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Cooking Temperature Is a Key Determinant of in Vitro Meat Protein Digestion Rate: Investigation of Underlying Mechanisms

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ABSTRACT: The present study aimed to evaluate the digestion rate and nutritional quality of pig muscle proteins in relation to different meat processes (aging, mincing, and cooking). Under our experimental conditions, aging and mincing had little impact on protein digestion. Heat treatments had different temperature-dependent effects on the meat protein digestion rate and degradation potential. At 70 °C, the proteins underwent denaturation that enhanced the speed of pepsin digestion by increasing enzyme accessibility to protein cleavage sites. Above 100 °C, oxidation-related protein aggregation slowed pepsin digestion but improved meat protein overall digestibility. The digestion parameters defined here open new insights on the dynamics governing the in vitro digestion of meat protein. However, the effect of cooking temperature on protein digestion observed in vitro needs to be confirmed in vivo.

KEYWORDS: *meat protein, oxidation, aggregation, in vitro digestion, processing*

INTRODUCTION

Consumers are aware of the impact of food on their health. In addition to food hygiene and sensory aspects, nutritional quality is becoming an increasingly important factor in food choices, especially against a background of increasing demand for convenience foods.

Meat is regularly given a "negative" health image. Epidemiological studies report that red meat is linked to cardiovascular disease and colon cancer.[1] The basis of these links has recently been challenged,[2,3] and even the link between meat cooking and risk of colorectal cancer is now being called into question.[4] A consensus has emerged that moderate meat consumption as part of a balanced diet is unlikely to increase the risk of cardiovascular disease or colon cancer. Meat is also a major source of proteins and a valuable source of micronutrients (iron, zinc, selenium, and vitamins B6 and B12), providing all of the body's essential amino acids. However, the nutritional quality of a protein source cannot be determined by amino acid composition alone; other factors such as digestibility in the small intestine, which determines amino acid bioavailability, and protein digestion rate, which shapes whole-body metabolism of the absorbed amino acids,[5] must also be taken into account. Few studies have focused on the determining of these parameters for meat products in human diet. It has been reported that grilled beef steak has high protein digestibility in the small intestine (about 95%) and high speed of digestion,[6,7] but it remains unknown how these parameters are affected by different meat-processing practices.

Meat storage and technological treatments such as mincing, salting, or cooking all assist in achieving a safe and palatable product; however, these treatments can play a significant role in the development of oxidation and denaturation processes, because they affect not just the structural properties of the meat[8] but also the physical−chemical state of the meat proteins and the bioavailability of their constitutive amino acids.[9−11] The oxidation of protein amino acid residues generates oxidation products, protein−protein cross-linkages, and protein fragmentation.[12,13] Furthermore, Schiff bases can also form in meat via the interaction between proteins and aldehyde products formed during lipid oxidation[14] or from reducing sugars.[15] It has been shown that meat protein aggregation during cooking is linked to the increase in protein surface hydrophobicity (i.e., protein insolubility[11,16]), which can reduce the rate of protein digestion by digestive tract enzymes.[9−11,17,18] Due to the complexity of in vivo approaches designed for studying digestion,[19] the ability to estimate meat protein digestion parameters in vitro is extremely valuable for assessing the nutritional qualities of meat products. Studies have tackled this issue over the past few years,[9,17,18,20,21] but without determining the kinetics involved. Furthermore, the connection between digestion and chemical oxidation of meat proteins is not yet fully elucidated, and results frequently diverge. The present study aimed to evaluate the digestion rate and nutritional quality of pig muscle proteins in relation to different meat processes (aging, mincing, and cooking). In vitro digestion parameters (initial slope, maximal degradation, and half-life time) are presented and discussed in connection with antioxidant composition, glycolytic potential, lipid and protein oxidation, denaturation, and aggregation.

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MATERIALS AND METHODS

Animals and Aging. The experiment was carried out on 12 male commercial Basque pigs (6 months old) slaughtered at about 100 kg liveweight in a commercial slaughterhouse. After 24 h, the Longissimus dorsi muscle was removed from each carcass. One part of the muscle was frozen in liquid nitrogen and stored at −80 °C (D1 samples), whereas the other part was kept for 3 days at 4 °C under air-permeable film before being frozen in liquid nitrogen and stored at −80 °C until sample preparation and analysis (D4 samples).

Sample Preparation and Heating Procedure. The heating procedure was performed on only D4 samples. Three temperatures were applied: 70, 100, and 140 °C. These temperatures were chosen to represent cooked ham, boiled meat, and grilled meat. Thawed meat samples (3 g/tube) were placed in polypropylene test tubes (inner diameter = 10 mm, thickness = 1 mm) and heated for 30 min in a digital temperature-controlled dry bath (BT3-heater from Prolabo). The heat treatment was then immediately stopped by placing the samples on ice. Samples were weighed and immediately frozen at −80 °C. Cooking loss (CL) was calculated as the ratio of postcooking weight to precooking weight, expressed as a percentage (% CL = [(thawed weight − cooked weight)/thawed weight] × 100).

A mincing procedure was also applied to evaluate the combined effect of mincing and heating. Six grams of frozen muscle cut into 10−12 pieces of around 5 mm each was minced using a Waring blender (Waring, Hartford, CT, USA) by applying four 5 s pulses and then kept for 1 day at 4 °C under air-permeable film before being cooked as described above. For technical reasons, only the two extreme temperatures (70 and 140 °C) were applied on these minced meat samples.

