UF UNIVERSITY of FLORIDA

George A. Smathers Libraries
Public Services Division/Access Services
Interlibrary Loan

245 Library West
PO Box 117001
Gainesville, FL 32611-7001
352-273-2535
352-392-7598 Fax
illiad.uflib.ufl.edu/illiad/ill.html

Electronic Delivery Cover Sheet

If you receive a copy that is missing pages, smudged or unreadable, please contact the UF ILL Office so we may obtai clean copy for you as quickly as possible.

Journal Title: Archives of Insect Biochemistry and Physiology

Volume: 18
Issue: 2
Month/Year: 1991
Pages: 105-117

Article Author: Ward, G. B., Mayer, R. T.

Article Title: Gut chitin synthase and sterols from larvae of Diaprepes-abbreviatus (Coleoptera, Curculionidae)

Imprint: Cab Abstracts

Archives of Insect Biochemistry and Physiology 18:105–117 (1991)

Gut Chitin Synthase and Sterols From Larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae)

Gordon B. Ward, Richard T. Mayer, Mark F. Feldlaufer, and James A. Svoboda

U.S. Horticultural Research Laboratory, Agriculture Research Service, U.S. Department of Agriculture, Orlando, Florida (G.B.W., R.T.M.); Insect Neurobiology and Hormone Laboratory, Agricultural Research Service, Beltsville Agricultural Research Center, Beltsville, Maryland (M.F.F., J.A.S.)

Gut chitin synthase was characterized and the sterols and ecdysteroids in the sugarcane rootstalk borer weevil, *Diaprepes abbreviatus*, were identified. An in vitro cell-free chitin synthase assay was developed using larval gut tissues from *D. abbreviatus*. Subcellular fractionation experiments showed that the majority of chitin synthase activity was located in 10,000*g* pellets. The gut chitin synthase requires Mg^{2+} to be fully active: 7–8-fold increases in activity were obtained with 10 mM Mg^{2+} present in reaction mixture. Calcium also stimulated activity (4–5-fold with 10 mM Ca^{2+}), while Cu^{+2} completely inhibited at 1 mM. Other monovalent and divalent cations had little or no effect on activity. The pH and temperature optima were 7 and 25°C, respectively. Gut chitin synthesis was activated ca. 50% by trypsin treatments. GlcNAc stimulated chitin synthase activity, but Glc, GlcN and glycerin did not. Polyoxin D, UDP, and ADP inhibited the chitin synthase reaction with I_{50}'s of 75 μM, 2.3 mM, and 3.6 mM, respectively. Nikkomycin Z was a potent inhibitor of chitin synthase (91% inhibition at 10 μM). Tunicamycin and diflubenzuron had no effect on the enzyme. The apparent Km and V_{max} for the gut chitin synthase were, respectively, 122.5 ± 7.4 μM and 426 ± 19.7 pmol/h/mg protein utilizing UDP-GlcNAc as the substrate. Sterol analyses indicated that cholesterol was the major dietary and larval sterol. HPLC/RIA data indicated that 20-hydroxyecdysone was the major molting hormone.

Key words: chitin synthesis inhibitors, ecdysteroids, molting hormone

Acknowledgments: We thank Mr. Alex Wang for his technical assistance.

Received November 13, 1990; accepted May 30, 1991.

Address reprint requests to Richard T. Mayer, USDA, ARS, SAA, USHRL, 2120 Camden Road, Orlando, FL 32803-1419.

Mention of a trademark, warranty, proprietary product, or vendor does not constitute a guarantee by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

Gordon B. Ward is now at USDA, ARS, NAA, Plum Island Animal Disease Center, P.O. Box 848, Greenport, NY 11944-0848.

INTRODUCTION

Insect chitin synthase is located primarily in the intestine, where it aids in forming the peritrophic membrane, and in the integumental epidermis, where it synthesizes cuticular chitin [1–5]. Deposition of chitin in insects is controlled by ecdysteroids, in particular 20-hydroxyecdysone [1–10]. Chitin synthesis is the target for a number of insecticides [1–5], but very little is known about the mode of action of these compounds. There are reports on the effects of these insecticides in vivo, as well as in vitro, where organ cultures of gut cardiae [6,7], cuticle [8], imaginal discs [9], or cell lines [10] were utilized. However, there are very few studies of in vitro cell-free chitin synthase. The first report on a cell-free, in vitro chitin synthesis assay was by Jaworski et al. [11]. Since then, only a few other systems have been studied [12–14]. Cuticular chitin synthase from the dipteran *Stomoxys calcitrans* was isolated and partially characterized by Mayer et al. [12]. Mitsui et al. [13] studied lepidopteran cuticular chitin synthase from *Mamestra brassicae*. Reports on cell-free gut chitin synthases from *Tribolium castaneum* have been made by Cohen and Casida [14].

