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Novel insecticide polymer chemistry to reduce the enzymatic digestion of a protein pesticide, trypsin modulating oostatic factor (TMOF)

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ABSTRACT

A limiting factor 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 movement across the midgut ventriculus. Trypsin modulating oostatic factor (TMOF) is a *per os* mosquito peptidic larvacide which moves across the digestive system and binds to receptors on the hemolymph side of the gut where the hormone inhibits protease synthesis and food utilization ultimately causing death. In the current study, the *in vitro* degradation of TMOF by the digestive enzyme, leucine aminopeptidase, was inhibited by conjugation of TMOF-K with aliphatic polyethylene glycol (PEG) polymers. Structure activity studies demonstrated a correlation between the molecular weight of the PEG polymer and resistance to digestion and show proof of concept that aliphatic-PEG protein polymerization can be used to prevent protease degradation of a protein insecticide.

1. Introduction

Insect pests have a significant impact on the production of food and fiber [1] and human health [2–8]. Thus, the management of insect pest populations is critical to the sustainability of agriculture and the reduction of diseases transmission. An important tool in insect pest management is insecticides, which traditionally have been small molecules including organochlorines, organophosphates, carbamates, pyrethroids, neonicotinoids, and others. Increasing insect resistance to these insecticides and the possible negative environmental and health impact of at least some of these compounds has resulted in a greater interest in the development of protein insecticides like the highly successful delta-endotoxin of *Bacillus thuringiensis* [9]. Over the past 20 years, numerous proteins and peptides that exhibit insecticidal activity have been discovered [10,11]. Examples include the artificial elevation of juvenile hormone esterase [12,13], the scorpion toxin from *Amdroctonus australis* (AaIT) [14,15], the neurotoxin from the yellow scorpion, *Leuirus quinquestriatus hebraeus* (LqhalphaIT), snail venom from *Conus querciones*, depressive insect toxins BjIT2 from *Buthotus judaicus* and LqhIT2 from *Leiurus quinquestriatus hebraeus* [10], and others. For example, trypsin modulating oostatic factor (TMOF), originally isolated from the ovary of adult *Aedes aegypti*, is a decapeptide hormone of the sequence H-Tyr-Asp-Pro-Ala-Pro_6-OH. TMOF is released into hemolymph 24–48 h after blood feeding, binds to specific gut epithelial cell receptors and inhibits trypsin biosynthesis by exerting a translational control on trypsin mRNA [16,17]. When mosquito larvae ingest TMOF, protein digestion is inhibited, and larvae die from starvation. TMOF and its analogs also inhibit trypsin biosynthesis of other insect species. For example, Nauen et al. have detected that TMOF significantly reduced protein digestion in larvae of the tobacco budworm, *Heliothis virescens* [18]. Roe et al. found that TMOF protein can be digested by proteases as well [19], suggesting that metabolism can reduced its insecticidal activity.

A major limitation to the application of peptidic insecticides is their facile metabolism in the gut lumen and the penetration

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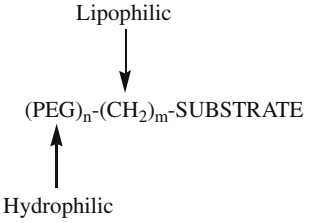
barrier of cell membranes and the insect midgut ventriculus. Modification of proteins to increase their stability in the digestive system and enhance penetration across the gut into hemolymph could increase the prospects for their use as insecticides. A number of compounds have been used for protein stabilization including polymers [20–22] and small molecules [23,24]. Boccu et al. [25] have reported that a protein conjugated to polyethylene glycol (PEG) increases stability against denaturation and enzymatic digestion. PEG polymers conjugated to insulin substantially enhanced resistance to aggregation as well as eliminated protein antigenicity and antibody recognition sites [26]. Approaches to enhance cell membrane penetration include the use of liposomes [27] and encapsulation [28]. However, many of these substrates are unstable in biological systems. Research on the application of these approaches to develop novel protein insecticides was not previously examined.

Our work has focused on the development of chemical delivery systems for insecticides that increase the stability of the peptide and facilitate their accumulation across the insect gut lumen in hemolymph. The strategy includes a hydrophilic PEG polymer that inhibits the degradation of peptides and proteins, with possible linkage of a lipophilic moiety (alkyl groups) to enhance the movement of TMOF across the digestive system as described before for other 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)). As shown in Scheme 1, the relative hydrophilicity and lipophilicity can be controlled by varying "n" and "m". In a preliminary report, we described that one PEG polymer conjugated to TMOF, TMOF-K(CH_3-$(PEG)_7$-*O*-$(CH_2)_2$C(O)), can enhance protein accumulation from the gut into hemolymph and enhance the insecticide toxicity [29]. Herein, we report on the preparation of a series of PEG polymers conjugated to TMOF with various PEG molecular weights "n" (i.e., the number of PEG repeat units is varied) and the first examination of the effect of this polymerization on the *in vitro* degradation by proteases of a protein insecticide. The resistance of PEG polymers conjugated to TMOF to enzymatic degradation and the influence of the PEG molecular weight on resistance is discussed.

