated that phospholipids were now widely recognized as the main substrates in muscle tissue for lipolysis and oxidation reactions and responsible for many of the oxidative off odors. Research has also pointed to phospholipids as the potential initiator of lipid oxidation reactions (Love and Pearson, 1971; Meynier et al., 1999). Phospholipid or membrane lipid removal should produce a more stable protein isolate.

This manuscript describes a process whereby proteins from two distinctly different types of chicken muscle (breast, mostly light muscle fibers; thigh and leg, mixture of dark and light muscle fibers) were solubilized under conditions of low ionic strength, low pH and recaptured using isoelectric precipitation. Recovered proteins had reduced lipid and phospholipid content and retained the ability to form a gel.

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## Materials and Methods

### *Materials*

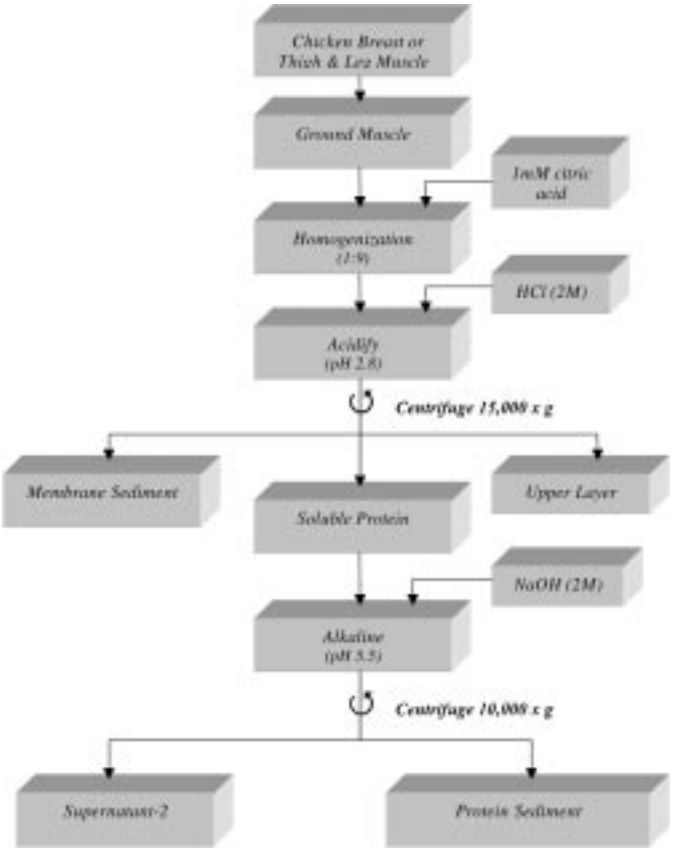
Hybrid hens (*Rhode Island Red/White*) were obtained at Longwood Farm, Reading, MA and transported to the laboratory where they were asphyxiated using carbon dioxide. Muscle from the breast and thigh and leg was stored on the skeletal frame until postmortem resolution of rigor had occurred, prior to being excised from the carcasses and well iced. Pre-cast, linear gradient (4–20%) Mini Plus SepraGel Tris-HCl electrophoresis gels and Pro-Blue stain were obtained from Owl Separation Systems (Woburn, MA). Molecular weight SDS-PAGE standards myosin, rabbit muscle, 205kD; β-galactosidase, E-coli, 116 kD; phosphorylase b, rabbit muscle, 97.4 kD; albumin, bovine, 66 kD; albumin, egg, 45 kD; and carbonic anhydrase, bovine erythrocytes, 29 kD, were obtained from Sigma Chemical (St. Louis, MO). All other chemical were of reagent grade.

### *Methods*

#### *Acid Solubilization-Precipitation Process*

Chicken breast and thigh muscle, well trimmed of lipid, was used as the starting material for the acid solubilzation/precipitation process, diagrammed in Fig. 1. The tissue was ground to approximately 6 mm using a Kitchen-Aid grinder (St. Joseph, MI) prior to being combined with a 1 mM citric acid solution, at a ratio of 1:9. The mixture of fish muscle and water was homogenized using a Polytron at speed 76 for 2 minutes. The resultant homogenate was adjusted to pH 2.8 using 2 M hydrochloric acid added drop-wise. The homogenate was centrifuged using a Model L-65B, or a L8-55M ultracentrifuge (Beckman, Palo Alto, CA) and a No. 19 rotor (10,000 RPM) for 30 minutes with a resultant g-force of 15,000. Three distinct phases were produced by the centrifugation step: an upper layer, a membrane sediment layer and a middle aqueous protein solution, which contained the proteins. The protein solution was separated from the other layers and adjusted to approximately pH 5.5 using 2 M NaOH added drop-wise. As the pH increased, thread-like aggregates became appar-

