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TRYPSIN MODULATING OOSTATIC FACTOR (TMOF): AN ENVIRONMENTALLY SAFE INSECTICIDE

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Abstract

Trypsin modulating oostatic factor (TMOF) acts in adult female mosquitoes to reduce expression of trypsin and inhibit protein digestion. When fed to mosquito larvae, TMOF also inhibits digestion, causing death by starvation. *In vitro* tests designed to mimic the effect of digestive enzymes on TMOF show that the enzyme leucine aminopeptidase rapidly degraded TMOF from its active form to inactive products. *In vivo* tests of oral toxicity in mouse and duck models and dermal toxicity in a rabbit model indicate that TMOF had no detectable deleterious effects upon ingestion or dermal application. Finally, chronic toxicity testing with *Daphnia magna* indicated that at high levels of exposure, TMOF was not toxic to daphnids, and caused no reduction in number of molts, time to first brood, number of brood, or size of the adult daphnid. Based on these end point indices of toxicology, TMOF appeared to be an environmentally safe insecticide. Chemical and peptidic analogs are under development as TMOF mimics designed to have activity against Lepidopteran pests of agricultural crops, including cotton.

Introduction

Trypsin modulating oostatic factor (TMOF) is a peptidic hormone, originally isolated from the ovaries of adult female mosquitoes (Borovsky, 1985). Upon completing digestion of the blood meal, TMOF levels increase, causing a decrease in the activity of trypsin and other digestive enzymes (Borovsky, 1988). When isolated from ovaries and injected back into female mosquitoes, TMOF inhibits both egg development and blood meal digestion (Borovsky, 1985). Synthetic TMOF fed to mosquito larvae reduces the activity of digestive enzymes in the larvae, leading to their death by starvation (U.S. Patent Number: 5,629,196). Therefore, TMOF is under development as a mosquito larval control method.

The toxicological effects of TMOF in non-target species was required as a prelude to EPA registration of TMOF as a pesticide. A combination of *in vitro* and *in vivo* techniques were used to assay the toxicity of TMOF to mammals, birds and daphnids. We examined *in vitro* degradation of TMOF with digestive enzymes to study the results of a possible accidental ingestion. The *in vitro* experimental results were extended to *in vivo* analysis of oral or dermal exposure to TMOF. Mice and mallard ducks were treated with TMOF orally by gavage, and TMOF was applied to the epidermis of rabbits. In these *in vivo* studies animal models were observed for signs of TMOF-related toxicity. Finally, daphnids were assayed for the effects of chronic exposure to TMOF in the rearing water.

Materials and Methods

Test Materials

TMOF TGAI and KM71H control yeast were provided by Biotechnology Research Institute (Montreal, Canada). TMOF TGAI is a *Pichia pastoris* cell line modified to express the decapeptide, TMOF. KM71H is the control yeast strain. Following fermentation *Pichia* cells are killed by heating the fermented culture for 3 hours at 75°C (no viable cells survive).

Synthetic TMOF was purchased from SYNPEP Corporation, Dublin, CA.

In Vitro Digestion of TMOF

Leucine aminopeptidase (Biozyme, San Diego, CA) was activated and digests of synthetic TMOF were performed according to Biozyme's directions. The digestion was analyzed by Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC)

on a Microsorb-MV 5µm C18 100 Å column. Separation was accomplished using a linear gradient of 10 – 50% acetonitrile in 0.1% TFA/water over 16 minutes at a flow rate of 1.0 mL/min. Absorbance was monitored at 220 nm.

Mouse Acute Oral Toxicity

Acute oral toxicity testing of TMOF was conducted according to EPA OPPTS 870.1100 Guidelines. Testing was done under the auspices and with the approval of the North Carolina State University Institutional Animal Care and Use Committee (Protocol number 99-042-B, approved 29 April 1999, amended and approved 16 November 2000). Briefly, sixteen young adult mice of the CD-1 strain (males and females) were acclimated to local conditions for five days. Mice were dosed orally by gavage with the test material, TMOF TGAI or the control, KM71H, at 2000 mg/kg body weight. General observations were made daily. Weights were taken weekly. At the conclusion of the observation period, mice were euthanized and gross necropsy was performed.

