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Phospholipase A_2 in Salivary Glands Isolated from Tobacco Hornworms, *Manduca sexta*

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Abstract

We describe a phospholipase A_2 (PLA_2) associated with the salivary glands of tobacco hornworms, *Manduca sexta*. This enzyme is able to hydrolyze arachidonic acid from the *sn*-2 position of PLs. Addition of the calcium chelator, EGTA, or calcium, to the Tris reaction buffer impaired the PLA_2 activity, from which we infer the enzyme requires very low concentrations of calcium or perhaps other ions for optimal activity. PLA_2 activity was sensitive to protein concentration (highest activity at 25 μg protein per μl), reaction time (optimal at 30 min), buffer pH (optimal at pH 8–10), and reaction temperature (optimal range 18–38°C). The salivary gland PLA_2 was sensitive to the site-specific inhibitor, oleyloxyethylphosphorylcholine and stable to freezing at –80°C, but not –20°C. The biological significance of this enzyme may relate to hydrolysis of fatty acid moieties from dietary PLs for absorption by midgut epithelia. This salivary gland enzyme may also be responsible for killing foodborne bacteria.

Keywords: *Manduca sexta*, salivary gland, phospholipase A_2, nutrition, digestion, essential fatty acids, eicosanoids

1. Introduction

Certain polyunsaturated fatty acids (PUFAs) are essential nutrients for most animals, including insects (Dadd, 1985). Exceptions include those species of insects (Blomquist et al., 1982; Cripps et al., 1986) and other invertebrates (Weinert et al., 1993) which are able to biosynthesize 18:2n-6 from 18:1n-9 by action of a Δ-12 desaturase (fatty acid notation described in Stanley- Samuelson et al., 1988; Stanley, 2004). PUFAs act in two areas of animal biology. One, they serve as important structural components of biomembranes. Two, certain C20 PUFAs, which can be derived from their C18 counterparts via various elongation/desaturation steps (Stanley-Samuelson et al., 1988), are required for biosynthesis of prostaglandins and other eicosanoids, important signal moieties in vertebrates and invertebrates (Stanley, 2000, 2004).

In general, PUFAs are most abundant in the phospholipid (PL) and glycolipid fractions of animal and plant tissues. The fatty acids connected to dietary PLs must be hydrolyzed from their glycerol moieties before absorption into midgut epithelia. C18 and C20 PUFAs are usually linked to the *sn*-2, rather than *sn*-1, position of dietary PLs, from which these fatty acids are hydrolyzed by action of phospholipase A_2s (PLA_2s; Balsinde et al., 1999; Dennis, 1997; Six and Dennis, 2000). We are interested in PLA_2s from insect sources other than venom because these enzymes act in important areas of insect physiology, including PL digestion and eicosanoid biosynthesis. So far we have characterized digestive PLA_2s associated with midgut contents of several insect species, including the tiger beetle (*Cicindela circumpicta*), the robber fly (*Asilis* sp.; Uscian et al., 1995), the tobacco hornworm (*Manduca sexta*; Rana et al., 1998), larval mosquitoes (*Aedes aegypti*; Nor Aliza and Stanley, 1998), and larvae of the primary screwworm (*Cochlioomyia hominivorax*, Nor Aliza et al., 1999). These enzymes are considerably different from one another, and some differ from mammalian digestive PLA_2s. Based on finding substantial differences among PLA_2s associated with this small sampling of insect species, it may be supposed that digestive PLA_2s will prove to be extremely variable among insects.

We also investigated a PLA_2 associated with the oral secretions of adult burying beetles (*Nicrophorus marginatus*; Rana et al., 1997). We found that the oral secretions are the virtual sole source of PLA_2 activity in the burying beetle alimentary canals. On the basis of this finding, we hypothesized that insect salivary glands express considerable PLA_2 activities. We used salivary glands from tobacco hornworms to test our hypothesis. Here we report that hornworm salivary glands express substantial PLA_2 activity.

2. Materials and methods

2.1. Insects

Eggs of the tobacco hornworm (*M. sexta*) were purchased from Carolina Biological Supply Co. (Burlington, North Carolina, USA). Larvae were reared on a standard culture medium under semisterile conditions (Rana et al., 1998). Three- to four-day old fifth instar larvae were used in all experiments, except in studies on the effect of starvation on PLA_2 activity.

