Exploring the effects of incorporating egg powder from hens immunized with anti-phospholipase α2 to ground striploin on its shelf-life

by

Carolina Velasco Ayala

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Elizabeth A. E. Boyle

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Abstract

Lipid oxidation is the primary non-microbial degradation mechanism in beef. Phospholipase α2 (PLA2) may enhance this reaction during postmortem storage and retail display by degrading phospholipids into free unsaturated fatty acids and lysophospholipids. Antiphospholipase α2 (aPLA2) could potentially inhibit PLA2 by binding to it before PLA2 degrades phospholipids. Past research has shown that aPLA2 can be mass-produced in eggs from hens immunized against PLA2, and the resulting egg can be spray -or freeze-dried as egg powder to preserve the antibody activity. The present study aimed to determine the effects of incorporating three different levels of egg powder containing antiphospholipase α2 (aPLA2) and assess its potential to extend ground striploin shelf-life by inhibiting phospholipase α2 (PLA2). Enzymatic activity of aPLA2 used in this study was confirmed through an enzyme-linked immunosorbent assay (ELISA). Ten vacuum-packaged USDA choice striploins were obtained from different carcasses 2 d postmortem. On the following day, each loin was ground, divided into 4 batches, hand mixed with 0, 0.4, 0.8, or 1.6% of dried egg powder containing aPLA2, vacuum packaged, and held for 14 d at 2°C ± 2°C. Afterwards, treatments were divided into 114 g batches, formed into patties, randomly assigned into three display times (0, 4, and 7 display days), and displayed under fluorescent lighting at 2°C ± 2°C in a coffin-style retail case in aerobic packaging. Percent visual discoloration and instrumental color measurements were taken on d 7 patties for all treatments. At the end of each designated display period, patties were vacuum packaged and stored at -80°C until pulverized with nitrogen. Proximate analysis (moisture, protein, and fat), phospholipid composition through thin-layer chromatography (TLC), and fatty acid (FA) profile were measured on d 0 samples from all treatments. Lipid oxidation, antioxidant capacity (through oxygen radical absorbance capacity, ORAC), and pH

were measured on d 0, 4, 7 samples from all treatments. The aPLA2 egg powder additions had no effect on discoloration or instrumental color measurements ($P<0.05$), although throughout the 7 d of retail display, a* and b* values decreased, and visual discoloration increased ($P<0.05$). The L* values were unaffected by display day or treatment ($P>0.05$). The addition of aPLA2 egg powder decreased the moisture content for the 1.6% treatment when compared to other treatments ($P<0.05$), but protein and fat percentages did not differ across treatments ($P>0.05$). Lipid oxidation increased ($P<0.05$) for all treatments throughout the 7 d display periods. Additionally, striploin patties containing 1.6% egg powder had higher ($P<0.05$) lipid oxidation than the rest of the treatments. The results for TLC phospholipid classification showed no difference for sphingomyelin (SM) across treatments ($P>0.05$). Phosphatidylethanolamine relative percentage was higher for the control when compared to the 1.6% treatment ($P<0.05$) and phosphatidylcholine relative percentage was higher for the 1.6% treatment compared to the control and 0.4% treatment ($P<0.05$). Display day pH had a main effect ($P<0.05$), increasing on d 7. Additionally, pH increased as more aPLA2 egg powder was incorporated into ground striploin ($P<0.05$). The addition of 1.6% egg powder to ground striploin increased the relative percentage of C11-18:1 trans, C18:2, C18:3, C20:1, and C22:6, but decreased the relative percentage of C17:0, and C17:1 when compared to other treatments ($P<0.05$). Interestingly, the 0.8% treatment showed a higher hydrophilic antioxidant capacity than the control and the 1.6% treatment. Adding aPLA2 egg powder had an effect on striploin a* values over time, moisture content, fatty acid profile, phospholipid composition, lipid oxidation, and hydrophilic antioxidant capacity. However, these reactions could be attributed to components introduced by the egg powder, not necessarily due to the aPLA2 content. The binding of aPLA2 to PLA2 was

demonstrated through ELISA, but in this study, the efficacy of aPLA2 activity did not decrease ground striploin lipid oxidation.

Table of Contents

List of Figures viii
List of Tables ix
List of Abbreviations x
Acknowledgements xiii
Dedication xv
Chapter 1 - Lipid oxidation in meat and phospholipase α2 mechanisms 1
1. Introduction 1
2. Lipids in meat 2
2.1 Lipid classifications 2
2.1.1 Non-polar lipids 2
2.1.2 Polar lipids 3
3. Lipid oxidation 5
3.1 Mechanisms for lipid oxidation 5
3.2 Antioxidants 8
3.2.1 Antioxidants mechanisms 8
3.2.2 Antioxidants in meat 9
4. Phospholipase α2 10
4.1 The sPLA2 family 11
4.1.1 Inhibitors of the sPLA2 family 12
4.2 The cPLA2 family 13
4.2.1 Inhibitors of the cPLA2 family 14
4.3 The iPLA2 family 14
4.3.1 Inhibitors of the iPLA2 family 15
4.4 The PAF-AH family 15
4.4.1 Inhibitors of the PAF-AH family 16
4.5 The Lysosomal PLA2 family 16
4.6 Additional PLA2 inhibitors 16
5. Conclusion 17
7. References 18

Chapter 2 - Exploring the effects of incorporating egg powder from immunized hens with antiphospholipase α2 to ground striploin on its shelf life 25
Abstract 25
1. Introduction 26
2. Materials and methods 28
2.1 Egg powder fabrication 28
2.2 Enzyme-Linked Immunosorbent Assay (ELISA) 28
2.3 Sample collection, fabrication, and treatment application 29
2.4 Color descriptors in simulated retail display 30
2.5 Proximate analysis 31
2.5.1 Moisture analysis 31
2.5.2 Protein analysis 31
2.5.3 Lipid analysis 31
2.6 Lipid oxidation 32
2.7 Phospholipid extraction and Thin-Layer Chromatography (TLC) 32
2.7.1 Solid-Phase Extraction (SPE) of lipid classes 32
2.7.2 Thin-layer chromatography 33
2.8 pH analysis 33
2.9 Fatty acid analysis 34
2.10 Antioxidant capacity through Oxygen Radical Absorbance Capacity (ORAC) 35
2.11 Statistic analysis 36
3. Results and discussion 37
3.1 Color descriptors in simulated retail display 37
3.2 Moisture, protein, and fat content 38
3.3 Lipid oxidation 39
3.4 Thin-layer chromatography of phospholipids 40
3.5 pH results 40
3.6 Fatty acid profile 41
3.7 Oxygen radical absorbance capacity (ORAC) 42
Implications 43
References 45

List of Figures

Figure 1. Standard curve from enzyme-linked immunosorbent assay used to calculate the titer of extracted antiphospholipase α2 from egg powder. Results yielded an approximate titer of 16 mg of antibody/kg egg power. 54

Figure 2. Schematic display of the sample preparation process for each striploin. 54

Figure 3. A) Main effect of display days for discoloration %. B) No main effect of display days for L*. C) Treatment × display day interaction for a*; the superscripts represent the display effect only. D) Main effect of display days for b*. The treatments consisted of beef patties added with 0, 0.4, 0.8 or 1.6% of egg powder containing antiphospholipase α2. $^{a-h}$ Values with different superscripts indicate a difference at $P<0.05$..... 55

Figure 4 Representative Thin-Layer Chromatography plate. PE = phosphatidylethanolamine; PC = phosphatidylcholine; SM = sphingomyelin; LPC = lysophosphatidylcholine. 56

List of Tables

Table 1. Main effect of treatments for proximate analysis, phospholipid profile, lipid oxidation, and antioxidant capacity of beef patties added with 0%, 0.4%, 0.8%, and 1.6% of egg powder containing antiphospholipase α2. 57

Table 2. Main effect of display days for lipid oxidation and pH of beef patties added with 0%, 0.4%, 0.8%, and 1.6% of egg powder containing antiphospholipase α2. 58

Table 3. Comparison of fatty acid profiles of beef patties added with 0%, 0.4%, 0.8%, and 1.6% of egg powder containing aPLA2. 59

List of Abbreviations

AA	Arachidonic acid
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
aiPLA2	Acidic Ca2+ independent PLA2
ATK	Arachidonyl trifluoromethyl ketone
AUC	Area under the curve
BCA	Bicinchoninic acid assay
BEL	Bromoenol lactone
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
Ca^{2+}	Calcium
CAT	Catalase
CP	Diphosphatidylglycerol, Cardiolipin
cPLA2	Cytosolic PLA2
DHA	Docosahexaenoic acid
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EP	Egg powder
EPA	Ecopentanoic acid
ETYA	5,8,11,14-Eicosatetraynoic acid
FA	Fatty acid
FFA	Free fatty acid
FID	Flame ionization detector

GC	Gas chromatography
GSH-Px	Glutathione peroxidase
HELSS	Tetrahydro-3-(1-naphtalenyl)-2H-pyran-2-one
iPLA2	Calcium-independent PLA2
LPC	Lysophosphatidylcholine
Lp-PLA2	Lipoprotein-associates PLA2
MDA	Malondialdehyde
MUFA	Monounsaturated fatty acid
ORAC	Oxygen radical absorbance capacity
PA	Phosphatidic acid
PAF-Ahs	Platelet-activating factor acetylhydrolases
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PLA1	Phospholipase α1
PLA2	Phospholipase α2
PS	Phosphatidylserine
PSE	Pale, soft, exudative
PUFA	Polyunsaturated fatty acid
RMCD	Randomly methylated-cyclodextrin
ROS	Reactive oxygen species
SM	Sphingomyelin
SOD	Superoxide dismutase
SPE	Solid-phase extraction

sPLA2	Secreted PLA2
TBARS	2- thiobarbituric acid reactive substances
TBHQ	Tert-butylhydroxyquinone
TE	Trolox equivalent
TLC	Thin-layer chromatography

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Dedication

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Chapter 1 - Lipid oxidation in meat and phospholipase α2 mechanisms

1. Introduction

The appalling statistics for food waste are well established. In 2021, the United Nations Environment Programme reported an estimated 845 million metric tons of food waste worldwide. Even with this vast amount of unconsumed food, the authors of the report state that previous approximations have been significantly underestimated (Forbes et al., 2021), implying that the problem is of a greater magnitude than what it is usually considered.

Lipid oxidation in meat poses a great problem for meat's shelf life. As the main non-microbial cause of meat degradation, lipid oxidation causes a reduction of nutritional value through the loss of vitamins and essential fatty acids, a gradual reduction of sensory quality through changes in color, texture, and the generation of rancid odor and flavor, ultimately impacting consumer acceptance (Domínguez et al., 2019; Frankel E, 2012; Kumar et al., 2015). The unfavorable reaction starts with unsaturated fatty acids interacting with molecular oxygen via free radical mechanism. This onset triggers a cascade of effects that produce hydroperoxides, lipid oxidation primary products, that will, in turn, react to create undesired byproducts responsible for sensory and nutritional degradation (Domínguez et al., 2019).

