INVISIBLE PROTEINS: NOVEL DELIVERY SYSTEM FOR THE STABILIZATION AND ENHANCED ACTIVITY OF PROTEIN INSECTICIDES

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Abstract

Limiting factors in the use of proteins as insecticides, especially when the site of action is in the insect hemocoel, is protease degradation in the digestive system and hemolymph and constrained movement of proteins across the cuticle and midgut. We present data of a novel polymer chemistry that renders proteins essentially invisible to proteases and enhances their accumulation across the insect cuticle and digestive system into hemolymph. Proof of concept was conducted with two model systems, insulin (a nontoxic protein in insect systems) and trypsin modulating oostatic factor (TMOF). The latter is a *per os* peptidic mosquito larvacide which crosses the gut and binds to receptors on the hemolymph side of the digestive system inhibiting gut protease synthesis and food utilization. Polyethylene glycol (PEG) polymers were used including a polydisperse PEG-hexanoate (PEG350) moiety and three monodisperse PEG units $CH_3(OCH_2CH_2)_n$-O-propionyl- with n = 3 {PEG333 or PEG(3)}, 7 {PEG509 or PEG(7)} or 11 {PEG685 or PEG(11)}. The chemistry for the synthesis of PEG-bovine insulin polymers is described. Feeding PEG-conjugated (two variants of PEG) and free insulin to 5th stadium tobacco budworms in meal pads reveals a higher level of the PEG-conjugated insulin in the hemolymph than unconjugated insulin. In addition, the PEG-insulin conjugates crossed the insect cuticle while native insulin does not. When PEGylated TMOF-K was fed to larval mosquitoes, the LC50s were reduced by as much as an order of magnitude compared to those of free TMOF-K and TMOF. The larvae did not feed and were reduced in size typical of starvation and the expected toxicology of TMOF. PEG-TMOF-K *per os* also reduced the growth of neonates of the tbw as compared to TMOF-K with no activity (data not shown). The *in vitro* degradation of TMOF-K by the digestive enzyme leucine aminopeptidase was inhibited by conjugation of TMOF-K with PEG polymers. Under identical degradation conditions, the substrates that remained after 60 min of incubation for TMOF-K, TMOF-K(methyl(ethyleneglycol)$_3$-O-propionyl), TMOF-K(methyl(ethyleneglycol)$_7$-O-propionyl) and TMOF-K(methyl(ethyleneglycol)$_{11}$-O-propionyl) were in the ratio of 1 : 3 : 12 : 30. These studies demonstrated a positive correlation between the molecular weight of the PEG polymer and resistance to digestion. As expected, no degradation of TMOF-K or the PEGylated TMOF-K conjugates occurs in the absence of enzyme. The rate of degradation of TMOF-K also increases with increasing concentration of the enzyme as would be expected for enzymatic reactions where the substrate is in excess and the formation of an enzyme-substrate complex follows traditional Michaelis-Menton kinetics. However, at concentrations up to 300 U/mL of enzyme stock solution, TMOF-K(methyl(ethyleneglycol)$_{11}$-O-propionyl) was not degraded.

Introduction

Advances in biotechnology and the potential for practical benefits render the use of proteins as selective insecticides an attractive target. However, the application of proteins as insecticides is limited by protease degradation in the digestive system and hemolymph, especially when the site of action is in the insect hemocoel, as well as limited movement of proteins across the cuticle and midgut. Jeffers and Roe (2008) recently reviewed the state of the art of protein movement across the digestive system of insects including approaches used to enhance protein accumulation in these systems. In general, several compounds have been investigated for protein stabilization including polymers (Abuchowski and Davis 1981, Marshall and Rabinowitz 1976, Yang and Lopina 2006) and small molecules (Igarashi 1990, Mill et al. 1977). Approaches to enhance movement of compounds across unit membranes include the use of liposomes (Chien, 1992) and encapsulation (Saffran et al., 1986). While these techniques have merit, they

are also unstable once entering the organism. Boucu et al. (1982) reported that proteins conjugated to polyethylene glycol (PEG) results in an increase in stability against denaturation and enzymatic digestion. Several patents {N.N. Ekwuribe in US Patents 5,359,030 (1994), 5,438,040 (1995), and 5,681,811 (1997); and Ekwuribe et al. 6,191,105 (2001)} showed that the combination of hydrophilic PEG linked to a lipophilic moiety (alkyl groups) could both stabilize proteins and enhance protein movement. This approach was investigated for the development of an oral insulin to treat human and animal diabetes. The use of these PEG polymers was considered in the current study to determine if this might stabilize proteins and enhance the accumulation of proteins across the cuticle or digestive system into hemolymph of insects. If successful, this technique could be used as a novel insecticidal delivery system for proteins that are only active once they enter the insect hemocoel.