Diaprepes abbreviatus (Coleoptera: Curculionidae), the sugarcane rootstalk borer, is a serious pest of both sugarcane and citrus [15]. The larvae of this insect are capable of girdling the roots of citrus and killing the tree. Being a root-feeding insect, *Diaprepes abbreviatus* is difficult to control and little is known of its physiology, biochemistry, and susceptibility to pesticides. Presently, no pesticides are registered for larval control of this species due to concerns for groundwater contamination. Chitin synthesis inhibitors may be useful in controlling the insect as they pose less of an environmental risk.

We have investigated chitin synthesis in the gut of larval *Diaprepes abbreviatus* and developed an in vitro assay system for studying kinetics and inhibition of chitin synthase. Also, we have characterized the ecdysteroids that are present in the larvae to provide some insight as to which ecdysteroids may be involved with the chitin deposition process.

MATERIALS AND METHODS

Insects

Third- to fourth-generation laboratory-reared *Diaprepes abbreviatus* adults were fed fresh citrus leaves and allowed to oviposit between wax paper sheets. The newly hatched larvae were reared at 27 + 2°C on artificial diet #1675 from Bio Serve Inc. (Frenchtown, NJ) as described by Beavers [16]. Larvae of at least 300 mg were used for isolations of chitin synthase and analysis of ecdysteroids.

Chemicals

Tritiated uridine diphosphate N-acetyl-D-glucosamine, [glucosamine-6^{3}H (N)] (26.8 Ci/mmol), was purchased from New England Nuclear (Wilmington, DE). Nikkomycin Z was purchased from Calbiochem (La Jolla, CA). Diflubenzuron was obtained through Thompson Hayward Chem. Corp. (Kansas City, KS). Other chemicals were supplied by Sigma Chemical Co. (St. Louis, MO) and Boehringer Mannheim Biochemicals (Indianapolis, IN).

Isolation of Gut Chitin Synthase

Insects were removed from food 2–3 days prior to dissection so that they could purge their gut. The larvae were chilled on ice and the guts removed and placed into ice-cold homogenization buffer (50 mM MOPS, pH 7, 1 mM DTT*, 1.25 mM PMSF). The guts were opened and rinsed extensively with ice-cold buffer. All subsequent operations were conducted at 4°C. Washed guts were homogenized in cold homogenization buffer (10 guts/ml) for 30 s, using a Brinkman Polytron with a chilled PTA 20S generator. The homogenate was centrifuged at 1,000*g* for 15 min; the supernatant was transferred to another centrifuge tube and then centrifuged at 10,000*g* for 20 min. A 100,000*g* pellet and supernatant were obtained by centrifugation of the 10,000*g* supernatant for 1 h. The pellets were washed by resuspension in homogenization buffer that was of a volume equal to the supernatant and recentrifuged at the speeds and times at which they were originally obtained.

Enzyme Assay

Unless otherwise specified, the reaction mixture consisted of 250 μg of 10,000g pellet protein and enough 50 mM MOPS buffer (pH 7.0) containing 10 mM $MgCl_2$ and 1 mM DTT to make the volume 475 μl. The reaction mixtures were contained in 13 × 100-mm glass test tubes. To each of the assay mixtures was added 25 μl of substrate solution (0.1 μCi of UDP-[^{3}H]-GlcNAc plus an appropriate amount of unlabeled UDP-GlcNAc) for a final concentration of 100 μM UDP-GlcNAc. The assays were conducted at 25°C for 1.5 h. The reactions were terminated by addition of 2 ml of 50% KOH (w/w) to each of the tubes. The tubes were heated subsequently at 110°C for 4 h. The digests were neutralized with 10N acetic acid and filtered through a Gelman A/E glass fiber filter and washed with two alternate washes of 300 ml water and 50 ml ethanol. Radioactivity in the dried filters was measured in 10 ml of Ecoscint A liquid scintillation fluid (National Diagnostics, Manville, NJ).

Enzyme Activation by Trypsin

Other chitin synthases have been reported to be activated by proteases [12,14]. Therefore, the *D. abbreviatus* enzyme was incubated with trypsin to determine if activation occurred. Type III trypsin (Sigma) was utilized and possessed 10,200 BAEE units per mg of protein. One BAEE unit = ΔA_{253} of 0.001 per min with BAEE as the substrate at pH 7.6 at 25°C. The 10,000*g* pellet was isolated using a mixture of protease inhibitors in the homogenization buffer. These included SBTI (0.25 mg/ml), TLCK (100 μM), aprotinin (4 μg/ml), and PMSF (1.25 mM)—in the extraction buffer. An aliquot of the pellet suspension was tested using azocoll (Sigma) as a substrate to ensure that proteolytic activity was inhibited. Generally, greater than 95% of the proteolytic activity was inhibited. The isolated pellet was then washed by resuspending the pellets in inhibitor-free buffer and recentrifugation at 10,000*g* for 10 min prior to protease activation experiments. Trypsin was added to a portion of the pellet and its activity against

*Abbreviations used: BAEE = Nα-benzoyl-L-arginine ethyl ester; CHAPS = 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DTT = dithiothreitol; I_{50} = concentration giving 50% inhibition; MOPS = 4-morpholinepropanesulfonic acid; PMSF = phenylmethylsulfonyl fluoride; TLCK = N-tosyl-L-lysine chloromethyl ketone; SBTI = soybean trypsin inhibitor.

azocoll was tested to ensure that the suspensions were protease inhibitor-free. Protease activation of chitin synthase was determined by preincubating varying amounts of trypsin with the chitin synthase reaction mixture for 15 min at 25°C. TLCK (100 μM final) was added to inhibit proteolytic activity, and the pellet was then assayed for chitin synthase activity.

