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Research Article

A specificity of microstructural and biochemical changes during ripening of dark, firm and dry sheep meat

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Abstract

The objective of this study was to recognize the specificity of microstructural changes of DFD sheep m. *Longissimus dorsi* during its ripening, and to try to explain them by biochemical and physicochemical amendments of meat. The fresh (4 h *post mortem*) DFD sheep m. *Longissimus dorsi* with pH_1 = 6.95 stored at 2 ± 2°C was used. The microstructure, degree of proteolysis, color properties, water holding capacity and pH of DFD sheep meat were evaluated during 5d of storage. The shortenings of the sarcomeres and typical alternation of light and dark bands were established after 48h storage of DFD sheep muscles. Simultaneously, pH and water holding capacity decreased with 16.5% and 50.6% resp. ($p \leq 0.05$), accompanied with the lowest protein solubility, and a disappearance of myosin heavy chains. On the 3d *post mortem* sarcomeres were partly regenerated and their native structure was recovered, A- and I-disks were visible, and Z-lines were undamaged. On the 4d *post mortem* was found large gap formation in sarcomeres and difficulty recognizable A- and I-disks. On the 5d *post mortem* the pH and water holding capacity were similar ($p > 0.05$) with the initial values but free amino nitrogen decreased with 34.5%.

Keywords: DFD sheep, microstructure, SDS-PAGE, soluble proteins, water holding capacity

Abbreviations:
CIE - International Commission of Illumination;
pH - potential of hydrogen - the negative of the base 10 logarithm of the activity of the hydrogen ion;
SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis;
SAS - Statistical Analysis System;
UV-VIS – ultraviolet-visible spectroscopy

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Introduction

Some research has been done to highlight post mortem changes which lead to conversion from muscle tissue to "meat" (Bond and Warner 2007; Hwang et al. 2003; Chen et al. 2011; Pearce et al. 2011; Veiseth et al. 2004). Those post mortal amendments is basically associated with the glycolysis process (Huff-Lonergan and Lonergan 2005). As a result the pH, and the water holding capacity of meat decrease, and the muscle tissue constrict because of the actomyosin formation. A phase of the so-called *"rigor mortis"* develops (Pearce et al. 2011). With the beginning of meat aging the proteolysis is activated, and the pH and water holding capacity increases again. As a result of the relation between water mobility and meat structure (Bertram et al. 2002) the muscle tissue ability to retain the water is restored. The actomyosin complex dissociates, the myofibrilllar structure weakens again and the meat tenderness increases (Huff-Lonergan and Lonergan 2005). However, in the literature there can be found numerous studies focused on the *post mortem* tenderization of sheep meat, about its post-exsanguination (Farouk et al. 1992), and calcium ions infusion (Polidori et al. 2000), factors contributing to proteolysis and disruption of myofibrillar proteins (Hopkins and Thompson 2002), and the use of biophysical methods related to the meat structure (Damez and Clerjon 2008). The duration of the maturation phase depends of many factors: animal breed, gender, age, feeding regime and processing conditions such as carcass suspension and electrical stimulation (Geay et al. 2004; Vierira et al. 2007). Another important factor influencing the meat quality is stress (Braggins, 1996; Ferguson and Warner 2008 and the dark, firm and dry meat, associated with it, especially in older cows and sheep at the end of their fertile period (Miranda de la Lama et al. 2009). The quality of the edibility of the meat is connected to their microstructure (Shimokomaki et al. 2012).

There is not enough information on the microstructural changes during DFD sheep aging. Considering all these aspects, the objective of this study was to specify the microstructural changes in DFD sheep *m. Longissimus dorsi* during its maturation and to try to explain them in the light of biochemical and physicochemical changes in meat.

Materials and Methods

Sheep meat. 21 three years old females from Synthetic population of Bulgarian dairy sheep being reared on pasture in 2014 were used to determine DFD meat quality characteristics. Sheep with a live weight of about 35 kg were used for slaughter. The sheep were killed after having been hungry but being given water for 21h after one hour of transport.

The fresh (4 h *post mortem*) sheep *m. Longissimus dorsi* with pH = 6.95 were supplied by the company Untempt Ltd, village of Voyvodinovo, District of Plovdiv, Bulgaria, where the slaughter of the sheep had taken place. The fresh sheep *m. Longissimus dorsi* was measured immediately after meat processing (sample K0, 4 h *post mortem*). The sheep *m. Longissimus dorsi* was chilled to 4ºC in the center and stored for 5 d at 0-4ºC. The samples (*m. Longissimus dorsi*) were measured on the first (sample K1), second (sample K2), third (sample K3), forth (sample K4) and fifth (sample K5) day of the experiment.

