# *IN VITRO* PEPSIN RATE OF DIGESTION INCREASED BY HEATING: ASSOCIATED PROTEOMIC MARKERS

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***Abstract*— The meat undergoes numerous transformations before being consumed which may alter the biochemical composition and structure of proteins, and hence change their nutritional properties. The aim of the study was to evaluate the effect of ageing and heating on *in vitro* pepsin digestion rate and to find proteomic markers associated to this parameter. LD pig muscles were aged for 4 days and heated at 70°C. Proteome analysis of myofibrillar proteins was performed at Day 1. *In vitro* pepsin digestion rate was measured as well as physicochemical analysis (carbonyls, hydrophobicity, aggregation, TBARS...), SOD, Catalase, glycolytic potential. This study demonstrated that a temperature of 70°C increased the *in vitro* pepsin digestion rate, unlike ageing. This can be possibly explained by the conformational changes of the proteins occuring, leading to a better access for the protease to its cutting sites. At this temperature, less protein oxidation was noted compared to lipid oxidation. Numerous spots were correlated to the *in vitro* pepsin digestion rate for the different conditions (raw, aged and heated meat).**

***Index Terms*— ageing, denaturation, heating, *in vitro* digestion rate, oxidation, proteome**

## I. INTRODUCTION

For many years, the research effort on the quality of meat has focused on the sensory and technological dimensions of quality with special emphasis on the search of biological determinants (Sayd et al., 2006; Laville et al., 2007; Kwasiborski, et al., 2008). Nowadays consumers are aware of the impact of food on their health. Thus, in addition to sanitary and sensorial aspects, the nutritional quality becomes an important factor in the choice of food, especially in a context of an increasing demand of more elaborated foodstuffs. The meat is a complex type of food which undergoes numerous transformations before being consumed (maturation, preservation, mincing, cooking,…), technological processes which may alter the biochemical composition and structure of proteins, and hence may change their nutritional properties. Recent studies showed that meat cooked at high temperature induced oxidation by the formation of carbonyls groups, disulfure bridges and also protein denaturation (Santé-Lhoutellier, Engel, Aubry & Gatellier, 2008; Santé-Lhoutellier, Astruc, Marinova, Greve & Gatellier, 2008). Lower temperatures are often used for pork. The aim of the study was to evaluate the effect of ageing and heating on pepsin digestion rate and to find proteomic markers associated to this parameter.

## II. MATERIALS AND METHODS

The experiment was carried out on 12 pigs, slaughtered at about 140kg live weight in a commercial slaughterplant. After one day, the muscle *Longissimus dorsi* was removed from each carcass. One part of the muscles was frozen in liquid nitrogen and stored at -80°C (samples Day 1), while the other part was stored for 3 days at 4°C under air permeable film before being frozen and stored at -80°C until analysed (samples D4). The heating procedure was only performed on samples aged 4 days. The meat (3 g) was confined in polypropylene tubes and heated at 70°C during 30min in a digital temperature-controlled dry bath (BT3-heater, Prolabo). Then the samples were immediately put in ice. Cooking loss was estimated by sample weight before and after cooking and was expressed as percentage. Biochemical analyses were performed on raw meat at Day 1 and Day 4 and on cooked meat at Day 4. Protein carbonyl groups were evaluated by the method of Oliver, Alin, Moerman, Goldstein, & Stadtman (1987) with slight modifications. Hydrophobicity of myofibrillar proteins was determined using the hydrophobic chromophore bromophenol blue (BPB) according to Chelh, Gatellier, & Santé-Lhoutellier (2006) with slight modifications. Lipid oxidation was measured by the ThioBarbituric Acid Reactive Substances (TBARS) method according to the method Lynch & Frei (1993) modified by Mercier, Gatellier, Viau, Remignon, & Renerre (1998). Glycogen and lactate in the muscle were determined according to Dalrymple & Hamm (1973) and Bergmeyer (1974) and the glycolytic potential was calculated according to Monin & Sellier (1985). Protein aggregates were analysed by granulometry on the total

