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Does *N*-hydroxyglycine inhibit plant and fungal laccases?

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Abstract

The effect of *N*-hydroxyglycine on the oxidation of substrates, syringaldazine, tolidine, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) and 2,6-dimethoxyphenol, by fungal and plant laccases was examined. At μM concentrations, *N*-hydroxyglycine decolorized solutions of substrates oxidized enzymatically by laccase or chemically by sodium periodate. This discoloration, or bleaching, could be mistaken for inhibition of laccase activity if N-hydroxyglycine was added to assays for laccase that monitored colored products. *N*-hydroxyglycine also affected oxygen consumption assays when some of these substrates were oxidized enzymatically or chemically. Spectral scans of the products formed during enzymatic or chemical oxidation of the substrates indicated that addition of *N*-hydroxyglycine caused a general decrease in absorption. Except for 2,6-dimethoxyphenol, no formation of new absorption peaks was noted. These results suggest that *N*-hydroxyglycine may not be a ''classical'' enzyme inhibitor of laccase, but that this compound interferes with both spectrophotometric and oxygen uptake enzyme assays for laccase.

Keywords: Plant; Fungal; Laccase; *N*-hydroxyglycine; Inhibition

1. Introduction

Laccase (EC 1.10.3.2; benzendiol:oxygen oxidoreductase) is an extracellular Cu-containing enzyme found in fungi, plants, and some bacteria (Mayer & Harel, 1979; Mayer, 1987; O'Malley, Whetten, Bao, Chen & Sederoff, 1993; Ferrar, Barberel, Ginger & Walker, 1995; Yaropolov, Skorobogat'ko, Vartanov & Varfolomeyev, 1994; Thurston, 1994; Dean & Eriksson, 1994; Call & Mucke, 1997). In the presence of a suitable electron donor, the enzyme catalyzes the four-electron reduction of molecular oxygen to water. Mono/di/polyphenols, aminophenols, aromatic amines, methoxyphenols, ascorbic acid, and even some inorganic ions can act as artificial electron donors and become oxidized in the process of reducing molecular oxygen. Most, if not all, white rot fungi excrete laccases that can degrade lignin, a polyphenolic component of plant cell walls. In the presence of a ''mediator'', fungal laccases have also been shown to oxidize nonphenolic compounds as well as to participate in delignification (Call & Mucke, 1997). Other roles assigned to fungal laccases include involvement in fruit body development, spore/conidia pigmentation, phytopathogenicity, and detoxification (Mayer & Harel, 1979; Mayer, 1987; Ferrar et al., 1995; Thurston, 1994; Call & Mucke, 1997). In contrast, plant laccases are believed to be involved in lignification rather than lignin degradation (O'Malley et al., 1993; Dean & Eriksson, 1994).

Even though a wide variety of compounds can be used to monitor laccase activity, there are few specific or selective inhibitors of the enzyme. Because laccase contains Cu ions that act to accept and transfer electrons in the reduction of molecular oxygen, it is susceptible to agents that chelate or reduce copper. Some of these agents include CN^-, sodium azide, diethyldithiocarbamate, EDTA, and thioglycolic acid. Desferal, a transition metal ion chelator, has been reported to inhibit laccase, but the I_{50} for desferal was approximately 30 mM (De Pinto & Ros Barcelo,

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1996). CTAB, a quaternary ammonium cationic detergent, has also been shown to inhibit laccases from a variety of sources (Ferrar et al., 1995; Walker & McCallion, 1980). However, the K_i values for CTAB were in a range of approximately 1–20 mM. These large I_{50} and K_i values seem to indicate a lack of specificity compared to other enzyme inhibitors. In contrast, some halides, especially F^-, are potent inhibitors of fungal laccases (Xu, 1996).

Recently, *N*-hydroxyglycine (HG), a natural product isolated from *Penicillim citrinum*, was reported to be a specific inhibitor of *Coriolus versicolor* laccase (Murao et al., 1992). Murao et al. (1992) indicated that *N*-hydroxyglycine did not inhibit tyrosinase, ascorbate oxidase, bilirubin oxidase or plant phenol oxidase, but that it was a potent (I_{50} of 0.1 μmol) and selective inhibitor for *C. versicolor* laccase. Since that initial report, other investigators have used *N*-hydroxyglycine to inhibit laccase. For example, HG inhibited laccase from *P. oryzae* and *Azospirillum lipoferum* using *o*- and *p*-aminophenol and syringaldazine as substrates (Faure, Bouillant & Bally, 1995; Jacoud, Faure, Effosse, Wadoux & Bouillant, 1995). I_{50} values ranged from 50 to 800 μM for HG inhibition of laccase in these reports. Flurkey, Ratcliff, Lopez, Kuglin and Dawley (1995) also reported the use of HG to selectively discriminate between laccase and tyrosinase activities and to inhibit laccases in *Agaricus bisporus*, Crimini, Enoki, Oyster, and Shiitake mushrooms.

