Changes of phospholipase A₂ and C activities during dry-cured duck processing and their relationship with intramuscular phospholipid degradation

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1. Introduction

Traditional Chinese dry-cured duck is a well known local delicacy in China and Southeast Asia, due to its tasty flavour and texture, and it has a history of over 300 years (Li, 1988). In Nanjing city alone, about five million dry-cured ducks are consumed annually (Li, 1988; Zhang et al., 2013). Similar to dry-cured Jinhua ham, dry-cured duck is produced by dry curing, marinating, piling and drying naturally, but the period of its production is shorter than that of hams (Xu, Xu, Zhou, Wang, & Li, 2008).

The biochemical changes that occur during the processing of dry-cured meat are directly associated with the final flavour of the product. Flavour development in meat and meat products are reported to be associated with lipids composition, the extent of lipolysis and oxidation of lipids and free fatty acids during processing (Chizzolini, Novelli, & Zanardi, 1998). The phospholipid (PL) fraction is an important group of lipids in meat and meat products because of its high sensitivity to lipolysis and oxidation. This is due to its high proportion of long chain polyunsaturated fatty acids and its close contact with catalysts of lipid lipolysis or oxidation in the aqueous phase of the muscle cell (Pérez-Palacios, Ruiz, Dewettinck, Le, & Antequera, 2010). Therefore, the research concerning the composition and changes of intramuscular phospholipids was of particular interest.

Phospholipids hydrolysis, as the first stage and main cause of phospholipid degradation, is generally attributed to phospholipases. The lipolytic enzyme activities (such as neutral lipase, acid lipase and total phospholipase) have been studied in some dry-cured products, and these enzymes remained active during the entire process (Jin et al., 2010; Motilva, Toldrá, & Flores, 1992). However, the studies on the activities of the phospholipases subgroups in dry-cured meat products are rare. Phospholipases are classified into A₁, A₂, C and D according to the ester bonds they hydrolyse. Among them, PLAs and PLC are more intensively studied in meat tissues; they cleave the phospholipids into free fatty acids (FFA), lysophospholipids and diacylglycerol etc. However, the data related to muscle PLAs and PLC and the factors affecting the activities of these enzymes are very limited. A number of methods have been applied to measure the activity of phospholipases. Titrimetric and colorimetric procedures are commonly used in studying muscle or meat samples, but they were affected by various factors, while the method using radioactive substrate could provide a more
sensitive and accurate assay of phospholipase activities (Van den Bosch & Aarsman, 1979). The knowledge of the phospholipase activities during processing is essential for improving the quality of end products and further complicated overall lipid degradation mechanism. Therefore, the objective of this study was to evaluate the changes of phospholipase A<sub>2</sub> and C activities during dry-cured duck processing and their relationship with intramuscular phospholipid degradation.

2. Materials and methods

2.1. Sampling and processing conditions

Thirty-six lean-type Cherry Valley Pekin ducks (Anas platyrhynchos) with the average weight of approximately 2.0 kg were slaughtered humanely in a commercial meat processing plant (Jiangsu furun Food Ltd., Xuzhou, China). After chilling for 2 h, dry-cured ducks were processed as follows: duck carcasses were dry-salted for 1 day (salt content: 6.5% of carcass weight), marinated in brine for 1 day (saturated salt solution), piled for 2 days and then dried at 2–10 °C in a well-ventilated room for 6–12 days. At the end of each processing stage (including raw), six carcasses were selected for lipids and assayed for phospholipase A<sub>2</sub> and C activities. The leg muscles were removed from the carcasses and trimmed of all visible subcutaneous fat and connective tissues, then stored at −40 °C.

