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A LIGHT MICROSCOPY STUDY ON CONNECTIVE TISSUE DECOMPOSITION IN BOVINE *M.LONGISSIMUS DORSI* DURING THE FIRST WEEK *POST MORTEM*

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Introduction

Tenderness is an important factor for the consumer`s acceptance of meat, and postmortem storage of meat is known to increase tenderness. Tenderness is determined by the structural properties of myofibers and connective tissue and the processes going on in the tissue. In a recent light microscopy study, splitting of bovine *M. longissimus dorsi* was reported to occur inside the myofibers as well as in the connective tissue between the myofibers and between the myofiber-perimysium during the first 7 days after slaughter (Ofstad et al. 2005). Degradation of meat is reported to occur in the Z-lines of the myofibres (Taylor et al, 1995). Ruptures and splitting in the connective tissue is considered a result of tissue decomposition. In a recent study on bovine *M. longissimus dorsi* we reported that decomposition of connective tissue could be detected as early as 24 hours after slaughter by light microscopy on Haematoxylin eosin stained cryo-sections (Hannesson et al. 2003). Furthermore, proteoglycan aggregates of high molecular size carrying chondroitin/dermatan sulfate glycosaminoglycans (GAGs) and hyaluronic acid, were involved in the process as judged by gel chromatography and PAGE. Ruptures and splitting in the connective tissue is considered as a parameter for tissue decomposition. In the present study we have focused on the splits occurring in the connective tissue; in the myofibre/myofibre attachment as well as in the myofibre/perimysium attachment. The amount of splitting was calculated and the expression and localization of sulfated glycosaminoglycan (GAG) structures carried by the large chondroitin sulfate proteoglycans (CSPGs) studied by immunohistochemistry using monoclonal antibodies (mAbs). MAbs against the large heparan sulfate proteoglycan of basement membranes, perlecan were also included. The Warner Bratzler values for the different meat samples are included.

Objectives

To study components involved in myofiber/ connective tissue detachments in bovine skeletal muscle aged for 7 days. The focus is on the expression and distribution of unsulfated and 6-sulfated chondroitin sulfate and the large basement membran heparan sulfate PG, perlecan, and possible changes in the expression of these epitopes after 7 days storage.

Methodology

Animals: Specification and handling of the animals and the method used for WB-measurements were the same as described by Aass et al.(2005).

Preparation of samples for microscopy: Plastic and cryo-sections were taken on muscle samples collected from the medial central part of bovine *M.longissimus dorsi* adjacent to the pieces used for WB after removal of the epimysium. Pieces of approximately 4 x 4 x 3 mm were cut at different locations.

Plastic–sections. Fixation was carried out in 2.5%glutaraldehyde in cacodylate buffer and embedded in plastic resin, Historesin (Ofstad et al.1993). Cross-sections (3μm) were then stained in 0.1% Toluidine Blue O (Sigma/Aldrich) dissolved in aqueous sodium acetate (1g/100ml)

Splitting was calculated after measuring the number of splits between the myofibers and myofiber/perimysium (Ofstad et al. 2005). No efforts were done to evaluate the length or with of the splits.

Cryosections: The pieces were embedded in O.C.T. compound (Tissue Tek 4583, Miles Inc., Diagnostic Division, Elkhart, USA) for 30 minutes and finally immersed in liquid nitrogen. The frozen samples were stored at -80 C

Immunohistology

Enzyme treatment: To generate the antigenic epitopes for detection of the different sulfate structures present in the CS/DS chains, the samples were digested with a drop of chondroitinase ABC lyase from *Proteus vulgaris* (0.5 units/ml) (EC 4.2.2.4, Sigma Chemical Comp. St. Louis, MO, USA) in 0.1 M Tris-HCl buffer pH 8 for 4 h at 37C. (Yamagata *et al.* 1968). This method produces a terminal disaccharide, consisting of an

unsaturated uronic or iduronic acid residue adjacent to the N-acetylgalactosamine that may be unsulfated or sulfated in the C4 or C6 positions (Couchman et al. 1984)

Antibodies: For detection of N-acetylgalactosamine sulfated in C6 (C6S), the mAb 3B3 was used. For detection of unsulfated N-acetylgalactosamine (C0S) the mAb 1B5 was used (Caterson et al.1985).

