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Effect of Pentoxifylline on Chitinolytic Enzyme Activity in the Eastern Subterranean Termite (Isoptera: Rhinotermitidae)¹

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Abstract A two-molecule chitinolytic enzyme system (endo- and exo-chitinase) hydrolyzes and degrades the chitin polymers. Therefore, it is imperative to discover novel compounds for inhibiting chitinolytic enzymes to prevent insect growth. This research examined the effect of pentoxifylline (a dimethylxanthine chitinase inhibitor) on inhibition of endo- and exo-chitinolytic enzyme activities in eastern subterranean termite, *Reticulitermes flavipes* (Kollar). Enzyme activities were compared with amounts of treated diet consumed by termites and percent mortality observed over time. Pentoxifylline affected in vitro endo-chitinase activity in a concentration-dependent manner, while having minimal to no effect on in vitro exo-chitinase enzyme activity. However, pentoxifylline treatment affected in vivo endo- and exo-chitinase enzyme activity and caused measurable termite mortality. Moreover, pentoxifylline concentrations did not deter the amount of diet consumed by termites, thereby suggesting that it is palatable. The results of this study support further exploration into termiticidal activity and potential use of pentoxifylline for termite control.

Key Words *Reticulitermes flavipes*, pentoxifylline, chitinase inhibitor

Chitin, a β -(1,4)-linked polymer of *N*-acetyl-D-glucosamine (NAGlc), is one of the most abundant biomolecules (Dahiya et al. 2006). In insects, chitin is found in cuticle of the exoskeleton and the peritrophic membrane lining of midgut. Chitinolytic enzymes are responsible for hydrolysis of chitin linear polymers into NAGlc monomers. Degradation of chitinous structures is carried out by a two-component chitinolytic enzyme system. Insect chitinases (EC 3.2.1.14; Family 18 glycosylhydrolase) act almost exclusively as endo-chitinases by binding and hydrolyzing β -(1,4)-linkages at sites within the chitin polymer (Arakane and Muthukrishnan 2010, Shi et al. 2007). Insect exo-chitinase activity is primarily carried out by β -*N*-acetyl-D-glucosaminidase (NAGase) (EC 3.2.1.52; Family 20 glycosylhydrolase, NAGase). The rapid breakdown of chitin fibers result from the combined action of endo-chitinases and NAGases and ultimately form dimer and trimer oligomers of *N*-acetyl glucosamine (NAG), which are then immediately processed to monomeric NAG by NAGase (Fukamizo and Kramer 1985, Shi et al. 2007).

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Due to the importance of chitin in insects, it is a highly selective target for pest management strategies (Cohen 1993, Hirose et al. 2010). Subterranean termites are destructive pests throughout many temperate regions, with annual worldwide control and repair costs estimated to be US\$32 billion (Rust and Su 2012). Due to their cryptic and destructive nature, elimination of invading subterranean termite colonies within a structure is the ultimate goal of any control practice. One pest management tactic used for control subterranean termite control is a monitoring–baiting program (Grace et al. 2002). For >15 yr, commercialized baiting systems containing benzoylphenylurea (BPU) insecticidal compounds such as hexaflumuron, noviflumuron, and diflubenzuron have been used to control or eliminate invading subterranean termite colonies (DeMark et al. 1995, Prabhakaran 2001, Su 2003, Getty et al. 2005). The BPU active ingredients function as chitin synthesis inhibitors, which disrupt normal molting and alimentary tract (including peritrophic membrane) physiology. The BPU insecticides are relatively nontoxic to mammals due to robust target site protein binding specificity and rapid catabolism to less toxic compounds (Bayoumi et al. 2003).

To achieve physiological homeostasis during periods of digestion (peritrophic membrane) or ecdysis (cuticle molting), chitin synthesis and chitin degradation must be firmly coordinated (Merzendorfer and Zimoch 2003). Insecticidal activity by inhibition of chitin synthesis has been well studied (Deul et al. 1978, Cutler et al. 2005), whereas insecticidal efficacy by inhibition of chitinolytic enzymes remains an area of potential insecticide discovery (Tabudravu et al. 2002). Although few specific NAGase inhibitors have been described, two potent NAGase inhibitors are streptozotocin and *O*-(2-acetamido-2-deoxy- β -glucopyranosylidene)amino-*N*-phenylcarbamate (Horsch et al. 1991, Konrad et al. 2001). Endo-chitinase inhibitors such as allosamidin, argifin, argidin, cyclic dipeptides, and psammaplin A have been studied to evaluate the toxic effects (via topical application and feeding bioassays) in insects–arthropods, including the parasitic blow fly (*Lucile cuprina* Wiedemann), southern cattle tick (*Boophilus microplus* Canestrini), silkworm (*Bombyx mori* L.), webbing clothes moth (*Tineola bisselliella* Hummel), the American cockroach (*Periplaneta americana* L.), the German cockroach (*Blattella germanica* L.), and the green peach aphid or peach-potato aphid (*Myzus persicae* Sulzer) (Sakuda et al. 1987, Londershausen et al. 1996, Blattner et al. 1997, Arai et al. 2000, Shiomi et al. 2000, Houston et al. 2002, Saguez et al. 2006, Huang 2012). However, production costs and availability make these chemicals impractical for implementation into an insecticide baiting program.