2.2. Chemicals

L-α-1-palmitoyl-2-[14C]arachidonyl-sn-glycero-phosphatidylcholine (2-[1-14C]AA-GPC, 58.2 mCi/mmol) was purchased from PerkinElmer (USA). Fatty acids C14:0, C14:1, C16:0, C16:1, C18:0, C18:1, C18:2, C20:2, C20:4, C22:5 and C22:6 standards were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO); and methanol, isopropyl alcohol, chloroform, n-hexane, acetic acid, diethyl ether, 2,2-dimethoxypropane, BF<sub>3</sub>, NaCl, Triton X-100 and CaCl<sub>2</sub> were of analytic grade.

2.3. Crude enzyme extraction and PL<sub>A2</sub> and PLC activities assay

The crude enzyme was extracted according to the method described by Jeong et al. (2011) with some modifications. Immediately after overnight storage at −40 °C, 30 g of muscle sample was homogenised with 100 ml Tris–HCl buffer (100 mM, pH 8.0) at 20000 rpm on ice using an Ultra Turrax (T25, IKA, Germany). The homogenate was then centrifuged for 20 min at 12000g at 4 °C (Allegra 64R, Beckman, USA), the supernatant was collected and filtered through four-layer gauze.

The PL<sub>A2</sub> activity was assayed using the method of Bell, Kennerly, Standford, and Majerus (1979) and Jeong et al. (2011). The reaction was performed in 60 μl of the standard reaction buffer (150 mM Tris–HCl [pH 7.5], 0.33% Triton X-100 and 1 mM CaCl<sub>2</sub>), 40 μl of crude enzyme sample and 20 μl of the diluted substrate (2-[1-14C]AA-GPC, 0.45 nM). The mixture was incubated at 37 °C for 30 min. The assay was subsequently terminated by adding 560 μl of the terminating reagent (n-heptane/methanol/isopropyl alcohol 4/2/1 [V/V]) and 110 μl of water. The mixture was vortexed and centrifuged at 12000g, 4 °C for 2 min (Allegra 64R, Beckman, USA). Then, 150 μl of the upper phase were transferred to a new microtube, to which 800 μl of n-heptane and silica gel (~20 mg) was added. The sample was vortex-mixed and centrifuged again at 12000g, 4 °C for 3 min. An aliquot (500 μl) of the supernatant was pooled and mixed with 3.5 ml of the β-scintillation solution (Insta Gel-XF). The radioactivity was counted with a liquid scintillation counter (LS6500, Beckman-Coulter, Fullerton, expressed as disintegrations per minute (DPM).

The PLC activity was determined by the procedures of Kuriko and Matsuda (1976) and Cao, Zheng, Wang, Rui, and Tang (2009) using p-Nitrophenylphosphorylcholine (NPPC; Aldrich) as the substrate. The reaction was carried out at 37 °C in a cuvette with a 10 nm optical path length, and the rate of NPPC hydrolysis by PLC was monitored at various time intervals by measurement of p-Nitrophenol release at 410 nm. A basal reaction mixture consisted of 1 ml of 20 mM NPPC, 1 ml of 0.25 M Tris-HCl [pH 7.2] in 60% sorbitol, and 0.5 ml of crude enzyme. The PL<sub>A2</sub> and PLC activities were defined as the relative activity (%) as compared to that in the raw duck samples.

2.4. Determination of composition and fatty acid profiles of lipids

Total lipids were extracted from muscle samples according to the method of Folch, Lees, & Sloane-Stanley (1957) with small modifications. Briefly, 10.0 g of muscle sample was homogenized with 200 ml of chloroform/methanol (2/1, V/V) solution at 1500 rpm using an Ultra Turrax. The homogenate was allowed to stand for 1 h and then passed through two layers of filters. After that, 0.2-fold of its volume of a solution containing 7.3 g/l NaCl, and 0.5 g/l CaCl<sub>2</sub> was added to the filtrate. The mixture was centrifuged for 15 min at 3000 rpm (Allegra 64R, Beckman, USA) and the lower phase was dried under vacuum on a rotary evaporator (RE-85C, Yarong, China) in a 44 °C water bath and then stored at −40 °C.

