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EFFECTS OF SALTING ON MICROSTRUCTURE AND PROTEIN SECONDARY STRUCTURE AS MEASURED BY FT-IR MICROSPECTROSCOPY IN PORCINE M.SEMITENDINOSUS OF DIFFERENT QUALITY

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Introduction

Meat muscle that is immersed in salt solution will swell first (NaCl> 1%) and again shrink, when the salt concentration in the muscle exceeds 6% NaCl. This is mainly due to a swelling and shrinking of muscle cells. Cross-sectional areas of myofibers will therefore display microstructural changes depending on existing salt levels and the degree of swelling or dehydration of the myofibrils. Swelling and water expelling will depend on the post mortem status of the meat as well as the type of muscle and fibres selected for the experiment (Offer et al., 1989; Knight et al., 1989; Egelandsdal et al. 1995).

FT-IR microspectroscopy, i.e., the combination of infrared spectroscopy and microscopy, has great potential for chemical analysis of tissue on microscopic scale. Chemical changes, as changes in secondary structure of proteins (e.g. from native to aggregated proteins) can be studied spatially resolved on microscopic level, e.g., for connective tissue and myofiber cells, separately. Conventional histological techniques do not allow the direct detection of these types of changes. In addition the chemical methods commonly used to identify altered protein structures give no information about their spatial distribution necessary to explain salt uptake/water holding properties. Kirschner et al. (2004) applied FT-IR microspectroscopy to follow denaturation during heating in beef muscle tissue.

A combination of both, histological and microspectroscopic techniques, enables us to relate chemical changes to structural changes.

Objectives

The scope of this study was to investigate effects of salting on microstructure and protein secondary structure of pork muscle tissue by light microscopy and FT-IR microspectroscopy with respect to different raw meat qualities.

Methodology

A variation in meat quality was introduced by an experimental design including different pre-slaughter treatments of the animals. The pre-slaughter treatment was as follows: (1) treadmill and electronic stunning (Bertram et al. 2004) (2) injection of adrenaline 15 h prior slaughter (Henckel et al. 2000) and (3) a control animal without special treatment. Samples of *M. semitendinosus* of 4 cm x 4 cm x 4 cm were excised and placed into brine solutions of different concentrations: 0.9% ("1"), 3% ("2"), 6% ("3") and 9% ("4") (10 mM Na-acetate buffer pH 5.5 with 0.05% NaN_3 to prevent microbial growth). They were kept in the brine for 8 days at 4 °C. Samples for microspectroscopy were excised, embedded in O.C.T. compound (Tissue-Tek, Electron Microscopy Sciences, Hatfiles, USA) and frozen in liquid nitrogen before ("0"-samples) and after salting. They were kept at –80 °C until sectioning, which was performed transversely to fiber direction on a cryostat (Leica Germany). 8 μm thick sections were prepared and thaw-mounted on infrared transparent 2 mm thick CaF_2 slides for FT-IR microscopic measurements and parallel sections were collected for light microscopy. The latter sections were stained according to a standard procedure with Hematoxylin Erythrosine.

FT-IR spectra were acquired with an IRScope II coupled to an Equinox 55 (BRUKER OPTICS, Germany). The spectra were scatter-corrected using extended multiplicative signal correction (EMSC). EMSC is a pre-processing method that allows the separation of physical light-scattering effects (e.g. sample thickness) from chemical absorbance effects in spectra (Kohler et al. 2005).

The Unscrambler® version 9.1 and in-house developed algorithms written in C++ (CAMO Process AS, Norway, 2004) were used for spectral processing and multivariate analysis of the data.

Results & Discussion

Fig.1 shows selected light microscopic images of Hematoxylin Erythrosine stained-sections (differences in color intensity may be due to differences in pH value and salt content of the samples). The selected regions shown in the images correspond to areas in which FT-IR spectra were collected. The upper panel presents sections from samples of the control animal, the middle from the treadmill-treated pig, and the lower from the adrenaline-treated animal. The images in the left column represent samples taken 24 hours after slaughter followed to the right by columns for salted samples with the salt concentration indicated in the figure. The unsalted samples already reveal structural differences among the treatments: In the control and adrenaline treated samples the myofibres are well attached, whereas in the treadmill-sample the myofibres are detached and the extracellular area is extended. The adrenaline-sample has a more bluish color than the two other samples. This may be due to the higher pH value of this sample. During salting the fibers first swelled (diameter increases) and thereafter shrunk at NaCl $\geq$ 6 %. At the highest salt concentration (images the column furthest to the right) the control and treadmill samples appear shrunken and more edgy in shape. In contrast, the adrenaline samples seemed to keep an apparently intact microstructure. However, at the high salt concentration it was difficult to distinguish between individual fibers and the fibres were amorphous.