Rao et al. (2005a) screened a number of marketed drug molecules and found that the methylxanthine derivative, pentoxifylline, inhibited the Family 18 chitinases. Pentoxifylline (1-(5-oxohexyl)-3,7-dimethylxanthine) is the active ingredient in a drug manufactured by Aventis Inc. (Greenville, DE) under the brand name Trental® that is marketed to improve blood flow, reduce fibrotic lesions induced by radiation therapy, and treat nausea, headaches, and alcoholic liver disease (Delanian et al. 2005). Aside from the Family 18 chitinase inhibition, pentoxifylline also inhibits phosphodiesterase activity (nonselectively activating protein kinase A), as well as production of tumor necrosis factors and leukotrienes (thereby reducing innate immune response) (Marques et al. 1999, Essayan 2001, Peters-Golden et al. 2005). Due to its low production costs and widespread availability, pentoxifylline is an excellent candidate for exploring its insecticidal activity.

Pentoxifylline toxicity via ingestion against the eastern subterranean termite, *Reticulitermes flavipes* (Kollar), has been established (Husen and Kamble 2013, 2014). The time required to reach 50% test population mortalities in response to pentoxifylline treatment via an ingested diet at 3.5 to 70 $\mu\text{g/ml}$ (12.5 to 250 μM) ranged from 32.2 to 44.6 d of feeding on treated diet and significantly differed from untreated control diet. This study examined pentoxifylline treatment effects on eastern subterranean termite endo- and exo-chitinolytic enzyme activities. Enzyme activity levels were compared between amounts of treated diet consumed and percent mortality observed over time.

Materials and Methods

Termites. Termite-infested logs were removed from Wilderness Park Recreation Area (Lincoln, NE) in August 2011 and April 2012, transported to the laboratory, and stored in Roughneck trash cans (121 L; Rubbermaid, Huntersville, NC) under high relative humidity. Prior to experimental use, termites were extracted from the logs and maintained in Plexiglas containers (35 \times 25 \times 10 cm) with moistened substrate (sand, debris from log) and a corrugated cardboard food source. Termites were taxonomically identified to be *R. flavipes* using soldier morphology (Husen et al. 2006, Nutting 1990, Weesner 1965). No-choice pentoxifylline feeding bioassays and corresponding enzyme activity assays were conducted on termites from the same colony. Colony distinction was established by microsatellite genotyping (Vargo 2000). Undifferentiated third- to fifth-instar termite workers were used for all bioassays.

Chemical. Pentoxifylline (solid, $\text{C}_{13}\text{H}_{18}\text{N}_4\text{O}_3$, molecular weight 278.31; Sigma Aldrich, St. Louis, MO; Product no. P1784, Lot no. 059K1682) was used for termite feeding bioassays. Pentoxifylline was dissolved in High Performance Liquid Chromatography (HPLC)-grade acetone (Sigma Aldrich; Product no. 270725, Lot no. MKBD5310) for termite diet treatment. Endo- and exo-chitinase enzyme activities were assayed using a Chitinase Assay Kit (Sigma Aldrich; Product no. CS0980, Lot no. 118K4077).

Pentoxifylline treatment of filter paper diet. Whatman® grade 4 qualitative filter paper disks (55-mm diameter, Whatman, Kent, United Kingdom) were treated with 3 ml of control (acetone only) or pentoxifylline–acetone solution by applying 1.5 ml on each side of the disk. Pentoxifylline was incorporated into diet at 0, 3.5, 7, 14, 28, and 70 $\mu\text{g/ml}$ (0, 12.5, 25, 50, 100, and 250 μM) concentrations. After treatment, the filter paper disks were placed in Takealong® plastic storage containers (669 ml; Rubbermaid) and into a chemical hood overnight at room temperature until the acetone had completely evaporated. The disks were then stored in airtight plastic containers at 4°C until needed in experimental units.

No-choice feeding test. No-choice feeding tests were used to determine diet consumption and termiticidal effects of pentoxifylline treatments. Each experimental unit consisted of a plastic petri dish (100 \times 15 mm; BD Falcon, Becton, Dickenson, and Company, Franklin Lakes, NJ), 20 g of distilled water washed, and oven-dried sand moistened with 4 ml of deionized water, 40 worker termites plus 1–2 soldier termites, and a filter paper diet. Five experimental unit replicates were conducted at each concentration of pentoxifylline for each of five sampling time intervals.

At the onset of the feeding bioassays, experimental units were constructed and stored in 26.5-L Bella plastic storage units (Bella Contemporary Storage, Leominster, MA). High levels of humidity were maintained within the containers by placing moistened paper towels above and below the experimental units. Throughout the course of the bioassays, paper towels were moistened every 2 d. The experimental units were placed in a growth chamber at 23°C and complete darkness for 24 h to allow termites to acclimate to the petri plate units. After the acclimation period, pentoxifylline-treated or control diet was added to each unit.

