

Selection and Improvement of Lignin-Degrading Microorganisms: Potential Strategy Based on Lignin Model-Amino Acid Adducts

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The purpose of this investigation was to test a potential strategy for the ligninase-dependent selection of lignin-degrading microorganisms. The strategy involves covalently bonding amino acids to lignin model compounds in such a way that ligninase-catalyzed cleavage of the models releases the amino acids for growth nitrogen. Here we describe the synthesis of glycine-*N*-2-(3,4-dimethoxyphenyl)ethane-2-ol (I) and demonstrate that growth (as measured by mycelial nitrogen content) of the known lignin-degrading basidiomycete *Phanerochaete chrysosporium* Burds. with compound I as the nitrogen source depends on its production of ligninase. Ligninase is shown to catalyze the oxidative C—C cleavage of compound I, releasing glycine, formaldehyde, and veratraldehyde at a 1:1:1 stoichiometry. *P. chrysosporium* utilizes compound I as a nitrogen source, but only after the cultures enter secondary metabolism (day 3 of growth), at which time the ligninase and the other components of the ligninolytic system (lignin → CO₂) are expressed. Compound I and related adducts have potential not only in the isolation of lignin-degrading microbes but, perhaps of equal importance, in strain improvement.

Many if not most lignin-degrading microorganisms fail to grow on lignin as sole carbon source, precluding their isolation from the environment via standard enrichment procedures (13). We have devised a strategy which should circumvent this difficulty. The strategy involves covalently bonding amino acids to lignin model compounds in such a way that ligninase-catalyzed cleavage of the new adduct releases the amino acids for growth nitrogen. Here we describe the synthesis of one such adduct and show that ligninase cleaves it, releasing the amino acid as predicted. Growth of the lignin-degrading fungus *Phanerochaete chrysosporium* Burds. with the adduct as nitrogen source is shown to depend on its production of ligninase.

Ligninase is a recently discovered peroxidase that catalyzes the H₂O₂-dependent oxidation of lignin and lignin model compounds (7, 23). Recent work with model compounds indicates that substrates can be oxidized by one (9, 11, 22) or two (25) electrons. With lignin model compounds containing alkyl side chains, the predominant reaction is C_α—C_β cleavage, producing an aromatic aldehyde from the C_α-bearing moiety and a C_β-hydroxylated product (24) (Fig. 1A). This cleavage by ligninase results in partial depolymerization of methylated lignin (23). C_α—C_β cleavage is known from other studies to be a major reaction of lignin degradation by the basidiomycetes (3).

We have taken advantage of this ligninase-catalyzed C_α—C_β cleavage to design lignin model amino acid adducts which predictably would be degraded by the enzyme to yield the amino acid, an aromatic aldehyde, and formaldehyde. The first compound synthesized, and the one described here, is glycine-*N*-2-(3,4-dimethoxyphenyl)ethane-2-ol (I).

MATERIALS AND METHODS

Chemicals. All purchased chemicals were reagent grade. 1-(3,4-Dimethoxyphenyl)-2-bromoethanone was synthesized

by brominating 1-(3,4-dimethoxyphenyl)ethanone with Br₂ (18); the product was crystallized from ethanol. Glycine-*N*-2-(3,4-dimethoxyphenyl)ethane-2-ol (structure shown in Fig. 1B) was synthesized from glycine ethyl ester (Sigma Chemical Co., St. Louis, Mo.) and the above product as follows (cf. reference 19). (i) The free base of the glycine ethyl ester was obtained by adding 12 ml of 30% NaOH (0.84 equivalent) to 15 g of glycine ethyl ester hydrochloride (in 5 ml of H₂O) at 4°C. Excess granular potassium carbonate was then added, and this solution was extracted three times with equal volumes (30 ml) of ether. The ether extract was dried over anhydrous sodium sulfate and evaporated to a final volume of 40 ml, which contained 7.96 g of the free glycine. (ii) With stirring, at room temperature, 9.55 g of 1-(3,4-dimethoxyphenyl)-2-bromoethanone in 150 ml of ether was added over 15 min. The solution was stirred for 2 h, during which glycine ethyl ester hydrobromide precipitated. The mixture was then chilled on ice, and the hydrobromide was filtered off. The ether was evaporated to give a yellow syrup containing the ethyl ester of glycine-*N*-2-(3,4-dimethoxyphenyl)ethane-2-one. (iii) The ethyl ester was hydrolyzed by refluxing the solution in 30 ml of 6 N HCl for 30 min. The reaction was then cooled and extracted three times with 1 volume of chloroform. The aqueous solution was decolorized with activated charcoal, and the water was removed by vacuum evaporation to yield glycine-*N*-2-(3,4-dimethoxyphenyl)ethane-2-one. (iv) The corresponding alcohol was produced by reduction with sodium borohydride in 50% ethanol. The mixture was adjusted to pH 5 with concentrated HCl, and the water evaporated to give a solid; the product was crystallized from water at pH 6 (mp, 190.5 to 193°C) (2.34 g; 8.7% yield). Nuclear magnetic resonance spectra were as follows: ¹H-nuclear magnetic resonance spectrum (²H₂O) (ppm)—3.23 (2H, d, -NH-CH₂-CHOH-, *J* = 6.2), 3.57 (2H, s, -NH-CH₂-COOH), 3.74 (3H, s, Ar-OCH₃), 3.76 (3H, s, Ar-OCH₃), 4.92 (1H, t, Ar-CHOH-CH₂-, *J* = 6.5), 6.95 (3H, d, aromatic, *J* = 7.4); ¹³C-nuclear magnetic

