Partial Purification and Properties of an Inducible Uridine 5′-Diphosphate-Glucose:Salicylic Acid Glucosyltransferase from Oat Roots

Nasser Yalpani, Margaret Schulz, Michael P. Davis, and Nelson E. Balke
Department of Agronomy, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

A salicylic acid (SA)-inducible uridine 5′-diphosphate (UDP)-glucose:SA 3-O-glucosyltransferase was extracted from oat (Avena sativa L. cv Dal) roots. Reverse phase high-performance liquid chromatography or anion exchange chromatography was used to separate SA from the product, β-O-glucosylsalicylic acid. The soluble enzyme was purified 176-fold with 5% recovery using a combination of pH fractionation, anion exchange, gel filtration, and chromatofocusing chromatography. The partially purified protein had a native molecular weight of about 50,000, an apparent isoelectric point at pH 5.0, and maximum activity at pH 5.5. The enzyme had a K_m of 0.28 mM for UDP-glucose and was highly specific for this sugar donor. More than 20 hydroxybenzoic and hydroxycinnamic acid derivatives were assayed as potential glucose acceptors. UDP-glucose:SA 3-O-glucosyltransferase activity was highly specific toward SA (K_m = 0.16 mM). The enzyme was inhibited by UDP and uridine 5′-monophosphate but not by up to 7.5 mM uridine 5′-triphosphate but not by up to 7.5 mM uridine 5′-triphosphate.

SA is widely distributed in plants (27), where it may be involved in the regulation of a number of physiological processes. Increases in endogenous levels of SA have been shown to trigger heat production in inflorescences of certain thermogenic species (26) and may also induce resistance to plant pathogens (21, 22, 33). Exogenously applied SA has been shown to stimulate flowering in some plants (6) and inhibit ethylene biosynthesis (19) and stomatal closure (25). High concentrations of SA, as have been found in the rhizosphere under decomposing rye, maize, and other species, may inhibit the growth of other plants (5, 24). This toxicity may be due to SA-induced disruption of membrane integrity and inhibition of ion (K^+, Cl^-, PO_4^{2-}) absorption (9, 11, 20).

Conjugation reactions, notably those catalyzed by glucosyltransferases, play a key role not only in the removal of plant hormone molecules from the active pool (28) but also in the detoxification of xenobiotics such as herbicides (18). Oat roots can recover from the toxic effect of SA on ion absorption (11). Upon incubation of oat roots in a solution of SA, a GTase that catalyzes the conjugation of glucose to SA is induced (2). Hydrolysis studies revealed that in oat roots only one SA conjugate, GSA, is formed. This compound was found to be less inhibitory to Cl^- absorption than SA. Thus, the GTase may detoxify SA.

In an earlier communication, the partial purification and some properties of this GTase were reported (2). Here, we present a more effective purification protocol and further characterization of this enzyme.

MATERIALS AND METHODS

Plant Material

Oat (Avena sativa L. cv Dal) seeds were germinated on cheesecloth stretched over a 4-L beaker containing 3 L of continuously aerated 10 mM CaSO_4 in the dark at room temperature.

Induction of GTase

Roots from 5-d-old seedlings were excised, rinsed with distilled H_2O, and incubated in induction buffer (0.5 mM KCl, 0.25 mM CaSO_4, 0.5 mM SA, 25 mM Mes/Tris [pH 6.5]) using 30 mL g^-1 of roots at 25°C for 12 h. The induction was stopped by decanting the induction buffer, washing the roots in ice-cold distilled H_2O for 2 min, blotting them dry, and freezing them with liquid N_2. Roots could be stored at ~80°C for several months without loss of GTase activity.

Partial Purification of GTase

All procedures were performed at 0 to 4°C. Induced roots (200 g) were submerged in liquid N_2 and homogenized with a mortar and pestle. The powder was thawed in 300 mL of TM buffer containing 10% (w/w root) Dowex-1 and 1% (w/w root) insoluble PVP. The brei was passed through four
layers of cheesecloth and the filtrate centrifuged at 15,000g for 20 min. The pH of the supernatant (crude GTase) was lowered to 5.1 with 0.1 M HCl and the mixture allowed to equilibrate for 40 min. Insoluble proteins were removed by centrifugation (15,000g, 20 min) and discarded. The supernatant was brought to pH 7.0 with 0.1 M KOH. Overnight dialysis against TM buffer to reduce the concentration of charged low mol wt solutes, the extract was loaded onto a DEAE-Sepharose column (2.5 × 17 cm, Pharmacia) equilibrated with TM buffer. After unbound proteins were eluted, GTase eluted with 0.18 to 0.22 M KCl during a linear 200-mL, 0.05 to 0.25 M KCl gradient in TM buffer pumped through the column at 0.3 mL min⁻¹. Fractions containing GTase activity were pooled and concentrated to 5 mL by ultrafiltration on an Om-1000 membrane (Pharmacia).

