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8 # Biochemical characterization of chitin synthase activity and inhibition in the
9 # African malaria mosquito, *Anopheles gambiae*

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16 **Running Head:** *Mosquito chitin synthase activity and inhibition*

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Abstract

Chitin synthase (CHS) is an important enzyme catalyzing the formation of chitin polymers in all chitin containing organisms and a potential target site for insect pest control. However, our understanding of biochemical properties of insect chitin synthases has been very limited. We here report enzymatic and inhibitory properties of chitin synthase prepared from the African malaria mosquito, *Anopheles gambiae*. Our study, which represents the first time to use a nonradioactive method to assay chitin synthase activity in an insect species, determined the optimal conditions for measuring the enzyme activity, including pH, temperature, and concentrations of the substrate UDP–*N*-acetyl-D-glucosamine (GlcNAc) and Mg^{++}. The optimal pH was about 6.5-7.0, and the highest activity was detected at temperatures between 37 and 44˚C. Dithithreitol is required to prevent melanization of the enzyme extract. CHS activity was enhanced at low concentration of GlcNAc, but inhibited at high concentrations. Proteolytic activation of the activity is significant both in the 500xg supernatant and the 40,000xg pellet. Our study revealed only slight *in vitro* inhibition of *An. gambiae* CHS activity by diflubenzuron and nikkomycin Z at the highest concentration (2.5 μM) examined. There was no *in vitro* inhibition by polyoxin D at any concentration examined. Furthermore, we did not observe any *in vivo* inhibition of chitin synthase activity by any of these chemicals at any concentration examined. Our results suggest that the inhibition of chitin synthesis by these chemicals is not due to direct inhibition of chitin synthase in *An. gambiae*.

Key words: *Anophele gambiae*, chitin synthase, diflubenzuron, enzyme inhibition

48 **Introduction**

49 Chitin, a linear polysaccharide of N-acetyl-β-D-glucosamine residues joined by β -1,4 glycosidic
50 linkages, is the second most abundant biological polymer after cellulose (Merzendorfer, 2006;
51 Kramer & Muthukrishnan, 2005). It is widely distributed in arthropods, fungi, nematodes and
52 other Phyla such as annelids, molluscs and coelenterates. In arthropods, chitin is a vital
53 component of the cuticular exoskeleton and thus is crucial for growth and development
54 (Merzendorfer & Zimoch, 2003). Chitin is also found in internal structures of many insects and
55 other arthropods, including the cuticular linings of trachea and in the peritrophic matrixes (PM)
56 lining the gut epithelium (Richards, 1951; Hunt, 1970; Cohen, 2001).

57 Chitin production in arthropods is a complicated process and a series of biochemical
58 pathways are involved in individual chitin polymer biosynthesis in which the terminal step is
59 catalyzed by chitin synthase (CHS, EC2.4.1.16), which is a large transmembrane protein that
60 belongs to the family of *β*-glycosyltransferases. CHS catalyzies the transfer of sugar moieties
61 from activated sugar donors to specific acceptors in all chitin-containing organisms. In insects,
62 chitin synthase contains multiple transmembrane helices reflecting their association with either
63 the plasma membrane or intracellular vesicles such as chitosomes (Tellam *et al*., 2000).

64 As insect and fungi growth and development depend on precisely tuned expression of
65 CHS (Arakane *et al*., 20005, 2008; Merzendorfer, 2006) and chitin is not synthesized by
66 vertebrates, CHS presents an attractive target for combating insect pests and fungi-born diseases
67 (Merzendorfer, 2006). For example, peptidyl nucleosides including polyoxins and nikkomycins
68 are anti-fungi agents which competitively inhibit CHS in fungi and insects (Cohen & Casida,
69 1980b; Zhang & Miller, 1999; Ruiz-Herrera & San-Blas, 2003), whereas benzylphenoloureas
70 (BPUs) such as diflubenzuron are highly effective insecticides which inhibit chitin synthesis in

71 insects (Post & Vincent, 1973; Ishaaya & Casida, 1974; Post *et al*., 1974). Diflubenzuron has
72 been widely used to control various agricultural and public health pests such as mosquitoes and
73 fly larvae since the 1970's. It is extremely toxic to young larvae of many mosquito species
74 (Eisler, 1992; Baruah & Das, 1996; Ali *et al*., 1999; Zhang & Zhu, 2006; Zhu et *al*., 2007).
75 However, the exact mechanisms of chitin synthesis inhibition are still elusive. It is
76 uncertain as to whether BPUs can directly inhibit insect chitin synthase because different studies
77 have yielded inconsistent results. In cell-free chitin synthesizing systems, for example, the BPUs
78 do not inhibit chitin synthesis (Cohen & Casida, 1980b; Mayer *et al*., 1981) or block the chitin
79 biosynthetic pathway between glucose and UDP-GlcNAc in intact larvae (Post *et al*., 1974). In
80 contrast, Nakagawa *et al*. (1993) showed that diflubenzuron and polyoxin D clearly inhibited the
81 incorporation of [^{3}H]-*N*-acetylglucosamine into chitin in isolated intact integument from newly
82 molted American cockroaches. Horst (1981) reported that diflubenzuron can dramatically inhibit
83 CHS activity (approximately 90%) in the crude microsomes and membrane fractions prepared
84 from brine shrimp larvae.
85 CHS activity is often measured by a radioactive assay using [^{14}C] UDP –*N*-acetyl-D-
86 glucosamine (GlcNAc) as a substrate followed by quantization of insoluble ^{14}C-labeled chitin
87 after acid precipitation. The observation that wheat germ agglutinin (WGA) specifically binds to
88 chitin polymer at multiple sites led to the development of a nonradioactive, high throughput
89 screening for antimicrobial agents acting on chitin synthases (Lucero *et al*., 2002). The
90 sensitivity of this nonradioactive assay method was reported to be similar or even slightly higher
91 than that of the radioactive assay. Also, the method was compatible with a variety of assay
92 conditions, performed using low-cost, widely available commercial reagents, and most helpful
93 when multiple determinations of several samples are required. In this study, we characterized the