Prior to the addition of diet, each piece of filter paper was weighed (pretreatment diet weight) using an Ohaus GA110 digital scale (Ohaus Inc., Parsippany, NJ). After diet was added, experimental units were returned to the growth chamber set at 23°C for the duration of the study. Five experimental units from each pentoxifylline concentration were removed from the test container at 1-, 2-, 3-, 4-, and 5-week intervals. At each of these sampling intervals, live termites were counted to determine mortality over time. A termite was considered dead when it was placed on its back and displayed lack of appendage movement when prodded (Mao et al. 2011). Experimental units were observed every 3 d, when dead termites were removed and recorded in an effort to reduce further mortality resulting from cannibalism and/or microbial growth (bacterial or fungal). At all sampling intervals, each diet disk was cleaned with a soft artist brush, air-dried, and then reweighed to calculate diet consumption by the termites (posttreatment diet weight).

In vivo chitinolytic enzyme assays. At the conclusion of each feeding bioassay sampling interval, live termites from all feeding bioassay experimental unit replicates within a pentoxifylline treatment concentration were sorted into a plastic petri dish. From the remaining live termites, three groups of five individual termite workers were randomly chosen (three biological replicates at six treatment concentrations at five sampling intervals) for endo- and exo-chitinase enzyme assays. Termites from each replicate (group of five termites) were homogenized in a microcentrifuge tube using a battery-powered micropestle in 20 μL of ice-cold 0.1 M potassium phosphate (pH 7.6). Samples were then centrifuged at $13,200 \times g$ for 15 min at 4°C, and the resulting supernatant was used for in vivo enzyme assays.

Exo-chitinase (NAGase) activity and endo-chitinase (chitinase) activities were determined based on the enzymatic hydrolysis of *p*-nitrophenol–modified substrates (4-nitrophenyl *N*-acetyl- β -D-glucosaminide and 4-nitrophenyl β -D-*N,N,N'*-triacetylchitotriose, respectively). Hydrolytic action by exo- or endo-chitinase releases the *p*-nitrophenol from the substrate that upon ionization at a basic pH can be measured colorimetrically at 405 nm using a spectrophotometer (Duo-Chuan et al. 2005). Substrates were initially tested using a positive control chitinase from *Trichoderma viride* Pers prior to testing on termites.

Exo-chitinase activity reactions consisted of 1 μL of termite total protein extract and 99 μL of substrate solution (1 mg/ml of 4-nitrophenyl *N*-acetyl- β -D-glucosaminide in 100 mM sodium acetate buffer, pH 5.0). Endo-chitinase activity reactions consisted of 5 μL of termite enzyme extract and 95 μL of substrate solution (0.2 mg/ml 4-nitrophenyl β -D-*N,N,N'*-triacetylchitotriose in 100 mM sodium acetate buffer, pH 5.0). Reactions were incubated at 37°C for 30 min and stopped by adding 200 μL of 400 μM sodium carbonate. Prior to measuring absorbance, endo- and exo-chitinase reactions were diluted with 400 and 800 μL of deionized water, respectively. At each sampling interval–pentoxifylline treatment concentration,

enzyme assays from each biological replicate were performed in triplicate. Enzyme assay controls included blank reactions (substrate solution only) and a standard solution (50 μM *p*-nitrophenol). One activity unit of exo- or endo-chitinase activity was defined as the amount of enzyme activity required to release 1 μmol of *p*-nitrophenol from its substrate per minute at pH 5.0 and 37°C.

In vivo Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) in-gel endo-chitinase assays. An in-gel activity assay was performed using the supernatant obtained from techniques described above to assess the endo-chitinase activities and were measured colorimetrically. A Laemmli-based SDS-PAGE gel system (10% resolving gel, 8% stacking gel) was modified by incorporating 0.02% glycol chitin into the resolving gels (Laemmli 1970). Glycol chitosan (Sigma Aldrich; Product no. G7753, Lot no. 089K1076) was acetylated to glycol chitin (1% stock solution) according to the protocol of Trudel and Asselin (1989). The SDS-PAGE gels were multicast to ensure consistency, and resolved using a mini Protean 3 gel electrophoresis unit (Bio Rad Life Sciences, Hercules, CA). Samples consisted of 10 μL of termite total protein extract plus 10 μL of 2 \times Laemmli SDS-PAGE load buffer; and 15 μL was loaded per lane and electrophoresed at 100 mV for 100 min.

One replica gel was stained with Coomassie blue, while the other was stained for endo-chitinase enzyme activity. Endo-chitinase enzyme activity staining was achieved by immersion in 100 mM sodium acetate buffer (pH 5.0) for 1 h at room temperature, washed with fresh buffer, incubated at 4°C overnight, washed again, and chitinolytic enzyme activity was reconstituted by incubation at 37°C for 2 h. After incubation, gels were stained in 0.01% (w/v) Calcofluor White M2R (solid, fluorescent brightener 28; Sigma Aldrich; Product no. F3543, Lot no. 079K1546) for 15 min, destained in deionized water for 1 h, and activity was visualized using an ultraviolet transilluminator and photographed with a Bio Rad Gel Doc system (Bio Rad Life Sciences) (Kim et al. 2003, Shen and Jacobs-Lorena 1997).

