Chapter 7

Electrophoresis as a Useful Tool in Studying the Quality of Meat Products

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1. Introduction

The study of meat quality usually involves a great number of methodologies at the laboratory. Thus, new technology and automatic equipments are used in combination to well-known methodologies in order to improve the study of the constituents of a given sample. Several methodologies are usually carried out in combination, especially when composition and functionality of food compounds are studied.

Meat and meat products are mainly composed of water, proteins, lipids, minerals and carbohydrates (Bender, 1992). Most sensory and functional properties depend on the quantity and quality of these compounds. In the last years, several studies aimed its efforts on the improvement of sensory quality of meat products by means of the incorporation of additives (Szerman et al., 2012).

At present, ready-to-cook and pre-cooked foods are installed in life. Regarding meat market, *sous vide* processing appears to be an interesting option for offering different kinds of beef products for consumption. It consists of the cooking/pasteurization process to a raw material packaged in a heat-stable vacuum container. The products are stored at 0-3 °C and are able to be re-heated and consumed even after 5 weeks (Vaudagna et al., 2002). Besides the enhancement of shelf life, several other advantages have been associated to *sous vide* processing, such as increased flavour profile, increased tenderness and nutritional loss reduction (Church & Parsons, 2000; Vaudagna et al., 2002).

The *sous vide* system has been extensively studied in meat and meat products (Church & Parsons, 2000; García-Segovia et al., 2007). Nevertheless, there is still a lack of knowledge regarding the application of this mild cooking system to whole-muscle beef. Moreover, this pre-cooked system presents important technological inconvenients like juice retention inside the packaging, which would affect profitability and sensorial and nutritional characteristics

of the final product (Church & Parsons, 2000; Vaudagna et al., 2002). On this regard, additives such as sodium chloride and alkaline phosphates have being frequently used for meat products manufacturing in order to increase water holding capacity (WHC) of meat and, consequently, reducing cooking weight loss (Baublits et al., 2006; Pietrasik & Shand, 2004, 2005; Vaudagna et al., 2008). Results obtained showed that the incorporation of NaCl (0.70% or 1.20% w/w) plus TPP (0.25% w/w), and posterior cooking at mild temperatures (60-65 °C) increase the WHC of *Semitendinosus* muscles and reduce cooking weight losses. The increased WHC would be responsible for the tenderness increment found, probably as a consequence of the swelling of myofibrils. Nevertheless, the exact physicochemical events involved have not been completely elucidated. Thus, the study of the structure and functionality of meat proteins emerges as an important issue to take into account in order to elucidate the effect of additives incorporated into meat products and the mechanisms involved in the improvement of sensorial and physical properties.

Regarding meat proteins, it is known that they have a widely range of size between approximately 20 kDa to 3,000 kDa (Warris, 2000). Among them, high molecular weight proteins display a key role in conferring functional properties to meat (Warris, 2000). Its approach usually represents a challenge at the laboratory. Myosin, actin, titin and nebulin are four of the major proteins of the skeletal sarcomere, and possibly the most important myofibrillar proteins having interactions with each other in the muscular tissue. Myosin (aprox. 540 kDa) and actin (aprox. 43 kDa) are the most noticeable contractile proteins of the thick and thin filaments, respectively (Clark et al., 2002). Titin (aprox. 3,000 kDa) and nebulin (aprox. 800 kDa) are two of the giant myofibrillar proteins, acting as protein rulers by regulating the assembly of myosin and actin filaments in the sarcomere (Wang, 1996). At present, several methods from different sources have been reported for their isolation. However, most of them are complex, tedious and mainly focused on the isolation of one protein at a time.

Currently, electrophoresis represents one of the most and reliable methods used in studying meat proteins in the laboratory. It does not require expensive equipment and its outcomes allow to be analyzed by means of several techniques like densitometry and differential scanning calorimetry (DSC), among others. In general terms, electrophoresis refers to the migration of charged particles in a particular medium under the influence of an electric field. It constitutes a powerful and broadly used method for the analysis of complex mixtures of analytes from different sources. Separated molecules can be visualized by means of specific staining. One remarkable advantage is that the dried support medium can be finally kept as a permanent record. It has been stated that the migration rate (cm/s) of a given particle submitted to electrophoresis relies on several factors related to the shape, charge and size of the particle and to the electrical field strength and the temperature of the system as well. Thus, the mobility of the particle –migration rate per unit of field strength (V/cm)- results directly proportional to net charge of the particle and inversely proportional to its molecular size at a given viscosity of the electrophoretic support (Karcher & Landers, 2006). Several support mediums had been described. Among them, polyacrylamide gels (PAGE) offer several advantages when compared to others matrixes, especially when

proteins mixtures are been analyzed (Karcher & Landers, 2006). In this case, protein separation takes place on the basis of charge-to-mass-ratio and molecular size. The addition of sodium dodecyl sulphate to the system (SDS) denatures proteins conferring to them a constant charge-to-mass ratio. Thus, under described conditions, proteins separation only depends on its relative molecular weights (MW). Gradient SDS-PAGE is usually recommended when proteins mixture spans wide MW range. In this case, the decreasing pore size also contributes to sharpen the proteins bands.

2. Practical applications

A great deal of attention has been paid to the most important proteins of the myofibrillar system because of their potential role in meat processing. Several authors have succeeded in the isolation and/or enrichment of some of these proteins (Ho et al., 1994; Pan & Damodaran, 1994; Wang, 1982; Wang & Greaser, 1985). Nevertheless, most of the described methodologies use to be difficult to carry out because of their complexity, associated in part with the exerted interactions among these proteins throughout the myofibrils (Houmeida et al., 1995; Linke et al., 2002).

The purpose of the present chapter is to compile own published data which illustrate the usefulness of electrophoresis analysis in meat research, either used alone or in combination to other techniques. The first part describes a simple methodology to simultaneously co-enrich myosin, actin, titin and nebulin from beef muscles. It is believed that the described method will provide enriched myofibrillar proteins, which could be used for more specific analysis, like microscopy studies, immunological and/or differential scanning calorimetric analysis. The second component of this chapter is focused on the study of the effect of saline additives on muscle proteins structure to improve meat quality. Since DSC has become one of the most useful techniques for studying the thermal behavior of components of biological systems, its approach in combination to SDS-PAGE is also described. The methodologies used provide useful information in order to explain the improvement of sensorial properties observed in meat treated with saline additives and submitted to *sous vide* system.