Antioxidant Enzyme Activities. After thawing, muscle samples (1 g) were homogenized with 10 mL of 50 mM phosphate buffer (pH 7.0) in an Ultra Turrax homogenizer (IKA T25 Digital, Staufen, Germany), centrifuged at 4000*g* for 15 min at 4 °C, and the supernatant was collected. Protein concentration in supernatant was determined according to the biuret method. Total superoxide dismutase (SOD) activity (Cu−Zn-SOD and Mn-SOD) was measured, in duplicate, according to the method of Marklund and Marklund[22] using inhibition of pyrogallol autoxidation in a basic medium. One unit (U) was taken as the activity that inhibits pyrogallol autoxidation by 50%. Catalase activity was measured, in duplicate, by the rate of decomposition of H_2O_2 at 240 nm according to the method of Aebi[23] and expressed as nanomoles of decomposed H_2O_2 per minute per milligram of protein.

Muscle Glycogen, Lactate, and Glycolytic Potential. Glycogen, glucose, glucose-6-phosphate, and lactate from muscle homogenate were measured, in duplicate, by enzymatic methods according to the methods of Dalrymple and Hamm[24] and Bergmeyer,[25] with slight modifications. Muscle tissue (1 g) was homogenized with 10 mL of 0.5 M perchloric acid. Aliquots of homogenate (0.5 mL) were taken for enzymatic determination of glycogen, glucose, and glucose-6-phosphate after glycogen hydrolysis with amyloglucosidase. Lactic acid was determined in the supernatant from homogenate centrifuged at 4000*g* for 20 min at 4 °C and expressed in micromoles per gram of fresh tissue. Glycolytic potential (GP, i.e., the amount of compound that can be transformed into lactic acid) was calculated at D1 and D4 using the following formula:[26]

$$GP = 2 \times ([\text{glycogen}] + [\text{glucose-6-phosphate}] + [\text{glucose}]) + [\text{lactic acid}]$$

GP was expressed as micromoles of equivalent lactate per gram of fresh tissue.

Biochemical Parameters. All biochemical parameters were assessed on either fresh or cooked samples in duplicate.

Determination of Carbonyl Content. Protein carbonyl groups were evaluated according to the method of Oliver et al.,[27] with slight modifications. A 1 g muscle sample was homogenized in 10 mL of 0.15 M KCl + 0.1 mM BHT for 60 s in an Ultra Turrax homogenizer. Carbonyl groups were detected by reactivity with 2,4-dinitrophenylhydrazine (DNPH), leading to the formation of protein hydrazones. Carbonyl content was determined in fresh and cooked meat and expressed as nanomoles of DNPH fixed per milligram of protein.

Determination of Protein Surface Hydrophobicity. The hydrophobicity of myofibrillar proteins was determined using hydrophobic chromophore bromophenol blue (BPB) as described in Chelh et al.,[28] with slight modifications. This method is based on fixing BPB with insoluble myofibrillar proteins and quantifying the amount of bound BPB. Myofibrillar proteins were suspended at a concentration of 3 mg/mL in 20 mM phosphate buffer at pH 6. One milliliter of myofibril suspension was added to 300 μL of 1 mg/mL BPB (in distilled water) and mixed well. A myofibril-free control sample (blank) was performed by adding 300 μL of 1 mg/mL BPB (in distilled water) to 1 mL of 20 mM phosphate buffer at pH 6. Samples and control samples were continually shaken at room temperature for 10 min and then centrifuged at 4000*g* for 15 min at 4 °C. Supernatant absorbance corresponding to free BPB was measured at 595 nm against a phosphate buffer blank. The amount of bound BPB (μg) was calculated as the difference between total and free BPB and used as an index of hydrophobicity. Each determination was performed in duplicate. Bound BPB (μg) = 300 μg × (OD control − OD sample)/OD control.

Determination of Lipid Oxidation. Lipid oxidation was measured by using the thiobarbituric acid-reactive substances (TBA-RS) method according to Lynch and Frei,[29] with slight modifications. Muscle samples (1 g) were homogenized with 10 mL of 0.15 M KCl + 0.1 mM butyl hydroxytoluene (BHT) in an Ultra Turrax homogenizer. Homogenates (0.5 mL) were incubated with 1% (w/v) 2-thiobarbituric acid in 50 mM NaOH (0.25 mL) and 2.8% (w/v) trichloroacetic acid (0.25 mL) for 10 min in a boiling water bath. After cooling at room temperature for 30 min, the pink chromogen was extracted with *n*-butanol (2 mL), and its absorbance was measured at 535 nm against an *n*-butanol blank. TBA-RS concentrations were calculated using 1,1,3,3-tetraethoxypropane (0−0.8 μM) as standard and expressed as milligrams of malondialdehyde (MDA) per kilogram of meat (TBA units).

Measurements of Protein Aggregation. A meat sample (0.5 g) was homogenized in 5 mL of a 0.15 M ice-cooled KCl solution containing 0.1 mM of BHT, using a an Ultra Turrax homogenizer. Homogenization was performed via a short burst (20 s) at low speed (1100 rpm) to minimize protein denaturation. The protein concentration of the meat extract was then estimated by using the biuret method and adjusted to 3 mg/mL with KCl + BHT solution. The lipids were removed from this whole meat extract by adding 2 volumes of butanol/di-isopropyl ether (40:60 v/v) to 1 volume of meat extract. The mixture was stirred for 30 min at 4 °C and then centrifuged at 4000*g* for 15 min at 4 °C to separate the aqueous phase from the organic phase. The organic phase was removed by careful suction with a micropipet.

