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DIGESTIVE PHOSPHOLIPASE A_2 IN INSECTS

by

Rico Lamoste Rana

A DISSERTATION

Presented to the Faculty of

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Major: Entomology

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Digestive Phospholipase A2 in Insects

BY

Rico Lamoste Rana

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GRADUATE COLLEGE
UNIVERSITY OF NEBRASKA

DIGESTIVE PHOSPHOLIPASE A_2 IN INSECTS

Rico Lamoste Rana, Ph.D.

University of Nebraska, 1999

Advisor: David W. Stanley

We hypothesized that phospholipase A_2 (PLA_2) is a common feature of insect digestive physiology in insects. PLA_2 hydrolyzes polyunsaturated fatty acids (PUFAs) associated with the *sn*-2 position of phospholipids (PLs). We conducted experiments to determine whether insects produce a digestive PLA_2, and investigated its biochemical and physiological characteristics.

Oral secretions of adult burying beetles, *Nicrophorus marginatus*, display a calcium-dependent PLA_2 similar to its mammalian counterparts. However, PLA_2 activity in midgut contents of the tobacco hornworm larvae, *Manduca sexta*, is independent of calcium. PLA_2 activity in both insects is sensitive to reaction pH, protein concentration, substrate concentration, temperature, and incubation time.

We also found that fed hornworms expressed higher PLA_2 activity than starved ones. Based on *in vitro* assay, midguts isolated from *M. sexta* are competent to secrete PLA_2 into the incubation medium. Two-hour incubations with buffer amended with selected diet component resulted in increased secretion of digestive PLA_2, while small increases in secretion were detected when the buffer was amended with a specific PL, phosphatidylcholine. Highest secretion of the enzyme was detected from the middle

region and lowest secretion from the anterior midgut region. Because isolated midguts responded to food chemicals with increased secretion of digestive PLA_2, we suggest the secretion is regulated by a prandial and/or paracrine mechanism, as suggested for digestive proteases in other insect species.

Our efforts to partially purify the digestive PLA_2 yielded three interesting points. First, upon elution from superose-12 size-exclusion column, fractions containing the highest enzyme activity was determined to be about 24 kDa, which is higher than the mammalian secretory PLA_2. Second, in the absence of ammonium sulfate precipitation, we obtained more than 100% recovery of enzyme activity, which is equivalent to a 1.5-fold purification of the enzyme. And third, three major proteins were displayed upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the most active fraction. One of which was identified to be trypsin (43 kDa), based on its similarities with trypsins identified from *Manduca*.

DEDICATION

I would like to dedicate this work to my family, for their love and support throughout these years. Studying overseas has been a challenging ordeal for me and my Nanay, Tatay, and the rest of the family. Their patience, trust and prayers have kept me going despite the distance. I love them very much!

RLR

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INTRODUCTION

INTRODUCTION

The insect midgut is the principal site for digestive and absorptive processes

The alimentary canal of insects is generally subdivided into three major sections. First, the foregut or stomodaeum includes the mouth, pharynx, esophagus, crop, and proventriculus. It is derived from the invaginations of the embryonic ectoderm and lined with a cuticle. Second, the midgut or mesenteron includes the midgut ceca and ventriculus. It is sometimes lined with a cell-thick peritrophic membrane and the only section of the alimentary canal that is of endodermal in origin. Third, the hindgut or proctodaeum is comprised of the Malpighian tubules, pylorus, ileum, colon, rectum, and anus, and, like the foregut, is lined with a cuticle (Chapman, 1985a; McFarlane, 1985). Of these three sections, the midgut of most insect species is the primary site for secretion of digestive enzymes, digestion of food, and absorption of nutrients. The midgut of lepidopterous larvae also regulates transport of ions. For example, potassium is actively transported from the hemolymph side to the lumen side of isolated midguts of *Hyalophora cecropia* (Harvey and Nedergaard, 1964), *Manduca sexta* (Blankemeyer, 1976), *Bombyx mori*, *Macrothylatia rubia*, and *Philosamia cynthia* (Giordana and Sacchi, 1977), while magnesium (Wood *et al.*, 1975) and calcium (Wood and Harvey, 1976) are actively transported in the opposite direction.