2.2. Reagents

Radioactive phosphatidylcholine (1-palmitoyl, 2-arachidonyl[arachidonyl-1-^{14}C], 1.85 GBq/mmol) and oleyloxyethylphosphorylcholine (OOPC) were purchased from New England Nuclear (Boston, Massachusetts, USA) and BioMol Inc. (Plymouth Meeting, Pennsylvania, USA), respectively. The same buffer (0.1 M Tris [hydroxymethyl]aminoethane, pH 9; Sigma, St. Louis, Missouri, USA) was used for dissections, homogenizations, and incubations, except in studies on Ca^{2+}, as described in section 3. All solvents were reagent grade from Fischer Scientific or Mallinckrodt.

2.3. PLA_2 source preparation

Hornworms were anesthetized on ice, and the salivary glands were isolated under saline. We used six pairs of glands per assay. Samples were homogenized with approximately 500 μl buffer in a glass tissue grinder. The homogenates were centrifuged at 735 × *g* for 3 min, then at 11,750 × *g* for 10 min. The resulting supernatants were used as enzyme sources.

2.4. PLA_2 activity assay

Radioactive PL substrate was prepared in the form of vesicles to access enzyme activity as described elsewhere (Nor Aliza and Stanley, 1998). A stock solution of substrate was prepared by adding four parts of solvent (toluene:ethanol; 1/1, v/v) to one part of radioactive phosphatidylcholine (0.05 μCi/μl). Five microliters of the substrate were dispensed into each 1.5 ml microcentrifuge tube. Solvents were removed under N_2 and 80 μl of 0.1 M Tris buffer was added to each tube. For studies on Ca^{2+}, amended buffers were used as indicated in section 3. The tubes were vortexed for 15 s to form vesicles. Substrate vesicle tubes were stored at 4°C for 1–2 h before use.

The PLA_2 assays were initiated by adding enzyme source to the 1.5 ml tubes containing substrate vesicles, and vortexing the tubes for 15 s. In our standard assay conditions, we used 125 μg of protein incubated in 250 μl total volume for 30 min at 28°C. The effects of protein concentration, incubation time, temperature, pH, and Ca^{2+} dependency were investigated by varying each parameter, as indicated in section 3.

The reactions were terminated by adding extraction solvent (600 μl chloroform:methanol; 2:1, v/v) acidified with 100 μl 1.0 N HCl. Arachidonic acid (20 nmol in 10 μl chloroform) was added to each tube as a carrier and free fatty acid standard. Each tube was vortexed for 15 s and centrifuged at 11,750 × *g* for 2 min. The lower organic phase was transferred to another 1.5 ml microcentrifuge tube and two more extraction steps followed with 500 μl chloroform.

2.5. Affinity column chromatography

Because some PLA_2s from mammalian sources interact with heparin, we determined whether the salivary gland PLA_2 similarly interacts with heparin. Routine enzyme source preparations (1.5 ml samples) were loaded onto a 5 ml HiTrap Heparin Column (Pharmacia Biotech AB, purchased from Sigma-Aldrich, St. Louis, Missouri). The column was eluted with four salt concentrations, Tris buffer (5 ml), Tris buffer plus 0.15 M NaCl (3 ml), Tris plus 1.05 M NaCl (3 ml), and Tris plus 1.5 M NaCl (3 ml). After determining protein concentrations, PLA_2 activity was determined in each fraction.

2.6. Chromatography and liquid scintillation counting

The samples were dried under N_2, dissolved in 100 μl chloroform, and applied to thin layer chromatography (TLC) plates (20 × 20 cm silica gel G, 0.25 mm; Sigma). The TLC plates were developed in petroleum ether:diethyl ether:glacial acetic acid (80:20:1, v/v) and exposed to iodine vapors to visualize the fractions corresponding to free fatty acids. Peaks of radioactivity associated with PL and free fatty acids were localized on the TLC plates using a Bioscan 200 Imaging Scanner (Bioscan, Washington, DC, USA). The PL and free fatty acid fractions were transferred into liquid scintillation vials. The radioactivity in each fraction was estimated by adding Ecolite scintillation medium (ICN Biomedicals, Irvine, California, USA) then counting on an LKB Wallac 1209 Rackbeta Liquid Scintillation Counter (Pharmacia, Turku, Finland) at 96% counting efficiency for ^{14}C.

