**A comparison of endogenous and microbial proteolytic activities during fast
fermentation of silver carp inoculated with *Lactobacillus Plantarum***

Running title: Proteolytic activities in fermented fish surimi

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Abstract The study was aimed to investigate different roles of endogenous and *Lactobacillus Plantarum* proteases during fast fermentation of silver carp. The results show that endogenous proteases could degrade both sarcoplasmic and myofibrillar proteins. In contrast, *Lactobacillus Plantarum* had low proteinase activities and could only hydrolyze sarcoplasmic peptides. This indicates that gel properties could be mainly affected by endogenous proteolysis while microbial proteolysis contributed to the production of smaller peptides and free amino acids which may be related to flavor and taste. Texture and free amino acid analyses verified these hypotheses. It shows that endogenous lysosomal proteases were the major contributors for the decrease of gel strength while *Lactobacillus Plantarum* proteolytic activities could lead to the increase of aspartic acid, glutamic acid, and alanine, which may result in umami and sweet taste; and also lead to a rise in some amino acids which were volatile compounds precursors.

Keywords fast fish fermentation; endogenous; *Lactobacillus Plantarum*; proteolytic activities; gel properties; taste formation.

24

25 **1. Introduction**

26 Fish is highly perishable and application of fermentation in fish processing would provide a
27 method both to preserve fish and modify their sensory and functional properties (Riebroy, Benjakul,
28 & Visessanguan, 2008; Xu, Xia, Yang, Kim, & Nie, 2010). Traditional techniques for fermentation
29 of fish are conducted as spontaneous processes at household or small scale levels. This natural
30 maturation is time consuming and the quality is highly variable based on the climate. In order to
31 realize industrialization, standardized methods should be established. Since a long time
32 fermentation may increase the cost and protein and lipid oxidation during this long period may lead
33 to health risk, a preliminary trial in our lab was made to shorten the fermentation time. This was
34 based on the microbial, physical and chemical properties of fermented silver carp surimi (Xu, Xia,
35 Yang, Kim, & Nie, 2010). However, the respective roles of endogenous and microbial proteases in
36 this fast fermentation were not clear.

37 Proteolytic activities play an important role in a product's organoleptic qualities, such as gel
38 properties and taste development (An, Peters, & Seymour, 1996; Molly, Demeyer, Johansson,
39 Raemaekers, Ghistelinck, & Geenen, 1997). A better understanding of endogenous and microbial
40 proteolytic activities would provide a theoretical foundation to control proteolysis and thus help to
41 produce a high-quality product. Proteolytic activities in fermented dairy food (Khalid & Marth,
42 1990) or meat product (Hierro, de la Hoz, & Ordóñez, 1999) were investigated systematically in
43 previous literature. However, that in the fermentation of fish, especially fast fermentation was not
44 fully understood. Since different materials could have different proteolysis systems, an investigation
45 in a fermented fish product is needed. In addition, in the studies of fermented meat products,
46 different views are given on the respective roles of endogenous and microbial proteolytic activities.

One view is that meat proteins are firstly hydrolyzed by endogenous enzymes such as collagenases, calpains and cathepsins and finally degraded into smaller peptides and free amino acids by bacterial proteases (Molly, Demeyer, Johansson, Raemaekers, Ghistelinck, & Geenen, 1997), while in other studies, lactic acid bacteria could hydrolyze high-molecular-weight protein and therefore could have influence on the quality of products at the beginning of fermentation (Hughes, Kerry, Arendt, Kenneally, McSweeney, & O'Neill, 2002; Ohashi & Negishi, 2014; Sanz, Fadda, Vignolo, Aristoy, Oliver, & Toldrá, 1999). What happens in fast fermentation of fish remains to be known.

Sarcoplasmic and myofibrillar proteins which are important components of fish muscle protein have an important role in functional and structural properties of fish products. Content of α -amino nitrogen could show amount of small peptides and free amino acids and thus help to determine aminopeptidase activities in the system. Addition of specific protease inhibitors could discriminate respective roles of different endogenous enzymes on gel strength. Free amino acid measurement would help to analyze taste development. *Lactobacillus Plantarum* was used because it is a common microorganism which has been found in different fermented fish and fish products from China, Thailand, Philippines and Israel, etc. (Gelman, Drabkin, & Glatman, 2000; Olympia, Fukuda, Ono, Kaneko, & Takano, 1995; Paludan-Müller, Huss, & Gram, 1999; Zeng et al., 2014). The strain used in this study was obtained in our lab and showed good potential to produce high-quality fermented fish product (Zeng et al., 2014). The objective of this study was therefore to investigate contributions of endogenous and *Lactobacillus Plantarum* proteases during fast fermentation.

2. Materials and methods

2.1 Materials

69 Fresh silver carp (*Hypophthalmichthys molitrix*, 2-3kg/fish) was obtained from a local
70 supermarket near Jiangnan University in the spring. They were immediately decapitated,
71 eviscerated and transported to our lab after purchase. Dorsal muscle was used.

