



Tyrosine β -Glucosyltransferase in the Tobacco Hornworm, *Manduca sexta* (L.): Properties, Tissue Localization, and Developmental Profile

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Tyrosine β -glucosyltransferase activity in larval tissues of *Manduca sexta* was determined by reversed-phase HPLC to quantify the product tyrosine glucoside [β -D-glucopyranosyl-*O*-L-tyrosine, (TG)] formed in incubation mixtures. Synthesis of TG occurred only in fat body preparations, with most of the enzyme activity (95%) in the 15,000 g pellet of homogenates. Other tissues were devoid of activity but did support glucosylation of phenolic substrates other than tyrosine. Activity was greatest with uridine 5'-diphosphoglucose (UDPG) as the glucose donor. However, thymidine 5'-diphosphoglucose (dTDPG) afforded approximately one fourth the amount of tyrosine glucosylation provided by UDPG. Magnesium ions at concentrations up to 15 mM stimulated activity, whereas Ca^{2+} and Mn^{2+} were only slightly stimulatory at 5 mM but progressively inhibited TG synthesis at higher concentrations as did Co^{2+} . Maximal rates of glucosylation occurred in the pH range 7.5–9.0. The enzyme was highly specific for tyrosine, because no glucosylation occurred for a number of tyrosine derivatives. Several natural and synthetic mono- and diphenols were found to be weak to moderate inhibitors of the enzyme. In a study of tyrosine β -glucosyltransferase activity during development, no TG synthesis occurred in enzyme preparations from first, second, third, or fourth larval instars, although glucosylation of *p*-nitrophenol and 4-hydroxycoumarin did occur. Activity was not detected in the fat body of newly ecdysed fifth instars, but low levels were observed 36 h later. The rate of tyrosine glucosylation continued to increase to a peak at 4 days, but then decreased to very low levels after cessation of larval feeding. Low activity was also observed in the pharate adult fat body about 1 day before eclosion. Therefore, tyrosine β -glucosyltransferase activity occurs primarily in the fat body of fifth stadium larvae for synthesis of the pupal cuticle tanning precursor tyrosine glucoside.

Conjugation β -Glucoside Fat body Tobacco hornworm *Manduca sexta* Tyrosine UDP-glucosyltransferase Sclerotization

INTRODUCTION

Tyrosine is the precursor of *o*-diphenolics and their quinonoid derivatives which are involved in the sclerotization or tanning and, often, pigmentation of insect cuticle (see reviews by Kramer and Hopkins, 1987; Hopkins and Kramer, 1991, 1992). Although tyrosine is sparingly soluble in aqueous media, concentrations in hemolymph from several insects indicate its abundance for cuticle tanning at levels several fold higher than its solubility

limit. In addition to this apparently free or weakly bound form of tyrosine, larvae of both Lepidoptera and Diptera have been shown to sequester it in even greater concentrations as conjugates with glucose or phosphate. Thus, tyrosine is not only rendered more soluble as a conjugated storage form, but is also protected from competing metabolic pathways until the time of larval-pupal or puparial transformation when hydrolysis regenerates free tyrosine for use in cuticle tanning. The types of tyrosine conjugates appear to be species specific. Either β -glucoside, phosphate ester, or β -alanine peptidal conjugates of tyrosine are found among Diptera, but Lepidoptera seem to resort solely to the β -glucoside form (Kramer and Hopkins, 1987).

In the tobacco hornworm, *Manduca sexta*, the regulation of tyrosine glucoside (TG) synthesis and hydroly-

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sis is under hormonal control. Decline in juvenile hormone titer after the last larval ecdysis initiates TG synthesis (Ahmed *et al.*, 1985), while the major pulse of 20-hydroxyecdysone triggers hydrolysis of TG by a hydrolase (β -glucosidase) in the fat body (Ahmed *et al.*, 1983b). In this study, we investigated the properties, tissue localization, and developmental activity of the glucosyltransferase (TG synthetase) that catalyzes the biosynthesis of TG in *M. sexta*.

MATERIALS AND METHOD

Insects

Larvae of *M. sexta* were reared from eggs at $27 \pm 1^\circ\text{C}$ with a photoperiod of 16L:8D as previously described (Bell and Joachim, 1976).

Chemicals

All chemicals were from Sigma Chemical Co., St Louis, MO, with the exceptions: 3-nitro-L-tyrosine, Chemical Dynamics, South Plainfield NJ; 3-chloro-L-tyrosine, K and K Labs, Hollywood, CA; 3-iodo-L-tyrosine, Calbiochem, La Jolla, CA; catechol, Fisher Scientific, Springfield, NJ; and HPLC grade acetonitrile, Fisher Scientific, St Louis, MO. Tyrosine β -glucoside was synthesized according to Lu *et al.* (1982).