To determine proof of concept, PEG polymers were conjugated to bovine insulin (the latter is known not to be toxic to insects) and to investigate movement of this modified protein across the cuticle and digestive system. In addition, PEG-based polymers conjugated to the decadpeptide TMOF (trypsin modulating oostatic factor) were synthesized to determine if this modification could enhance the insecticidal activity of TMOF. TMOF is a *per os* peptidic mosquito larvacide that crosses the gut and binds to receptors on the hemolymph side of the digestive system inhibiting gut protease synthesis and food utilization (Borovsky et al. 1994, Borovsky 2003). TMOF is also active in larval Lepidoptera (Nauen et al., 2001). Finally, the metabolism of conjugated TMOF by a model protease, leucine aminopeptidase, was investigated to determine the effect of polymer conjugation on protein degradation including the influence of polymer molecular weight. This presentation reports on the progress of this research.

Materials and Methods

Synthesis of PEGylated Insulin and TMOF

The routes for the syntheses of PEG-conjugated insulin and TMOF are shown in Schemes 1 and 2. PEG polymers with variable molecular weight were conjugated to commercially available bovine insulin or TFA-TMOF-K. In the case of the latter, the PEGylated TMOF-K conjugates were deprotected to release TFA prior to studies of the biological activity. The conjugation of PEG to TMOF was made on the polyproline end of the decapeptide since previous structure activity studies have shown that this part of the hormone is less important in insecticidal activity. Bulk samples of product were purified using preparative scale RP-HPLC, and the ESI-MS data were consistent with the production of the PEG-tethered products as described.

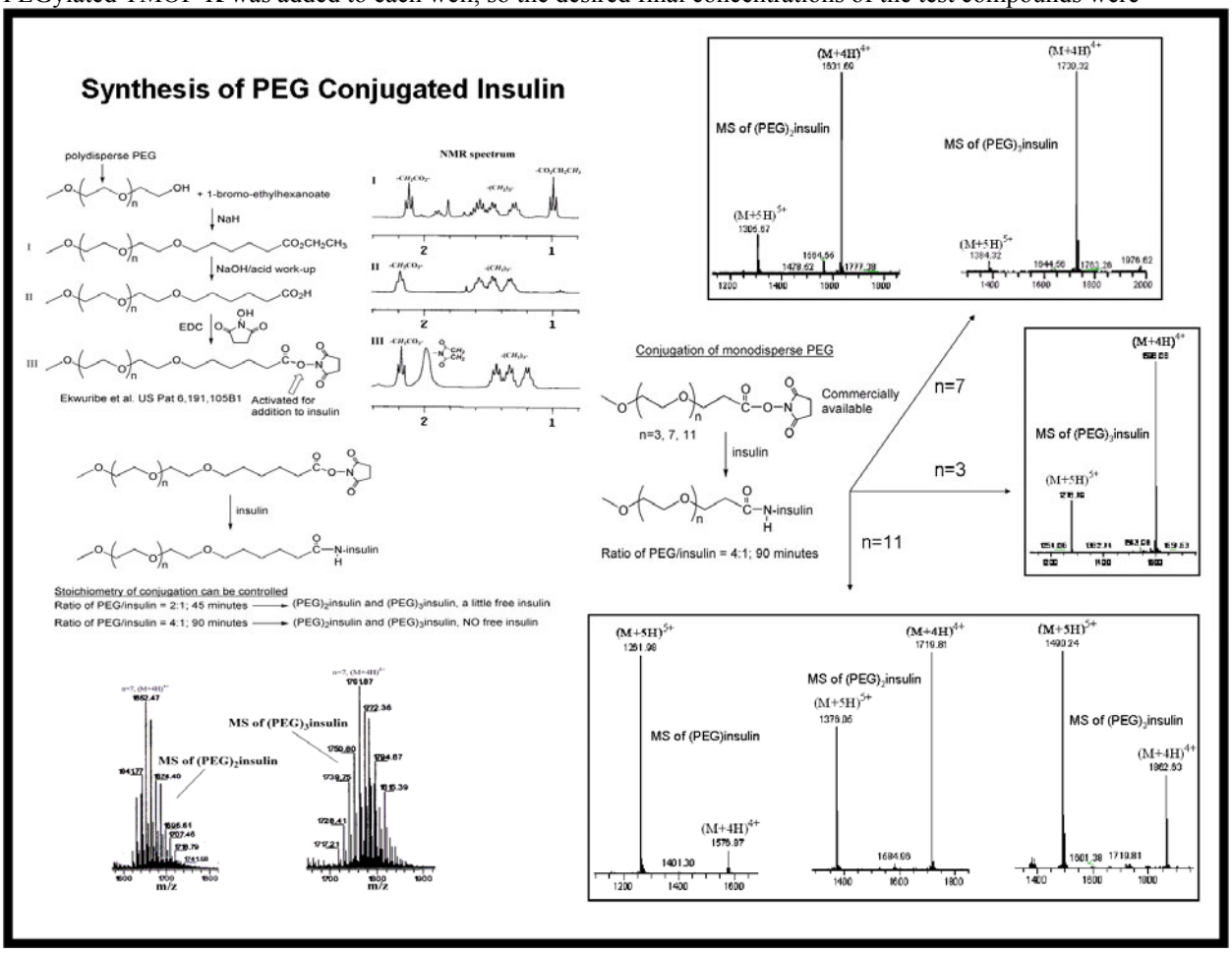
Movement of PEGylated Insulin Across Digestive System and Cuticle