In vitro chitinolytic enzyme assays. Two replicate groups of 25 worker termites (control termites feeding on untreated diet) were homogenized in a microcentrifuge tube using a battery-powered micropestle in 100 μL of ice-cold 0.1 M potassium phosphate (pH 7.6). Samples were then centrifuged at 13,200 $\times g$ for 15 min at 4°C, with the resulting supernatant being used for assays.

Enzyme samples consisted of 10 μL of protein extract, 8 μL of 0.1 M potassium phosphate (pH 7.6), and 2 μL of pentoxifylline (dissolved in acetone) at 0, 28 $\mu\text{g}/\text{ml}$, 280 $\mu\text{g}/\text{ml}$, 2.8 mg/ml , 28 mg/ml , 70 mg/ml , 140 mg/ml , and 280 mg/ml concentrations (0, 100 μM , 1 mM , 10 mM , 100 mM , 250 mM , 500 mM , and 1 M) and were incubated overnight at 4°C prior to use in enzymatic activity assays. In vitro endo- and exo-chitinase activity assays were performed using the same substrates and same experimental procedures as in the in vivo enzyme activity assays. Individual assays within each group replicate were performed in duplicate.

Statistical analyses. Statistical analyses were conducted at 1-, 2-, 3-, 4-, and 5-week (no-choice feeding test) intervals to compare termite mortality, diet consumption, and in vivo endo- and exo-chitinase enzyme activity in response to pentoxifylline-treated diet. Percent mortality was statistically analyzed using the arcsine of the square root transformation (percent mortality transformed to normal distribution) (Yamamura 2002). Transformed percent mortalities were subjected to analysis of variance (ANOVA) using the PROC MIXED procedure to detect

significant differences at $\alpha = 0.05$. Differences between treatment means were evaluated by paired *t*-tests (SAS Institute 2003). Diet consumption in response to pentoxifylline treatment was also analyzed by ANOVA using the PROC MIXED procedure. Means were separated using Fisher least significant difference (LSD) procedure with statistical differences tested by paired *t*-tests ($P \leq 0.05$) (SAS Institute 2003). Endo- and exo-chitinase (in vivo and in vitro) colorimetric enzyme assay data were sorted by sampling time interval and analyzed by ANOVA followed by LSD *t*-tests ($P \leq 0.10$) (SAS Institute 2003). In all statistical tests, ANOVAs were necessary to show significant fixed effects prior to mean separation testing.

Results

Termite mortality response to pentoxifylline-treated diet. Significant mortality resulted from feeding on pentoxifylline-treated diets at the highest concentrations tested, even as early as 1 and 2 weeks when compared to feeding on control diet (Table 1). Specifically, at 1 week, mortality in the pentoxifylline treatments (28 and 70 $\mu\text{g/ml}$) was significantly higher than in the control. Similarly, at 2 weeks, pentoxifylline concentrations of 14 $\mu\text{g/ml}$ or above caused significantly higher mortality than in the control (Table 1). All pentoxifylline-treated diet concentrations resulted in significantly greater mortality versus control at 3 weeks. At 4 and 5 weeks, a general trend of increased mortality in response to pentoxifylline treatment was observed. For example, only the 70 $\mu\text{g/ml}$ (4 weeks) and the 28 $\mu\text{g/ml}$ (5 weeks) pentoxifylline treatments displayed significantly greater mortality than the control treatment (Table 1). Other mean mortality resulting from pentoxifylline treatments were greater than the control, but means were not statistically separable due to high variance about the means (Table 1).

Consumption response to pentoxifylline-treated diet. The treatment comparisons of diet consumption found no significant difference between the pentoxifylline treatment and the control across all time intervals (Table 2). Diet consumption generally decreased as pentoxifylline treatment concentration increased. Consumption levels decreased (not significantly) in the majority of treated diets (3.5 to 28 $\mu\text{g/ml}$ pentoxifylline) from 3 to 4 weeks (Table 2). Only the 70 $\mu\text{g/ml}$ pentoxifylline treatment had less consumption than the control treatment at all sampling intervals. However, when mortality (in experimental units) was taken into account despite variability, it is clear that pentoxifylline does not deter feeding.

In vivo chitinolytic activity assays. When examining in vivo endo-chitinase enzyme activity in response to pentoxifylline treatment, a significant fixed-effect interaction occurred between treatment and time ($F = 19.71$; $df = 20, 150$; $P < 0.0001$). At 1 week, mean percentage activity reduction (normalized to control) ranged from 37.1% to 52.6% (Table 3). Activity resulting from pentoxifylline treatment was significantly reduced in the 3.5 $\mu\text{g/ml}$ ($P < 0.0994$), 7 $\mu\text{g/ml}$ ($P < 0.0927$), and 70 $\mu\text{g/ml}$ ($P < 0.0997$) treatments when compared to activity from the control (Fig. 1). At 2 weeks, mean percent activity reduction ranged from 29.9% to 52.8% (Table 3). Significant endo-chitinase activity reductions resulted from the 14 $\mu\text{g/ml}$ ($P < 0.0883$), 28 $\mu\text{g/ml}$ ($P < 0.0969$), and 70 $\mu\text{g/ml}$ ($P < 0.0493$) pentoxifylline treatments (Fig. 1). At 3 weeks, mean percent activity reduction reached its highest levels throughout the course of the study, ranging from 53.7% to

Table 1. Mortality of eastern subterranean termite workers exposed to pentoxifylline (PX)-treated diet in no-choice feeding tests.

