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High hydrostatic pressure processing of beef patties: Effects of pressure level and sodium tripolyphosphate and sodium chloride concentrations on thermal and aggregative properties of proteins

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ABSTRACT

Beef patties added with sodium tripolyphosphate (STPP; 0, 0.25 or 0.5%) and/or NaCl (0, 1 or 2%) were treated at 200 or 300 MPa (5 min, 5 °C) or kept refrigerated (non-pressurized). In non-pressurized patties, NaCl-solubilized proteins were denatured, whereas STPP-solubilized proteins remained in native state. At 200 MPa, myosin head was more sensitive to high hydrostatic pressure (HHP) than actin. 1% NaCl favored HHP-induced denaturation of myosin head and actin, whereas 0.25% STPP protected against that effect. At 300 MPa, STPP favored HHP-induced denaturation of myosin head, actin and other proteins. The effect of STPP at 200 MPa may depend on the presence of specific binding sites for STPP anion, which would be destroyed at 300 MPa. Insoluble aggregates were formed at 300 MPa in samples without salts. Salts minimized protein aggregation was observed at 300 MPa. Noticeable differences in thermal and aggregative behavior occurred whether HHP level was 200 or 300 MPa.
Industrial relevance: Currently, the reduction of sodium content in the manufacture of meat products is a hot topic and it is expected that this issue will become more relevant in the next years, as response to consumers' demands. Soluble high hydrostatic pressure-denatured beef proteins may provide interesting texture and technological properties to meat products with reduced salt content.

1. Introduction

Since the demand for healthy products has significantly increased in the last years, food manufacturers are interested in developing new products that respond to these demands. Traditionally, NaCl and sodium tripolyphosphate (STPP) are additives used in the manufacture of meat products. These additives are involved in the extraction and solubilization of myofibrillar proteins, which form a gel upon heating and led to a compact and uniform structure with improved water-retention (Desmond, 2006; Feiner, 2006; Sun & Holley, 2011; Tornberg, 2005). However, NaCl is associated with hypertension (Chen & Trout, 1991; Ruusunen & Puolanne, 2005) and polyphosphates are currently perceived as negative among consumers because of the clean labeling issue (Yusop, O'Sullivan, & Kerry, 2011).

During the last decade, several meat products treated by high hydrostatic pressure (HHP) such as pork and turkey cooked ham, dry-cured pork ham, prosciutto, sausages, marinated turkey or pork meat, ready-to-eat meats have been commercialized in Europe, Japan and North America (Sun & Holley, 2010). The main objective of the application of HHP in those products is the inactivation of pathogen microorganisms (such as *Listeria monocytogenes* and *Salmonella* spp.) and the extension of shelf-life. In addition, HHP processing causes physicochemical changes in meat proteins, such as depolymerization of F-actin, dissociation of actomyosin, solubilization of myofibrillar proteins and even their aggregation at pressures between 100 and 300 MPa (Buckow, Sikes, & Tume, 2013; Iwasaki, Noshiroya, Saitoh, Okano, & Yamamoto, 2006; Macfarlane, 1985). Those changes, which depend on the characteristics of the system and the conditions of processing (Fernández-Martín, Cofrades, Carballo, & Jiménez-Colmenero, 2002), improve meat binding properties and partially compensate the reduction of NaCl and STPP concentrations (Monahan & Troy, 1997). Several authors studied the application of HHP treatments (100–350 MPa), before or after, for the manufacture of low sodium content meat products (Crehan, Troy, & Buckley, 2000; Ferrari, Szerman, Sanow, Sancho, & Vaudagna, 2012; O'Flynn, Cruz-Romero, Troy, Mullen, & Kerry, 2014; Sikes, Tobin, & Tume, 2009; Villamonte, Simonin, Duranton, Chéret, & de Lamballerie, 2013). The application of HHP

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increased hardness values of different meat products such as beef and pork batters and patties (Ferrari et al., 2012; Sikes et al., 2009; Villamonte et al., 2013). Villamonte et al. (2013) concluded that this hardening effect was associated with the denaturation of myofibrillar proteins and the formation of a new protein component in pork batters. These authors also observed a synergic effect of NaCl and STPP on water binding capacity, enhanced by HHP. Moreover, Sikes et al. (2009) obtained similar cooking weight losses in beef batters formulated with 1% NaCl and HHP-treated at 200 MPa and those formulated with 2% NaCl and non-pressurized. However, we found that cooking weight loss increased when pressure level increased from 100 to 300 MPa in beef patties with low NaCl content (1% NaCl; 0.25% STPP) (Ferrari et al., 2012). Although the effects of the addition of NaCl and STPP on myofibrillar proteins have been largely studied (Barbut & Findlay, 1991; Findlay & Barbut, 1992; Kijowski & Mast, 1988; Paterson, Parrish, & Stromer, 1988; Pighin, Sancho, & Gonzalez, 2008; Xiong, Lou, Wang, Moody, & Harmon, 2000), the effects of those salts in combination with HHP processing on the solubilization and aggregation of myofibrillar proteins are still not well understood. Since some heterogeneous results were reported, a molecular characterization will provide a better understanding of the system.

The aim of this study was to evaluate the combined effect of salt concentration (NaCl and STPP) and HHP pressure levels on thermal and aggregative properties of beef proteins.

2. Materials and methods

2.1. Materials

Fresh beef shoulder clods (muscles *trapezius*, *deltoideus*, *latissimus dorsi*, *infraspinatus*, *triceps brachii*, *anconeus internus*, *anconeus extens*, *teres major* and *tensor fasciae antebrachii*) were obtained from a local market (COTO CICSA, Buenos Aires, Argentina). Meat pieces were vacuum-packed and stored at 1.0 ± 1.0 °C for 48 h. Muscles were defatted, and fat was conserved for patty preparation. After that, the pH of the pieces was measured using a puncture electrode (TESTO model 230, Sparta, NJ, USA), and those with normal pH (between 5.4 and 5.7) were selected. Then, meat and fat were vacuum-packed in Cryovac BB2800CB bags (permeability to: O_2 30 cm^3 m^{-2} 24 h^{-1} bar^{-1}; CO_2 150 cm^3 m^{-2} 24 h^{-1} bar^{-1}; water vapor 20 g 24 h^{-1} m^{-2}; Sealed Air Co., Buenos Aires, Argentina) and refrigerated at 1.0 ± 1.0 °C for 24 h.

