Changes of endogenous enzymes and physiochemical indicators in the process of dry-salted Decapterus maruadsi

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ABSTRACT
In order to evaluate the relationship between of endogenous enzymes and physiochemical indicators during the processing of dry-salted Decapterus maruadsi produced by the traditional method. The evolution of the endogenous enzymes (cathepsin B, cathepsin L, cathepsin H, acid lipase, neutral lipase, and phospholipase) and physical and chemical parameters (moisture content, salt content and pH) of dry-salted Decapterus maruadsi during processing was investigated. Principal component analysis and Pearson correlation coefficient analysis were used to explore the main factors influencing the endogenous enzyme activity and the interactions between endogenous enzymes. The following results were observed during the processing of dry-salted Decapterus maruadsi: the salt content could stimulate cathepsin (L and B) activity and inhibit lipase activity; a lower moisture content could inhibit lipase activity, while the inhibitory effect on cathepsin was inconspicuous; pH had a minor influence on the endogenous enzyme activities; cathepsin B and L could partially inhibit lipase activity.

1. Introduction
Fish are rich in nutrients, such as protein. However, fish are highly perishable. To extend the edible storage period and increase the flavor of fish, they are often turned into dry-salted fish products (Boeri et al., 2011). Related research has been conducted on dry-salted shark (Mujaffar & Sankat, 2005, 2006) and dry-pickled cod fish (Lauritzen et al., 2004). Consumers widely accept these products due to their nutritional richness and peculiar sensory properties (texture and flavor). The formation of unique flavor compounds during manufacturing require a complex sequence of chemical and biochemical reactions, such as lipid oxidation and maillard reactions (Careri et al., 2005; Ventanas et al., 1992). Lipid and protein degradation products are the main substrates of flavor compounds, and it has been suggested that lipases (acid lipase, neutral lipase and phospholipase) (Flores, Aristoy, Antequera, Barat, & Toldrá, 2012; Huang, Li, Huang, Li, & Sun, 2014; Motilva, Toldrá, & Flores, 1997, 1993; Zhong et al., 2012) might be wholly or partly responsible for lipid hydrolysis and proteolysis. Studies have found that during the processing of dry-cured hams cathepsin (B, L, H) (Flores et al., 2012; Flores, Aristoy, Antequera, Barat, & Toldrá, 2009) and lipase (acid lipase, neutral lipase and phospholipase) (Flores et al., 2012; Vestergaard, Virgili, & Virgili, 2000) have a certain activity and the degradation of protein and fat is correlated with cathepsin and lipase. Research on salted fish products has mainly focused on quality and safety (Wu et al., 2014, 2011) in the production process, such as the microbe, amine, and nitrite contents and...
the evolution of protein degradation and lipolysis-oxidation (Cai et al., 2014). Therefore, the exploration of the degradation mechanisms of proteins and lipids is relatively weak. Up till now, the evolution of endogenous enzymes (cathepsin and lipase) during the processing of traditional China dry-salted fish has not been investigated, and the mutual influence between two types of enzyme activity changes has not been reported. Our study aims were to evaluate the changes of endogenous enzymes (cathepsin B, cathepsin L, cathepsin H, acid lipase, neutral lipase, phospholipase) and physiochemical indicators (water content, salt content and pH) and the relationship between of them in the process of dry-salted Decepterus maraudsi produced by the traditional method. Principal component analysis and the Pearson correlation coefficient were used to explore the main factors that could influence the endogenous enzyme activity, as well as the interactions between the endogenous enzymes during the processing of dry-salted fish.

2. Materials and methods

2.1. Processing of dry-salted fish and sampling

Chilled round scad (Decepterus maraudsi; iced fresh fish) were purchased from a local supermarket (live weight: 100 ± 5 g). They were prepared by adding 20% salt (w/w) using the following process steps. The salt was poured evenly on the fish. Then, the fish were covered in saturated salt water and placed in an incubator at 4°C for 36 h. After the wet-cure procedure, the fish were soaked in water for 1 h, and the desalination step was repeated 3 to 4 times. Then, the fish were air-dried at 28 ± 2°C with 10% relative humidity (60 h, air flow rate: 6 m/s) until the water content was approximately 40%. Specimens were sampled at five points: raw fish (sample A), end of salting (sample B), after desalting (sample C), the medium- to low-temperature heat pump drying (sample D), and finished product (sample E). Approximately 10 fish were sampled at each point, and the samples were vacuum packed and stored at −80°C until analysis.

