

STRUCTURAL ELEMENTS OF LIPIDIC INHIBITORS OF PHOSPHOLIPASE A<sub>2</sub>

A Thesis  
by  
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## **Abstract**

### STRUCTURAL ELEMENTS OF LIPIDIC INHIBITORS OF PHOSPHOLIPASE A<sub>2</sub>

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Phospholipases A<sub>2</sub> (PLA<sub>2</sub>) are a family of enzymes that release fatty acids from phospholipids and play varied and important roles in the biology of all organisms. Secreted phospholipases A<sub>2</sub> (sPLA<sub>2</sub>) are the largest subfamily of PLA<sub>2</sub>. sPLA<sub>2</sub> expression levels have been positively correlated to the severity of multiple inflammatory diseases. Many sPLA<sub>2</sub> inhibitors have been developed and tested, however, none have shown to be clinically effective and specific against individual enzymes within this family. In this study we discovered an inhibition phenomenon in a widely used sPLA<sub>2</sub> enzymatic assay. The enzymatic rate of reaction of sPLA<sub>2</sub> group IIA from *Crotalus adamanteus* was significantly reduced when negatively charged lipids were added at very low mole fractions. This inhibition phenomenon did not occur if the lipid added was either positively charged or polar but neutral. These results suggest that the standard assay should be re-optimized to prevent this nonspecific inhibition and that the literature may need to be reevaluated.

## **Acknowledgments**

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I want to thank the graduate students and faculty in the Biology Department at Appalachian State University for their support as a community. The people in the department create an environment that seems more like a family than a workplace. I would specifically like to thank Brewer Logan, Dakota Goad, Katie Hoffman, and the undergrads Meagan Colie and Madison Dietrich in Dr. Venable's lab for helping to keep me sane during my time here. Without all of you my days would not have been as fun and enjoyable as they were.

Finally, I would like to thank the rest of my family and friends for supporting me though this endeavor and for always being by my side. Without all of you I don't think I

would have made it this far; even if most of you didn't understand what I was talking about most of the time.

## **Dedication**

Michael and Amanda Gress: For always being there for me when I needed it and even more so when I didn't. You always thought of what was best for me even when you didn't know how it would affect you or your family. You took me in and supported me in a trying time in my life and you turned it around. I wouldn't be who I am today if it were not for the both of you. Thank you both for your love and support. To you, I dedicate this thesis.

## Table of Contents

Abstract.....	iv
Acknowledgments.....	v
Dedication.....	vii
Foreword.....	ix
Introduction.....	1
Materials and Methods.....	8
Results and Discussion.....	11
Conclusion.....	24
References.....	25
Vita.....	28



## **Foreword**

This research is to be submitted to *BBA Molecular and Cell Biology of Lipids*, a peer-reviewed journal owned by Elsevier Publishing Company. This thesis has been written in accordance to the citation and formatting requirements of said publication.

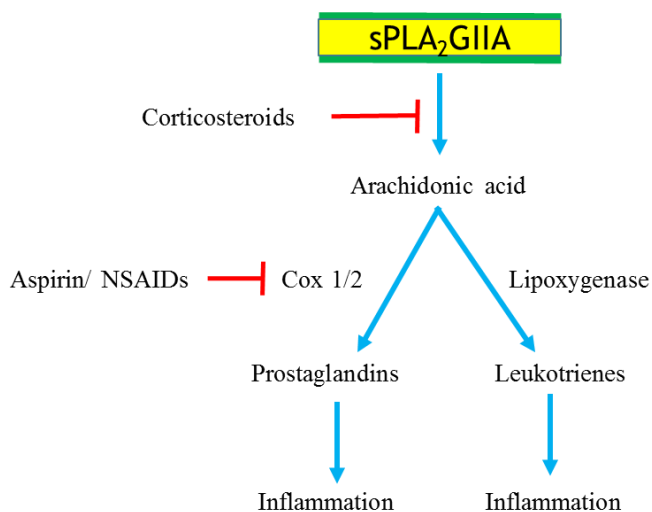
## Introduction

Phospholipases A<sub>2</sub> (PLA<sub>2</sub>s) are a superfamily of enzymes responsible for the hydrolysis of the fatty acid at the *sn*-2 glycerol position of membrane associated phospholipids. This hydrolysis results in a lysophospholipid and a free fatty acid; both of which can be used in various biological processes such as the formation of inflammatory eicosanoids (1, 2). Groups of PLA<sub>2</sub>s are denoted by roman numerals (PLA<sub>2</sub>GI, II, III...). Each group is separated according to unique features such as catalytic residue makeup, Ca<sup>2+</sup> dependence, structure, and function. Each group contains various isoforms denoted by capital letters (PLA<sub>2</sub>GIA, B, C...). Each group can be vastly different from one another, but each isoform within the group is structurally/functionally similar. Researchers also prefix each PLA<sub>2</sub> name with a lowercase letter that signifies a specific characteristic. The four characteristic group prefixes are secreted (s-), cytosolic (c-), Ca<sup>2+</sup> independent (i-), and lipoprotein-associated (Lp-PLA<sub>2</sub>).

Secreted PLA<sub>2</sub>s (sPLA<sub>2</sub>s) are the largest family of phospholipases A<sub>2</sub>. They are small secreted proteins 14-18 kDa in size, contain a highly conserved Ca<sup>2+</sup> binding domain, have a histidine (His) and aspartate (Asp) dyad in the active site, and have six conserved disulfide bonds. Due to the Ca<sup>2+</sup>-binding domain these enzymes are activated at millimolar concentrations of Ca<sup>2+</sup> and thus are only catalytically active in the extracellular space after secretion. They are also very stable due to their small size and number of disulfide bonds, making them relatively easy to handle *in vitro*. Some organisms including humans express more than one group/isoform of sPLA<sub>2</sub>s in their tissues; this allows different tissues to have their own sPLA<sub>2</sub> expression pattern and allow them to carry out specific functions with little

interference. The following sPLA<sub>2</sub>s are normally expressed in the human body: sPLA<sub>2</sub>GIB, IIA, IIF, III, V, and X.

Over the past few decades researchers have come to understand the structure, function, and expression patterns of sPLA<sub>2</sub>s. One of the most well-known and studied human sPLA<sub>2</sub>s is sPLA<sub>2</sub>GIIA. sPLA<sub>2</sub>GIIA was originally characterized as a “bactericidal sPLA<sub>2</sub>” due to its high affinity for phosphatidylethanolamine (PE) which makes up a lot of gram-positive bacterial membranes (1). This is also supported by the fact that sPLA<sub>2</sub>GIIA is highly expressed in intestinal Paneth cells and tear glands which are commonly exposed to high levels of bacteria (2). Recently, this enzyme has been associated with inflammation due to its ability to release arachidonic acid (AA) from phosphatidylcholine (PC) (Figure 1), the



**Figure 1. Production of Arachidonic Acid by sPLA<sub>2</sub>GIIA.** sPLA<sub>2</sub>GIIA can degrade membrane bound phosphatidylcholine to release arachidonic acid. Arachidonic acid can then be used in the Cox 1/2 and Lipoxigenase pathways to produce inflammatory eicosanoids such as prostaglandins, leukotrienes, thromboxanes, etc. Current inhibitors of this pathway include Aspirin, NSAIDs, and corticosteroids.

former of which can then be used in the formation of inflammatory signaling molecules called eicosanoids which are known to play a role in inducing inflammation, pain, fever, and modulating aspects of the immune system (3, 4). It is also understood that these enzymes have been correlated with the severity of multiple inflammatory diseases such as

cardiovascular disease (5), rheumatoid arthritis (6, 7), and cancer (8, 9). In many of these diseases, overexpression of sPLA<sub>2</sub>s have been positively correlated with disease progression or severity. For example, elevated levels of sPLA<sub>2</sub>GIIA in the blood has been used as a reliable biomarker for atherosclerosis for over a decade (10, 11). Although these correlations can be helpful when determining the presence of a disease it is still not known whether or not treating these high sPLA<sub>2</sub> levels will effectively treat these diseases in humans. However, studies have shown that by decreasing or knocking out the expression of various sPLA<sub>2</sub>s, treatment and/or prevention of some inflammatory diseases are possible in mice.