2.7. Characterizing the salivary gland PLA2

These experiments used salivary glands plus contents for PLA_2 sources. Pools of six pairs of salivary glands were processed for enzyme assays. The influence of protein concentration, incubation time, pH, temperature, and OOPC on PLA_2 activity was assessed by varying each of the parameters as reported in Section 3. OOPC is a site-specific inhibitor of mammalian digestive PLA_2s.

The influence of Ca^{2+} on PLA_2 activity was assessed by preparing three buffers. Buffer 1 was Tris buffer with no additions; buffer 2 was Tris buffer amended with 5 mM $CaCl_2$; buffer 3 was Tris buffer amended with 5 mM ethylene-glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA). Separate salivary gland preparations (six pairs per reaction) were dissected and processed for PLA_2 activity assays in each buffer.

2.8. The influence of fasting on PLA2 activity

To test influence of starvation on PLA_2 activity, newly molted fifth instar larvae were placed in diet-free cups and fasted for 2 and 4 days. After the fasting periods, the salivary glands were processed for PLA_2 activity.

2.9. Statistical analysis

Data were analyzed using the analysis of variance in the general linear models (GLM) procedure, and mean comparisons were made using least significant difference (LSD) test (SAS Institute, 1989).

3. Results

3.1. Influence of calcium on salivary gland PLA_2 activity

PLA_2 activity reactions conducted in Tris buffer amended with 5 mM calcium yielded approximately 60 pmol/mg protein per h (fig. 1). PLA_2 activity was significantly increased (approximately doubled) in reactions conducted in Tris buffer without added calcium. Reactions conducted in Tris buffer plus EGTA yielded about 80 pmol/mg protein per h, not significantly more activity compared to results obtained in experiments with added calcium. As a practical matter, all subsequent experiments were carried out with untreated Tris buffer.

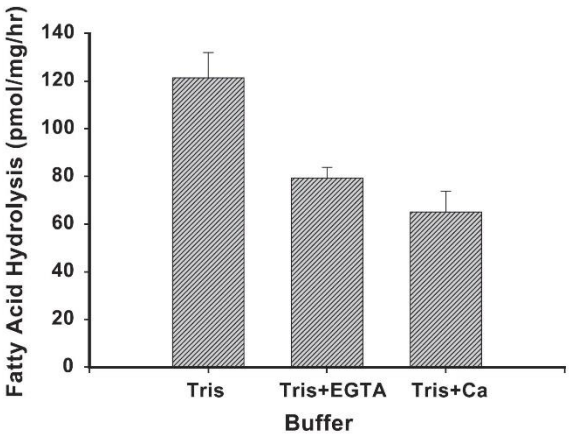

Figure 1. The influence of buffer amendments on tobacco hornworm (*M. sexta*) salivary gland PLA_2 activity. PLA_2 activity in salivary gland preparations was assessed as described in section 2, and PLA_2 activity was significantly higher in reactions conducted in Tris buffer with no amendments. Each bar represents the mean of three replicates, and the error bars indicate 1 S.E.M.

3.2. PLA_2 activity is not due to other PL-digesting pathways

Recovery of free radioactive fatty acid is usually interpreted as PLA_2 activity. However, an apparent PLA_2 activity can follow from other PL-digesting enzymes. In two sequential steps, phospholipase C could hydrolyze the base and associated phosphate group from the PL backbone, leaving diacylglycerol with a radioactive fatty acid in the *sn*-2 position. A subsequent lipase step could hydrolyze the radioactive fatty acid, which would indicate PLA_2 activity. The presence of radioactivity in the diacylglycerol fraction would indicate likely phospholipase C activity. Assessing radioactivity in the diacylglycerol fraction by liquid scintillation counting revealed a very small amount of radioactivity, approximately 0.6% (table 1). We infer the PLA_2 activity we observed in hornworm salivary gland preparations is due to a PLA_2 and not an alternative pathway.

Table 1. Phospholipase, PLA_2, and PLC, activities in tobacco hornworm (*M. sexta*) salivary gland preparations

Lipid fraction	PLA_2 activity (pmol/mg protein per h)	Indicated enzyme activity
Triacylglycerol	0.55 ± 0.07	Fatty acid incorporation
Free fatty acid	627.9 ± 89.7	PLA_2 activity
Diacylglycerol	4.1 ± 1.1	PLC activity

PLA_2 activity reactions using salivary gland preparations were conducted as described in section 2. Values represent means of three replicates, ± 1 S.E.M.