Tobacco budworms, *Heliothis virescens*, were obtained as fourth instars on artificial diet (Burton, 1970) in 28 mL plastic cups (Solo, Woodridge, IL, USA) from the Department of Entomology at North Carolina State University (Raleigh, NC, USA). Bioassays were conducted with larvae that had completed their feeding in the fourth stadium, were removed from the diet, and were allowed to undergo ecdysis. Feeding was conducted using hydrateable meal pads and hemolymph collected as described before from this laboratory (Jeffers et al., 2005). For studies of the movement of insulin across the cuticle, the compound was dissolved in DMSO at the level of 5 mg/mL and topically applied on the dorsum of day 0 fifth stadium larvae. Hemolymph was collected as described before (Jeffers et al., 2005). For the feeding and topical studies, hemolymph was collected 6 and 4 h, respectively, after the initiation of the treatment. Insulin levels in hemolymph were measured by ELISA using a commercial assay (Mercodia, Uppsala, Sweden).

Insecticidal Activity of PEGylated TMOF-K Conjugates Against Mosquitoes

Yellow fever mosquitoes, *Aedes aegypti* were obtained from Dr. Charles Apperson (Department of Entomology, NCSU). The colony was originally collected by Tulane University in New Orleans, LA and was reared at NCSU under the conditions of 27°C, 75% relative humidity, and 14:10 light:dark (included in the light phase are two 30 minute crepuscular periods). The median lethal concentration (LC50) for TMOF, TMOF-K, and PEGylated TMOF-K was estimated for larvae of *A. aegypti* using a static five day bioassay modified from an existing protocol from this laboratory (Vanderherchen, 2005). Day 1 first stadium *A. aegypti* were transferred singly to individual wells of a 96-well microtiter plate in approximately 20 µL of sterile distilled water. All water was removed and 150 µL of a 0.15% 1:2 yeast (Brewers-debittered, inactive; ICN Biochemicals, Cleveland, OH): liver (MP Biomedicals, Solon,

Ohio) solution was immediately added to each well. An aqueous solution (50 µL) of TMOF, TMOF-K, or PEGylated TMOF-K was added to each well, so the desired final concentrations of the test compounds were

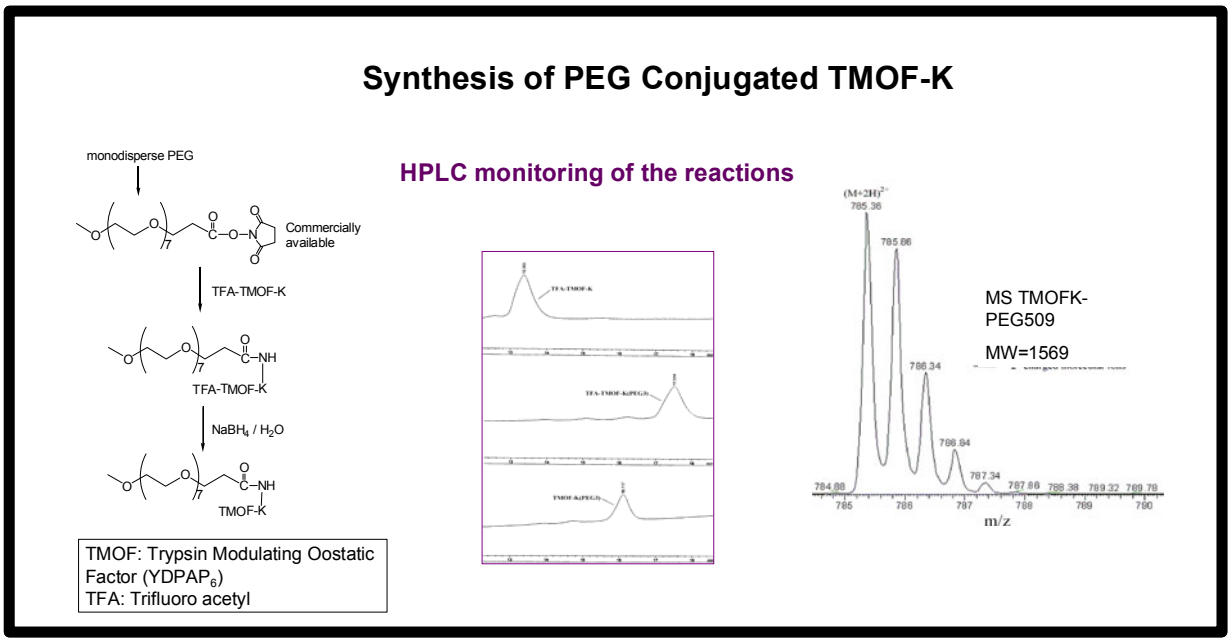


Scheme 1. Synthesis of insulin-PEG conjugates.

obtained in a total volume of 200 µL per well. Plates were covered with plastic transparent lids and placed in sealed plastic containers. The floors of the containers were covered with water before sealing. Larvae were incubated at 27°C, 75% relative humidity, and 14:10 light:dark phase as described before. Mortality was determined at 24 h intervals for five days and was defined as the cessation of contraction of dorsal longitudinal muscles and failure to respond to a blunt probe in 10 sec. In all LC50 estimations, treatments were replicated three times with 10 insects per replicate. Similar studies were conducted with the Asian tiger mosquito, *Aedes albopictus*, also provided by the Dearstyne Laboratory. The LC50 was calculated by log-probit analysis (SAS, Cary, NC).