The salts used were NaCl (Dos Anclas, Buenos Aires, Argentina) and STPP (N 15-16 Chemische Fabrik Budenheim R.A. Oetker, Budenheim, Germany).

2.2. Product manufacturing

Patties were prepared with the following composition: lean meat, 80% (w/w); fat, 10% (w/w); water, 10% (w/w) and NaCl (0, 1 or 2%) and/or STPP (0, 0.25 or 0.5%). The percentage of meat was modified according to the salt concentrations in the formulation. First, lean beef and fat were separately minced using a 4 mm plate in a Hobart meat grinder (Hobart Corp., Troy, Ohio, USA). During mincing, temperature was monitored using a puncture thermometer (Testo model 230, Sparta, NJ, USA), and it was lower than 8 °C during this step. After mixing lean meat and fat by hand, the mixture was minced through a 4 mm plate in a Hobart meat grinder (Hobart Corp., Troy, Ohio, USA). Then, STPP (dry powder) was added and manually mixed for 5 min. Finally, NaCl (previously dissolved in water at 8 °C) was incorporated and the mix was mixed by hand for 5 min. After that, portions of 140 g were formed into patties between grease proof papers using a manual patty press (100 mm diameter). Patties were stored at −20 °C for 24 h. Two smaller patties (50 mm diameter each) were obtained from each patty using a punch because the diameter of the HHP canister was 70 mm. After that, patties were vacuum-packed in Cryovac BB2800CB bags and stored at 1.0 ± 1.0 °C for 24 h. Henceforth, "control patties" corresponded to patties without salts non-subjected to HHP treatments.

2.3. High hydrostatic pressure treatments

Vacuum-packed patties were subjected to 200 or 300 MPa for a holding time of 5 min. HHP treatments were applied in a High Pressure System Stansted Fluid Power Ltd. model Iso-Lab FPG9400:922 (Stansted, United Kingdom), with a vessel working volume of 2 dm^3 (maximum working pressure: 900 MPa; temperature range: −20–120 °C). A mixture of propylene glycol and water (30:70) was used as compression fluid. Pressurization rate was 300 MPa min^{-1}. Conditioning temperature of vessel and initial temperature of compression fluid were 5 °C. The adiabatic heating induced an increase of fluid temperature that reached a maximum (10 °C) at 300 MPa. Patties (non-pressurized or HHP-treated) were stored at −40 °C for a maximum storage time of 4 months. Before testing, patties were thawed at 4 °C overnight.

2.4. Sample analysis

2.4.1. Protein content

The protein content of beef patties was determined by the Kjeldahl method (AOAC, 1990), using an N × 6.25 factor for calculation (2200 Kjeltec Auto Distillation, Foss Tecator, Hillerod, Denmark).

2.4.2. Thermal analysis

The thermal properties of beef patties were studied using a Perkin-Elmer Pyris-1 differential scanning calorimeter (Waltham, MA, USA). Indium was used as standard for temperature and heat flow calibration.

A sample of 19 to 24 mg of each patty, accurately weighed (Mettler Toledo H54, ±0.01 mg), was placed into an aluminum pan, which was hermetically sealed and equilibrated for 2 min at the initial scanning temperature. An empty pan was used as reference. The temperature increased from 20 to 90 °C at 5 °C min^{-1}. Changes of thermal denaturation enthalpy (ΔH) of total proteins were estimated as the area (heat flow vs. time) between the DSC curve and a straight line extended from the onset to the final temperatures of all detected transitions. The temperature of maximum heat absorption (Td) and the ΔH were determined by Origin Pro 8 software (Northampton, MA, USA); ΔH was expressed as J g^{-1} of protein. Peak Fit software V4.0 (Jandel Scientific Software, Chicago, IL, USA) was used to deconvolute the curves and calculate the percentage of ΔH corresponding to each individual transition. The ΔH values for myosin head and actin were reported because their identification and individualization was evident. The degree of denaturation was calculated as 100 multiplied by 1 minus the ratio between ΔH1 and ΔH2, where ΔH1 was the ΔH after HHP treatment and/or salt addition and ΔH2 was the ΔH for control or non-pressurized patties with salts addition. The degree of denaturation was calculated only in those cases where ΔH1 and ΔH2 were significantly different ($p < 0.05$). DSC analysis was carried out in quadruplicate for each treatment.

2.4.3. Salt-soluble protein extraction

Proteins were extracted from raw beef patties following the procedure of Wang, Smith, and Steffe (1990) with modifications. Extraction was carried out in a two-step procedure: the first step consisted in stirring (20 min at 4 °C) 0.2 g of chopped patty to 0.8 mL of 0.1 M NaCl, 0.05 M Na_2HPO_4/NaH_2PO_4 buffer, pH 6.5 (B0.1). Then, centrifugation was carried out (13,000 ×*g* for 15 min, 10 °C, Aircooled Microlitre Centrifuge Z 233 MK-2 Hermle, Gosheim, Germany). Supernatant, which contained proteins soluble in B0.1, was used for SDS-PAGE analysis. The second step of extraction consisted in exposing the pellet obtained after centrifugation to 0.8 mL of 0.6 M NaCl, 0.05 M Na_2HPO_4/NaH_2PO_4 buffer, pH 6.5 (B0.6) or to 0.8 mL of 0.6 M NaCl + 1% (w/v) SDS, 0.05 M Na_2HPO_4/NaH_2PO_4 buffer, pH 6.5 (B0.6-SDS). The second extraction was also carried out for 20 min at 4 °C followed by a centrifugation in the same conditions indicated above. The supernatants, which contained proteins soluble in B0.6 or B0.6-SDS, were used for SDS-PAGE analysis.