3.3. Characterization of the salivary gland PLA2

The influence of protein concentration on PLA_2 activity is displayed in figure 2. PLA_2 activity increased with increasing protein concentration at 5, 10, 25, and 125 Ag/Al to a maximum of about 65 pmol/h. Reactions conducted with higher protein concentrations did not yield higher PLA2 activity. We routinely used 125 μg protein per μl in subsequent experiments.

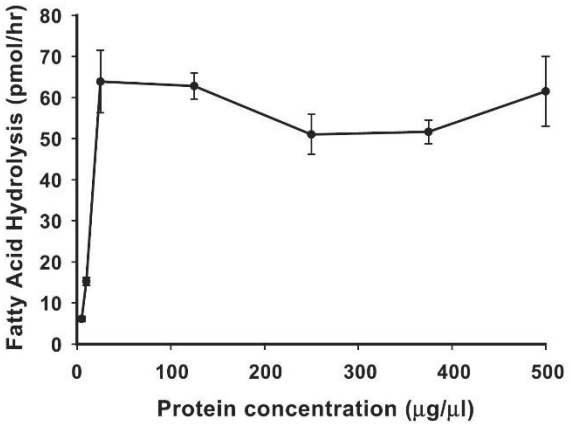

Figure 2. The influence of salivary gland protein concentration on tobacco hornworm (*M. sexta*) salivary gland PLA_2 activity. PLA_2 activity in salivary gland preparations was assessed as described in section 2. Each point represents the mean of three replicates, and the error bars indicate 1 S.E.M.

Free radioactive fatty acid accumulated with increasing reaction time in an approximately linear manner over the first 30 min (fig. 3). The reactions appeared to proceed more slowly over the following 30 min. We used 30 min reaction periods in the following experiments.

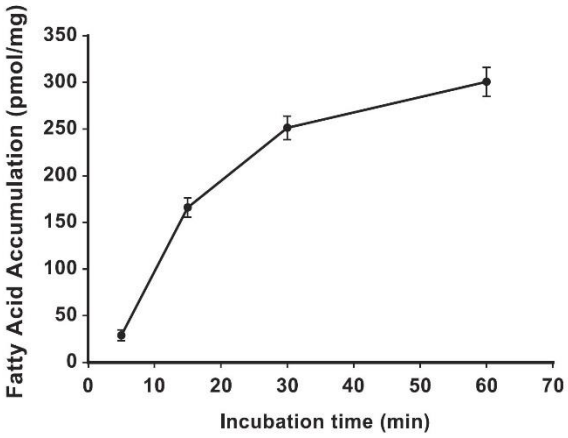

Figure 3. Progress of the tobacco hornworm (*M. sexta*) salivary gland PLA_2 reaction. PLA_2 activity in salivary gland preparations was assessed as described in section 2. Each point represents the mean of three replicates, and the error bars indicate 1 S.E.M.

The hornworm salivary gland PLA_2 was sensitive to reaction pH (fig. 4). Reactions at pH 6 and 7 yielded relatively low rates of fatty acid hydrolysis (< 200 pmol/mg per h). Reaction rates were significantly higher ($P < 0.05$) at pH 8 and above (> 500 pmol/mg per h). Optimal reaction conditions for the salivary gland PLA_2 appeared to be approximately pH 8; however, reactions at pH 9 and 10 did not yield significantly lower rates. We used pH 9 for all other experiments, consistent with the high alkaline conditions of lepidopteran alimentary canals (Dow, 1986).

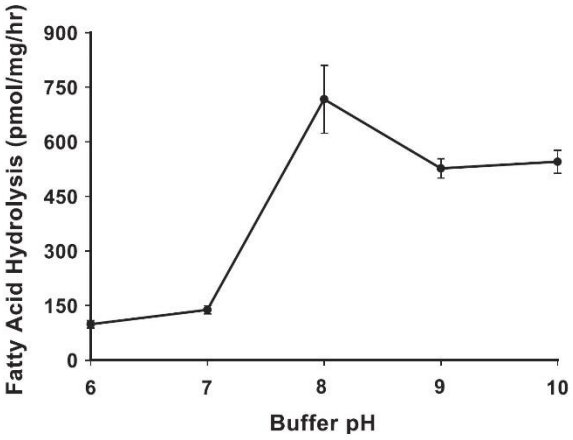

Figure 4. The influence of reaction buffer pH on tobacco hornworm (*M. sexta*) salivary gland PLA_2 activity. PLA_2 activity in salivary gland preparations was assessed as described in section 2 and PLA_2 activity was significantly higher in reactions conducted at pH 8 or above than in reaction conducted at lower pH values. Each point represents the mean of three replicates, and the error bars indicate 1 S.E.M.