Enzyme preparation

1st, 2nd and 3rd instars were homogenized as whole larvae. 4th and 5th instar larvae were immobilized on crushed ice before dissection, washed briefly under distilled water, and then dissected. Tissues were collected under an ice-cold buffer composed of 0.1 M sodium phosphate, pH 7.5, containing 5 mM diethyldithiocarbamic acid (DEDTCA) or 0.125 M KCl (when the effect of pH and buffers was studied). To determine tissue localization of TG synthetase, the labial glands, fat body, midgut, hindgut, Malpighian tubules, and epidermis with muscles were isolated from 3–4 day-old 5th instar larvae. Otherwise, the fat bodies of 4th instars and of 3–4-day-old 5th instars were collected as the enzyme source for kinetic, pH, cofactor, and inhibitor studies. Fat bodies were taken from larvae of specified age during development of the 5th instar. Tissues from 3–7 larvae were pooled and homogenized for 1 min with ice-cold dissection buffer in glass tissue grinders. Aliquots of the whole homogenates were centrifuged at 15,000 *g* for 30 min, and the pellets were suspended in dissection buffer or medium. Supernatants were usually discarded, except when fat body fractional distribution of TG synthetase was investigated. All procedures were carried out at 5°C .

Enzyme assay

The typical incubation mixture used to investigate TG synthetase in larval instars, tissues, and centrifugal fractions of tissue homogenates contained 7.5 mM uridine 5'-diphosphoglucose (UDPG), 25 mM MgCl_2 , 1 mM glu-

conolactone, 3 mM tyrosine, and 2.5 mM DEDTCA in 0.1 M phosphate buffer, pH 7.5, in a final vol of 0.2 ml. Tyrosine was predissolved in warm 1 mM HCl before being transferred to the mixtures. The reaction was initiated by adding 0.1–0.8 mg protein of the enzyme source to the mixture with shaking in a water bath either at 30° or 37°C . After an incubation time that varied according to the experiment, the reaction was terminated by adding 0.1 ml 9% HClO_4 ; the mixture was cooled in crushed ice and centrifuged to remove denatured protein. Supernatants were saved for HPLC analyses. Control incubations in which UDPG or tyrosine was deleted were run for each enzyme source from larval or tissue preparations. Properties of TG synthetase from 3–4-day-old 5th instars and during fifth instar development were studied by similar procedures with modifications as described in the results section. Data were collected from incubations of enzyme from one or two different homogenates of pooled fat body samples depending upon the experiment. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA).

Inhibitor studies

Inhibitors were investigated using a series of monophenols and diphenols. The 15,000 *g* pellet from the fat body homogenate pooled from six 3–4-day-old 5th instars was used as the enzyme source. The incubation mixture contained 3 mM tyrosine, 15 mM MgCl_2 , 2.5 mM CoCl_2 , 1 mM gluconolactone, and 10 mM UDPG. Each inhibitor was prepared in 1 mM HCl and transferred to the mixture to yield 3 mM final concentration, except for epinephrine and norepinephrine, which were 0.5 mM. *N*- β -alanyldopamine (NBAD) and *p*-nitrophenol (PNP) were investigated in a series of concentrations. The reaction was started by adding 0.1 mg protein of the enzyme source, the mixture was incubated for 30 min at 37°C , and supernatants were analyzed by HPLC using the mobile phase containing acetonitrile.

Substrate specificity

In studying the potential for glucosylation of tyrosyl-tyrosine, *N*-acetyltyrosine, tyramine, 3-iodo-, 3-chloro-, and 3-nitrotyrosine by TG synthetase, tyrosine was replaced by these compounds at 3 mM final concentration in the incubation mixtures. Control incubations in which UDPG was not included were run for each substrate. A tyrosine incubation was also done to confirm the presence of enzymatic activity in the 15,000 *g* pellet of fat body pooled from seven 3–4-day-old 5th instars.

HPLC analysis

Reversed-phase HPLC was done using a C18 $5\mu\text{m}$ spherical particle 4.6×250 cm column with a flow rate of 1 ml/min at 30°C and absorbance detection at 225 nm to separate and quantify TG concentrations. The mobile phase primarily used was 0.1 M sodium phosphate buffer, pH 2.5. The retention times for TG and tyrosine

were 7.3 and 9.9 min, respectively. A second mobile phase, composed of 7.5% acetonitrile, 0.26 mM sodium lauryl sulfate, and 0.1M phosphoric acid adjusted to pH 2.5, was used to resolve TG from interfering endogenous compounds or from glucosides of plant phenolics. The retention times for TG and tyrosine in this mobile phase were 12.5 and 45 min, respectively.

RESULTS

Tissue and subcellular distribution

The fat body was the only tissue active in the β -glucosylation of tyrosine. Enzyme preparations from labial glands, midgut, Malpighian tubules, hindgut; epidermis, and muscle did not support any TG synthesis in the complete incubation mixtures. However, 15,000 g fractions from all tissues were active in the β -glucosylation of two other phenolic compounds, *p*-nitrophenol and 4-hydroxycoumarin. The cell particulate fraction collected in the 15,000 g pellet from fat body crude homogenate contained almost all of the glucosyltransferase activity that catalyzed synthesis of TG, whereas the supernatant of the same homogenate contained less than 5% of total TG synthetase activity. Therefore, subsequent investigation of TG synthesis was carried out using a suspension of the 15,000 g pellet of fat body homogenates as the enzyme source.

