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¹ Effect of Cooking on *in Vitro* Digestion of Pork Proteins: ² A Peptidomic Perspective

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⁷  Supporting Information

⁸ **ABSTRACT:** This study was designed to investigate the effect of cooking on *in vitro* digestibility and peptide profiling of pork
⁹ protein. We simulated gastrointestinal digestion of cooked pork that was treated with pepsin alone or followed by trypsin
¹⁰ treatment. Digested products were identified using matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry
¹¹ and liquid chromatography–mass spectrometry analyses. Cooking led to a reduction ($p < 0.05$) in digestibility and band
¹² intensities on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. Peptide profiling and identification analyses also
¹³ showed significant difference ($p < 0.05$) in the *m/z* ranges and number of peptides from the pepsin-digested products between
¹⁴ raw (4 °C) and very well done samples (100 °C). Peptides sequenced from pepsin-digested samples under lower degrees of
¹⁵ doneness disappeared as the temperature increased. Meanwhile, the trypsin cleavages appeared more consistent among different
¹⁶ degrees of cooking. Further work may be needed to evaluate the bioavailability of the digested products under different cooking
¹⁷ temperatures.

¹⁸ **KEYWORDS:** cooking, *in vitro* digestibility, peptidomics, sequencing

19 ■ INTRODUCTION

20 Cooking is the way to convert raw meat into edible meat. The
 21 degree of cooking affects palatability and consumer acceptability
 22 differing with primal cuts and cooking methods.^{1–4} Generally
 23 speaking, people in western countries prefer roasting and grilling
 24 with medium doneness, but Asian people prefer stewing and stir-
 25 frying with very well doneness. In recent years, the degree of
 26 doneness has been associated with the production of heterocyclic
 27 aromatic amines and the risk of colorectal cancer in the clinic.^{5,6}
 28 However, meat has played and will continue to play a crucial role
 29 in the supply of high-quality protein, vitamins, and minerals.⁷
 30 Notably, it should be paid more attention to how cooking affects
 31 the bioavailability of nutrients in meat, especially proteins.
 32 However, few data are available.

33 A French group have performed much work on the effect
 34 of cooking on *in vitro* digestibility of meat, and they found that
 35 heat-induced oxidation and increased surface hydrophobicity of
 36 myofibrillar proteins could result in protein aggregation, which
 37 further decreased the pepsin digestion rate.^{8–11} However, the
 38 nutritional quality of meat proteins should not only be associated
 39 with digestibility or amino acid composition but also with peptide
 40 size and sequences,^{12–14} which may affect protein digestibility
 41 after cooking. It is known that oligopeptides can be degraded by
 42 the brush border peptide hydrolases and taken up into intestinal
 43 epithelial cells where the size of oligopeptides may be critical.¹⁵
 44 It is crucial to characterize the digested products from meat
 45 proteins when they go through the stomach and the gut to
 46 evaluate the effect of cooking on meat protein bioavailability.¹⁶

47 The objective of the present study was to characterize the
 48 *in vitro* digested products with pepsin and trypsin from cooked
 49 pork under different cooking temperatures by mimicking
 50 gastrointestinal digestion in the human body.

■ MATERIALS AND METHODS

Samples and Cooking Procedure. Pork longissimus dorsi muscles ($n = 8$; muscle pH 5.83 ± 0.18) were obtained at 36 h post-mortem from a commercial meat packing company. After all visible fat and connective tissue were removed, each pork muscle was cut vertically into five 2 cm thick pieces (weighing 90–110 g each). Three of these pieces were packed in retort pouch and cooked in a 72 °C water bath (Julabo, TW 20, Germany) to different center temperatures, i.e., 60 °C (cooking time of 12–16 min), 65 °C (cooking time of 21–29 min), and 70 °C (cooking time of 33–37 min), individually. The fourth piece was packed in retort pouch and cooked in a 100 °C water bath (Julabo, TW 20, Germany) to the center temperature of nearest to 100 °C (cooking time of 3 h). The fifth piece was not cooked (4 °C) as the control (raw). During cooking, the center temperature of the samples was monitored using a digital, hand-held thermometer (Testo108, Testo AG, Germany). After cooking, the samples were cooled to the room temperature (20 ± 2 °C). Eight repeats were applied for each temperature point.

In Vitro Digestion. Cooked meat was *in-vitro*-digested according to the procedure by Escudero et al., with some modifications.¹⁴ Briefly, meat samples (0.5 g, each) were homogenized in 2 mL of double-distilled water for 2×30 s at 9500 rpm and 2×30 s at 13 500 rpm with 30 s cooling between bursts. The homogenate was adjusted to pH 2.0 with 1 mol/L HCl, and pepsin (≥ 400 units/mg of protein, from porcine gastric mucosa, product number P7125, Sigma-Aldrich, St. Louis, MO) was added 0.016 g each at a ratio of 1:31.25 of the substrate based on the weight of meat. The mixture was incubated at 37 °C for 2 h with continuous shaking, and then the enzyme was inactivated by adjusting the pH to 7.5 with 1 mol/L NaOH on ice. A total of 1 mL of the resulting

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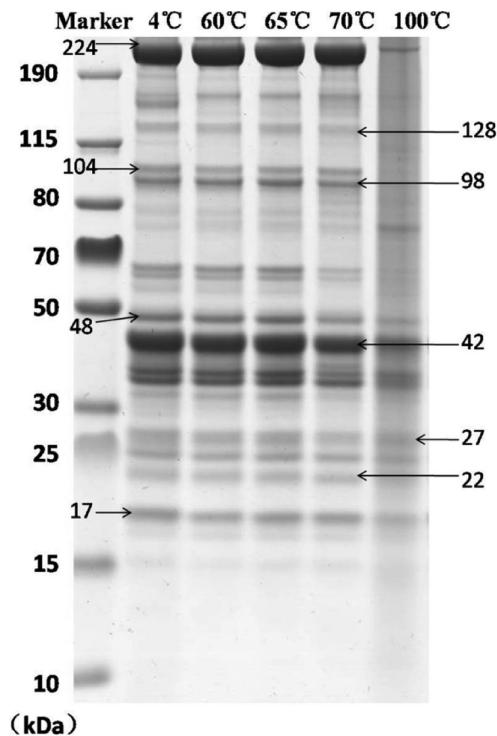


Figure 1. SDS-PAGE profiling of total proteins from cooked pork under different cooking temperatures. Proteins of bands with different numbers on the right five lanes were quantified and identified, with more details seen in Table 1.

digestion mixture was removed for peptide profiling, and the remaining (approximately 1.5 mL) was kept at 37 °C for trypsin digestion. Trypsin (1645 units/mg of protein, from porcine pancreas, lyophilized powder, type II-S, product number T7409, Sigma-Aldrich, St. Louis, MO) was added 0.006 g each at a ratio of 1:50 substrate based on the mass of meat left. The reaction mixture was maintained under the same conditions as above. After 2 h of trypsin digestion, enzyme activity was terminated by heating at 95 °C for 5 min and then 1 mL of the resulting digestion

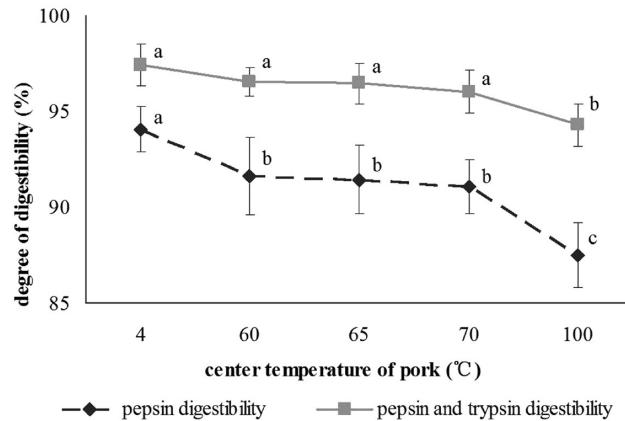


Figure 2. *In vitro* digestibility of pork under different cooking temperatures. Diamond, samples were treated with pepsin; black box, samples were treated with pepsin followed by trypsin. Different letters indicate statistically significant difference among the values.

mixture was taken out for peptide profiling. Both the pepsin digests and pepsin/trypsin digests were deproteinized by adding three volumes of ethanol and storing for 12 h at 4 °C. The samples were then centrifuged at 10000g for 20 min at 4 °C. The supernatant and precipitate were separated and stored at -18 °C.

