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# INACTIVATION OF RED BEET β-GLUCAN SYNTHASE BY NATIVE AND OXIDIZED PHENOLIC COMPOUNDS

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**Key Word Index**—Glucan synthase; polyphenoloxidase; tyrosinase; phenolic compounds; red beet root; microsomal membranes.

**Abstract**—The effects of phenolic compounds on glucan synthase, a membrane-bound enzyme from red beet root, were examined. Different classes of phenolic compounds were screened in the absence and presence of polyphenoloxidase (PPO). At levels less than 1 mM, inactivation occurred with many of the compounds tested. However, in most cases oxidation by PPO was required. Coumarin, previously demonstrated to interfere with cell wall polysaccharide biosynthesis, was not inhibitory. Mechanistic studies utilizing catechol showed that phenolic inactivation could be protected against by PPO inhibitors and thiol protective reagents. However, once inactivation occurred, it could not be reversed. Ommission of thiols and polyvinylpyrolidone from homogenization buffers did not reduce glucan synthase levels of microsomal preparations. It appears that glucan synthase, a membrane-bound enzyme, is as susceptible to phenolic effects as cytosolic enzymes and *in situ* inactivation is a function of the availability of both endogenous phenolics and PPO.

## INTRODUCTION

It has long been recognized that phenolic compounds can influence the activity of soluble enzymes. Enzymatic activity was shown to be effectively inhibited by phenolic derivatives generated through the action of polyphenoloxidase [1, 2]. These derivatives were found to covalently bind to functional residues in proteins such as sulphydryl and primary amino groups [3–6]. Lysine residues, in particular, were identified as the target sites for polyphenoloxidase-generated chlorogenoquinone [7, 8]. To minimize phenolic oxidation, the inclusion of polyvinyl pyrrolidone-40 (PVP) in whole tissue homogenates was proposed [9]. Thiol protectants such as dithiothreitol are also routinely included for this purpose [2, 10, 11].

In recent years, much interest has been focused on the characterization and isolation of membrane-bound enzymes. Although PVP and thiol protectants are routinely included in homogenization media, the susceptibility of integral membrane enzymes to phenolic inactivation has not been investigated. The objective of this study was to determine conditions under which a membrane-bound enzyme from a higher plant, glucan synthase, is susceptible to phenolic inactivation and to ascertain whether the mechanism of inactivation of a membrane enzyme by phenolics is comparable to that of soluble enzymes.

## RESULTS

### *Buffer selection*

Glucan synthase assays are traditionally conducted in the presence of Tris buffers. Since Tris is a primary amine, it can potentially react with phenolic oxidation products and mask the effects of phenolic compounds on enzyme activities. For this reason, the compatability of glucan synthase with other buffer systems was investigated. Mops and Hepes buffer had no adverse effects on glucan synthase activity. Phosphate buffer, however, reduced the rate of glucose incorporation by *ca* one-third.

In a preliminary phenolic inhibitor screening experiment using catechol and PPO in the presence of Tris, complete inhibition of glucan synthase was only observed at concentrations of 10 mM and above. When Mops and Hepes or phosphate buffers were substituted for Tris, complete inhibition was obtained at catechol concentrations as low as 0.1 mM. The use of Tris was therefore discontinued and all subsequent screenings were conducted in Hepes.

### *Effects of phenolic compounds and their oxidation products on glucan synthase activity*

The effects of phenolic compounds were tested in the presence and absence of PPO. Since red beet root tissue contains PPO activity, it was necessary to ascertain its presence in the microsomal fraction. Microsomal preparations were invariably found to contain activity at levels ranging from 0.68 to 3.34 absorbance units per mg protein. An attempt was made to remove this activity by two washing treatments known to remove adsorbed soluble proteins from membrane fractions [12, 13]. Neither 0.25 M KI or 0.15 M KCl was able to reduce microsomal PPO levels. These reduced glucan synthase activity by 51% and 77% activity, respectively.