Avian Oral Toxicity

Avian oral toxicity testing of TMOF was conducted according to EPA OPPTS 885.4050 Guidelines. Testing was done under the auspices and with the approval of the North Carolina State University Institutional Animal Care and Use Committee (Protocol number 99-042-B, approved 29 April 1999, amended and approved 16 November 2000). Briefly, ducklings were acclimated to local conditions for five days. Ducklings then were dosed orally by gavage with the test material, TMOF TGAI (thirty ducklings) or the control, KM71H (ten ducklings) at 1250 mg/kg body weight once daily for five days. General observations were made daily. Weights were taken weekly. At the conclusion of the observation period, ducks were euthanized and gross necropsy was performed.

Rabbit Acute Dermal Toxicity

Acute dermal toxicity testing of TMOF was conducted according to EPA OPPTS 870.1200 Guidelines. Testing was done under the auspices and with the approval of the North Carolina State University Institutional Animal Care and Use Committee (Protocol number 99-042-B, approved 29 April 1999, amended and approved 16 November 2000). Briefly, ten New Zealand White rabbits (males and females) were acclimated to local conditions for five days. Rabbits were shaved 24 hours prior to dermal application of TMOF TGAI. TMOF TGAI was mixed with water to make a slurry that was applied to the shaved area and covered with a gauze bandage. Dermal application jackets (Lomir Biomedical, Inc. Malone, NY) were used to keep TMOF TGAI in place for at least 24 hours. After dermal treatment, TMOF TGAI was removed by washing with sterile water. General observations were made daily. Weights were taken weekly. At the conclusion of the observation period, rabbits were euthanized and subjected to gross necropsy.

Daphnid Chronic Toxicity

Daphnid chronic toxicity testing of TMOF TGAI was conducted according to EPA OPPTS 850.1300 Guidelines. Briefly, one daphnid (*Daphnia magna*) neonate was placed in each of ten beakers per treatment. TMOF TGAI or the control, KM71H were added to the culture media at a final concentration of 3×10^6 cells/mL. A second control group was not treated with any additional substances. Beakers were observed daily for molts and offspring. If present, these were removed and counted. At the conclusion of the observation period, each adult daphnid was measured from the top of the head capsule to the base of the tail spine for statistical analysis of overall growth.

Results and Discussion

***In Vitro* Digestion of TMOF**

In order to investigate its potential for degradation to biologically inactive products (as measured by mosquito larval assay, D. Borovsky, pers. comm.) by animal digestion, synthetic TMOF was exposed to the digestive enzyme, leucine aminopeptidase (LAP). LAP is an exopeptidase that cleaves the amino-terminal peptide bond of a polypeptide. Thus, it was expected TMOF would be degraded one amino acid at a time from the amino-terminal end (Table 1). Synthetic TMOF was incubated *in vitro* with LAP for increasing times up to 24 hours followed by analysis on RP-HPLC. The decapeptide, TMOF, has a characteristic elution time (T=10.36 minutes) on RP-HPLC. The products of digestion were identified by retention time on RP-HPLC and co-injection with known standards (Table 1). The results of a typical TMOF digestion are shown in Figure 1. The TMOF concentration rapidly decreased (almost 90% gone) within 2 hours after enzyme addition. The intermediates Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro ($DPAP_6$) and Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro (PAP_6) were not separable by RP-HPLC under these conditions. The $DPAP_6$/PAP_6 intermediates increased rapidly, then the peak height and area decreased as the Ala-Pro-Pro-Pro-Pro-Pro-Pro (AP_6) product increased. AP_6 reached a maximum sometime between 6 and 24 hours. AP_6 did not decline in abundance during the time frame of this experiment. Proline in the penultimate position in AP6 most likely interfered with its digestion by LAP (Hanson and Frohne, 1976). AP_6 has a LC50 (against mosquito larvae) of greater than 1 mM (D. Borovsky, pers. comm.) and is thus considered inactive. Therefore, according to RP-HPLC analysis, TMOF was rapidly degraded to inactive products by the digestive enzyme, LAP.