Granulometry measurements were performed in triplicate on 6 mg of proteins in 1.5 mL of this meat extract using a Sysmex flow particle image analyzer (FPIA-3000; Malvern Instruments Ltd., Malvern, U.K.) in low-power field mode according to the method of Promeyrat et al.[16] The sample was injected into a sample chamber and homogenized by a mixing rotor. Particle numbers were measured, and particle size and form were evaluated by equivalent circle (EC) diameter, circularity, and Feret aspect ratio.

In Vitro Digestibility. *In Vitro Pepsin Digestion.* Myofibrillar proteins were suspended in 33 mM glycine buffer, pH 1.8, and the final concentration was adjusted to 0.75 mg/mL. Proteins were hydrolyzed by gastric pepsin (porcine gastric mucosa, EC 3.4.23.1, ref P7012, Sigma) at a concentration of 10 U/mg of myofibrillar proteins for 90 min at 37 °C. Digestion was terminated by the addition of 15% (final concentration) trichloroacetic acid at various time points (0, 5, 10, 15, 20, 25, 30, 40, 60, and 90 min). Samples were then placed on ice for 1 h. After centrifugation at 4000*g* for 15 min at 4 °C, hydrolyzed peptide content in the supernatant was measured at 280 nm.

All of the analyses were performed in duplicate.

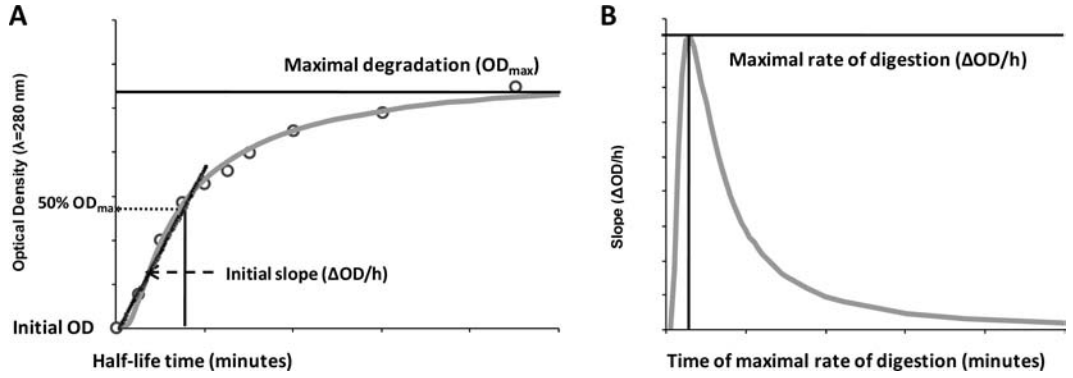

Figure 1. Examples of the digestion curves used to determine digestion parameters: (A) measured values (circles) fitted by eq 1 (curve) (graph shows four parameters: initial slope (ΔOD/h), initial OD, maximal degradation, and half-life time, as determined using the curve equation); (B) digestion rate calculated from eq 2 (graph shows two parameters: maximal rate of digestion and time to reach maximal rate of digestion, as determined using the derivate curve).

Table 1. Effect of Aging on Antioxidant Enzyme Activities, Glycolytic Potential, Biochemical Parameters, Granulometry Parameters, and in Vitro Digestion Parameters

	day 1[a]	day 4[a]	ρ_A[b]
antioxidant enzyme activities			
superoxide dismutase (U)	1.08 ± 0.06	1.26 ± 0.09	NS
catalase (nmol H_2O_2 min^{-1} (mg protein)$^{-1}$)	2081 ± 189	2134 ± 132	NS
glycolytic potential			
glycolytic potential (μmol equiv lactate/g)	110.5 ± 2.5	108.6 ± 4.8	NS
residual glycogen (μmol/g)	9.3 ± 0.9	8.3 ± 1.1	NS
lactate (μmol/g)	91.8 ± 1.9	92.1 ± 3.6	NS
biochemical parameters			
carbonyls (nmol DNPH/mg protein)	1.35 ± 0.06 b	1.92 ± 0.04 a	***
myofibrillar protein hydrophobicity (μg bound BPB)	67.7 ± 3.1	61.7 ± 4.3	NS
TBA-RS (mg MDA/kg meat)	0.10 ± 0.03 b	0.26 ± 0.05 a	*
granulometry parameters			
particle number	25294 ± 3323 b	34916 ± 2690 a	*
EC diameter (μm)	13.4 ± 0.3	13.6 ± 0.1	NS
circularity	0.748 ± 0.008 b	0.788 ± 0.004 a	***
Feret aspect ratio	0.730 ± 0.004 b	0.752 ± 0.002 a	***
in vitro pepsin digestion			
initial slope (ΔOD/h)	0.094 ± 0.002 a	0.086 ± 0.002 b	*
half-life time (min)	22.6 ± 0.7 b	24.8 ± 0.4 a	*
maximal degradation (OD_{max})	0.065 ± 0.002	0.065 ± 0.002	NS
maximal rate of digestion (ΔOD/h)	0.138 ± 0.003 a	0.126 ± 0.003 b	*
time to maximal rate of digestion (min)	7.8 ± 0.2 b	8.6 ± 0.2 a	*
in vitro trypsin and α-chymotrypsin digestion			
initial OD	0.087 ± 0.001	0.088 ± 0.001	NS
initial slope (ΔOD/h)	0.206 ± 0.005	0.210 ± 0.006	NS
half-life time (min)	6.4 ± 0.3	6.4 ± 0.3	NS
maximal degradation (OD_{max})	0.088 ± 0.003	0.090 ± 0.003	NS
maximal rate of digestion (ΔOD/h)	0.65 ± 0.02	0.67 ± 0.02	NS
time to maximal rate of digestion (min)	2.3 ± 0.1	2.3 ± 0.1	NS

[a]Values are the mean ± SEM of 12 pigs. Values followed by different letters (a, b) show significant differences between treatments ($p < 0.05$). [b]$\rho_{(A=ageing)}$ value from the ANOVA analysis represents the level of significance between days 1 and 4 and is annotated as $\rho > 0.05$, NS; $\rho < 0.05$, *; and $\rho < 0.001$, ***.