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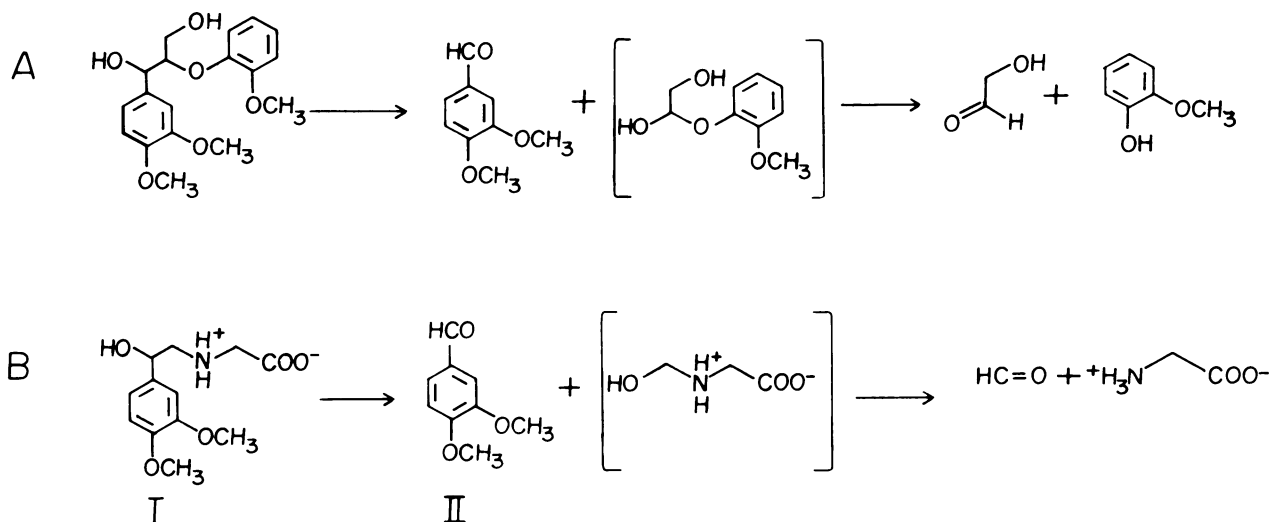


FIG. 1. Scheme showing products formed from ligninase-catalyzed oxidation of a β -O-4 dimeric model of lignin (A) and the glycine adduct (B). Proposed intermediates are shown in brackets.

resonance spectrum ($^2H_2O:[^2H(U)]-CH_3OH$, 1:1) (ppm)—50.4 (Ar-CHOH-CH₂), 54.53 (-NH-CH₂-COOH), 56.70 (-OCH₃-), 69.73 (Ar-CHOH-), 110.75 (Ar, C₂), 113.11 (Ar, C₃), 119.78 (Ar, C₆), 134.07 (Ar, C₁), 149.01 plus 149.83 (Ar, C₃ plus C₄). Nuclear magnetic spectra was obtained with a Bruker (Billerica, Mass.) 250-MHz nuclear magnetic resonance spectrophotometer. The mass spectrum (chemical ionization, methane) showed the following, m/z (%): 256 (M⁺, 58.9), 239 (17.3), 238 (100), 167 (17.1). Mass spectra were obtained with a Finnigan MAT (San Jose, Calif.) mass spectrometer.