72 Bovine serum albumin (BSA), hemoglobin (Hb), trans-epoxysuccinyl-L-leucyl-amido
73 (4-guanidino) butane (E-64), aprotinin, pepstatin A, and leupeptin hydrochloride were purchased
74 from Sigma Chemical Co. (St. Louis, MO). Molecular weight marker and loading buffer (2×) for
75 sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were products of
76 Beyotime Biotechnology (Shanghai, China). Penicillin, streptomycin, amphotericin were bought
77 from Zhongnuo Pharmaceutical Co., Ltd (Shijiazhuang, China), Shenzhen Huayao Nanfang
78 Pharmaceutical Co., Ltd. (Shengzhen, China) and Shanghai Jinsui Bio-Technology Co., Ltd
79 (Shanghai, China), respectively. D-gluconic acid δ -lactone (GDL) was bought from Shanghai
80 Luzhou Food Additive Co., Ltd. (Shanghai, China). All the other chemical regents were of
81 analytical grade.

82 83 2.2 Preparation of samples

84 Three groups were designed, i.e., acidified control group (AG), endogenous proteases group
85 (EG), and endogenous plus microbial proteases group (EMG). Samples in all the groups were
86 incubated at 30 °C for 48 hours. Common formula was as follows: 2 g NaCl, 2.5 g GDL, antibiotics
87 (penicillin 8,000 I.U., streptomycin 20 mg, amphotericin 50 mg) in 100 g fish mince. To AG,
88 protease inhibitors (100 μ mol leupeptin and 1 μ mol pepstatin A) were added; to EG, no more
89 ingredients were added; to EMG, crude protease extract from *Lactobacillus Plantarum* was added.
90 The crude extract was the supernatant from starter culture after centrifugation at 10,000 g, 4 °C for
91 15 min. The starter culture was prepared as described by Zeng, Xia, Yang, and Jiang (2013).

92

93 2.3 *Proteolytic activities assay*

94 The proteolytic activities were measured as described by Sovik and Rustad (2004) with slight
95 modification. Samples of three groups taken out at 0, 12, 24, 36, 48 h were homogenized in 2
96 volumes of distilled water at 12,000 rpm for 30 seconds and then stirred occasionally for 30 min at
97 refrigerated temperature. After centrifuged for 20 min (14,600 g, 4 °C), the supernatant was filtered
98 through three-layer-cloth and kept as crude enzyme extract. Each 1 mL extract was added to 1 mL
99 1% (w/v) Hb and then the mixture was incubated at 30 °C for 1 hour. The reaction was terminated
100 by adding 4 mL 5% (w/v) trichloroacetic acid (TCA). After kept at 4 °C for 30 minutes, the mixture
101 was filtered and a sum of peptide and amino acid concentration in the filtration was measured by
102 Lowry method (1951). One unit (U) was defined as mg Hb cut per g water soluble protein per hour.
103 The water soluble protein was measured by Biuret method (Gornall, Bardawill, & David, 1949).

104

105 2.4 *Electrophoresis analysis*

106 The extraction of two types of proteins was carried as described by Nie, Lin, and Zhang (2014)
107 with different buffer. Fresh dorsal muscle was homogenized with 5 volumes of 50 mmol/L
108 phosphate buffer (pH 7.0) and then kept for 30 min (4 °C). After centrifuged for 20 min (8000 g, 4
109 °C), the supernatant was filtered through three-layer-cloth and used as sarcoplasmic proteins. The
110 precipitate was resolved in 10 volumes of 50 mmol/L phosphate buffer (pH 7.0) containing 0.6
111 mol/L of NaCl. The following steps were carried out as described above. This supernatant was used
112 as myofibrillar proteins.

113 Three groups – AG, EG, and EMG – were designed with the formula the same as described
114 above in Method 2.2 with the change of “in 100 g fish mince” to “in 100 mL solution”. Samples of

all groups were incubated at 30 °C for 2 days and electrophoresis analysis of samples on 0 and 2nd day were performed. For each sample on 2nd day, precipitate occurred and therefore supernatant and precipitate were separated for experiment. Supernatant was obtained by centrifugation at 12,000 g, 4 °C for 20 minutes. Precipitate was treated with 9 volumes of 5% (w/v) sodium dodecyl sulfate (SDS) at 100 °C for 1 hour, centrifuged at 12,000 g, 4 °C for 20 minutes and the supernatant was collected for use. Both supernatant and treated precipitate solution were adjusted to 4 mg/mL, filtered through 0.45 µm filtration membrane, and mixed with loading buffer (2×). Before SDS-PAGE, the loading mixtures were boiled for 5 minutes. Each 10 µL of loading sample was used on 4% stacking gel and 12% resolving gel. After electrophoresis, gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 40% (v/v) ethanol and 10% (v/v) acetic acid for 4 hours, and then destained with a solution of 40% (v/v) ethanol and 10% (v/v) acetic acid for 1-2 hours.