protein extract with a first step of delipidation according to Promeyrat et al (2010). Surface hydrophobicity of the total protein was evaluated. The antioxidant potential was evaluated on raw meat by measuring activities of Superoxyde Dismutase (SOD) and Catalase according to Gatellier, Mercier, & Renerre (2004). *In vitro* rate of digestion of myofibrillar protein by pepsin was performed. Myofibrillar proteins were washed in 33mM glycine buffer at pH 1.8 and the final concentration was adjusted at 0.8 mg/ml. Proteins were digested by gastic pepsin (Porcine gastric mucosa, Sigma) at the concentration 50U/mg myofibrillar proteins during 120min at 37°C. Digestion was terminated by addition of 15% (final concentration) trichloroacetic acid (TCA) at various times (0, 5, 10, 15, 20, 25, 30, 40, 60, 90, 120min). After centrifugation for 15min at 4000g, the content of hydrolysed peptides in the supernatant was measured at 280nm and expressed as optical density units by hour (ΔOD/hour). The proteomic analysis was performed on the myofibrillar proteins from samples Day 1. Muscle was homogenised using a glass bead agitator MM2 (Retsch, Haan, Germany) in 40mM Tris-HCl (pH 8), 2mM EDTA and a protease inhibitors cocktail (Sigma) at 4°C at the ratio of 1:4 (w/v) for 45min. The homogenate was centrifuged at 10°C for 30min at 10000g. After discarding the supernatant, the pellet was then homogenised during 10min at 4°C, centrifuged at 10°C for 15min at 10000g and the supernatant was discarded. The pellet was then washed three times. The pellet was dissolved in 7M urea, 2M thiourea, 4% CHAPS, a protease inhibitors cocktail, 100mM EDTA and 1% DTT at 4°C at the ratio 1:4 (w/v) for 30min, centrifuged at 10°C for 15min at 10000g. The supernatant, referred to as the myofibrillar extract, was stored at -80°C. The protein concentration was determined by the RCDC assay (Biorad). One milligram of myofibrillar proteins was solubilised in a rehydratation solution (7M urea, 2M thiourea, 2% CHAPS, a protease inhibitors cocktail, 1%DTT, ampholytes and bromophenol blue). Samples were loaded onto immobilized pH gradient strips (pH 3-10 NL, 17cm, Biorad), and isoelectric focusing was performed using a Protean IEF cell system (Biorad). Gels were passively rehydrated for 16h. Rapid voltage ramping was subsequently applied to reach a total of 85kVh. Focused IPG strips were equilibrated for 15min in 6M urea, 30% glycerol, 2% SDS, 50mM Tris pH 8.8, 1% DTT and then for 20min in the same solution where DTT was replaced by 2.5% iodoacetamide and bromophenol blue were added. After equilibration, proteins were separated in the second dimension on 11% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with the Protean II XL system (Biorad) at constant 15mA by gel for 1 hour at 40V and 110V until the run was completed. Gels were Coomassie blue (colloidal blue G250) stained and analysed using Progenesis Samespots Nonlinear software.

Analysis of variance (ANOVA) was performed using the General Linear Model (GLM) procedure of SAS system. The linear model included fixed effects of ageing and treatment. When significant effect of ageing and treatment were encountered, least squares means were compared using LSMEANS with PDIFF option and TUKEY adjustment. Principal Component Analysis was performed using Statistica 7.1 (StatSoft), Pearson correlation coefficients were calculated between the *in vitro* rate of digestion, the physicochemical parameters and the proteomic data.

## III. RESULTS AND DISCUSSION

**Table 1:** *In vitro* digestion rate of proteins by pepsin, physico-chemical and granulometry parameters, glycolytic potential, SOD and Catalase, TBA-RS and cooking loss of raw muscle *Longissimus dorsi* (Day 1 and Day 4) and on cooked muscle (Day 4 – 70 C) muscle LD.