The Amide I band at the frequency region from 1700-1600 cm-1 is the most prominent feature in the FT-IR spectrum of the muscle fibers. This band is mainly due to the carbonyl stretching vibration with minor contribution of C-N stretching and N-H bending vibrations and it mostly depends on the secondary structure of the protein backbone. Therefore it is commonly used for secondary structure analysis of proteins (Barth and Zscherp 2002). To gain a better resolution the second derivative was applied to the spectra. In Fig. 2 the second derivative spectrum of the Amide I region is shown exemplary for the "0"-sample of the control animal. The band at 1653 cm-1 is most likely referring to α-helical structures in the myofibrillar proteins. Jackson and Mantsch (1995) assign bands from 1610 cm-1 to 1628 cm-1 to denatured aggregated β-sheet components, while they relate the band between 1630-1640 cm-1 to antiparallel β-sheet structures. In Fig. 2 bands that are possibly related to denatured aggregated β-sheet structures are found at 1619 cm-1 and 1628 cm-1 and the band at 1638 cm–1 could be related to antiparallel β-sheets. Due to transition dipole coupling in β-conformational structures, the bands found at the higher wavenumbers, 1682 cm–1 and 1693 cm–1, may be the weaker components of the antiparallel β-sheet structures and the denatured aggregated β-sheet components, respectively.

In order to analyze the FT-IR spectra a principal component analysis (PCA) was carried out with the second derivative spectra (average spectra resulting from 12 spectra taken from two areas on the tissue section) in the spectral region from 1700-1600 cm-1. One sample outlier was identified and removed.

The score plot of the first two principal components (PCs) is presented in Fig.3a. The first PC results from variations in the raw material showing differences between adrenaline (red), control (blue) and treadmill (green) animal. Compared to the other two groups the treadmill group reveals a much larger variation over PC1. PC2 gives the variation in the salt content of the samples (labels "1" to "4" indicate the salt concentration). The explained variance for PC1 and PC2 is 64% and 23%, respectively. By comparing the x-loading plots (Fig. 3b) with the score plot for PC1 and PC2, one can find which samples are related to certain wavenumbers. For example the frequency range around 1628 cm–1, which might be corresponding to denatured aggregated β-sheet, could be associated with the treadmill samples. The band at 1652 cm-1 (α-helix) might be related to samples low in salt content and adrenaline samples show correlation to antiparallel β-sheet components. These results indicate that raw quality of pork muscle as well as salt concentration leads to differences in the protein secondary structure as measured by FT-IR spectroscopy.

Conclusions

Pork muscle samples displayed microstructural differences related to raw material and salt content. FT-IR spectroscopy revealed corresponding changes in the protein secondary structure. Corresponding differences were found. This suggests that the microscopic changes are inherent with a change at the molecular level.