In Vitro Metabolism of PEGylated TMOF by Leucine Aminopeptidase

Leucine aminopeptidase was activated according to the manufacturer's directions. In a test tube, the following were added via pipet: 0.04 mL of manganese (II) chloride aqueous solution (0.025 M), 0.25 mL of Tris buffer (0.5 M, pH 8.5) and 0.01 mL of the enzyme stock solution. The mixture was incubated at 40 °C for approximately 1 h. Activated leucine aminopeptidase (0.01 mL) was added to the mixture of TMOF-K or PEG conjugated TMOF-K (0.3 mM, 0.25 mL), Tris buffer (0.5 M, 0.02 mL, pH 8.5), and magnesium (II) chloride aqueous solution (0.0625 M, 0.02 mL). The mixture was then incubated at 40 °C for variable time periods, and the reactions quenched by addition of reagent grade trifluoroacetic acid to a final concentration of 1% followed by filtration through a 0.45 µm millipore filter. The filtrates were analyzed by RP-HPLC with a Microsorb-MV column (C-18, 5 microns, 250 × 4.6 mm; flow rate, 1 mL/min). Detection was by UV at 220 nm. The following gradients were used: (a) for the analysis of TMOF-K and TMOF-K(methyl(ethyleneglycol)$_{11}$-O-propionyl) when the concentration of enzyme stock solution



Scheme 1. Representative synthesis of a PEGylated TMOF-K conjugate. Mass spectrometry data on the right show the anticipated peaks for the dicationic substrate $\{(M+2H)^{2+}\}$ where M = PEGylated TMOF-K.

was lower than 300 U /mL, 10% to 35% acetonitrile (0.1% trifluoroacetic acid) in water (0.1% trifluoroacetic acid) from 0 to 30 min; (b) for the analysis of TMOF-K(methyl(ethyleneglycol)$_3$-O-propionyl), 10% to 35% acetonitrile (0.1% trifluoroacetic acid) in water (0.1% trifluoroacetic acid) from 0 to 50 min; (c) for the analysis of TMOF-K(methyl(ethyleneglycol)$_7$-O-propionyl), 10% to 30% acetonitrile (0.1% trifluoroacetic acid) in water (0.1% trifluoroacetic acid) from 0 to 120 min; and (d) for the analysis of TMOF-K(methyl(ethyleneglycol)11-O-propionyl) when the concentration of enzyme stock solution was higher than 500 U /mL, 10% to 25% acetonitrile (0.1% trifluoroacetic acid) in water (0.1% trifluoroacetic acid) from 0 to 180 min. The degradation products were also analyzed by ESI/MS to confirm structure.

Results and Discussion

Movement of a model protein across the insect digestive system and cuticle.

The ability for proteins to move across the digestive system of insects and other arthropods was recently reviewed by Jeffers and Roe (2008). In addition to intact movement of proteins normally found as part of the insect diet, proteins artificially added to an insect meal can also transition into the hemolymph. These results suggest that the mechanism of movement is not exclusively receptor mediated, and the consensus view is that proteins move across the cells lining the gut without the need for the formation of vacuoles. The rate of movement in some cases is unrelated to molecular size, and proteins retain their primary and multimeric structure as well as function when they enter the hemolymph. For BSA (bovine serum albumin), the same multimeric forms of the protein (monomer, dimer and trimers) were found in the same relative proportions both in the diet and hemolymph (Jeffers et al., 2005).

In the current study it was not surprising that the movement of bovine insulin from the artificial diet of the tobacco budworm into hemolymph was found (Fig. 1). Bovine insulin was used in these studies because of its expected benign effects on the physiology of the insect. The accumulation of insulin in the blood was enhanced by pegylation for both polymers tested, PEG350- and PEG333-insulin (Fig. 1). If the assumption is made that the protein does not affect digestive function or the overall physiology of the insects, then this increased accumulation in hemolymph can