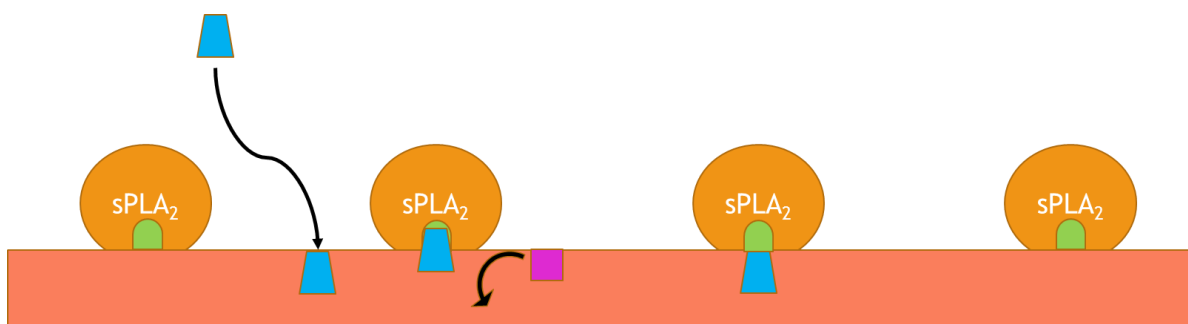
In 2010 researchers found that transgenic mice that overexpress the sPLA<sub>2</sub>GIIA enzyme have a significantly increased susceptibility to K/BxN autoantibody induced arthritis. They also found that sPLA<sub>2</sub>GIIA knockout mice were protected from the same induced arthritis model compared to wild-type mice (6). Another study showed that transgenic mice that overexpress sPLA<sub>2</sub>GIIF were more sensitive to a 7, 12-Dimethylbenzanthracene (DMBA) chemical carcinogenesis model and developed larger skin tumors than wild-type mice. sPLA<sub>2</sub>GIIF knockout mice, again, were found to be significantly more resistant and developed fewer skin tumors (12, 13). These studies give evidence that inflammatory diseases that overexpress sPLA<sub>2</sub>s could potentially be treated by genetic regulation or specific and effective sPLA<sub>2</sub> inhibitors.

While there are various other examples of sPLA<sub>2</sub> knockout mice showing protection or resistance to inflammatory disease models, there are also studies that show overexpression of sPLA<sub>2</sub>s can be beneficial to some diseases. Gastric cancer patients whose tumors express high levels of sPLA<sub>2</sub>GIIA have been found to have a significantly improved rate of survival and decreased metastasis versus those patients whose tumors express low levels of

sPLA<sub>2</sub>GIIA (14). sPLA<sub>2</sub>GV knockout mice show exacerbated symptoms in a mouse model with induced arthritis, and more interestingly, these symptoms can be somewhat rescued via the introduction of exogenous sPLA<sub>2</sub>GV (15). While the evidence for the therapeutic potential of sPLA<sub>2</sub> inhibitors is probable the contravening roles of various sPLA<sub>2</sub>s even within the same disease, such as sPLA<sub>2</sub>GIIA and V in arthritis, make it troublesome to implement. This is mainly due to a lack of specific and effective inhibitors.

There have been many effective sPLA<sub>2</sub> inhibitors over the years. Many have made it to clinical trials, but none have made it to market. This is mainly due to non-specific inhibition. YM-26734 and KH064 are two effective inhibitors of sPLA<sub>2</sub>s that have been shown to decrease inflammation in rat ear edema (16), protect against intestinal reperfusion injury (17), attenuate NF-κB activity in lung cancer cells (18), and protect against diet-induced metabolic syndrome in rats (19). However, these inhibitors target many sPLA<sub>2</sub>s and can thus have multiple side effects (20, 21). As a result, these drugs have mostly been relegated to the lab rather than going on to become treatments. Varespladib, a sPLA<sub>2</sub>GIIA inhibitor, failed to reduce rheumatoid arthritis in a double blind study during clinical trials (22). This has been attributed to non-specific inhibition of sPLA<sub>2</sub>GV, which has been shown to reduce arthritis in arthritic-induced mouse models (15). In another clinical trial for cardiovascular disease Varespladib showed a 90% reduction in sPLA<sub>2</sub>GIIA activity and was effective in reducing LDL cholesterol, but later showed increased incidence of myocardial infarction and total cardiovascular events during phase 3 trials. Again this has been attributed to non-specific inhibition of sPLA<sub>2</sub>GV and/or group X since Varespladib inhibits these enzymes at similar concentrations (23, 24). These studies show that sPLA<sub>2</sub> inhibitors do have therapeutic potential for some inflammatory diseases, but the creation of specific inhibitors is

at this point lacking. The mechanism by which most sPLA<sub>2</sub> inhibitors function, Varespladib included, is through competitive inhibition. Researchers use amphipathic molecules that integrate into the membrane and when brought into the catalytic site are not able to be hydrolyzed by the enzyme. This effectively extends the time between enzyme substrate interactions and decreases the overall reaction rate (Figure 2). The more time the inhibitor spends in the catalytic site, the lower the dissociation constant ( $K_D$ ), the more effective the inhibitor. Thus, the lack of specific inhibitors is mainly due to how similar in structure and function sPLA<sub>2</sub>s are to one another.

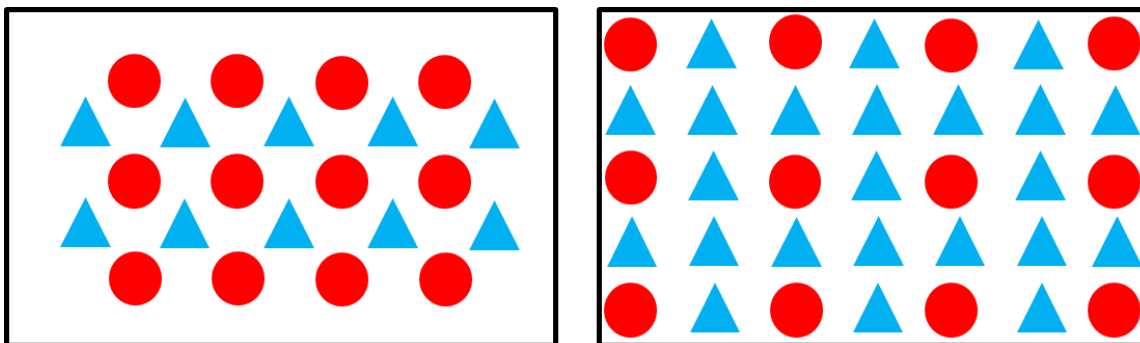


**Figure 2. Competitive inhibition of an sPLA<sub>2</sub>.** Once the competitive inhibitor (blue) is integrated into the membrane (pink) the sPLA<sub>2</sub> will bring the non-hydrolysable inhibitor into the active site (green) for a period of time preventing the binding of substrate (purple). Eventually, the enzyme will release the inhibitor back into the membrane and continue its function.

sPLA<sub>2</sub>GV and IIA have high homology (~40%) with the difference coming from group V lacking one of the disulfide bonds found within group IIA. Although these groups are very similar the difference in one disulfide bond gives each enzyme varying affinities towards their substrates. Group V is able to hydrolyze phosphatidylcholine more effectively than group IIA, which conversely has a higher affinity towards phosphatidylethanolamine (25). Isoforms IIA, IIC, IID, IIE, and IIF only differ by C-terminal extensions of varying length and composition (26). While these differences are notable the active site within these enzymes containing the His-Asp catalytic dyad are almost identical. This homology,

particularly in the active site, is what makes finding specific inhibitors of each enzyme remarkably difficult.