126

2.5 Determination of α -amino nitrogen (AAN)

Samples of sarcoplasmic and myofibrillar proteins on 0 day and supernatant on 2nd day were used. The content of α -amino nitrogen was determined by Sorensen method (Taylor, 1957) with slight modification. Briefly, each sample was diluted 140 folds with distilled water and 70 mL of each was titrated with 0.05 mol/L NaOH to the end pH of 8.2. The level mark was recorded as V_1 . A formalin solution (37%, 10 mL) was then added and the mixture was titrated again with NaOH to the end pH of 9.2. This level mark was recorded as V_2 . Subsequently, distilled water in replacement of sample was performed in the same procedures and V_0 and V_0' were recorded respectively. The AAN content was calculated as follows:

$$AAN(mg \cdot 100mL^{-1}) = \frac{[(V_2 - V_1) - (V_0' - V_0)] \times C_{NaOH} \times M_{Nitrogen}}{70mL} \times 140folds \times 100$$

137

138 2.6 *Effect of different endogenous proteases on gel strength*

139 Pepstatin, E-64, aprotinin and EDTA were used as different specific protease inhibitors and
140 their concentrations were 25, 10, 10, 20 $\mu\text{mol/kg}$, respectively. Other ingredients are shown in
141 common formula in Method 2.2. All the samples were incubated at 30 °C for 48 hours. Blank was
142 sample without any inhibitors.

143 Samples were trimmed to the same size before textural measurement by a Universal TA-XT2i
144 texture analyzer (Stable Micro Systems, Surrey, UK). The probe was a P/0.25S spherical plunger.
145 Pre-test, test, and post-test speeds were all 1 cm/s, the strain was 80%, and a 1 kg load-cell was
146 used for calibrating the instrument. Breaking force (g) and deformation (mm) were recorded at
147 breaking point. The gel strength was given as the product of breaking force and deformation. All
148 determinations were performed in triplicate.

149

150 2.7 *Free amino acids analysis*

151 Free amino acids in fresh fish muscle and another two groups of fish surimi (EG and EMG)
152 taken out after two days were measured as described by Zeng, Xia, Yang, and Jiang (2013).
153 Samples (5 g) were homogenized with 5% (w/v) TCA and made up to 25 mL with the same TCA
154 solution. The mixture was then treated by ultrasonic wave for 30 min. After standing for 1 h, it
155 was filtered with two-layer filter paper. The supernatant after centrifugation at 10000 rpm for 5
156 min was kept as loading sample. A high-performance liquid chromatography (HPLC) (Agilent
157 1100 series, Palo Alto, CA, USA) equipped with a Hypersil ODS C18 column was used for
158 determination of free amino acid content. The column was 250×4.6 mm (5 μm particle size), and
159 the temperature was controlled at 40 °C. Two eluents were used in the solvent system: Buffer A

(7.35 mM sodium acetate/triethylamine/tetrahydrofuran, 500/0.12/2.5, v/v/v, pH 7.2) and buffer B (7.35 mM sodium acetate/methanol/acetonitrile, 1/2/2, v/v/v, pH 7.2). To separate different amino acids, the flow rate was set and gradient was performed as follows: Initial-27.5 min 1.0 mL/min with 92% A and 8% B; 27.5-31.5 min 1.0 mL/min with 40% A and 60% B; 31.5-34 min 1.5 mL/min with 100% B to wash; 34-35.5 min 1.0 mL/min with 100% B to wash and finally reequilibrate by 1.0 mL/min with 92% A and 8% B. O-Phthaldialdehyde (OPA) was used as the pre-column derivation reagent and the detection wavelengths were set at UV 338nm (amino acids except for proline) and 262nm (proline).

2.8 Data analysis

SPSS 11.0 for windows software was used for statistical analysis (SPSS Inc., Chicago, Illinois, USA). Analysis of variance (ANOVA) was calculated for data processing. Significant differences were defined at $P < 0.05$. Comparisons of group means were obtained using Duncan's multiple range tests.

3. Results and discussion

3.1 Comparison of endogenous and microbial proteolytic activities

As can be seen in Fig.1, endogenous proteolytic activity (EG) grew gradually as incubation time increased. The rapid increase of proteolytic activity was accompanied with the fast decline in pH during the first 12 hours, indicating that acid could activate endogenous proteases. However, little difference was found between EG and EMG, implying that proteinases in *Lactobacillus Plantarum* may be very low. In other literature, lactic acid bacteria were also thought to have limited ability for proteolysis, while micrococci, staphylococci, proteus, bacillus, coliforms were the

183 main contributors for hydrolysis during fermentation (Chaves-López, Paparella, Tofalo, & Suzzi,
184 2011; Durá, Flores, & Toldrá, 2004; Kröckel, 1995). However, some *Lactobacillus Plantarum*
185 strain had high proteolytic activities (Fadda, Sanz, Vignolo, Aristoy, Oliver, & Toldrá, 1999b)
186 which may be due to different sources. According to Fadda, Vignolo, Holgado, and Oliver (1998),
187 large amounts of free amino acids were produced during 48-72 h of sausage fermentation inoculated
188 with *Lactobacillus Plantarum* and this bacterium had dipeptidyl peptidases, tripeptidases and
189 aminopeptidases. Therefore, even if the strain of *Lactobacillus Plantarum* in our study had low
190 ability for the initial degradation of protein, it may have influence on the secondary hydrolysis of
191 small peptides which would be investigated in the following experiment.

