

Protein Extraction From Chicken Myofibrils Irrigated with Various Polyphosphate and NaCl Solutions

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ABSTRACT: Physical changes in chicken gastrocnemius myofibrils incubated in 0.1 to 1.0 M NaCl solutions with or without 10 mM ortho-(P), pyro-(PP), tripoly-(TPP) or hexameta- (HMP) phosphate at pH 6.0 were examined by phase-contrast microscopy, electrophoresis, and solubility. PP and TPP performed similarly in promoting protein extraction, P had no apparent effect, and HMP exhibited an intermediate effect. PP, TPP, and HMP treatments markedly improved protein solubility in 0.3 and 0.4 M NaCl through the release of myosin, but the phosphate effect diminished in ≥ 0.6 M NaCl. Overall, phosphates influenced the ultrastructure of myofibrils and extraction of their constituents in the order: PP $\approx$ TPP > HMP > P $\approx$ nonphosphate control.

Key Words: myofibril, protein extraction, polyphosphate, phase contrast microscopy

Introduction

PHOSPHATES HAVE THE ABILITY TO ENHANCE WATER-HOLDING in muscle tissues and to modify textural properties of comminuted meat products (Trout and Schmidt 1983; Patterson and others 1988). When used in conjunction with salt (NaCl), phosphates can markedly improve cooking yield and palatability (juiciness) of meat and poultry products (Froning and Sackett 1985; Xiong and Kupski 1999b). The strong hydration-promoting effect of phosphates makes them excellent ingredients for reduced-fat, water-added muscle foods. Among the phosphates most widely used in the meat industry are alkaline or acidic types of di- and tripolyphosphates and, to a lesser extent, cyclic phosphate compounds (Dziezak 1990).

Improved water-retention of meats treated with phosphates has been recognized for more than half a century. Early studies that demonstrated the beneficial effect of phosphates had been conducted primarily with sausage and canned meats (Bendall 1954). Hamm and Grau (1958) and Hellendoorn (1962) showed an increased water-binding capacity of raw muscle after it was blended in pyrophosphate (PP) and tripolyphosphate (TPP) solutions. The specific activity of both phosphates seemed to be highly dependent on pH; in general, a pH 6 or higher was required to produce a maximum hydration effect. On the other hand, tetrametaphosphate (TMP), hexametaphosphate (HMP), and orthophosphate (P) were found to have less or no effect on water-holding capacity of meat (Hamm 1960; Hellendoorn 1962). Possible mechanisms by which phosphates enhance the water-binding ability of meat have been reviewed (Hamm 1970), and they were ascribed to the ionic effect and pH alteration contributed by phosphates, and for PP, also the dissociation of the actomyosin complex (Bendall 1954). Our recent study suggested that differences in the specific activity among various phosphates were also related to their ability to penetrate or diffuse into the muscle matrix (Xiong and Kupski 1999a,b).

Much of the work cited above had been undertaken with whole or fabricated muscle tissues. In fresh meat, approximately 85% of the water is located inside muscle cells, primarily between thick and thin filaments (Hamm 1986). Although myofibrils are the primary site for intracellular water, many other

cellular components may also contribute to water-binding in the meat. Entrapment and mobility of the water present outside the cells (about 15%) could be affected by many environmental factors, and its exact content could vary with different processing treatments. Therefore, in order to define the hydration mechanism of meat marinated in different phosphate solutions with different ionic and pH conditions, it is important to identify the contribution by the individual muscle components, for example, myofibrils.

Ishiwata (1981) and Offer and Trinick (1983) presented some of the initial work that examined the effect of pyrophosphate on myofibril hydration. Using phase-contrast microscopy, the authors elucidated salient phosphate-induced structural changes in the myofibril architecture. Several similar studies were published subsequently (Patterson and others 1988; Parsons and Knight 1990). Despite these previous investigations, which have been done on mammalian muscles, mechanisms underlying the effects of different phosphate compounds on muscle hydration are not entirely understood. For example, information is absent from the literature comparing the effects of different phosphates commonly used in processed muscle foods (tripolyphosphate and hexametaphosphate in particular) on the hydration properties of myofibrils under various ionic strength conditions. The objective of the present study was to elucidate structural changes in chicken myofibrils and extraction of the individual myofibrillar components when exposed to various commercial phosphates at different salt (NaCl) levels.

