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

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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 500×g supernatant and the 40,000×g 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 μmol/L) 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*.

Keywords

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

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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 catalyzies 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.*, 2005, 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 benzylphenolureas (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 dithiothretol (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 Zhang and Zhu (2006).

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

Fifty mosquito pupae were homogenized in 1.0 mL of 50 mmol/L Tris-HCI buffer (pH 7.5) containing 20 mmol/L of DTT and 1 mmol/L of $MgCl_2$ 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 H_2O) 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 mmol/L 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 mmol/L GlcNAc, 1 mmol/L UDP–GlcNAc in 50 mmol/L Tris-HCI 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 °C 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 r/m. 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/hour. 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 mmol/L) was prepared in acetone, whereas polyoxin D (1 mmol/L) and nikkomycin Z (1 mmol/L) were prepared in the solvent of acetone: water (1:1). Before use, diflubenzuron was further diluted to 25, 5, 1 and 0.2 μmol/L by using acetone, whereas polyoxin D and Nikkomycin Z were diluted to 25, 5, and 1 μmol/L 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 difubenzuron in the reaction mixtures were 2.5, 0.5, and 0.1, 0.02 μmol/L, whereas polyoxin D and Nikkomycin Z were 2.5, 0.5 and 0.1 μmol/L. Same volume of its own solvent was used as control. The mixture was incubated with shaking at 100 r/m 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 diflubenruon, 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 mmol/L significantly increased CHS activity, whereas 10.0 mmol/L or higher significantly inhibited CHS activity (Fig. 1B).

Effects of UDP-GlcNAc and GlcNAc on CHS activity

The addition of 0.5 mmol/L 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 mmol/L) 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 500×g 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 & 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 40 000×g 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 μmol/L) 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

Ali A, Chowdhury MA, Hossain MI, Mahmud-UI-Ameen, Habiba DB, Aslam AF. Laboratory evaluation of selected larvicides and insect growth regulators against field-collected *Culex quinquefascialus* larvae from urban Dhaka, Bangladesh. Journal of the American Mosquito Control Association. 1999; 15:43–47. [PubMed: 10342267]

Arakane Y, Muthukrishnan S, Kramer KJ, Specht CA, Tomoyasu Y, Lorenzen MD, Kanost M, Beeman RW. The *Tribolium* chitin synthase genes *TcCHS1* and *TcCHS2* are specialized for synthesis of epidermal cuticle and midgut peritrophic matrix, respectively. Insect Molecular Biology. 2005; 14:453–463. [PubMed: 16164601]

Arakane Y, Specht CA, Kramer KJ, Muthukrishnan S, Beeman RW. Chitin synthases are required for survival, fecundity and egg hatch in the red flour beetle, *Tribolium castaneum*. Insect Biochemistry and Molecular Biology. 2008; 38:959–962. [PubMed: 18718535]

Baruah I, Das SC. Evaluation of methoprene (Altosid) and diflubenzuron (Dimilin) for control of mosquito breeding in Tezpur (Assam). Indian journal of malariology. 1996; 33:61–66. [PubMed: 8952169]

Broehan G, Zimoch L, Wessels A, Ertas B, Merzendorfer H. A chymotrypsin-like serine protease interacts with the chitin synthase from the midgut of the tobacco hornworm. Journal of Experimental Biology. 2007; 210:3636–3643. [PubMed: 17921165]

Cabib E, Farkas V. The control of morphogenesis: an enzymatic mechanism for the initiation of septum formation in yeast. Proceedings of the National Academy of Sciences of the United States of America. 1971; 68:2052–2056. [PubMed: 4943783]

Cohen E. Chitin synthetase activity and inhibition in different insect microsomal preparations. EXS. 1985; 41:470–472.

Cohen E. Chitin synthesis and inhibition: a revisit. Pest Management Science. 2001; 57:946–950. [PubMed: 11695188]

Cohen E, Casida JE. Properties of *Tribolium* gut chitin synthetase. Pesticide Biochemistry and Physiology. 1980a; 13:121–128.

Cohen E, Casida JE. Inhibition of *Tribolium* gut synthetase. Pesticide Biochemistry and Physiology. 1980b; 13:129–136.

Eisler, R. Contaminant Hazard Reviews Report. Vol. 25. U.S. Fish and Wildlife Service; Laurel, MD: 1992. Diflubenzuron hazards to fish, wildlife, and invertebrates: A synoptic review.

