Study on endogenous protease and protein degradation of dry-salted Decapterus maruadsi

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ABSTRACT

Extensive proteolysis and formation of flavor substances occur during the processing of dry-salted fish due to the action of endogenous proteases. This study analyzed the evolution of the endogenous proteases and the relationship between endogenous protease levels and protein degradation during processing. Cathepsin B and L activity increased significantly during the last two stages, while cathepsin H activity decreased non-significantly (residual activity was 92.27%) compared to initial activity levels in fresh fish. Dipeptidyl peptidase (DPP I, IV) activity decreased overall. The activity of three aminopeptidases (arginine aminopeptidase; alanyl aminopeptidase; leucyl aminopeptidase) initially decreased (P < 0.05) then significantly increased (P < 0.05) during the final stage. Principal component analysis indicated that cathepsins B and L had larger influences than cathepsin H on protein degradation and flavor substance formation in dry-salted fish, but the influence of dipeptidyl peptidases and aminopeptidases on protein degradation and flavor substance formation was limited.

1. Introduction

Due to its unique flavor and resistance to storage, dry-salted fish has been favored by consumers deeply. The flavors in dry-salted fish come from variety pathways, such as lipolysis-oxidation, protein degradation, and the Maillard reaction. In the processing of ham, protein degradation can produce large amounts of peptides and free amino acids (FAAs). Small peptides and FAAs can cause bitterness, sweetness, or freshness, and some FAAs and small peptides are precursors to compounds that give ham its flavor (Careri et al., 1993). The endogenous proteases in pickled products that can degrade proteins into polypeptides and FAAs are cathepsins, calpains, dipeptidases, and aminopeptidases (Gallego, Mora, Fraser, Aristoy, & Toldrá, 2014; Toldra, Aristoy, & Flores, 2000). Endogenous cytoplasmic calpains (both u- and m-calpains) and lysosomal cathepsins B, D, H, and L play important roles in post-mortem proteolysis of myofibrils and concomitant meat softening (Ahmeda, Donkora, Street, & Vasiljevic, 2015). Cathepsin B plays an important role in textural changes in fish tissue. Chéret, Delbarre-Ladrat, Lamballerie-Anton, and Verrez Bagnis (2007) found that the activity of cathepsins B and L was 29.7 and 4 times higher in fish muscle than in bovine meat, respectively. Calpain and cathepsin D were not active during ham processing; however, the activities of cathepsins B, L, and H were detected throughout the course of processing (Fidel & David, 1988; Sárraga, Gil, & García-Regueiro, 1993). Four major dipeptidases (DPP I, II, III, and IV) have been identified from porcine skeletal muscle. DPP I and IV had high activity during the processing of ham (Angel & Toldra, 2001). Aminopeptidases in ham could degrade proteins and peptides to FAAs (Toldra, Flores, & Sanz, 1997). Toldrá et al. (1992) found that alanyl aminopeptidase (AAP), arginine aminopeptidase (RAP), leucyl aminopeptidase (LAP), tyrosinophilin aminopeptidase and pyroglutamime aminopeptidase had a high activity in the curing phase of ham. Toldra, Cerverro, and Part (1993) found that AAP, RAP, and...
LAP remained highly active throughout ham processing by simulating the processing conditions of dry-cured ham.

Based on the above discussion, the research into endogenous proteases related to protein degradation and flavor substance formation mainly focused on the processing of dry-salted fish. However, there have been no reports on the role of endogenous protease activity in aquatic products. The objective of our study was to analyze the evolution of the endogenous proteases (cathepsin B, cathepsin L, cathepsin H, DPP I, DPP IV, AAP, RAP, LAP), the parameters of physical and chemical indicators (water content, salt content, and pH values), the level of protein degradation indicators (nitrogen, PN; non-protein nitrogen, NPN; protein hydrolysis rate, Pi), and FAA during the processing of dry-salted fish. Furthermore, the relationship between endogenous proteinases activity and protein degradation in dry-salted fish was explored by principal component analysis (PCA) using SPSS.