192

193 3.2 Hydrolysis of sarcoplasmic and myofibrillar proteins

194 The bands were the same among AG, EG, and EMG at the beginning of incubation (Fig.2A),
195 indicating that the addition of microbial crude enzyme extract and protease inhibitors did not affect
196 the electrophoresis analysis. As shown in Fig.2A, protein components whose molecular weights >
197 90 kDa disappeared and bands of other components lightened after two days in the supernatant of
198 AG. In addition, the lightened bands were recovered in the precipitate (Fig.2B). This suggests that
199 certain hydrolysis or aggregation of sarcoplasmic proteins was induced by acid. In Fig.2A, it could
200 be seen that the widest band at 39~35 kDa which was referred to be two components, creatine
201 kinase and aldolase (Ladtrat, Verrez-Bagnis, Noël, & Fleurence, 2003), became less colored in AG.
202 Meanwhile, proteins of ~31 and ~18 kDa which were indicated as glyceraldehyde-3-phosphate
203 dehydrogenase and nucleoside diphosphate kinase (Ladtrat, Verrez-Bagnis, Noël, & Fleurence, 2003;
204 Spaziani, Torre, & Stecchini, 2009) were formed after 2-day incubation. From this, it could be
205 inferred that some sarcoplasmic proteins (SP) could be degraded and small peptides could be

206 formed by acid hydrolysis. Unlike AG, bands of EG and EMG in supernatant on 2nd day were very
207 light, especially in EMG, no bands could be seen by eye. This indicates higher degree of hydrolysis
208 caused by both proteases. Combined with the result of proteolytic activities, it could be inferred that
209 muscle proteinases initiated the first breakdown of sarcoplasmic proteins and *Lactobacillus*
210 *Plantarum* hydrolyzed the oligopeptides afterwards.

211 In precipitate of SP (Fig.2B), little difference were found between AG and EG, while EMG
212 had new bands at ~61 and ~53 kDa and intensified bands at ~27 kDa and below 22 kDa. This
213 indicates more degradation by *Lactobacillus Plantarum* peptidases and the produced peptides could
214 aggregate under acid conditions. However, bands of EG were no more than that in AG, suggesting
215 peptides produced by endogenous proteolysis could not induce cross-linking at low pH. This may
216 be due to different cleavage sites. Some endopeptidases, such as glutamic acid endopeptidase, could
217 cleave hydrophilic residues and keep most hydrophobic residues remained (Doucet, Otter, Gauthier,
218 & Foegeding, 2003). Therefore, hydrophobic interaction could be enhanced and aggregation could
219 be formed when pH decreases towards pI and electrostatic repulsion weakens. Endopeptidases in
220 *Lactobacillus Plantarum* were found to cleave some hydrophilic residues like glutamate, histidine
221 and lysine (Fadda, Sanz, Vignolo, Aristoy, Oliver, & Toldrá, 1999a). Therefore, many hydrophobic
222 groups may be exposed by moderate microbial hydrolysis from *Lactobacillus Plantarum* and thus
223 precipitate could be formed. On the contrary, endogenous proteases were indicated as non-specific,
224 containing both exo- and endo- peptidases (Toldrá, 2006). They could cleave both hydrophilic and
225 hydrophobic residues, thus not leading to aggregation under acid conditions.

226 As shown in myofibrillar proteins (Fig.2C and 2D), myosin heavy chain (MHC), actin and
227 myosin light chain-3 (MLC-3) decreased significantly in the supernatant of AG after two days and
228 meanwhile the lightened bands were recovered in the precipitate. This was similar as that in SP,

229 indicating that MHC, actin, and MLC-3 could be precipitated by acid. In Fig.2C, both MHC and
230 actin disappeared in EG and EMG after 2-day incubation. Troponin-T (TN-T), tropomyosin (TM)
231 and MLC-2 remained in the supernatant of EG, while only one intensified band near TN-T occurred
232 in the supernatant of EMG. Intensified bands at lower molecular weight may be caused by
233 co-migration of smaller protein and peptides from large protein, such as combination of TN-T and
234 fragments from myosin (Spaziani, Torre, & Stecchini, 2009). The degradation of α -actinin may be
235 attributed to cathepsin B, L and D while TM could be degraded by cathepsin L (Ladrat,
236 Verrez-Bagnis, Noël, & Fleurence, 2003).