94 enzymatic and inhibitory properties of the chitin synthase prepared from the African malaria
95 mosquito (*Anopheles gambiae*) by using the nonradioactive assay technique.

96 Materials and methods

97 *Materials*

98 Reagents were purchased from various companies, and each is listed with the company
99 name and catalog number in the parentheses as follows: Trypsin (Sigma-Aldrich, St. Louis, MO;
100 T-1426), soybean trypsin inhibitor (STI) (Fluka BioChemika, WA; 13168), chitin (Sigma-
101 Aldrich; C-9752), protease inhibitor cocktail (Sigma-Aldrich; P-8215), wheat germ agglutinin
102 (WGA) (Bector Lab, Inc. Buringame, CA; L-1020), wheat germ agglutinin-conjugated
103 horseradish peroxidase (WGA-HRP) (Sigma-Aldrich; L-3892), *N*-acetylglucosamine (GlcNAc)
104 (Sigma-Aldrich; A-8625), UDP–GlcNAc (Sigma-Aldrich; U-4375), BCA protein assay kit
105 (Sigma-Aldrich; B-9643), high-sensitivity peroxidase substrate mixtures Colorburst Blue
106 (Alercheck, Inc., Springvale, ME; 90101), 96-well microtiter plates (Corning Incorporated,
107 Acton, MA; 3595), diflubenzuron (Chem Service, West Chester, PA; PS-1028AJ), nikkomycin Z
108 and polyoxin D (Calbiochem, San Diego, CA; 481995 and 529313, respectively), and
109 dithiothretol (DTT) (Sigma-Aldrich; D0632-5G).

110 *Mosquito rearing*

111 A colony of *An. gambiae* obtained from the Malaria Research and Reference Reagent
112 Resource Center (MR4) (Manassas, VA) was maintained in the Department of Entomology at
113 Kansas State University (Manhattan, KS) since 2007 by using the same methods as described by
114 Zhang and Zhu (2006).

115 *Crude enzyme preparation, protein content assay, and pretreatment of the enzyme*

116 Fifty mosquito pupae were homogenized in 1.0 ml of 50 mM Tris-HCI buffer (pH 7.5)
117 containing 20 mM of DTT and 1 mM of $MgCl_2$ for 60s by using a glass-pestle homogenizer.
118 Another 0.5 ml same buffer was used to rinse the homogenizer and combined with the
119 homogenate. The combined homogenate was then centrifuged at 500×g for 10 min to remove
120 unbroken cells, nuclei and debris. The supernatant was carefully transferred to a new tube and
121 used as crude enzyme for following analysis. To obtain the 40,000×g fractions, the supernatant
122 were centrifuged at 40,000×g for 10 min. Then the supernatant was carefully removed and the
123 pellet was resuspended in the same volume of the same buffer. All preparations were conducted
124 on ice or at 4 °C. Protein determination was carried out in microtiter plate using bovine serum
125 albumin as standard by using the BCA method. To pretreat the enzyme, 10 µl of trypsin solution
126 (2 µg/µl in buffer) was added to 250 µl enzyme preparations in a glass tube and incubate for 10
127 min at 30°C followed by addition of 10 µl of STI solution (3 µg/µl in buffer). Ten µl of buffer
128 instead of 10 µl trypsin solution was used as control without trypsin treatment.

129 *CHS activity assays*

130 The assay was based on Lucero *et al*. (2002) with some modifications. In brief, 100 µl of
131 WGA solutions (50 µg/ml in deionized H_2O) were added to each well of the microtiter plate
132 followed by a 16-h incubation at room temperature (23-25 ^oC). WGA solutions were removed
133 by vigorously shaking of the plate content. To wash the plate, the empty plate was immersed in
134 a basket of tap water followed by empty the water in the wells by shaking. This washing was
135 repeated two more times to remove the unbound WGA completely. After washing, the wells
136 were blocked by adding 300 µl of bovine serum albumin (BSA) blocking buffer (20 mg/ml BSA
137 in 50 mM Tris–HCl, pH 7.5) and incubated for 3 h at room temperature. After incubation, the

138 blocking solutions were emptied by shaking. Fifty µl of reaction mixture (5 mM GlcNAc, 1 mM

139 UDP–GlcNAc in 50 mM Tris-HCI buffer, 7.5) were added to the appropriate wells followed by

140 the addition of extraction buffer and pretreated enzyme (20 µl) to a final volume of 100 µl. For

141 each assay the corresponding boiled enzyme preparations (95 ºC for 10 min) was used to assess

142 the background readings.