Results and Discussion

Myofibril structural changes

Myofibrils prepared in this study had different diameters (thickness), indicating that some samples (the thick ones) were probably composed of two or more myofibrils. Despite the size variation, all myofibril samples showed a striation pattern characteristic of skeletal muscle fibers which are comprised of repeated A-bands (the rectangularly shaped dark region) and Z-lines (Fig. 1). In the absence of phosphates, no appreciable swelling or any other change was noted at salt (NaCl) concen-

trations of 0.4 M or less. When the NaCl concentration of the irrigation solutions was increased to 0.5 M, myofibrils started to enlarge transversely. Protein extraction became significantly noticeable at 0.6 M NaCl, which was evidenced by the reduction in the A-band intensity. It was also obvious that protein extraction occurred throughout the entire length of the A-band. At 0.9 and 1.0 M NaCl, resolution of myofibrils was poor, apparently due to a high refractive index of the salt solution. Hence, micrographs of myofibrils irrigated at these salt concentrations were not presented.

With phosphates, extraction of proteins followed two distinct patterns: 1) extraction throughout the A-band for treatments with P, PP(-Mg²⁺), and HMP; and 2) extraction starting from both ends of the A-band for treatments with PP(+Mg²⁺) and TPP. The structural changes and protein extraction for the first phosphate treatment group were essentially similar to those in the non-phosphate control sample except that PP(-Mg²⁺) and HMP treatments resulted in more abrupt and more extensive swelling as the NaCl level was raised from 0.5 to 0.6 M. When the irrigation buffer contained 10 mM PP with Mg²⁺, or 10 mM TPP, swelling of the fibrils occurred remarkably as the NaCl level was increased from 0.4 to 0.5 M. This major structural alteration was accompanied by an abrupt extraction of proteins starting from both sides of the A-band. The center of the A-band formed a line which gradually thinned as the salt con-

centration increased further, but it never totally disappeared even at 1.0 N NaCl (result not shown due to loss of micrograph resolution). Some proteins located around the H-zone were apparently resistant to extraction. As the extraction progressed, the Z-line became diffuse but seemed to remain intact. The Z-disk is a highly complex matrix system which consists of many structural proteins, such as zeugmatin, α -actinin, cap-Z, desmin, filamin, as well as titin (Swartz and others 1994). Electrophoresis did indicate that α -actinin was extracted (shown later) and its release would probably allow the Z-disk to expand (swell) at high salt concentrations.

The action of PP on the chicken muscle was consistent with that found on mammalian species, including rabbit and beef (Ishiwata 1981; Offer and Trinick 1983; Patterson and others 1988). The PP-induced change in the protein extraction pattern, that is, extraction starting from both sides of the A-band, may be explained by the biochemical role of PP. At the ends (A-I junctions) of the A-band in postrigor muscle fiber, thick (myosin) and thin (actin) myofilaments are cross-linked through the actomyosin bridges. PP has been shown to be capable of dissociating the actomyosin complex (Granicher and Portzehl 1964; Nauss and others 1969). The ATP-like property would enable PP to extract myosin and, therefore, disintegrate the A-band from the outside of the thick filaments. Dissociation of the actomyosin complex would also enable the myofibril lattices to expand laterally, thereby allowing an increased water uptake. The ability of TPP to influence protein extraction was very similar to PP. This was not surprising because TPP is dephosphorylated to PP, most likely by a phosphatase which may be present in myofibril preparations (Torley and Young 1995) or perhaps simply by the myosin head ATPase, because like PP, TPP has a high affinity for binding to the S1 subfragment around the myosin-actin binding site (Muhlrad and others 1991). Hence, the role of PP or TPP should be viewed not only as an ionic species to dissociate myofilaments and depolarize the thick filaments, as found with high concentrations of NaCl (Hanson and Huxley 1955), but also as a dissociating agent to remove the actomyosin structural barricade.

