

Effect of Chinese rice wine on proteolytic characterization originating from endogenous proteases in topmouth culter (*Culter alburnus*)

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In this study we investigated the influence of rice wine (Jinbiao, Yinbiao, Wunianchen, Nv'erhong) on the activity of endogenous proteases, myofibrillar degradation and quality characteristics in topmouth culter muscle. Rice wines had pH of about 4.3, and micromolar level of calcium ions. Nv'erhong (NEH) had the highest total phenolics content, while Wunianchen (WNC) showed the strongest total antioxidant capacity. WNC showed the considerable inhibitory action on the endogenous proteases, and delayed the degradation of myosin heavy chain and α -actinin, which occurred more quickly in other groups, especially in blank and alcohol controls. Total volatile base nitrogen production and texture profile for the fish fillets confirmed the improvement role of rice wine on the quality. In general, rice wine can inhibit the endogenous proteases and reduce the extent of myofibrillar degradation on the combined role of acidic pH and antioxidative components, resulting in the maintaining of good quality of the fish.

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15 ABSTRACT:

16 In this study we investigated the influence of rice wine (Jinbiao, Yinbiao, Wunianchen,
17 Nv'erhong) on the activity of endogenous proteases, myofibrillar degradation and quality
18 characteristics in topmouth culter muscle. Rice wines had pH of about 4.3, and micromolar level
19 of calcium ions. Nv'erhong (NEH) had the highest total phenolics content, while Wunianchen
20 (WNC) showed the strongest total antioxidant capacity. WNC showed the considerable inhibitory
21 action on the endogenous proteases, and delayed the degradation of myosin heavy chain and α -
22 actinin, which occurred more quickly in other groups, especially in blank and alcohol controls.
23 Total volatile base nitrogen production and texture profile for the fish fillets confirmed the
24 improvement role of rice wine on the quality. In general, rice wine can inhibit the endogenous
25 proteases and reduce the extent of myofibrillar degradation on the combined role of acidic pH and
26 antioxidative components, resulting in the maintaining of good quality of the fish.

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28 *Keywords:* Rice wine; Topmouth culter; Endogenous proteases; Myofibril degradation; Quality

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30 1. Introduction

31 Topmouth culter (*Culter alburnus*) is one of the high-valued freshwater fishes in China,
32 especially in Lake Taihu region. Topmouth culter is well accepted by consumers, due to its
33 abundant nutrients, delicious taste and delicate texture (Qiu, Cao, & Liu, 2015). The cultured
34 production and consumption has greatly increased over the past decades, making the species to be
35 an important commercial freshwater fish in China.

36 Like other freshwater fish varieties, topmouth culter is susceptible to muscle softening and
37 difficult to keep fresh during post-harvest storage. So at present the species are mainly consumed
38 freshly and alive in China, and only a very small portion are salted and air-cured to produce a
39 traditional fish product. Salting and drying are two effective techniques commonly used in food
40 industry for preservation and processing purpose, due to the mechanism of water loss and salt
41 uptake. However, such traditional products could induce the significant physicochemical changes
42 of protein, lipid and other components, and the consequent deterioration of overall quality
43 including the flavor, texture and color (Chaijan, 2011). Despite its economic and ecological
44 importance, studies on this species has not been widely carried out. Early researches mostly
45 focused on the genetic structure and diversity of its wild and cultured populations (Qi, Qin, & Xie,
46 2015; Wang, Chen, Yang, Hou, He, Gu, et al., 2007). Few study has been conducted to develop
47 the manufacturing technology of the species. In order to develop products with good quality close
48 to fresh cooked fishes, it is important to resolve the unfavorable muscle softening and quality
49 deterioration problems at the stage of post-rigor or post-mortem.

50 Endogenous proteases, including cytosolic calpains and lysosomal cathepsins, especially
51 cathepsins B, D, and L, are reported to be involved in the degradation of myofibrillar proteins and
52 the loss of fish freshness during post-mortem storage and processing (Ahmed, Donkor, Street, &
53 Vasiljevic, 2015; Gaarder, Bahuaud, Veiseth-Kent, Morkore, & Thomassen, 2012). Endogenous
54 proteases have been thoroughly investigated in mammals. By contrast, a limited number of studies
55 are carried out on fish species. Until now, the effects of endogenous enzymes on topmouth culter
56 muscle proteins, as well as their inhibitors of the enzymes, have not been investigated. In scientific
57 researches, some substances, such as phenanthroline, antipan, EDTA, E-64 [L-trans-epoxy-
58 succinylleucylamido (4-guanidino) butane], were used to rapidly inactivate calpains and cathepsins
59 (Ayensa, Montero, Borderías, & Hurtado, 2002; Wang, Vang, Pedersen, Martinez, & Olsen, 2011).
60 However, since some of these inhibitors are not food-approved, it would be necessary to explore
61 food-grade additives that could safely and economically used as inhibitors in food application
62 (Kang & Lanier, 1999).