Influence of incubation time on TG synthesis

TG rates of synthesis in the complete incubation mixture using 3 mM tyrosine were linear through 45 min ($0.81 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$), but was slightly lower at 60 min ($0.75 \text{ nmol min}^{-1} \text{ mg}^{-1}$). Some tyrosine precipitated in the incubation mixture by 60 min and may have reduced the availability of substrate causing a lower glucosylation rate. Lower concentrations of tyrosine (0.5–2 mM) yielded linear rates of TG synthesis through 60 min. Therefore, subsequent experiments usually were carried out with a 30–60-min reaction time using a 3 mM final tyrosine concentration. However, in some experiments lower tyrosine concentrations were used.

Determination of apparent kinetic parameters

Specific activities of TG formation at initial tyrosine concentrations of 0.5, 1, 2, and 3 mM were 0.24, 0.39, 0.67, and $0.81 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$, respectively. The K_m was 3.1 mM tyrosine and the V_{max} was $1.7 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ (Fig. 1). The solubility limit of tyrosine prevented using initial substrate concentrations above the K_m value.

Utilization of glucosyl nucleotides as glucose donors

TG synthetase exhibited its maximal activity in the complete incubation mixture with UDPG as the glucose donor (Fig. 2). However, appreciable synthesis was achieved with dTDPG, a synthetic nucleotide with a β -glucosidic linkage instead of the usual α -bond found in other

glucosylated nucleotides. The enzyme activity using dTDPG as a substrate was approx. one fourth of that obtained using UDPG. This ratio of activity was maintained with different levels of enzyme in the incubation mixtures. No TG synthesis was observed when cytidine 5'-diphosphoglucose (CDPG) was substituted for UDPG in the incubation mixtures, whereas very low activities of about $5 \text{ pmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ were detected with either adenosine 5'-diphosphoglucose (ADPG) or guanosine 5'-diphosphoglucose (GDPG). No kinetic parameters were determined for the co-substrate UDPG, which was added at a final concentration of 7.5 mM to the incubation mixtures unless otherwise noted.

Effect of pH and buffers

Enzyme activity was detectable at pH 4.5, reached maximal activity at pH 7.5–9.0, and then decreased from pH 9–10.5 (Fig. 3). Piperazine-*N,N*-bis-(2-ethanesulfonic acid) monosodium (PIPES) and glycine-NaOH buffers gave the highest enzyme activity in the optimal pH range followed by Tris-HCl. Although TG synthetase activity was lower in sodium phosphate buffer, 0.1 M phosphate at pH 7.5 was used in most experiments in this study because of its strong buffering capacity at physiological pH. Also, a more alkaline buffer was avoided, because tyrosine tends to undergo oxidation at higher pHs.

Effect of divalent cations

Highest TG synthetase activity occurred with 15 mM Mg^{2+} (Fig. 4). Higher Mg^{2+} concentrations resulted in a gradual decrease in activity. At 5 mM, Mn^{2+} and Ca^{2+} slightly stimulated activity, but both cations were inhibitory at 25 mM and higher concentrations. Co^{2+} had no effect at 5 mM but progressively inhibited TG synthesis at higher levels. Hg^{2+} was a strong inhibitor at 2.5 mM, eliminating all enzyme activity. Initial experiments described in this study were performed using 25 mM Mg^{2+} , but subsequent experiments were conducted using 15 mM Mg^{2+} plus 2.5 mM Co^{2+} . When diethyldithiocarbamic acid (DEDTCA) was added to the incubation mixture to inhibit phenoloxidases, a new product was synthesized that appeared to be the glucoside conjugate of this compound. However, the product was not formed when Co^{2+} was added to the incubation mixture, probably because Co^{2+} complexes with DEDTCA and precipitates it. Therefore, 2.5 mM Co^{2+} was added to incubation mixtures containing DEDTCA. No difference in the rates of TG synthesis between incubations with and without Co^{2+} occurred, indicating that neither Co^{2+} nor glucosylation of DEDTCA affected TG synthesis.

Effect of gluconic acid lactone

Gluconic acid lactone is a strong competitive inhibitor of β -glucosidases that has been added to incubation mixtures at 5 mM to inhibit endogenous β -glucosidases that might otherwise hydrolyze glucosylated products (Ahmad and Hopkins, 1992). Enzymatic TG formation rates were improved to 105% that of control when 1.25–