Whatever the mechanism, the enhanced protein extraction from the A-I band junction by the presence of PP and TPP suggested that the myosin-actin cross-bridge provided a physicochemical constraint to extraction (Offer and Trinick 1983). Other possible structural factors thought to restrain myosin from extraction include several transverse proteins located along the thick filaments, that is, C-, H-, X- and M-line proteins (Offer and Trinick 1983), and proteins that make up the Z-disks, for example, titin (Patterson and others 1988). However, the involvement of C- and X-proteins seemed questionable as polyclonal antibody binding tests showed minimal interference of these proteins with myosin extraction (Parsons and Knight 1990). On the other hand, there is a lack of direct evidence for specific interaction of HMP or P with myosin head. Being a cyclic bulky compound, HMP was shown to be less able to diffuse into muscle (Xiong and Kupski 1999a), thereby reducing its ability to cause myofibrils to swell and to imbibe water.

It is worth noting that Mg²⁺ seemed to play a critical role in the dissociation of actomyosin by PP. Without Mg²⁺, PP had little effect on myofibril swelling and myosin extraction (Fig. 1), probably due to its inability to split the actomyosin cross-bridges (Granicher and Portzehl 1964). If the action of PP is indeed similar to that of ATP, whose hydrolysis by myosin ATPase requires Mg²⁺, then the Mg²⁺-dependence by PP can be understood.

Protein solubility

To quantitate the effect of phosphates on myofibrillar protein extraction, protein concentrations of the extracts were measured. At 0 and 0.1 M NaCl, the "extracts" essentially contained

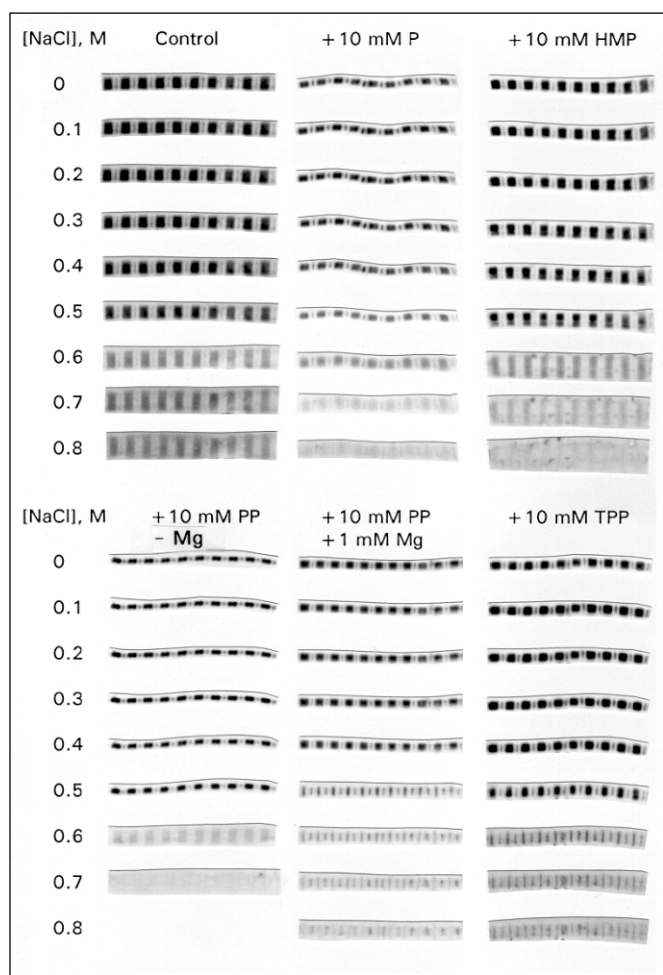


Fig. 1—Typical phase-contrast micrographs of chicken gastrocnemius muscle myofibrils after 3-min irrigation with various concentrations of NaCl in the presence of 10 mM sodium phosphates. Control = no phosphate, P = orthophosphate, PP = pyrophosphate (without or with 1 mM Mg²⁺), TPP = tripolyphosphate, and HMP = hexametaphosphate. Magnification = 1250 $\times$.

