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Tissue polyunsaturated fatty acids and a digestive phospholipase A_2 in the primary screwworm, *Cochliomyia hominivorax*

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Abstract

We report on the presence of arachidonic acid in larval and adult tissues of the primary screwworm, *Cochliomyia hominivorax* and of the secondary screwworm, *C. macellaria*. Arachidonic acid is present in the phospholipids of whole animal extracts of both species. This fatty acid appears to be accumulated during the larval stages, because proportions of arachidonic acid were higher in adults than in larvae. These insects probably obtain the arachidonic acid from dietary phospholipids. We also report on a phospholipase A_2 activity in midgut preparations from third instars of the primary screwworm. Phospholipase A_2 is responsible for hydrolyzing fatty acids from the *sn*-2 position of dietary phospholipids to release essential fatty acids. The screwworm enzyme is similar to mammalian digestive phospholipase A_2s because it depends on calcium for high catalytic activity, it is sensitive to the site-specific inhibitor oleyloxyethylphosphorylcholine, and it interacts with heparin. We further characterized the screwworm midgut phospholipase A_2 by altering the reaction conditions, including reaction time, radioactive substrate concentration, protein concentration, pH and temperature. We speculate that the biological significance of this enzyme relates to acquiring essential fatty acids, including arachidonic acid, from dietary phospholipids.

Keywords: Screwworm; *Cochliomyia hominivorax*; Polyunsaturated fatty acids; Phospholipids; Phospholipase A_2; Insect digestion

1. Introduction

The significance of C20 polyunsaturated fatty acids (PUFAs) and eicosanoids in the biology of invertebrates is gaining considerable attention (Stanley and Howard, 1998; Stanley, 1999). Eicosanoid is a general term for all oxygenated metabolites of arachidonic acid and two other C20 PUFAs. The structures and biosynthetic pathways of these molecules are illustrated in several reviews (Stanley-Samuelson, 1994; Stanley, 1999). Most of our understanding of eicosanoids is linked to their clinical significance in human and veterinary medicine, and recognition of the broader significance of these compounds represents a substantial shift in appreciation. Stanley and Howard (1998) proposed that eicosanoids should be regarded under a biological, rather than the more widely held medical paradigm. The main tenet of the biological paradigm lies in recognition that eicosanoids act as lipid mediators in the earliest forms of animal life, and throughout ensuing evolution eicosanoids were recruited in a bewildering array of functions. We imagine that eicosanoids act in all animals, as detailed by Stanley (1999).

The point might be more easily embraced if eicosanoids and C20 PUFAs were present in high quantities in insects, the largest assemblage of invertebrates. Contrarily, most analyses of insect tissue lipids reveal that arachidonic acid, the principle substrate for eicosanoid biosynthesis, and other C20 PUFAs, are present in very low abundance in most terrestrial insect species. These components are often overlooked in routine analyses. This is particularly so for many dipterans. For example, in their very careful analysis of houseflies, *Musca domestica*, Wakayama et al. (1985) recorded arachidonic acid at about 0.04% of adult phospholipid (PL) fatty acids. Similarly, Rapport et al. (1984) were unable to detect any C20 PUFAs in their analyses of fruit fly, *Dro-*

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sophila melanogaster, tissue PL fatty acids. It would appear that C20 PUFAs occur in only vanishing traces in dipteran tissue lipids.

The situation is rather different for at least some of the hematophagous insects. In his lengthy research program on mosquito nutritional requirements, Dadd (1983) found that mosquitoes differ from all known animals, except a tick species, in that they require dietary arachidonic acid, or certain other C20 PUFAs, during their larval stages to develop into normal adulthood. Stanley-Samuelson and Dadd (1981) and Dadd et al. (1987, 1988) later determined that arachidonic acid is present in substantial abundance in mosquito tissue PLs. Similarly, Gadelhak et al. (1995) recorded substantial proportions of arachidonic acid in PLs prepared from stable flies, *Stomoxys calcitrans*. While arachidonic acid is accumulated from blood meals by adult stable flies, the authors concluded that, as in mosquitoes, the larval stages of stable flies are more important for accumulation of C20 PUFAs. Adult female ticks, *Amblyomma americanum*, accumulate substantial proportions of arachidonic acid into salivary gland phospholipids during their lengthy feeding periods (Shipley et al., 1993). Like mosquitoes, *A. americanum* is regarded as unable to biosynthesize arachidonic acid from C18 PUFAs (Bowman et al., 1995).

More recently, we have turned our attention to the primary screwworm, *Cochliomyia hominivorax*, a parasitic insect which feeds on living mammalian tissue, rather than blood per se. Primary screwworms infest the bodies of mammals, usually through wound sites or body openings, producing a disease state known as myiasis. Females of this species deposit their eggs in rows near the edges of wounds. Larvae emerge from eggs after 12–24 h, and make their way into a wound, where they remain in characteristic head-down postures. The insects feed on tissues within the wound. The screwworms develop to the third and last larval stadium, then crawl out of the wound and drop to the ground, burrow under the surface layer and pupate. Adults emerge from the ground, and feed on nectar while they develop to the reproductive phases of adulthood (Laake et al., 1936; Thomas, 1993).

