Exploring the potential effect of phospholipase A2 antibody to extend beef shelf-life in a beef liposome model system

by

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Abstract

Phospholipase-A_2 (PLA2) is a ubiquitous enzyme that cleaves a fatty acid tail at the sn-2 position from a phospholipid (PL) in cell membranes. The resulting free fatty acids (FFA) are typically polyunsaturated fatty acids (PUFA) which are prone to lipid oxidation when exposed to pro-oxidants such as light and oxygen. Therefore, the objective of this study was to elucidate the interaction of PLA2 and a PLA2 antibody (aPLA2) on PL hydrolysis utilizing a beef liposome model system and to understand how the altered PL composition in the presence of myoglobin may affect lipid oxidation and antioxidant capacity in a retail display setting. The PL was extracted from 10 United States Department of Agriculture choice beef loins at 3d post-mortem, and PL from each steak was further split into six different treatments: 1) PL (10 mg/ml of PL); 2) aPLA10 (PL+10 µg/ml of aPLA2); 3) aPLA20 (PL+20 µg/ml of aPLA2); 4) PLA2 (PL+4 µg/ml of PLA2); 5) PLA2+aPLA10 (PL+PLA2+10 µg/ml of aPLA2); 6) PLA2+aPLA20 (PL+PLA2+20 µg/ml of aPLA2). After execution of the liposome system, an aliquot was immediately taken for PL profile analysis, FFA analysis and product ion analysis by mass spectrometry. Eighty µM of bovine myoglobin was added to the remaining samples and exposed to retail display conditions (2±2°C; 2300 lx) for 7d. At 0, 1, 4, and 7d, aliquots were taken for hydrophilic and lipophilic oxygen radical absorbance capacity (ORAC) and lipid oxidation analysis (TBARS). As expected, the PL composition was significantly altered by the PLA2 treatments, and the generation of FFAs was evident. The PLA2 treatments had significantly less relative % of total phosphatidylcholine (PC), ether-linked PC (ePC), and phosphatidylethanolamine (PE) than treatments without PLA2 ($P < 0.05$). Individual PL species, notably PC 36:2, 36:3, 36.4, ePC 32:4, 36:3, 36:4, PE 36:2 and 38:4 also showed greater relative % in PLA2 treatments as compared to treatments with PLA2. Product Ion analysis revealed that

the major FAs involved in these altered PL were 16:0, 18:0, 18:1, 18:2 and 20:4. The FFA profile showed that treatments containing PLA2, regardless of the addition of aPLA2, had greater amounts of 16:1, 18:1, 18:2, 20:4, and 20:5 ($P < 0.01$), but no treatment difference were found for any of the saturated FFA such as 18:0 and 16:0 ($P > 0.05$). The PLA2 treatments also showed greater relative % of total lysophosphatidylcholine (LPC) and LPC 16:0, 16:1, 18:0, and 18:1 than treatments without PLA2 ($P < 0.01$). There was no apparent inhibition effect from aPLA2 as there was no difference between PLA2 and aPLA+PLA2 treatments in formation of FFA and in the relative % of total PL classes ($P > 0.05$). In addition, the altered PL composition also influenced ORAC and TBARS values. There were treatment x retail display effects for hydrophilic and lipophilic ORAC ($P < 0.01$), as well as in TBARS ($P < 0.01$). For hydrophilic ORAC, samples with PLA2 had higher antioxidant capacity than samples without PLA2 ($P < 0.01$). For lipophilic ORAC, samples with PLA2 also showed higher antioxidant capacity than treatments with no PLA2 at 0 d ($P < 0.01$), but the enhanced antioxidant capacity from the PLA2 samples faded after just 1d of retail display. For TBARS, PLA2 treatments had higher lipid oxidation than treatments without PLA2 added throughout the entire retail display period ($P < 0.01$). Interestingly, not only did 7d aPLA10 and aPLA20 have less lipid oxidation than PL only and all PLA2 treatments, but 7d aPLA10 and aPLA20 also had less oxidation compared to those from 4 d PLA2 ($P < 0.01$). This study confirmed that the hydrolysis of PL can generate extensive amount of FFAs and potentially influence lipid oxidation in meat during the retail display period. Although no inhibition effect was observed for PLA2 by the addition of aPLA2, aPLA2 by itself seemed to influence lipid oxidation with PLA2 exhibited antioxidant capacity. Further research is needed to better understand the mechanisms of aPLA2 to PLA2 interaction to elucidate the potential benefits of aPLA2 in a meat system.

Table of Contents

List of Abbreviations

AAPH	2,2’-Azobis(2-amidinopropane) dihydrochloride
aPLA2	Phospholipase A2 Antibody
CDP	Cytidine diphosphate
CMP	Cytidine monophosphate
cPLA2	Cytosolic Phospholipase A2
DAG	Diacylglycerols
ELISA	Enzyme-linked immunosorbent assay
ePC	Ether-linked phosphatidylcholine
ePE	Ether-linked phosphatidylethanolamine
ESI	Electrospray-ionization
FA	Fatty acids
FFA	Free fatty acids
GSH-Px	Glutathione peroxidase
HDL	High-density lipoprotein
i-face	Interfacial binding zone
iPLA2	Calcium-independent phospholipase A2
LDL	Low-density lipoprotein
LPC	Lyso-phosphatidylcholine
LPE	Lyso-phosphatidylethanolamine
LPG	Lyso-phosphatidylglycerol
LPLA2	Lysosomal phospholipase A2
LUV	Large unilamellar vesicles
LysoPL	Lyso-phospholipid
MAP	Modified atmosphere packaging
MLV	Multilamellar vesicles
MUFA	Mono-unsaturated fatty acid
MUFFA	Mono-unsaturated free fatty acid
NL	Neutral lipids
ORAC	Oxygen radical absorbance capacity

PAF-PLA2	Platelet activating factor phospholipase A2
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PL	Phospholipid
PLA1	Phospholipase A1
PLA2	Phospholipase A2
PLA2R	Phospholipase A2 receptor
PS	Phosphatidylserine
PSE	Pale, soft, exudative
PUFA	Polyunsaturated fatty acids
PUFFA	Polyunsaturated free fatty acids
QC	Quality control
RMCD	Randomly methylated, -cyclodextrin
ROS	Reactive oxygen species
SFA	Saturated fatty acids
SFFA	Saturated free fatty acids
sPLA2	Secreted-phospholipase A2
TAG	Triglycerides
TBARS	2-thiobarbituric acid reactive substances
TE	Trolox equivalent
TL	Total lipid
USDA	United states department of agriculture
WCM	Washed cod muscle
WHC	Water holding capacity

List of Figures

List of Tables

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Dedication

I dedicate this to my grandparents. Grandma, Grandpa, Popo and GoongGoong. I am who I am today because of all of you. And I couldn’t be happier about it.

Chapter 1 - Review of Literature

Introduction

The composition of meat consists of three major macronutrients: water, protein, and fat. Meat constitutes approximately 70-75% water and 20-22% protein, while fat, has the most variability of the major macronutrients (Wood, 2017). Lipids are generally split into two groups, polar and neutral, which each have different functions within the biological system (Willian, 2013). No matter which lipid group, the basic unit of lipids are fatty acids, which are variable in length and the degree of saturation on their carbon chains (Lichtenstein, 2013). The composition of fatty acids can influence the sensory and shelf-life of meat products through lipid oxidation in which oxidation products can negatively affect meat odor, taste, and color and ultimately influences consumers' purchasing decision (Barden & Decker, 2016; Dinh et al., 2021; Domínguez et al., 2019).

Lipid oxidation is the process of lipids reacting with reactive oxygen species and undergoes a free radical chain reaction forming primary and secondary products (B. Min & D. Ahn, 2005). Lipid oxidation in meat occurs when it is exposed to various free radical generating events such as oxygen, heat, and light (Domínguez et al., 2019). Therefore, processes such as cutting, grinding, displaying, and cooking can all lead to accelerated oxidation of the meat product (Amaral et al., 2018). In reality, the process of lipid oxidation starts as soon as the animal is harvested and continues until the product is finally consumed (Domínguez et al., 2019).

There are different methods that can prevent lipid oxidation such as the application of antioxidants in the form of enzymes, peptides, and vitamins (Domínguez et al., 2019). In this literature review, we will look at meat lipids, lipid oxidation, and various methods that may

mitigate lipid oxidation to improve on shelf-life and quality characteristics of fresh meat products to avoid unnecessary food wastage.

Lipid Components in meat

Fatty Acids

Fatty acids (FA) are the basic lipid components that vary in length and saturation, resulting in differences in functionality. Fatty acids are hydrocarbon chains that are either saturated (SFA), containing no double bonds, or unsaturated, containing one or more double bonds (Willian, 2013). Unsaturated fatty acids can contain a single double bond, which is known as monounsaturated fatty acid (MUFA), or multiple double bonds, which is also known as polyunsaturated fatty acid (PUFA) (Willian, 2013). Fatty acids can also vary in length from 2-8 carbon atoms all the way to 20 or more carbon atoms within their chain (Willian, 2013). Meat shelf-life is strongly influenced by fatty acid structure. In general, unsaturated fatty acids are more prone to lipid oxidation than SFA, and the rate of oxidation is positively correlated with number of double bonds (Amaral et al., 2018). The composition of fatty acids varies among livestock species. Monogastric species such as pigs have higher levels of PUFA, while ruminants such as cattle and sheep contain more SFA mostly due to differing digestive systems (Wood et al., 2008). Many studies have shown that the FA profile of the meat from monogastric species can easily be altered by modification of their diets (Kouba et al., 2003; Stephenson et al., 2016; Wood et al., 2004). This is due to PUFA consumed by monogastric species are not subjected to biohydrogenation, while PUFA that are fed to the ruminants are typically biohydrogenized to SFA and MUFA in the rumen by rumen microbials (Maia et al., 2010). Despite that, when the diet is high in PUFA, a small fraction of PUFA may escape biohydrogenation without further

modifications and enter the small intestine to be absorbed and increase the overall PUFA content in beef lipid composition (Maia et al., 2010). Burnett et al. (2020) showed feeding fish oils and flaxseed feed additives, a diet high in Omega-3 FA, to beef cattle increased the amounts of eicosapentaenoic acid (20:5n-3), docosahexaenoic acid (22:6n-3), and alpha-linolenic acid (18:3n-3) in various beef cuts. Besides the overall FA composition of species, FA acid composition is also different among lipid classes, giving them different functions within the cellular system (Calder, 2015).

Neutral Lipids

Neutral lipids (NL) consist of triacylglycerols (TAG), diacylglycerols (DAG) and cholesterol. These lipids are called neutral because they are non-polar, meaning the charge is evenly distributed throughout the structure (Akoh, 2017; Wilde, 2014). Their basic functions and roles in meat will be briefly discussed.

Triacylglycerols (TAG) and Diacylglycerols (DAG)

A TAG has three fatty acids esterified to a glycerol backbone and acts as the main storage unit of fatty acids. Fatty acids attach at three distinct positions of the glycerol: sn-1, sn-2, and sn-3 (Lichtenstein, 2013). Within a living animal, there are various deposits of triglycerides, such as intermuscular, intramuscular, visceral, and subcutaneous. As TAGs make up most of the fat within an animal, it is used as insulation and more importantly as an energy reservoir (Lichtenstein, 2013). In beef TAGs, SFA (16:0 and 18:0) and MUFA (18:1n-9) account for approximately 80% of total TAG fatty acids while 2% of the portion are dominantly 18:2n-6 and 18:3n-3 PUFA (Scollan et al., 2006). As mentioned above, monogastric animal fat composition

is widely influenced by the diet, with neutral lipids being influenced more so than their polar lipid counterpart (Raes et al., 2004). On average, monogastric triglyceride composition consists of 7-15% PUFA with the FA composition dependent on diet (Raes et al., 2004).

As the name implies, DAGs contain only two fatty acids attached to the glycerol backbone. In living organisms, DAGs are essential for their signaling properties such as immune cell activation, regulation, and function (Singh & Kambayashi, 2016). Diacylglycerols can be generated through de novo synthesis or degradation of TAGs, as DAGs are intermediate steps to both processes (Eichmann & Lass, 2015). DAGs are also building blocks for phospholipids (Eichmann & Lass, 2015), which will be briefly discussed in the glycerophospholipid section.

Cholesterol

Cholesterol is another major lipid in meat and typically, red meat contains around 50 – 100 mg of cholesterol per 100 g of wet muscle tissue (Rhee et al., 1982; Swize et al., 1992; Willian, 2013). Cholesterol in living animals is an essential components of cell membranes and neural signal transmission, and is a precursor for steroid hormones (Lichtenstein, 2013). Cholesterol is found in two forms, nonesterified (free cholesterol) or esterified, with the latter containing a fatty acid attached to the sterol ring structure (Lichtenstein, 2013). Cholesterol is a major component of lipoproteins and are commonly associated with them (Khan, 2006). The two major types of lipoproteins are low-density lipoproteins (LDL) and high-density lipoproteins (HDL), which are typically known as “bad” and “good” cholesterol, respectively. The “bad” LDL carry the majority of blood cholesterol which can block blood vessels in the form of plaque and is susceptible to oxidation (Khan, 2006). The “good” HDL will remove cholesterol from the bloodstream and transport it into the liver for disposal or reuse (Marcus, 2013).

Polar Lipids

Polar lipids are considered amphiphilic compounds as the fatty acid tails are hydrophobic, whereas the head is hydrophilic giving the molecule polarity (Li et al., 2019). The major groups of polar lipids are glycerophospholipids, lysoglycerophospholipid, and sphingolipids.

Glycerophospholipids

Glycerophospholipids (PL) are the main components of cellular membranes (Lichtenstein, 2013). Glycerophospholipids also act as metabolic intermediates and messengers depending on head group and fatty acid composition (El-Bacha & Torres, 2016; Zhou & Rakariyatham, 2019). Glycerophospholipids differ from TAGs as they only have two fatty acids at the sn-1 and sn-2 position whereas the sn-3 position of the glycerol is attached to a polar head group (Lichtenstein, 2013) which also determines there PL class. For example, when choline is attached to the glycerol head at the sn-3 position, the PL class is named phosphatidylcholine (PC). Common PL classes found are PC, phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI). These common PLs have ester bonds which separate them from another class of PLs known as ether-linked. Ether-linked phospholipids have an alkyl chain attached to the sn-1 position by an ether bond, rather than conventional phospholipids having chains attached via ester bonds at both the sn-1 and -2 positions (Dean & Lodhi, 2018). This change in bond allows the sn-1 and sn-2 chains of the ether-linked PL to become parallel and allow for tighter spacing, causing for rigidity of cellular membranes which is important for the membranes in the myelin sheath (Wallner & Schmitz, 2011). Ether-linked PL have also been looked at as a potential antioxidant. It is thought that the ether-bond could be used as a free

radical scavenger to prevent the oxidation of PUFA (Broniec et al., 2011; Engelmann, 2004; Wallner & Schmitz, 2011).