2. Materials and methods

2.1. Salted fish processing and sampling

Chilled round scad (Decapterus maruadis) were purchased from a local supermarket with live weights of 100 ± 5 g. Dry-salted (20% salt (w/w)) fish was prepared as follows. Salt was evenly spread on the fish, saturated salt water was used to cover the fish, and they were placed in an incubator at 4°C for 36 h. After the wet-cured procedure was complete, the fish were soaked in water for 1 h. This desalination process was repeated for a total of three or four water soaks. Then, fish were air-dried at 28 ± 2°C with 10% relative humidity (flowing air speed, 6 m/s) until the water content of the salted fish was approximately 40%. Specimens were sampled at five points: raw fish (sample A), end of salting (sample B), after desalination (sample C), during low temperature heat pump drying (sample D), and the finished product (sample E). Approximately 10 fish were sampled at each point. Samples were vacuum-packed and stored at −80°C until analysis.

2.2. Endogenous proteinases extraction and activity assays

2.2.1. Cathepsin (B and L) activity assay

A crude cathepsin solution was extracted according to the method described by Garcia-Garrido, Quiles-Zafra, Tapiador, and Luque (2000), and Rosell and Toldrá (1998). The activity of cathepsin B and activity of cathepsins B and L combined were measured as described by Zhao, Zhou, Tian, et al. (2005), and Ge, Xu, Xia, Jiang and Jiang (2016) with few modifications.

A portion of the enzyme solution (0.5 mL) was diluted with 0.25 mL reaction buffer (cathepsin B buffer: pH 6.0, containing 352 mmol/L KH₂PO₄, 4 mmol/L Na₂EDTA, 48 mmol/L Na₂HPO₄, 8 mmol/L L-Cys; cathepsin B + L buffer: pH 5.5, containing 340 mmol/L NaAc, 4 mmol/L Na₂EDTA, 60 mmol/L acetic acid, 8 mmol/L DTT). The homogenous solution was preheated to 40°C for 5 min using a water bath. Finally, 0.5 mL of substrate buffer (cathepsin B: Z-Arg-Arg-7-amido-4-methylcoumarin; cathepsin B + L: Z-Phe-Arg-7-amido-4-methylcoumarin; 20 µmol/L; Sigma, St. Louis, MO, USA) was added. After incubation at 40°C for 15 min, the reaction was quenched with 2 mL of termination reagent (pH 4.3, 100 mmol/L CH₃COONa, 70 mmol/L acetic acid, 30 mmol/L NaAc). For the control group, the termination reagent was added before the other solutions. Fluorescence was measured using a fluorescence spectrophotometer (Cary Eclipse, Varian, America) (λex = 340 nm, λem = 440 nm). The activity of cathepsin L = (cathepsin B + L) − (cathepsin B).

2.2.2. Cathepsin H activity assay

L-Arg-AMC can be hydrolyzed by aminopeptidase and cathepsin H, and cysteine protease-specific irreversible inhibitors (E-64) can inhibit cathepsin H activity. The determination of cathepsin H is slightly different than the others. The procedure is as follows: group (A): A portion of the enzyme solution (0.5 mL) was diluted with 20 µL E-64 (1 mmol/L) and 0.25 mL reaction buffer (cathepsin H buffer containing 200 mmol/L KH₂PO₄, 200 mmol/L Na₂HPO₄, 4 mmol/L Na₂EDTA, 8 mmol/L L-Cys; pH 6.8). The homogenous solution was preheated for 60 min at 40°C using a water bath; group (B): A portion of the enzyme solution (0.5 mL) was diluted with 0.25 mL cathepsin H buffer, and the homogenous solution was preheated for 5 min at 40°C using a water bath. Then, 0.5 mL of substrate buffer (cathepsin H: L-Arg-7-amido-4-methylcoumarin 20 µmol/L; Sigma, St. Louis, MO, USA) was added to groups (A) and (B). The next steps are the same as above (2.2.2). Cathepsin H activity = group (A) − group (B).