Transmission electron microscopy (TEM). Samples for the microstructural observations were done as described by Kołczak et al. (2003). The samples were observed through a JEM-1200EX/ASID transmission electron microscope (Jeol, Japan) at 12000x and photographed on film that was scanned at 600dpi on a flatbed transparency scanner.

Extraction of myofibrillar proteins. The extraction was carried out with PBS buffer (49 mM $Na_2HPO_4.7H_2O$; 4.5 mM $NaH_2PO_4.H_2O$ and KCl, ionic strength 0.55), according to the procedure described by Khan (1962) with some modifications. Muscle tissue (2.5g) was homogenized with 48.5cm^3 PBS buffer. After 12h

at 0-4ºC the homogenate was centrofugated at 3000 g for 15min.

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out (Fritz et al. 1989) using 10% gels at a constant voltage mode (200V) in an Omni PAGA Electrophoresis system (Cleaver Scientific Ltd.). Protein markers ware obtained from Precision plus Protein TM Standards (Bio-Rad Laboratories, Inc. Hercules, CA 94547, USA).

Measurement of soluble myofibrillar proteins. The myofibrillar proteins concentration was determined by the method described by Lowry et al. (1951). The absorbance was measured after 30min with double beam UV-VIS spectrophotometer Cam-spec model M550 (Camspec Ltd, Cambridge, UK) at 750nm.

Measurement of free amino nitrogen. The modified titration method of Sorensen was used for determination of free amino nitrogen in samples (Lorenzo et al. 2008). The absorption was measure with double beam UV-VIS spectrophotometer Camspec model M550 (Camspec Ltd, Cambridge, UK) at 570nm.

pH determination. pH of the samples was determined by pH-meter Microsyst MS 2004 (Microsyst, Plovdiv, Bulgaria), equipped by combined pH electrode Sensorex combination recorder S 450 CD (Sensorex pH Electrode Station, Garden Grove, CA, USA) (Young et al. 2004).

Water holding capacity. Water holding capacity was measured according to Modzelewska-Kapitula and Cierach (2009) procedure.

Color properties establishment. Colorimeter Konica Minolta model CR-410 (Konica Minolta Holding, Inc., Ewing, New Jersey, USA) was used to evaluate the lightness (L* value), red component (a* value), and yellow component (b* value) of the color (Hunt et al. 2012). Further, the changes of the color properties of surface cross-sectional views of the samples smoked beef strip loins were held on the 6th day of storage captured in dynamics during the 60 min of air exposure.

Statistical analysis. The data of different samples were analysed independently by SAS software (SAS Institute, Inc. 1990). The Student-Newman-Keuls multiple range test was used to com-pare differences among means. Mean values and standard errors of the mean were reported. Significant differences at $p \leq 0.05$ were defined.

Results and Discussion

Effect of ripening on muscle microstructure

The native structure of myofibrils of DFD sheep muscle *Longissimus dorsi* on 4h *post mortem* (K0) was preserved (Fig. 1). The sarcomeres were clearly identifiable, A-bands and I-bands, H-zones, M-lines and Z -lines were visible, without fragmentations. Myofibrils were with native structure and "garland" form. After 1d *post mortem* at 0-4°C (K2) a certain shortening of the sarcomeres due to ongoing rigor mortis was found (Fig. 1). Individual sarcomeres, A-zones and I-bands were difficult to recognize. The pH, the water holding capacity and the protein solubility (Table 1) of DFD sheep at 48h were minimal and corresponded with the changes observed. After the *rigor mortis* goes away (K3) sarcomeres had partly regenerated their native structure and it was recovered. A- and I-disks were visible, Z-lines were undamaged.

Those ultrastructural changes were connected to the increase of pH, the water holding capacity, the protein solubility and the free amino nitrogen content (Table 1). The sarcomeres with large gap formation and local destructions in sarcolemma were found in DFD sheep at 4d (K4) post mortem (0-4°C). Z-lines were partly disrupted, A- and I-disks were difficult to recognize. Probably the Z-disk fragmentation is the main factor contributing to meat tenderization (Koohmaraie 1994). The disruption between Z-disks obtained on the 4d *post mortem* at 0-4°C (Fig. 1) and the increase of polypeptides with molecular weight of 28-32kDa (Fig.1) confirmed the proteolytic changes during

DFD sheep aging. On the 5d *post mortem* due to deep autolytic changes the sarcomere structure was largely lost. As a result of proteolysis the typical dark and light banding was not visible, the Z-lines were totally disrupted, the H-zones and M-lines had completely disappeared. This deep myofibrillar degradation was confirmed by the data established for the free amino nitrogen content on the 5d *post mortem* (Table 1). Our results are in accordance with those reported by Nagaraj et al. (2005) about the microstructure and the protein changes during the aging of goat meat but differ from those reported for normal sheep meat from Hopkins and Thompson (2002).