A major liberator of free fatty acids is the enzyme family phospholipase (Wu et al., 2021). The subfamily phospholipase α2 (PLA2) is known to cleave off unsaturated fatty acids from a phospholipid's sn-2 position, releasing a lysophospholipid and a free fatty acid. Different groups within this family work under diverse conditions in a varied range of living organisms (Murakami et al., 2011). Multiple inhibitors have been studied because of the beneficial consequences of muting PLA2's enzymatic activity (Nikolaou et al., 2019). The present work

aims to relate the pathways of PLA2 with possible inhibitors as well as the effects of these two in living organisms and in meat oxidation.

2. Lipids in meat

Generally, meat composition comprises 75% water, 19% protein, 2.5% fat, 1.2% carbohydrates, and 1.65% nitrogen compounds (excluding protein) (Beltrán & Bellés, 2018). However small the lipidic percentage seems to be, it is the substrate for numerous reactions in biological systems. The range of functionality is attributed to the wide range of lipid types and properties.

2.1 Lipid classifications

Lipids are a complex macromolecule group classified based on their solubility. Non-polar lipids are soluble in organic solvents and comprise two main groups: triacylglycerol and diacylglycerols, and free fatty acids. Polar lipids, in opposition, are only soluble in polar solvents like water. These classes and subclasses of lipids participate in different reactions and cover a wide range of functions, as discussed below (Frankel, 2012c).

2.1.1 Non-polar lipids

Triacylglycerols. Also referred to as triglycerides, triacylglycerols are the main lipid component in food (Frankel, 2012c). These lipids are considered animals' primary energy storage form. Their structure comprises a glycerol head esterified to three fatty acids, which accommodate in three available positions referred to as sn-positions (Frankel, 2012c). Diacylglycerols are sometimes grouped in this classification since the difference is one less fatty acid chain attached to the glycerol head.

Free fatty acids. Fatty acids are chains of aliphatic carboxylic acids. Their carbon-based chain can be either saturated or unsaturated (Frankel, 2012c). This property grants different characteristics that will influence physical structure, functionality, and participation in chemical reactions. A saturated fatty acid will create a more rigid lipid matrix, will not be as prone to lipid oxidation, and will usually have a higher melting point. Unsaturated fatty acids can be monounsaturated, meaning there is only one double bond in the carbon chain, and they behave mostly like saturated acids. Polyunsaturated fatty acids, conversely, are common substrates for lipid oxidation due to their unstable instaurations (Campbell & Farrell, 2015).

2.1.2 Polar lipids

Phospholipids. Phosphoglycerides, or phospholipids, are polar lipids of high interest for lipid oxidation. Phospholipids' structure is of biological importance because of their amphipathic characteristics. They are composed of a hydrophilic head (a 3-glycerophosphoric acid) and a hydrophobic tail (two fatty acid chains) which are esterified to an alcohol group. Because of this, phospholipids are the primary component of cell membranes, as they enable the selective passing of substances in and out of the cells (El-Bacha & Torres, 2015; Frankel, 2012c).

Like triacylglycerols, the position of the fatty acids is described as sn-positions. The sn-1 position of animal origin is occupied mainly by saturated fatty acids, the sn-2 position hosts polyunsaturated fatty acids, and the sn-3 position is for the link of the phosphate residue (Frankel, 2012c; Narváez-Rivas & León-Camacho, 2015). The fatty acid chain in the sn-2 position is commonly a substrate for lipid oxidation reactions because of its unsaturation. Phospholipases can hydrolyze both sn-positions; phospholipase α1 cleaves off the fatty acid in the sn-1 position, while phospholipase α2 hydrolyzes the fatty acid in the sn-2 position (Frankel, 2012c).

Moreover, phospholipids are divided into two categories: glycerophospholipids and sphingolipids. The former category comprises lipids usually composed of two fatty acids, a glycerol molecule, a phosphate group (phosphoric acid), and an alcohol group that will bond with an organic group (Montealegre et al., 2014). The phospholipid subclass will receive a different name depending on the organic group it attaches to. Common glycerophospholipids include phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), diphosphatidylglycerol or cardiolipin (CP), and on the rare occasion that one fatty acid is cleaved off from a PC, a lysophosphatidylcholine (LPC) is formed (El-Bacha & Torres, 2015; Montealegre et al., 2014; Uphoff et al., 2008). Sphingolipids also bind to organic groups that assign a different nomenclature; binding to chlorine will form sphingomyelin (SM), and when binding to hydrogen, a ceramide is formed (El-Bacha & Torres, 2015).

Some lipids are of high interest when observing lipid oxidation. Phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin are some examples of such lipids. Gobley identified phosphatidylcholine in the 1840s as a significant constituent of egg yolk (El-Bacha & Torres, 2015; Stewart & Day, 2012). Phosphatidylcholine is the main component of mammalian cell membranes (Uphoff et al., 2008). It is vulnerable to lipid oxidation as it is described as a "sink" for free radicals because of its zwitterionic nature (Stewart & Day, 2012; Uphoff et al., 2008). Because of this, some studies have been made to assess PC's antioxidant properties; a study on corn oil concluded that, under different conditions, PC could be a catalyst or a non-contributor to lipid oxidation (Kim et al., 2015). Phosphatidylethanolamine was described for the first time in 1884, and it is synthesized chiefly from PS decarboxylation. Like PCs, PEs are structural phospholipids in cell membranes (Vance et al., 2008). PCs generally have more saturations than PE (MacGibbon & Taylor, 2011). This fact would make PC more susceptible to

lipid oxidation; however, they are both considered synergists in the reinforcement of antioxidants (Frankel, 2012c). SMs are also related to lipid oxidation because of their zwitterionic nature and are considered signaling lipids (Bolander, 2004). They are associated with cell stress, as they are broken down under low cellular viability and can be re-generated if a stress response is activated (Uphoff et al., 2008).

To summarize, lipids have different characteristics and classifications. Some of these characteristics attribute a higher or lower proficiency to participating in oxidation. The general mechanisms for lipid oxidation are described ahead.

3. Lipid oxidation

Simply, lipid oxidation is the reaction between unsaturated free fatty acids and molecular oxygen through a free radical mechanism. As stated before, lipid oxidation brings undesirable effects on meat, such as degradation of nutritional value, loss of essential fatty acids, and unfavorable changes in color, texture, and flavor (Domínguez et al., 2019). Different pathways can lead to lipid oxidation; the general mechanisms are described below.

3.1 Mechanisms for lipid oxidation

There are different mechanisms in which lipid oxidation can occur: autoxidation, enzyme-catalyzed oxidation, and photooxidation. Autoxidation is the most common process happening in meat (Domínguez et al., 2019) Autoxidation is the reaction that refers to the direct interaction between molecular oxygen and organic compounds, like free fatty acids (Frankel, 2012b). This process is generally described as a simplified three-step reaction. Initiation is the

first step in autoxidation; an unsaturated lipid (LH) loses a hydrogen radical (H•), forming an alkyl radical of unsaturated lipid (L•) in the presence of an initiator (I), as shown below.

$$LH \xrightarrow{I} IH + L^{\bullet} \quad (1)$$

There are different initiators; while specific oxygen molecule accommodations cannot interact directly with unsaturated fatty acids, the presence of heat, redox metals, and light sensitizers aid as initiators. Moreover, oxygen molecules with favorable accommodation are usually responsible for rapid hydroperoxide formation under photosensitized conditions. These quickly formed hydroperoxides serve as important initiators for further free radical reactions. Hydroperoxides are the primary lipid oxidation product (Frankel, 2012b).

The second phase of lipid oxidation is propagation. This phase can be summarized in two general reactions. The first reaction is the rapid formation of peroxyl radicals (LOO•) from the interaction of molecular oxygen and an alkyl radical (L•) (Frankel, 2012b).

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (2)$$

As shown below, the peroxyl radicals will interact with other unsaturated lipids in a hydrogen transfer to form more hydroperoxides (Frankel, 2012b).

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \quad (3)$$

Propagation forms hydroperoxides at a slow rate because of the hydrogen transfer reaction; in exchange, the reaction becomes selective to weakly bonded hydrogens (Frankel, 2012b), such as the ones in covalent bonds from unsaturated fatty acids. Since this phase produces substrates for itself through these two reactions, it becomes repetitive and promotes more lipid oxidation, hence the name.

The final phase for lipid oxidation is known as termination. Lipid oxidation can end through different pathways. Once the concentration of peroxyl radicals reaches high levels, two

LOO• can interact with each other to form non-radical products (4), thus ending lipid oxidation. Another possibility is having a reaction between two LOO• to form an unstable intermediate that decomposes into non-radical products and an oxygen molecule (5) (Frankel, 2012b).

$$LOO^{\bullet} + LOO^{\bullet} \rightarrow non - radical\ products \quad (4)$$

$$LOO^{\bullet} + LOO^{\bullet} \rightarrow [LOOOOL] \rightarrow non - radical\ products \quad (5)$$

Though these two reactions are based on the principle of high substrate concentration, other reactions include introducing other components like metals. Moreover, reactions at different temperatures and pressures can stop lipid oxidation reactions; one of high importance is the use of antioxidants, in which the antioxidant donates a proton to a peroxyl radical (6) (Frankel, 2012b) Antioxidants will be further discussed later on in this review.

$$LOO^{\bullet} + AH \leftrightarrow LOOH + A^{\bullet} \quad (6)$$

While autoxidation is the primary mechanism for lipid oxidation, lipid photooxidation must also be considered, as many meat products are subject to retail display. Lipid photooxidation happens when sensitizers, such as myoglobin or hemoglobin, absorb light energy. This reaction also produces hydroperoxides, which ultimately trigger the propagation phase of free radical chain reactions (Domínguez et al., 2019). Similarly, enzymatic lipid oxidation differs slightly from autoxidation. Instead of a free radical initiating lipid oxidation, an enzyme will catalyze the reaction that forms hydroperoxides (Domínguez et al., 2019), prompting the propagation of lipid oxidation.

In meat, oxidative stability can be affected in three main stages. After slaughter, anaerobic conditions imply loss of intrinsic antioxidant mechanisms and enzymes, and prooxidants can be present. Additionally, the composition of the meat and factors like myoglobin/hemoglobin content can also promote or retard lipid oxidation. During processing,

parameters like temperature, pressure, oxygen presence, unit operations, and antioxidant treatments can alter the oxidative stability of meat. Finally, during storage, atmospheric conditions, packaging conditions, temperature, lighting conditions, and antioxidant treatments are all factors that affect meat's stability (Kumar et al., 2015).