2. Materials and methods

2.1. Chemicals

TMOF-K {TMOF ($YDPAP_6$) = trypsin modulating oostatic factor} was obtained from SynPep Corporation (Dublin, CA). TFA–TMOF-K (TFA = trifluoroacetyl) was from Elim BioPharmaceuticals, Inc. (Hayward, CA). Methyl(ethyleneglycol)$_n$-*O*-propionyl-*N*-hydroxy-succinimide esters (n = 3,7,11) were purchased from Pierce Biotechnology, Inc. (Rockfold, IL). HPLC-grade water and acetonitrile with 0.1% trifluoroacetic acid were obtained from Sigma–Aldrich (St. Louis, MO). Leucine aminopeptidase from porcine kidney (EC 3.4.11.1, 12 U/mg solid; 1 U is the amount of enzyme which catalyzes the hydrolysis of 1 μmol L-leucinamide per min at 25 °C at pH 8.5) was purchased from Calzyme Laboratories, Inc. (San Luis Obispo, CA).



Scheme 1. Conjugation of proteins/peptides with polymeric delivery systems can potentially enhance stability and facilitate transport. Potential structure active moieties that affect the isooctane:water co-efficient are shown.

2.2. Instrumentation

Electron spray ion mass spectrometry (ESI–MS) analysis was performed on a LTQ linear ion trap mass spectrometer (Thermo, San Jose, CA). Analytical Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) for analysis of reactions was performed using an HP 1090 System (Hewlett–Packard, Palo Alto, CA). RP-HPLC for analysis of degradation products was performed using an HP 1100 System (Hewlett–Packard, Palo Alto, CA). Preparative-scale RP-HPLC for purification of products from our polymer synthesis was performed by a LabAlliance Prep 200 HPLC System (LabAlliance, State College, PA).

2.3. General synthetic procedure of PEGylated TMOF-K

To a round-bottomed flask charged with TFA–TMOF-K (50 mg/mL buffer) and carbonate buffer (0.1 M, pH 9, 1 mL), 2.5 mol of methyl(ethyleneglycol)$_n$-*O*-propionyl-*N*-hydroxy-succinimide esters (n = 3,7 or 11) were added. The resulting solution was stirred at room temperature and monitored by analytical RP-HPLC with a Microsorb-MV column (C-18, 5 μm, 250 × 4.6 mm; flow rate, 1 mL/min). Detection was by UV at 220 nm. The following gradients of water with 0.1% trifluoroacetic acid and acetonitrile with 0.1% trifluoroacetic acid were used: (a) for analysis of TMOF-K(methyl(ethyleneglycol)$_7$-*O*-propionyl), 10–50% acetonitrile in water from 0 to 16 min and (b) for the analysis of other PEG–TMOF conjugates, 10–50% acetonitrile in water from 0 to 30 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, 5 μm, 250 × 21.4 mm; flow rate 20 mL/min). Detection was by UV at 220 nm. The following gradients were used: (a) for TMOF-K(methyl(ethyleneglycol)$_7$-*O*-propionyl), 10% acetonitrile (0.1% trifluoroacetic acid) in water (0.1% trifluoroacetic acid) from 0 to 5 min and 10–50% acetonitrile (0.1% trifluoroacetic acid) in water (0.1% trifluoroacetic acid) from 5 to 21 min; and (b) for the other reactions, 10% acetonitrile (0.1% trifluoroacetic acid) in water (0.1% trifluoroacetic acid) from 0 to 5 min and 10–50% acetonitrile in water from 5 to 35 min. The appropriate fractions of pegylated TFA–TMOF-K were collected. The solvent volume of the isolated solution was reduced to approximately 5 mL *in vacuo* at 35 °C. 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 (HPLC conditions were identical to those described above) to confirm complete reaction (~8 h). Once HPLC analysis showed complete reaction, the samples were purified by preparative-scale RP-HPLC (HPLC conditions were identical to those described above), lyophilized, reconstituted with deionized water, and stored at −80 °C until used for other studies. The molecular weights of all products were determined by ESI–MS at the Genomic Sciences Laboratory, North Carolina State University (Raleigh, NC). Table 1 provides the molecular ions found for the TMOF conjugates and Fig. 1 the mass spectrum of TFA–TMOF-K(methyl(ethyleneglycol)$_3$-*O*-propionyl) and TMOF-K(methyl(ethyleneglycol)$_3$-*O*-propionyl).

2.4. In vitro degradation of TMOF conjugates

Leucine aminopeptidase was activated according to the manufacturer's directions. In a test tube, the following were pipetted: 0.04 mL of manganese 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