| | Raw meat | | Cooked meat 30 min | Significance | |
|---|---|---|---|---|---|
| | Day 1 | Day 4 | Day 4 - 70°C | $p_{(D1-D4)}$ | $p_{(D4 - D4 (70°C))}$ |
| Pepsin rate of digestion (ΔOD*10/h) | 2.508 ± 0.051 $^{b}$ | 2.526 ± 0.064 $^{b}$ | 3.330 ± 0.096 $^{a}$ | NS | <0.0001 |
| Carbonyls (nmol of DNPH/mg of protein) | 1.345 ± 0.017 $^{c}$ | 1.923 ± 0.040 $^{a}$ | 1.662 ± 0.057 $^{b}$ | <0.0001 | 0.0051 |
| Total delipided protein hydrophobicity (µg bound BPB ) | 181.216 ± 5.430 $^{b}$ | 179.802 ± 5.655 $^{b}$ | 228.717 ± 6.549 $^{a}$ | NS | <0.0001 |
| Myofibrillar protein hydrophobicity (µg bound BPB ) | 67.665 ± 3.094 $^{b}$ | 61.667 ± 4.282 $^{b}$ | 222.336 ± 3.679 $^{a}$ | NS | <0.0001 |
| **Granulometry parameters** | | | | | |
| Particles number | 25293.69 ± 3322.69 $^{ab}$ | 34915.73± 2690.05 $^{a}$ | 16763.77 ± 4120.54 $^{b}$ | NS | 0.0030 |
| EC Diameter (µm) | 13.432 ± 0.280 $^{a}$ | 13.597 ± 0.135 $^{a}$ | 8.830 ± 0.232 $^{b}$ | NS | <0.0001 |
| Circularity | 0.748 ± 0.008 $^{c}$ | 0.788 ± 0.004 $^{b}$ | 0.821 ± 0.008 $^{a}$ | 0.0017 | 0.0117 |
| Aspect Feret Ratio | 0.730 ± 0.004 $^{b}$ | 0.752 ± 0.002 $^{a}$ | 0.749 ± 0.005 $^{a}$ | 0.0022 | NS |
| SOD (I.U.) | 1.079 ± 0.056 | 1.259 ± 0.087 | | NS | |
| Catalase (nmol $H_2O_2$/min/mg prot) | 2080.890 ± 188.995 | 2134.367 ± 131.768 | | NS | |
| Glycolytic Potential (µmol/g) | 110.450 ± 2.520 | 108.580 ± 4.754 | | NS | |
| Glycogen (µmol eq lactate/g) | 9.3222 ± 0.9131 | 8.253 ± 1.094 | | NS | |
| Lactate (µmol/g) | 91.805 ± 1.879 | 92.074 ± 3.623 | | NS | |
| TBA-RS (mg MDA/kg of meat) | 0.098 ± 0.025 $^{b}$ | 0.263 ± 0.053 $^{b}$ | 0.963 ± 0.161 $^{a}$ | NS | <0.0001 |
| Cooking loss (%) | | | 21.51 ± 0.78 | | |

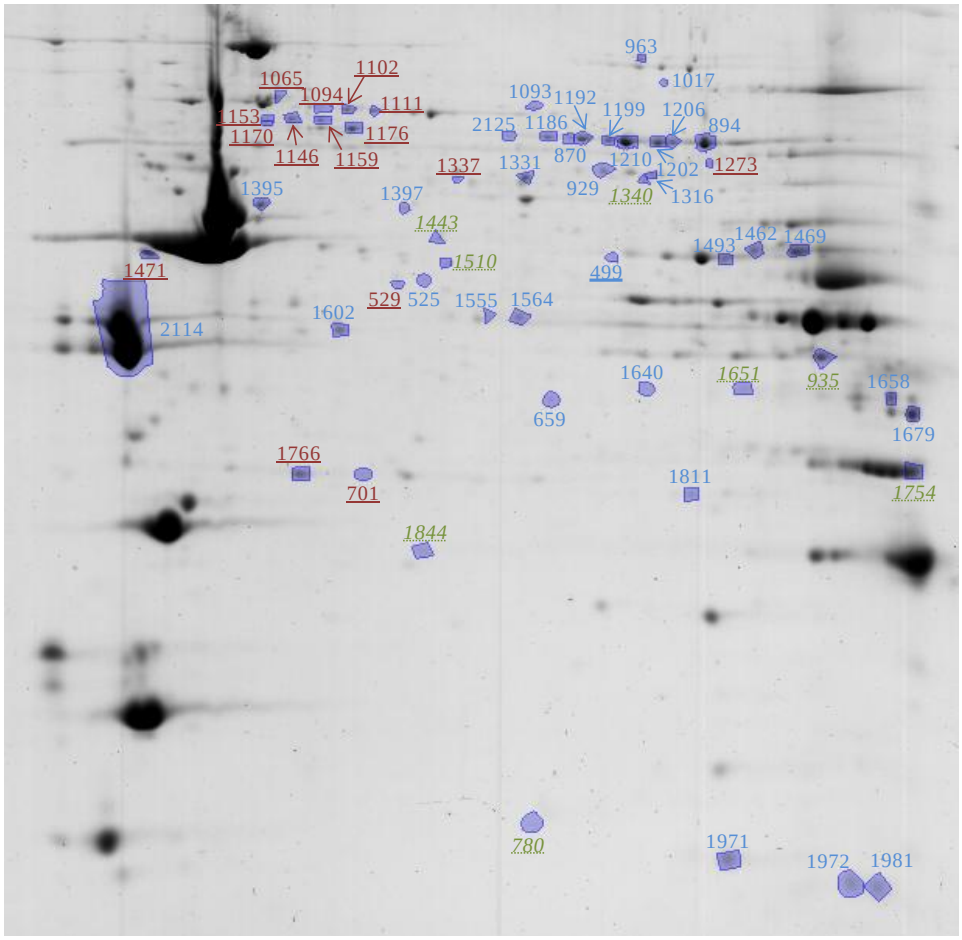
Values are means ± SEM. *p* value from the ANOVA analysis. $^{a, b, c}$ show significant differences between treatment groups ($p$<0.05)

