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Protease activity and the ultrastructure of broiler chicken PSE (pale, soft, exudative) meat

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ABSTRACT

The biological cause of broiler PSE meat seems to be an excessive release of Ca^{2+}, promoted by a genetic mutation of ryanodine receptors located in the sarcoplasmic reticulum of skeletal muscle cells. Excessive Ca^{2+}, associated with protein denaturation in meat, enhances protease activity and influences the functional properties of PSE meat. Twenty-four-hour *post-mortem Pectoralis major* m. samples exhibited lower values for pH, water-holding capacity, and shear force than did control samples, in contrast to colour (L^*) and cooking loss values. Protease activity, measured as myofibril fragmentation index, presented higher values in PSE meat than in control samples. Ultrastructural examination revealed shrinking and depolymerisation of myofilaments and Z-lines disorganisation within the sarcomere in PSE meat. Intense calpain activity was also observed, indicating that the process may initiate at the filaments, because of protein denaturation, and spread through Z-lines, resulting in the collapse of the sarcomere structure.

1. Introduction

The causes and consequences of broiler breast PSE meat have recently been the subject of experimental studies by several research groups (Barbut et al., 2008; Olivo & Shimokomaki, 2006; Swatland, 2008). The addition of vitamin E to the birds' diet successfully reduced the formation of PSE meat (Olivo, Soares, Ida, & Shimokomaki, 2001) and increased phospholipase A2 activity (Soares et al., 2003), which may enhance lipid oxidation in PSE meat (Soares et al., 2009). Showering just before slaughtering at processing plants calms the birds and contributes to Ca^{2+} homoeostasis (Guarnieri et al., 2004). Transportation conditions between the farm and abattoir can influence the formation of PSE meat (Langer, Soares, Rossa, Shimokomaki, & Ida, 2008; Mitchell & Kettlewell, 1998). Ultrastructural studies have revealed that a robust sarcomere shrinking within the muscle fibrils takes place in PSE (Barbut, Zhang, & Marcone, 2005; Guarnieri et al., 2004). There is growing evidence that the exposure of birds to thermal stress just before sacrifice is one of the causes of PSE meat (Barbut, 1998; Langer et al., accepted for publication; Mitchell & Kettlewell, 1998).

Protease activities have been shown to affect tenderness during meat maturation and the calpain system is known to initiate enzymatic digestion in the myofibrillar framework (Goll, Boehm, Geesink, & Thompson, 1997). In fact, Lee, Santé-Lhoutellier, Vigouroux, Briand, and Briand (2008) reported that calpain enzymatic activities in broilers and mammals are somehow different since, in the former, it takes 6 h for μ-calpain activity to start developing tender meat. Under commercial conditions, 24 h at 2 ± 2 °C are necessary for complete broiler meat maturation (Kriese et al., 2007), while mammal meat requires over 24 h (Wheeler & Koohmaraie, 1994). The present work investigated the protease activities in PSE broiler breast meat by means of tenderness, biochemical properties, and ultrastructural profiles.

2. Materials and methods

2.1. Sample preparation

Fillet meat (*Pectoralis major* m.) samples were obtained from 42-day old broilers of either sex from a commercial plant located in south Brazil. The animals were slaughtered according to the standard industry practice, essentially consisting of electrical stunning, bleeding, defeathering (for birds), evisceration, water-cooling the carcass, deboning, and refrigeration (Maganhini et al., 2007; Northcutt, 2001). The time from slaughtering to sample collection was about 1.5 h.