Neutral lipid, FFA and phospholipids were separated from total lipids according to the procedure of Xu et al. (2008). Briefly, 100.0 mg of total lipid extract was dissolved in 2.0 ml of chloroform, and 1.0 ml of the solution was transferred into an aminopropyl-silica minicolumn (500 MG, VARIAN, USA) that was activated with 2.0 ml of chloroform before transfer. The minicolumn was washed with 5.0 ml of chloroform/2-propanol (2/1, V/V) to obtain a neutral lipid, and then FFA was eluted with 5.0 ml of 2% acetic acid in diethyl ether (V/V). Finally, phospholipids were eluted with 5.0 ml of methanol. Neutral lipid, FFA and phospholipids elute was evaporated to remove the solvent. The amounts of neutral lipid, FFA and phospholipids were gravimetrically determined and the results were expressed as a percent of total lipid weight obtained.

The neutral lipid, FFA and phospholipids residue were mixed with 3.0 ml of 0.5 mol/l NaOH/methanol, then placed in a 50 °C water bath for 20 min. After that, 3.0 ml 14% BF<sub>3</sub>/methanol was added, the mixture was methylated at 60 °C for 30 min. At the same time, 0.5 ml of 2.2-dimethoxypropane was added to remove water that is produced during methylation. After cooling, 1.0 ml of water and 1.0 ml of n-hexane were added and they were shaken vigorously. The resulting mixture was allowed to stand for 1 h and then the upper organic phase was dried by rotary evaporation under N<sub>2</sub>. The residue was dissolved in 0.4 ml of hexane for GC analysis.

The methylated fatty acids were analysed with a gas chromatograph (GC–2010 Plus, Shimadzu, Japan) equipped with a flame ionisation detector and a split injector. One point 5 ml of the sample was injected onto a capillary column (SP-2560, 100 m × 0.25 mm × 0.20 μm, Varian, USA). The oven temperature increased from 140 to 220 °C at 5 °C/min and maintained for 30 min at 220 °C. The detector temperature was maintained at 285 °C. The carrier gas used was nitrogen and its pressure was maintained at 90 KPa. The peaks were identified by comparing their retention times with those of the standards. The relative percentages of fatty acids were determined by the peak areas (Gandemer, 2002; Gandemer, Moran-Mahé, Meynier, & Lepercq, 1991).
2.5. Statistical analysis

Statistical analysis of the differences between each group was evaluated by one-way analysis of variance (ANOVA) using the SPSS 18.0. The correlation coefficient was estimated with the Pearson correlation coefficient option of SPSS 18.0 (Argyrous, 2011). Differences were regarded as significant at $P < 0.05$. All data were expressed as mean ± standard error.

3. Results and discussion

3.1. Changes and discussion of lipid composition

Table 1 shows the contents of the different lipid composition (neural lipid, free fatty acids and phospholipids) expressed as percentages of the total lipids. Phospholipids accounted for 49.25% of the total lipids in raw duck muscle followed by neutral lipid and free fatty acids. The percentages of free fatty acids showed a great increase through the processing of traditional Chinese dry-cured duck which is also observed in the other dry-cured meat products (Hernandez, Navarro, & Toldra, 1999; Navarro, Nadal, Izquierdo, & Flores, 1997; Qiu, Zhao, Sun, Zhou, & Cui, 2013). The percentage of neutral lipid was found to increase significantly at the piling and drying stages ($P < 0.05$), in contrast, a significant decrease in the percentage of phospholipids ($P < 0.05$) was detected. During the whole process, the percentage of phospholipids and free fatty acids was negatively correlated ($R = -0.787$, $P < 0.01$), while the neutral lipid and free fatty acids positively correlated ($P < 0.01$), suggesting that the hydrolysis of phospholipids mainly occurred during this process. Both neutral lipids and phospholipids could contribute to the generation of FFA catalysed by lipases and phospholipases respectively; however in most cases, phospholipids are the main substrates for lipolysis in dry-cured meat products (Buscailhon, Gandelmer, & Monin, 1994). As the muscle enzyme systems play an important role in the generation of free fatty acids (Motilva et al., 1992), the increase in the amount of free fatty acids could be the result of the action of lipolytic enzymes.