Mammalian tissue lipids are rich in arachidonic acid, and screwworms have the potential to accumulate substantial amounts of this and other C20 PUFAs during the parasitic phase of their lives. To determine whether this is so, we analyzed the tissue lipids prepared from whole larvae, pupae and adults of the primary screwworm. For comparison, we also analyzed similar preparations from the secondary screwworm, *C. macellaria*, a sibling species which feeds on carrion, rather than living tissue. Here we report substantial proportions of arachidonic acid in primary screwworm tissue lipids, and smaller proportions in secondary screwworms.

Most of the arachidonic acid associated with mammalian tissue lipids is linked to cellular PLs, and digestive phospholipases are essential enzymes in processing dietary PLs. C18 and C20 PUFAs are typically associated with the *sn*-2 position of PLs, and phospholipase A_2 (PLA_2) is the enzyme that hydrolyzes fatty acids from the *sn*-2 position of dietary PLs (Dennis, 1994; Waite, 1987). While well understood in mammalian physiology, we have relatively little information on the presence and characteristics of insect digestive PLA_2s. Recognizing the relatively high proportions of arachidonic acid in primary screwworm tissue lipids, we considered a digestive PLA_2 in screwworm midgut contents. In this paper we also report on the partial enrichment and characterization of a digestive PLA_2 in the midguts of third instars of the primary screwworm.

2. Materials and methods

2.1. Chemicals

Radioactive phosphatidylcholine (1-palmitoyl, 2-arachidonyl[arachidonyl-1-^{14}C], 1.85 GBq/mmol) and oleyloxyethylphosphorylcholine (OOPC) were purchased from New England Nuclear (Boston, MA) and BioMol Inc. (Plymouth Meeting, PA), respectively. The same buffer (0.1 M Tris[hydroxymethyl]aminomethane, pH 9; Sigma Chemical Co., St Louis, MO) was used for dissections, homogenizations and incubations, except in studies on Ca^{2+}, as described in Section 3.

For the analysis of tissue fatty acids, fatty acid methyl esters (FAMEs) were purchased from Sigma. All solvents were reagents grade from Fisher Scientific or Mallinckrodt.

2.2. Insects

Primary screwworms, *C. hominivorax*, (Costa Rican strain CR 92) were reared in a gelled diet in the Screwworm Biocontainment Facility, USDA/ARS, Lincoln, NE. The diet was composed of 70 g dried whole bovine blood, 30 g dried whole egg, 30 g dried nonfat milk, and 12 g acrylamide–acrylate polymer gelling agent per liter of water. Secondary screwworms, *C. macellaria*, were collected in Nebraska, and maintained in routine culture on ground beef (90% lean) in the USDA/ARS facility.

2.3. Preparing fatty acid methyl esters

Lipid extraction, separation and analysis followed our routine protocols (Howard and Stanley-Samuelson, 1996). Individual insects were suspended in 2 ml of chloroform:methanol (2:1, v/v) amended with 50 μl of 2% butylated hydroxytoluene to minimize autoxidation of PUFAs. Total lipids were extracted three times as

described. The total lipid extracts were applied to thin-layer chromatography (TLC) plates (20×20 cm, 0.25 mm silica gel G, from Aldrich). The plates were developed in petroleum ether:diethyl ether:acetic acid (80:20:1, v/v). Bands corresponding to TGs and PLs were scraped from the plates and transferred to 15-ml screw cap reaction tubes with Teflon-lined screw caps. The lipid fractions were transmethylated by refluxing in 3 ml of acidified methanol for 90 min. After cooling, the FAMEs were extracted three times in petroleum ether. The samples were dried by evaporating the petroleum ether, then added to 25–100 μl of isooctane for gas chromatographic analysis.

2.4. Gas chromatography and gas chromatography–mass spectrometry

The FAMEs were analyzed on a Hewlett-Packard 5890 gas chromatograph (San Fernando, CA) equipped with a Supelcowax 10 capillary column (30 m by 0.25 mm, 0.25 μm film thickness, Supelco, Inc., Bellefonte, PA), a flame ionization detector, and a Hewlett-Packard 3390A recording integrator. Injections were conducted in split mode (45:1), and the FAMEs were separated by temperature programming from 150 to 230°C at 2°C/min. The carrier gas was helium, flowing at 0.6 ml/min. The components were identified by comparing their chromatographic behaviors to the behaviors of authentic standards (Supelco, Inc.).

The fatty acid identifications were confirmed by gas chromatography–mass spectrometry. The FAME samples were analyzed using a Hewlett-Packard 6890 gas chromatograph (injections done in split mode, 50:1) interfaced with a Hewlett-Packard 5973 mass selective detector. The detector was operated in electron impact mode at 70 eV. The chromatograph was equipped with a column identical to the column just described, and separations were conducted with temperature programming from 160 to 230°C at 2°C/min. The carrier gas was ultrapure helium at 1 ml/min. Identifications were confirmed by analyzing total ion mass spectra of the FAMEs (McCloskey, 1970) and by comparing chromatographic retention times and mass spectra with authentic standards from Supelco.

2.5. PLA_2 source preparation

The alimentary canals were dissected from third instars by making a small cut in the caudal end of the larvae, then gently pulling the entire alimentary canal from the larvae. The tissues were collected into tubes containing Tris buffer amended with 10 μl of phenylthiourea (0.02 mM) to prevent melanization. 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.