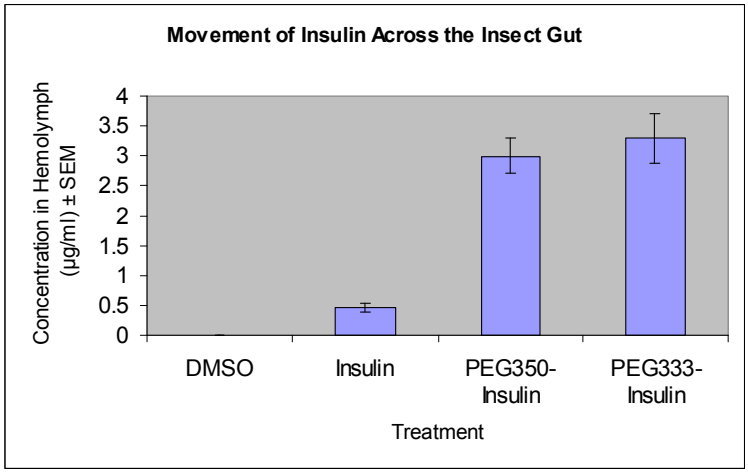


Figure 1. Movement of insulin and PEG-insulin conjugates across the digestive system {PEG350 is the polydisperse PEG-hexanoate substrate and PEG333 is methyl(ethyleneglycol)$_3$-O-propionyl}.

be attributed to a decreased degradation rate of the protein in the gut lumen, an increased rate of movement across the digestive system, a decrease degradation rate in blood, a decreased clearance rate from the hemolymph, or a combination of one or more of these factors. As will be shown later in this paper, we have evidence that the degradation rate *in vitro* by a model proteases is decreased with other PEGylated proteins.

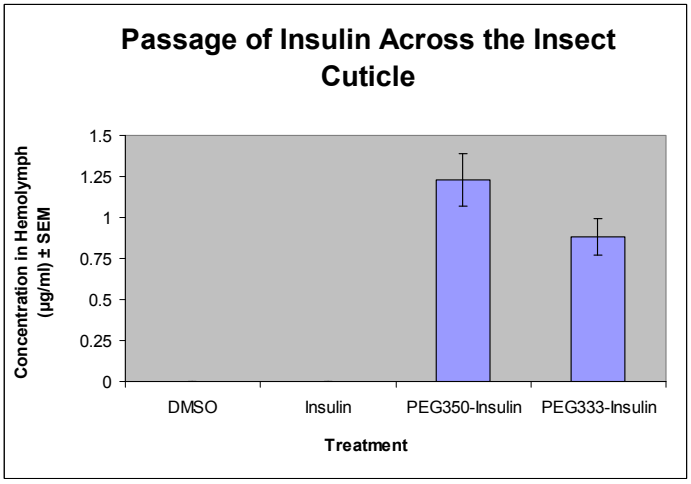


Figure 2. Movement of insulin across insect cuticle {PEG350 is the polydisperse PEG-hexanoate substrate and PEG333 is methyl(ethyleneglycol)$_3$-O-propionyl}.

Data suggest that conjugation of PEG also enhances the movement of insulin across the insect cuticle. When insulin was applied in DMSO topically, no protein could be found in the blood. However, the dorsal application of PEG350- and PEG333-insulin resulted in detectable protein in the hemolymph. These results were unexpected and the method of transport and site of movement is unknown. Whether the protein is moving across the cuticle on the dorsum or is being transferred at joints, at the base of setae, through spiracles or other sites is not known. The placement of the treatment on the dorsum was made to minimize oral introduction. However, we cannot rule out transfer of the protein to the rearing cup and then by some other method including oral from the rearing cup into hemolymph. For both the feeding and topical studies, special care was made to ensure that contamination of the hemolymph did not occur from the surface of the insect during bleeding.

One problem in the studies just described is that the attachment of PEG polymers to insulin can affect antibody-antigen interaction, the important recognition factor in ELISAs. Additional physical methods like LC-MS or MS-MS will be needed in the future to confirm the reported results.

Effect of PEGylation on the Insecticidal Activity of TMOF

The median lethal concentration (LC50) for TMOF, TMOF-K, and PEGylated TMOF-K was estimated for day 1 first stadium larvae of the yellow fever mosquito, *A. aegypti,* using a static 5-day bioassay. The following concentrations were used to assess the LC50 of TMOF for *A. aegypti*: 0.3, 0.4, 0.5 and 0.6 mM. The following concentrations were used to assess the LC50 of TMOF-K for *A. aegypti*: 1.2, 1.0, 0.8, 0.6 and 0.4 mM. The following concentrations were used to assess the LC50 of PEGylated TMOF-K for *A. aegypti*: 0.15, 0.125, 0.1, 0.075 and 0.05 mM. Ten mosquito larvae were used for each treatment, and the assays were replicated three times. The LC50 values for TMOF, TMOF-K, and PEGylated TMOF-K conjugates of *A. aegypti* were 0.5, 0.8, 0.08 mM, respectively (Fig. 3). The attachment of PEG to TMOF-K increased the mosquito insecticidal activity of the protein for *A. aegypti*.

Results

LC50s after 5 day test

	Aedes aegypti ***(n=10) x 3***	***Aedes albopictus*** ***(n=10) x 3***
TMOF	**0.46mM**	**0.7mM**
TMOFK	**0.80mM**	**1.5mM**
TMOFK-PEG(7)	**0.08mM**	**0.3mM**

Figure 3. Medium lethal dose of compounds tested against two species of larval mosquitoes. {TMOFKPEG(7)= TMOF-K(methyl(ethyleneglycol)$_7$-O-propionyl)}.