143 The plate was covered with a sealer and incubated at 37°C for 60 min by shaking at 100

144 rpm using incubator shaker (New Brunswick Scientific; I2400). To stop the reaction, the plate

145 was emptied and washed as described above for five times, followed by the addition of 200 µl

146 WGA–HRP (0.5 µg/ml, in blocking buffer) and incubation for 15 min at 30°C with gentle

147 shaking at 100 rpm. The plate was emptied by vigorous shaking followed by five times washing

148 as described above. Finally, 100 µl peroxidase substrate reagents were added to each well and

149 the optical density (OD) at 600 nm was determined immediately for 3 min. The content of

150 GlcNAc and the chitin synthase activity in the treatments were calculated by using a standard

151 curve. The standard curve was prepared following the same procedure as described by Lucero *et*

152 *al*. (2002). The specific enzyme activity was expressed as nmol GlcNAc.mg^{-1}. $hour^{-1}$. Each

153 experiment was repeated 3-4 times, each with triplicate determinations.

154 *In vitro and in vivo inhibition assay*

155 For *in vitro* inhibition assay, diflubenzuron stock solution (1 mM) was prepared in

156 acetone, whereas polyoxin D (1 mM) and nikkomycin Z (1 mM) were prepared in the solvent of

157 acetone: water (1:1). Before use, diflubenzuron was further diluted to 25, 5, 1 and 0.2 µM by

158 using acetone, whereas polyoxin D and Nikkomycin Z were diluted to 25, 5, and 1 µM by using

159 acetone:water=1:1. Five µl of each solution was added to 25 µl crude enzyme and 20 µl

160 extraction buffer. The final concentrations of difubenzuron in the reaction mixtures were 2.5,

161 0.5, and 0.1, 0.02 μM, whereas polyoxin D and Nikkomycin Z were 2.5, 0.5 and 0.1 μM. Same
162 volume of its own solvent was used as control. The mixture was incubated with shaking at 100
163 rpm at 37 ºC for 20 min and the subsequent procedures were same as the enzyme activity assay.
164 For *in vivo* assay, a series of dilutions of diflubenruon, nikkomycin Z, and polyoxin D
165 were made using acetone. Twenty μl of each chemical were added to a 500-ml glass beaker
166 containing 15 mosquito pupae of 9-h old in 100 ml distilled water and 1 ml fish food. The final
167 concentrations of diflubenzuron were 25, 50, 100, 250, and 500 μg/L, whereas nikkomycin Z and
168 polyoxin D were 100 and 500 μg/L. Same volume of acetone was used as control. After a 24-h
169 (L:D, 16:8) exposure at 25 °C, the pupal mortality was examined and the surviving pupae were
170 collected for crude enzyme preparation followed by enzyme assays based on the same procedure
171 described above. Each control and treatment was repeated four times.

172 *Statistical analysis for enzyme activity*

173 In each assay, the treatment showing the highest specific activity was used as a reference
174 to calculate relative activities in other treatments. The relative activity in percentage was firstly
175 transformed into arcsine square root before one-way ANOVA. Fisher's least significant
176 difference (LSD) multiple comparisons were then used to separate the means of the transformed
177 relative activity or specific enzyme activity among the treatments.

178 **Results**

179 *Chitin synthase activity in mosquito pupae*

180 Previous study showed that both two CHS genes, *AgCHS1* and AgCHS2, are highly
181 expressed in the pupal stage (Zhang *et al*., 2010), implying an intensive synthesis of chitin in the
182 pupal stage. Thus, we used mosquito pupae to prepare the enzyme for all assays in this study. To

183 make a standard curve, homogeneous chitin suspension in acetic acid was used for the assay. A
184 high linear correlation is observed when initial rate was plotted as a function of chitin amounts
185 (Fig. S1). Using this standard curve, we examined CHS activity in the crude enzyme. As shown
186 in Fig. S2, CHS activity linearly increased when low amount of enzyme used and reached a
187 plateau phase as the amount of the enzyme increased further. Thus, specific CHS activity of the
188 crude enzyme could be determined by using the data within the linear phase.

189 *Effects of dithiothreitol (DTT) and* Mg^{++} *on CHS activity*

190 To prevent the enzyme oxidation, we added DTT into the buffer for enzyme extraction.
191 Without using DTT, the crude enzyme turned black within a minute and no chitin synthase
192 activity can be detected by using the oxidized enzyme in the subsequent assay. It is obvious that
193 low concentration is not enough to inhibit the enzyme oxidation completely. However, decreased
194 CHS activity was observed when the concentration of DTT was too high (Fig. 1A). Divalent
195 cations have been reported to stimulate CHS activity in insect and other systems. We found that
196 low concentration of Mg^{++} at 1.0-4.0 mM significantly increased CHS activity, whereas 10.0
197 mM or higher significantly inhibited CHS activity (Fig. 1B).