63 Rice wine is one of the famous brew drinks in the world and enjoys a great popularity throughout
64 China with a high reputation as the “National Wine”. Chinese rice wine is made from high-starchy
65 cereal grains, and fermented via a complex starter culture “Wheat Qu” including various yeasts,
66 fungi and bacteria (Liu, Mao, Liu, Meng, Ji, Zhou, et al., 2015; Pan, Tang, Chen, Wu, & Han,
67 2013; Park, Liu, Park, & Ni, 2016). In China, rice wine is used widely in traditional medicine and
68 aquatic food cookery. Many researchers reported that rice wine had strong antioxidant property
69 and a high content of phenolic compounds, which are supposed to attribute to the functional
70 therapeutic and nutritional activity. To the best of our knowledge, there is no report about the effect

71 of rice wine on the inhibition of endogenous enzymes during the post-mortem stage. In order to
72 understand the role of rice wine in Chinese cookery, the present paper attempts to elucidate the
73 effect of rice wine on the proteolytic activity of cathepsins and calpains on myofibrillar, and the
74 consequent quality change of topmouth culter in the early stages of processing or storage.

75 **2. Materials and methods**

76 *2.1. Materials*

77 Fresh topmouth culter (*Culter alburnus*), 850 ± 150 g each, were purchased from local market
78 (Nanjing, Jiangsu, China) and transported on ice to the lab within 30 min. The fish were
79 immediately slaughtered by deheading, and then scaled, gutted, and filleted. The fillets were
80 minced using a meat bone separator (Shandong, China) with a 2 mm diameter perforations. The
81 minced sample was stored at 4 °C until used, but not longer than 1 h.

82 *2.2. Preparation of sarcoplasmic and myofibrillar proteins*

83 The extraction of sarcoplasmic and myofibrillar proteins were carried out according to a
84 previously described method (Nie, Lin, & Zhang, 2014) with slight modifications. The minced
85 fish samples (10 g) were homogenized with a homogenizer (T25, IKA, Germany) in 30 ml distilled
86 water at 12,000 rpm for 30 s. The supernatant was recovered from the homogenate after
87 centrifugation at 10,000 rpm, 4 °C for 15 min. The precipitate was further homogenized in 20 ml
88 distilled water and centrifuged as above. The supernatants were pooled as sarcoplasmic protein

89 extraction containing endogenous proteases. The residue was taken for the further extraction of
90 the myofibrillar protein, using 0.05 M potassium phosphate buffer (pH 7.2) containing 0.6 M NaCl,
91 through the same procedure described as above. The supernatants obtained were combined as
92 myofibrillar protein extraction. Protein content was determined by Bradford assay kit (Jiancheng
93 Bioengineering Institute, Nanjing, China).

94 *2.3. Proteolytic digestion*

95 Sarcoplasmic protein extraction (50 ml) was added with different kinds of rice wine (5 mL) and
96 incubated at 37 °C. Four kinds of rice wine with the alcohol content of 15% were purchased from
97 local supermarket, including Jinbiao Hejiu (JB), Yinbiao Hejiu (YB), Wunianchen rice wine
98 (WNC), Nv'erhong rice wine (NEH). The control (CK) was carried out without any rice wine but
99 with the distilled water instead. In addition, ethanol was diluted to the content of 15% (AL) by
100 distilled water and used to eliminate the effect of alcohol in the experiments. Two aliquots were
101 taken at specific intervals of 0, 15 and 30 min, 1, 2, 4, 10 and 20 h. One aliquot was used to
102 determine the endogenous protease activity. The other aliquot was mixed with myofibrillar protein
103 extraction at a ratio of 1:1 at 37 °C for 2 h, followed by the performance of SDS-PAGE to check
104 the digestion of myofibrils.

105 *2.4. Assay of protease activity*

106 The activities of four endogenous proteases (calpains, and cathepsins B, D, and L) in
107 sarcoplasmic extraction, were determined using the fluorometric assay kits (Catalog #K240-100,

108 K140-100, K143-100, K142-100, respectively, Biovision, USA) according to the kit directions.
109 The fluorescence absorbance was recorded by a fluorometer (Infinite M200 Pro, Tecan,
110 Switzerland). The activity was expressed by comparing the relative fluorescent unit with the level
111 of the initial untreated control.

112 2.5. SDS-PAGE electrophoresis

113 The digestion mixture (50 μ L) of sarcoplasmic and myofibrillar proteins were boiled for 5 min
114 with the equal volume of the loading buffer (WB2001, NCM Biotech, Suzhou, China) containing
115 10 mM Tris-HCl, 1 mM EDTA, 5% SDS, 10% β -mercaptoethanol, and 0.005% bromophenol blue,
116 pH 6.8. Samples (10 μ L) were loaded and SDS-PAGE was performed on 5% stacking gel and 8%
117 separating gel. The electrophoresis was done with a Mini-PROTEAN Tetra Cell (BIO-RAD, USA)
118 at a constant voltage of 120 V. After migration, the gels were stained in Coomassie Brilliant Blue
119 R-250 (0.1%, in 25% methanol and 10% acetic acid) for 2 h, and subsequently destained overnight
120 in distilled water. Molecular weight markers (RTD6105, Tiangen Co., Nanjing, China) were
121 loaded simultaneously for molecular mass identification. Image of the gels were captured using
122 Gel Image System (GIS3500, Tanon Co., Shanghai, China).