Cathepsin activity was calculated using a standard curve. One unit of activity (U) was defined as the amount of enzyme hydrolyzing 1 mmol of 4-methylumbelliferol-oleate per min at 40°C. Enzyme activity is expressed in (U/g fish).

2.2.3. Dipeptidyl peptidase I and IV activity assay

A crude dipeptidyl peptidase (DPP) solution was extracted according to the method described by Sentandreu and Toldrà (2001). The activities of DPP I and IV were measured as described by Bolumar, Bindricha, Toepfl, Toldrá, and Volker Heinz (2014) and Zhao, Zhou, Tian, et al. (2005), with little modification. A portion of the enzyme solution (0.5 mL) was diluted with 0.25 mL of reaction buffer (DPP I: pH 8.0, 50 mmol/L sodium citrate buffer containing 0.5 mmol/L DTT; DPP IV: pH 8.0, 200 mmol/L Tris–HCl buffer; containing 5 mmol/L DTT). The homogenous solution was preheated for 5 min at 40°C using a water bath. Finally, 0.5 mL of substrate buffer (DPP I: H-Gly-Arg-7-amido-4-methylcoumarin, 200 mmol/L Tris–HCl: H-Gly-Pro-7-amido-4-methylcoumarin, 20 µmol/L; Sigma, St. Louis, MO, USA) was added. After incubation at 40°C for 15 min, the reaction was quenched with 5 mL anhydrous ethanol. For the control group, the ethanol was added before the other solutions. Fluorescence was measured using a fluorescence spectrophotometer (λex = 340 nm, λem = 440 nm).

2.2.4. Aminopeptidase activity assay

A crude aminopeptidase solution was extracted according to the method described by Rosell and Toldrá (1998). Aminopeptidase (RAP; AAP; LAP) activity was measured as described by Bolumar et al. (2014), with little modification. A portion of the enzyme solution (0.5 mL) was diluted with 0.25 mL reaction buffer (AAP: pH 7.0, 100 mmol/L sodium phosphate buffer containing 0.33% of 30% Brij 35, 5 mmol/L CaCl₂, 1 mmol/L DTT; RAP: pH 6.5, 50 mmol/L sodium phosphate buffer containing 0.33% of 30% Brij 35, 1 mmol/L DTT, 5 mmol/L MgCl₂). The homogenous solution was preheated for 5 min at 37°C using a water bath. Finally, 0.5 mL of substrate buffer (AAP: Ala-7-amido-4-methylcoumarin; RAP: Arg-7-amido-4-
methylcoumarin; LAP: Leu-7-amido-4-methylcoumarin, 20 μmol/L; Sigma, St. Louis, MO, USA) was added. After incubation at 37°C for 15 min, the reaction was quenched with 5 mL anhydrous ethanol. For the control group, the ethanol was added before other solutions. Fluorescence was measured using a fluorescence spectrophotometer (λex = 340 nm, λem = 440 nm).

Proteases 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 min at 40°C. Enzyme activities are expressed in (U)/g fish.

2.3. Determination of protein degradation index and FAAs

NPN (%): A portion of the sample (2.00 g) was mixed with 20 mL of ultra-pure water. The solution was heat shocked at 40°C, then 20 mL of 10% TCA solution was added. The new homogenate was continuously shocked for 40 min then centrifuged (10,000g) for 15 min at 4°C using a refrigerated centrifuge (Sigma 3k30, Germany). The supernatants were filtered through glass wool, and the filtrates were collected for further assays. The NPN content of the filtrate was assayed with 3 g catalyst and 12 mL of H2SO4 using a protein auto-analyzer. Total nitrogen (TN; %): A 2.00-g portion of the sample was assayed with 3 g catalyst and 12 mL of H2SO4 using a protein autoanalyzer. Protein Degradation Index (PI) = NPN (%)/TN(%) × 100. FAAs were measured as described by Stavropoulou, Borremans, De Vuyst, De Smet, and Leroy (2015) and Berardo et al. (2017).