198 *Effects of UDP-GlcNAc and GlcNAc on CHS activity*

199 The addition of 0.5 mM UDP-GlcNAc to the reaction mixture slightly increased the CHS
200 activity, whereas high concentration of UDP-GlcNAc significantly inhibited CHS activity (Fig.
201 1C). Similarly, GlcNAc at low concentration in the reaction mixture enhanced CHS activity but
202 at high concentration (>10 mM) it inhibited CHS activity (Fig. 1D).

203 *Optimal pH and temperature for CHS activity*

204 To determine the optimal pH and temperature for CHS activity, we examined the CHS
205 activity at different pH and temperature conditions using buffer Tris-HCI. Optimal pH condition
206 appeared to be pH 6.5-7.0 (Fig. 2A), whereas optimal temperature ranged between 37 and 44˚C
207 (Fig. 2B).

208 *Proteolytic activation of CHS activity*

209 To evaluate the effect of proteolysis, we measured CHS activity in the presence of
210 trypsin. CHS activity was increased by about 1.2- and 1.7-fold in the 500xg crude enzyme and
211 the successive 40,000g fractions, respectively, as compared to those of controls without the
212 addition of trypsin (Fig. 3).

213 *Effect of chitin synthesis inhibitors on CHS activity*

214 The larvae of *An. gambiae* were highly susceptible to diflubenzuron. Exposure of the
215 third-instar larvae to diflubenzuron at 50 μg/L resulted in about 60% mortality in 48 h, whereas
216 the classical CHS inhibitors nikkomycin Z and polyoxin D, well established inhibitors of fungal
217 enzymes, exhibited virtually no mortality at 500 μg/L (Table 1). We further examined whether
218 these chitin synthesis inhibitors can inhibit CHS *in vitro*. Very limited inhibition was observed at
219 high concentrations for diflubenzuron and nikkomycin Z, whereas no inhibition on CHS activity
220 was observed for polyoxin D (Fig. 4A). We further exposed the 9-h pupae to these three
221 chemicals under the sublethal concentrations with the mortality within range 0-4.4% (data not
222 shown). The surviving mosquito pupae were collected at 24 h and the crude enzyme were
223 prepared from these pupae. In contrast to the *in vitro* assay, no *in vivo* inhibition to CHS activity
224 was observed in any of these treatments (Fig. 4B).

225 ## Discussion

226 Insects have two chitin synthases encoded by two different genes, including *CHS1* (also known
227 as *CHS A*) and *CHS2* (also known as *CHS B*). *CHS1* is exclusively expressed in the epidermis
228 underlying the cuticular exoskeleton and related ectodermal cells such as tracheal cells, whereas
229 *CHS2* is expressed in midgut epithelial cells and responsible for the synthesis of the PM-
230 associated chitin (Merzendorfer and Zimoch, 2003; Arakane et al., 2005, 2008; Zimoch et al.,
231 2005). Similarly, two CHS genes were also identified in *An. gambiae* (Zhang et al., 2012). Thus,
232 the measurement of CHS activity using crude enzyme preparations in this study comes from the
233 total activity of the two enzymes. However, as the expression of CHS1 is much higher than that
234 of CHS2 (Zhang et al., 2012), therefore the enzyme activity we obtained in this study mainly
235 represents CHS1 activity.

236 Lucero *et al*. (2002) reported the first alternative to the radioactive assay for CHS activity
237 used since 1957 (Glaser & Brown, 1957) and successfully applied the assay for measuring fungal
238 CHS activity. In the current study, we first adapted and applied this method for measuring insect
239 CHS activity. The assay provides us a convenient, rapid, cheap and high throughput method for
240 CHS activity assay. Also, the high sensitivity of the assay allows testing of multiple samples
241 containing low amounts of active enzyme. A comparison between two methods showed that this
242 method is even more sensitive as compared with the conventional radioactive method (Lucero *et*
243 *al*., 2002).

244 WGA is a chitin-binding lectin with high affinity and specificity for GlcNAc. However, it
245 has been reported that the specificity of this chitin binding assay is lower than the radioactive
246 assay (Kramer & Muthukrishnan, 2005). To avoid the effect brought by non-specific binding
247 and the effect by endogenous chitin in the crude enzyme, the same amount of the boiled enzyme

248 was used as a validation control in this study. No significant differences were observed in the
249 boiled control as the amount of the enzyme was increased. This result indicated that the effects
250 of the endogenous chitin and the non-specific binding were negligible in our assay. Thus, the
251 activity detected in the crude enzyme from mosquito pupae reflects the catalytic activity other
252 than artifacts brought by non-specific bindings or endogenous chitin (Fig. S2).