Chitin Synthase Product Characterization

The isolated alkaline-treated product of the chitin synthase reaction was digested with *Streptomyces griseus* chitinase (10 mg/ml) in 300 mM sodium acetate buffer (pH 5.5) + 300 mM NaCl at 37°C for 48 h. Similar product preparations without chitinase served as controls. The reaction mixture was stopped by addition of 95% ethanol (66% final). It was then filtered as described above for the chitin synthase reaction, and the radioactivity of the filter-trapped material determined. The filtrate was evaporated and the residue was dissolved in a minimum amount of 20 mM NaOH (ca. 75 μl). The soluble products were chromatographed on a Dionex HPLC equipped with a CarboPac column (PA1, 4 × 250 mm). Products were eluted isocratically with 20 mM NaOH at 1 ml/min and detected using a pulsed amphoteric detector. Fractions (0.5 ml) were collected, neutralized with HCl, and the radioactivity determined by liquid scintillation counting. In some instances, aliquots of the chitinase product were also digested in 6 N HCl for 6 h at 100°C to obtain a more complete digestion. Carbohydrates were identified by comparison with standards.

Detergent Solubilization

Suspensions of 10,000*g* pellets were prepared from *D. abbreviatus* as described above. Various detergents were dissolved in 50 mM MOPS (pH 7) and varying amounts added to 10,000*g* pellet suspensions (250 μg protein each). The final concentration was 5 μg protein/μl detergent buffer. The suspension was gently stirred for 1 h at 4°C. The suspension was then centrifuged at 10,000*g* for 20 min at 4°C. The supernatant was drawn off and the volume measured. The pellet was resuspended in an equal volume of buffered detergent and both the supernatant and the suspended pellet were assayed for enzyme activity. Protein assays using the Bradford method [17] and bovine serum albumin as the standard were performed on aliquots of the supernatant and the pellet to determine the specific activity of the solubilized protein.

Ecdysteroid Extraction

D. abbreviatus larvae (100 g) were homogenized in 100% methanol (2 × 400 ml), 75% methanol:water (1 × 250 ml), and after filtering, the filtrates were combined and dried in vacuo. The residue (13.73 g) was partitioned between 70% methanol:water and n-hexane (countersaturated; 100 ml each phase); the aqueous phase being passed over three additional portions of hexane and the initial hexane back-extracted with another portion of 70% methanol. The dried organic residue (1.22 g) was reserved for sterol analyses, while the dried methanolic residue (12.5 g) was further purified to separate the ecdysteroids.

Sterol Purification and Analyses

The hexane phase from the initial partition was used to determine the relative percentages of neutral, free sterols in *D. abbreviatus* larvae. In addition,

the diet that larvae were fed was extracted with chloroform:methanol (2:1) and analyzed for sterol content so comparisons could be made between dietary sterols and larval sterols. Saponification procedures and alumina column chromatography of neutral sterols have been previously reported [18]. Qualitative and quantitative analyses of sterols were carried out by capillary GLC [18].

Isolation and Purification of Ecdysteroids

The methanolic residue was further purified by apportioning between n-butanol and water (countersaturated; 75 ml each phase). After five transfers of butanol over three separatory funnels containing water, the butanolic residue, which contained free ecdysteroids, yielded 130 mg of material upon drying. This residue was fractionated on a silica gel column (20 g; 20 mm i.d.) packed in a chloroform slurry and eluted with 200 ml each of increasing concentrations of ethanol in chloroform (5%, 15%, 25%, and 40%). The column was stripped with 200 ml methanol. RIA (see below) of individual column fractions indicated immunoactivity in the 15% and 25% fractions. These fractions were combined, dried, and chromatographed on a C18 Sep-Pak (Waters Assoc., Milford, MA) as previously described [19,20].

High Performance Liquid Chromatography

A Spectra-Physics solvent delivery system was used in conjunction with a Waters Model 481 UV detector, which monitored the eluant at 242 nm. The residue from the 60% methanol:water Sep-Pak fraction was injected in methanol and fractionated as previously described [21] on i) a C_8 column eluting with 35% methanol:water; and ii) a silica column eluting with methylene chloride:2-propanol:water (125:25:2). In both instances, 1-ml fractions were collected for RIA analysis.