These conclusions were supported by analysis of LAP digests of synthetic TMOF using liquid chromatography/mass spectroscopy (LC/MS, data not shown). LAP digests of synthetic TMOF were subjected to LC/MS, looking for the presence and abundance of TMOF and its degradation products; $DPAP_6$, PAP_6 and AP_6. The results confirmed that TMOF was rapidly degraded to a level undetectable by HPLC. The intermediates $DPAP_6$ and PAP_6 increased in abundance, then decreased to undetectable levels, leaving AP_6 as the largest detectable molecule. Thus, by a combination of RP-HPLC and LC/MS, we showed that TMOF was rapidly degraded by LAP to its predicted products. Because the end point, AP_6, is inactive against mosquito larvae (D. Borovsky, pers. comm.), we conclude that enzymes of the animal digestive system are sufficient to degrade TMOF to inactive products in the event of accidental ingestion.

Mouse Acute Oral Toxicity

The toxicity of ingested TMOF TGAI was tested in mice. Young adult mice (males and females) of the CD-1 strain were dosed at 2000 mg TMOF TGAI or KM71H control yeast/kg body weight orally by gavage. Clinical observations were made daily and included analysis of mouse behavior and body condition as described in EPA OPPTS 870.1100 Guidelines. Mice were weighed weekly. At the end of the experiment, mice were euthanized and gross necropsies were performed. Over the course of treatment, there were neither negative effects detected through daily clinical observations, nor was there any mortality. Two tailed t-test of weights of KM71H control treated mice against TMOF TGAI treated mice (males and females separately) showed no significant differences between treatments at the 0.05 level (see Table 2). Finally, gross necropsy of treated mice showed no macroscopic lesions. These data provide no indication that TMOF TGAI is toxic to mice when dosed orally by gavage at high dose (2000 mg/kg body weight).

Avian Oral Toxicity

Avian oral toxicology was tested in mallard ducklings. Ducks were dosed at 1250 mg TMOF TGAI or KM71H control yeast/kg body weight once daily for five consecutive days as described in Materials and Methods. Clinical observations were made daily and included analysis of behavior and body condition as described in EPA OPPTS 885.4050 Guidelines. Ducklings were weighed weekly. At the end of the experiment, ducks were euthanized and gross necropsies were performed. Three ducklings died during the thirty-day study. Two were dosed with negative control yeast and one was from the TMOF TGAI treatment. Gross necropsy revealed the cause of death to be accidental gavage to the lungs instead of the crop. Thus, the three duckling deaths were not TMOF-related. There was no additional loss through mortality of any of the ducks. There were no TMOF-related negative effects detected through daily clinical observations. Table 2 shows the weights of ducks treated with TMOF TGAI or control yeast Two tailed t-test of duck weights showed no significant difference (at the 0.05 level) between TMOF TGAI or KM71H treated ducks. Finally, gross necropsy of treated ducks showed no macroscopic lesions of the cranial, thoracic and abdominal cavities. These data provide no indication that TMOF TGAI is toxic to ducks when dosed orally by gavage at high dose (1250 mg/kg body weight for five consecutive days).

Rabbit Acute Dermal Toxicity

Acute dermal toxicology of TMOF TGAI was tested on New Zealand White male and female rabbits. Clinical observations were made daily and included analysis of behavior and body condition as described in EPA OPPTS 870.1200 Guidelines. Rabbits were weighed weekly. At the end of the experiment, rabbits were euthanized and gross necropsies were performed. Over the course of the experiment, there were neither negative effects detected through daily clinical observations, nor was there any mortality. Finally, gross necropsy of treated rabbits showed no macroscopic lesions of the skin, abdominal and thoracic viscera, brain, eyes and bone marrow. These data provide no indication that TMOF TGAI is toxic to rabbits when dosed dermally at high dose (2000 mg/kg body weight).

