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A non-venomous sPLA$_2$ of a lepidopteran insect: Its physiological functions in development and immunity

Mohammad Vatanparast[a], Shabbir Ahmed[a], Salvador Herrero[b], Yonggyun Kim[a,*]

[a] Department of Plant Medicals, Andong National University, Andong, 36729, South Korea
[b] Department of Genetics, Universitat de València, Dr Moliner 50, 46100, Burjassot, Valencia, Spain

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ABSTRACT

Eicosanoids are oxygenated C20 polyunsaturated fatty acids that mediate various physiological processes in insects. Eicosanoid biosynthesis begins with a C20 precursor, arachidonic acid (5,8,11,14-eicosatetraenoic acid: AA). AA is usually released from phospholipids at *sn-2* position by catalytic activity of phospholipase A$_2$ (PLA$_2$). Although various PLA$_2$s classified into 16 gene families (= Groups) are known in various biological systems, few PLA$_2$s are known in insects. Only two PLA$_2$s involved in intracellular calcium independent PLA$_2$ (iPLA$_2$) group have been identified in lepidopteran insects with well known eicosanoid physiology. This study reports the first secretory PLA$_2$ (sPLA$_2$) in lepidopteran insects. A partial open reading frame (ORF) of PLA$_2$ was obtained by interrogating *Spodoptera exigua* transcriptome. Subsequent 3′-RACE resulted in a full ORF (*Se-sPLA2A*) encoding 194 amino acid sequence containing signal peptide, calcium-binding domain, and catalytic site. Phylogenetic analysis indicated that Se-sPLA$_2$A was clustered with other Group III sPLA$_2$s. *Se-sPLA$_2$A* was expressed in most larval instars except late last instar. Its expression was inducible by immune challenge and juvenile hormone analog injection. RNA interference of *Se-sPLA$_2$A* significantly suppressed cellular immunity and impaired larval development. These results suggest that non-venomous sPLA$_2$ plays a crucial role in immune and developmental processes in *S. exigua*, a lepidopteran insect.

1. Introduction

Eicosanoids are a group of oxygenated C20 polyunsaturated fatty acids (PUFAs). These lipid signal molecules can mediate various physiological processes in vertebrates and invertebrates (Kim et al., 2018). Eicosanoids are classified into three subgroups: prostaglandin (PG), leukotriene (LT), and epoxyeicosatrienoic acid (EET). In mammals, biosynthesis of eicosanoids begins with hydrolysis of a C20 PUFA (usually arachidonic acid: AA) from the *sn-2* position of phospholipids (PLs) by phospholipase A$_2$ (PLA$_2$). Free AA is then oxygenated by cyclooxygenase (COX), leading to various PGs. It is also oxygenated by lipoxygenases (LOXs) to generate LTs or other products. In addition, it can be oxygenated by position-specific cytochrome P$_{450}$ monooxygenases to produce epoxygenated products. PGs and LOX products play crucial roles in mediating excretion, reproduction, and immune responses in insects (Stanley and Kim, 2014). Increased EET levels can lead to up-regulated expression levels of some antimicrobial peptide genes (AMPs) in mosquitoes that are resistant to malarial parasites (Xu et al., 2016). Thus, all three major eicosanoid groups (PGs, LTs, and EETs) play crucial signaling roles in insect immunity, in which PLA$_2$ catalyzes the committed step for biosynthesis of eicosanoids and it has been regarded as a molecular target controlled by various endogenous and exogenous regulators to alter physiological status (Stanley, 2006; Kim et al., 2018).

Many PLA$_2$ genes are present in biological systems. Based on their amino acid sequences, molecular weights, disulfide bonds, and Ca^{2+} requirements, they are separated into at least 16 gene families (called Groups I-XVI) (Vasquez et al., 2018). PLA$_2$s mediate diverse biological functions such as lipid digestion, cellular membrane remodeling, signal transduction, host immune defense, and production of various lipid mediators in mammalian studies (Valentin and Lambeau, 2000). For convenience, these diverse PLA$_2$s are largely classified into three types: secretory PLA$_2$ (sPLA$_2$), Ca^{2+}-dependent cellular PLA$_2$ (cPLA$_2$) and Ca^{2+}-independent cellular PLA$_2$ (iPLA$_2$) (Burke and Dennis, 2009). sPLA$_2$s found in venoms, pancreatic juice, and arthritic synovial fluid were the first known PLA$_2$s. They are characterized by a small size (13–15 kDa), six conserved cysteines, histidine-containing active site, and Ca^{2+}-dependent activity (Gelb et al., 1995). In insects, sPLA$_2$s have been discovered in hymenopteran venoms (Dudler et al., 1992; Xin et al., 2009; Baek and Lee, 2010). Four non-venomous sPLA$_2$s encoded

* Corresponding author.
E-mail address: hosanna@anu.ac.kr (Y. Kim).