3.2 Antioxidants

The mechanisms mentioned above for lipid oxidation might differ on how lipid oxidation starts; however, free radicals will be involved eventually. Free radicals are highly reactive; nonetheless, they can be inhibited by antioxidants. Antioxidants essentially are radical acceptors that react with other free radicals (Frankel, 2012c).

3.2.1 Antioxidants mechanisms

Briefly, antioxidants can act through different mechanisms. Chain-breaking antioxidants (AH) act by hindering chain propagation or initiation through hydrogen-atom transferring and by donating atoms (7) (Frankel, 2012a).

$$L^{\bullet} + AH \rightarrow LH + A^{\bullet} \qquad (7)$$

Antioxidants can also inhibit lipid oxidation by mechanisms that do not interfere with free-radical chains. Initiator inhibitors and preventive antioxidants are usually metal inactivators, such as ethylenediamine tetraacetic acid (EDTA), that deactivate metal ions that would otherwise initiate lipid oxidation. Hydroperoxide destroyers are antioxidants that inhibit the decomposition of hydroperoxides into reactive substances by forming stable or inactive products. Phenols like Trolox, a water-soluble carboxylic acid, are examples of this type of antioxidant. Additional antioxidants include ultraviolet light deactivators and synergists/ multi-component systems (Frankel, 2012a).

3.2.2 Antioxidants in meat

Antioxidants are also classified by source as natural or synthetic antioxidants. Moreover, natural antioxidants can be enzymatic or non-enzymatic (Atta et al., 2017). Meat inherently has some antioxidant capacity, mainly attributed to three particular enzymes: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), which would fall under the natural enzymatic antioxidants category. These enzymes either defend cells against reactive oxygen species (ROS) and free radicals or reduce hydrogen peroxide molecules in the cytoplasm. Nevertheless, these enzymes act at a low level, and studies have shown that their activity is reduced post-mortem in beef muscles (Pastsart et al., 2013). Their role is mainly observed as cellular component defenders in live organisms (Atta et al., 2017). For this motive, external antioxidants are commonly added to meat and meat products to delay lipid oxidation. Non-enzymatic natural antioxidants include phenolic compounds, such as tocopherols and phenolic acids. These antioxidants regularly work by scavenging free radicals, chelating prooxidants, and chelating metals, among other pathways. Naturally sourced antioxidants that have been studied in meat and meat products include fruits or fruit byproducts like date pits, plums, grapes, and pomegranates (Amany et al., 2012; Bañón et al., 2007; Karre et al., 2013). Spices and herbs such as lemon leaves, rosemary, and oregano (Akarpat et al., 2008; Chan et al., 2007; Han & Rhee, 2005; Karre et al., 2013; Movileanu et al., 2013) have also been used. While synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butylhydroxyquinone (TBHQ) are useful to the meat industry, they are not well received by consumers because of the shift for more "natural" products (Atta et al., 2017; Kumar et al., 2015). It can be confidently stated that antioxidants will be studied continuously in the meat

science industry. Researchers are looking for novel ways to limit lipid oxidation; antibodies and enzymes are being considered as possible alternatives.

4. Phospholipase α2

Phospholipases α (PLA2) are ubiquitous enzymes in plants and mammals (Murakami et al., 2020; Ryu, 2004). This superfamily is conformed by hydrolytic enzymes acting on ester bonds (EC 3.1.1). It is divided into two subtypes, phospholipase α1 (PLA1) and phospholipase α2 (PLA2), both of which hydrolyze a carboxylic-ester from a phospholipid yielding a lysophospholipid and a free fatty acid. The difference between these subtypes is the ester bond from phospholipids on which they act; PLA1 cleaves off fatty acids from the sn-1 position, while PLA2 cleaves off fatty acids from the sn-2 position (Ryu, 2004).

The PLA2 superfamily participates in a wide variety of biological functions. As it releases free fatty acids and lysophospholipids, it also produces lipid mediators, aids in membrane remodeling, and disturbance of PLA2-mediated metabolism is regularly related to diverse diseases (Murakami et al., 2020; Six & Dennis, 2000). Just in meat and meat products, PLA2s have been implied as lipid antioxidants in fish and pork, pale, soft, exudative (PSE) promotors in pork and broilers, and restrictors for weight intake in beef (Chen et al., 2010; Cook, 2004; Lourenço Soares et al., 2009; Mercadante et al., 2015; Soares et al., 2003; Tatiyaborworntham & Richards, 2017; Whalin et al., 2022).

PLA2s' vast range of functionality could be attributed to the different families that compose the PLA2 superfamily; each has its own set of characteristics that define its functionality in diverse biological processes. The classification of families regularly includes various groups, and the five main ones would be secreted PLA2 (sPLA2), cytosolic PLA2

(cPLA2), calcium-independent PLA2 (iPLA2), platelet-activating factor acetylhydrolases (PAF-AHs), and lysosomal PLA2 (Murakami et al., 2011). The sPLA2, cPLA2, and iPLA2 families work primarily on phospholipids (Murakami et al., 2011), and some are generally stimulated by concentrations of PC and release amino acids mostly from PC and PE (Bolander, 2004). Different families have particularities which will be discussed below.

4.1 The sPLA2 family

The sPLA2 family is known for its low molecular mass and calcium (Ca^{2+}) dependency. This family was first observed in snake venom and cows' pancreases (Burke and Dennis, 2009). Nowadays, it is known to be present in multiple organisms such as yeasts, nematodes, flies, and plants (Murakami et al., 2011). As the name implies, these enzymes are secreted; therefore, their primary targets reside in extracellular space, such as membranous and soluble phospholipids. Residues from sPLA2s' enzymatic activity generally include arachidonic acid (AA), ω-3 fatty acids like ecopentanoic acid (EPA) and docosahexaenoic acid (DHA), and lysophospholipids like LPC (Murakami et al., 2011). These enzymes work with a conserved His-Asp catalytic dyad in their active site under high concentrations of Ca^{2+}.

Different groups within the sPLA2 family have dissimilar substrate specificity. Groups sPLA2-IB, -IIA, and –IIE do not show preference over fatty acids species, while the sPLA2 group is partial to fatty acids with a lower profile of unsaturation, and groups -IID, -IIF, -III, and –X often work with polyunsaturated fatty acids (Murakami et al., 2020).

Moreover, the differing preferences for the sPLA2 family provide flexibility to participate in biological functions. The sPLA2-IB group is usually referred to as a pancreatic digestive enzyme. It shows a higher binding force to anionic phospholipids than to

phosphatidylcholine; however, enzymes work on both and yield mainly LPC (Murakami et al., 2011, 2020). While this enzyme can help in some systems as a defense against larvae, overstimulation can also promote diet-induced obesity, insulin resistance, and atherosclerosis because of its digestive activities (Hui, 2019; Murakami et al., 2020). This range portrays an essential fact about enzymes; their functions are necessary, and their impact can vary according to substrate availability and environmental conditions. Group IIA sPLA2s are known for their bacterial and inflammatory activities. These enzymes can degrade bacterial membranes by hydrolyzing PEs of organisms like *Staphylococcus aureus* (Murakami et al., 2020) Conversely, sPLA2-IIDs show anti-inflammatory properties. Said "resolving sPLA2s" hydrolyze PE to release anti-inflammatory ω3 polyunsaturated fatty acids (PUFA). Other sPLA2s, like –IIF, -V, and X, participate in the hydrolysis of plasma to release free fatty acids, which inhibit parasite growth in vitro (Murakami et al., 2020). Whether related to obesity, colon cancer, aortic protection, sperm activation, or asthma (Murakami et al., 2020), sPLA2s vary widely in functionality, making them thrive in different environments.

4.1.1 Inhibitors of the sPLA2 family

Indoles are ubiquitous molecules in nature capable of binding various receptors with high affinity (Humphrey & Kuethe, 2006). These molecules are also known to be potent sPLA2 inhibitors and are used for pharmaceutical purposes to prevent disease models in experimental animals. The use of indoles is specific for different models. As stated before, different sPLA2s serve a wide range of functions, so the specificity of use is well researched before implementation to avoid undesired side effects. Some examples of these inhibitors include Indoxam, Me-Indoxam, and Varespladib (Murakami et al., 2011). Animal sPLA2 inhibitors like aristolochic acid in high concentrations, manoalide, quercetin, flavonoids like rutin, and

5,8,11,14-eicosatetraynoic acid (ETYA) have also been reported (Böhl et al., 2006; Lindahl & Tagesson, 1997; Ryu, 2004).

4.2 The cPLA2 family

The intracellular cytosolic PLA2 family is exclusive to vertebrates, it employs a Ser/Asp catalytic dyad in its active site, and it is known to have a C2 domain at its N-terminal region in almost all its enzymes. This domain is of importance because it causes a Ca^{2+} dependent association with membranes (Murakami et al., 2020). The cPLA2 family can catalyze diverse reactions, acting as PLA2, PLA1, lysophospholipases, transacylases, and lipases (Murakami et al., 2011). Said functionalities have been commonly observed in mice and humans (Burke and Dennis, 2009), beef, and rabbits (Gelb et al., 1994). Similar to sPLA2s, cPLA2s are subdivided into categories. In the case of the latter, the division is marked through the six isoforms (α, β, γ, δ, ε, and ζ). Some cPLA2s are particular to certain phospholipids; cPLA2α, for example, is specific to phospholipids containing AA or EPA (Murakami et al., 2020). In human platelets, cPLA2αs are responsible for lipid mediation generation and energy flux and produce lipid mediators in numerous pathogeneses. Unlike other cPLA2s, cPLA2γ lacks the C2 domain; hence it does not require Ca^{2+} to function. It is considered to play a role in lipid droplet formation. In the presence of viruses, such as Hepatitis C (HCV), cPLA2γs is upregulated to aid in the virus' assembly. Other relations have been studied between cPLA2 groups and biological reactions; cPLA2δ shows connections with psoriasis, cPLA2ε is enhanced by PS in the plasma membrane and organelles from endocytic pathways, and cPLA2ζ is related to heart health (Murakami et al., 2020). Evidently, functional versatility is apparent in this family.

4.2.1 Inhibitors of the cPLA2 family

Allergen-induced airway hyper-responsiveness and airway inflammation caused by cPLA2α has been inhibited by arachidonyl trifluoromethyl ketone (ATK). Other developed inhibitors of cPLA2 include pyrrolidine-based inhibitors, indole-based inhibitors, indolylpropanone inhibitors, propanone inhibitors, and 2-oxoamide inhibitors (Murakami et al., 2011).