All of the reports describing the use of HG as an inhibitor of laccase used colorimetric or spectrophotometric assay methods to monitor laccase activity. In the analysis of tyrosinase, another Cu containing oxido-reductase, Kahn and co-workers have shown that some types of compounds can interfere with color formation in spectrophotometric tyrosinase assays, but have little or no effect on oxygen consumption assays (Kahn, Schved & Lindner, 1993; Kahn, Lindner & Zakin, 1995). Interference was thought to occur through reactions with product or product intermediates. In the course of synthesizing HG and derivatives of HG as potential inhibitors of laccase, we noticed some unexpected behavior of HG in solution and when added to enzyme assays to inhibit laccase activity. In this report, we show that HG has little, if any, inhibitory effect on laccase. Instead this compound interferes with formation of colored product in spectrophotometric assays and can also interfere in some oxygen consumption assays for laccase.

2. Results and discussion

2.1. Synthesis of N-hydroxyglycine

Initially, we attempted to prepare N-hydroxyglycine (HG) by the method of Goto, Kawakita, Okutani and Miki (1986), but in our hands the final step led only to a mixture of intractable products. We then attempted to synthesize HG using the method of Jahngen and Rossomando (1982) which involves the sodium cyanoborohydride reduction of glyoxalic acid oxime. The product obtained by this method was contaminated with a large amount of an unidentified impurity. A minor modification in the original procedure circumvented the problem; we used only half the amount of solvent so that much of the product crystallized out of solution during the reaction.

Table 1
Effect of HG on the oxidation of syringalazine and tolidine by plant and fungal laccases

Enzyme source	Enzyme activity (A/min/ml)			
	Syringaldazine		*o*-tolidine	
	−HG	+HG	−HG	+HG
P. oryzae (ICN)	0.96	0.29	0.03	0
P. oryzae (Sigma)	0.22	0.06	0.33	0
C. hirsutus	22.0	0.07	8.7	0.13
A. bisporus	0.1	0	6.67	0
R. vernicifera	0.25	0.04	0.21	0

2.2. Effect of HG on laccase activity

Several compounds were examined for potential use as substrates in the inhibition studies using HG. Some of these substrates included guaiacol, *p*-phenylenediamine, catechol, pyrogallol, dopa, syringaldazine (SYR), o-tolidine (TOL), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and 2,6-dimethoxyphenol (DMP). SYR, TOL, ABTS, and DMP were chosen as substrates in our studies because: (1) they are frequently used as substrates for laccase; (2) they can be monitored at different wavelengths because of characteristic product absorption spectra; (3) the oxidized substrates yield different absorption spectra distinct from the starting material; and (4) each oxidized product has a different color. When SYR and TOL were used as substrates, they were oxidized to different extents by three types of fungal laccases and one plant laccase (Table 1). *C. hirsutus* laccase showed greatest activity with SYR while low activities with SYR were observed using the other laccases. *A. bisporus* and *C. hirsutus* laccase showed high activity with TOL as the substrate while *P. oryzae* and *Rhus vernicifera* laccases showed much lower activities with TOL. *C. hirsutus* and *A. bisporus* laccase were used in subsequent studies because of their greater activity with SYR,TOL and also ABTS and DMP (data not shown). When the laccases were assayed with SYR or TOL in the presence of HG, an apparent decrease in activity was observed

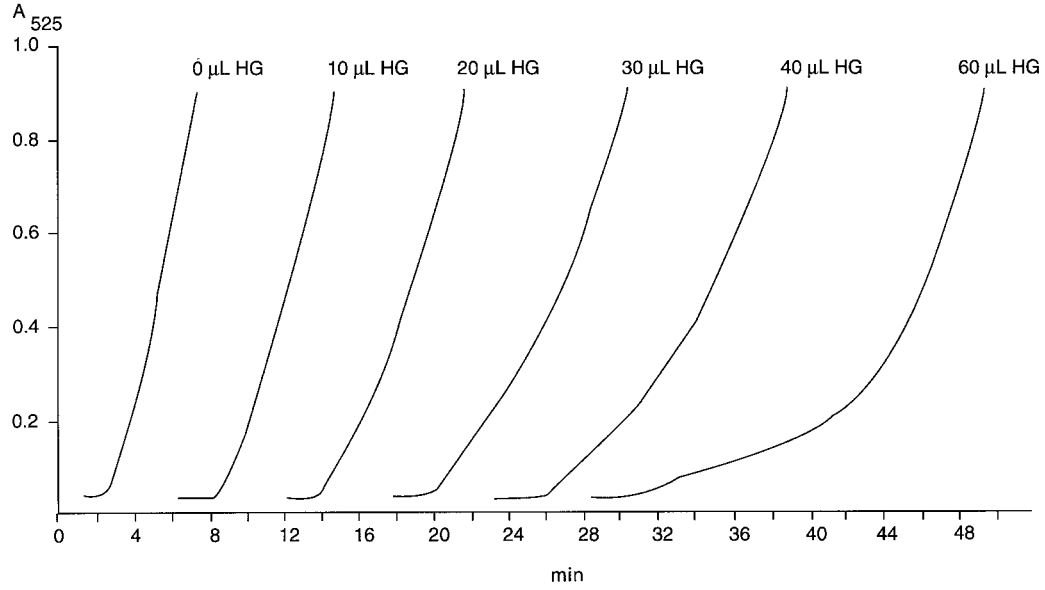

Fig. 1. Effect of HG on the enzymatic oxidation of SYR by *C. hirsutus* laccase. *C. hirsutus*, 20 μl, laccase (1 mg solid/ml; 1 mg protein/ml) was incubated with 0.2 ml of 0.5 mM SYR (0.1 μmole) and 1.8 ml of acetate buffer (pH 5.0). Various amounts (0.05 μmole/10 μl) of 5 mM HG dissolved in acetate buffer were added at zero time. Rates of the individual reactions at 525 nm were determined in A/min between 0 and 0.2 A and 0.6 to 0.9 A.

compared to when HG was absent. This decrease was noted if changes in absorbance were measured during the first few min of the reaction, assuming these first few min represent the initial rate of the reaction.