253 Both Mg^{++} and UDP-GlcNAc were found to stimulate CHS activity at low concentrations
254 but inhibit the activity at high concentrations (Figs. 1B and 1C). Relative high CHS activity was
255 also observed even if Mg^{++} and UDP-GlcNAc were not added (Figs. 1B and 1C). These results
256 suggest that the endogenous Mg^{++} and substrate UDP-GlcNAc in the crude enzyme preparations
257 could sustain the CHS activity and thus produce chitin to some extent. Nevertheless, proper
258 levels of Mg^{++} and substrate UDP-GlcNAc are required to CHS to catalyze chitin synthesis. As
259 one mechanism of enzyme activity regulation, substrate inhibition has also been found in other
260 enzyme systems (Shafferman *et al*., 1992).

261 GlcNAc, a molecule that is described as an allosteric activator of fungal CHS activity
262 (Merz *et al*., 1999) has been reported to inhibit enzyme activity at relatively low concentrations
263 (1 mM) for *Manduca sexta* (Zimoch *et al*., 2005) as well as for the stable fly (Mayer *et al*.,
264 1980). Interestingly, we found that GlcNAc stimulated CHS activity at 2.5 mM but inhibited
265 enzyme activity at higher concentrations (Fig. 1D). The mechanism underling the stimulation by
266 GlcNAc for *An. gambiae* CHS remains elusive. To date, the allosteric activation for CHS was
267 only reported in fungal CHS but not in insect CHS.

268 High CHS activity was observed at temperatures between 37 and 44˚C (Fig. 2B). In *M.*
269 *sexta*, high incorporation of UDP-GlcNAc was observed at about 30˚C, and decreased at about
270 40˚C (Zimoch *et al*., 2005). In general, CHS activity depends on the membrane fluidity, and thus

271 CHS activity decreases as temperature increases to >30˚C. Obviously, our results are not
272 consistent with what was observed in *M. sexta* CHS, but may suggest insect species-dependent
273 variations with respect to optimal temperature for CHS activity.

274 The addition of trypsin not only enhanced the enzyme activity in the crude enzyme
275 preparations but also in the 40,000xg fractions (Fig. 3). To date, very limited information on
276 CHS regulation is available. As a post-translational regulation, the addition of trypsin to cell-free
277 extracts leads to the stimulation of chitin synthesis in fungal and insect systems (Cabib & Farkas,
278 1971*;* Cohen & Casida, 1980a; Mayer *et al.*1980; Ward *et al*., 1991; Zimoch *et al*., 2005),
279 suggesting that inactive CHS is synthesized as a zymogen. However, the *in vivo* activation
280 factors of CHS remains to be elusive. In some fungal systems, proteolytic fragments associated
281 with CHS activity have been identified (Kang *et al*., 1984; Machida & Saito, 1993; Uchida *et al*.,
282 1996). In *M. sexta*, trypsin stimulates chitin synthesis in crude midgut extracts but not in
283 membrane fractions. Trypsin-dependent activation was recovered when the soluble fraction was
284 added to the membrane fractions, suggesting that CHS is not directly affected by trypsin but by
285 an unknown soluble factor (Zimoch *et al*., 2005). However, it is difficult to reveal such a soluble
286 factor as the components of the supernatant are complicated. Later, a chymotrypsin-like protease
287 (CTLP1) that interacts with the extracellular carboxyl-terminal domain of CHS2 *in vitro* was
288 identified. Highly conserved trypsin cleavage presented in the CTLP1 amino acid sequence
289 suggests that the CTLP1 precursor is activated by trypsin although direct evidence is still
290 missing. It was further suggested that CTLP1 activated by trypsin could stimulate CHS activity
291 (Broehan *et al*., 2007). A recent study showed that an active, oligomeric CHS complex can be
292 purified from the midgut of the tobacco hornworm (Maue *et al*., 2009). By using purified

293 enzymes, it will be very helpful to study the properties of the CHS and its mechanisms of post-

294 translational regulation.

295 Our study revealed only slight *in vitro* inhibition of *An. gambiae* CHS activity by

296 diflubenzuron and nikkomycin Z at the highest concentration (2.5 μM) examined (Fig. 4A).

297 There was no *in vitro* inhibition by polyoxin D at any concentration examined. Furthermore, no

298 *in vivo* inhibition was observed by any of these chemicals at any concentration examined (Fig.

299 4B). Indeed, there has generally been lack of report showing that these chemicals are capable of

300 inhibiting CHS activity *in vitro* to date. The only report of CHS inhibition by diflubenzuron in

301 insects was observed in American cockroaches, in which the isolated intact integument from

302 newly molted cockroaches was used to examining the incorporation of [^{3}H]-N-

303 acetylglucosamine into chitin (Nakagawa *et al*., 1993). It would be interesting to know whether

304 diflubenzuron can inhibit the incorporation of UDP-GlcNAc into chitin polymers by using the

305 isolated intact integument in *An. gambiae*. Nevertheless, the inhibition of chitin synthesis by

306 diflubenzuron could be due to its effects on other steps of chitin biosynthetic pathways instead of

307 direct inhibition of CHS. Further studies by using purified CHS may help clarify this issue.

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322 University Museum of Entomological and Prairie Arthropod Research, Manhattan, Kansas.