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Tables and Figures

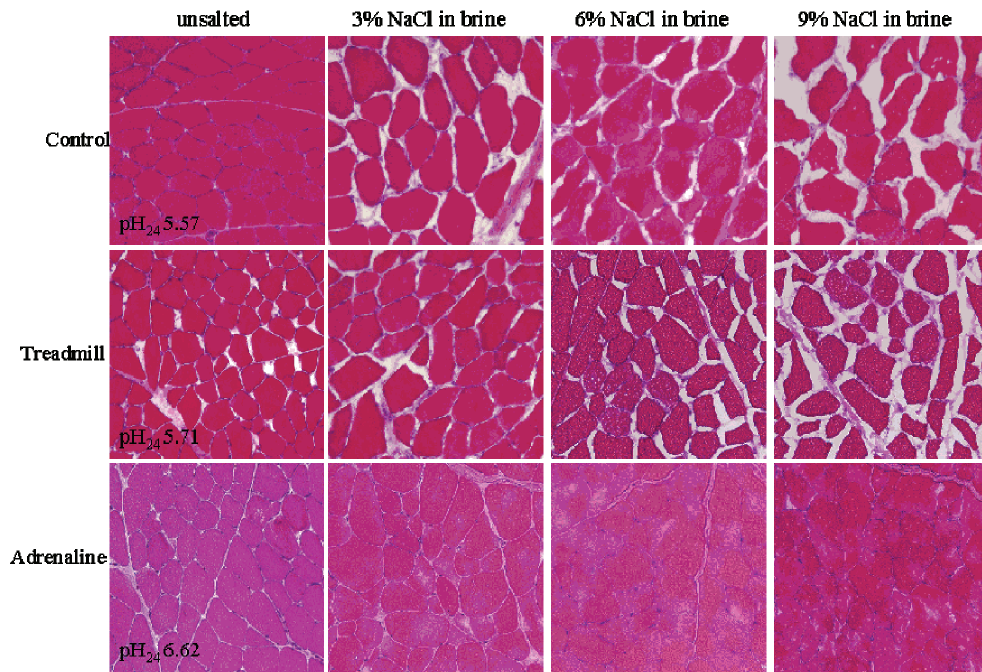

Fig. 1: Selected Hematoxylin Erythrosine images of porcine *M. semitendinosus*. FT-IR spectra were collected in the same areas.

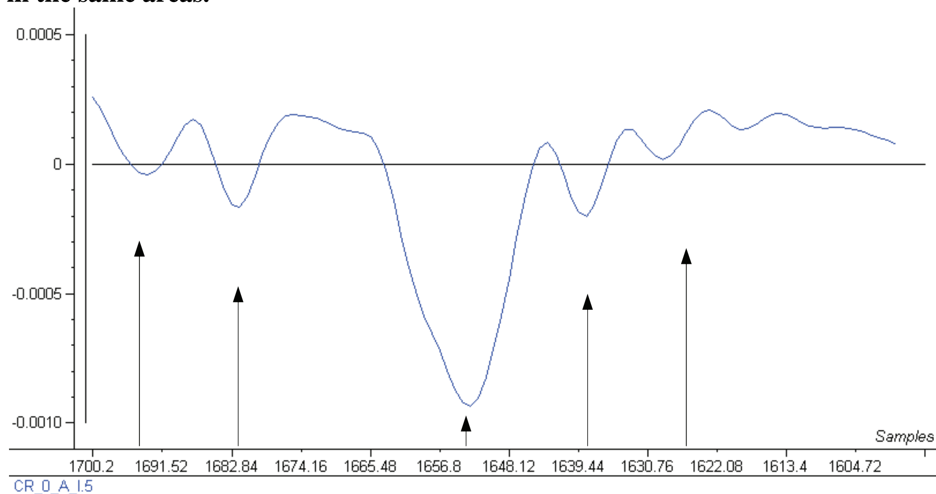

Fig. 2: 2nd derivative spectrum (average of 12 spectra) of unsalted control sample in the wavenumber range 1700-1600 cm^{-1} (shown on the x-axis). The arrows indicate wavenumbers described in the text.

