ABSTRACT

JEFFERS, LAURA ANN. Novel System for the Stabilization and Delivery of Proteins to the Insect Hemocoel through Conjugation with Aliphatic Polyethylene Glycol. (Under the direction of Dr. R. Michael Roe.)

Despite the numerous insecticidal proteins identified, there are few commercially-successful protein insecticides. Oral delivery of proteins is difficult due to their degradation by digestive endo- and exo-peptidases and their limited movement across the gut epithelium. PEGylation, the process of covalently attaching polyethylene glycol (PEG) polymer chains to another molecule, has been used in the pharmaceutical industry for decades to deliver proteins across the digestive system of humans. In this study, two PEGylated insulins and a PEGylated insecticidal decapetide, Trypsin Modulating Oostatic Factor (TMOF), were created to determine if PEGylation could reduce the rate of degradation and enhance the accumulation of the parent compounds from the diet or cuticle in the insect hemocoel. The chemistry for the synthesis of monodispersed aliphatic TMOF-K-PEG7P, polydispersed aliphatic PEG350-insulin and monodispersed aliphatic PEG333-insulin are described herein. The PEGylation of insulin yields a 6.7 and 7.3 fold increase in appearance of insulin species in the hemolymph of Heliothis virescens larvae after feeding for the PEG350 and PEG333 chemistries, respectively. When insulin is topically applied to the dorsum of H. virescens, no insulin is found in the hemolymph. However, after topical application of the PEGylated insulins, insulin species were detected in the hemolymph. After injections of insulin into the hemocoel of 4th stadium H.virescens, insulin is completely cleared from the hemolymph in 120
minutes. In comparison, when PEG350-insulin and PEG333-insulin are injected into the hemocoel, insulin species were still present in the hemolymph 300 and 240 minutes after injection, respectively, translating to a 3.3 and 2.7 fold increases in the length of time insulin remains in the hemolymph after injection. Conjugation of TMOF to polyethylene glycol increased its insecticidal effects against several insect species. For example, the addition of lysine to TMOF reduced its per os activity relative to the parent TMOF, but conjugation of TMOF-K with methyl(ethyleneglycol)-O-propionyl increased it toxicity 5.8 and 10.1 fold above that of TMOF and TMOF-K for Aedes aegypti. However, unlike the PEGylated insulin species, no TMOF, TMOF-K, or PEGylated TMOF-K was detected in the hemolymph after topical application to the cuticle.
Novel System for the Stabilization and Delivery of Proteins to the Insect Hemocoel through Conjugation with Aliphatic Polyethylene Glycol

by
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BIOGRAPHY

After finishing her undergraduate degrees in chemistry and biochemistry from North Carolina State University, Laura worked in insecticide development and modes of action with Rhone Poulenc, an Agricultural Chemical Company in Research Triangle Park, NC. After a particularly nasty merger, Laura left industry to go back to graduate school and earn a Master’s degree in entomology, examining the movement of proteins across the gut of insects. Following that line of research, her PhD dissertation investigated the use of protein delivery systems to enhance the movement of proteins across the gut for possible pest control applications. In 2007, Laura made the jump from physiology to regulatory science when she started working at the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) Plant Protection and Quarantine (PPQ) Center for Plant Health Science and Technology (CPHST) as a biosurveillance analyst. For the past 4 years, she has worked as a Pest Exclusion Specialist with CPHST’s Treatment Quality Assurance Unit (TQAU). With TQAU, Laura spends most of her time resolving issues regarding quarantine phytosanitary treatments, such as fumigation and irradiation.

Laura lives in Raleigh with her husband, Brad Cooper; three boisterous labradors, Bridie, Neeps, and Tater; and 5 mischievous Buff Orpingtons. When she isn’t sweeping up the dog hair, Laura enjoys traveling, a passion she inherited from her globe-trotting parents, Jim and Grace Jeffers. She also enjoys gardening, swimming, and pretending she is good at home improvement projects.
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### Review: The Movement of Proteins across the Insect and Tick Digestive System

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### Enhanced Activity of an Insecticidal Protein, Trypsin Modulating Oostatic Factor (TMOF), through Conjugation with Aliphatic Polyethylene Glycol

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Review: The movement of proteins across the insect and tick digestive system

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Abstract

The movement of intact proteins across the digestive system was shown in a number of different bloodfeeding and nonbloodfeeding insects in the orders Blattaria, Coleoptera, Diptera, Hemiptera, Lepidoptera, Orthoptera, Neuroptera and Siphonaptera as well as in two tick families Ixodidae and Argasidae. Protein movement was observed for both normal dietary and xenobiotic proteins, which suggest the mechanism for transfer is not substrate specific. The number of studies on the mechanism of movement is limited. The research so far suggests that movement can occur by either a transcellular or intercellular pathway in the ventriculus with most of the research describing the former. Transfer is by continuous diffusion with no evidence of pinocytosis or vesicular transport common in mammalian systems. Proteins can move across the digestive system without modification of their primary or multimeric structure and with retention of their functional characteristics. Accumulation in the hemolymph is the result of the protein degradation rate in the gut and hemolymph and transfer rate across the digestive system and can be highly variable depending on species. Research on the development of delivery systems to enhance protein movement across the insect digestive system is in its infancy. The approaches so far considered with some success include the use of lipophilic-PEG polymers, the development of fusion proteins with lectins, reduced gut protease activity and the development of amphiphilic peptidic analogs. Additional research on understanding the basic mechanisms of protein delivery across the insect digestive system, the importance of structure-activity in this transfer and the development of technology to improve movement across the gut could be
highly significant to the future of protein and nucleic acid-based insecticide development as well as traditional chemical insecticidal technologies.
1. Introduction

Insecticides like the organochlorines, organophosphates, pyrethroids, JH mimics, neonicotinoids and others have traditionally been lipophilic compounds that easily move across the insect cuticle or digestive system to their site of action. However, the successful development of the delta-endotoxin from *Bacillus thuringiensis* (*Bt*) as an insecticide (Gill et al., 1992; Fischhoff, 1996; Gould et al., 1997; Gould, 1998; Christou et al., 2006) has revolutionized our thinking about the use of proteins for insect control, and it is clear that this trend will only increase in the future (Christou et al., 2006). A number of proteins are being considered for their potential use as insecticides: insect hormones (Roe and Venkatesh, 1990; Hammock and Philpott, 1992; Borovsky et al., 1990; Bonning et al., 1999; Fitches et al., 2002; Borovsky, 2003; Gäde and Goldsworthy, 2003); various mite (Tomalski et al., 1988, 1989, 1993; Tomalski and Miller, 1991; Burden et al., 2000), scorpion (Zlotkin et al. 1971, 1991, 2000; Stankiewicz et al., 1996; Wudayagiri et al., 2001; Inceoglu et al., 2003; Karbat et al., 2004; Gurevitz et al., 2006; Trung et al., 2006; Banerjee et al., 2006) and spider (Lipkin et al., 2002; Fitches et al., 2004; Tedford et al., 2004; Wullschleger et al., 2004; Down et al., 2006; de Lima et al., 2007; Rohou et al., 2007) toxins; protease inhibitors (Murdock and Shade, 2002; Haq et al., 2004); plant toxic proteins, including vegetative insecticidal proteins (Estruch et al., 1996; Warren, 1997; Yu et al., 1997; Zhu et al., 2006) and lectins (Peumans and Van Damme, 1995; Carlini and Grossi-de-Sá, 2002; Murdock and Shade, 2002); as well as others. One of the major obstacles in using proteins with sites of action accessible from the insect hemocoel is delivery across the insect cuticle and digestive system. The insect outer epicuticle is highly lipophilic and the exocuticle heavily sclerotized, which serves as a
barrier for protein movement. Oral delivery of proteins is difficult due to their degradation by digestive endo- and exo-peptidases and their limited movement across the gut epithelium. The *Bt* delta-endotoxin was successful because it acts directly on the lining of the gut (Gill et al., 1992) rather than requiring transport into the insect hemolymph.

Our understanding of protein movement across the insect digestive system is in its infancy and yet basic research in this area and the discovery of methods to enhance this movement is critical to future development of protein insecticides. Numerous papers and reviews have been published on the use of host antibodies to control ticks (Foy et al., 2002; Nuttall et al., 2006; Pruett, 1999; Wang and Nuttall, 1999; Wikel, 1996; Willadsen and Jongejan, 1999); this research will be discussed only in brief here. No reviews have focused on protein movement either in the Acari or insects. This paper considers our current understanding of protein movement across the gut of insects and ticks and methods to enhance this movement.
2. Protein movement across the insect/tick digestive system

2.1 Protein movement across the digestive system of bloodfeeding insects

Wigglesworth (1943) was one of the first researchers to demonstrate the passage of intact proteins across the insect digestive system. He found that a small amount of ingested hemoglobin was absorbed across the gut of *Rhodnius prolixus* into the hemolymph. Since then numerous arthropods have been shown to have intact host proteins in their hemolymph after feeding (Table 1).

In flies, Nogge and Giannetti (1979) demonstrated that intact human albumin and fragments of immunoglobulins appeared in the hemolymph of *Glossina morsitans*, the tsetse fly, after feeding on human blood. The presence of serum proteins in the hemolymph of *G. morsitans* after oral administration was determined by immunodiffusion in agar gels using the method of Schneweis and Nahmias (1971). Collected hemolymph was allowed to diffuse against anti-albumin IgG, anti-human IgG (complete) and anti-human IgG (Fab fragments). No intact IgG was detected in the hemolymph, but Fab fragments and albumin were present. Nogge and Giannetti (1980) found that about 0.035% of the albumin added to a bloodmeal reached the hemolymph unchanged.

Using ELISA and Western blots, Allingham et al. (1992) were able to detect intact host IgG in the hemolymph of blood-fed buffalo flies, *Haematobia irritans*. Heparinized ovine or bovine blood was fed to *H. irritans* continuously or for a single 10-min period. A single protein, an uncharacterized bovine IgG with a molecular weight of 150 kDa was detected in the hemolymph of the blood fed flies. Because it co-migrated with bovine IgG, the hemolymph protein was apparently intact. Ninety four percent of flies observed to take a
blood meal had detectable amounts of IgG in the hemolymph after feeding. The concentration of IgG in pooled hemolymph of flies allowed continuous access to a blood meal ranged from 2.1 to 7.3 μg/mL hemolymph.

Proteins also can move across the digestive system of mosquitoes. Vaughan and Azad (1988) found that anti-Rickettsia typhi antibody was present in hemolymph of four mosquito species after feeding on rats immunized with an R. typhi extract. Anti-R. typhi was present in the hemolymph of Anopheles stephensi at 3, 6, and 18 h after feeding, An. gambiae at 3, 6, 18 and 24 h after feeding, An. albimanus at 3, 6, 18 and 24 h after feeding, and Culex pipiens at 3 h after feeding. In these experiments, hemolymph was collected by hemocoel perfusion. Selected control experiments were conducted to make sure that this method of hemolymph collection produced the same results as hemolymph directly collected from mosquito legs amputated at the coxa-trochanter joint and bled into a droplet of saline. In a similar study with Aedes aegypti, host antibodies were found sequestered in oocytes 48 h after the blood meal (Ramasamy et al., 1988).

Vaughan et al. (1998) also fed heparinized cat blood to the cat flea, Ctenocephalides felis, using membrane feeders. Cat IgG detected by ELISA and Western blots was found to be present in hemolymph of engorged female fleas 1 h after ingestion at the level of 35 ± 14 μg/mL (average ± SD). Following a single blood meal, 100% of both male and female fleas had detectable cat IgG in their hemolymph 1 h after feeding. Cat IgG was only present in 50% of the flea hemolymph tested at 3 h after ingestion and in 10% of samples at 18 h after ingestion. Fleas were also allowed access to blood over a 72 h period, and the cat IgG concentration remained fairly constant (3-16 pg per flea) over the entire period. It is clear
from all of these studies that proteins of different types can move across the insect digestive system of blood feeding insects from different orders, and these proteins to some extent maintain both their native structure and function.

2.2 Protein movement across the digestive system of non-bloodfeeding insects

There is a growing body of evidence that the movement of intact proteins across the digestive system from the diet is not unique to blood feeding arthropods. Ben-Yakir and Shochat (1996) studied the fate of ingested anti-ovalbumin IgG in the lepidopteran, the European corn borer, *Ostrinia nubilalis*. When the larvae were allowed to feed continuously for 24, 48 and 96 h on artificial diet containing 1,200 µg anti-ovalbumin per g diet, the concentration of IgG in hemolymph was 2.40 ± 0.98, 2.06 ± 1.14, and 1.64 ± 1.32 µg/ml (average ± SD), respectively. The transfer rate from the diet into the hemolymph in this experiment was in the ratio of approximately 1:500 (wt:wt). After 48 h of feeding on anti-ovalbumin diet, the concentration of IgG in the midgut was 673 ± 447 µg/g. When the larvae were transferred to artificial diet without anti-ovalbumin and incubated for 6 or 18 h, the concentration of IgG in the midgut decreased to 204 ± 153 and 64 ± 38 µg/g, respectively. The IgG concentration in the hemolymph was shown to have a significant positive correlation and linear relationship (r = 0.96) with the IgG concentration in the gut, suggesting that blood titers are affected in a dose dependent manner.

Mulberry leaf urease was also found to pass through the gut wall of the silkworm, *Bombyx mori*, and into the hemolymph without change (Hirayama et al., 2000; Sugimura et al., 2001; Kurahashi et al., 2005). When silkworms were allowed to feed on mulberry leaves,
urease activity was detected in the hemolymph from the beginning of spinning to the pharate adult stage. However, silkworms feeding on an artificial diet without urease did not have detectable urease activity in their hemolymph, suggesting that the enzyme can be absorbed across the gut from mulberry leaves (Yamada et al., 1984). To determine if the hemolymph urease and leaf urease were similar, the enzyme was purified from the hemolymph of spinning larvae reared on mulberry leaves and compared to mulberry leaf urease. Both enzymes co-migrated with an apparent molecular weight of 90.5 kDa on SDS-PAGE. The hemolymph urease also cross reacted equally with the mulberry urease using four out of six monoclonal antibodies raised against jack bean seed urease, and the $K_m$ for urea and the optimum pH for activity were similar for the two enzymes. Finally, comparing the sequence of the first 20 amino acids on the N-terminus, the ureases were identical from both sources (Hirayama et al., 2000). It was hypothesized that the silkworm larvae selectively transported the plant urease across the gut and that it was important in hemolymph nitrogen metabolism (Kurahasi et al., 2005).

Movement of protein has also been investigated in other insects outside of the Lepidoptera. FITC-Casein (a digestible protein with a fluorescein isocyanate conjugate label) and green fluorescent protein (GFP, a non-digestible protein) were fed to the western tarnished plant bug, *Lygus hesperus*, using a sachet system (Habibi et al., 2002). Starved, adult females were fed on sachets containing 1% FITC-casein for 2 h and then moved to control sachets without casein. Hemolymph and gut samples were collected at several time points after the transfer. In other experiments, the insects were fed 0.05% GFP for 3 h and then transferred to control sachets. Intact FITC-casein was detected in the hemolymph at 2
and 4 h after the onset of feeding, and ingested GFP was detected in the hemolymph at 3 and 6 h. By 12 h, FITC-casein and GFP were not detected in any tissue as holoproteins. Smaller fragments of FITC-casein were detected at 2 and 4 h after the onset of feeding in the hemolymph and gut, but by 12 h no fragments were detected in the gut or hemolymph. No degradation productions of GFP were found in any tissue at any time point. Using immunohistological images, GFP was shown to bind to the gut epithelial cell brush border membranes.

Using oral toxicity assays, column chromatography, and microscopic autoradiography of a native and radioiodinated scorpion venom neurotoxin, AaIt was shown to cross the gut of the fly, Sarcophaga falculata. At 10 µg/100mg of body weight, AaIt was shown to induce paralysis of flies within 1-2 h after oral administration. Oral toxicity was shown to be 0.14% of the injected toxicity. Five percent of the ingested radioactivity appeared in the hemolymph 85 min after feeding. Most of this polypeptide was degraded, but 0.3% of intact toxin remained (Zlotkin et al., 1992). In similar studies, 2.2% of a radiolabeled cobra venom neurotoxin was also shown to move across the gut and into the hemolymph (Primor et al., 1980; Primor and Zlotkin, 1980; Fishman et al., 1984).

The movement of lectins has been examined in a number of insects (Table 1). In tomato moth larvae, Lacanobia oleracea, snowdrop and jackbean lectins were shown to accumulate in the gut, hemolymph and malpighian tubules and the jackbean lectin in the fat body after feeding (Fitches et al., 1998, 2001a, 2001b). In the rice brown plant hopper, Nilaparvata lugens, snowdrop lectin was shown to accumulate in the hemolymph, fat body and ovarioles (Powell et al., 1998). Lectins have also been found to move across the
digestive system of some aphid predators. Snowdrop lectin was detected in the hemolymph of *Chrysoperla carnea* and *Adalia bipunctata* but not in *Coccinella septempunctata* (Hogervorst et al., 2006). Cowpea seed vicilins, which are similar to lectins, have also been shown to move intact across the gut after feeding and are sequestered by various organs such as the fat body, malpighian tubules and hemolymph in the beetle, *Callosobruchus maculatus* (Uchôa et al., 2006). It is interesting that vicilin can be sequestered in the larval stage and retained through the adult stage.