**FIGURE 1.**




*Generalized schematic of the acid solubilization/precipitation process used in obtaining functional, chicken protein isolates.*

ent. At the isoelectric point of the proteins, a large mass of these aggregates developed. The aggregates, which are approximately 95% moisture, were de-watered using centrifugation at 10,000 g-force for 20-30 minutes in the ultracentrifuges described above.

*Manufacture of Protein Gels*

Precipitated, de-watered protein (iso-electric) was mixed with cryoprotectants (4% sucrose, 4% sorbitol, and 0.3% sodium tripolyphosphate) and were blended into the dewatered mince using a Model R301 chilled food processor (Robot Coupe, Inc., Ridgeland, MS) for 30 seconds. The pH was adjusted to between 7.1–7.2 using 0.35% $NaHCO_3$ prior to freezing at -80°C.

Frozen surimi was tempered at refrigerated temperatures for 1 hour, adjusted to 80% moisture with distilled, deionized water and chopped with 2% crystallized sodium chloride using a Robo-Coupe R301 Ultra chopper (Robo-Coupe, Inc., Ridgeland, MS) for 2 minutes. Equipment was pre-chilled and chopping took place in a refrigerated room (6°C) to prevent the surimi from exceeding 10°C while chopping. Product was stuffed into stainless steel tubes (19 d x 175 l mm) and sealed on both ends. The tubes were heated in water baths at 90°C for 30 minutes. Heated product was cooled in ice for approximately 15 to 30 minutes. Cooked gels were stored in polyethylene bags at 6°C for 48 hours prior to analysis.

*Stress and Strain at Breakage*

Stress and strain at structural failure was determined on the gels using the procedure of Wu et al., 1985. Sections of gels were machined to achieve a concave shape with a mid-section diameter of 10 mm. Diameter was checked with a caliper and adjustments to the shaper were performed if needed. Twisting of the gel was performed on a Haake rotovisco PK plate on a Brookfield DV-II viscometer. Rheological values as described by Lanier et al., 1991 were obtained using a computer-aided software program linked to the computer and viscometer (North Carolina State University, 1985).

*Protein*

Protein was quantified using a Biuret reaction method as described in Torten and Whitaker (1969). A detergent sodium deoxycholate (10%) was added (0.5–1.0 ml) to the incubating biuret protein solution to clarify for absorbance measurement at 540 nm. Appropriate dilution was adjusted for in the calculations. Standard curves were constructed using bovine serum albumin. Protein solubility was determined as protein concentration in the first centrifugation supernatant divided by protein concentration in the homogenate, multiplied by 100.

*Electrophoresis*

Soluble and homogenate proteins were separated according to the electrophoresis procedure described by Laemelli (1970). Pre-cast mini linear gradient gels 4–20% (Owl Separation Systems, Woburn, MA) were used to separate proteins on a vertical PAGE Mini Device (Daiichi Scientific, Toyko, Japan) with a constant current of 30 mA per gel. A modified sample buffer containing 40 mM EDTA and 160 mM DTT was prepared and heated to 100°C for 2 min following the procedure of Wang (1982). Protein bands were fixed using a 1 hr incubation in 12% trichloroacetic acid, followed by overnight staining using Pro-Blue. Scanning of the stained gels was accomplished using a Hoefer Model GS 300 Scanning Densitometer (Hoefer Sci., San Francisco, CA) in the transmittance mode with accompanying Model 365W Densitometer Analysis software for protein quantification. A standard curve was constructed using high molecular weight SDS-PAGE standards on a linear gradient as described by Hames (1981).