2.4.4. Molecular characterization by using SDS-PAGE

Electrophoretic profiles were analyzed by SDS-PAGE. Slab gels from 5 to 15% acrylamide linear gradient were prepared by pumping acrylamide solutions from two communicating vessels. Also, continuous gels were prepared (stacking 4% and running 12% acrylamide) according to Laemmli (1970). Samples from salt-soluble extraction were diluted 1:1 with a Tris–HCl glycerol-containing buffer at pH 8.8. Then, 4 μL of diluted samples were loaded per lane. HMW (53–212 kDa) and LMW (14.4–97 kDa) markers were used (GE healthcare, Buckinghamshire, UK). Electrophoresis was carried out at constant current of 30 mA (Mini Protean III system Bio-Rad, Hercules, CA, USA). Gels were stained with Coomassie Brilliant Blue dye solution.

2.5. Statistical analysis

Analysis of variance (ANOVA) was applied to evaluate the effects of pressure levels and salt concentrations on the Tds. Then, differences among the mean values of Tds (each peak was analyzed individually) were assessed using a multiple comparison Bonferroni test ($p \leq 0.05$). Also, ANOVA of ΔH values was carried out to evaluated the effects and interactions of factors (pressure levels and salt concentrations) and differences among means were assessed using a multiple comparison Bonferroni test ($p = 0.05$). Data were analyzed using Infostat software version 2011 (Di Rienzo et al., 2011).

3. Results and discussion

3.1. Thermal analysis

Fig. 1 shows a thermogram of a control beef patty, with 4 transitions that were partially overlapped. The peaks obtained by deconvolution are also depicted.

The analysis of thermal behavior of meat proteins is complex because of the coexistence of different types of proteins (e.g. collagen, sarcoplasmic and myofibrillar) which are sensitive to the presence of other compounds (e.g. salts). Thermograms are also affected by heating rate, type of meat and muscle, and its thermal history (frozen, refrigerated, etc.) (Findlay, Stanley, & Gullett, 1986; Stabursvik & Martens, 1980).

Several authors reported that, under most of the conditions of pH and ionic strength used in meat processing, myosin presents two thermal transitions: the first one between 45 and 54 °C, which corresponds to myosin head denaturation, and the second one between 54 and 65 °C, which corresponds to myosin rod denaturation (Lorinczy & Belagyi, 1995; Sikes et al., 2009; Stabursvik & Martens, 1980; Wright & Wilding, 1984). Denaturation of sarcoplasmic proteins exhibited two

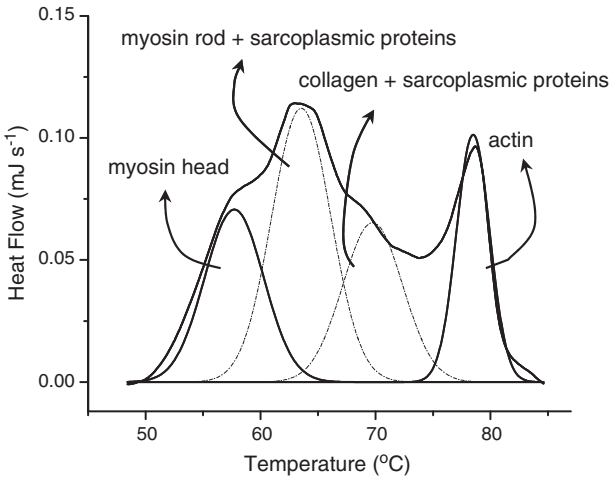

Fig. 1. Thermogram and deconvoluted peaks of control patty.

transitions: the first one between 54 and 62 °C, and the second one between 68 and 70 °C, whereas collagen denaturation exhibited one transition near 67 °C (Stabursvik & Martens, 1980). Several authors found that actin transition occurred between 75 and 80 °C, and comprised G-actin monomers, fragments of F-actin and actomyosin (Pighin et al., 2008; Sikes et al., 2009; Stabursvik & Martens, 1980).

Taking into account these data and the different factors that affect Td, we attributed our observed transitions of control patties as follows: myosin head at 57.5 ± 0.2 °C, myosin rod and first transition of sarcoplasmic proteins at 63.8 ± 0.2 °C, second transition of sarcoplasmic proteins and collagen at 68.8 ± 0.1 °C, and actin at 79.2 ± 0.1 °C (Fig. 1 and Table 1).

3.1.1. Effects of salts in non-pressurized patties

Fig. 2a shows the thermal behavior of non-pressurized patties formulated with different concentrations of STPP and/or NaCl. Table 2 presents the ΔH values related to the transitions of total proteins and Table 3 presents the ΔH values related to the individual transitions of myosin head and actin.

The ΔH of total proteins significantly decreased ($p < 0.05$) in the presence of NaCl, but showed no modifications in the presence of 0.25% STPP. The denaturation degree was 26% at 1% NaCl, which increased up to 42% at 0.50% STPP + 2% NaCl. In addition, the presence of 0.25% STPP decreased the denaturation degree from 26 to 16% when 1% NaCl was added. The effects of the simultaneous presence of STPP and NaCl on denaturation depended on salt concentrations.

The presence of salts also modified the Tds (Table 1). The addition of 0.25% STPP promoted the appearance of a peak at 61.8 ± 0.2 °C, which was originated as a split of the peak at 63.8 °C of control patties. We concluded that because the area of the two transitions (peaks at 61.8 and 64.3 °C) was equal to the area of the original peak at 63.8 °C in control patties (data not shown). Kijowski and Mast (1988) found that 0.25 and 0.5% STPP increased the Td of one of the myosin peaks. Thus, the Td of myosin rod increased towards 64.3 °C and the Td of sarcoplasmic proteins decreased towards 61.8 °C. The addition of 0.25% STPP caused no modifications of the Tds of myosin head, collagen or actin (Table 1). However, the ΔH of myosin head significantly increased ($p < 0.05$) whereas the ΔH of actin was not modified (Table 3).

