

## MEAT PROTEOLYSIS BY PEPSIN HIGHLIGHTED BY MALDI IMAGING MASS SPECTROMETRY

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**Abstract – The aim of this study was to highlight the *in situ* hydrolysis of proteins to peptides on a muscle tissue section. In this context, a bovine muscle was incubated in a pepsin solution, cryo-fixed and sectioned. MALDI (Matrix Assisted Laser Desorption Ionization) Mass spectrometry imaging was used to obtain the ionic map of proteins directly on the muscle section. This method allowed us to localize the appearance and disappearance of proteins or fragments of proteins. The results give new insights into the mechanisms of enzyme action within muscle structure.**

### I. INTRODUCTION

Among muscle proteins, the contractile ones are the most relevant regarding nutritional considerations. They are localized in the intracellular space of muscle fibers, which are surrounded by connective tissue. Despite the poor nutritional value of intramuscular connective tissue, it might act as a barrier for digestive proteases to contractile proteins.

Meat ageing is applied to improve its organoleptic quality, in particular meat tenderness. In this context, the muscles are kept at 4°C until postmortem proteolysis leads to the breakdown of muscle structure, that improve muscle proteins bioaccessibility and therefore their muscle protein digestibility (Astruc et al. 2012). Meat ageing was described to improve *in vitro* digestibility of myofibrillar pork proteins (Bax et al., 2013). This highlights the effect of meat structure on the bio-accessibility of the digestive enzymes to myofibrillar proteins. However the role of the extracellular matrix as a barrier requires to be explored using a method avoiding proteins extraction and preserving the original form of the meat structure

The study aimed to monitor *in situ* the hydrolysis of proteins into peptides using mass spectrometry imaging.

### II. MATERIALS AND METHODS

#### Sample preparation

Bovine semitendinosus muscle was sampled and cut into 1.5 cm<sup>3</sup> pieces. Incubation was performed at 37°C for 2 hours in a solution of pepsin at 125 U / mg of proteins. The tubes were placed in ice to stop the enzyme action. The muscle pieces were then cryo-fixed in isopentane cooled by liquid nitrogen (-196 °C) and stored at -80°C until use.

#### Tissue Preparation

Histological sections (10 µm) were performed using a cryostat (Microm, Francheville, France) at -20°C, and were collected on conductive indium-tin-oxide glass slides (Bruker Daltonics, Bremen, Germany) for MALDI imaging. Muscle sections were subjected to washing steps using 70% and 95% Ethanol/H<sub>2</sub>O to deplete lipids, and were dried in a desiccator in 30 minutes. Serial sections were collected on glass slides for Hematoxylin-Eosin staining.

#### Maldi-MSI Sample Preparation

The matrix was applied using the ImagePrep station (Bruker Daltonics) with standard protocols. For protein imaging, the matrix was sinapinic acid (SA) at 10 mg/ml in water/acetonitrile 60:40 (v/v) with 0.2% trifluoroacetic acid.

#### Maldi-MSI Instrumentation

The MALDI spectra were acquired on an Autoflex Speed MALDI-TOF/TOF mass spectrometer with a Smartbeam laser using FlexControl 3.4 and FlexImaging 3.0 software packages (Bruker Daltonics). For protein imaging, ions were detected in positive linear mode at a mass range of m/z 2000 – 30000 with a sampling rate of 0.63 GS/s. The lateral resolution was set to 100 µm and a total of 500 laser shots were accumulated per pixel at

constant laser power. A protein standard (Bruker Daltonics, Bremen, Germany) was employed for external calibration of spectra, which was done externally on the same target before each measurement.

### Spectra analysis

Spectra from each imaging sequence were baseline-subtracted (TopHat) and smoothed (0.5  $m/z$  Savitzky–Golay window, 2 cycles), and peaks were chosen (thresholds of 1.5 S/N, total possible peaks at 1000, peak width 1.0 Da) by a batch processing macro written in FlexAnalysis software (Bruker Daltonics). Principal Component Analyses (PCA) were performed using ClinProTools-3.0 (Bruker).

## III. RESULTS AND DISCUSSION

The study aimed to link the muscular structure and the action of pepsin. To achieve this point, MALDI mass spectrometry imaging was performed. This method allows to detect proteins and/or protein fragments *in situ*, directly on a tissue section. The protein molecular map was acquired from  $m/z$  2000 to  $m/z$  30000; Fig. 1 represents the mean spectrum of the entire section.