Treatment	Mean % Termite Mortality (\pm SEM)*				
	1 Week	2 Weeks	3 Weeks	4 Weeks	5 Weeks
Control	2.0 \pm 2.5a	3.0 \pm 2.5a	4.0 \pm 3.7a	3.0 \pm 2.5a	3.0 \pm 4.0a
3.5 mg/ml PX	7.0 \pm 5.1ab	21.0 \pm 11.1ab	22.0 \pm 5.1b	28.0 \pm 13.3ab	28.0 \pm 8.7ab
7 mg/ml PX	14.0 \pm 3.7ab	27.0 \pm 9.8ab	26.0 \pm 5.8b	32.0 \pm 27.5ab	40.0 \pm 14.8ab
14 mg/ml PX	16.0 \pm 5.8ab	30.0 \pm 8.4b	40.0 \pm 10.0b	42.0 \pm 19.1ab	55.0 \pm 24.5ab
28 mg/ml PX	24.0 \pm 6.6b	32.0 \pm 4.0b	50.0 \pm 15.8b	41.0 \pm 30.2ab	51.0 \pm 10.2b
70 mg/ml PX	24.0 \pm 5.8b	31.0 \pm 7.4b	45.0 \pm 18.4b	44.0 \pm 15.0b	54.0 \pm 25.2a

* Means within the same column followed by different letters are significantly different based on paired *t*-tests ($P < 0.05$).

Table 2. Mean pentoxifylline (PX)-treated diet consumption by eastern subterranean termite workers in no-choice feeding tests.

Treatment	Mean Diet Consumption (\pm SEM) (mg)*				
	1 Week	2 Weeks	3 Weeks	4 Weeks	5 Weeks
Control	3.3 \pm 1.6a	12.1 \pm 1.8a	16.4 \pm 1.9a	21.2 \pm 4.4a	26.9 \pm 7.2a
3.5 mg/ml PX	5.2 \pm 0.8a	12.1 \pm 2.4a	20.6 \pm 8.0a	20.4 \pm 4.3a	30.6 \pm 3.2a
7 mg/ml PX	3.6 \pm 1.4a	8.8 \pm 1.7a	17.3 \pm 4.6a	14.6 \pm 1.6a	22.5 \pm 6.9a
14 mg/ml PX	3.7 \pm 0.7a	8.6 \pm 2.5a	18.8 \pm 2.3a	16.9 \pm 4.8a	28.0 \pm 4.0a
28 mg/ml PX	2.7 \pm 0.6a	9.0 \pm 2.5a	17.5 \pm 2.2a	14.5 \pm 6.5a	28.9 \pm 7.1a
70 mg/ml PX	2.0 \pm 1.1a	8.9 \pm 6.3a	10.1 \pm 4.9a	19.78 \pm 3.1a	20.5 \pm 4.3a

* Means within the same column followed by different letters are significantly different based on paired *t*-tests ($P < 0.05$).

Table 3. In vivo eastern subterranean termite chitinolytic enzyme activity percent reduction in response to ingestion of pentoxifylline (PX)-treated diet.

Treatment	Mean Endo-Chitinase % Activity Reduction*				
	1 Week	2 Weeks	3 Weeks	4 Weeks	5 Weeks
3.5 mg/ml PX	52.6	31.2	61.9	4.3	41.5
7 mg/ml PX	38.7	29.9	53.7	11.8	38.1
14 mg/ml PX	37.1	36.6	61.8	18.2	33.1
28 mg/ml PX	40.6	47.2	64.5	28.8	36.9
70 mg/ml PX	51.6	52.8	57.4	39.1	27
Treatment	Mean Exo-Chitinase % Activity Reduction*				
	1 Week	2 Weeks	3 Weeks	4 Weeks	5 Weeks
3.5 mg/ml PX	59.9	44.7	36.7	10.8	35.2
7 mg/ml PX	44.8	36	38	21.5	33.8
14 mg/ml PX	65.6	56.1	44.5	11.9	41.5
28 mg/ml PX	53.7	63.3	41.9	16.2	44.4
70 mg/ml PX	57.2	49	42.4	20.5	41

* Mean endo- and exo-chitinase percent activity reductions normalized to activity of control treatment.