2.4. Determination of physiochemical index

Moisture content was measured as described by ISO 1442:1997 (E). Salt content was measured as described by the ISO recommended methods 1841-1 (ISO, 1996E). Measurements of pH were taken as described by Jin et al. (2010).

2.5 Statistical analysis

All the data in this study were analyzed using a one-way variance with SPSS statistical software (Version 13, SPSS Inc., Chicago, IL, USA). The level of significance was set at P < 0.05. The relationship between enzyme activity and protein degradation during salted fish processing was analyzed by PCA using SPSS statistical software.

3. Results and discussion

3.1. Endogenous proteinases activity changes during the process of dry-salted fish

The changes in endogenous proteinases activity in the processing of dry-salted fish are shown in Table 1. Cathepsin B, L, and H demonstrated activity at all stages. In the final product, the activity of cathepsins B and L increased significantly. This was due to the lysosomes in fish tissue being more likely to rupture, which resulted in more lysosomal proteases being release. Cathepsin H activity decreased but not significantly (residual activity was 92.27%) compared to initial activity levels in fresh fish. Sárraga et al. (1993) found that the activities of cathepsins B, L, and H had decreased substantially by the end of the ham processing (residual activity is only 5–15%).

Cathepsin B activity changed, but not significantly (P > 0.05), over stages A, B, and C and then increased significantly (P < 0.05) over stages D and E. Cathepsin L activity increased significantly (P < 0.05) at stage B, decreased at stage C, then increased substantially (P < 0.05) over stages D and E. Cathepsin H activity decreased significantly at stage B, then increased gradually in the following steps. The activity of cathepsin B in the process was the most stable. Parreho, Cussó, Gil, and Sárraga (1994) found similar results during the manufacturing process of Spanish dry-cured ham. Zhao, Zhou, Wang, et al. (2005) found that cathepsin L was more stable than cathepsin B during the processing of Jinhua ham. In our study, cathepsin H demonstrated the highest activity throughout the entire processing, followed by cathepsin B, with cathepsin B exhibiting the lowest. In contrast to our conclusion, Flores, Aristoy, Antequera, Barat, and Toldrá (2012) and Flores, Aristoy, Antequera, Barat, and Toldrá (2009) found that cathepsin B + L demonstrated the highest activity, followed by cathepsin B + H, and cathepsin H was found to have the lowest activity throughout the entire process of manufacturing Iberian dry-cured ham.

The physicochemical factors (such as temperature, salt content, and pH) have complex effects on DPP I and IV during the processing of Jinhua ham, and the enzyme activity is controlled by many factors (Zhao, Zhou, Tian, et al., 2005). As seen in Table 1, although the processing of dry-salted fish could inhibit DPP I and IV activity, DPP I and IV demonstrated activity at all stages. The development of DPP I and IV activity in processing was different. DPP I activity significantly decreased at stage C (P < 0.05) and remained stable at stage C, D, and E. DPP IV activity significantly decreased at stage B (P < 0.05) and then

| Table 1. Changes in cathepsin (B, L, H), dipetidyl peptidase, and aminopeptidase activities during processing. |
|---------------------------------------------------------------|
| **Index (U/g fish)** | **Stage A** | **Stage B** | **Stage C** | **Stage D** | **Stage E** |
| B  | 0.81 ± 0.03c | 0.81 ± 0.03c | 0.83 ± 0.01c | 1.06 ± 0.03ab | 1.19 ± 0.04a |
| L  | 0.62 ± 0.02c | 1.18 ± 0.01ab | 0.95 ± 0.04b | 1.11 ± 0.02ab | 1.31 ± 0.15a |
| H  | 2.85 ± 0.23a | 1.63 ± 0.04c | 2.15 ± 0.04b | 2.60 ± 0.07ab | 2.64 ± 0.13ab |
| DPP-I | 1.19 ± 0.02a | 1.08 ± 0.06a | 0.59 ± 0.1b | 0.35 ± 0.02b | 0.59 ± 0.02b |
| DPP-IV | 1.72 ± 0.19a | 0.52 ± 0.03b | 0.54 ± 0.01b | 0.43 ± 0.01b | 0.73 ± 0.04b |
| AAP | 4.74 ± 0.26a | 1.82 ± 0.12cd | 3.35 ± 0.40b | 1.66 ± 0.07d | 2.56 ± 0.05bc |
| LAP | 5.10 ± 0.02a | 1.45 ± 0.05e | 2.57 ± 0.11c | 1.71 ± 0.03d | 3.18 ± 0.02b |
| RAP | 0.96 ± 0.01a | 0.21 ± 0.01d | 0.42 ± 0.07c | 0.27 ± 0.03cd | 0.72 ± 0.01b |