The two main pathways PL are synthesized utilizing DAG are the cytidine diphosphate (CDP)-DAG and the Kennedy pathway (Gibellini & Smith, 2010; Han & Carman, 2013). The cytidine monophosphate (CMP) moiety of the CDP-DAG is replaced by a serine to generate PS which can then be decarboxylated to PE and furthermore methylated to PC (Han & Carman, 2013). A PI can also be synthesized through this pathway when the CMP moiety is displaced by inositol instead of serine (Han & Carman, 2013). In the Kennedy pathway, DAG is utilized as the lipid anchor along with CDP-choline or CDP-ethanolamine to form PC and PE respectively, with CMP being a byproduct (Gibellini & Smith, 2010).

Sphingolipid

Sphingolipids are another type of polar lipid, and sphingolipids have a sphingosine as the alcohol group whereas the glycerol is the alcohol for glycerophospholipids (El-Bacha & Torres, 2016). Sphingolipids contain a ceramide backbone which typically contains a saturated fatty acid, and the major phosphosphingolipid is sphingomyelin (Litwack, 2018). Sphingomyelin is an important brain sphingolipid (Litwack, 2018) due to their structural role in the myelin sheath (Jana & Pahan, 2010). Sphingomyelin has an important role in HDL and reverse cholesterol transport (Martínez-Beamonte et al., 2013).

Lysoglycerophospholipid

The class of lysoglycerophospholipids (LysoPL) are glycerophospholipids with one fatty acid removed, being left with one single FA chain (either in sn-1 or sn-2 position) and are

generated via lipid oxidation (Balsinde et al., 1999) or enzymatic hydrolyzation (Hao et al., 2020). The LysoPLs are typically generated corresponding to the PL oxidized or hydrolyzed (Li et al., 2016). For example, in the inflammatory response, arachidonic acid will be cleaved from PC via phospholipase A2 at the sn-2 position, to generate lysophosphatidylcholine (LPC), and the arachidonic acid will be further converted to pro-inflammatory mediators (Li et al., 2016). In the case of meat, the cleaved FA may be subjected to lipid oxidation and reduce meat shelf-life. PL hydrolysis may be more prone to lipid oxidation as the cleaved FA are typically PUFA, which their high potential for lipid oxidation will be discussed in subsequent sections.

Lipid Oxidation

Lipid oxidation is the process of reactive oxygen species (ROS) and other free radicals sequestering an electron from the lipid, resulting in oxidative degradation of lipid (B. Min & D. Ahn, 2005). Lipid oxidation influences important facets of meat quality such as flavor and shelf-life. The two main mechanisms of lipid oxidation that will be covered is autoxidation and photo-oxidation. By understanding the mechanisms behind lipid oxidation, we may find ways to decrease the rate of lipid oxidation in meat.

Mechanisms of lipid oxidation

The three main steps of lipid oxidation are: initiation, propagation, and termination. The difference between autoxidation and photo-oxidation lies within the initiation step whereas the subsequent steps are similar. In autooxidation, initiation begins when a hydrogen atom from a methylene group of a FA is taken by ROS (B. Min & D. Ahn, 2005). This attack will stabilize the free radical, but will further result in an unpaired electron, known as a lipid or alkyl radical

(Domínguez et al., 2019). In addition, the lipid radical will attempt to stabilize itself by forming conjugated dienes along its carbon chain (B. Min & D. Ahn, 2005). Photo-oxidation has a different initiation mechanism compared to autooxidation. Photo-oxidation involves the interaction of photosensitizers, light, and meat. Photosensitizers in meat come in the form of myoglobin, hemoglobin, and riboflavin (Frankel, 2012). Photosensitizers will absorb visible light or near-UV light to move from the singlet excited state, to a triplet excited state which allows them to interact with different substrates (Frankel, 2012). Photosensitizers in the triplet state can initiate lipid oxidation in two main pathways which can occur simultaneously (Tsubone et al., 2021). In the first pathway, the triplet photosensitizers can directly react with the hydrogen atom or through an electron transfer on lipid radicals, similar to that in autoxidation (Frankel, 2012). In the second pathway, triplet photosensitizers transfer energy to molecular oxygen bringing it to a highly reactive oxygen species which can then initiate lipid oxidation to generate lipid radicals (Frankel, 2012; Tsubone et al., 2021). Autooxidation is promoted by light, heat, oxygen, and metals, such as iron and copper while photo-oxidation is mainly promoted by light and oxygen (Domínguez et al., 2019).

Next, the propagation stage is referred to as a “chain reaction”. The resulting lipid radicals will react with atmospheric oxygen to generate peroxy radicals (B. Min & D. Ahn, 2005). These peroxy radicals are highly reactive and will sequester hydrogen atoms from another FA. The result of this step generates a hydroperoxide and another lipid radical that can react with oxygen to form more peroxy radicals resulting in a chain reaction (B. Min & D. Ahn, 2005). Hydroperoxides themselves are odorless and flavorless molecules, but can be degraded into numerous secondary lipid oxidation products such as ketones, aldehydes, alcohols, hydrocarbons, and furans which are important in the flavor and odor of meat products (Frankel,

1987). The final stage of lipid oxidation is termination. In termination, radicals will react with either radicals or non-radicals such as antioxidants, to produce non-radical products (Barden & Decker, 2016). Antioxidants have been widely used to prevent and reduce lipid oxidation, and most of them work by having phenolic structures which can sequester free radicals of their unpaired electron, stabilizing them and prevent from attacking lipid substrate for further oxidation (Amaral et al., 2018; Domínguez et al., 2019; Zeb, 2020).

In initiation, there is a lag phase as lipid radicals will be neutralized by the antioxidants within the system and not yet contribute to the rancidity of products (Barden & Decker, 2016). Until this antioxidant pool succumbs, only then will the main substrate of lipid oxidation and secondary products form (Barden & Decker, 2016). It is interesting to note that the initiation step started with ROS whereas the step of propagation begins with lipid-to-lipid interactions. This brings up the importance of endogenous and extrinsic factors of lipid oxidation in meat products.

Factors affecting lipid oxidation

Fatty acid composition and lipid oxidation

Fatty acid profiles affect the oxidation within meat products. In general, unsaturated fatty acids are more prone to lipid oxidation than SFA, and the rate of oxidation will significantly increase with the increasing number of double bonds (Amaral et al., 2018). This is because carbon-hydrogen bonds of the methylene in between two double bonds of the carbon chain have the weakest bond strength leaving it susceptible to ROS attack as opposed to fully saturated carbons near no double bonds (B. Min & D. Ahn, 2005). Therefore, meat products with more PUFA have shown to be more prone to lipid oxidation than SFA (Arab-Tehrany et al., 2012; Kouba et al., 2003; Wood et al., 2008; Wood et al., 2004). Ahn et al. (1996) fed pork four levels

of 18:3n3 (0, 1.5, 2.5 3.5%) using flaxseeds to increase the proportion of PUFA ratio. Specifically, 18:3n3, 20:3n3, 20:5n3 and 22:5n3 PUFAs were increased (Ahn et al., 1996) within pork loins, and they found that the elevated levels of PUFA increased lipid oxidation products as compared the control. In beef, Conte et al. (2019) fed a concentrate diet containing 20% of extruded linseeds and saw an increase in PUFA content within the longissimus thoracis, specifically 18:3n3. As expected, the linseed diet showed higher levels of lipid oxidation compared to the control diet after a two day storage period, and the gap further widens after six days of storage (Conte et al., 2019). Therefore, producers should closely monitor animals' diets to avoid excessive alteration of FA composition, or mitigate the effect of unsaturated FA with the addition of antioxidants in animals' diet (Huang & Ahn, 2019; Wood et al., 2008).

Retail display on lipid oxidation

Photo-oxidation is influenced by light and oxygen which is concerning as meat is commonly exposed to both factors while under retail display. It is well documented that as meat sits in retail display, lipid oxidation increases (Cooper et al., 2017; Domínguez et al., 2019; Martin et al., 2013). When looking at light exposure, differing light sources do not seem to influence lipid oxidation differently (Cooper et al., 2017). Using three different light sources (LED, low-UV fluorescent, and high-UV fluorescent) and oxygen permeable overwrap, Cooper et al. (2017) displayed beef triceps brachii steaks at 2℃ for 1, 3, 5, and 7 days. Just after three days of retail display, lipid oxidation products exceeded the threshold in which consumers find acceptable (Cooper et al., 2017). The two main packing conditions commonly utilized are modified atmosphere packaging (MAP), which typically involve high oxygen content, and vacuum packaging, which aims to remove as much oxygen as possible. When comparing the two

packaging types under retail display conditions (2±°C, 7.0 lux fluorescent light) for 9 days, Kim et al. (2010) found that high oxygen packaging, significantly increased in lipid oxidation values while vacuum packaging showed no change in lipid oxidation values. Therefore, packaging techniques that minimize oxygen exposure such as vacuum packaging or the addition of oxygen scavenging tools can minimize lipid oxidation in meat (Fang et al., 2017; Huang & Ahn, 2019; Wood et al., 2008).

Antioxidants and lipid oxidation

It is known that antioxidants can effectively prevent oxidation. Common antioxidants are vitamin E, vitamin C, and carotenoids (Li & Liu, 2012). Of the three, the most common antioxidant used to reduce lipid oxidation is vitamin E, which is also referred to as α-tocopherol (Li & Liu, 2012). Vitamin E is commonly found within green pastures and forage-based feeds (Li & Liu, 2012), and the incorporation of vitamin E-rich diet has shown to carry over well into the muscle. The final meat products show a decrease in lipid oxidation and increase in lipid stability as demonstrated in many studies (Bellés et al., 2019; Gatellier et al., 2001; Mercier et al., 1998; Wood et al., 2004). Finally, O'Grady et al. (2000) mixed a solution of vitamin E and olive oil with ground beef packaged in MAP with oxygen, and it was found that ground beef mixed with vitamin E had lower lipid oxidation at 10 days of cold storage as compared to the control ground beef.

Vitamin E from feed is typically deposited into the cellular membranes (Domínguez et al., 2019). Vitamin E works as a chain breaker of lipid oxidation due to its ability to react and stabilize peroxyl radicals much faster than lipid radicals can attack lipid substrates (Schneider,

2005). Due to this ability, vitamin E may also play a role in maintaining the structural integrity of phospholipid membranes (Descalzo & Sancho, 2008).

Phospholipase A2

Phospholipase is a ubiquitous group of enzymes that hydrolyze PL and each phospholipase family will catalyze reactions at different locations (Wilton, 2008). For example, phospholipase D will remove the base group of a PL while phospholipase A2 (PLA2) will cleave off a fatty acid tail at the sn-2 position of a PL (Wilton, 2008). This makes PLA2 an enzyme of interest regarding lipid oxidation due to the formation of FFA in PL hydrolysis. The basic mechanisms of how PLA2 works, the effect PLA2 has on meat quality, and the potential to inhibit PLA2 will be discussed.

Phospholipase A2 classifications and mechanism

While all PLA2 are characterized by their ability to cleave FA at the sn-2 position, there are roughly 5 different classes of mammalian PLA2: secreted (sPLA2), cytosolic (cPLA2), calcium-independent (iPLA2), lysosomal (LPLA2), and platelet activating factor (PAF-PLA2) (Murakami, 2017; Murakami et al., 2011). Each class also has subspecies which will not be covered here as it is beyond the scope of this review. The two most common and documented classes are the sPLA2 and cPLA2 (Dennis et al., 2011). The sPLA2 are typically small (12-15 kDa), calcium dependent and are named ‘secreted’ as it is mostly a secreted enzyme from the pancreas (Dennis, 1994). The family of sPLA2 strictly hydrolyze the sn-2 position, but are not specific to which FA they remove (Murakami, 2017). The sPLA2 typically prefer phosphatidylglycerol (PG), PE and PS, but some subspecies of sPLA2 prefer to hydrolyze PC

(Stremmel et al., 2016; Veldhuizen et al., 1998). The cPLA2 are found within the cytosol of cells and are much larger (61-114 kDa) (Ghosh et al., 2006). Although found in the cytosol, cPLA2 will translocate to the cellular membrane upon calcium activation (Ghosh et al., 2006; Murakami, 2017). The cPLA2 enzyme, in accordance with normal PLA2 activities, can catalyze multiple reactions such as cleavage at the sn-1 position similar to that of phospholipase A1 (PLA1) (Ghosh et al., 2006). The cPLA2 shows a preference for cleaving PC and the FA 20:4 (Ghosh et al., 2006). Its preference for FA 20:4 makes it a key component in pathological processes such as inflammation, which will be further discussed in subsequent sections (Ghosh et al., 2006). The iPLA2 family consists of larger enzymes (84-90 kDa) that do not require calcium for activation and are localized in the cytosol, but can translocate to the golgi, endoplasmic reticulum, and mitochondria (Balsinde & Balboa, 2005; Balsinde et al., 2006). This class exhibits no specificity to FA at the sn-2 position nor head group at the sn-3 position, but has also shown the ability to catalyze multiple reactions similar to that of cPLA2 (Balsinde et al., 2006). Due to their multi-functionality and location, iPLA2 are a key component of phospholipid homeostasis (Balsinde & Balboa, 2005) and membrane remodeling (Balsinde & Balboa, 2005; Ramanadham et al., 2015). LPLA2 are around 45 kDa and found in the lysosome of cells (Dennis et al., 2011). The LPLA2 class is not calcium dependent, but in the presence of calcium, activity is enhanced (Dennis et al., 2011). LPLA2 does not show specificity for FA, but has a preference for the PE and PC head groups (Shayman & Tesmer, 2019). Found in the lysosome, LPLA2 functions to localize macrophages for immune response through PL catabolism (Shayman et al., 2011; Shayman & Tesmer, 2019). Finally, PAF-PLA2 catalyzes the removal of the acetyl group from the sn-2 position of a PAF (Dennis et al., 2011). A PAF are like PL and contain choline as their head group (Ashraf & Nookala, 2021). These PAF-PLA2s are specific to PAFs and are not

calcium dependent (Stafforini, 2009). PAFs are one of the mediators in the inflammation pathway which will be discussed in more detail below (Dennis et al., 2011).

Although the specific functions of PLA2 vary with class, PLA2 in general are responsible for 2 major functions: aiding in digestibility and participating in inflammation (Wilton, 2008). One of the earliest roles PLA2 were found to have was as a digestive enzyme (Dennis et al., 2011). Dietary PLA2 are secreted from the pancreas into the digestive tract, where they will hydrolyze dietary PL and the PL in bile, allowing for absorption of FFA and lysoPL into the body (Murakami et al., 2011).

The second major function is in the inflammatory response where PLA2s' preference to remove proinflammatory FA such as 20:4 and 18:2 from the sn-2 position is crucial (Dennis et al., 2011). These two fatty acids are important in the inflammatory pathway as they are converted to inflammatory mediators such as prostaglandin, leukotrienes, thromboxanes, which are known as eicosanoids (Norman & Henry, 2015). For example, when a tissue is damaged and foreign microbials are noticed, the inflammatory response will initiate (Nathan & Ding, 2010). In the onset of inflammation, PLA2 will be signaled to release 20:4 or 18:2 that will be converted to eicosanoids which improve immune response to the damaged tissue through vasodilation of vessels (Agarwal et al., 2009). The other product of PL hydrolysis, lysoPL also plays a role in the inflammatory response (Dennis et al., 2011). The LysoPL is a diverse messenger in inflammation, which can signal for wound healing through macrophages and growth factors (Gräler & Goetzl, 2002).