no protein, and at 0.2 M NaCl, protein solubilities of all treatments were also marginal. When the NaCl concentration was raised to 0.3 M, however, the presence of PP(+Mg²⁺), TPP, and also HMP markedly enhanced ($P < 0.05$) the protein solubility

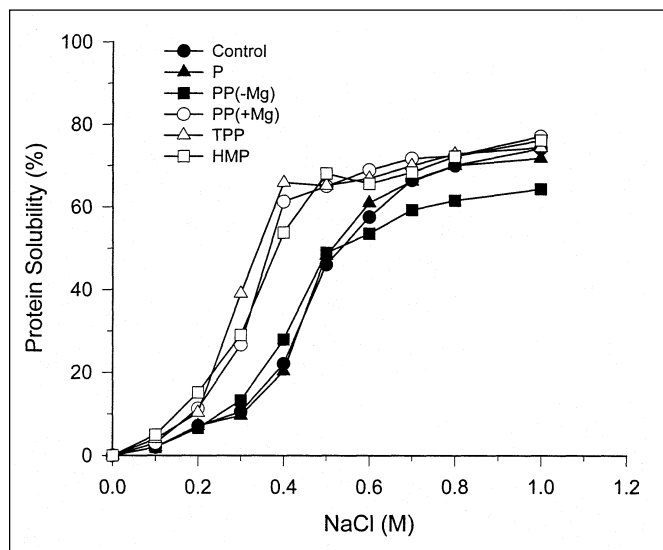


Fig. 2—Influence of sodium phosphates (10 mM) on solubility of chicken gastrocnemius myofibrillar proteins at various concentrations of NaCl at pH 6.0. Control = no phosphate, P = orthophosphate, PP = pyrophosphate (without or with 1 mM Mg²⁺), TPP = tripolyphosphate, and HMP = hexametaphosphate.

(27% to 39%) as compared with control and P or PP(-Mg²⁺) treatment (9% to 11%) (Fig. 2). Unlike PP(+Mg²⁺) or TPP, HMP increased protein solubility although it caused no preferable protein extraction from the ends of the A-I band junction. The solubility difference between the two treatment groups continued to exist, reaching a maximum (about 40%; $P < 0.05$) at 0.4 M NaCl, until the NaCl concentration was raised to about 0.7 M where the effect of phosphates diminished and maximal fibril swelling was essentially attained (Fig. 1). These results were generally consistent with those reported by Fukazawa and others (1961) who demonstrated that the effectiveness of phosphates in facilitating protein extraction from whole muscle with a 0.6 M NaCl Weber-Edsall solution followed the order of: PP > TPP > HMP at pH 6.4 and 7.0. The effect of PP on protein solubility was also dependent upon Mg²⁺ (Fig. 2), which was in good agreement with the microscopic observation (Fig. 1). Although solubility of proteins increased markedly in the 0.2 to 0.3 M NaCl concentration with the presence of phosphates, the microscopic examination failed to clearly detect protein extraction. A possible explanation would be that in addition to those individual proteins that were truly soluble in the extract, some myofibril fragments, which may be somewhat hydrated, become less able to form sediment (that is, more "soluble") under the mild centrifugation conditions employed in the solubility test.