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in the genome of *Tribolium castaneum* are expressed in hemocytes. They can mediate cellular immune responses (Shrestha et al., 2010). iPLA$_2$s are similar to cPLA$_2$s in their active sites. However, their enzymatic activities are independent of Ca^{2+}. Instead of a C2 domain present in cPLA$_2$, iPLA$_2$ possesses 7–8 ankyrin repeats for protein interactions (Six and Dennis, 2003). iPLA$_2$s are relatively large molecules (85–88 kDa) without calcium dependency. The first insect iPLA$_2$ (SeiPLA$_2$A) was identified in a lepidopteran insect, *Spodoptera exigua.* It has functional association with cellular immune response (Park et al., 2015). Phylogenetic analysis showed that SeiPLA$_2$A is clustered with Group VI subgroup A in PLA$_2$ superfamily and characterized by multiple ankyrin repeats in the N-terminal region with a consensus lipase motif (GTSTG) in the C-terminal region (Winstead et al., 2000). Another type of iPLA$_2$ has also been identified from *S. exigua* (SeiPLA$_2$B) (Sadekuzzaman et al., 2017). SeiPLA$_2$B possesses a lipase catalytic site. However, it does not have ankyrin repeats in the amino terminal region, unlike SeiPLA$_2$A. Phylogenetic analysis indicated that SeiPLA$_2$B was clustered with Group VI subgroup F, unlike SeiPLA$_2$A. Many cPLA$_2$s prefer AA-containing substrates. They are central enzymes mediating the generation of eicosanoids. In vertebrates, cPLA$_2$s mediate inflammatory processes (Kramer and Sharp, 1997). cPLA$_2$s are large proteins (61–114 kDa) with a Ca^{2+}-binding C2 domain and a serine/aspartic acid dyad-containing active site (Ghosh et al., 2006).

Immune responses mediated by eicosanoids are well known in lepidopteran insects, suggesting a crucial role of PLA$_2$. However, no sPLA$_2$ has been identified in lepidopteran insects. The presence of sPLA$_2$ has been supported by several evidences. Rana and Stanley (1999) have detected the secretion of PLA$_2$ from the midgut epithelium of *Manduca sexta* using *in vitro* organ culture system. Immune responses of several lepidopteran insects are crippled after treatment with inhibitor targeting sPLA$_2$ (Stanley and Kim, 2014). Cellular immune responses of *M. sexta* hemocytes require PLA$_2$ activity that is susceptible to sPLA$_2$-specific inhibitor (Park et al., 2005). Bacterial challenge can increase PLA$_2$ activity of *M. sexta* hemocytes (Tunaz et al., 2003). Injection of a sPLA$_2$ inhibitor increases the susceptibility of *Lymantria dispar* or *S. frugiperda* to a baculovirus (Stanley and Shapiro, 2007, 2009). In *S. exigua*, PLA$_2$ sensitive to sPLA$_2$ inhibitor exhibits development- and tissue-specific activity patterns (Sadekuzzaman and Kim, 2017). Insect pathogenic bacteria *Photorhabdus* and *Xenorhabdus* exert immunosuppressive actions by inhibiting sPLA$_2$s (Park et al., 2005; Kim et al., 2005). These findings suggest that sPLA$_2$ is present in lepidopteran insects.

The objective of this study was to identify lepidopteran sPLA$_2$ from the beet armyworm, *S. exigua*. We choose *S. exigua* as the lepidopteran insect in this study because eicosanoid-mediated physiological processes have been well-studied in *S. exigua*. First, the presence of sPLA$_2$ activity in *S. exigua* has been documented (Park and Kim, 2003; Sadekuzzaman and Kim, 2017). Second, eicosanoids released from the catalytic activity of sPLA$_2$ can mediate both cellular and humoral immune responses in *S. exigua* (Shrestha and Kim, 2009). Third, *S. exigua* PLA$_2$ activities are inducible in fat body and hemocytes in response to microbial challenge, suggesting the presence of immune-associated PLA$_2$ (Park and Kim, 2012). Here, we report a lepidopteran sPLA$_2$ and its physiological functions associated with both immunity and development.