Insect hormones can also move across the digestive system and into hemolymph (Zhu et al., 2001). Radiolabeled trypsin modulating oostatic factor of the flesh fly *Sarcophaga bullata* (Neb-TMOF) was introduced to the foregut of *Schistocerca gregaria* using Teflon capillary tubing (by intubation) to examine whether the peptide can cross the gut epithelium. Hemolymph samples were collected from small wounds made near the hind legs 5 to 120 min after the introduction of the hormone into the foregut. Five min after intubation, approximately 1% of the original radioactive Neb-TMOF was detected in the hemolymph by HPLC analysis. The highest percentage of Neb-TMOF, 5.0 ± 2.3% (average ± SD), was detected in hemolymph after 30 min. The amount of intact Neb-TMOF gradually decreased thereafter but was still present after 2 h. Amaranth dye known not to be absorbed across the insect gut, was also not detected in hemolymph in control experiments, indicating that the Neb-TMOF must be moving across the intact gut epithelium.

Recently, Jeffers et al. (2005) investigated the movement of bovine serum albumin (BSA) and anti-bovine serum albumin (anti-BSA) across the digestive system of fourth instars of the tobacco budworm, *Heliothis virescens*. After 8 and 16 h of feeding on artificial
diet containing anti-BSA, the concentration of the antibody in hemolymph was 2,430 ± 125 (average ± 1 SEM) and 3,459 ± 105 ng/mL, respectively. After 8 and 16 h of feeding on artificial diet containing BSA, the concentration of BSA in hemolymph was 1,547 ± 132 and 1,623 ± 122 ng/mL, respectively. The concentration in the diet compared to that of the hemolymph was in the ratio of approximately 1:300 for anti-BSA and 1:500 for BSA and was similar in concentration to the studies of Ben-Yakir and Shochat (1996) for ingested anti-ovalbumin IgG in the European corn borer. In the experiments with the tobacco budworm, special care was taken to prevent contamination of the hemolymph during bleeding by any BSA or anti-BSA proteins that may have contaminated the surface of the insect during feeding. Also as determined from topical treatments with anti-BSA and BSA, these proteins did not move across the cuticle into hemolymph. It is interesting that all three multimeric forms of BSA were detected in the hemolymph after feeding and in the same ratios as found in the diet as determined by Western blotting. In addition, anti-BSA retained both its structure and function, and even though it had a larger molecular weight than BSA, was found in a higher concentration in the hemolymph. These experiments indicated that proteins can move across the gut from the diet maintaining their primary and multimeric structure as well as their function. Since BSA and anti-BSA can move across the digestive system and the fact that these proteins are not normally in the diet of the tobacco budworm, it appears that the mechanism for protein transport across the gut is not specific to dietary proteins as was suggested for urease in the silkworm discussed earlier. It also appears from these studies that protein size did not affect the rate of accumulation.
Jeffers et al. (2005) also showed that anti-BSA and BSA titers decreased after injection into the hemolymph of larvae and when added to hemolymph in vitro. Apparently, the amount of protein found in hemolymph is a function of its rate of absorption across the gut and its rate of removal from hemolymph. Therefore, the development of methods to maximize the accumulation of xenobiotic proteins across the gut or cuticle would be benefited by both increasing the rate of transfer and reducing the rate of removal (or degradation) in hemolymph. The movement of BSA and anti-BSA across the digestive system and into the hemolymph is not limited to the tobacco budworm and is variable within the Lepidoptera. Jeffers et al. (2005) also showed transport for *Heliocoverpa zea, Acheta domesticus* and *Gromphadorhina portentosa* but not *Manduca sexta*. This variability between arthropod species have been found by other researchers as will be discussed later.

### 2.3 Protein movement across the digestive system of ticks

Chinzei and Minoura (1987) fed either rabbit or human IgG through an artificial membrane for 1-2 h to the soft tick, *O. moubata*. Hemolymph was collected from female ticks 7 d after feeding and applied to Ouchterlony plates. The hemolymph of ticks engorged on rabbit IgG formed an immunoprecipitin line between the rabbit IgG and anti-rabbit IgG. Similarly the hemolymph of ticks engorged on human IgG reacted with anti-human IgG to form an immunoprecipitin. These observations demonstrate that IgG in the tick hemolymph is immunologically identical to the IgG found in the blood meal and suggests that female ticks directly absorb host IgG through the gut wall into the hemocoel with the retention of antibody function. This has also been reported to occur in insects as previously discussed.

Jasinskas et al. (2000) used capillary feeding to introduce compounds into the midgut of the hard tick, *A. americanum*. The proteins studied were mouse, donkey and human IgG, mouse serum albumin, chloramphenicol acetyltransferase, and human IgM (labeled with $^{125}$I, $^{14}$C or biotin). The entry of the immunoglobulins into the hemolymph was greater (6%) after 6 h than the smaller proteins, chloramphenicol acetyltransferase and albumin (3% and 1%, respectively). Comparing the uptake of different types of IgG into the hemolymph, these results indicate uptake is not specific for mouse, donkey or human IgG.

Ben-Yakir (1989) measured host IgG concentration in the hemolymph of female hard and soft ticks. *H. excavatum, R. sanguineus, O. tholozani and O. moubata* were allowed to feed on rabbits that had been immunized with ovalbumin. *Argas persicus* were allowed to feed on a chicken that had been immunized with cytochrome C. Hemolymph samples were taken 24 h after removal from their hosts. Host IgG was detected in the hemolymph of *H. excavatum, R. sanguineus* and *O. moubata* but not *O. tholozani* and *A. persicus*. Using
Protein A, the host IgG found in hemolymph was shown to be intact in all three species. It is also clear that an animal host can develop resistance to tick engorgement. Ticks that feed on resistant hosts show slowed development, decreased fecundity and increased mortality (Ben-Yakir, 1989).

Recently, two studies used a proteomics approach to characterize proteins in the tick salivary gland. Host proteins and antibodies were found in the saliva of *I. scapularis* (Valenzuela et al., 2002), *A. americanum* and *A. maculatum* (Madden et al., 2002), suggesting that these proteins may cross the digestive system and migrate to the salivary glands.

From the studies conducted so far, it appears that proteins of different types can move unchanged across the digestive system of several insects and ticks and accumulate in hemolymph. Protein movement is not restricted to blood feeding arthropods and can be variable occurring in one species but not present in a related species. The fact that movement occurs with proteins not normally found in the diet of some insects (Jeffers et al., 2005; Habibi et al., 2002) as well as normal diet proteins (Nogge and Giannetti, 1980; Ben-Yakir, 1989) suggests that at least some transport is not protein specific (discussed in more detail next). It is not clear whether the appearance of proteins that cross the digestive system into the hemolymph is functionally significant in several cases that have been examined and whether there are specific mechanisms for the utilization of these intact proteins.
3. Mechanisms for protein movement across the insect/tick digestive system

The movement of intact macromolecules (including proteins) across the digestive system and into the circulatory system of mammals was documented as early as 1936 by Verzár and McDougall. Macromolecular transport across the mammalian digestive system has been discussed in several reviews and papers (Walker et al., 1972; Silk, 1980; Udall and Walker, 1982; Gardner, 1984; Kleinman and Walker, 1984; Silk et al., 1985; Alpers, 1986; Gardner, 1988; Weiner, 1988; Pácha, 2000; Ziv and Bendayan, 2000). The transport of large compounds like proteins across the stomach and small intestine is important during early postnatal life to facilitate the absorption of growth factors, antigens and immunoglobulin from maternal colostrum and milk. In early mammalian development, the transport of these macromolecules occurs in enterocytes and follows two pathways: specific receptor-mediated and nonspecific transcytosis (Pácha, 2000). In specific receptor-mediated transepithelial transport of immunoglobulins, the IgG present in milk or colostrum bind to a specific receptor in the apical membrane; the complex is endocytosed, moved through the cell within a transport vesicle and secreted into the contraluminal compartment. In some mammals, it is possible to observe a massive absorption of IgG via an alternative nonselective endocytic pathway. In this system, intraluminal macromolecules are endocytosed, partially destroyed and partially transported across the enterocytes. The high permeability of the intestinal epithelium for large compounds declines after birth in a process called gut closure (Pácha, 2000). However, the mature mammalian small intestine retains the ability to absorb these compounds at a lower level than in early postnatal life (Danforth and Moore, 1959; Bockman

In comparison to mammals, very little is known about the movement of compounds like proteins across the insect gut. Hatfield (1988) found mouse IgG persisted in *Aedes aegypti* mosquito bloodmeals for 2-3 d after ingestion. Immunoenzyme labeling showed that mouse IgG bound to the midgut epithelium of *A. aegypti* after feeding. Immunogold labeling of thin sections showed mouse IgG in the cytoplasm of the midgut epithelium microvilli, and the protein was found by ELISA in the mosquito hemolymph. The IgG was not found bound to any other tissue besides the gut. Hatfield proposed that IgG might actively and selectively move through the midgut cells. Alternatively, IgG may passively be transferred between epithelial cells. This is described as a “leaky” midgut, the same mechanism by which arboviruses move from the bloodmeal into hemolymph (Boorman, 1960; Miles et al., 1973; Hardy et al., 1983).

Using electron microscopy and electron-dense tracers in ingested food, Modespacher et al. (1986) examined the movement of intact horseradish peroxidase (HRP) across the midgut of the tsetse fly. This research appears to support the hypothesis of a "leaky" midgut. After feeding the flies on human serum containing horseradish peroxidase, they found that HRP moved into the intercellular clefts of the anterior midgut at 0.5 h after feeding and was in these clefts halfway through the length of the midgut after 1 h. One h after feeding, HRP was present in the basal labyrinth of the midgut epithelium in these same midgut regions. After 2 h, most basal parts of the basal labyrinth were preferentially labeled as well as the basal lamina, indicating HRP had reached the hemolymph. No HRP was found in the
posterior-part of the midgut, and the authors found no indication that pinocytosis was the mode of transport.

Fishman and Zlotkin (1984) introduced HRP (0.03-0.3 mg per insect) to the flesh fly, *Sarcophaga falculata*, and movement across the midgut followed by light and electron microscopy. After crossing the peritrophic membrane and spreading into the intercellular spaces of the midgut epithelium, it was shown that HRP entered the epithelial gut cells of *S. falculata* through microvilli 30 min after feeding. This was followed by diffusion across the cytoplasm of the cell. Thirty to 60 min after oral application, 40% of the sections from the midgut demonstrated the absence of HRP in the apical region of the midgut epithelial cells and the accumulation of HRP at the basal regions. Finally the HRP was shown to cross the inner plasma membrane of the midgut epithelium, the basal membrane and the layer of connective tissue covering the gut muscles. The continuous diffusion throughout the cytoplasm indicated that pinocytotic uptake or vesicular transport, common in mammalian systems, was unlikely.

Most recently, Casartelli et al. (2005) examined the *in vitro* absorption of albumin across the gut of *Bombyx mori*. Transport appeared to occur through a transcellular pathway. This is corroborated by FITC-albumin being detected as a widespread cytoplasmic fluorescence of the midgut epithelium and was not found in the paracellular spaces.

With the limited number of studies that have been conducted so far on protein movement across the insect digestive system, it appears that two routes are possible, movement through the ventricular epithelial cells and one report of movement through the intercellular spaces of the midgut. An explanation of the molecular mechanism for this
movement is currently unavailable. It appears there is no evidence for pinocytosis as a method for trans-epithelial movement of protein into the hemolymph.

There is no direct evidence for receptor mediated specific protein transport except for that discussed so far for urease absorption (Kurahasi et al., 2005). There have been a number of examples of apparent non-specific transport across the insect digestive system of proteins not normally found in the diet (Ben-Yakir and Shochat, 1996; Habibi et al., 2002; Zhu et al., 2001; Jeffers et al., 2005), which might argue against a highly specific, receptor mediated transfer system. Whether in some cases these are artifacts resulting from the introduction of high levels of foreign proteins in the diet and would not be typical of most normal diet proteins needs further examination. The evidence so far suggests that protein transport into the hemolymph occurs across the anterior midgut but this is based on work from a single insect species. The digestion of proteins and the uptake of amino acids by insects and ticks have been reviewed before (Casartelli et al., 2001; Giordana et al., 2002, 2003; Neal et al., 1996; Reuveni and Dunn, 1993; Rhodes et al., 1996; Salvucci et al., 1998; Schneider et al., 1986; Shinbo et al., 1996; Wolfersberger, 1996; Wolfersberger, 2000; Woods and Kingsolver, 1999; Zhou et al., 2004).

The kinetics of protein movement across the digestive system and that related to the biology of the organism is worth considering as well. In insects like the cat flea, C. felis, 100% of the males and females contained cat immunoglobulin G in their hemolymph 1 h after a single feeding on heparinized cat blood (Vaughan et al., 1998). Only 50% of the insects contained detectable cat IgG after three hours while fleas provided a continuous blood source maintained constant levels of the protein. However, in the American dog tick, D.
variabilis, Rat IgG was found in hemolymph of flat nymphs 2 months after they had fed as larvae and even after they had molted to nymphs (Vaughan et al., 2002). It is apparent from these two examples that the strategies for the uptake of proteins from the diet and/or their sequestration and turnover in the hemolymph can be quite different depending on the life strategy of the organism. It was interesting in the dog tick that the rat IgG in hemolymph was completely replaced by the rabbit IgG during nymphal feeding by a mechanism not well understood (Vaughan et al., 2002). In the tick, *A. americanum*, the uptake of IgG from different hosts in hemolymph was greater than that of other smaller proteins like albumin or choramphenicol acetyltransferase, suggesting in this case that a possible specific mechanism of uptake is possible (Jasinskas et al., 2000) as was discussed before for insects (Kurahasi et al., 2005). Although this review is focused on protein movement across the digestive system, it is obvious that protein sequestration and degradation in the hemocoel and the organs thereof in addition to the life strategy of the organism all play a role in how different proteins might move intact across the arthropod gut.
4. Developing Technology for Enhanced Movement of Proteins across the Gut

From the research so far, it is clear that proteins can move across the digestive system of insects and ticks, presumably by intra- and possibly inter-cellular transport mechanisms. To enhance this movement and better understand mechanisms, structure-activity studies are needed to identify the important characteristics of protein structure that govern this transfer rate; little work has been conducted in this area. In addition, understanding the mechanism of transport would be helpful. Despite the paucity of information in these areas, some significant progress has been made in developing methods to enhance protein movement across the gut (Table 2).

4.1 Production of insecticidal proteins using baculoviruses

One method investigated to circumvent the problem of protein delivery has been the use of transgenic baculoviruses to move the genetic information for specific protein synthesis into the insect hemocoel and into insect cells. The use of baculoviruses for insect control has been reviewed before (Maeda, 1989, 1995; Bonning and Hammock, 1996; Moscardi, 1999) and will be discussed only in brief here. With the baculovirus expression system, the cellular machinery of the insect is used to synthesize the protein to be delivered, producing a highly selective and safe method for insect control. The drawbacks have been the speed of toxic action, the difficulty in insecticide production and reduced residual activity in the environment once released (Duffey et al., 1995; Bonning and Hammock, 1996). Although the commercialization of baculoviruses for insect control appears doubtful at this time, one outcome of this research has been the discovery of many highly insect specific, highly active
protein toxins that could be used as insecticides. The limitations have been that the site of action of these proteins is the insect hemocoel and their limited delivery rate across the insect cuticle and digestive system. Despite these limitations, new technologies for protein delivery are emerging which may have a significant impact in insect control (Table 2).

4.2. Protein lipophilic-polyethylene glycol polymers

Important advances have been made in the pharmaceutical industry for the delivery of proteins across the digestive system of humans for the treatment of diseases using covalently bound protein lipophilic-polyethylene glycol (PEG) polymers, a process called pegylation (Zalipsky and Harris, 1997; Veronese, 2001; Roberts et al., 2002; Harris and Veronese, 2002; Yowell and Blackwell, 2002). Simply formulating PEG with Cry1Ac protein toxin increased its activity against insects by feeding >900-fold (Olsen and Daly, 2000). In vertebrate animal systems, there is considerable evidence that covalent protein-PEG and protein-lipophilic PEG polymers can increase protein transport across biological membranes, decrease proteolytic degradation and increase accumulation in the circulatory system (Bailon et al., 1998; Still, 2002; Clement et al., 2004). Roe and Brandt (2003) using a similar approached were able to enhance the accumulation of a model protein, human insulin, across the insect digestive system and across the outside insect cuticle into hemolymph. Insulin normally does not move across the insect cuticle when applied topically. These researchers proposed using this approach to enhance the delivery of chemical, protein and nucleic acid-based insecticides. The feasibility of this approach for insect control using proteins was recently demonstrated. Polymers of the insecticidal hormone, TMOF, were found to accumulate in hemolymph of
larval tobacco budworms after feeding and increased the toxicity of TMOF approximately 10-fold against mosquito larvae (Jeffers and Roe, unpublished). It appears that the synthesis of the TMOF-lipophilic PEG polymers did not prevent the binding of the hormone to its receptor on the hemolymph-side of the digestive system in mosquitoes or the PEG polymer was released before binding. This same approach could be used to improve the delivery of many different types of insecticidal compounds to their site of action. Additional research is needed to further validate this approach in insects.