123 2.6. Quality characteristics of the fish muscle

124 The fish fillets were mixed well with rice wine at a ratio of 50:1 (W/V), and stored at room
125 temperature for 20 h. Fish fillet samples (10 g) were mixed with 90 ml distilled water and
126 homogenized at 12,000 rpm for 30 s. The mixture was centrifuged at 10,000 rpm, 4 $^{\circ}$ C for 15 min.

127 The supernatant was subjected to pH and TVB-N determination. pH was measured using a digital
128 pH meter (Mettler Toledo FE20, Switzerland) with electrode LE438. TVB-N was determined
129 according to the method of the Chinese standard (SC/T 3032-2007). The method is based on water
130 vapour distillation and extraction of volatile base, followed by the titration with standard
131 hydrochloric acid. The contents are expressed as milligrams per 100 g fish muscles.

132 Texture profile analysis (TPA) were performed using a Texture Analyser (TVT-300 XP, FTC
133 Ltd., America) according to a modified procedure of Duangmal and Taluengphol (2009). The fish
134 fillet samples of 2 cm in diameter and 2 cm in height were equilibrated at 25 °C for 30 min and
135 tested at the same temperature. Texture Analyser was equipped with a 5 kg load cell. The resistance
136 force (g) and deformation (mm) were recorded using a flat-ended cylindrical probe (30mm
137 diameter, type P/30) at a test rate of 1 mm/s. The force required to press the cylinder down to 50%
138 of shape deformation was used to describe the textural parameter fillet firmness. Data were
139 analysed using Texture Expert version 7.6. The values given were the means of 10 replications at
140 different sites on the sample.

141 2.7. *Determination* of physiochemical indices of rice wine

142 pH of rice wine was measured directly using a digital pH meter (Mettler Toledo FE20,
143 Switzerland). Total calcium (Ca) content was determined according to the method of Chinese
144 national standard (GB/T 5009) using atomic absorption spectrophotometer (AA 320N, Shanghai,
145 China). Total phenolics content (TPC) of rice wine was determined by the Folin-Ciocalteu method
146 (Liu, Dong, Chen, Jiang, Lv, & Yan, 2007) using gallic acid as the standard. Total antioxidant

147 capacity (T-AOC) based on linoleic acid peroxidation was measured using the assay kit (Jiancheng
148 Bioengineering Institute, Nanjing, China).

149 2.8. *Statistical analysis*

150 The results were expressed as means \pm SD of three parallel replicates. Analysis of variance
151 (ANOVA) was done by SPSS 13.0 software (Chicago, IL, USA). A significance level of 5% was
152 adopted for all comparisons.

153 3. Results and Discussions

154 3.1. *Physicochemical properties of rice wine*

155 Rice wine is widely consumed in China in a variety of ways besides drinking, e.g. as cooking
156 condiment and medical supplement (Chang, Jang, Lin, & Duan, 2016; Li, Shen, & Meng, 2013).
157 Many evidences indicate that rice wine has health-promoting effects, which may be related to the
158 antioxidant activity and phenolic compounds (Kim, Lee, Lee, Choi, & Lee, 2004; Que, Mao, &
159 Pan, 2006). Positive correlations between phenolic compounds and antioxidant activity have been
160 also proven in many studies (Liu, Dong, Chen, Jiang, Lv, & Yan, 2007; Que, Mao, & Pan, 2006).
161 However, up to date, researches about rice wine as a traditional drinking are focused on the modern
162 fermentation technology. Comprehensive studies about the nutrients and constituents in rice wine
163 and their role on the inhibition of endogenous proteases were limited.

164 Table 1 presented the physicochemical indices of rice wines used in this study. Rice wines with

165 different brands had similar pH of about 4.3. Calcium contents in rice wines differ significantly.
166 NEH presented the highest calcium content of 123.7 $\mu\text{g}/\text{mL}$, followed by WNC, JB, and YB
167 (103.6, 102.5 and 89.3 $\mu\text{g}/\text{mL}$, respectively). TPC and T-AOC followed the similar order, except
168 that WNC had a stronger antioxidant capacity than NEH. Since phenolic compounds are well
169 known as the powerful antioxidants *in vitro*, the inconsistency between the value orders of TPC
170 and T-AOC in our study is the result of the specific compound profiles. Many researches have
171 supported the point. Xu et al. (2015) determined the TPC of different fermented wines with the
172 range of 479.67-597.30 mg GAE/L, and total phenolic acid amounts of 167.21-261.18 $\mu\text{g}/\text{mL}$. Wu
173 et al. (2017) detected the total phenolics content of 433.62 mg GAE/L in a local rice wine, and
174 found that the phenolic profile was characterized by high contents of syringic acid, (+)-catechin,
175 and protocatechuic acid and low content of other phenolic compounds. Total antioxidant activity
176 determined by the three different methods also differs significantly, 92.08 mg TEAC/L with DPPH
177 assay, 101.18 mg TEAC/L with ABTS assay, and 143.19 mg TEAC/L with FRAP assay. Different
178 total phenolics, antioxidant activity and other physicochemical component pattern may result from
179 many factors, e.g. raw rice materials, traditional steam cooking, storing for aging, analytical
180 methods, etc.