Glaser L, Brown DH. The synthesis of chitin in cell-free extracts. Journal of Biological Chemistry. 1957; 228:729–742. [PubMed: 13475355]

Horst MN. The biosynthesis of crustacean chitin by a microsomal enzyme from larval brine shrimp. Journal of Biological Chemistry. 1981; 256:1412–1419. [PubMed: 6450211]

Hunt, S. Polysaccharide-protein complexes in invertebrates. Academic Press; New York, NY: 1970.

Ishaaya I, Casida JE. Dietary TH-6040 alters cuticle composition and enzyme activity of housefly larval cuticle. Pesticide Biochemistry and Physiology. 1974; 4:484–490.

Kang MS, Elango N, Mattia E, Au-Young J, Robbins PW, Cabib E. Isolation of chitin synthetase from *Saccharomyces cerevisiae*. Purification of an enzyme by entrapment in the reaction product. Journal of Biological Chemistry. 1984; 259:14966–14972. [PubMed: 6238967]

Kramer, KJ.; Muthukrishnan, S. Chitin metabolism in insects. In: Gilbert, LI.; Iatrou, K.; Gill, SS., editors. Comprehensive MolecularInsect Science. Vol. 4. Elsevier; New York: 2005. p. 111-144.

Lucero HA, Kuranda MJ, Bulik DA. A nonradioactive, high throughput assay for chitin synthase activity. Analytical Biochemistry. 2002; 305:97–105. [PubMed: 12018950]

Machida S, Saito M. Purification and characterization of membrane-bound chitin synthase. Journal of Biological Chemistry. 1993; 268:1702–1707. [PubMed: 8420947]

Maue L, Meissner D, Merzendorfer H. Purification of an active, oligomeric chitin synthase complex from the midgut of the tobacco hornworm. Insect Biochemistry and Molecular Biology. 2009; 39:654–659. [PubMed: 19576988]

Mayer RT, Chen AC, DeLoach JR. Characterization of a chitin synthase from the stable fly, *Stomoxys calcitrans* (L.). Insect Biochemistry and Molecular Biology. 1980; 10:549–556.

Mayer RT, Chen AC, DeLoach JR. Chitin biosynthesis inhibiting insect growth regulators do not inhibit chitin synthase. Experientia. 1981; 37:337–338.

Merz RA, Horsch M, Nyhlen LE, Rast DM. Biochemistry of chitin synthase. Exs. 1999; 87:9–37. [PubMed: 10906949]

Merzendorfe H. Insect chitin synthases: A review. Journal of Comparative Physiology B. 2006; 176:1–15.

Merzendorfer H, Zimoch L. Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. Journal of Experimental Biology. 2003; 206:4393–4412. [PubMed: 14610026]

Nakagawa Y, Matsumura F, Hashino Y. Effect of diflubenzuron on incorporation of [3H]-N-acetylglucosamine ([^{3}H]NAGA) into chitin in the intact integument from the newly molted American cockroach *Periplaneta americana*. Comparative Biochemistry and Physiology C. 1993; 106C:711–715.

Post LC, de Jong BJ, Vincent WR. 1-(2,6-Disubstituted benzoyl)-3-phenylurea insecticides: inhibitors of chitin synthesis. Pesticide Biochemistry and Physiology. 1974; 4:473–483.

Post LC, Vincent WR. A new insecticide inhibits chitin synthesis. Naturwissenschaften. 1973; 60:431–432. [PubMed: 4772134]

Richards, AG. The integument of arthropods. University of Minnesota Press; Minneapolis, MN: 1951.

Ruiz-Herrera J, San-Blas G. Chitin synthesis as target for antifungal drugs. Current Drug Targets – Infectious Disorders. 2003; 3:77–91. [PubMed: 12570735]

Shafferman A, Velan B, Ordentlich A, Kronman C, Grosfeld H, Leitner M, Flashner Y, Cohen S, Barak D, Ariel N. Substrate inhibition of acetylcholinesterase: Residues affecting singal transduction from the surface to the catalytic center. EMBO Journal. 1992; 11:3561–3568. [PubMed: 1396557]

Tellam RL, Vuocolo T, Johnson SE, Jarmey J, Pearson RD. Insect chitin synthase: cDNA sequence, gene organization and expression. European Journal of Biochemistry. 2000; 267:6025–6042. [PubMed: 10998064]

Uchida Y, Shimmi O, Sudoh M, Arisawa M, Yamada-Okabe H. Characterization of chitin synthase 2 of *Saccharomyces cerevisiae*. II: Both full size and processed enzymes are active for chitin synthesis. Journal of Biochemistry. 1996; 119:659–666. [PubMed: 8743566]

van Eck WH. Mode of action of two benzoylphenyl ureas as inhibitors of chitin synthesis in insects. Insect Biochemistry. 1979; 9:295–300.