237 Unlike sarcoplasmic proteins, no new bands occurred in precipitate of myofibrillar proteins
238 (MP) in EG and EMG compared to AG (Fig.2D), suggesting that peptides produced from MP by
239 proteolytic degradation could not aggregate under acid conditions. Since a better ability to
240 aggregate results in a better ability to form gel, hydrolysis of MP by endogenous and microbial
241 enzymes could lead to a weakening of gel strength, which is known as “modori”. Besides, actin in
242 the precipitate were significantly fewer in EG and EMG than that in AG, indicating that degradation
243 of actin was mainly caused by proteolytic hydrolysis rather than acid hydrolysis. In contrast, MHC
244 in the precipitate of all three groups was fewer than that in fresh MP. This indicates that MHC could
245 be hydrolyzed by both acid and proteases. In addition, little difference in supernatant and precipitate
246 could be seen in EG and EMG, which implies that microbial proteolytic activities had limited
247 influence on degradation of myofibrillar peptides. Considering that myofibrillar protein is closely
248 related to gel properties, microbial proteases may have less effect on gel strength. The difference
249 between EG and AG in supernatant (Fig.2C) shows that endogenous proteases could have
250 significant influence on gel properties.

251

252 3.3 Production of α -amino nitrogen (AAN)

253 According to SDS-PAGE, some bands disappeared in supernatant were a result of
254 acid-induced precipitate. Other weakened bands may be caused by degradation into smaller
255 peptides which were not in the range of electrophoresis graph. This could be verified in ANN assay.
256 In Fig.3, the production of low molecular weight peptides was in different patterns between
257 hydrolysis of SP and MP. Both endogenous and microbial proteases contributed to small peptides
258 from SP, while only endogenous proteolysis increased MP degradation. What's more, as can be
259 seen in Fig.3, α -amino nitrogen increased after 2 days in SP of AG but not in MP, and little
260 difference was found between MP-EG and MP-EMG. This is in agreement with the SDS-PAGE
261 results. Acid and microorganisms could cause partial hydrolysis of SP or SP products but not MP.
262 As claimed by other previous studies, endogenous tri-peptidylpeptidases (I and II),
263 di-peptidylpeptidases (I, II, III and IV), alanyl, arginyl, methionyl, leucyl and pyroglutamyl
264 aminopeptidases in meat could cause the accumulation of amino acid nitrogen (Toldrá, 2006), while
265 *Lactobacillus* had relatively high aminopeptidase activities rather than proteinase activities which
266 could also contribute to production of TCA-soluble peptides and amino acid nitrogen (Nie, Lin, &
267 Zhang, 2014).

268

269 3.4 Effect of endogenous proteases on gel strength

270 Pepstatin A, E-64, aprotinin, and EDTA are protease inhibitors of cathepsin D, caspases (such
271 as cathepsin B, L and H), serine proteases and metalloproteinases (such as calpains and some types
272 of collagenases), respectively. The blank with no protease inhibitors inside had all endogenous
273 proteolytic activities while protease inhibition groups had all other endogenous proteolytic activities

274 except for inhibited one. Therefore, the lower gel strength of the blank than the specific protease
275 inhibition group could be regarded as the participation of this protease.

276 As can be seen in Fig.4, each group except for “Inhibition by Pepstatin A” group differed from
277 the blank during the first 15 hours of incubation, indicating that caspases, serine proteases and
278 metalloproteinases participated in hydrolysis of protein at the beginning of fermentation, leading to
279 a decrease in gel strength. Meanwhile, cathepsin D was not activated at the initial stage of
280 incubation. Besides, metalloproteinases could be found as the most important factor for modori at
281 the beginning. As the incubation time increased, the difference between the lysosomal enzymes
282 inhibition groups (inhibited by “Pepstatin A” and “E-64”) and the blank increased, suggesting that
283 lysosomal enzymes played an important role in modori and this influence was enhanced at the later
284 stage of incubation under acid conditions. Comparing each group after 48 h of incubation, the
285 impacts of serine proteases and metalloproteinases were smaller than lysosomal proteases.

286 In other studies, calpains, collagenases, cathepsin B and L, and serine proteases were thought
287 to degrade proteins during heat-processing and storage (An, Peters, & Seymour, 1996; Hultmann &
288 Rustad, 2002), while cathepsin D was believed to have little effect at post-mortem stage since it had
289 high activities only between pH 3.0-5.0 and pH of fish after death could not be such low (Ahmed,
290 Donkor, Street, & Vasiljevic, 2015). In this study, cathepsin D had little hydrolytic activity at the
291 initial stage when pH was not low enough, while other proteases could decrease the gel strength.
292 This was in consistency with previous studies. At the later stage, the decrease activities of serine
293 proteases and metalloproteinases may be due to irreversible inactivation by long exposure to acid,
294 while the increase influence of lysosomal proteases on gel strength may be due to increased
295 sensitivity to proteins after hydrolysis of proteins by calpains (Gaarder, Bahuaud, Veiseth-Kent,

296 Mørkøre, & Thomassen, 2012) and activation of enzymes by acid (Liu, Yin, Zhang, Li, & Ma,
297 2006).

298

299 3.5 Effect of microbial proteases on the change of free amino acids

300 The changes of free amino acids are shown in Fig.5. Aspartic acid, glutamic acid, alanine,
301 tyrosine, valine, methionine, phenylalanine, and leucine increased significantly as affected by
302 proteases from *Lactobacillus Plantarum*, while arginine and proline decreased a little, compared
303 with endogenous proteases group. It could also be seen in Fig.5 that the predominant amino acids
304 were histidine, glycine, alanine, glutamic acid, phenylalanine and leucine. This is in agreement with
305 Candogan, Wardlaw, and Acion (2009) who also found these amino acids dominated during
306 fermentation and drying. In addition, trace serine was found in both their and our studies. The
307 increase of alanine might be due to carnosine degradation, while lysine, arginine, and leucine were
308 mainly from myofibrillar proteins (Fadda, Sanz, Vignolo, Aristoy, Oliver, & Toldrá, 1999a).