2.2. Lipase extraction and activity assay

2.2.1. Crude enzyme solution extraction

Crude lipase solution was extracted according to the method described by Hernández and Toldrá (1998) with slight modification. The samples were thawed, the skin and bone were removed, and the meat was finely chopped. Five grams of chopped sample was homogenized (6 × 10 s at 18,000 rpm, cooled with ice) in 25 mL of 50 mM phosphate buffer [pH 7.5; 5 mM of ethylene glycol-bis (2-aminoethyl ether)-N, N, N', N'-tetraacetatic acid (EGTA)] using a Polytron homogenizer (IKA T25, Germany). The homogenate was stirred for 1 h and then centrifuged (10,000 g) in a refrigerated centrifuge (sigma 3k30, Germany) for 30 min at 4°C. The supernatant was filtered using glass wool, and the filtrate was collected as crude lipase solution for further assays. The protein concentration was determined by the biuret method.

2.2.2. Neutral lipase activity determination

The neutral lipase activity was measured as described by Motilva et al. (1992) and Vestergaard et al. (2000) with slight modifications. A portion of the enzyme solution (0.1 mL) was diluted with 2.8 mL of 0.22 M Tris/HCl buffer (pH 7.5; 0.05% (w/v) Triton X-100), and 0.1 mL of substrate buffer (1.0 mM 4-methylumbelliferyl-oleate, Sigma, St. Louis, MO, USA) was added. After incubation at 37°C for 30 min, the samples were immediately cooled in an ice-water mixture and analyzed within a minute. Fluorescence was measured using a fluorescence spectrophotometer (Cary Eclipse, Varian, America) (λ_ex = 344 nm, λ_em = 445 nm).

2.2.3. Acid lipase activity determination

Acid lipase activity was measured as described by Motilva et al. (1992) and Vestergaard et al. (2000) with some minor modifications. A portion of the enzyme solution (0.1 mL) was diluted with 2.8 mL of 0.1 mM disodium phosphate/0.05 M citric acid buffer [pH 5.0, 0.05% (w/v) Triton X-100 and 0.8 mg·mL⁻¹ bovine serum albumin (BSA)], and 0.1 mL of substrate buffer (1.0 mmol·L⁻¹ 4-methylumbelliferyl-oleate) was added. After incubation at 37°C for 30 min, the reaction was stopped using 0.5 mL of 1 M HCl, and the fluorescence was measured using a fluorescence spectrophotometer (Cary Eclipse, Varian, America) (λ_ex = 328 nm, λ_em = 470 nm).

The lipases activity was calculated using a standard curve. One unit of activity (U) was defined as the amount of enzyme hydrolyzing 1 nmol 4-methylumbelliferyl-oleate per h at 37°C, and the enzyme activity was expressed as U·g⁻¹ protein.

2.3. Cathepsin extraction and activity assay

2.3.1. Crude enzyme solution extraction

Crude cathepsin solution was extracted according to the methods described by Garcia-Garrido, Quiles-Zafría, Tapiador, and LuqueD (2000), Virgili, Parolari, Schivazappa, Soresi-Bordini, and Borri (1995) and Rosell and Toldrá (1998) with slight modification. After removing the skin and bone, thawed samples were finely chopped. Five grams of chopped sample was homogenized (6 × 10 s at 15,000 × g, cooled with ice) in 20 mL of 50 mM phosphate buffer [pH 5.0; 50 mM NaAc; 100 mM NaCl; 1 mM EDTA; 0.2% (w/v) Triton X-100] using a Polytron homogenizer (IKA T25, Germany). The homogenate was stirred for 1 h at 4°C and then centrifuged (10,000 × g) in a refrigerated centrifuge (sigma 3k30, Germany) for 45 min at 4°C. The supernatant was filtered using glass wool, and the filtrate was collected (diluted to 50 mL with extraction buffer) as the crude cathepsin solution for further assays.

2.3.2. Cathepsin activity determination

The cathepsin B and B-L activity was measured as described by Barrett (1980), Toldrá et al. (1993) and Zhao et al. (2005) with slight modifications. A portion of the enzyme solution
(0.5 mL) was diluted with 0.25 mL of reaction buffer (cathexin B buffer: pH 6.0, containing 352 mM KH₂PO₄, 4 mM Na₂EDTA, 48 mM Na₂HPO₄, 4 mM L-Cys; cathexin B+L buffer: pH 5.5, containing 340 mM NaAc, 4 mM Na₂EDTA, 60 mM HAc, 8 mM DTT) and heated in a 40°C water bath for 5 min, and 0.5 mL of substrate buffer (cathexin B: Z-Arg-Arg-7-amido-4-methylcoumarin; cathexin B+L: Z-Phe-Arg-7-amido-4-methylcoumarin, 20 µmol·L⁻¹; Sigma, St. Louis, MO, USA) was added. After incubation at 40°C for 15 min, the reaction was stopped with 2 mL termination reagent (pH 4.3, 100 mM CICH₂COONa, 70 mM HAc, 30 mM NaAc; Control group: termination reagent was added before adding the other solution), and the fluorescence was measured using a fluorescence spectrophotometer (Cary Eclipse, Varian, America) (λex = 340 nm, λem = 440 nm). The activity of cathepsin L = (cathexin B+L) – (cathexin B).