4.3. Lectins

Lectins are another pharmaceutical delivery tool that has found an application in insect control. Lectins are proteins that recognize and bind to sugar complexes attached to proteins and lipids (Bies et al., 2003). The binding of lectins to the epithelium of the gut and their movement across the digestive system was previously discussed in this review. Using the mannose-specific snowdrop lectin (GNA), researchers were successful in delivering functional allatostatin (Fitches et al., 2002), an insecticidal spider venom toxin (Fitches et al., 2004; Down et al., 2006) and a lepidopteran-specific scorpion toxin (Trung et al., 2007) into the insect hemocoel.

When Fitches et al. (2002) fed a GNA/Manduca sexta allatostatin fusion protein produced in *Escherichia coli* to the tomato moth, *Lancanobia oleracea*, the protein inhibited feeding and growth in fifth stadium larvae at the level of 1.5 or 0.5% of the total dietary protein. GNA, allatostatin, or a mixture of GNA and allatostatin at similar concentrations had no deleterious effects. There were elevated levels of material that reacted with an
antibody to *M. sexta* allatostatin found in the hemolymph of larvae fed the fusion protein, which suggested that the fusion protein had crossed the gut.

Fitches et al. (2004) made a similar construct with an insecticidal spider venom toxin (*Segestria florentina* toxin 1, SFI1), this time expressed in *Pichia pastoris*. When this fusion protein was fed to first stadium larvae of *L. oleracea*, 100% mortality was observed after 6 d when the protein was 2.5% of the total dietary protein. The fusion protein also exhibited toxicity towards fourth and fifth stadium larvae, causing 90% growth reduction over a 4 d period. Larvae fed GNA or the spider toxin exhibited no acute toxicity. In addition, Down et al. (2006) demonstrated toxicity in the peach-potato aphid, *Myzus persicae*, and the rice brown planthopper, *Nilaparvata lugens*, using the SFI1/GNA fusion protein. Evidence of the SFI1/GNA fusion protein was found in the hemolymph of *N. parvata* and *L. oleracea* using Western blots (Fitches et al., 2004; Down et al., 2006).

Most recently, a fusion protein was constructed between GNA and a lepidopteran-specific toxin (ButalT) from the South Indian red scorpion (*Mesobuthus tamulus*) expressed in *P. pastoris* (Trung et al., 2006). Injections of ButalT and the fusion protein into fifth instars of *L. oleracea* caused immediate paralysis leading to decreased growth and mortality. When third instars were fed diet containing the fusion protein at 2.5 and 4.5% of the total dietary protein, there were delayed effects of reduced growth leading to mortality. Again, the intact fusion protein was detected in the hemolymph after feeding by Western blotting. Interestingly, it has been claimed that the scorpion toxin, ButalT, is lepidopteran-specific, but the fusion protein may have a wider range of toxicity. When the fusion protein was fed to *N. lugens*, the mortality for the fusion protein was significantly higher than that for GNA alone.
These discoveries open the door to a transgenic approach for the development of protein toxins for insect control that are not restricted to sites of action in the insect gut lining.

4.3. Protease inhibitors

In 1954, Lipke published the first description of a soybean protease inhibitor and its effects on the red flour beetle, Tribolium cofusum, leading to the discovery and cloning of the gene. Researchers have long hoped that protease inhibitors could be used to control insects on plants. Murdock and Shade (2002) suggested several reasons why this approach may not work. For example, the insect resistance imparted by protease inhibitors is only moderate, with the most common effect being slowed development. In order to achieve even this level of protection the concentration of the inhibitor must be high, >1% of the soluble plant protein. In addition, insects use several enzymes to digest protein, and they can compensate for the inhibition of one protease by increasing the expression of others. Also, since most crops have multiple pests, it would be difficult to find a single protease inhibitor with broad-spectrum activity. There could also be non-target effects in humans and animals if the inhibitors are to general in their spectrum of activity.

The study of protease inhibitors may also be relevant for other reasons. Two factors can affect the rate of transfer of proteins across the insect/tick digestive system: the rate of protein degradation in the digestive system and the rate of movement across the gut. Although the consideration of protease inhibitors alone for insect control has been considered, the use of protease inhibitors to enhance the delivery of insecticidal proteins appears to be novel. However, many of the arguments for why this approach would not be
Effective as an insecticide alone (Murdock and Shade, 2002) would also apply to their co-application with insecticidal proteins. No research is yet available that directly examines the use of protease inhibitors to enhance protein transfer across the insect gut. However, Nachman et al. (1999, 2002) indirectly examined this question. They were able to improve protein delivery by the development of peptidase-resistant neuropeptides. The primary site of degradation of pyrokinin/PBAN is the peptide bond between the active core proline and arginine. By incorporating modified proline residues, they were able to obtain resistance against tissue-bound peptidases. The analogs demonstrated biological activity per os, presumed in part from their reduced midgut degradation. Further research is needed to better understand the effect of protease inhibition on protein movement across the insect digestive system.

4.4. Amphiphilic proteins

Enhanced delivery of proteins also has been achieved by the development of amphiphilic pseudopeptide analogs of the neuropeptide, pyrokinin/PBAN (Nachman et al., 1998, 1999, 2002). Since the insect cuticle contains a nonpolar lipid matrix, polar compounds (like most proteins and peptides) do not readily move across this surface. The modification of insecticidal peptides to add amphiphilicity might allow cuticle penetration while at the same time, permit sufficient water solubility to allow delivery to the target site in the insect hemocoel. The same principle was used for the development of lipophilic-PEG protein polymers discussed before. A combination of this approach and the addition of modified amino acids to decrease protease degradation did not improve the topical activity of
pyrokinin/PBAN but were successful in increasing oral activity. It was suggested that the success in part was dependent on the ability of the modified peptides to penetrate the foregut cuticle and avoid the hostile environment of the midgut (Nachman et al., 2002).

In conclusion, the movement of intact proteins across the digestive system of insects and ticks is an understudied area of research. It appears that proteins of different types can move intact across the digestive system, regardless of whether the protein would be native or foreign to the diet, in different ticks and bloodfeeding/nonbloodfeeding insects. The specific mechanisms by which proteins are able to move intact across the digestive system are not known. Proteins appear to move across the digestive system by both transcellular and intercellular pathways, although most of the studies so far describe the former. Movement appears to be continuous and not by pinocytosis. Research on the development of delivery systems to enhance protein movement across the insect digestive system is in its infancy. The approaches so far considered with some success include the use of lipophilic-PEG polymers, the development of fusion proteins with lectins, reduced gut protease activity and the development of amphiphilic peptidic analogs. Additional research on understanding the basic mechanisms of protein delivery across the insect digestive system, the importance of structure-activity in this transfer and the development of technology to improve gut transfer of compounds could be highly significant to the future of protein and nucleic acid-based insecticides as well as traditional chemical insecticidal technologies.
5. Acknowledgments

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Table 1 (continued)
Protein movement across the insect and tick digestive system

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Enhanced Activity of an Insecticidal Protein, Trypsin Modulating Oostatic Factor (TMOF), through Conjugation with Aliphatic Poly(ethylene glycol)

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Abstract

BACKGROUND: Trypsin modulating oostatic factor (TMOF), a decapeptide (Tyr-Asp-Pro-Ala-Pro$_6$), isolated from the ovaries of the adult yellow fever mosquito, *Aedes aegypti*, regulates trypsin biosynthesis. TMOF *per os* is insecticidal to larval mosquitoes and a good model for the development of technologies to enhance protein insecticide activity by reduced catabolism and/or enhanced delivery to the target.

RESULTS: TFA-TMOF-K (TFA = trifluoro acetyl) allowed the specific conjugation of monodispersed, aliphatic polyethylene glycol (PEG) to the amino group of lysine producing TMOF-K- (methyl(ethyleneglycol)$_7$-O-propionyl)(TMOF-K-PEG$_7$P). The addition of lysine to TMOF reduced its *per os* larval mosquitocidal activity relative to the parent TMOF but conjugation of TMOF-K with (methyl(ethyleneglycol)$_7$-O-propionyl) increased its toxicity above that of TMOF and TMOF-K by 5.8- and 10.1-fold for *Ae. aegypti*. Enhanced insecticidal activity also was found for larval *Ae. albopictus* and for neonates of *Heliothis virescens* and *Heliocoverpa zea*. Only TMOF-K was found by MS/MS in the hemolymph for *H. virescens* fed on TMOF-K-PEG$_7$P. No TMOF, TMOF-K or PEGylated TMOF-K was detected in the hemolymph after topical applications.

CONCLUSIONS: This research suggests that aliphatic PEG polymers can be used as a new method for increasing the activity of insecticidal proteins.
Keywords: Trypsin modulating oostatic factor, TMOF, PEGylation, polyethylene glycol, mosquito, tobacco budworm, cotton bollworm, protein delivery
1 INTRODUCTION

Insecticides have historically been lipophilic compounds like the organochlorines, organophosphates, pyrethroids and others that easily move across the insect cuticle or digestive system to their site of action. With the development of the insecticide from the delta-endotoxin of Bacillus thuringiensis (Bt), the use of proteins for insect control has garnered substantial interest. For example, insect hormones; mite, scorpion and spider toxins; protease inhibitors; and plant toxic proteins including vegetative insecticidal proteins and lectins are being considered for their potential use as insecticides. In addition, there are a number of studies on the use of host antibodies to control ectoparasites such as ticks.

The major hurdle in using proteins with sites of action accessible from the insect hemocoel is delivery across the insect cuticle and digestive system. The insect outer epicuticle is highly lipophilic and the exocuticle heavily sclerotized which serves as a barrier for protein movement. Oral delivery of proteins is also difficult due to their degradation by digestive proteases and their limited movement across the gut epithelium. In addition, xenobiotic metabolism can limit activity once the compound is in the hemolymph. The Bt delta-endotoxin is a successful insecticide because it acts directly on the lining of the gut rather than requiring transport into the insect hemolymph. Even in this case, there is the concern that enhanced gut digestion could be a potential mechanism of resistance to Bt. Basic research to limit protein degradation and enhance movement across the insect digestive system will be critical to future development of protein insecticides.
Jeffers and Roe\textsuperscript{20} reviewed for the first time protein movement across the gut of insects and ticks. This movement has been demonstrated in a number of different blood-feeding and non-blood-feeding insects as well as in two tick families, the Ixodidae and Argasidae. Macromolecule movement across the digestive system and into the circulatory system of mammals was reviewed in detail earlier\textsuperscript{21-23}. In brief, this transport occurs across the stomach and small intestine and is important during early postnatal life to facilitate the absorption of growth factors, antigens and immunoglobulin from maternal colostrum and milk. In early mammalian development, the transport of these macromolecules occurs in enterocytes and follows two pathways, specific receptor-mediated and non-specific transcytosis.\textsuperscript{23} In specific receptor-mediated trans-epithelial transport of immunoglobulins, the IgG present in milk or colostrum bind to a specific receptor in the apical membrane; the complex is endocytosed, moved through the cell within a transport vesicle and secreted into the contraluminal compartment. In some mammals, it is possible to observe a massive absorption of IgG via an alternative non-selective endocytotic pathway. In this system, intraluminal macromolecules are endocytosed, partially destroyed and partially transported across the enterocytes. The high permeability of the intestinal epithelium for large compounds declines after birth in a process called gut closure.\textsuperscript{23}

The limited research on protein movement across the insect digestive system suggests two routes are possible, movement through the ventricular epithelial cells and movement through the intercellular spaces of the midgut\textsuperscript{20,24-26}. To our knowledge, there is no evidence for pinocytosis. There also is no direct evidence for receptor-mediated specific protein transport except for urease absorption.\textsuperscript{27} However, there have been a number of examples of
apparent non-specific transport across the insect digestive system of proteins not normally found in the diet\textsuperscript{20,28-29}, which argues against a receptor-mediated transfer system in insects. The evidence so far suggests that protein transport into the hemolymph occurs across the anterior midgut\textsuperscript{25}, but this is based on work from a single insect species. Proteins can move across the digestive system without modification of their primary or multimeric structure and with retention of their functional characteristics.\textsuperscript{20,29}

Jeffers and Roe\textsuperscript{20} reviewed the strategies used in insects to enhance protein movement across the digestive system of insects and/or to reduce xenobiotic metabolism. These approaches include the production of fusion proteins with lectins, the reduction of gut protease activity and the development of amphiphilic peptidic analogs. Research in this area in insects has been modest, especially as compared to similar efforts for drug development and for the discovery of new modes of action for protein insecticides. Most recently, Shen et al.\textsuperscript{30} conducted structure activity studies to show that optimized aliphatic-PEG conjugates to the mosquito peptidic hormone, trypsin modulating oostatic factor (TMOF), can greatly reduce \textit{in vitro} metabolism of the protein by a model protease. TMOF (Tyr-Asp-Pro-Ala-Pro\textsubscript{6}) is an insect hormone originally isolated from the ovaries of the yellow fever mosquito, \textit{Aedes aegypti} Linnaeus (Diptera: Culicidae), that regulates trypsin biosynthesis.\textsuperscript{32} The hormone binds to a TMOF receptor on the hemolymph side of the gut which reduces the level of the major digestive enzyme, trypsin.\textsuperscript{33} and when introduced orally can move across the digestive system into the hemolymph.\textsuperscript{34} TMOF or peptidic analogs of TMOF have been shown to inhibit trypsin biosynthesis in other medically important Diptera and Siphonaptera\textsuperscript{35} as well as agriculturally important species of Coleoptera\textsuperscript{36} and Lepidoptera.\textsuperscript{37}
Orally introduced TMOF reduces feeding in mosquito larvae which results in starvation, prevention of weight gain and death. The current study is to determine for the first time proof of concept that the conjugation of a model insecticidal peptide, an aliphatic PEG polymer of TMOF, can be used as a general method to enhance protein toxicity in insects.
2 MATERIALS AND METHODS

2.1 Synthesis of the aliphatic PEG polymer of TMOF-K (TMOF-K-PEG$_7$P)

TFA-TMOF-K (TFA = trifluoro acetyl; TMOF (YDPAP$_6$) = trypsin modulating oostatic factor) and TMOF were obtained from the American Peptide Company (Sunnyvale, CA). The monodisperse methyl(ethyleneglycol)$_7$O-propionyl-N-hydroxy- succinimide ester was purchased from Pierce Biotechnology, Inc. (Rockfold, IL).

The synthesis of TMOF-K-PEG$_7$P was described in detail before from our laboratories.$^{30}$ In brief, 2.5 moles of methyl(ethyleneglycol)$_7$O-propionyl-N-hydroxy-succinimide ester were added to a round-bottom flask charged with TFA-TMOF-K (50 mg/mL buffer) and carbonate buffer (0.1 M, pH 9, 1 mL). The resulting solution was stirred at room temperature and monitored by analytical RP-HPLC (220 nm). After HPLC analysis showed complete consumption of starting material (as indicated by disappearance of a peak at approximately 30 min), the product was purified by preparative RP-HPLC. The volume of the appropriate fractions of PEGylated TFA-TMOF-K were reduced to approximately 5 mL in vacuo at 35 ºC. To the concentrated solution, 50 molar equivalents of NaBH$_4$ were added. The resulting solution was stirred at room temperature and monitored by analytical RP-HPLC as mentioned earlier to confirm complete reaction (~ 8 h). Once HPLC analysis showed complete reaction, the sample was purified by preparative-scale RP-HPLC, lyophilized, reconstituted with deionized water, and stored at -80 ºC until needed for other studies. The molecular weights of the product were determined by ESI-MS at the Genomic Sciences Laboratory, North Carolina State University (Raleigh, NC).
2.2 Insects

Tobacco budworms, *Heliothis virescens* Fabricius (Lepidoptera: Noctuidae) and cotton bollworms, *Heliocoverpa zea* Boddie (Lepidoptera: Noctuidae) were obtained from the North Carolina State University (NCSU) Department of Entomology (Raleigh, NC, USA) and routinely reared on artificial diet. The budworm strain is HV97, which was established from field collections from tobacco plants in North Carolina in 1996 and 1997. The bollworms were collected from cotton plants in Plymouth, NC in 1996.

Yellow fever mosquitoes, *Aedes aegypti* Linnaeus (Diptera: Culicidae), and Asian tiger mosquitoes, *Aedes albopictus* Skuse (Diptera: Culicidae), were obtained from Dr. Charles Apperson (Department of Entomology, NCSU). The colonies were originally collected from the field in New Orleans, LA and reared in the laboratory at 27°C, 75% relative humidity, and 14:10 h (light:dark; included in the light phase are two 30 min crepuscular periods).