309 It is known that glutamic acid and aspartic acid have umami taste and alanine has sweet taste
310 (Fadda, Sanz, Vignolo, Aristoy, Oliver, & Toldrá, 1999b). Meanwhile, hydrophobic amino acids are
311 thought to have bitter flavor characteristics, such as valine (Beriaín, Lizaso, & Chasco, 2000).
312 However, these “bitter” amino acids could be tasted as other flavors with the co-effect by other
313 amino acids or under different concentrations. For instance, in some cases, methionine and leucine
314 are associated with sea flavor and sweet flavor, respectively (Beriaín, Lizaso, & Chasco, 2000). In
315 our study, the amino acids with desirable taste, i.e., alanine, aspartic acid and glutamic acid,
316 increased significantly, producing a special fermentation flavor. However, some “bitter” amino
317 acids also increased. It is interesting to know that some of them do not necessarily have an
318 unpleasant flavor in a product which has been explained above and some of them such as the

319 branched amino acids (valine, leucine and isoleucine) and proline and tyrosine could transform into
320 volatile compounds (Beriaín, Lizaso, & Chasco, 2000; Fadda, Sanz, Vignolo, Aristoy, Oliver, &
321 Toldrá, 1999b). In our study, in spite of proline, these amino acids did not decrease which may be
322 due to shortened fermentation time and less microbial activities. Therefore, desirable aroma may be
323 lacked. In order to enhance flavor and taste, exogenous enzymes such as Flavourzyme and fungal
324 protease EPg222 (Benito, Rodríguez, Martín, Aranda, & Córdoba, 2004; Feng, Qiao, Zou, Huang,
325 Kang, & Zhou, 2014) are recommended to be added in.

326 It should be noticed that the generation of hydrophilic peptides which was not investigated in
327 our study could also attribute to desirable flavors (Fadda, Sanz, Vignolo, Aristoy, Oliver, & Toldrá,
328 1999b). This needs further investigation.

329

330 **4. Conclusions**

331 It could be concluded that *Lactobacillus Plantarum* proteases could hydrolyze sarcoplasmic
332 peptides while endogenous proteases could hydrolyze both sarcoplasmic and myofibrillar proteins.
333 The activity of proteinases in *Lactobacillus Plantarum* was low, but high aminopeptidases could
334 degrade peptides from sarcoplasmic proteins into small fragments which could aggregate under acid
335 condition and were related to pleasant taste and formation of volatile compounds. Endogenous
336 proteases only induced modori phenomenon during fermentation. Among these proteases, calpains
337 and serine proteases had negative influence on the gel strength only at the initial stage of
338 fermentation, while lysosomal proteases whose activities dominated in the last stage affected gel
339 strength the most during 2-day fermentation.

340

341 **Acknowledgements**

342 This research was financially supported by the earmarked fund for China Agriculture Research
343 System (CARS-46), NSF 31171709, CXLX12_0736, and the Natural Science Foundation of
344 Jiangsu Province (Grants NO. BK20150152).

345

346 **References**

347 Ahmed, Z., Donkor, O., Street, W. A., & Vasiljevic, T. (2015). Calpains-and cathepsins-induced
348 myofibrillar changes in post-mortem fish: Impact on structural softening and release of
349 bioactive peptides. *Trends in Food Science & Technology*, 45, 130-146.

350 An, H., Peters, M. Y., & Seymour, T. A. (1996). Roles of endogenous enzymes in surimi gelation.
351 *Trends in Food Science & Technology*, 7, 321-327.

352 Benito, M. A. J., Rodríguez, M., Martín, A., Aranda, E., & Córdoba, J. J. (2004). Effect of the
353 fungal protease EPg222 on the sensory characteristics of dry fermented sausage
354 “salchichón” ripened with commercial starter cultures. *Meat Science*, 67, 497-505.

355 Beriain, M., Lizaso, G., & Chasco, J. (2000). Free amino acids and proteolysis involved in
356 ‘salchichon’ processing. *Food Control*, 11, 41-47.

357 Candogan, K., Wardlaw, F., & Acton, J. C. (2009). Effect of starter culture on proteolytic changes
358 during processing of fermented beef sausages. *Food Chemistry*, 116, 731-737.

359 Chaves-López, C., Paparella, A., Tofalo, R., & Suzzi, G. (2011). Proteolytic activity of
360 *Saccharomyces cerevisiae* strains associated with Italian dry-fermented sausages in a model
361 system. *International Journal of Food Microbiology*, 150, 50-58.