Electrophoresis

SDS-PAGE was performed to determine the composition of the protein extracts obtained under various salt and phosphate treatment conditions (Fig. 3). Extracts obtained at 0 and 0.1 M NaCl contained too little protein to be sensitively analyzed, and hence, they were not included in the electrophoretograms. Irri-

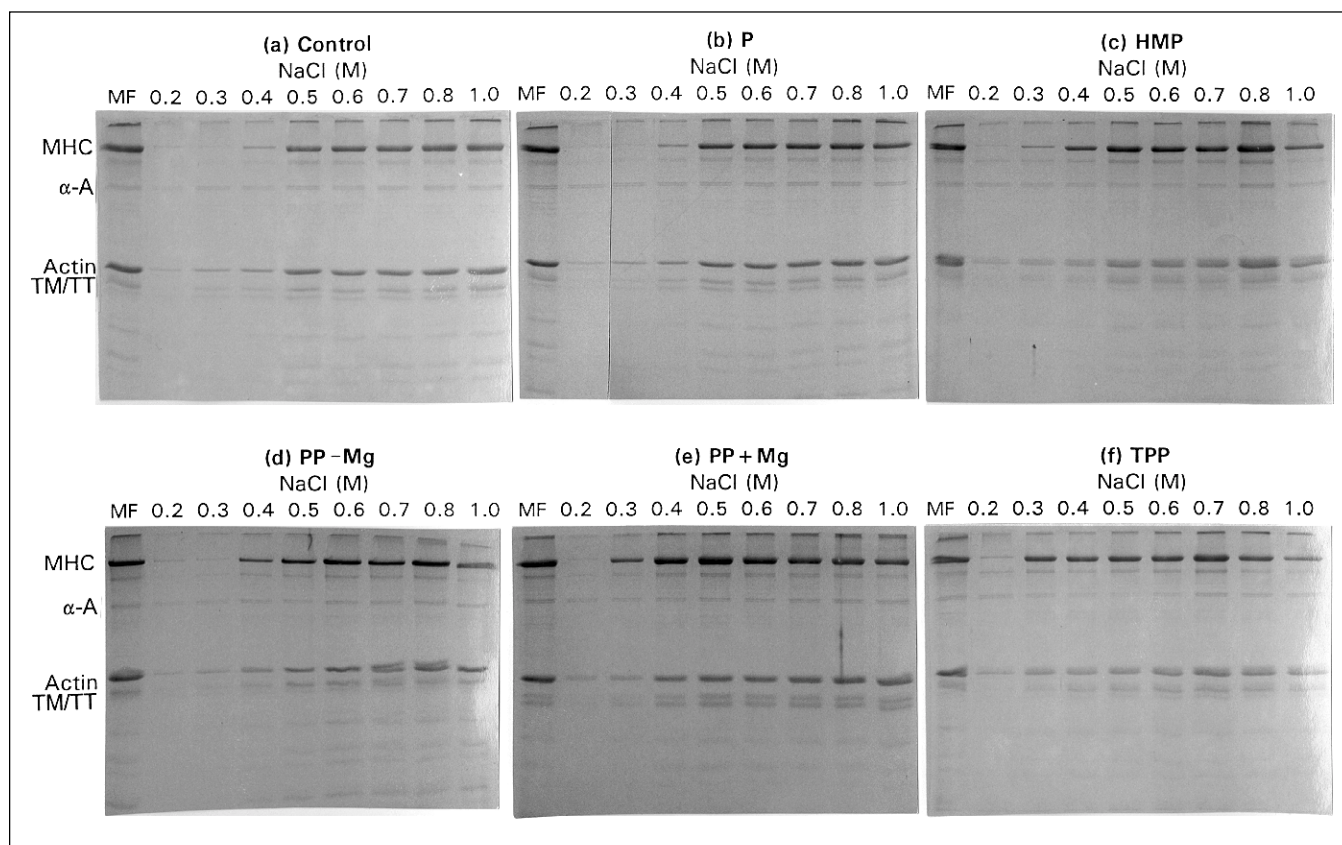


Fig. 3—Typical SDS-PAGE patterns of proteins extracted from chicken gastrocnemius myofibrils at various concentrations of NaCl at pH 6.0 in the presence of different sodium phosphates. MF = whole myofibrils. Band MHC = myosin heavy chain; band α-A = α-actinin; and band TM/TT = tropomyosin/troponin-T. Control = no phosphate, P = orthophosphate; PP = pyrophosphate (without or with 1 mM Mg²⁺); TPP = tripolyphosphate; and HMP = hexametaphosphate.