2.3 Mosquito assays

The median lethal concentrations (LC₅₀S) for TMOF, TMOF-K and TMOF-K-PEG₇P were estimated for larvae of *Ae. aegypti* and *Ae. albopictus* using a static 5-day bioassay modified from an existing protocol.³⁸ Day 1 first stadium larvae were transferred singly to individual wells of a 96-well microtiter plate (Falcon, Franklin Lakes, NJ, USA) in approximately 20 µL of sterile distilled water. All water was removed and 150 µL of a 0.15% solution of 1:2 of yeast (Brewers-debittered, inactive (ICN Biochemicals, Cleveland, OH):liver (MP Biomedicals, Solon, Ohio) in distilled water was immediately added to each well. Fifty µL
of TMOF, TMOF-K or TMOF-K-PEG7P in distilled water were then added to separate wells, so the desired final concentration of the test compounds were obtained in a total volume of 200 µL per well. Fifty µL of distilled water only was used to determine the control mortality. Other controls will be discussed in detail in the Results and Discussion. Microtiter plates were covered with plastic transparent lids and placed in sealed plastic containers. The floors of the plastic containers were covered with water before sealing to maintain high humidity and reduce evaporation from the microtiter plate wells. Larvae were kept at 27°C, 75% relative humidity, and 14:10 h (light:dark; included in the light phase are two 30 min crepuscular periods). Larval mortality (cessation of contraction of dorsal longitudinal muscles and failure to respond to a blunt probe in 10 sec) was monitored every 24 h for five days. In all LC50 estimations, treatments and controls were replicated three times with 10 insects per replicate.

2.4 Protein stability in the mosquito bioassay rearing water

One hundred and fifty µL of 0.15% solution of 1:2 of yeast:liver in distilled water was added to each well of a 96-well microtiter plate (plate 1). Then, 50 µL aliquots of the TMOF, TMOF-K or TMOF-K-PEG7P in distilled water was added to separate wells of this plate so the desired final concentration of the test compounds were obtained in a total volume of 200 µL per well. The control was 50 µL of distilled water only. As above, the plate was covered with a plastic transparent lid, placed in a sealed plastic container, and pre-incubated for 120 h in the absence of mosquito larvae under the environmental conditions as described earlier for the determination of LC50s. After this incubation, day 1 first stadium Ae. aegypti were
transferred to wells of a second 96-well microtiter plate (1 larva/well) in approximately 20 µL of sterile distilled water. All water was removed, and the incubated media from each well in plate 1 was transferred in toto to the corresponding wells in plate 2. The second plate was covered with a plastic transparent lid, placed in a sealed plastic container and incubated as described above. Larvae were monitored every 24 h for five days for mortality. These results were compared to the mortality data obtained for the determination of the LC50s for TMOF, TMOF-K or TMOF-K-PEG7P without pre-incubation.

2.5 Neonate lepidopteran feeding assay

Feeding assays were conducted using a 16-well feeding disruption test (FDT) plate described by Roe et al.39. In brief, each well has a 100 µL reservoir containing the same volume of a hydratable caterpillar diet positioned in the bottom center of a larger insect rearing chamber. The insect is confined to the rearing chamber at the well-top with an adhesive, vented lid. Before the insect is transferred to the FDT plate wells, the meal pad is hydrated from the top with 80 µL of distilled water with or without test materials dissolved in this water. The hydratable meal pad contains a blue dye which colors the feces, indicating that the larva has fed on the diet. Eighty µL of TMOF (4.8, 4.4, 4.0, 3.6, 3.2, 2.8, 2.4, 2.0 mM), TMOF-K (4.8, 4.4, 4.0, 3.6, 3.2, 2.8, 2.4, 2.0 mM) or TMOF-K-PEG7P (1.3, 1.2, 1.1, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4 mM) in distilled water was added to the top surface of the hydratable meal pad and the meal pad was incubated at room temperature for 30 min to completely rehydrate the diet. The controls were hydrated with distilled water only. After hydration, newly hatched *H. virescens* or *H. zea* larvae (<24 h post hatch) were placed in the wells of the plate using a
camel hair brush (1 larva per well). The plates were immediately covered with Bio-CV-16 perforated plastic covers (C-D International, Pittman, NJ) and incubated at 27 ± 1 °C and 60 ± 10% relative humidity (14:10 h, light:dark) for 4 d. The percent reduction in feeding compared to control larvae fed in FDT plates hydrated with water only was calculated. The amount of feeding was determined by counting the number of blue fecal pellets produced which has been shown before to be an index of the amount of feeding.39-40

2.6 Protein stability in hydrated meal pads assessed by bioassay
Mealpads hydrated with TMOF, TMOF-K or TMOF-K-PEG7P in distilled water were pre-incubated for 96 h (before the addition of insects) under the same conditions as described earlier for this assay type. After pre-incubation, newly hatched *H. virescens* larvae (<24 h post hatch) were placed in the wells of the FDT plate (1 larva per well). Then the plates were covered with plastic lids and incubated at 27 ± 1 °C and 60 ± 10% relative humidity (14:10 h, light:dark) for 4 d. The percent reduction in feeding from that of the control (with no TMOF, TMOF-K or TMOF-K-PEG7P) was compared to the percent reduced feeding without preincubation (described earlier) to determine if there was any reduction in the TMOF, TMOF-K or TMOF-K-PEG7P bioassay activity resulting from the preincubation.

2.7 Gut protein penetration in larval budworms
Solo cups (28 mL; Woodridge, IL, USA) containing approximately 2 mL of artificial diet41 were frozen at −80 °C for 48 h and then lyophilized on a Virtis Bench Top 6 freeze-dryer (Virtis, Gardiner, NY, USA; cold trap = −70 °C, ≈200 mTorr, ambient temperature = 23 °C)
for 48 h. The lyophilized diet was then divided by hand with a razor blade into 80–120 mg meal pads, which were stored in vacuum sealed PAKVF4-TE SipSeal packets containing Silica Gel SECA-PAX (Impak Corp., Los Angeles, CA) to remove any moisture.

Bioassays to examine penetration across the digestive system were conducted with *H. virescens* larvae that had completed the feeding stage of the 4th stadium. The larvae were removed from the diet and placed individually into 28 mL Solo cups without diet, and the cups sealed with a paper lid. The larvae were stored for 6 h under standard rearing conditions as described previously. After the starvation period, only larvae that had completed ecdysis to the fifth stadium as determined by the presence of a shed, fourth-stadium head capsule were used.

Next, lyophilized meal pads (80–120 mg) were placed into 28 mL plastic cups (1 meal pad per cup) and hydrated with 1.0 mg/mL solutions of TMOF, TMOF-K or TMOF-K-PEG7P in distilled water. The volume of water used was equivalent to that removed by lyophilization and based on the dry weight of each meal pad. Starved, fifth stadium, day 0 *H. virescens* larvae were transferred to the hydrated meal pads (one larva per cup), and the cups sealed with a paper lid. The larvae were kept under standard rearing conditions as described previously. The larvae were observed after 30 min, and those that had not initiated feeding were removed from the test. Larvae were observed again after 6 h, and those that had not consumed most of the meal pad were discarded.

After 6 h, larvae were removed from the diet. Any feces or diet on larvae was removed with a camel-hair brush. Each larva was then rinsed with a stream of 2–3 mL of phosphate buffer (Dulbecco’s phoshsate buffer, pH 7.4, 0.008 M sodium phosphate, 0.002 M...
potassium phosphate, 0.14 M sodium chloride, 0.01 M potassium chloride (Pierce, Rockford, IL, USA) with 0.05% Tween 20 (Fisher, Pittsburgh, PA, USA)) and blotted dry on a Kimwipe (Kimberly Clark, Roswell, GA, USA). Jeffers et al. found that this cleaning was adequate in removing any potential contaminates from the artificial diet that might absorb to the surface of the insect during feeding. Hemolymph was collected from a cut thoracic leg using scissors and transferred as a small drop to the surface of Parafilm (Parafilm, Menasha, WI, USA). The scissors were rinsed with buffer and dried with a Kimwipe between larvae to prevent cross-contamination. Five μL of the hemolymph from the Parafilm was aliquoted into 45 μL of cold distilled water (4 °C) in a 2.0 mL microcentrifuge tube and immediately vortexed. All samples were stored at −80 °C until assayed. Freezing or thawing had no effect on assay results.

Samples were analyzed using a Thermo LQT ion trap mass spectrometer and a synergi Hydro RP column of dimensions 150 x 1 mm (reversed phase C18 column), operating at a flow rate of 50 uL/min. The gradient used for the 30 min run is 0.1% trifluoroacetic acid, 0.05% heptafluorobutyric acid in water to the same ion-pairs in acetonitrile. Data are presented using Thermo Xcalibur 1.4 software.

2.8 Cuticle protein penetration in larval budworms

Five μL of a 5.0 mg/mL solution of TMOF, TMOF-K, and TMOF-K-PEG7P in DMSO was topically applied to the dorsum of the abdomen of fifth stadium, day 0 tobacco budworms (staged as described previously). Treated larvae were then transferred to a 28 mL Solo cup (1 larva per cup) without food. The larvae were incubated at standard rearing conditions as
described previously. After 4 h, larvae were removed and any feces on the surface of the larvae were removed with a camel-hair brush. Each larva was then rinsed with a stream of 2–3 mL of buffer and blotted dry with a Kimwipe as described earlier. Hemolymph was collected and diluted into 45 μL of cold distilled water as described before. All samples were stored at −80 °C until assayed. Samples were analyzed using LC-MS also as described previously for gut protein penetration in larval budworms.

2.9 Data analysis

Regression analysis was used to approximate the LC\textsubscript{50} and 95% confidence intervals for each compound in the mosquito bioassays by plotting probit mortality versus log dose\textsuperscript{42} using the SAS procedure PROC PROBIT\textsuperscript{43}. Similar methods were used to determine the effective concentration that reduced fecal production by 50% (FD\textsubscript{50}) by plotting probit percentage fecal reduction versus log dose. The variance-covariance matrix was estimated by the methods of Steel and Torrie\textsuperscript{44}. Calculations were made in Excel Spreadsheets.\textsuperscript{45} Analysis of variance (PROC ANOVA; SAS\textsuperscript{®} ver. 9.1, SAS Institute, Cary, NC) was used to determine if significant differences in \textit{Ae. aegypti} larval mortality existed between un-incubated water and water pre-incubated with TMOF, TMOF-K or TMOF-K-PEG\textsubscript{7}P for 120 h. The same methods were used to examine differences in fecal production for lepidopteran larvae provided un-incubated diet treated with TMOF, TMOF-K or TMOF-K-PEG\textsubscript{7}P and diet incubated with the same proteins for 96 h.
3 RESULTS AND DISCUSSION

3.1 Synthesis of TMOF-K (methyl(ethylene glycol)$_7$-O-propionyl)

The overall objective was to develop a chemically modified protein-based insecticide that increases the stability of the protein, potentially facilitates accumulation across the insect digestive system or cuticle into hemolymph, and/or enhances its biological activity. The strategy in the current paper includes the use of a hydrophilic polyethylene glycol (PEG) polymer that inhibits the degradation of peptides and proteins and a lipophilic moiety (alkyl groups) to potentially enhance the movement of TMOF across the digestive system and/or cuticle. As shown in Scheme 1, the relative hydrophilicity and lipophilicity can be controlled by manipulation of the length of the –OCH$_2$CH$_2$ (hydrophilic) and CH$_2$ (hydrophobic) portions by varying "n" and "m", respectively. We have previously reported that PEGylation of TMOF-K inhibits in vitro enzymatic digestion of TMOF by a model protease, leucine aminopeptidase. Having established proof of principle vis-à-vis protein protection by aliphatic PEG conjugation, the current study examines for the first time the potential impact of this polymeric system on the enhancement of insecticidal activity.

TMOF is a decapeptide of primary structure Y-D-P-A-P-P-P-P-P-P. It is possible to attach polymers at either the C-terminus (proline), the carboxyl group on the aspartic acid, and the N-terminus (tyrosine) (Scheme 2). However, maintaining the integrity of the N-terminus end of the peptide was suggested to be important for the retention of the larval mosquitocidal activity of TMOF (personal communication, Alan Brandt). As a result, we developed a synthetic strategy to place the PEG polymer at the proline terminus, the greatest
distance from the insecticidal motif (Scheme 3). Starting with TMOF that is protected at the amino terminus with TFA (trifluoroacetyl) in combination with the addition of a carboxyl terminal lysine to give TFA-TMOF-K (TFA-YDPAPPPPPPK), we synthesized a "PEGylated" TMOF-K. The lysine provides a free amino group on the C-terminus of TMOF, which allows site-specific conjugation of the polymers. In an effort to facilitate control over the polymer properties and to simplify chromatography and MS analyses, we selected a relatively low molecular weight, monodisperse polyethylene glycol with seven –OCH₂CH₂ repeat units. Further structure activity studies will be needed to better understand the importance of the balance between hydrophilicity and lipophilicity and sites of conjugation on insecticidal activity. The reaction of the activated PEG with the TFA-protected TMOF-K (shown in Scheme 3) produced the desired TFA-protected PEG-TMOF-K conjugate which appeared as a single peak on HPLC (Fig. 1B); the product structure was verified by MS (Fig. 2). Deprotection to remove the TFA group produced TMOF-K (methyl(ethyleneglycol)₇-O-propionyl), which also reveals a single peak by HPLC (Fig. 1C); the molecular ions for TMOF-K-PEG₇P are shown in Table 1. TMOF-K-PEG₇P for the bioassays that follow was purified in bulk by preparative HPLC.

3.2 Enhanced mosquitocidal activity of TMOF-K-PEG₇P

The median lethal concentration (LC₅₀) for TMOF, TMOF-K, and TMOF-K-PEG₇P was estimated for Day 1 first stadium Ae. aegypti using a static 5-day bioassay where the compound to be tested was added to larval rearing water. The following concentrations were used to estimate the Ae. aegypti LC₅₀s: for TMOF, 0.6, 0.5, 0.4 and 0.3 mM; for TMOF-K,
1.2, 1.0, 0.8, 0.6 and 0.4 mM; and for TMOF-K-PEG7P, 0.15, 0.125, 0.1, 0.075, and 0.05 mM. Ten mosquito larvae were used per treatment, and the assays were replicated three times. The LC50s were 0.46, 0.81 and 0.08 mM, respectively (Table 2). The addition of lysine to the C-terminus of TMOF significantly reduced the mosquitocidal activity relative to the parent TMOF by a factor of 1.8. However, the addition of (methyl(ethyleneglycol)-O-propionyl) to the C-terminal lysine of TMOF-K increased the activity by 10.1-fold over that of TMOF-K and 5.8-fold over that of TMOF.

For comparative studies, the median lethal concentration for TMOF, TMOF-K and TMOF-K-PEG7P was also estimated for Day 1 first stadium *Aedes albopictus*. The following concentrations were used to assess the LC50: for TMOF, 0.6, 0.5, 0.4 and 0.3 mM; for TMOF-K, 1.2, 1.0, 0.8, 0.6 and 0.4 mM; and for TMOF-K-PEG7P, 0.15, 0.125, 0.1, 0.075 and 0.05 mM. Again, ten mosquito larvae were used for each treatment, and the assays were replicated three times. The LC50s were 0.60, 1.23 and 0.18 mM, respectively (Table 3). As before for *Ae. aegypti*, the addition of the lysine to TMOF reduced the insecticidal activity by 2.1 fold. The addition of -methyl(ethyleneglycol)-O-propionyl) to TMOF-K increased the mosquitocidal activity over that of TMOF-K by 6.8-fold, and TMOF-K-PEG7P was 3.3-fold more active than TMOF.

Even though the compounds tested (Tables 2 and 3) were greater than 99% pure, a number of control experiments were conducted with both *Ae. aegypti* and *Ae. albopictus* to confirm that the enhanced mortality of TMOF-K-PEG7P was not due to minor impurities resulting from the synthesis or purification of the product. Three replicates with 10 insects per replicate were conducted for each species, sample and concentration tested. The
additions to the bioassay in 50 μL were conducted exactly as described before and which produced the results in Tables 2 and 3. The controls conducted were as follows: distilled water; polydisperse PEG-hexanoate (CH₃(OCH₂CH₂)₃-O-propionyl (0.05, 0.075, 0.1, 0.125 and 0.15 mM)); polydisperse PEG-hexanoate (CH₃(OCH₂CH₂)₃-O-propionyl bovine insulin (0.05, 0.075, 0.1, 0.125 and 0.15 mM) synthesized as shown in Schemes); TFA (0.025, 0.0375 and 0.05%, wt/vol final concentration in assay well); acetonitrile (0.025, 0.0375 and 0.05%, wt/vol final concentration in assay well); NaBH₄ (0.0625, 0.125 and 0.25 mg/mL final concentration in well); and HPLC product-free elution buffer for purification of TMOF-K-PEG₇P. In all of these experiments, no mortality was found at 120 h post-treatment, showing it was unlikely that the enhanced mortality noted for TMOF-K-PEG₇P in Tables 2 and 3 were the result of minor impurities from our synthesis or the solvents used in the purification of the product. The lack of insecticidal activity of polydisperse PEG-hexanoate CH₃(OCH₂CH₂)₃-O-propionyl bovine insulin demonstrates that the toxicity of TMOF-K-PEG₇P is not likely the result of the general PEGylation of a non-toxic protein but specific to the known mosquito mode of action of TMOF. We have shown before that simple mixing of PEG with proteins like bovine serum albumin (BSA) do not enhance the per os accumulation of the protein in lepidopteran hemolymph (Jeffers and Roe, unpublished) where BSA concentration is a function of the rate of absorption from the gut and metabolism and/or sequestration from the hemolymph.20,29-30

The increase in insecticidal activity over that of TMOF-K and even over that of TMOF by the addition of -(methyl(ethyleneglycol)₇-O-propionyl) provides the first proof of concept that aliphatic PEG polymers can be used to enhance the insecticidal activity of a
protein. There are a limited number of other approaches used in insects to improve the biological activity of proteins, i.e., the production of lectin fusion proteins, use of gut protease inhibitors and the development of amphiphilic peptidic analogs. The interesting aspect of the use of aliphatic PEG covalently bound to proteins is that it provides protection from protein degradation that was previously demonstrated for TMOF and at the same time has the potential of increasing the rate of movement of proteins across the digestive system. The strategy includes a hydrophilic PEG polymer that inhibits protease degradation with a lipophilic moiety (alkyl groups) to enhance movement across the digestive system as described before for non-insecticidal proteins (N. N. Ekwuribe in US Patents 5,359,030 (1994), 5,438,040 (1995), and 5,681,811 (1997); and Ekwuribe et al. in 6,191,105 (2001)).