4.3 The iPLA2 family

The Ca^{2+} independent PLA2 (iPLA2) is also known as the patatin-like phospholipase (PNPLA) family since they all have a patatin domain (Murakami et al., 2020). These enzymes work through a catalytic Ser at their active site (Burke & Dennis, 2009). The classification of this group as phospholipases has been questioned, as newer research shows more lipase-like activity in the majority of these enzymes (Murakami et al., 2011). Similar to sPLA2s, these enzymes are present in eukaryotes like yeasts, flies, and vertebrates. They have roles in homeostatic lipid metabolisms rendering them essential to life. Different groups contribute diverse functionalities. The subgroup iPLA2β is known to have poor fatty acid selectivity and is associated with the plasmatic membrane, mitochondria, endoplasmic reticulum, and the nuclear envelope. Processes in male fertility, neuronal disorders, metabolic diseases, and inflammation are all examples in which iPLA2β shows enzymatic importance. Its inhibition could cause lipid peroxidation deficiency, which could lead to impaired neurotransmission in organisms such as *Drosophila*. Lipid mediators can be generated by iPLA2γ, which is different from other subgroups in the sense that it can act as a PLA1 when phospholipids of sn-2 positions are abundant. Some groups, like iPLA2δ (PNPLA6) and iPLA2θ (PNPLA7), have lysophospholitic activity; they cleave off

both sn-1 and sn-2 fatty acids. Disruption of these two subgroups can lead to motor neuron diseases. In meat, iPLA2 has been associated with a higher incidence of PSE pork (Chen et al., 2010). There are more groups of iPLA2 enzymes, all with different characteristics, showing once more the versatility even within enzyme subfamilies (Murakami et al., 2020).

4.3.1 Inhibitors of the iPLA2 family

Briefly, iPLA2 is inhibited by bromoenol lactone (BEL) and antisense oligonucleotides, which may decrease lysophosphatidylcholine levels. Additionally, iPLA2-aided cell proliferation can be compromised when substrate imbalances alter the enzyme's activity. Moreover, calmodulin has been reported to work as a regulatory substance for iPLA2β; according to the cell's necessities, it binds or unbinds to halter or promote enzymatic activity (Murakami et al., 2011). This example displays the importance of the conditions in cells, or systems, for enzyme functionality. Other inhibitors of iPLA2 include tetrahydro-3-(1-naphtalenyl)-2H-pyran-2-one (HELSS) and ETYA (Ryu, 2004).

4.4 The PAF-AH family

Platelet-activating factor acetylhydrolases are classified into two PLA2 groups, and they both hydrolyze acetyl groups from the sn-2 position of platelet-activating factors through a catalytic serine (Burke & Dennis, 2009). PAH-AHs can be either extracellular or intracellular enzymes. Their functions aid in immunological responses involving plasma. Lipoprotein-associates PLA2 (Lp-PLA2) belongs to this subfamily and is related to allergic reactions and atherosclerosis due to its capability to hydrolyze oxidized phospholipids (Murakami et al., 2020).

4.4.1 Inhibitors of the PAF-AH family

Like all enzymes, regulation of enzymatic activity is crucial in biological systems. Darapladib, a plasma-type PAF-AH inhibitor, reduces LCP content in lesions and has been shown to hinder the development of atherosclerosis diseases in hypercholesterolemic swine (Murakami et al., 2011).

4.5 The Lysosomal PLA2 family

This family group is known by various names, including LPLA2 and group XV PLA2. It shows catalytic activity under slightly acidic conditions and can hydrolyze both sn-fatty acid positions in phospholipids. This ability renders the family a contributor to phospholipid degradation in lysosomes. Enzymes in this family, like acidic Ca^{2+} independent PLA2 (aiPLA2), are related to biological processes ranging from physiological turnover to sperm fertilizing competence (Murakami et al., 2020).

4.6 Additional PLA2 inhibitors

It is important to remark that substrate availability or residue concentrations can also alter enzymatic activity. PLA2 activity is not only dependent on the hydrolysis of the sn-2 positioned fatty acid but also on the concentration of available phospholipids. In other words, interfacial binding of the enzyme to liposomal substrates also alters enzymatic activity (Böhl et al., 2006). The families of PLA2s dependent on calcium could be inhibited by molecules that alter calcium release or the availability in cells, such as pyrrophenones (Nikolaou et al., 2019). Antibodies have been used in the past to attenuate PLA activity. In the 1980s, antibodies were studied to reduce allergenic reactions to bee venom triggered by PLAs (Wetterwalda et al., 1985), and

monoclonal antibodies were used against rat PLA2 as snake venom remedies (Murakami et al., 1988). PLA2 inhibitors are of great interest in pharmaceutical industries because of their potential uses (Nikolaou et al., 2019).

The meat industry could potentially benefit from said inhibition. Egg yolk aPLA2 is already being fed to animals because it binds to microbial and mucosal phospholipases preventing the cleavage of AA from the sn-2 positions, causing a greater uptake of feed (Cook, 2004). Further studies could focus on the potential benefits of inhibiting PLA2, like reducing lipid oxidation, in meat.

5. Conclusion

Lipids determine a wide range of biological functions in different organisms and systems. Lipid profiles and metabolic enzymes, such as PLA2, deserve the focus received because of the potential uses their regulation could produce. Lipid oxidation is undesired, remarkably so in meat products, which is why antibodies that could work as antioxidants need to continue being explored.

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Chapter 2 - Exploring the effects of incorporating egg powder from immunized hens with antiphospholipase α2 to ground striploin on its shelf life

Abstract

The present study aimed to determine the effects of incorporating different levels of egg powder (EP) containing phospholipase α2 antibody (aPLA2) to assess its ability to extend ground striploin shelf-life by inhibiting phospholipase α2 (PLA2) activity. Ten USDA choice striploins were ground, and ground striploin from each striploin was divided into 4 equal batches, mixed with 0, 0.4, 0.8, or 1.6% dried EP containing aPLA2 (w/w). The treatment batches were further divided into 3 display times (0, 4, and 7 d) for retail display. Color descriptors during retail display, lipid oxidation, pH, and antioxidant capacity were measured over retail display. Proximate analysis, and phospholipid and fatty acid (FA) profiles were also measured. Percent visual discoloration and instrumental color measurements were unaffected by aPLA2 EP treatments ($P>0.05$). There was a more lipid oxidation for the 1.6% treatment compared to the 0% treatment ($P<0.05$). The pH values increased with aPLA2 EP content ($P<0.05$). The 1.6% treatment had a higher relative percentage of phosphatidylcholine (PC) compared to the 0% treatment ($P<0.05$), but the lack of lysophosphatidylcholine (LPC) generation on the 0% treatment reflects a lack of detectable level of PLA2 activity. Moreover, the FA profile was altered by the EP addition, as the 1.6% treatment showed a higher relative percentage of FA 11-8:1, 18:2, 20:1, and 22:6 ($P<0.05$). The 0.8% treatment showed a higher antioxidant activity

(*P<0.05*) than the 0% treatment. In this study, the addition of EP containing aPLA2 did not demonstrate any effect to extend striploin shelf-life when incorporated into ground striploin.

Keywords: Lipid oxidation, Phospholipid composition, Fatty acid profile, Discoloration, Antioxidant capacity

1. Introduction

Global food waste is on the rise; ironically, world hunger is still occurring at the same time (Forbes et al., 2021; Karwowska et al., 2021). Meat is a prime subject of food waste in developed countries because consumers have a preference for a bright cherry-red coloration for meat (Ramanathan et al., 2021). A recent study showed that consumers were 25% less likely to purchase a beef product when as little as 20% discoloration was perceived (Lybarger, 2022). When consumers avoid purchasing a meat product with minor discoloration, grocery stores are forced to discount or dispose of the undesired meat (Domínguez et al., 2019). In 2019 alone, a total of $14.2 billion was lost globally due to discoloration (Ramanathan et al., 2021).

A key driver of discoloration in meat and meat products is lipid oxidation, the main non-microbial cause of degradation (Domínguez et al., 2019). Conjugated dienes from unsaturated lipids interact with reactive oxygen species to produce primary and secondary lipid oxidation products (Frankel, 2012b, 2012a) which then participate in subsequent reactions resulting in various meat quality issues such as off-flavors, nutritional value reduction and/or discoloration (Domínguez et al., 2019; Frankel, 2012a; Karre et al., 2013). Therefore, it has always been a priority for the meat industry to develop technology to hinder lipid oxidation, meat discoloration, and ultimately, meat waste.

Phospholipids are known to contain highly unsaturated long FA at their sn-2 position (El-Bacha & Torres, 2016). Chao et al. (2020) hypothesized that phospholipase α2 (PLA2) enzymes could be responsible for phospholipid degradation in aged pork. The PLA2 group of ubiquitous enzymes are found in animals (Murakami et al., 2020) and plants (Ryu, 2004). Chun (2022) confirmed that PLA2s are extremely efficient in cleaving off the FA chain from the sn-2 position of glycerophospholipids, particularly the phosphatidylcholine (PC), in a beef liposome model system. The fact that PLA2 acts as a free fatty acid (FFA) liberator may instigate further implications on lipid oxidation in beef.

The inhibition of PLA2 has been studied for decades in the biomedical field for its relation to inflammatory diseases in humans (Nikolaou et al., 2019). In more recent years, the livestock industry has shown an increased interest in this field by investigating the effect of inhibiting PLA2 by antibody supplementation on the growth performance of steers (Mercadante et al., 2015), broilers (Cook, 2004), and fish (Barry & Yang, 2008). These studies have demonstrated improved growth performance for the respective livestock species when inflammation reactions stimulated by PLA2 are halted by a PLA2 antibody (Cook, 2011).

Furthermore, passive immunity of PLA2 can easily be achieved by immunization of avian maternal bodies, which enables the commercial production of immune-boosted eggs containing high concentration of antiphospholipase α2 (aPLA2) (Cook & Trott, 2010). Furthermore, the development of immune-boosted EP presents the possibility of aPLA2 inclusion in a meat system, which could diminish the degradation of phospholipids by PLA2, thus reducing lipid oxidation. Therefore, the present study aimed to investigate the effects of adding EP from hens immunized with aPLA2 to ground striploin on meat shelf-life.

2. Materials and methods

2.1 Egg powder fabrication

The procedures for obtaining the EP containing aPLA2 were followed according to the methods described by Chun (2022). Briefly, Single Comb White Leghorn laying hens were injected with 0.6 mg sPLA2-IB (10,000 U/g; Bioseutica, Lugano, Switzerland) in three separate injections. Eggs were collected after the final injection for six weeks, and the eggs were dried into powders using a spray dryer (Mini-Glatt 3 Fluid Bed Dryer; Glatt, Binzen, Germany).

