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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ABSTRACT:

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.

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Materials

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

2.2. Preparation of sarcoplasmic and myofibrillar proteins

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

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

2.5. SDS-PAGE electrophoresis

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

2.6. Quality characteristics of the fish muscle

The fish fillets were mixed well with rice wine at a ratio of 50:1 (W/V), and stored at room temperature for 20 h. Fish fillet samples (10 g) were mixed with 90 ml distilled water and homogenized at 12,000 rpm for 30 s. The mixture was centrifuged at 10,000 rpm, 4 ºC 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 ºC 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 ± 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

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

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

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

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

3.2.1. Calpains

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

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

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

3.2.2. Cathepsin B and L

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

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

than that of WNC.

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

3.2.3 Cathepsin D

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

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

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

3.3. Proteolytic profiles

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

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

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

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

3.4. Quality of fish muscle

pH values of topmouth culter fillets during storage are presented in Fig. 3 A. The initial pH of fresh fillets was 6.79~6.83. During 20 h of storage, pH decreased for all samples. pH decreased slightly to 6.35 and 6.43 for CK and AL, respectively. Because of the permeation of rice wine (pH below 4.5, shown in Table 1), pH showed a significant decrease below 5.0 for the four rice wine 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

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

Conflict of interest

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

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Figure 1(on next page)

Changes of endogenous protease activity with different treatments

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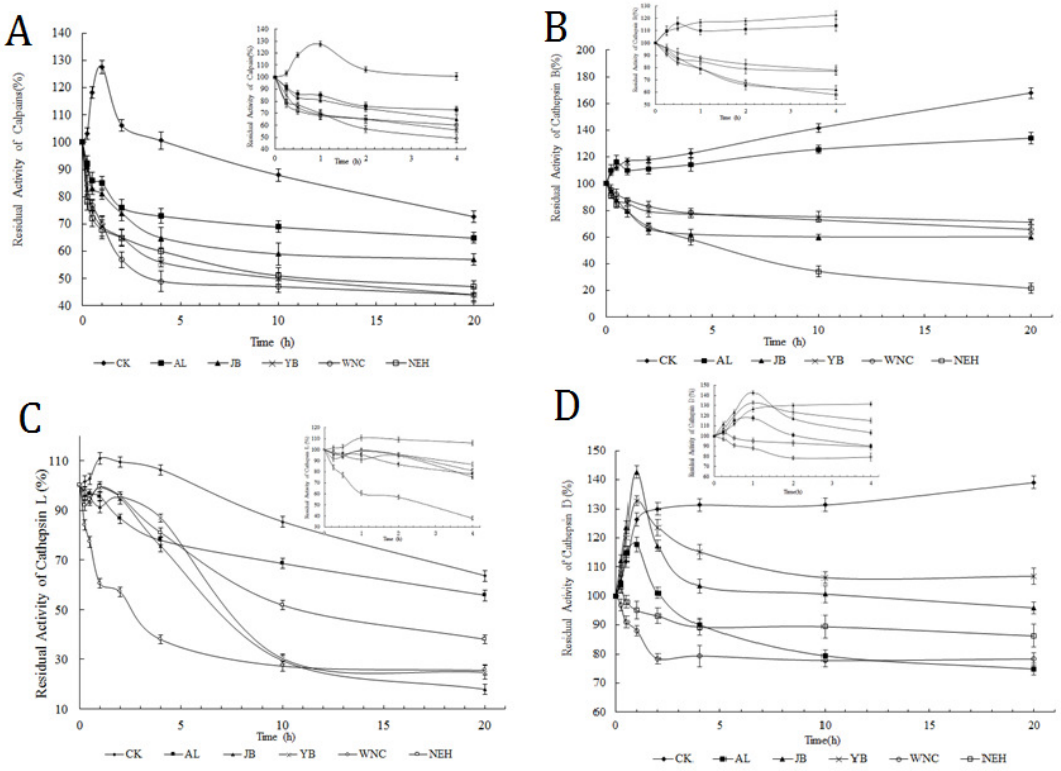

Fig. 1. Changes of endogenous protease activity with different treatments. Each bar is presented with standard error. A: Calpain; B: Cathepsin B; C: Cathepsin L; D: Cathepsin D. CK, control; AL, alcohol control; JB, Jinbiao Hejiu; YB, Yinbiao Hejiu; WNC, Wunianchen rice wine; NEH, Nv'erhong rice wine.