PLA<sub>2</sub>s in general are interfacial enzymes because they have to interact with a 2D amphipathic structure such as a membrane, vesicle, or micelle in order to reach their substrate. Interfacial enzyme kinetics tend to be more complicated than matrix enzymes due to this interaction. Matrix enzymes are able to find their substrates in solution, and therefore, the standard reaction rates are easily determined because each enzyme is exposed to the same environment. Each interfacial enzyme, however, is not exposed to the same environment since substrate concentration, non-substrate concentration, membrane type, and membrane size from one interface to another can vary greatly. As these factors change so does the enzymatic rate of reaction. This issue has led to the development of kinetic models. The surface dilution model estimates the effect of heterogeneously mixed interfaces on enzymatic rates of reaction (27, 28). For example, researchers looked at the differences in reaction rate of the cobra venom sPLA<sub>2</sub> when it was introduced to various concentrations of Triton X-100 and phosphatidylcholine in mixed micelles (27, 29). These studies found that as the ratio of Triton X-100 to substrate increased, the reaction rate of cobra sPLA<sub>2</sub> severely decreased even though there was no direct change in overall substrate concentration (Figure 3). This decrease in enzyme activity was due to a decrease in the number of enzyme/substrate interactions overall. This surface dilution effect can represent either a decrease in substrate concentration or an increase in non-hydrolysable molecules within a micelle. Thus, inhibition studies must be performed without surface dilution having an effect on enzymatic reaction rates as to not give a false positive.



**Figure 3. Surface Dilution Example.** From left to right is an example of surface dilution occurring on the same area of a micelle (black box) when sPLA<sub>2</sub> substrate (red circle) is diluted with Triton X-100 (blue triangle).

sPLA<sub>2</sub> reaction rates can also be slowed, or show a false positive, by a change in micelle structure. Fortunately, nonionic detergents do not distort micelle structure when above their critical micelle concentration. This means that with the use of nonionic detergents, like Triton X-100, the addition of lipids at small mole fractions would not have a significant effect on micelle structure and thus should not affect reaction rates. Another benefit of nonionic detergents is that as lipids are added they become uniformly solubilized into a homogeneous micelle mixture (30). This homogeneity allows for even distribution of lipids so that variation between micelles is low and inhibitor studies can be performed without surface dilution or micelle distortion leading to skewed results.

Specific, effective, and natural inhibitors of sPLA<sub>2</sub>s are needed in order to further our understanding of these enzymes as well as to determine if their inhibition within inflammatory diseases could yield effective treatments. During the initial phase of this study, in which a potential new inhibitor was being tested, an unknown inhibition phenomenon was found. When natural lipid extracts were added to a sPLA<sub>2</sub> assay in concentrations that cannot cause surface dilution, substantial inhibition was observed. The subsequent study set out to characterize the chemical structural elements that may be responsible for this inhibition.



## Materials and Methods

### 2.1 *sPLA<sub>2</sub>* Assay Stock Reagents

All chemicals are from Millipore Sigma unless otherwise stated. Assay buffer stock (25 mM Tris-HCl, 10mM CaCl<sub>2</sub>, 100 mM KCl, 0.3 mM Triton X-100, pH 7.5) and Tris-HCl stock (0.4 M Tris-HCl, pH 8.0) was stored at 4°C. Aliquots of *Crotalus adamanteus* (eastern diamondback rattlesnake) sPLA<sub>2</sub>GIIA (0.025mg/mL) were stored at -20°C. 1,2-dithioheptanoyl-sn-glycero-3-phosphorylcholine (diheptanoyl-thio-PC) (10 mM) and 1-palmitoyl-2-thio-arachidonoyl-sn-glycero-3-phosphorylcholine (arachidonoyl-thio-PC) (10 mM) in 9:1 chloroform: methanol were stored at -20°C. Bovine serum albumin (BSA) in assay buffer solution (1mg/mL) along with Ellman's reagent (5-5'-dithio-bis-(2-nitrobenzoic acid)/DTNB (30mM)) were prepared weekly and stored at 4°C between assays. Before assays an aliquot of either diheptanoyl-thio-PC or arachidonoyl-thio-PC substrate was evaporated under a stream of nitrogen and re-suspended/water bath sonicated with BSA and assay buffer solution to a final concentration of 5 mM. Lipid additives (dioctanoyl-PC, egg-PC, oleic acid, heptanoic acid, deoxycholic acid, tween 20, or hexylamine) were evaporated under a stream of nitrogen and re-suspended in DMSO: ethanol (EtOH) (1:1) by vortexing and water bath sonication. Dilution to achieve needed total mole fractions were made in the assay buffer described above. Total mole fraction = [additive]/ [additive + substrate + detergent].

### 2.2 *Diheptanoyl-Thio-PC sPLA<sub>2</sub>* Assay

This assay was based on the sPLA<sub>2</sub> assay protocol from Reynolds, Hughes, and Dennis (31). This sPLA<sub>2</sub> assay was performed in 96 microwell plates. Each well contained 85 µL of BSA (1mg/mL), 10 µL of 30 mM Ellman's reagent (DTNB), 50 µL of 5 mM

diheptanoyl-thio-PC substrate, and 5  $\mu$ L of either lipid additive or vehicle control (1:1 DMSO: EtOH). Negative control wells received 90  $\mu$ L BSA (1mg/mL). A final volume in each well of 155  $\mu$ L was reached after enzyme addition (5  $\mu$ L). Next, the plate was placed on a plate shaker for 5-10 minutes. After mixing, positive control and experimental groups were challenged with 5  $\mu$ L of 0.025mg/mL *Crotalus adamanteus* sPLA<sub>2</sub>GIIA mixed by pipetting then immediately placed on Molecular Devices M2 plate reader set to 414 nm at room temperature ( $\sim$ 23°C). Plates were read every 30 sec for 8 minutes. Each experimental assay was replicated in triplicate.

*Crotalus adamanteus* sPLA<sub>2</sub>GIIA enzyme, when added, will hydrolyze the *sn*-2 glycerol position of the thio-labeled substrate (diheptanoyl-thio-PC or arachidonoyl-thio-PC). In turn the sulfide on the lysophospholipid will react with Ellman's reagent to produce a 5-thio-2-nitrobenzoic acid. The production of 5-thio-2-nitrobenzoic acid leads to a measurable colorimetric change.

### 2.3 Arachidonoyl-Thio-PC cPLA<sub>2</sub> Assay

This procedure was based on a cPLA<sub>2</sub> assay kit procedure from Cayman Chemical (Item #765021). This assay was performed exactly as the diheptanoyl-thio-PC assay described above except the assay buffer stock was prepped with 8.0 mM Triton X-100, 2-deoxy-2-thio arachidonoyl PC was used as the substrate, and the temperature of the spectrophotometer was set at 37°C, unless otherwise stated.