*Lipid*

Total lipid was extracted from muscle and different phases of centrifuged muscle using a 1:1 ratio of cold chloroform/methanol as described by Lee et al. (1996) for use with tissue suspected of containing lipids of average polarity. Phospholipid was determined according to the method of Kovacs (1986) by placing approximately 300 µl of chloroform/methanol extracted lipid (accurately weighed) containing 1-20 µg phosphorus and ashed for 550°C for 2 hours. To prevent loss of product during the ashing step due to spattering, 1 ml of 10%

zinc acetate was used to cover sample. A phosphorus/molybdate/ascorbate chromophore was developed and detected at 820 nm. Calculation of phospholipid (PL) in the tissue sample was obtained using the formula:

$$\%PL = (\mu g \text{ phosphorus/vol sample}) \times (\text{vol of chloroform}) \times (750/30) \div \text{tissue (g)}$$

The conversion of lipid phosphorus to phospholipid content was estimated by using the average molecular weight of 750 daltons for phosphotidylcholine divided by the molecular weight of phosphorus of 30 daltons, phosphorus and phosphotidylcholine being equimolar.

*Chemical Analysis*

Ionic strength of the final soluble supernatant was determined using a YSI Model 35 Conductance meter (YSI, Yellow Springs, OH) equipped with a YSI Conductivity cell (K=1.0/cm). Samples were measured at 25°C. A standard curve was constructed using sodium chloride. Viscosity was measured at 4-6°C using a Brookfield "HAT" viscometer equipped with a series of spindles and varying speeds. A manufacture's chart allowed for the determination of viscosity when using different combinations of spindle and RPM. Color "L", "a", and "b" values were determined on fish gels and powders using a Hunter LabScan II colorimeter (Hunter Associates Laboratories, Reston, VA). A $D_{65}$ illuminant was utilized and reflected light was viewed at 10°. Moisture content was determined by weight difference between unheated samples and those that had been heated at 105°C overnight. For moisture adjustment purposes in surimi, a Cenco Moisture Balance (CSC Scientific Co., Inc.) was used that rapidly dried product using infra-red.

## Results and Discussion

Protein solubility for chicken breast (light) muscle at pH 2.8 and an ionic strength of 28 mM was 92.6% ± 5.5. This is comparable to most lean, white fish with a postmortem muscle pH around neutrality. The values for low pH solubility for thigh and leg muscle, which contains a majority of dark muscle fibers, was 71.1% ± 6.2 at 18.9 mM ionic strength and pH 2.8. Krishnamurthy et al. (1996) found that essentially all the proteins from chicken breast muscle could be solubilized at neutral pH and at physiological ionic strength or less. However, a critical order of extraction had to be followed whereby selective removal of certain polypeptides was found necessary to enable the total (> 90%) solubilization of the muscle proteins. This critical "unlocking" of key proteins to allow complete solubilization was also found to be true using Atlantic mackerel light muscle at neutral pH and ionic strengths around 150 mM and less (Feng and Hultin, 1997). No such selective removal of proteins was needed to solubilize proteins at low pH. Protein side chains on the acidic side of the isoelectric point gain a net positive effect, which increases as the pH become more acidic (Hamm, 1994). At pH values around 2.8 a majority of the proteins would have a positive charge. Two amino acid side chains found in great abundance in fish are glutamic acid ($pK_{a2}$ 4.25) and aspartic acid ($pK_{a2}$ 3.86) (Shahidi, 1994), which lose their negative charge as their environment becomes acidic. Like charges cause repulsive forces between neighboring proteins allowing the proteins to become surrounded by, or interact with the solvent and become solubilized. Salt, when added to the protein solutions at low pH, tends to shield the repulsive forces causing increased insolubilization. Using Atlantic cod muscle proteins at pH 3.0, we found almost complete solubilization of the protein (100–97.1%) occurs from 1–200 mM NaCl. Solubilization thereafter drops off somewhat in a steep manner. At 300 mM the solubility was at 86.8% followed by 43.4% and 0.1% at 400 mM and 500 mM, respectively.