The addition of 1% NaCl decreased the Tds of myosin head and actin (peaks 2 and 6, Table 1). The effect was more important on actin ($\Delta Td = 5.7$ °C) than on myosin head ($\Delta Td = 1.7$ °C). These results are similar to those reported by Kijowski and Mast (1988), Fernández-Martín et al. (2002) and Pighin et al. (2008). Also, myosin head and actin tended to denature with the addition of 1% NaCl (Table 3). Sikes et al. (2009) and Villamonte et al. (2013) reported a decrease in the ΔH of actin and no changes in the ΔH of myosin head in beef batters with 1.0% NaCl and pork batters with 1.5% NaCl, respectively. The Td of collagen and some sarcoplasmic proteins decreased 3.3 °C (peak 5, Table 1). Kijowski (1993), Penkova, Goshev, Gorinstein, and Nedkov (1999) and Tomaszewska-Gras and Konieczny (2010) also reported a decrease in Td of collagen between 2.7 and 5.3 °C due to NaCl presence (0.9–6%). Kijowski (1993) stated that this decrease was caused by swelling, which changes the water–protein interactions. The differences in sensitivity to NaCl may be due to the collagen origin or whether it was isolated and, in our case, to the presence of other proteins.

Regarding 0.25% STPP + 1% NaCl-patties, the destabilization induced by 1% NaCl on actin was significantly ($p < 0.05$) reduced by 0.25% STPP ($\Delta Td = 5.7$ vs. 4.5 °C, Table 1). It is important to remark that this stabilizing effect of STPP was only observed in the presence of 1% NaCl (Table 1). Since no denaturation of actin was evidenced (Table 3) STPP would protect actin towards NaCl-induced denaturation. Fernández-Martín et al. (2002) also reported that actin was more destabilized in the presence of 1.5% NaCl than in the presence of 1.5% NaCl + 0.3% STPP. On the other hand, the Tds of myosin head (peak 2) and collagen and some sarcoplasmic proteins (peak 5) were significantly ($p < 0.05$) lower than in control patties ($\Delta Td = 1.3$ and 2.4 °C, respectively).

Table 1
Temperature of maximum heat absorption (Td) of beef proteins in patties formulated with different STPP and/or NaCl concentrations and treated at different pressure levels for 5 min.

Formulation	Pressure level (MPa)	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6
Without salts	Non-pressurized	–	57.5 ± 0.2^{bc}	–	63.8 ± 0.2^{a}	68.8 ± 0.1^{ab}	79.2 ± 0.1^{a}
	200	–	56.7 ± 0.3^{bcd}	–	63.8 ± 0.2^{a}	68.9 ± 0.1^{ab}	78.1 ± 0.1^{b}
	300	51.3 ± 0.2^{abc}	–	–	–	65.2 ± 0.3^{d}	–
0.25% STPP	Non-pressurized	–	58.3 ± 0.1^{ab}	61.8 ± 0.2^{a}	64.3 ± 0.1^{a}	69.2 ± 0.2^{ab}	79.1 ± 0.1^{a}
	200	–	59.6 ± 0.4^{a}	–	64.5 ± 0.2^{a}	69.4 ± 0.2^{a}	79.3 ± 0.2^{a}
	300	52.1 ± 0.3^{ab}	–	60.6 ± 0.8^{ab}	–	65.4 ± 0.5^{cd}	–
1% NaCl	Non-pressurized	–	55.8 ± 0.4^{de}	–	61.2 ± 0.3^{cd}	65.5 ± 0.2^{cd}	73.5 ± 0.2^{d}
	200	–	54.0 ± 0.2^{f}	–	61.9 ± 0.1^{bc}	65.9 ± 0.1^{cd}	73.4 ± 0.2^{d}
	300	50.0 ± 0.6^{bc}	–	–	–	65.5 ± 0.2^{cd}	–
0.25% STPP + 1% NaCl	Non-pressurized	–	56.2 ± 0.2^{d}	–	62.2 ± 0.2^{bc}	66.4 ± 0.2^{cd}	74.7 ± 0.2^{c}
	200	–	56.7 ± 0.3^{cd}	–	62.7 ± 0.1^{b}	66.8 ± 0.1^{c}	74.4 ± 0.3^{cd}
	300	53.0 ± 0.5^{a}	–	–	62.1 ± 0.2^{bc}	66.5 ± 0.2^{c}	–
0.5% STPP + 2% NaCl	Non-pressurized	50.8 ± 0.4^{bc}	54.6 ± 0.3^{ef}	58.3 ± 0.2^{c}	61.5 ± 0.2^{cd}	68.2 ± 0.4^{b}	–
	200	49.9 ± 0.2^{c}		58.3 ± 0.1^{c}	62.0 ± 0.1^{bc}	68.2 ± 0.3^{b}	–
	300	50.5 ± 0.4^{bc}		59.2 ± 0.2^{bc}	60.4 ± 0.3^{d}	68.6 ± 0.2^{ab}	–

STPP: sodium tripolyphosphate; NaCl: sodium chloride; peak 1: salt- or HHP-induced structure; peak 2: myosin head; peak 3: sarcoplasmic proteins; peak 4: myosin rod and sarcoplasmic proteins; peak 5: collagen and sarcoplasmic proteins; peak 6: actin. $^{a–f}$Means values with different letters in the same column are significantly different ($p < 0.05$).

Besides, the decrease of collagen and some sarcoplasmic proteins Td (peak 5) was lesser than in the presence of 1% NaCl alone (3.3 °C).

Increasing the concentrations of salts, 0.50% STPP + 2% NaCl, decreased most of the Tds. The Td of actin decreased up to overlap with the transition of collagen and the resulting peak had a Td of 68.2 ± 0.4 °C. The Td of myosin head decreased to 54.6 ± 0.3 °C, and the peak of myosin rod and some sarcoplasmic proteins was split into two transitions of 58.3 ± 0.2 and 61.5 ± 0.2 °C (Table 1). The addition of 0.50% STPP + 2% NaCl caused 95% denaturation of myosin head in relation to control patties (Table 3). Actin also suffered an important degree of denaturation, but no percentage could be calculated because of the overlapping of transitions (Table 3). No protective effect of STPP was detected at 2% NaCl, which indicates that the mechanism involved in protein stabilization depended on salt concentrations. Also, a transition at 50.8 ± 0.4 °C (Table 1) with a ΔH value of 0.48 ± 0.07 J g^{-1} appeared in these samples. This transition reflects the presence of a structure, which may be formed by salt-extracted proteins. Villamonte et al. (2013) also reported a salt-induced structure that denatured at low temperature in pork proteins from batters.