For comparative studies, the median lethal concentration (LC50) for TMOF, TMOF-K, and PEGylated TMOF-K was also estimated for day 1 first stadium larvae of the Asian tiger mosquito, *Aedes albopictus*. The following concentrations were used to assess the LC50 of TMOF for *A. albopictus*: 0.3, 0.4, 0.5 and 0.6 mM. The following concentrations were used to assess the LC50 of TMOF-K for *A. albopictus*: 1.2, 1.0, 0.8, 0.6 and 0.4 mM. The following concentrations were used to assess the LC50 of PEGylated TMOF-K for *A. albopictus*: 0.15, 0.125, 0.1, 0.075 and 0.05 mM. Ten mosquito larvae were used for each treatment, and the assays were replicated three times. The LC50 values for TMOF, TMOF-K and PEGylated TMOF-K of *A. albopictus* were 0.7, 1.5, 0.3 mM, respectively (Fig. 3). The conjugation of PEG to TMOF-K also increased the insecticidal activity of the protein in *A. albopictus.*

These studies show for the first time that PEGylation of a protein can increase its insecticidal activity. Preliminary studies also show that PEGylation also can increase the effect of TMOF by feeding in artificial diet on neonate development of tobacco budworms. There are several explanations for this increased activity in mosquito hemolymph including enhanced accumulation in the hemocoel resulting from decreased degradation rates, increased movement across the gut or cuticle, a reduced clearance rate from the hemolymph in general, an enhanced delivery rate to the receptor site, or any combination of these factors. It is also not known whether PEGylated TMOF retains its biological activity or if the PEG polymer is removed by endogenous esterases in the biological system before eliciting its effect on larval digestion.

Effect of PEGylation on Reducing Protease Degradation of TMOF *In Vitro*

Our previous studies (Thompson et al., 2004) have revealed that TMOF is a substrate of leucine aminopeptidase, a proteolytic enzyme. Leucine aminopeptidase hydrolyzes the peptide bonds involving the leucine carbonyl.

However, it also catalyzes the hydrolytic release of other amino acids located at the *N*-terminal end of various peptides and proteins with free amino groups. In this respect, the first step in TMOF ($YDPAP_6$) degradation by leucine aminopeptidase *in vitro* produces $DPAP_6$ and tyrosine, which is followed by the sequential removal of additional amino acids on the *N*-terminus as described before from this lab (Thompson et al., 2004). In the current study, TMOF-K and PEGylated TMOF-K substrates were incubated with leucine aminopeptidase *in vitro* as a model system. These efforts were initiated to investigate the influence of the PEG polymer on protease degradation of TMOF-K and to establish proof of concept that this approach might be used as a novel method to stabilize insecticidal proteins to digestion. Incubation times and reactant concentrations were chosen to focus only on the removal of tyrosine to produce $DPAP_6K$ or the PEG conjugate of $DPAP_6K$.

TMOF-K ($YDPAP_6K$) (0.25 mM final concentration) was incubated *in vitro* with leucine aminopeptidase (0.11 U/mL final concentration, 100 U/mL stock solution concentration). The mixtures were analyzed by HPLC after 0, 15, 30, and 60 min of incubation. At the beginning of the incubation, only the peak of TMOF-K was detected by HPLC. The mixture was also analyzed by mass spectrometry. The peak of TMOF-K at 588.63 $\{(M+2H)^{2+}\}$ was detected with no other peaks found in the range (m/z) of 500 to 600. During the 60 min of incubation, the peak due to TMOF-K in the HPLC decreased in intensity and two new peaks due to tyrosine and $DPAP_6K$ appeared and increased. After 60 min of incubation, only 2.1% (Fig. 4) of the original TMOF-K remained (compared to the initial HPLC data). After 30 min of incubation, the mixture was analyzed by mass spectrometry. The peak of TMOF-K at 588.54 $\{(M+2H)^{2+}\}$ and a new peak due to $DPAP_6K$ at 507.02 $\{(M+2H)^{2+}\}$ were detected. No peaks were detected by HPLC upon injection of the enzyme and the reaction buffer in the absence of TMOF-K.

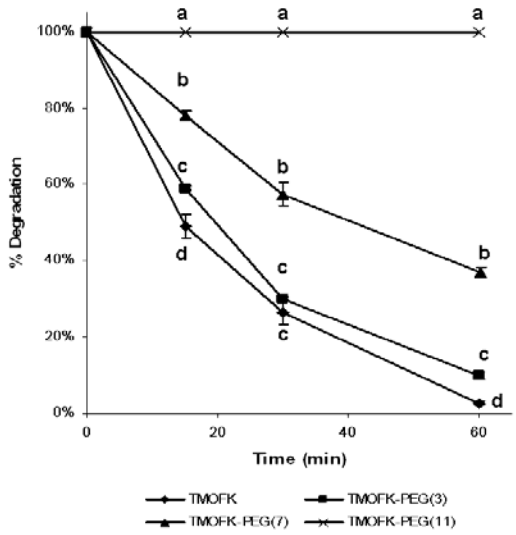


Figure 4. In vitro degradation of TMOF and PEGylated TMOF. Letters indicate statistical differences {alpha less than or equal to 0.05, n=2 (LSD)}. Error bars plus or minus one standard error of the mean {TMOF-K(methyl(ethyleneglycol)$_3$-O-propionyl) = TMOFKPEG(3), TMOF-K(methyl(ethyleneglycol)$_7$-O-propionyl) = TMOFKPEG(7) and TMOF-K(methyl(ethyleneglycol)$_{11}$-O-propionyl) = TMOFK-PEG(11)}.