Viscosities of the pH 2.8 chicken breast homogenate was 51.7 ± 0.3 mPa.s and the thigh and leg homogenate was 26.5 ± 1.2 mPa.s. In both homogenates the viscosity is high around pH 7.0 and decreased rapidly as the pH approaches the isoelectric point. Hamm (1994) found this to be true with many muscle proteins. On the acidic side of the isoelectric point, the viscosity increases until approximately pH 4.5–4.2. This may be an indication that the conformation of the protein is changing to a more unfolded configuration. In polymers, as the secondary structure opens, it allows more effective or hydrated volume to occur, which leads to increased viscosity (Hodge and Osman, 1976). A long, linear molecule will have a greater relative volume, hence greater viscosity than a compact molecule of the same molecular weight. Our viscosity results suggest possibly that thigh and leg muscle does not undergo partial unfolding to the same degree as the breast and hence its reduced solubility. Complete unfolding creates exposed hydrophobic domains, increasing the chances for hydrophobic interactions thus leading to insolubilization or precipitation (Pace, 1983).

A protein mass balance through the steps of the acid solubilization/precipitation process is shown in Table 1. Both breast and thigh and leg start the process at approximately equal protein concentrations. After the first centrifugation, substantially more protein is separated out with the membrane sediment using thigh and leg muscle as the starting material. It seems probable that the sediment layer would contain the more insoluble connective tissue proteins, found in higher amounts in thigh and leg muscle than in breast muscle. The upper layer contains small amounts of protein, which appear to be in an emulsified layer closely associated with the neutral lipid. Proteins found in the protein soluble layer were high in concentration. The extent of solubilization determines, in most part, the overall yield of protein isolate. One other aspect of yield is recovery. The main difference between solubility and recovery lies in protein that gets distributed in a "soft mass" fraction that sometimes forms just above the membrane sediment layer. While these proteins are mostly soluble, they are entrapped and accounted for in the sediment. Hultin et al., 1995 describe the phenomenon of solubility versus extractability, which is similar to the above.

In the second centrifugation, only slight amounts of protein were found in the supernatant. In processing these would constitute effluent contamination and would be desirous to be kept low. Adjustment of the proteins of both muscle types to their isoelectric point induced precipitation. Since the iso-

**TABLE 1.** Protein mass balance through selected steps in the acid solubilization/precipitation process.

| | | Centrifugation–1 | | | Centrifugation–2 | |
|---|---|---|---|---|---|---|
| | Muscle / Homogenate | Membrane Sediment | Upper Layer | Protein Solution | Supernatant-2 | Protein Sediment |
| Chicken Breast (Light) | 22.22 ± 1.36 (100) | 1.10 ± 0.16 (4.95) | 1.33 ± 0.43 (5.99) | 19.60 ± 0.80 (88.21) → | 1.04 ± 0.53 (4.68) | 18.56 ± 1.33 (83.53) |
| Chicken Thigh & Leg (Dark) | 22.95 ± 0.76 (100) | 6.77 ± 2.20 (29.50) | 0.44 ± 0.44 (1.9) | 16.84 ± 1.01 (73.38) → | 0.50 ± 0.12 (2.18) | 15.74 ± 0.78 (68.58) |

Mean in units of g/100 g starting material ± standard deviation.
Ground muscle was homogenized (1:9) in 1 mM citric acid pH 2.8. Protein was determined using a Biuret method described by Torten and Whitaker (1969).
Values in parenthesis are percentage protein relative to the starting muscle/homogenate.

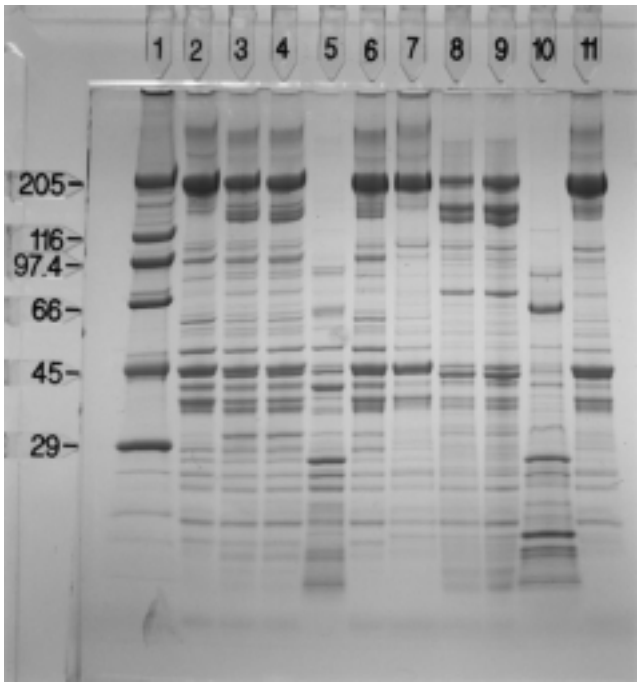
electric point may be different for the two different muscle types, slight adjustments in pH may further reduce the losses.