2.2 Enzyme-Linked Immunosorbent Assay (ELISA)

A crude solution containing aPLA2 was extracted from the EP as described by Bobeck (2007) with modifications. Briefly, 0.1 g of the antibody EP was homogenized in 1 mL of the acidified PBS (pH= 5) and stored under refrigeration overnight. The mixture was centrifuged, and the aqueous portion was removed and adjusted to a concentration of 1 mg of protein/mL using a bicinchoninic acid assay (BCA) kit (PierceTM BCA Protein Assay Kit; Thermo Scientific, Waltham, MA). The process was repeated with commercial EP for use as a control.

The activity of aPLA2 was evaluated through ELISA as described by Chun (2022) with modifications. Briefly, an immunoGrade 96 well plate (781722; Midwest Scientific, Valley Park, MO) was coated overnight with a 2.4% PLA2 in 50 mM $NaHCO_3$ and subsequently blocked with 1% bovine serum albumin in 1X PBS. The crude aPLA2 extraction and the controls were serial diluted to 1:5¬1:80 with the blocking solution. Purified anti-sPLA2-IB (Ab E Discovery, Waterloo, Wisconsin) was serial diluted with blocking solution and used to prepare a standard curve (1:1,000- 1:6,000 dilutions). Standards, controls, and samples were added into wells and incubated for 30 min at room temperature. Following the incubation, goat anti-chicken IgY with

horseradish peroxidase (A16054; Invitrogen) at 0.25 µg/mL was added to the wells and incubated at room temperature for another 30-min. Subsequently, 1-step TMB-ELISA (Turbo; Thermo Fisher Scientific, Waltham, MA) substrate solution was added to each well and incubated for 5 min at room temperature under darkness. To terminate the reaction, 0.5 M sulfuric acid was added to each well, and the optical density of the plate was read at 450 nm using a spectrophotometer (Eon; BioTek Instruments Inc., Winooski, Vermont). The crude extraction from the EP yielded 16 mg antibody/kg of EP, while the extracted protein from the control yielded no reactivity to PLA2, as seen in Figure 1.

2.3 Sample collection, fabrication, and treatment application

Boneless striploins were collected two d postmortem from ten USDA low Choice beef carcasses from a processing facility in Kansas. The striploins were vacuum packaged, transported to Kansas State University Meat Laboratory, and kept under refrigeration (2 ± 2°C) overnight. The following day, the posterior portion of each striploin containing *gluteus medius* was removed. The remaining striploin containing only *longissimus lumborum* was sliced into 2.54 cm steaks and trimmed to remove exterior fat and connective tissue. Each striploin was ground through a 0.32 cm grinder plate in a meat grinder (KG-12-FS, Pro-Cut, Waltham, MA). The grinder was cleaned out of any residual beef between each striploin. The ground striploin was mixed and separated into four 500-gram batches for each striploin.

Each batch of ground striploin from one striploin was assigned to one of 4 treatments: 1) ground striploin only containing 0% aPLA2 EP; 2) containing 0.4% aPLA2 EP; 3) containing 0.8% aPLA2 EP; and 4) containing 1.6% aPLA2 EP (w/w). The EP was hand mixed into each batch according to the assigned treatment, vacuumed packaged (75001840 3-Mil standard Nylon/

Polyethylene vacuum pouches, 0.2 cc/100 in^2/24h at 23°C and 0% RH; Clarity, Riverside, MO), shielded from light and stored under refrigeration at 2°C ± 2°C for 14 d. After the storage period, each batch of ground striploin was formed into four 114 g patties using a mold. The four patties from the same batch were subjected to one of three display times (0, 4, or 7 d). A schematic of the sample preparation process is illustrated in Figure 2. The patties designated for retail display were overwrapped with plastic food wrap (Polyvinyl chloride, 23,250 cc/m^2/24h at 23°C and 0% RH; Classic Sysco, Houston, TX), in styrofoam trays (17S; Genpak, Charlotte, NC) lined with a tray absorbent pad (Dri-Loc AC-25; Novapak, IL) and displayed under continuous fluorescent lighting (32 W Del-Warm White 3,000 K; Phillips Lighting Company, Somerset, NJ), averaging 2,143 ± 113 lx emission case-wide and 2 ± 2°C in a coffin-style retail case.

2.4 Color descriptors in simulated retail display

Percent visual discoloration was determined using a trained panel (N=7) on patties designated for 7 d of retail display. Panelists were trained by studying a visual discoloration guide which provided ten ground beef images ranging from 0% to 100% discoloration representing surface discoloration in increments of 10%. A percentage of 0% was used to indicate no visual discoloration, and 100% was used to indicate complete discoloration. On each day of the 7-d retail display period, panelists visually evaluated the samples and assessed the percentage of discoloration at the same time to minimize variation. Instrumental color measurements L* (lightness), a* (redness), and b* (yellowness) were measured with a Hunter Lab MiniScan EZ spectrophotometer (Model 2500L, Illuminant D65, 2.54cm aperture, 10° observer; Hunter Associates Laboratory Inc, Reston, VA) on the same set of patties. Samples were randomly rearranged daily to minimize any possible lighting location effects. Upon

termination of each sample's designated display period, patties were removed from the overwrapped packaging, vacuum packaged, and stored at -80°C until further processing. Finally, all ground striploin samples were frozen by submersion in liquid nitrogen and pulverized using commercial blenders (Model 51BL32, Warning Commercial; Torring, CT) packaged in Whirl-pak bags (Madison, WI) and stored at -80°C for lab analysis.

2.5 Proximate analysis

2.5.1 Moisture analysis

Proximate analyses were conducted on all d 0 samples from the four treatments. Moisture analysis was performed by weight differentiation following a modified 950.46 AOAC oven-drying method (AOAC International, 2000a).

2.5.2 Protein analysis

Protein content was measured with a LECO protein analyzer (CN828; LECO Corporation, St. Joseph, MI) according to a modified AOAC method 992.15 (AOAC International, 2000b). Ethylenediaminetetraacetic acid (EDTA, 9.56% nitrogen) was used to calibrate the instrument before analysis. The percentage of nitrogen obtained was multiplied by a factor of 6.25 to calculate the percentage of crude protein.

2.5.3 Lipid analysis

Lipids were extracted as described by (Folch et al., 1957) with modifications from d 0, 4, and 7 samples from all treatments. Briefly, samples, deionized water, chloroform, and methanol were added to each sample tube. The tubes were shaken and a 0.74% potassium chloride solution in ultrapure water was added. The mixture was centrifuged, and an aliquot of the bottom layer was collected into a 16×100 mm pre-labeled, dried, and weighted glass tube. Chloroform was

evaporated, and the evaporated-sample glass tubes were placed in a centrifuge vacuum dryer and weighed afterward. Lipid percentage was calculated and the lipid was resuspended in chloroform to achieve a 10 mg/mL concentration. The lipid stocks were stored in capped glass tubes sealed with parafilm in a -80°C freezer until FA analysis and phospholipid class profiling.

2.6 Lipid oxidation

For lipid oxidation evaluation, analyses were conducted on all d 0, 4, and 7 samples from the four treatments. Procedures follow the 2-thiobarbituric acid reactive substances (TBARS) methodology as described in Dahmer et al. (2022) with modifications. Briefly, a malondialdehyde (MDA) bis (diethyl acetal) standard curve was prepared (0-25µM). Pulverized samples were homogenized with a TBA/Trichloroacetic acid solution (20mM:15% in ultrapure water) and 0.3% of butylated hydroxytoluene (BHT) in ethanol. The content was centrifuged, filtered, incubated at 70°C in a water bath and cooled in cold water. Aliquots of 200 µL of samples and standards were plated into a 96 wells plate and read in a spectrophotometer at 532 nm (Eon; BioTek, Winooski, VT). The results for lipid oxidation from each sample were calculated and expressed as mg MDA/kg of muscle tissue.

2.7 Phospholipid extraction and Thin-Layer Chromatography (TLC)

2.7.1 Solid-Phase Extraction (SPE) of lipid classes

Phospholipid class profiling was conducted on d 0 samples. Procedures for SPE to separate lipid classes followed the methods described by (Legako et al., 2015) with modifications. The SPE columns (SPE NH2 500 mg/6 ml, JGFinneran, Vineland, NJ) were pre-conditioned with two rinses of hexane and chloroform. Following the pre-conditioning step, 5

mL of lipid stock was loaded into the column, and neutral lipids and FFA were eluted by chloroform and 2% acetic acid in diethyl ether, respectively. Phospholipids were eluted with methanol and evaporated using a vacuum evaporator (RapidVap; Labconco, Kansas City, MO). Once evaporated, the tubes were weighed, and the dried samples were resuspended in chloroform to achieve a 2 mg/mL concentration of phospholipids for thin-layer chromatography (TLC) analysis.

2.7.2 Thin-layer chromatography

The TLC was performed as described by Chao et al. (2017). TLC plates (PE SIL G, 250 µm layer; Whatman, Kent, England) were pre-cleaned with chloroform:methanol and dried before use. The TLC plate was activated by spraying 2.3% boric acid in ethanol. Twenty µg soy reference (containing 38, 30, 18, 7 and 7 % of PC, PE, PI, PA, and LPC, respectively; Soy Phospholipid Mixture; Avanti Polar Lipids Inc, Alabaster, AL) were used as a standard, and 60 µg of extracted phospholipids were spotted on a plate. The TLC running solvent (chloroform:ethanol:water:triethylamine, 30:32:7:35 by volume) was added to the TLC tank, and the spotted plates were placed in the solvent. Once the run was complete, the plates were sprayed with a 10% cupric sulfate in 10% phosphoric acid, dried, and heated in the oven at 180°C until spots appeared. The area of the spots was imaged with an iBright Imaging System (FL150; Thermo Fischer Scientific, Waltham, MA). The phospholipid classes were identified against the soy reference, and a relative percentage of each phospholipids class was calculated.

2.8 pH analysis

The pH values were measured for all samples as described by Hammond et al. (2022). Briefly, 5 g of raw pulverized muscle samples were homogenized with 50 mL of ultrapure water

for 20 sec at 10,000 rpm using a benchtop homogenizer (850; Fisher Scientific, Pittsburgh, PA). An InLab Science Pro-ISM probe (Mettler-Toledo, Switzerland) connected to a Seven Compact pH meter was calibrated with pH 4 and 7 standard solutions, and the pH of the samples was measured following the calibration.

2.9 Fatty acid analysis

The FA analysis was conducted on d 0 samples as described by Dahmer et al. (2022) with modifications. An aliquot of the lipid stock was diluted with chloroform to reach a 2 mg/mL lipid concentration. For FA methyl ester preparation, pentadecanoic acid (15:0) was added to each sample as an internal standard, and the solvent was evaporated under nitrogen. Subsequently, 3M methanolic hydrochloric acid was added to each tube to be heated. Ultrapure water and hexane were added, vortexed, and centrifuged. The top hexane layer was removed into a glass tube and evaporated to dryness. The content was redissolved in hexane and transferred to a Gas Chromatography (GC) vial with an insert.