The GTase-enriched solution was loaded onto a Sephacryl S-200 gel filtration column (2.5 × 95 cm) equilibrated in TM buffer and chromatographed at 0.65 mL min⁻¹. The gel filtration column was calibrated using Cyt c, carbonic anhydrase, ovalbumin, BSA, alcohol dehydrogenase, and α-amylase from Sigma. The mol wts of these proteins were assumed to be 12,400, 29,000, 44,000, 68,000, 150,000, and 200,000, respectively. Fractions eluted between 260 and 300 mL of TM buffer contained GTase activity, were pooled, and were concentrated by ultrafiltration. The concentrated GTase was transferred into BT buffer by passing it through a 1.5- × 6.0-cm Sephadex G-25-desalting column equilibrated in BT buffer. The entire protein fraction was loaded onto a Mono P HR 5/20 column (Pharmacia) that was equilibrated in BT buffer and linked to a Shimadzu gradient HPLC system. This chromatofocusing column was washed at 1 mL min⁻¹ with a solution of 10% (v/v) Polybuffer 74 (Pharmacia) at pH 4.0, containing 10% (v/v) glycerol, and 20 mM 2-mercaptoethanol, resulting in a pH gradient from pH 7.2 to 4.0. Fractions (1 mL) were collected in test tubes containing 1 mL of TM buffer to reduce the inhibitory effect of Polybuffer 74 on GTase activity and stability. Fractions eluting between pH 5.1 and 4.9 contained GTase activity and were combined. Polybuffer 74 was removed from the GTase by three cycles of concentration and redilution on an Om-100 ultrafiltration membrane. The GTase-enriched preparation was stored at −80°C overnight before being used in experiments to characterize the enzyme.

GTase Assay

The standard incubation mixture consisted of 40 μL of protein solution containing 2 to 120 μg total protein (depending on the purity of the GTase), 5 mM NH₄Cl, 10 mM UDP-glucose, and 0.4 mM SA in TM buffer in a final volume of 200 μL. Assays with radioactive SA also contained 1 kBq [7-¹⁴C]GSA (1.96 GBq mmol⁻¹) (New England Nuclear). Incubations were performed at 30°C and terminated by addition of 200 μL of methanol after 30 min. SA was separated from the product, GSA, by chromatography on either Polyamide 6 (Serva) or C₄ Ultrafiphere ODS (Beckman Instruments, Inc.) columns. For separations of [¹⁴C]GSA from [¹⁴C]GSA using Polyamide 6 columns, the entire 400 μL of methanolic reaction mixture was loaded onto a 0.8- × 2-cm polyamide column equilibrated in 10 mM Tris-Mes, pH 7.0. One milliliter of H₂O was passed through the column. GSA was then eluted with 5 mL of H₂O, collected in scintillation vials, and 14.5 mL of Ecolite (Westchem, San Diego, CA) scintillation cocktail was added. Radioactivity was determined with a scintillation counter (LKB 1217 Rackbeta). For the determination of substrate specificity, pH optimum, and kinetic constants, [¹⁴C]GSA was omitted from the incubation mixture. The methanolic reaction mixture was centrifuged (12,000g, 5 min) to remove precipitates, and a 50- to 100-μL aliquot was injected onto a C₁₈ column (4.6 × 150 mm, Ultrasphere ODS) connected to an HPLC (Beckman gradient liquid chromatograph, model 334, with a model 165 variable wavelength detector). A linear gradient of 1% (v/v) acetic acid in H₂O to 100% methanol at 1 mL min⁻¹ over 20 min was used to separate SA from GSA. Elution was monitored at 280 nm. GTase activity was quantified by measuring the decrease in the content of the phenolic substrate in the reaction mixture. Formation of the glucoside product was confirmed by hydrolysis of the material eluting in the product peak.