Ward GB, Mayer RT, Feldlaufer MF, Svoboda JA. Gut chitin synthase and sterols from larvae of *Diaprepes abbreviatus* (Coleoptera, Curculionidae). Archives of Insect Biochemistry and Physiology. 1991; 18:105–117.

Zhang D, Miller MJ. Polyoxins and nikkomycins: progress in synthetic and biological studies. Current Pharmaceutical Design. 1999; 5:73–99. [PubMed: 10066885]

Zhang JZ, Zhu KY. Characterization of a chitin synthase cDNA and its increased mRNA level associated with decreased chitin synthesis in *Anopheles quadrimaculatus* exposed to diflubenzuron. Insect Biochemistry and Molecular Biology. 2006; 36:712–725. [PubMed: 16935220]

Zhang X, Zhang JZ, Zhu KY. Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (*Anopheles gambiae*). Insect Molecular Biology. 2010; 19:683–693. [PubMed: 20629775]

Zhang X, Zhang JZ, Park Y, Zhu KY. Identification and characterization of two chitin synthase genes in African malaria mosquito, *Anopheles gambiae*. Insect Biochemistry and Molecular Biology. 2012; 42:674–682. [PubMed: 22683441]

Zhu KY, Heise S, Zhang JZ, Anderson TD, Starkey SR. Comparative studies on effects of three chitin synthesis inhibitors on common malaria mosquito (Diptera: Culicidae). Journal of Medical Entomology. 2007; 44:1047–1053. [PubMed: 18047205]

Zimoch L, Hogenkamp DG, Kramer KJ, Muthukrishnan S, Merzendorfer H. Regulation of chitin synthesis in the larval midgut of *Manduca sexta*. Insect Biochemistry and Molecular Biology. 2005; 35:515–527. [PubMed: 15857758]