3. Methodology

3.1. Materials

Beef *Semitendinosus* muscles were dissected from British breed steer carcasses 48 h post slaughter, following the Guidelines of National Sanitary Authority (SENASA, 1969). Slaughter procedure was carried out in a commercial meatpacking and processing plant with good manufacturing practices. High molecular weight (HMW) standard was purchased from Amersham-Pharmacia (Buckinghamshire, England). Bovine serum albumin (BSA, fraction V), enzyme inhibitors and mouse monoclonal anti-nebulin antibody were obtained from SIGMA (St. Louis, MO, USA). Goat anti-mouse IgG antibody conjugated to alkaline phosphatase was purchased from Bio-Rad (Cambridge, MA). All other reagents were of the purest grade available (analytical grade) and obtained from SIGMA or Bio-Rad.

Semitendinosus muscles were trimmed free of fat and connective tissue before measuring pH with a portable puncture pHmeter (Metrohm AG CH-9101, Herisau, Switzerland). Muscles with pH 24 h values ranging between 5.5 and 5.7 were selected for the studies carried out.

3.2. Methods

3.2.1. Isolation of titin, nebulin, MHC and actin

3.2.1.1. Separation of myofibrillar proteins

The separation of myofibrillar proteins of *Semitendinosus* muscles was conducted according to Boehm et al. (1998), and as detailed in Pighin & Gonzalez (2008). Protein concentration was assayed according to Lowry et al. (1951), using BSA as standard. Discontinuos SDS-PAGE (see details below) was performed to evaluate the protein profile. The rest of the extracted fraction was diluted as described in Pighin & Gonzalez (2008) to reach a final concentration of 5 g protein/L.

3.2.1.2. SDS-PAGE analysis and enrichment of titin, nebulin, MHC and actin

Partial isolation of proteins was achieved by means of ammonium sulfate salt precipitation, using four different saturation ranges: 0–20, 20–40, 40–60 and 60–100 g/L. After each precipitation step, protein solution was centrifuged at 23,000 x g for 20 min at 4 °C. The resulting precipitate was resuspended in SDS buffer (3% SDS, 10% glycerol, 1 mM EDTA, 5 mM DTT, 10 mM Tris-HCl, pH 8.0), and the saline extract was dialyzed overnight against buffer SDS (nitrocellulose membrane, MWCO: 12–14 kDa). Discontinuous SDS-PAGE (see details below) was used to monitor the protein composition of each precipitate.

SDS-PAGE analysis conducted according to Laemmli (1970) with some modifications was performed in order to monitor the protein profile of extracts and to continue the isolation of proteins. Electrophoresis was run in a lineal gradient of acrylamide concentration (3–12%) in a Mini Protean III system (Bio-Rad), at constant voltage (130 V) during 90 min. For comparison purposes, stacking and resolving gel solutions were prepared both with and without glycerol (50 mL/L). A HMW calibration stained kit was used to estimate the molecular weight (MW) of the different proteins. In order to monitor protein composition, each line was loaded with 50 mg of proteins, and with 120 mg for isolation purposes. Gels were stained with 1 g/L Coomassie Brilliant Blue R-250 solution. Images were captured with a Bio-Rad GS-800 Imaging Calibrated Densitometer and processed by Quantity One 1-D Analysis software (Bio-Rad, version 4.4.1) to determine relative front (Rf), MW and relative amount of each protein. When a protein was not accurately identified in the electrophoretic gel by staining, the Western blot technique was utilized as a tool to reveal the presence of this protein. This analysis was applied to nebulin. For this purpose, a non-stained SDS-PAGE gel was transferred overnight onto a nitrocellulose membrane (0.45-mm pore size) at 90 mA using Mini Trans-Blot Cell (Bio-Rad). Then, the presence of nebulin was detected with the commercial mouse monoclonal anti-nebulin clone NB2 antibody (diluted 1:400), followed by the secondary antibody anti-mouse IgG raised in goat and conjugated to alkaline phosphatase (dilution 1:1,000). Once the protein of interest was located on the

nitrocellulose membrane, its position on an electrophoretic gel could be established by comparison.

Once MHC, actin, nebulin and titin were identified, they were excised from a non-stained gel, and individually eluted in an Electro-Eluter model 422 (Bio-Rad, MWCO: 12–15 kDa) as detailed in Pighin & Gonzalez (2008). Finally, each protein was collected in a volume of approximately 500 mL, dialyzed overnight, lyophilized and stored at -80 °C. The purity of the isolated extracts was finally monitored by discontinuous SDS-PAGE as previously described.

3.2.2. Study of protein contribution to the effect of saline addition to meat

3.2.2.1. Incorporation of saline additives

Brines containing NaCl and/or TPP were incorporated into the selected muscles (10% w/w) with an automatic multi-needle injector (36 needles, Fricor, Buenos Aires, Argentina) to give the final concentrations (g/100 g injected muscle) of 0.70 NaCl; 0.25 TPP; 0.70 NaCl + 0.25 TPP, and 1.20 NaCl + 0.25 TPP. Non injected (NI) muscles were used as control. Injected and NI muscles were vacuum packaged (Cryovac CN510, Sealed Air Co., Buenos Aires, Argentina) and submitted to continuous tumbling at 5.0 rpm, 8 h, 2-4 °C (Lance Industries tumbler, model LT-15, Allenton, USA) in order to improve brine sorption and protein extraction. Two slices (2.0 cm wide) of each muscle were frozen at -80 °C until thermal analysis and the rest of the muscles were kept at 2-4 °C until protein extraction.

3.2.2.2. Myofibrillar and sarcoplasmic proteins extraction and myofibrils isolation

Myofibrillar proteins extraction was conducted as mentioned in **3.2.1.1.** After centrifugation, the supernatant containing sarcoplasmic proteins was separated and stored at -80 °C until electrophoretic analysis. The precipitate was suspended in SDS buffer and filtered through a home-made nylon strainer to remove connective tissue. An aliquot of the filtrate, which included proteins of the myofibrillar system, was subjected to SDS-PAGE analysis. Protein concentration of both sarcoplasmic and myofibrillar extracts was measured according to Lowry et al. (1951).

Myofibrils isolation from minced meat of the different saline treatments was conducted according to Culler et al. (1978), and as detailed in Pighin et al. (2008). The filtered myofibrils collected were used for DSC analysis. Protein concentration of isolated myofibrils and of whole muscles was determined by a macro-Kjeldahl method (Foss Tecator equipment, Sweden), with a conversion factor of N = 6.25.

