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Polymers for the stabilization and delivery of proteins topically and *per os* 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 fourth stadium tobacco budworms, *Heliothis virescens*, did not affect the levels of BSA in the hemolymph. Covalent conjugation of small proteins like the decapeptide trypsin modulating oostatic factor (TMOF) to polyethylene glycol was previously shown to protect the peptide from protease attack and enhance its accumulation in the insect hemocoel. Whether this polymer chemistry could do the same for larger proteins was examined. The chemistry for the synthesis of polydispersed aliphatic PEG350-insulin and monodispersed aliphatic PEG333-insulin are described herein. Insulin was used for this synthesis and not BSA to better control conjugation among the available free amine groups. When PEGylated insulin or free insulin were fed in artificial diet to fifth stadium budworms, greater concentrations of insulin using the PEGylated variants were found in the hemolymph than when free insulin was used (a 6.7 and 7.3-fold increase for the PEG350 and PEG333 conjugates, 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 were detected in the hemolymph. After injections of insulin into the hemocoel of fourth stadium *H. virescens*, insulin is completely cleared from the hemolymph in 120 min. In comparison, PEG350-insulin and PEG333-insulin were present in the hemolymph for 300 and 240 min after injection, respectively, translating to a 3.3 and 2.7-fold increase in the length of time insulin remains in the hemolymph after injection.

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1. Introduction

Interest in the use of proteins as insecticides stems from the successful development of the *Bacillus thuringiensis* (*Bt*) delta-endotoxin where the site of action is the insect midgut epithelium [1,2]. The oral delivery of insecticidal proteins which act in the insect hemocoel is more difficult due to protease degradation in the digestive system and their limited movement across the gut epithelium. In mammals, transport across the digestive system follows two pathways: specific-receptor mediated and non-specific transcytosis [3–5]. The movement of ingested, intact proteins across the digestive system into the hemolymph occurs in both blood-feeding and non-blood feeding insects, but the mechanism for this movement is not clear (reviewed by Jeffers and Roe [6]). Nogge and Giannetti [7] reported that intact human albumin appeared in the hemolymph of *Glossina morsitans* after feeding on human blood; they speculated, since blood is a poor energy source, human albumin absorption was a method of energy conservation. Similarly, Kurahashi et al. [8] reported that mulberry leaf urease move unchanged across the gut and into the hemolymph of *Bombyx mori*. The authors suggested that *B. mori* selectively

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transported the plant urease because it was important for hemolymph nitrogen metabolism. There are two possible routes for protein movement in insects: through the ventricular epithelial cells and through the intercellular spaces of the midgut. Only a small percentage of dietary proteins move unchanged from the diet to hemolymph; Jeffers et al. [9], for example, reported ratios of 1:500 and 1:300 for bovine serum albumin and anti-BSA, respectively, in the tobacco budworm. Enhancement of this movement and stabilization of proteins in the hemolymph could be important in the development of new protein insecticides where their site of action is in the hemocoel. The movement of proteins across the cuticle is not expected because of the high lipophilicity of the epicuticle.

Jeffers and Roe [6] reviewed several approaches that might be used 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 [10], and it was shown that dietary snowdrop and jackbean lectins accumulate in the gut, hemolymph and Malpighian tubules of *Lacanobia oleracea* after feeding [11–13]. Similarly, snowdrop lectin accumulated in the hemolymph, fat body, and ovarioles of *Nilaparvata lugens* after feeding [14]. Mannose-specific snowdrop lectin fusion proteins were used to deliver allatostatin [15], an insecticidal spider venom toxin [16,17], and a lepidopteran-specific scorpion toxin [18] to the insect hemocoel. The use of protease inhibitors like that from soybean [19] to prevent protein degradation in the insect digestive system and enhance protein absorption has also been examined [20]. Insects use multiple enzymes to digest proteins, and they can compensate for the inhibition of one enzyme by the increased expression of others. In addition, each crop has different insect pests with different proteases requiring a broad spectrum inhibitor to achieve insect control by protease inhibition. Finally, the concentration of inhibitor must be high in the insect's diet, approximately 1% of the soluble plant [20].

PEGylation, the process of covalently attaching polyethylene glycol (PEG) polymer chains to another molecule, has been used by the pharmaceutical industry to deliver proteins across the digestive system of humans [21–25] including insulin [26]. To determine if this system could be used in insects, Jeffers et al. [27] covalently bound a PEG polymer to the decapeptide trypsin modulating oostatic factor (TMOF). TMOF (Tyr-Asp-Pro-Ala-Pro_6) originally isolated from the ovaries of the adult yellow fever mosquito, *Aedes aegypti*, regulates trypsin biosynthesis [28] and is larvicidal *per os* by crossing into the hemocoel and turning off protein degradation in the gut. The addition of trifluoro acetyl and lysine to TMOF, yielding TFA-TMOF-K, allowed the specific conjugation of monodispersed and aliphatic PEG (mw 509) to the amino group of lysine. The median lethal concentration (LC_{50}) for *A. aegypti* (*per os*) for unaltered TMOF was 0.46 mM. By adding the lysine that allows the conjugation to PEG, the activity decreases (LC_{50} = 0.81 mM). However, the TMOF-K-PEG509 demonstrated increased activity 5.8 and 10.1-fold above that of TMOF and TMOF-K, respectively [27]. Similarly, Shen et al. [29] 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. [29] 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 protein that is larger than TMOF and also enhance its accumulation from the diet or cuticle into the insect hemocoel. As insulin is not insecticidal during the time course of our assay, we were able to examine treatment effects in the absence of insect toxicity.

2. Materials and methods

2.1. 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. Following their collection, the *H. virescens* and *H. zea* strains were reared in the laboratory on artificial diet. The *M. sexta* are the original Yamamoto [30] strain reared on artificial diet. Standard rearing and bioassays with these insects were conducted at 27 °C and 14:10 (L:D).

2.2. Preparation of diet for larval lepidopteran feeding assay

Plastic cups (30-mL transparent polypropylene; Solo Cup Company, Lake Forest, IL, USA) containing approximately 2 mL of artificial diet [31] 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 [32–35]. The lyophilized diet was then divided with a razor blade into 80–120 mg meal pads that were stored in the dark at room temperature and 0% humidity until needed. Test proteins dissolved in distilled water were added to meal pads at the time of meal pad hydration to produce a final concentration of 0.8 mg of test protein/g wet diet.

2.3. Stability of proteins in mealpads

The stability of BSA in our artificial diet was shown previously [9]. For insulin, mealpads were hydrated with insulin (described later) in distilled water and incubated for 0 and 24 h under standard rearing conditions described above. After incubation, the diet was homogenized in 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) for 10 s (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 min. The supernatant (50 μL) was added to 450 μL of PBS at 4 °C and stored at −80 °C until assayed.

2.4. Larval lepidopteran feeding assay

After completing the feeding stage in the fourth stadium, *H. virescens* larvae were removed from the diet and placed individually into Solo 30 mL plastic cups without diet. The cups were sealed with a paper lid and the larvae stored under standard rearing conditions for 6 h. 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 30 mL Solo 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 were sealed with a paper lid. The larvae were incubated under standard rearing conditions and examined after 30 min; those that had not initiated feeding were removed from

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