We consider that the effects of STPP and/or NaCl are the result of a combination of mechanisms, which depend on concentration (ionic strength), type of salt (kosmotropic–chaotropic effect) and specific interactions with active sites. Hamm (1972) proposed that negatively charged ions, such as chloride, are more strongly bounded to myofibrillar proteins than positively charged ones, such as sodium; chloride binding to filaments led to an increase in protein–protein repulsion. The mechanism proposed by Hamm may decrease the attractive interactions between polypeptides. This effect, which depends on salt concentration, may explain the decreases in the ΔH of actin and myosin head, since electrostatic interactions represent an important contribution to the ΔH. Stabursvik and Martens (1980) found that NaCl destabilized actin and this effect was dependent on its concentration, whereas NaH_2PO_4 and KH_2PO_4 stabilized this protein independently of $H_2PO_4^-$ concentration. Therefore, $H_2PO_4^-$ may exert a kosmotropic effect. Tripolyphosphate anion ($P_3O_{10}^{-5}$) may have, due to its structure similarity with $H_2PO_4^-$ and higher charge density, a more stabilizing activity than Cl^- in the Hofmeister series. This fact may explain the "protection" of STPP observed on the Td and ΔH of actin against the effect of 1% NaCl. On the other hand, phosphate analogs bind at the active site of myosin head in the position normally occupied by the terminal phosphate of ATP (Henry, Maruta, Ikebe, & Sykes, 1993; Kijowski & Mast, 1988). Shriver and Kamath (1990) reported that the binding of analogs of ATP modified thermal properties of myosin head. For these reasons, a specific binding of tripolyphosphate anion to myosin head may occur, which induces conformational changes that stabilize it in our samples. This stabilization would not be evident in the presence of 2% NaCl, where the denaturation of myosin head was almost complete with the disruption of the active site. Other explanation would be that at high STPP concentrations (0.5%), the specific binding sites may be saturated and an ionic effect (similar to that of chloride ion) may favor the unfolding of myosin head.

3.1.2. Effects of salts in patties treated at 200 MPa

The ΔH values of total proteins for 200 MPa patties presented no significant changes in comparison with their respective non-pressurized ones ($p > 0.05$, Table 2). However, the ΔH values of 200 MPa-patties added with 1% NaCl or 0.25% STPP + 1% NaCl tended to decrease in relation to non-pressurized ones (Table 2). Thus, the presence of 1% NaCl may increase the sensitivity of meat proteins to a 200 MPa treatment.

Myosin head in 200 MPa-patties without salts was 51% denatured (Table 3). The addition of 1% NaCl increased HHP-induced denaturation to 69%. In contrast, the addition of 0.25% STPP had a protective effect against HHP-induced denaturation, since no changes in ΔH were observed in 0.25% STPP and 0.25% STPP + 1% NaCl-patties in relation to non-pressurized ones (Table 3). No protective effect was found for 0.5% STPP + 2% NaCl-patties, because myosin head was already 90% denatured by salts in non-pressurized patties. The specific effect of STPP on the stability of myosin head observed in non-pressurized patties would also occur at 200 MPa.

Neither in patties without salts nor in patties with 0.25% STPP actin was denatured at 200 MPa. However, actin presented a 74% denaturation degree in 1% NaCl-added patties, whereas showed a 27% denaturation degree in 0.25% STPP + 1% NaCl-added patties. These results suggest a favoring effect of NaCl (74 vs. 0%) and a protective effect of STPP (27 vs. 74%) on actin towards HHP-induced denaturation.

By comparing the effect of 200 MPa on myosin head and actin, we conclude that in the absence of salts myosin head is more sensitive than actin to HHP-induced denaturation. The addition of 1% NaCl minimized this difference, and 0.25% STPP partially counteracted the effect of 1% NaCl in both proteins.

The sarcoplasmic protein transitions of non-pressurized patties, that appeared at 63.8, 61.8, 61.2 and 62.2 °C for control, 0.25% STPP, 1% NaCl, and 0.25% STPP + 1% NaCl-patties, respectively, were not detected or their areas became smaller in 200 MPa-patties (Fig. 2b–e). Marcos, Kerry, and Mullen (2010) reported that some components of sarcoplasmic proteins (such as a polypeptide of 88 kDa) were denatured at 200 MPa. However, those authors found that most of sarcoplasmic proteins were denatured when pressure level was higher than 200 MPa. Hence, we consider that STPP and NaCl favored the denaturation of some sarcoplasmic proteins at 200 MPa. On the other hand, the Td of sarcoplasmic proteins coincided with that of myosin rod for 1% NaCl and 0.25% STPP + 1% NaCl-patties, so the contribution of myosin rod cannot be discarded.