181 Besides phenolics, rice wine is a rich source of amino acids, proteins, oligosaccharides, organic
182 acid, vitamins, Maillard reaction products, γ -aminobutyric acid, and mineral elements (Wu, Long,
183 Xu, Wang, Xu, Jin, et al., 2015; Wu, Xu, Long, Wang, Xu, Jin, et al., 2015; Yu, Ding, & Mou,
184 2003), which also form the strong antioxidant capacity together.

185 3.2. *Effect of rice wine on the residual activity of endogenous proteases*

186 Figure 1 (A-D) show the residual activity of calpains, cathepsins B, D, and L, respectively,
187 during incubation with rice wines. The inset is the activity change in the initial 4 h.

188 3.2.1. *Calpains*

189 Calpain activity of CK group increased greatly up to 127% in the first 1 h of storage, quickly
190 decreased to 106% in 2 h, and then decreased to 72% at the end of 20 h storage (Fig. 1 A). During
191 the 20 h storage, the calpain activity decreased in all the other five groups with the addition of
192 alcohol control or rice wines, and changed much slowly after 4 h. The ultimate residual activities
193 of calpains after 20 h were 65%, 57%, 44%, 44% and 47% for AL, JB, YB, WNC and NEH,
194 respectively. The addition of rice wines significantly inhibited the calpain activity. Calpains are
195 neutral muscle proteinases, having optimal pH at 6.9-7.5. In our study, pH value of the extraction
196 of sarcoplasmic proteins was detected to be 6.89, which may be the reason for the activation of
197 calpains in CK group, resulting in the increase of activity in the first one hour. The addition of
198 acidic rice wines reduced pH values of the sarcoplasmic extraction, and caused the subsequent
199 inactivation of calpains. Pomponio et al. (2010) also observed a faster decrease in pH and a reduced
200 level of μ -calpain activity in porcine muscle.

201 In addition, calpains can be further subclassified into several isoforms and the endogenous
202 inhibitor calpastatin (Ahmed, Donkor, Street, & Vasiljevic, 2015). The two best-characterized
203 isoforms are μ -calpain and m-calpain, depending on the different calcium ion requirement (Saïdo,

204 Sorimachi, & Suzuki, 1994). The concentration of calcium ions plays an important role in the
205 regulation of calpain activity. The existence of calcium in rice wines (Table1) could cause the
206 activation of calpain, on the other hand, other factors, like phenolics and acidic pH, manifested
207 more inactive action on calpains, even eliminating the activating role of calcium. Gaarder et al.
208 (2012) found a significant increase in total calpastatin activity during the storage of super-chilled
209 and ice-stored Atlantic salmon fillets. The presence of inhibitor calpastatin could be a cause of the
210 decrease of activity occurred in the CK group after 1 h storage. In addition, the decrease of calpain
211 activity in AL group indicated the inhibitory role of alcohol on the calpains.

212 3.2.2. *Cathepsin B and L*

213 Two types of the change tendency of cathepsin B activity were shown obviously in Fig. 1 B.
214 One kind existed in CK and AL samples, exhibiting an increasing trend during the whole storage,
215 reaching up to 167% and 134%, respectively. On the contrary, in the samples treated with rice
216 wines, the activity of cathepsin B dropped continuously. NEH showed the greatest inhibitory effect
217 on cathepsin B, with the residual activity of 21%. Other rice wines, JB, YB and WNC, had similar
218 effects on the cathepsin B activity, with the residual activity ranging from 60% to 71%.

219 As shown in Fig. 1 C, Cathepsin L activity of CK group increased significantly to 110% in the
220 first 1 h, followed by a continuous drop to the residual activity of 63% at 20 h. The fastest decrease
221 in Cathepsin L activity occurred in the presence of WNC. The residual activity dropped to 38% in
222 the first 4 hours, and then decreased slowly to 25%. The lower ultimate residual activities, 17%
223 and 24%, were presented in the presence of JB and YB, but the decrease rates were much slower

224 than that of WNC.

