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

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**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: *Anopheles gambiae*, chitin synthase, diflubenzuron, enzyme inhibition
Introduction

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

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

As insect and fungi growth and development depend on precisely tuned expression of CHS (Arakane et al., 20005, 2008; Merzendorfer, 2006) and chitin is not synthesized by vertebrates, CHS presents an attractive target for combating insect pests and fungi-born diseases (Merzendorfer, 2006). For example, peptidyl nucleosides including polyoxins and nikkomycins are anti-fungi agents which competitively inhibit CHS in fungi and insects (Cohen & Casida, 1980b; Zhang & Miller, 1999; Ruiz-Herrera & San-Blas, 2003), whereas benzylphenoloureas (BPUs) such as diflubenzuron are highly effective insecticides which inhibit chitin synthesis in
insects (Post & Vincent, 1973; Ishaaya & Casida, 1974; Post et al., 1974). Diflubenzuron has been widely used to control various agricultural and public health pests such as mosquitoes and fly larvae since the 1970's. It is extremely toxic to young larvae of many mosquito species (Eisler, 1992; Baruah & Das, 1996; Ali et al., 1999; Zhang & Zhu, 2006; Zhu et al., 2007).

However, the exact mechanisms of chitin synthesis inhibition are still elusive. It is uncertain as to whether BPUs can directly inhibit insect chitin synthase because different studies have yielded inconsistent results. In cell-free chitin synthesizing systems, for example, the BPUs do not inhibit chitin synthesis (Cohen & Casida, 1980b; Mayer et al., 1981) or block the chitin biosynthetic pathway between glucose and UDP-GlcNAc in intact larvae (Post et al., 1974). In contrast, Nakagawa et al. (1993) showed that diflubenzuron and polyoxin D clearly inhibited the incorporation of $[^3]$H-$N$-acetylglucosamine into chitin in isolated intact integument from newly molted American cockroaches. Horst (1981) reported that diflubenzuron can dramatically inhibit CHS activity (approximately 90%) in the crude microsomes and membrane fractions prepared from brine shrimp larvae.

CHS activity is often measured by a radioactive assay using $[^{14}]$C UDP–$N$-acetyl-D-glucosamine (GlcNAc) as a substrate followed by quantization of insoluble $^{14}$C-labeled chitin after acid precipitation. The observation that wheat germ agglutinin (WGA) specifically binds to chitin polymer at multiple sites led to the development of a nonradioactive, high throughput screening for antimicrobial agents acting on chitin synthases (Lucero et al., 2002). The sensitivity of this nonradioactive assay method was reported to be similar or even slightly higher than that of the radioactive assay. Also, the method was compatible with a variety of assay conditions, performed using low-cost, widely available commercial reagents, and most helpful when multiple determinations of several samples are required. In this study, we characterized the
enzymatic and inhibitory properties of the chitin synthase prepared from the African malaria
mosquito (*Anopheles gambiae*) by using the nonradioactive assay technique.

**Materials and methods**

**Materials**

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

**Mosquito rearing**

A colony of *An. gambiae* obtained from the Malaria Research and Reference Reagent
Resource Center (MR4) (Manassas, VA) was maintained in the Department of Entomology at
Kansas State University (Manhattan, KS) since 2007 by using the same methods as described by
Crude enzyme preparation, protein content assay, and pretreatment of the enzyme

Fifty mosquito pupae were homogenized in 1.0 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 20 mM of DTT and 1 mM of MgCl₂ for 60s by using a glass-pestle homogenizer. Another 0.5 ml same buffer was used to rinse the homogenizer and combined with the homogenate. The combined homogenate was then centrifuged at 500×g for 10 min to remove unbroken cells, nuclei and debris. The supernatant was carefully transferred to a new tube and used as crude enzyme for following analysis. To obtain the 40,000×g fractions, the supernatant were centrifuged at 40,000×g for 10 min. Then the supernatant was carefully removed and the pellet was resuspended in the same volume of the same buffer. All preparations were conducted on ice or at 4 °C. Protein determination was carried out in microtiter plate using bovine serum albumin as standard by using the BCA method. To pretreat the enzyme, 10 µl of trypsin solution (2 µg/µl in buffer) was added to 250 µl enzyme preparations in a glass tube and incubate for 10 min at 30°C followed by addition of 10 µl of STI solution (3 µg/µl in buffer). Ten µl of buffer instead of 10 µl trypsin solution was used as control without trypsin treatment.