323

324

325 **Disclosure**

326 All authors have declared no conflicts of interest, including specific financial interests and
327 relationships and affiliations (other than those affiliations listed in the title page of the
328 manuscript) relevant to the subject of this manuscript.

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438

439 **Table 1** Toxicity of chitin synthesis inhibitors to third-instar mosquito larvae.

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Treatment		% Mortality at 24 h	% Mortality at 48 h
Chemical	Concentration (μg/L)	(Mean±SE) *	(Mean±SE) *
Control	0	0.0±0.00 d	0.0± 0.00 c
Diflubenzuron	6.25	2.5±1.67 d	4.9± 2.25 c
	12.5	26.3±8.29 c	27.5±8.66 b
	25	41.3±5.95 b	47.5±8.66 a
	50	57.5±2.89 a	60.0±2.36 a
Nikkomycin Z	50	0.0±0.00 d	0.0±0.00 c
	500	1.5±1.70 d	1.5±1.70 c
Polyoxin D	50	0.0±0.00 d	0.0±0.00 c
	500	2.1±1.41 d	2.1±1.41 c

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442 * Same letters indicate no significant difference within each column based on one-way ANOVA
443 followed by Fisher's LSD ($P \geq 0.05$).

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458 **Figure legends**

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460 **Fig. 1** The DTT, Mg^{++}, UDP-GlcNAc and GlcNAc dependency of CHS activity in mosquito
461 pupae. (A) Enzyme activity was dependent on DTT in the enzyme extraction buffer. (B) The
462 Mg^{++} dependent CHS activity. DTT and Mg^{++} were added to the Tris-HCI buffer for crude
463 enzyme preparation. The concentrations in this figure represented the concentrations in the
464 extraction buffer. UDP-GlcNAc (C) and GlcNAc (D) were added in the reaction mixture and the
465 concentrations in the figure represented the final concentration in the reaction system. The CHS
466 activity values are given in percent of maximal activity and as an average (±SEM) of three
467 independent replicates.

468

469 **Fig. 2** Effects of pH (A) and temperature (B) on the activity of CHS prepared from mosquito
470 pupae. The CHS activity values are given in percent of maximal activity and as an average
471 (±SEM) of three independent replicates.

472

473 **Fig. 3** Proteolytic activation of chitin synthesis in different enzyme preparations from the
474 mosquito pupae. Specific CHS activity was measured in the presence (Trp+) and absence (Trp-)
475 of trypsin in 500xg supernatant and successive 40,000xg pellet which was resuspended in
476 proportional volume of the extraction buffer. Asterisks indicate significant difference based on
477 Fisher's LSD ($P \geq 0.05$).

478

479 **Fig. 4** Comparisons of chitin synthase activity in the crude enzyme preparations following
480 incubation with various concentrations of three chitin synthesis inhibitors (A) and the crude

481 enzyme preparations from the pupae exposed to the three chitin synthesis inhibitors (B). DF:

482 diflubenzuron; PD: polyoxin D; NZ: nikkomycin Z. Same letters on the error bars indicate no

483 significant difference based on Fisher's LSD ($P \geq 0.05$).

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511 **Fig. 1**

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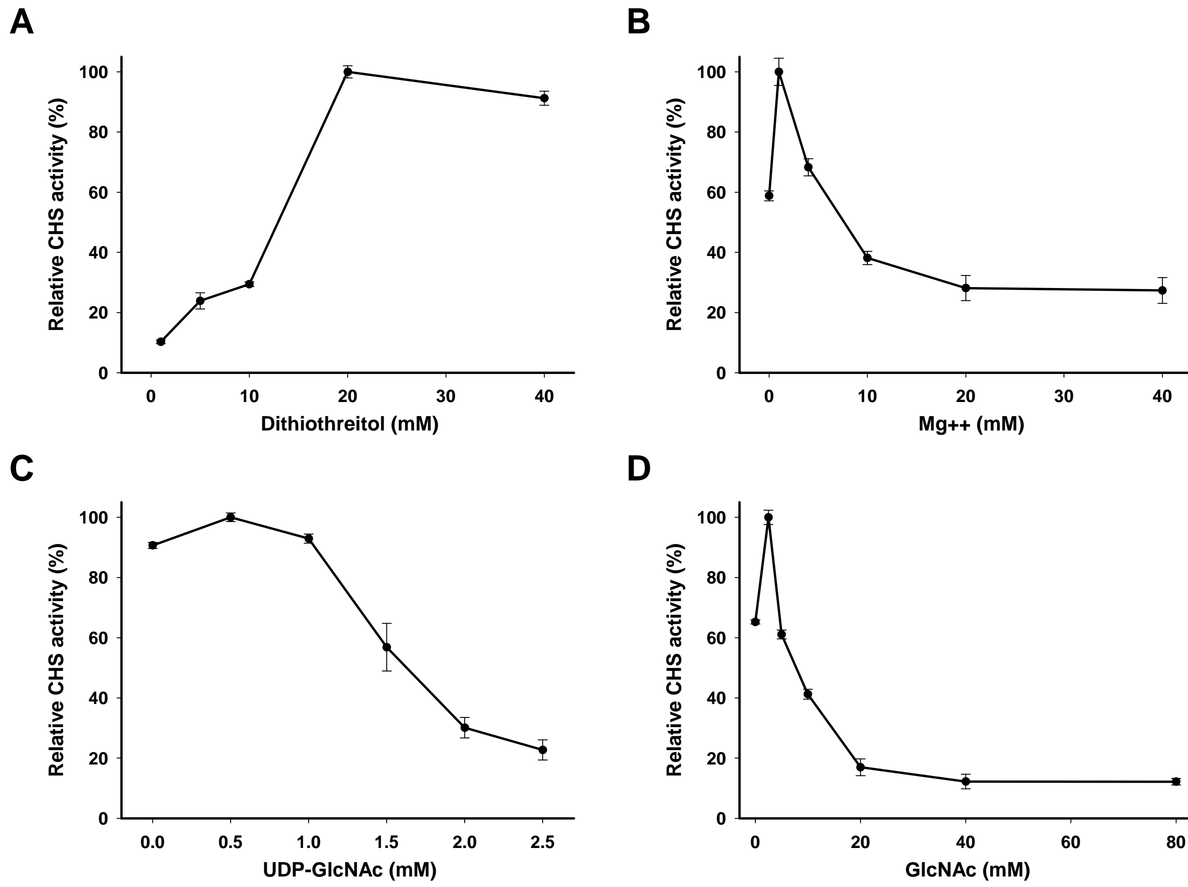