A


B

C

D
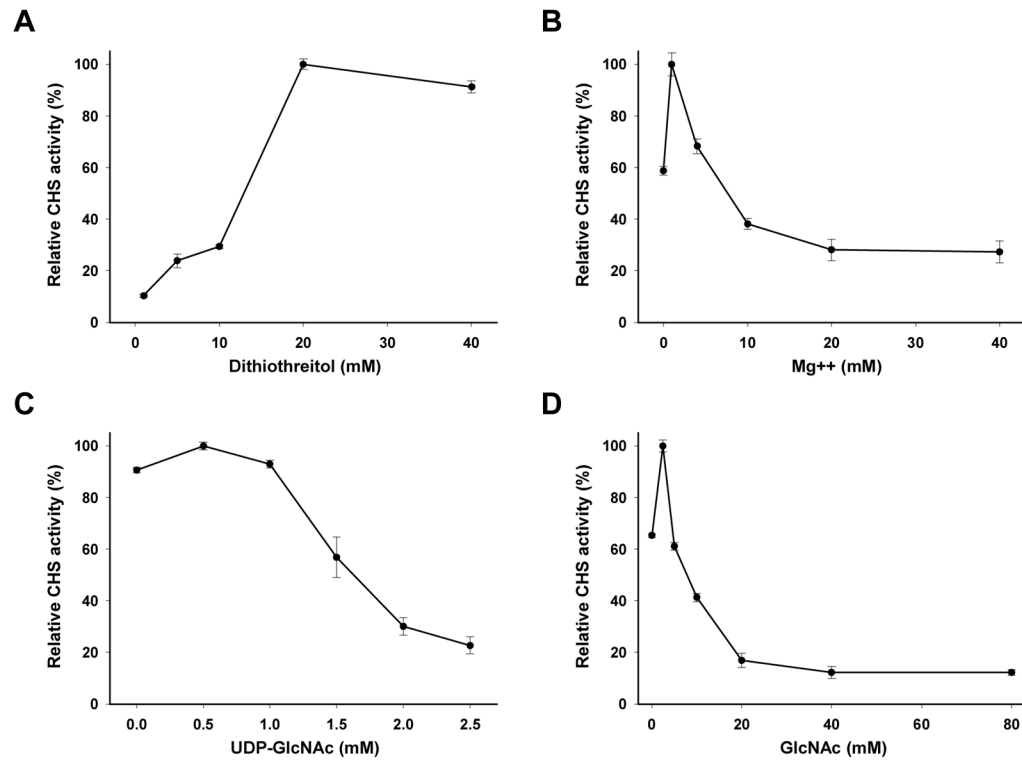
Fig. 1.
The DTT, Mg^{++}, UDP-GlcNAc and GlcNAc dependency of CHS activity in mosquito pupae. (A) Enzyme activity was dependent on DTT in the enzyme extraction buffer. (B) The Mg^{++} dependent CHS activity. DTT and Mg^{++} were added to the Tris-HCl buffer for crude enzyme preparation. The concentrations 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.
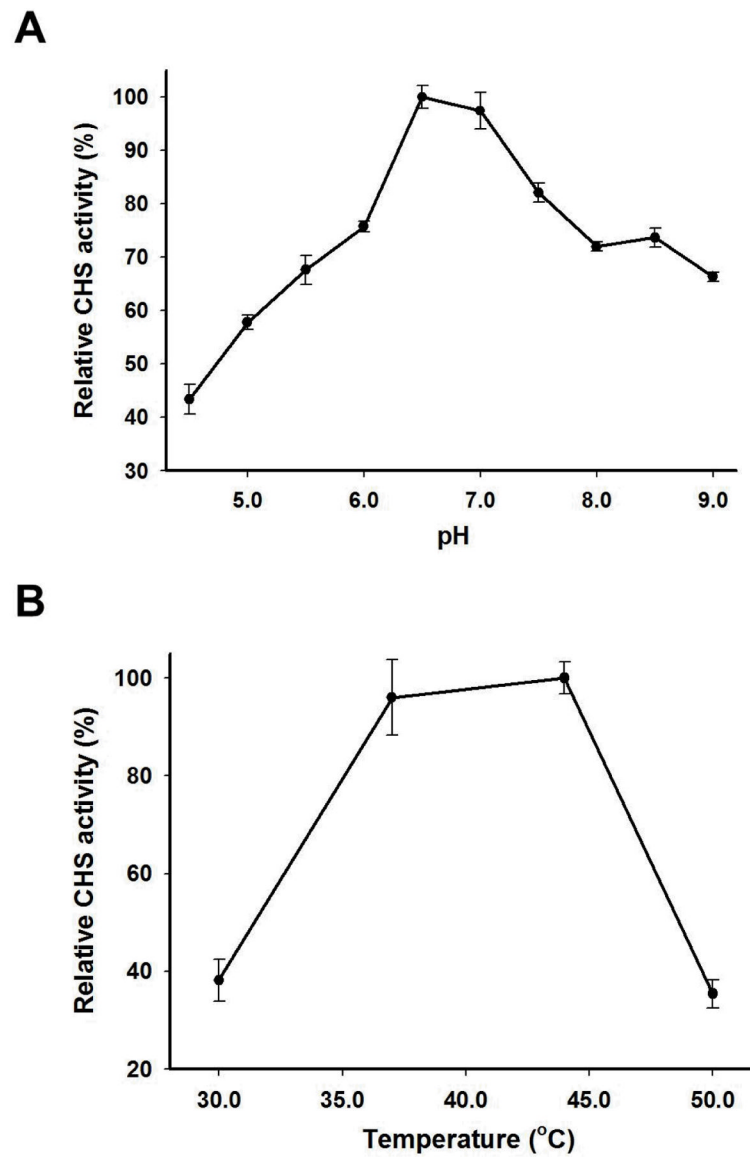
Fig. 2.
Effects of pH (A) and temperature (B) on the activity of CHS prepared from mosquito pupae. The CHS activity values are given in percent of maximal activity and as an average (±SEM) of three independent replicates.
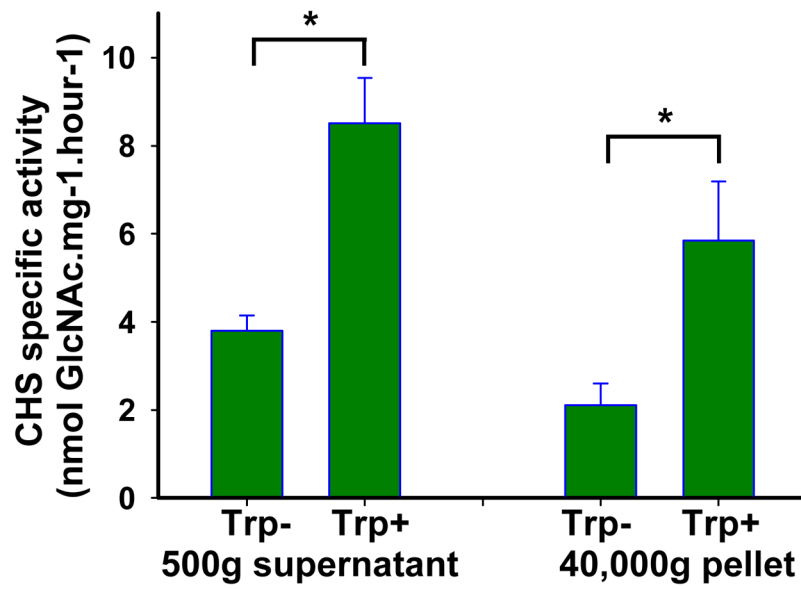
Fig. 3.
Proteolytic activation of chitin synthesis in different enzyme preparations from the mosquito pupae. Specific CHS activity was measured in the presence (Trp+) and absence (Trp−) of trypsin in 500×g supernatant and successive 40 000×g pellet which was resuspended in proportional volume of the extraction buffer. Asterisks indicate significant difference based on Fisher's LSD (P 0.05).