CHS activity assays

The assay was based on Lucero et al. (2002) with some modifications. In brief, 100 µl of WGA solutions (50 µg/ml in deionized H₂O) were added to each well of the microtiter plate followed by a 16-h incubation at room temperature (23-25 °C). WGA solutions were removed by vigorously shaking of the plate content. To wash the plate, the empty plate was immersed in a basket of tap water followed by empty the water in the wells by shaking. This washing was repeated two more times to remove the unbound WGA completely. After washing, the wells were blocked by adding 300 µl of bovine serum albumin (BSA) blocking buffer (20 mg/ml BSA in 50 mM Tris–HCl, pH 7.5) and incubated for 3 h at room temperature. After incubation, the
blocking solutions were emptied by shaking. Fifty µl of reaction mixture (5 mM GlcNAc, 1 mM UDP–GlcNAc in 50 mM Tris-HCl buffer, 7.5) were added to the appropriate wells followed by the addition of extraction buffer and pretreated enzyme (20 µl) to a final volume of 100 µl. For each assay the corresponding boiled enzyme preparations (95 ºC for 10 min) was used to assess the background readings.

The plate was covered with a sealer and incubated at 37°C for 60 min by shaking at 100 rpm using incubator shaker (New Brunswick Scientific; I2400). To stop the reaction, the plate was emptied and washed as described above for five times, followed by the addition of 200 µl WGA–HRP (0.5 µg/ml, in blocking buffer) and incubation for 15 min at 30°C with gentle shaking at 100 rpm. The plate was emptied by vigorous shaking followed by five times washing as described above. Finally, 100 µl peroxidase substrate reagents were added to each well and the optical density (OD) at 600 nm was determined immediately for 3 min. The content of GlcNAc and the chitin synthase activity in the treatments were calculated by using a standard curve. The standard curve was prepared following the same procedure as described by Lucero et al. (2002). The specific enzyme activity was expressed as nmol GlcNAc.mg⁻¹. hour⁻¹. Each experiment was repeated 3-4 times, each with triplicate determinations.

In vitro and in vivo inhibition assay

For in vitro inhibition assay, diflubenzuron stock solution (1 mM) was prepared in acetone, whereas polyoxin D (1 mM) and nikkomycin Z (1 mM) were prepared in the solvent of acetone: water (1:1). Before use, diflubenzuron was further diluted to 25, 5, 1 and 0.2 µM by using acetone, whereas polyoxin D and Nikkomycin Z were diluted to 25, 5, and 1 µM by using acetone:water=1:1. Five µl of each solution was added to 25 µl crude enzyme and 20 µl extraction buffer. The final concentrations of diflubenzuron in the reaction mixtures were 2.5,
0.5, and 0.1, 0.02 µM, whereas polyoxin D and Nikkomycin Z were 2.5, 0.5 and 0.1 µM. Same volume of its own solvent was used as control. The mixture was incubated with shaking at 100 rpm at 37 °C for 20 min and the subsequent procedures were same as the enzyme activity assay.

For in vivo assay, a series of dilutions of diflubenzuron, nikkomycin Z, and polyoxin D were made using acetone. Twenty µl of each chemical were added to a 500-ml glass beaker containing 15 mosquito pupae of 9-h old in 100 ml distilled water and 1 ml fish food. The final concentrations of diflubenzuron were 25, 50, 100, 250, and 500 µg/L, whereas nikkomycin Z and polyoxin D were 100 and 500 µg/L. Same volume of acetone was used as control. After a 24-h (L:D, 16:8) exposure at 25 °C, the pupal mortality was examined and the surviving pupae were collected for crude enzyme preparation followed by enzyme assays based on the same procedure described above. Each control and treatment was repeated four times.

Statistical analysis for enzyme activity

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

Results

Chitin synthase activity in mosquito pupae

Previous study showed that both two CHS genes, AgCHS1 and AgCHS2, are highly expressed in the pupal stage (Zhang et al., 2010), implying an intensive synthesis of chitin in the pupal stage. Thus, we used mosquito pupae to prepare the enzyme for all assays in this study. To
make a standard curve, homogeneous chitin suspension in acetic acid was used for the assay. A high linear correlation is observed when initial rate was plotted as a function of chitin amounts (Fig. S1). Using this standard curve, we examined CHS activity in the crude enzyme. As shown in Fig. S2, CHS activity linearly increased when low amount of enzyme used and reached a plateau phase as the amount of the enzyme increased further. Thus, specific CHS activity of the crude enzyme could be determined by using the data within the linear phase.