Radioimmunoassay

Antiserum (from rabbits injected with a carboxymethoxime derivative of 20-hydroxyecdysone) was a gift of Dr. Jan Koolman (Marburg, FRG). The labeled ligand was [23, 24-^{3}H]ecdysone (specific activity 55–60 Ci/mmol; Zoecon Corp., Palo Alto, CA), which was used to construct the standard curve. All assays were performed in triplicate as previously described [21,22], and the results were corrected for cross-reactivity.

RESULTS

Subcellular Fractionation

Gut homogenate was fractionated as described in Materials and Methods into 1,000*g*, 10,000*g*, 100,000*g* pellets and a 100,000*g* supernatant. A typical subcellular fractionation experiment is summarized in Table 1. The majority (56%) of the chitin synthase activity is located in the 10,000*g* pellet. Each of the other fractions contained 15% or less of the total activity. The 10,000*g* pellet also had the highest specific activity, incorporating 1,389 pmol GlcNAc/h/mg protein. The other membrane fractions, the 1,000*g* and 100,000*g* pellets, respectively, had specific activities 2.3- and 6-fold lower than the 10,000*g* pellet (Table 1).

TABLE 1. Subcellular Distribution of *D. abbreviatus* Gut Chitin Synthase*

Fraction	Protein (mg)	Specific activity[a]	Total activity[b]	Total (%)
Homogenate	61.2	185 ± 17	11,322	100.0
1,000*g* pellet	2.6	588 ± 23	1,529	13.5
10,000*g* pellet	4.6	1,389 ± 74	6,389	56.4
100,000*g* pellet	3.1	230 ± 3	713	6.2
100,000*g* supernatant	71.2	24 ± 2	1,709	15.0

*n = 4.
[a]pmol GlcNAc incorporated/h/mg protein.
[b]Total pmol GlcNAc incorporated/h.

pH and Buffer Optimization

Several buffers with overlapping pH ranges between 6.6 and 8 were used to determine the pH optimum of the enzyme. The pH optimum was 7. Both MOPS and Hepes were good buffers to use for the enzyme assay, while sodium phosphate buffer inhibited the enzyme. Since MOPS was used to isolate the enzyme, it was also used for the enzyme assay. Buffer concentration also affected chitin synthase activity; there is approximately a 33% increase in enzyme activity with 50 mM MOPS compared with 100 mM MOPS at pH 7. Therefore, 50 mM MOPS at pH 7 was chosen for subsequent assays.

Cation Dependence

Enzyme activity increased by the divalent cations Ca^{+2}, Mg^{+2} (Table 2). Gut chitin synthase from *D. abbreviatus* required Mg^{2+} to be fully active. Concentrations of 10 to 25 mM Mg^{2+} stimulated gut chitin synthase activity 7–8-fold over control values. Thus, all subsequent assay mixtures contained 10 mM Mg^{2+}. Cobalt and copper divalent ions, conversely, were inhibitory, while Fe^{+2} and Mn^{+2} had little effect on the enzyme activity. No increase in activity was observed when monovalent cations (Na+ and K+) up to 200 mM were added (Table 2).

TABLE 2. Effect of Cations on Gut Chitin Synthase Activity*

Cation	Concentration (mM)	Percentage of control
CA^{2+}	1	324 ± 108
	10	395 ± 133
	25	480 ± 157
Co^{2+}	1	145 ± 21
	5	39 ± 17
Cu^{2+}	1	0
Fe^{2+}	25	113 ± 31
Mg^{2+}	1	303 ± 215
	10	728 ± 187
	25	860 ± 564
Mn^{2+}	25	97 ± 8
Na^{+}	200	93 ± 12
K^{+}	200	108 ± 13

*n = ⩾ 4.

Temperature Optimum

Enzyme activity was tested at different temperatures (0–35°C). Maximal activity was at 25°C and this temperature was used for subsequent assay. Chitin synthase activity decreased by 8% at 20°C and by 34% at 30°C.

Enzyme Stability

The enzyme was stable to repeated freezing and thawing with liquid nitrogen, especially if 10% glycerol was included in the enzyme suspension. The activity after a single freezing was 93% of control, while activity after three to five freeze-thaw cycles decreased by no more than 10% of the original activity. Addition of glycerol up to 20% did not affect enzyme activity. The enzyme was not very stable with regard to long-term storage at −100°C. After 3, 5, and 14 days, the activity was 70, 58, and 21% of the original activity, respectively.

Identification of Enzyme Product

Digestion of the reaction product with 2 ml of 50% KOH for 4 h at 110°C before filtration was necessary, since the alkaline stable material constituted only 44% of the labeled product precipitated by 66% ethanol. Digesting the product longer than 4 h did not decrease the amount of recovered labeled product. The digests were neutralized with 10 N acetic acid before the filtration step to minimize chemiluminescence.