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523 **Fig. 2**

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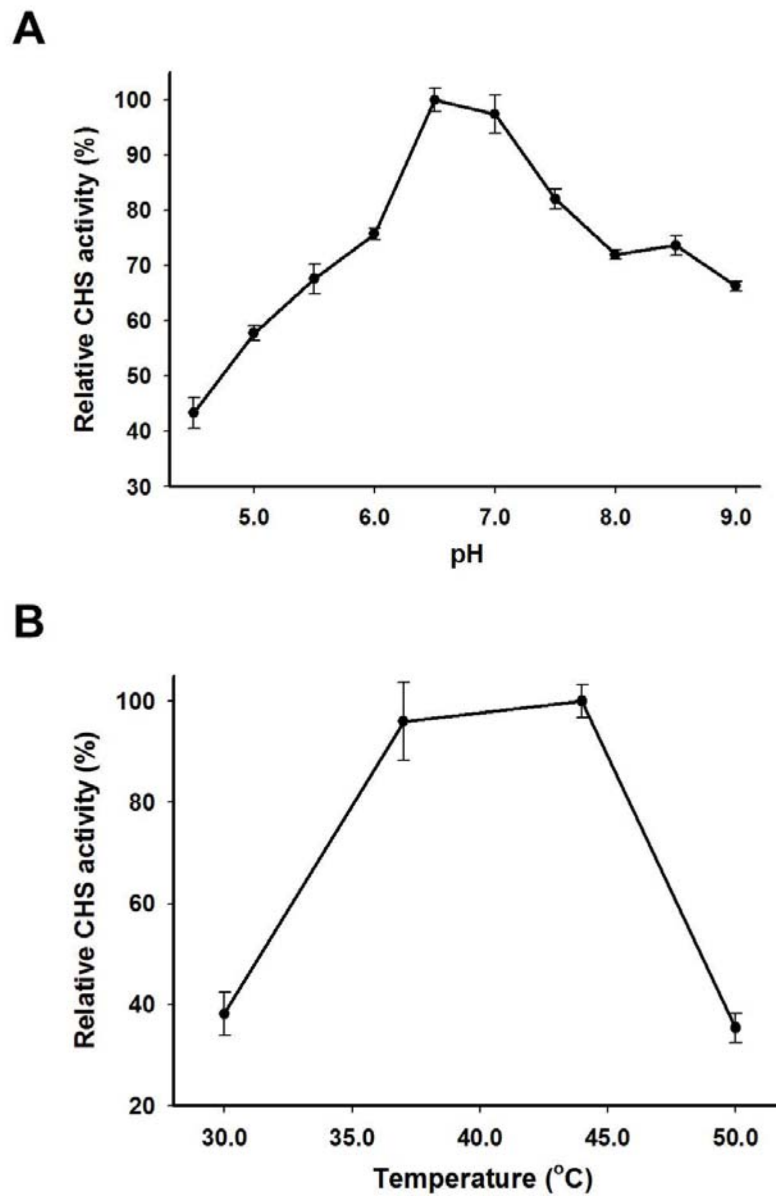