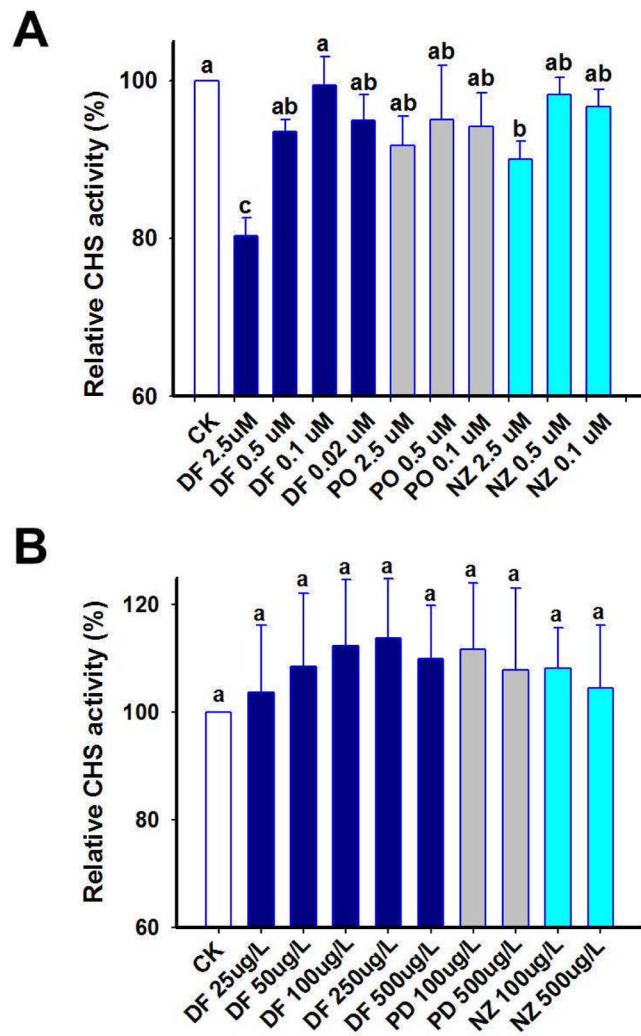
Fig. 4.
Comparisons of chitin synthase activity in the crude enzyme preparations following
incubation with various concentrations of three chitin synthesis inhibitors (A) and the crude
enzyme preparations from the pupae exposed to the three chitin synthesis inhibitors (B). DF:
diflubenzuron; PD: polyoxin D; NZ: nikkomycin Z. Same letters on the error bars indicate
no significant difference based on Fisher's LSD (P 0.05).

Table 1

Toxicity of chitin synthesis inhibitors to third-instar mosquito larvae.

Treatment		% Mortality at 24 h (Mean ± SE)[†]	% Mortality at 48 h (Mean ± SE) [†]
Chemical	Concentration (μg/L)		
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

[†]Same letters indicate no significant difference within each column based on one-way ANOVA followed by Fisher's LSD (P 0.05).