2.6. PLA_2 activity assay

Radioactive PL substrate was prepared in the form of vesicles to access enzyme activity, following the methods of Reynolds et al. (1991). Substrate (0.05 μCi/reaction) was dispensed into each Eppendorf tube, solvents were removed under N_2 and 80 μl of 0.1 M Tris buffer was added to each Eppendorf tube. For studies on Ca^{2+}, Tris buffers were amended with either calcium chloride (5 mM) or EGTA (5 mM) 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 the enzyme source to the Eppendorf tubes containing substrate vesicles, and vortexing the tubes for 15 s. In our standard assay conditions, we used 250 μg of protein incubated in 250 μl of total volume for 30 min at 28°C. The effects of Ca^{2+} dependency, incubation time, substrate concentration, protein concentration, pH, and temperature were investigated by varying each parameter, as indicated in Section 3.

The reactions were terminated by adding extraction solvent (500 μl of 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 735 *g* for 2 min. The lower organic phase was transferred to another Eppendorf tubes and two more extraction steps followed with 500 μl chloroform.

2.7. Chromatography and liquid scintillation counting

The samples were dried under N_2, dissolved in 100 μl chloroform, and applied to TLC plates (20×20 cm silica gel G, 0.25 mm; Sigma Chemical Co., St Louis, MO). 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 that corresponded to free fatty acids. Peaks of radioactivity associated with PL and free fatty acids were detected on a Bioscan 200 Imaging Scanner (Bioscan Inc., Washington, DC). 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 Inc., Irvine, CA) then counting on a LKB Wallac 1209 Rackbeta Liquid Scintillation Counter (Pharmacia, Turku, Finland) at 96% counting efficiency for ^{14}C.

2.8. Statistical analyses

Data were analyzed using the analysis of variance in the General Linear Models procedure, and mean comparisons were made using Least Significant Difference test (SAS Institute, 1989).

3. Results

3.1. Fatty acid compositions of screwworm tissue lipids

The fatty acid compositions of PLs and TGs prepared from third-stage larvae, pupae and adult males and females of the primary screwworms are presented in Tables 1 and 2. The fatty acid profiles of PLs and TGs prepared from the secondary screwworms collected in Jamaica and in Nebraska are fairly similar to the profiles from the primary screwworm, and these data are not repeated. Several comments are appropriate to all data. First, as seen in most analyses of animal tissue lipids, the quantitatively major fatty acids were 16:0, 16:1, 18:1, and 18:2n-6. Smaller proportions of other components were also detected. Second, the screwworms are similar to other dipteran species with relative high proportions of 16:1 in all analyses (12–25%). Third, while 18:2n-6 is a major component of most insect lipids, often comprising more than 50% of PL fatty acids, this compound made up less than 7% of the larval and pupal PLs in both species. Fourth, more components were recorded from PLs than TGs, and higher proportions of PUFAs were present in PLs than TGs.

Arachidonic acid was present in the tissue lipids of both species, albeit in different proportions. For primary screwworms, this component was present at about 1.5–1.8% of larval and pupal PLs and about 3% of adult PLs. Arachidonic acid was also present in TGs, at about 1% of larval and pupal TGs, about 0.6% of male TGs and nearly 2% of female TGs. The secondary screwworms yielded considerably less arachidonic acid. Arachidonic acid accounted for less than 1% of larval PL fatty acids, and was not detected in pupal lipids. The adults maintained higher arachidonic acid proportions, about 2% of male and female PLs. Arachidonic acid was not present in TGs from secondary screwworms.

Aside from the differences just mentioned, the overall fatty acid profiles from larval and pupal lipids from both species were fairly similar. We note another difference between the two species. One component, 20:2n-6, accounted for about 10% of the TG fatty acids from adults of secondary screwworms. This component was not detected in either lipid fraction prepared from primary screwworms.

3.2. Initial experiments and partial enrichment of the digestive PLA_2

Our initial experiment was designed to assess the presence of a digestive PLA_2 in the primary screwworm,

Table 1
Fatty acid composition, as proportions of total fatty acids (±SEM), in phospholipid and triacylglycerol fractions prepared from total lipid extracts from immature stages of the primary screwworm, *C. hominivorax* (Coq.)[a]

	Larva				Pupa			
Fatty acids	PLs		TGs		PLs		TGs	
12:0	–	–	0.13	(0.02)	–	–	0.40	(0.18)
14:0	0.79	(0.04)	2.64	(0.04)	0.90	(0.12)	2.26	(0.14)
15:0	0.13	(0.01)	0.16	(0.01)	–	–	–	–
16:0	22.58	(0.39)	28.28	(0.22)	31.56	(0.59)	25.87	(0.07)
16:1n-6	25.83	(0.43)	20.78	(0.83)	18.22	(1.58)	18.58	(1.88)
17:0	–	–	–	–	–	–	0.26	(0.02)
17:1	–	–	–	–	–	–	0.34	(0.02)
18:0	1.41	(0.03)	4.20	(0.09)	1.58	(0.03)	4.46	(0.03)
18:1	38.57	(0.37)	36.68	(0.65)	34.29	(0.82)	35.15	(0.89)
18:2	6.65	(0.09)	5.86	(0.15)	6.84	(0.79)	6.51	(0.73)
18:3n-6	0.14	(0.01)	–	–	–	–	–	–
18:3n-3	0.12	(0.03)	0.12	(0.01)	–	–	–	–
20:0	0.70	(0.03)	–	–	3.49	(1.69)	–	–
20:1	0.53	(0.02)	–	–	–	–	0.54	(0.10)
20:2	–	–	0.15	(0.02)	–	–	3.44	(0.62)
20:4n-6	1.53	(0.09)	1.01	(0.06)	1.85	(0.12)	1.40	(0.10)
20:5n-3	0.33	(0.02)	–	–	–	–	–	–
22:0	0.82	(0.01)	–	–	1.58	(0.04)	0.54	(0.12)