In Vitro Digestibility. Two portions of meat sample weighing 1 g were taken from each piece. One portion was only treated with pepsin, and the other one was treated with pepsin, followed by trypsin. The digestion procedures were the same as above. After digestion, the digest was centrifuged at 10000g for 20 min at 4 °C and the supernatant was discarded. The precipitate was dried until the weight was constant. The degree of digestibility was calculated by the weight change of insoluble protein as the following equation:

$$DT = \left(1 - \frac{W_i}{W_t} \right) \times 100\%$$

where DT is the digestibility, W_i is the weight of dried insoluble protein, and W_t is the total weight of meat before digestion.

Table 1. Identified Proteins and Relative Band Intensities on SDS-PAGE Gels (Mean \pm Standard Deviation)^a

protein name	M_{re}^b (kDa)	M_{rt}^c (kDa)	4 °C	60 °C	65 °C	70 °C	100 °C	accession number	sequence coverage (%)	score
myosin-1	220	224	6.32 \pm 0.89 a	5.82 \pm 0.80 ab	5.27 \pm 0.96 b	4.14 \pm 1.17 c	0.12 \pm 0.07 d	NP_001098421	3	429
myosin-2		224						NP_999301	3	429
myosin-binding protein C, fast type	137	128	0.71 \pm 0.26 a	0.63 \pm 0.09 a	0.62 \pm 0.11 a	0.51 \pm 0.11 b	nd	XP_003127419	4	121
α -actinin-3-like isoform 1	98	104	0.59 \pm 0.10 a	0.48 \pm 0.09 b	0.46 \pm 0.07 b	0.30 \pm 0.10 c	nd	XP_003122525	4	127
glycogen phosphorylase, muscle form isoform 1	90	98	1.12 \pm 0.21 a	1.08 \pm 0.14 a	1.01 \pm 0.15 a	0.63 \pm 0.32 b	nd	XP_003122636	9	352
β -enolase	48	48	2.20 \pm 1.26 a	1.45 \pm 0.14 b	1.43 \pm 0.15 b	1.46 \pm 0.20 b	0.37 \pm 0.13 c	NP_001037992	7	138
actin, α skeletal muscle	42	42	3.67 \pm 0.63 a	3.98 \pm 0.39 a	3.85 \pm 0.66 a	2.74 \pm 0.94 b	0.78 \pm 0.52 c	NP_776650	15	357
creatine kinase M type		43						NP_001123421	3	107
triosephosphate isomerase	27	27	0.72 \pm 0.16 a	0.71 \pm 0.09 a	0.70 \pm 0.17 a	0.64 \pm 0.16 a	0.51 \pm 0.10 b	-	-	-
myosin light chain 3	22	22	0.80 \pm 0.22 a	0.84 \pm 0.14 a	0.83 \pm 0.14 a	0.71 \pm 0.12 ab	0.60 \pm 0.24 b	-	-	-
myoglobin	18	17	1.71 \pm 0.28 a	1.51 \pm 0.21 ab	1.44 \pm 0.38 bc	1.29 \pm 0.30 c	0.56 \pm 0.31 d	-	-	-

^aMeans with different letters in the same row differ significantly ($p < 0.05$). nd = not detectable. Proteins marked with “-” are bands that could not be identified by the MALDI-ToF-ToF method but matched with Swiss-Prot. ^bEstimated molecular weights of the proteins. ^cTheoretical molecular weights of the identified proteins.

Gel Electrophoresis and Protein Identification. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed to characterize the total protein profiles before and after cooking to different center temperatures.

A total of 1 g of cooked meat was homogenized in 4 mL of extraction buffer (2% SDS and 0.01 mol/mL sodium phosphate buffer at pH 7.0) with an Ultra Turrax homogenizer (IKA T25 Digital, Germany). The homogenate was centrifuged (Allegra 64R, Beckman Coulter, Brea, CA) at 1500g for 15 min at 4 °C. The protein concentration of the supernatant was determined with a BCA protein assay kit (catalog number 23225, Thermo Scientific, Waltham, MA).

Appropriate volumes of protein samples were mixed with 12.5 μL of XT sample buffer (Bio-Rad, Hercules, CA) and made up to a total volume of 50 μL with ultrapure water to a final protein concentration of 0.5 μg/μL. Protein samples were denatured by heating at 95 °C for 5 min. A total of 10 μL of each sample was loaded on 4–12% Bis-Tris Criterion precast gels (catalog number 345-0125, Bio-Rad, Hercules, CA). The gels were run in 900 mL of XT MES running buffer (Bio-Rad, Hercules, CA) at 150 V for approximately 1 h, until the bromophenol blue dye front just disappeared. After electrophoresis, proteins were stained with Coomassie Blue R250 for 3 h and destained until the bands were clear.

Gel images were captured using an image scanner (GE Healthcare, Little Chalfont, U.K.), and the band intensities were quantified with the Quantity One software (Bio-Rad, Hercules, CA). The intensity of each band was calculated as its actual intensity relative to the intensity of the 190 kDa band in the prestained calibration marker (catalog number 161-0377, Bio-Rad, Hercules, CA).

A total of 12 different bands were picked up and further identified by matrix-assisted laser desorption/ionization–time-of-flight tandem mass spectrometry (MALDI–ToF MS–MS, ultrafleXtreme Bruker, Germany).¹⁷ In brief, protein bands were destained, cleaned, and digested in gel with trypsin (Promega, Madison, WI). The extracted peptide mixtures were analyzed by MALDI–ToF–ToF MS. All peptide spectra were matched with the online MASCOT program (<http://www.matrixscience.com>) against the Swiss-Prot databases. The data matching was performed with a peptide mass tolerance of 0.12 Da and ToF–ToF fragment ion mass tolerance of 0.6 Da. Carbamidomethyl of Cys was chosen as fixed modification; oxidation of Met and pyro-Glu formation of N-terminal Gln were chosen as dynamic modifications; and one missing tryptic cleavage was allowed. Hits were applied only if their MASCOT probability were less than 0.05.

Characterization of the Digested Products. MALDI–ToF MS Analysis. The ethanol-soluble fractions from the pepsin-treated and the pepsin/trypsin-treated samples were characterized by MALDI–ToF MS in duplicate. An aliquot (1 μL) of each sample was spotted on the target plate with an equal volume of matrix solution [α -cyano-4-hydroxycinnamic acid (CHCA), 0.005 g/mL prepared in 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA)]. The mixture was dried before analysis. Positive-ion (M^{+}) spectra were acquired in the linear MALDI–ToF MS mode (ultrafleXtreme Bruker, Germany). The m/z signals were recorded between 200 and 1500 for peptide identification.

The peak data of the MS spectra were subjected to hierarchical clustering using the ClinProTools 2.2 software (Bruker Daltonics, Germany). Principal component analysis (PCA) was performed using the unsupervised clustering mode.

Liquid Chromatography–Mass Spectrometry (LC–MS) Analysis. The ethanol-soluble fractions from the pepsin-treated and pepsin/trypsin-treated samples were also analyzed with hybrid quadrupole orbitrap mass spectrometer equipped with a nanoelectrospray ionization source with an online nano-LC system (Nano LC-Q-Exactive-MS-MS, Thermo Fisher Scientific, Waltham, MA). The ethanol-soluble fractions were filtered through ultra-0.5 mL centrifugal filter units with ultracel-3 membrane (Amicon Ultra, Millipore, Ireland) under centrifugation at 15000g for 15 min. Peptide mixtures were then fractionated on an online nano-LC system (EASY-nLC 1000, Thermo Scientific, Waltham, MA). Samples were loaded onto a C₁₈ column (2 cm long, 100 μm inner diameter, and 5 μm particle size) at a maximum pressure of 500 bar.