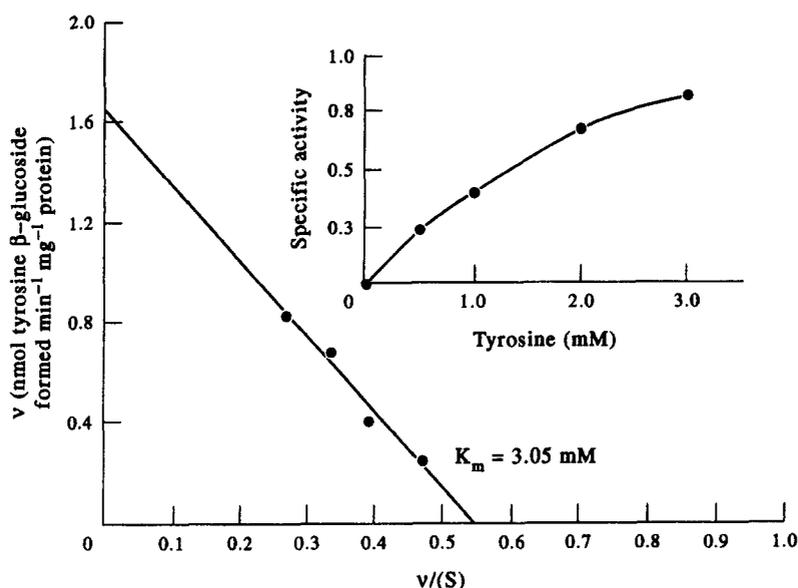


FIGURE 1. Kinetic parameters and specific activity (inset) for tyrosine β -glucosyltransferase in the fat body of *M. sexta*. Incubation conditions: tyrosine 3.0 mM, UDPG 7.5 mM, MgCl_2 25 mM, phosphate buffer 0.1 M (pH 7.5) at 37°C for 30 min. Enzyme source (approx. 0.1 mg protein) was from aliquots of a 15,000 g pellet obtained from fat body homogenate pooled from 7 5th instar larvae 3 days old.

2.5 mM gluconic acid lactone was added to the incubation mixtures. When the gluconic acid lactone concentration was increased to 25 mM, enzyme activity decreased to about 66% of the control activity (data not shown). Therefore, subsequent experiments were conducted with 1 mM gluconic acid lactone added to the incubation mixtures as a precaution to inhibit endogenous β -glucosidases in TG synthetase preparations.

Developmental activity of tyrosine β -glucosyltransferase

Using the optimized incubation conditions described previously, TG synthesis *in vitro* was examined throughout stages of larval growth. No TG synthesis was

detected in the 15,000 g pellet fraction of whole homogenates of 1st, 2nd, and 3rd larval instars approximately midway through feeding behavior in each stadium. However, the same enzyme preparations were active in the glucosylation of *p*-nitrophenol, indicating the presence of phenol β -glucosyltransferases (data not shown). The 15,000 g pellet fraction of fat body from the 4th larval instar midway through feeding also had no TG synthesis activity (Fig. 5). However, it was active in the β -glucosylation of *p*-nitrophenol and another phenolic substrate, 4-hydroxycoumarin. *p*-Nitrophenol and 4-hydroxycoumarin also were glucosylated by enzyme preparations from fat bodies of 0-, 1-, and 4-day-old 5th larval instars (7.4–

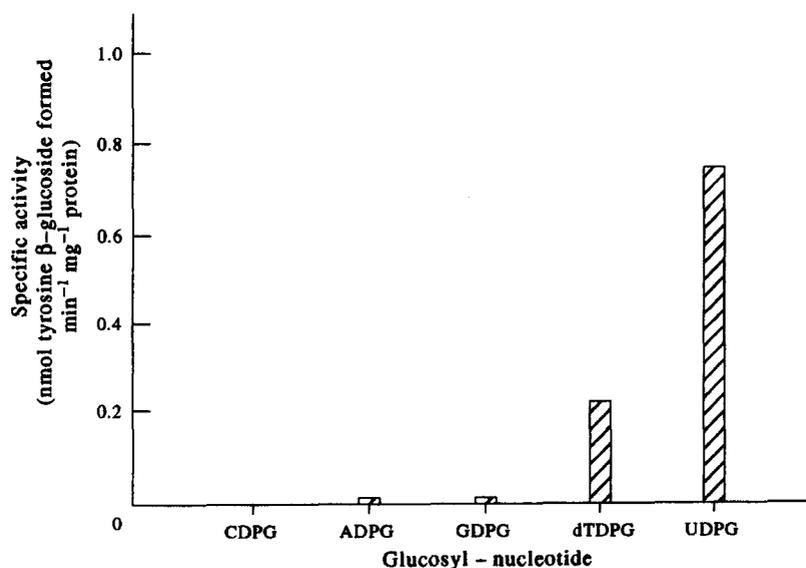


FIGURE 2. Utilization of glucosyl nucleotides as glucose donors for tyrosine β -glucosyltransferase in *M. sexta* fat body. Incubation conditions: tyrosine 3 mM, glucosyl nucleotide concentrations of 7.5 mM at 37°C for 60 min. Enzyme source and buffer components same as in Fig. 1.