a–d: Means that the values are not significantly different than other values with the same letter in different processing stages (P > 0.05); B, L, H means cathepsin B, L, and H activity in different processing stages; DPP-I, DPP-IV mean dipetidyl peptidase I and IV activity in different processing stages; RAP, AAP, and LAP mean arginine aminopeptidase, alanil aminopeptidase, and leucyl aminopeptidase activity in different processing stages.

a–d significa que los valores no son significativamente diferentes que otros valores con la misma letra en distintas etapas del procesamiento (p > 0.05); BLH significan actividad de B, L y H en distintas etapas del procesamiento; DPP-I, DPP-IV significan actividad de dipetidil peptidasa i y iv; RAP, AAP y LAP significan actividad de arginina aminopeptidasa, alanil aminopeptidasa y leucil aminopeptidase en distintas etapas del procesamiento.
increased at stage E. In general, DPP I and IV activity decreased over the whole processes, and the residual activities in final product was 49.58% and 42.44%, respectively. Flores et al. (2009) also reached a similar conclusion in their investigation into Iberian ham processing. DPP I activity in dry-salted JinHua ham increased significantly (i.e. 64% compared to raw meat), and the residual DPP IV activity was 4.81% in JinHua ham (Zhao, Zhou, Tian, et al., 2005).

All three of the aminopeptidases (AAP, LAP, RAP) demonstrated activity during the processing of dry-salted fish, and three kinds of enzyme activities were also detected in dry-cured ham (Flores et al., 2012; Toldra et al., 2000; Zhao et al., 2006, Zhao, Zhou, Tian, et al., 2005). The AAP, LAP, and RAP activities were 54%, 62.35%, and 75% compared with the levels in fresh fish. The results showed that RAP was the most stable during the processing of dry-salted fish. The changes in AAP, LAP, and RAP were similar during the manufacturing process. The three aminopeptidase activities initially decreased ($P < 0.05$) and then significantly increased ($P < 0.05$) by stage C. The activity in the samples decreased markedly at stage D and significantly increased ($P < 0.05$) by the final stage. These strong trends in aminopeptidase activity showed the environment during the processing of dry-salted fish greatly influenced the activity of aminopeptidases. Zhao et al. (2006) found that salinity, water, and temperature had a complex effect on aminopeptidase activity during the processing of Jinhua ham.

### 3.2. Changes in protein degradation and physicochemical index during the processing of dry-salted fish

Studies have found that the characteristic flavor of ham has a close relationship with peptide composition and content (Hansen-Miller & Hinrichsen, 1997). Severe protein hydrolysis causes hams to taste bitter because some small peptides with lipophilic side chains can be bitter (Sforza et al., 2001). The extent of protein hydrolysis can be expressed as a proteolytic index (PI). Table 2 shows the variation in NPN, total protein nitrogen (PN), and proteolysis index (PI) during the processing of dry-salted fish. The per cent PN gradually increased with the decreasing moisture content in the whole process. The per cent NPN increased with decreasing water content and protein hydrolysis. PI values increased throughout the whole process. In their study of Parma ham, Careri et al. (1993) found that insufficient protein degradation (PI < 22) made the ham aroma inadequate, and excessive protein degradation (PI > 30) gave hams a noticeable bitterness or metallic aftertaste. In our study, the PI value was 23.99 ($22 < PI < 30$) indicating no obvious bitterness or metallic aftertaste in the final product. In general, the moisture content decreased during the most of processing stages; in contrast, the salt content increased. Moisture content decreased from 77.18 ± 0.62% (fresh fish) to 45.44 ± 0.32% (final product); 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.