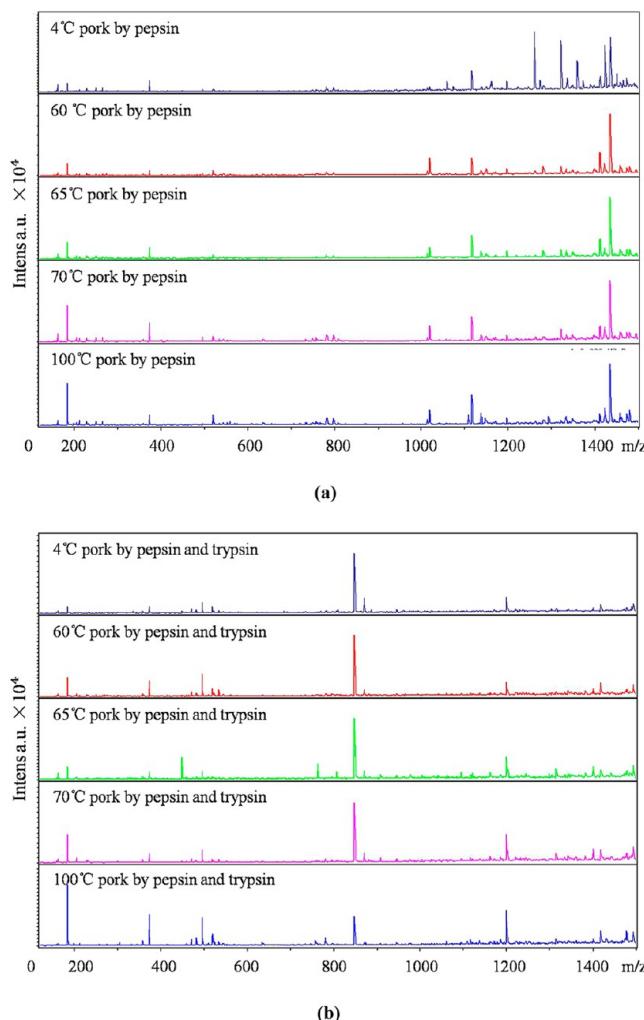


Figure 3. MALDI–ToF MS spectra of ethanol-soluble fragments from pork under different cooking temperatures. Each panel includes five spectra from 4, 60, 65, 70, and 100 °C samples: (a) pepsin treated and (b) pepsin and trypsin treated.

Peptides were separated using a mobile phase changing from 0.1% formic acid (FA) in water (buffer A) to 0.1% FA in ACN (buffer B). A step-gradient elution at a flow rate of 350 nL/min was applied with an increasing concentration of buffer B: (1) 10 min from 0 to 8%, (2) 25 min from 8 to 20%, (3) 15 min from 20 to 30%, (4) 5 min from 30 to 90%, and finally kept at 90% until the procedure completed. The hybrid quadrupole orbitrap mass spectrometer was operated in a data-dependent mode, and a scan cycle was initiated with a full-scan MS spectrum (from m/z 100 to 1500). The top 20 abundant ions were selected for collision-induced dissociation (CID) fragmentation in the linear ion trap, and the exclusion time was set as 60 s.

MS/MS spectra of peptides were matched using Proteome Discoverer (Thermo Fisher Scientific, Waltham, MA) against the Swiss-Prot *Sus scrofa* database (<http://www.uniprot.org/taxonomy/>). Data matching was performed with a fragment ion mass tolerance of 0.8 Da and a parent ion tolerance of 10 ppm. Oxidation of Met and pyro-Glu formation of N-terminal Gln were chosen as dynamic modifications, and two missing cleavages were allowed. Unspecific enzyme was used in the peptic peptide database search, while both unspecific enzyme and trypsin were used in the peptide search for pepsin/trypsin-treated samples. Peptide identification was accepted if they could be established at a PeptideProphet probability of greater than 95%. Protein matching was accepted if they could be established at the probability of greater than 95% and contained at least two identified spectra.

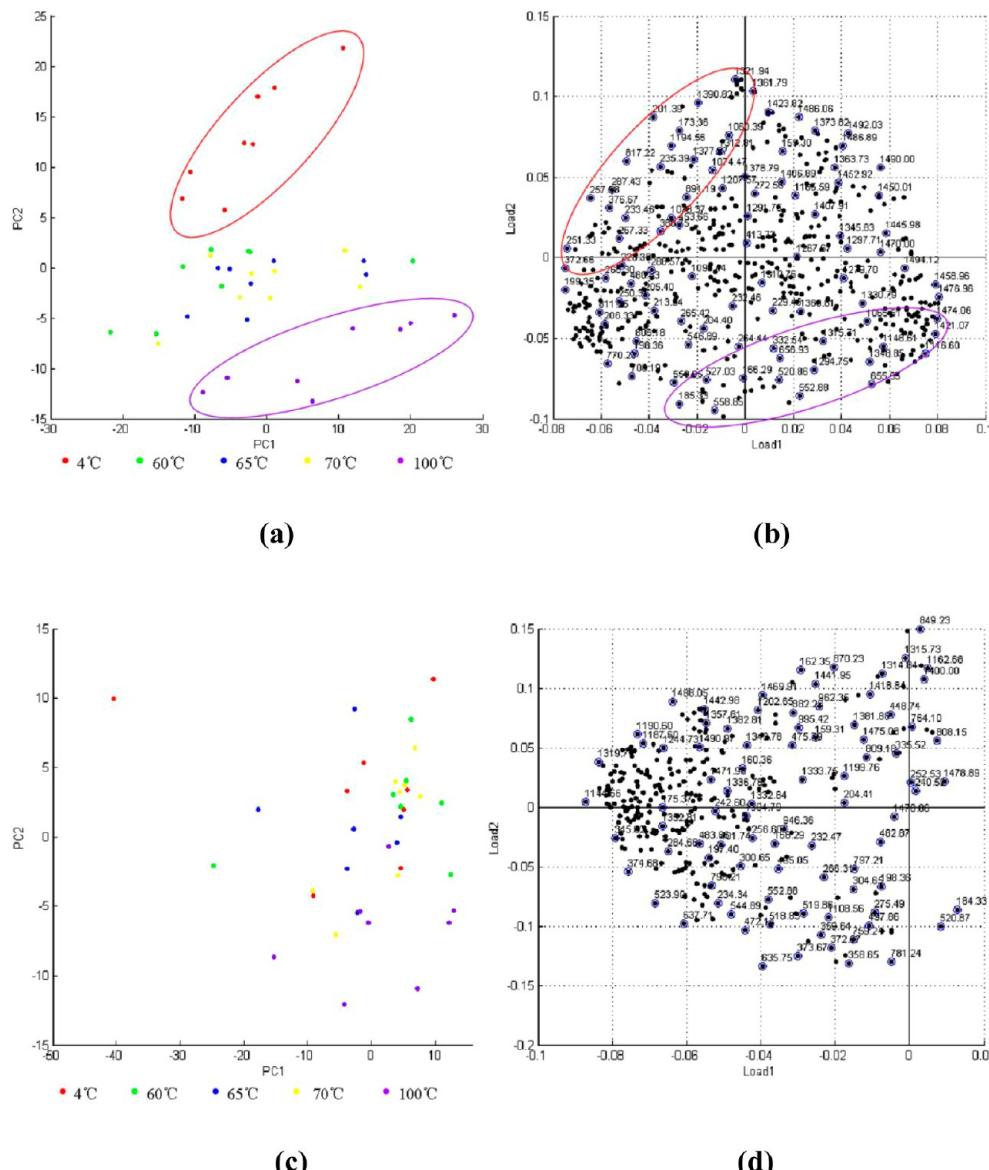


Figure 4. PCA plots of the peptides from cooked pork treated with pepsin and trypsin: (a and c) scores plot, treated with pepsin and pepsin/trypsin, respectively (red, green, blue, yellow, and purple plots represent 4, 60, 65, 70, and 100 °C treatment samples) and (b) and (d) loadings plot, treated with pepsin and pepsin/trypsin, respectively.

