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Analyzing pH-induced changes in a myofibril model system with vibrational and fluorescence spectroscopy

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ABSTRACT

The decline of pH and ultimate pH in meat postmortem greatly influences meat quality (e.g. water holding capacity). Four spectroscopic techniques, Raman, Fourier transform infrared (FT-IR), near infrared (NIR) and fluorescence spectroscopy, were used to study protein and amino acid modifications to determine pH-related changes in pork myofibril extracts at three different pH-levels, 5.3, 5.8 and 6.3. Protonation of side-chain carboxylic acids of aspartic and glutamic acid and changes in secondary structure, mainly the amide I–III peaks, were the most important features identified by Raman and FT-IR spectroscopy linked to changes in pH. Fluorescence spectroscopy identified tryptophan interaction with the molecular environment as the most important contributor to changes in the spectra. NIR spectroscopy gave no significant contributions to interpreting protein structure related to pH. Results from our study are useful for interpreting spectroscopic data from meat where pH is an important variable.

1. Introduction

The postmortem pH-decline in meat has been studied extensively, and has been shown to affect overall quality of meat and meat products significantly (Fischer, 2007). The main motivations for measuring pH in meat is that it is closely related to water-holding capacity (WHC) (Schafer, Rosenvold, Purslow, Andersen, & Henckel, 2002), it impacts the potential for proteolysis postmortem and subsequent changes in protein structure (Huff-Lonergan & Lonergan, 2005), and it influences shelf-life of meat (Blixt & Borch, 2002). WHC is an important quality parameter for meat as it influences total salable weight, eating quality (e.g. juiciness (Lawrie, 1985)) and nutritional value of meat (Savage, Warriss, & Jolley, 1990). Poor WHC causes high amounts of liquid exuding from the meat, which starts forming when muscles are contracting during the rigor mortis process postmortem, and continues for several days and weeks afterwards. The amount of drip formed is dependent on several factors, such as antemortem handling at the slaughterhouse (Henckel, Karlsson, Oksbjerg, & Soholm Petersen, 2000), rate of pH decline and ultimate pH postmortem (Bee, Anderson, Lonergan, & Huff-Lonergan, 2007), enzyme activity (Davis, Sebranek, Huff-Lonergan, & Lonergan, 2004), genetic predisposition and temperature during conditioning and storage (Cheng & Sun, 2008). Many of these factors are interconnected, and this signifies that WHC is a very complex quality trait in meat. Thus, there is a need for more knowledge about which mechanisms influence WHC in a significant way, and there is a demand for a method to predict WHC in the slaughterhouse. Spectroscopic analyses are prime candidates to contribute to advancing the knowledge of both these topics, because they are rapid techniques suitable for on-line measurements that can give specific biochemical information of meat.

Two of the most promising spectroscopic techniques in this regard are Raman and Fourier transform infrared (FT-IR) spectroscopy. Using these techniques, it is possible to extract information about secondary structure of proteins, protein and amino acid interaction with the environment and protonation of amino acids (Tu, 1986), all of which may be subjected to modification as pH changes and the proteins can subsequently act as pH-probes. By following the pH decline in meat postmortem and simultaneously record Raman spectra, Nache, Scheier, Schmidt, and Hitzmann (2015) was able to predict pH with cross validated coefficient of determination (r^2_{cv})of 0.97 and cross-validated root mean square error (RMSECV) of 0.06 pH units. Raman measurements at the slaughter line has not yielded as good results for predicting pH, but are showing some promise, with an r^2_{cv} of 0.55 and RMSECV of 0.09 pH units for pH at 35 min. postmortem and an r^2_{cv} of 0.31 and RMSECV of 0.05 pH units for pH at 24 h. postmortem in a recent study (Scheier, Scheeder, & Schmidt, 2015). FT-IR and Raman spectroscopy are also showing promise as methods of predicting WHC. Using FT-IR, Pedersen et al. was able to predict WHC with an r^2 of 0.89 and RMSECV of 0.86% in a research setting and an r^2 of 0.79 and RMSECV of 1.06% in an industrial trial (Pedersen, Morel, Andersen, & Balling Engelsen, 2003). Raman spectroscopy performed at 60–120 min. postmortem in a cooling room achieved an r^2_{cv} of 0.73 and a RMSECV of 1.0% for drip loss (Scheier, Bauer, & Schmidt, 2014), while measurements at 30–60 min.