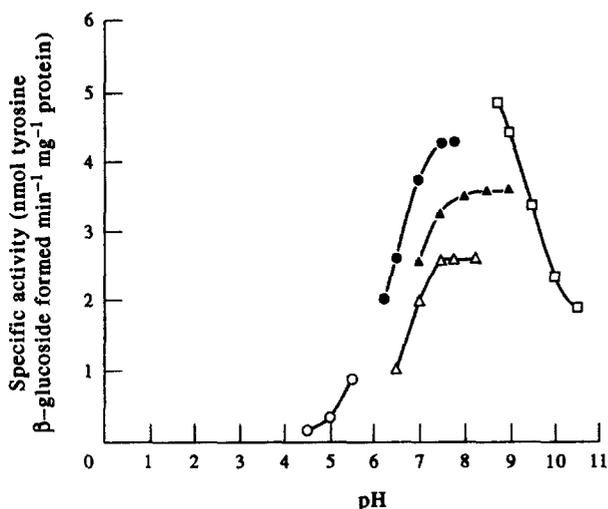


FIGURE 3. Effects of buffers and pH on tyrosine β -glucosyltransferase activity in *M. sexta* fat body. Incubation conditions: tyrosine 2.5 mM, UDPG 5 mM, $MgCl_2$ 25 mM at 30°C for 60 min. Buffers (0.1 M) were (○) acetate, (●) PIPES, (△) phosphate, (▲) Tris-HCl, (□) glycine-NaOH. Enzyme source (approx. 0.1 mg protein) was from aliquots of a 15,000 g pellet obtained from fat body homogenate pooled from 7 5th instar larvae 3 days old.

8.5 and 0.6–1.3 nmol min⁻¹ mg⁻¹ protein, respectively), whereas no TG synthesis occurred in either the 0- or 1-day fat-body preparations (Fig. 5). However, enzyme preparations of fat body from 4-day-old feeding 5th larval instars exhibited a relatively high rate of TG synthesis (5.87 nmol min⁻¹ mg⁻¹ protein, range = 0.9; $n = 2$).

A more detailed study was made of tyrosine β -glucosyltransferase activity in fat body during development of the 5th larval instar (Fig. 6). TG synthesis was detected at 36 h (12 pmol min⁻¹ mg⁻¹ protein), and activity remained at that level through 48 h. However, TG synthesis tripled by 54 h and rose steadily through 72 h. A

dramatic increase in synthesis occurred in 3.5- and 4-day old larval fat body to 2.8 and 5.0 nmol min⁻¹ mg⁻¹ protein, respectively. Activity dropped to 4.6 nmol min⁻¹ mg⁻¹ in 4.5-day-old larvae. Activity fell sharply to 0.2 nmol min⁻¹ mg⁻¹ 6 h later after gut purging and during the body wetting stage. On the fifth day after ecdysis, larvae were in wandering behavior, and glucosylation of tyrosine was 0.09–0.12 nmol min⁻¹ mg⁻¹ protein. Activity remained low thereafter. During the pupal stage, activity was not determined, but it was very low in the fat body of pharate adults 1 day before eclosion (22 days after last larval ecdysis).

Substrate specificity

When 3 mM concentrations of 3-chlorotyrosine, 3-iodotyrosine, 3-nitrotyrosine, *N*-acetyltyrosine, tyrosyltyrosine, or tyramine were substituted for tyrosine as the substrate in the complete incubation mixture, none of these tyrosine derivatives were glucosylated (data not shown). However, small amounts of tyrosine present as an impurity in some of those derivatives were glucosylated.

Inhibitors of tyrosine glucoside synthesis

Synthesis of TG was inhibited by the presence of certain phenolics in the complete incubation mixtures. Tyrosine derivatives, catecholamines, and a variety of synthetic and plant phenolics at various concentrations were weakly to moderately inhibitory to TG synthesis (Table 1). Hydroquinone (*p*-diphenol) and *p*-nitrophenol were the most inhibitory compounds tested (64–66% inhibition, range = 0.5–1.2% $n = 2$): Hydroquinone was 1.7 times more inhibitory than catechol and 2 times more than phenol. Methylation of a hydroxyl group of hydroquinone or catechol reduced the level of inhibition. Thus,