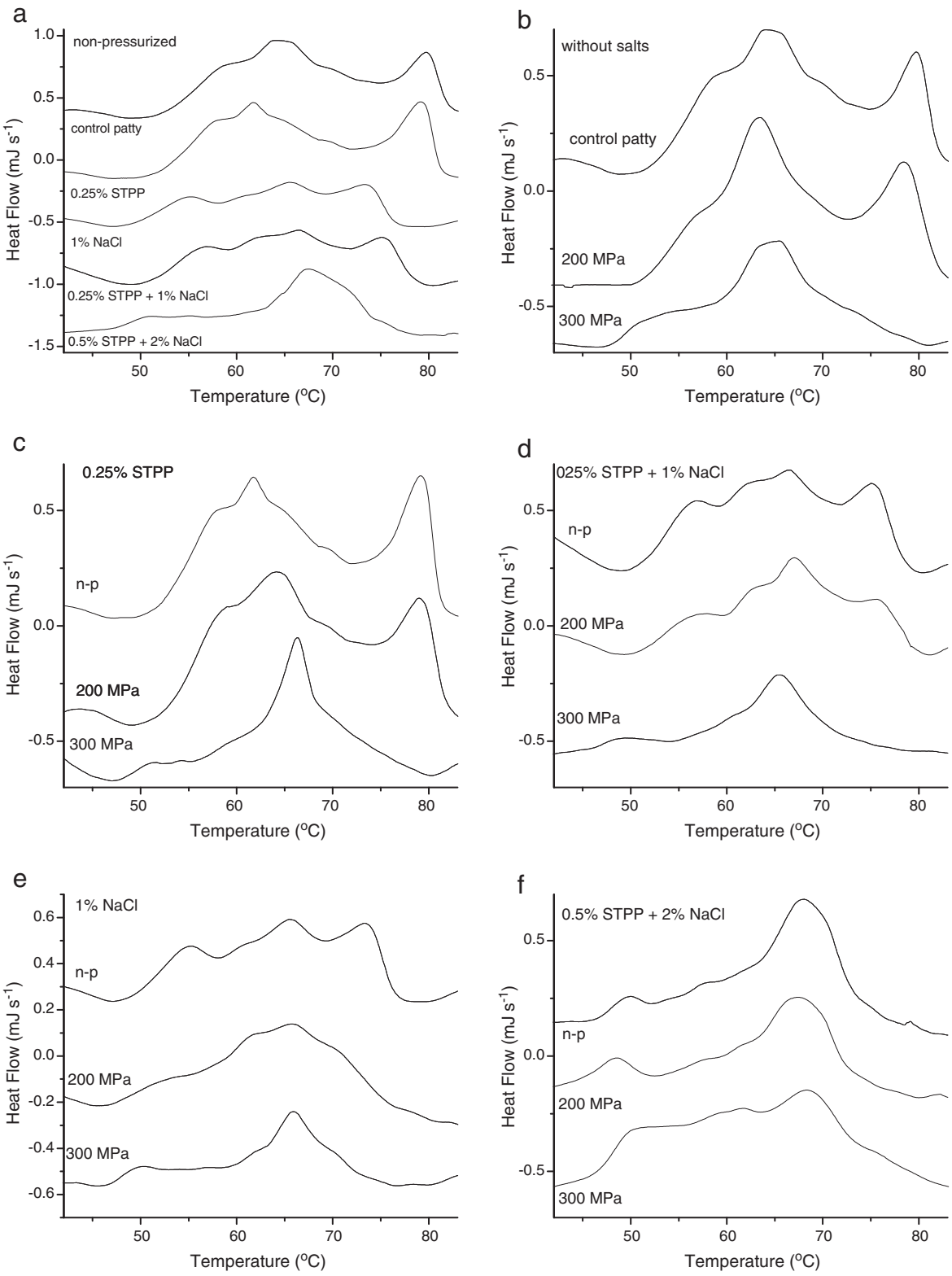

Fig. 2. Thermograms of beef patties formulated with different STPP and/or NaCl concentrations in non-pressurized (n-p), 200 or 300 MPa patties. Non-pressurized (a), non-pressurized or HHP-treated patties: without salts (b), 0.25% STPP-added (c), 1% NaCl-added (d), 0.25% STPP + 1% NaCl-added (e), and 0.5% STPP + 2% NaCl-added (f).

3.1.3. Effects of salts in patties treated at 300 MPa

The ΔH of total proteins of 300 MPa patties depended on the presence of salts (Table 2). The denaturation degrees were 66% for 0.25% STPP and 72% for 0.25% STPP + 1% NaCl-patties, which were higher than those obtained for patties without salts (37%) or with 1% NaCl (47%). The ΔH of 0.5% STPP + 2% NaCl-patties was higher after than before HHP treatment at 300 MPa (Table 2). This fact mainly occurred because of an increase of the ΔH of the transition observed at low temperature (50.5 ± 0.4 °C, Fig. 2f and Table 1) attributed to a salt-induced structure. It is noteworthy that no protective effect of 0.25%

Table 2
Denaturation enthalpies (J g^{-1} protein) for total proteins of beef patties formulated with different STPP and/or NaCl concentrations and treated at different pressure levels for 5 min.

Pressure level (MPa)	Without salts	0.25% STPP	1% NaCl	0.25% STPP + 1% NaCl	0.50% STPP + 2% NaCl
non-pressurized	10.83 ± 0.95^{ab}	12.53 ± 0.62^{a}	8.01 ± 0.51^{cdef}	9.03 ± 0.30^{bcde}	6.25 ± 0.15^{fg}
200	10.11 ± 0.73^{abcd}	10.42 ± 0.40^{abc}	5.13 ± 0.30^{fg}	6.82 ± 0.17^{efg}	6.33 ± 0.35^{fg}
300	6.82 ± 0.58^{defg}	4.30 ± 0.56^{fgh}	4.24 ± 0.30^{gh}	2.50 ± 0.39^{h}	7.42 ± 0.90^{cdefg}

STPP: sodium tripolyphosphate; NaCl: sodium chloride.
$^{a–h}$Means values with different letters are significantly different ($p < 0.05$).

STPP was observed, but the opposite effect seemed to occur. The effect of STPP detected at 200 MPa may depend on the presence of specific binding sites that were destroyed at 300 MPa (by a differential unfolding due to the higher pressure level). Thus, the overall effect at 300 MPa mainly depended on the concentration of salts but not on the type of ions or specific interactions. Villamonte et al. (2013) also found in pork batters, treated at 350 MPa, that the denaturation degree was higher in the presence of 0.25% STPP + 1.5% NaCl than in the presence of 1.5% NaCl alone (52% vs. 39%). In this study, the application of 300 MPa induced the appearance of a transition at low temperature (ca. 50 °C), whose area was higher for 0.5% STPP + 2% NaCl-patties. Villamonte et al. (2013) reported the formation of a similar structure and suggested that it was made up from myofibrillar proteins. These proteins may form aggregates stabilized by hydrogen bonds after the extraction by salts and HHP. Several authors proposed the formation of hydrogen bond-stabilized aggregates after HHP treatment in proteins from different sources, which were disassembled at low temperatures (Angsupanich, Edde, & Ledward, 1998; Boonyaratanakornkit, Park, & Clark, 2002; Ma & Ledward, 2004; Speroni, Jung, & De Lamballerie, 2010). The protein structures that resisted the treatment at 300 MPa seemed to correspond mainly to collagen (Fig. 2b–f). This finding is in agreement with the results of Sikes et al. (2009) and Villamonte et al. (2013). Collagen Td decreased in patties without salts or with 0.25% STPP (ΔTd = 3.6 and 3.8 °C, respectively, peak 5, Table 1) after 300 MPa. Potekhin, Senin, Abdurakhmanov, and Tiktopulo (2009) found that the change of volume (ΔV) of pork-skin collagen upon denaturation was a function of pressure level: ΔV was positive up to 324 ± 20 MPa whereas ΔV was negative at higher pressures. Since the increase in pressure level favors phenomena accompanied by negative ΔVs, those authors concluded that HHP favored collagen denaturation at pressure levels higher than 324 MPa.