Polypeptides associated with selected steps in the acid solubilization/precipitation process are shown in the SDS-PAGE results (Fig. 2). Both light and dark muscle contain large concentrations of myosin heavy chain (MW 205 kDa) and higher molecular weight proteins. Most likely the large molecular weight peptides (> 205 kDa) are the cytoskeletal proteins titin and nebulin. Reducing the pH to acidic levels produced additional bands at approximately 150 kDa for both breast and thigh and leg muscle. We have found the development of this peptide only occasionally in fish and it appeared to increase inversely proportionally to the myosin heavy chain band. This suggests that either acid or enzymic hydrolysis has occurred possibly using myosin as substrate. Takeda and Seki (1996) also found a 150 kDa MW fragment in Walleye Pollock surimi, and believed it was from the proteolytic digestion of myosin. The protein troponin-T was found to proportionally decrease in concentration as a 150 kDa peptide developed in Pollock surimi paste held at 30°C (Numakura et al., 1985). However, many authors have found no negative effect of the development of this proteolytic peptide on gelation quality (Kamath et al., 1985; Muramoto and Seki, 1989; Lee et al., 1990).

The second supernatant fraction contained no polypeptide greater than approximately 75 kDa in either the light or dark muscle. Also, small amounts of the contractile protein actin were found, predominantly in the breast muscle sample. Both protein sediments contained large concentrations of myosin and actin. Myosin is the myofibrillar protein most noted for imparting functionality to the final end products (Yamamoto et al., 1987; Park et al., 1997). The high molecular weight proteins (> 205 kDa) are also present in the final sediment. These are not commonly found in large amounts in the final sediments when Atlantic cod or mackerel are used as the starting muscle.

Initial muscle tissue and protein sediment was analyzed for major components and phospholipids (Table 2). Moisture

**FIGURE 2.**




*SDS-PAGE (4-20%, linear gradient) of chicken breast (light) and thigh and leg (dark) muscle proteins at selected steps in the acid solubilization/precipitation process.*

*Lane 1, molecular weight standards; lane 2, chicken breast muscle; lane 3, chicken breast homogenate (1:9) pH 2.8; lane 4, chicken breast soluble protein from centrifuged (15,000 x g) sample; lane 5, chicken breast supernatant-2 from de-watering centrifuge (10,000 x g); lane 6, chicken breast protein sediment; lane 7, chicken thigh & leg muscle; lane 8, chicken thigh & leg homogenate (1:9) pH 2.8; lane 9, chicken thigh & leg soluble protein from centrifuged (15,000 x g) sample; lane 10, chicken thigh & leg supernatant-2 from de-watering centrifuge (10,000 x g); lane 11 chicken thigh & leg protein sediment. Protein was applied to lanes at 15 mg/lane.*

**TABLE 2.** Physical characteristics of chicken breast and thigh & leg muscle and the functional protein isolates prepared from the two types of muscle using acid solubilization/precipitation.

| | Moisture (%) | Protein (%) | Lipid (%) | Phospholipid (mg/100g) |
|---|---|---|---|---|
| Chicken Breast | | | | |
| Muscle | 74.4 ± 0.3 | 23.6 ± 0.3 (92.2) | 1.39 ± 0.08 (5.43) | 822.6 ± 0.6 (3210) |
| Protein sediment | 76.2 ± 0.1 | 22.1 ± 0.4 (92.9) | 0.64 ± 0.03 (2.69) | 483.4 ± 34.8 (2030) |
| Chicken Thigh & Leg | | | | |
| Muscle | 75.7 ± 0.1 | 20.3 ± 1.0 (83.5) | 4.95 ± 0.02 (20.37) | 920.2 ± 42.5 (3790) |
| Protein sediment | 72.8 ± 0.4 | 26.8 ± 1.8 (98.5) | 1.12 ± 0.01 (4.12) | 500.6 ± 104.4 (1840) |