2.2. Biochemical and physicochemical parameters

2.2.1. pH and colour measurements

pH was measured by inserting electrodes into the meat samples (Boulianne & King, 1995; Maganhini et al., 2007), using a contact

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pH meter system (Testo 205). Analyses were performed in triplicate at 1.5 h *post-mortem*, as reported by Olivo et al. (2001). A Minolta CR400 colorimeter was used to evaluate the colour and L^* (lightness) of the posterior surface of intact skinless breast muscles at 24 h *post-mortem*. The L^* values were measured at three different sites on the same sample: the proximal extremity of the muscle, the distal extremity of the muscle and the medial side halfway between the proximal and distal extremities (Soares et al., 2003). The samples were kept frozen at −22 °C prior to further analyses. A similar technique was used for pork, applying the methodology of Warris and Brown (1987). The meat samples for histological evaluation were stored in a refrigerator and were collected at 0 and 72 h. Thirty samples were classified as control at initial $pH_{1.5}$ (>pH 6.0) and twenty samples as PSE at $pH_{1.5}$ (<6.0 pH).

2.2.2. Myofibril fragmentation index (MFI)

MFI was determined as the indirect measurement of calpain activity, according to Culler, Parrish, Smith, and Cross (1978). Four-gramme muscle samples, free of external fat and visible connective tissue, were homogenised for 30 s in 20 ml of MFI buffer (100 mM KCl, 20 mM potassium phosphate, 1 mM EDTA, 1 mM $MgCl_2$, and 1 mM NaN_3) at pH 7.0. The homogenate was centrifuged at $10 \times 10^3 g$ for 15 min at 2 °C. The supernatant was discarded and the pellet was resuspended in 20 ml of the MFI buffer and centrifuged at 1000*g* for 15 min at 2 °C. The supernatant was discarded and the pellet was suspended in 10 ml of MFI buffer. The myofibril suspension was strained to remove connective tissue and assayed for protein concentration using the Biuret method. Suspension aliquots were diluted in MFI buffer to a final protein concentration of 0.5 mg/ml and poured into a cuvette for immediate absorbance measurement at 540 nm with a spectrophotometer (Cintra 20). MFI was expressed as $A_{540\ nm} \times 200$.

2.2.3. Texture measurement

Tenderness was evaluated by measuring shear force, as described by Honikel (1998). The samples were packed in plastic bags and cooked in a water bath until the internal temperature was 75 °C. After refrigeration at 2 ± 2 °C for 12 h, the samples were cut into 1 cm^3 pieces, and analysed on a texturometer TATX-2i. The results were expressed in Newtons.

2.2.4. Water-holding capacity (WHC)

WHC was determined, based on the technique described by Hamm (1960). Twenty-four-hour *post-mortem* samples were collected from the cranial side of the breast fillets and cut into 2.0-g (±0.10) cubes. The samples were analysed in duplicate. They were first carefully placed between two filter papers and then left under a 10 kg weight for 5 min. The samples were weighed and WHC was determined by the exudated water weight via the following formula: $100 - [(W_i - W_f/W_i) \times 100]$, where W_i and W_f are the initial and final sample weights.

2.2.5. Cooking loss (CL)

CL was measured according to Honikel (1998). The samples were weighed (75 ± 5 g) before and after 15 min of cooking, which was the time required for the internal temperature to reach 75 °C.

2.3. Histological evaluation

2.3.1. Scanning electron microscopy

Histological evaluation by electron microscopy was described by Guarnieri et al. (2004). *Post-mortem* samples were fixed in 2% glutaraldehyde in a 0.14 M sodium cacodylate buffer at pH 7.4, containing 0.18 M sucrose. After washing in phosphate buffer, the samples were post-fixed in 1% osmium tetroxide in phosphate buffer for 2 h, followed by dehydration in acetone, and embedding in Araldite resin. Ultrathin sections (50 nm) were stained with saturated uranyl acetate in 50% ethanol and lead citrate for 1 h. The sample ultrastructure was observed with a JEOL JEM-1010 scanning electron microscope.

2.4. Statistical analysis

Statistical analysis was carried out using Statistica software, version 6.0 (Oklahoma, 2001). The Tukey test was used in the analysis between control and experimental samples, which were classified for PSE in relation to pH, colour, WHC, SF, and MFI. Correlation coefficients between these variables were generated using the Pearson correlation coefficient.