3.2. Molecular characterization of proteins from patties

Fig. 3 shows the electrophoretic profiles of proteins solubilized with different buffers from control patties or 300 MPa-patties formulated with different STPP and/or NaCl concentrations. Extractions with different buffers are useful to analyze solubility, aggregate formation and forces that stabilize the HHP-induced aggregates.

Sarcoplasmic proteins, which were extracted with B0.1, exhibited similar electrophoretic profiles for non-pressurized and 200 MPa-patties for all the evaluated formulations. The only exception was the increase of the intensity of a band at 32.5 kDa in patties without salts treated at 200 MPa (data not shown). Electrophoretic profile of patties without salts treated at 300 MPa showed the increase in the intensity of 35 and 21–22 kDa bands and the decrease in the intensity of 48, 31.5 and 28.5 kDa ones (Fig. 3a). Marcos et al. (2010) reported similar results on beef treated at 200 and 400 MPa. The intensities of the bands at 48 and 31.5 kDa showed no decrease in 300 MPa-patties added with 0.25% STPP, 1% NaCl or 0.25% STPP + 1% NaCl. Therefore, no HHP-induced aggregation of these polypeptides may occur in the presence of salts.

Profiles of myofibrillar proteins extracted with B0.6 showed no differences among different patty formulations in non-pressurized or 200 MPa-patties (data not shown). The NaCl concentration in B0.6 was high enough to extract myofibrillar proteins, and may have canceled the effects on solubilization achieved by the different NaCl and/or STPP concentrations added to patties. The identified bands were attributed to myosin heavy chain, C-protein, actin, tropomyosin, T-, I- and C-troponins and light myosin chains (Fig. 3b). Since electrophoretic profiles showed no differences, whereas denaturation degrees did showed differences between non-pressurized and 200 MPa-patties, we conclude that 200 MPa-induced denaturation led to no formation of aggregates. It is also possible that the treatment at 200 MPa induced the formation of aggregates, stabilized by weak interactions, which were disassembled by B0.6. Profiles of proteins extracted with B0.6 from 300 MPa-patties without salts presented a noticeable decrease in the intensity of myosin heavy chain, C-protein, actin and T- and C-troponin bands. This result indicates that those myofibrillar proteins were involved in insoluble aggregates. Besides, the degree of aggregation of these proteins was lesser in 0.25% STPP- or 1% NaCl-patties. Thus, the presence of STPP or NaCl avoided their HHP-induced aggregation. The application of 300 MPa in the presence of both STTP and NaCl led to aggregation of myosin heavy chain, T- and C-troponin, but minimized aggregation of actin. These findings indicate that the type of polypeptides present in HHP-induced aggregates strongly depended on patty formulation. No changes in light myosin chain bands were observed in 300 MPa-patties, regardless of formulation. Therefore, we suggest that the mechanism of myosin aggregation involved the dissociation of heavy chains from light chains, and then heavy chains formed aggregates. However, the aggregation of myosin light chains with myosin heavy chains – or other proteins – through bonds that were disrupted by extracting with B0.6 should not be discarded.

Beef proteins extracted by B0.6-SDS presented no differences among formulations in non-pressurized or 200 MPa-patties (data not shown). Profiles were similar to those obtained with B0.6, but the intensity of

Table 3
Denaturation enthalpy of myosin head and actin (J g^{-1} protein) from beef patties formulated with different STPP and/or NaCl concentrations and treated at different pressure levels for 5 min.

	Pressure level (MPa)	Without salts	0.25% STPP	1% NaCl	0.25% STPP + 1% NaCl	0.50% STPP + 2% NaCl
Myosin head	Non-pressurized	2.74 ± 0.31^{b}	3.89 ± 0.25^{a}	2.21 ± 0.31^{bc}	2.23 ± 0.13^{bc}	0.26 ± 0.04^{e}
	200	1.34 ± 0.13^{cde}	3.03 ± 0.27^{ab}	0.68 ± 0.10^{de}	1.67 ± 0.22^{cd}	0.26 ± 0.10^{e}
	300	0.61 ± 0.19^{de}	0.41 ± 0.05^{de}	0.30 ± 0.06^{de}	0.09 ± 0.04^{e}	0.53 ± 0.12^{de}
Actin	Non-pressurized	2.58 ± 0.24^{ab}	2.80 ± 0.22^{a}	1.81 ± 0.13^{bc}	2.17 ± 0.10^{abc}	ND
	200	2.47 ± 0.29^{abc}	2.35 ± 0.12^{abc}	0.46 ± 0.05^{d}	1.58 ± 0.13^{c}	ND
	300	0.20 ± 0.01^{d}	0.14 ± 0.05^{d}	0.10 ± 0.01^{d}	0.05 ± 0.01^{d}	ND

STPP: sodium tripolyphosphate; NaCl: sodium chloride. ND: No determination was possible because of peak overlapping.
$^{a–e}$Means values with different letters are significantly different ($p < 0.05$).