198 **Statistical Analysis.** The effects of cooking on *in vitro* digestibility
199 and SDS-PAGE band intensities were evaluated by one-way analysis of
200 variance and the Duncan's multiple-range test using the SAS program
201 (SAS Institute, Inc., Cary, NC). PCA on spectral data was described in
202 the MALDI-ToF MS Analysis section.

203 ■ RESULTS AND DISCUSSION

204 **Effect of Cooking on Pork Protein Profiling and *In Vitro***
205 **Digestibility.** Generally, band intensities on SDS-PAGE gels
206 decreased as the center temperature increased, especially for
207 100 °C samples (Figure 1). A possible explanation for the
208 decrease in band intensities is the aggregation of meat proteins.
209 Previous studies indicated that a high cooking temperature led to
210 increased oxidation and protein surface hydrophobicity of myofi-
211 brillar proteins^{8,9} and, as a result, increased protein aggregation.
212 Sarcoplasmic proteins, which contain various types of enzymes,
213 pigments, and regulatory proteins, may aggregate between
214 40 and 60 °C.¹⁸ The heat-induced protein aggregation normally

appeared with the decreased equivalent circle diameter and
215 number of particles.^{11,19} 216

In details, muscle proteins showed different responses to
217 cooking ($p < 0.05$) in terms of band intensities on SDS-PAGE
218 gels (Figure 1 and Table 1). Myosin heavy chain (224 kDa),
219 α -actinin (104 kDa), β -enolase (48 kDa), and myoglobin
220 (18 kDa) showed a distinct intolerance to heating in that their
221 band intensities dropped at 60 °C ($p < 0.05$). Myosin-binding
222 protein C (128 kDa), glycogen phosphorylase (98 kDa), and
223 actin/creatine kinase (42 kDa) had a bit stronger heat tolerance,
224 and the band intensities decreased significantly at 70 °C ($p < 0.05$).
225 A previous study has confirmed that myosin has endothermic
226 transitions at 54 °C, with a transition temperature of 77 °C for
227 actin.²⁰ However, triosephosphate isomerase and myosin light
228 chain 3 (22 kDa) were more tolerant to high-temperature cooking
229 (100 °C). Kajak-Siemaszko et al. showed a similar SDS-PAGE
230 pattern of meat proteins during cooking, but they did not show
231 qualitative and quantitative data.²¹ Our data suggest that the
232

233 different responses of meat proteins to cooking could be indicative
234 of different digestibilities.

235 Cooking had a significant effect ($p < 0.05$; Figure 2) on *in vitro*
236 digestibility of pork treated with pepsin and trypsin. When pork
237 was treated only with pepsin, cooking led to a distinct decrease
238 ($p < 0.05$) in digestibility between 60 °C and raw meat and
239 between 70 and 100 °C but there was no significant difference
240 ($p > 0.05$) between 60 and 70 °C. For pork treated with pepsin
241 followed by trypsin, only a high temperature (100 °C) resulted
242 in a significant decrease in digestibility. These results are in
243 accordance with previous studies in which the digestion rate
244 was applied to evaluate *in vitro* digestibility.^{11,19} In those studies,
245 the decrease in protein digestibility was associated with protein
246 aggregation. It is notable that the values of digestibility are
247 quite different from those by Santé-Lhoutellier et al.⁸ mainly
248 because of the different methodologies in which the OD₂₈₀ value
249 was applied to quantify peptides in that previous study, while we
250 calculated it on a dry weight basis.

251 Effect of Cooking on Peptide Profiling after Digestion.

252 As described above, cooking affected the extent of *in vitro*
253 digestion. However, to our knowledge, peptides in the digested
254 products under different cooking temperatures have never been
255 characterized. Therefore, two types of high-throughput methods,
256 including MALDI–ToF MS and nano-LC–MS–MS, were applied
257 to characterize the composition of digested products.

258 MALDI–ToF MS analysis revealed different peptide
259 profiles for the ethanol-soluble fractions from the samples with
260 different degrees of cooking (Figure 3). Meat cooked at higher
261 temperatures had relatively less abundant peptides with a *m/z*
262 range between 200 and 1200 after pepsin treatment (Figure 3a),
263 but the difference became weak after pepsin/trypsin treatment
264 (Figure 3b). This is in accordance with the changes of *in vitro*
265 digestibility.

266 PCA revealed significant differences in peptide composition
267 and abundance under different cooking temperatures. Again,
268 peptides from raw (4 °C) and very well done (100 °C) pork were
269 well-separated from the other three groups after pepsin digestion
270 (Figure 4a; scores plot) in that fragments from raw meat had
271 relatively smaller mass than the other groups (Figure 4b; loadings
272 plot). Peptides from 60, 65, and 70 °C samples could not be
273 well separated (Figure 4b). However, peptides from all of the
274 groups could not be separated at all after pepsin/trypsin digestion
275 (panels c and d of Figure 4). It is notable that the sensitivity
276 of MALDI–ToF MS seems not good enough to characterize
277 low-abundance peptides.

278 LC–MS analysis was performed to characterize peptides more
279 sensitively. According to a previous study, the size of peptides
280 from pepsin/pancratin-digested pork ranges from 6 to 16 amino
281 acids,¹⁴ and in the present study, the *m/z* search range was set
282 between 800 and 2000. A total of 1000 peptides were identified
283 from the pepsin-treated samples, and 1203 peptides were
284 identified from the pepsin/trypsin-treated samples. Of these
285 peptides, 554, 466, 484, 329, and 376 peptides were sequenced
286 from pepsin-digested samples for 4, 60, 65, 70, and 100 °C
287 groups, individually, and 662, 549, 520, 431, and 549 peptides
288 were sequenced from pepsin/trypsin-digested samples (Figure 5a)
289 from 4, 60, 65, 70, and 100 °C groups, respectively. When
290 peptides were categorized by *m/z* into three groups, 800–1000,
291 1000–1500 and 1500–2000, the majority of peptides have
292 a *m/z* of 1000–1500 or 1500–2000. The 70 °C samples had
293 the least peptides across three mass to charge ranges whether the
294 samples were treated with pepsin alone or followed by trypsin
295 (panels b and c of Figure 5). This could be due to the protein

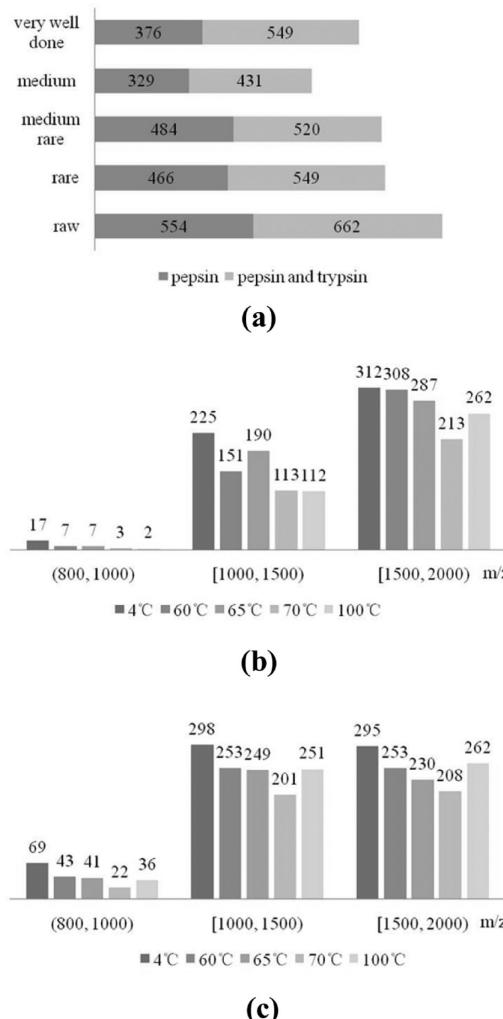


Figure 5. Number of identified peptides from cooked pork treated with pepsin and trypsin: (a) total number of identified peptides between 800 to 2000 Da, (b) number of the peptides from pepsin-digested samples categorized by size, and (c) number of the peptides from pepsin/trypsin-digested samples categorized by size.