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postmortem at the slaughter line achieved an r^2_{cv} of 0.52 and a RMSECV of 0.6% for drip loss (Scheier et al., 2015).

Near-infrared (NIR) spectroscopy has successfully been implemented in slaughterhouses to analyze main chemical composition of meat (Prieto, Roehe, Lavin, Batten, & Andres, 2009), and it has been investigated extensively as a method for predicting pH and WHC. In one recent study using a hyperspectral NIR spectroscopy laboratory set-up, Barbin, ElMasry, Sun, and Allen (2012) managed to predict pH with an r^2_{cv} of 0.86 and a RMSECV of 0.11 pH units and drip loss with an r^2_{cv} of 0.88 and a RMSECV of 0.73%. Another approach is to use an insertion probe NIR instrument, and Forrest et al. (2000) was able to predict drip loss with a correlation coefficient (r) of 0.84 and root mean square error of prediction (RMSEP) of 1.8% using such an instrument.

Fluorescence spectroscopy can give important information about the three amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) and their interactions with the molecular environment (Christensen, Norgaard, Bro, & Engelsen, 2006), thus giving a possible link between proteins and pH in meat. The research on fluorescence spectroscopy for measuring pH and WHC is not as thorough as for Raman, FT-IR and NIR, but there have been a few attempts to utilize this technique as well. An example is a study by Brondum et al. (2000), where drip loss was predicted with moderate success by laboratory fluorescence measurements giving an r of 0.68 and standard error of prediction (SEP) of 2.27%.

In many of the studies using Raman, FT-IR, NIR or fluorescence spectroscopy, the focus has been to predict pH and WHC, and the interpretation of the spectral features and what information the spectra can give about protein structure relevant for meat quality has not been emphasized. In meat samples, changes in α-helical secondary protein structure have been linked to changes pH for both Raman spectra (Scheier, Kohler, & Schmidt, 2014) and FT-IR spectra (Pedersen et al., 2003). In most studies with NIR and fluorescence related to pH and WHC an interpretation of the connection between spectra, protein structure and pH is lacking.

Thus, the aim of this study was to gain more detailed knowledge regarding the potential effects of pH on the spectroscopic properties of myofibrillar proteins. Since pH is known to influence WHC of meat, this knowledge may in the long run lay grounds for improved spectroscopic modelling/prediction of WHC. In order to avoid the influence of other chemical and physical features of muscle on the spectroscopic measurements, a myofibril model system, containing extracted myofibril proteins from pork meat, was used in this study to examine protein modifications at selected pH-levels relevant in postmortem meat by Raman, FT-IR, NIR and fluorescence spectroscopy. Similar model systems have been used by others to study the effect of e.g. proteolysis on muscle proteins (Koohmaraie, Schollmeyer, & Dutson, 1986) and the influence of protein oxidation on digestibility of meat proteins (Sante-Lhoutellier, Aubry, & Gatellier, 2007). By using myofibril extracts, it is possible to make samples with homogenous pH, obtain protein-specific spectroscopic influence and acquire a deeper understanding of protein-protein interactions related to pH in meat. This gives an opportunity to weight spectral channels in statistical models based on general protein characteristics related to pH, and it can give an important contribution to understanding some of the mechanisms of WHC in meat.

2. Materials and methods

2.1. Animals, myofibril isolation and sample preparation

Five pigs were slaughtered at a commercial abattoir in Tønsberg, Norway, following standard slaughtering procedures. The entire *M. longissimus dorsi* muscle was excised from the left side of each carcass approximately 24 h postmortem. The samples were stored at 4 °C and transported to Nofima AS, Ås, where the muscles were sliced, vacuum packed and stored at −20 °C.