Phospholipase A2 and meat quality

Cell membrane disruption through the hydrolysis of PL can cause detrimental meat quality issues. PLA2 has been associated with the development of pale, soft exudative (PSE) meat. PSE is characterized by pale raw meat color, soft texture and low water holding capacity (WHC), resulting in a watery appearance of meat likely to be rejected by consumers due to its unattractive appearance in retail display (Keenan, 2016). Cheah et al. (1995) hypothesized that the decrease in WHC of PSE meat may be caused by the destabilization of mitochondria cell membranes by PLA2 hydrolysis. This release of calcium can accelerate a rapid pH decrease and increase the use of ATP and muscle contraction resulting in a lower WHC (Ertbjerg & Puolanne, 2017; Hughes et al., 2014). Comparing PSE-prone pig species to non-PSE prone pigs, Cheah et al. (1995) found increased levels of PLA2 activity in the PSE-prone pigs; at the same time, PSE-prone pigs also showed increased levels of sarcoplasmic calcium indicating the release of calcium from endogenous sources, i.e. the mitochondria (Cheah et al., 1995). It is interesting to note that Chen et al. (2010) found that both PLA2 activity and lipid oxidation were higher in PSE pork loin than that of normal pork loins. Perhaps the liberation of FFA, particularly PUFA, from the hydrolysis of PL affects the lipid oxidation of meat products.

It was reported that PL contributes 90% towards lipid oxidation (B. Min & D. U. Ahn, 2005). Therefore, PLA2 as a prooxidant seems logical as the FA cleaved for the inflammatory pathway are PUFA (Dennis et al., 2011). However, studies have also note that PLA2 may have antioxidant properties. Shewfelt et al. (1981) incubated fish muscles with PLA2, and it showed an inhibitory effect on lipid oxidation when compared to the control treatments. Using washed cod muscle (WCM) in an *in vitro* system, Tatiyaborworntham and Richards (2018) induced lipid oxidation using hemoglobin. The inclusion of PLA2 at the early stages inhibited lipid

hydroperoxide formation, and PLA2 was able to remove lipid hydroperoxides from PL. A possible explanation for this is that PLA2 preferably hydrolyze oxidized FA from the PL membrane, and lipid oxidation on PL membranes also increases PLA2 activity (Salgo et al., 1993). The release of oxidized FA allows for synergistic mechanisms with naturally occurring antioxidant enzymes such as glutathione peroxidase (GSH-Px), superoxide dismutase, and catalase to detoxify the released FA (McLean et al., 1993). The three enzymes can remove ROS generated by lipid oxidation and convert them into water and oxygen (Domínguez et al., 2019). GSH-px can further reduce lipid hydroperoxides to alcohol, water, and oxidized glutathione (Domínguez et al., 2019). Finally, oxidized glutathione is converted back to glutathione with electron donors (Alanazi et al., 2015; Domínguez et al., 2019), thus allowing PLA2 to act as an antioxidant, as long as the endogenous enzymes are around to support it.

Inhibition of phospholipase A2

On the other hand, PLA2 inhibition can be beneficial for reasons that include reduction of PSE meat and increasing feed efficiency in live animals. There are many ways to mitigate the effect of PLA2 activity, such as inclusion of vitamin E and an antibody of PLA2 (aPLA2). Cheah et al. (1995) incorporated vitamin E into the diets of the PSE-prone pigs and found that it reduced PLA2 activity and calcium levels compared to the non-PSE pigs. It appears that the vitamin E inhibited PLA2 activity by stabilizing the mitochondrial membrane (Cheah et al., 1995). As vitamin E also reduced lipid oxidation, it would also prevent PLA2 from being activated as an antioxidant (Kwag et al., 2001). There is also evidence that vitamin E can directly bind to PLA2 and partially reduce its function through conformational changes of PLA2 (Chandra et al., 2002).

The use of aPLA2 in live animals is rather common as it is an effective way to improve feed efficiency through inhibiting gut inflammation (Cook, 2011). The aPLA2 can be mass produced through laying hens when immunized against PLA2 (Sand et al., 2016). The egg is then dried down to an egg powder, containing aPLA2 and used as a feed supplement (Cook, 2004). Gut inflammation is a natural response to protect the animal from food borne pathogens (Cook et al., 1993). This response will redirect nutrients to immune response rather than growth, reducing the feed efficiency (Cook, 2011). The use of aPLA2 have been shown in multiple studies to improve feed efficiency when added as a feed additive. Barry and Yang (2008) fed 0.30% aPLA2 egg powder to rainbow trout for 2 weeks and found the aPLA2 treatment can improve growth by 27.8% compared to the control. Cook (2002) fed day age chicks 0 or 0.5 g of dietary aPLA2 egg powder for 3 weeks. After 3 weeks, supplementation with aPLA2 improved weight gain by 5.4% compared to the control (Cook, 2002). Mercadante et al. (2015b) fed 0.6% aPLA2 supplements in a forage-based diet to beef over a 70-day growth period. It showed that aPLA2 treatments maintained similar average daily gain and body weight as the controls, but had a lower dry matter intake, indicating improved feed efficiency (Mercadante et al., 2015b). On the other hand, when implemented into a grain based diet, aPLA2 did not show any improvements (Mercadante et al., 2015a). Grain-based diets will typically lower rumen pH (Nagaraja & Titgemeyer, 2007). Even though egg antibodies are able to retain near 85% of activity at a pH of 2 for 1 hour (Cook & Trott, 2010), the effects of longer exposure to low pH has not been researched.

The hydrolysis of PLA2 and the formation of FFA affecting meat shelf-life is not well understood. FFA, especially PUFA, are strong prooxidants that will contribute to lipid oxidation (Waraho et al., 2011). Endogenous antioxidants are great at keeping lipid oxidation at bay, but

when depleted or outnumbered by lipid radicals, hydroperoxides will begin to form (Barden & Decker, 2016; Cadenas & Sies, 1998). The discoloration of meat is influenced by the products of hydroperoxide degradation as they will destabilize color pigments, such as myoglobin, which allows them to be oxidized, fostering the change from a bright cherry red to brown (Faustman et al., 2010). Consumers will associate bright cherry red steaks with wholesomeness, and discoloration of products will be rejected (Suman et al., 2014). The aPLA2 antibody has shown success in preventing PLA2 activity in the digestive tracts of animals, yet the effect on the use of aPLA2 to inhibit PLA2 activity in meat has not yet been researched. The mechanism of aPLA2 preventing PLA2 hydrolysis and preventing FFA generation may prove beneficial in extending the shelf-life of meat with minimal antioxidant presence in the meat system.

Conclusion

Lipid oxidation can be detrimental to meat shelf-life due to its influence on flavor and color, and lipid oxidation is influenced by multiple factors such as lipid content and composition. However, not much attention has been given to the effect of PL on lipid oxidation until recently. PL contains a higher amount of PUFA compared to the neutral lipids. In the absence of antioxidants, the FFA released by PLA2 hydrolysis may partake in lipid oxidation when exposed to prooxidants such as light and oxygen. aPLA2 has shown to inhibit PLA2 activity in the digestive tract of animals but its mechanism in a meat system requires further clarification. Therefore, the objectives of this study are to elucidate the effect of PLA2 and aPLA2 from egg powder on PL hydrolysis utilizing a beef liposome model system and to understand how the altered PL composition and the presence of PLA2 and aPLA2 affect lipid oxidation and antioxidant capacity in a retail display setting.

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Chapter 2 - Exploring the Potential Effect of Phospholipase A2 antibody to extend beef shelf-life in a beef liposome model system

Abstract

The objective of this study was to elucidate the effect of phospholipase A_2 (PLA2) and a PLA2 antibody (aPLA2) on phospholipid (PL) hydrolysis utilizing a beef liposome model system and to understand how the altered PL composition may affect lipid oxidation and antioxidant capacity of beef in a retail display setting. Various combinations of PLA2 and aPLA2 were introduced to PL of a beef liposome model system and exposed to a retail display. Mass spectrometry was used to analyze the PL and free fatty acid (FFA) profiles. Antioxidant capacity and lipid oxidation were measured for the liposome system throughout retail display. Key PL classes were reduced and the release of highly unsaturated FFAs was increased with the inclusion of PLA2 in the treatments ($P<0.05$). There was no inhibition of PL hydrolysis with the addition of aPLA2. Interestingly, PLA2 showed strong antioxidant capacity in the liposome system ($P<0.01$), but the samples from PLA2 treatments still increased in lipid oxidation throughout the retail display ($P<0.01$). Finally, aPLA2 treatments demonstrated potential to decrease lipid oxidation ($P<0.01$).

Keywords: PhospholipaseA2, Anti-phospholipaseA2, Phospholipid, Antioxidant Capacity, Lipid oxidation, Lipidomics

Introduction

Phospholipase-A_2 (PLA2) is a ubiquitous enzyme in living organisms that serves two main functions: lipid digestion and inflammation initiation (Murakami et al., 2011). PLA2 hydrolyzes dietary phospholipid (PL) at the sn-2 position into free fatty acids (FFA) and lysophospholipids (LysoPL) which becomes available for absorption into the body. It is common to find inflammatory initiators like arachidonic acid (20:4) or linoleic acid (18:2) located at the sn-2 position on PL (Norman & Henry, 2015). Upon release by PLA2 and oxidation, these proinflammatory fatty acids (FA) may further generate inflammatory mediators and reactive oxygen species (ROS), thus leading to oxidative stress (Agarwal et al., 2009; Chatterjee, 2016).

Although PLA2 is essential in maintaining lipid homeostasis, this enzyme is also linked to meat quality defects such as pale, soft, and exudative meat (PSE). PSE meat is characterized by pale meat color, soft texture and a low water holding capacity (WHC) (Keenan, 2016). When comparing PSE prone to non-PSE prone pigs, Cheah et al. (1995) found that the PSE-prone pigs had higher levels of PLA2 activity. While PSE is mostly a concern in pigs, it has been shown in beef products as well but at a much lower rate (Aalhus et al., 1998). Either way, the general characteristics of PSE and causes of PSE between the two species remain similar. The hydrolysis by PLA2 could rupture the mitochondria's cellular membrane leading to extensive release of calcium and accelerate the rigor mortis process, which in turn significantly decreases the water holding capacity of meat (Cheah et al., 1995; Chen et al., 2010; Lee et al., 2011). Nevertheless, a previous study has shown PLA2 may have an antioxidant effect as PLA2 prefers to cleave oxidized FA in the cell membrane when natural antioxidants are around to reduce the oxidized FA (Tatiyaborworntham & Richards, 2018). These natural antioxidants include glutathione peroxidase (GSH-Px), superoxide dismutase, and catalase which work synergistically to reduce

ROS and lipid products into water, oxygen, and alcohol (Domínguez et al., 2019). However, it is possible that the cleaved oxidized FFA without the presence of antioxidants could contribute to lipid oxidation propagation, leading to a negative effect on color and flavor of retail displayed meat (Domínguez et al., 2019).

A commercially available egg powder containing an antibody against PLA2 (aPLA2) has been used as a feed supplement to improve growth efficiency for different species such as fish (Barry & Yang, 2008), poultry (Cook, 2002), and cattle (Mercadante et al., 2015b). The aPLA2 egg powder improves feed efficiency by preventing gut inflammation through the inhibition of PLA2 cleaving inflammatory initiators (Cook, 2011). This egg powder can be mass produced by immunizing laying hens against PLA2, and the eggs will yield ~0.4 mg/g of antibody per egg yolk when the laying hens are injected with 200 µg of immunizer (Sand et al., 2016). The eggs produced can then be spray-dried into a powder to further extend the shelf-life (Sand et al., 2016).

We hypothesize that the aPLA2 from the egg powder can inhibit PLA2 activity, thus preventing the hydrolysis of PL and the formation of FFA during postmortem storage of meat. The economic loss of meat disposal due to shelf-life was estimated in to be ~$3 billion in 2019 in the USA (Ramanathan et al., 2021), and the use of aPLA2 egg powder in a meat system may provide a novel way to further improve meat shelf-life. However, little is known about the effect of aPLA2 inhibition on PLA2 in a meat system. Therefore, the objective of this study was to elucidate the effect of PLA2 and aPLA2 from egg powder on PL hydrolysis utilizing a beef liposome model system and to understand how the altered PL composition and the presence of PLA2 and aPLA2 may affect lipid oxidation and antioxidant capacity in a retail display setting.

Materials and Methods

PLA2 and aPLA2 collection and extraction

All procedures involving chickens were approved by the University of Wisconsin College of Agricultural and Life Sciences Animal Care and Use Committee. The egg powder containing aPLA2 was produced following methods described by Sand et al. (2016) with modifications. Briefly, each breast of a white leghorn laying hen was injected with 0.5 mL Freund's complete adjuvant (FCA) containing 0.1 mg of emulsified sPLA2-IB (10,000 U/g; Bioseutica, Zeewolde, Netherlands) for a total of 0.2 mg of sPLA2-IB in 1 mL of FCA for each hen. Two subsequent injections containing 0.2 mg of sPLA2-IB emulsified with 1 mL of Freund's incomplete adjuvant (FIA) was used two and four weeks after the initial injection, respectively. Eggs were collected after the third injection for six weeks. The entire egg (yolk and whites) where dried using a Mini-Glatt 3 Fluid Bed Dryer (Glatt, Binzen, Germany). The egg powder and PLA2 (Bioseutica) was shipped to Kansas State University from the University of Wisconsin-Madison.

The aPLA2 was extracted from the egg powder following methods of Pauly et al. (2011) with modifications. Briefly, 30 mL of 1X PBS and 3.5% of polyethylene glycol (PEG 6000) was added to five g of egg powder aPLA2 and shaken for 10 min using a Wrist Action Shaker (Model 75; Burrell Corporation, Pittsburg, Pennsylvania). After shaking, samples were centrifuged at 13,000 x g at 4°C for 20 min. The supernatant was filtered through Whatman #1 filter paper, and 8.5% of PEG 6000 was added per volume amount. The shaking and centrifugation were repeated, the supernatant was discarded, and the pellet was redissolved in 10 mL of 1X PBS and 12% of PEG 6000 was added per volume amount. The shaking and

centrifugation process was repeated one more time, and the supernatant was discarded. The pellet was resuspended in 800 µL of 1X PBS, and the extract was dialyzed using a 10 kDa molecular weight cutoff dialysis cassette (87730; Thermo Fisher Scientific, Waltham, MA) against 0.1% saline solution overnight, and the solution was swapped to 1X PBS for a three hours. The dialyzed aPLA2 extract was collected from the cassette and stored at -20℃. The protein concentration of aPLA2 was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and diluted to one mg/mL using 1X PBS. The same procedure was performed on commercially available spray dried whole egg powder from unimmunized hens (Judee's, Plain City, Ohio) to be used as the control for the enzyme-linked immunosorbent assay (ELISA).