2.3. Effect of HG on the enzymatic oxidation of SYR

If one assumes that this apparent inhibitory effect by HG is real, then I_{50} values for HG can be determined for different substrates and different laccases. As illustrated in Fig. 1, *C. hirsutus* laccase was assayed in the presence of a constant amount of SYR and varied amounts of added HG. We observed that a distinct lag period occurred in all assays. This lag period, the time in which it took before color began to develop, seemed to increase with increasing [HG]. The initial rate measurements (0–0.2 A) decreased as the [HG] was increased in the assays. If one used these initial rate measurements to determine I_{50} values, an I_{50} of 50 μM for SYR and 65 μM for TOL was obtained (data not shown). These I_{50} values are similar to those reported elsewhere (Murao et al., 1992; Faure et al., 1995). However, if the reaction was allowed to proceed for a longer period of time, color formation began again and the rate of color formation approached the rate in the absence of added HG (rates measured at 0.4–0.8 A/time). This suggested that small amounts of HG may not be inhibiting the enzyme but changing the product into a non-colored material.

To determine if HG had an effect on the product formation, successive amounts of HG were added to an enzyme reaction containing *C. hirsutus* laccase and SYR, and the reaction monitored for a longer period of time. As seen in Fig. 2(A), excess HG added at time zero inhibited the reaction for approximately 12 min, at which time a pink color began to form. We let this reaction proceed until an absorbance of approximately 0.7 at 525 nm was reached and then more HG was added. An immediate decrease in absorbance, "bleaching of product", took place and the absorbance dropped to a baseline level. After some time (10–12 min), the color began forming again but did not reach as high an absorbance as earlier. Addition of more HG caused bleaching and an immediate decrease in absorbance.

This process is better illustrated in Fig. 2(B) in which smaller amounts or HG were added to a *C. hirsutus* laccase catalyzed reaction in progress. We allowed the reaction to proceed until an absorbance of approximately 0.55 was reached. At this time small amounts of HG were added. Addition of HG caused an immediate decrease in absorbance; this decrease reached a minimum and color formation began again. It appeared that HG was consumed in some process and the rate of product formation proceeded as before. This cycle could be repeated several times. Doubling the amount of HG added, decreased the absorbance by a factor of approximately 2. Upon depletion, product formation resumed and could be "bleached" in a repetitive fashion several more times. Eventually, with enough added HG, the amount of product formed began to decline, perhaps due to eventual substrate de-