3.2. Changes of relative activities of phospholipase A2 and C

Figs. 1 and 2 show the changes of phospholipase A2 and C activities during traditional Chinese dry-cured duck processing. The phospholipase A2 activities significantly decreased over the whole process ($P < 0.05$). The phospholipase C activity increased significantly ($P < 0.05$) at the dry-salting stage initially, and decreased between dry salting and drying, then it markedly increased ($P < 0.05$) during air-drying from 6 to 12 days. The phospholipase activities in the final products were found to be higher than those observed by Zhou and Zhao (2007) and Jin et al. (2010) who reported the total phospholipases retained 7.56% and 9.1% of the initial activity in the end products respectively. The reduced activity of lipolytic enzymes as drying progresses might be due to the decreased water activity. Variables such as drying temperature, salt content and water activity could influence the enzyme activities in dry-cured meat products (Toldra, 2006), but up to now, compared to total phospholipases activities, very little is known about the activities of phospholipase A2 and C in skeletal muscles and the regulation of their activities during dry-cured meat processing.

3.3. Changes in fatty acid profiles of different lipid fraction

Polyunsaturated fatty acids (PUFA) of FFA and neutral lipids (Tables 2 and 3) increased significantly during the entire process ($P < 0.05$), and it is strongly correlated with decreased PUFA of phospholipids fraction (Table 4, $R = -0.841$, $P < 0.05$, $R = -0.946$, $P < 0.01$, respectively). Concerning each single fatty acid, the linoleic (C18:2) and arachidonic acid (C20:4) of the phospholipid decreased markedly ($P < 0.05$), whereas they increased significantly in free fatty acids ($P < 0.05$), which is likely due to the lipolysis of phospholipids. It has been indicated that the enzymes have a selective activity according to carbon chain length and degree of unsaturation of fatty acids. Our result is in agreement with the study of lipids changes in dry-cured meat (Lorenzo, Bermudez, & Franco, 2013; Qiu et al., 2013; Yang, Ma, & Qiao, 2005). But some other studies indicated that lipolysis in phospholipid was not specific to the fatty acid chain length or unsaturation during the processing of dry cured hams (Buscailhon et al., 1994) and pork loins (Hernandez et al., 1999). The difference between the present study and mentioned litera-

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Table 1

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Changes in lipid composition during traditional Chinese dry-cured duck processing (g/100 g lipids).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raw</td>
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<tr>
<td></td>
<td>Neutral lipid</td>
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<td></td>
<td>FFA</td>
</tr>
<tr>
<td></td>
<td>Phospholipids</td>
</tr>
</tbody>
</table>

* Means in the same row with different letters differ significantly ($P < 0.05$).
changes in fatty acid profiles of neutral lipid during traditional Chinese dry-cured duck processing (%)

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Raw</th>
<th>Dry-salted</th>
<th>Marinated</th>
<th>Piled</th>
<th>Dried for 6 days</th>
<th>Dried for 12 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUFA/SFA</td>
<td>0.42</td>
<td>0.42</td>
<td>0.42</td>
<td>0.46</td>
<td>0.50</td>
<td>0.51</td>
</tr>
<tr>
<td>SFA</td>
<td>0.57</td>
<td>0.76</td>
<td>0.77</td>
<td>0.77</td>
<td>0.75</td>
<td>0.75</td>
</tr>
</tbody>
</table>

a Means in the same row with different letters differ significantly (P < 0.05).

### Table 3
Changes in free fatty acid composition during traditional Chinese dry-cured duck processing (%).

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Raw</th>
<th>Dry-salted</th>
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<td>0.75</td>
<td>0.75</td>
</tr>
</tbody>
</table>

a Means in the same row with different letters differ significantly (P < 0.05).