2.3.3. Cathepsin h activity determination

L-Arg-AMC can be hydrolyzed by aminopeptidase and cathepsin H, and cysteine protease specific irreversible inhibitors (E-64) can inhibit the activity of cathepsin H. The determination of cathepsin H was slightly different from that of the other cathepsins. The specific steps were as follows. Group (A): A portion of the enzyme solution (0.5 mL) was diluted with 20 µl E-64 (1 mM) and 0.25 mL reaction buffer (cathexin H buffer containing 200 mM KH₂PO₄, 200 mM Na₂HPO₄, 4 mM Na₂EDTA, 8 mM L-Cys; pH 6.8) and heated in a 40°C water bath for 60 min. Group (B): A portion of the enzyme solution (0.5 mL) was diluted with and 0.25 mL of cathexin H buffer and heated in a 40°C water bath for 5 min. Then, 0.5 mL of substrate buffer (cathexin H: L-Arg-7-amido-4-methylcoumarin 20 µmol·L⁻¹; Sigma, St. Louis, MO, USA) was added to group (A) and (B). The following steps were the same as those in section 2.3.2. The activity of cathepsin H = group (A) – group (B).

The cathepsin activity was calculated using a standard curve. One unit of activity (U) was defined as the amount of enzyme hydrolyzing 1 nmol AMC per min at 40°C. The enzyme activity was expressed as U · g⁻¹ fish.

2.4. Physicochemical analysis

The moisture content was measured according to ISO 1442:1997(E), the salt content was measured according to ISO recommended method 1841-1 (ISO, 1996E), and the pH was measured as described by Jin et al. (2010).

2.5. Statistical analysis

All experiments were repeated 3 times. The endogenous enzyme activities (cathexin B, L, H; lipase (acid lipase, neutral lipase, phospholipase)) and physicochemical indicators (moisture content, salt content, pH) at the different stages of processing were analyzed using SPSS statistical software (Version 13, SPSS Inc., Chicago, IL, USA). The level of significance was set to p < 0.05. The relationships among enzyme activities and physicochemical indicators during dry-salted fish processing were analyzed by principal component analysis and Pearson correlation analysis using SPSS statistical software.

3. Results and discussion

3.1. Changes in the physicochemical parameters during the processing of dry-salted decapterus maruadsi

The changes in the physicochemical parameters of Decapterus maruadsi during dry-salted processing are shown in Table 1. In general, the moisture content decreased during the most of processing stages; in contrast, the salt content increased. The moisture content significantly (P < 0.05) decreased from 77.18 ± 0.62% (fresh fish) to 67.1 ± 0.05% during the salting and air-drying stages. The salt content increased significantly (P < 0.05) during the salting and air-drying stages. The average salt content of the final product was 8.55 ± 0.1 g/100 g fish, which is higher than the salt content of other dry-cured meat products, such as dry-cured ham (6–8%; Motliva & Toldrá, 1993). The salt content during the salting stage and the organization characteristics of the raw materials resulted in the high salt content in the final product. Moon and Yoon (2016) reported the total and water weight decreased and the salt weight increased with decreasing sea cucumber initial weight. The evolution of the pH (6.3–6.5) was non-significant (P > 0.05) during most of the processes, with the exception of the curing phase, where it decreased significantly (P < 0.05) compared with fresh fish. Huang et al. (2014) found that the pH value barely changed during bacon processing; it ranged from 5.5 to 6.0, which was lower than the pH measured in our study.

3.2. Changes in cathepsin (B, L, H) during the processing of dry-salted decapterus maruadsi

As shown in Figure 1, cathepsin (B, L, H) demonstrated activity throughout the entire dry-salted fish processing. The activity of cathepsin B and L in the final product increased significantly (by 46.91% and 113.11%, respectively) compared to their initial activities in fresh fish; in contrast, cathepsin H activity decreased by 7.37%. The activity of cathepsin (B, H) increased significantly (P < 0.05) during most stages, except the salting stage. The cathepsin L activity increased (P < 0.05) first, decreased during desalination, and then increased markedly (P < 0.05) in the final two stages. Toldrá et al. (1993) found that the cathepsin (B, L, H) activity decreased seriously (residual activity of only 5% to 18%) during the processing of dry-salted fish.