2-D PAGE

The partially purified GTase preparation obtained after chromatofocusing, as described above, was fractionated by 2-D PAGE according to the procedures of O’Farrell (23) with the following modifications. Proteins were partitioned into phenol and pelleted in 1% (v/v) 2-mercaptoethanol in methanol containing 0.1 M ammonium acetate (13). They were solubilized with O’Farrell’s lysis buffer (1 μL μg⁻¹ of protein) (23) modified to contain 3.2% (v/v) BioLyte 4/6 and 0.8% (v/v) BioLyte 3/10 (Bio-Rad). Five micrograms of protein were loaded on focused, first-dimension, IEF tube gels (1.6 × 70 mm). The upper, cathodic buffer was 0.1 M NaOH; the anodic buffer was 0.1 M H₂PO₄. Electrophoresis was performed at 200 V for 15 h followed by 750 V for 1 h. Under these conditions, a linear pH gradient from pH 7.3 to 4.5 was obtained. The apparent pH profile was determined by dividing a focused IEF gel into 10 segments and soaking each segment in 2 mL of 1 M KCl for 8 h before pH measurement. IEF gels were prepared for second-dimension SDS-PAGE using the method of Görg et al. (10). The SDS gels were 1.5 mm thick. Each 60-mm, 10% (w/v) acrylamide separating gel was overlayed with a 10-mm stacking gel that was 4% (w/v) acrylamide. Electrophoresis was performed at a constant 150 V using a Bio-Rad minigel apparatus. Bio-Rad low mol wt protein standards and oat root proteins were silver stained (4).

Protein Determination

Protein was determined using Bio-Rad protein dye reagent and BSA as the standard.

RESULTS

Enzyme Purification

A 15,000g supernatant of an oat root homogenate was used for GTase purification. Lowering the pH of the supernatant from 7 to 5 precipitated a portion of the unwanted proteins. The extract was titrated back to pH 7 and fraction-
UDP-GLUCOSE:SALICYLIC ACID GLUCOSYLTRANSFERASE

Table I. Purification of GTase from Oat Roots

GTase was extracted from 200 g of oat roots and activity determined using polyamide columns as detailed in "Materials and Methods."

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Activity (milliunits)</th>
<th>Specific Activity (milliunits/mg protein)</th>
<th>Purification Fold</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>498</td>
<td>2.0</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>pH fractionated</td>
<td>245</td>
<td>3.3</td>
<td>1.7</td>
<td>49</td>
</tr>
<tr>
<td>DEAE</td>
<td>161</td>
<td>8.5</td>
<td>4.3</td>
<td>32</td>
</tr>
<tr>
<td>S-200</td>
<td>104</td>
<td>22.0</td>
<td>11.1</td>
<td>21</td>
</tr>
<tr>
<td>Mono P</td>
<td>26</td>
<td>353.0</td>
<td>176.0</td>
<td>5</td>
</tr>
</tbody>
</table>

GTase was eluted with a KCl gradient and further purified by gel filtration chromatography (Fig. 1B). Peak enzyme fractions from this purification step were loaded onto a chromatofocusing column and eluted with a pH 7.2 to 4.0 gradient (Fig. 1C). This procedure resulted in 176-fold purification and 5% recovery of GTase activity (Table I). On the basis of its recovery and its specific activity before and after partial purification, we estimate that the GTase represents less than 0.6% of the total proteins in the crude extract. Thus, it is a minor cell constituent.

Comparison of silver-stained, 2-D electrophoresis gels of crude with partially purified GTase illustrated the purification.
accomplished (Fig. 2). At least 30 peptides were detectable in the GTase preparation after chromatofocusing.

Physical Properties

Throughout the purification protocol, we used 2-mercaptoethanol, leupeptin, and glycerol in the buffers, because preliminary experiments had shown that they protected the enzyme from loss of activity. The GTase was eluted from a Sephacryl S-200 gel filtration column with a retention volume corresponding to proteins with an $M_r$ of about 50,000 (Fig. 1B). Based on its elution during chromatofocusing, the apparent isoelectric point of the GTase was at pH 5.0 (Fig. 1C). For this partially purified extract, one peptide was just detectable in the region of 2-D PAGE gels where proteins with an $M_r$ of 50,000 and an isoelectric point of 5.0 would be expected; however, enrichment of peptides with $M_r$s between 25,000 and 30,000 was also noted (Fig. 2B). It was possible that, under the conditions used for 2-D PAGE, the GTase does not move to its native isoelectric point and that it dissociates into subunits that have a different isoelectric point. Whereas some glucosyltransferases are monomeric (1), others consist of a dimer of low mol wt peptides (12, 14, 15). Positive identification of the peptide(s) representing the GTase on the gels was, therefore, not possible.