362 Doucet, D., Otter, D. E., Gauthier, S. F., & Foegeding, E. A. (2003). Enzyme-induced gelation of
363 extensively hydrolyzed whey proteins by alcalase: peptide identification and determination
364 of enzyme specificity. *Journal of Agricultural and Food Chemistry*, 51, 6300-6308.

365 Durá, M., Flores, M., & Toldrá, F. (2004). Effect of *Debaryomyces* spp. on the proteolysis of
366 dry-fermented sausages. *Meat Science*, 68, 319-328.

367 Fadda, S., Sanz, Y., Vignolo, G., Aristoy, M.-C., Oliver, G., & Toldrá, F. (1999a). Characterization
368 of Muscle Sarcoplasmic and Myofibrillar Protein Hydrolysis Caused by *Lactobacillus*
369 *plantarum*. *Applied and Environmental Microbiology*, 65, 3540-3546.

370 Fadda, S., Sanz, Y., Vignolo, G., Aristoy, M.-C., Oliver, G., & Toldrá, F. (1999b). Hydrolysis of
371 pork muscle sarcoplasmic proteins by *Lactobacillus curvatus* and *Lactobacillus sake*.
372 *Applied and Environmental Microbiology*, 65, 578-584.

373 Fadda, S., Vignolo, G., Holgado, A. P. R., & Oliver, G. (1998). Proteolytic activity of *Lactobacillus*
374 strains isolated from dryfermented sausages on muscle sarcoplasmic proteins. *Meat Science*,
375 49, 11-18.

376 Feng, L., Qiao, Y., Zou, Y., Huang, M., Kang, Z., & Zhou, G. (2014). Effect of Flavourzyme on
377 proteolysis, antioxidant capacity and sensory attributes of Chinese sausage. *Meat Science*,
378 98, 34-40.

379 Gaarder, M., Bahuaud, D., Veiseth-Kent, E., Mørkøre, T., & Thomassen, M. (2012). Relevance of
380 calpain and calpastatin activity for texture in super-chilled and ice-stored Atlantic salmon
381 (*Salmo salar* L.) fillets. *Food Chemistry*, 132, 9-17.

382 Gelman, A., Drabkin, V., & Glatman, L. (2000). Evaluation of lactic acid bacteria, isolated from
383 lightly preserved fish products, as starter cultures for new fish-based food products.
384 *Innovative Food Science & Emerging Technologies*, 1, 219-226.

385 Gornall, A. G., Bardawill, C. J., & David, M. M. (1949). Determination of serum proteins by means
386 of the biuret reaction. *Journal of Biological Chemistry*, 177, 751-766.

387 Hierro, E., de la Hoz, L., & Ordóñez, J. A. (1999). Contribution of the microbial and meat
388 endogenous enzymes to the free amino acid and amine contents of dry fermented sausages.
389 *Journal of Agricultural and Food Chemistry*, 47, 1156-1161.

390 Hughes, M., Kerry, J., Arendt, E., Kenneally, P., McSweeney, P., & O'Neill, E. (2002).
391 Characterization of proteolysis during the ripening of semi-dry fermented sausages. *Meat*
392 *Science*, 62, 205-216.

393 Hultmann, L., & Rustad, T. (2002). Textural changes during iced storage of salmon (*Salmo salar*)
394 and cod (*Gadus morhua*). *Journal of Aquatic Food Product Technology*, 11, 105-123.

395 Khalid, N. M., & Marth, E. H. (1990). Lactobacilli—their enzymes and role in ripening and
396 spoilage of cheese: a review. *Journal of Dairy Science*, 73, 2669-2684.

397 Kröckel, L. 1995. Bacterial fermentation of meats. *Fermented Meats*. G. Campbell-Platt and P. E.
398 Cook, Springer US: 69-109.

399 Ladrat, C., Verrez-Bagnis, V., Noël, J., & Fleurence, J. (2003). In vitro proteolysis of myofibrillar
400 and sarcoplasmic proteins of white muscle of sea bass (*Dicentrarchus labrax L.*): effects of
401 cathepsins B, D and L. *Food Chemistry*, 81, 517-525.

402 Liu, H., Yin, L., Zhang, N., Li, S., & Ma, C. (2006). Purification and characterization of cathepsin L
403 from the muscle of silver carp (*Hypophthalmichthys molitrix*). *Journal of Agricultural and*
404 *Food Chemistry*, 54, 9584-9591.

405 Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the
406 Folin phenol reagent. *Journal of Biological Chemistry*, 193, 265-275.

407 Molly, K., Demeyer, D., Johansson, G., Raemaekers, M., Ghistelinck, M., & Geenen, I. (1997). The
408 importance of meat enzymes in ripening and flavour generation in dry fermented sausages.
409 First results of a European project. *Food Chemistry*, 59, 539-545.

410 Nie, X., Lin, S., & Zhang, Q. (2014). Proteolytic characterisation in grass carp sausage inoculated
 411 with *Lactobacillus plantarum* and *Pediococcus pentosaceus*. *Food Chemistry*, 145, 840-844.