3. Results and discussion

3.1. Biochemical parameters

Table 1 shows the values of the differences in biochemical and physicochemical parameters between the control and PSE broiler breast meat samples: pH values of samples at 1.5 h and 24 h *post-mortem*, and only at 24 h *post-mortem* for WHC, CL, MFI, and SF. The pH and L^* values are significantly different and typical for control and PSE meats, as previously reported (Barbut, 1997; Olivo et al., 2001; Van Laack, Liu, Smith, & Loveday, 2000). While the control sample pH decreased gradually throughout the *post-mortem* duration, the pH in PSE meat samples did not decrease significantly ($p = 0.32$), due to the rapid pH decrease at the initial *rigor mortis* stage, which means that the residual muscle glycogen was depleted. L^* values, for both control and PSE meat samples, agree with previously observed values for WHC ($p \leqslant 0.01$) and CL ($p \leqslant 0.05$), due to myofibril protein denaturation, as originally discussed in Guarnieri et al. (2004) and Olivo et al. (2001).

Table 1 also shows the MFI values, which indirectly measure protease activity and can be correlated with the meat SF. Proteolysis is significantly higher in PSE samples (11.0%) than in control samples, giving rise to about 25.0% greater tenderness in PSE samples.

As reported elsewhere (Kriese et al., 2007), there is a significant negative correlation ($R = -0.42$, $p \leqslant 0.05$) between MFI and SF values in refrigerated samples, suggesting that, as protease activity increases, breast meat becomes more tender. The calpain enzyme system digested the sarcomere structure (Guarnieri et al., 2004; Kriese et al., 2007), improving meat tenderness in both control

Table 1
Comparison of biochemical and physicochemical parameters between control and PSE broiler breast meat (*Pectoralis major* m.) samples.

Parameters	Control ($n = 30$)	PSE ($n = 20$)
$pH_{1.5}$	6.49^a	5.81^b
pH_{24}	5.89^a	5.77^b
L^*	52.00^a	57.37^b
WHC (%)	69.12^a	65.66^b
CL (%)	24.01^A	25.59^B
MFI	103.86^a	117.40^b
SF (N)	58.09^A	43.46^B

$pH_{1.5}$: pH 1.5 h; pH_{24}: pH 24 h, L^*: colour, WHC: water-holding capacity, CL: cooking loss, MFI: myofibril fragment index.
$pH_{1.5}$ and pH_{24} pH values measured 1 h 30 min and 24 h *post-mortem*, respectively. WHC = water-holding capacity, CL = cooking loss, MFI = myofibrillar fragmentation index, SF = shear force, N = Newton.
[a–b] Means within each line with different superscripts are significantly different ($p \leqslant 0.01$).
[A–B] Means within each line with different superscripts are significantly different ($p \leqslant 0.05$).

and PSE samples. Increased protease activity in PSE samples might be due to the high concentration of intracellular Ca^{2+}. A concentration of 10 μm would be sufficient to enhance the activity of the endogenous muscle proteases system and, in fact, it was previously demonstrated that the Ca^{2+} concentration is about 1.4–1.8 times higher in PSE samples throughout the broiler life span (Soares et al., 2003). This higher ion concentration should primarily enhance the μ-calpain activity, at least in the first 6 h *post-mortem* when, according to Lee et al. (2008), the activity of this enzyme ceases, due to autolysis. The action of another calpain system component, μ/m, begins (Lee et al., 2008) and further proteasomal activity takes place during the meat maturation process (Lee et al., 2008).

Correlations between the different measurements, such as $pH_{1.5}$, pH_{24}, L^*, CRA, PPC, MFI, and FC, are listed in Table 2. A significant negative Pearson correlation is observed between $pH_{1.5}$ and CL (−0.35, $p < 0.05$) and L^* (−0.57, $p < 0.01$), and a positive correlation to CRA (0.66, $p < 0.01$), indicating that, the lower the $pH_{1.5}$, the higher is the impairment of the functional properties of the meat protein. This behaviour is also observed for L^* values in relation to CL (0.42, $p < 0.01$) and WHC (−0.49, $p < 0.01$), suggesting that, as meat becomes paler, the CL increases, while the WHC decreases. Finally, MFI values correlated positively with L^* (0.53, $p < 0.01$) and PPC (0.39, $p < 0.05$), and negatively with WHC (−0.45, $p < 0.01$) and SF (−0.42, $p < 0.05$), indicating that the higher is the proteolysis, the higher the impairment of the functional properties of meat.