[a] Data based on three separate gas–liquid chromatographic analyses. Fatty acid identity was confirmed by gas liquid chromatography–mass spectrometry. (PLs=phospholipids; TGs=triacylglycerols.)

Table 2
Fatty acid composition, as proportions of total fatty acids (±SEM), in phospholipid and triacylglycerol fractions prepared from total lipid extracts from adults of the primary screwworm, *C. hominivorax* (Coq.)[a]

	Male				Female			
Fatty acids	PLs		TGs		PLs		TGs	
12:0	–	–	–	–	–	–	–	–
14:0	0.45	(0.05)	2.70	(0.24)	0.86	(0.47)	2.1	(0.25)
15:0	–	–	–	–	–	–	–	–
16:0	24.05	(0.60)	27.37	(1.20)	23.10	(0.19)	24.57	(0.87)
16:1	13.86	(0.88)	17.82	(1.18)	12.82	(0.79)	17.13	(1.40)
17:0	–	–	–	–	–	–	–	–
17:1	–	–	–	–	–	–	–	–
18:0	2.62	(0.68)	5.21	(0.30)	2.63	(0.62)	5.44	(0.66)
18:1	34.56	(0.59)	39.04	(2.11)	35.80	(0.59)	40.61	(0.87)
18:2	20.62	(0.66)	7.28	(0.49)	21.58	(0.19)	7.94	(0.27)
18:3n-6	–	–	–	–	–	–	–	–
18:3n-3	0.75	(0.14)	–	–	–	–	–	–
20:0	–	–	–	–	–	–	–	–
20:1	–	–	–	–	–	–	0.49	(0.00)
20:2	–	–	–	–	–	–	–	–
20:4n-6	3.07	(0.09)	0.57	(0.06)	3.50	(0.40)	1.80	(1.01)
20:5n-3	–	–	–	–	–	–	–	–
22:0	–	–	–	–	–	–	–	–

[a] Data based on three separate gas–liquid chromatographic analyses. Fatty acid identity was confirmed by gas liquid chromatography–mass spectrometry. (PLs=phospholipids; TGs=triacylglycerols.)

using the reaction conditions we had established for tobacco hornworms, *Manduca sexta*, and larval mosquitoes, *Aedes aegypti*. Unfractionated homogenates of midguts and associated midgut contents yielded very low PLA_2 activity, less than 2 pmol/mg protein/min, compared to about 80 pmol/mg protein/min with the mosquito PLA_2 (Nor Aliza and Stanley, 1998). This finding supported the idea that screwworms express a digestive PLA_2, however, the low specific activity indicated an enrichment step would be required before attempting further characterization.

Because we had achieved a partial enrichment of the tobacco hornworm digestive PLA_2 on ammonium sulfate precipitation (Rana et al., 1998), we subjected screwworm midgut homogenates to the same treatment. We did not recover PLA_2 activity, and concluded ammonium sulfate precipitation was not an effective first step. Many PLA_2s from mammalian sources interact with heparin, which can be used to partially enrich the PLA_2 by affinity chromatography. We loaded 0.5-ml samples of filtered midgut homogenates (using a 0.45-μ membrane) onto a heparin affinity column (1 ml HiTrap Heparin, Pharmacia Biotech AB, purchased from Sigma Chemical Co., St Louis, MO). After a 30-min incubation period, the column was sequentially washed with four buffers, first with Tris homogenization buffer, second with 0.15 M NaCl, third with 1.05 M NaCl and finally with 1.5 M NaCl. Highest specific activity was recovered in the 1.05 M NaCl fraction (about 8 pmol/mg protein/min), indicating this step effectively enriched the target PLA_2 to allow further characterization.

Enrichment on the affinity column may contribute to the analysis of this enzyme in two ways. First, the enrichment step effectively eliminates some of the protein provided in protein-rich diets from the midgut contents. Second, we had noted that PLA_2 activity in the midgut homogenates was not stable to overnight storage in the refrigerator nor at −20°C. On the other hand, after the affinity chromatography step, the 1.05 M NaCl fractions were stable to long term storage, which is typical of secretory PLA_2s. We suggest that the enrichment step separates the PLA_2 from digestive proteases, which degrade the PLA_2 during storage. The high salt concentration of the elution buffer may also act to stabilize the protein. For all subsequent work with the screwworm digestive PLA_2, freshly prepared midgut homogenates were immediately processed through the heparin columns.