Substrate Specificity

The partially purified enzyme exhibited high substrate specificity. UDP-glucose was the favored sugar donor (Table II). When GDP-glucose was used instead of UDP-glucose, 83% less GSA was formed. Other nucleotide diphosphate sugars could not donate sugars to SA. Also, SA was the favored sugar acceptor (Table III). Other phenolic compounds similar in structure to SA were not glucosylated by the GTase in the presence of UDP-glucose. Thus, the GTase appears to be specific for UDP-glucose as the sugar donor and for SA as the glucose acceptor.

Other Properties

Properties of the GTase were examined using the partially purified enzyme preparation. Under standard assay conditions, the rate of product formation was linear with incubation times up to 60 min and concentrations up to 120 $\mu$g of protein per assay. GTase activity was not significantly affected by the divalent cations Ca$^{2+}$, Cu$^{2+}$, Fe$^{3+}$, Mg$^{2+}$, or Mn$^{2+}$ (1 mm) (data not shown). Product formation was maximal at 43°C. An Arrhenius plot of the log of the reaction rate against the reciprocal of temperature was linear between 15 and 43°C (Fig. 3). The decline in $V_{\text{max}}$ at temperatures exceeding 43°C may reflect GTase denaturation. The calculated apparent energy of activation was 7.5 kcal mol$^{-1}$. Optimum catalysis was observed at pH 5.5 (Fig. 4). Kinetic constants for the partially purified enzyme were determined by assaying GTase activity in the presence of a range of substrate concentrations. The apparent $K_m$ for SA was 0.16 mm and for UDP-glucose 0.28 mm when assayed in the presence of 10 mm UDP-glucose and 0.4 mm SA, respectively.

Consistent with UDP-glucose being the preferred sugar donor, we observed inhibition of enzyme activity by UDP, a product of the GTase reaction, but not by ≤7.5 mm UMP (Fig. 5), ADP, or ATP (data not shown). Uridine triphosphate was a stronger inhibitor of GTase than UDP was (Fig. 5). When the concentration of the substrate UDP-glucose was varied in the presence of 0.4 mm SA (Fig. 6), the data yielded a nonlinear secondary Lineweaver-Burk plot for UDP inhibition (Fig. 6, inset). This result suggested a mixed type of inhibition. In contrast, competitive inhibition by UDP was observed when the concentration of the substrate SA was varied in the presence of fixed concentrations of UDP-glucose (Fig. 7). In this case, the apparent $K_m$ for UDP was determined to be 0.25 mm.

DISCUSSION

Many of the properties of the enzyme described in this report are shared by other plant glucosyltransferases. The GTase from oats has an apparent $M_r$ of 50,000 (Fig. 1B) and an isoelectric point near 5 (Fig. 1C). Transferases catalyzing the O-glucosylation of flavonoids and anthroquinones from a number of species have $M_r$s ranging from 42,000 to 61,000 (12, 15, 16) and also have acidic isoelectric points near pH 5.0 (12, 14, 16). A preference for UDP-glucose as the sugar donor is quite typical for plant glucosyltransferases (7) and was also observed with the oat root GTase (Table II). The estimated energy of activation of the reaction catalyzed by this enzyme, 7.5 kcal mol$^{-1}$, is similar for a glucosyltransferase from papaya fruits (15). The apparent $K_m$ of the GTase for UDP-glucose, its preferred sugar donor ($K_m = 0.28$ mm), is of the same order of magnitude as for glucosyltransferases from soybean and Lithospermum, where the reported $K_m$s for UDP-glucose were 0.3 and 0.27 mm, respectively (3, 14). This high $K_m$ value may reflect the presence of high concentrations of this cosubstrate in plant tissues (30). Because UDP-glucose is involved in numerous cellular processes, it is unlikely to be a regulatory molecule for the reaction catalyzed by the GTase.

In contrast to our data with the oat GTase (Figs. 6 and 7),
Table III. Glucose Acceptor Specificity of Partially Purified GTase

Acceptors-glucose conjugate and free acceptor were separated by reverse phase HPLC and detected at 280 nm following incubation of the enzyme with acceptor (0.4 mM) and UDP-glucose at 1.0 mM. Quantitation of GTase activity was based on disappearance of phenolic substrate, which was accompanied by the appearance of a product peak. Conjugate formation was confirmed by hydrolysis of the eluted product.