aggregation associated with the resistance to the proteolysis of 296 digestive enzymes.^{9,11,19} We also found that peptide sequences 297 from pepsin-treated samples were different from those from 298 pepsin/trypsin-treated samples (see the Supporting Information), 299 which could be attributed to the stochasticity of active sites 300 of pepsin.²²

Peptides were matched with porcine muscle proteins against 302 the Swiss-Prot *S. scrofa* database. High-abundance peptides were 303 mainly from several myofibrillar proteins (myosin, actin, 304 tropomyosin, and titin) and sarcoplasmic proteins (phosphorylase, 305 glyceraldehyde-3-phosphate dehydrogenase, creatine kinase, 306 6-phosphofructokinase, etc.). Notably, 16–21% of identified 307 peptides came from myosin heavy chain. This gives a good 308 explanation for the decrease in band intensities on SDS–PAGE 309 gels (Figure 1). To explore the effect of cooking on the digestion 310 pattern of these proteins, we localized identified sequences to 311 the intact protein sequence chains. Figure 6 is an example of 312 myosin heavy chain (myosin-4). Generally, peptides identified 313 from pepsin-digested samples under lower temperature dis- 314 appeared as the temperature increased. Meanwhile, in compar- 315 ion to the pepsin cleavages (a1–a3 of Figure 6), the pepsin/ 316 trypsin cleavages appeared more consistent among samples under 317

4 °C MSSDQEMAIFGEAAPYLRKSEKERIEAQNKPFDAKTSVFAEPKE SFVKGTQSREGGKTVKTEAGATLTVKEDQVFPMNPPKJDKI
 60 °C MSSDQEMAIFGEAAPYLRKSEKERIEAQNKPFDAKTSVFAEPKE SFVKGTQSREGGKTVKTEAGATLTVKEDQVFPMNPPKJDKI
 65 °C MSSDQEMAIFGEAAPYLRKSEKERIEAQNKPFDAKTSVFAEPKE SFVKGTQSREGGKTVKTEAGATLTVKEDQVFPMNPPKJDKI
 70 °C MSSDQEMAIFGEAAPYLRKSEKERIEAQNKPFDAKTSVFAEPKE SFVKGTQSREGGKTVKTEAGATLTVKEDQVFPMNPPKJDKI
 100 °C MSSDQEMAIFGEAAPYLRKSEKERIEAQNKPFDAKTSVFAEPKE SFVKGTQSREGGKTVKTEAGATLTVKEDQVFPMNPPKJDKI

4 °C EDMAMMTHLHEPAVLYNLKERYAAWMIYTYSLFCVTVPYKWL PVYNAEVVTAYRGKKRQEAPPHIFSISDNAYQFMLTDRENQSIL
 60 °C EDMAMMTHLHEPAVLYNLKERYAAWMIYTYSLFCVTVPYKWL PVYNAEVVTAYRGKKRQEAPPHIFSISDNAYQFMLTDRENQSIL
 65 °C EDMAMMTHLHEPAVLYNLKERYAAWMIYTYSLFCVTVPYKWL PVYNAEVVTAYRGKKRQEAPPHIFSISDNAYQFMLTDRENQSIL
 70 °C EDMAMMTHLHEPAVLYNLKERYAAWMIYTYSLFCVTVPYKWL PVYNAEVVTAYRGKKRQEAPPHIFSISDNAYQFMLTDRENQSIL
 100 °C EDMAMMTHLHEPAVLYNLKERYAAWMIYTYSLFCVTVPYKWL PVYNAEVVTAYRGKKRQEAPPHIFSISDNAYQFMLTDRENQSIL

4 °C ITGESGAGKTNTKRVIQYFATIAVTGEKKKEPTPGKMQGL EDQIISANPLLEAFGNAKTVRNDNSRGKFIRIHFGTTGKLASAD
 60 °C ITGESGAGKTNTKRVIQYFATIAVTGEKKKEPTPGKMQGL EDQIISANPLLEAFGNAKTVRNDNSRGKFIRIHFGTTGKLASAD
 65 °C ITGESGAGKTNTKRVIQYFATIAVTGEKKKEPTPGKMQGL EDQIISANPLLEAFGNAKTVRNDNSRGKFIRIHFGTTGKLASAD
 70 °C ITGESGAGKTNTKRVIQYFATIAVTGEKKKEPTPGKMQGL EDQIISANPLLEAFGNAKTVRNDNSRGKFIRIHFGTTGKLASAD
 100 °C ITGESGAGKTNTKRVIQYFATIAVTGEKKKEPTPGKMQGL EDQIISANPLLEAFGNAKTVRNDNSRGKFIRIHFGTTGKLASAD

4 °C IETYLLEKSRVTFQLKAERSYHIFYQIMSNKKPELIELMLITTPY DYAFVSQGEITVPSIDQEELMATDSAIEILGFTSDERSIYKLTGAV
 60 °C IETYLLEKSRVTFQLKAERSYHIFYQIMSNKKPELIELMLITTPY DYAFVSQGEITVPSIDQEELMATDSAIEILGFTSDERSIYKLTGAV
 65 °C IETYLLEKSRVTFQLKAERSYHIFYQIMSNKKPELIELMLITTPY DYAFVSQGEITVPSIDQEELMATDSAIEILGFTSDERSIYKLTGAV
 70 °C IETYLLEKSRVTFQLKAERSYHIFYQIMSNKKPELIELMLITTPY DYAFVSQGEITVPSIDQEELMATDSAIEILGFTSDERSIYKLTGAV
 100 °C IETYLLEKSRVTFQLKAERSYHIFYQIMSNKKPELIELMLITTPY DYAFVSQGEITVPSIDQEELMATDSAIEILGFTSDERSIYKLTGAV

4 °C MHYGNLKFQKQKQREEQAEPDGTEVADKAAYLQGLNSADLLKAL CYPRVKVGNEFTKGQTVQQVYNAVGALAKAVYDKMFLWMVT
 60 °C MHYGNLKFQKQKQREEQAEPDGTEVADKAAYLQGLNSADLLKAL CYPRVKVGNEFTKGQTVQQVYNAVGALAKAVYDKMFLWMVT
 65 °C MHYGNLKFQKQKQREEQAEPDGTEVADKAAYLQGLNSADLLKAL CYPRVKVGNEFTKGQTVQQVYNAVGALAKAVYDKMFLWMVT
 70 °C MHYGNLKFQKQKQREEQAEPDGTEVADKAAYLQGLNSADLLKAL CYPRVKVGNEFTKGQTVQQVYNAVGALAKAVYDKMFLWMVT
 100 °C MHYGNLKFQKQKQREEQAEPDGTEVADKAAYLQGLNSADLLKAL CYPRVKVGNEFTKGQTVQQVYNAVGALAKAVYDKMFLWMVT

4 °C RINQQLDTKQPRQYFIGVLDIAGFEIFDFNSLEQLCINFTEKLNQQF FNHHMFVLEQEYKKEGIEWEFIDFGMDLAACIELIEKPMGIFSILE
 60 °C RINQQLDTKQPRQYFIGVLDIAGFEIFDFNSLEQLCINFTEKLNQQF FNHHMFVLEQEYKKEGIEWEFIDFGMDLAACIELIEKPMGIFSILE
 65 °C RINQQLDTKQPRQYFIGVLDIAGFEIFDFNSLEQLCINFTEKLNQQF FNHHMFVLEQEYKKEGIEWEFIDFGMDLAACIELIEKPMGIFSILE
 70 °C RINQQLDTKQPRQYFIGVLDIAGFEIFDFNSLEQLCINFTEKLNQQF FNHHMFVLEQEYKKEGIEWEFIDFGMDLAACIELIEKPMGIFSILE
 100 °C RINQQLDTKQPRQYFIGVLDIAGFEIFDFNSLEQLCINFTEKLNQQF FNHHMFVLEQEYKKEGIEWEFIDFGMDLAACIELIEKPMGIFSILE