Enzyme-Linked Immunosorbent Assay (ELISA) of extracted antibodies

ELISA was performed to confirm the specificity and titer of the extracted aPLA2 following methods described by Bobeck (2007) with modifications. An immunoGrade 96 well plate (781722; Midwest Scientific, Valley Park, Missouri) was coated overnight at 4℃ with 100 µL of PLA2 coating buffer containing 2.4% PLA2 in 50 mM $NaHCO_3$ (pH 8.5). The following day, the coating solution was discarded, plate was washed 2X with a wash buffer (1X PBS + 0.05% Tween-20; pH 7.4) and blocked with a blocking solution (1% bovine serum albumin in 1X PBS) for one hour at room temperature.

After blocking, the plate was washed with washing buffer two more times. Samples (1 mg/mL) and controls (1 mg/mL) were serial diluted 1:50 – 1:1,600 with the blocking solution. The standard curve was performed using purified anti-sPLA2-IB (1 mg/mL; Ab E Discovery, Waterloo, Wisconsin) and serial diluted 1:1,000 – 1:32,000 with blocking solution. The diluted standards, controls, and samples were added to their respective wells in duplicates and incubated

for 30 min at room temperature. At the end of the incubation, the plate was washed 6X with wash buffer. A detection antibody (goat anti-chicken IgY with horseradish peroxidase, 1mg/mL; A16054; Invitrogen) was added to blocking solution for a concentration of 0.25 µg/mL of secondary antibody. The detection antibody was then added to the wells at 100 µl/well and incubated for 30 min at room temperature. Upon the completion of the detection antibody incubation, the plate was washed 8X with wash buffer, and 120 µL of 1-Step TMB-ELISA TURBO substrate solution (Thermo Fisher Scientific) was added to each well and incubated for 5 min at room temperature under darkness. Following the addition of substrate solution, 50 µL of stop solution (0.5M sulfuric acid) was added to each well and optical density was read at 450 nm using a spectrophotometer (Eon; BioTek Instruments Inc., Winooski, Vermont). A four-parameter logistic curve (figure 1) was used for the standard curve. The titer of the egg powder was 435 mg antibody/kg egg powder, whereas the extracted protein from the control showed no binding activity to PLA2.

Sample Collection and Lipid extraction

Ten longissimus lumborum (loin) from the left side of United States Department of Agriculture (USDA) high-choice beef carcasses were collected from a Midwest meat processor at 2 d post-mortem. The loins were vacuum packaged and transported to Kansas State University (KSU) meat laboratory. The following day (3 d postmortem), a 2.54 cm face was removed from the anterior end and discarded before two 2.54 cm thick steaks were removed, vacuum packaged and stored at -20°C until lipid extraction.

Lipids were extracted according to methods described by Bligh and Dyer (1959) with modifications. Sixty grams of lean from frozen beef loins were homogenized in 30 mL of

chloroform and 60 ml of methanol for 2 x 15 s bursts at 12,000 rpm using homogenizer (Fisherbrand 850 homogenizer, Thermo Fisher Scientific) equipped with a large generator probe (15-340-182; Thermo Fisher Scientific). Another 30 mL of chloroform was added and further homogenized for 15 s. Homogenate was transferred to amber bottles and 60 mL of 0.8% KCl solution in MQ water was added, and samples were shaken for 4 min using a Wrist Action Shaker (Model; 75 Burrell Corporation, Pittsburg, PA). Homogenate was filtered through Whatman #1 filter paper with assistance of vacuum. Filtrate was transferred to polypropylene copolymer Nalgene centrifuge bottles (3141-0500; Thermo Fisher Scientific) and centrifuged at 1,000 x g for five min. The top layer of methanol/water was aspirated and discarded, and the bottom layer of chloroform was collected into pre-dried and weighed 25 x 150 mm glass tubes. Chloroform was evaporated under nitrogen gas at room temperature and appropriate amounts of chloroform was added to each dried sample to achieve a 150 mg/mL total lipid (TL) stock and stored at -80°C.

Fractionation of Lipid Classes

Solid Phase Extraction was carried out according to methods described by Legako et al. (2015) with modifications to fractionate the TL into neutral lipids (NL), FFA, and PL using aminopropyl silica packed cartridges (Sep-Pak Vac 10g; Waters Corporation, Milford, MA, USA). Each cartridge was pre-conditioned with 2 x 35 mL of hexane followed by 2 x 35 mL of chloroform. Two hundred and fifty mg of TL was loaded into the cartridges, and the NL was eluted with 5 x 25 mL of chloroform. The FFA was eluted with 5 x 25 mL of 2% acetic acid in diethyl ether, and PL was eluted with 5 x 25 mL of methanol. The methanol containing PL from each sample was captured into pre-weighed and pre-dried 25 x 150 mm glass tubes, and

methanol was evaporated using a vacuum evaporator (RapidVap, Labconco; Kansas City, Mo) set at 25% speed, 120 mbar vacuum, at room temperature for ~6 h. Each sample PL was redissolved in chloroform to achieve a 25 mg/mL PL stock and stored at -80°C until ready to prepare the liposome model system.

Preparation of Liposome and Liposome Model System

Ten mL of Tris/$CaCl_2$ buffer (200 mM/10 mM; pH 8.0) was added to 200 mg of PL extracted from each beef loin sample and shaken for 30 min using a Wrist Action Shaker (Burrel Corporation) to form multilamellar vesicles (MLV). Using a lipid extruder (THE EXTRUDER, Lipex Biomembranes Inc. Vancouver, BC), large unilamellar vesicles (LUV) were prepared by passing the MLV through a polycarbonate membrane filter (pore size = 0.1 µm) 10X. The prepared PL from each beef loin sample was aliquoted and combined with the respective PLA2 and aPLA2 treatments in Tris/$CaCl_2$ buffer resulting the following six treatments (all in 2.5 mL of total volume):

1) PL – Tris/$CaCl_2$ buffer containing 10 mg/mL of PL with no added PLA2 or aPLA2.
2) aPLA10 –Tris/$CaCl_2$ buffer containing 10 mg/mL of PL with 10 µg/mL of aPLA2.
3) aPLA20 –Tris/$CaCl_2$ buffer containing 10 mg/mL of PL with 20 µg/mL of aPLA2.
4) PLA2 – Tris/$CaCl_2$ buffer containing 10 mg/mL of PL with 4 µg/mL of PLA2 only.
5) PLA2+aPLA10 – Tris/$CaCl_2$ buffer containing 10 mg/mL of PL with 4 µg/mL of PLA2 and 10 µg/mL of aPLA2.
6) PLA2+aPLA20 – Tris/$CaCl_2$ buffer containing 10 mg/mL of PL with 4 µg/mL of PLA2 and 20 µg/mL of aPLA2.

It is important to note that any treatment containing for PLA2 and aPLA2 was prepared beforehand with Tris/$CaCl_2$ buffer and incubated with no light at room temperature for one hour to ensure proper binding between PLA2 and aPLA2.

After respective treatments were prepared, samples were incubated at 37°C for two hours in an incubator (Symphony, VWR, Radnor, Pennsylvania). After incubation, 2 mL were taken out and transferred to 12 x 75 mm glass test tubes containing 2.8 mg of beef myoglobin (IBOMBLY1GM; Innovative Research Inc., Novi, Michigan) to achieve a 80 µM concentration of beef myoglobin per tube. The remaining 0.5 mL aliquots were saved for PL extraction for PL and FFA profiling. To the remaining 2 mL of liposome aliquot containing the myoglobin, air permeable Polyvinyl chloride meat wrap (HIYG Gold Stretch Meat, Berry Plastics Corp. Evansville, IN) was wrapped over the opening of each tube and placed under fluorescent lighting at 2,300 lux for 7 days at 2 ± 2°C. At 0, 1, 4, and 7 days, 0.2 mL aliquots were taken from each sample for lipid oxidation and oxygen radical absorbance capacity (ORAC) analysis.

Extraction of Phospholipids from Model System

The lipid in the 0.5 mL aliquots from the model system was re-extracted following the method described by Bligh and Dyer (1959) with modifications. To each aliquot, 3.75 mL of 1:2 chloroform:methanol (v/v) was added and incubated at 4°C for 18 hours. After the incubation, 1.25 mL of chloroform and 1.25 mL of 0.8% KCl in ultrapure water was added and shaken for one min using a Rapidvap evaporator (Labconco) set at 25% speed, room temperature and no vacuum. Following shaking, samples were centrifuged at 1,000 x g for five min. The bottom chloroform layer was removed into a pre-dried and weighed 12 x 75 mm glass tube. Another

1.25 mL of chloroform was added to the remaining top layer, and the process was repeated with the bottom chloroform removed into the same pre-dried tube. This step was repeated once more and the pooled chloroform was dried under nitrogen, and the tubes containing the dried lipid was dried again using a vacuum dryer (CentriVap DNA Vacuum Concentrator, Labconco) for 1 hour. The PL was weighed and redissolved in chloroform to achieve a 2 mg/mL and stored at -80°C until lipidomic analysis.

Lipidomic Analysis

Preparation of phospholipid samples for lipidomic analysis

An aliquot of 5 µl of extracted PL diluted in chloroform equivalent to 10 µg of PL diluted in chloroform was added to amber vials (11 mm; MicroLiter, Wheaton, Millville, New Jersey) with snap caps (MicroLiter). One µl of PL internal standards were added to each vial containing the following quantities: 0.66 nmol of di14:0- phosphatidylcholine (PC), 0.66 nmol of di24:1-PC, 0.66 nmol of 13:0- lysophosphatidylcholine (LPC), 0.66 nmol of 19:0-LPC, 0.36 nmol of di14:0- PE, 0.36 nmol of di24:1-PE, 0.36 nmol of 14:0- lysophosphatidylethanolamine (LPE), 0.36 nmol of 18:0- LPE, 0.36 nmol of di14:0- PG, 0.36 nmol of di24:1- PG, 0.36 nmol of 14:0-lysophosphatidylglycerol (LPG), 0.36 nmol of 18:0- LPG, 0.36 nmol of di14:0- phosphatidic acid (PA), 0.36 nmol of di20:0- phytanoyl-PA, 0.24 nmol of di14:0- PS, 0.24 nmol of di20:0-phosphatidylserine (PS), 0.20 nmol of 16:0–18:0- phosphatidylinositol (PI), and 0.16 nmol of di18:0- PI. The PL internal standard mixture was purchased from Kansas Lipidomics Research Center, and the mixture was prepared using the methods similar to those described in Welti et al. (2002). Finally, 1.2 mL of a solvent (chloroform: methanol: 300 mM ammonium acetate in water; 300:665:35; v/v/v) was added to each vial. The FFA samples were prepared the same as

the PL samples with the exception of two µl FA internal standard containing 0.1 mmol of 15:0 was added instead of the PL internal standards.

Phospholipid analysis and free fatty analysis

Electrospray ionization (ESI)-triple quadrupole mass spectrometry

Prepared samples for both PL and FFA profile were sent to Kansas Lipidomics Research Center for MS/MS analysis. Samples were introduced by continuous infusion into the ESI source of a triple-quadrupole mass spectrometer (Xevo TQ-S, Waters Corporation) using a 2777 autosampler (Waters Corporation). The source temperature was 150℃, desolvation temperature was 250℃ and applied to the electrospray capillary, and the cone voltage was 40V. The collision gas was set at "low". Curtain gas and two ion source gases were set at 20 and 45 psi respectively. Sequential precursor and neutral loss scans of the samples produce a series of spectra with each spectrum revealing a set of lipid species containing a common head group or fatty acid fragment. The intact ion analyzed, fragment type, scan mode, polarity, collision energy, and scan duration to detect PL species are described in table 1. Ether-linked PC and PE (ePC and ePE) species were determined in relation to the same internal standards as other PC and PE species. The identification of the most abundant PL molecular species (total acyl carbons: total double bonds) was based on their mass to charge ratio (Table 1). All PL molecular species were detected using previous scans (Brügger et al., 1997; Welti et al., 2002; Xiao et al., 2010).

The FFA were detected using a Xevo TQS electrospray ionization mass spectrometer (Waters) in a MS1 scan quantified as fatty acyl anions in negative mode with relevant settings: cone voltage of -17 V, mass range 150 – 450 u, collision energy of 5 V, and scan duration of 3 s.

The identification of the most abundant FFA species (acyl group) was based on their mass to charge ratio (Table 2).

The lipid peak areas in each PL class and FFA were uploaded to an online processing software for direct-infusion mass spectral data for lipid profile (LipidomeDB Data Calculation Environment; Zhou et al. (2011). The LipidomeDB software corrected signals for isotopic overlaps. The "A+2" peak of one species may produce a signal at the same mass as the main ("A") peak of another (less unsaturated) lipid species. Therefore, the contribution of signal from the "A+2" peak must be subtracted. Additionally, a linear calibration curve (intensity vs. mass/charge) was fitted to the signals of the internal standards and was used to correct for mass-dependent variation in instrument response. The corrected signals for the targeted lipids were converted to nmol based on the signals from the 2 internal standards and the corresponding internal standard amounts specified by the user (Zhou et al., 2011).

Finally, each apparent lipid molecular species is displayed as total acyl carbons: total double bonds. Each lipid class was expressed as mole percent (mol%; distribution of each PL species in relative % of total PL) of total lipid analyzed. To calculate mol%, we multiplied each nanomolar value x 100% and divided by the total of the nanomolar amounts of the lipids analyzed. In addition, FFA (total acyl carbons: total double bonds) was normalized to the dried lipid weight for each sample and expressed as nmol FFA/mg of PL.

Background subtraction and preparation of quality control (QC) samples

An "internal standards only" sample was inserted for every 10 samples, and the mass spectra were acquired on the "internal standards only" samples to correct for chemical or instrumental noise in the samples. The molar amount of each lipid metabolite detected in the

"internal standards only" samples was treated as background contamination and was subtracted from the molar amount of each lipid metabolite in the next 10 samples. Furthermore, 12 QC samples were prepared for this study. The QC samples were prepared by pooling a 4 μL of lipid extract from each sample. The values for the first three QC samples were eliminated due to potential instrument instability when the instrument was first started. Lipid analytes in which the variation (standard deviation divided by mean of the amount of the analyte in the QC) were > 30% were removed from the data.

Product ion analysis

Further characterization of the following major phospholipid species was performed using product-ion analysis to reveal the potential fatty acid compositions of the following molecular species: PC 32:1, PC 34:3, PC 36:5, PC 36:4, PC 36:3, PC 36:2, PC 36:1, PE, 36:3, PE 36:2, PE 38:5, PE 38:4, ePC 34:4, ePC 34:2, ePC 34:1, ePC 36:5, ePC 36:4, ePC 36:3, ePC 36:2, and, ePC 36:1. Fatty acids anions from the selected PL of pooled samples were identified using the appropriate negative ion precursors: PE were analyzed as $[M -H]^-$ ions, and PC was analyzed as $[M + OAc]^-$. The ePC species were analyzed similar to PC. Specific running conditions and masses used for product-ion analysis were described by Devaiah et al. (2006). These PL species were selected for product-ion analysis because they either represent a significant portion of total PL, or their contents were altered by the treatments.