In Vitro Trypsin and α-Chymotrypsin Digestion. Protein hydrolysis by trypsin (E.C. 3.4.21.4, ref T0303, Sigma) and α-chymotrypsin (E.C. 3.4.21.1, ref C4129, Sigma) was preceded by pepsin digestion for 60 min at 37 °C as described above in order to mimic the gastric step. Pepsin activity was stopped by rising the pH with fresh glycine buffer (33 mM, pH 10.3), and cooling the samples on ice for 30 min. The final pH was about 8. Trypsin (6.55 U/mg of protein) and α-chymotrypsin (0.33 U/mg of protein) were then added in the tubes and placed at 37 °C for 4 h. Digestion was terminated by addition of 15% (final concentration) trichloroacetic acid at various time points (0, 5, 12, 21, 30, 45, 60, 90, 120, 180, 240 min). Samples were then placed on ice for 1 h. After centrifugation at 4000 *g* for 15 min at 4 °C, hydrolyzed peptide content in the supernatant was measured at 280 nm.

All of the analyses were performed in duplicate.

Determination of in Vitro Parameters of Digestion Kinetics. The following mathematical model adapted from Gatellier and Santé-

Table 2. Effect of Mincing and Temperature on Biochemical Parameters, Granulometry Parameters, and in Vitro Digestion Parameters

	70 °C[a]	70 °C minced[a]	140 °C[a]	140 °C minced[a]	ρ_M[b]	ρ_T[b]	ρ_I[b]
cooking loss (%)	21.5 ± 0.8 c	17.8 ± 1.2 d	58.2 ± 0.5 a	54.1 ± 0.3 b	***	***	NS
biochemical parameters							
carbonyls (nmol of DNPH/mg of protein)	1.66 ± 0.06 b	1.98 ± 0.04 b	3.14 ± 0.19 a	2.75 ± 0.13 a	NS	***	NS
myofibrillar protein hydrophobicity (μg bound BPB)	222.3 ± 3.7	225.6 ± 5.9	229.2 ± 5.6	219.1 ± 6.1	NS	NS	NS
TBA-RS (mg MDA/kg of meat)	0.96 ± 0.16 a	1.13 ± 0.15 a	0.30 ± 0.06 b	0.39 ± 0.07 b	NS	***	NS
granulometry parameters							
particle number	16764 ± 4121	20514 ± 4791	8653 ± 1707	11662 ± 1348	NS	NS	NS
EC diameter (μm)	8.8 ± 0.2	9.5 ± 0.3	8.6 ± 0.2	9.4 ± 0.3	NS	**	NS
circularity	0.821 ± 0.008 c	0.855 ± 0.004 ab	0.848 ± 0.005 b	0.874 ± 0.005 a	***	***	NS
feret aspect ratio	0.749 ± 0.005 b	0.767 ± 0.003 a	0.760 ± 0.004 ab	0.775 ± 0.004 a	***	*	NS
in vitro pepsin digestion							
initial slope (ΔOD/h)	0.107 ± 0.003 a	0.094 ± 0.004 b	0.065 ± 0.002 c	0.057 ± 0.003 c	**	***	NS
half-life time (min)	30.6 ± 1.1 b	33.5 ± 1.1 b	45.2 ± 0.8 a	49.3 ± 1.4 a	**	***	NS
maximal degradation (OD_{max})	0.106 ± 0.005 b	0.106 ± 0.005 b	0.122 ± 0.004 a	0.126 ± 0.004 a	NS	***	NS
maximal rate of digestion (ΔOD/h)	0.163 ± 0.004 a	0.148 ± 0.005 a	0.126 ± 0.003 b	0.121 ± 0.004 b	*	***	NS
time to maximal rate of digestion (min)	10.6 ± 0.4 b	11.7 ± 0.4 b	15.7 ± 0.3 a	17.2 ± 0.5 a	**	***	NS
in vitro trypsin and α-chymotrypsin digestion							
initial OD	0.105 ± 0.002 ab	0.099 ± 0.002 ab	0.089 ± 0.003 b	0.080 ± 0.002 c	**	***	NS
initial slope (ΔOD/h)	0.363 ± 0.014	0.374 ± 0.011	0.392 ± 0.009	0.376 ± 0.008	NS	NS	NS
half-life time (min)	9.4 ± 0.6	11.3 ± 1.1	12.8 ± 1.5	14.0 ± 1.3	NS	NS	NS
maximal degradation (OD_{max})	0.17 ± 0.01	0.19 ± 0.01	0.21 ± 0.01	0.21 ± 0.01	NS	NS	NS
maximal rate of digestion (ΔOD/h)	0.88 ± 0.03 a	0.83 ± 0.03 ab	0.82 ± 0.03 ab	0.74 ± 0.03 b	*	*	NS
time to maximal rate of digestion (min)	3.4 ± 0.2	4.0 ± 0.4	4.5 ± 0.5	5.0 ± 0.4	NS	NS	NS

[a]Values are the mean ± SEM of 12 pigs. Values with different letters (a–d) show significant differences between treatments ($p < 0.05$). [b]$\rho_{(M=mincing\ ;\ T=temperature\ ;\ I=interaction)}$ values from the ANOVA analysis represent the level of significance between 70 °C, 70 °C minced, 140 °C, and 140 °C minced and are annotated $\rho > 0.05$, NS; $\rho < 0.05$, *; $\rho < 0.01$, **; and $\rho < 0.001$, ***.