Product identification. Products from ligninase-catalyzed oxidation of the glycine adduct were identified from reaction mixtures containing 1.5 μ M ligninase (extinction coefficient at 409 nm = 168 $mM^{-1} cm^{-1}$ [25]), 0.4 mM H₂O₂, and 1 mM adduct in 50 mM sodium tartrate, pH 3.5. The reaction was initiated by H₂O₂ addition. The reaction mixture was periodically sampled (as specified in the figure legends) by removing 1.1-ml aliquots and stopping the reaction by adding 25 μ l of 100 mM sodium azide. Veratraldehyde was identified by exhibiting a similar retention time on high-pressure liquid chromatography as compared with a standard and quantitated by its A₃₁₀ (extinction coefficient at 310 nm = 9,300 $M^{-1} cm^{-1}$). Glycine was identified by its R_f in chromatography (compared with an authentic standard) as its 1-fluoro-2,4-dinitrobenzene derivative (6). It was quantitated by chromophore formation with picric acid (21). Formaldehyde was identified and quantitated by the method of Nash (20).

Enzyme and cultures. *P. chrysosporium* (ATCC 24725) was grown and its ligninase was isolated and purified as previously described (14). The ligninase (H8 [14]) activity was assayed by veratryl alcohol oxidation (14). Preparations had a minimum turnover number of 3.5 s⁻¹ (assayed at pH 3.5; cf. reference 25).

The effect of the adduct on cultures was assessed with 10-ml cultures grown in 125-ml Erlenmeyer flasks. Culture medium was as described by Kirk et al. (14). The cultures were flushed with pure oxygen on the day of inoculation and every third day thereafter. The cultures were sampled periodically ($n = 4$) for dry-weight analysis and mycelial nitrogen content via micro-Kjeldahl techniques.

RESULTS

Incubation of the glycine adduct with ligninase (+H₂O₂ and O₂) yields veratraldehyde (II), formaldehyde, and glycine (Fig. 1B). We found essentially a 1:1:1 stoichiometry in product formation with respect to time (Fig. 2). The scheme shown in Fig. 1B is consistent with the product profile and is based on previous work with the arylglycerol- β -aryl ether and diarylpropane-1,2-diol types of dimeric lignin models (8, 9, 17, 23).

The ability of the ligninase to catalyze the cleavage of compound I would suggest that the adduct can serve as a ligninase-dependent growth substrate. This would be true if the liberated amino acid, but not the uncleaved adduct, can serve as a nitrogen source. To test this possibility, we added spores of *P. chrysosporium* to a basal medium containing varying amounts of the adduct and no or 1.2 mM NH₄⁺. It should be noted that the ligninolytic system, including the

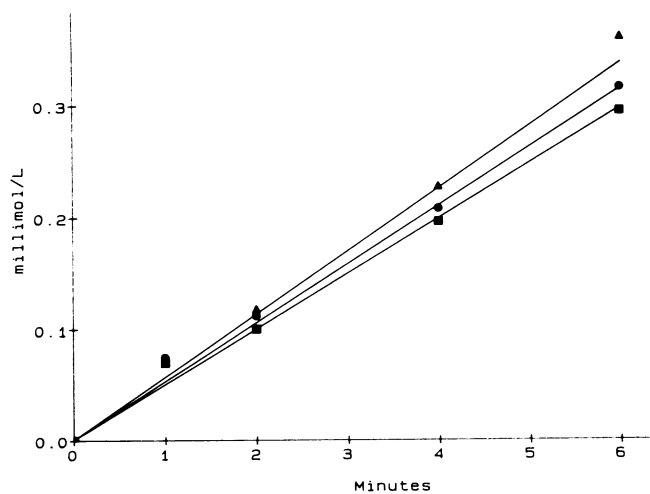


FIG. 2. Time course of veratraldehyde (●), glycine (■), and formaldehyde (▲) formation from ligninase-catalyzed oxidation of glycine adduct. Details of reaction mixture and methodology are described in Materials and Methods.

ligninase, is produced only during secondary (idiophasic) metabolism (4, 24), which is brought about by nitrogen (or carbon or sulfur) starvation (10, 12). Consequently, the medium used here was growth limiting in NH_4^+ -nitrogen, when present. Total mycelial nitrogen provided the measure of growth. (Mycelial weight is not strictly proportional to growth with *P. chrysosporium* because an extracellular polysaccharide is synthesized irrespective of growth [5]).

No growth occurred in cultures supplied with the glycine adduct as the sole nitrogen source (not shown). In contrast, cultures were able to grow with glycine as the sole nitrogen source (not shown), indicating that free glycine was not released from compound I under these control conditions. When the cultures were grown with NH_4^+ as the sole nitrogen source, growth increased rapidly until day 2, after which no further growth occurred (Fig. 3); the nitrogen is known to be growth limiting within 2 days under the conditions used (12). Cultures grown with both NH_4^+ and varying amounts of the glycine adduct showed an increase after day 2 proportional to the total amount of nitrogen supplied (Fig. 3). This clearly reflects the utilization of the adduct as a growth nitrogen substrate. It is significant to note that the increase in mycelial nitrogen content was not observed during primary metabolism; the increase occurred only during secondary metabolism. These data indicate that the effect of the adduct was not observed when NH_4^+ served as growth nitrogen source (prior to day 3) and are consistent with the secondary metabolic (ligninase-dependent) nature of the adduct utilization. Other work has shown that the ligninase is produced only during secondary metabolism (4, 24).