The elimination of endogenous PPO activity without adversely affecting glucan synthase could be accomplished by the addition of thiol compounds, ascorbic acid, and bovine serum albumin. In assays where it was desirable to eliminate the effects of endogenous PPO,

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membranes were incubated with phenolic compounds and 0.5 mM thioglycolic acid.

Phenolic screening assays are shown in Figs 1–5. Many of the phenolic compounds tested inactivated glucan synthase, but mainly after they were oxidized. Some of the oxidized phenolic compounds found to be highly inhibitory were (numbers in parentheses are approximate $I_{50}$ values in decreasing order of potency): catechin (50 $\mu$m) (Fig. 3), epicatechin (50 $\mu$M) (Fig. 3), chlorogenic acid (50 $\mu$M) (Fig. 2), caffeic acid (50 $\mu$M) (Fig. 2), catechol

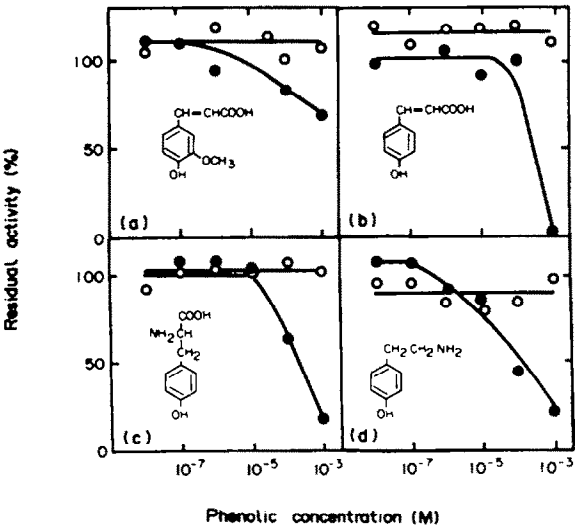


Fig. 1. Concentration effects of some monohydroxy phenolic compounds on glucan synthase activity. A. Ferulic acid; B. coumaric acid; C. tyrosine and D. tyramine. ●——●, Preincubation mixtures containing phenolic compound, added mushroom tyrosinase and microsomes; O——O, preincubation mixtures containing phenolic compound, membranes and 0.5 mM thioglycolic acid.

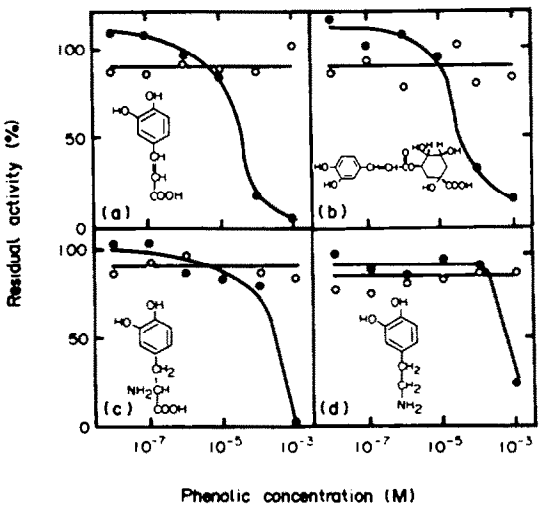


Fig. 2. Concentration effects of some dihydroxy phenolic compounds on glucan synthase activity. A. Caffeic acid; B. chlorogenic acid; C. L-DOPA and D. dopamine. Symbols: See Fig. 1.