TMOF is an insect hormone produced by the ovaries of adult Ae. aegypti and secreted into hemolymph where it regulates digestion. The hormone binds to a TMOF receptor on the hemolymph side of the gut reducing the level of the major digestive enzyme, trypsin. When TMOF is added to rearing water, mosquito larvae acquire the protein by feeding. TMOF moves across the digestive system epithelium, binds to its receptor on the hemolymph side of the gut, and starvation occurs due to reduced protease synthesis; the insect is unable to gain weight, and the larva eventually dies from starvation. As shown in Fig. 3, TMOF-K-PEG7P produced a similar effect to that of starvation in our bioassay system, while the fed control and the fed larvae treated with a non-toxic dose of TMOF-K consumed food and grew normally during the course of the 5 day assay. These results suggest that the mechanism of action of TMOF-K-PEG7P is the same as that for TMOF. If our hypothesis is
correct relative to the mode of action of TMOF-K-PEG₇P, then TMOF-K-PEG₇P and/or TMOF-K is binding to the TMOF receptor to elicit the lethal response.

Since the compounds tested were added to non-sterile rearing water that contained mosquitoes and food, studies were also conducted to examine the effect of a 5 day pre-incubation period in rearing water on mosquitocidal activity. The same static 5-day LC₅₀ bioassay was used to determine insecticidal activity for *Ae. aegypti* after the preincubation period. The following concentrations were used to estimate LC₅₀s: for TMOF, 0.3, 0.4, 0.5 and 0.6 mM; for TMOF-K, 0.4, 0.6, 0.8, 1.0 and 1.2 mM; and for TMOF-K-PEG₇, 0.05, 0.075, 0.1, 0.125 and 0.15 mM. Ten mosquito larvae were used for each treatment, and the assays were replicated twice. The LC₅₀s for TMOF, TMOF-K and TMOF-K-PEG₇P were not significantly different from the treatments without the pre-incubation period (Table 2), suggesting that the compounds in the bioassay were not affected by rearing water alone. The interaction of mosquito larvae with the rearing water and this effect on toxicity was not evaluated.

TMOF, TMOF-K and TMOF-K-PEG₇P may exist in the rearing water either in solution, as a suspension and/or bound to the mosquito food. There are two possible routes of entry into the mosquito from the rearing water, either by movement across the cuticle and/or by movement across the digestive system. In studies discussed later, no movement of TMOF-K-PEG₇P was detected across the cuticle when the compound was topically applied to larvae of the tobacco budworm; whether this is also the case for our 5 day, static mosquito bioassay is not known. If entry into mosquito larvae was by feeding, the increased activity associated with PEGylation could be attributed to (i) increased uptake by feeding, (ii)
decreased degradation of TMOF-K-PEG7P in the digestive system and/or hemolymph, (iii)
enhanced movement across the digestive system into the mosquito hemocoel, (iv) improved
targeting of the gut TMOF receptors, (v) enhanced receptor activation, and (vi) a
combination of these effects. Although our previous work\textsuperscript{30} showed that PEGylation of
TMOF can greatly reduce protease degradation \textit{in vitro} using leucine aminopeptidase,
whether this occurs in an insect system has not yet been examined.

3.3 Enhanced larval lepidopteran activity of TMOF-K-PEG7P

In addition to mosquitoes, TMOF or peptidic analogs of TMOF also inhibit trypsin
biosynthesis in other medically important Diptera and Siphonaptera\textsuperscript{35} as well as agriculturally
important species in the Coleoptera\textsuperscript{36} and Lepidoptera.\textsuperscript{37} With this in mind, further studies
were conducted to determine whether PEGylation could enhance the insecticidal activity of
TMOF in neonate larvae of \textit{H. virescens}. These studies were conducted by the addition of
the compound to be tested dissolved in distilled water to 100 µL hydrateable, lepidopteran
meal pads (containing a blue indicator dye) in 16-well FDT plates; these plates were
originally developed for insecticide resistance monitoring.\textsuperscript{39} The effect of the test compound
on feeding is measured by counting the number of blue fecal pellets as an index of food
consumption after incubation of neonates in the plates for 4 d. The dose for a 50% reduction
in feeding (feeding dose, FD\textsubscript{50}) was estimated with the following concentrations of test
material: for TMOF and TMOF-K, 2.0, 2.4, 2.8, 3.2, 3.6, 4.0, 4.4 and 4.8 mM and for
TMOF-K-PEG7P, 2.0, 2.4, 2.8, 3.2, 3.6, 4.0, 4.4 and 4.8 mM. Four larvae were used for each
treatment, and the assays were replicated three times. The FD\textsubscript{50} values for TMOF, TMOF-K
and PEGylated TMOF-K of *H. virescens* were 4.57, 4.49 and 1.10 mM, respectively. PEGylation of TMOF-K reduced the FD₅₀, 4.1 fold. The FD₅₀ for TMOF, TMOF-K and TMOF-K-PEG₇P was also estimated for neonates of the bollworm using the same concentrations as described for *H. virescens*. Four larvae were used for each treatment, and the assays were replicated three times. The FD₅₀s for TMOF, TMOF-K and TMOF-K-PEG₇P were 4.62, 4.72 and 1.13 mM, respectively (Table 5), a 4.2 fold reduction in the FD₅₀ upon PEGylation of TMOF-K.

In our mosquito bioassays, the addition of lysine to TMOF decreased its mosquitocidal activity. However, in the studies with the budworm and bollworm, the addition of lysine to the C-terminus of TMOF resulted in no significant difference between the FD₅₀ for that of TMOF and TMOF-K; in this case, the end point being measured was reduced feeding as determined by reduced fecal production. The PEGylation of TMOF-K to produce TMOF-K-PEG₇P increased the activity of the hormone by approximately 4-fold for the budworm and bollworm, providing additional evidence that this approach can be used to enhance protein activity in both larval mosquitoes and caterpillars.

In Nauen et al.³⁷, injections of TMOF into third instars of the tobacco budworm inhibited 50% of the gut trypsin biosynthesis at 0.42 µM. They also looked at other TMOF analogs, i.e., YDPAP, FDPAP, DPAP and DPA (but not TMOF-K), and found them to be less active than TMOF with 50% reduction occurring at concentrations in the range of 46 to 190 µM. These researchers concluded that a TMOF-like hormone may be present in the budworm to regulate protein digestion; they found a factor with TMOF like activity in the hemolymph, but to date the structure of the hormone has not yet been identified.
We have shown before that proteins like bovine insulin and anti-bovine insulin antibody are stable in our hydrated meal pads. In order to determine the same for TMOF and our TMOF analogs, the test compounds were pre-incubated in the hydrated meals pads for 5 d prior to conducting the 4-day FD$_{50}$ bioassay with tobacco budworm neonates, under the same environmental conditions for both the preincubation and incubation. The following concentrations were used: for TMOF and TMOF-K, 2.0, 2.4, 2.8, 3.2, 3.6, 4.0, 4.4 and 4.8 mM and for TMOF-K-PEG$_7$P, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2 and 1.3 mM. Four larvae were used for each treatment, and the assays were replicated two times. The FD$_{50}$s for TMOF, TMOF-K and TMOF-K-PEG$_7$P were 4.51, 4.55, and 1.11 mM, respectively (Table 4). The lack of statistically significant differences in the FD$_{50}$s for the preincubation versus no preincubation for TMOF, TMOF-K and TMOF-K-PEG$_7$P (Table 4) suggests that the compounds were stable in the meal pads during the course of the bioassay.

3.4 Movement of TMOF-K-PEG$_7$P across the larval lepidopteran digestive system and cuticle

We have shown in this paper that the addition of -(methyl(ethyleneglycol)$_7$-O-propionyl) to the lysine on TMOF-K increases the mosquitocidal activity of TMOF-K and improves TMOF-K inhibition of neonate lepidopteran feeding as measured by reduced fecal production. In the neonate bioassay that was conducted, not only do the larvae feed on the diet containing TMOF-K-PEG$_7$P, but they can also crawl on the diet surface. Therefore, in both the mosquito bioassays and the neonate, lepidopteran feeding bioassay, one potential route of movement of TMOF-K-PEG$_7$P into hemolymph is across the cuticle. Jeffers and
Roe (unpublished) have found that topically applied aliphatic PEGylated bovine insulin in DMSO appears in the hemolymph of budworm larvae while bovine insulin alone does not. To investigate whether this occurs for TMOF-K-PEG7P, 5 μL of a 5.0 mg/mL solution of the compound in DMSO was topically applied to the dorsum of the abdomen of fifth stadium, day 0 tobacco budworms. After 4 h, hemolymph was collected from a cut thoracic leg taking special precautions to prevent any contamination of the sample from TMOF-K-PEG7P that might be on the surface of the cuticle. Fig. 4 is the full ms scan from 420.00 to 1500.00 of this hemolymph. No molecular ions were found corresponding to TMOF-K-PEG7P or TMOF-K (=(M+2H)²⁺ of 588.63). These results suggest that the movement of TMOF-K-PEG7P into the hemolymph probably does not occur across the cuticle in the neonate lepidopteran feeding studies.

As discussed before, consumption of TMOF-K-PEG7P by mosquitoes and now for caterpillars could result in the appearance of TMOF-K-PEG7P and/or TMOF-K in the hemolymph. Insect hemolymph has high esterase activity, and the amide ester in TMOF-K-PEG7P should be susceptible to enzymatic nucleophilic attack on the carbonyl by esterases as one example. This would result in the production of TMOF-K. Fifth stadium, day 0 budworm larvae were allowed to feed on lyophilized meal pads rehydrated with a 1.0 mg/mL solution of PEGylated TMOF-K. After 6 h, hemolymph was collected from a cut thoracic leg, again being careful not to contaminate the sample with potential test compound on the cuticle surface. The spectrum in Fig. 5 shows the identical LC-MS/MS from hemolymph to that of an authentic TMOF-K standard (not shown). TMOF-K-PEG7P was not found in the hemolymph. Three explanations are possible then for the effect of PEGylation on the FD₅₀s
in *H. virescens* (Table 4) and *H. zea* (Table 5): (i) aliphatic PEGylation is enhancing the accumulation of TMOF-K in the hemolymph, (ii) (methyl(ethyleneglycol)$_7$-O-propionyl) released from TMOF-K-PEG$_7$P produces the change in the FD$_{50}$, and (iii) a combination of (i) and (ii). At least in the mosquito bioassays discussed earlier, we found no evidence that aliphatic-PEG or starting materials for the synthesis of TMOF-K-PEG$_7$P were toxic.

In summary, proof of concept was demonstrated that the aliphatic PEGylation of a peptidic insecticide, TMOF-K, increased its insecticidal activity and suppression of growth in two species of larval mosquitoes when added to rearing water and increased its suppression of feeding on neonates of two lepidopteran species when added to artificial diet. The route of entry of TMOF-K-PEG$_7$P in these studies is likely across the digestive system as appears to be the case for studies conducted with the tobacco budworm. Also, free TMOF-K was found in the hemolymph of the tobacco budworm and not TMOF-K-PEG$_7$P, which suggest that the insect has a mechanism for regenerating the peptide from the PEG-protein polymer. TMOF-K-PEG$_7$P was shown before to inhibit protease degradation of TMOF-K *in vitro* by leucine aminopeptidase.$^{30}$ The exact mechanism for the increased insecticidal activity *in vivo* is not clear but must involve either an enhanced accumulation of the active in the hemolymph, increased delivery to the target and/or an enhancement of the response after binding to the receptor. This research suggests that aliphatic PEG polymers can be used as a new method for increasing the insecticidal activity of proteins.
ACKNOWLEDGEMENTS

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REFERENCES


Scheme 1. Protein conjugated with polyethylene glycol via the methylene chain provides control over the hydrophilic/lipophilic balance.
Scheme 2. Polymers to function as delivery systems can potentially be attached to TMOF at C-terminus or N-terminus.
Table 1. ESI-MS results of TMOF conjugates

<table>
<thead>
<tr>
<th>TMOF conjugates</th>
<th>Molecular Ion Peaks</th>
<th>ESI-MS m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFA-TMOF-K-PEG&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>785.36 (M+2H)&lt;sup&gt;2+&lt;/sup&gt;</td>
</tr>
<tr>
<td>TMOF-K-PEG</td>
<td></td>
<td>748.2 (M+2H)&lt;sup&gt;2+&lt;/sup&gt;, 762.3 (M+2NH&lt;sub&gt;4&lt;/sub&gt;)&lt;sup&gt;2+&lt;/sup&gt;, 1489.4 (M+H)&lt;sup&gt;+&lt;/sup&gt;, 1506.4 (M+NH&lt;sub&gt;4&lt;/sub&gt;)&lt;sup&gt;+&lt;/sup&gt;</td>
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<sup>a</sup> PEG = {methyl(ethyleneglycol)<sub>7</sub>-O-propionyl}
Table 2. Median lethal concentration for *Aedes aegypti* treated with TMOF, TMOF-K or TMOF-K-PEG7P without and with pre-incubation

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>LC50 (mM)</th>
<th>95% FL (mM)</th>
<th>χ²</th>
<th>Slope ± SE</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aedes aegypti</em></td>
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<tr>
<td>TMOF</td>
<td>120</td>
<td>0.46 A, a</td>
<td>0.43 – 0.50</td>
<td>36.26</td>
<td>7.71 ± 0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>TMOF-K</td>
<td>90</td>
<td>0.81 B, a</td>
<td>0.66 – 1.08</td>
<td>6.72</td>
<td>3.91 ± 0.19</td>
<td>0.99</td>
</tr>
<tr>
<td>TMOF-K-PEG7P</td>
<td>120</td>
<td>0.08 C, a</td>
<td>0.07 – 0.10</td>
<td>20.08</td>
<td>3.82 ± 0.71</td>
<td>0.99</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (pre-incubated diet)</td>
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<tr>
<td>TMOF</td>
<td>120</td>
<td>0.45 A, a</td>
<td>0.42 – 0.49</td>
<td>38.66</td>
<td>8.13 ± 0.86</td>
<td>0.99</td>
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<tr>
<td>TMOF-K</td>
<td>90</td>
<td>0.81 B, a</td>
<td>0.69 – 0.97</td>
<td>9.60</td>
<td>4.76 ± 0.15</td>
<td>0.99</td>
</tr>
<tr>
<td>TMOF-K-PEG7P</td>
<td>150</td>
<td>0.08 C, a</td>
<td>0.05 – 0.11</td>
<td>18.57</td>
<td>8.15 ± 2.74</td>
<td>0.99</td>
</tr>
</tbody>
</table>

a Number of larvae per compound  

b Median lethal concentration from probit analysis (SAS® ver. 9.1, SAS Institute, Cary, NC)  
c Fiducial limits from probit analysis (SAS® ver. 9.1, SAS Institute, Cary, NC)  
d χ², Wald chi-square value from probit analysis (SAS® ver. 9.1, SAS Institute, Cary, NC)  
e Slope and standard error of the regression line from probit analysis  
f LC50 values followed by a different uppercase letter differ in significance based on Fiducial limits  
g LC50 values followed by a different lowercase letter differ in significance for the same protein in un-incubated diet and diet pre-incubated with the protein (SAS® ver. 9.1, SAS Institute, Cary, NC)
### Table 3. Median lethal concentration for *Aedes albopictus* treated with TMOF, TMOF-K or TMOF-K-PEG7P

<table>
<thead>
<tr>
<th>Compound</th>
<th>n&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; (mM)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>95% FL&lt;sup&gt;c&lt;/sup&gt; (mM)</th>
<th>χ&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Slope ± SE&lt;sup&gt;e&lt;/sup&gt;</th>
<th>r&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td><em>Aedes albopictus</em></td>
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<td></td>
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<tr>
<td>TMOF</td>
<td>180</td>
<td>0.60 A</td>
<td>0.56 – 0.62</td>
<td>57.08</td>
<td>13.13 ± 1.08</td>
<td>0.94</td>
</tr>
<tr>
<td>TMOF-K</td>
<td>180</td>
<td>1.23 B</td>
<td>1.17 – 1.29</td>
<td>50.56</td>
<td>21.79 ± 3.69</td>
<td>0.92</td>
</tr>
<tr>
<td>TMOF-K-PEG&lt;sub&gt;7&lt;/sub&gt;P</td>
<td>180</td>
<td>0.18 C</td>
<td>0.16 – 0.19</td>
<td>26.88</td>
<td>17.58 ± 2.16</td>
<td>0.97</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of larvae per compound