Reaction temperature exerted considerable influence on the salivary gland PLA_2 activity (fig. 5). We recorded substantial PLA_2 activity in the range of 18–38°C (> 500 pmol/mg per h). Significantly lower reaction rates were obtained in reactions conducted at 8°C and 58°C. PLA_2 activity was virtually abolished at 78°C. We used 28°C in our standardized protocols.

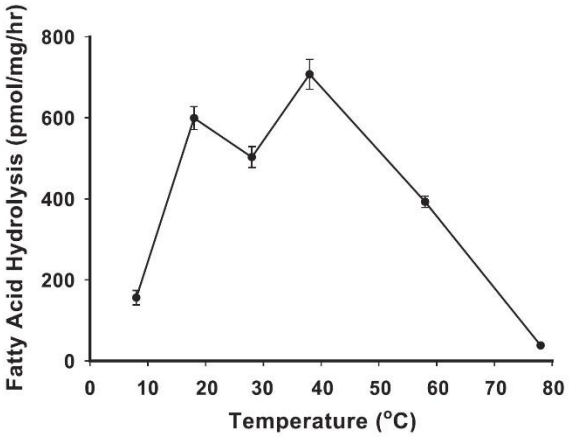

Figure 5. The influence of temperature on tobacco hornworm (*M. sexta*) salivary gland PLA_2 activity. PLA_2 activity in salivary gland preparations was assessed as described in section 2. Each point represents the mean of three replicates, and the error bars indicate 1 S.E.M.

The salivary gland PLA_2 was inhibited in reactions conducted in the presence of OOPC, a site-specific inhibitor of PLA_2 activity (fig. 6). Reactions with 50 μM OOPC slightly lowered enzyme activity but not significantly less than positive control reactions. The PLA_2 activity was significantly reduced to less than half the control values in reactions with 100 AM or higher OOPC.

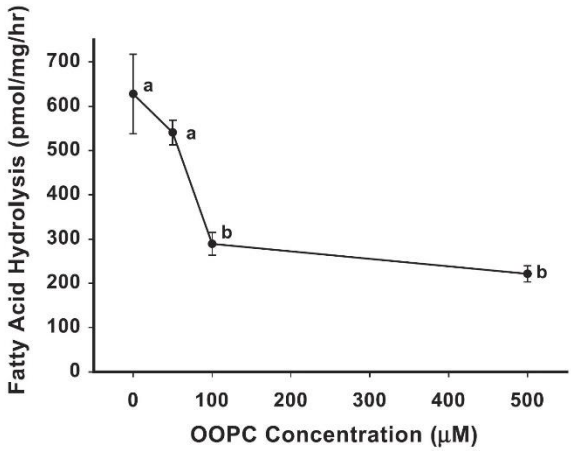

Figure 6. The influence of the site-specific inhibitor OOPC on tobacco hornworm salivary gland PLA_2 activity. PLA_2 activity in salivary gland preparations was assessed as described in section 2. Each point represents the mean of three replicates, and the error bars indicate 1 S.E.M. Points annotated with the same letter are not significantly different ($P < 0.05$).

We considered the freezing stability of the salivary gland PLA_2 (fig. 7). Compared to fresh samples, 1 week storage at –20°C resulted in a nearly 70% loss of enzyme activity. Storage at –80°C, however, was not deleterious to the PLA_2 activity.

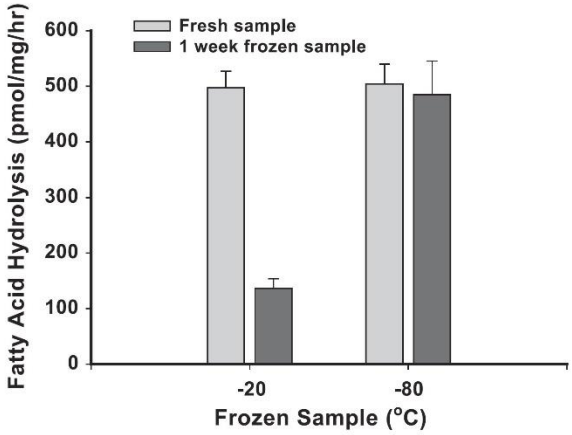

Figure 7. The influence of storage at two temperatures on PLA_2 activity in tobacco hornworm (*M. sexta*) salivary gland preparations. PLA_2 activity in salivary gland preparations was assessed as described in section 2. Histogram bars represent the mean of three replicates, and the error bars indicate 1 S.E.M.