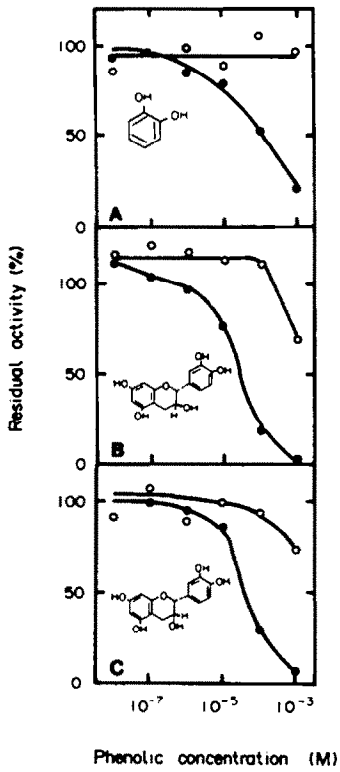


Fig. 3. Concentration effects of catechins on glucan synthase activity. A. Catechol; B. catechin and C. epicatechin. Symbols: see Fig. 1.

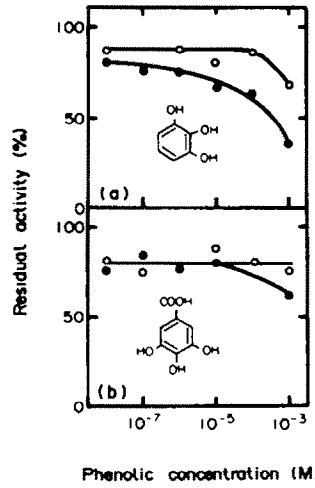


Fig. 4. Concentration effects of some trihydroxy phenolic compounds on glucan synthase. A. Pyrogallol and B. gallic acid. Symbols: see Fig. 1.

(100 $\mu$M) (Fig. 3), tyrosine (100 $\mu$M) (Fig. 1), tyramine (100 $\mu$M) (Fig. 1), pyrogallol (1 mM) (Fig. 4), L-DOPA (1 mM) (Fig. 2), dopamine (1 mM) (Fig. 2), *p*-coumaric acid (1 mM) (Fig. 1) and ferulic acid (10 mM) (Fig. 1). Gallic acid was slightly inhibitory at 1 mM. The unoxidized forms of catechin, epicatechin, and pyrogallol were slightly inhibitory at 1 mM. Gossypol, quercetin,

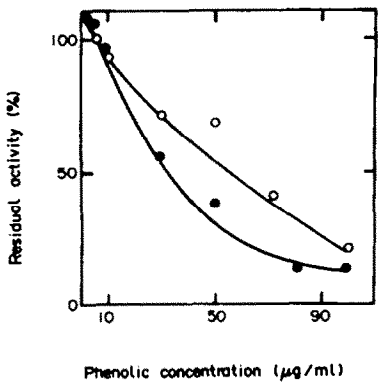


Fig. 5. Concentration effect of tannic acid on glucan synthase. Symbols: see Fig. 1.

ellagic acid, cinnamic acid, coumarin, and resorcinol had no inhibitory effects on glucan synthase activity (data not shown).

Tannic acid, being an impure preparation, was assayed over a concentration range from 0.1 to 100 μg/ml (Fig. 5). Loss of glucan synthase activity occurred in either the presence of excess mushroom tyrosinase or absence of PPO and proceeded gradually throughout the concentration range. Oxidation of tannic acid enhanced its inhibitory effects.

### *Mechanism of glucan synthase inactivation by catechol*

*Effect of preincubation and contribution of endogenous PPO.* With soluble enzymes, maximal inhibitory effects were observed when oxidized phenolic compounds were allowed to preincubate with the enzyme prior to the addition of substrate [1]. This was also found to be the case with glucan synthase. Fig. 6A shows the time course of [$^{14}$C]-glucose incorporation in a control and with membranes incubated with catechol in the presence of both endogenous (microsomal) PPO and exogenously added mushroom tyrosinase. In the presence of mushroom tyrosinase and catechol, glucan synthase was completely inactivated (Fig. 6A). The addition of mushroom tyrosinase and catechol to a glucan synthase reaction mixture at 5 min resulted in the almost instant termination of the reaction (Fig. 7). In the presence of endogenous PPO only, the rate of incorporation of [$^{14}$C]-glucose into catechol-treated membranes was significantly reduced relative to the control (Fig. 6A), but complete inactivation did not occur. Since this inhibition could be protected against by the addition of PPO inhibitors of the free-radical scavenger type (Table 1), it was concluded that inactivation of glucan synthase activity resulted from the interaction of phenolic oxidation products with the enzyme.