<sup>b</sup> Median lethal concentration from probit analysis (SAS® ver. 9.1, SAS Institute, Cary, NC)

<sup>c</sup> Fiducial limits from probit analysis (SAS® ver. 9.1, SAS Institute, Cary, NC)

<sup>d</sup> χ<sup>2</sup>, Wald chi-square value from probit analysis (SAS® ver. 9.1, SAS Institute, Cary, NC)

<sup>e</sup> Slope and standard error of the regression line from probit analysis

<sup>f</sup> LC<sub>50</sub> values followed by a different uppercase letter differ in significance based on Fiducial limits
Table 4. Median effective concentration of diet treated with TMOF, TMOF-K or TMOF-K-PEG7P and diet pre-incubated with the same proteins for 96 h for *Heliothis virescens*

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>FD50 (mM)</th>
<th>95% FL (mM)</th>
<th>$\chi^2$</th>
<th>Slope ± SE</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Heliothis virescens</em></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TMOF</td>
<td>60</td>
<td>4.57 A, a</td>
<td>4.38 – 4.86</td>
<td>52.50</td>
<td>7.27 ± 0.60</td>
<td>0.96</td>
</tr>
<tr>
<td>TMOF-K</td>
<td>72</td>
<td>4.49 A, a</td>
<td>4.20 – 5.09</td>
<td>34.33</td>
<td>7.14 ± 0.32</td>
<td>0.98</td>
</tr>
<tr>
<td>TMOFK-PEG7P</td>
<td>84</td>
<td>1.10 B, a</td>
<td>1.05 – 1.17</td>
<td>100.24</td>
<td>4.78 ± 0.43</td>
<td>0.91</td>
</tr>
<tr>
<td><em>(pre-incubated diet)</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMOF</td>
<td>60</td>
<td>4.51 A, a</td>
<td>4.34 – 4.75</td>
<td>62.05</td>
<td>8.07 ± 0.72</td>
<td>0.97</td>
</tr>
<tr>
<td>TMOF-K</td>
<td>72</td>
<td>4.55 A, a</td>
<td>4.36 – 4.82</td>
<td>82.71</td>
<td>7.04 ± 0.28</td>
<td>0.98</td>
</tr>
<tr>
<td>TMOFK-PEG7P</td>
<td>108</td>
<td>1.11 B, a</td>
<td>1.05 – 1.18</td>
<td>125.47</td>
<td>4.20 ± 0.31</td>
<td>0.96</td>
</tr>
</tbody>
</table>

a Number of larvae per compound
b Median concentration for 50% reduction in fecal production from probit analysis (SAS® ver. 9.1, SAS Institute, Cary, NC)
c Fiducial limits from probit analysis (SAS® ver. 9.1, SAS Institute, Cary, NC)
d $\chi^2$, Wald chi-square value from probit analysis (SAS® ver. 9.1, SAS Institute, Cary, NC)
e Slope and standard error of the regression line from probit analysis
f LC50 values followed by a different uppercase letter differ in significance based on Fiducial limits
g LC50 values followed by a different lowercase letter differ in significance for the same protein in un-incubated diet and diet pre-incubated with the protein (SAS® ver. 9.1, SAS Institute, Cary, NC)
Table 5. Median effective concentration of diet treated with TMOF, TMOF-K or TMOF-K-PEG7P for 96 h for *Helicoverpa zea*

<table>
<thead>
<tr>
<th>Compound</th>
<th>n(^a)</th>
<th>FD(_{50}) (mM)(^b)</th>
<th>95% FL(^c) (mM)</th>
<th>(\chi^2)(^d)</th>
<th>Slope ± SE(^e)</th>
<th>r(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Helicoverpa zea</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMOF</td>
<td>60</td>
<td>4.62 A(^f)</td>
<td>4.42 – 4.94</td>
<td>50.47</td>
<td>7.32 ± 0.39</td>
<td>0.98</td>
</tr>
<tr>
<td>TMOF-K</td>
<td>72</td>
<td>4.72 A</td>
<td>4.48 – 5.09</td>
<td>68.62</td>
<td>6.29 ± 0.76</td>
<td>0.95</td>
</tr>
<tr>
<td>TMOF-K-PEG7P</td>
<td>96</td>
<td>1.13 B</td>
<td>1.07 – 1.21</td>
<td>94.15</td>
<td>4.63 ± 0.43</td>
<td>0.91</td>
</tr>
</tbody>
</table>

\(^a\) Number of larvae per compound  
\(^b\) Median concentration for 50% reduction in fecal production from probit analysis (SAS\(^\circledR\) ver. 9.1, SAS Institute, Cary, NC)  
\(^c\) Fiducial limits from probit analysis (SAS\(^\circledR\) ver. 9.1, SAS Institute, Cary, NC)  
\(^d\) \(\chi^2\), Wald chi-square value from probit analysis (SAS\(^\circledR\) ver. 9.1, SAS Institute, Cary, NC)  
\(^e\) Slope and standard error of the regression line from probit analysis  
\(^f\) LC\(_{50}\) values followed by a different uppercase letter differ in significance based on Fiducial limits
Figure 1. The reaction stoichiometry and reaction times were optimized for 100% product formation. Optimization was verified by HPLC as shown.
Figure 2. Mass spectrometry of the anticipated peaks for the TFA-protected dicationic product \( \{(M+2H)^{2+}\} \) where \( M = \text{TFA-TMOF-K (methyl(ethyleneglycol)7-O-propionyl)} \) shown in Scheme 3.
Figure 3. Yellow fever mosquito, *Aedes aegypti*, larvae on day 4 of the 5-day LC\textsubscript{50} bioassay. A, fed control; B, fed and treated with 0.08 mM TMOF-K; C, fed and treated with 0.08 mM TMOF-K-PEG\textsubscript{7}P; and D, starved.
Figure 4. Molecular ions in the range of 420.00 to 1500.00 from hemolymph of tobacco budworms treated topically with TMOF-K-PEG7P in DMSO.
Figure 5. LC-MS/MS for TMOF-K found in the hemolymph of *Heliothis virescens* larvae fed on artificial diet containing TMOF-K-PEG₇P.
Novel System for the Stabilization and Delivery of Proteins to the Insect Hemocoel through Conjugation with Aliphatic Polyethylene Glycol

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ABSTRACT

Co-feeding of aliphatic polyethylene glycol (PEG), phospholipase A2, anionic and ionic detergents, and amphipathic glycoside with bovine serum albumin (BSA) as a model protein to 4th stadium tobacco budworms, Heliothis virescens, did not affect the levels of BSA in the hemolymph. Conjugation of the decapetide, TMOF (trypsin modulating oostatic factor), to polyethylene glycol was previously shown to protect the peptide from protease attack and enhance its insecticidal effects against Aedes aegypti and Aedes albopictus. The chemistry for the synthesis of polydispersed aliphatic PEG350-insulin and monodispersed aliphatic PEG333-insulin are described herein, a larger protein than TMOF. When PEGylated insulins or insulin were fed in artificial diet to 5th stadium Heliothis virescens, greater amounts of insulin from the former were found in the hemolymph. The PEGylation of insulin yields a 6.7 and 7.3 fold increases in appearance of insulin in the hemolymph of Heliothis virescens larvae for the PEG350 and PEG333 chemistries, respectively. When insulin is topically applied to the dorsum of H. virescens, no insulin is found in the hemolymph. However, after topical application of the PEGylated insulins, PEG350-insulin and PEG333-insulin was detected in the hemolymph. After injections of the insulin species into the hemocoel of 4th stadium H.virescens, insulin is completely cleared from the hemolymph in 120 minutes. In comparison, PEG350-insulin and PEG333-insulin were still present in the hemolymph 300 and 240 minutes after injection, respectively, translating to a 3.3 and 2.7 fold increases in the length of time insulin remains in the hemolymph after injection.
Keywords: insulin, *Heliothis virescens*, PEGylation, polyethylene glycol, protein delivery
INTRODUCTION

The development of the delta-endotoxin of *Bacillus thuringiensis* (*Bt*) changed our thinking about the use of proteins for insect control. Unfortunately, despite the numerous proteins exhibiting insecticidal properties identified in the last few decades, there have been few commercially-successful protein insecticides. Traditionally, insecticides, such as organochlorines, organophosphates, and pyrethroids, are lipophilic compounds, easily moving across the insect cuticle and digestive system. However, oral delivery of insecticidal proteins is difficult due to protease degradation in the digestive system and limited movement across the gut epithelium. The success of *Bt* is due to the location of the site of action on the midgut epithelium; the toxin binds to the site disrupting digestion and eventually causing death (Gill et al., 1992; Fischhoff, 1996).

Despite the inhospitable conditions in the insect gut, the movement of ingested, intact proteins across the digestive system into the hemolymph has been reported in several blood-feeding and non-blood feeding insects (Jeffers and Roe, 2008). However, the reason for this phenomenon is not known. In mammals, the transfer of intact macromolecules across the digestive system is well documented and has been discussed in several reviews (Gardner, 1988; Weiner, 1988; Pácha, 2000). This mechanism is important during early post-natal development, allowing the absorption of antigens, immunoglobulins, and growth factors from maternal colostrum and milk. The transport follows two pathways: specific-receptor mediated and non-specific transcytosis (Pácha, 2000).
It is unclear why and how proteins move intact across the insect digestive system. Nogge and Giannetti (1979) reported that intact human albumin appeared in the hemolymph of Glossina morsitans after feeding on human blood and speculated since blood is a poor energy source, human albumin absorption was a method of energy conservation. Similarly, Kurahashi et al. (2005) reported that mulberry leaf urease move unchanged across the gut and into the hemolymph of Bombyx mori. The authors suggested that B. mori selectively transported the plant urease because it was important for hemolymph nitrogen metabolism.

There are two possible mechanisms for the movement of intact proteins across the insect gut: movement through the ventricular epithelial cells and movement through the intercellular spaces of the midgut (reviewed by Jeffers and Roe, 2008). Only a small percentage of dietary proteins move unchanged from the diet to hemolymph; Jeffers et al. (2005), for example, reported ratios of 1:500 and 1:300 for bovine serum albumin and anti-BSA. Enhancement of this movement or stabilization of proteins in the hemocoel could potentially be used to increase the toxicity of insect toxins in the diet. Jeffers and Roe (2008) reviewed several approaches to enhance protein movement across the insect digestive system including lectin fusion proteins, reduction of gut protease activity, and protein lipophilic-polyethylene glycol (PEG) polymers.

Lectins bind to sugar complexes attached to lipids and proteins (Bies et al., 2004). It has been reported that dietary snowdrop and jackbean lectins accumulate in the gut, hemolymph and malpighian tubules of Lacanobia oleracea after feeding (Fitches and Gatehouse, 1998;
Fitches et al. 2001a; Fitches et al., 2001b). Similarly, snowdrop lectin accumulated in the hemolymph, fat body, and ovarioles of *Nilaparvata lugens* after feeding (Powell et al., 1998). This mechanism was exploited when researchers used mannose-specific snowdrop lectin fusion proteins to deliver allatostatin (Fitches et al., 2002), an insecticidal spider venom toxin (Fitches et al., 2004; Down et al., 2006), and a lepidopteran-specific scorpion toxin (Trung et al., 2006) to the insect hemocoel.

Lipke et al. (1954) provided the first descriptions of the soybean protease inhibitor and its effects on *Tribolium confusum*. Since then, researchers have examined the use of protease inhibitors for insect control. However, Murdock and Shade (2002) discussed the many reasons why this approach will not work. Apparently, insects use multiple enzymes to digest proteins. When one is inhibited, the insect compensates by increasing the expression of others. Also, most crops have multiple insect pests; it will be difficult to find a single protease inhibitor with broad spectrum activity to many different insect species, sometimes from different families and even orders. Finally, to achieve insect control by protease inhibition, the concentration of inhibitor must be high, approximately 1% of the soluble plant protein; this would be difficult to achieve using plant transgenesis.

PEGylation, the process of covalently attaching polyethylene glycol (PEG) polymer chains to another molecule, has been used in the pharmaceutical industry for decades to deliver proteins across the digestive system of humans (Zalipsky and Harris, 1997; Veronese, 2001; Roberts et al., 2002; Harris and Veronese, 2002; Yowell and Blackwell, 2002). To determine
if this system could be used in insects, Jeffers et al. (2011) covalently bound a PEG polymer to the decapeptide, trypsin modulating oostatic factor (TMOF), to determine if this would increase its insecticidal activity. TMOF (Tyr-Asp-Pro-Ala-Pro₆) originally isolated from the ovaries of the adult yellow fever mosquito, *Aedes aegypti*, regulates trypsin biosynthesis (Borovsky, 2003). The addition of trifluoro acetyl and lysine to TMOF, yielding TFA-TMOF-K, allows the specific conjugation of monodispersed, aliphatic PEG (mw 509) to the amino group of lysine. The median lethal concentration (LC₅₀) for *A. aegypti* (*per os*) for unaltered TMOF is 0.46 mM. By adding the lysine that allows the conjugation to PEG, the activity decreases (LC₅₀ 0.81 mM). However, the TMOF-K-PEG509 demonstrated increased activity 5.8 and 10.1 fold above that of TMOF and TMOF-K (Jeffers et al., 2011). Similarly, Shen et al. (2009) demonstrated that the conjugation of TMOF-K to PEG polymers inhibited its degradation by the digestive enzyme, leucine aminopeptidase. Using structure activity studies, Shen et al. (2009) confirmed a correlation between PEG molecular weight and reduced protease degradation.

In this study, two PEGylated insulins, PEG350-insulin and PEG333-insulin, were synthesized to determine if PEGylation could reduce the degradation rate of a larger protein than TMOF and also enhance its accumulation from the diet or cuticle into the insect hemocoel. As insulin does not possess any insecticidal effects (at the concentrations in this study), the authors were able to examine treatment effects in the absence of insect toxic activity. Similar to PEGylated TMOF studied before (Jeffers et al., 2011), the poly (ethylene glycol) moiety in PEG333-insulin is monodisperse. A second PEGylated insulin, PEG350-
insulin, was synthesized using a mixture of PEGs with an average molecular weight of 350 (polydisperse), resulting in a mixture of conjugates with different molecular weight oligomers attached to insulin. Unlike TFA-TMOF-K where there is only 1 attachment site (per molecule) for the activated PEG to covalently bind, insulin is comprised of numerous amino acids containing free amines. Fortunately, the quaternary structure of insulin shields most of these sites from conjugation with PEG. However, there are 3 accessible sites, an asparagine at the carboxy terminus of the alpha chain, a phenylalanine at the amino terminus of the beta chain, and a lysine at the penultimate position at the carboxy terminus of the beta chain. Depending on the stoichiometry of the reaction, the placement and the number of the PEG polymers on the insulin can be altered.
MATERIALS AND METHODS

Insects

Tobacco budworms, *Heliothis virescens* Fabricius (Lepidoptera: Noctuidae), cotton bollworms, *Heliocoverpa zea* Boddie (Lepidoptera: Noctuidae) and tobacco hornworm, *Manduca sexta* Linneaus (Lepidoptera: Sphingidae) were obtained from the North Carolina State University (NCSU) Department of Entomology insectary (Raleigh, NC, USA). The *H. virescens* strain is HV97, which was established from field collections from tobacco plants in North Carolina in 1996 and 1997. The *H. zea* were obtained from cotton plants in Plymouth, NC in 1996. The *H. virescens* and *H. zea* strains were reared in the laboratory on artificial diet since their collection from the field. The *M. sexta* are the original Yamamoto (1969) strain and were reared on artificial diet since their arrival at the NCSU insectary.

Preparation of Larval Lepidoptera Feeding Assay Diet

Solo cups containing approximately 2 ml of artificial diet (Burton, 1970) were frozen at $-80^\circ$C for 48 h and then lyophilized on a Virtis Bench Top 6 freeze-dryer (Virtis, Gardiner, NY, USA; cold trap = $-70^\circ$C, ≈200 mTorr, ambient temperature = 23 $^\circ$C) for 48 h (Roe et al., 1999; Roe et al., 2000; Bailey et al., 2001). The lyophilized diet was then divided with a razor blade into 80–120 mg meal pads which were stored in the dark at room temperature and 0% humidity until needed.
Stability of Proteins in Mealpads

The stability of BSA in our artificial diet was shown before (Jeffers et al., 2005). For insulin, mealpads were hydrated with insulin in distilled water and incubated for 0 and 24 hours under the same conditions as described for the feeding bioassay. After incubation, the diet was homogenized in PBS for 10 seconds (4 °C) at full speed with a polytron PT10/35 homogenizer with PTA 10 generator (Brinkmann, Westbury, NY). The generator probe was rinsed at full speed in PBS between samples to prevent cross-contamination. A 500 μl aliquot of the homogenate was centrifuged at 960 g (4 °C) for 1 minute. Fifty μl of the supernatant was added to 450 μl of PBS at 4 °C and stored at -80 °C until assayed.