<table>
<thead>
<tr>
<th>Glucose-Acceptor</th>
<th>Substituents</th>
<th>GTase Activity % of SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoic acid</td>
<td>COOH</td>
<td>0</td>
</tr>
<tr>
<td>α-Hydroxybenzoic acid (SA)</td>
<td>COOH OH</td>
<td>100*</td>
</tr>
<tr>
<td>m-Hydroxybenzoic acid</td>
<td>COOH OH</td>
<td>9</td>
</tr>
<tr>
<td>p-Hydroxybenzoic acid</td>
<td>COOH OH</td>
<td>0</td>
</tr>
<tr>
<td>Methylsalicylate</td>
<td>COOCH₃ OH</td>
<td>0</td>
</tr>
<tr>
<td>Saligenin</td>
<td>CH₂OH OH</td>
<td>0</td>
</tr>
<tr>
<td>α-Methoxyphenol</td>
<td>OCH₃ OH</td>
<td>0</td>
</tr>
<tr>
<td>Phenol</td>
<td>OH</td>
<td>0</td>
</tr>
<tr>
<td>p-Nitrophenol</td>
<td>OH NO₂</td>
<td>12</td>
</tr>
<tr>
<td>m-Hydroxybenzaldehyde</td>
<td>COH OH</td>
<td>0</td>
</tr>
<tr>
<td>p-Hydroxybenzaldehyde</td>
<td>COH OH</td>
<td>0</td>
</tr>
<tr>
<td>Vanillin</td>
<td>CHO OCH₃ OH</td>
<td>9</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>COOH OCH₃ OCH₃</td>
<td>0</td>
</tr>
<tr>
<td>Syringaldehyde</td>
<td>CHO OCH₃ OCH₃</td>
<td>4</td>
</tr>
<tr>
<td>trans-Cinnamic acid</td>
<td>CH=CHCOOH</td>
<td>0</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>CH=CHCOOH OH</td>
<td>13</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>CH=CHCOOH OCH₃ OH</td>
<td>0</td>
</tr>
</tbody>
</table>

*GTase activity was 0.56 milliunits assay⁻¹ when SA was used.

Figure 3. Arrhenius plot of GTase activity at temperatures from 15 to 50°C. T, Temperature; µU, microunits.

Figure 4. Activity of partially purified GTase at pH values from 4.0 to 8.0. Activity was assayed in either acetate or Tris-Mes buffer (25 mM) for samples at pH 4 to 5.5 and pH 6.0 to 8.0, respectively. Results are means ± se. µU, Microunits.
UDP is a noncompetitive inhibitor with respect to its phenolic substrate and a competitive inhibitor with respect to UDP-glucose for a UDP-glucose:
\[ \text{UDP-glucose:p-hydroxybenzoate glucosyltransferase from Lithospermum} \]
cell cultures (3). More kinetic work with the GTase is needed to resolve the basis for these differences. Also, whereas the oat GTase (Table III) and many other glucosyltransferases show a high degree of specificity for their phenolic substrates (3, 31), a UDP-glucose:cinnamic acid glucosyltransferase from sweet potatoes catalyzes the conjugation of a range of phenolics (29).

Conjugation of SA by the GTase may play not only a role in the deactivation of exogenously supplied SA but may also be important for the regulation of endogenous, constitutively 

present SA in plants. SA has been isolated from a wide range of plant taxa (27). Oat seedling roots that have not been exposed to exogenous SA contain 500 ng of SA g\(^{-1}\) fresh weight (N. Yalpani, unpublished data) which, incidentally, may explain the high \(K_m\) value of the GTase for SA (\(K_m = 0.16 \text{ mM}\)). Furthermore, enzymes that catalyze the formation of GSA are not unique to oats. Tanaka et al. (32) prepared a crude extract of a similar, SA-inducible enzyme from *Mallotus* suspension cultures. Feeding studies with \(^{14}\)C-labeled benzoic acid resulted in the synthesis of significant amounts of labeled GSA along with smaller amounts of SA in *Helianthus* hypocotyls (17). An accumulation of GSA in vivo also accompanies an increase in the endogenous levels of SA during the resistance response of tobacco to virus infection (8). Insights into possible physiological roles of SA will contribute to our understanding of the significance of the GTase in plant tissues.

**LITERATURE CITED**


5. Chou CH, Patrick ZA (1976) Identification and phytotoxic ac-