Values are means ± standard deviation on a wet weight basis. Parentheses presented on a dry basis. Protein was determined using the Biuret method as described by Torten and Whitaker (1969). Lipid was determined using a 1:1 chloroform:methanol extraction solution as described by Lee et al. (1996). Phospholipid was estimated as phosphorus determined by the dry ashing method of Kovacs (1986) and the assumption that the average MW of phosphotidylcholine was 750 daltons.

content in the protein sediments was found to be similar to the initial starting muscle. In extracting protein using the standard surimi washing procedure around neutral pH, 0.1–0.2% salt is usually added to enable de-watering of tissue (Lee, 1986). However, at the isoelectric point water binding by the proteins is at a minimum, therefore de-watering of the acid solubilized protein is relatively easy. Protein contents on dry basis of both protein sediments increased compared to the initial muscle. This was due in part to the decrease found in the total lipid content. The acid solubilization process removed approximately 50% of the initial lipid from chicken breast muscle and 80% from the thigh and leg muscle. Phospholipids are difficult to remove from muscle tissue and usually require the use of polar organic solvents, such as methanol (Bligh and Dyer, 1959). Using acid solubilization and 15,000 x g centrifugation, approximately 37% and 51% of the phospholipid was removed from breast and thigh and leg muscle, respectively. This is lower than the removal amounts of phospholipids we found for fish, which are usually in the 85–90% range (Hultin and Kelleher, 1999). Perhaps, chicken muscle being more dense than fish muscle, interferes with the removal of phospholipids using acid solubilization.

Quality values of gels prepared from protein sediments are shown in Table 3. Stress and strain values for breast muscle are consistent with a sample that is quite firm (stress) and elastic (strain). Chung et al. (1993) stated that a value of 1.9 or greater is an indication of a Grade A, high quality gel. Thigh and leg muscle had moderate firmness and a low elastic value, representative of a fair quality gel. One possible explanation for the poorer quality may lie in the solubility results. Thigh and leg muscle proteins were 29% insoluble under low pH, low ionic strength conditions. In Fig. 2, lane 9 it appears that there is less myosin heavy chain (MW 205 kDa) in the soluble fraction of thigh and leg muscle than in the breast muscle soluble fraction (lane 4). Myosin may be selectively less soluble under these low pH conditions. Myosin is believed to be the protein most responsible for muscle protein functionality ( Park et al., 1997).

Color values of the final gels made from the two types of muscle are also shown in Table 3. Values for breast muscle

**TABLE 3.** Gelation and color values of chicken protein isolates prepared using acid solubilization/precipitation**.**

| | | | Color | | |
|---|---|---|---|---|---|
| | Stress (kPa) | Strain | "L" | "a" | "b" |
| Chicken Breast (Light) | 62.74 ± 2.18 | 2.19 ± 0.06 | 83.93 ± 1.57 | –1.85 ± 0.16 | 10.57 ± 0.45 |
| Chicken Thigh & Leg (Dark) | 24.01 ± 1.37 | 1.24 ± 0.12 | 69.02 ± 0.36 | –0.17 ± 0.06 | 12.79 ± 0.06 |

Stress and strain at structural failure was determined using the torsion method described by Wu et al., 1985. Color values were obtained using a Hunter LabScan II colorimeter as described in Methods.

are consistent with a light sample. A high "b" value may indicate that oxidation may have occurred during the process. Srinivasan and Hultin (1997) found that "b" values of washed cod muscle increased when the sample was subjected to a free radical oxidizing system. Color values for the thigh and leg muscle are indicative of a somewhat dark sample with a tan hue.

## Conclusions

Protein solubility at pH 2.8 for chicken breast (light) muscle was 92.6% ± 5.5, while thigh and leg muscle, which contains a majority of dark muscle fibers, was 71.1% ± 6.2. Acceptable yields of protein isolates were obtained using breast and thigh and leg muscle as the starting muscle source. Using high centrifugal force, substantial amounts of lipid and phospholipid were separated and removed from the proteins. Breast muscle responded better than thigh and leg muscle in almost all categories that were tested, such as solubility, yield and functionality. However, a higher percentage of both initial lipid and phospholipid was extracted from thigh and leg (dark) muscle than breast (light) muscle.

## Acknowledgements

This work was supported in part by the Massachusetts Agricultural Experiment Station. The views expressed herein are those of the authors and do not necessarily reflect the views of the Department of Agriculture or any of its sub-agencies. The authors would like to thank Dr. Yuming Feng for his helpful contributions.

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