### Table 4
Changes in fatty acid profiles of phospholipids during traditional Chinese dry-cured duck processing [%].

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Raw</th>
<th>Dry-salted</th>
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</tr>
</tbody>
</table>

a Means in the same row with different letters differ significantly (P < 0.05).

### Table 5
Changes in fatty acid composition during traditional Chinese dry-cured duck processing (%).

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Raw</th>
<th>Dry-salted</th>
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<th>Piled</th>
<th>Dried for 6 days</th>
<th>Dried for 12 days</th>
</tr>
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### 3.4. The relationship among fatty acid profiles, PLA2 and PLC activities and intramuscular phospholipid degradation

The relative activities of PLA2, highly correlated with the decline of phospholipids (R = −0.996, P < 0.01), and the increase of free fatty acids (R = 0.967, P < 0.01). Significant correlations were also

ture is probably due to the different origins of experimental materials and also to the different processing conditions. Linoleic acid (C18:2) is among the moderately mobilized fatty acids and arachidonic acid (C20:4) is classified as a highly mobilized fatty acid, which is proposed by Raclot when he studied lipolysis in Adipose tissue (Raclot, 2003).
obtained between the relative activities of PLC and the decrease of phospholipids ($R = -0.9558$, $P < 0.05$), the increase of neutral lipid ($R = 0.990$, $P < 0.01$). The slowing-down decrease in phospholipids and increase in FFA corresponded to the decreased activity of PLA2. The increasing rate of neutral lipids content became slower in the early processing stages and faster between 6 and 12 days of drying, which might result from the changes in PLC activity. Correlation analysis indicated that PLA2 and PLC may contribute to the degradation of intramuscular phospholipids during the processing of traditional Chinese dry-cured duck.

PLA2 comprises a set of extracellular or intracellular enzymes and catalyze the selective hydrolysis of the sn-2 acyl ester bond in 1,2-diacyl-sn-glycero-3 phospholipids, resulting in the formation of free fatty acids such as arachidonic acid (C20:4) and lyso-phospholipids (Burke & Dennis, 2009). There is in fact a cytosolic PLA2 that preferentially hydrolyses phospholipids with arachidonic acid in the sn-2 position (Balsinde, Winstead, & Dennis, 2002). In our study, a distinct decline ($P < 0.05$) of the percentages of arachidonic acid in phospholipids was observed, concomitant with a marked increase ($P < 0.05$) in free fatty acids. This further confirmed that phospholipids were hydrolysed by PLA2 during the traditional Chinese dry-cured duck processing.

PLC cleaves the phosphodiester bond of membrane phospholipids, resulting in the formation of diacylglycerol (Bunney & Katan, 2011; Rebecchi & Pentalya, 2000). Since PUFAs are high in phospholipids, the diacylglycerol which is derived from the lipolysis of phospholipids would also have a high content of PUFAs. We found an obvious increase ($P < 0.05$) of the percentages of PUFAs in neutral lipid, which was similar to the study by Qiu et al. (2013). In combination with the results of Gandermer, (2002), who reported that the percentages of diacylglycerol increased during the processing of Serrano dry cured ham, the increased diacylglycerol content in dry-cured duck could attribute to PLC on the lipolysis of intramuscular phospholipids.

In summary, lipolysis of phospholipids was observed during the processing of dry-cured duck. PLA2 and PLC activities declined with the processing time, but they retained 83.70% and 86.78% of their initial activities respectively at the end of the drying process. PUFAs of FFA and neutral lipid gradually increased during the whole process whereas PUFAs of phospholipids decreased, suggesting the production of FFA and diacylglycerol catalyzed by PLA2 and PLC. The significant correlation between phospholipase activities and phospholipids degradation and increase of FFA further confirmed that PLA2 and PLC play important roles in intramuscular phospholipids lipolysis.

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