The effect of preincubating catechol and UDPG in the presence and absence of mushroom tyrosinase where the glucan synthase reaction was initiated by the addition of microsomal membranes (no preincubation) is shown in Fig 6B. With mushroom tyrosinase present, a small amount of [$^{14}$C]-glucose was incorporated however, the reaction rapidly ceased. With membranes and no added mushroom tyrosinase, incorporation during the first 15 min was comparable to the control. By 30 min, enzymatic activity began to diminish.

Two control experiments to demonstrate that UDPG did not participate in the PPO reaction were performed. The first (Fig. 6B) shows that preincubation of mushroom tyrosinase with UDPG had no effect on reaction rate. The second showed that when UDPG, mushroom tyrosinase and catechol were preincubated together, the time course of [$^{14}$C]-glucose incorporation was identical to the same reaction initiated by a mixture of UDPG and membranes (data not shown). Had UDPG participated in the reaction between catechol and mushroom tyrosinase, a significant

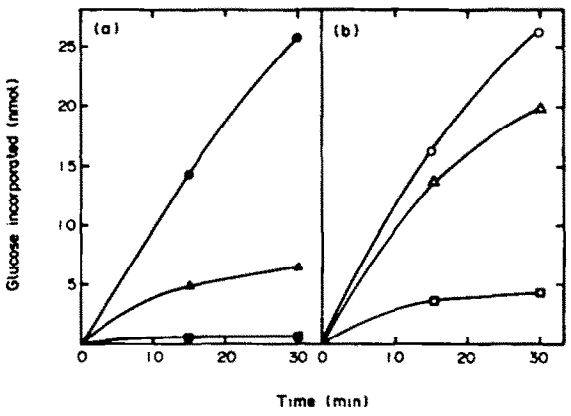


Fig. 6. Time courses of glucan biosynthesis in the absence and presence of catechol. A. Membranes were pre-incubated 15 min with catechol alone (▲) and with mushroom tyrosinase plus catechol (■). Reactions were initiated with UDPG. In the control experiment, catechol and mushroom tyrosinase were omitted (●). B. UDPG pre-incubated 15 min with catechol alone (Δ) and mushroom tyrosinase plus catechol (□). These reactions were initiated by the addition of membranes. In the control, UDPG was pre-incubated for 30 min with mushroom tyrosinase (O) before initiation of the reaction with membranes.

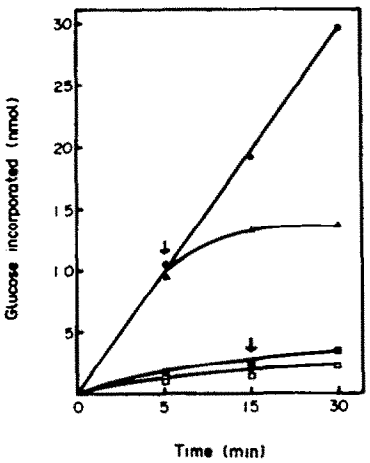


Fig. 7. Attempt to relieve catechol inhibition by thioglycolate addition midway through the glucan synthase reaction. Points denoted by squares represent time courses where mushroom tyrosinase and catechol were held 15 min, and were then incubated with membranes 15 min before initiation with UDPG. □——□, Reaction mixture brought to 0.5 mM thioglycolic acid at 15 min; ■——■, no thioglycolate added; ●——●, control; Δ——Δ, 1 mM catechol and 10 μg mushroom tyrosinase added at 5 min.