Lhoutellier[30] was used to fit the measurements for pepsin or trypsin/α-chymotrypsin digestions (Figure 1)

$$OD = OD_{max} \times \exp\left(-\frac{B}{time}\right) \quad (1)$$

where B = (half-life time)·ln(2).

For each digestion trial, primary parameter pairs (OD_{max} and half-life time) were estimated by minimizing the sum of squares of the differences between the calculated OD values and measured values (Figure 1A). OD_{max} represents the degradation potential, that is, the OD value that would be obtained after an infinite digestion time. The half-life time is the time needed to produce half the amount of hydrolyzed peptides in comparison to OD_{max}.

To better analyze the dynamics of the digestion process, the instantaneous rate of digestion was calculated from the derivative of eq 1

$$\frac{d(OD)}{d(time)} = 60 \times OD_{max} \times B \times \frac{1}{(time)^2} \times \exp\left(-\frac{B}{time}\right) \quad (2)$$

where B = (half-life time)·ln(2).

Figure 1B shows an example of the curve of the instantaneous digestion rate (ΔOD/h) versus time; curve shape was the same for all digestion trials. After a short ramp-up period, the digestion rate peaks at a maximal value and then decreases exponentially to become almost nil at the end of the incubation. Three parameters were determined to characterize this curve: maximal rate of digestion; time required to reach the maximal rate of digestion; and initial slope (ΔOD/h), which was calculated as the mean value over the first 15 min for pepsin reactions and over the first 21 min for trypsin/α-chymotrypsin reactions.

The initial rate of digestion is conventionally calculated by linear regression from the values measured during the fast digestion step (Figure 1A).[30] However, as the derivative curve (Figure 1B) shows that there is no linear part, the initial slope was calculated as the mean value of the first 15 min for pepsin reactions and of the first 21 min for trypsin/α-chymotrypsin reactions. The initial slope value was found to be similar to the initial rate but was less affected by experimental errors in OD determinations because it is derived from the estimated OD_{max}/B, which integrates all of the measurements rather than just a selected four or five.

Trypsin/α-chymotrypsin digestion was assessed using another parameter, that is, initial OD. This value was measured when trypsin/α-chymotrypsin digestion was begun (time 0) and represents the level of hydrolyzed proteins obtained after 1 h of pepsin digestion.

Statistical Methods. Analysis of variance (ANOVA) was performed using the general linear model procedure of the SAS software package (version 8.1, STAT, SAS Institute, Cary, NC, USA). Three linear models were performed: (i) including aging, (ii) including mincing with temperature (at 70 and 140 °C) as fixed effects, and (iii) including temperature. When significant effects were found for aging, mincing, or temperature, least-squares means were compared by computing LSMEANS with the PDIFF option followed by Tukey adjustment. Furthermore, a principal component analysis was performed on the entire data set using STATISTICA software (version 7.1, Stat Soft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Effect of Aging. Table 1 summarizes the measurements on antioxidant status, glycolytic potential, biochemical parameters, granulometry parameters, and the in vitro pepsin and trypsin/α-chymotrypsin digestion parameters.

SOD and catalase antioxidant enzyme activities reflected muscle antioxidant status before the technological treatments. Both enzymes help regulate oxidative stress by eliminating the toxic superoxide ions, first by forming hydrogen peroxide and second by forming water and O_2. SOD activity and catalase activity did not vary during meat aging. Consequently, aging for

Table 3. Effect of Temperature on Biochemical Parameters, Granulometry Parameters, and in Vitro Digestion Parameters