For detection of perlecan mAb A7L6 (Chemicon International LTD, Hofheim, Germany) was used. Perlecan clone A7L6 reacts with perlecan core domain IV (Couchman and Ljubimov, 1989).

For immunostaining, an immunoperoxidase system Vectastain Universal Elita ABC kit (Vector Laboratories, Inc., Burlingame, CA) was used according to the manufacturers recommendations. Before immunostaining, the 5 μm cross-sections were fixed in 8% formaldehyde in phosphate buffered saline (PBS, pH 7.4) for 5 min. Unspecific binding sites were blocked using 5% bovine serum albumin (Sigma-Aldrich Chemie) in PBS added normal serum from horse (Vectastain Universal Elita ABC kit. The sections were then incubated over night at 4 °C with the following mAbs; 1B5 (1:200), 3B3 (1:200) and A7L6 (diluted 1:100).The mAbs were diluted in PBS added 5% BSA and 0.005 % Tween-20 (Sigma-Aldrich). The cover slips were photographed in a Leica DMLB microscope (Leica Microsystems Nussloch GmbH, Germany) by a Spot RT Color Camera (Diagnostic Instruments inc. Burroughs Sterling Heights, Michigan).

Results & Discussion

The results obtained on plastic sections from M.*longissimus dorsi* after 7 days storage after slaughter, using toluidine blue, are shown in figure 1 a and b. Figure 1a represents the tougher muscle with a Warner Bratzler (WB) value of 44.7, whereas figure 1b represents the tender muscle with a WB value of 90.1. An extensive breakdown of the connective tissue was seen in the tender muscle (Fig 1b). In the tough muscle the decomposition was hardly visible (Fig 1a). The decomposition appeared in the endomysial area, between the individual myofibers as well as between the myofibers and the perimysium as illustrated with arrows in figure 1b. The present result shows a tough and a tender muscle selected from a population of 28 animals, consisting of animals with a wide range in tenderness scores (Ofstad et al. 2005). In this population 65% of the counted myofibers showed detachment with one or more of the adjacent myofibers whereas 38 % of the myofibers lining the perimysia showed detachment. The results indicate that the myofiber-perimysium attachment is more resistant to decomposition compared to the myofibre-endomysium attachments. Large individual variations in the ability to split were observed between the animals.

To examine possible components involved in the detachments, proteoglycans (PGs) carrying chondroitin/dermatan glycosaminoglycan (GAG) chains with either unsulfated (C0S) or 6-sulfated (C6S) N-acetylgalactosamine residues were selected. The large PG-HA aggregates shown to decompose in a previous study after gelfiltration and PAGE of tissue extracts (Hannesson et al.2003) carry C0S and C6S epitopes (results not shown).

The muscle samples in the present study were collected from an animal exhibiting a WB score decreasing from 90 to 48 after 7 days of storage. Samples taken some hours after slaughter (day 0) and stored for 7 days were compared. The results obtained using the mAb against unsulfated PG epitopes are illustrated in figure 2a and b. At day 0 a distinct and consistent staining is observed in the endomysia (E) (Fig.2a). A somewhat weaker staining is observed between the myofibers and the perimysium. After 7 days the staining is no longer consistent and several splits are seen between the myofibers, on both sides of the structure carrying the unsulfated GAG epitopes (Fig. 2b). Some detachments are furthermore evident between the myofiber and the perimysium, the latter showing almost no stain except for the nerve (N).