An Agilent 7683 autosampler (6890N GC system; Agilent Technologies, Santa Clara, CA) was used to inject 1 µL of the prepared sample into Agilent 6890N GC coupled to a Flame Ionization Detector (FID; Agilent Technologies, Santa Clara, CA). The GC was fitted with a DB-23 capillary column (60 m in length, 250 µm in internal diameter, and 0.25 µm of film thickness). Helium was used as the carrier gas at a flow rate of 1.5 mL/min. The GC oven temperature ramp was operated as described in Dahmer et al. (2022) as follows: 1) an initial temperature of 150 °C with a hold of 1 min; 2) an increase of 25 °C/min until a 175 °C temperature was achieved; 3) the temperature rate increased at 4°C/min to reach 230°C and holding for 9 min. The total run time was 24.75 min. The FID detector was operated at 260 °C.

The hydrogen flow to the detector was 30 mL/min, and the airflow was 400 mL/min. The sampling rate of the FID was 20 Hz. Individual FA peaks were processed through Agilent Chemstation software (Agilent) and expressed as relative percentages of total FA. A commercial standard (37-component FAME mix; Supelco, Bellefonte, PA) was used to identify the FA.

2.10 Antioxidant capacity through Oxygen Radical Absorbance Capacity (ORAC)

The methodology used for ORAC followed the methods described by Huang et al. (2002b) with modifications on all d 0, 4, and 7 samples from the four treatments. For Lipophilic ORAC, samples were placed in homogenization bead-filled tubes with hexane (Bead Blaster 24; Benchmark, Sayreville, NJ). The tubes were shaken and centrifuged. The hexane layer was collected into a glass tube, and the hexane glass tubes were evaporated to dryness under nitrogen in a nitrogen flow evaporator (Reacti-Vap III #TS-18826; Thermo Scientific, Waltham, MA). The dried tubes were resuspended in 750 µL of 7% randomly methylated-cyclodextrin (RMCD, solution in 50:50 acetone:water).

For hydrophilic ORAC, 80:20 water:ethanol solution was added to the tube containing meat sample previously been extracted with hexane. The mixture was homogenized, centrifuged, and 750 µL of supernatant were transferred into another microcentrifuge tube. This mixture was diluted by a dilution factor of 20 with an 80% water/20% ethanol solution.

Trolox (Sigma-Aldrich, St. Louis, Missouri) standard curves (6.25 µM to 100 µM) were prepared with a 7% randomly methylated,-cyclodextrin (RMCD) solution in 50:50 acetone:water or 75 mM phosphate buffer (pH of 7.4) for lipophilic and hydrophilic ORAC, respectively. The buffers were used as blanks for their corresponding treatment. A fluorescein solution (1:1,000 in 75 mM phosphate buffer) was added to all wells of a non-adhering black 96-well microplate

(655906; Greiner bio-one, Kremsmünster, Austria). Twenty-five µL of blanks, standard dilutions, and samples were pipetted into the microplate, then incubated in a Synergy HTX Multi-mode Reader for 30 min (BioTek Instruments, Inc., Winooski, VT). Following the incubation period, 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was added to each experimental well. Finally, the fluorescence of each well was measured in the microplate reader from the bottom of the plate at an excitation wavelength of 485 nm and an emission wavelength of 528 nm every 60 sec for 120 min. ORAC results were obtained by calculating the area under the curve (AUC) and Net AUC. Once the net AUC was obtained, the Trolox standard was plotted as a function of Trolox concentration to calculate the sample concentration in µmol/L. The results were reported as µmol of Trolox equivalent (TE) per g of meat.

2.11 Statistic analysis

Proximate analysis, phospholipid profile, pH analysis, and FA profile were analyzed as completely randomized designs, and lipid oxidation and ORAC analysis were analyzed as a split-plot design with treatments as the whole plot factor, and the retail display days as the sub-plot with a treatments × display days interaction. Carcasses were considered as the experimental units. Finally, meat color data were analyzed as a split-plot repeated-measures design with treatments as the whole-plot and retail display day as the repeated-measures. The Toeplitz covariance structure was selected based on the best fit model. Multiple comparisons were obtained through Tukey Method, and mean separation was conducted using least squared means procedures. All data points were analyzed through SAS software using the GLIMMIX procedure (version 9.4, SAS Institute, Cary, NC). Degrees of Freedom were obtained thorough the

Kenward-Roger Method. Finally, a 5% level of significance was used to detect differences among means.

3. Results and discussion

3.1 Color descriptors in simulated retail display

There was no treatments × display days interaction nor a main effect for treatments for discoloration ($P>0.05$). However, there was a main effect for display days for discoloration ($P<0.01$; Figure 3A). As expected, as display days increased, more discoloration was observed for the striploin patties regardless of the treatments ($P<0.01$). There was no treatments × display days interaction for the instrumental color measurements for L*. Moreover, L* values were unaffected by treatments or display days ($P>0.05$; Figure 3B). The values for a* showed an interaction between treatments and display days ($P<0.05$; Figure 3C). Generally, a* values decreased as the display days progressed ($P<0.05$). On d 3, 5, and 7 of the retail display, samples from treatments 0% tended to have higher a* values than those from 1.6% ($P<0.10$). There was no treatments × display days interaction for b* values, but they showed a main effect for display days; as days progressed, a lower b* was obtained ($P<0.05$; Figure 3D).

The enzyme PLA2 is a free fatty acid (FFA) liberator from phospholipids, and most of the FFA released from the sn-2 position is highly unsaturated (Burke & Dennis, 2009; Murakami et al., 2020). As unsaturated FA are highly susceptible to lipid oxidation, PLA2 could negatively impact discoloration (Suman et al., 2014). Nevertheless, a few previous studies have found a color-stabilizing effect when PLA2 was added into the meat system. In pork sausage, PLA2 has shown a color stabilizing effect when added with rosemary extract (Whalin et al., 2022).

Furthermore, Govindarajan et al. (1977) added PLA2 into ground beef and observed an inhibition of myoglobin oxidation apparent to a trained panel.

However, the presence of higher amounts of native PLA2 has indeed been shown to negatively impact meat color, such as in the case for pale, soft, exudative (PSE) animals. Soares et al. (2003) showed a strong relationship between discoloration and PLA2 content in heat-stressed chickens, and the same group of authors hypothesized that PLA2 is related to meat color destabilization in PSE broilers (Soares et al., 2009). Furthermore, Chen et al. (2010) compared PSE and regular pork and found higher PLA2 levels and L* value for the PSE pork; however, no correlation was found between those two characteristics.

Meat color depends on myoglobin's oxidation state (Tewari et al., 2001), which the formation of metmyoglobin increases over time during retail display when myoglobin is oxidized (Suman & Joseph, 2013). This study is the first one to the authors' knowledge to investigate the effect of adding aPLA2 on color stability. The unexpected null differences in perceived discoloration for treatments tied in with the lack of concrete differences across treatments in instrumental color measurements L*, a*, and b*, demonstrating the incorporation of aPLA2 through EP likely is not effective in preserving ground striploin color during retail display. In fact, the tendency found for a decrease over time in a* for the 1.6% treatment demonstrated a potential negative impact of incorporating aPLA2-containing EP into ground striploin.

3.2 Moisture, protein, and fat content

Moisture content was lower for the 1.6% treatment when compared to all other treatments ($P<0.01$; Table 1). There was no difference in protein and fat content among the treatments ($P>0.05$; Table 1). The moisture result could be attributed to the low moisture level of spray-

dried EP, which whole dried eggs contain ~4% moisture (USDA, 2019a). The addition of 1.6% dried EP was enough to decrease the moisture level of the patties by ~1%. Nevertheless, the protein and fat contents for all treatments matched the ones expected for beef choice loins, according to the USDA (2019b), at ~ 22%., and ~6%, respectively.

3.3 Lipid oxidation

There was no treatments × display days interaction for lipid oxidation ($P>0.05$). As patties continued in retail display, lipid oxidation values increased over time ($P<0.05$; Table 2). The 1.6% treatment has higher lipid oxidation values compared to those from the other treatments ($P<0.05$; Table 2).

Different authors attribute discoloration to myoglobin oxidation and production of off-flavors to lipid oxidation; however, both reactions are concurrent in beef and have been considered somewhat intertwined (Ramanathan et al., 2021). Wang et al. (2021) showed a moderate positive correlation between the rate of lipid oxidation and visual discoloration during retail display. The PLA2 has been reported as an antioxidant in cod and trout liposome model studies (Tatiyaborworntham et al., 2021; Tatiyaborworntham & Richards, 2018) and pork sausage (Whalin et al., 2022). Conversely, PLA2 has also been reported as a lipid oxidation promotor in PSE pork (Chen et al., 2010). In this study, we hypothesized that lipid oxidation of striploins might be reduced by adding aPLA2 into it, with the thought that aPLA2 may prevent the generation of FFA in postmortem meat. Although Chun (2022) observed a reduction in lipid oxidation for treatments containing aPLA2 compared to the PLA2 only treatment in a beef liposome study, our results did not see such effect in the actual ground striploin model.

3.4 Thin-layer chromatography of phospholipids

Sphingomyelin (SM), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) were the only three phospholipid classes identified by the samples by TLC (Table 1, Figure 4). There were no differences for SM across treatments ($P>0.05$). The 1.6% treatment showed higher relative % for PC and a lower relative % for PE when compared to 0% and 0.4% EP inclusion treatments ($P<0.05$). However, treatments 0, 0.4, and 0.8% did not differ in PC or PE relative % ($P>0.05$).

The PLA2 is known to hydrolyze primarily PCs and PEs to generate FFA and lysophosphatidylcholines (LPC) or lysophosphatidylethanolamines (Bolander, 2004; Burke & Dennis, 2009; Murakami et al., 2011). Therefore, FFA and LPC levels are often used as markers for PLA2 activity; however, there was no detectable production of these LPCs in this study through TLC. Arguably, this lack of LPC production and the higher content for PC in the treatments with aPLA2 EP could signify preservation of the phospholipid because of aPLA2 activity. However, Chun (2022) observed no effect on PC preservation and LPC production in treatments with PLA2 and aPLA2 in a beef liposome treatment. Therefore, the total lack of LPC production in this study would suggest that there wasn't enough PLA2 activity in the samples to be detected by TLC in the first place. Finally, a possible explanation for the noted increase in PC for 1.6% treatment is the inherent high PC concentration in the EP (Blesso, 2015; Palacios & Wang, 2005).