The digestive PLA_2s associated with preparations from mosquito larvae and tobacco hornworms differ from all known secretory PLA_2s because these two enzymes do not require the presence of calcium for full catalytic activity. In contrast, reactions performed in the presence of calcium yielded lower PLA_2 activity than reactions in the presence of a calcium chelator (Nor Aliza and Stanley, 1998; Rana et al., 1998). On the basis of these findings, we assessed the influence of calcium on the screwworm preparations. We found that added calcium (5 mM) did not yield significantly increased PLA_2 activity in crude fractions (Fig. 1). Alternatively, reactions with crude homogenates in the presence of 5 mM EGTA yielded virtually no PLA_2 activity

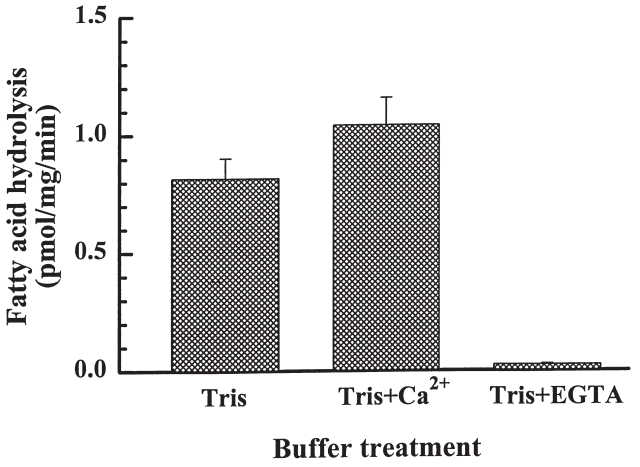

Fig. 1. The influence of Ca^{2+} and the Ca^{2+}-chelator EGTA on PLA_2 activity in third instar primary screwworm midgut preparations. Enzyme activities were determined in unfractionated samples prepared in three separate buffers, as indicated. The histogram bars represent means of three replicates, and the error bars represent 1 SEM.

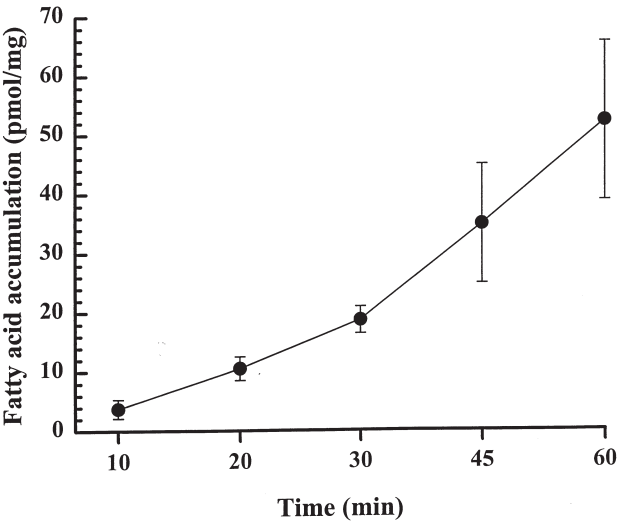

Fig. 2. Progress of the primary screwworm digestive PLA_2 reaction. PLA_2 activity in third instar midgut preparations enriched on affinity column chromatography was assessed as described in Section 2. Each point represents the mean of three replicates, and the error bars indicate 1 SEM.

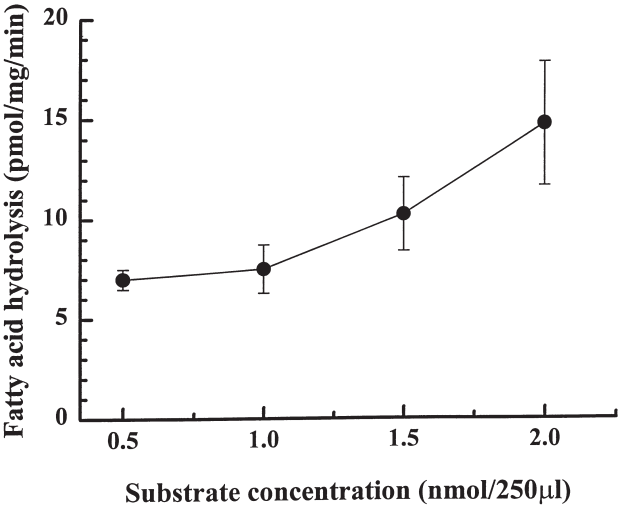

Fig. 3. The influence of radioactive substrate concentration on PLA_2 activity in third instar primary screwworm midgut preparations. PLA_2 activity in third instar midgut preparations enriched on affinity column chromatography was assessed as described in Section 2. Each point represents the mean of three replicates, and the error bars indicate 1 SEM.

(significantly different from other treatments at $P<0.05$), suggesting the screwworm digestive PLA_2 requires calcium for catalytic activity, as seen in all mammalian and venom-associated secretory PLA_2s. The screwworm culture medium provides copious milk solids, which provide abundant calcium. We suggest that the added calcium did not enhance the screwworm PLA_2 activity because a sufficiency of calcium was already present. All subsequent PLA_2 reactions were conducted in Tris buffer without added calcium.

3.3. A characterization of the primary screwworm PLA_2 activity

The accumulation of free arachidonic acid, the reaction product, increased with reaction time from 10 to 60 min (Fig. 2). As seen in the other insect digestive PLA_2s, the screwworm PLA_2 was not inhibited by the accumulated reaction product during the 1-h incubations. We used 45-min incubations in subsequent experiments.