Table 2
Pearson correlation between the various chicken *P. major* m. biochemical and physicochemical parameters 24 h *post-mortem*.

	FC	MFI	PPC	CRA	L^*	pH_{24}
$pH_{1.5}$	0.19	−0.16	−0.35*	0.66**	−0.57**	0.50**
pH_{24}	−0.33	−0.06	−0.29*	0.29*	−0.31*	
L^*	−0.35*	0.53**	0.42**	−0.49**		
CRA	0.25	−0.45**	−0.48**			
PPC	−0.04	0.39*				
MFI	−0.42*					

* $p < 0.05$.
** $p < 0.01$.

3.2. Histological evaluation

3.2.1. Scanning electron microscopy

Fig. 1 A1 shows micrographs from the first *post-mortem* hours of the control samples. The myofilament structure is normal, without any apparent destructive changes. Bands I and A, zone H and lines Z and M are evident. However, at 72 h *post-mortem*, this pattern is somewhat altered and, apparently, the myofibril sarcomere is depolymerised and some lacunas are evident within the bands, indicating protein fragmentation (Fig. 1 A2). Conversely, in Fig. 1 B1, PSE samples show complete sarcomere disorganisation, the typical dark and light pattern is not evident, and the Z-lines seem more pronounced. Myosin filaments predominate within sarcomeres, even touching the Z-lines. Open spaces are visible and muscle contraction is evident, drawing some sarcomere components towards the Z-lines, which increases their density, as described by Guarnieri et al. (2004). However, by comparing Fig. 1 A1–A2, the calpain systems seem to initiate their activity in PSE samples much earlier than in the control samples. This can be explained by an increase in the concentration of Ca^{2+} in the PSE samples, probably even before slaughtering. This hypothesis is supported by consistently lower pH values with respect to control samples (Soares et al., 2003). In fact, the excess Ca^{2+} within the tissue at this stage enhances the μ-calpain activity prematurely in birds when compared to mammals. In mammals, a certain amount of time is necessary to initiate the maturation assembly, presumably because the Ca^{2+} concentration is not high enough to induce muscle catabolism (Lee et al., 2008). It is fair to point out that there is probably a specific relationship between the initiation of the enzyme activity