Table 1. Effect of PPO inhibitors on glucan synthase inhibition*

| Addition | Concentration† (mM) | Ethanol insoluble glucan (nmol. incorporated/ml)§ |
|---|---|---|
| None‡ | - | 12.1 |
| Thioglycolic acid | 0.5 | 52.6 |
| Dithioerythritol | 0.125 | 54.5 |
| Thiourea | 5.0 | 46.7 |
| Cysteine | 0.125 | 42.6 |
| Ascorbic acid | 0.25 | 40.5 |
| Bovine serum albumin | 10 (mg/ml) | 40.0 |

*Membranes were preincubated for 15 min with the PPO inhibitor, then preincubated an additional 15 min with catechol. The reaction was initiated with substrate.

†These concentrations are the minimal levels giving complete inhibition of PPO.

‡Range of PPO activity observed in 10 different microsomal preparations without PPO inhibitors was 3.4–16.7 Δ Abs/min/ml.

§Reaction mixtures were sampled for ethanol insoluble glucan 30 min after initiation.

difference between the two assays would have been expected.

*Irreversibility of inactivation.* To determine whether the glucan synthase inactivation by catechol was reversible, several experiments were conducted. In the first (Fig. 7), glucan synthase was inactivated by a pretreatment with 1 mM catechol and 10 μg of mushroom tyrosinase. The reaction was initiated by UDPG and at 15 min into the time course, thioglycolic acid was added. No stimulation of activity was observed. Similarly a second addition of UDPG at 15 min also had no effect (data not shown). Membranes treated with catechol and mushroom tyrosinase and subsequently repurified from soluble oxidation products did not possess activity. Activity loss remained at 90% with membranes initially exposed to oxidized catechol or when repurified.

The kinetics of catechol inhibition were determined. A Lineweaver–Burk plot of untreated microsomal membranes and membranes incubated with 125 μM catechol showed the inhibition to be of the mixed type [14]. Treatment of membranes with 125 μM catechol produced approximately 50% inhibition of glucan synthase and increased the apparent $K_m$ for UDPG from 0.73 mM to 1.44 mM.

## DISCUSSION

In this study, exposure of glucan synthase, a plasma membrane-bound enzyme, to a diversity of phenolic compounds at μ- and mM levels under oxidizing and non-oxidizing conditions was investigated. Screenings in the presence and absence of polyphenoloxidase demonstrated that this membrane-bound enzyme, like many soluble enzymes, was relatively resistant to inactivation by unoxidized phenolic compounds. Using catechol as a model phenolic compound, mechanistic studies demonstrated the potential irreversibility of inhibition by phenolic compounds under oxidizing conditions and the likelihood that such inhibition involves covalent modification of glucan synthase at multiple sites.

To conduct assays under non-oxidizing conditions, it was necessary to remove endogenous PPO activity from microsomal preparations. Salt washing, which has been shown to remove ionically-bound soluble contaminants from membrane and cell wall preparations [12, 13], did not remove PPO and suggests that red beet root contains a membrane-bound form of PPO. Though a great proportion of cellular PPO is soluble, several membrane-bound forms of PPO have been identified [15–19] which are inhibited by thiol compounds, ascorbic acid, and bovine serum albumin (Table 1), all well characterized PPO inhibitors that function as free radical scavengers [10, 20]. Inclusion of thioglycolic acid in assay mixtures enabled the screening of phenolic compounds to be conducted under non-oxidizing conditions (Figs 1–5).

There appears to be a correlation between susceptibility to PPO oxidation and inhibition. With the phenolics that were inhibitory to glucan synthase in the presence of PPO, a significant browning reaction characteristic of PPO oxidation was observed. The inability to determine the inhibitory nature of ellagic acid, quercetin, and gossypol in either their native or oxidized states may have been due to their relative insolubility in aqueous systems. Covalent modification of proteins by phenolic oxidation products has been previously observed [9, 21–24] and would explain the irreversible inactivation of glucan synthase by oxidized catechol. Neither addition of thioglycolic acid (Fig. 7) or fresh substrate (data not shown) to glucan synthase mixtures nor washing membranes free of oxidized catechol restored activity.