Oxygen Radical Absorbance Capacity (ORAC)

The ORAC procedure was performed according to methods by Huang et al. (2002) with modifications. To prepare the lipophilic portion of ORAC, one ml of hexane was added to 200

µL of aliquoted sample from the liposome system. Samples were vortexed for 45 s and shaken for one hour at room temperature using the RapidVap evaporator (Labconco) set to 12% speed and no vacuum. After shaking, samples were centrifuged at 3,000 x g for 10 min, and the hexane layer was collected and evaporated under nitrogen. Seven hundred fifty µL of 7% randomly methylated, -cyclodextrin (RMCD) in 50:50; acetone:water was added to redissolve the lipophilic portion. For the hydrophilic portion, one mL of 15% TCA in 80% water/20% ethanol was added to the same aliquoted samples following the removal of the hexane layer. The sample was vortexed, centrifuged at 12,000 x g for 10 min, and 750 µL of the supernatant was collected. Both the lipophilic and hydrophilic portions were diluted 1:5 with 7% RMCD or 1:20 with 80% water/20% ethanol solution respectively. The prepared samples were then stored at -80℃ until analysis.

Samples, standards, and blanks were added to a black 96-well plate (655906; Greiner bio-one, Kremsmünster, Austria) in triplicates. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Sigma Aldrich, St. Louis, Missouri) was serial diluted to 6.25 – 100 µM using RMCD or 80%/20% water/ethanol for lipophilic or hydrophilic portions of the analysis, respectively. Following sample addition, the plates were incubated at 37℃ for 30 min, and 25 µL of 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) solution was added. A multi-mode plate microplate reader (Synergy HTX; BioTek Instruments Inc.) was used with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Fluorescence of each well was measured from the bottom every 60 sec for 120 min for a total of 120 measurements. The ORAC values were measured as the net area under the curve (AUC). The net AUC is calculated by subtracting the AUC of the samples from the AUC of the blank. A standard curve was obtained by plotting Trolox concentrations against their average net

AUC. A standard curve of Trolox concentrations (0-100 µM) were prepared with the respective buffers used for hydrophilic or lipophilic ORAC. The final unit was calculated as µmol of Trolox equivalent (TE) per mg of PL.

Lipid Oxidation

Lipid oxidation was measured by 2-thiobarbituric acid reactive substance (TBARS) analysis. Four hundred µL of thiobarbituric acid:trichloroacetic acid solution (TBA/TCA; 20mM:15% in ultrapure water) and 20 µL of 3% butylated hydroxytoluene (BHT) in ethanol were added to the 200 µl of aliquoted samples. Samples were vortexed for 5 s, centrifuged at 2,000 x g for five min and the supernatant were transferred to 12 x 75 mm glass tubes. Six hundred µL of n-butanol was added to each sample, vortexed, and centrifuged at 2,000 x g for five min. Two hundred µL of the butanol layer was pipetted into their respective wells of a 96 well plate and read in a spectrophotometer (Eon; BioTek Instruments Inc., Winooski, Vermont) at 532 nm. A standard curve containing 0 – 25 µM of malondialdehyde (MDA) bis was used for the calculation of sample MDA concentration, and the final sample was expressed as µg of MDA per mg of PL.

Statistics

PL and FFA profile were analyzed as a completely randomized design, and the lipid oxidation and ORAC analysis were analyzed as a split-plot design with treatment as the whole plot factor and the day of retail display as the sub-plot factor with a retail display by treatment interaction. Each animal used as the experimental unit. Animals within treatments was considered the whole-plot error term, and display day by treatments was considered the split-plot

error term. All data were analyzed using the GLIMMIX procedure of SAS (version 9.4, SAS Institute, Cary, NC). A contrast statement was used to compare results for treatments with or without PLA2. Tukey method were used for multiple comparisons, and separation of means was conducted using LSMEANS procedure (least significant differences) at $P < 0.05$. A macro was created in SAS for performing the PL profile analyses automatically and repeatedly due to high number of data points.

Results and Discussion

Lipidomics

Phospholipid Profile Analysis

The PL profile of the beef liposome system is shown in Figure 2. The most abundant PL classes were PC, ePC, PE and SM which represented 33.6, 36.1, 10.5, and 12.8 % of the total lipids analyzed respectively, followed by ePE and LPC, which represented 1.8 and 4.1% respectively. Minor PL classes such as PG, PI, PA, PS, ether-linked PS, and LPE collectively accounted for 1% of total PL. Among the most abundant PL classes found in this study, PC, ePC and PE mol % were altered by treatments (Figure 3; $P < 0.01$). The aPLA20 treatment had higher relative % of total PC compared to those from PLA2 treatments (PLA2, PLA2+aPLA10, and PLA2+aPLA20; $P < 0.01$). However, the total PC of PLA2 treatments were not different than those from PL and aPLA10 ($P > 0.05$). In addition, treatments containing PLA2 had lower percentages of individual PC species such as PC 32:1, 32:2, 34:3, 36:1, 36:2, 36:3, 36:4, and 36:5 than treatments without PLA2 (Figure 4; $P < 0.01$). In total ePC, treatments with PLA2 had a lower percentage of total ePC than treatments without PLA2 (Figure 3; $P < 0.01$). In addition, treatments with PLA2 had a lower percentage of individual ePC species such as ePC 34:1, 34:2,

34:4, 36:1, 36:2, 36:3, 36:4, and 36:5 than those from treatments without PLA2 (Figure 5; $P < 0.01$). It is interesting to note that there are also PC and ePC species that showed no hydrolysis from PLA2 at all, particularly the ones with saturated FA only such as 32:0, 34:0, 36:0, and 38:0 ($P > 0.05$). Treatments with PLA2 showed a lower percentage of total PE than treatments without PLA2 (Figure 3; $P < 0.01$), and individual PE species affected by PLA2 hydrolysis were PE 36:3, 36:2, 38:5, 38:4, and 40:5 (Figure 6; $P < 0.01$).

Utilizing product ion analysis, the FA composition for the most abundant PC, ePC and PE species were revealed (Table 3). The most abundant molecular species in PC found in this study were 34:3 (composed of 72% of 16:1/18:2), 36:2 [equal distribution of 18:1/18:1 (47%) and 18:0/18:2 (43%)], 36:3 (composed of 83% of 18:1/18:2) and 36:4 (composed of 65% of 16:0/20:4). The most abundant ePC molecular species were 34:2 [equal distribution of 18:0/18:2 (44%) and 18:1/18:1 (41%)], 36:3 [18:1/18:2 (78%)] , 36:4 [18:2/18:2 (46%), and equal distribution of 16:1/20:3 (22%) and 16:0/20:4 (21%)], and finally 36:5 (comprised of 78% of 16:1/20:4). The highest abundant PE species were 36:2 (composed of 87% of 18:0/18:2) and 38:4 (composed of 88% of 18:0/20:4).

The PL profiling data are consistent in that PC and PE make up the majority of the PL in animals as illustrated in many other studies (Lordan et al., 2017; van der Veen et al., 2017). Larick and Turner (1989) found that PC accounted for nearly 60%, while PE was approximately 8% of total PL in the pectoralis major muscle of Angus and Angus x Hereford steers. The ability of PLA2 to hydrolyze PL into LysoPL and FFA has been well documented (Dennis, 1994; Kudo & Murakami, 2002). Therefore, it was expected to see PL classes/species alterations from the PLA2 treatments as seen in this study. However, the clear hydrolysis of preference for ePC over PC found in this study is not quite understood. Opposite of our finding, Hayashi et al. (2022)

found that group V sPLA2 (sPLA2-V) showed preference for conventional PC over ePC. The ePC contains an ether bond at the sn-1 position whereas the conventional PC has an ester bond at that same position (Burke & Dennis, 2009), and Hayashi et al. (2022) found that the lack of carbonyl oxygen on the chain at the sn-1 position of the ePC impeded on the binding capability of sPLA2-V to the lipid substrate. While the overall folding and structure of sPLA2 are highly conserved, the interfacial binding zones (i-face) between the sPLA2 species can vary significantly (Winget et al., 2006). The specific PLA2 used in this study is a secretory PLA2 group IB (sPLA2-IB). The sPLA-IB has more cationic amino acids in the i-face making allowing for higher affinity for anionic head groups, while sPLA2-V has more a polar amino acid, allowing it to better bind to PL with zwitterionic head groups (Singer et al., 2002). The variation in PLA2 i-faces may be the reason why the sPLA2 used this study showed a preference for ePC over PC. Finally, the sPLA2-IB has a preference for anionic PLs, which explained the noted PE alterations as seen in this study (Dennis et al., 2011).

In this study, PL species containing at least one MUFA or PUFA, particularly 18:2 and 20:4 were more effectively hydrolyzed by PLA2 compared to the PL species containing SFA only. There is a body of evidence that PLA2 prefers to hydrolyze PL tails containing unsaturated FAs (Lambeau & Gelb, 2008). Diez et al. (1994) utilized sPLA2 and mammalian membrane substrate to confirm sPLA2 preference of PL classes and species and found sPLA2 can hydrolyze a multitude of PL classes containing a variety of FA. Although sPLA2 did not show a FA tail preference for PC hydrolysis, sPLA2 prioritized PE hydrolysis from species containing 20:4 in that same study (Diez et al., 1994). Furthermore, Hayashi et al. (2021) investigated the cytosolic PLA2 (cPLA2), calcium-independent PLA2 (iPLA2) and sPLA2's abilities to hydrolyze PLs at the sn-2 position. They found that cPLA2 preferred to hydrolyze PL species

containing 20:4, while sPLA2 showed stark preferences towards PL species containing 18:2 and 18:1 (Hayashi et al., 2021). It is thought that PLA2's preference for unsaturated FAs stems from their biological functions involving the inflammatory response (Dennis et al., 2011; Lambeau & Gelb, 2008). Notably, 18:2 and 20:4 are important precursors to inflammatory mediators known as eicosanoid (Norman & Henry, 2015). At the onset of cell damage or detection of foreign microbials, cytokines will signal for PLA2 synthesis, resulting in the release of 18:2 and 20:4 (Nathan & Ding, 2010; Pruzanski & Vadas, 1991).

PLA2 hydrolysis products: FFA and Lyso-phospholipids

All notable FFA identified are listed in Table 2. As expected, treatments with PLA2 significantly increased the generation of monounsaturated FFA (MUFFA), polyunsaturated FFA (PUFFA) and total FFA in the beef liposome system ($P < 0.01$). Interestingly, the total amounts of saturated FFA (SFFA) were not affected by PLA2 addition ($P > 0.05$). When looking at individual FFA species, the treatments with PLA2 had higher amounts of FFA 16:1, 17:1, 18:1, 18:2, 18:3, 19:1, 20:2, 20:3, 20:4, 20:5, 22:0, 22:4, 22:5, and 22:6 than treatments without PLA2 ($P < 0.01$). However, there was no difference between the treatments for FFA 16:0, 18:0, and 19:0 ($P > 0.05$). On top of FFA, the other major by-product of PLA2 hydrolysis found in this study was lysophosphatidylcholine (LPC). As expected, the relative % of total LPC increased with PLA2 treatments as compared to treatments without PLA2 (Figure 3; $P < 0.01$). Treatments with PLA2 had higher relative % of individual LPC species, specifically LPC 16:0, 16:1, 18:0, and 18:1 than treatments without PLA2 (Figure 7; $P < 0.01$). Interestingly, in each of these LPC species, the treatment aPLA20 had less relative % of LPC species than the other treatments

(Figure 7; $P < 0.01$), while PLA2+aPLA20 samples had the highest relative % of LPC 16:0, 16:1, and 18:0 among the treatments ($P < 0.01$).

On top of the involvement of inflammatory pathway as mentioned earlier, sPLA2 functions as a digestive enzyme, which it will hydrolyze any PL that enters into the GI system (Dennis et al., 2011; Murakami et al., 2011). Due to the need for this specific function, sPLA2 contains both aliphatic and aromatic residues in the active site allowing for more diversity when cleaving off FAs from PL (Mouchlis et al., 2018). While PLA2 may have its pro-inflammatory aspects, studies have also shown it may have anti-inflammatory effects as it also generates 20:5, 22:6 and notably 18:1 (Murakami et al., 2011). These FAs, 18:1, 20:5, and 22:6 have been well documented to be anti-inflammatory due to their ability to reduce eicosanoid production (Calder, 2013; Medeiros-de-Moraes et al., 2018).

The FFA data agreed with our product ion analysis data demonstrating that 18:1, 18:2 and 20:4 are the major FA at the sn-2 position of PL from this study. It is well established that PL has a preference to incorporate highly unsaturated FAs at the sn-2 position to allow for membrane fluidity, which also explained why minimal SFA was released by PLA2 in this study as the sn-2 position of a PL is rarely occupied by SFA (El-Bacha & Torres, 2016). Studies have shown that 18:2 and 20:4 are the most abundant PUFA in conventional beef lipid due to the animals' grain-based diet sources (Daley et al., 2010; Dannenberger et al., 2007). In addition, during de novo synthesis of PLs, 18:1 will be more readily incorporated into the sn-2 position than 20:4 (Yamashita et al., 2017). Through PLA2 action, 18:1 will be released and 20:4 will be incorporated by a process commonly known as the Lands cycle (Shindou et al., 2009). Therefore, the extensive release of 18:1, 18:2 and 20:4 from PLA2 hydrolysis found in this study was not unexpected as these FA are more readily available at the sn-2 position of a PL (Mouchlis et al.,

2018). It is also common for the sn-1 position of PC to contain SFA or MUFA (El-Bacha & Torres, 2016), which explained the increase in LPC 16:0, 16:1, 18:0 and 18:1 after PLA2 hydrolysis. Finally, FAs 16:0, 16:1, 18:0 and 18:1 are the most abundant FAs in ruminants, which again explained the dominance of these FAs at the sn-1 position (Daley et al., 2010; Quehenberger et al., 2010).

Besides the apparent effect of PLA2 on LPC production, it also appeared that the aPLA20 treatment showed some conflicting results in the formation for LPC species. The lecithin:cholesterol acyltransferase (LCAT) is activated by membrane bond apolipoprotein A1 (apoA1) to degrade PC to LPC and utilizes the FA at the sn-2 position to generate cholesterol ethers (Semenkovich et al., 2016). In addition, apoA1 antibodies are known to prevent apoA1's ability to bind and activate to LCAT (Meng et al., 1993). Perhaps, aPLA2 demonstrated cross reactivity with a multitude of antigens, including apoA1, thus preventing LCAT from degrading PC in this study. Interestingly, the PLA2+aPLA20 treatment showed a general increase in LPC production indicating this treatment had higher hydrolysis of PC species. Cavigiolio and Jayaraman (2014) found that PLA2 are capable of hydrolyzing apoA1. It is possible that aPLA2 bonded to PLA2 and inhibited the hydrolysis of apoA1, which further stimulated LCAT activity in this instance.

aPLA2 effect on PLA2 activity

The inhibition of PLA2 by aPLA2 was not apparent in this study as there was no difference between PLA2 and the two PLA2+aPLA treatments in relative % of total ePC, PC, and PE species (Figure 3; $P > 0.05$), as well as in FFA and LPC specie generation (Table 2 and

Figure 7 respectively; $P > 0.05$) even though ELISA confirmed aPLA2 activity and binding to PLA2 (Figure 1).