225 Cathepsins were reported to be associated with post-mortem myofibrillar proteolysis and tissue
226 softening. Among them, cathepsin B and L were two major cysteine proteases with pH optima of
227 6.5 ~ 7.0 (Chéret, Delbarreladrat, Lamballerieanton, & Verrezbagnis, 2007; Shahidi & Kamil,
228 2001). Since their pH optima are close to the original fish muscle pH, the activities of cathepsin B
229 and L usually increase with post-mortem time. Similar change tendency of cathepsin B and L
230 during post-mortem storage was also observed in other researchers' study (Duun & Rustad, 2008;
231 Gaarder, Bahuaud, Veiseth-Kent, Morkore, & Thomassen, 2012; Hu, Morioka, Chen, Liu, & Ye,
232 2015; Wang, Zhang, Deng, Xu, Liu, Geng, et al., 2016).

233 3.2.3 Cathepsin D

234 Interestingly, different treatments resulted in different cathepsin D change tendency in this study
235 (Fig. 1 D). In CK group, cathepsin D activity increased quickly to 126%, and then increased slowly
236 to 139% until the end of storage. AL, JB and YB groups presented a profile of increasing in the
237 first 1 h, followed by a significant decrease. The ultimate cathepsin D activity in JB and YB groups
238 were still close to the initial level, while that in AL group dropped to 75%. In general, cathepsin
239 D activity in WNC and NEH groups showed an overall decrease tendency, except that the activity
240 in NEH group had a slight increase in the first 15 min. For all the groups, the activity did not
241 change significantly after 4h.

242 Unlike cathepsin B and L, cathepsin D is an aspartic proteinase, having its pH optimum within
243 acidic range below 5.0 (Ahmed, Donkor, Street, & Vasiljevic, 2015; Shahidi & Kamil, 2001).

244 Considering this, it seemed that the acidic property of rice wine should have activated cathepsin D
245 in post-mortem proteolysis. However, the fact of the overall drop of cathepsin D activity in WNC
246 and NEH groups indicated that cathepsin D may be more susceptible to other inhibitory factors,
247 e.g., phenolics, than to pH.

248 3.3. *Proteolytic profiles*

249 Since the respective contributions of endogenous proteases to myofibrillar protein degradation
250 are still unclear, and many researches have proved that post-mortem protein degradation is the
251 result of the synergistic action of calpains and cathepsins on the myofibrillar (Ahmed, Donkor,
252 Street, & Vasiljevic, 2015; Delbarre-Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2004), crude
253 endogenous proteases extract was used to evaluate the proteolysis in our study. SDS-PAGE was
254 performed on myofibrillar fractions in topmouth culter fish muscles (Fig. 2). The degradation of
255 muscle proteins could be observed in SDS-PAGE patterns as changes in band intensity,
256 disappearance of bands or occurrence of new bands. WNC showed the best inhibitory activity on
257 the proteolysis of myofibrils, where no detectable change was found even after 10 h of storage,
258 and just a slight faint of myosin heavy chain (MHC, ~200 kDa) occurred at 20 h. Compared with
259 those in rice wine groups, MHCs in CK and AL were much more sensitive and almost completely
260 degraded into fragments of about 130-150 kDa. The result indicated that alcohol had no ideal effect
261 on the inhibition of post-mortem MHC degradation, although that AL treatment could also inhibit
262 the endogenous protease activity to a certain extent (Table 1), e.g. calpains and cathepsin L. These
263 results may also indicate that calpains and cathepsin L played less contributions to the

264 fragmentation of myofibrils. In addition, MHC in CK and AL samples was rapidly degraded as
265 early as the beginning of storage, while the addition of rice wine delayed the degradation
266 significantly, which occurred after the storage of 1 h (JB, YB and NEH) and 10 h (WNC),
267 respectively.

268 In the case of CK, AL, JB, and YB groups, α -actinin (~100 kDa) was degraded as indicated by
269 the faint of the band intensity and occurrence of 70 kDa bands. α -Actinin is a key component of
270 muscle Z-disk connecting neighbouring sarcomeres, so its release or degradation would result in
271 a looser structure and softer texture (Ahmed, Donkor, Street, & Vasiljevic, 2015; Godiksen,
272 Morzel, Hyldig, & Jessen, 2009).

273 Previous results reported that protein bands including MHC, α -actinin, desmin, actin, troponin
274 T, tropomyosin can be degraded by endogenous proteases (Delbarre-Ladrat, Verrez-Bagnis, Noël,
275 & Fleurence, 2004; Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2003). This work showed that only
276 MHC and α -actinin in topmouth culter were susceptible during the storage, while very little
277 degradation was observed in other proteins with low molecular weights.

278 3.4. *Quality of fish muscle*

279 pH values of topmouth culter fillets during storage are presented in Fig. 3 A. The initial pH of
280 fresh fillets was 6.79~6.83. During 20 h of storage, pH decreased for all samples. pH decreased
281 slightly to 6.35 and 6.43 for CK and AL, respectively. Because of the permeation of rice wine (pH
282 below 4.5, shown in Table 1), pH showed a significant decrease below 5.0 for the four rice wine
283 treated samples after incubation for about 1 h. The result confirms the hypothesis that pH plays an

284 important role on the inhibition of the endogenous proteases.