Daphnid Chronic Toxicity

TMOF TGAI was tested for toxicity to daphnids. TMOF TGAI was added to the rearing water at 1×10^6 cells/mL. Daphnids were observed daily for mortality, number of molts and number of offspring produced. At the end of the experiment, adult daphnid length was measured. Over the course of the experiment, there was no mortality in any of the treatments. The number of molts (Figure 2) and time to first brood (data not shown) were not significantly different in any of the treatments. By the final three days of observation (day 19 – 21) Control yeast and TMOF TGAI yeast treated daphnids produced more neonates than daphnia in the water control (Figure 3, two-tailed t-test, $P<0.05$). Although there was no significant difference in the length of control yeast and TMOF TGAI treated daphnids, these adults were significantly larger than daphnids from the water control ($P<0.05$, data not shown). It is possible that the yeast provided additional nutrition to daphnids. Experiments are ongoing to determine if daphnid culture might be improved by the addition of yeast to the feeding regime. These data provide no indication that TMOF TGAI is toxic to *Daphnia magna* when exposed to a high concentration (1.0×10^6 cells/mL).

Summary and Future Prospects

TMOF has been shown, by a combination of *in vitro* and *in vivo* methods, to be safe to mammals, birds and *Daphnia*. These data provide strong evidence that TMOF when formulated for delivery as a pesticide is unlikely to have any environmental or

human toxicological effects. Currently, studies are underway to extend the range of the TMOF molecule to include pests of agricultural importance (see Vanderherchen et al., 2002). Using the compound, TMOF, as a starting point, structure-activity studies were conducted on the TMOF molecule. The aim of these studies is to synthesize new compounds that would have similar pesticidal action but would be effective against different insects, primarily those of agricultural importance.

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Table 1. Amino acid sequence of TMOF and its leucine aminopeptidase digestion products.

TMOF and its Polypeptide Degradation Products	RP-HPLC Elution Time (min)
Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro (TMOF)	10.36
Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro ($DPAP_6$)	9.73
Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro (PAP_6)	9.73
Ala-Pro-Pro-Pro-Pro-Pro-Pro (AP_6)	8.66

Table 2. Mouse weights after treatment with TMOF TGAI (TMOF) or negative control yeast, KM71H (Control) by gavage. Weights are given ± one standard error of the mean. Days are counted beginning at the day of treatment (Day 0).

	Day 0		Day 6		Day 13		Day 19	
	Control	**TMOF**	**Control**	**TMOF**	**Control**	**TMOF**	**Control**	**TMOF**
Females	24.5±0.9	23.3±0.3	22.9±1.7	21.8±0.9	28.1±1.0	27.6±1.0	27.3±1.3	27.4±1.1
Males	28.3±1.3	29.0±0.3	26.5±0.5	26.2±0.7	33.7±0.4	32.7±1.1	33.4±1.2	32.3±1.2

Table 3. Duck weights after treatment with TMOF TGAI (TMOF) or negative control yeast, KM71H (Control) by gavage. Weights are given ± one standard error of the mean. Days are counted beginning at the day of treatment (Day0).

	Day 0	Day 5	Day 12	Day 19	Day 26	Day 33	Day 40
Females (TMOF)	80.0±3.5	204.4±9.1	411.0±14.6	598.0±24.8	733.0±26.0	882.0±20.7	996.0±19.0
Females (Control)	73.8±6.6	185.0±8.1	356.8±21.4	580.0±43.6	665.0±53.5	843.8±71.8	986.3±90.0
Males (TMOF)	81.1±3.6	206.3±10.3	405.4±17.4	620.0±23.0	766.4±22.4	910.7±21.4	1032.1±25.0
Males (Control)	80.5±10.8	221.5±20.2	368.8±29.7	583.8±38.3	723.8±45.3	907.5±42.2	1085.0±54.4

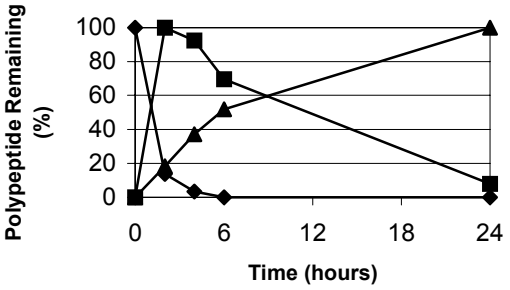

Figure 1. Percent Polypeptide Remaining. The decline of the polypeptide hormone, TMOF (◆) upon digestion with the enzyme leucine aminopeptidase is shown as percent remaining (relative to the maximum detected) over time. As TMOF declines in abundance, the products $DPAP_6$ and PAP_6 increase (■), then decrease over time. The final product, AP_6 (▲) accumulates over the 24 hours of the assay.