Monoclonal antibodies against PLA2 have shown to be generated in rats when immunized with PLA2 (Murakami et al., 1988). Murakami et al. (1988) generated nine different antibodies against platelet PLA2. All nine antibodies were able to recognize and bind to the rat platelet PLA2, but only one antibody was able to show a 50% inhibition of PLA2 activity (Murakami et al., 1988). In addition, Rodriguez et al. (2006) found that antibodies produced from rabbits to combat PLA2 in snake venom showed binding and cross reactivity between various snake venoms, but no more than 50% inhibition of PLA2 activity. Many studies have also investigated the utilization of aPLA2 to improve feed efficiency in various species through the inhibition of gut inflammation (Barry & Yang, 2008; Cook, 2002; Mercadante et al., 2015a). Gut inflammation is a natural response to protect the animal from foodborne pathogens, which inflammation will redirect nutrients to immune response rather than growth, thus reducing the feed efficiency (Cook, 2011). Mercadante et al. (2015b) fed 0.6% aPLA2 supplements in a forage-based diet to beef cattle over a 70-day growth period. The aPLA2 treatments maintained similar average daily gain and body weight as the controls, but improved feed efficiency (Mercadante et al., 2015b). However, when implemented into a grain based diet, aPLA2 failed to show any improvements in feed efficiency (Mercadante et al., 2015a). Even though aPLA2 has been shown to retain nearly 85% of its activity at a pH 2 for one hour (Cook & Trott, 2010), Mercadante et al. (2015a) hypothesized that the change in rumen pH from the grain-based diet may have reduced aPLA2 binding.

Based on the findings from those studies, we hypothesize two potential mechanisms to explain for the lack of aPLA2 inhibition effect for PLA2: 1) As previously discussed, the two

main domains of PLA2 are the i-face and the catalytic site. Although the antibodies recognized PLA2 in this study, it is possible that the binding site of the antibody is located at a different part other than the catalytic site (Murakami et al., 1988); or 2) aPLA2 works by preventing PLA2 from interacting with other cellular components such as transmembrane PLA2 receptors (PLA2R), but not its PL substrate. The PLA2R regulates PLA2 function by elevating the inflammatory responses (Hanasaki & Arita, 2002). Hanasaki et al. (1997) induced PLA2R deficient mice with septic shock, which the PLA2R deficient mice showed lower pro-inflammatory cytokines production compared to the wild-type mice. Perhaps, aPLA2 was never going to inhibit PLA2's ability to hydrolyze PL in this liposome system, but potentially reduce the pro-inflammatory effect of PLA2 as seen later in the lipid oxidation section.

Oxygen Radical Absorbance Capacity (ORAC)

Hydrophilic and Lipophilic ORAC

There was a treatment x display interaction for hydrophilic ORAC (Table 4; $P < 0.01$). In hydrophilic ORAC, samples with PLA2 had higher antioxidant capacity than samples without PLA2 (Figure 8; $P < 0.01$). Samples without PLA2 and samples with PLA2 only increased in antioxidant capacity in the hydrophilic portion after 4 d of retail display ($P < 0.01$) and remained stable till the end of the 7 d retail display. On the other hand, PLA2+aPLA10 and PLA2+aPLA20 had higher antioxidant capacity in the hydrophilic portion at 0 d of retail display and remained stable throughout the retail display. There was also a display x treatment interaction for lipophilic ORAC (Table 4; $P < 0.01$). In lipophilic ORAC, samples with PLA2 showed higher antioxidant capacity than treatments with no PLA2 at 0 d (Figure 9; $P < 0.01$), but the enhanced antioxidant capacity from the PLA2 samples were depleted after 1 d and stayed

stable through the rest of the 7-d display period ($P > 0.05$). The aPLA20 treatment also showed slightly higher antioxidant capacity in the lipophilic portion than PL only samples at 0 d ($P < 0.01$), but this enhancement also quickly mitigated after 1 d of display ($P > 0.05$).

The addition of PLA2 into the liposome system did not create a prooxidative system due to the enhancement of the antioxidant capacity in the hydrophilic portion to combat the extensive FFA production. Tatiyaborworntham and Richards (2018) showed that in the presence of electron donors, PLA2 can exhibit an antioxidant effect as PLA2 preferably hydrolyzes oxidized FA. The removed oxidized FFA can later be stabilized by the surrounding electron donors such as glutathione peroxidase (GSH-px), catalase, and potentially hemoglobin and myoglobin (Tatiyaborworntham & Richards, 2018). With the addition of myoglobin in the current liposome system, it is possible that there was an interaction between myoglobin and the oxidized FA removed by PLA2. Perhaps, the myoglobin was able to act as an electron donor that sequestered the free radicals before they could damage the fluorescent probe in the ORAC assay (Huang et al., 2005). It was interesting to note that PLA2 treatments showed greater antioxidant capacity than treatments without PLA2 only at 0 d of retail display in the lipophilic portion, which is likely due to the presence of fat-soluble antioxidants such as α-tocopherol and ß-carotene embedded in the beef PL (Descalzo et al., 2005; Wu et al., 2008). As the PLA2 removed oxidized FA from the PL, the fat-soluble antioxidants could serve as electron donors to stabilize the oxidized FA. However, the free radical scavenging of ability of the PLA2 treatments was depleted after just 1 d of retail display, which indicated the level of PL-embedded antioxidant was minute.

Lipid Oxidation (TBARS)

There was a display x treatment interaction for lipid oxidation (Table 4; $P < 0.01$). For all treatments, lipid oxidation increased throughout the retail display period ($P < 0.01$). In general, PLA2 treatments showed higher lipid oxidation than treatments without PLA2 added throughout the entire retail display period ($P < 0.01$; Figure 10). Interestingly, aPLA10 and aPLA20 samples had less lipid oxidation than the PLA2 treatments and the PL only control on d 4 and 7 of retail display ($P < 0.01$). Finally, d4 PLA2+aPLA20 samples were the only ones that demonstrated a clear lipid oxidation inhibition effect from the addition of aPLA2 to a beef liposome system containing PLA2 ($P < 0.05$).

Although the PLA2 treatment group had greater antioxidant capacity, the extensive production of proinflammatory FFA due to PLA2 hydrolysis still negatively influenced lipid oxidation. The long chain unsaturated FAs are known to be the forefront of MDA production, a secondary lipid oxidation product (Ayala et al., 2014; Domínguez et al., 2019). In the current study, the release of the long chain FFAs such as 18:2, 20:4 and 22:6 due to the addition of PLA2 concurs with our findings in the increase of lipid oxidation values.

Although it was clear that the aPLA2 treatments did not effectively inhibit the hydrolysis of PL from PLA2, the aPLA10, aPLA20 and PLA2+aPLA20 treatments showed less lipid oxidation on certain time points of retail display compared to treatments without aPLA2 added. Non-enzymatic proteins are known to have antioxidant activities through free radical scavenging and reduction of lipid hydroperoxides (Elias et al., 2008). Elias et al. (2005) measured the antioxidant capabilities of whey proteins in a fish oil/water emulsion system and 750 µg/mL of whey protein where able to inhibit TBARS and lipid hydroperoxide formation by 77 and 93% respectively when compared to the control at d 4 of the model system (Elias et al., 2005).

Nevertheless, lipid oxidation increased after d 4 due to oxidatively labile amino acids such as cysteine and tryptophan being depleted after 4 d into the model system, which indicated they played a role as antioxidant during the lag phase of lipid oxidation (Elias et al., 2005). Avian immunoglobins contain cysteine rich regions in the complimentary determining regions (Harley, 2016; Wu et al., 2012). The cysteines from the avian aPLA2 from this study were exposed to circulating free radicals and potentially combating lipid oxidation. In our liposome system, we added 10 μg/mL of aPLA2 in the aPLA10 treatments and 20 μg/mL of aPLA2 in the aPLA20 treatments, which both demonstrated inhibition of lipid oxidation effect without the addition of PLA2. In the presence of PLA2, it is possible that the additional FFA in the system required more free proteins (at least 20 μg/mL) to demonstrate a lipid oxidation inhibition effect as seen at 4 d with PLA2+aPLA20.

Conclusion

In this study, there was a clear alteration in PL composition from the addition of PLA2. In the PLA2 treatments, there were less diacylglycerol-containing PL classes, higher LPC and an increase in MUFFA, PUFFA and total FFA. This change in lipid composition significantly influenced the antioxidant capacities and lipid oxidation while under retail display. Although PLA2 may possess an antioxidant effect, the extensive release of FFA from PL hydrolysis likely overcame the antioxidant effect and could be detrimental to the oxidative stability of meat. Although the binding of aPLA2 to PLA2 was demonstrated through ELISA, PLA2 inhibition was not observed. Despite that, aPLA2 appeared to show some antioxidant effects through decreasing LPC generation and lipid oxidation which may be resulted from the increase in

protein concentration. Nevertheless, further research on the binding mechanisms of aPLA2 to PLA2 is needed to further elucidate the potential benefits of aPLA2 in a meat system.

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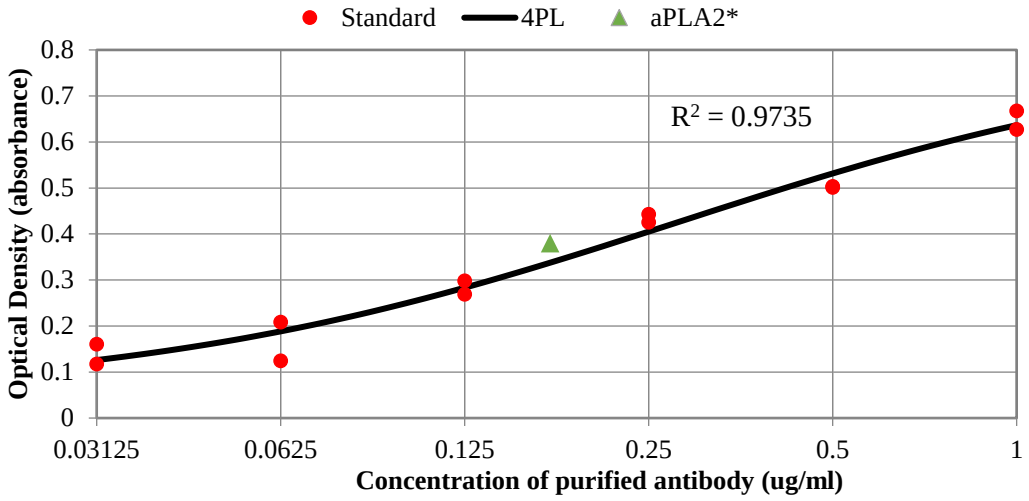

Figure 2.1 Four parameter logistic standard curve of purified aPLA2 antibody from enzyme-linked immunosorbent assay to calculate the titer of extracted egg powder aPLA2. * 1/200 dilution of 1 mg/mL extracted egg powder aPLA2 indicating the titer of the egg powder was 435 mg antibody/kg egg powder.

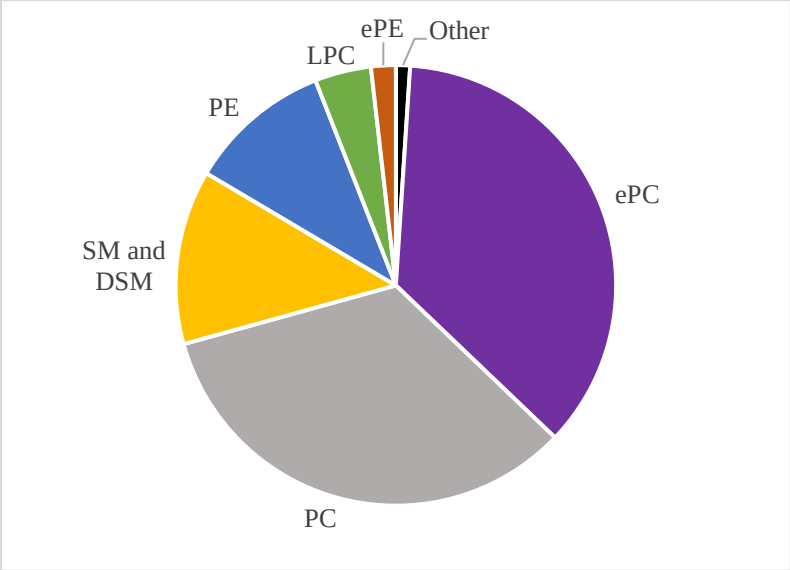

Figure 2.2 Phospholipid classes (mol % of total phospholipids) in extracted lipids from the beef liposome model system. PC = phosphatidylcholine; ePC = ether-linked PC; PE = phosphatidylethanolamine; ePE = ether-linked PE; SM = sphingomyelin; DSM = dihydro-SM LPC = lyso-PC. Other contains PG = phosphatidylglycerol; PS = phosphatidylserine; PI = phosphatidylinositol; PA = phosphatidic acid; LPE = lyso-PE; ePS = ether-linked PS.

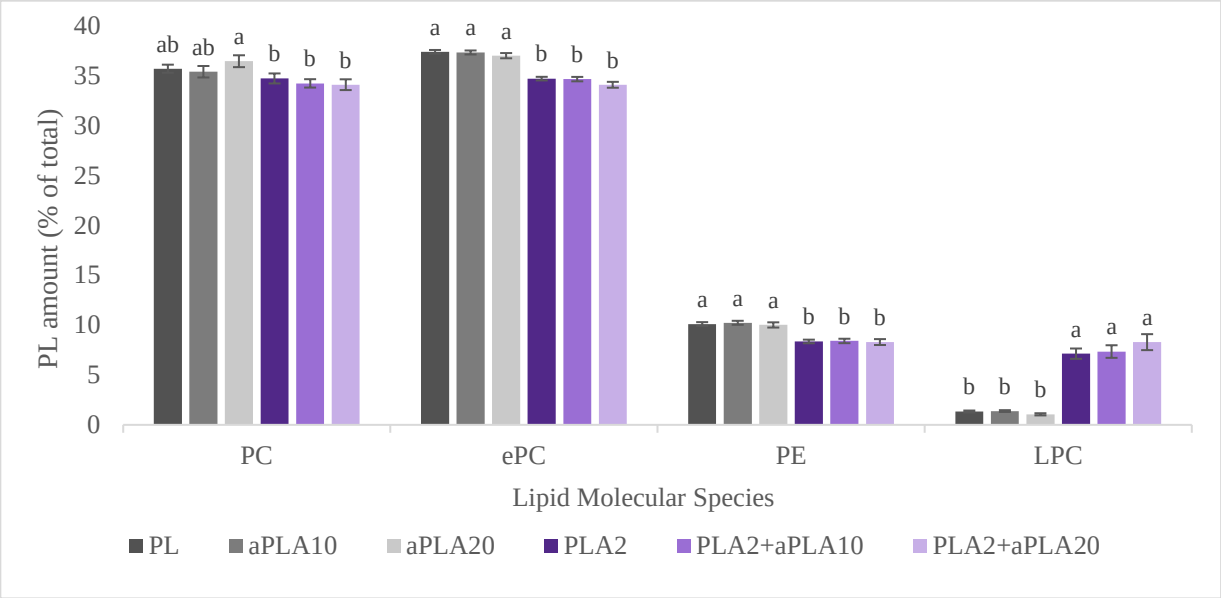

Figure 2.3. Effects of treatments on relative % of phospholipid classes of total PL from a beef liposome model system treated with various combinations of PLA2 and aPLA2. Each bar represents the mean ± standard error; n=60 (six treatments and 10 replications). Means with different letters within a lipid class differ significantly at $P < 0.05$. PC = phosphatidylcholine; ePC = ether-linked PC; PE = phosphatidylethanolamine; LPC = lyso-PC.