gation treatments can be grouped based on their resulting electrophoretic patterns: 1) control and P; 2) HMP and PP(-Mg²⁺); and 3) PP(+Mg²⁺) and TPP. For control and P treatment within 0.2 to 0.4 M NaCl, actin and α -actinin seemed to be the most extractable proteins. Tropomyosin/troponin-T started to be extracted at 0.3 M NaCl; for myosin, noticeable extraction was detected at 0.4 M NaCl. However, it was not until 0.5 M NaCl when significant extraction of both myosin and actin began to occur. Extraction of the two major proteins and other myofibrillar components gradually increased at higher salt levels. The effects of the polyphosphate treatments varied. The electrophoretic patterns of HMP and PP(-Mg²⁺) were essentially identical, that is, myosin was readily extracted at 0.4 M NaCl, appearing as a dark band (heavy chain). For PP(+Mg²⁺) and TPP, a pronounced protein extraction began at 0.3 M NaCl and the extract mainly consisted of myosin, especially for the TPP-treated samples. These results were in agreement with the microscopic observations (Fig. 1) and generally corroborated the solubility measurement (Fig. 2). It is of interest to note that HMP, which

caused more extensive myofibril swelling at ≥ 0.6 M NaCl (Fig. 1) than the control and which enhanced protein solubility as effectively as PP(+Mg²⁺) and TPP (Fig. 2), required a higher ionic strength (0.4 M NaCl vs. 0.3 M NaCl) to extract myosin when compared to PP(+Mg²⁺) and TPP. This may be attributed to the inability of HMP to cleave the actomyosin cross-bridges as indicated previously.

Conclusion

PHOSPHATE-INDUCED CHANGES IN THE MYOFIBRIL STRUCTURE, which vary between phosphates as shown in this study, may explain the different hydration properties of chicken muscle when marinated with different phosphate solutions as reported in the literature. In particular, the study contributes to the understanding of the mechanism of muscle hydration induced by different polyphosphates, and validates that pyrophosphate, tripolyphosphate, and to a lesser extent, hexameta-phosphate, are excellent functional ingredients for use in meat products to enhance cooking yield and juiciness.

Materials and Methods

Preparation of myofibrils

Myofibrils were isolated from postrigor (36–40 h postmortem) gastrocnemius muscle of 6 chicken broilers (6 weeks old) according to the procedure of Parsons and Knight (1990) with modifications. Each isolation was prepared from one individual bird, and gastrocnemius muscles from both legs were combined. Minced muscle (20 g) was suspended in 200 mL rigor buffer (0.1 M KCl, 2 mM MgCl₂, 1 mM EGTA, 0.5 mM dithiothreitol, and 10 mM K₂HPO₄, pH 7.0) by homogenization in a Waring blender at low speed setting for 15 s. The homogenates were centrifuged (2 °C) at 2000 g for 10 min. After decanting the supernatant, pellets were resuspended under the same conditions above. This last step (homogenization followed by centrifugation) was repeated three more times. Prior to the final centrifugation, the myofibril suspension was passed through a strainer. To prepare myofibrils used for pyrophosphate treatment in the absence of magnesium, myofibrils were further washed in a rigor buffer (without Mg²⁺) for four additional times. After determination of the protein concentration by the Biuret method (Gornall and others 1949), the final myofibril pellets were slowly stirred into the rigor buffer to give a protein concentration of about 5 mg/mL. This dilute myofibril suspension was kept on ice and analyzed within 3 days.