Effect of ripening on the muscle tissue proteolysis

On the 4 h *post mortem* (K0) due to relatively high pH (6.95) and high water holding capacity (19.62%) (Table 1) the structure of the muscle tissue was with an open grid (Okeudo and Moss 2005; Bond and Warner 2007) and the solubility of the proteins was high (Table 1). During the next 48h the solubility of proteins ($p < 0.05$) decreases 5.78 times compared to K0. The results correspond with the lowest pH and water holding capacity (K2) and confirmed the course of rigor mortis. After 48 h *post mortem* (K3, K4, K5) under the action of the endogenous tissue enzymes autolytic changes take place in meat (Hopkins and Thompson, 2002) and as a result the solubility of the proteins increased significantly ($p < 0.05$). At the 5 d of DFD sheep storage (0-4°C) the extracted soluble proteins (K4) were not statistically significantly different ($p > 0.05$) from the ones established in K0 (Table 1). During the first 24h *post mortem* at 0-4°C no significant ($p > 0.05$) some changes in the free amino nitrogen content were found (Table 1). A significant decrease by 30.8% in the free amino nitrogen content was observed in sample K2 (2d *post mortem*), probably due to the autolytic changes (Hopkins and Thompson 2002) flowed after *rigor mortis*. At the end of the experiment (K4, K5) the free amino nitrogen content increased with 34.7% and 44.7% respectively ($p < 0.05$) which is an indication of strong proteolysis changes in DFD sheep (Geay et al. 2001). SDS-PAGE electrophoresis (Fig. 2) showed that 200kDa polypeptides – myosin heavy chains (MHC) were identified in the initial period post mortem (K0). Till the end of the experiment (K5) polypeptides with this molecular weight were not found. After the fourth day (K4) of storage at 0-4°C due to *post mortem* aging and proteolysis the amount of 25 to 35 kDa polypeptides increased (Fig. 2). It is known that troponin-T degradation came to polypeptides with molecular mass 28 and 32KDa (Huff-Lonergan and Lonergan 1999). The increase of 25-35 kDa polypeptides (Fig. 2) was also associated to meat tenderization (Koohmaraie and Darrel 1995) and was an indicator for proteolytic changes in DFD sheep (Veiseth et al., 2004; Chen et al. 2011). Similar changes are reported by Newton and Gill (1978) and confirmed in DFD beef (Dransfield 1981).

Effect of aging on the pH and water holding capacity of sheep meat

At the 4h the pH value of DFD sheep (6.95) was very close to neutral (Table 1) and significantly decreased with 16.5% ($p < 0.05$) after the second day of storage. This was an indirect evidence for the *rigor mortis* occurrence. After the third day (K3) of the meat aging the pH of DFD sheep increased ($p < 0.05$) and this trend remains until the end of the experiment (5d storage at 0-4ºC). The changes in the water holding capacity of sheep correspond with the data established for meat pH (Table 1). In comparison to sample K0 (4h) the water holding capacity in samples K1 and K2 decreases with 42.0% and 50.6% resp. ($p < 0.05$). After 96 h the water holding capacity of samples K4 and K5 increased with 9.5% and 10.3% respectively ($p > 0.05$). A close relation between the ultimate pH, the water holding capacity and the tenderness of DFD sheep confirm the findings of Bouton et al. (1971) for mutton.