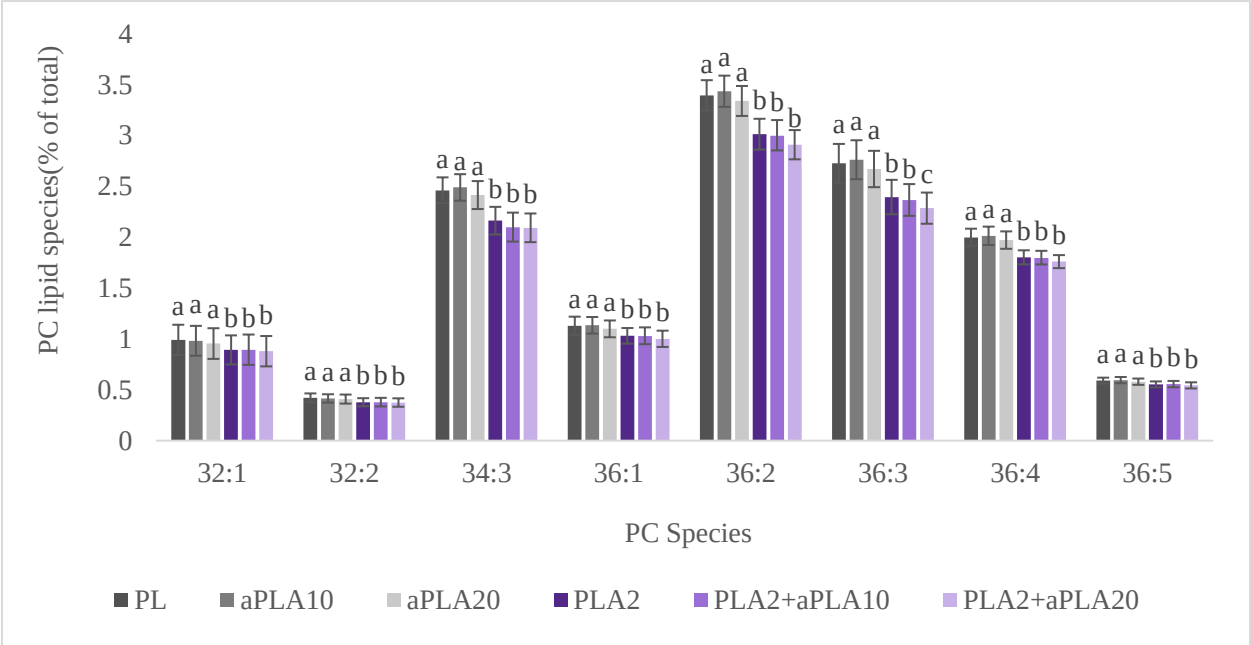

Figure 2.4 Effects of treatments on relative % of phosphatidylcholine (PC) molecular species (total acyl carbons: total carbon bonds) from the beef liposome model system treated with various combinations of PLA2 and aPLA2. Each bar represents the mean ± standard error; n=60 (six treatments and 10 replications). Means with different letters within a PC molecular species, differ significantly at $P < 0.05$.

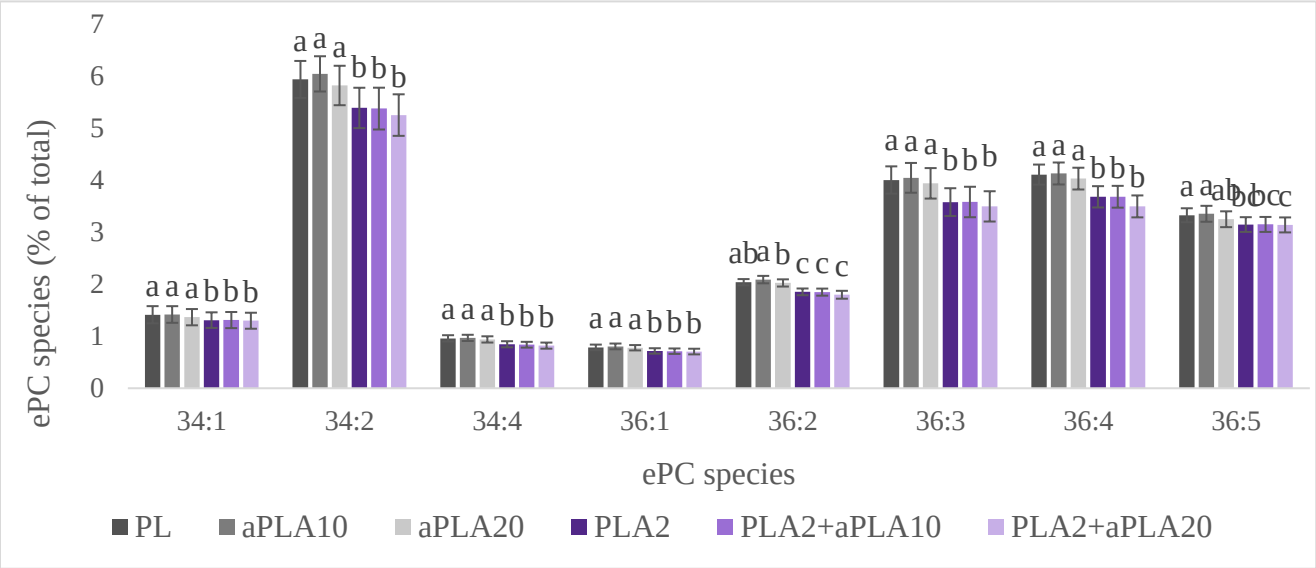

Figure 2.5 Effects of treatments on relative % of ether-linked phosphatidylcholine (ePC) molecular species (total acyl carbons: total carbon bonds) from the beef liposome model system treated with various combinations of PLA2 and aPLA2. Each bar represents the mean ± standard error; n=60 (six treatments and 10 replications). Means with different letters within an ePC molecular specie, differ significantly at $P < 0.05$.

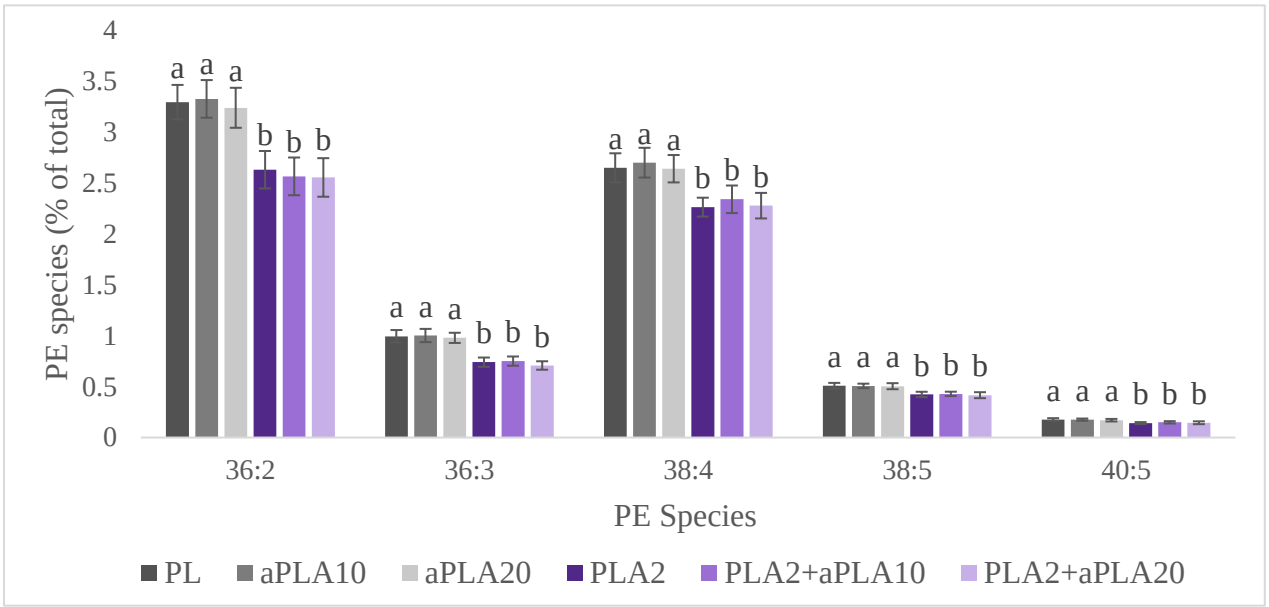

Figure 2.6 Effects of treatments on relative % of phosphatidylethanolamine (PE) molecular species (total acyl carbons: total carbon bonds) from the beef liposome model system treated with various combinations of PLA2 and aPLA2. Each bar represents the mean ± standard error; n=60 (six treatments and 10 replications). Means with different letters within a PE molecular species, differ significantly at $P < 0.05$.

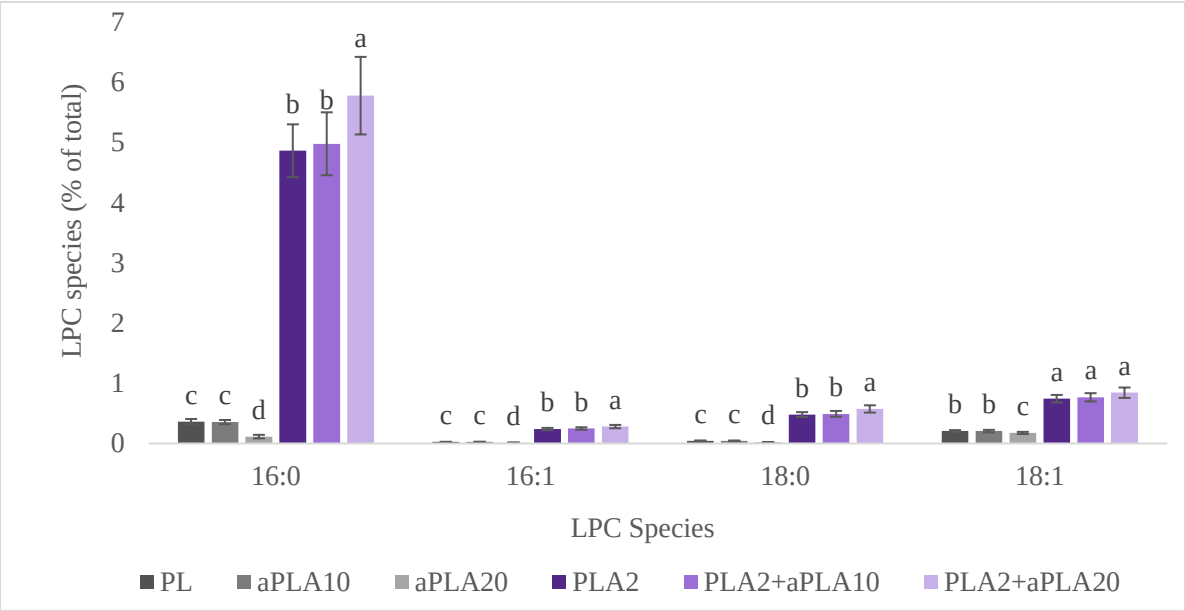

Figure 2.7 Effects of treatments on relative % of lyso-phosphatidylcholine (LPC) molecular species (total acyl carbons: total carbon bonds) from the beef liposome model system treated with various combinations of PLA2 and aPLA2. Each bar represents the mean ± standard error; n=60 (six treatments and 10 replications). Means with different letters within a LPC molecular species, differ significantly at $P < 0.05$.

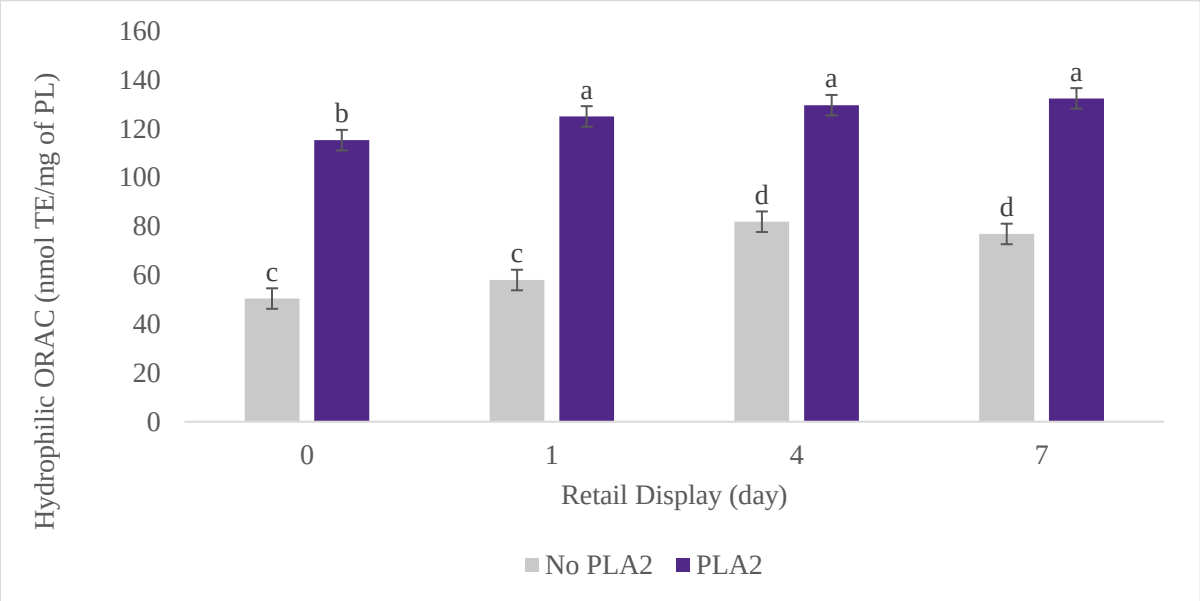

Figure 2.8 Effects of PLA2 and retail display on hydrophilic ORAC of beef liposome model system (n=240). Each bar represents a mean ± standard error. Means with different letters differ significantly at $P < 0.05$.