The chitin synthase product was isolated by alkaline digestion and filtration as described in Materials and Methods. The isolated product was subjected to a chitinase treatment to determine if the product could be further digested. After 48 h of incubation with chitinase, the reaction was stopped and the mixture was filtered. The radioactivity on the filters was less than 10% of the starting material for the chitinase-treated samples, whereas the control filters retained greater than 85% of the starting material. Recovery of total radioactivity exceeded the radioactivity measured on the untreated filter (ca. 120%), probably due to greater efficiency of counting the soluble product than the insoluble, filter-trapped product.

The filtrate of the chitinase digestive product was separated on HPLC and fractions collected for counting (Fig. 1). Most of the radioactivity was found in

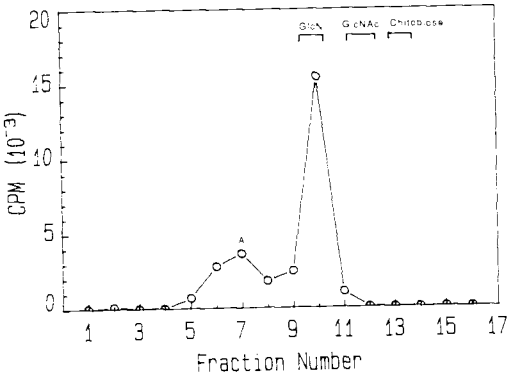

Fig. 1. HPLC separation of the chitinase digested product. Conditions for the separation are as described in Materials and Methods.

fraction 10, which eluted with the glucosamine standard. This was expected, because alkaline treatment of chitin causes deacetylation and the formation of chitosan. The broad peak formed by fractions 6 to 8 was assumed to be a disaccharide of GlcN. This assumption was based on the observation that when the product was further digested with HCl, the radioactivity in fractions 6–8 disappeared and the radioactivity in peak 10 increased.

Protein Dependence

The reaction was linear at protein concentrations of 150–1,200 μg protein per ml.

Detergent Solubilization

The detergents, CHAPS and N-octyl-B-glucoside (nonionic), were tested as enzyme solubilization agents. CHAPS did not affect the activity of soluble or insoluble chitin synthase. At concentrations above 0.3%, CHAPS inhibited the enzyme. N-Octyl-β-glucoside did not solubilize active chitin synthase. It did, however, produce a chitin-synthase-enriched pellet. Comparison of activity and protein content of the pellets remaining after extraction with 0.01% and 0.3% N-octyl-β-glucoside showed a 5.75-fold increase in activity and a 3.2-fold decrease in protein content, which resulted in a 18.5-fold increase in specific activity. This increase may not be due only to solubilization of protein. We found a 2-fold increase in specific activity when the enzyme was incubated with 0.3% N-octyl-β-glucoside as compared with 0.01%. This may indicate that the substrate was more available to the enzyme in the presence of detergent.

Time Course of the Reaction

The time course of the reaction at 25°C is shown in Figure 2. There is a 15–30-min lag period before the reaction becomes linear, and the reaction reached a plateau at 120 min. The sigmoidal appearance of the graph between

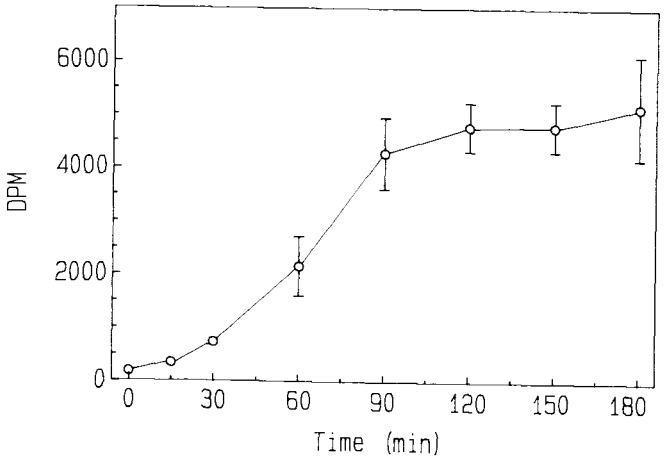

Fig. 2. Time course of the *Diaprepes abbreviatus* gut chitin synthase reaction. Assays were conducted at 25°C using 250 μg of protein, 10 mM Mg^{++}, and 2 μmol UDP-GlcNAc with 0.2 μCi[^{3}H]UDP-GlcNAc in a total volume of 500 μl.

0 and 30 min is probably due to activation by a protease in the 10,000*g* pellet. Therefore, an incubation time of 90 min was chosen.

Proteolytic Activation

Preincubation of the 10,000*g* pellet with trypsin increased the activity of the enzyme (Table 3). Maximum activation (146% of control) was obtained using 20 units trypsin/250 μg protein. Enzyme activation dropped off with increased trypsin and fell below control levels when 80 units trypsin were used; this was probably due to chitin synthase degradation by trypsin.