3.2.2.3. SDS-PAGE analysis and thermal analysis

Electrophoretic profile of protein extracts was monitored by discontinuous SDS-PAGE (Laemmli, 1970), carried out under the same conditions previously described in **3.2.1.2.**

A Perkin-Elmer Pyris-1 differential scanning calorimeter (DSC) was employed to study the thermal properties of proteins of the whole muscle and of the isolated myofibrils. For

temperature and heat flow calibration, Indium was used as standard (melting point 156.6 °C, enthalpy 28.46 J/g). Five to fifteen milligrams of each sample, accurately weighed, were placed into an aluminum pan, which was hermetically sealed and equilibrated for 2 min at the initial scanning temperature. During the study, a heating rate of 10 °C /min was applied over a 20-90 °C temperature range. All samples were re-scanned (submission to a second thermal analysis) to check for the irreversibility of the denaturation process. Endotherm (mW vs. temperature), denaturation enthalpy (DH) and endothermic peak (Td) were obtained using the software Pyris-7 (Perkin-Elmer). The total enthalpy change (DHT) was estimated by the algebraic sum of the individual denaturation enthalpies.

3.2.2.4. Statistical analysis

The experiments described were conducted in duplicate. The study of saline incorporation included 5 treatments (NI; 0.70% NaCl; 0.25% TPP; 0.70% NaCl + 0.25% TPP; 1.20% NaCl + 0.25% TPP). A set of five muscles were assigned at random to each treatment. SDS-PAGE analysis and DSC measurements were performed in all muscles included in the treatments. Analysis of variance (ANOVA) for DSC measurements was conducted using the statistical package SAS (version 8, SAS Institute Inc., 2004, Cary, NC). Duncan´s Multiple Range Test (with a significance of a = 0.05) was used to determine differences among treatments.

4. Results and discussion

4.1. Isolation of titin, nebulin, MHC and actin

4.1.1. Separation of myofibrillar proteins

Figure 1 shows the electrophoretic pattern of supernatant and precipitate fractions obtained after the extraction of myofibrillar proteins. It can be seen (lane 2) that most of the soluble proteins (28–70 kDa) remained in the supernatant fraction. Instead, proteins of the precipitate (lane 3) have a wider MW range (25–2,500 kDa). The precipitate included almost exclusively myofibrillar proteins, yielding 64 g of extracted protein per kilogram of muscular tissue. It can also be observed that the most intense band is MHC (MW: 220 kDa, Rf: 0.47), which represents approximately 35 % of total myofibrillar proteins extracted. Actin (MW: 43 kDa) is the second most intense (20 %) having one of the largest Rf (0.92). Titin band represents approximately 4 % of the proteins extracted. It has the lowest Rf (0.06) in the SDS-PAGE analysis with an estimated MW of 2,500 kDa. A similar MW for titin was found by Kellermayer & Grama (2002) and Kurzban & Wang (1988). A comparable electrophoretic pattern of myofibrillar proteins has been reported previously by Chen et al. (2005).

Nebulin could not be precisely identified on the present SDS-PAGE due to the presence of a set of several proteins in the range of 600–800 kDa. Therefore, the use of monoclonal antibodies appeared to be a useful tool to corroborate nebulin localization in the gel. Figure 2 shows the Western blot analysis of protein extract (precipitate fraction). Only one band was detected by the commercial kit against nebulin, with an Rf of 0.20 and an estimated MW of 710 kDa. It is important to denote that the MW of titin and nebulin were calculated in

approximation because of the absence of suitable HMW standards for SDS-PAGE. Nevertheless, the MW found for nebulin is consistent with previous published data reporting a value of 600– 900 kDa for this protein (Locker & Wild, 1986; Wang, 1982).

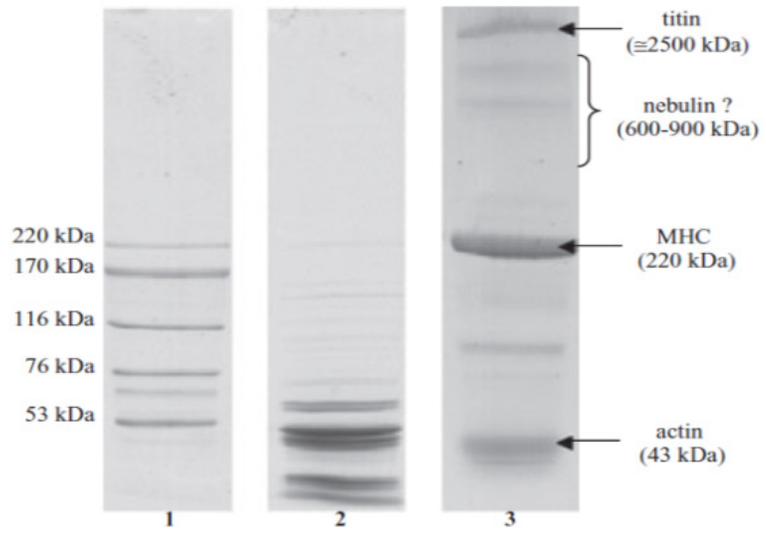

Figure 1. Myofibrillar protein isolation and SDS-PAGE (3–12%) analysis of protein fractions Lane 1 = HMW standard. Lane 2 = supernatant. Lane 3 = precipitate. (Pighin & Gonzalez, 2008)

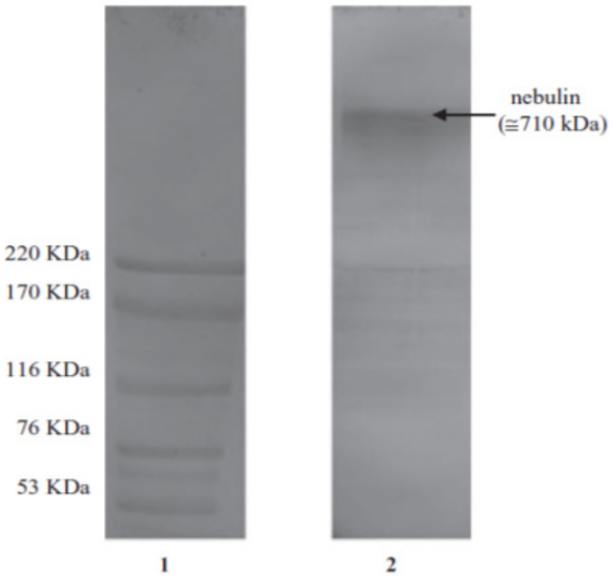

Figure 2. Western blot analysis of myofibrillar protein extract Lane 1 = HMW standard. Lane 2 = myofibrillar protein extract. (Pighin & Gonzalez, 2008)

It is important to remark that the use of gradients is usually recommended when SDS-PAGE analysis is applied to high-MW proteins, in order to improve separation and resolution of bands. The addition of glycerol has been reported to facilitate gradient formation and also to improve resolution of the SDS-PAGE technique (Sobieszek, 1994). However, Chen et al.