285 Changes of TVB-N are shown in Fig. 3 B. TVB-N is an important parameter used to evaluate
286 fish freshness and quality. The content increased rapidly to 87.36 ± 4.11 mg/100g for control
287 samples at the end of storage. The treatments of rice wine, as well as alcohol, produced a significant
288 inhibitory effect on TVB-N production. The ultimate TVB-N content for the rice wine treated
289 samples were 38.72-44.32 mg/100g, significantly lower than that in CK group. AL group also
290 exhibited a distinct inhibitory effect on the increase of TVB-N, indicating that alcohol could also
291 restrain the microbiological and autolytic activity in the fish muscle during the storage.

292 Texture profile analysis, including hardness, springiness and chewiness, is shown in Table 2.
293 The maximum hardness, springiness and chewiness values were observed in fresh fish fillets at 0
294 h. The values decreased dramatically in CK and AL groups. Hardness, the measurement of the
295 force necessary to attain a given deformation, decreased from the initial 2.86 g to 1.92 g and 1.98
296 g for CK and AL groups, respectively, and still above 2.0 g for all the rice wine treated samples.
297 A similar but more severe decreasing trend was observed for chewiness. The decrease in chewiness
298 reached up to 73.1% (219.77 mJ residual) for CK sample. A different behavior was observed for
299 springiness change. The springiness for all rice wine treated samples increased, especially in JB
300 and NEH groups. The results indicated that the addition of rice wine could effectively delay the
301 myofibrillar degradation and texture softening of fish muscle.

302 4. Conclusions

303 In this paper, we have focused on the residual activity of four endogenous proteases and the

304 degradation of myofibrillar proteins in topmouth culter muscle after the treatment of rice wines, in
305 order to elucidate the role of Chinese rice wine on the traditional aquatic cookery. Acidic rice wine
306 has a rich amount of calcium and phenolics with good antioxidant activity. In control samples,
307 cathepsin B and D activity increased during storage, while cathepsin L and calpain activity
308 increased in the first 1 h and thereafter decreased to below their initial level. The activity of all the
309 tested proteases decreased significantly in the rice wine treated groups, except that cathepsin D in
310 JB and YB groups was activated at first and then inhibited with a final residual activity close to
311 the initial level. In general, WNC showed an overall better inhibitory action on the proteases with
312 a faster rate. From the complex inhibition pattern of the endogenous proteases, it is difficult to
313 draw conclusions about the respective importance of these endogenous proteases on myofibril
314 fragmentation, and the respective contribution of inhibitory factors in rice wine. SDS-PAGE of
315 myofibril fragmentation and quality characteristic evaluation of fish fillets confirmed the results
316 from enzyme assay. Compared with other rice wines, WNC delayed the breakdown of myosin
317 heavy chain and α -actinin significantly, which are important to muscle softening. The addition of
318 rice wine could also maintain the hardness and chewiness of fish fillets, and increase the
319 springiness of the fish muscle, as well as inhibit the TVB-N production. Based on findings in this
320 study, it is recommended that Chinese rice wine is very useful for the inhibition of endogenous
321 proteases, and maintaining of fish sensory quality. Future study will be conducted using respective
322 phenolic components and pH factors, as well as the individual purified proteinase, to elucidate the
323 mechanism of rice wine on the proteolysis.

324 **Conflict of interest**

325 All authors have read the manuscript, and have no any conflict of interests.

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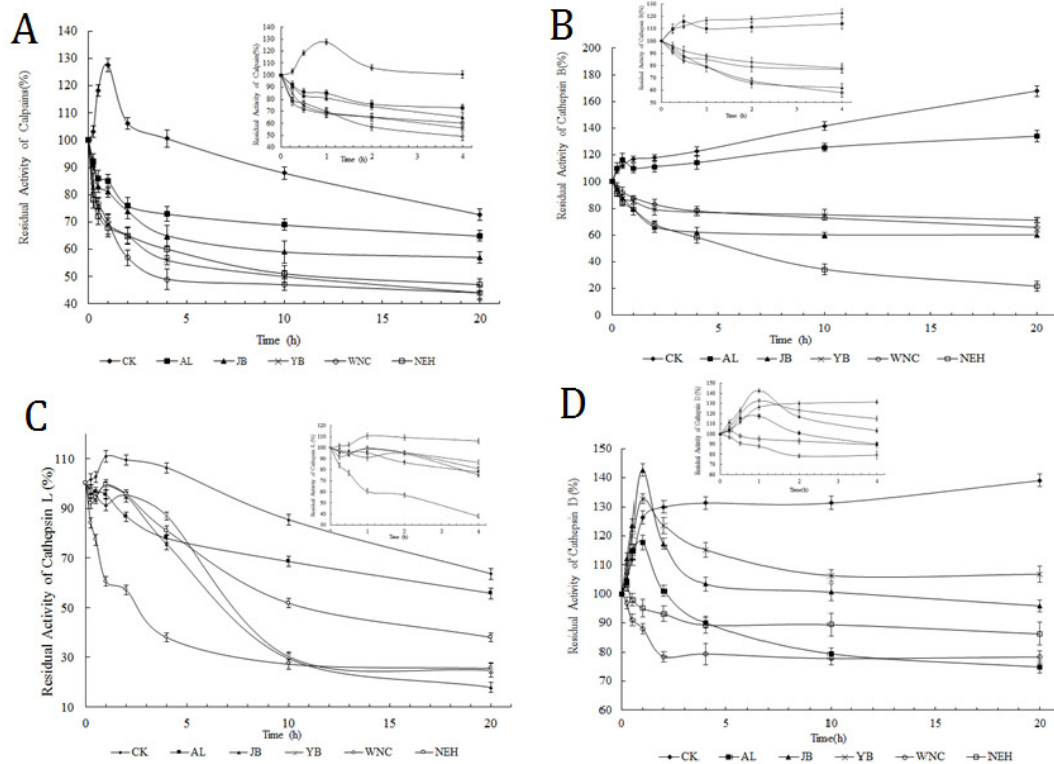
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412