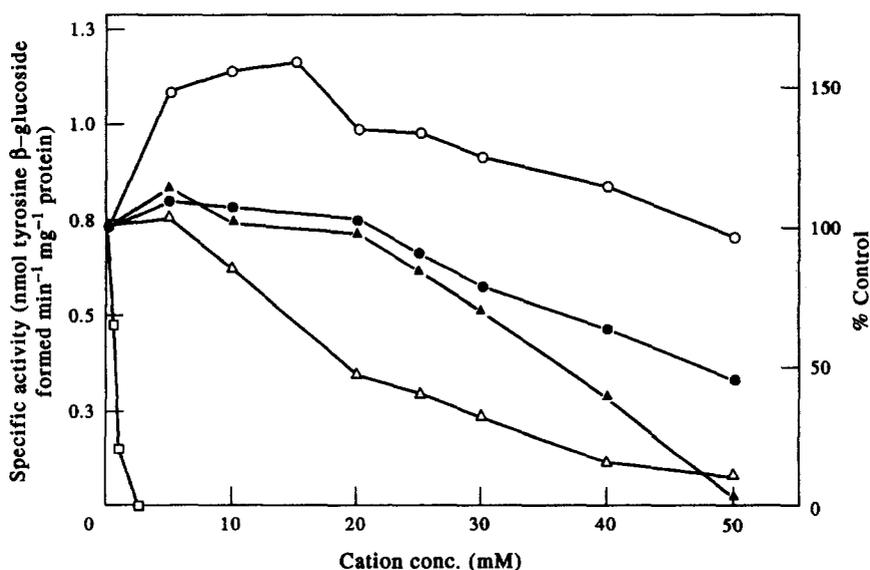


FIGURE 4. Effects of divalent cations on tyrosine β -glucosyltransferase activity in *M. sexta* fat body. Incubation conditions were tyrosine 3 mM, UDPG 7.5 mM, 0.1 M phosphate buffer, (pH 7.5) containing 2.5 mM DEDTCA at 37°C for a 45 min. Cations were (○) Mg^{2+} , (●) Ca^{2+} , (△) Co^{2+} , (▲) Mn^{2+} , (□) Hg^{2+} . Enzyme source (approx. 0.1 mg protein) was from aliquots of a 15,000 g pellet obtained from fat body homogenate pooled from 7 5th instar larvae 3 days old.

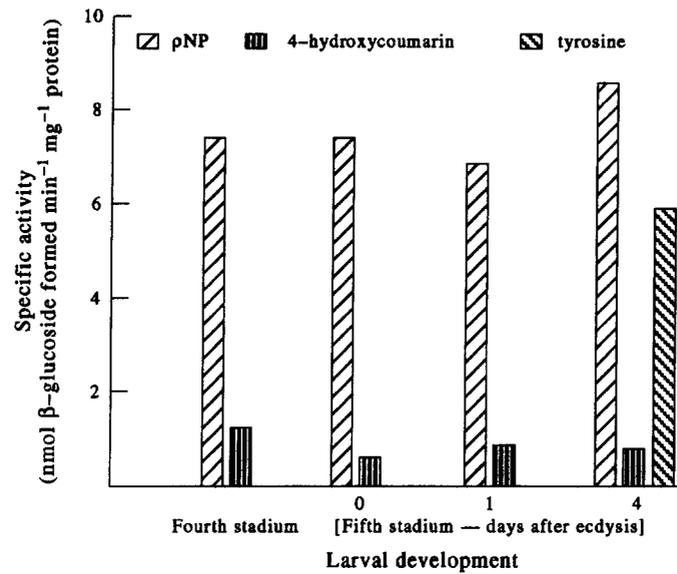


FIGURE 5. Tyrosine β -glucosyltransferase and phenol β -glucosyltransferase activity in *M. sexta* fat bodies from 4th and 5th stadium larvae. Incubation conditions were UDPG 5 mM, $MgCl_2$ 25 mM, 0.1 M phosphate buffer (pH 7.5) at 30°C for 60 min. Substrates were *p*-nitrophenol (pNP, 5 mM), 4-hydroxycoumarin (5 mM), and tyrosine (2.5 mM). Enzyme source (approx. 0.1 mg protein) was from aliquots of a 15,000 g pellet obtained from fat body homogenate pooled from 6 larvae at each stage.

p-methoxyphenol was 0.7 times as inhibitory as its parent hydroquinone, and guaiacol was 0.6 times as inhibitory as catechol. However, *p*-methoxyphenol was 2 times more inhibitory than guaiacol. Substitution on the aromatic ring lowered the inhibitory effect of the phenol, except in the case of *p*-nitrophenol. Guaiacol was 1.7 times more inhibitory than its aldehyde vanillin, and catechol was 2 times more inhibitory than dopamine. Inhibition by phenol was 3.3 times that of tyramine. Acylation of the side chain of dopamine did not seem to have an effect, because dopamine, *N*-acetyldopamine (NADA), and *N*- β -alanyldopamine (NBAD) were of similar inhibitory magnitude. However, hydroxylation of

the side chain drastically reduced the level of inhibition. Norepinephrine and epinephrine were among the least inhibitory compounds. Tyrosine derivatives generally were poor inhibitors of TG synthesis, although 3-chlorotyrosine was as inhibitory as dopamine.

DISCUSSION

Conjugation of phenols with glucose is ubiquitous in insects (Smith, 1968; Ahmad *et al.*, 1986; Ahmad and Hopkins, 1993b). It plays an important role in the detoxification of phenolic xenobiotics and allelochemicals that insects unavoidably encounter in their environment