(2005) did not find a great improvement of the gel system with the use of this reagent. Present research supports the first statement, given the fact that the addition of glycerol both to the resolving and the stacking gel solutions has successfully improved band resolution (comparison data not shown).

4.1.2. Enrichment of titin, nebulin, MHC and actin

The isolation of myofibrillar proteins requires time-consuming procedures that usually separate them individually. Thus, myosin isolation generally involves the removal of sarcoplasmic proteins by means of washings with diluted phosphate buffer and subsequent protein extraction with specific solutions containing elevated amounts of KCl (Dudziak & Foegeding, 1988; Hermanson et al., 1986). Actin isolation requires a prior separation of G-actin by breaking intermolecular linkages of F-actin. One of the most commonly used agents is acetone, which can break the linkages without denaturing the protein (Syrovy, 1984). After that, a second isolation stage involves several steps including washing, filtration, centrifugation, polymerization/depolymerization cycles, dialysis and centrifugation (Pardee & Spudich, 1982; Spudich & Watt, 1971). Gel filtration chromatography can also be employed for further actin purification (Kuroda, 1982). More recently, Perez-Juan et al. (2007) have succeeded in the isolation of actomyosin and actin in an interesting attempt to amalgamate myofibrillar protein isolations in a single extraction process.

High-molecular weight proteins (i.e. titin and nebulin) extraction specifically involves the addition of protease inhibitors to avoid degradation during processing. The isolation methodology initiates with myofibrils extraction and posterior protein–SDS solubilization, followed by the precipitation of titin/nebulin–SDS complex by salt fractionation. For further purification, the use of gel filtration is recommended because of the great size of these proteins (Wang, 1982). A more exhaustive extraction of titin was proposed by Pospiech et al. (2002), which demanded several centrifugation steps followed by suspension/homogenization of the sediments in specific solutions, dialysis, and hydroxyapatite and ion-exchange chromatography. Previous description shows the requirement of time and effort to achieve the isolation of one or another myofibrillar protein. For that reason, the present research was focused on looking for a more simple methodological strategy.

Salting out fractionation is believed to be a useful step for protein isolation because the procedure is inexpensive and relatively easy to apply. Sodium chloride had been proposed for the precipitation of the giant proteins, titin and nebulin (Wang, 1982; Wang & Wright, 1988). However, in the present research, the use of ammonium sulfate as precipitating agent produced a proper isolation of these two proteins and also of myosin and actin. In order to select the appropriate saturation range, different ammonium sulfate ranges were assayed: 0–20, 20–40, 40–60 and 60–100 g/L. After each centrifugation step, the protein profile of the precipitated fractions was monitored by SDS-PAGE. The different saturation ranges showed the following protein pattern (Fig. 3): the band corresponding to MHC (MW: 220 kDa) was the most noticeable one, showing similar concentrations in all saline ranges (see numbers between brackets). Actin (MW: 43 kDa) was also detected in all fractions, being its intensity

higher in the saline ranges of 40–60 g/L and 60–100 g/L. Protein profiles of the ranges 0–20 g/L and 20–40 g/L were similar, except for the amount of the titin band (2,500 kDa), which is about four times higher in the second saline range. A saturation range of 40–60 g/L showed that the amount of titin and nebulin extracted is the highest. A higher saturation range (60–100 g/L) did not include the giant proteins in the precipitated fraction.

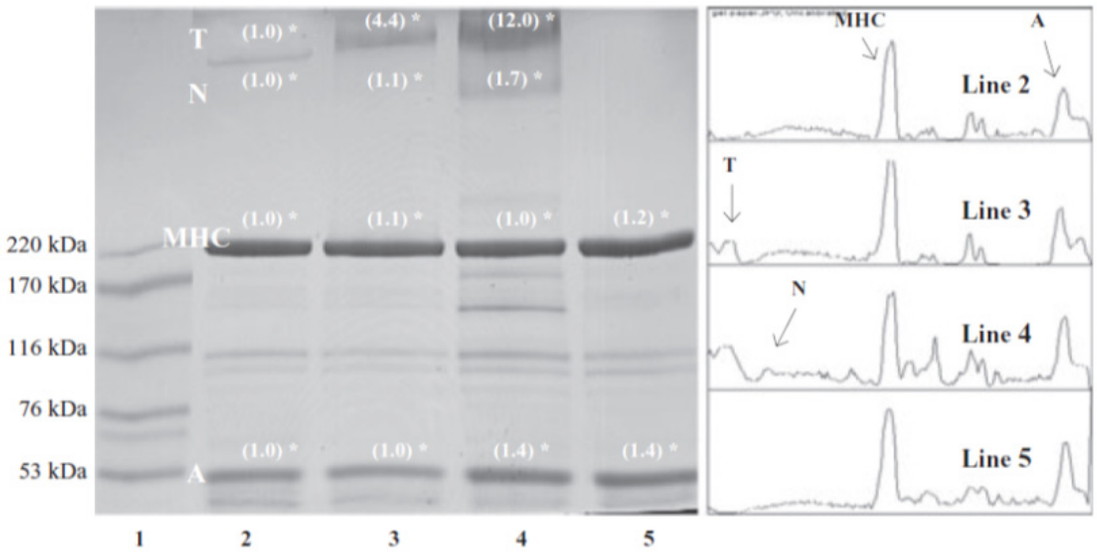

Figure 3. SDS-PAGE analysis of the different ammonium sulfate precipitates
Lane 1 = HMW standard. Lane 2 = range 0–20 g/L. Lane 3 = range 20–40 g/L. Lane 4 = range 40–60 g/L. Lane 5 = range 60–100 g/L. T, titin; N, nebulin; MHC, myosin heavy chain; A, actin. *Numbers between brackets mean times increment. (Pighin & Gonzalez, 2008)

If this protein profile (Fig. 3) is densitometrically compared to the myofibrillar protein extract (Fig. 1), it becomes evident that titin and nebulin have been greatly enriched in the saline range of 40–60 g/L, having 3.6 and 4.0 times increment, respectively.

Even though the analysis of the gel in Fig. 3 indicates that the saline ranges of 20–40 g/L and 40–60 g/L were appropriate for isolation and enrichment of titin, and 40–60 g/L was so for the enrichment of nebulin, the unified range of 20–60 g/L appeared to be an interesting option to increase the amount of these proteins in subsequent experiments. Instead, the saline range of 60–100 g/L was suggested to isolate actin and MHC. Once the saline precipitation ranges were selected, SDS-PAGE was chosen to continue the purification protocol. For this purpose, each ammonium sulfate precipitate was resuspended and electrophoretically developed in an SDS-PAGE gel (3–12%). After the identification of the proteins on the gel by means of its Rf´s, they were first excised, and then submitted to electroelution.