Figure 1 (on next page)

Changes of endogenous protease activity with different treatments



1

2 Fig. 1. Changes of endogenous protease activity with different treatments. Each bar is

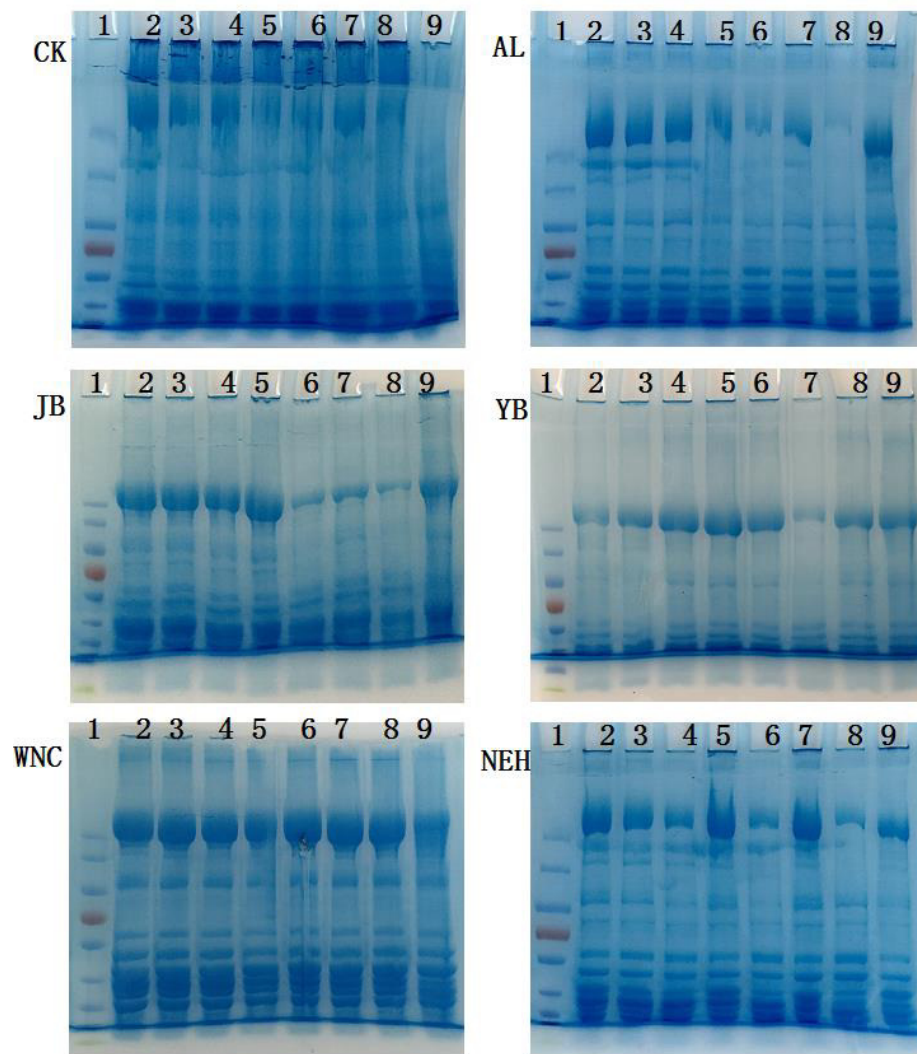
3 presented with standard error. A: Calpain; B: Cathepsin B; C: Cathepsin L; D:

4 Cathepsin D. CK, control; AL, alcohol control; JB, Jinbiao Hejiu; YB, Yinbiao Hejiu;

5 WNC, Wunianchen rice wine; NEH, Nv'erhong rice wine.

Figure 2 (on next page)

SDS-PAGE of myofibrillar protein with different treatments



1

2 Fig. 2. SDS-PAGE of myofibrillar protein with different treatments. CK, control; AL,

3 alcohol control; JB, Jinbiao Hejiu; YB, Yinbiao Hejiu; WNC, Wunianchen rice wine;