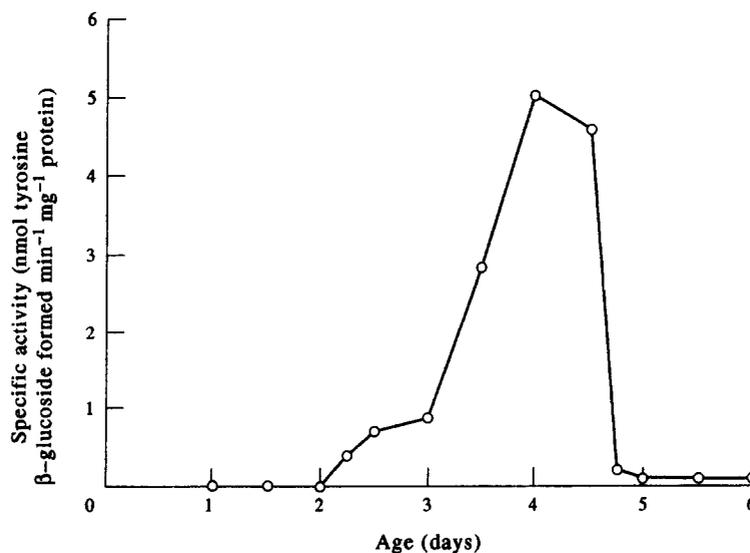


FIGURE 6. Tyrosine β -glucosyltransferase activity in larval fat body during the 5th stadium of *M. sexta*. Incubation conditions: tyrosine 3 mM, UDPG 7.5 mM, $MgCl_2$ 15 mM, $CoCl_2$ 2.5 mM, guconolactone 1 mM, 0.1 M phosphate buffer (pH 7.5) with 2.5 mM DEDTCA at 37°C for 45 min. Enzyme source (0.15–0.5 mg protein) were from aliquots of 15,000 g pellets from fat body homogenates pooled from 3–4 larvae at each age.

TABLE 1. Inhibition of tyrosine β -glucoside synthesis by various phenolic compounds*

Compound	Concentration (mM)	Inhibition (%)
Monophenols		
Tyramine	3.0	9.4
4-Hydroxycoumarin	3.0	11.8
Vanillin	3.0	12.5
Guaiacol	3.0	21.0
Phenol	3.0	31.1
<i>p</i> -Methoxyphenol	3.0	42.1
<i>p</i> -Nitrophenol	0.25	12.2
	1.0	35.2
	3.0	66.3
3-Iodotyrosine	3.0	5.1
3-Nitrotyrosine	3.0	8.1
3-Chlorotyrosine	3.0	18.7
<i>N</i> -Acetyltirosine	3.0	12.8
Diphenols		
Epinephrine	0.5	5.6
Norepinephrine	0.5	6.6
L-Dopa	3.0	10.6
<i>N</i> - β -Alanyldopamine	0.5	5.7
	1.5	14.5
	3.0	18.0
Dopamine	3.0	18.7
<i>N</i> -Acetyldopamine	3.0	19.0
Catechol	3.0	36.9
Hydroquinone	3.0	63.6

*Enzyme source was the 15,000 g pellet from a fat body homogenate pooled from 6 5th instar larvae 3–4 days old. See Materials and Methods for experiment details.

(Ahmad and Hopkins, 1992, 1993a,b). Sequestration of plant phenolics as β -glucosides in insects also has been shown to facilitate storage of defensive compounds or cuticular pigments or perhaps other metabolic needs (Andersen *et al.*, 1988; Hopkins and Ahmad, 1991). Various endogenous phenolics derived from intermediary metabolism in insects are conjugated with glucose, either as a means of inactivation or for subsequent physiological functions (Takahashi and Ohnishi, 1966; Murdock *et al.*, 1970; Hopkins *et al.*, 1984; Yago and Kawasaki, 1984; Thompson *et al.*, 1987; Real and Ferré, 1989). Thus, β -glucosylation of phenolic substrates of both endogenous and exogenous origins for detoxication, excretion, transport, sequestration, or other needs during the course of development may require β -glucosyltransferases with differing properties, substrate specificities, and tissue localization. The present study demonstrates at least two types of glucosyltransferases in *M. sexta*, one specific for tyrosine and the other with a broader specificity for phenolic compounds.

The results of this study demonstrate that TG synthesis in the last larval instar of *M. sexta* is mediated by a specific β -glucosyltransferase found only in the fat body. This enzyme bears some similarities to other phenol β -glucosyltransferases (PGT) in *M. sexta* tissues. Substantial β -glucoside formation of various substrates is obtained with

dTDPG as a glucose donor and the ratios of activity with dTDPG to that with UDPG are similar between those fat body enzymes. However, PGT could transfer glucose from GDPG with *p*-nitrophenol (PNP) as an acceptor at higher rates than the enzyme responsible for TG synthesis (Ahmad and Hopkins, 1992). All β -glucosyltransferases were associated with the 15,000 g particulate fraction, but although PGTs were distributed widely in tissues, the one active towards tyrosine was found only in the fat body. The enzymes have a similar optimal pH range (7.5–9.0), but the effect of buffers on enzyme activity differs. Phosphate buffer was better than Tris for PNP glucosylation, but the reverse was true for tyrosine glucosylation. Mg^{2+} was stimulatory to both enzymes, but it was the only divalent cation that stimulated TG synthesis. Co^{2+} was stimulatory to PNP glucosylation up to 25 mM, but inhibitory to tyrosine glucosylation above 5 mM. Ca^{2+} and Mn^{2+} were as effective as Mg^{2+} for PNP glucosylation at 5 mM, but those ions had little effect on tyrosine glucosylation at that concentration and were inhibitory at higher levels. Whether the differential effects of cations were due only to differing enzyme characteristics is not known. However, these ionic effects may be useful to increase or suppress one type of glucosyltransferase during the purification and characterization of other types of glucosyltransferases.