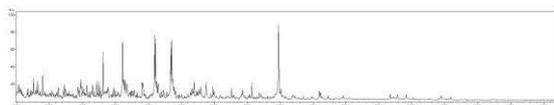


Figure 1. Mean spectrum of proteins acquired from  $m/z$  2000 to  $m/z$  30000 directly on semitendinosus bovine muscle.

A co-registration of images between the Hematoxylin-Eosin stained section and the section used for MALDI imaging allowed to define two parts within the tissue incubated in pepsin: a lighter and a darker one (Fig. 2). The hypothesis was as follows: the difference of staining was due to the enzyme action on the meat structure.

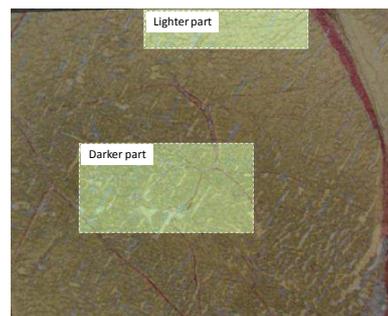


Figure 2. Co-registration of images between the Hematoxylin-Eosin stained section and the section used for MALDI of muscle tissue incubated in pepsin.

To validate this hypothesis, the spectra from each part (area within dashes on Fig.2) was extracted in order to visualize the mean spectrum from each area (Fig.3).

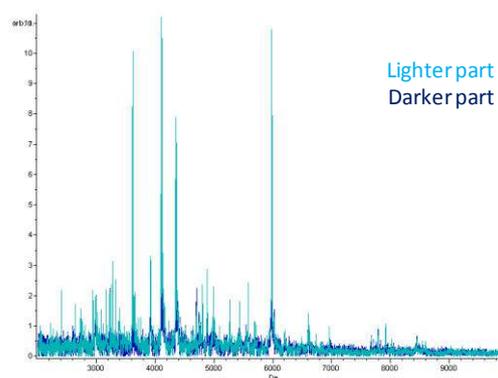


Figure 2. Mean spectra of proteins extracted from the lighter and the darker parts of the muscle tissue.

Principal component analysis was used to determine the sub-population within the spectra collection.

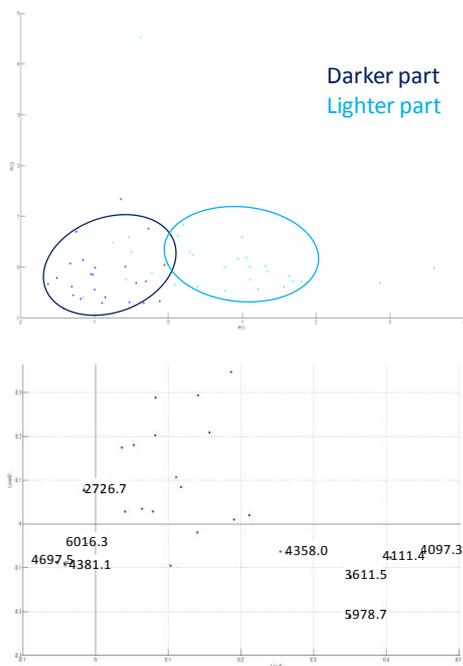


Figure 2. Principal component analysis

The results showed two sub-populations represented by spectrum of each tissue area respectively. The darker part was associated with  $m/z$  2726.7, 6016.3, 4697.5, and 4381.1. The lighter part was associated with  $m/z$  4358.0, 3611.5, 4111.4, 4097.3, and 5978.7. The first cluster of  $m/z$  seems to be linked to the non-degraded tissue part, and so may be the proteins non hydrolyzed by pepsin unlike in the lighter part. The second cluster of  $m/z$  may be the products of hydrolysis, resulting on the action of pepsin on the proteins.

#### IV. CONCLUSION

The results give new insights into the mechanisms of enzyme action within muscle structure. MALDI mass spectrometry imaging succeeded in studying in situ the action of pepsin within the tissue for the first time. Our results revealed specific  $m/z$  values from digested area and from non-digested area respectively. The next step of this study is to identify these protein ions, and then to identify the products and the protein targets of pepsin in bovine *semitendinosus* muscle.

#### ACKNOWLEDGEMENTS

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