Enzyme Kinetics

Kinetic parameters of the chitin synthase were analyzed using a Lineweaver-Burk plot (Fig. 3). The apparent Km and V_{max} for the enzyme are 122.5 ± 7.4 μM and 426 ± 19.7 pmol/h/mg protein, respectively.

Effects of Carbohydrates on Chitin Synthase Activity

The results summarized in Table 4 show that GlcNAc stimulated chitin synthase activity. Although GlcNAc stimulated activity, a clear dose-response rela-

TABLE 3. Trypsin Activation of Chitin Synthase*,[a]

Units of trypsin	Percentage of control
20	146 ± 5
40	109 ± 5
60	99 ± 12
80	65 ± 12

*After extraction of the 10,000*g* pellet with protease inhibitors (PMSF, TLCK, SBTI) 250 μg pellet protein was incubated with trypsin for 15 min at 30°C followed by addition of TLCK to stop the reaction. The pellet was then assayed for chitin synthase activity.
[a]n = 3.

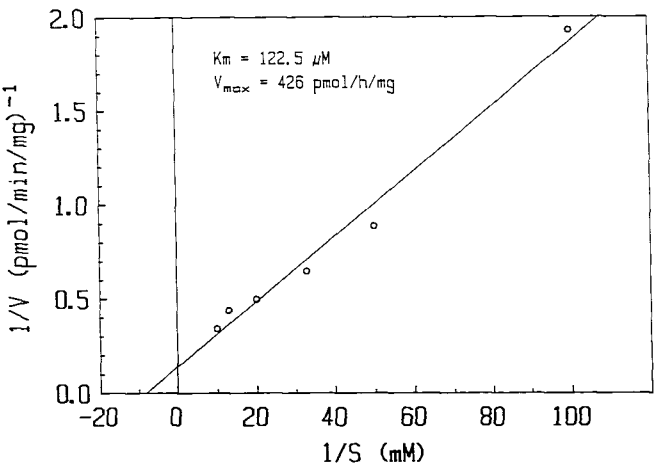

Fig. 3. A Lineweaver-Burke kinetic plot of *Diaprepes abbreviatus* gut chitin synthase. Assays were conducted for 30 min at 25°C using 250 μg of protein and 2 μmol UDP-GlcNAc with 0.2 μCi [^{3}H]UDP-GlcNAc in a total volume of 500 μl. S = UDP-GlcNAc.

TABLE 4. Effect of Carbohydrates on Gut Chitin Synthase Activity*

Carbohydrate	Concentration (mM)	Percentage of control
Glucose	50	99 ± 16
Glucosamine	50	97 ± 18
N-acetylglucosamine	1	229 ± 160
	5	269 ± 102
	10	174 ± 28
	50	222 ± 66
Glycerol	100	100 ± 18

*n = ≥ 5.

tionship could not be established. Glucose, glucosamine, in concentrations up to 50 mm, and glycerol up to 100 mm had no effect on the gut chitin synthase activity.

Effects of Inhibitors on Chitin Synthase Activity

Polyoxin D and nikkomycin Z, which are similar in structure to UDP-GLcNAc, have been reported as being effective inhibitors of chitin synthase [12,23,24] (Table 5). Probit analysis of the polyoxin D results gave an I_{50} of 75 μM. Nikkomycin Z inhibited *D. abbreviatus* gut chitin synthase effectively at 10 μM (91% inhibition). UDP and ADP also inhibited the enzyme with I_{50}'s of 2.3 and 3.6 mM, respectively. Neither tunicamycin nor diflubenzuron inhibited chitin synthase activity.

Sterol Analyses

Cholesterol was the predominant sterol in both the diet and larvae (Table 6). Cholesterol accounted for 83% of the sterols found in the diet and over

TABLE 5. Effect of Compounds on Gut Chitin Synthase Activity*

Compound	Concentration	Inhibition (%)	I_{50}
Nikkomycin Z	100 μM	100	
	10 μM	91 ± 4	
Polyoxin D	5 mM	98 ± 3	75 μM
	1 mM	91 ± 4	
	500 μM	61 ± 10	
	50 μM	40 ± 7	
	5 μM	15 ± 5	
Tunicamycin	5mM	9 ± 9	
	1 mM	0	
UDP	50 mM	99 ± 1	2.3 mM
	10 mM	92 ± 9	
	5 mM	77 ± 11	
	1 mM	19 ± 10	
ADP	50 mM	93 ± 4	3.6 mM
	10 mM	83 ± 5	
	5 mM	52 ± 11	
	1 mM	20 ± 6	
Diflubenzuron	130 μM	3 ± 8	

*n = ≥ 4.

TABLE 6. Relative Percentages of Dietary and Larval Sterols

Sterol	Diet	Larva
Cholesterol	83.0	90.5
22-Dehydrocholesterol	1.6	0.4
Desmosterol	1.4	< 0.1
Campesterol	2.7	1.9
Sitosterol	8.9	7.1

90% of those in the larvae. Other sterols identified were 22-dehydrocholesterol, desmosterol, campesterol, and sitosterol.