The same experiments were performed with the PEGylated TMOF-K conjugates TMOF-K(methyl(ethyleneglycol)$_3$-O-propionyl) and TMOF-K(methyl(ethyleneglycol)$_7$-O-propionyl). At the beginning of the incubation periods, the HPLC data revealed single peaks for PEGylated TMOF-K substrates. At this time analysis, mass spectrometry revealed peaks due to TMOF-K(methyl(ethyleneglycol)$_3$-O-propionyl) and TMOF-K(methyl(ethyleneglycol)$_7$-O-propionyl) in the range (m/z) of 600 to 700 and 690 to 790. TMOF-K(methyl(ethyleneglycol)$_3$-O-propionyl) and TMOF-K(methyl(ethyleneglycol)$_7$-O-propionyl) were degraded by leucine aminopeptidase at longer incubation times, and -$DPAP_6K$(methyl(ethyleneglycol)$_3$-O-propionyl) and $DPAP_6K$(methyl(ethyleneglycol)$_7$-O-propionyl) were produced during the degradation. After 30 min of incubation of TMOF-K(methyl(ethyleneglycol)$_3$-O-propionyl), new peaks due to the formation of $DPAP_6K$(methyl(ethyleneglycol)$_3$-O-propionyl) were detected by mass spectrometry. Similarly, after 30 min of incubation for TMOF-K(methyl(ethyleneglycol)$_7$-O-propionyl), new peaks due to $DPAP_6K$(methyl(ethyleneglycol)$_7$-O-propiony) were detected by mass spectrometry. After 60 min of

incubation of TMOF-K(methyl(ethyleneglycol)$_3$-O-propionyl) and TMOF-K(methyl(ethyleneglycol)$_7$-O-propionyl) there were 10.8% and 38.0% of the original substrates remaining, respectively (Fig. 4).

For the degradation of TMOF-K(methyl(ethyleneglycol)$_{11}$-O-propionyl) by leucine aminopeptidase, HPLC results showed that there was little change after 60 min of incubation with leucine aminopeptidase. The peaks due to TMOF-K(methyl(ethyleneglycol)$_{11}$-O-propionyl) were detected at the beginning of the degradation and at 30 min of incubation by mass spectrometry. However, only a slight amount of DPAP$_6$K(methyl(ethyleneglycol)$_{11}$-O-propionyl) was detected in the mixture after 30 min of incubation at the range (m/z) of 780 to 880 by mass spectrometry. Thus, the degradation of TMOF-K(methyl(ethyleneglycol)$_{11}$-O-propionyl) is substantially inhibited relative to the rate of degradation of TMOF-K, TMOF-K(methyl(ethyleneglycol)$_3$-O-propionyl) and TMOF-K(methyl(ethyleneglycol)$_7$-O-propionyl) (Fig. 4).

Leucine aminopeptidase is an amino peptidase that requires the presence of a free amino group in the substrates (Smith and Polglase, 1949). The free amino group is essential to form an enzyme-substrate complex in the presence of Mn^{2+} (Smith and Spackman, 1955). Because the PEG polymers were connected to the lysine residue of TMOF-K at the *C*-terminal, the *N*-terminal of the TMOF-K and PEGylated TMOF-K had the same amino acid sequence. Thus, PEGylation should not alter the pathway for degradation of TMOF-K. Consistent with this suggestion, the HPLC and MS results demonstrate that the degradation products of TMOF-K and PEGylated TMOF-K substrates are compounds resulting from loss of a tyrosine at the *N*-terminal of these substrates. YDPAP$_6$K (H or PEG) substrates were degraded to DPAP$_6$K (H or PEG). Thus, the results suggest that PEGylation of TMOF-K does not change the mechanism of the TMOF-K degradation. In contrast, although the pathway of the TMOF-K degradation was the same, the rate of the degradation varied substantially. As shown in Fig. 4, the rates of the degradation of PEGylated TMOF-K were lower than that of unconjugated TMOF-K. PEGylation inhibits the degradation of TMOF-K by leucine aminopeptidase. Furthermore, an inverse correlation between the molecular weight of the PEG polymers and rate of degradation was established (Fig. 4). The PEG polymer with the highest molecular weight conjugated to TMOF-K, the TMOF-K(methyl(ethyleneglycol)$_{11}$-O-propionyl) substrate, was not degraded under the same conditions and time constraints as other TMOF conjugates. From these data, we suggest that the conjugation of PEG to TMOF-K inhibits binding of the enzyme and the substrates and/or substrate hydrolysis and release from the enzyme. Thus, the overall substrate turnover is reduced. Further studies are needed to understand the exact mechanism for reduced degradation rates. Because the PEGylation suppresses overall enzyme catalysis, this strategy is applicable as a novel method to inhibit protease degradation of peptidic insecticides both in the insect gut, hemolymph and other tissues and potentially enhance toxicity by reducing the rate of xenobiotic metabolism. It appears at least for TMOF that larger PEG polymers provide greater protection.