### Statistical Analysis

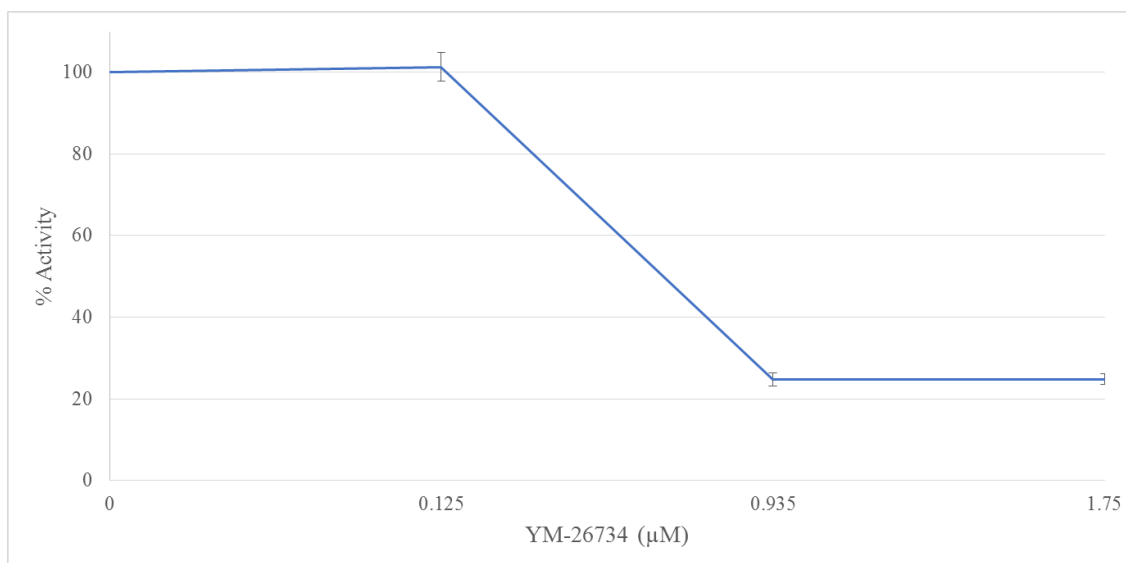
Percent activity values were determined by comparing the slope of the experimental groups with the slope of the control group. The statistical analysis of this data was performed

in Microsoft Excel. An analysis of variance (F-test) was used to determine if two sets of data at the same mole fraction/concentration contained equal variances. A Student T-test was used to signify the differences in percent activity between two experimental groups at a significance level of  $p < 0.05$ .

## Results and Discussion

### 3.1 PLA<sub>2</sub> Assay Functionality

The diheptanoyl-thio-PC (sPLA<sub>2</sub>) assay was tested with a known inhibitor, YM-26734, to determine if the assay was functioning similar to Myake, A. et al. (16). YM-26734 has an average IC<sub>50</sub> of 0.085 μM with a range from 0.056-0.129 μM against rabbit sPLA<sub>2</sub>GIIA. *Crotalus adamanteus* sPLA<sub>2</sub>GIIA should act similar to rabbit sPLA<sub>2</sub>GIIA since both mammals and snakes have similar sPLA<sub>2</sub> crystal structures (32). Since the standard diheptanoyl-thio-PC assay required a higher enzyme concentration a proportionate amount of YM-26734 was used, thus the inhibitor concentrations 0.125, 0.935, and 1.75 μM were tested (Figure 4). An IC<sub>50</sub> of 0.67 μM was calculated. Since this was near published values it was concluded that the assay and response to lipidic inhibitors was functioning similarly to the rabbit enzyme in other labs (16).

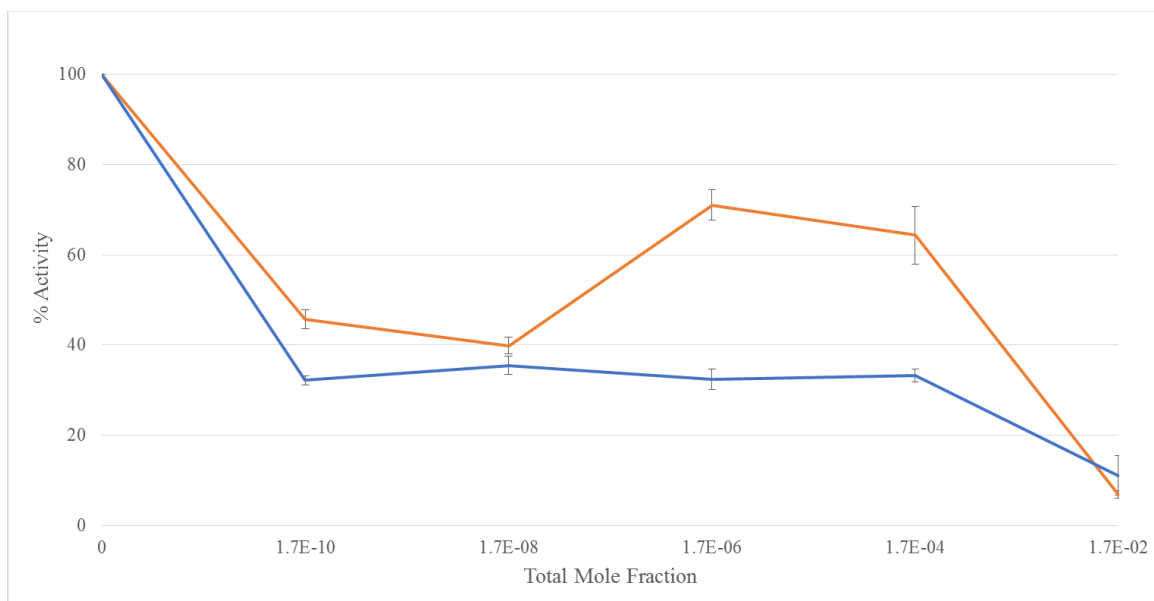


**Figure 4. Inhibition of sPLA<sub>2</sub> by YM-26734.** Percent activity curve for known inhibitor YM-26734 (blue). YM-26734 inhibited sPLA<sub>2</sub>GIIA from *Crotalus adamanteus* with an IC<sub>50</sub> of ~0.67 μM. Values represented as averages ± standard deviation and n = 3.

### 3.2 Effect of Dioctanoyl-PC and Egg-PC on PLA<sub>2</sub> Activity in Diheptanoyl-Thio-PC Assay

Dioctanoyl-PC (DC<sub>8</sub>PC) was added to the sPLA<sub>2</sub> assay in small molar fractions as a potential control for future natural lipidic inhibitors. DC<sub>8</sub>PC was added as stated in section 2.2 with mole fraction relative to total lipid present (total mole fraction). At a mole fraction of  $1.7 \times 10^{-2}$  DC<sub>8</sub>PC decreased enzymatic activity by 85% compared to control (Figure 5). At mole fractions of  $1.7 \times 10^{-4}$ ,  $1.7 \times 10^{-6}$ ,  $1.7 \times 10^{-8}$ , and  $1.7 \times 10^{-10}$  DC<sub>8</sub>PC had less of an effect on sPLA<sub>2</sub>GIIA with a decrease in activity of around 65%. The “inhibitory effect” of DC<sub>8</sub>PC should not have been as drastic as was observed according to the surface dilution model (28). At these low mole fractions there was more of an effect than would be predicted if surface dilution or affinity was the main contributor. Indeed, when adding DC<sub>8</sub>PC at one molecule in  $10^{10}$  there was a 65% inhibition. At this concentration there should be undetectable surface dilution or occupation of the enzyme by unlabeled substrate. The “inhibitory effect” did subside after a mole fraction of  $1.7 \times 10^{-15}$  was achieved ( $98.1 \pm 2.99\%$  of control).

In order to determine if the same “inhibitory effect” would be seen with longer and unsaturated fatty acid chains, egg-phosphatidylcholine (egg-PC, 18 carbon) was added in the same mole fractions as DC<sub>8</sub>PC (8 carbon) in hopes that the resulting increase in fluidity would allow the enzyme to run at 100% activity. At  $1.7 \times 10^{-2}$  mole fraction egg-PC yielded the same result as DC<sub>8</sub>PC at the same mole fraction (Figure 5). As the concentration increased, the sPLA<sub>2</sub>GIIA activity decreased, similar to DC<sub>8</sub>PC. Moreover, with egg-PC, the activity still did not reach expected levels of 100% activity.