A meat slice from each pig was thawed in room temperature prior to isolation. Connective tissue and fat was removed before ~20 g of each sample was sliced in small cubes and transferred to a 250 ml centrifuge tube. 160 ml pyrophosphate relaxing buffer (PRB) (2 mM $Na_4P_2O_7$, 2 mM $MgCl_2$, 2 mM triethylene glycol diamine tetraacetic acid, 10 mM Tris(hydroxymethyl)aminomethane maleate salt, 0.5 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, pH 6.8) was added to the tube and sample was homogenized using a polytron at a speed of 15,000 rpm for 30 s three times with a 30 s resting step in between. The homogenate was centrifuged at 1000*g* for 10 min at 4 °C. After centrifugation, the homogenate was washed as follows: discarded the supernatant, added 200 ml extraction buffer (PRB without $Na_4P_2O_7$ and PMSF), rigorously shaking the homogenate prior to centrifugation as described earlier. The supernatant was discarded, 200 ml extraction buffer was added and the homogenate was rigorously shaken, before the homogenate was filtered through a sieve (400-μm mask width for sample 1 to 4, 710-μm for sample 5) to remove connective tissue, and once more centrifuged as described earlier. After another step of washing, the supernatant was discarded and 200 ml of Triton X-100 buffer (extraction buffer supplemented with 0.02% w/v Triton X-100) was added, the sample was rigorously shaken and centrifuged as described earlier. The samples were washed three times and finally stored at −20 °C in 42.5% (v/v) glycerol in extraction buffer. Protein concentration in each of the myofibril extracts was measured by the Biuret method (Gornall, Bardawill, & David, 1949).

Myofibril extracts were thawed in room temperature, then transferred to a centrifuge tube and subsequently washed three times in extraction buffer (using the same volume of extraction buffer as sample volume after removal of the supernatant) to remove glycerol. Each sample was diluted to a protein concentration of ~20 mg/ml with extraction buffer. The diluted sample was distributed in three test tubes, each containing 15 ml. A Beckman Φ31 pH Meter (Brea, CA, USA) was used to measure and adjust pH in each tube to 5.3, 5.8 or 6.3 (±0.1) with 3 N HCl. The pH-adjusted samples were distributed in three new test tubes, each containing 5 ml sample, giving 45 samples all together. All samples were incubated for 2 h at 4 °C on a rotating test tube holder at 40 rpm. After measuring pH, all samples were analyzed by Raman, FT-IR, NIR and fluorescence spectroscopy. The experiment was conducted over three days, where samples from two pigs were prepared and analyzed day one and day two, and one pig on day three.

2.2. Spectroscopy and data analysis

2.2.1. Raman microspectroscopy

A droplet from each sample was placed on an aluminum plate with a plastic Pasteur pipette and left to dry for 2 h at room temperature prior to Raman spectroscopic analysis.

Raman spectra were recorded on a LabRam HR 800 Raman microscope (Horiba Scientific, France). The Raman system was equipped with a 785 nm laser used for excitation and was coupled confocally to a spectrograph with a focal length of 800 mm equipped with a grating of 600 g/mm. The laser light was tightly focused using a Fluotar ×50 objective (Leica, Germany, 0.55 NA). Scattered Raman photons from the sample were collected in the backscattered geometry by the same microscope objective and collected by the spectrometer. The confocal hole was set at 1000 μm. The spectrometer was equipped with an air-cooled deep depletion CCD array detector (1024 × 256 pixels). The laser power at the sample surface was approx. 90 mW, and for all samples an exposure time of 5 times 5 s was used in the range 300–1800 cm^{-1}. The spectra were calibrated to a standard silicon reference peak at 520.7 cm^{-1}. Three spectra were recorded from different locations on the dried droplet for each sample. Data acquisition and instrument control was carried out by using LabSpec software version 5.93.20 (HORIBA Jobin Yvon SAS).