Ultrastructure of the insect midgut epithelium

The midgut epithelium in most insects is morphologically and, perhaps, functionally differentiated along its length. However, the apparent regional differentiation

and cellular characteristics present in these regions may not always confer functionality. Structure-function relationships may not be discerned by ultrastructural studies alone. Histochemical localization studies of enzyme activity may yield some light in identifying specific midgut structures directly involved in food digestion. Cioffi (1979) divided the midgut of lepidopterous larvae of the tobacco hornworm (*M. sexta*) into three regions, namely: anterior, middle, and posterior region. He separated the midgut according to the pattern of folding of the epithelial sheet, which gives the tissue a corrugated appearance, and also by variations in cellular structure occurring along its length. A detailed microscopic examination of the midgut epithelium revealed cell types distinct among lepidopterans: columnar and goblet cells. Columnar cells are the predominant digestive and absorptive epithelial cells in the midgut. They are characterized by the presence of dense brush border of highly folded microvilli in the apical plasma membrane. On the other hand, the goblet cells have a flat nucleus lying in the basal cytoplasm and contain a large cavity with an apical opening into the lumen of the midgut (Endo and Nishiitsutsuji-Uwo, 1981). These cells are believed to be the principal agent of active potassium transport from the hemolymph to the lumen of the midgut (Anderson and Harvey, 1966). Both the columnar and goblet cells could form a basis for the functional role of the midgut regions in digestive, secretory, storage, and absorptive processes.

Some distinct differences occur in cellular structures along the midgut. For instance, the ultrastructure of the columnar cell changes gradually from the anterior to the posterior midgut region, while the goblet cells show a sudden change from one type to another. A common feature of the goblet cell cytoplasm in the three midgut regions

includes the presence of mitochondria, golgi complexes, rough endoplasmic reticula, and polyribosomes. Goblet cells usually occupy the bulk of the anterior and middle midgut epithelium. The projections of the goblet cell apical membrane contain elongated mitochondrion, a feature unique to arthropod epithelia, and characteristic of active ion-transporting tissues (Klein *et al.*, 1991). However, at the posterior midgut, the goblet cells occupy less than 50% of the midgut cell and the apical membrane projections lack mitochondria. Anderson and Harvey (1966) first suggested that the apical membrane of the goblet cell was the site of active potassium transport. Since their pioneering work on *Cecropia* midgut, several attempts were made to positively identify the transporting cell in other insects (Harvey and Zerahn, 1969; Harvey and Wood, 1972; Wood and Harvey, 1975; Wieczorek *et al.*, 1986; 1989; 1991). Studies of the nature of the K^+ pump were possible with the development of a procedure to isolate plasma membrane fractions from the posterior midgut (Cioffi and Wolfersberger, 1983). They found that the goblet cell apical membrane (GCAM) contained considerable K^+-stimulated V-ATPase activity. Immunohistochemical studies using antibodies raised against the V-ATPase showed that the V-ATPase activity is confined to the GCAM throughout the midgut regions. The K^+ pump of the GCAM vesicles consists of two molecular components, a primary H^+ pump and a secondary K^+/H^+ antiport (Wieczorek *et al.*, 1991; Azuma *et al.*, 1995). This is the first instance in which a V-ATPase was found to energize a secondary active transport in animal plasma membranes. When ATP is present and K^+ absent, the GCAM vesicles developed a pH gradient of 2-3 units and an electrical potential difference, suggesting that the primary transporter is an electrogenic proton pump. The presence of K^+ dissipated

both the electrical and chemical gradients, but stimulated ATP hydrolysis (Wieczorek *et al.*, 1991). The dissipation of the gradients is consistent with a K^+/H^+ exchange; the stimulation of ATP hydrolysis would reflect the sensitivity of the electrogenic H^+ pump to the H^+ gradient. When a K^+ gradient is present across the vesicle membrane, both acidification and charge transfer took place in the absence of ATP, consistent with K^+/H^+ exchange driven by the K^+ gradient. These findings indicate that the presence of a K^+/H^+ antiport modifies the composition and charge transfer. Using static measurements in vesicles, Azuma *et al.* (1995) showed the stoichiometry of the antiport to be 2 H^+ per K^+.