Using the antibody against the C6S epitopes a similar staining pattern as with C0S is observed with a distinct and consistent staining of the endomysia at day 0 (Fig.3a) After storage splitting of myofibers is evident. Furthermore, some endomysia still exhibit color whereas in others expression of 6-sulfated epitopes cannot be detected. The change in the staining pattern and the localization of the splitting in the sections after storage support the previous biochemical analysis that CS/DSPGs may be involved in post mortem tenderization of beef.

The staining pattern obtained using the basement membrane heparan sulfate PG, perlecan, is shown in figure 4 a and b. In the samples collected at day 0 a distinct and consistent color is lining all myofibers. After 7 days a weaker staining is evident in the areas with intermyofibrillar ruptures compared to more intact areas (see asterisks Fig. 4b). In the area without visible detachments a borderline of strong stain is present between myofiber and perimysium (see arrow Fig.4b). Furthermore, a strong coloration is seen in the walls of the blood vessels (arteries (A) and veins (V)). The importance of perlecan for basement membrane integrity is clearly demonstrated in perlecan knock out

mice (Costell et al., 1999). The perlecan null mutations were embryonic lethal due to an abnormal development of the heart and deterioration of the basement membranes in regions with increased mechanical stress, such as the contracting myocardium and the expanding brain vesicle. In addition, a defective collagenous network was present in the cartilage of perlecan null mutants (Arikawa-Hirasawa et al 1999).

In addition to the ruptures between the myofibers and between the myofibers and the perimysium, ruptures (R) are visible in the perimysium proper (Fig.4b). Ofstad et al. (2005) have reported using transmission electron microscopy an extensive breakdown of the extracellular matrix between the collagen fibers in fish white muscle. This was most evident in fish species with gaping problems as Atlantic cod. Nishimura et al. (1995) demonstrated that a breakdown had occurred in between the collagen fibers in beef conditioned for 21 days using microscopy and maceration techniques.

According to the different localizations of the ruptures, several components and processes may be involved in meat tenderization. Further studies are necessary to clarify the processes involved.

Conclusions

Decomposition of connective tissue in bovine meat occurs during the first 7 days of post mortem storage. Splitting occurs in several locations; between the myofibers, between the myofibers and the perimysium and in the perimysium proper.

The area between the myofibers appeared more vulnerable for splitting than the area between the myofibers and the perimysium.

A change in the expression pattern of unsulfated- and 6-sulfated CS/DS PGs and perlecan were evident after 7days storage, indicating a role for the proteoglycans in the loss of adhesion.