The rate of *in vitro* digestion by pepsin was only affected by heating treatment. An increase of 28 % was noted. The carbonyls content increased during ageing and decreased slightly after a heating treatment at 70°C. On the contrary surface hydrophobicity of myofibrillar proteins and of total delipided protein extract remained stable during ageing. A increase of 80% to 300% was found according the protein fraction studied (total extract and myofibrillar proteins, respectively) when the meat was heated. These protein modifications could have created news interactions inside the

molecule as well as outside, At 70°C, the heating treatment has induced a protein denaturation leading to conformational changes and an increase of hydrophobic sites at the protein surface. Consequently, the protein structure changes occuring at 70°C would have improved the accessibility for the pepsin to its cutting sites (the aromatic amino acids: Phe, Tyr, Trp) by favouring exposed hydrophobic sites.

Moreover the granulometry parameters showed a decrease of the particules number and the EC diameter after heating treatment, which can be explained by modified interactions between the particules. The increase of the circularity and the Aspect Feret Ratio reinforce this hypothesis. In other words, all these parameters underlined aggregation phenomena induced by the heating treatment and at a lower level by ageing. An increase of the circularity express the loss of the fibrous form of myofibrils protein. Similarly, Promeyrat et al. (2010) reported an lesser increase of circularity for meat heated at 100°C.

SOD, Catalase and the glycolytic potential remained the same during ageing. Our results agreed with those reported by Renerre et al. (1996) for beef meat aged 10 days. We did not evaluate these parameters after heating because of the thermal lability of the studied enzymes. Contrary to the proteins oxidation, lipid oxidation increased during ageing and rose sharply after heating at 70°C. The amount of oxidized lipids was multiplied by 10. The cooking loss averaged 22% after heating treatment at 70°C during 30 min, which is in agreement with others studies for the same temperature (Aasslyng et al., 2003; Garcia-Segovia et al., 2007)