Figure 4 shows the SDS-PAGE of each electroeluted protein (lanes 1–4) and the Western blot analysis of nebulin (lane 5). It can be seen that MHC (lane 2) was obtained in an almost pure state. Actin and titin (lanes 3 and 4) were obtained enriched but not in a pure state. The reason for this result is that actin and titin concentrations obtained after the salt precipitation

and the electrophoresis steps were low and made difficult the removal of a thinner band from the electrophoretic gel to improve their isolation by electroelution. Nebulin band -stained with Coomassie Brilliant Blue dye- was not accompanied by any other protein band (data not shown). However, the band was vaguely stained and it required the identification and confirmation by the previously mentioned immunoblotting technique (lane 5).

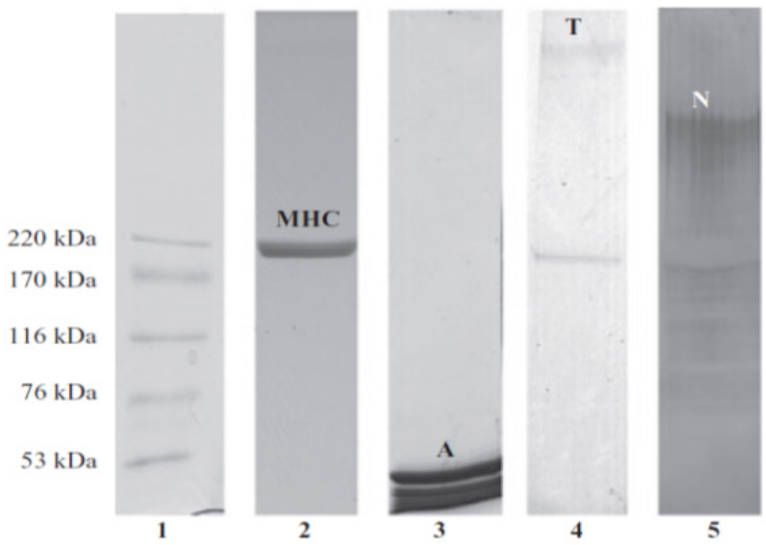

Figure 4. SDS-PAGE and Western blot analysis of the electroeluated proteins
Lane 1 = HMW standard. Lane 2 = myosin heavy chain extract (SDS-PAGE analysis). Lane 3 = actin (A) extract (SDS-PAGE analysis). Lane 4 = titin (T) extract (SDS-PAGE analysis). Lane 5 = nebulin (N) extract (Western blot analysis).
(Pighin & Gonzalez, 2008)

4.2. Study of protein contribution to the effect of saline addition to meat

4.2.1. Electrophoretic analysis of proteins extracts

The electrophoretic analysis of sarcoplasmic proteins extracts belonging to saline treated muscles are shown in Fig. 5. As can be seen, proteins bands ranged between 30 and 75 kDa. It is worth noting that the intensity of the band with an estimated MW of 70 kDa was increased about 2.5 times in samples of muscles containing NaCl alone or combined with TPP (see arrows, lanes 3-A, 3-B and 4-B) with respect to NI. Even more, an additional band with an estimated MW of 65 kDa was revealed in these saline treated muscles (see dashed arrows, lanes 3-A, 3-B and 4-B). On the contrary, the presence of TPP alone did not modify the protein pattern found in NI muscles (lanes 2-A and 2-B). These findings suggest that the presence of NaCl collaborates in the solubilization of myofibrillar proteins, releasing them to the soluble fraction.

Myofibrillar proteins were separated by SDS-PAGE into a broad range of molecular weight (Fig. 6). Bands corresponding to MHC (MW 220 kDa, see arrow) and actin (MW 46 kDa, see dashed arrow) were the most noticeable ones. No differences among treatments were verified by means of densitometric analysis. This result does not agree with the one previously shown in Fig. 5, which had suggested an augmented solubilization of myofibrillar proteins as a

consequence of the application of NaCl into muscles. Hence, an attenuated intensity of any of the bands in Fig. 6 was expected. It is probable that the increment of 70 kDa or the appearance of 65 kDa band found in Fig. 5 is a result of the degradation of myofibrillar proteins.

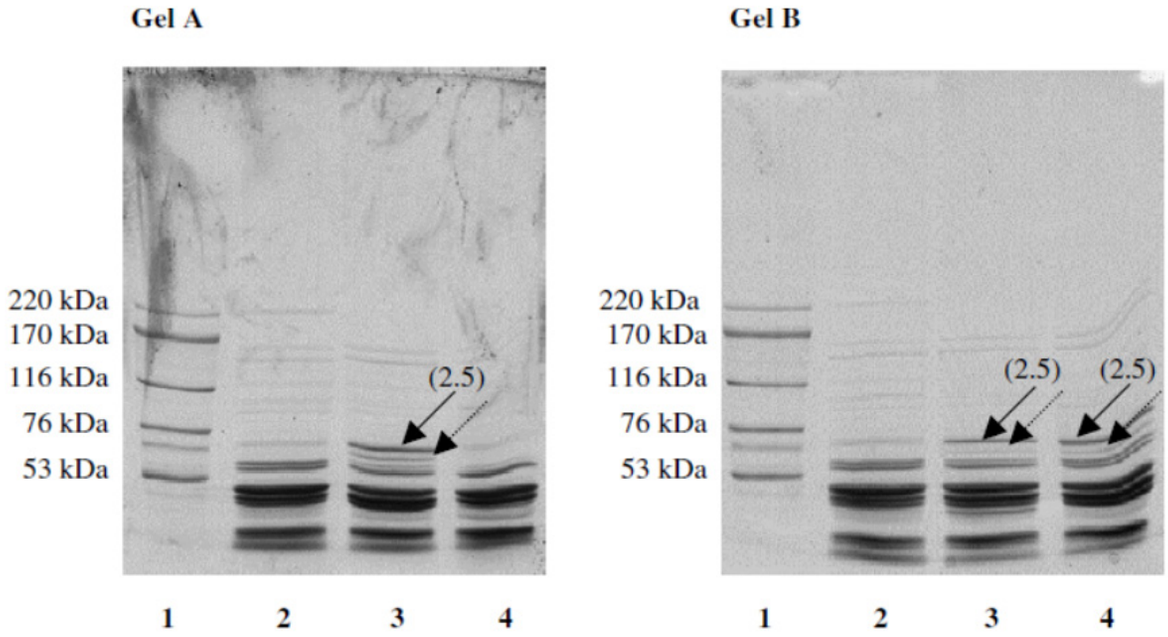

Figure 5. SDS-PAGE analysis of sarcoplasmic proteins obtained from muscles treated with NaCl and/or TPP
Gel A: Lane 1, HMW-SDS; Lane 2, NI; Lane 3, 0.70 % NaCl; Lane 4, 0.25 % TPP.
Gel B: Lane 1, HMW-SDS; Lane 2, NI; Lane 3, 0.70 % NaCl + 0.25 % TPP; Lane 4, 1.20 % NaCl + 0.25 % TPP.
(Pighin et al., 2008)