Larval Lepidoptera Feeding Assay

H. virescens were obtained as fourth instars on artificial diet (Burton, 1970) in 28 ml plastic cups (Solo, Woodridge, IL, USA). After completing the feeding stage in the fourth stadium, larvae were removed from the diet and placed individually into 28 ml plastic cups without diet. The cups were sealed with a paper lid. The larvae were stored under standard Lepidoptera rearing conditions as described previously for six hours. After the starvation period, only larvae that had completed ecdysis to the fifth stadium as determined by the presence of a shed, fourth-stadium head capsule were used in the studies that follow.
Lyophilized meal pads were placed into 28 ml plastic cups and hydrated with the test solution. Fifth stadium, day 0 *H. virescens* larvae were transferred to the hydrated meal pads (one larva per cup), and the cups sealed with a paper lid. The larvae were kept under standard Lepidoptera rearing conditions as described previously. The larvae were observed after 30 min, and those that had not begun feeding were removed from the test. Larvae were observed again after 6 hours, and those that had not consumed most of the meal pad were discarded.

After 6 hours, larvae were removed from the diet. Any fras or diet on larvae was removed with a camel-hair brush. Each larva was then rinsed with a stream of 2–3 ml of PBST [PBS (Dulbecco’s phosphate buffer solution, pH 7.4, 0.008 M sodium phosphate, 0.002 M potassium phosphate, 0.014 sodium chloride, 0.01 M potassium chloride; Pierce, Rockford, IL) with 0.05% Tween 20 (Fisher, Pittsburgh, PA)] and blotted dry on a Kimwipe (Kimberly Clark, Roswell, GA). Hemolymph was collected from a cut thoracic leg using scissors and transferred as a small drop to the surface of Parafilm (Parafilm, Menasha, WI). The scissors were rinsed with PBST and dried with a Kimwipe between larvae to prevent cross-contamination. Using a 10 μl pipettman (Rainin, Emeryville, CA), 5.0 μl of the hemolymph from the Parafilm was aliquoted into 45 μl of distilled water (4 °C) in a 2.0 ml microcentrifuge tube and immediately vortexed. All samples were stored at −80 °C until assayed. Freezing or thawing had no effect on assay results.

In order to verify that the external washes were adequate in removing all of the diet contaminates from the cuticle, after the 6 hour incubation, larvae were subjected to an
additional 300 µL stream of PBST. The wash was collected and stored at -80 °C until assayed. Feeding assays were also conducted using 4th stadium, day 0 *M. sexta* and 5th stadium, day 0 *H. zea*.

**Non-Synthetic Approaches to Enhance Protein Movement**

Previous research by Jeffers et al. (2005) showed that bovine serum albumin (BSA) in artificial diet, was able to cross the digestive system intact, even maintaining it multimeric configuration. In order to determine if the movement of BSA across the insect digestive system can be increased with enzymatic gut epithelium disruption or detergent, fourth stadium, day 0 *Heliothis virescens* larvae were fed a diet containing BSA (0.8 mg/g diet; Fisher, Pittsburgh, PA) or on diet containing BSA (0.8 mg/g diet) and PEG350 (0.5 mg/g diet; Sigma-Aldrich, Saint Louis, MO; polydisperse, average molecular weight 350), phospholipase A2 (an enzyme that hydrolizes phospholipids; 0.5 mg/g diet; Sigma-Aldrich, Saint Louis, MO; 5-20% protein), sodium deoxycholate (an anionic detergent; 1 mg/g diet; Sigma-Aldrich, Saint Louis, MO; 97% purity), Tween 20 (an ionic detergent; 1 mg/g diet; Sigma-Aldrich, Saint Louis, MO; ≥ 40% lauric acid), or saponin from Quillaga bark (an amphipathic glycoside; 0.5 mg/g diet; Sigma-Aldrich, Saint Louis, MO; 20-35% sapogenin content). The concentration of the additive to BSA was the largest possible that would not alter rate of feeding (when compared to larvae feeding on control diet with BSA only). After 8 hours of feeding, hemolymph was collected from a cut thoracic leg and analyzed for the presence of BSA by ELISA as described before by Jeffers et al. (2005).
General Synthetic Procedure of PEG350-Insulin (Scheme 1)

Preparation of methyl(ethyleneglycol)γ-O-ethyl hexanoate

A solution of methyl(ethyleneglycol)γOH (25g: 7142 mmol) in dry tetrahydrofuran (THF) (52 ml) was added dropwise to a stirred suspension of NaH (3.057g: 76.4 mmol, of 60% oil dispersion) in dry THF (71ml). During the addition the reaction temperature was kept below 20°C. After the addition, the resulting solution was stirred for 3 hours. At the end of this period, 1-bromo ethyl hexanoate (15.93 g: 71.4 mmol), dissolved in dry THF (20 ml) was added dropwise, and the mixture continues to stir for 6-8 hours. The solvent was removed under reduced pressure and the residue was treated with cold water (100 ml), and extracted with ethyl acetate (3 times, 30 ml). The combined organic layer was washed sequentially with water (1 time, 20 ml), brine (1 time, 20 ml), dried over MgSO4 and the solvent was evaporated at reduced pressure to yield methyl(ethyleneglycol)γ-O-ethyl hexanoate as a pale yellow oil. The oil was chromatographed on silica gel and eluted firstly with ethyl acetate-hexane (1:1) which removes mineral oil and bromo ethylhexanoate along with other smaller fractions of polymer. Later elution with dichloromethane-methanol (80:20) yields pure methyl(ethyleneglycol)γ-O-ethyl hexanoate. The structure was confirmed by NMR.
Preparation of methyl(ethyleneglycol)\textsubscript{7}-O-hexanoic acid

The ester (25g: 50.813 mmol) was stirred with 1N NaOH (76.21 ml) at room temperature for 3 hours. The aqueous layer was cooled to 0°C by adding ice, acidified with 6N HCl to pH 2-3, and extracted with dichloromethane (3 times, 25 ml). The combined organic layer was washed with water (1 time, 20 ml), brine (1 time, 20 ml), dried over MgSO\textsubscript{4}, filtered, and evaporated to leave an oil. The product was purified by column chromatography on silica gel. Elution with pure ethyl acetate removes the impurities. Further elution with dichloromethane-methanol (80:20) gave pure methyl(ethyleneglycol)\textsubscript{7}-O-hexanoic acid. The structure was confirmed by NMR.

Preparation of methyl(ethyleneglycol)\textsubscript{7}-O-hexanoic acid-N-hydroxy-succinimide ester

To a stirring solution of methyl(ethyleneglycol)\textsubscript{7}-O-hexanoic acid (25g: 53.87 mmol) in dry methylene chloride (134.69 ml) under nitrogen atmosphere, solid N-hydroxysuccinimide (6.20 g: 53.87 mmol) was added. The stirring solution, solid EDC (11.36 g: 59.26 mmol) was added in portion and the solution was stirred at room temperature for 4 hours. At the end of this period the solution was treated with water (2 times, 20 ml), 1 N HCl (1 time, 20 ml), water (1 time, 20 ml), 1 N sodium bicarbonate (2 times, 20 ml), water (1 times, 20 ml), 1 N HCl (1 time, 20 ml), water (1 time, 20 ml), and brine. The organic layer was dried over MgSO\textsubscript{4}, filtered and evaporated under reduced pressure at room temperature to give pure activated ester. The structure was confirmed by NMR.
Conjugation of methyl(ethyleneglycol)\(_7\)-O-hexanoic acid NHS to insulin

Bovine insulin (Fisher; Pittsburgh, PA; 27 USP units/mg) was dissolved in dimethyl sulfoxide at 25°C and reacted with 1.2 molar equivalent of activated methyl(ethyleneglycol)\(_7\)-O-hexanoic acid for 45 minutes at 25°C. The product mixture was purified by dialysis on a 3,500 mw cut off membrane at 5°C. The solution was lyophilized and structure was confirmed by LC/MS (Notch patent).

**General Synthetic Procedure of PEG333-Insulin (Scheme 2)**

For the synthesis of insulin (methyl(ethyleneglycol)\(_7\)-O-propionyl)(PEG333-insulin), to a round-bottom flask charged with insulin (50 mg/mL buffer) and carbonate buffer (0.1 M, pH 9, 1 mL), 2.5 moles of monodisperse methyl(ethyleneglycol)\(_7\)-O-propionyl-N-hydroxy-succinimide ester (Pierce Biotechnology, Inc.; Rockfold, IL) were added. The resulting solution was stirred at room temperature and monitored by analytical RP-HPLC with a Microsorb-MV column (C-18, 5µ, 250 × 4.6 mm; flow rate, 1 mL/min). Detection was by UV at 220 nm. The following gradients of HPLC-grade water with 0.1% trifluoroacetic acid (Sigma-Aldrich; Saint Louis, MO) and acetonitrile with 0.1% trifluoroacetic acid (Sigma-Aldrich; Saint Louis, MO) were used: 10% to 50% acetonitrile in water from 0 to 16 min. After HPLC analysis showed complete consumption of starting material (as indicated by disappearance of a peak at approximately 30 min), the product was purified by preparative RP-HPLC with a Microsorb-MV column (C-18, 5µ, 250 × 21.4 mm; flow rate 20 mL/min). Detection was by UV at 220 nm. The following gradients were used: 10% acetonitrile (0.1%
trifluoroacetic acid) in water (0.1% trifluoroacetic acid) from 0 to 5 min and 10% to 50% acetonitrile (0.1% trifluoroacetic acid) in water (0.1% trifluoroacetic acid) from 5 to 21 min. The appropriate fractions of PEGylated insulin were collected. The solvent volume of the isolated solution was reduced to approximately 5 mL in vacuo at 35 ºC. To the concentrated solution, 50 molar equivalents of NaBH₄ were added. The resulting solution was stirred at room temperature and monitored by analytical RP-HPLC (HPLC conditions were identical to those described above) to confirm complete reaction (~ 8 h). Once HPLC analysis showed complete reaction, the sample was purified by preparative-scale RP-HPLC (HPLC conditions were identical to those described above), lyophilized, reconstituted with deionized water, and stored at -80 ºC until needed for other studies. The molecular weights of the product were confirmed by ESI-MS.
**Larval Topical Cuticle Assay**

Five μl of a 5.0 mg/ml solution of insulin, PEG350-insulin, and PEG333-insulin in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Saint Louis, MO; ≥ 99.9% purity) was topically applied using a 10 μl pipettman to the dorsum of the abdomen of fifth stadium, day 0 tobacco *H. virescens* (staged as described previously). Treated larvae were then transferred to a 28 ml cup (1 larva per cup) without food. The larvae were incubated at standard Lepidoptera rearing conditions as described previously. After 4 hours, larvae were removed from the cup. Any fras on larvae was removed with a camel-hair brush. Each larva was then rinsed with a stream of 2–3 ml of PBST and blotted dry on a Kimwipe. Hemolymph was collected from a cut thoracic leg using scissors and transferred as a small drop to the surface of Parafilm. The scissors were rinsed with PBST and dried with a Kimwipe between larvae to prevent cross-contamination. Using a 10 μl pipettman, 5.0 μl of the hemolymph from the Parafilm was aliquoted into 45 μl of distilled water (4 °C) in a 2.0 ml microcentrifuge tube and immediately vortexed. All samples were stored at −80 °C until assayed. Freezing or thawing had no effect on assay results.

**Injection into the Insect Hemocoel**

One μl of a 20 mg/ml solution of insulin, PEG350-insulin, or PEG333-insulin in PBS or PBS alone (control) was injected into fourth stadium, day 0 *H. virescens*. The injections were made dorsolaterally, two or three segments from the posterior of the abdomen using a
repeating dispenser fitted with a 10 μl glass syringe with a 33 gauge needle (Hamilton, Reno, NV). The needle was inserted into the larvae just under the epidermis with the end of the needle extending at least two segments anterior to the insertion site. After injection, the needle was left in place for 5 seconds. The needle was removed, and any larvae that demonstrated bleeding were discarded. Injected larvae were placed into 28 ml diet cups without food and incubated at 25 °C. Hemolymph was collected at 5, 15, 30, 60, 90, 120, 180, 240, 300, 360, 420, and 480 minutes after injection as described earlier. All samples were stored at −80 °C until assayed. Freezing or thawing had no effect on assay results.

**Incubations in Plasma**

Hemolymph was collected from fourth stadium, day 0 *H. virescens* larvae as described earlier and pooled in a 2.0 ml microcentrifuge tube containing 5 mg phenylthiourea (PTU; Sigma-Aldrich, Saint Louis, MO) on ice. The tube was shaken repeatedly during hemolymph collection to dissolve the PTU, and then after collection, the tube was centrifuged at 1000 g for 5 minutes at 25° C. Aliquots (19.8 μl) of the supernatant (plasma) at 25° C were added to 0.2 ml microcentrifuge tubes to 0.2 μl of a 20 mg/ml solution of insulin, 0.2 μl of a 20 mg/ml solution of PEG350-insulin, 0.2 μl of a 20 mg/ml solution of PEG333-insulin, or 0.2 μl of PBS only (control) and incubated for 5, 15, 30, 60, 90, or 120 minutes at the same temperature. After incubation, 5 μl of the hemolymph from each sample was added to 495 μl of PBS (4° C) and immediately vortexed. All samples were stored at −80 °C until assayed. Freezing or thawing had no effect on assay results.
Presence of Insulin in Feces

After feeding on mealpads rehydrated with solutions of insulin, PEG350-insulin, or PEG333-insulin for 6 hours, *H. virescens* larvae were removed from rearing cups and washed as previously described. Larvae were transferred to new rearing cups without diet and returned to standard rearing conditions. After 4 hours, the feces was collected and frozen at -80 °C for 24 hours followed by lyophilization for 12 hours. The feces was weighed and homogenized as previously described. The homogenate was transferred to a microcentrifuge tube and centrifuged or 2 minutes at 960 g (4 °C). The supernatant was stored at -80 °C until assayed.

Enzyme-linked Immunoabsorbant Assay (ELISA) for Insulin

A Mercodia (Uppsala, Sweden) Bovine Insulin ELISA was used to determine the concentration of insulin in samples collected. The ELISA is a solid phase 2-site enzyme immunoassay based on the direct sandwich technique. For this technique, 25 µL of sample and 50 µL of enzyme conjugate (peroxidase conjugated mouse monoclonal anti-insulin) were added to each well of the 96-well Mercodia microtiter plate (coated with mouse monoclonal anti-insulin with a different antigenic site than the conjugated anti-insulin). After a 2 hour incubation at 25°C on a shaker, the plates were emptied and rinsed 6 times with 350 µL of Mercodia wash buffer per well. Afterward, 200 µL of 3,3’,5,5’-tetramethylbenzidine substrate solution was added to each well and incubated for 15 minutes at 25°C on a shaker. Then 50 µL of stop solution (0.5 M H_2SO_4) was added to each well, and the absorbance was
read at 450 nm on a Molecular Devices Corp. plate reader (Downington, PA). Results were compared to a standard curve generated by known concentrations of insulin in PBS. Also, 50 µL of ng/mL solution of ovalbumin (Fisher, Pittsburgh, PA) in PBS was used as a negative control and 50 µL of PBS was used as a blank. We were unable to obtain a direct measure of insulin or conjugated insulin using LC MS or LC MS/MS because of the complex protein makeup of the different samples under study. Therefore, the proportion of free insulin, conjugated insulin and degradation products is unknown. However, previous feeding studies have shown that only TMOF is found in the hemolymph after feeding *H. virescens* PEGylated TMOF (Jeffers et al., 2011). Therefore, we believe that the same will be true for PEGylated insulin. PEGylated insulin was marginally less responsive to the Mercodia insulin ELISA. Therefore, if any PEGylated insulin was in the hemolymph, then the values in this study are under-reported.

**Enzyme-linked Immunoabsorbant Assay (ELISA) for BSA**

For BSA ELISA methodology, please see Jeffers et al., 2005
RESULTS AND DISCUSSION

Feeding Bioassays Using Hydrated Mealpads

It was critical to studies of protein movement across the lepidopteran digestive system to have a simple method of protein incorporation into small amount of artificial insect diet. This was especially important to minimize the cost of purified proteins and conjugates of these proteins that were under study. Roe et al. (2000, 2003) and Bailey et al. (2001) developed a technique of using dehydrated mealpads for monitoring lepidopteran resistance to $Bt$ protein. Bailey et al. (1998) showed the addition of proteins to the mealpad matrix resulted in a uniform dispersal of the test protein in the diet. In addition, the insect growth and development on this hydrated mealpad system was identical to that of standard artificial rearing diet, even when the dehydrated pads had been stored for months at room temperature.

Once added to the mealpad, it is also important that test proteins are not subject to degradation during the course of the bioassay. Jeffers et al. (2005) noted that at room temperature, bovine serum albumin (BSA) and anti-BSA polyclonal antibody retained their concentration, multimeric structure and functional characteristics for at least 24 hours in the same hydrated mealpad matrix that was used in our current work. Western blots showed even the retention of the BSA multimeric structure and for anti-BSA, no change in its immunochemical activity. For the current experiments, it was necessary to establish the same stability for insulin and the insulin conjugates that were synthesized. Solutions of
insulin, PEG350-insulin and PEG333-insulin were added to lyophilized mealpads and incubated for 0 and 24 hours under the same conditions as described in the feeding bioassay. There were no changes in the concentration of insulin, PEG350-insulin and PEG333-insulin in the hydrateable mealpads after a 24 hour incubation (t-test, $\alpha = 0.05$).