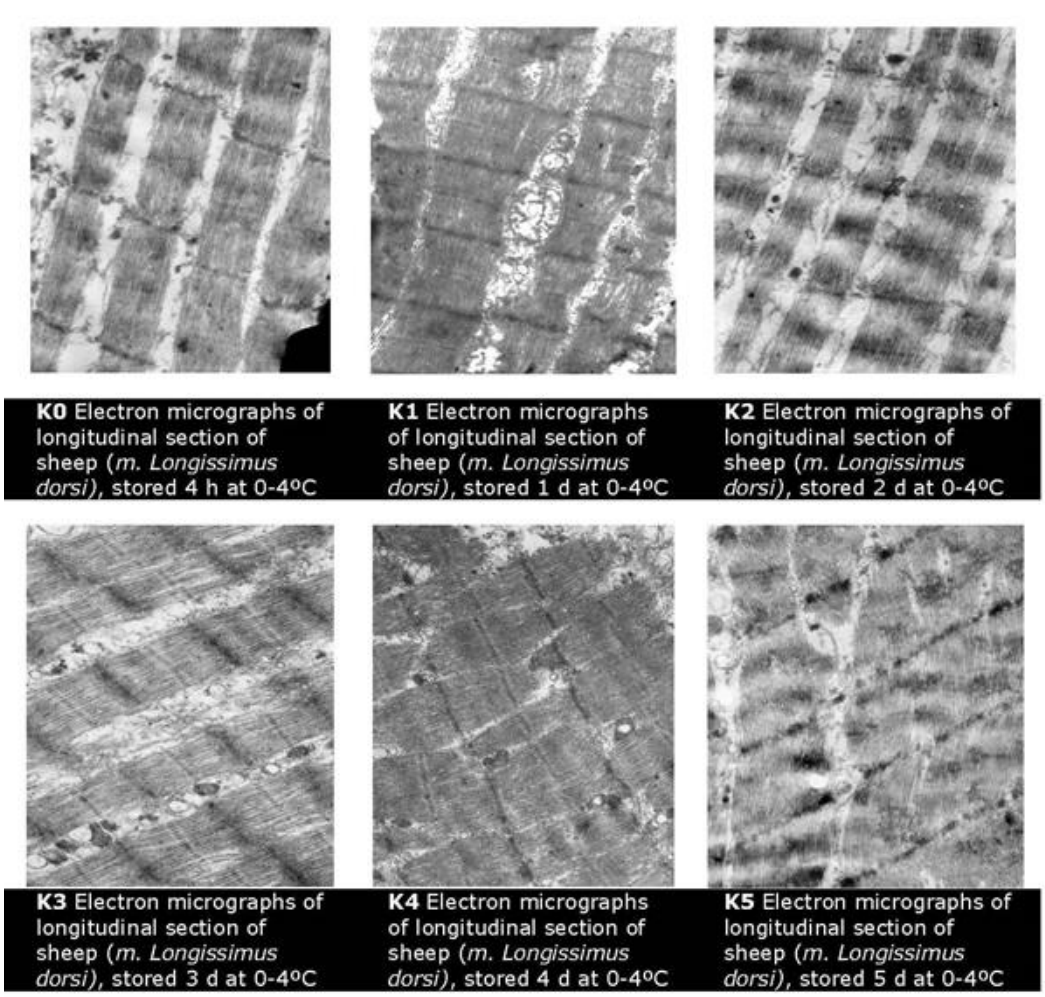

Figure 1. Electron micrographs of longitudinal section of sheep m. Longissimus dorsi, 12000x

Table 1. Changes of the pH values, water holding capacity, free amino nitrogen and soluble protein fraction during the 5 d storage of the sheep meat at 0-4ºC.

Mean value ± SD, $^{a, b, c, d, e}$ - Different letters on the means denote statistical differences amongst samples ($p \leq 0.05$)

Samples	K0 4 h p.m.	K1 1 d p.m.	K2 2 d p.m.	K3 3 d p.m.	K4 4 d p.m.	K5 5 d p.m.
pH	6.95±0.02^{d}	6.20±0.02^{b}	5.80±0.05^{a}	6.80±0.08^{c}	6.90±0.06^{c,d}	7.05±0.07^{e}
Water holding capacity, %	19.62±1.47^{b}	11.38±1.67^{a}	9.69±1.42^{a}	18.81±2.33^{b}	21.49±1.09^{c}	21.65±1.18^{c}
Free amino nitrogen, mg/100g	9.91±0.53^{a}	13.33±1.32^{b}	14.32±1.81^{b}	14.83±0.54^{b,}	15.18±0.23^{c}	17.91±1.53^{d}
Soluble proteins, mg/ml	1.33±0.22^{d}	0.79±0.03^{c}	0.23±0.05^{a}	0.46±0.03^{b}	0.85±0.06^{c}	1.18±0.07^{d}