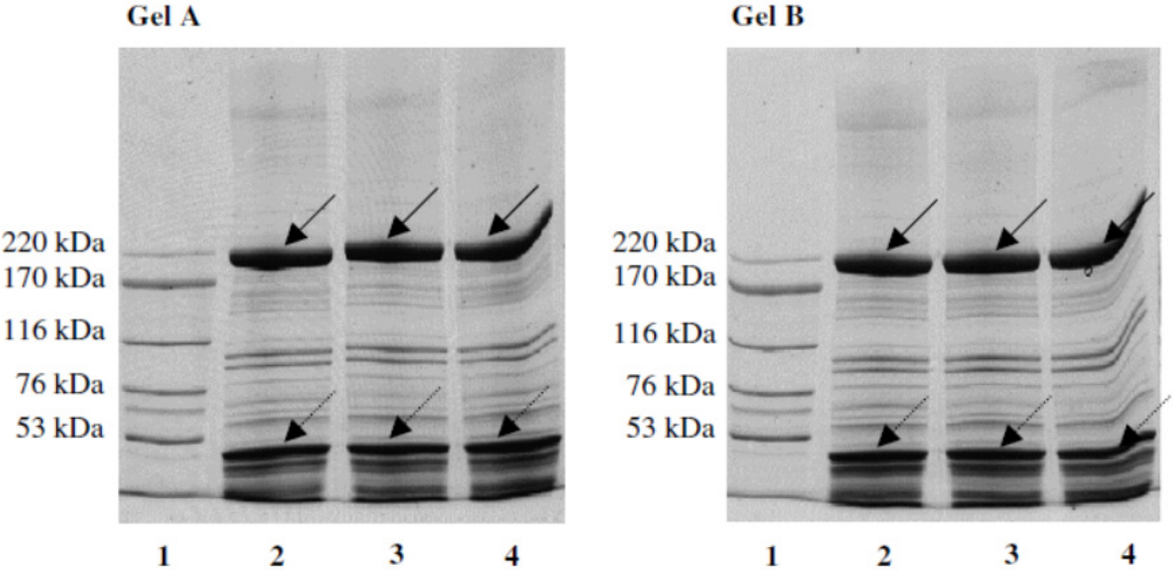

Figure 6. SDS-PAGE analysis of myofibrillar proteins extracted from muscles treated with NaCl and/or TPP
Gel A: Lane 1, HMW-SDS; Lane 2, NI; Lane 3, 0.70 % NaCl; Lane 4, 0.25 % TPP.
Gel B: Lane 1, HMW-SDS; Lane 2, NI; Lane 3, 0.70 % NaCl + 0.25 % TPP; Lane 4, 1.20 % NaCl + 0.25 % TPP.
(Pighin et al., 2008)

4.2.2. Thermal analysis of whole muscle

Thermal analysis of NI whole muscles (Fig. 7) showed three characteristic endothermic peaks. Published data allowed to relate them to myosin and its subunits denaturation (I; 57.0 °C), sarcoplasmic proteins and collagen denaturation (II; 67.4 °C) and actin denaturation (III; 80.3 °C) (Graiver et al., 2006; Kijowski & Mast, 1988; Stabursvik & Martens, 1980). Enthalpies involved in each thermal transition were 1.32, 0.65 y 1.74 J/g protein, respectively. Re-scanned samples did not show any thermal response, corroborating the irreversibility of the transitions. The calculated DHT of NI muscles was 3.71 J/g protein. It can be seen in Table 1 that NaCl treatment (0.70 %) significantly reduced myosin DH and actin Td. Only slight decreases were found in myosin Td and actin DH. These results make evident that actin was destabilized by NaCl, becoming the more susceptible protein to thermal denaturation (decreased Td). Instead, the effect of NaCl upon myosin could be seen on the DH, which reduction could be related to the decrease of hydrogen bonds and/or to the increment of protein aggregation (exothermic event) exerted by the salt. Total enthalpy (DHT) for NaCl-treated muscles (calculated by adding DH of individual values related to transitions I, II, and III) decreased from 3.71 J/g protein (NI muscle) to 3.04 J/g protein, demonstrating system instability.

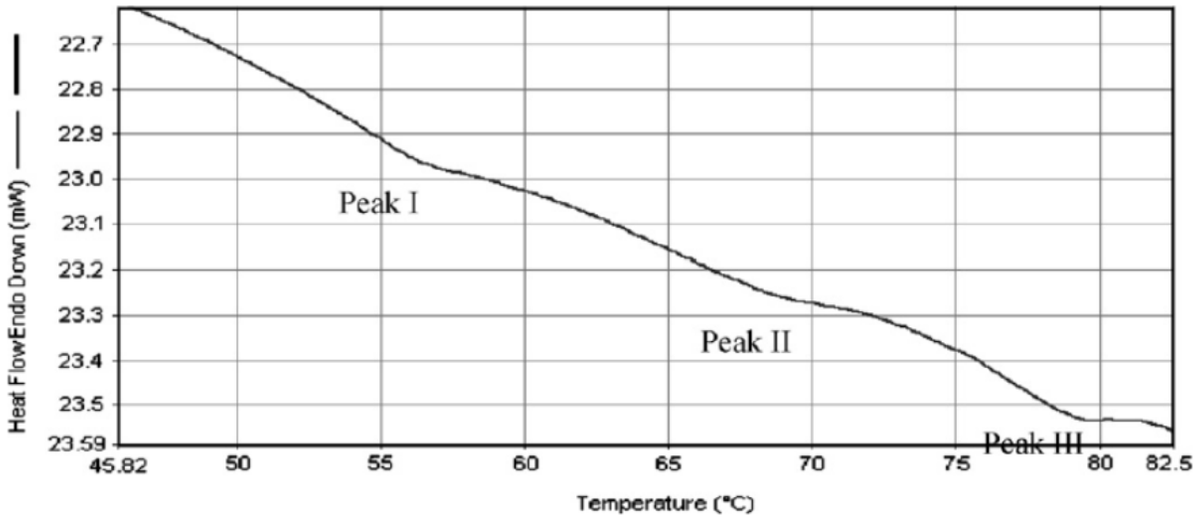

Figure 7. Thermal analysis of NI whole muscles
(Pighin et al., 2008)