Ecdysteroid Analysis

Ecdysteroids were analyzed by normal and reversed-phase HPLC as described in Materials and Methods. RIA of the collected fractions indicated that an ecdysteroid-like immunoreactive compound had the same retention time as 20-hydroxyecdysone standard. Calculations, after correcting for cross-reactivity, indicated that 20-hydroxyecdysone was present in larvae at a concentration of 1.68 ng per g fresh weight. Little or no immunoactivity was detected in the ecdysone region of the chromatograms.

DISCUSSION

Few cell-free assays for chitin synthases from insect integument [12, 23] and insect intestine [14] have been reported. An extensive characterization of the integumental chitin synthase was reported by Mayer et al. [12] using *Stomoxys calcitrans* pupae. The only characterizations of intestinal chitin synthase other than the present study were by Cohen and Casida [14,24] using *Tribolium castaneum* larvae. Most reports have concentrated on the effect of chitin synthesis inhibitors on enzyme activity. In vitro incubation of chitin synthase with benzoylphenylurea pesticides, such as diflubenzuron, did not inhibit either integumental [12,23,25] or intestinal [24] chitin synthases. Conversely, competitive inhibitors such as polyoxin D and nikkomycin Z inhibited the enzyme [12,23,25]. We also found that polyoxin D and nikkomycin Z inhibited *D. abbreviatus* gut chitin synthase. *Hyalophora cecropia* integumental chitin synthase, however, was very poorly or completely unaffected by polyoxin D and nikkomycin Z [23].

Inhibition of chitin synthase by nucleotides such as UDP, UTP, CDP, and CTP has been reported previously [12,23,24], and we showed also that UDP inhibited the *D. abbreviatus* gut enzyme. It was surprising, however, that ADP inhibited the reaction as this nucleotide had never been reported as an inhibitor of insect chitin synthases.

In both of the gut chitin synthase preparations studied [13, this report] and integumental chitin synthase from *T. ni* [23], GlcNAc was strongly stimulatory, whereas in the integumental chitin synthase of *S. calcitrans*, it was quite inhibitory [11]. Glc had no effect on any chitin synthase system assayed. Cohen and Casida [23] found GlcN to be inhibitory, while we found that it had no effect if care was taken to adjust the pH of the assay mixture.

The fungal chitin synthase systems appear to consist of zymogens. Conclusive evidence for a zymogen form of insect chitin synthase has not been reported. However, activation of insect chitin synthase activity by trypsin suggests a zymogenic form.

Since there are so few chitin synthases that have been characterized from insects, generalizations about the integumental and intestinal forms are difficult. For example, the effect of divalent cations has been variable between insect chitin synthase systems. The *S. calcitrans* system was unaffected by divalent cations. The *T. castaneum* system was stimulated by a number of cations such as Mg^{+2}, Mn^{+2} and Co^{+2}, but inhibited by Cu^{+2} [14]. The *D. abbreviatus* system was stimulated by Mg^{+2} and Ca^{+2} but inhibited by Mn^{+2}, Co^{+2}, and Cu^{+2}.

Ecdysone and 20-hydroxyecdysone are required to initiate chitin deposition in most insects [1–5], with the latter being the more biologically active. Karlson et al. [26] has suggested that 20-hydroxyecdysone acts primarily at the transcriptional level by means of gene activation. Our investigations indicate that 20-hydroxyecdysone is the major molting hormone in larvae of *D. abbreviatus* and is present at levels of 1–2 ng per g fresh tissue. 20-Hydroxyecdysone has been isolated from pupae of the scolytid, *Xyleborus ferrugineus*, at considerably higher concentrations [27], though the amount in *D. abbreviatus* is not dissimilar to that found in fourth-instar *Leptinotarsa decemlineata* [28]. Attempts were made to induce molting by injection of ecdysone and 20-hydroxyecdysone (1–10 μg ecdysteroid/larva) without success (data not shown). It may be that the ecdysteroids are being rapidly metabolized and not reaching the target sites.

As more information becomes available on insect chitin synthase systems, perhaps broader conclusions can be drawn about the similarities and differences between integumental and intestinal chitin synthase. We know that integumental chitin synthase is active only during certain periods of development, but intestinal chitin synthase is active during the entire larval development period. Whether the same enzyme is present in both tissues or if a different means of regulation accounts for these differences is not known. Therefore, it is important to study the isolated enzyme from both tissues of the same species of insect.