The radioactive substrate concentration influenced the PLA_2 activity (Fig. 3). At 0.5 and 1.0 nmol/reaction, we obtained approximately 7 pmol/mg/min of enzyme activity, which was roughly doubled at 2.0 nmol/reaction. Although reactions in the presence of higher radioactive substrate concentration yielded significantly more enzyme activity, we used 1.0 nmol/reaction in subsequent work.

The influence of protein concentration on PLA_2 activity is shown in Fig. 4. PLA_2 activity increased in a linear way up to 250 μg protein/reaction, then plateaued. We used 250 μg protein/reaction in all subsequent experiments.

The influence of pH on the screwworm digestive PLA_2 activity is displayed in Fig. 5. Over the range of pH 5–10, enzyme activity at pH 9 was significantly higher than all other pH values (LSD, $P<0.0005$). In particular, PLA_2 activity was substantially reduced at pH 8 and below. We used pH 9 for all subsequent experiments.

The screwworm digestive PLA_2 was sensitive to temperature (Fig. 6). Highest activity, approximately 12 pmol/mg/min, obtained at 30°C. PLA_2 activity was significantly reduced at 20 and 40°C, and virtually abolished at higher temperatures.

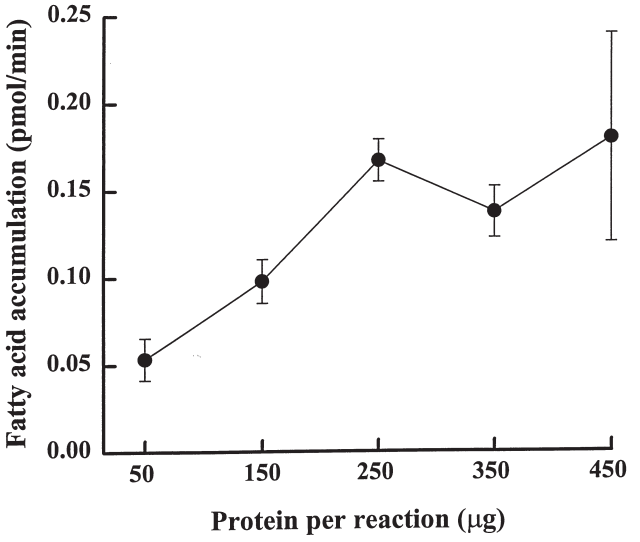

Fig. 4. The influence of midgut protein concentration on primary screwworm digestive PLA_2 activity. PLA_2 activity in third instar midgut preparations enriched on affinity column chromatography was assessed as described in Section 2. Each point represents the mean of three replicates, and the error bars indicate 1 SEM.

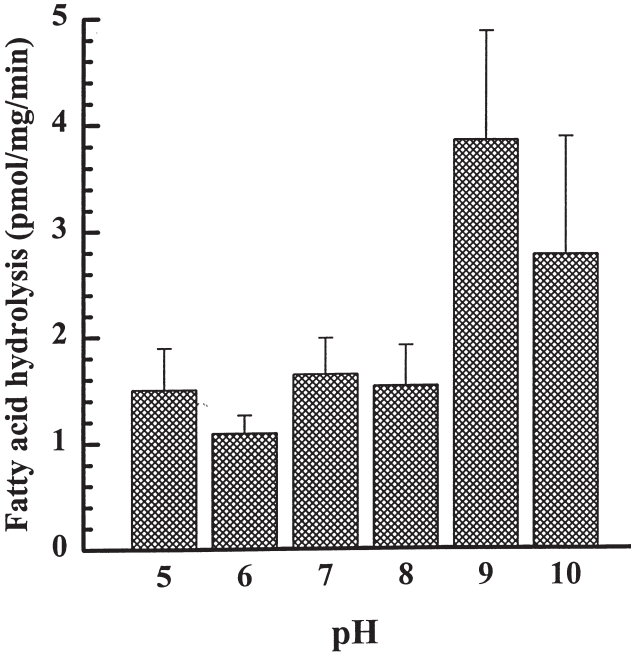

Fig. 5. The influence of pH on primary screwworm digestive PLA_2 activity. PLA_2 activity in third instar midgut preparations enriched on affinity column chromatography was assessed as described in Section 2. Each point represents the mean of three replicates, and the error bars indicate 1 SEM.

Mammalian digestive PLA_2s are inhibited in the presence of some phospholipid analogues, including OOPC. By contrast, the digestive PLA_2s associated with mosquito and tobacco hornworm midgut contents are not influenced by OOPC, even at very high concentrations (Rana et al., 1997; Nor Aliza and Stanley, 1998). PLA_2 activity in the screwworm preparations was sensitive to OOPC. As seen in preparations from mammalian sources, PLA_2 activity decreased in the presence of increasing concentration of OOPC in the range of 5–500 μM (Fig. 7).

We have noted that an apparent PLA_2 activity can follow from other enzymatic pathways (Nor Aliza and Stanley, 1998). For example, phospholipase C could hydrolyze the choline base and its associated phosphate

Fig. 6. The influence of temperature on primary screwworm digestive PLA_2 activity. PLA_2 activity in larval midgut preparations enriched on affinity column chromatography was assessed as described in Section 2. Each point represents the mean of three replicates, and the error bars indicate 1 SEM.