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

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

Effects of UDP-GlcNAc and GlcNAc on CHS activity

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

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

Proteolytic activation of CHS activity

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

Effect of chitin synthesis inhibitors on CHS activity

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

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

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

WGA is a chitin-binding lectin with high affinity and specificity for GlcNAc. However, it has been reported that the specificity of this chitin binding assay is lower than the radioactive assay (Kramer & Muthukrishnan, 2005). To avoid the effect brought by non-specific binding and the effect by endogenous chitin in the crude enzyme, the same amount of the boiled enzyme
was used as a validation control in this study. No significant differences were observed in the boiled control as the amount of the enzyme was increased. This result indicated that the effects of the endogenous chitin and the non-specific binding were negligible in our assay. Thus, the activity detected in the crude enzyme from mosquito pupae reflects the catalytic activity other than artifacts brought by non-specific bindings or endogenous chitin (Fig. S2).

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

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

High CHS activity was observed at temperatures between 37 and 44°C (Fig. 2B). In *M. sexta*, high incorporation of UDP-GlcNAc was observed at about 30°C, and decreased at about 40°C (Zimoch et al., 2005). In general, CHS activity depends on the membrane fluidity, and thus
CHS activity decreases as temperature increases to >30°C. Obviously, our results are not consistent with what was observed in *M. sexta* CHS, but may suggest insect species-dependent variations with respect to optimal temperature for CHS activity.

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

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 (Fig. 4A). There was no *in vitro* inhibition by polyoxin D at any concentration examined. Furthermore, no *in vivo* inhibition was observed by any of these chemicals at any concentration examined (Fig. 4B). Indeed, there has generally been lack of report showing that these chemicals are capable of inhibiting CHS activity *in vitro* to date. The only report of CHS inhibition by diflubenzuron in insects was observed in American cockroaches, in which the isolated intact integument from newly molted cockroaches was used to examining the incorporation of $[^3]$H-N-acetylglucosamine into chitin (Nakagawa *et al.*, 1993). It would be interesting to know whether diflubenzuron can inhibit the incorporation of UDP-GlcNAc into chitin polymers by using the isolated intact integument in *An. gambiae*. Nevertheless, the inhibition of chitin synthesis by diflubenzuron could be due to its effects on other steps of chitin biosynthetic pathways instead of direct inhibition of CHS. Further studies by using purified CHS may help clarify this issue.
Acknowledgements

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Disclosure

All authors have declared no conflicts of interest, including specific financial interests and relationships and affiliations (other than those affiliations listed in the title page of the manuscript) relevant to the subject of this manuscript.
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**Table 1** Toxicity of chitin synthesis inhibitors to third-instar mosquito larvae.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration (μg/L)</th>
<th>% Mortality at 24 h (Mean±SE) *</th>
<th>% Mortality at 48 h (Mean±SE) *</th>
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<td>Control</td>
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<td>0.0± 0.00 c</td>
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<tr>
<td>Diflubenzuron</td>
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<td>4.9± 2.25 c</td>
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<td>12.5</td>
<td>26.3±8.29 c</td>
<td>27.5±8.66 b</td>
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<td></td>
<td>25</td>
<td>41.3±5.95 b</td>
<td>47.5±8.66 a</td>
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<tr>
<td></td>
<td>50</td>
<td>57.5±2.89 a</td>
<td>60.0±2.36 a</td>
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<tr>
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<td>0.0±0.00 c</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>1.5±1.70 d</td>
<td>1.5±1.70 c</td>
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<tr>
<td>Polyoxin D</td>
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<td>0.0±0.00 c</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>2.1±1.41 d</td>
<td>2.1±1.41 c</td>
</tr>
</tbody>
</table>

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

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

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

**Fig. 3** Proteolytic activation of chitin synthesis in different enzyme preparations from the mosquito pupae. Specific CHS activity was measured in the presence (Trp+) and absence (Trp-) of trypsin in 500xg supernatant and successive 40,000xg pellet which was resuspended in proportional volume of the extraction buffer. Asterisks indicate significant difference based on Fisher’s LSD ($P_{\geq0.05}$).

**Fig. 4** Comparisons of chitin synthase activity in the crude enzyme preparations following incubation with various concentrations of three chitin synthesis inhibitors (A) and the crude
enzyme preparations from the pupae exposed to the three chitin synthesis inhibitors (B). DF: diflubenzuron; PD: polyoxin D; NZ: nikkomycin Z. Same letters on the error bars indicate no significant difference based on Fisher’s LSD ($P \geq 0.05$).
Fig. 1

A

Dithiothreitol (mM)

Relative CHS activity (%)

B

Mg++ (mM)

Relative CHS activity (%)

C

UDP-GlcNAc (mM)

Relative CHS activity (%)

D

GlcNAc (mM)

Relative CHS activity (%)
Fig. 3

CHS specific activity
(nmol GlcNAc.mg-1.hour-1)

Trp-    Trp+               Trp-     Trp+

500g supernatant          40,000g pellet

*  *

*  *