412 Ohashi, K., & Negishi, H. (2014). Hydrolysis of pork myofibrillar proteins during fermentation
 413 using starter cultures of *Lactobacillus bulgaricus* and *Streptococcus Thermophilus*. *Food*
 414 *Science and Technology Research*, 20, 679-685.

415 Olympia, M., Fukuda, H., Ono, H., Kaneko, Y., & Takano, M. (1995). Characterization of
 416 starch-hydrolyzing lactic acid bacteria isolated from a fermented fish and rice food, “Burong
 417 Isda”, and its amylolytic enzyme. *Journal of Fermentation and Bioengineering*, 80,
 418 124-130.

419 Paludan-Müller, C., Huss, H. H., & Gram, L. (1999). Characterization of lactic acid bacteria
 420 isolated from a Thai low-salt fermented fish product and the role of garlic as substrate for
 421 fermentation. *International Journal of Food Microbiology*, 46, 219-229.

422 Riebroy, S., Benjakul, S., & Visessanguan, W. (2008). Properties and acceptability of Som-fug, a
 423 Thai fermented fish mince, inoculated with lactic acid bacteria starters. *LWT-Food Science*
 424 *and Technology*, 41, 569-580.

425 Sanz, Y., Fadda, S., Vignolo, G., Aristoy, M.-C., Oliver, G., & Toldrá, F. (1999). Hydrolysis of
 426 muscle myofibrillar proteins by *Lactobacillus curvatus* and *Lactobacillus sake*.
 427 *International Journal of Food Microbiology*, 53, 115-125.

428 Sovik, S. L., & Rustad, T. (2004). Proteolytic activity in byproducts from cod species caught at
 429 three different fishing grounds. *Journal of Agricultural and Food Chemistry*, 53, 452-458.

430 Spaziani, M., Torre, M. D., & Stecchini, M. L. (2009). Changes of physicochemical,
 431 microbiological, and textural properties during ripening of Italian low-acid sausages.
 432 Proteolysis, sensory and volatile profiles. *Meat Science*, 81, 77-85.

433 Taylor, W. (1957). Formol titration: an evaluation of its various modifications. *Analyst*, 82,
434 488-498.

435 Toldrá, F. (2006). The role of muscle enzymes in dry-cured meat products with different drying
436 conditions. *Trends in Food Science & Technology*, 17, 164-168.

437 Xu, Y. S., Xia, W. S., Yang, F., Kim, J. M., & Nie, X. H. (2010). Effect of fermentation
438 temperature on the microbial and physicochemical properties of silver carp sausages
439 inoculated with *Pediococcus pentosaceus*. *Food Chemistry*, 118, 512-518.

440 Zeng, X., et al. (2014). Technological properties of *Lactobacillus plantarum* strains isolated from
441 Chinese traditional low salt fermented whole fish. *Food Control*, 40, 351-358.

442 Zeng, X., Xia, W., Yang, F., & Jiang, Q. (2013). Changes of biogenic amines in Chinese low-salt
443 fermented fish pieces (Suan yu) inoculated with mixed starter cultures. *International*
444 *Journal of Food Science & Technology*, 48, 685-692.

1 **Figure captions**

2 **Fig. 1** The change of pH (dash lines) and endogenous and microbial proteolytic activities (solid
3 lines) during the incubation in a GDL acidified system

4 **Fig. 2** Products produced by endogenous and microbial proteolytic activities in supernatant (A) and
5 precipitate (B) of sarcoplasmic protein; supernatant (C) and precipitate (D) of myofibrillar protein.
6 M: marker. MP: myofibrillar protein. AG: acidified group. EG: endogenous proteases group. EMG:
7 endogenous plus microbial proteases group. Subscript number 0: before incubation. Subscript
8 number 2: after incubation at 30 °C for 2 days. ①: creatine kinase; ②: aldolase; ③:
9 glyceraldehyde-3-phosphate dehydrogenase; ④: nucleoside diphosphate kinase. i : myosin heavy
10 chain (MHC); ii : α -actinin; iii: desmin; iv: actin; v : troponin-T (TN-T); vi: tropomyosin (TM);
11 vii: myosin light chain-1 (MLC-1); viii: troponin-I; ix: troponin-C; x: myosin light chain-2 (MLC-2);
12 I: myosin light chain-3 (MLC-3)

13 **Fig. 3** The production of α -amino nitrogen (AAN) by endogenous and microbial proteolytic
14 activities. SP: sarcoplasmic protein. MP: myofibrillar protein. AG: acidified group. EG: endogenous
15 proteases group. EMG: endogenous plus microbial proteases group.

16 **Fig. 4** Effect of different endogenous proteases on gel strength during the incubation in a GDL
17 acidified system

18 **Fig. 5** Effect of microbial proteases on the change of free amino acids. Fresh: dorsal muscle from
19 fresh fish. EG: endogenous proteases group. EMG: endogenous plus microbial proteases group.

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Fig. 1

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Fig. 2

Fig. 3

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