	raw[a]	70 °C[a]	100 °C[a]	140 °C[a]	ρ_T[b]
cooking losses (%)		21.5 ± 0.8 c	31.6 ± 0.7 b	58.2 ± 0.5 a	***
biochemical parameters					
carbonyls (nmol DNPH/mg protein)	1.92 ± 0.04 c	1.66 ± 0.06 c	2.29 ± 0.05 b	3.14 ± 0.19 a	***
myofibrillar protein hydrophobicity (μg bound BPB)	61.7 ± 4.3 c	222.3 ± 3.7 b	236.8 ± 5.1 a	229.2 ± 5.6 ab	***
TBA-RS (mg MDA/kg of meat)	0.26 ± 0.05 c	0.96 ± 0.16 a	0.66 ± 0.09 b	0.30 ± 0.06 c	***
granulometry parameters					
particle number	34916 ± 2690 a	16764 ± 4121 b	10563 ± 2741 b	8653 ± 1707 b	***
EC diameter (μm)	13.6 ± 0.1 a	8.8 ± 0.2 c	10.1 ± 0.5 b	8.6 ± 0.2 c	***
circularity	0.788 ± 0.004 c	0.821 ± 0.008 b	0.843 ± 0.005 a	0.848 ± 0.005 a	***
feret aspect ratio	0.752 ± 0.002	0.749 ± 0.005	0.761 ± 0.004	0.760 ± 0.004	NS
in vitro pepsin digestion					
initial slope (ΔOD/h)	0.086 ± 0.002 b	0.107 ± 0.003 a	0.064 ± 0.003 c	0.065 ± 0.002 c	***
half-life time (min)	24.8 ± 0.4 c	30.6 ± 1.1 b	46.1 ± 1.4 a	45.2 ± 0.8 a	***
maximal degradation (OD_{max})	0.065 ± 0.002 c	0.106 ± 0.005 b	0.124 ± 0.003 a	0.122 ± 0.004 a	***
maximal rate of digestion (ΔOD/h)	0.126 ± 0.003 b	0.163 ± 0.004 a	0.127 ± 0.002 b	0.126 ± 0.003 b	***
time to maximal rate of digestion (min)	8.6 ± 0.2 c	10.6 ± 0.4 b	16.1 ± 0.5 a	15.7 ± 0.3 a	***
in vitro trypsin and α-chymotrypsin digestion					
initial OD	0.088 ± 0.001 b	0.105 ± 0.002 a	0.086 ± 0.002 b	0.089 ± 0.003 b	***
initial slope (ΔOD/h)	0.210 ± 0.006 c	0.363 ± 0.014 b	0.381 ± 0.008 ab	0.392 ± 0.009 a	***
half-life time (min)	6.4 ± 0.3 b	9.4 ± 0.6 b	13.3 ± 1.3 a	12.8 ± 1.5 a	***
maximal degradation (OD_{max})	0.09 ± 0.003 c	0.17 ± 0.01 b	0.21 ± 0.01 a	0.21 ± 0.01 a	***
maximal rate of digestion (ΔOD/h)	0.67 ± 0.02 c	0.88 ± 0.03 a	0.77 ± 0.03 b	0.82 ± 0.03 ab	***
time to maximal rate of digestion (min)	2.3 ± 0.1 b	3.4 ± 0.2 b	4.7 ± 0.4 a	4.5 ± 0.5 a	***

[a]Values are the mean ± SEM of 12 pigs. Values with different letters (a–c) show significant differences between treatments ($p < 0.05$). [b]$\rho_{(T=temperature)}$ values from the ANOVA analysis represent the level of significance between raw meat and meat cooked at 70, 100, and 140 °C and are annotated $\rho > 0.05$, NS; and $\rho < 0.001$, ***.

a few days did not appear to modify muscle antioxidant status. These values are comparable to those obtained by Renerre et al.[31] on bovine Longissimus muscle during storage.

Glycolytic potential measurements were used to gauge the potential for postslaughter tissue acidification. Residual glycogen content and lactic acid content did not vary during aging. Measured glycolytic potential was similar to the values reported by Talmant et al.[32] for commercial pig breeds.

Concerning the biochemical parameters (i.e., lipid and protein oxidation and protein hydrophobicity), aging resulted in at least a 40% increase in carbonyl groups and TBA-RS rose sharply to 260%. Aging had no effect on protein hydrophobicity. Aging had no effect on protein denaturation but promoted both lipid and protein oxidation. In an in vitro study, Santé-Lhoutellier et al.[18] reported that protein hydrophobicity was enhanced in the presence of oxidants such as Fe^{2+}/H_2O_2 at concentrations above 0.5 M. However, this level of oxidant concentration is seldom found in meat during aging.

In the myofibril extract, aging had no effect on particle diameter but increased particle numbers, circularity, and Feret aspect ratio, thus revealing that the particles undergo proteolysis.

In vitro pepsin digestion parameters showed a decrease in initial slope and in maximal rate of digestion, whereas there was no change in half-life time or time to maximal rate of digestion with aging. Thus, aging slightly slowed pepsin digestion rate but had no impact on maximal degradation. The endogenous proteolysis occurring within the muscle during aging, as shown above via the increase in particle numbers, did not appear to promote digestion by gastric enzymes. This could be due to the fact that the pork meat was aged for only a short period, and we cannot exclude that longer aging could have led to more peptides or protein fragments being produced. Aging had no effect on digestion by intestinal enzymes.

To summarize, aging promoted both protein and lipid oxidation and tended to speed protein aggregation. However, the impact on digestion parameters remained low.

Effect of Mincing. Mincing is a common process in the meat industry. Table 2 summarizes the effects of mincing on the parameters studied.

Water losses measured on unminced and minced samples averaged 21.5 and 17.8%, respectively, after cooking at 70 °C and 58.2 and 54.1%, respectively, after cooking at 140 °C. The results show that water losses were significantly lower in minced samples. This can be explained by the fact that the mincing process led to around 4% of water losses occurring during the 24 h in storage prior to cooking. Cooking loss is a result of coshrinkage of connective tissue and muscle fibers occurring during heating, which causes thermal denaturation of meat proteins. This denaturation is visible in the secondary protein structure as an increase in aggregated β-sheet structure and a decrease in α-helices.[33]

Mincing alone was expected to significantly amplify protein and lipid oxidation by increasing the meat surface area exposed to contact with oxygen, yet surprisingly no difference was observed between unminced and minced samples. One of several plausible explanations is that the 24 h postmincing storage period could be too short to initiate oxidation reactions.[34] Note also that protein hydrophobicity remained unaffected.