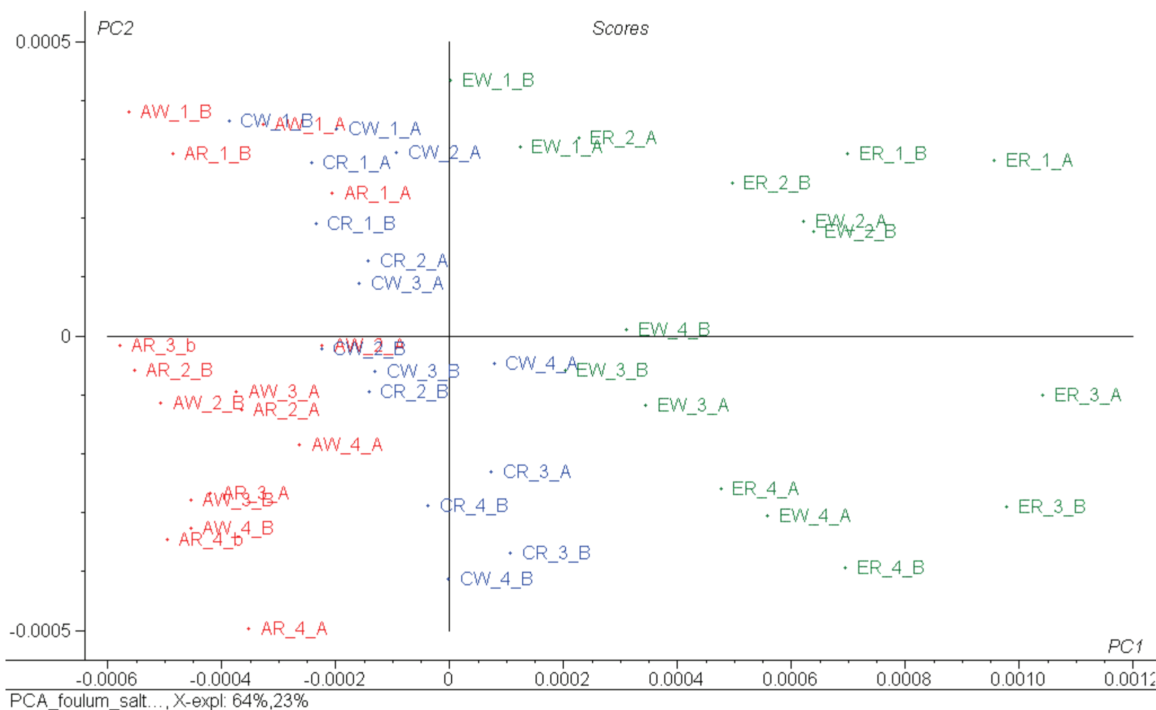

Fig. 3a: PCA score plot (PCA run for 1700-1600 cm^{-1}) with 64% and 23% explained variance for PC1 and PC2 respectively.

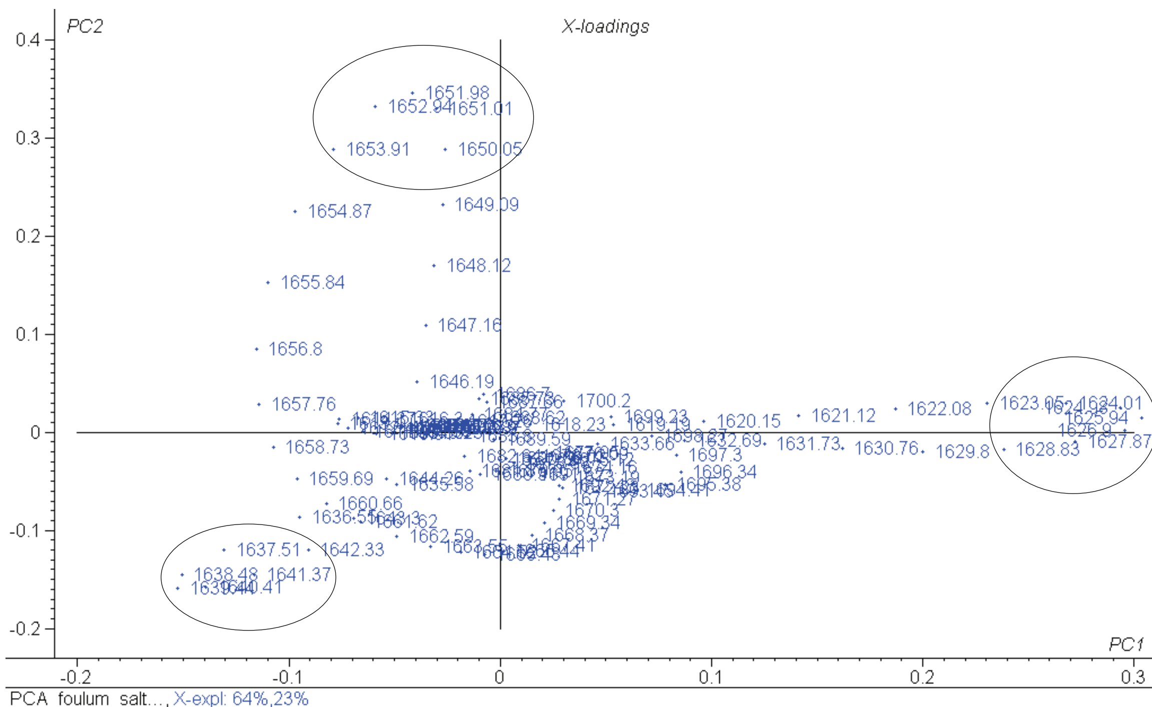

Fig. 3b: x-loading plot corresponding to score plot shown Fig. 3a. Wavenumber ranges indicated are described in the text.