2. Materials and methods

2.1. Insect rearing

Beet armyworm larvae were reared with an artificial diet as described previously (Goh et al., 1990) under controlled condition (25 °C, 16:8 h of L:D photoperiod, and 60 ± 5% relative humidity). Adults were fed with 10% sucrose solution. Larval instars (L1-L5) were determined based on head capsule sizes (Goh et al., 1990).

2.2. Total PLA$_2$ enzyme activity

Total PLA$_2$ activity was measured using a pyrene-labeled surrogate substrate in the presence of bovine serum albumin (BSA) by spectrofluorometry (Radvanyi et al., 1989; Seo et al., 2012). Enzyme samples were prepared by isolating hemolymph from 10 individuals of L4 larvae per replication. The hemolymph was centrifuged at 800 × *g* for 3 min at 4 °C and the supernatant (= plasma) protein concentration was determined by Bradford (1972) assay using BSA as a standard. PLA$_2$ reaction mixture contained 1.5 μL of 10% BSA, 1 μL of 1 M $CaCl_2$, 1 μL of pyrene-labeled phosphatidylcholine (PC) (10 mM in ethanol), and 100 μg of enzyme preparation (3 μL) dissolving in 142.5 μL of 100 mM phosphate-buffered saline (PBS, pH 7.4). A spectrofluorometer (VICTOR multi-label Plate reader, PerkinElmer, Waltham, MA, USA) was used to measure fluorescence intensities at wavelengths of Ex_{345} and Em_{395}. Enzyme activity was calculated by change in fluorescence per min. The specific activity of enzyme was obtained by dividing fluorescence change by protein amount used as enzyme source of the reaction (data presented as ΔFLU/min/μg). Each treatment was replicated with three biologically independent enzyme preparations using different larval samples.

2.3. sPLA$_2$ enzymatic activity

To measure sPLA$_2$ activity, a commercial assay kit (sPLA$_2$ Assay Kit, Cayman Chemical, Ann Arbor, MI, USA) was used with diheptanoyl thio-PC as enzyme substrate. Briefly, L5 larvae of *S. exigua* were injected with *Escherichia coli* bacteria (10^4 cells/μL) in 1 μL volume using a microsyringe (Hamilton, Reno, NV, USA) equipped with a 26-gauge needle and incubated at 25 °C. At 8 h post injection (PI), hemolymph was collected from 10 larvae per experimental unit. Hemocytes and plasma were separated by centrifugation at 800 × *g* for 3 min at 4 °C. Hemocytes were then homogenized in PBS with a polytron (Ultra-Turrax T8, Ika Laboratory, Staufen, Germany). After centrifugation at 14,000 × *g* for 5 min at 4 °C, protein concentration was determined by Bradford (1972) assay using BSA as standard. sPLA$_2$ enzyme activities were measured using sPLA$_2$ Assay Kit with Ellman's reagent [5,5′-dithio-bis-(2-nitrobenzoic acid), DTNB] to create 5-thio-2-nitrobenzoic acid, a colored product. DTNB was prepared in 10 mM in 0.4 M Tris buffer (pH 8.0). Diheptanoyl thiophosphatidylcholine was used for substrate in 1.66 mM. Assay buffer used 25 mM Tris (pH 7.5) containing 10 mM $CaCl_2$, 100 mM KCl, and 0.3 mM Triton X-100. A reaction volume was 225 μL, which contained 10 μL plasma sample, 10 μL DTNB, 5 μL assay buffer, and 200 μL substrate. For negative control, the same volume of reaction mixture consisted of 10 μL DTNB, 15 μL assay buffer, and 200 μL substrate. Changes in absorbance at wavelength of 405 nm of the reaction product were measured and plotted to obtain the slope of a linear portion of the curve. Absorbance for non-enzymatic blank controls was calculated and subtracted from sample wells. The actual extinction coefficient for DTNB was 13.6 $mM^{-1}cm^{-1}$. However, this value was adjusted to 10.66 mM^{-1} considering the path-length of the solution in the well (0.784 cm). The following formula was used to calculate PLA$_2$ activity:

$$\text{sPLA}_2\ \text{activity(pmol/min/ml)} = \frac{\Delta\text{A405 per min}}{10.66\text{mM}^{-1}} \times \frac{0.225\text{ml}}{0.01\text{ml}} \times 10^6$$

Specific enzyme activity (pmol/min/μg) was calculated by dividing absorbance change by protein amount used as enzyme source for the reaction. A spectrofluorometer (VICTOR multi-label Plate reader, PerkinElmer, Waltham, MA, USA) was used to measure enzyme activity. Each treatment was replicated with three biologically independent enzyme preparations using different larval samples.

2.4. Bioinformatics

Phylogenetic trees were constructed with Neighbor-joining method