**Figure 5.** DC<sub>8</sub>PC and egg-PC's effect on *Crotalus adamanteus* sPLA<sub>2</sub>GIIA. Molar fractions of dioctanoyl-PC (blue) and egg-PC (orange) were added to the assay to determine their effect on sPLA<sub>2</sub>GIIA percentage of activity. Values represented as average  $\pm$  standard deviation (error bars) and n = 3.

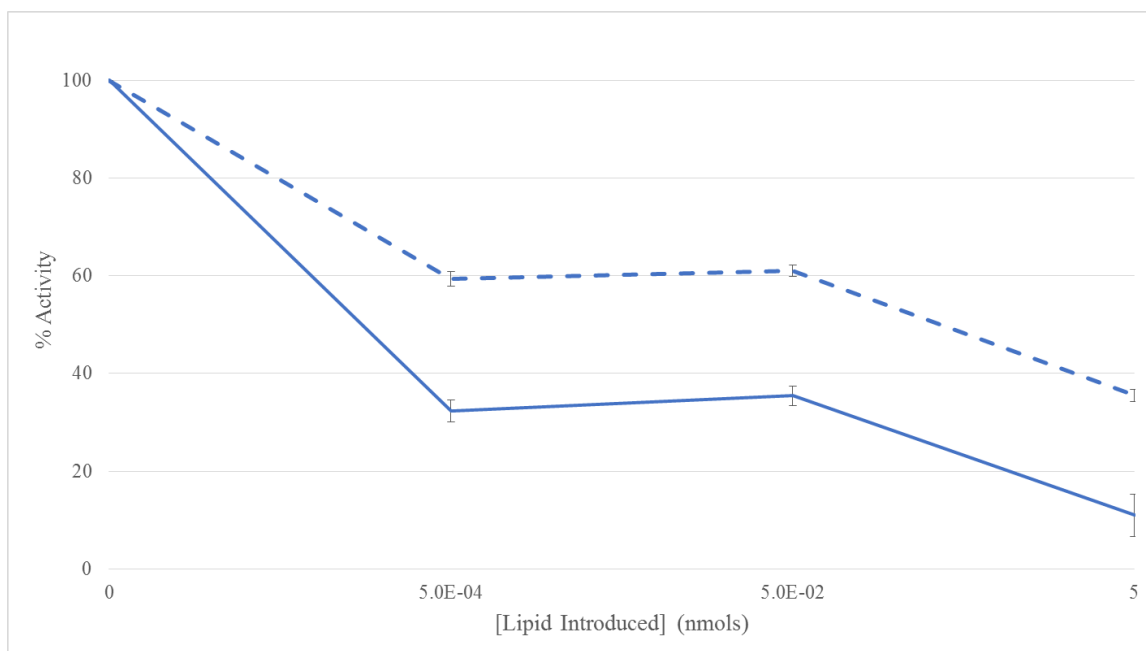
### 3.3 Effect of Dioctanoyl-PC and Egg-PC on PLA<sub>2</sub> Activity in Arachidonoyl-Thio-PC Assay

Next, we wanted to see if the same inhibition would occur with a long chain substrate. The three higher concentrations of DC<sub>8</sub>PC and egg-PC from the diheptanoyl-thio-PC assay (see 3.2) were tested again here using long-chain (2-deoxy-2-thio arachidonoyl PC) as the labeled substrate with the same enzyme, *Crotalus* sPLA<sub>2</sub>GIIA as above. The assay with this substrate requires changes in Triton X-100 concentration (8.0 mM) and temperature (37°C). These conditions were tested to determine if the “inhibitory effect” seen previously (Section 3.2) was due to an unfavorable enzyme environment. Had the inhibition been due to the substrate's short fatty acids (8 carbons), then the arachidonoyl-thio-PC assay (20 carbons) should not show any inhibition. Total mole fraction changes in relation to the concentration of detergent in the assay (see equation in section 2.1). Since the diheptanoyl-thio-PC and arachidonoyl-thio-PC assays differ in Triton X-100 concentration the total mole fraction would be different and a direct comparison could not be made, thus it was decided to keep

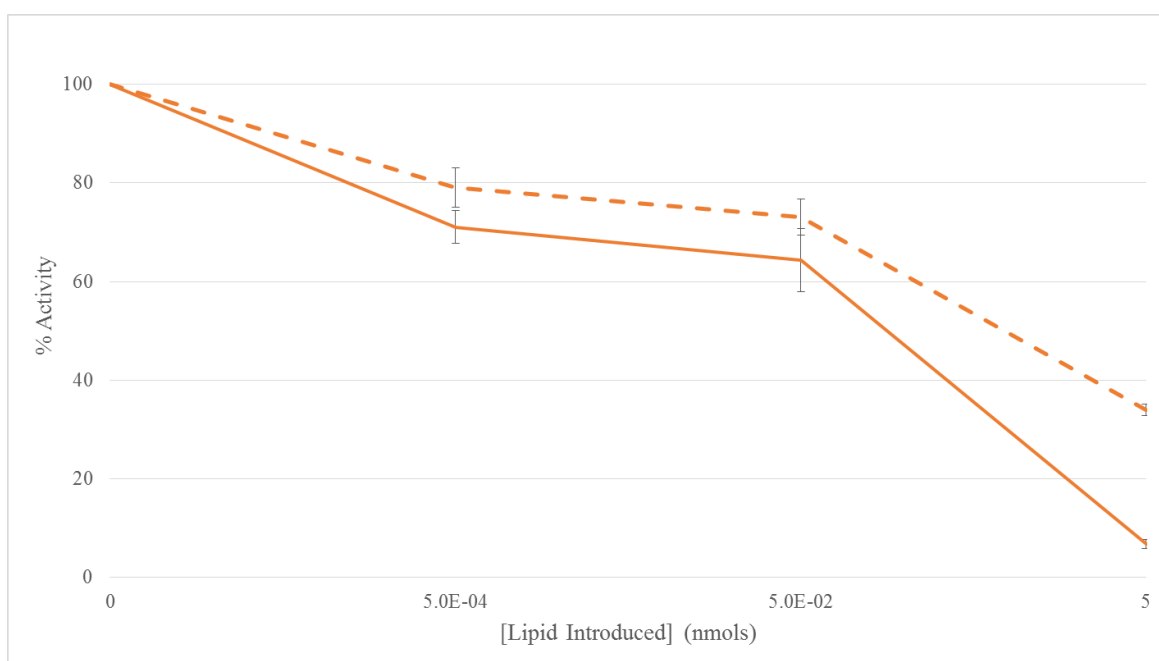
the concentration/number of DC<sub>8</sub>PC and egg-PC added to the assays the same across subsequent tests; thus the concentration of lipid introduced was used as an x-axis for comparison purposes.

DC<sub>8</sub>PC caused significantly less reduction of activity in the arachidonoyl-thio-PC assay than in the diheptanoyl-thio-PC assay (Figure 6). sPLA<sub>2</sub> activity was 24-28% higher in the arachidonoyl-thio-PC assay (dashed vs solid) at all three tested concentrations ( $p = 7.5 \times 10^{-4}$ ,  $1.3 \times 10^{-5}$ , and  $5.4 \times 10^{-5}$ ). However, activity still failed to reach expected values of 100% activity at any mole fraction.

The difference in inhibition by egg-PC between diheptanoyl-thio-PC and arachidonoyl-thio-PC was statistically significant at the lowest ( $5 \times 10^{-4}$  nmol) and highest (5 nmol) concentrations of lipid introduced ( $p = 0.027$  and  $5.4 \times 10^{-6}$ , respectively), but not at the intermediate concentration ( $5 \times 10^{-2}$  nmols) ( $p = 0.055$ ) (Figure 7). Overall, the “inhibitory effect” was still present with the addition of either lipid in both assay conditions although the arachidonoyl-thio-PC did show less of a reduction. A change in assay conditions inherent between the two assays may lead to expected values of 100% activity.