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Tables and Figures

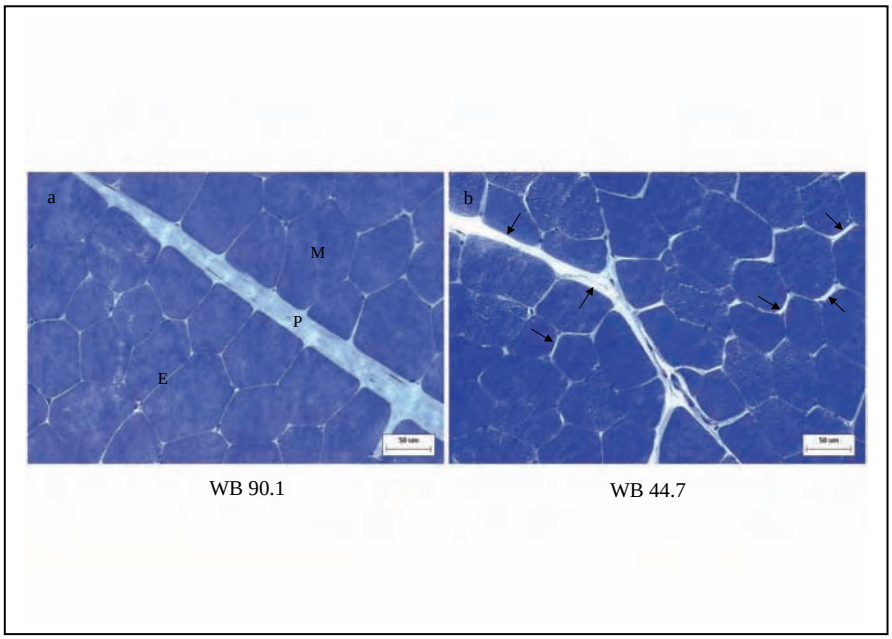

Figure 1a and b show the plastic embedded sections from bovine *M .longissimus dorsi* obtained after aging for 7 days. The sections were stained by toluidine blue. 1a represents the tougher beef, whereas 1b represents the tender beef (WB 90.1 and 44.7). M, P and E indicate the myofiber, peri- and endomysium, respectively. The arrows show the ruptures between the myofibers and between the myofibers and the perimysium.

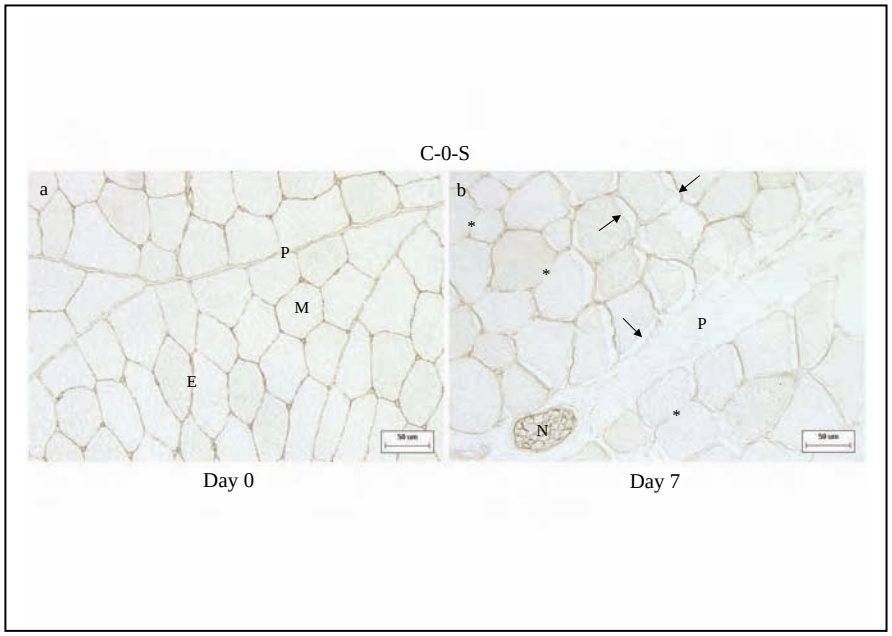

Figure 2a and b show the cryo-sections stained with mAbs against unsulfated GAG epitopes (C-0-S). 2a represents beef obtained day 0 showing a distinct and consistent staining around all the myofibers. 2b represents beef after 7 days aging and splitting is evident in different areas indicated by arrows. The asterix shows the endomysial areas with no visible C0S epitopes. N represents a nerve, P the unstained perimysium.