Another important feature of the midgut regions described by Cioffi (1979) is the distinct projections of the apical surface of the columnar cells. These projections constitute a dense brush border of microvilli which changes from the anterior to posterior end of the midgut. These microvilli are generally branched in the anterior region, and tend to fuse with adjacent projections forming a dense interconnecting network. Large, membrane-bound vesicles break off from the surface of this structure, and small golgi vesicles are the only cell organelle present at the apical border of the cell. The microvilli at the apical surface of the columnar cells become less pronounced in the middle region until, by the posterior region, where these structures become more distinct. Each projection is a distinct structure not connected to adjacent microvilli along its entire length. Beneath the posterior microvilli are large whorls of rough endoplasmic reticulum, golgi complexes, stores of glycogen granules, and few mitochondria. The occurrence of highly organized stacks of rough endoplasmic reticulum and its associated golgi complexes and mitochondria may be associated with storage, lysosomal, or secretory

vesicles (Andries, 1977; Billingsley and Downe, 1986). In the mosquito, *Aedes aegypti*, the whorls themselves may store intracellular, membrane-bound ribosomes, to which associated mRNA may be protected from degradation. This arrangement allows cells to respond readily to changes in lumen content by *de novo* synthesis of mRNA (Felix *et al.*, 1991).

In addition to columnar and goblet cells, lepidopteran midguts also contain endocrine and regenerative cells, which may be more distinct from cells found in other insect orders. Pyramidal or bowl-shaped endocrine cells, or basal-granulated cells, are dispersed in the epithelia of insect midguts, extending toward the lumen side. The cells contain several well-developed golgi complexes undergoing sequential stages of granule development, and the nucleus usually occur as a distorted sphere in shape (Endo and Nishiitsutsuji-Uwo, 1981). Discovery of different types of endocrine cells was possible through ultrastructural and immunohistochemical studies utilizing antibodies raised against vertebrate hormones. According to these studies, the abundance and distribution of endocrine cells are not only species-specific, but stage-specific as well (Iwanaga *et al.*, 1981; Schoofs *et al.*, 1988). These cells occur as an "open" type, where the basal lamina is in direct contact with the midgut lumen through an apical cell process, which is covered by a tuft of microvilli (Endo and Nishiitsutsuji-Uwo, 1981; Brown *et al.*, 1985; Sivasubramanian, 1992). Also, "closed" type endocrine cells were observed in midguts of hematophagous species (Billingsley and Downe, 1986; Glättli *et al.*, 1987). These cells are embedded in the epithelium and lack luminal contact. These endocrine cells function as primary sensors. The "open" type registers the nutrient content of the gut, while the

"closed" type complements the nervous perception of gut tension (Fujita and Kobayashi, 1977; Fujita *et al.*, 1988). Endocrine cells are usually more common in the posterior end of the midgut (Brown *et al.*, 1985; Billingsley and Downe, 1986).

Conversely, regenerative cells are small and either singly dispersed in the epithelium (Endo and Nishiitsutsuji-Uwo, 1980) or lie as a mass like those in the cockroach midgut (Nishiitsutsuji-Uwo and Endo, 1981). They are relatively undifferentiated cells with dense cytoplasm, few organelles, and lack the elaborations at the apical and basal membranes described for columnar, goblet or endocrine cells (Billingsley and Lehane, 1996). Regenerative cells have no known digestive function, but play a role in regeneration of midgut epithelium in some insect species during metamorphosis (Hecker, 1977; Billingsley and Downe, 1986) or following injury (Spies and Spence, 1985). They give rise to new columnar, goblet, and possibly endocrine cells during the larval-larval ecdysis. At the early pharate pupal stage, the cells begin to proliferate and differentiate into only tall columnar cells replacing the old epithelial cells during larval-pupal ecdysis (Waku and Sumimoto, 1971). Short and cuboidal regenerative cells eventually replace the old pupal cells during pupal-adult ecdysis. Regenerative cells may be absent in midguts of some fly larvae (Terra *et al.*, 1988) or adults (Böhringer-Schweizer, 1977).

Diversity in digestive enzymes reflects the variety of insect diets

Nutrition may be considered the source of the chemical substances required by an organism for maintenance and reproduction. The molecular interconversions of