**Figure 6. DC<sub>8</sub>PC in diheptanoyl-thio-PC and arachidonoyl-thio-PC assay conditions.** Differences in percent activity for DC<sub>8</sub>PC (blue) in diheptanoyl-thio-PC (solid) and arachidonoyl-thio-PC (dashed) assays. Higher values indicate less inhibition of the enzyme. Values represented as averages  $\pm$  standard deviation and  $n = 3$ .



**Figure 7. Egg-PC in diheptanoyl-thio-PC and arachidonoyl-thio-PC assay conditions.** Differences in percent activity for egg-PC (orange) in diheptanoyl-thio-PC (solid) and arachidonoyl-thio-PC (dashed) assays. Higher values indicate less inhibition of the enzyme. Values represented as averages  $\pm$  standard deviation and  $n = 3$ .



### 3.4 Effect of Arachidonoyl-Thio-PC Assay conditions on PLA<sub>2</sub> Activity

The differences in effect between of DC<sub>8</sub>PC and egg-PC in the arachidonoyl-thio-PC and diheptanoyl-thio-PC assays, described in section 3.3, could have been due to changes in Triton X-100 concentration, temperature, or the substrate itself. The effects of each variation in conditions between the arachidonoyl-thio-PC and diheptanoyl-thio-PC assays were separately examined while keeping the concentrations of added DC<sub>8</sub>PC and egg-PC constant.

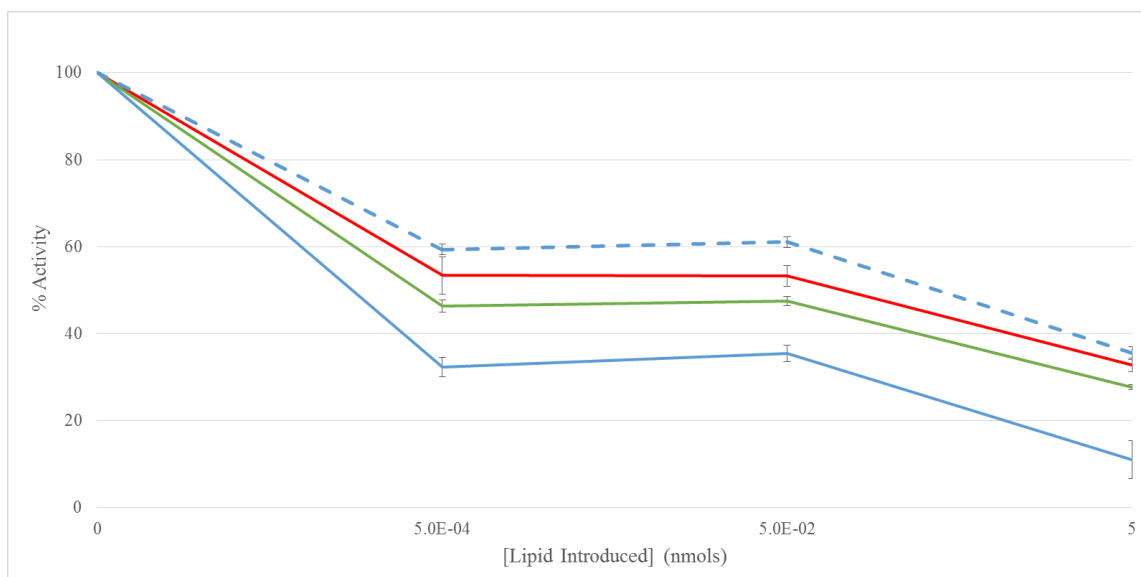
Triton X-100 concentration was the first variable examined. In the diheptanoyl-thio-PC assay Triton X-100 was 0.3 mM, however, this was increased to 8.0 mM for the arachidonoyl-thio-PC assay (see Figure 6 and Sections 2.1 and 2.3), thus the same diheptanoyl-thio-PC assay (challenged with DC<sub>8</sub>PC) was performed except a concentration of 8.0 mM Triton X-100 was used. This increase in detergent increased sPLA<sub>2</sub> activity in the diheptanoyl-thio-PC assay by approximately 15% at each concentration (Figure 8). This increase in detergent should have reduced percent activity according to the surface dilution model as the substrate molecules become more spread out in the membrane (29). However, here it is shown that an increase in detergent alone significantly increased activity. This increase in activity may be explained by the “surface binding model” in which the increase in detergent leads to the formation of more micelle surface area (33). Since there was an increase in micelle number more enzymes were able to bind more effectively and therefore the inhibitory effect of dioctanoyl-PC on the assay was lessened resulting in an increase in overall percentage of activity.

The second variable to be examined was temperature. While the diheptanoyl-thio-PC assay was performed at room temperature (23°C) the arachidonoyl-thio-PC assay was performed at human body temperature (37°C). As shown in Figure 8, temperature does have

a significant effect at all three concentrations tested. Phospholipases A<sub>2</sub> along with other enzymes function more effectively at warmer temperatures, and thus a rise in percent activity was expected. Another factor is an expected increase in micelle membrane fluidity and lateral lipid diffusion. The overall increase in activity seen in Figure 8 is likely a combination of the two effects. However, since degradation of enzyme would occur at higher assay temperatures increasing the temperature of the assay would not be a viable option for reaching 100% activity.

Finally, the effect of substrate fatty chain can be seen when comparing the standard arachidonoyl-thio-PC assay to the temperature and detergent varied diheptanoyl-thio-PC assay. Although the percentage of activity between the two are similar there is a significant difference between arachidonoyl and diheptanoyl substrates at concentrations  $5.0 \times 10^{-4}$  and  $5.0 \times 10^{-2}$  ( $p = 0.045$  and  $6.9 \times 10^{-3}$ , respectively) (Figure 8). This difference is due to the difference in fatty acid length. The heptanoylacyl groups are saturated and short (7 carbons) while arachidonoyl groups are polyunsaturated and long (20 carbons). Although both of these molecules are good substrates for the sPLA<sub>2</sub>GIIA used, this difference might be due to a slight difference in substrate affinity or due to the difference in fatty acid length (31, 34).

With the condition effects established we examined the effect of various structural elements of substrate analogs. As observed with the diheptanoyl-thio-PC assay, the arachidonoyl-thio-PC showed a large decrease in activity with the addition of DC<sub>8</sub>PC even at these low concentrations. Since there was a difference of activity between the two substrates alone, regardless of assay conditions, fatty acid length may be affecting micelle structure in some way to cause this decrease in activity seen in all tests thus far.