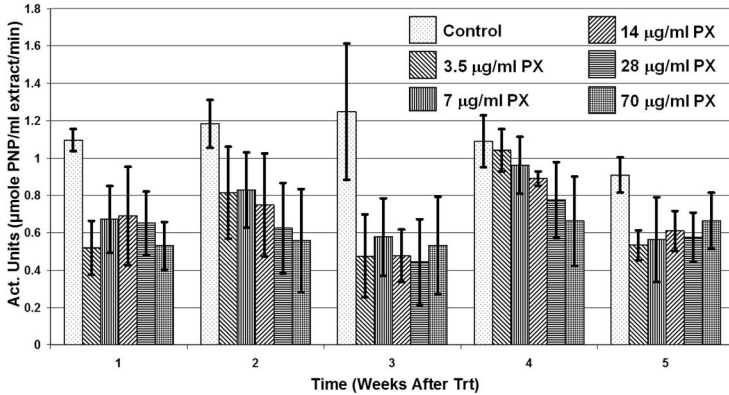


Fig. 1. Mean in vivo endo-chitinase activity in eastern subterranean termites fed pentoxifylline (PX)-treated diets. Error bars represent standard error of the mean. Activity units expressed as 1 μmol *p*-nitrophenol (PNP) released/min/ml of termite extract at 37°C and pH 5.0. Absorbance was measured colorimetrically at 405 nm and compared to PNP standard.

64.5%. Endo-chitinase enzyme activity was significantly reduced at all pentoxifylline concentrations when compared to the control (3.5 $\mu\text{g/ml}$, $P < 0.0683$; 7 $\mu\text{g/ml}$, $P < 0.0564$; 14 $\mu\text{g/ml}$, $P < 0.0711$; 28 $\mu\text{g/ml}$, $P < 0.0645$; 70 $\mu\text{g/ml}$, $P < 0.0658$) (Fig. 1). At 4 and 5 weeks, mean endo-chitinase activity resulting from pentoxifylline treatments was lower than that of the control treatment (ranging from 4.33% to 41.5% activity reduction). However, no significant activity differences were observed between treated and untreated diets.

Analyses of in vivo exo-chitinase enzyme activities in response to pentoxifylline treatment showed a significant interaction between treatment type and time ($F = 3.11$; $df = 20, 150$; $P < 0.0001$). At 1, 2, and 3 weeks, exo-chitinase activity was significantly reduced at all pentoxifylline treatment concentrations (all treatments versus control, $P < 0.005$) (Fig. 2). Percent reductions in mean exo-chitinase activity ranged from 44.8 to 65.6 (1 week), 36.0 to 63.3 (2 weeks), and 36.7 to 44.5 (3 weeks) (Table 3). At 4 weeks, mean percent exo-chitinase activity reduction ranged from 10.8% to 21.4%, but no significant differences were observed between treated and control diets. At 5 weeks, pentoxifylline treatments reduced exo-chitinase activity by 33.7% to 44.4% (Table 3). The 14 $\mu\text{g/ml}$, 28 $\mu\text{g/ml}$, and 70 $\mu\text{g/ml}$ pentoxifylline-treated diet significantly reduced exo-chitinase activity compared to the control treatment (all $P < 0.005$ to 0.001).

In vivo SDS-PAGE in-gel endo-chitinase assays. The SDS-PAGE in-gel chitinase assays showed that numerous endo-chitinase isoforms exist in the whole-termite total protein extract, with enzymes ranging in size from 40 to 100 kD (Fig. 3). When comparing treatment concentrations at one time point or across all sampling intervals, inhibition patterns were variable and no specific isoforms were consistently inhibited.

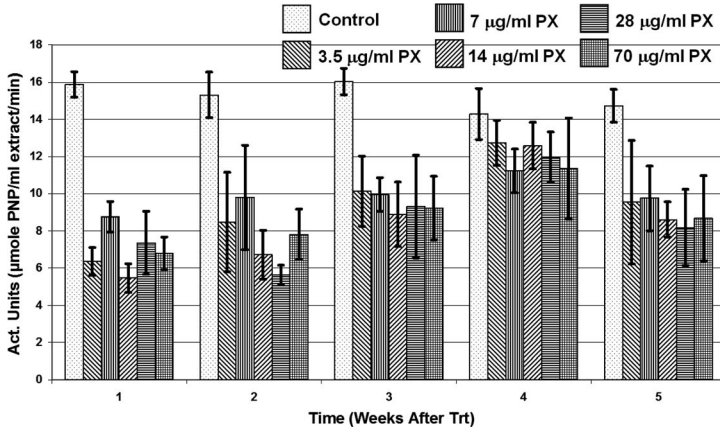


Fig. 2. Mean in vivo exo-chitinase (*N*-acetyl-glucosaminidase) activity within eastern subterranean termites fed pentoxifylline (PX)-treated diets. Error bars represent standard error of the mean. Activity units expressed as 1 μmol *p*-nitrophenol (PNP) released/min/ml of termite extract at 37°C and pH 5.0. Absorbance was measured colorimetrically at 405 nm and compared to PNP standard.