Treatment	Peak I (myosin)		Peak II (sarcoplasmic proteins/collagen)		Peak III (actin)		
	T_d (°C)	ΔH (J/g protein)	T_d (°C)	ΔH (J/g protein)	T_d (°C)	ΔH (J/g protein)	ΔH_T (J/g protein)
NI	57.0 ab	1.32 a	67.4 c	0.65 ab	80.3 a	1.74 a	3.71
0.70% NaCl	56.4 b	0.70 b	67.1 c	0.73 a	77.2 c	1.61 a	3.04
0.25% TPP	57.6 a	1.75 a	70.9 a	0.50 bc	80.8 a	2.03 a	4.28
0.70% NaCl + 0.25% TPP	58.1 a	1.79 a	69.7 ab	0.38 c	78.5 b	1.66 a	3.83
1.20% NaCl + 0.25% TPP	56.3 b	1.40 a	68.6 bc	0.51 bc	76.8 c	0.84 b	2.75

Table 1. Endothermic peaks (Td) and denaturation enthalpies (DH) of whole muscles treated with NaCl and/or TPP
a-c Means within a column having different letters are significantly different ($p < 0.05$).
(Pighin et al., 2008)

The described findings reveal an important effect of the salt in decreasing the thermal stability of proteins. In order to support this fact, it was proposed that addition of neutral salt causes anions to compete with water molecules for specific sites of proteins, altering its hydration properties, and consequently requiring lower denaturation energies (Von Hippel & Wong, 1965). Treatment with TTP (0.25 %) significantly increased sarcoplasmic proteins/collagen Td. Also, Td of myosin and DH of actin were slightly increased. Whole muscle treated with 0.25 % TPP increased the DHT from 3.71 (NI muscles) to 4.28 J/g protein. These findings show an important stabilizing effect exerted by TPP, probably by altering hydrophobic interactions rather than affecting pH or ionic strength (Trout & Schmidt, 1986). With regard to the effect of phosphates on meat, Kijowski & Mast (1988) studied the effect of different phosphates (sodium pyrophosphate or sodium tripolyphosphate) on chicken breast muscles and its isolated myofibrils. They stated that low levels of sodium pyrophosphate or sodium tripolyphosphate (0.25 % or 0.50 %) caused the maximum stabilization (DHT) of chicken muscle and isolated myofibrils by means of myosin stabilization (increased DH). These results agree with present ones, even they were obtained in beef muscles.

When both additives were used together (0.70 % NaCl + 0.25 % TPP), Td corresponding to sarcoplasmic proteins/collagen was significantly increased in comparison to NI muscles, suggesting stabilization of the related proteins. This stabilization went along with a significant decreased of the DH, probably associated to an increment of the exothermic protein aggregation. On the contrary, actin Td showed a significant reduction in comparison to NI. Destabilization of actin in the presence of NaCl and phosphates was also described by Kijowski & Mast (1988) in chicken breast muscles treated with 2 % NaCl and 0.25/0.50 % pyrophosphate. When the effect of this combination of salts is compared to the one previously described, 0.70 % NaCl effect, it can be speculated that two effects appear to coexist: the same stabilizing effect of TPP on sarcoplasmic proteins/collagen and the destabilizing effect of NaCl on actin. Even though, the last effect is smaller in magnitude than the one produced by NaCl alone. It is important to denote that DHT of the muscle treated with the combination of salts (0.70 % NaCl + 0.25 % TPP) was slightly higher than the NI one (3.83 vs. 3.71 J/g protein, respectively). Comparing the DHT obtained individually for NaCl and TPP treatments, it can be seen that the effect of the addition of both salts is contrary to the effect of NaCl alone and in the same direction as TPP. Hence, the final increase could be assigned to the increase (even non significant) of myosin DH induced by TPP. Even more, when NaCl was raised to 1.20%, a maximal drop in actin stability was achieved (higher reduction of Td and DH). This event was accompanied by a maximal drop in DHT (from 3.71 to 2.75 J/g protein). It becomes evident that the increased amount of NaCl in the brine produced a destabilizing effect similar to the one found for NaCl alone, which also turned to be predominant to expenses of the effect of TPP.

Regarding this issue, Findlay & Barbut (1992) had stated that the addition of NaCl (0.5-2.0 %) reduced myosin Td and conversely TPP (0.2-0.6 %) increased its stability. Additionally, DHT varied depending on salts concentration. At low NaCl levels (less than 1.00 %) the effect of TTP on the increment of DHT was predominant. Instead, as NaCl

increased the phosphate effect was minimized. In accordance with such observations, present results indicate that NaCl exerts a destabilizing effect in the presence of TPP (0.25 %) that becomes stronger as NaCl level raises.

4.2.3. Thermal analysis of isolated myofibrils

Thermal analysis of isolated myofibrils from NI muscle showed only two endothermic peaks (Fig. 8), corresponding to denaturation enthalpies of myosin and its subunits (I: 58.1 °C) and actin (II: 73.5 °C). Data obtained from Table 2 showed a significant decrease of myosin and actin DH in myofibrils isolated from 0.70 % NaCl-treated muscles and washed in the same brine solution. No changes in Td of these proteins were found. The destabilizing effect of NaCl was in agreement with data obtained from the whole muscle. TPP (0.25 %) treatment produced a significant increase of both, myosin and actin Td. In contrast, DH of myosin was strongly reduced ($p < 0.05$) by the treatment. These findings indicate that the salt is capable of stabilizing actin and myosin structures. However, the smaller energy required to denature myosin (lower DH) suggests a small amount of protein available in the myofibril fraction. Supporting this comment, it had been proposed that phosphates can induce myosin solubilization by promoting both, thick filament depolymerisation and actomyosin dissociation (Granicher & Portzehl, 1964; Xiong, 2005). Even though, an increased protein aggregation (exothermic event) taking place immediately after myosin denaturation, might not be discarded. In the case of whole muscle treated with TPP, myosin and actin Td did not suffer any change and their DH were only slightly increased. Therefore, it can be affirmed that the effect of TPP on the whole muscle and myofibrils is almost alike.