Irrigation of myofibrils

Myofibrils were irrigated according to Parsons and Knight (1990) with some modifications. An irrigation channel was devised as such: two 12.7 mm wide strips of a double-sided adhesive tape were stuck to the upper face of a 76.2 × 25.4 mm glass microscope slide to form a well 1 to 2 mm wide and about 200 μ m deep. A 22 × 22 mm coverslip was pressed into position on the tape strips. The superfluous tape was trimmed off with a surgical blade along the edge of the coverslip. On one end of the channel, a pool of about 12 mm wide, 18 mm long, and 500 μ m deep was made with a tape.

Irrigation solutions contained various concentrations of NaCl (0.1 to 1.0 M) with or without 10 mM P, PP (with or without 1 mM MgCl₂), TPP, and HMP in a 10 mM [piperazine-N-N'-bis-(2-ethanesulfonic acid)] (PIPES) buffer at pH 6.0. Immediately before irrigation, myofibril samples were diluted to a protein concentration of 0.3–0.5 mg/mL with rigor

buffer. A small drop of this suspension was applied to one end of the channel on the slide and taken up to the full length of the channel by capillary force. One drop of rigor buffer was then applied to each end of the channel to prevent dehydration. The slide was inverted for about 2 min to allow myofibrils to settle on and attach to the underside of the coverslip. After the suitable myofibrils were located and fixed under the microscope objective, some irrigation solution was applied to the pool. The amount was just enough for 3 min irrigation and was established from a preliminary experiment. The solution was drawn through the channel by a filter paper wick to the other end. When the filter paper touched the edge of the coverslip, timing was started. Irrigation was performed at room temperature (22 $\pm$ 1 °C).

Salt- and phosphate-induced changes in the myofibril ultrastructure were observed using a Nikon photomicroscope equipped with a 100 $\times$ oil immersion phase-contrast objective and a broadband green filter. Images were recorded at 250 $\times$ by automatic exposure on a Kodak film. Myofibrils were photographed before and after 3 min irrigation by which maximal fiber swelling occurred as determined from a preliminary study.

Protein solubility

Myofibrils were suspended (1 mg/mL protein) in the same salt and phosphate solutions (pH 6.0) as those used for the microscopic observation indicated above. After incubation at 2° C overnight, the protein suspensions were centrifuged (2 °C at 5000 g for 15 min. Protein solubility was defined as the protein concentration in the supernatant divided by the protein concentration of the uncentrifuged suspension. The protein concentration was determined by the Biuret method (Gornall and others 1949).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on the extracted protein samples (supernatants from the solubility test) essentially according to Laemmli (1970) using a 3% acrylamide stacking gel and a 10% acrylamide separating gel prepared with an SE 250 Mighty Small II slab gel electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) as described by Robe and Xiong (1992). Electrophoresis was run with a PS 1500 DC power supply (Hoefer Scientific Instruments, San

Francisco, Calif.) at constant currents. The initial current was 20 mA per gel; after samples entered the separating gel, the current was raised to 40 mA per gel. All protein samples (1 µg protein/µL) were treated with 5% 2-mercaptoethanol, and 20 µg of protein was loaded to each sample well.

Statistical analysis

The six myofibril isolates, prepared from individual chick-

ens (replications), were treated with different phosphate solutions. A typical representation of phase contrast microscopic examinations and that of electrophoretic patterns of extracted proteins are reported. Statistical analysis was conducted for protein solubility which was determined with triplicate samples in each replication. Treatment means of protein solubility were separated by the Student's t-test (Snedecor and Cochran 1989).