In vitro chitinolytic activity assays. Within the in vitro endo-chitinase activity assays, a significant treatment effect was seen ($F = 1.27$; $df = 7, 24$; $P < 0.0573$). Endo-chitinase percent activity reductions (normalized to control activity) ranged from 6.0% to 31.72% within in vitro pentoxifylline treatments (100 μM to 1 M) (Table 4). When compared endo-chitinase activity to the control, significant endo-chitinase inhibition occurred in response to the 70 mg/ml ($t = 1.78$; $df = 24$; $P < 0.0885$), 140 mg/ml ($t = 2.12$; $df = 24$; $P < 0.0447$), and 280 mg/ml pentoxifylline treatments ($t = 2.34$; $df = 24$; $P < 0.0281$). Within pentoxifylline treatments, the 280 mg/ml (highest in vitro concentration tested) treatment significantly inhibited endo-chitinase enzyme activity when compared to the 28 $\mu\text{g/ml}$ treatment (lowest in vitro concentration tested) ($t = 1.81$; $df = 24$; $P < 0.0833$). No other significant differences of activity inhibition were observed within pentoxifylline treatments.

Analyses of in vitro exo-chitinase enzyme activity in response to pentoxifylline treatments showed no significant fixed effect of treatment on enzyme activity ($F = 0.56$; $df = 7, 24$; $P < 0.7782$) at all concentrations tested. Percent exo-chitinase inhibitions ranged from 0.1656% to 4.945% (Table 4).

Discussion

Chitin synthesis and degradation are essential metabolic pathways in arthropods, and especially important in insect inhibition of the peritrophic membrane and cuticle (Spindler and Spindler-Barth 1994). Thus, chitin metabolism has great potential as a target site for reduced-risk (towards vertebrates) insecticide development (Merzendorfer and Zimoch 2003). Chitin synthesis-inhibiting insecticides have been the subject of considerable research and commercial use over

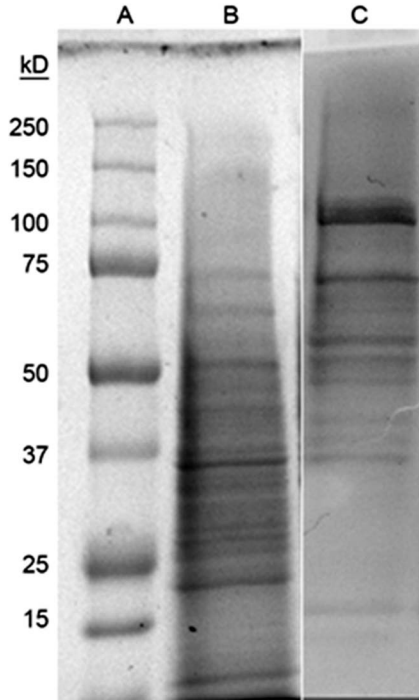


Fig. 3. In-gel endo-chitinase enzyme assay reveals a complex mixture of chitin-degrading enzymes. Termite total protein extracts were resolved on Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels supplemented with 0.02% glycol chitin. Replica gels were stained with Coomassie blue for total protein (A and B) or 0.01% Calcofluor White M2R for chitinolytic activity (C). Chitin-degrading enzymes detected ranged in size from approximately 40 to 100 kD. Lane A, molecular weight markers; lanes B and C, total termite protein extracts.

the last 25 yr. However, research into commercial application of chitinolytic-inhibiting insecticides has been largely unexplored. Recently, chitinase inhibitors have been shown to be potent aphid larvicides when ingested through treated diet (Saguez et al. 2006). Additional research confirmed chitinase inhibitor-treated diet toxicity in larval third- to fifth-instar workers of the eastern subterranean termite, *R. flavipes* (Husen 2012). We examined the effect of pentoxifylline, a Family 18 chitinase inhibitor, on eastern subterranean termite chitinolytic (endo- and exo-chitinase) enzyme activities via pentoxifylline-incorporated diet.

In response to pentoxifylline treatment, we hypothesized that termite mortality would increase, while treated diet consumption would decrease over time in a concentration-dependent manner. Generally, pentoxifylline ingestion significantly impacted termite mortality, and it was positively correlated with concentration.

Table 4. Mean (\pm SEM) percentage in vitro eastern subterranean termite chitinolytic enzyme activity reduction in response to pentoxifylline (PX) treatments.

Treatment	% Endo-Chitinase Inhibition*
28 μ g/ml PX	6.0 \pm 3.6 a
280 μ g/ml PX	10.2 \pm 1.2 a
2.8 mg/ml PX	14.5 \pm 2.4 a
28 mg/ml PX	16.3 \pm 3.6 a
70 mg/ml PX	23.6 \pm 2.4 a
140 mg/ml PX	27.9 \pm 3.8 a
280 mg/ml PX	31.7 \pm 4.0 b
	% Exo-Chitinase Inhibition*
28 μ g/ml PX	0.2 \pm 0.2 a
280 μ g/ml PX	0 a
2.8 mg/ml PX	0.3 \pm 0.3 a
28 mg/ml PX	0 a
70 mg/ml PX	0.6 \pm 0.6 a
140 mg/ml PX	2.5 \pm 2.5 a
280 mg/ml PX	4.9 \pm 1.2 a

* Mean endo- and exo-chitinase percent activity inhibitions are normalized to activity of control treatment. Means within the same column followed by different letters are significantly different based on paired *t*-tests ($P < 0.05$).

However, pentoxifylline-treated diet consumption did not follow this trend. Pentoxifylline treatment increased diet consumption, as at some sampling intervals more diet was consumed in treated versus control units; despite the increased mortality (Table 2). Pentoxifylline treatment did not deter feeding, indicating that pentoxifylline is palatable to eastern subterranean termites.