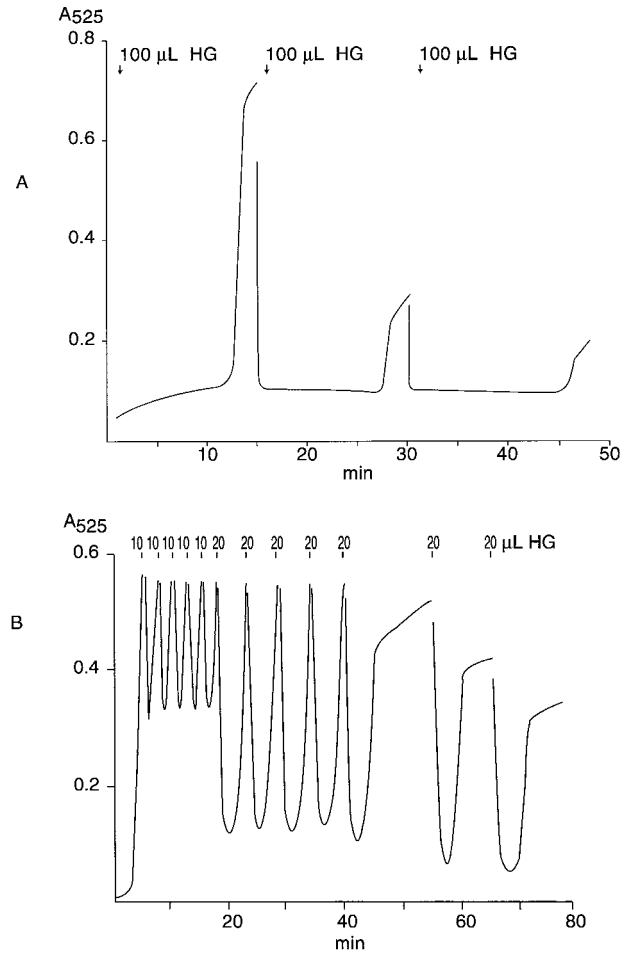

Fig. 2. Effect of HG on spectrophotometric enzyme assays monitoring the oxidation of SYR by *C. hirsutus* laccase. (A) 25 µl of *C. hirsutus* laccase (1 mg solid/ml; 1 mg protein/ml) was incubated with 0.1 ml of 0.5 mM SYR (0.05 µmole), 1.8 ml of acetate buffer and 0.1 ml of 5 mM HG (0.5 µmole) dissolved in acetate buffer. After the absorbance increased and began to taper off, a second aliquot of 5 mM HG was added. This process was repeated a third time. (B) 10 µl of *C. hirsutus* laccase was added to 1.8 ml acetate buffer and 0.1 ml of 0.5 mM SYR (0.05 µmole) and the absorbance monitored with time. The absorbance was allowed to increase to approximately 0.55 A, at which time 10 µl of 5 mM HG (0.05 µmole/10 µl) was added. The process was repeated several times with 10 and 20 µl additions of HG.

pletion or decreased enzyme activity. A similar phenomenon was also observed using SYR, TOL, ABTS, DMP, and quaiacol and *A. bisporus* laccase with additions of HG. *N*-hydroxylglycine added to a reaction in progress with these substrates bleached product/color formation almost immediately (data not shown).

2.4. Effect of HG on the chemical oxidation of various laccase substrates

If HG is interfering with the formation of enzymatically generated colored oxidation products, a similar phenomenon should be observed with chemical oxidation of laccase substrates. Four laccase substrates, SYR, TOL, ABTS, and DMP, were oxidized in the presence of sodium periodate. In the absence of HG, each compound was oxidized to a colored product and the rate of color formation was monitored with time. In the presence of large amounts of added HG at time zero, the rate of product formation decreased substantially. Adding HG to the reaction in progress decreased the rate of product as indicated by a decrease in absorbance. Eventual loss of color or decreased color was reached using all four laccase substrates. These results indicated an enzymatic reaction is not needed for HG to have a "bleaching" effect on oxidized laccase substrates.

2.5. Effect of HG on oxygen uptake during enzymatic oxidation of laccase substrates

Laccase activity can also be monitored by following oxygen uptake during the enzymatic reaction. HG can apparently affect spectrophotometric assays for laccase by reducing the amount of colored product, but can it affect oxygen uptake assays? To examine this possibility, enzymatic oxidation of SYR, TOL, ABTS, and DMP was followed using a Clark oxygen electrode after successive additions of HG (Fig. 3). Addition of small amounts of HG had little effect on decreasing oxygen uptake in assays using SYR, TOL, DMP and ABTS as substrates with *A. bisporus* laccase. In fact, HG caused an apparent increase in oxygen consumption using SYR, DMP, and ABTS as substrates when HG was added in amounts exceeding the amount of substrate. If HG were behaving as a laccase inhibitor, one would expect a significant decrease, not an increase, in oxygen consumption. However, we observed little to no decrease in oxygen consumption under these conditions. Because the oxygen electrode reaction chambers were transparent, we observed that addition of HG caused an immediate and/or fast bleaching of any colored product formed without a corresponding decrease in oxygen consumption. Therefore, product or color disappeared with HG addition but oxygen consumption stayed the same or actually increased. We also observed that excessive amounts of added HG during later times of the reactions caused a large increase in oxygen consumption (data not shown).

2.6. Effect of HG on oxygen uptake during chemical oxidation of laccase substrates

If HG causes an apparent increase in O_2 consumption in enzymatic assays then a similar increase might be observed in chemical oxidation of laccase sub-