| Spots | Treatment | Correlation coefficient R |
|---|---|---|
| 525 | Day 1 | -0.6538 |
| 659 | Day 1 | -0.5888 |
| 870 | Day 1 | -0.5922 |
| 894 | Day 1 | -0.6012 |
| 929 | Day 1 | -0.6426 |
| 963 | Day 1 | -0.6955 |
| 1017 | Day 1 | -0.602 |
| 1093 | Day 1 | 0.7306 |
| 1186 | Day 1 | -0.7723 |
| 1192 | Day 1 | -0.7581 |
| 1199 | Day 1 | -0.5903 |
| 1202 | Day 1 | -0.6254 |
| 1206 | Day 1 | -0.7284 |
| 1210 | Day 1 | -0.6986 |
| 1316 | Day 1 | -0.5771 |
| 1331 | Day 1 | -0.7052 |
| 1395 | Day 1 | 0.5993 |
| 1397 | Day 1 | -0.6011 |
| 1462 | Day 1 | -0.6416 |
| 1469 | Day 1 | -0.6333 |
| 1493 | Day 1 | -0.6957 |
| 1555 | Day 1 | -0.9223 |
| 1564 | Day 1 | -0.7667 |
| 1602 | Day 1 | 0.5804 |
| 1640 | Day 1 | 0.6004 |
| 1658 | Day 1 | -0.5789 |
| 1679 | Day 1 | -0.5791 |
| 1811 | Day 1 | -0.6132 |
| 1971 | Day 1 | -0.7326 |
| 1972 | Day 1 | -0.7266 |
| 1981 | Day 1 | -0.6409 |
| 2114 | Day 1 | 0.5801 |
| 2125 | Day 1 | -0.7564 |
| 499 | Day 1/ Day 4 | 0.6251/ 0.6549 |
| 529 | Day 4 | 0.6587 |
| 701 | Day 4 | -0.8284 |
| 1065 | Day 4 | -0.6687 |
| 1094 | Day 4 | -0.6955 |
| 1102 | Day 4 | -0.6654 |
| 1111 | Day 4 | -0.5863 |
| 1146 | Day 4 | 0.6617 |
| 1153 | Day 4 | 0.6481 |
| 1159 | Day 4 | 0.6475 |
| 1170 | Day 4 | 0.594 |
| 1176 | Day 4 | -0.5931 |
| 1273 | Day 4 | 0.5960 |
| 1337 | Day 4 | 0.6332 |
| 1471 | Day 4 | 0.6394 |
| 1766 | Day 4 | -0.7929 |
| 780 | 70°C | 0.6355 |
| 935 | 70°C | -0.6234 |
| 1340 | 70°C | -0.5862 |
| 1443 | 70°C | -0.6112 |
| 1510 | 70°C | -0.6521 |
| 1651 | 70°C | -0.6290 |
| 1754 | 70°C | 0.6674 |
| 1844 | 70°C | 0.6586 |

**Figure 1:** 2-D gel map (strips pH 3-10 NL, 11% SDS-PAGE gel) of myofibrillar proteins of pig *Longissimus dorsi* muscle extracted at Day 1. Spots show proteins which are correlated (opposite table) with the proteolysis rate of pepsin on raw meat at Day 1, at Day 4 (underlined) and on cooked meat at 70 C (italic and dot lined)

Figure 1 shows the spots correlated with the rate of pepsin digestion on raw meat (Day 1), after ageing (Day 4) and on cooked meat (70°C). A total of 57 spots were correlated with the rate of pepsin digestion: 34 at Day 1, 15 at Day 4 on raw meat and 8 spots on cooked meat at 70°C. The spots positively correlated with the pepsin digestion rate at Day 1 and Day 4 were mostly found on the acidic zone of the gel (left part) whereas the spots negatively correlated with the pepsin digestion rate at Day 1 and Day 4 were found in the basic zone of the gel. Most of the spots correlated with the pepsin digestion rate at Day 1 were on the right part of the gel and spread from the top to the bottom of the gel. On the contrary, the spots correlated with the pepsin digestion rate at Day 4 were grouped on the left corner, showing molecular weights between 30kDa to 100kDa. One spot (# 499) was correlated with both the pepsin digestion rate at Day 1 and Day 4. No specific position was found for the spots correlated with the pepsin digestion rate after cooking (70°C). It is noteworthy that the number of spots correlated with pepsin rate of digestion decreased with *post mortem* time and with heating treatment, which could be explained by *post mortem* modification : proteolysis during ageing and protein denaturation after heating treatment.

## IV. CONCLUSION

This study demonstrated that a temperature of 70°C increased the *in vitro* pepsin digestion rate, unlike ageing. This can be possibly explained by the conformational changes of the proteins occuring, leading to a better access for the protease to its cutting sites. At this temperature, less protein oxidation was noted compared to lipid oxidation. Numerous spots were correlated to the *in vitro* pepsin digestion rate for the different conditions (raw, aged and heated meat). Furthermore, the number of spots correlated with pepsin rate of digestion decreased with *post mortem* time and with heating treatment, which could be explained by *post mortem* modification: proteolysis during ageing and protein denaturation after heating treatment. Identification of spots of interest is in progress. Further work would be needed to validate our findings with different pepsin levels.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