Within living termites (insect plus symbionts), numerous endo-chitinase and most likely exo-chitinase isoforms exist (Fig. 3). Insect endo-chitinases range in size from 40 to 85 kD (Merzendorfer and Zimoch 2003). In-gel endo-chitinase activity assays from total termite protein extract showed endo-chitinase activity ranging from 40 to 100 kD, suggesting that termite plus microorganism activity are present. In vivo, pentoxifylline treatments significantly reduced both endo- and exo-chitinase activities versus the control treatment (no within-pentoxifylline treatment differences) (Figs. 1, 2; Table 3). However, in vitro pentoxifylline significantly reduced endo-chitinase activity (Table 4), but showed no effect on exo-chitinase activity. Pentoxifylline competitively inhibits Family 18 glycosylhydrolases (endo-chitinases) by forming extensive stacking interactions with conserved tryptophan

residues of the enzyme active site. The mode of inhibitory action of pentoxifylline mimics that of allosamidin on fungal, bacterial, and human chitinases (Rao et al. 2005a, 2005b). Similar to the *in vitro* activity results, allosamidin and its analogue isoallosamidin were shown to significantly affect endo-chitinase activity, while having minimal to no effect on exo-chitinase activity in a *Chironomus* midge cell line (Spindler and Spindler-Barth 1994).

Pentoxifylline treatment significantly reduced both endo- and exo-chitinase enzyme activities *in vivo*. The reduction of *in vivo* endo-chitinase activity was expected; however, the reduction of exo-chitinase activity was unexpected. Activity of both types of chitinolytic enzymes were significantly reduced by 1 week of feeding on treated diet and remained at/or near the same level of inhibition throughout the course of the study (Figs. 1, 2). Peak percent enzyme activity reductions were at 1 and 3 weeks of feeding for exo- and endo-chitinase, respectively (Table 3).

When examining the *in vivo* chitinolytic enzyme activities in the context of diet consumption and termite mortality, two questions emerge. Why does increased pentoxifylline-treated diet consumption not correlate with reduced enzyme activity, and why does mortality increase in a concentration-dependent manner, yet reductions of chitinolytic enzyme activities remain relatively constant?

One possible explanation for both of these questions is the potential effect of pentoxifylline treatment on microorganismal symbionts within the subterranean termite. In a worker subterranean termite hindgut alone, there are an estimated 350,000 bacterial cells (primarily *Streptococcus*, *Bacteroides*, and *Enterbacteriaceae* species) and tens of thousands of individual protists (primarily *Dinenympha*, *Pyrronympha*, and *Trichonympha* species), which are specialized to live in the anaerobic conditions of the termite hindgut (Lewis and Forschler 2010, Schultz and Breznak 1978, Yamaoka et al. 1986). Pentoxifylline and other chitinase-inhibiting molecules are active against bacterial, protist, fungal, insect, crustacean, and human chitinases (Rao et al. 2005a, 2005b). Lewis and Forschler (2010) reported significant impacts on protist populations within eastern subterranean termites fed diet containing one of five commercially used chitin synthesis-inhibiting insecticide active ingredients. Total protist population reduced by $\geq 30\%$ at 3 d of feeding exposure with $>50\%$ total loss by 3 weeks of feeding (four of the five chitin synthesis inhibitors tested) (Lewis and Forschler 2010).

Based on *in vivo* activity results of this study, it is possible that chitinase inhibitors similarly affect bacterial and protist populations. Chitinolytic enzyme activities (both endo- and exo-) seen *in vivo* may directly reflect not only *in vitro* enzyme inhibition (Table 4), but the loss of many endo- and exo-chitinase expressing organisms within the termite itself. Many of the anaerobic bacteria and protists within the termite hindgut play a critical role in cellulose digestion and absorption (Inoue et al. 1997). The loss of these microorganisms would have a significant impact on termite energy metabolism, and the increased consumption of treated diet may possibly be an effort to overcome this loss of nutrient absorption. Whereas, the concentration-dependent trend in termite mortality may be related to the rate at which pentoxifylline defaunates the termite hindgut.

In conclusion, at the whole-termite level numerous endo-chitinase isoforms exist. Pentoxifylline affects *in vitro* endo-chitinase activity (in a concentration-dependent manner) while having minimal to no effect on *in vitro* exo-chitinase enzyme activity.

It affects in vivo endo- and exo-chitinase enzyme activity (but enzyme activity does not decrease in relation to pentoxifylline concentration). It is toxic to termites (overall mortality) in a concentration-dependent manner, and does not affect the amount of diet consumed, suggesting it is palatable to the termites. The results of this study support further exploration and potential use of pentoxifylline for termite control. Future directions of this research might include analyzing in vitro chitinolytic enzyme activity on separate gut fractions (foregut + midgut = termite; hindgut = symbionts) to explore the action of pentoxifylline on termite peritrophic membrane disruption and chitinolytic inhibition of important symbionts. Further research should be undertaken to quantify pentoxifylline treatment effects on bacterial cell numbers and protist species diversity–population sizes in the termite hindgut.

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