3.5 pH results

There was no treatments × display days interaction for pH ($P>0.05$). There were main effects for both treatments and display days for pH ($P<0.01$). Day 7 samples had a higher pH

value than those from samples that were only displayed for 0 and 4 d ($P<0.01$, Table 2). Additionally, the pH value increased as the aPLA2 EP percentage increased ($P<0.01$, Table 1). While there was a slight increase in pH on d 7, the values of pH obtained in this study are in the expected range for ground beef and steaks, as shown in Ramanathan et al. (2021) and Ramanathan et al. (2019). The difference in pH among the treatments was likely attributed to the EP's pH. In this study, the pH of the EP was 8.0. The pH of dried EP varies depending on the egg's components included in the power and manufacturing processes, usually between pH 7–9.5 (Vargas-del-Río et al., 2022).

The optimum pH for PLA2 activity ranges around 8.0 (Oliveira et al., 2002; Ponce-Soto et al., 2002). Nevertheless, Tatiyaborworntham et al. (2021) demonstrated PLA2 enzymatic activity at a pH of 6.5 in a liposome model system using washed cod muscle. Moreover, it is not well established regarding the optimum activity for aPLA2, but aPLA2 activity delivered through EP has been reported at pH values as low as 2.0 (Cook & Trott, 2010). Enzymatic activity is known to be driven by environmental factors such as pH as their molecular structure and interaction with other molecules depend on hydrogen bonds (Campbell & Farrell, 2015). Therefore, it is possible the lack of PLA2 and aPLA2 activity in this study is partly resulted from the low pH of ground striploin.

3.6 Fatty acid profile

There were observed differences for specific (FA) types, as listed in Table 3. The treatments with 1.6% aPLA2 EP additions had higher amounts of FA vaccenic acid (11-18:1 trans), linoleic acid (18:2), eicosenoic acid (20:1), and docosahexaenoic acid (22:6) and a lower content for FA heptadecanoic acid (17:0) and heptadecenoic acid (17:1) when compared to the

0% treatment ($P<0.05$). Moreover, there were no differences among the treatments for total saturated FA (SFA), monounsaturated FA (MUFA), or polyunsaturated fatty FA (PUFA) ($P>0.05$).

Dennis (1973) stated that PLA2 follows Michaelis-Menten kinetics, in which enzymatic activity depends on substrate availability, and Murakami et al. (2020) showed that sarcoplasmic PLA2 prefers phospholipids with FA 18:1 and 18:2. It is well established that non-ruminants such as pigs have much higher proportions of PUFAs in adipose and muscle tissue than cattle, as they digest most lipids without altering their saturation levels (Wood et al., 2008). Thus, it could be explained that as cattle have a lower content of unsaturated FA than pork or fish, PLA2 activity in cattle may not be as involved as seen in pork for postmortem phospholipid hydrolysis (Chao et al., 2020).

Dried EP is known to contain high levels of 18:1, 18:2, and 22:6 (Javed et al., 2018; Pirkwieser et al., 2022). Accordingly, EP incorporation altered the FA composition of the striploin patties, increasing some unsaturated FA content. Thus, it is reasonable to attribute a higher lipid oxidation on the 1.6% treatment to the higher presence of those unsaturated FA, even if there was no overall difference for PUFAs.

3.7 Oxygen radical absorbance capacity (ORAC)

There was no treatments × display days interaction for lipophilic nor hydrophilic ORAC ($P>0.05$). There was a main effect for treatments for the hydrophilic ORAC ($P<0.05$; Table 1). Interestingly, the 0.8% treatment showed a higher antioxidant capacity than the 1.6% treatment and the 0% treatment ($P<0.05$), but the 0%, 0.4%, and 1.6% treatment showed no differences in hydrophilic ORAC ($P<0.05$). Similar behavior was seen for lipophilic ORAC; treatments 0.4%

and 1.6% were similar to other treatments, but the 0% treatment tended to have lower lipophilic antioxidant capacity compared to the 0.8% treatment (*P=0.07*; Table 1).

Antioxidant capacity was obtained through ORAC analysis, as it has been reported to be an adequate indicator of both oxidation inhibition time and degree of inhibition by measuring peroxide radical scavenging capacity (Echegaray et al., 2021; Huang et al., 2002a). The EP addition was the most likely culprit for the observed differences in antioxidant capacity among the treatments. Hydrophilic antioxidants in eggs include peptides and vitamin C (Remanan & Wu, 2014; Zhang et al., 2019). Similarly, eggs can contain a wide variety of lipophilic antioxidants such as carotenoids, free amino acids, ovalbumin, vitamin E, selenium, and iodine (Nimalaratne & Wu, 2015). Finally, amphiphilic egg lecithin can also act as an antioxidant (Ribeiro et al., 2019).

Studies have shown that the activity of some antioxidants such as PLA2 are dose-dependent, with higher level limiting its effect (Tatiyaborworntham et al., 2021; Tatiyaborworntham & Richards, 2018). Therefore, it is possible the aPLA2 EP demonstrated a similar behavior on dose dependency. In fact, the spike in antioxidant activity of 0.8% treatment could be the reason that 0.8% treatment had lower lipid oxidation level than the 1.6% treatment, given the similarities in their FA profile.

Implications

Adding EP containing aPLA2 affected moisture content, FA profile, phospholipid composition, lipid oxidation, and antioxidant capacity. However, these differences could be the result from the introduction by the EP and not necessarily from the aPLA2. The binding of aPLA2 to PLA2 was demonstrated through ELISA, but the lack of LPC production across all

treatments brought up the questionable relevance of PLA2 in lipid oxidation for postmortem beef. Further research on aPLA2's ability to inhibit lipid oxidation should focus on livestock species with higher overall unsaturated FA like poultry, pork, or fish.

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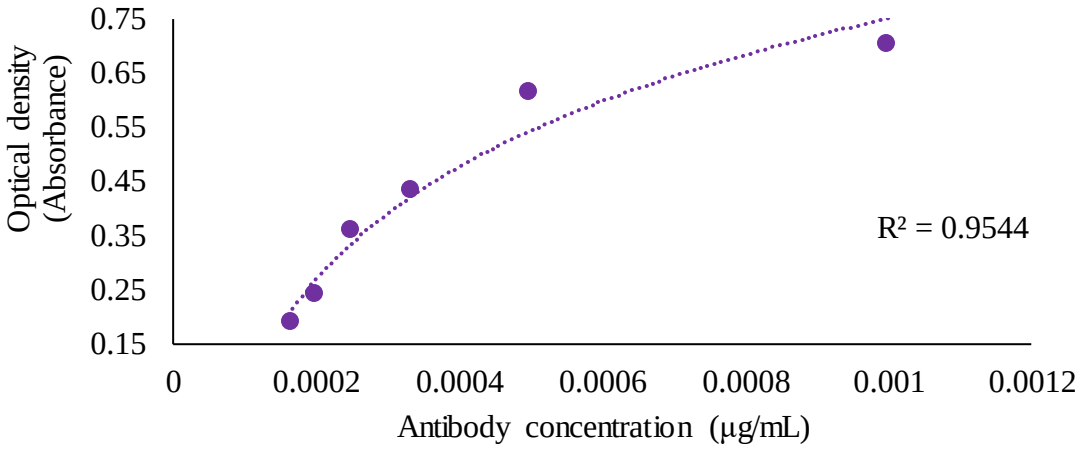

Figure 1. Standard curve from enzyme-linked immunosorbent assay used to calculate the titer of extracted antiphospholipase α2 from egg powder. Results yielded an approximate titer of 16 mg of antibody/kg egg power.

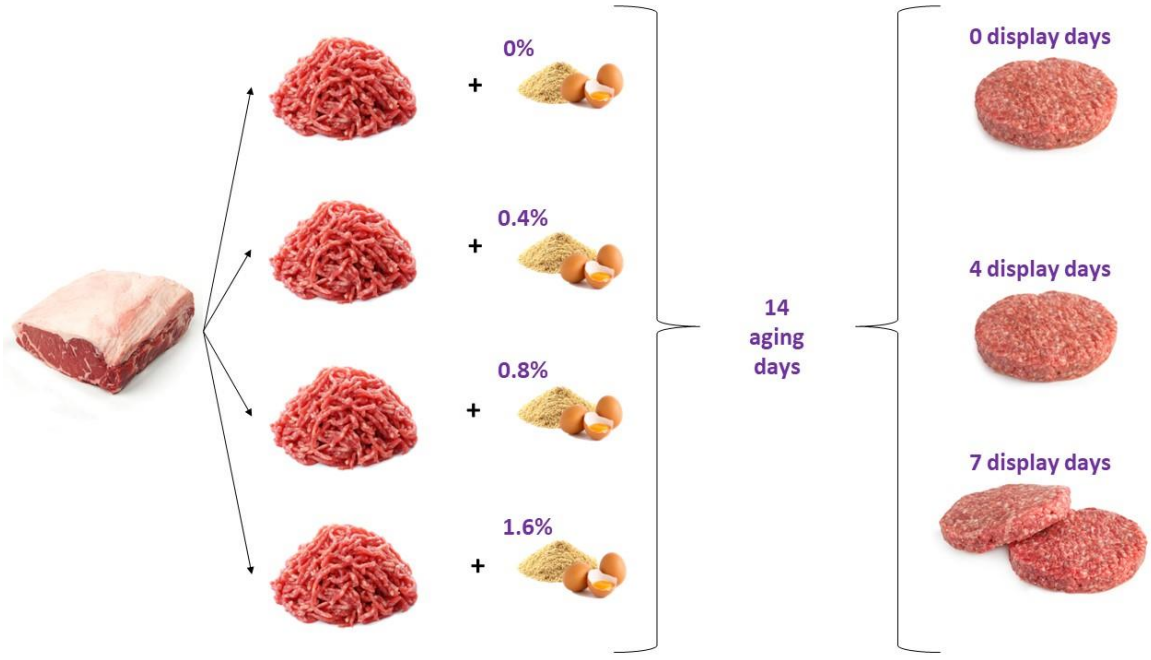

Figure 2. Schematic display of the sample preparation process for each striploin.