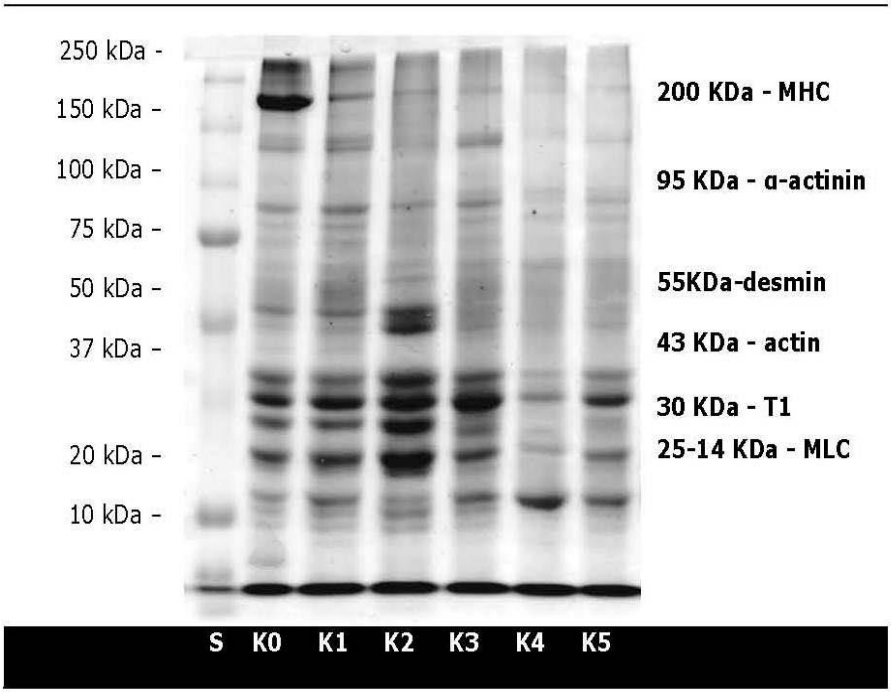

Figure 2. SDS-PAGE electrophoresis of sheep proteins during the 5 d storage period at 0-4ºC

Table 2. Changes of the color characteristics (L*, a*, b*) of the sheep meat cut surface during 5 d storage at 0-4ºC

Mean value ± SD, [a, b, c, d, e] - Different letters on the means denote statistical differences amongst samples ($p \leq 0.05$)

Samples	K0 4 h p.m.	K1 1 d p.m.	K2 2 d p.m.	K3 3 d p.m.	K4 4 d p.m.	K5 5 d p.m.
L*(C)	39.65 ± 0.15^{d}	39.36 ± 0.08^{c}	39.06 ± 0.20^{c}	38.32 ± 0.15^{b}	38.16 ± 0.23^{b}	35.77 ± 0.18^{a}
a*(C)	20.01 ± 0.73^{c}	19.32 ± 0.42^{a}	18.88 ± 0.31^{b}	18.39 ± 0.82^{b}	18.24 ± 0.84^{b}	17.75 ± 0.33^{a}
b*(C)	4.56 ± 0.32^{a}	5.97 ± 0.44^{b}	6.00 ± 0.65^{b}	6.05 ± 0.52^{b}	7.06 ± 0.31^{c}	7.25 ± 0.62^{c}

Effect of aging on the color characteristics of sheep meat

On the second day of storage at 0-4°C (Table 2) a statistically significant decrease in the color brightness of DFD sheep (L*) ($p < 0.05$) was found. For the next two days the estimated changes in L* color component (samples K4 and K5) were minimal ($p > 0.05$). For the studied period (5d, 0-4°C) the color brightness (L*) statistically significantly decreased with 9.8% ($p < 0.05$) and the red color component (a*) of DFD sheep decreased with 11.3% ($p < 0.05$) (Table 2). One possible reason for the observed color changes was the iron oxidation from ferrous (Fe^{2+}) to ferric (Fe^{3+}) form, and the formation of oxymyoglobin – compound with bright red color on the meat surface formed after interaction with oxygen from the air (Geay et al. 2001).

The opposite trend was established in the yellow color component (b*). On the 5 d (K5) of DFD sheep storage at 0-4ºC (Table. 2) an increase by 9.9% ($p < 0.05$) was observed. Our results are in

agreement with the determined good correlations between the ultimate pH and some quality traits of sheep meat (Bouton and Shorthose 1969).

Conclusion

There is a strong relation between the muscle microstructure and the biochemical changes in the process of DFD sheep aging. During the early period (4h) *post mortem* the meat myofibrillar structure was found with open grid and the highest protein solubility. This may be explained with the fact that the myosin heavy chains were identified up to 24h *post mortem*. With decreasing of the DFD sheep pH at 48h *post mortem* the *rigor mortis* takes place. The sarcomere shortening is a consequence of an actomyosin formation and a decrease of protein solubility. During this period the water holding capacity was minimal but not the free amino nitrogen content itself. With the passing of *rigor mortis* on 3d post mortem the pH increased. As a result of autolytic changes the microstructure was recovered which enabled the myofibrillar swelling and the water holding capacity increased again. The increase of the polypeptides with molecular weight of 28-32kDa and the Z-disk fragmentation after 4d *post mortem* confirmed the proteolytic changes and tenderization during DFD sheep aging.

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