Table 1. Changes in the physicochemical indexes of Decapterus maruadsi during dry-salted processing.

| Index          | Stage A   | Stage B   | Stage C   | Stage D   | Stage E   |
|---------------|-----------|-----------|-----------|-----------|-----------|
| Salt content  | 0.18 ± 0.05d | 6.41 ± 0.05b | 5.25 ± 0.39c | 6.65 ± 0.07b | 8.55 ± 0.1a |
| Moisture content | 77.18 ± 0.62a | 66.71 ± 0.07c | 73.56 ± 0.39b | 62.41 ± 0.53d | 45.44 ± 0.32e |
| pH            | 6.44 ± 0.01a | 6.35 ± 0.02b | 6.44 ± 0.02a | 6.44 ± 0.01a | 6.5 ± 0.01a |

a-e The same letter indicates that there is no significant difference (p > 0.05).

a-e La misma letra indica que no hay una diferencia significativa (p > 0.05).
3.3. Changes in lipase activity during the processing of dry-salted Decapterus maruadsi

Lipase (acid lipase, neutral lipase, phospholipase) demonstrated activity throughout the entire processing of dry-salted fish, as shown in Figure 2. Lipase activity has also been observed in other dry-pickled meat products (Flores et al., 2012; Huang et al., 2014; Ripollés, Bastianello, Campagnol, Armenteros, Aristoy, & Toldrá, 2011). The lipase activity decreased substantially in the final products compared to the initial activity in the fresh fish. The acid lipase properties remained relatively stable throughout the process. Ripollés et al. (2011) made the same conclusion in the study of ham, where the acid lipase remained stable during the first 50 days of ham processing. The evolution trends of neutral lipase and phospholipase were the same throughout the process. The activities of neutral lipase and phospholipase decreased significantly (P < 0.05) during most stages, except desalination. The acid lipase activity decreased (P < 0.05) first, increased after the soak desalination process, reached its maximum during stage D, and then decreased markedly (P < 0.05) in the final stage. Neutral lipase demonstrated the highest activity throughout the entire processing, followed by acid lipase, with phospholipase exhibiting the lowest activity. Wang et al. (2016) also reported that neutral lipase was the main hydrolytic enzyme in fat hydrolysis throughout the entire processing of dry-cured goose. The relationship between endogenous lipase and the physical and chemical indicators will be explored by principal component analysis and Pearson correlation analysis in the following sections.
Table 2. Index of principal component characteristic values and their cumulative contribution to the total variance for the endogenous enzymes and the physicochemical parameters of dry-salted Decapterus maruadsi. Using Kaiser rule extracting principal component.

| Principle component | Eigenvalue | Contribution (%) | Cumulative eigenvalue | Cumulative contribution (%) |
|---------------------|------------|------------------|-----------------------|-----------------------------|
| 1                   | 6.283      | 69.814           | 6.283                 | 69.814                      |
| 2                   | 2.307      | 25.633           | 8.590                 | 95.447                      |

Figure 3. Loading plots of the principal components for the endogenous enzymes and physicochemical properties of dry-salted Decapterus maruadsi.

Figure 3 shows that acid lipase, neutral lipase, phospholipase and the moisture content were in group (I); cathepsin B, cathepsin L and the salt content were in group (II); and cathepsin H and the pH were in group (III). In groups (I) and (II), there are high positive loadings for acid lipase, neutral lipase, phospholipase and moisture content (0.881, 0.988, 0.991 and 0.930, respectively), and the high negative loadings of cathepsin B, cathepsin L and the salt content (−0.858, 0.926 and −0.935, respectively) in principal component 1 indicated that moisture content and salt content had a significantly positive effect on maintaining the stability of the lipase and cathepsin (B, L) activities, respectively, throughout the process. Groups (I) and (II) distributed in the opposite area (group (III)), confirming the results. Huang et al. (2014) also reported that the decreased moisture content could inactivate the lipase in traditional Chinese smoke-cured bacon. The impacts of the comprehensive changes in the external environment (salt concentration, temperature, moisture content) on the cathepsin activity were extremely complex (Zhao et al., 2005). Therefore, the inhibitory effect of the moisture content on cathepsin was unremarkable in our experiment. The pH presented a non-significant negative correlation with lipase and a non-significant positive correlation with cathepsin. Although the pH is located in group (III) with cathepsin H in Figure 3, there is a long distance between the two factors, indicating that pH has little influence on endogenous enzyme activity.