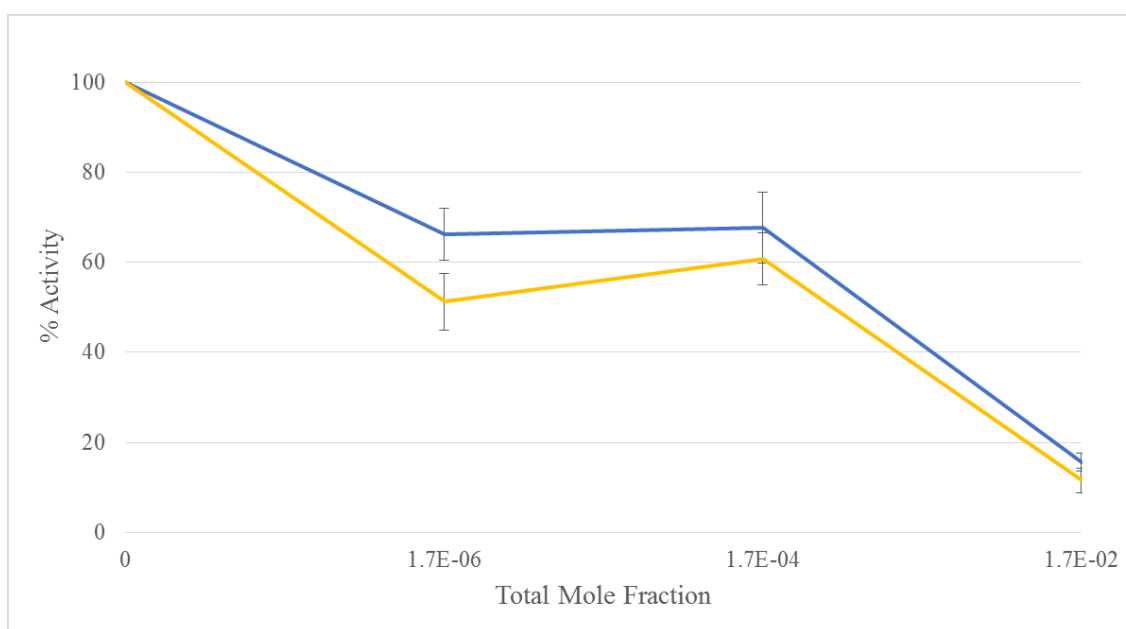
**Figure 8. Effects of Assay Conditions on PLA<sub>2</sub> Activity.** DC<sub>8</sub>PC in diheptanoyl-thio-PC (solid blue) and arachidonoyl-thio-PC (dashed blue) assays were carried over from figure 6 as a reference. DC<sub>8</sub>PC in diheptanoyl-thio-PC assay with a rise in Triton X-100 concentration from 0.3 mM to 8.0 mM (green). DC<sub>8</sub>PC in diheptanoyl-thio-PC assay with an increase in Triton X-100 concentration (8.0 mM) and temperature (from 23°C to 37°C) (red). DC<sub>8</sub>PC in arachidonoyl-thio-PC assay with an increase in Triton X-100 concentration (8.0 mM), temperature (37°C), with arachidonoyl-thio-PC substrate (dashed blue). Values represented as averages  $\pm$  standard deviation and n = 3.

### 3.5 Effects of Fatty Acid Length on PLA<sub>2</sub> Activity

In Section 3.4, the increase in fatty acid length and unsaturation of arachidonoyl-thio-PC vs diheptanoyl-thio-PC, was shown to have lessened the “inhibitory effect” of DC<sub>8</sub>PC on the assay. We set out to determine if the fatty chains themselves were causing this phenomenon. We used oleic acid (18 carbons) and heptanoic acid (7 carbons). Fatty acids have the added benefit of not being a potential substrate for the enzyme unlike DC<sub>8</sub>PC or egg-PC. This prevents any decrease in activity to be attributed as enzyme affinity towards an unlabeled substrate. In order to directly maintain the fatty acids at the same concentration as DC<sub>8</sub>PC and egg-PC the concentrations of fatty acids were two times the PC concentrations, *i.e.* two fatty acids would substitute for one phospholipid.

At  $1.7 \times 10^{-2}$  and  $1.7 \times 10^{-4}$  mole fraction both heptanoic acid and oleic acid had similar inhibitory effects on sPLA<sub>2</sub> activity (Figure 9). At  $1.7 \times 10^{-6}$  mole fraction however heptanoic

acid continued to follow the same trend as egg-PC, but oleic acid decreased in percent activity and was significantly different from heptanoic acid ( $p = 1.9 \times 10^{-2}$ ). Overall, we can infer that fatty acid length is not the critical factor responsible for the inhibitory phenomenon. These results also tell us that the difference seen in Figure 8 between the two substrates may be due to affinity for the enzyme and not due to fatty acid length. Oleic and heptanoic acids showed the same activity trend as egg-PC *i.e.* all three showed ~40% reduction in activity (Reference Figure 5). Therefore, with this data it can be concluded that fatty acid length and presence of a phosphorylcholine group are not the major contributing factors to this “inhibitory effect.”



**Figure 9. Effects of Fatty Acid Length on PLA<sub>2</sub> Activity.** Oleic acid (yellow) and heptanoic acid (light blue) were tested in the diheptanoyl-thio-PC assay. Values represented as averages  $\pm$  standard deviations with  $n = 3$ .

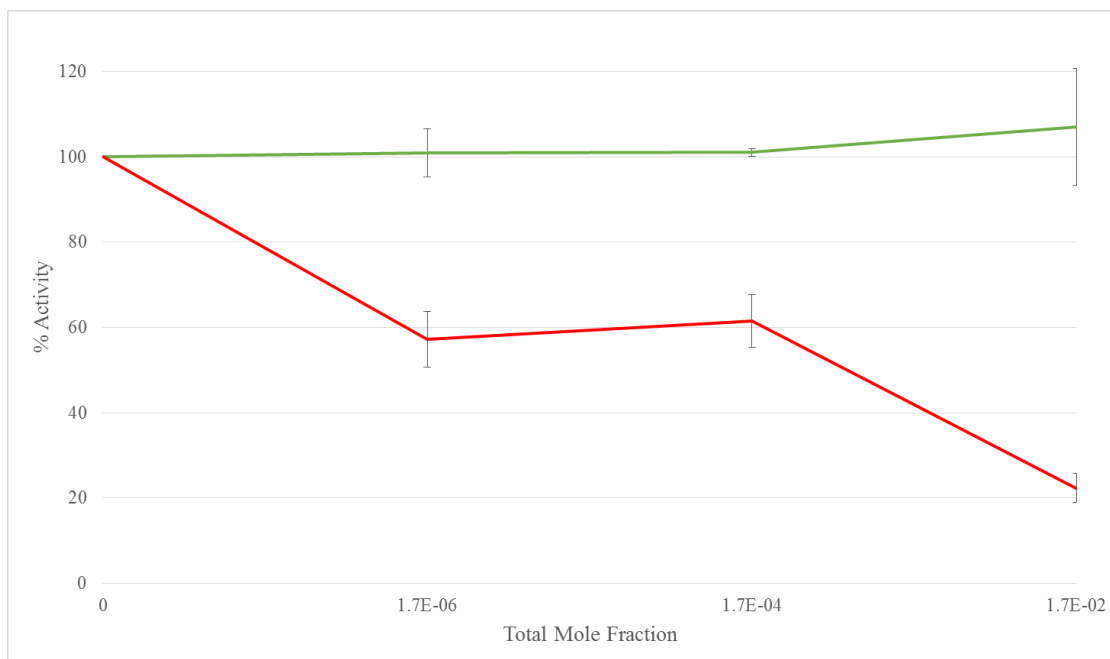
### 3.6 Effects of Ionic and non-Ionic Detergents on PLA<sub>2</sub> Activity

Next, we wanted to determine if the charge of the lipid additives is a key factor in sPLA<sub>2</sub> inhibition. The ionic detergent deoxycholic acid and the non-ionic detergent tween 20 were chosen to represent a negative and positively charged additive, respectively. The non-

ionic detergent, Triton X-100, was used in the standard diheptanoyl-thio-PC assay. In Figure 8, where the Triton X-100 concentration was increased from 0.3 mM to 8.0 mM there was no variation to the control (mole fraction of 0), thus Triton X-100 was inadvertently shown to not have an inhibitory effect when added to the assay.