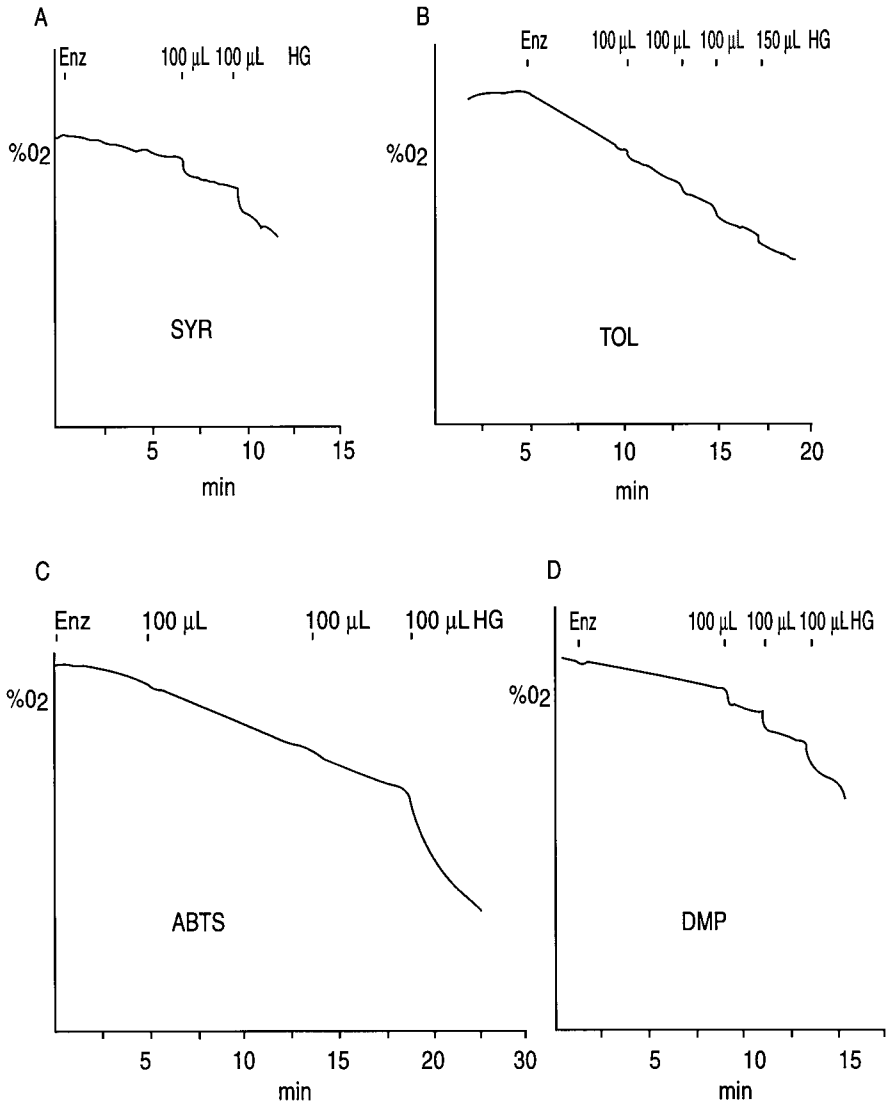

Fig. 3. Effect of HG on enzymatic oxygen uptake assays for laccase with different substrates. (A) 0.1 ml of SYR (0.1 µmole), 3.9 ml of acetate buffer, and 30 µl of *A. bisporus* laccase (2 mg protein/ml) were mixed in the electrode cell. Indicated amounts of 5 mM HG (0.5 µmole/0.1 ml) were added at the designated times. (B) 25 µl of 40 mM tolidine (1 µmole), 3.85 ml of acetate buffer, and 30 µl of *A.bisporus* laccase were mixed in the electrode cell. At the times indicated, specified amounts of 10 mM HG (1 µmole/01. ml) were added. (C) 3.6 ml of 0.5 mM ABTS (1.8 µmole) in acetate buffer and 30 µl of *A. bisporus* laccase were mixed in the electrode cell. At indicated times, 0.1 ml of 10 mM HG (1 µmole) was added. (D) 0.2 ml of 5 mM DMP (1 µmole) in acetate buffer, 3.6 ml of acetate buffer, and 10 µl of *A. bisporus* laccase were mixed in the electrode cell. At selected intervals, 0.1 ml of mM HG (0.5 µmole) was added. Oxygen uptake was followed with time using a Clark oxygen electrode.

strates, SYR, TOL, and ABTS, by sodium periodate. Oxygen consumption was monitored in buffered substrates containing HG that were oxidized with sodium periodate. In general, addition of HG showed a slight increase or no change in oxygen consumption. When excess periodate was added, there was immediate formation of a colored product. If more HG was added, the intensity of the color decreased. In all later additions, little to no change in O_2 consumption was noted and no decrease in oxygen consumption was observed. Similarly, addition of HG to buffered DMP caused a small increase in oxygen uptake but no change in color. In contrast to the above, periodate addition to the DMP reaction caused an immediate coloration that was not blocked by HG.

2.7. Incubation experiments with laccase and HG

If the literature values of the I_{50} for HG are in the micromolar range, one might expect the enzyme to

have a low K_i for HG and thus significant inhibition should occur if the enzyme was incubated with large concentrations of HG. Even on dilution, the enzyme might be expected to be significantly inhibited. To test this, *A. bisporus* laccase (0.2 μM final concentration) was incubated with 1 mM HG (5 μM after dilution) for 30 min. Control experiments contained enzyme added to buffer. At the end of 30 min, tolidine and buffer were added, and the enzyme assayed spectrophotometrically. Enzyme activity decreased slightly from 8.5 units/ml to 8.0 units/ml when incubated in the absence of HG compared to the presence of HG. Although this does not prove lack of inhibition by HG, it does suggest that incubation with 1 mM solution should have resulted in a greater diminution of enzyme activity.

2.8. Effect of HG on the absorption spectra during enzymatic oxidation of laccase substrates

The effect of HG on the spectral products generated by enzymatic oxidation of SYR, TOL, DMP, and ABTS was also examined using scanning absorption spectrophotometry. Oxidation of SYR by *C. hirsutus* laccase showed an increase in absorbance at 525 nm with time as expected. Addition of HG after the reaction was in progress for 5 min caused the disappearance of this absorption with time (Fig. 4(A)), while addition of HG at time zero prevented the appearance of this absorption peak for at least 16 min (Fig. 4(B)). Similar results were observed for the effect of HG on the enzymatic oxidation of TOL (Fig. 4(C) and (D)). In addition, a second absorption peak at 366 increased