3.5. Pearson correlation coefficient analysis of the endogenous enzymes and physicochemical indicators

The relationship between the endogenous enzymes and the physicochemical indicators during dry-salted fish processing were evaluated by Pearson correlation coefficient analysis using SPSS statistical software; the results are shown in Table 3.

3.5.1. Correlation analysis of the physicochemical indicators and endogenous enzymes

Table 3 shows that the salt content and water content were strongly correlated with cathepsin (B, L) and lipase. The salt content presented a significant positive correlation (0.981; \( P < 0.01 \)) with cathepsin L, and a non-significant positive correlation (0.679; \( P > 0.05 \)) with cathepsin B. In contrast, the salt content showed significant negative correlations with acid lipase (−0.946; \( P < 0.01 \)), phospholipase (−0.961; \( P < 0.01 \)) and neutral lipase (−0.928; \( P < 0.05 \)) and a non-significant negative correlation with cathepsin H (−0.334; \( P > 0.05 \)). Through single-factor and control experiments, Roicé, Toldrá, and Flores (1991) found that salt notably inhibited the activities of cathepsin B and L. However, García-Garrido et al. (2000) observed that salt levels were only weakly correlated with residual enzyme (cathepsin L and B) activity in Spanish dry-cured ham. Our Pearson correlation analysis showed that increasing the salt content in the machining process can stimulate and enhance the activity of cathepsin L and B. Therefore, the comprehensive effects of different raw materials and processing conditions on the activity of cathepsin are not the same. An increase in salt content had a significant inhibitory effect on the endogenous lipase activity, and the inhibitory effect on cathepsin H was inconspicuous. Toldrá (1998) and Toldrá (2006) reported that an increased salt concentration could inactivate neutral lipase and phospholipase and have an inconspicuous effect on acid lipase. Jin et al. (2010) found that the acid lipase and phospholipase activities decreased significantly with increasing salt concentration in dry-cured ham.

The moisture content presented a significant negative correlation with cathepsin B and L (−0.892 and −0.886; \( P < 0.05 \)) and a minor negative correlation with cathepsin H (−0.034; \( P > 0.05 \)). In addition, the moisture content showed a positive correlation with phospholipase (0.876; \( P < 0.05 \)), neutral lipase (0.905; \( P < 0.05 \)) and acid lipase (0.680; \( P > 0.05 \)). The Pearson correlation analysis showed that lower moisture content in the machining process can inhibit the activity of lipase, but the inhibitory effect on cathepsin was inconspicuous. In Figure 3, the moisture content is located in group (I) and lipase and cathepsin (B, L) are distributed in the opposite area (group (II)), confirming the results. Huang et al. (2014) also reported that the decreased moisture content could inactivate the lipase in traditional Chinese smoke-cured bacon. The impacts of the comprehensive changes in the external environment (salt concentration, temperature, moisture content) on the cathepsin activity were extremely complex (Zhao et al., 2005). Therefore, the inhibitory effect of the moisture content on cathepsin was unremarkable in our experiment. The pH presented a non-significant negative correlation with lipase and a non-significant positive correlation with cathepsin. Although the pH is located in group (III) with cathepsin H in Figure 3, there is a long distance between the two factors, indicating that pH has little influence on endogenous enzyme activity.

Table 3. Correlation analysis of lipase and cathepsin

| Enzymes | Lipase | Cathepsin B | Cathepsin L |
|---------|--------|-------------|-------------|
| Lipase  | 1.000  | 0.892       | 0.886       |
| Cathepsin B | 0.892 | 1.000       | 0.679       |
| Cathepsin L | 0.886 | 0.679       | 1.000       |
fish. In contrast, cathepsin H activity and lipase activity decreased substantially in the final products compared to the initial activity in the fresh fish. Principal component analysis and Pearson correlation analysis proved that during the processing of dry-salted *Decapterus maruadsi* an increased salt content could enhance the activity of cathepsin L and B, and inhibited lipase activity and a lower moisture content could inhibit lipase activity, but its inhibitory effect on cathepsin was inconspicuous and pH had little influence on the endogenous enzyme activities. In addition, cathepsin B and L partially inhibited lipase activity, and the influence of cathepsin H on lipase was minor.

### Disclosure statement
No potential conflict of interest was reported by the authors.

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