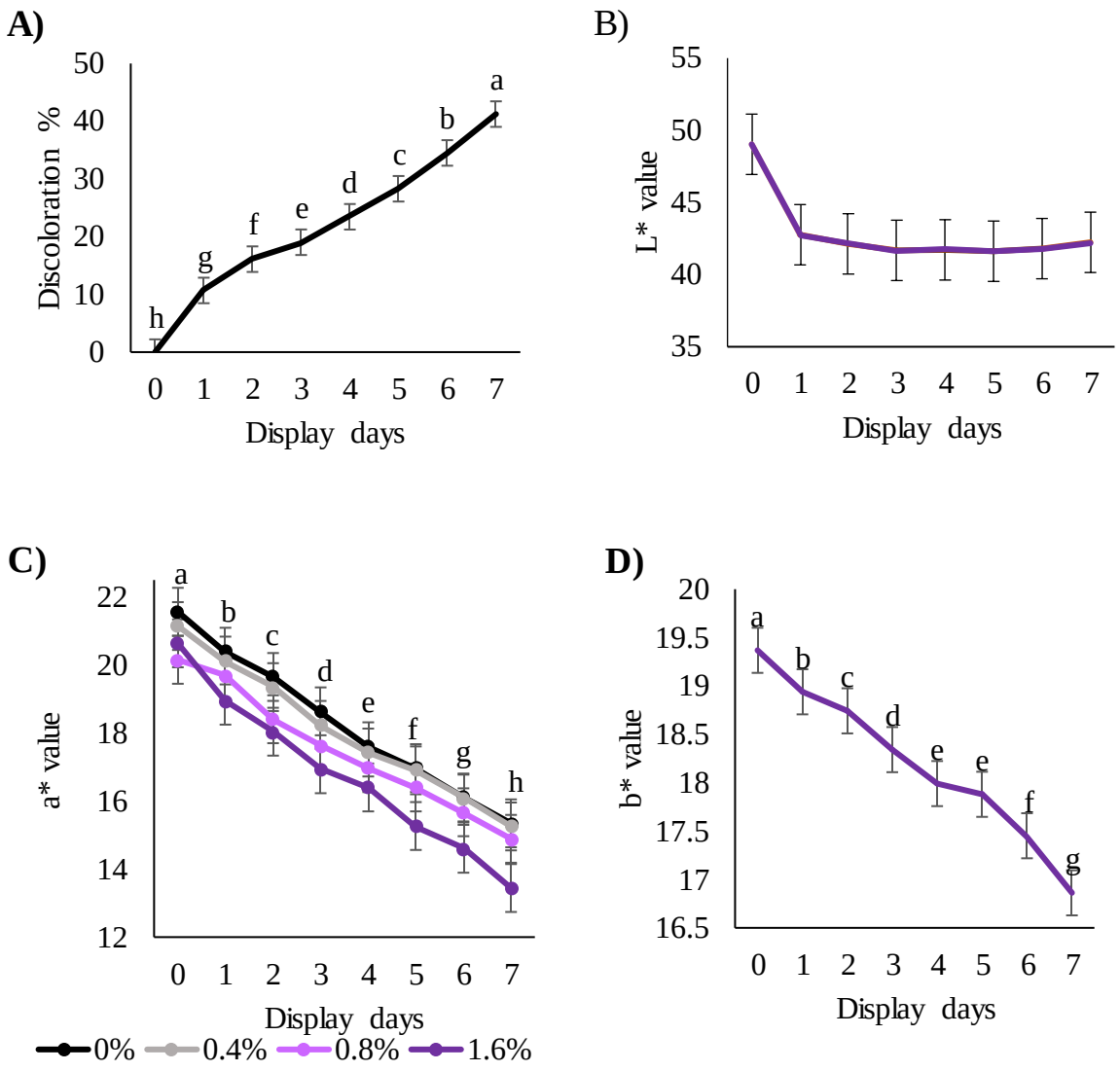

Figure 3. A) Main effect of display days for discoloration %. B) No main effect of display days for L*. C) Treatment × display day interaction for a*; the superscripts represent the display effect only. D) Main effect of display days for b*. The treatments consisted of beef patties added with 0, 0.4, 0.8 or 1.6% of egg powder containing antiphospholipase α2. [a-h] Values with different superscripts indicate a difference at $P<0.05$.

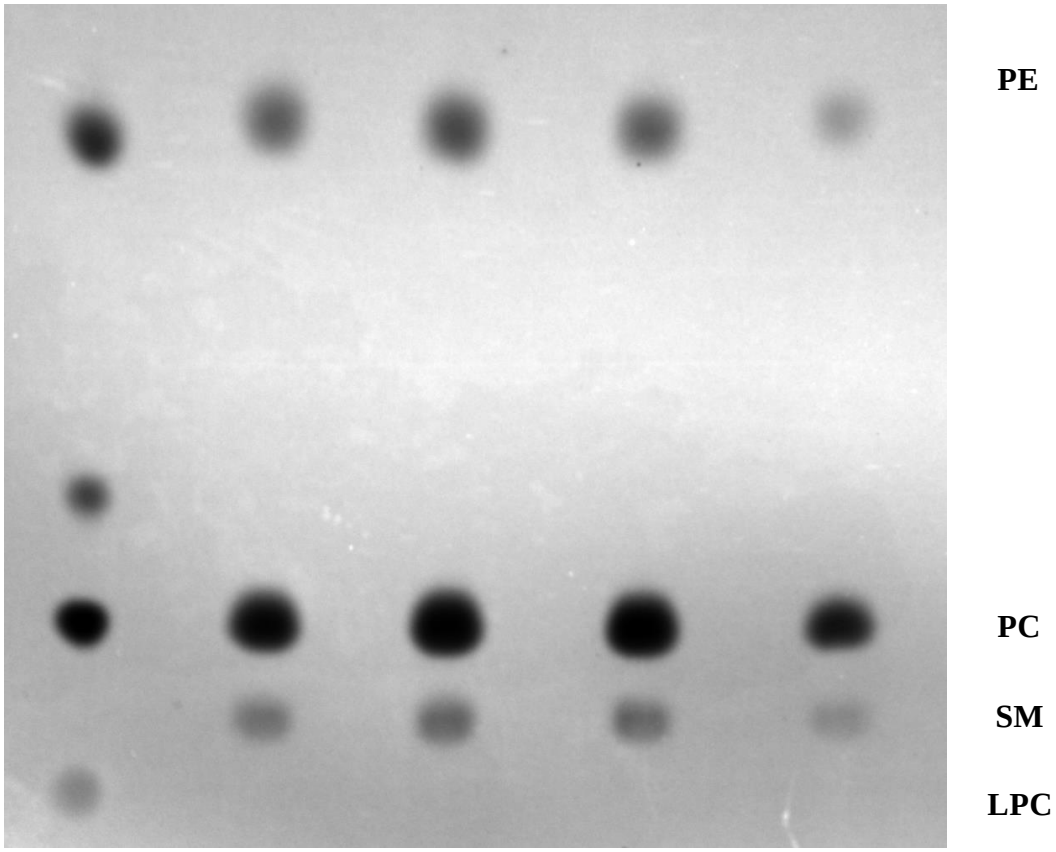

Figure 4 Representative image of a thin-layer chromatography plate for phospholipid identification. PE = phosphatidylethanolamine; PC = phosphatidylcholine; SM = sphingomyelin; LPC = lysophosphatidylcholine.

Table 1. Main effect of treatments for proximate analysis, phospholipid profile, lipid oxidation, and antioxidant capacity of beef patties added with 0%, 0.4%, 0.8%, and 1.6% of egg powder containing antiphospholipase α2.

% Egg powder	Treatments				SEM	*P-value*
	0%	0.4%	0.8%	1.6%		
Moisture (%)	71.19^{a}	70.93^{a}	70.82^{a}	70.17^{b}	0.19	<0.01
Protein (%)	22.44	22.35	22.57	22.43	0.43	0.54
Fat (%)	6.39	6.47	6.70	7.04	0.15	0.45
Phospholipid profile						
SM (relative %)	15.01	11.49	11.11	10.38	3.01	0.52
PE (relative %)	29.35^{a}	30.16^{a}	24.86^{ab}	18.73^{b}	2.83	<0.01
PC (relative %)	54.98^{b}	58.35^{b}	63.60^{ab}	70.59^{a}	3.62	<0.05
Lipid oxidation, mg MDA/ kg of meat	1.10^{b}	1.11^{b}	1.11^{b}	1.41^{a}	0.10	<0.01
pH	5.51^{d}	5.54^{c}	5.57^{b}	5.60^{a}	0.01	<0.01
Hydrophilic ORAC, TE/g of meat	17.06^{b}	17.47^{ab}	17.97^{a}	17.06^{b}	0.34	<0.01
Lipophilic ORAC, TE/g of meat	0.69	0.73	0.80	0.75	0.04	0.07

ORAC = oxygen radical absorbance capacity, SM = sphingomyelin; PE = phosphatidylethanolamine; PC = phosphatidylcholine, MDA = malondialdehyde, TE = Trolox equivalents. ^{abcd} Values with different superscripts indicate a difference within each row

Table 2. Main effect of display days for lipid oxidation and pH of beef patties added with 0%, 0.4%, 0.8%, and 1.6% of egg powder containing antiphospholipase α2.

	Display days				
	0	4	7	SEM	*P-value*
Lipid oxidation, mg MDA/ kg of meat	0.44^{c}	1.33^{b}	1.77^{a}	0.10	<0.01
pH	5.51^{b}	5.54^{b}	5.57^{a}	0.01	<0.01

MDA = malondialdehyde. ^{abc} Values with different superscripts indicate a difference within each row.

Table 3. Comparison of fatty acid profiles of beef patties added with 0%, 0.4%, 0.8%, and 1.6% of egg powder containing aPLA2.

	Treatment					
Fatty acid	0%	0.40%	0.80%	1.60%	SEM	*P-value*
14:0	2.60	2.38	2.31	2.36	0.15	0.12
14:1	0.62	0.55	0.54	0.54	0.05	0.14
16:0	29.24	29.12	29.26	29.20	0.39	0.94
16:1	3.44	3.37	3.07	3.04	0.22	0.24
17:0	1.01^{a}	0.98^{b}	0.96^{bc}	0.94^{c}	0.06	<0.01
17:1	0.77^{a}	0.74^{ab}	0.73^{bc}	0.71^{c}	0.05	<0.01
18:0	13.22	13.17	13.22	13.10	0.29	0.54
18:1	37.91	37.98	38.16	37.75	0.72	0.79
9-18:1 trans	1.89	1.83	1.78	1.77	0.13	0.11
11-18:1 trans	1.34^{b}	1.37^{ab}	1.39^{a}	1.38^{a}	0.03	0.03
18:2	4.93^{b}	5.38^{ab}	5.52^{ab}	6.05^{a}	0.45	0.02
18:3	0.20	0.20	0.21	0.21	0.01	0.18
20:1	0.15^{b}	0.16^{a}	0.16^{a}	0.16^{a}	0.01	0.03
20:3	0.43	0.43	0.41	0.40	0.05	0.72
20:4	1.53	1.59	1.56	1.62	0.20	0.93
20:5	0.11	0.11	0.11	0.11	0.02	0.93
22:4	0.24	0.25	0.24	0.23	0.03	0.92
22:5	0.33	0.32	0.30	0.30	0.04	0.60
22:6	0.03^{d}	0.05^{c}	0.08^{b}	0.11^{a}	0.01	<0.01
SFA	46.07	45.65	45.75	45.61	0.53	0.52
MUFA	46.12	46.00	45.82	45.36	0.77	0.43
PUFA	7.81	8.35	8.43	9.03	0.77	0.23

SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids. ^{abcd} Values with different superscripts indicate a difference within each row.