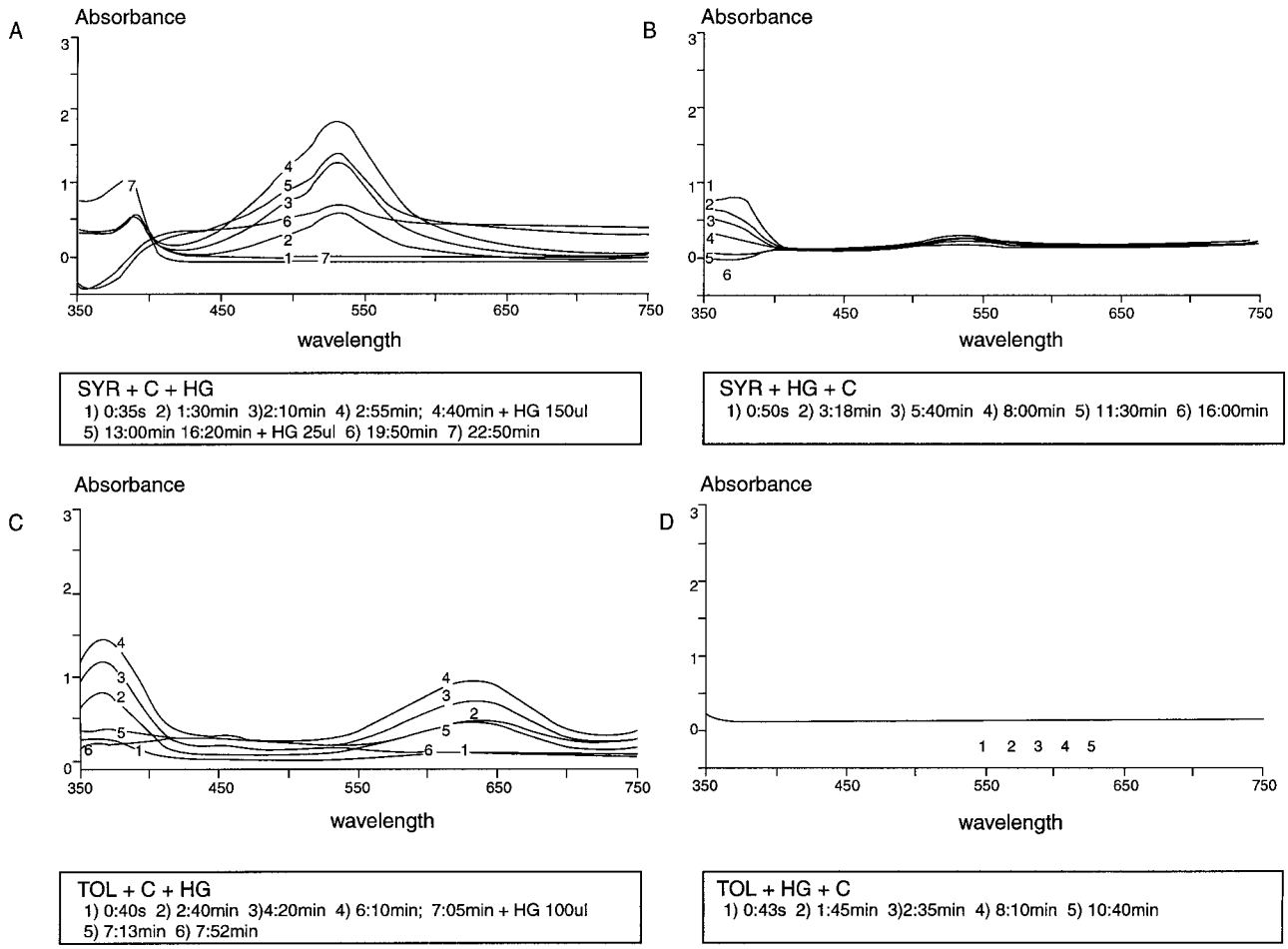

Fig. 4. Effect of HG on the absorption spectra of substrates oxidized enzymatically by laccase. HG (final concentration 1.25 mM) was added to buffer containing SYR (final concentration 0.17 mM) at time zero (B) or after the reaction was initiated with *C. hirsutus* laccase (A). HG (final concentration 1.0 mM) was added to buffer containing TOL (final concentration 2.0 mM) at time zero (D) or after the reaction was initiated with *C. hirsutus* laccase (C). HG (final concentration 0.91 mM) was added to buffer containing ABTS (final concentration 1.8 mM) at time zero (F) or after the reaction was initiated with *C. hirsutus* laccase (E). HG (final concentration 1.54 mM) was added to buffer containing DMP (final concentration 1.54 mM) at time zero (H) or after the reaction was initiated with *C. hirsutus* laccase (G). Spectral scans were carried out at time intervals indicated in the boxes.

and decreased with HG addition after the reaction was initiated (Fig. 4(C)). Again, similar results were observed for the effects of HG on the products from enzymatic oxidation of ABTS (Fig. 4(E) and (F)). However, if the reaction was allowed to continue for a long period of time, approximately 40 min, the absorbance and absorption peak at 420 nm began to increase again (Fig. 4(E)). Oxidation of DMP by laccase caused an increase in absorbance at 470 nm (Fig. 4(G)). In contrast, HG addition appeared to cause the appearance of a new absorption peak at 390 nm that was generated irrespective whether HG was added at time zero or after the reaction was initiated (Fig. 4(H)). This suggests that HG may be reacting with the product of DMP oxidation to generate a different compound.