Although earlier developmental stages had fat body β -glucosyltransferases active towards PNP and 4-hydroxycoumarin, activity towards tyrosine appeared only after day 1 following the last larval ecdysis. Therefore, a specific tyrosine β -glucosyltransferase is apparently synthesized in the fat body during the feeding period of the 5th instar, and that enzyme is responsible for the accumulation of large concentrations of TG in hemolymph during this stage (Ahmed *et al.*, 1983a). Juvenile hormone (JH) was found to be a negative effector for tyrosine glucoside synthesis, and its disappearance after ecdysis of the last larval instar was a necessary prerequisite for TG synthesis (Ahmed *et al.*, 1985).

Having a β -glucosyltransferase with high specificity towards tyrosine would be advantageous for the insect because exogenous phenolic compounds may be abundant in tissues during the feeding period. The ingestion of a variety of plant phenolics in the natural diet would interfere with TG glucosylation, if the enzyme had a broad specificity for phenolic substrates. Inhibition studies indicated that the rate of TG synthesis could be affected by simple phenolics of plant origin. To minimize the interference by dietary phenolics with TG synthesis, *M. sexta* appears to utilize two mechanisms: (1) a wide tissue distribution of β -glucosyltransferases active towards plant phenolics and (2) a high enzyme affinity and specificity for those substrates. We found that all tissues except hemolymph are active in the glucosylation of PNP (Ahmad and Hopkins, 1992). This compound is also an inhibitor of TG synthesis. It is interesting to note that simple diphenolics, such as hydroquinone and catechol, were glucosylated at the highest rate by the labial glands,

which are tubular organs of significant mass in this species positioned next to the gut (Ahmad and Hopkins, 1993a). Thus, gut, labial glands, fat body, and epidermis may be tissues with defensive functions that protect TG synthesis by having β -glucosyltransferases with high activity towards plant phenolics. With apparent K_m s for PNP by the labial gland and fat body PGTs of approx. 0.1 mM (Ahmad and Hopkins, 1992), the glucosylation system for detoxification would efficiently eliminate interfering phenolics at relatively low concentrations. On the other hand, catecholamines that are moderately inhibitory to TG synthesis *in vitro* are present at very low concentrations in the hemolymph *in vivo* during active TG synthesis (Hopkins and Kramer, 1991). Krueger *et al.* (1989) showed that NBAD was synthesized and stored in the integument during the period of active TG synthesis by the fat body. Only when larvae had initiated wandering behavior and TG synthesis was shut down did NBAD synthesis by the fat body increase. This mechanism and timing may prevent competition with TG synthesis.

The sharp decline in TG synthesis coincides with the first release of 20-hydroxyecdysone (20-HE) (Bollenbacher *et al.*, 1981). This event occurs on day 4 after the last larval ecdysis and triggers cessation of feeding, purging of gut contents, and initiation of wandering behavior. The second major release of 20-HE that occurs 2 days later initiates apolysis and production of pupal cuticle. Injection of 20-HE in isolated abdomens of larval *M. sexta*, which mimics the second release 2 days after beginning of wandering, did not immediately lower TG levels in hemolymph (Ahmed *et al.*, 1983b). Two days after 20-HE injection, TG titers dropped because of increasing activity of TG hydrolase, which is regulated by 20-HE. These results indicated that 20-HE does not directly inhibit or deactivate the enzyme responsible for TG synthesis, because a higher concentration of the hormone did not inhibit the residual TG synthesis activity. Thus, the first release of the hormone probably leads to a reduction of TG synthesis by turning off expression of the glucosyltransferase gene, coupled with degradation and turnover of the enzyme. Phenol β -glucosyltransferase activity towards PNP, on the other hand, also declined after cessation of feeding, but not to levels as low as TG synthetase (data not shown).

Integrating our results with those of Ahmed *et al.* (1983a,b, 1985), the fat body appears to be the main site of regulation of tyrosine and TG titers in the hemolymph of the last larval instar of *M. sexta*. Decreasing levels of JH titers after the last larval ecdysis result in a gradual increase in tyrosine β -glucosyltransferase activity in the fat body and the appearance of TG in the hemolymph. Activity then increases sharply on the third day after ecdysis, reaches a maximum level on day 4, and then declines, probably because of events triggered by the first pulse of 20-HE. During this time, TG reaches maximum concentrations in the hemolymph (Ahmed *et al.*, 1983a). Low levels of TG synthetase occur during body wetting

behavior a few hours before wandering behavior on the fifth day after the last larval ecdysis and are maintained thereafter. At the same time, the first minor pulse of the molting hormone causes the synthesis or activation of TG hydrolase present in the fat body (Ahmed *et al.*, 1983b). β -Glucosidase activity begins to increase at the onset of wandering behavior, rises sharply after tanning begins in pharate pupal cuticle (brown thoracic bar stage), reaches a peak of activity at ecdysis, and then decreases to its initial low level 1-day after pupal ecdysis. Levels of TG in hemolymph fall as tyrosine levels rise during the period of high β -glucosidase activity and low TG synthetase activity during pharate pupal development. Tyrosine then enters the metabolic pathways that supply the *N*-acyldopamine precursors for quinonoid cuticular tanning agents (Hopkins and Kramer, 1991, 1992).

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