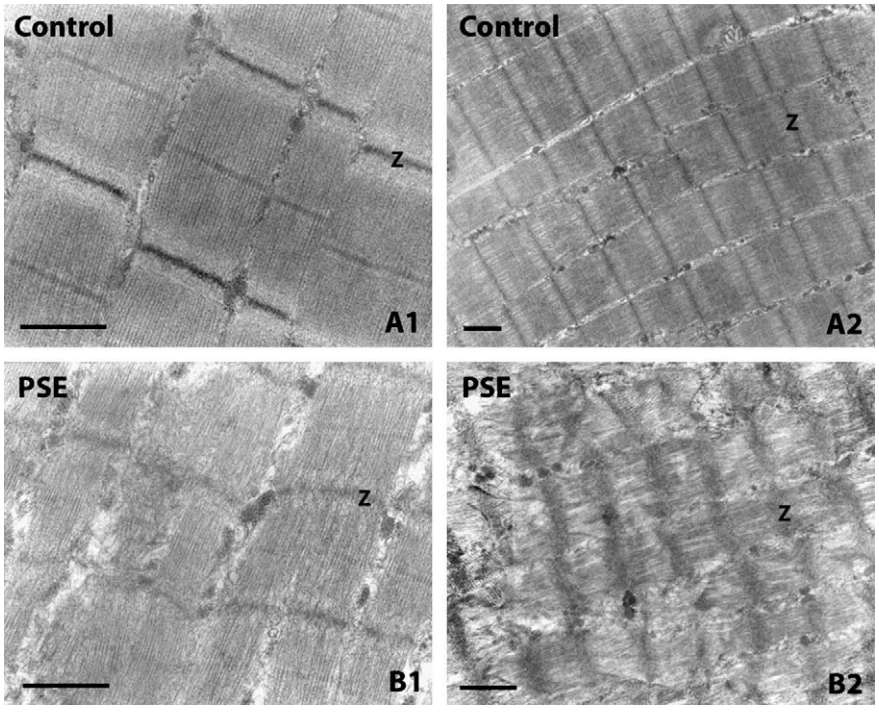

Fig. 1. Longitudinal electron micrograph of *Pectoralis major* m. from control group 1.5 h (A1) and 72 h (A2) *post-mortem* and PSE group 1.5 h (B1) and 72 h (B2) *post-mortem*. Note a disorganisation of A and I bands in (B1 and B2). The M bands disappeared and a super contraction of the Z (Z) line is evident. Magnifications: A1 25,000×, A2 10,000×, B1 25,000× and B2 15,000×. Bar = 700 nm.

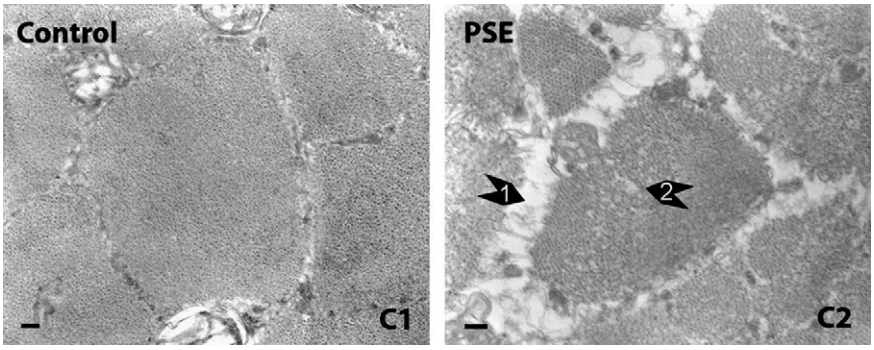

Fig. 2. Electron micrograph of a transverse section of 72 h *post-mortem* broiler *Pectoralis major* m. Control sample (C1) and PSE meat sample (C2). Arrow 1: endomysium. Arrow 2: intracellular space. Magnifications: C1 20,000× and C2 25,000×. Bar = 200 nm.

and animal species. It is clear that, of all the proteases present in skeletal muscle tissue, μ-calpain is the only enzyme capable of acting on myofibril proteins since, at physiological pH, the lysosome enzymes are inactive. Furthermore, proteasomes cannot digest myofibril components, since they need to be unfolded (Koohmaraie & Geesink, 2006). Thus, it is the calpain system that acts first to degrade the sarcomere entities.

Fig. 2 shows transversal sections of 24 h *post-mortem* Control (C1) and PSE (C2) samples. Open spaces are clearly seen in the endomysial network (arrow 1); faint collagen fibres can be noticed in PSE samples. Open spaces are observable, both intramuscularly and within the endomysium. Muscle cell shrinking (about 10.0%) occurs due to the low pH and relatively high temperature conditions, which denature muscle proteins and impair their functional properties, thus preventing intra- and intermuscular water retention. Simultaneously, the higher protease activities might render these proteins further non-functional and synergetically promote an increased loss of water. It is reasonable to believe that, in this region, the water starts to move from the intracellular compartment to the endomysium (Fig. 2 C2), and finally to the surface of the meat. Obviously, control samples present muscle fibres with entirely filled endomysial networks with noticeable collagen fibres.

4. Conclusions

Excessive Ca^{2+} concentration during the installation of broiler PSE meat promotes high protease activity, possibly before the broiler slaughtering process, affecting the integrity of the muscle fibre structure and thus impairing the meat protein functionality.

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