2.9. Effect of HG on the absorption spectra during chemical oxidation of laccase substrates

The effect of HG on the absorption spectra of the products derived from chemical oxidation of SYR, TOL, ABTS, and DMP was also examined. HG addition at time zero prevented an increase in absorbance at the wavelengths usually used to monitor SYR (525 nm), TOL (630 nm), ABTS (420 nm), and DMP (470 nm) oxidation. *N*-hydroxylglycine addition after the chemical oxidation was initiated caused the absorbance to decrease at these wavelengths, in effect causing a "bleaching" of color. Similarly, HG decreased the absorbance/absorption peak at 470 nm during oxidation of DMP if added at some later time. Much like enzymatic oxidation of DMP, addition of HG at time zero almost prevented absorbance increases at 470 nm but showed an increase in the absorption peak at 390 nm.

2.10. Does HG inhibit laccase activity?

All spectral changes suggest that HG is reacting with products from the enzymatic or chemical oxidation of SYR, TOL, ABTS, and DMP. Thus, it

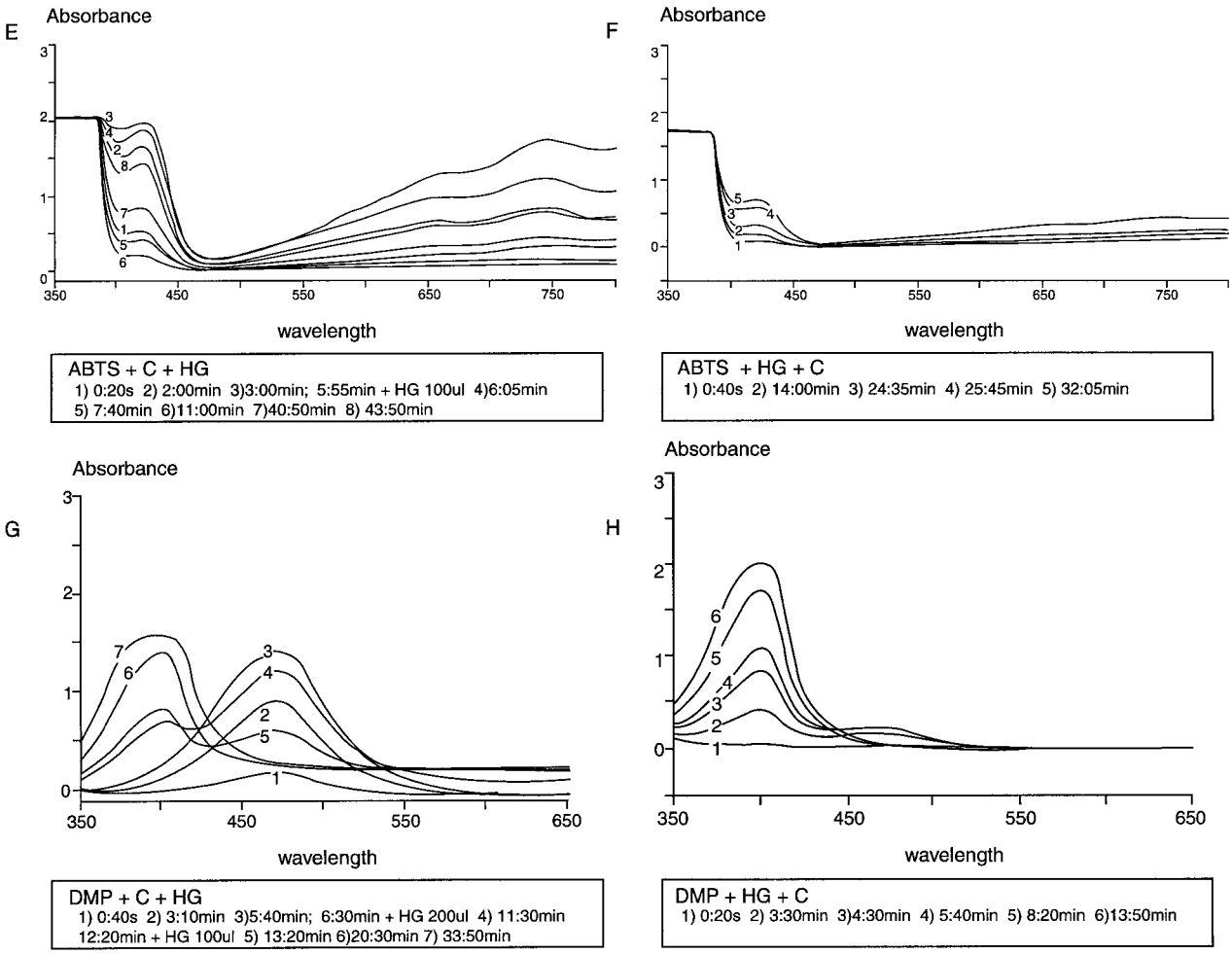

Fig. 4 (*continued*)

appears that HG causes a decrease in the absorption at those wavelengths used to monitor laccase activity, resulting in the "apparent" but unreal inhibition of enzymatic activity. Although definite evidence showing that HG inhibits laccase is not available, there appears to be ample evidence that HG can interfere with the product or intermediates in the reactions catalyzed by laccase. In this sense, HG does not behave as a classically defined enzyme inhibitor. Until definitive experiments to determine if HG can inhibit laccase, such as equilibrium dialysis or radio-isotope tagged HG binding studies, can be carried out, the current study suggests that HG does not inhibit laccase by acting as a classical enzyme inhibitor.