3.4. Affinity column chromatography

After chromatography on a HiTrap Heparin column, 85% of the PLA_2 activity was recovered in the Tris wash, indicating that the salivary gland PLA_2 did not interact with the heparin column (data not shown).

3.5. The influence of fasting on salivary gland PLA_2 activity

We considered the influence of fasting on the salivary gland PLA_2 activity. It can be seen in figure 8 that fasting for 2 days resulted in a significant loss of PLA_2 activity, from > 600 pmol/mg per h to about 400 pmol/mg per h. After 4 days fasting, however, we did not record a substantial loss of PLA_2 activity. We take these data to suggest that feeding history does not influence expression of the salivary gland PLA_2.

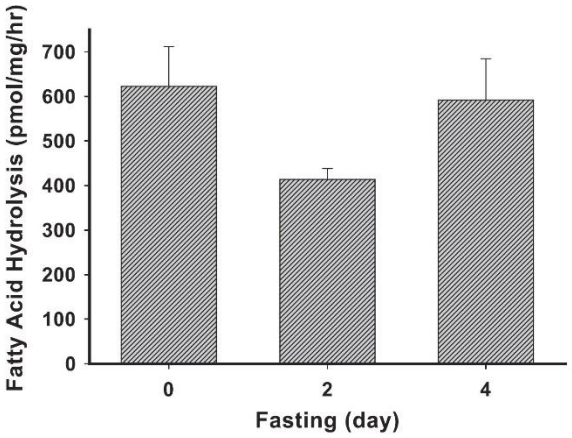

Figure 8. The influence of fasting on PLA_2 activity in tobacco hornworm (*M. sexta*) salivary gland preparations. PLA_2 activity in salivary gland preparations was assessed as described in section 2. Histogram bars represent the mean of three replicates, and the error bars indicate 1 S.E.M.

4. Discussion

In this paper we report on a PLA_2 associated with the salivary glands of the tobacco hornworm, *M. sexta*. Inquiry into the biochemical and biological features of PLA_2s from insect sources other than venoms remains in an early phase. So far, PLA_2s from midgut contents of tiger beetles, mosquito larvae, screwworms, and tobacco hornworms, plus a PLA_2 associated with oral secretions of adult burying beetles have been characterized to a limited extent (Nor Aliza and Stanley, 1998; Nor Aliza et al., 1999; Rana et al., 1997, 1998; Uscian et al., 1995). Aside from these secretory enzymes, putative intracellular PLA_2s have been described for tobacco hornworm fat body (Uscian and Stanley-Samuelson, 1993) and hemocytes (Schleusener and Stanley-Samuelson, 1996). The hemocyte PLA_2 appeared to operate in eicosanoid biosynthesis because it exhibited a marked preference for arachidonyl-containing substrates (Schleusener and Stanley-Samuelson, 1996), and increased enzyme activity was stimulated by bacterial challenge (Tunaz et al., 2003). The salivary gland PLA_2

also is able to hydrolyze 20:4n-6 from the *sn*-2 position of substrate PLs, although substrate specificities of PLA_2s from insect sources have not yet been considered in detail.

We now have information on the PLA_2s associated with tobacco hornworm fat body, hemocytes, salivary glands, and midgut contents. These enzymes differ with respect to OOPC sensitivity. Reactions in the presence of OOPC yielded reduced enzyme activity with salivary gland and hemocyte preparations and OOPC did not influence PLA_2 activity in preparations of midgut contents. The PLA_2s from all four tissues appear to be calcium-independent, although the enzymes may depend on the presence of other ions, as seen for a recombinant human cytosolic PLA_2 (Reynolds et al., 1993).

The PLA_2 in screwworm midgut contents was partially enriched by affinity column chromatography using a heparin column (Nor Aliza, 2001). We considered the possibility that the hornworm salivary gland PLA_2 similarly may be enriched by heparin chromatography. Our results indicate that most PLA_2 activity was recovered in the first column wash, from which we suggest that this enzyme does not interact with heparin. These data provide further evidence that the PLA_2s from insect sources are considerably different from one another.