4 °C EECMPKATDTSFKNKLYEQHLGKSNNFQPKPAKGKAEEAHFSI HYAGTVDYNTGWLKDKNKDPLNETVVGLYQKSSVKTLaFLFAER
 60 °C EECMPKATDTSFKNKLYEQHLGKSNNFQPKPAKGKAEEAHFSI HYAGTVDYNTGWLKDKNKDPLNETVVGLYQKSSVKTLaFLFAER
 65 °C EECMPKATDTSFKNKLYEQHLGKSNNFQPKPAKGKAEEAHFSI HYAGTVDYNTGWLKDKNKDPLNETVVGLYQKSSVKTLaFLFAER
 70 °C EECMPKATDTSFKNKLYEQHLGKSNNFQPKPAKGKAEEAHFSI HYAGTVDYNTGWLKDKNKDPLNETVVGLYQKSSVKTLaFLFAER
 100 °C EECMPKATDTSFKNKLYEQHLGKSNNFQPKPAKGKAEEAHFSI HYAGTVDYNTGWLKDKNKDPLNETVVGLYQKSSVKTLaFLFAER

4 °C QSSEEGGTTKGGKKKGSSFQTVSALFRENLNKLMTNLRSTHPHV. RCIIIPNETKTPGAMEHEELVHLQRLCNGVLEGIRICRKGFPSRILYAD
 60 °C QSSEEGGTTKGGKKKGSSFQTVSALFRENLNKLMTNLRSTHPHV. RCIIIPNETKTPGAMEHEELVHLQRLCNGVLEGIRICRKGFPSRILYAD
 65 °C QSSEEGGTTKGGKKKGSSFQTVSALFRENLNKLMTNLRSTHPHV. RCIIIPNETKTPGAMEHEELVHLQRLCNGVLEGIRICRKGFPSRILYAD
 70 °C QSSEEGGTTKGGKKKGSSFQTVSALFRENLNKLMTNLRSTHPHV. RCIIIPNETKTPGAMEHEELVHLQRLCNGVLEGIRICRKGFPSRILYAD
 100 °C QSSEEGGTTKGGKKKGSSFQTVSALFRENLNKLMTNLRSTHPHV. RCIIIPNETKTPGAMEHEELVHLQRLCNGVLEGIRICRKGFPSRILYAD

4 °C FKQRYKVLNASAIPEGQFIDSKKASEKLLGSIDIDHTQYKFGHTKV FFKAGLLGTLEEMRDEK
 60 °C FKQRYKVLNASAIPEGQFIDSKKASEKLLGSIDIDHTQYKFGHTKV FFKAGLLGTLEEMRDEK
 65 °C FKQRYKVLNASAIPEGQFIDSKKASEKLLGSIDIDHTQYKFGHTKV FFKAGLLGTLEEMRDEK
 70 °C FKQRYKVLNASAIPEGQFIDSKKASEKLLGSIDIDHTQYKFGHTKV FFKAGLLGTLEEMRDEK
 100 °C FKQRYKVLNASAIPEGQFIDSKKASEKLLGSIDIDHTQYKFGHTKV FFKAGLLGTLEEMRDEK

(a1)

Figure 6. continued

4 °C FCIQYNIRAFMNVKHWPWMKLYFKIKPLL_{ksaeteke manmk} EEF EKT KED LAK SE AKR KE LE EK MVAL MQE KNDLQLQVQAEA
 60 °C FCIQYNIRAFMNVKHWPWMKLYFKIKPLL_{ksaeteke manmk} EEF EKT KED LAK SE AKR KE LE EK MVAL MQE KNDLQLQVQAEA
 65 °C FCIQYNIRAFMNVKHWPWMKLYFKIKPLL_{ksaeteke manmk} EEF EKT KED LAK SE AKR KE LE EK MVAL MQE KNDLQLQVQAEA
 70 °C FCIQYNIRAFMNVKHWPWMKLYFKIKPLL_{ksaeteke manmk} EEF EKT KED LAK SE AKR KE LE EK MVAL MQE KNDLQLQVQAEA
 100 °C FCIQYNIRAFMNVKHWPWMKLYFKIKPLL_{ksaeteke manmk} EEF EKT KED LAK SE AKR KE LE EK MVAL MQE KNDLQLQVQAEA

4 °C DGLADAAEERCDQLIKTKIQL_{EA}KIKEVTERAEDEEINAELTAKK RKEDECSELKKDIDDLTLAKVEKEKHATENKVNLTEEMAG
 60 °C DGLADAAEERCDQLIKTKIQL_{EA}KIKEVTERAEDEEINAELTAKK RKEDECSELKKDIDDLTLAKVEKEKHATENKVNLTEEMAG
 65 °C DGLADAAEERCDQLIKTKIQL_{EA}KIKEVTERAEDEEINAELTAKK RKEDECSELKKDIDDLTLAKVEKEKHATENKVNLTEEMAG
 70 °C DGLADAAEERCDQLIKTKIQL_{EA}KIKEVTERAEDEEINAELTAKK RKEDECSELKKDIDDLTLAKVEKEKHATENKVNLTEEMAG
 100 °C DGLADAAEERCDQLIKTKIQL_{EA}KIKEVTERAEDEEINAELTAKK RKEDECSELKKDIDDLTLAKVEKEKHATENKVNLTEEMAG

4 °C LDENIAKLTKKEKKALQEAHQTLDDLQAEEDKVNTLTKAKTKLE QQVDDLEGSLEQEKKLRLMDLERAKRKLEGDLKL_{AQESTMDIEND}
 60 °C LDENIAKLTKKEKKALQEAHQTLDDLQAEEDKVNTLTKAKTKLE QQVDDLEGSLEQEKKLRLMDLERAKRKLEGDLKL_{AQESTMDIEND}
 65 °C LDENIAKLTKKEKKALQEAHQTLDDLQAEEDKVNTLTKAKTKLE QQVDDLEGSLEQEKKLRLMDLERAKRKLEGDLKL_{AQESTMDIEND}
 70 °C LDENIAKLTKKEKKALQEAHQTLDDLQAEEDKVNTLTKAKTKLE QQVDDLEGSLEQEKKLRLMDLERAKRKLEGDLKL_{AQESTMDIEND}
 100 °C LDENIAKLTKKEKKALQEAHQTLDDLQAEEDKVNTLTKAKTKLE QQVDDLEGSLEQEKKLRLMDLERAKRKLEGDLKL_{AQESTMDIEND}

4 °C KQQLDEKLKKKEFEMSNLQSKIEDEQALAMQLQKKIKELQARTE ELEEEIEAERASRAKAEKQRSDL SRELEEI SERLLEAGGATSQIE
 60 °C KQQLDEKLKKKEFEMSNLQSKIEDEQALAMQLQKKIKELQARTE ELEEEIEAERASRAKAEKQRSDL SRELEEI SERLLEAGGATSQIE
 65 °C KQQLDEKLKKKEFEMSNLQSKIEDEQALAMQLQKKIKELQARTE ELEEEIEAERASRAKAEKQRSDL SRELEEI SERLLEAGGATSQIE
 70 °C KQQLDEKLKKKEFEMSNLQSKIEDEQALAMQLQKKIKELQARTE ELEEEIEAERASRAKAEKQRSDL SRELEEI SERLLEAGGATSQIE
 100 °C KQQLDEKLKKKEFEMSNLQSKIEDEQALAMQLQKKIKELQARTE ELEEEIEAERASRAKAEKQRSDL SRELEEI SERLLEAGGATSQIE