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Figure 2(on next page)

SDS-PAGE of myofibrillar protein with different treatments

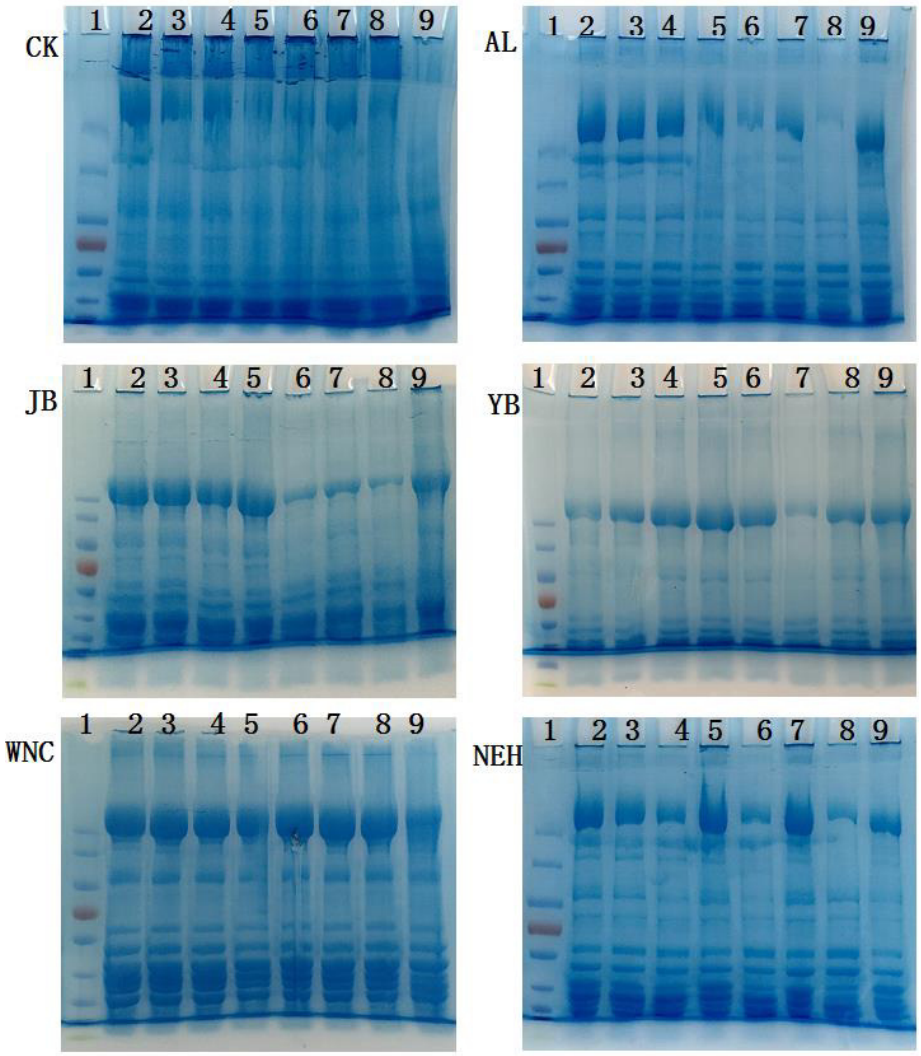

Fig. 2. SDS-PAGE of myofibrillar protein with different treatments. CK, control; AL, alcohol control; JB, Jinbiao Hejiu; YB, Yinbiao Hejiu; WNC, Wunianchen rice wine; NEH, Nv'erhong rice wine. Lane 1: MW marker; Lane 2: 0 h storage; Lane 3: 15 min storage; Lane 4: 30 min storage; Lane 5: 1 h storage; Lane 6: 2 h storage; Lane 7: 4 h storage; Lane 8: 10 h storage; Lane 9: 20 h storage. Molecular weight standard from top to bottom of gel: 170, 130, 100, 70, 55, 40, 35, 25, and 15 kDa.

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Table 1(on next page)

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

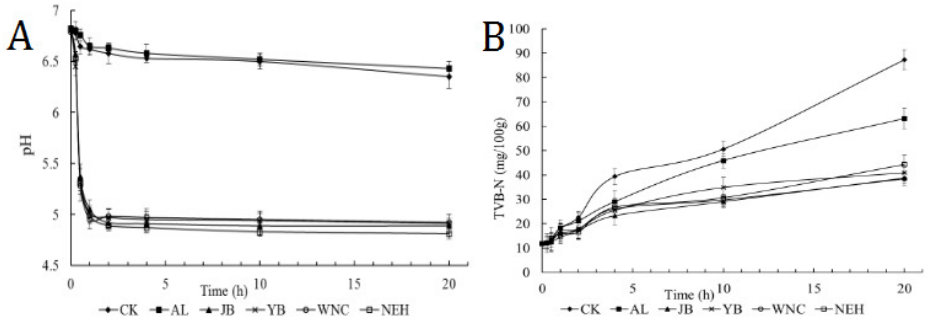

Fig. 3. Changes in pH (A) and TVB-N (B) of topmouth culter fillets with different treatments during incubation. CK, control; AL, alcohol control; JB, Jinbiao Hejiu; YB, Yinbiao Hejiu; WNC, Wunianchen rice wine; NEH, Nv'erhong rice wine.

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Table 2(on next page)

Physiochemical properties of different rice wine

Table 1 Physiochemical properties of different rice wine

Rice wine	pH	Ca (μg/ml)	TPC (μg 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]

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

Table 3(on next page)

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

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

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