4 NEH, Nv'erhong rice wine. Lane 1: MW marker; Lane 2: 0 h storage; Lane 3: 15 min

5 storage; Lane 4: 30 min storage; Lane 5: 1 h storage; Lane 6: 2 h storage; Lane 7: 4 h

6 storage; Lane 8: 10 h storage; Lane 9: 20 h storage. Molecular weight standard from

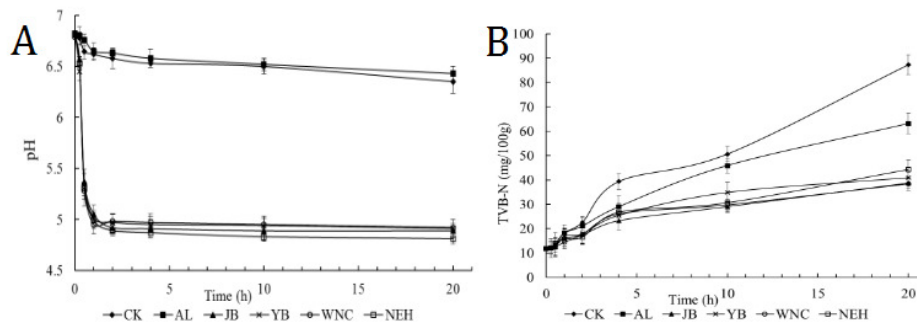
7 top to bottom of gel: 170, 130, 100, 70, 55, 40, 35, 25, and 15 kDa.

8

Table 1 (on next page)

Changes in pH (A) and TVB-N (B) of topmouth culter fillets with different treatments during incubation

1



2

3 Fig. 3. Changes in pH (A) and TVB-N (B) of topmouth culter fillets with different
4 treatments during incubation. CK, control; AL, alcohol control; JB, Jinbiao Hejiu; YB,
5 Yinbiao Hejiu; WNC, Wunianchen rice wine; NEH, Nv'erhong rice wine.

6

Table 2 (on next page)

Physiochemical properties of different rice wine

1 Table 1 Physiochemical properties of different rice wine

| Rice wine | pH | Ca ($\mu\text{g/ml}$) | TPC ($\mu\text{g GAE/ml}$) | T-AOC |
|-----------|------|-------------------------|------------------------------|--------------------|
| JB | 4.23 | 102.5 ± 2.13^b | 552.75 ± 7.15^b | 78.56 ± 2.13^b |
| YB | 4.39 | 89.3 ± 1.58^a | 464.91 ± 6.04^a | 62.17 ± 1.58^a |
| WNC | 4.27 | 103.6 ± 2.67^b | 567.61 ± 7.59^b | 87.29 ± 2.67^c |
| NEH | 4.29 | 123.7 ± 2.05^c | 583.38 ± 5.89^c | 85.46 ± 2.05^c |

2 Values expressed as means \pm standard deviation ($n=3$). Data with different letters in the same
3 column are significantly different ($P<0.05$). JB, Jinbiao Hejiu; YB, Yinbiao Hejiu; WNC,
4 Wunianchen rice wine; NEH, Nv'erhong rice wine; TPC, total phenolic content; T-AOC, total
5 antioxidant capacity.

Table 3 (on next page)

Hardness, springiness and chewiness of fish fillets with various treatments after 20h storage

1 Table 2 Hardness, springiness and chewiness of fish fillets with various treatments after 20h
 2 storage

| Samples | Hardness (g) | Springiness (mm) | Chewiness(mJ) |
|---------|--------------------------|--------------------------|----------------------------|
| 0h | 2.86 ± 0.03 ^a | 0.53 ± 0.04 ^b | 816.90 ± 4.08 ^a |
| CK | 1.92 ± 0.07 ^f | 0.34 ± 0.03 ^d | 219.77 ± 7.33 ^g |
| AL | 1.98 ± 0.11 ^f | 0.44 ± 0.03 ^c | 559.97 ± 7.53 ^f |
| JB | 2.08 ± 0.02 ^e | 0.65 ± 0.02 ^a | 604.07 ± 8.24 ^e |
| YB | 2.14 ± 0.05 ^d | 0.56 ± 0.05 ^b | 646.13 ± 6.93 ^d |
| WNC | 2.53 ± 0.04 ^b | 0.55 ± 0.03 ^b | 743.68 ± 5.72 ^b |
| NEH | 2.33 ± 0.06 ^c | 0.61 ± 0.03 ^a | 689.79 ± 6.83 ^c |

3 Values expressed as means ± standard deviation ($n=3$). Data with different letters in the same
 4 column are significantly different ($P<0.05$). CK, control; AL, alcohol control; JB, Jinbiao Hejiu;
 5 YB, Yinbiao Hejiu; WNC, Wunianchen rice wine; NEH, Nv'erhong rice wine.

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