LITERATURE CITED

1. Chen AC, Mayer RT: Insecticides: Effects on the cuticle. In: Comprehensive Insect Physiology, Biochemistry and Pharmacology. Kerkut GA, Gilbert LI, eds. Pergamon Press, Oxford, Vol 12, pp 57–77 (1985).
2. Kramer KJ, Dziadik-Turner C, Kogat D: Chitin metabolism in insects. In: Comprehensive Insect Physiology, Biochemistry and Pharmacology. Kerkut GA, Gilbert LI, eds. Pergamon Press, Oxford Vol 3, pp 75–115 (1985).
3. Marks EP, Leighton T, Leighton F: Modes of action of chitin inhibitors. In: Insecticide Mode of Action. Coats J. ed. Academic Press, New York, pp 281–313 (1982).
4. Cohen E: Chitin biochemistry: Synthesis and inhibition. Annu Rev Entomol 32, 71 (1987).
5. Mayer RT, Cunningham G, Gupton J: Insecticides based on differences in metabolic pathways. In: Safer Insecticides: Development and Use. Hodgson E, Kuhr RJ, eds. Marcel Dekker, Inc., New York, pp 209–255 (1990).
6. Becker B: Effects of 20-hydroxyecdysone, juvenile hormone, dimilin and captan on in vitro synthesis of peritrophic membranes in *Calliphora erythrocephala*. J Insect Physiol 24, 699 (1978).

7. Becker B: Effects of polyoxin D on *in vitro* synthesis of peritrophic membranes in *Calliphora erythrocephala*. Insect Biochem *10*, 101 (1980).
8. Vardanis A: An *in vitro* assay system for chitin synthesis in insect tissue. Life Sci *19*, 1949 (1976).
9. Oberlander H, Ferkovich SM, Van Essen F, Leach CE: Chitin biosynthesis in imaginal disks cultured in vitro. Rouxs Arch Dev Biol *185*, 95–98 (1978).
10. Ward GB, Newman SM, Klosterman HJ, Marks EP: Effect of 20-hydroxyecdysone and diflubenzuron on chitin production by a cockroach cell line. In Vitro Cell Dev Biol *24*, 326 (1988).
11. Jaworski EG, Wang L, Marco GJ: The synthesis of chitin in cell-free extracts of *Prodenia eridania*. Nature *198*, 170 (1963).
12. Mayer RT, Chen AC, DeLoach JR: Characterization of a chitin synthase from the stable fly, *Stomoxy calcitrans* (L.). Insect Biochem *10*, 549–556.
13. Mitsui T, Nobusawa, Fukami J: Inhibition of chitin synthesis by diflubenzuron in *Mamestra brassicae* L. J Pestic Sci *6*, 155–161 (1981).
14. Cohen E, Casida JE: Properties of *Tribolium* gut chitin synthetase. Pestic Biochem Physiol *13*, 121–138 (1980).
15. Schroeder WJ, Beavers JB: Citrus root weevils in Florida: Identification, biology and control. Proc Int Soc Citriculture *2*, 498–500 (1971).
16. Beavers JB: Biology of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) reared on an artificial diet. Florida Entomol *65*, 263–269 (1982).
17. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72*, 248 (1976).
18. Svoboda JA, Lusby WR: Sterols of phytophagous and omnivorous species of hymenoptera. Arch Insect Biochem Physiol *3*, 13–18 (1986).
19. Thompson MJ, Weirich GF, Svoboda JA: Ecdysone 3-epimerase. Methods Enzymol *111*, 437–442 (1985).
20. Lafton R, Pennetier J-L, Andrianjafintrimo M, Claret J, Modde J-F, Blais C: Sample processing for high performance liquid chromatograph of ecdysteroids. J Chromatogr *236*, 137 (1982).
21. Feldlaufer MF, Herbert EW, Svoboda JA, Thompson MJ, Lusby WR: Makisterone A: The major ecdysteroids from the pupa of the honey bee, *Apis mellifera*. Insect Biochem *15*, 597 (1985).
22. Feldlaufer MF, Lusby WR, Svoboda JA, Thompson MJ: Identification of 20-hydroxyecdysone and ecdysone from the pupa of the gypsy moth, *Lymantria dispar*. Arch Insect Biochem Physiol *1*, 323–330 (1984).
23. Cohen E, Casida JE: Properties and inhibition of insect integumental chitin synthetase. Pestic Biochem Physiol *17*, 301–306 (1982).
24. Cohen E, Casida JE: Inhibition of *Tribolium* gut chitin synthetase. Pestic Biochem Physiol *13*, 129–136 (1980).
25. Mayer RT, Chen AC, DeLoach JR: Chitin synthesis inhibiting insect growth regulators do not inhibit chitin synthase. Experientia *37*, 337 (1981).
26. Karlson P, Doenecke D, Sekeris CE: Intracellular mechanism of hormone action. Comp Biochem Physiol [A] *25*, 1 (1975).
27. Rao KDP, Norris DM, Chu HM: Ecdysteroids during pupal develpoment of female *Xyleborus ferrugineus*. Entomol Exp Appl *30*, 151 (1981).
28. Hsiao TH, Hsaio C, DeWilde J: Molting hormone titer changes and their significance during development of the Colorado beetle, *Leptinotarsa decemlineata*. J Insect Physiol *22*, 1257 (1976).