Tannins are well known as inhibitors of enzyme activity by protein crosslinking [25–28] ultimately leading to precipitation [9, 29]. Tannic acid may function to inhibit glucan synthase by crosslinking it to other proteins within the lipid bilayer, limiting the lateral movement of glucan synthase through the membrane.

Catechin, epicatechin, pyrogallol and tannic acid were exceptions to the oxidation requirement since inactivation of glucan synthase occurred under conditions where PPO was inhibited (Figs 3–5). This suggests the non-covalent complexation of these phenolic compounds with catalytically important regions of glucan synthase, as has been shown with other systems [30]. Covalent modification may act in concert with non-covalent effects. The addition of mushroom tyrosinase to catechin, epicatechin, pyrogallol, and tannic acid markedly lowered the concentrations at which inhibition of glucan synthase occurred.

It is interesting to note that red beet glucan synthase activity was unaffected by coumarin, a compound shown elsewhere to interfere with cellulose biosynthesis [31]. This raises many questions regarding the nature of coumarin's effects. Glucan synthase activity assayed *in vitro* may not be the same activity responsible for cellulose biosynthesis *in vivo*, coumarin may act by affecting glucan synthase substrate production, or coumarin's effect may be confined only to certain plants.

Kinetic studies showed that inhibition by catechol under oxidizing conditions was mixed, suggesting that phenolic oxidation products are combining with residues both at the active site and elsewhere [14]. Glucan synthase is known to be inhibited by $HgCl_2$ [32], suggesting that a sulphydryl group is essential for its activity. Thus, it is possible that oxidized phenolics react with sulphydryl residues.

The physiological significance of phenolic compounds as inhibitors of enzymatic activity is still not clearly understood. The extent to which phenolic compounds behave as enzyme regulators *in vivo* is dependent upon the further identification of specific biochemical systems controlled by phenolic compounds or their oxidized derivatives.

## EXPERIMENTAL

*Isolation of microsomal membranes.* Microsomes were isolated by differential centrifugation [12, 32] as follows: Fresh beet roots (*Beta vulgaris* L.) were obtained from local markets. The tops were removed and the roots stored in moist vermiculite at 4° until needed, but not for longer than 3 weeks. Before homogenization, beets were surface sterilized in a 10% hypochlorite solution for 10 min. After rinsing with sterilized water, the beets were peeled, diced and 100 g were homogenized in 50 ml of homogenization buffer in a juice extractor. The homogenization buffer consisted of 250 mM sucrose, 3 mM EDTA, 0.5% PVP-40, 1 mM dithioerythritol, and 70 mM Tris–HCl, pH 8.0. The homogenate was filtered through two layers of Miracloth and centrifuged at 13000 *g* for 15 min. The pellet was discarded and the supernatant centrifuged for 30 min at 80000 *g*. The microsomal pellet was washed once in 0.8 ml of a resuspension buffer containing 250 mM sucrose, 1 mM dithioerythritol, and 1 mM Tris–Mes, pH 7.2, and recentrifuged at 80000 *g* for 30 min. The pellets were combined and brought to protein concentrations ranging between 3 and 7 mg/ml in resuspension buffer containing 15% (v/v) glycerol. Aliquots were distributed among 1.5 ml microcentrifuge tubes and stored at −80° until use.

*Standard glucan synthase assay.* Unless indicated otherwise, glucan synthase assay mixtures consisted of 50 mM Hepes–NaOH, pH 7.0, 5 mM $MgCl_2$, 5 mM cellobiose, 1 mM [$^{14}$C]-UDPG (0.1 ci/mol.) and 50 μg of microsomal protein in a final vol of 100 μl. The assay mixtures were incubated for 5 min at 30° and the reactions were terminated by heating at 90–100° for 10 min. Incorporation of glucose into EtOH-insoluble products was measured as described below.