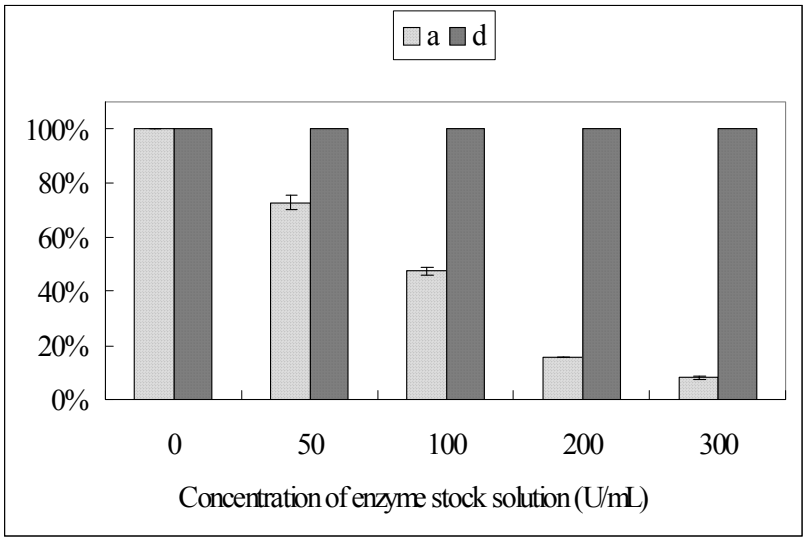


Figure 5. Effect of enzyme concentration on protein degradation *in vitro*. Error bars plus or minus one standard error of mean {a: TMOF-K, d. TMOF-K(methyl(ethyleneglycol)$_{11}$-*O*-propionyl)}.

The *in vitro* degradation of TMOF-K and TMOF-K(methyl(ethyleneglycol)$_{11}$-O-propionyl) (0.25 mM final concentration) at various concentrations of leucine aminopeptidase (0 U/mL, 50 U/mL, 100 U/mL, 200 U/mL and 300 U/L enzyme stock solution) was studied, where each concentration was incubated for 15 min (Fig. 5). No degradation of TMOF-K or the PEGylated TMOF-K conjugates occurred in the absence of enzyme. As anticipated, the rate of degradation of TMOF-K increased with increasing concentration of the enzyme, as would be expected for enzymatic reactions where the substrate is in excess and the formation of an enzyme-substrate complex follows traditional Michaelis-Menton kinetics. However, at concentrations up to 300 U/mL of enzyme stock solution, TMOF-K(methyl(ethyleneglycol)$_{11}$-O-propionyl) was not degraded suggesting that enzymatic turnover for the TMOF polymer is low. The mechanism for this reduced degradation rate over than of TMOF-K is not known. In order to observe degradation of TMOF-K(methyl(ethyleneglycol)$_{11}$-O-propionyl) after 15 min of incubation, concentrations of enzyme stock solution 500 U/mL and greater were required. Degradation studies of TMOF-K(methyl(ethyleneglycol)$_{11}$-O-propionyl) at 500 U, 1000 U and 2000 U enzyme stock solution were also conducted (data not shown). Peaks of DPAP$_6$K(methyl(ethyleneglycol)$_{11}$-O-propionyl) were detected by mass spectrometry at 792.27 $\{(M+2H)^{2+}\}$ when TMOF-K was incubated with leucine aminopeptidase at 1000 U/mL enzyme stock solution. These results demonstrate that TMOF-K(methyl(ethyleneglycol)$_{11}$-O-propionyl) was hydrolyzed by leucine aminopeptidase through the same mechanism as other TMOF conjugates.

Summary

Studies show that aliphatic PEG polymers of a model protein like insulin, which is expected to have minimal or no effect on the physiology of the insect, enhance the accumulation of the protein across the insect digestive system and cuticle in the hemolymph. Aliphatic PEG polymers also enhance the insecticidal activity of the protein TMOF against two species of mosquitoes. *In vitro* degradation studies of PEGylated TMOF with a model protease, leucine aminopeptidase, reduced the hormone metabolism. The larger the PEG polymer, the greater the reduction in turnover rate. These studies *in toto* suggest that PEG polymers can be used to stabilize protein insecticides, enhance their accumulation in the hemocoel and improve insecticidal activity, possible even for proteins that normally would not be able to cross the gut or cuticle barrier.

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