Although the PLA_2s from midgut contents and salivary gland preparations remain to be purified, we recorded differences in enzyme activities. Under optimal reaction conditions, PLA_2 activities were considerably higher in midgut preparations than in salivary gland preparations. The midgut PLA_2 preferred higher pH conditions, with optimal activity at pH 9 and 10. We registered little PLA_2 activity at pH 8 with midgut preparations, while pH 8 may be optimal for the salivary gland preparations. Hornworm feeding history exerted more influence on PLA_2 activity in midgut than in salivary gland. After 2 days fasting, PLA_2 activity in midgut contents was significantly reduced by about 33%, while fasting did not influence salivary gland PLA_2 activity. Finally, the PLA_2s from midgut and salivary gland exhibit different stabilities to freezing. The midgut contents enzyme could be stored for at least 2 weeks at −20°C with no loss of activity, while most PLA_2 activity was lost after storing the salivary gland preparations for 1 week at the same temperature.

We have commented on the potential biological significance of PLA_2s in lipid digestion (Uscian et al., 1995). In the most obvious role, PLA_2 facilitates fatty acid absorption by midgut epithelium by catalyzing the hydrolysis of fatty acids from the *sn*-2 position of dietary PLs. The second role of PLA_2 in digestion stems from the need to emulsify dietary lipids to facilitate enzymatic digestion. Mammals secrete bile salts to help emulsify dietary lipids. Insects do not secrete bile salts, and it is thought that the lysophospholipids which result from PLA_2 action on dietary PLs act as emulsifiers in insect lipid digestive physiology.

Here, we speculate on a possible third biological role of PLA_2 associated with insect salivary glands and midguts. The insect midgut is a major avenue of entry into the hemocoel for pathogenic and nonpathogenic microbes (Keddie et al., 1989). Some forms of secretory PLA_2, particularly the human IIA PLA_2, are able to directly attack bacterial cells. Indeed, Beers et al. (2002) suggested that killing bacteria is the major biological role of the Group IIA PLAs. Koduri et al. (2002) assessed the bactericidal activity of a wide range of human and mouse secretory PLA_2s. They showed that the Group IIA PLA_2s are the most potent of all secretory PLA_2s in their antibacterial activities; to the extent that the hornworm salivary gland PLA_2 act like known bactericidal PLA_2s from mammalian sources,

we suggest that a biological significance of insect salivary and digestive PLA_2s is their ability to kill food-borne bacterial cells.

In their treatments of the superfamily of PLA_2s, Balsinde et al. (1999) recognized 10 groups, which quickly grew to 12 (Six and Dennis, 2000) and will undoubtedly continue to grow. Their updated classification goes beyond traditional characteristics, such as PLA_2 location (secreted or cytosolic), molecular weight, calcium requirements, and number of internal disulfide linkages. Aside from catalyzing the hydrolysis of the center ester bond of natural substrate PLs, the classification depends on knowing the complete amino acid sequence of the protein. Until the PLA_2s from nonvenom insect sources are sequenced, we cannot attempt meaningful comparisons with the established classification. We predict, however, that sequence data will reveal additional novel members of the superfamily.

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References

Balsinde, J., Balboa, M. A., Insel, P. A., Dennis, E. A., 1999. Regulation and inhibition of phospholipase A_2. Annu. Rev. Pharmacol. Toxicol. 39, 175–189.

Beers, S. A., Buckland, A. G., Koduri, R. S., Cho, W., Gelb, M. H., Wilton, D. C., 2002. The antibacterial properties of secreted phospholipases A_2. J. Biol. Chem. 277, 1788–1793.

Blomquist, G. J., Dwyer, L. A., Chu, A. J., Ryan, R. O., de Renobales, M., 1982. Biosynthesis of linoleic acid in a termite, cockroach and cricket. Insect Biochem. 12, 349–353.

Cripps, C., Blomquist, G. J., de Renobales, M., 1986. De novo biosynthesis of linoleic acid in insects. Biochim. Biophys. Acta 876, 572–580.

Dadd, R. H., 1985. Nutrition: organisms. In: Kerkut, G. A., Gilbert, L. I. (Eds.), Comprehensive Insect Physiology Biochemistry and Pharmacology, vol. 4. Pergamon Press, Oxford, pp. 313–390.

Dennis, E. A., 1997. The growing phospholipase A_2 superfamily of signal transduction enzymes. Trends Biochem. Sci. 22, 1–2.