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531 **Fig. 3**

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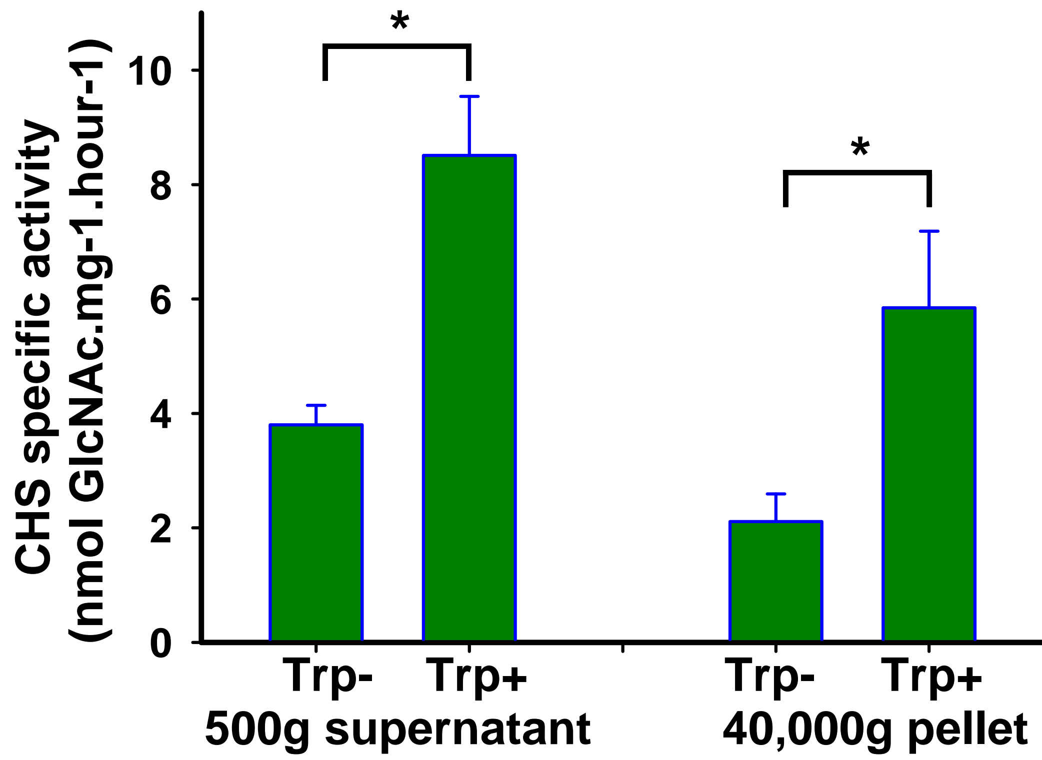

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544 **Fig. 4**

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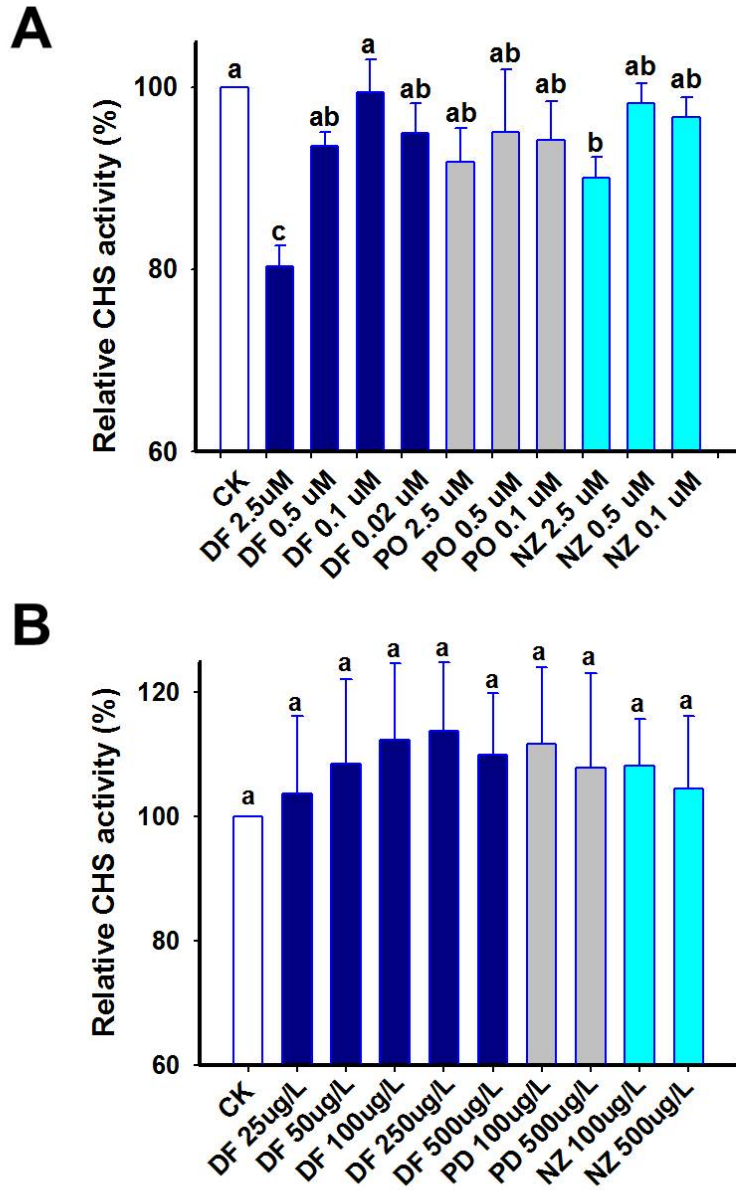

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