Mincing was not found to result in variation in particle numbers or diameter, but circularity and Feret aspect ratio both decreased. This could be explained by mechanical dispersion of the compounds. Concerning the effects of mincing on in vitro digestion parameters, mincing led to a 10% drop in initial slope

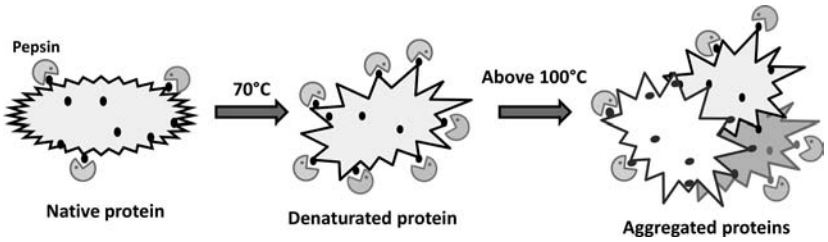

Figure 2. Proposed mechanism of pepsin action on raw (native) protein and proteins heated at different cooking temperatures.

and a maximal rate of pepsin digestion and, consequently, a rise in the half-life time and time to reach maximal rate. These results show a reduction of degradation potential of protein by pepsin, following mincing.

The only mincing-related differences recorded after trypsin/α-chymotrypsin digestion were a lower initial OD, which is consistent with the pattern previously observed for pepsin digestion, and a decrease in maximal rate of digestion.

Effect of Heating. The effects of heating are presented in Table 3.

Water losses measured on samples after cooking at 70, 100, and 140 °C averaged 21.5, 31.6, and 58.2%, respectively.

Under our experimental conditions, heating above 100 °C led to an increase in carbonyl groups. Gatellier et al.[35] also found that carbonyl content remained stable in beef heated to 65 and 96 °C.

Surface hydrophobicity of myofibrillar proteins increased dramatically with heating, without heating temperature-related differences. Chelh et al.[28] showed that even after a 30 min heat treatment, most BPB was already bound to the proteins at 60 °C. At 100 °C, Santé-Lhoutellier et al.[11] showed that protein denaturation peaked after just 5 min.

These data reflected a low exposure of hydrophobic sites in raw meat that subsequently increased at higher temperatures, which may lead to protein aggregation. The review by Morris et al.[36] together with research by Cellmer et al.[37] has comprehensively described the aggregation mechanisms through which globular proteins merge into amorphous or fibrillar aggregates.

TBA-RS content increased by around 270% after heat treatment at 70 °C but decreased at higher temperatures. This two-phase pattern has been reported in earlier work.[38] The decrease is thought to be due to the breakdown of malondialdehyde (MDA) into volatile compounds or to their reaction with protein chains, leading to the formation of Schiff bases, which are compounds known to play a role in protein aggregation.

Heat treatment had a progressive effect on particle numbers, which decreased around 75% at a treatment temperature of 140 °C, as well as on particle diameter, which decreased 30−40%. The main effect was observed when raw meat was compared to heated meat. These findings further emphasized the occurrence of a heating-induced particle aggregation and compaction effect. Philo and Arakawa[39] proposed a mechanism of aggregation that leads to the irreversible formation of oligomers and consequently lower particle numbers. Two main steps were described according to the molecular interactions taking place: the first involves conformational changes or partial protein unfolding leading to probably irreversible oligomers, whereas the second involves chemical reactions such as oxidation or deamidation that also lead to protein aggregation. In the present study, protein denaturation occurred at a temperature of 70 °C, as shown by the increase in protein surface hydrophobicity. This modification remained the major protein change. At higher temperatures, the proteins were further modified by oxidation, which promotes aggregation. This hypothesis is further supported by increases in circularity and Feret aspect ratio. The more fibrous-shaped particles in the raw sample will tend to respond to heating by folding and agglomerating, mainly through hydrophobic interactions, until they form circular densely packed aggregates.[16] Working at the ultrastructural level, Astruc et al.[8] demonstrated the presence of granular aggregates within muscle cells close to the sarcolemma and very likely from the myofibrils.

Pepsin digestion parameters showed that heating at 70 °C increased initial slope compared to raw meat. After higher temperature treatments, the initial slope dropped back to the same level as that measured in the original raw meat. Half-life time increased progressively according to temperature and maximum degradation rate. In comparison with raw meat, half-life time increased by around 25 and 85% after cooking at 70 and 100 °C, respectively, and did not increase further at 140 °C. Evenepoel et al.[40] reported similar results with raw and cooked egg. Heating egg protein doubled almost the duration in gastric emptying compared to raw egg protein. In the present study, we found that the heat treatments result in protein denaturation, characterized by visible protein conformation changes that maximize protein surface exposure to hydrophobic zones and thus promote the bioaccessibility of pepsin to cleavage sites, although only up to a certain limit, as at higher temperatures the aggregation process appears to outpace the thermal denaturation process. To summarize, pepsin cleaves hydrophobic aromatic amino acids such as phenylalanine, tyrosine, and tryptophan. Pepsin activity appears to be temperature-dependent. Santé-Lhoutellier et al.[11] found a sharp decrease in the rate of myofibrillar protein proteolysis by gastric pepsin heated at 100 °C and reported that this increase was correlated to the increase in carbonyl content. Studying phaseolins from beans autoclaved at 121 °C, Montoya et al.[20] did not find any changes in the degree of in vitro pepsin hydrolysis compared to raw phaseolins. Studying Cantonese sausages, Sun et al.[21] reported that the rate of pepsin hydrolysis decreased with heating duration, but comparison remains difficult, because the temperature applied was fairly low (under 50 °C) and Cantonese sausage can be considered a meat emulsion that includes both pro-oxidants (21% lipids) and antioxidants such as polyphenol from wine, as well as sodium nitrite.

Figure 2 proposes a mechanism of pepsin action based on our results. In a first step, heating produced protein conformational changes, enhancing the hydrolyzability by pepsin, and in a second step, protein oxidation processes were further added, leading to protein aggregation and a decrease of protein hydrolyzability by pepsin. These findings

were confirmed by the parameters calculated using hydrolysis equations. The maximum rate of digestion and time to reach maximal rate followed the same profile as half-life time and maximum degradation rate. These results suggest that degradation potential is greater in cooked meat but requires a longer digestion time.