4 °C MNKKREA EF QKM RR DLEEATLQHEATAA ALR KK HAD SV AEL GE QIDNLQ RV KQ KLE KE K SEL KM EIDD L AS NM ET V SKA KG N LE KM
 60 °C MNKKREA EF QKM RR DLEEATLQHEATAA ALR KK HAD SV AEL GE QIDNLQ RV KQ KLE KE K SEL KM EIDD L AS NM ET V SKA KG N LE KM
 65 °C MNKKREA EF QKM RR DLEEATLQHEATAA ALR KK HAD SV AEL GE QIDNLQ RV KQ KLE KE K SEL KM EIDD L AS NM ET V SKA KG N LE KM
 70 °C MNKKREA EF QKM RR DLEEATLQHEATAA ALR KK HAD SV AEL GE QIDNLQ RV KQ KLE KE K SEL KM EIDD L AS NM ET V SKA KG N LE KM
 100 °C MNKKREA EF QKM RR DLEEATLQHEATAA ALR KK HAD SV AEL GE QIDNLQ RV KQ KLE KE K SEL KM EIDD L AS NM ET V SKA KG N LE KM

(a2)

Figure 6. continued

4 °C	CRTLEDQLSEVKTKEEEHQRLINELSAQKARIQTESGEFSRQLD EKEALSQLSRGKQAFQQIEELKRQLEEETAKSALAHAVQSS
60 °C	CRTLEDQLSEVKTKEEEHQRLINELSAQKARIQTESGEFSRQLD EKEALSQLSRGKQAFQQIEELKRQLEEETAKSALAHAVQSS
65 °C	CRTLEDQLSEVKTKEEEHQRLINELSAQKARIQTESGEFSRQLD EKEALSQLSRGKQAFQQIEELKRQLEEETAKSALAHAVQSS
70 °C	CRTLEDQLSEVKTKEEEHQRLINELSAQKARIQTESGEFSRQLD EKEALSQLSRGKQAFQQIEELKRQLEEETAKSALAHAVQSS
100 °C	CRTLEDQLSEVKTKEEEHQRLINELSAQKARLQTESGEFSRQLD EKEALSQLSRGKQAFQQIEELKRQLEEETAKSALAHAVQSS
4 °C	RHDCDLLREQYEEEQEAQAKELQRAMSKANSEVAQWRKYETD AIQRTEELEAKKLAQRLQDAEEHVAVNAKCASTLETKQRL
60 °C	RHDCDLLREQYEEEQEAQAKELQRAMSKANSEVAQWRKYETD AIQRTEELEAKKLAQRLQDAEEHVAVNAKCASTLETKQRL
65 °C	RHDCDLLREQYEEEQEAQAKELQRAMSKANSEVAQWRKYETD AIQRTEELEAKKLAQRLQDAEEHVAVNAKCASTLETKQRL
70 °C	RHDCDLLREQYEEEQEAQAKELQRAMSKANSEVAQWRKYETD AIQRTEELEAKKLAQRLQDAEEHVAVNAKCASTLETKQRL
100 °C	RHDCDLLREQYEEEQEAQAKELQRAMSKANSEVAQWRKYETD AIQRTEELEAKKLAQRLQDAEEHVAVNAKCASTLETKQRL
4 °C	QNEVEDLMDVERSNAACAA D DKKQRNF D KILA E W H KYETQA E ASQ K ES S L T ELFKVNAYEESLDQ L ETLKRENKNLQQ
60 °C	QNEVEDLMDVERSNAACAA D DKKQRNF D KILA E W H KYETQA E ASQ K ES S L T ELFKVNAYEESLDQ L ETLKRENKNLQQ
65 °C	QNEVEDLMDVERSNAACAA D DKKQRNF D KILA E W H KYETQA E ASQ K ES S L T ELFKVNAYEESLDQ L ETLKRENKNLQQ
70 °C	QNEVEDLMDVERSNAACAA D DKKQRNF D KILA E W H KYETQA E ASQ K ES S L T ELFKVNAYEESLDQ L ETLKRENKNLQQ
100 °C	QNEVEDLMDVERSNAACAA D DKKQRNF D KILA E W H KYETQA E ASQ K ES S L T ELFKVNAYEESLDQ L ETLKRENKNLQQ
4 °C	EISDLTEQIAEGGKHIIHELEVKVKKQIEQKSELQA A EEASLEHEEGKILRIQ L ENQVKSEIDRKIAEKDEEIDQMCRNHRV V SMQS
60 °C	EISDLTEQIAEGGKHIIHELEVKVKKQIEQKSELQA A EEASLEHEEGKILRIQ L ENQVKSEIDRKIAEKDEEIDQMCRNHRV V SMQS
65 °C	EISDLTEQIAEGGKHIIHELEVKVKKQIEQKSELQA A EEASLEHEEGKILRIQ L ENQVKSEIDRKIAEKDEEIDQMCRNHRV V SMQS
70 °C	EISDLTEQIAEGGKHIIHELEVKVKKQIEQKSELQA A EEASLEHEEGKILRIQ L ENQVKSEIDRKIAEKDEEIDQMCRNHRV V SMQS
100 °C	EISDLTEQIAEGGKHIIHELEVKVKKQIEQKSELQA A EEASLEHEEGKILRIQ L ENQVKSEIDRKIAEKDEEIDQMCRNHRV V SMQS
4 °C	TLDAEIRSRNDALRIKKM E GLNEMEIQLNHANRQATEAIRNLR NTQGV L KDTQ L H L DDA I R G Q D DLKEQLAMVER R ANLMQAEIEEL
60 °C	TLDAEIRSRNDALRIKKM E GLNEMEIQLNHANRQATEAIRNLR NTQGV L KDTQ L H L DDA I R G Q D DLKEQLAMVER R ANLMQAEIEEL
65 °C	TLDAEIRSRNDALRIKKM E GLNEMEIQLNHANRQATEAIRNLR NTQGV L KDTQ L H L DDA I R G Q D DLKEQLAMVER R ANLMQAEIEEL
70 °C	TLDAEIRSRNDALRIKKM E GLNEMEIQLNHANRQATEAIRNLR NTQGV L KDTQ L H L DDA I R G Q D DLKEQLAMVER R ANLMQAEIEEL
100 °C	TLDAEIRSRNDALRIKKM E GLNEMEIQLNHANRQATEAIRNLR NTQGV L KDTQ L H L DDA I R G Q D DLKEQLAMVER R ANLMQAEIEEL
4 °C	RASLEQTERSRRVAEQELLDASERVLQLHTQNTSLINTKKKLET D SIQGEMEDIVQEARNAAEKKAKITDAAMMAEFLKKEQDTS A
60 °C	RASLEQTERSRRVAEQELLDASERVLQLHTQNTSLINTKKKLET D SIQGEMEDIVQEARNAAEKKAKITDAAMMAEFLKKEQDTS A
65 °C	RASLEQTERSRRVAEQELLDASERVLQLHTQNTSLINTKKKLET D SIQGEMEDIVQEARNAAEKKAKITDAAMMAEFLKKEQDTS A
70 °C	RASLEQTERSRRVAEQELLDASERVLQLHTQNTSLINTKKKLET D SIQGEMEDIVQEARNAAEKKAKITDAAMMAEFLKKEQDTS A
100 °C	RASLEQTERSRRVAEQELLDASERVLQLHTQNTSLINTKKKLET D SIQGEMEDIVQEARNAAEKKAKITDAAMMAEFLKKEQDTS A
4 °C	LERMKKNMEQTVKDQLQHRLDEAEQLALKGGKKQI Q KLEARVRELENEVENEQKRNV E AVKGLRKHERRV K ELTYQTEEDRK N V L R L Q
60 °C	LERMKKNMEQTVKDQLQHRLDEAEQLALKGGKKQI Q KLEARVRELENEVENEQKRNV E AVKGLRKHERRV K ELTYQTEEDRK N V L R L Q
65 °C	LERMKKNMEQTVKDQLQHRLDEAEQLALKGGKKQI Q KLEARVRELENEVENEQKRNV E AVKGLRKHERRV K ELTYQTEEDRK N V L R L Q
70 °C	LERMKKNMEQTVKDQLQHRLDEAEQLALKGGKKQI Q KLEARVRELENEVENEQKRNV E AVKGLRKHERRV K ELTYQTEEDRK N V L R L Q
100 °C	LERMKKNMEQTVKDQLQHRLDEAEQLALKGGKKQI Q KLEARVRELENEVENEQKRNV E AVKGLRKHERRV K ELTYQTEEDRK N V L R L Q
4 °C	DLVDKLQSKVKAYKRQAA E EEEQSNVNL S FRKLQ H E E EE A ER ADIAESQVNKL R V K SREVHTKVISEE
60 °C	DLVDKLQSKVKAYKRQAA E EEEQSNVNL S FRKLQ H E E EE A ER ADIAESQVNKL R V K SREVHTKVISEE
65 °C	DLVDKLQSKVKAYKRQAA E EEEQSNVNL S FRKLQ H E E EE A ER ADIAESQVNKL R V K SREVHTKVISEE
70 °C	DLVDKLQSKVKAYKRQAA E EEEQSNVNL S FRKLQ H E E EE A ER ADIAESQVNKL R V K SREVHTKVISEE
100 °C	DLVDKLQSKVKAYKRQAA E EEEQSNVNL S FRKLQ H E E EE A ER ADIAESQVNKL R V K SREVHTKVISEE