**Enhancement of Protein Accumulation Across the Gut with Phospholipase and Surfactants**

As was discussed in the introduction and reviewed in detail by Jeffers and Roe (2008), proteins move intact from the digestive system into the hemolymph in a variety of insects. It appears this movement occurs across the ventricular epithelial cells of the insect midgut and not between the cells, although more work is needed to understand the mechanism. It also appears, at least in some cases, this movement is not target specific since Jeffers et al. (2005) found that BSA, not normally expected in the diet of caterpillars, moved from artificial diet into the hemolymph.

Experiments were conducted here to begin understanding what factors might enhance this movement of BSA. *H. virescens* larvae were fed BSA mixed with PEG350 (a polydispersed polyethylene glycol), phospholipase A2, the anionic detergent (sodium deoxycholate), the ionic detergent (Tween 20) or Saponin (an amphipathic glycoside). Since these additives to BSA at high doses can inhibit feeding, we used the maximum dose that did not reduce feeding when compared to artificial diet without the additives. In all cases, there was no
increase in the accumulation of BSA in the hemolymph when compared to larvae feeding on diet with BSA only (Figure 1). There are several possible explanations for these results. It is possible that at the concentrations chosen, the additives just do not affect the mechanism of movement of BSA across the insect gut. Although it is well known that phospholipase A2 and detergents can disrupt cell membranes like that of the insect midgut, at the concentrations needed to prevent anti-feeding activity, this disruption may have been minimal. It also possible that the rate limiting step in the accumulation of BSA in the hemolymph is not its rate of movement from the gut, but its rate of degradation in the gut and/or hemolymph.

Larval Lepidoptera Feeding Assay with Insulin and PEGylated Insulins

The movement of intact proteins (both foreign and innate) across the Lepidopteran digestive system has been studied in a number of insects and in ticks (Ben-Yakir and Shochat, 1996; Casartelli et al., 2005; Fitches and Gatehouse, 1998; Fitches et al., 2001a, b; Hirayama et al., 2000; Jeffers et al., 2005; Kurahashi et al., 2005; Sugimura et al., 2001). Shen et al. (2009) found that PEGylation of a decapeptide, TMOF, decreased its metabolism by a model protease in vitro, and Jeffers et al. (2011) found that the addition of aliphatic PEG to TMOF also enhance its insecticidal activity to larval mosquitoes, presumably by decreasing its degradation rate in the insect and/or enhancing its movement across the digestive system. Changes in in vivo degradation of TMOF because of PEGylation and accumulation in the
hemolymph could not easily be determined because of the small size and aquatic habitat of mosquitoes.

In the current study, we were interested in investigating whether PEGylation of a larger protein would affect degradation \textit{in vivo} and/or increase accumulation in the hemolymph of larval Lepidoptera. To achieve this objective, we examined the movement of insulin, PEG350-insulin and PEG333-insulin across the gut after feeding (Table 1). Insulin should not produce significant adverse changes to the insect, and in fact, none of the treatments produce mortality or obvious changes in food consumption, growth, development or behavior. With fifth stadium, day 0 \textit{H. virescens} larvae, the hemolymph insulin concentrations for the insulin, PEG350-insulin, and PEG333-insulin were 0.45, 3.0 and 3.3 µg/mL, respectively. This translates to a 6.7 and 7.3 fold increases in appearance of insulin in the hemolymph for the PEG350 and PEG333 chemistries, respectively. Similar results were found when fifth stadium day 0 \textit{H. zea} larvae, the concentrations for insulin, PEG350-insulin, and PEG333-insulin in the hemolymph were 0.50, 3.2 and 3.1 µg/mL, respectively (Table 1). This translates to 6.4 and 6.2 fold increases in insulin equivalents in the hemolymph for the PEG350 and PEG333 chemistries, respectively. Jeffers et al. (2005) found that \textit{M. sexta} larvae fed on mealpads hydrated with solutions of BSA and anti-BSA, statistically insignificant amounts of BSA and no detectable anti-BSA was present in the hemolymph after 8 hours. Interestingly, 4th stadium, day 0 \textit{M. sexta} larvae fed on mealpads rehydrated with insulin and PEGylated (6 hour incubation), no insulin protein was detected after feeding like that for BSA, but PEG350-insulin and PEG333-insulin were detected in the hemolymph.
at 2.0 and 2.1 µg/mL, respectively (Table 1). It is clear from these studies in three different insect species that the conjugation of insulin with aliphatic PEG results in higher levels of insulin in insect hemolymph. Whether that is result of increased movement across the gut, increased stability of the protein against enzymatic degradation in the gut and/or hemolymph, and/or reduced movement of the protein from hemolymph either into different tissues and/or due to excretion is unclear.

In Jeffers et al. (2005), when *H. virescens* larvae fed on mealpads hydrated with solutions of BSA and anti-BSA, neither protein was detectable in feces. To see if PEGylation would increase the stability of a protein in the digestive system enough to allow detection in feces, similar experiments were conducted with insulin and our PEGylated insulin. After feeding on mealpads rehydrated with solutions of insulin, PEG350-insulin, or PEG333-insulin for 6 hours, *H. virescens* larvae were transferred to new rearing cups without diet and returned to standard rearing conditions. After 4 hours, the feces were collected and analyzed; no insulin was detected.

**Movement of PEGylated Insulins Across the Cuticle**

During the 6 hour feeding assays, the budworm, bollworm and hornworm larvae spend the majority of the time on or curled around the diet surface. Because, during these times, the insect cuticle is in direct contact with the diet, it is possible that proteins could enter the insect hemolymph through the cuticle. Jeffers et al. (2005) reported that after a 4 hour topical
application to the cuticle, BSA or anti-BSA was not found in the hemolymph of *H. virescens*. To determine if the same is true for PEGylated proteins, solutions of insulin, PEG350-insulin, and PEG333-insulin in DMSO was topically applied to the dorsum of the abdomen of fifth stadium, day 0 *H. virescens*, and hemolymph was collected after 4 hours. As expected based on our earlier work with BSA and anti-BSA, larvae treated with insulin had no detectable insulin in their hemolymph (Figure 2). However, both the PEG350-insulin and PEG333-insulin treatments resulted in the detection of insulin in the hemolymph at concentrations of 1.2 and 0.88 µg/mL, respectively (Figure 2). Note that the insect was washed to remove any possible contamination of the hemolymph by treatment proteins that might be absorbed to the cuticle. The method of washing was the same as that described in Jeffers et al. (2005) after feeding on artificial diet containing BSA and anti-BSA, where the final wash did not have any detectable levels of the protein. Also if hemolymph contamination is the source of detectable insulin in the PEGylated treatments, the same might be expected for the native insulin treatment; this was not the case. It also is unlikely that the insulin in the hemolymph is from oral exposure because the treatment was made in a location where the larval could not reach *per os*. It is possible that the topical treatment was transferred to the diet or rearing cup and then obtained *per os*, although again this did not occur for the native insulin. The idea that a protein could be applied topically and move across the cuticle by PEGylation could be translational and provide a method for the use of topically applied protein toxins where their mode of action might be in the insect hemocoel.
Degradation of Insulin and PEGylated Insulins in the Insect Hemolymph

It is well known that the clearance of the protein from the hemolymph is a combination of enzymatic degradation and tissue sequestration. When injected into the hemolymph of the blood-feeding insect *Rhodinus prolixus*, hemoglobin was denatured and oxidized to kathemoglobin and eventually sequestered by the ovaries, salivary glands and pericardial cells (Wigglesworth, 1943). Similarly, through receptor-mediated endocytosis, juvenile hormone esterase in the hemolymph was sequestered by the pericardial cells of *M. sexta*, and eventually the enzyme was degraded in the lysosomes (Ichinose et al., 1992; Bonning et al., 1997). Also as mentioned earlier, it was shown that dietary snowdrop and jackbean lectins are sequestered in the malpighian tubules of *Lacanobia oleracea* after feeding (Fitches and Gatehouse, 1998; Fitches et al. 2001a; Fitches et al., 2001b), and snowdrop lectin is sequestered in the fat body and ovarioles of *Nilaparvata lugens* after feeding (Powell et al., 1998). Jeffers et al. (2005) found the same to be true for foreign proteins not normally found in the diet of the insect. In-vivo injections of BSA and anti-BSA into the hemolymph were compared to the addition of BSA and anti-BSA to pooled plasma to examine the possible mechanisms for the turnover of proteins in hemolymph. The rate of degradation for the proteins was greater *in vivo* than *in vitro*, which in addition to enzymatic degradation, showed that sequestration is a factor in the disappearance of proteins from hemolymph. Jeffers et al. (2005) also concluded that non-blood-feeding insects must have a general mechanism for the clearance of foreign proteins entering the hemolymph from the digestive
system. Finally, Shen et al. (2009) found that PEGylation of the decapeptide, TMOF, reduced the in vitro enzymatic degradation of the protein by a model protease.

In order to determine the effects of protein PEGylation on in vivo hemolymph protein turnover in insects, insulin, PEG350-insulin and PEG333-insulin were injected into the hemolymph of fourth stadium, day 0 H. virescens. One hundred and twenty minutes after injection for the insulin treatment, no insulin was detected in the hemolymph (Figure 3). In comparison, for the PEG350 insulin and PEG333 insulin treatments, insulin was still present in the hemolymph 300 and 240 minutes after injection, respectively (Figure 3). This translates to 3.3 and 2.7 fold increases in the length of time insulin remains in the hemolymph after injection for the PEG350 and PEG333 chemistries, respectively. The rate of degradation of the PEGylated insulins was significantly shorter than that of insulin (weighted linear regression: $\alpha = 0.05$, $P < 0.0001$ for PEG350-insulin and PEG333-insulin). This strongly supports the theory that PEGylation of a protein protects it from enzymatic degradation.

To further examine the issue of degradation versus sequestration, insulin, PEG350-insulin and PEG333-insulin in different experiments were added to the pooled plasma of fourth stadium, day 0 H. virescens. After 120 minutes, the concentrations of insulin, PEG350-insulin, or PEG333-insulin were 15.0, 50.6 and 60.6 µg/mL (Figure 4). As with Jeffers et al. (2005) for BSA and anti-BSA, the rate of degradation of insulin, PEG350-insulin and PEG333-insulin was also significantly greater in vivo than in vitro (compare Figure 3 with
Figure 4)(weighted linear regression analysis: $\alpha = 0.05$, $P < 0.0001$ for insulin, PEG350-insulin, and PEG333-insulin). These results suggest that PEGylated proteins are sequestered by hemocoel tissues and that PEGylation also reduced the rate of degradation in insect hemolymph.

In summary, we reported before (Shen et al. 2009; Jeffers et al. 2011) that the covalent synthesis of aliphatic PEG to a small peptide, the decapeptide TMOF, reduced hydrolysis by a model protease and enhanced its insecticidal activity against larval mosquitoes, presumably by greater accumulation of TMOF in hemolymph. In the current study, we found that PEGylation with both monodispersed and polydispersed PEG to a larger peptide, insulin, also reduced its turnover rate both \textit{in vitro} and \textit{in vivo} in insect hemolymph, increased the accumulation of insulin in the hemolymph when administered \textit{per os} in three larval lepidopteran species and also allowed the protein to enter into the hemolymph across the insect cuticle. Co-feeding of insulin with PEG 350, phospholipase-A2 or different surfactants had no effects on insulin accumulation. The work is translational in showing that PEGylation of proteins like similar research in drug development is a potential method to enhance the delivery or toxic proteins into the insect hemocoel, even by spraying. As with the pharmaceutical industry, PEGylation is a potential method to enhance the delivery of toxic proteins into the insect hemocoel, through feeding or direct contact.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Effects of co-feeding bovine serum albumin (BSA) with one of several enhancers [PEG350 (0.5 mg/g diet); Phospholipase A2, an enzyme that hydrolizes phospholipids into fatty acids and other lipophilic substances (0.5 mg/g diet); sodium deoxycholate, an anionic detergent (1 mg/g diet); Tween 20, an ionic detergent (1 mg/g diet); or saponin from Quillaga bark, an amphipathic glycoside (0.5 mg/g diet)] in artificial diet on BSA accumulation across the digestive system in the hemolymph of larval *H. virescens*. Results are the average of (n = 10 or 20); ± 1 SEM.

Figure 2. Accumulation of insulin in hemolymph from across the insect cuticle. Solutions of insulin, PEG350-insulin and PEG333-insulin were topically applied to the dorsum of the abdomen of fifth stadium, day 0 *H. virescens* larvae. After 4 hours, larvae were rinsed, and hemolymph was collected from a cut thoracic leg. Results are the average of (n=10) ± 1 SEM.

Figure 3. *In-vivo* injections into the insect hemocoel. Solutions of insulin, PEG350-insulin, and PEG333-insulin were injected into fourth stadium, Day 0 *Heliothis virescens* larvae. Hemolymph was collected from a cut thoracic leg at 5, 15, 30, 60, 90, 120, 180, 240, 300 and 360 minutes after injection. Results are the average of (n=10) ± 1 SEM.

Figure 4. Incubations in insect plasma. Solutions of insulin, PEG350-insulin, and PEG333-insulin were added to pooled plasma from fourth stadium, Day 0 *Heliothis virescens* larvae
and incubated at 25 °C at 5, 15, 30, 60, 90 and 120 minutes after addition. Results are the average of (n=10) ± 1 SEM.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
TABLE LEGENDS

TABLE 1. Hemolymph accumulation across the insect digestive system for *H. virescens*, *H. zea* and *M. sexta* ((n=10 or 15) ± 1 SEM).
Table 1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( n^3 )</th>
<th>Concentration (( \mu g/mL )) ( \pm ) SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Heliothis virescens</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>15</td>
<td>0.45 ± 0.07 A(^c)</td>
</tr>
<tr>
<td>PEG350-Insulin</td>
<td>15</td>
<td>3.0 ± 0.3 B</td>
</tr>
<tr>
<td>PEG333-Insulin</td>
<td>15</td>
<td>3.3 ± 0.3 B</td>
</tr>
<tr>
<td><em>Helicoverpa zea</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>10</td>
<td>0.49 ± 0.07 A(^A)</td>
</tr>
<tr>
<td>PEG350-Insulin</td>
<td>10</td>
<td>3.2 ± 0.4 B</td>
</tr>
<tr>
<td>PEG333-Insulin</td>
<td>10</td>
<td>3.1 ± 0.4 B</td>
</tr>
<tr>
<td><em>Manduca sexta</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>10</td>
<td>0 C</td>
</tr>
<tr>
<td>PEG350-Insulin</td>
<td>10</td>
<td>2.0 ± 0.5 D</td>
</tr>
<tr>
<td>PEG333-Insulin</td>
<td>10</td>
<td>2.1 ± 0.6 D</td>
</tr>
</tbody>
</table>

\(^a\) Number of larvae per compound.

\(^b\) Concentration (\( \mu g \) insulin equivalents/mL) for the insulin, PEG350-insulin or PEG333-insulin treatments.

\(^c\) Concentrations for each insect followed by a different uppercase letter are statistically different based on T-tests (\( \alpha = 0.05 \)).
SCHEME LEGENDS

Scheme 1. Synthesis of polydispersed PEGylated insulin

Scheme 2. Synthesis of monodispersed PEGylated insulin
Scheme 1.

polydisperse PEG

\[
\begin{align*}
\text{OH} & \quad \text{+ 1-bromo-ethylhexanoate} \\
\text{CO}_2\text{CH}_2\text{CH}_3 & \quad \text{NaH} \\
\text{CO}_2\text{H} & \quad \text{NaOH/acid work-up} \\
\text{O} & \quad \text{EDC} \\
\text{N} & \quad \text{Activated for addition to insulin}
\end{align*}
\]

Ekwuribe et al. US Pat 6,191,105B1

Stoichiometry of conjugation can be controlled
Ratio of PEG/insulin = 2:1; 45 minutes \(\rightarrow\) (PEG)\(_2\)insulin and (PEG)\(_3\)insulin, a little free insulin
Ratio of PEG/insulin = 4:1; 60 minutes \(\rightarrow\) (PEG)\(_2\)insulin and (PEG)\(_3\)insulin, NO free insulin

MS of (PEG)\(_3\)insulin

MS of (PEG)\(_2\)insulin
Scheme 2.

Conjugation of monodisperse PEG

\[ \text{Commercially available} \]

Ratio of PEG/insulin = 4:1; 90 minutes
APPENDIX
Appendix A. Comparative Feeding Studies

Passage of (A) anti-BSA and (B) BSA across the digestive system into hemolymph after feeding. Fourth stadium, larval tobacco budworms, tobacco hornworms, and corn earworms, and adult female house crickets and Madagascar hissing cockroaches were fed diet containing anti-BSA or BSA (0.8 mg per gram diet) for 8 h. Hemolymph was collected and analyzed for the presence of anti-BSA or BSA by ELISA. Results are the average ± 1 SEM (ND = not detected). For materials and methods, please refer to