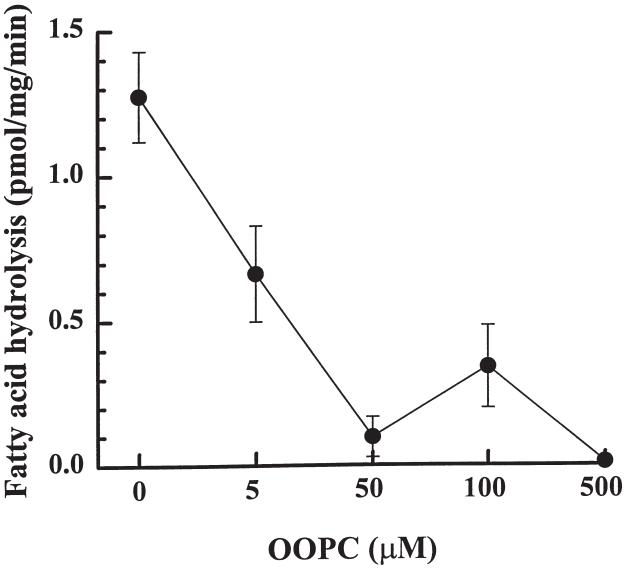

Fig. 7. The influence of the site-specific inhibitor OOPC on primary screwworm PLA_2 activity. PLA_2 activity in third instar midgut preparations enriched on affinity column chromatography was assessed as described in Section 2. Each point represents the mean of three replicates, and the error bars indicate 1 SEM.

group from the PL backbone, leaving a diacylglycerol with a radioactive fatty acid associated with the *sn*-2 position. A subsequent lipase or esterase step could hydrolyze the radioactive fatty acid, yielding free radioactive fatty acid product that is indistinguishable from the product of a single PLA_2 step. Our routine thin-layer separations of products from PLA_2 reactions did not reveal radioactivity in the diacylglycerol fraction, indicating the activity we observed was due to a PLA_2 step and not to an alternative pathway. The data are similar to published radiochromatograms (Nor Aliza and Stanley, 1998), and are not repeated here.

4. Discussion

The information in this report documents the fatty acid compositions of PLs and TGs prepared from larvae, pupae, and adults of the primary and secondary screwworms. Dipterans differ from other insect groups in their fatty acid compositions with characteristically high proportions of 16:1 in their tissue lipids (Stanley-Samuelson and Dadd, 1983). Some features of the fatty acid profiles of juvenile and adult screwworms are in line with the general dipteran pattern. We recorded 16:1 in relatively high proportions of screwworm fatty acids, ranging from 13 to 25% of the PL and TG fatty acids of both species. For larvae and pupae of both screwworm species, we determined 18:2n-6 at about 3–6% of total PL and TG fatty acids. This differs from the background of animal lipids and other insects, in which 18:2n-6 is more abundant in PLs. In contrast to larvae and pupae, 18:2n-6 is considerably more abundant in PLs prepared from adults of both screwworm species. Stanley-Samuelson et al. (1988) suggested that fatty acid patterns represent the outcomes of complex metabolic events. We infer from the differing patterns recorded for juvenile and adult screwworms that screwworms undergo considerable changes in lipid metabolism in the pupal–adult transition.

Most terrestrial insects maintain very low proportions of 20:4n-6 in their tissue lipids, often seen only as traces (Stanley-Samuelson, 1994). For example, we recorded less than 1% of 20:4n-6 in PLs prepared from Malpighian tubules from larval, pupal and adults of the tenebrionid beetle, *Zophobas atratus* (Howard and Stanley-Samuelson, 1996). In their work on houseflies, Wakayama et al. (1985) recorded 20:4n-6 at 0.04% of total fatty acids associated with PLs from newly emerged males. The accumulation of tissue arachidonic acid is otherwise for screwworms and certain other dipterans. Arachidonic acid was present in PLs and TGs of primary screwworm larvae and pupae at about 1–2% of total fatty acids. Again, the adults were different from the juveniles. Arachidonic acid was enriched in PLs from adults (about 3–4% of total fatty acids). Compared to primary screwworms, lower proportions of arachidonic acid were present in tissue lipids of secondary screwworms. In larvae, arachidonic acid made up 0.4% of PL fatty acids, and was not detected in TGs. No fatty acid components beyond 18:3n-3 were present in pupal PLs or TGs.

We considered the possibility that geographical races of secondary screwworms would generate differing fatty acid compositions. Secondary screwworms collected from the field in Jamaica yielded fatty acid patterns quite similar to the patterns registered from secondary screwworms collected in Nebraska. Hence, environmental and geographical differences between Nebraska and Jamaica did not influence secondary screwworm fatty acid metabolism.