*Measurement of EtOH-insoluble glucan.* Glucose incorporation into EtOH-insoluble products was measured by the method of ref. [33]. Eighty μl of each assay mixture was spotted on a Whatman GF/A filter disk (2.4 cm diameter), dried, and placed in an 18 × 150 mm test tube. Excess UDPG was removed by washing the filter disks successively with 66% EtOH (w/v) containing 0.85 mM EDTA, 66% EtOH (w/v), and 70% EtOH (w/v). The filters were then rinsed in $Me_2CO$ and dried. The disks were placed in scintillation vials, and 10 ml of Liquiscint (National Diagnostics, Somerville, NJ) was added. The vials were then counted on a Packard 3255 Tri-Carb liquid scintillation spectrometer.

*Incubation of phenolic compounds with membranes.* Standard screening experiments in which exogenous PPO was supplied were conducted as follows: To a 20 μl soln containing 0.5 μmol. cellobiose, 0.5 μmol. $MgCl_2$ and 5.0 μmol. Hepes–NaOH, pH 7.0, a 50 μl soln containing the phenolic compound and 10 μg of mushroom tyrosinase was added. After a 15 min preincubation period, 50 μg of microsomal protein in a vol. of 20 μl was added. This mixture was incubated for an additional 15 min and the glucan synthase reaction was then initiated by the addition of 10 μl 10 mM [$^{14}$C]-UDPG. Reactions were terminated by heating at 90–100° for 10 min. Incorporation of glucose into EtOH-insoluble products was measured as described above. Phenolic concentrations given under various experiments represent their final concentration in 100 μl assay mixtures.

Phenolic screenings in which endogenous PPO was inhibited by thiolgycolic acid were conducted as described in the previous paragraph except 0.05 μmol. of thioglycolic acid was substituted for exogenous PPO.

*Time course assays.* Unless otherwise indicated, time course assays were conducted as follows: A 260 μl soln containing 3.25 μmol cellobiose, 3.25 μmol $MgCl_2$, 32.5 μmol Hepes–NaOH buffer, pH 7.0, 0.65 μmol catechol, and 65 μg mushroom tyrosinase was incubated 15 min. Microsomal protein (0.33 mg) was added and the vol brought to 585 μl. The mixture was incubated for an additional 15 min after which 65 μl 10 mM [$^{14}$C]-UDPG was added to begin the reaction. At defined intervals, duplicate 100 μl aliquots were removed and immediately plunged into a 90° water bath to terminate the reaction. The tubes were cooled and 80 μl aliquots withdrawn, spotted on filters and washed with EtOH as described above.

In the buffer screening experiment, phenolic compounds and mushroom tyrosinase were omitted and the various buffers were substituted.

In some experiments, UDPG was substituted for membranes during the second 15 min incubation. In these mixtures, reactions were initiated by the addition of microsomal protein.

*Kinetics of catechol inhibition of glucan synthase.* This was examined by means of Lineweaver–Burk plots of the rate data obtained when untreated microsomal membranes and membranes incubated with 125 μM catechol were incubated with a range of UDPG concentrations. Catechol was preincubated for 15 min with 10 μg mushroom tyrosinase, then preincubated for an additional 15 min with 50 μg microsomal protein. Reactions were initiated with UDPG (0.25–2 mM). Each data point was done in triplicate. Lines were fitted using weighted linear regression analysis [34].

*Polyphenoloxidase assay.* Endogenous PPO in the microsomal preparations was measured spectrophotometrically [35]. The assay mixture contained 840 μl 50 mM phosphate buffer, pH 6.2, and 160 μl 0.1 M catechol. The vol. was brought to 1.0 ml by the addition of microsomal membranes to initiate the reaction. The increase in absorbance at 420 nm was monitored over a period of 4 min [36]. PPO inhibitors, when assayed for their effect on endogenous PPO, were added to the assay mixture before initiation of the reaction with microsomes.

*Protein determination.* Protein concentrations were determined using the Bradford method [37] with BSA as the standard.

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