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Quantification of peptides released during *in vitro* digestion of cooked meat

T. Sayd [a], C. Chambon [b], V. Santé-Lhoutellier [a,*]

[a] INRA UR 370, F 63122 Saint Genès Champanelle, France
[b] INRA UR 370 & PFEM cp, F 63122 Saint Genès Champanelle, France

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ABSTRACT

We aimed to identify and quantify the peptides generated during *in vitro* digestion of cooked meat by liquid chromatography coupled with high resolution mass spectrometer. A total of 940 non-redundant peptides in the gastric compartment and 989 non-redundant peptides in the intestinal compartment were quantified and identified. Among the 71 different proteins identified, 43 meat proteins were found in the two digestive compartments, 20 proteins were specific to the gastric compartment and 8 proteins to the intestinal compartment. In terms of estimation, the proteins involved in muscle contraction and structure were preferentially enzymatically hydrolyzed in the small intestine. The effect of cooking provided different but less clear patterns of digestion. To the best of our knowledge, this constitutes the highest number of peptides identified in beef meat digests and provides a comprehensive database for meat protein digestion associated with cooking conditions. Such quantitative and qualitative differences may have important nutritional consequences.

1. Introduction

Compared to vegetables and cereals, animal proteins have a higher protein content and a pattern of essential amino acids adapted to human requirements. But, the nutritional quality of a protein is also determined by its digestibility in the small intestine, which determines amino acid bioavailability. Moreover the protein digestion rate takes part of the nutritional quality since in the case of elderly, it is a limiting factor for *post prandial* muscle anabolism.

Tracking the fate of food proteins in the digestive tract leads to better understanding of the role of food structure in the bioavailability of food nutrients. In addition, this should eventually lead to formulating foods with improved positive health effects and reduced adverse health effects. The numerous scientific papers focusing on the digestion of food proteins reflect the interest devoted to this issue in parallel with the evolution of mass spectrometry developments (Mamone, Picariello, Caira, Addeo, & Ferranti, 2009; Picariello, Mamone, Nitride, Addeo, & Ferranti, 2013). Moreover, improvements in the sensitivity, mass accuracy and resolution of modern mass spectrometers have greatly increased the popularity of proteomics as an approach for describing protein digestion (Herrero, Simo, Garcia-Canas, Ibanez, & Cifuentes, 2012). Interestingly, the nutritional quality of proteins also refers to its ability to produce, during the digestion peptides, which certain can modulate bioactive activity such as antihypertension, antimicrobial resistance, etc. (Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011; Sanchez Rivera, Martinez-Maqueda, Cruz-Huerta, Miralles, & Recio, 2014).

Recent studies on the digestion of cooked meat using simple static *in vitro* assays have shown that cooking temperature affects the digestion rate of proteins more than digestibility (Bax et al., 2012, 2013). Bax et al. (2012) proposed a mechanism for explaining the increase in the rate of digestion observed when meat is cooked at 70 °C. Around this range of temperature, protein denaturation leads to significant changes in the conformation of proteins, favoring the bioaccessibility of the digestive proteases to their cleaving sites. An *in vivo* study with mini pigs confirmed the interest of cooking meat at a temperature under 75 °C to increase the *post* prandial occurrence of indispensable amino acids in the plasma (Bax et al., 2013). Protein oxidation superseded at higher temperatures, leading to protein aggregation (Bax et al., 2012). The impact of heat-induced structures on purified proteins from egg and milk (ovalbumine, lactoglobuline) and the ability to release peptides have been well characterized by the extent of proteolysis (Macierzanka et al., 2012; Nyemb, Guérin-Dubiard, et al., 2014) and the quantification of the peptides released (Nyemb, Jardin, et al., 2014). In this context studying the release of peptides from

* Corresponding author.
E-mail address: veronique.sante@clermont.inra.fr (V. Santé-Lhoutellier).

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a complex and structured matrix like meat remains a challenging issue To the best of our knowledge no characterization of meat proteins in different cooking conditions and the identification of their hydrolysates upon digestion has been published using a comprehensive approach. Here, we aimed to characterize the *in vitro* fate of meat proteins subjected to 3 cooking conditions in order to better understand the impact of processing on the nutritional quality of meat proteins. For this purpose, our goal was to identify and quantify all the peptides released using high resolution mass spectrometer, and therefore provide a, experimental dataset for the scientific community. In addition, we aimed to gain better understanding in the mechanisms underlying bioaccessibility of proteins to digestive enzymes.

2. Material and methods

2.1. Meat

2.1.1. Meat preparation

The meat was obtained from a 15 month-old Charolais bull. The *semimembranosus* muscles were excised from the carcass at the abattoir and aged for 15 days under vacuum. The meat was cut into slices 1 cm thick, put in a plastic bag, sealed and subjected to different cooking conditions. For each cooking condition, 8 slices weighing an average of 120 g were prepared. The temperature was recorded using a thermocouple device inserted in the core of the meat slices. Each slice was bagged and cooked in a water bath at 55 °C for 5 min, 70 °C for 30 min and 90 °C for 30 min. The duration of cooking started as soon as the core temperature was reached. The cooked meat was minced (8-mm grid) in order to mimic the step of chewing and 20 g samples of cooked meat were placed in a sealed bag at −80 °C until *in vitro* digestion.