Deoxycholic acid followed a similar “inhibitory” pattern to that of egg-PC in which both deoxycholic acid (Figure 10), heptanoic acid, and oleic acid (Figure 9) had similar percent activities at each mole fraction. Tween 20, interestingly, did not inhibit the reaction but slightly increased the reaction rate at the highest mole fraction ( $1.7 \times 10^{-2}$ ), although not significantly (Figure 10). This result departed from all of the amphipathic molecules tested thus far. This may be due to its lack of charge. Deoxycholic acid, oleic acid, heptanoic acid, dioctanoyl-PC, and egg-PC all have partial or full ionic charges, and more specifically exhibit negative charges (at pH = 7.0-7.4). Presumably, the addition of these charges affect the overall surface charge which can significantly impact the physical interaction of an enzyme with its interface. sPLA<sub>2</sub>s utilize hydrophobic and hydrophilic amino acids in order to incorporate themselves into a lipid interface as well as interact with an interface’s surface. If an interface’s surface charge changes significantly enough, then the amino acids associated with the interface may not be able to interact as effectively (33). If the surface charge is preventing the binding of sPLA<sub>2</sub>GIIA here, then we would expect to see a decrease in reaction rate or “inhibitory effect”. This seems to be what we have seen thus far. However, if a lipid was incorporated and did not interfere with surface charge, then we would not expect to see a decrease in reaction rate. In Figure 10 we see that tween 20, which is non-ionic, does not reduce reaction rate. This lack of “inhibitory effect” is attributed to a lack of change in

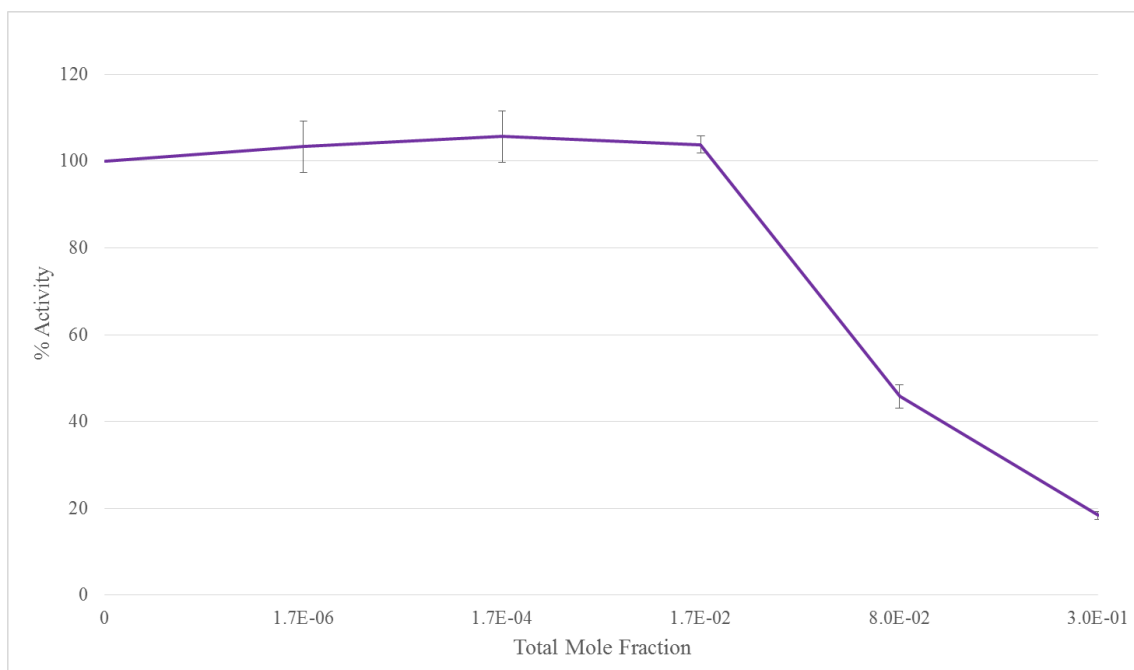
overall surface charge. However, historically sPLA<sub>2</sub>s have been shown to prefer binding to anionic surfaces, thus a binding study would likely clarify this phenomenon (35).



**Figure 10. Effects of Fatty Chain Charge on sPLA<sub>2</sub> Activity.** Effects of deoxycholic acid (red) and tween 20 (green) on diheptanoyl-thio-PC assay. Values represented as averages  $\pm$  standard deviation and  $n = 3$ .

In order to further support that charge as the basis for the “inhibitory effect” we tested the fatty amine, hexylamine, containing six carbons and a cationic primary amine.

Hexylamine, at low mole fractions, showed no significant effect on sPLA<sub>2</sub> activity, similar to results with tween 20 (Figure 11 and 10, respectively). At the higher mole fractions of  $8 \times 10^{-2}$  and  $3 \times 10^{-1}$  (50% and 90% of [substrate]), hexylamine had percent activities at  $45.8 \pm 2.67\%$  and  $18.4 \pm 0.90\%$  respectively, consistent with that of surface dilution (Figure 11). Therefore, when non-ionic or positively charged lipids are incorporated at low mole fraction a reduction in reaction rate is not seen. This evidence supports the conclusion that the negative charge is responsible for the decrease in reaction rate of sPLA<sub>2</sub>.



**Figure 11. Effects of Hexylamine on sPLA<sub>2</sub> Activity.** Effects of hexylamine (purple) on diheptanoyl-thio-PC assay. Values represented as averages  $\pm$  standard deviation and  $n = 3$ .

### 3.7 Future Directions

Since the results seen here do not match up with the expected results according to various models established in the literature future research should be focused on the characterization of this phenomenon. One potential future direction is the testing of other lipid additives in this assay to expand on the tests performed here. Such tests could include adding lipids that are more positively or negatively charged to the assay and determining their effect on the rate of reaction. Another such test could determine if the molecular size of the lipid additive would have an effect on the rate of reaction. These tests would not only confirm the presence of this phenomenon, but would also expand the understanding behind the mechanism by which it is acting.

A second future direction would be the testing of this phenomenon in other sPLA<sub>2</sub> assays such as the EnzChek™ (Product #E10217) assay which utilizes a fluorescent detection method. Since that technique is substantially different if the inhibition phenomenon

were to still be present then it would suggest that the effect is more generally applicable. Conversely, if the inhibition phenomenon seen here did not occur in the EnzChek™ assay with the same lipid additives, then this phenomenon could be due to the specifics of the thiolipid assay.

The structure of the micelles themselves could be imaged under scanning or transition electron microscopy before and after the addition of these additives in order to determine if the overall structure of the micelle changes. Along with these techniques a radioactive labeled lipid could be added to a micelle and imaged to determine if and how the lipid becomes incorporated within the existing micelle. This test would also be beneficial by showing whether or not the lipid additives are aggregating on the surface of the micelle. If this were to occur then an sPLA<sub>2</sub> enzyme may become lodged in this “lipid aggregate” which would affect enzyme activity.



## Conclusion

In this study, a large “inhibitory effect” on PLA<sub>2</sub> activity was seen when lipids were added at molar fractions below what surface dilution models can explain. This was seen with phospholipids, fatty acids, and detergents. Evidence points to this being due to a change in micelle surface charge, more specifically a negative charge. Past literature suggests that phospholipases A<sub>2</sub> typically have a higher affinity for anionic lipid interfaces, and the addition of lipids at these mole fractions should not affect the physical characteristics of a micelle such as size, charge, or area per molecule (32, 34). However, this study suggests that these subjects be revisited and further investigated. Thus, it is important to recognize these type of effects when performing future inhibition studies on interfacial enzymes such as sPLA<sub>2</sub>s.

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### **Vita**

James Lee Foley was born in Concord, North Carolina, to James John Foley and Michelle Leigh Foley. James was awarded a Bachelor of Science in Cell and Molecular Biology from Appalachian State University in May of 2016. He received his Master of Science with a concentration in Cell and Molecular Biology in December of 2018. James currently aspires of going into the field of biomedical research.