References

- Bendall JR. 1954. The swelling effect of polyphosphates on lean meat. *J. Sci. Food Agric.* 5:468-475.
- Dziezak JD. 1990. Phosphates improve many food. *Food Technol.* 44(4):80-82, 85-86, 90, 92.
- Frøning GW, Sackett B. 1985. Effect of salt and phosphates during tumbling of turkey breast muscle on meat characteristics. *Poult. Sci.* 64:1328-1333.
- Fukazawa T, Hashimoto Y, Yasui T. 1961. The relationship between the components of myofibrillar protein and the effect of various phosphates that influence the binding quality of sausage. *J. Food Sci.* 26:550-555.
- Gornall AG, Bardawill CJ, David MM. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* 177:751-766.
- Granicher D, Portzehl H. 1964. The influence of magnesium and calcium pyrophosphates chelates of free magnesium ions, free calcium ions and free pyrophosphate ions on the dissociation of actomyosin in solution. *Biochim. Biophys. Acta* 86:567-578.
- Hamm R. 1960. Biochemistry of meat hydration. *Adv. Food Res.* 10:355-463.
- Hamm R. 1970. Interactions between phosphates and meat proteins. In: J.M. DeMann and P. Melnychyn (Eds.). *Symposium: Phosphates in Food Processing*. Westport, CT: AVI Publishing Co. p. 65-82.
- Hamm R. 1986. Functional properties of the myofibrillar system and their measurements. In: Bechtel, PJ (Ed.). *Muscle as Food*. San Diego, CA: Academic Press, Inc. p.135-199.
- Hamm R, Grau, R. 1958. The water binding capacity of mammalian muscle. IV. The action of salts of weak acids. *Z. Lebensm. Untersuch. Forsch.* 108:280-293.
- Hanson J, Huxley HE. 1955. The structural basis of contraction in striated muscle. *Symp. Soc. Exp. Biol.* 9:228-264.
- Hellendoorn EW. 1962. Water-binding capacity of meat as affected by phosphates. *Food Technol.* 16(9):119-124.
- Ishiwata S. 1981. Melting from both ends of an A-band in a myofibril. Observation with a phase-contrast microscope. *J. Biochem.* 89:1647-1650.
- Laemmli, UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Muhlrad A, Peyser YM., Ringel I. 1991. Effect of actin, ATP, phosphates, and pH on vana-date-induced photocleavage of myosin subfragment 1. *Biochemistry* 30:958-965.
- Nauss KM., Kitagawa S, Gergely J. 1969. Pyrophosphate binding to and adenosine triphosphatase activity of myosin and its proteolytic fragments. *J. Biol. Chem.* 244:755-765.
- Offer G, Trinick J. 1983. On the mechanism of water holding in meat: the swelling and shrinking of myofibrils. *Meat Sci.* 8:245-281.
- Parsons N, Knight P. 1990. Origin of variable extraction of myosin from myofibrils treated with salt and pyrophosphate. *J. Sci. Food Agric.* 51:71-90.
- Patterson BC, Parrish FC., Jr., Stromer MH. 1988. Effects of salt and pyrophosphate on the physical and chemical properties of beef muscle. *J. Food Sci.* 53:1258-1265.
- Robe GH, Xiong YL. 1992. Phosphates and muscle fiber type influence thermal transitions in porcine salt-soluble protein aggregation. *J. Food Sci.* 57:1304-1307, 1310.
- Snedecor GW, Cochran WG. 1989. *Statistical Methods*, 8th ed. Iowa State University Press. Ames, IA.
- Swartz DR, Lim SS, Fassel T, Greaser ML. 1994. Mechanisms of myofibril assembly. *Proceed. Recip. Meat Conf.* 47:141-153.
- Torley PJ, Young OA. 1995. Rheological changes during isothermal holding of salted beef homogenates. *Meat Sci.* 39:23-34.
- Trout GR, Schmidt GR. 1983. Utilization of phosphates in meat products. *Recip. Meat Conf. Proceed.* 36:24-27.
- Xiong YL, Kupski DR. 1999a. Monitoring phosphate marinade penetration in tumbled chicken filets using a thin-slicing, dye-tracing method. *Poult. Sci.* 78:1048-1052.
- Xiong YL, Kupski DR. 1999b. Time-dependent marinade absorption and retention, cooking yield, and palatability of chicken filets marinated in various phosphate solutions. *Poult. Sci.* 78:1053-1059.
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