(a3)

Figure 6. continued

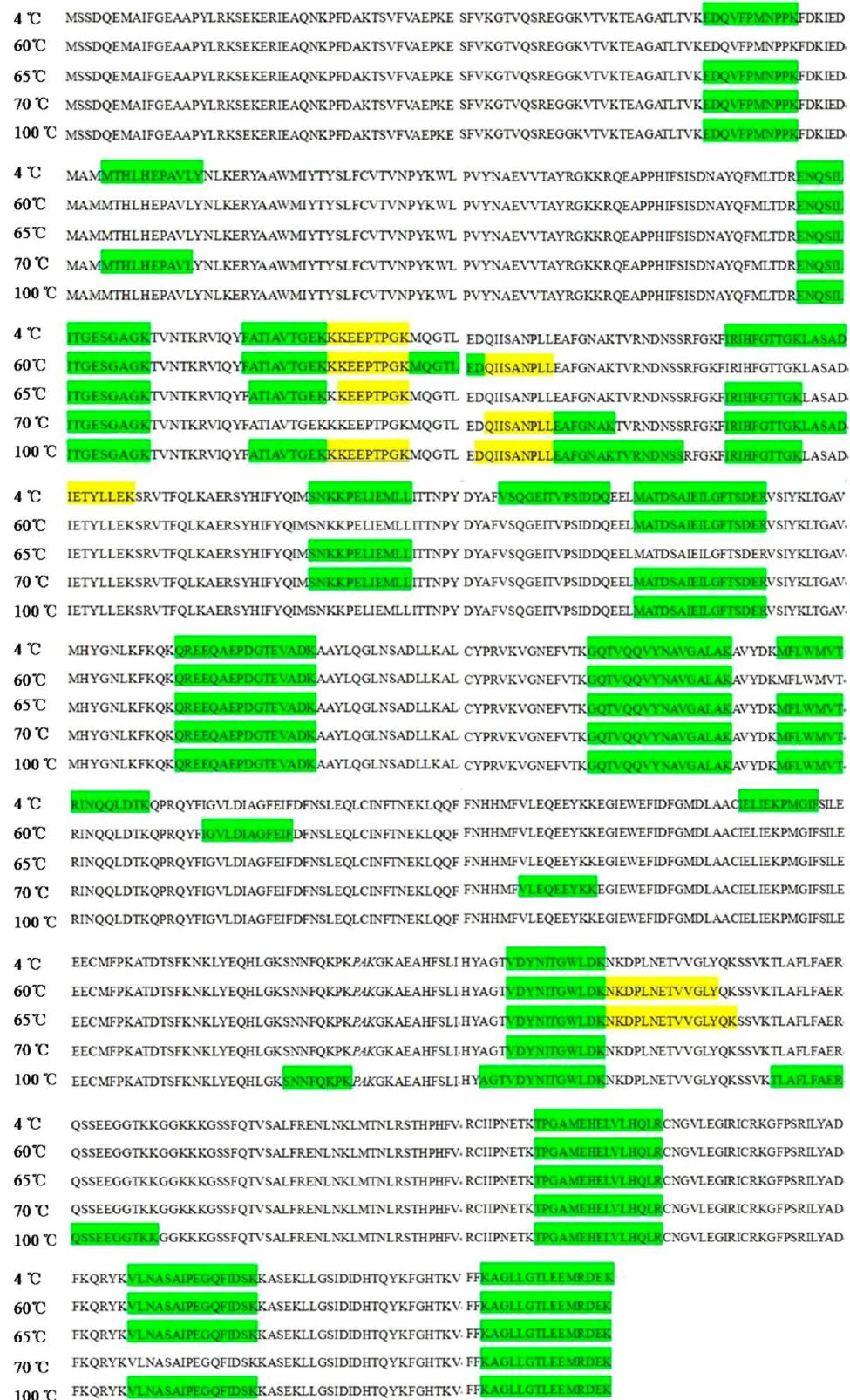


Figure 6. continued



(b2)

Figure 6. continued



(b3)

Figure 6. Localization of peptides from myosin-4 in response to cooking: (a1) peptides from pepsin-digested samples located at myosin head-like domain (positions 1–781), (a2) peptides from pepsin-digested samples located at myosin coiled tail domain (positions 812–1250), (a3) peptides from pepsin-digested samples located at myosin coiled tail domain (positions 1251–1937), (b1) peptides from pepsin/trypsin-digested samples located at myosin head-like domain (positions 1–781), (b2) peptides from pepsin/trypsin-digested samples located at myosin coiled tail domain (positions 812–1250), and (b3) peptides from pepsin/trypsin-digested samples located at myosin coiled tail domain (positions 1251–1937). Different colors of highlights were used to distinguish the peptides just next to each other.

318 different cooking temperatures (b1–b3 of Figure 6). For
319 pepsin-digested samples, 10 peptides appeared in all five
320 groups, while 33 peptides appeared in trypsin digestion with

321 the carbon-terminal cleavage sites as Lys and Arg. Although it
322 could be due to the trypsin peptides better identified in the
323 database search than pepsin, this indicates that cooking may
324

324 change the conformation of proteins but the cleavage sites of
325 digestive enzymes change little.

326 In summary, cooking had a significant effect on *in vitro*
327 digestibility and pepsin digestion but not for pepsin/trypsin
328 digestion. Characterized peptides showed the stochasticity of
329 pepsin cleavage and the stability of trypsin cleavage. Although
330 there was no significant difference in peptide profiles after
331 two-step digestion, it could be significant for absorption and
332 bioavailability in the live body, and additionally, some of the
333 peptides could have bioactive functions.

334 ■ ASSOCIATED CONTENT

335 ■ Supporting Information

336 LC-MS information on peptide sequences from pepsin-treated
337 and pepsin/trypsin-treated samples. This material is available
338 free of charge via the Internet at <http://pubs.acs.org>.

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351 University, for assisting in MALDI-ToF MS analysis.

352 ■ ABBREVIATIONS USED

353 SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electro-
354 phoresis; MALDI-ToF MS, matrix-assisted laser desorption/
355 ionization-time-of-flight mass spectrometry; Nano LC-Q-Exactive-
356 MS-MS, hybrid quadrupole orbitrap mass spectrometer equipped
357 with a nanoelectrospray ionization source with an online nano-LC
358 system

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