Dow, J. A. T., 1986. Insect midgut function. Adv. Insect Physiol. 19, 187–328.

Keddie, B. A., Aponte, G. W., Volkman, L. E., 1989. The pathway of infection of Autographa californica nuclear polyhedrosis virus in an insect host. Science 243, 1728–1730.

Koduri, R. S., Grönroos, J. O., Laine, V. J. O., Le Calvez, C., Lambeau, G., Nevalainen, T. J., Gelb, M. H., 2002. Bactericidal properties of human and murine groups I, II, V, X and XII secreted phospholipases A_2. J. Biol. Chem. 277, 5849–5857.

Nor Aliza, A. R., 2001. A Digestive Phospholipase A_2 and Prostaglandin Biosynthesis in the Myiasis-Causing Screwworm, *Cochliomyia hominivorax*. University of Nebraska–Lincoln. Ph.D. Dissertation.

Nor Aliza, A. R., Stanley, D. W., 1998. A digestive phospholipase A_2 in larvae mosquitoes, *Aedes aegypti*. Insect Biochem. Mol. Biol. 28, 561–569.

Nor Aliza, A. R., Rana, R. L., Skoda, S. R., Berkebile, D. R., Stanley, D. W., 1999. Tissue polyunsaturated fatty acids and a digestive phospholipase A_2 in the primary screwworm, *Cochliomyia hominivorax*. Insect Biochem. Mol. Biol. 29, 1029–1038.

Rana, R. L., Hoback, W. W., Nor Aliza, A. R., Bedick, J. C., Stanley, D. W., 1997. Pre-oral digestion: a phospholipase A_2 associated with oral secretions in adult burying beetles, *Nicrophorus marginatus*. Comp. Biochem. Physiol. B 118, 375–380.

Rana, R. L., Sarath, G., Stanley, D. W., 1998. A digestive phospholipase A_2 in midguts of tobacco hornworms, *Manduca sexta* L. J. Insect Physiol. 44, 297–303.

Reynolds, L. J., Hughes, L. L., Louis, A. I., Kramer, R. M., Dennis, E. A., 1993. Metal ion and salt effects on the phospholipase A_2, lysophospholipase, and transacylase activities of human cytosolic phospholipase A_2. Biochim. Biophys. Acta 1167, 272–280.

SAS Institute, 1989. 4th ed. SAS/STATR User's Guide, Version 6, vol. 2. SAS Institute, Cary, NC.

Schleusener, D. R., Stanley-Samuelson, D. W., 1996. Phospholipase A_2 in hemocytes of the tobacco hornworm, *Manduca sexta*. Arch. Insect Biochem. Physiol. 33, 63–74.

Six, D. A., Dennis, E. A., 2000. The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. Biochim. Biophys. Acta 1488, 1–19.

Stanley, D. W., 2000. Eicosanoids in Invertebrate Signal Transduction Systems. Princeton University Press, Princeton, NJ.

Stanley, D. W., 2004. Eicosanoids. In: Gilbert, L. I., Iatrou, K., Gill, S. (Eds.), Comprehensive Insect Physiology, Biochemistry, Pharmacology and Molecular Biology. Elsevier, London (in press).

Stanley-Samuelson, D. W., Jurenka, R. A., Cripps, C., Blomquist, G. J., de Renobales, M., 1988. Fatty acids in insects: composition, metabolism, and biological significance. Arch. Insect Biochem. Physiol. 9, 1–33.

Tunaz, H., Park, Y., Bòyòkgòzel, K., Bedick, J. C., Nor Aliza, A. R., Stanley, D. W., 2003. Eicosanoids in insect immunity: bacterial infection stimulates hemocytic phospholipase A_2 activity in tobacco hornworms. Arch. Insect Biochem. Physiol. 52, 1–6.

Uscian, J. M., Stanley-Samuelson, D. W., 1993. Phospholipase A_2 activity in the fat body of the tobacco hornworm *Manduca sexta*. Arch. Insect Biochem. Physiol. 24, 187–201.

Uscian, J. M., Miller, J. S., Sarath, G., Stanley-Samuelson, D. W., 1995. A digestive phospholipase A_2 in the tiger beetle *Cicindela circumpicta*. J. Insect Physiol. 41, 135–141.

Weinert, J., Blomquist, G. J., Borgeson, C.E., 1993. De novo biosynthesis of linoleic acid in two non-insect invertebrates: the land slug and the garden snail. Experientia 49, 919–921.