The relatively high abundance of arachidonic acid in screwworm lipids could, of course, be simple reflections of dietary inputs. However, comparisons of the fatty acid compositions of insect tissue lipids and the corresponding diets of the insects suggest that insects express fatty acid incorporation systems that are selective. The selectivity produces tissue lipid fatty acid patterns that are quite different from the diets. For example, in work on fatty acid compositions in tissues of the tobacco hornworm, we found higher proportions of arachidonic acid in the hornworm diet and in hornworm hemolymph than in the tissue lipids (Ogg and Stanley-Samuelson, 1992). We investigated this point in more detail by tracing the incorporation of radioactive fatty acids into hornworm hemocytes (Gadelhak and Stanley-Samuelson, 1994). When present in substantial quantities, free arachidonic acid can be deleterious to cells, and the hemocytes rapidly incorporated radioactive arachidonic acid into all cellular lipid pools. Soon after the initial, rapid incorporation, however, the arachidonic acid was selectively removed from hemocyte PLs, apparently to maintain very low levels of this component in PLs. We infer from these observations that high levels of arachidonic acid in some diets, such as mammalian blood or tissues, does not necessarily result in accumulation in tissue lipids. In particular, the relatively high proportions of arachidonic acid in PLs from primary screwworms, stable flies and mosquitoes are not passive consequences of diet.

We take an alternative view that some dipterans accumulate PUFAs for special purposes. For example, mosquitoes require dietary arachidonic acid during their larval phases to complete development to normal adults (Dadd, 1983). Part, but not all, of the arachidonic acid requirements could be spared by dietary PGs, from which Dadd inferred that arachidonic acid served multiple roles in the physiology of mosquitoes, some involving PG biosynthesis and others relating to structural components of cellular membranes (Dadd and Kleinjan, 1988). Similarly, adult stable flies maintain substantial proportions (1–5% of total fatty acids) of arachidonic acid in their tissue PLs (Gadelhak et al., 1995). As in

mosquitoes, it appears that the larval stages are most important for accumulating this component. The biological significance of the arachidonic acid may relate to nutritional interactions between blood-feeding insects and their hosts. Certain PGs act as anti-hemostatic factors in vertebrates. Adult female mosquitoes and adult stable flies of both sexes are hematophagous; perhaps the arachidonic acid is converted into PGs which facilitate blood feeding in these insects, as suggested for ticks (Sauer et al., 1993).

By comparison to the hematophagous and parasitic dipterans, arachidonic acid is generally not recorded in fatty acid analyses of other dipterans. This is so for the olive fruit fly, *Dacus oleae* (Madariaga et al., 1974), the Mediterranean fruit fly, *Ceratitis capitata* (Madariaga et al., 1972), and the fruit fly *Drosophila melanogaster* (Rapport et al., 1984). The results with *D. melanogaster* remain unclear, however, because Pages et al. (1986) recorded PG biosynthesis, which depends on the presence of arachidonic acid, in preparations from *D. melanogaster*. Stanley-Samuelson (1994) suggested that C20 PUFAs occur in these insects in very low levels, below the ranges of detectability on normal gas chromatography. These low catalytic levels would support PG biosynthesis for local physiological purposes.

Here, we also report on a digestive PLA_2 in midgut preparations from third instar screwworms. We now have basic descriptive information on digestive PLA_2s from six insect species, including two predacious insects, the tiger beetle, *C. circumpicta*, and a robber fly, *Asilis* sp. (Uscian et al., 1995), the burying beetle, *N. marginatus* (Rana et al., 1997), a lepidopteran, *M. sexta* (Rana et al., 1998), a mosquito, *A. aegypti* (Nor Aliza and Stanley, 1998) and now the primary screwworm. We perceive fundamental differences among the PLA_2s from these sources. The PLA_2s from the tiger beetle, robber fly and screwworms require calcium for full catalytic activity and they are sensitive to the site-specific PLA_2 inhibitor, OOPC. We gather there are structural similarities between these insect enzymes and their mammalian counterparts, which also require calcium (Waite, 1987). The PLA_2 associated with oral secretions of adult burying beetles was sensitive to the presence of the calcium chelator EGTA (Rana et al., 1997), albeit not to the extent seen in the tiger beetle, robber fly and screwworms. The PLA_2s from mosquito larvae and tobacco hornworms differed considerably from the others because enzyme activity increased, rather than decreased, in the presence of EGTA and the enzymes were not sensitive to OOPC. Moreover, the PLA_2 from screwworms interacted with heparin while the digestive PLA_2 from the tobacco hornworms did not (Rana, unpublished observation).

As to the usual biophysical parameters, the digestive PLA_2s from all insect sources we have examined so far require fairly high pH for optimal catalytic activity. This is in accord with expectation, because most insect midguts maintain alkaline conditions. The digestive PLA_2s from most insect sources are fairly stable to temperature, however, the screwworm enzyme exhibited a relatively narrow temperature optimum. This may relate to the feeding ecology of screwworms, which live under stable temperature conditions within mammalian tissues.

We also note considerable differences in specific activities among these preparations under similar reaction conditions. Unfractionated larval mosquito preparations yielded approximately 80 pmol/mg protein/min of PLA_2 activity (Nor Aliza and Stanley, 1998), compared to about 40 pmol/mg protein/min with similar preparations of burying beetle oral secretions (Rana et al., 1997) and of tobacco hornworm midgut contents (Rana et al., 1998). The PLA_2-enriched preparations from primary screwworm midguts yielded approximately 8 pmol/mg protein/min of PLA_2 activity, which could be pushed higher with increased substrate concentrations. These enzyme activity values are normalized to protein concentrations in midgut contents, which undoubtedly varies with dietary inputs. Hence, these differences in specific activities may be more apparent than real. We are currently working to purify a digestive PLA_2 from tobacco hornworms, after which we anticipate learning more on this point.

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