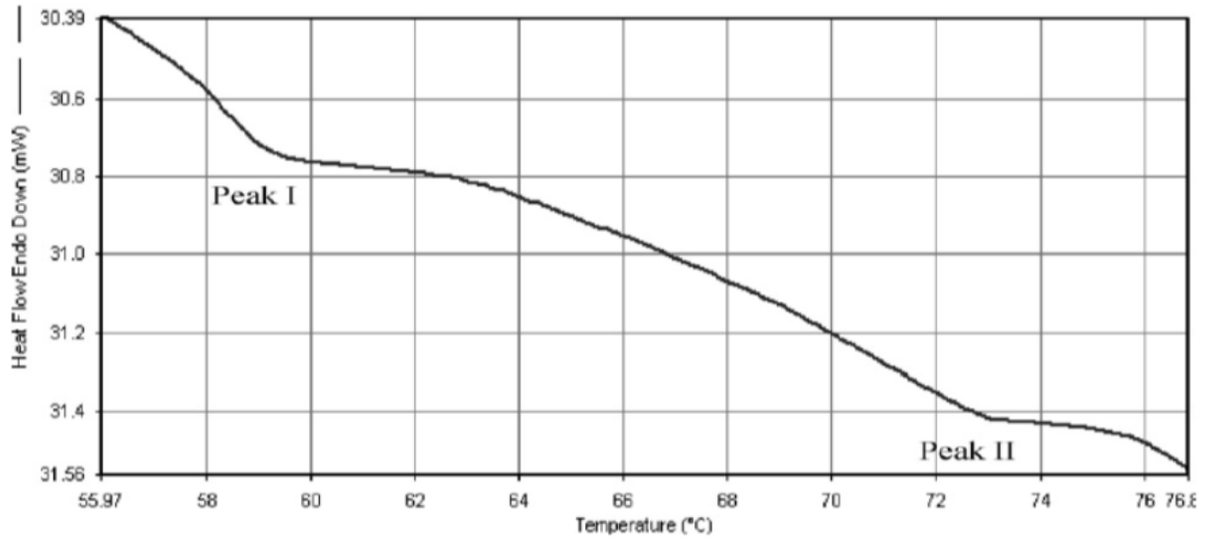

Figure 8. Thermal analysis of myofibrils isolated from NI muscles (Pighin et al., 2008)

When NaCl and TPP were used together (0.70 % NaCl + 0.25 % TPP) both, myosin and actin Td of isolated myofibrils significantly decreased ($p < 0.05$). Enthalpy of the proteins was also

decreased, being the myosin DH reduced in almost 2.60 J/g protein with respect to NI muscles (NI: 3.87 vs. 1.27 J/g protein; $p < 0.05$). These findings confirm the destabilizing effect of NaCl previously described for actin in the whole muscle. Even more, this effect seems to be stronger in the myofibril fraction since myosin transition was also affected. Further increments of NaCl (1.20 %) -in the presence of TPP- produced similar effects on both proteins, with the exception of an intensified effect on actin DH. It is important to remark that the effect of both additives together was definitively higher than the effect of any of the additives individually. Offer & Knight (1998) proposed that polyphosphates -at the concentrations used in the industry- may assist NaCl in causing depolymerisation of thick filaments and dissociation of actomyosin, facilitating in consequence the myosin extraction. The action of NaCl as a mild structure-breaker aided by the presence of TPP could be the reason of the significant reduction of protein stability found.

Treatment	Peak I (myosin)		Peak II (actin)	
	T_d (°C)	ΔH (J/g protein)	T_d (°C)	ΔH (J/g protein)
NI	58.1 b	3.87 a	73.5 b	2.10 a
0.70% NaCl	57.9 b	2.16 b	74.9 b	1.44 c
0.25% TPP	63.2 a	0.73 d	83.4 a	1.97 ab
0.70% NaCl + 0.25% TPP	51.5 c	1.27 c	68.7 c	1.53 bc
1.20% NaCl + 0.25% TPP	51.1 c	1.56 c	67.6 c	0.80 d

Table 2. Endothermic peaks (Td) and denaturation enthalpies (DH) of myofibrils isolated from muscles treated with NaCl and/or TPP
a-d Means within a column having different letters are significantly different ($p < 0.05$).
(Pighin et al., 2008)

As it was mentioned, the physicochemical mechanisms involved in water binding and retention in the protein matrix of cooked meat were not completely elucidated. These results showed that the combined presence of NaCl (0.70 % or 1.20 %) and TPP (0.25 %) changes the heat susceptibility of muscle proteins (especially actin) so that they denature and coagulate at a lower cooking temperature. These modifications suggest the occurrence of protein conformational changes due to the salts, probably by altering hydrophobic and electrostatic interactions that stabilize the protein structure (Franks & Eagland, 1975). Evidently, these changes collaborate in the binding and posterior retention of water inside the tissue by any of the mechanisms proposed for the action of NaCl and polyphosphates (Offer & Knight, 1998). Increased solubilization of proteins found in whole muscles treated with NaCl (alone or in combination with TPP) could be related to the removal of transverse myofibrillar proteins which may act as structural constrains to myosin extraction. Also, some other low molecular weight myofibrillar proteins could be removed. The softening of the structure together with conformational modifications of myofibrillar proteins would allow entrapping water inside the tissue. This event would be the reason of the increased WHC of *Semitendinosus* muscle found by Vaudagna et al. (2008).

5. Conclusion

The isolation protocol previously described in this chapter has been demonstrated to be a successful methodology for individually isolating four of the main myofibrillar proteins.

Despite the proteins were not isolated in a complete pure state, this approach seems to be useful for using them for further analysis. The estimation of proteins' Rf by means of an immunological method looks like an interesting tool to complement the identification of proteins in unclear electrophoretic patterns.

The thermal behavior of the myofibrillar proteins is quite involved in the thermal behavior of whole muscle. NaCl incorporation into meat leads to an important protein destabilizing effect upon myofibrillar proteins, even at relative low doses when compared to industrial usage. The use of NaCl in combination with TPP produces a destabilizing global effect, suggesting that TPP may assist NaCl in acting as structure-breaker. The increase of WHC of *Semitendinosus* muscles injected with saline additives and submitted to *sous vide* cooking would be mainly associated to the conformational modifications of myofibrillar proteins and to the weakening of myofibrillar structures due to protein removal.

Taken together, the use of gel electrophoresis demonstrated to be a useful tool not only in isolating major muscle proteins but also in contributing to study the effect of saline additives in meat, in order to improve sensorial properties. Its combination with advanced technologies like DSC represents an interesting issue to look forward in the meat science approach.

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