3. Experimental

3.1. Materials and equipment

Pyricularia oryzae laccase was obtained from Sigma (lot 87F-7704 and 119F-798, St. Louis, MO) and ICN (lot 61938, Costa Mesa, CA). *C. hirsutus* laccase was purchased from Calbiochem (lot 185891, San Diego, CA), and *Rhus vernicifera* laccase was from Sigma (lot 96H06761). Extracellular culture filtrates from *A. bisporus*, containing laccase, were a gift from R. Kerrigan (Sylvan Spawn, Worthington, PA). Guaiacol, *o*-tolidine, syringaldazine, 4-aminophenol, 1-hydroxybenzotriazole, catechol, dopa, and pyrogallol were obtained from Sigma (St. Louis, MO). 2,6-dimethoxyphenol and 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) were purchased from Sigma-Fluka (Milwaukee, WI). Sodium periodate and hydroxylamine hydrochloride were from Fisher Scientific (St. Louis, MO). All other chemicals were of reagent grade.

3.2. Synthesis and characterization of N-hydroxylglycine

N-hydroxyglycine was prepared by a modification of a procedure reported by Jahngen and Rossomando (1982). To a stirring solution of sodium cyanoborohydride (0.63 g, 10 mmol) and bromocresol green (5 mg) in 10 ml of methanol, glyoxalic acid oxime (0.89 g, 10 mmol) was added at room temperature. This solution was stirred for 3 h, and during that time 12 M HCl was added dropwise as needed to maintain a yellow color; this typically took about 10 drops over the 3 h period. At about 10 min into the reaction time, a precipitate began to form. At the end of the reaction time, the crystalline product was recovered by vacuum filtration. The compound was recrystalized by dissolving the product in about 4 to 5 ml of hot water (some bubbling occurred), cooling to room, and then cooling with an ice bath to give, after drying in vacuo, 0.3 g (33%) *N*-hydroxyglycine: mp 140 °C(dec); IR (KBr) 3105, 1601, 1536, 1483, 1399, 1310 cm^{-1}; ^{1}H-NMR δ D_2O) 3.87 (—CH_2—); ^{13}C-NMR δ (D_2O) 56.0, 173.0; MS (FAB) m/z $(M-1)^{-1}$)90. ^{1}H and ^{13}C-NMR spectra were recorded at 250 MHZ. Chemical shifts were reported in ppm downfield from sodium trimethylsilylpropanoate. High resolution fast atom bombardment mass spectra analysis was carried out by the Nebraska Center for Mass Spectrometry. To confirm the identity of HG, X-ray crystallography was carried out by John Huffman at the Indiana University Department of Chemistry Molecular Structure Center (data not shown).

The crude reaction mixture from which the *N*-hydroxyglycine had been filtered was evaporated to dryness in vacuo at 40 °C to give a mixture of *N*-hydroxyglycine and a substantial amount of a least one unidentified compound.

3.3. Enzyme assays

Oxidations of SYR, TOL, DMP, and ABTS were monitored spectrophotometrically in 0.1 M sodium acetate (pH 5.0) at 525, 630, 470, and 420 nm, respectively as described by Flurkey et al. (1995) and Miller, Kuglin, Gallagher and Flunkey (1997) Sanchez-Amat and Solano (1997) and Palmieri et al. (1997). Stock solutions of syringaldazine and *o*-tolidine were prepared in 95% ethanol. DMP and ABTS stock solutions were prepared fresh in 0.1 M acetate buffer pH 5.0. Changes in color/absorbance were followed using a Hitachi 100-60 recording spectrophotometer connected to a strip chart recorder. Initial rates were calculated from the linear portion of the absorbance vs time curves. Specific conditions for each experiment are noted in the legends. One unit of activity was defined as a change in one absorbance unit per min. Oxidation of substrates was also monitored using oxygen consumption assays with a Clark oxygen electrode connected to a YSI model 5300 biological oxygen meter. Assays were conducted in a circulating water bath at 23–24 ° C. Changes in oxygen were based on an air saturated buffer solution as a reference at 100%.

3.4. Chemical oxidation assays

Oxidations of SYR, TOL, DMP, and ABTS were carried out by additions of a stock solution of sodium periodate (5 mM) dissolved in acetate buffer. Changes in color/absorbance were observed visually and were also monitored spectrophotometrically as above. Changes in oxygen consumption, after chemical oxidation of the substrates, were monitored as above.

3.5. Scanning spectrophotometry

Changes in the chemical or enzymatic spectra from oxidation of SYR, TOL, DMP, and ABTS were monitored by scanning absorption spectrophotometry. Spectra were recorded using a Cary 5 UV-VIS-NIR spectrophotometer from 350 to 750 nm at a scan rate of 1000 nm/min. Reactions contained HG added at zero time or added at some time after the reaction was initiated. Enzymatic reactions used *C. hirsutus* laccase while chemical oxidation reactions used sodium periodate. Specific conditions are noted in the legends.

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