Focusing on trypsin and α-chymotrypsin digestion, the first parameter measured was initial OD, which gives the level of degradation achieved during the prior pepsin digestion phase. The only difference found in terms of initial OD was under heat treatments at 70 °C, which led to 19% higher OD than in raw samples or samples heated at temperatures over 70 °C. This means that at a set time interval (in this case a 60 min pepsin digestion), the heat treatment at 70 °C has a greater degradation potential. The initial slope increased by 70% under heat treatment at 70 °C and by >82% at 100 and 140 °C. Half-life time and time to maximal degradation rate increased by 50% after cooking at 70 °C and by >100% after cooking at 100 and 140 °C. After cooking, the degradation potential (OD_{max}) increased by >85% compared to raw samples, which translates to an improved amino acid availability. This digestion profile with trypsin and α-chymotrypsin appears to mirror the pepsin digestion profile. In an in vivo study on egg proteins, Evenepoel et al.[40] reported a 77% increase in true ileal digestibility for cooked eggs together with improved protein assimilation. Montoya et al.[20] showed a dramatic increase in in vitro pancreatin hydrolysis of phaseolins with heating. Their results suggest the improved degree of hydrolysis is mainly due to structural changes and that pepsin plays a role at the initiation stage. In conclusion, our findings show that heating conditions tend to have effects on trypsin/α-chymotrypsin digestion similar to those on pepsin digestion: the heat treatment improves the degradation potential of meat, although only at longer digestion times. It is, however, noteworthy that the digestion rate is faster with trypsin/α-chymotrypsin compared to pepsin. The mathematical model developed here (see Materials and Methods) made it possible to evaluate the time needed to obtain different degrees of protein hydrolysis. For example, with pepsin, it would take 25 min to reach 50% raw meat protein hydrolysis compared to 31 and 45 min for meat cooked at 70 and 140 °C, respectively. For trypsin/α-chymotrypsin, it would take 6 min to reach 50% raw meat protein hydrolysis compared to 9 and 13 min for meat cooked at 70 and 140 °C, respectively.

Pooling the full set of biochemical, granulometry, and digestion data enabled us to run a principal component analysis, which revealed that data could be distinguished into three separate groups (Figure 3). On the first axis, raw meat was opposed to the cooked meat whatever the temperature (increased circularity was opposed to number of particles and EC diameter). BPB and carbonyls were positively associated with the first axis. On the second axis, the group of meats cooked at 70 °C was opposed to the group of meats cooked at either 100 or 140 °C (initial slope of pepsin and maximal rate both digestive proteases were associated positively to the second axis). The effect of mincing remained negligible compared to heat treatment.

This study elucidated the hypothesis that technological processes can affect meat protein digestion parameters and consequently meat protein nutrition value. The results demonstrate that heating temperature modified the physico-chemical state of myofibrillar proteins. We highlighted two different physical−chemical mechanisms according to heating

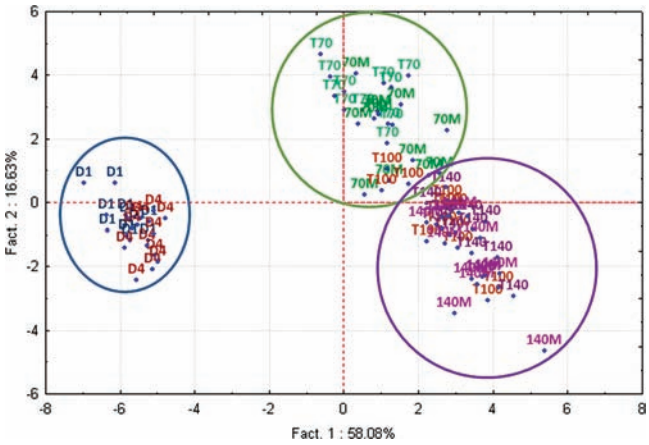

Figure 3. Principal component analysis plot: factor 1 versus factor 2 of all treatments. Abbreviations for raw meat: D1, day 1; D4, day 4 (blue circle). Abbreviations for cooked meat: T70, 70 °C unminced; 70 M, 70 °C minced (green circle); T100, 100 °C unminced; T140, 140 °C unminced; 140 M, 140 °C minced (purple circle).

temperature. The first mechanism occurs at a temperature of 70 °C, when the proteins undergo conformational changes that increase their hydrolyzability by pepsin. The second mechanism is triggered at higher temperatures (i.e., 100 °C), at which the dominant effects are oxidation-related. In general, heat treatment improves meat degradation potential, although only at longer digestion times. The digestion parameters defined (maximal slope, half-life time, and maximal degradation) show that it is possible to grasp a dynamic dimension of the in vitro digestion model. Moreover, this in vitro approach makes it possible to compare a broad series of processing conditions. However, in vitro digestion approaches do have their limitations, especially in terms of intestinal absorption factors. Complementary in vivo studies will bridge many of these limitations.

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ABBREVIATIONS USED

D1, day 1; D4, day 4; CL, cooking loss; SOD, superoxide dismutase; GP, glycolytic potential; BHT, butyl hydroxytoluene; DNPH, 2,4-dinitrophenylhydrazine; BPB, bromophenol blue; OD, optical density; TBA-RS, thiobarbituric acid-reactive substances; MDA, malondialdehyde; EC, equivalent circle.

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