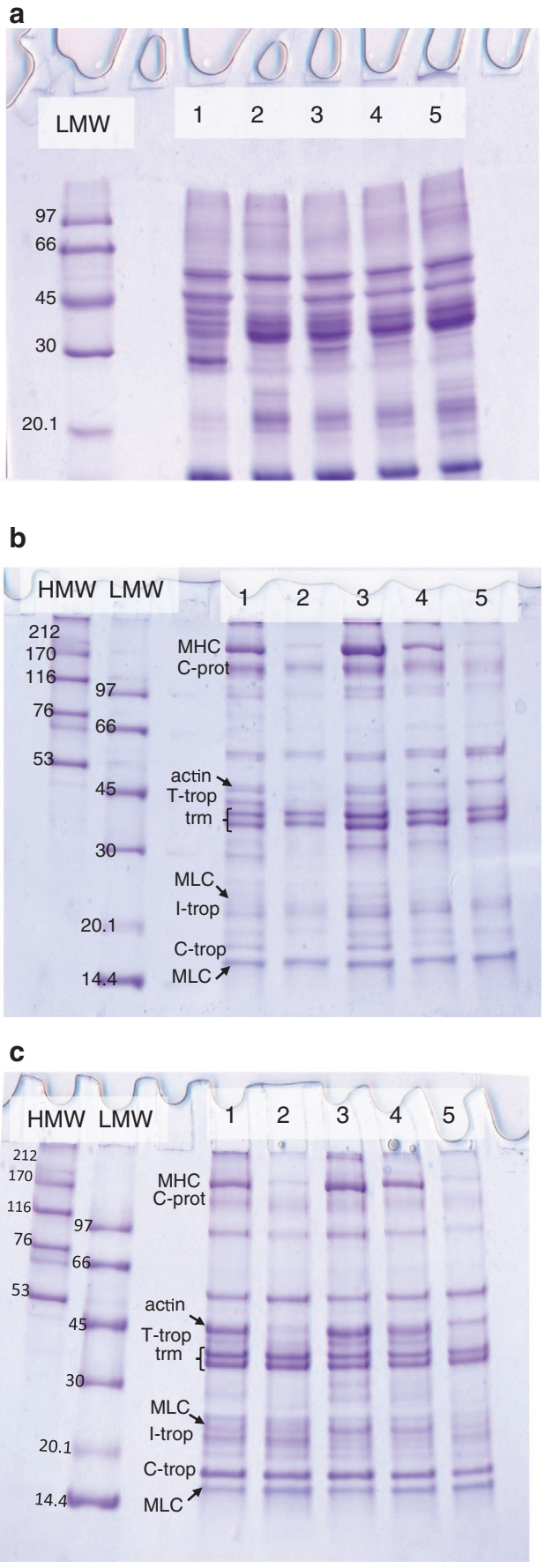

Fig. 3. Electrophoretic profiles of proteins extracted with B0.1 (a), B0.6 (b) and B0.6 + SDS (c) from beef patties formulated with different STPP and/or NaCl concentrations in control or 300 MPa patties. Lane 1: control patty; lane 2: without salts at 300 MPa; lane 3: 0.25% STPP at 300 MPa; lane 4: 1% NaCl at 300 MPa and lane 5: 0.25% STPP + 1% NaCl at 300 MPa. LMW: low molecular weight markers, HMW: high molecular weight markers. MHC: myosin heavy chain, C-prot: C-protein, T-trop: T-troponin, trm: tropomyosin, MLH: myosin light chain, I-trop: I-troponin, C-trop: C-troponin.

all bands was higher, mainly C-troponin and actin (Fig. 3c). In 300 MPa-patties without salts, the presence of SDS scarcely solubilized myosin heavy chain and actin. However, these proteins were released from the matrix in 0.25% STPP or 1% NaCl-patties. As observed with B0.6 extraction, actin was lesser involved in the formation of insoluble aggregates in the presence of 0.25% STPP + 1% NaCl than in 300 MPa-patties without salts. C-troponin band was more intense, regardless of formulation, so the aggregates in which it was involved at 300 MPa were disassembled by SDS. These findings reinforce the idea that salts modified HHP-induced aggregation and suggest that HHP-induced aggregates were stabilized by hydrogen bonds and hydrophobic interactions.

The aggregative behavior of myofibrillar and sarcoplasmic proteins showed noticeable differences in 200 or 300 MPa-patties. The presence of salts decreased the aggregation of both sarcoplasmic and myofibrillar proteins and/or decreased the strength of bonds that stabilized the aggregates. These effects were more noticeable in myofibrillar proteins than in sarcoplasmic ones.

Our results suggest that HHP induced the formation of different aggregates from myofibrillar proteins. Differences depended on whether proteins were still forming myofibrils (resulting in insoluble aggregates) or whether they had been already extracted by STPP and/or NaCl (resulting in aggregates that may be solubilized and/or disassembled by B0.6). Since these soluble aggregates were disassembled by a high ionic strength buffer and some of them denatured at low temperature (51.4 ± 0.5 °C), we hypothesize that they were stabilized by hydrogen bonds and/or electrostatic interactions.

4. Conclusions

The evaluated treatments caused important changes in thermal and aggregative properties of myofibrillar proteins. The effects of NaCl and STPP on thermal behavior are a result of a combination of mechanisms which depend on type of salt (kosmotropic–chaotropic effect), concentration (ionic strength), and specific interactions with active sites. We conclude that the obtained NaCl-soluble and STPP-soluble proteins were different, while NaCl alone (1%) caused the solubilization of proteins which resulted partially denatured; STPP alone (0.25%) caused the solubilization of proteins which remained in a native state. Pressure levels play an important role in protein denaturation since important changes were observed between 200 and 300 MPa, e.g. STPP protected against denaturation (at 200 MPa) or favored denaturation (at 300 MPa). HHP induced the formation of different types of aggregates which depend on whether proteins were still forming myofibrils (resulting in insoluble aggregates) or whether they had been already extracted by STPP and/or NaCl (soluble aggregates). Since these soluble aggregates were disassembled by a high ionic strength buffer and some of them denatured at low temperature (51.4 ± 0.5 °C), they may be stabilized by hydrogen bonds and/or electrostatic interactions.

According to these results, the application of HHP treatments (200 or 300 MPa) to meat products with reduced salt content may cause important changes on their texture and technological properties.

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