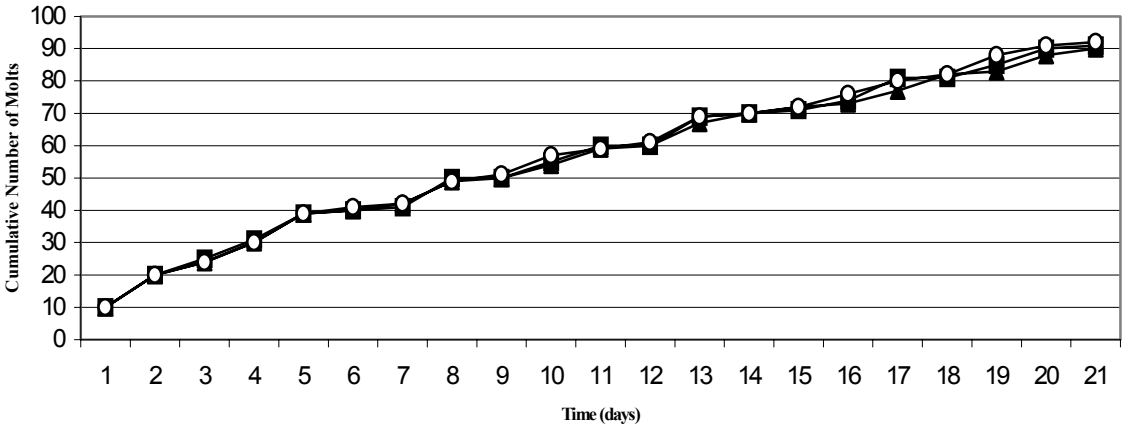

Figure 2. Daphnid Cumulative Molts. The total number of molts (± one standard error of the mean) per treatment (ten daphnids per treatment) is shown. The treatments are TMOF TGAI (○) negative control yeast, KM71H (▲) or a water control (■).

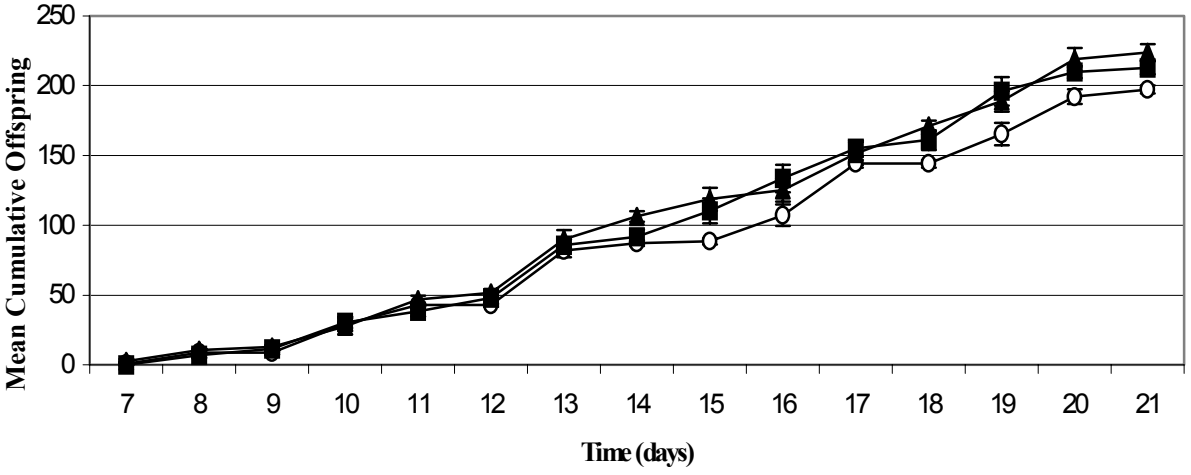

Figure 3. Cumulative Reproduction of Daphnia. The average number of neonates (± one standard error of the mean) produced per daphid is shown. The treatments are TMOF TGAI (■) negative control yeast, KM71H (▲) or a water control (◦).