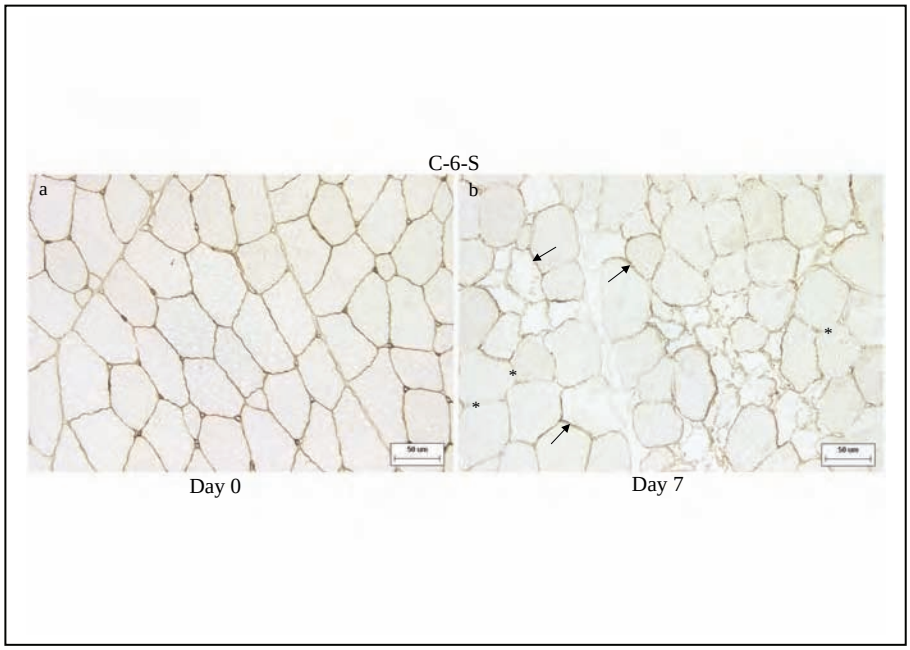

Figure 3a and b show the cryo-sections stained with mAbs against 6- sulfated GAG epitopes (C-6-S). 3a represents beef obtained day 0 whereas 2b represent beef after 7 days aging. In 3a a distinct and consisting stain is lining the myofibers. At day 7 the expression of the epitopes is still strong in some areas (arrows) but lacking in others (asterix).

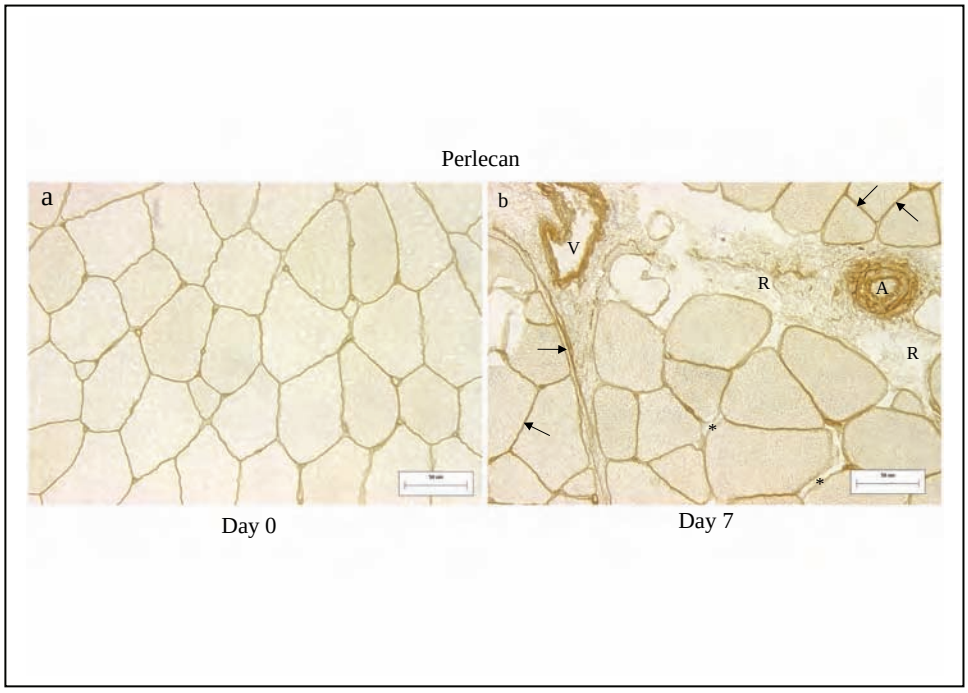

Figure 4a and b show the sections obtained at day 0 and day 7 using the mAb against perlecan. The areas without visible detachments exhibit still strong and consistent and V represent arteriol and veinole, respectively. In addition ruptures are visible in the connective tissue proper.