2.1.2. In vitro digestion model

For each cooking condition, *in vitro* digestion of meat was carried out and repeated four times. The *in vitro* digestion model consisted of sequential digestion mimicking the gastric and intestinal compartments at 37 °C. To mimic the gastric compartment, 20 g of ground cooked meat was placed in a beaker filled with 200 ml of water at pH 2 and pepsin (125 U/mg protein, Sigma) and subjected to gentle stirring. The pH was recorded over 2 h. Aliquots of 2 ml were taken at 5 min, 30 min, 60 min and 120 min and put in hemolysis tubes. To stop hydrolysis the aliquots were immediately put on ice and the proteins were precipitated with cold trichloroacetic acid (15% final concentration). After 1 h, the tubes were centrifuged at 4000*g* at 4 °C for 15 min. After the collection of the last aliquot at 120 min, the pH of the digestive solution described above was raised by adding 50 mM of bicarbonate buffer (pH 8.2) with a ratio of 1:1.6 (v/v) to obtain a final pH of 7. Then, trypsin (34.5 U/mg protein, Sigma) and chymotrypsin (0.44 U/mg protein, Sigma) were added to this medium to mimic the intestinal compartment. After 120 min, a 2 ml aliquot was taken. To stop hydrolysis the aliquot was immediately put on ice and the proteins were precipitated with cold trichloroacetic acid (15% final concentration) for 1 h. Then the tubes were centrifuged at 4000*g* at 4 °C for 15 min.

2.1.3. Peptide extraction

Peptide extraction was conducted for the gastric and intestinal aliquots. Peptide extraction was performed using porous silica nanoparticles MCM-41 (Sigma) according to Tian et al. (2009), with some modifications. Twenty five mg of MCM-41 nanoparticles were hydrated with 1 ml of TCA 3%. The resulting slurry was mixed and processed ultrasonically. Immediately, 1 ml of the gastric or intestinal sample resulting from the TCA precipitation described above was added and shaken for 2 h at 4 °C. Then the suspension was centrifuged for 15 min at 4000*g* and the supernatant was removed. The silica nanoparticles were then washed 3 times with 1 ml H_2O. The peptides retained on the MCM-41 porous silica nanoparticles were eluted with 1 ml of acetonitrile 80%. To create a reference spectrum for each compartment, the 48 gastric samples were mixed at equal volume (50 µl) to constitute a "gastric sample mix" and the 12 intestinal samples were mixed at equal volume (50 µl) to constitute an "intestinal sample mix". The samples were kept at −20 °C until used.

2.1.4. Liquid chromatography and mass spectrometry

An UltiMate 3000 Rapid Separation LC (RSLC) system (ThermoFisher-Dionex, Villebon sur Yvette, France) was used to separate the peptides extracted. Buffer A (0.5% trifluoroacetic acid in water) and buffer B (0.5% trifluoroacetic acid in 80% acetonitrile, 20% H2O) were used as mobile phases for gradient separation. 10 µl of extracted digestates was automatically loaded onto a commercial C18 reversed phase column (Phenomenex, Aeris peptide, C18, 250 × 2.1 mm) with 4% buffer B at a flow rate of 0.5 ml/min, followed by three step gradient separation. The 41 min-gradient was 30 min from 4% B to 35% B, 10 min to 70% B, 1 min to 100% and then maintained for 5 min. The column was equilibrated for 10 min with 4% B prior to the next analysis.

The eluted peptides from the column were electro sprayed through a capillary tip for ESI in a LTQ-Orbitrap Velos (ThermoFisher, Villebon sur Yvette, France). The LTQ-Orbitrap VELOS was operated in data-dependent top five mode. Thus the five most intense peaks over the range m/z 300–1400 (mass resolution 30,000, in orbitrap) with charge state $\geqslant 2$ were fragmented and dynamic exclusion was activated. Therefore, after 2 MS/MS on the same ion (tolerance 10 ppm), the latter was excluded for 120 s. Next, raw files were processed for quantification with Progenesis QI (nonlinear Dynamics, Waters) software.

2.1.5. Label-free peptide quantification and identification

The spectra (Thermo raw files) acquired from gastric and intestinal samples were loaded separately into the Progenesis QI software (Waters). The following procedure was applied for both analyses (gastric and intestinal): the profile data of the MS scans and MS/MS scans were transformed into peak lists with Progenesis QI using a personal peak-modeling algorithm, giving positional information (m/z and retention time) and peptide abundance. To compare all the expression profiles and compensate for between-run variation, a "gastric sample mix" and an "intestinal sample mix" were set as reference in order to align the retention time of all other mixes using automatic alignment. Then, the features with only one charge were excluded from the analysis. A normalization factor was calculated to correct the experimental variation. Then the "within subject" analysis design was chosen. A single subject was sampled from the cooked temperature group and the gastric digestion duration. A similar analysis was performed for the intestinal compartment. The MSMS list of all the peaks was exported from the Progenesis QI software as the mascot file (.mgf) and used for peptide identification with MASCOT (V 2.2) in the Swissprot_*Bos taurus* database (89,800 seq). There was no specific enzyme cleavage and a 10 ppm Da peptide mass tolerance and a 0.8 Da fragment mass tolerance were used, and two missed cleavages and methionine oxidation were set as variable modifications. Only unique peptides from a unique protein accession number with a mascot ion score of $\geqslant 41$ were considered as validated ($p < 0.05$). Protein abundance was calculated by summing the peptide abundances allocated to the respective protein. We chose to set a minimum of two unique peptides to validate protein identification. In the case of myosin heavy chain, for which 3 isoforms are present in the semimembranosus muscle (Oe, Nakajima, Muroya,