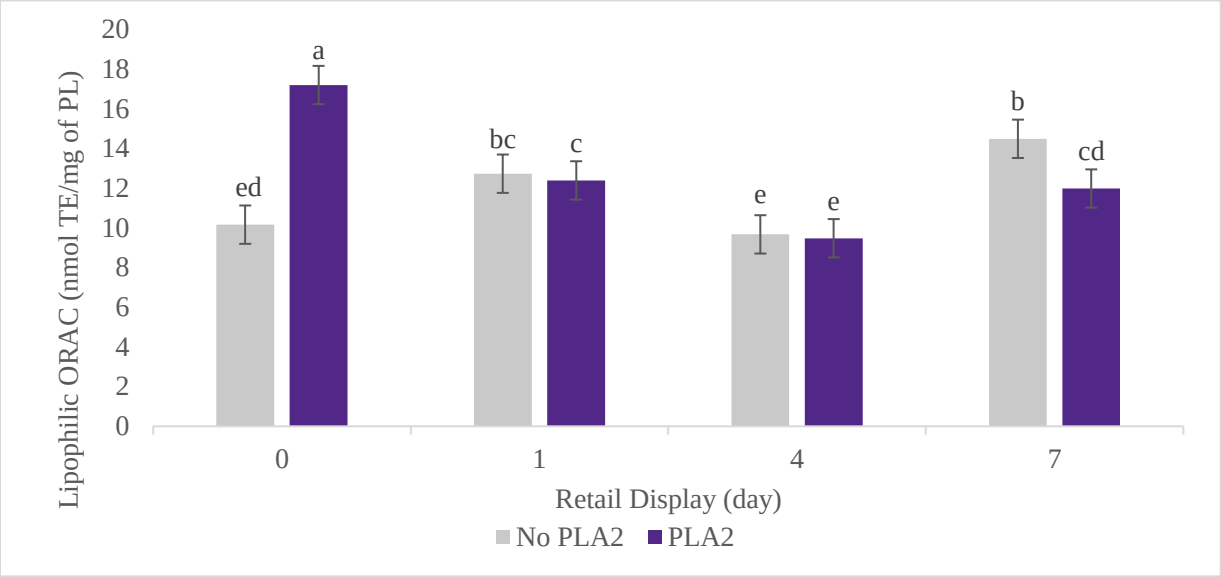

Figure 2.9 Effects of PLA2 and retail display on lipophilic ORAC of beef liposome model system (n=240). Each bar represents a mean ± standard error. Means with different letters differ significantly at $P < 0.05$.

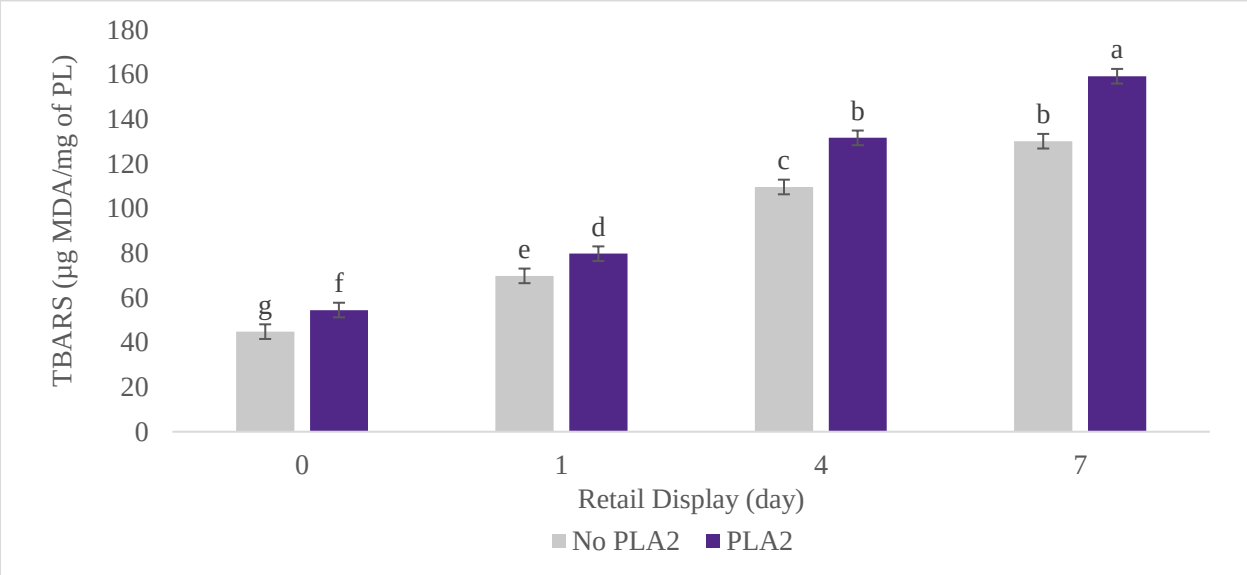

Figure 2.10 Effects of PLA2 and retail display on lipid oxidation of beef liposome model system (n=240). Each bar represents a mean ± standard error. Means with different letters differ significantly at $P < 0.05$.

Table 2.1 Mass spectrometry analysis parameters used for PL profiling.

Class	PA	PC,LPC,SM	PE and LPE	PI	PS	PG
Intact ion analyzed	$(M + NH_4)^+$	$(M + H)^+$	$(M + H)^+$	$(M + NH_4)^+$	$(M + H)^+$	$(M + NH_4)^+$
Fragment Type	Head Group	Head Group	Head Group	Head Group	Head Group	Head Group
Scan mode	Neutral loss of 115.00	Precursors of 187.07	Neutral loss of 141.02	Neutral loss of 277.00	Neutral loss of 185.00	Neutral loss of 189.04
Polarity	+	+	+	+	+	+
Mass/charge range	606-784	450-960	422-894	840-1000	675-1000	680-858
Collision energy (V)	5	28	12	17	13	8
Declustering potential (V)	100	100	100	100	100	100

PA = phosphatidic acid; PC = phosphatidylcholine; LPC = lysophosphatidylcholine; SM = sphingomyelin; PE = phosphatidylethanolamine; LPE = lysophosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PG = phosphatidylglycerol

Table 2.2 Free fatty acid composition identified from a beef liposome model system (n=60) treated with various combinations of PLA2 and aPLA2[1]

[2]FFA (nmol/mg lipid)	[3]*m/z* ratio	Treatments							
		PL	aPLA10	aPLA20	PLA2	PLA2+aPLA10	PLA2+aPLA20	SEM	P-value
14:0	227.20	0.46	0.43	0.55	0.50	0.62	0.58	0.09	0.27
16:0	255.23	4.06	3.56	8.67	5.19	8.02	7.49	1.86	0.22
16:1	253.22	0.14^{b}	0.16^{b}	0.22^{b}	1.91^{a}	2.05^{a}	2.23^{a}	0.25	< 0.01
17:0	269.25	0.34^{b}	0.36^{b}	0.50^{ab}	0.50^{ab}	0.62^{a}	0.56^{a}	0.07	0.02
17:1	267.23	0.10^{b}	0.12^{b}	0.10^{b}	1.25^{a}	1.35^{a}	1.41^{a}	0.16	< 0.01
18:0	283.26	2.13	2.16	2.97	2.42	3.12	2.70	0.48	0.45
18:1	281.25	0.39^{b}	0.44^{b}	0.76^{b}	35.20^{a}	37.32^{a}	40.47^{a}	4.09	< 0.01
18:2	279.23	0.75^{b}	0.80^{b}	0.60^{b}	108.81^{a}	108.49^{a}	117.41^{a}	9.30	< 0.01
18:3	277.22	0.00^{b}	0.00^{b}	0.00^{b}	2.31^{a}	2.26^{a}	2.45^{a}	0.25	< 0.01
19:0	297.28	0.39	0.45	0.59	1.40	1.23	0.76	0.43	0.42
19:1	295.26	0.04^{b}	0.06^{b}	0.09^{b}	0.20^{a}	0.22^{a}	0.21^{a}	0.02	< 0.01
20:0	311.29	0.07^{c}	0.09^{c}	0.10^{bc}	0.16^{ab}	0.22^{a}	0.13^{bc}	0.03	< 0.01
20:1	309.28	0.20^{bc}	0.13^{c}	0.23^{bc}	0.26^{bc}	0.43^{a}	0.29^{b}	0.06	0.02
20:2	307.26	0.00^{b}	0.00^{b}	0.00^{b}	0.31^{a}	0.30^{a}	0.30^{a}	0.05	< 0.01
20:3	305.25	0.00^{b}	0.00^{b}	0.00^{b}	6.43^{a}	6.31^{a}	6.83^{a}	0.70	< 0.01
20:4	303.23	0.17^{b}	0.19^{b}	0.11^{b}	39.42^{a}	39.65^{a}	42.43^{a}	4.09	< 0.01
20:5	301.22	0.00^{b}	0.00^{b}	0.00^{b}	2.95^{a}	2.86^{a}	3.07^{a}	0.35	< 0.01
21:0	325.31	0.03^{a}	0.05^{cd}	0.04^{cd}	0.10^{a}	0.14^{a}	0.09^{bc}	0.03	< 0.01
21:1	323.29	0.02	0.00	0.01	0.00	0.00	0.00	0.01	0.43
22:0	339.33	0.02^{c}	0.04^{c}	0.03^{c}	0.29^{b}	0.43^{a}	0.32^{b}	0.05	< 0.01
22:1	337.31	0.24^{bc}	0.07^{c}	0.57^{abc}	0.50^{abc}	0.85^{a}	0.71^{ab}	0.19	0.04
22:2	335.29	0.00	0.00	0.00	0.01	0.02	0.03	0.01	0.11
22:3	333.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.59
22:4	331.26	0.00^{b}	0.00^{b}	0.00^{b}	3.28^{a}	3.34^{a}	3.56^{a}	0.44	< 0.01
22:5	329.25	0.04^{b}	0.04^{b}	0.11^{b}	5.88^{a}	5.75^{a}	6.17^{a}	0.50	< 0.01
22:6	327.23	0.00^{b}	0.00^{b}	0.00^{b}	0.57^{a}	0.58^{a}	0.63^{a}	0.07	< 0.01
Total									
SFA		7.60	7.23	13.73	10.72	14.69	12.87	2.71	0.21

MUFA	1.13[b]	0.98[b]	1.98[b]	39.31[a]	42.21[a]	45.32[a]	4.45	< 0.01
PUFA	6.44[b]	6.18[b]	5.16[b]	175.63[a]	175.92[a]	189.24[a]	14.99	< 0.01
FFA	15.17[b]	14.39[b]	20.86[b]	225.66[a]	232.81[a]	247.42[a]	18.21	< 0.01

[1] Aliquots from the beef liposome model system were analyzed for fatty acid composition at the Kansas Lipidomics Research Center at Kansas State University (Manhattan, KS).

[2] Free fatty acids (total acyl carbons: total double bonds)

[3] Mass/charge ratio used to identify fatty acids

Table 2.3. Fatty acid combinations identified by product-ion analysis for the most common PC, ePC, and PE species found in this study.

Apparent molecular species (Total acyl carbons: total double bonds)	Fatty acid combinations identified	Relative abundance (%)
PC		
32:1	16:0/16:1	59.45
34:3	16:1/18:2	72.35
	16:0/18:3	22.20
36:1	18:0/18:1	81.70
36:2	18:1/18:1	47.22
	18:0/18:2	43.62
	16:0/20:2	3.80
36:3	18:1/18:2	83.39
	16:0/20:3	10.56
	18:0/18:3	6.05
36:4	16:0/20:4	65.32
	18:2/18:2	19.00
	18:1/18:3	7.90
36:5	16:1/20:4	69.36
	16:0/20:5	18.13
ePC		
34:1	18:1/16:1	40.16
34:2	18:0/18:2	44.57
	18:1/18:1	41.14
34:4	18:2/18:2	47.12
	18:3/18:1	33.93
36:1	18:0/18:1	54.64
36:2	18:0/18:2	44.22
	18:1/18:1	37.82
36:3	18:1/18:2	78.13
	16:0/20:3	9.51
36:4	18:2/18:2	46.10
	16:1/20:3	22.73
	16:0/20:4	21.60
36:5	16:1/20:4	78.69
PE		
36:2	18:0/18:2	87.44
	18:1/18:1	9.89
36:3	18:1/18:2	87.65
	18:0/18:3	8.75
38:4	18:0/20:4	88.37
	18:1/20:3	4.97
38:5	18:1/20:4	60.34
	18:0/20:5	18.23
	16:1/22:4	8.37

	16:0/22:5	6.50

PC = Phosphatidylcholine; ePC = Ether-linked PC; PE = Phosphatidylethanolamine

Table 2.4 Interaction of treatment x retail display on hydrophilic and lipophilic ORAC[1] and TBARS[2] (n=240) of a beef liposome model system treated with various combinations of PLA2 and aPLA2.

		No PLA2			PLA2 (4 µg/mL)				
Assay	Days[3]	PL	aPLA10	aPLA20	PLA2	PLA2+aPLA10	PLA2+aPLA20	SEM	P-value
Hydrophilic ORAC, nmol TE/mg of PL								5.42	< 0.01
	0	55.35^{Cc}	44.22^{Cc}	51.71^{Bc}	104.20^{Cb}	123.10^{ABa}	118.50^{ABab}		
	1	63.19^{BCb}	58.46^{Bb}	52.42^{Bb}	127.00^{Ba}	117.20^{Ba}	130.90^{Aa}		
	4	84.86^{Ac}	82.07^{Ac}	78.77^{Ac}	141.80^{Aa}	130.10^{ABab}	116.80^{Bb}		
	7	75.45^{ABb}	76.50^{Ab}	78.70^{Ab}	134.30^{ABa}	133.30^{Aa}	129.60^{ABa}		
Lipophilic ORAC, nmol TE/mg of PL								1.53	< 0.01
	0	7.44^{Bc}	10.76^{Abc}	12.31^{Bb}	16.86^{Aa}	16.75^{Aa}	18.01^{Aa}		
	1	13.50^{Aa}	11.23^{Aa}	13.49^{ABa}	10.17^{Ba}	13.46^{ABa}	13.57^{Ba}		
	4	10.97^{ABa}	10.31^{Aa}	7.76^{Ca}	10.52^{Ba}	8.21^{Ba}	9.73^{Ca}		
	7	12.50^{Aab}	13.29^{Aab}	16.02^{Aa}	13.32^{ABab}	12.95^{ABab}	9.69^{Cb}		
TBARS, µg MDA/mg of PL								6.21	< 0.01
	0	47.04^{Dab}	43.66^{Db}	43.77^{Db}	56.71^{Da}	56.30^{Da}	50.47^{Dab}		
	1	74.48^{Cabc}	68.35^{Cc}	66.52^{Cc}	80.48^{Ca}	81.28^{Ca}	77.40^{Cab}		
	4	119.90^{Bb}	105.20^{Bc}	103.50^{Bc}	138.10^{Ba}	131.80^{Bab}	125.00^{Bb}		
	7	142.20^{Ab}	127.20^{Ac}	120.90^{Ac}	162.70^{Aa}	160.70^{Aa}	154.00^{Aa}		

[A-D] Within a column of an assay, means without a common superscript differ at $P < 0.05$.

[a-c] Within a row, means with different superscripts differ at $P < 0.05$.

[1] Oxygen radical absorbance capacity assay to measure antioxidant capacity.

[2] 2-thiobarbituric reactive substances assay to measure lipid oxidation.

[3] Liposome model system was exposed to retail display and aliquots were taken on 0, 1, 4 and 7 days.