### 3.3. The change in FAAs during the processing of dry-salted fish

For some water-soluble FAAs (Thr, Ser, Glu, Pro, Gly, Ala, Met, Lys, His, Arg) dissolved into saturated brine, TFAA content significantly ($P < 0.05$) decrease at stage B and C (Table 3). However, TFAA content increased significantly ($P < 0.05$) during stages D and E, indicating that the FAAs in dry-salted fish were mainly produced in the last two stages. The content of caspase, leucine, and arginine was 3.03, 4.26, and 5.30 times higher in final product than in fresh fish. In the study of ham, Toldra et al. (1997) found that FAAs were mainly produced by the hydrolysis of peptides and proteins under the action of aminopeptidase. FAAs were the precursor substances of ham flavoring substances (such as sour, sweet, and bitter) and therefore have a strong correlation with the formation of ham flavoring substances (García, Díez, & Zumalacárregui, 1998; Toldra et al., 2000). Alanine has a sweet taste, glutamic acid and aspartic acid taste umami. Short peptides contained methionine, leucine, and isoleucine could hydrolyze and release of FAAs thus reduce bitter taste. The percentage of flavored FAAs (PFAA) that contribute to the taste increased steadily with the processing time. PFAA was 2.90 times higher in final product than in fresh fish. The level of FAA (methionine, leucine, and isoleucine) that contributes to the reduction of BFAA increased in stages B, D, and E. The level of fresh amino acids (DFAA) increased during the last three stages. DFAA was 2.73 times higher in final product than in fresh fish, which can enhance the umami taste of dry-salted fish. The level of sweet amino acids (SFAA) increased significantly in the early stage of drying (stage D). And, SFAA was 2.58 times higher in final product than in fresh fish, which can enhance the sweet taste of dry-salted fish.

### Table 2. Changing of protein degradation and physicochemical index of Decapterus maruadsi during different methods of dry salted processing.

| Index   | Stage A | Stage B | Stage C | Stage D | Stage E |
|---------|---------|---------|---------|---------|---------|
| TN      | 3.97 ± 0.18c | 3.64 ± 0.22c | 3.46 ± 0.04c | 6.02 ± 0.03b | 7.83 ± 0.37a |
| NPN     | 0.56 ± 0.07c | 0.53 ± 0.01c | 0.53 ± 0.04c | 1.16 ± 0.01b | 1.88 ± 0.10a |
| PI      | 14.04 ± 1.13c | 14.53 ± 0.50c | 15.42 ± 1.05c | 19.25 ± 0.04b | 23.99 ± 1.04a |
| SC (%)  | 0.18 ± 0.05d | 6.41 ± 0.05b | 5.25 ± 0.39c | 6.65 ± 0.07b | 8.55 ± 0.1a  |
| MC (%)  | 77.18 ± 0.62a | 66.71 ± 0.07b | 73.56 ± 0.39c | 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 | 6.3 ± 0.01a  |

a-d means that the values are not significantly different than other values with the same letter in different processing stages ($P > 0.05$). NPN means non-protein nitrogen content percentage in different processing stages. TN means total nitrogen content percentage in different processing stages. PI means Protein Degradation Index in different processing stages. SC means salt content percentage in different processing stages. MC means moisture content percentage in different processing stages. 

a-d significa que los valores no son significativamente diferentes de otros valores con la misma letra en distintas etapas del procesamiento ($p > 0.05$). NPN significa el porcentaje de contenido de nitrógeno en distintas etapas del procesamiento. TN significa el porcentaje del contenido total de nitrógeno en distintas etapas del procesamiento. PI significa índice de degradación proteínica en distintas etapas del procesamiento. SC significa el porcentaje del contenido de sal en distintas etapas del procesamiento. MC significa el porcentaje del contenido de humedad en distintas etapas del procesamiento.
3.4. Relationships between endogenous proteinase, protein degradation, and physicochemical index during dry-salted fish processing

The relationship between endogenous proteinases, protein degradation, physicochemical index, and FAA during dry-salted fish processing was evaluated by PCA. Two principal components (principal component 1: 63.064%; principal component 2: 27.722%) were extracted, explaining 90.786% of the total variance. Figure 1 shows the loadings of different variables in scatter plots of principal component 1 and principal component 2. The relationship among all variables could be explained by their location in the loading plots.

3.4.1. The relationship between endogenous proteinases and protein degradation

As shown in Figure 1, cathepsin B and L, protein degradation indicators (PI, NPN), and FAA (TFAA, BFAA, DFAA, SFAA, and PFAA) are clustered together (I). In Figure 1, we observed positive loadings of NPN (0.939), PI (0.966), BFAA (0.942), PFAA (0.986), SFAA (0.857), DFAA (0.826), and TFAA (0.867) and positive loadings of cathepsin B (0.968) and cathepsin L (0.812) in principal component 1. The dipeptidases (DPP I and IV) and aminopeptidase (AAP, RAP, LAP) formed a cluster (II) in a separate region with negative loadings (DPP I, -0.749; DPP IV, -0.532; AAP, -0.615; LAP, -0.360; RAP, -0.165). Cathepsin H is located in the region between I and II. Thus, cathepsin B and L were more closely related to protein degradation and the generation of FAA than cathepsin H in dry-salted fish processing. Parreño et al. (1994) also found that the contribution of cathepsin H to the proteolysis produced during ham-manufacturing processes was very low. The role of DPP I and IV and aminopeptidases AAP, RAP, and LAP on protein degradation and flavor formation in dry-salted fish was limited. Similar results were reported by Virgili, Parolari, Schizavazza, Soresi, and Borri (1995) who found a significant correlation between NPN and cathepsin B during the processing of Parma ham. Garcia-Garrido et al. (2000) found that the activity of cathepsins B + L was the main reason for the increased level of NPN during the processing of Spanish ham. During the processing of Jinhua ham (Zhao et al., 2005), similar results were reported by Zhao, Zhou, Tian, et al., 2005, Zhao, Zhou, Wang, et al., 2005, RAP and LAP were found to have a strong correlation with the release of FAA; however the role of AAP was limited. In addition, DPP I could promote the release of polypeptides, while DPP IV played a minor role.

3.4.2. The relationship between physicochemical index and protein degradation

Salt content (SC) and cathepsins B and L were clustered in the same group (I) in Figure 1. However, DPP I, DPP IV, AAP, RAP, and LAP activities did not cluster with SC. The results showed that SC had a major effect on the activity of cathepsins B and L (especially the activity of cathepsin L). Elías, Fidel, and José (1991) found that a certain concentration of salt content could not inhibit the activity of cathepsins B and L by single-factor and control experiments. In the processing of Spanish ham, Garcia-Garrido et al. (2000) found that NaCl affected NPN levels by affecting the activity of cathepsin. The PCA results in Section 3.4.1 showed that cathepsins B and L could promote the degradation of proteins, so it can be inferred that the salt content can promote protein degradation and flavor component formation by promoting the activity of cathepsins B and L during the processing of dry-salted fish. Moisture content (MC) and DPP I were in the same area (III); however, MC did not cluster with other endogenous proteinases in Figure 1 suggesting that MC in the process of dry-salted fish was not significant to the protein.
4. Conclusion

XPCA indicated that cathepsins B and L had larger influences than cathepsin H on protein degradation and flavor substance formation in dry-salted fish, but the influence of DPPs and aminopeptidases on protein degradation and flavor substance formation was limited. Certain concentrations of salt could promote the degradation of proteins and the formation of flavor substances. Water content and pH had no significant effect on protein degradation or FAA formation.

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