The addition of the adduct to cultures also resulted in a large increase in ligninase activity (Fig. 4). This increase, like growth, was proportional to the amount of adduct added. Activity reached a peak on day 4 for control cultures and on day 7 for cultures containing the adduct. We attribute this increase in ligninase activity in the adduct-containing cultures to two factors: the slow feed of nitrogen provided by the adduct which allowed simultaneous growth and second-

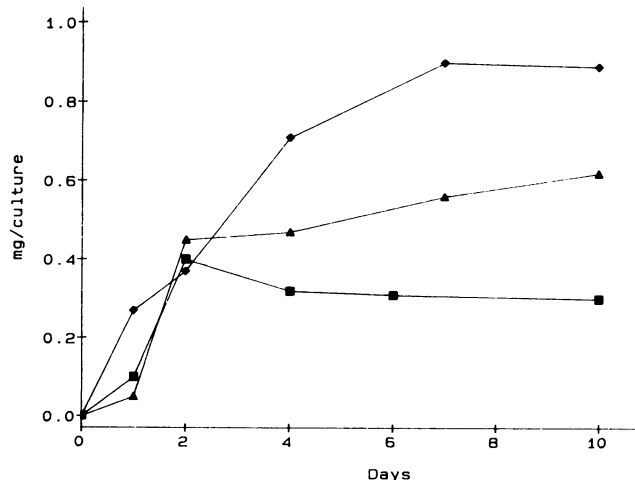


FIG. 3. Effect of nitrogen source on mycelial nitrogen content. At specified days, quadruplet cultures were pooled and the nitrogen content of the cultures was determined as described in Materials and Methods. Nitrogen source: 1.2 mM NH_4Cl (■), 1.2 mM NH_4Cl plus 1.2 mM glycine adduct (▲), and 1.2 mM NH_4Cl plus 2.4 mM glycine adduct (◆). Because cultures containing only glycine adduct did not grow, their nitrogen content was not determined.

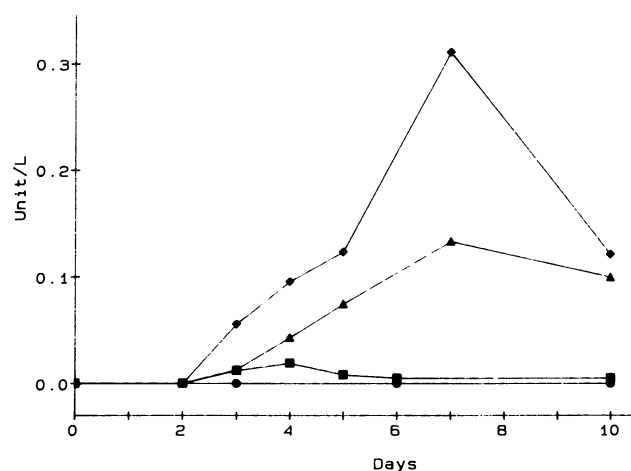


FIG. 4. Effect of nitrogen source on ligninase activity. The ligninase activity of the cultures shown in Fig. 3 were determined as described in Materials and Methods. Nitrogen source: 1.2 mM glycine adduct (●), 1.2 mM NH_4Cl (■), 1.2 mM NH_4Cl plus 1.2 mM glycine adduct (▲), and 1.2 mM NH_4Cl plus 2.4 mM glycine adduct (◆).

ary metabolism, precluding nitrogen repression; and the stimulation of ligninase activity by the releasing veratraldehyde (II), which is rapidly reduced to veratryl alcohol (2), a compound known to increase ligninase activity (14). The predominant ligninases produced by adduct-fed cultures were H2 and H8 (data not shown).

DISCUSSION

Adducts such as compound I have potential in the primary screening of lignin-degrading organisms from the environment. The methodology described for the synthesis of the glycine adducts works also with leucine (see below) and should be applicable to other amino acids. A possible selection strategy might be to use a mixture of adducts. With this mixture as the main or only nitrogen source, the medium (containing readily used carbon sources) would select for organisms which possess ligninase activity. This novel strategy thus might provide an alternative to the standard strategy of using the substrate as sole carbon source, which, as pointed out, is infeasible with lignin. Rather than requiring that the organism degrade lignin for growth, this selection strategy would require only that the organism be able to degrade the adducts and use the liberated amino acids as nitrogen source, as seen here with the glycine adduct and *P. chrysosporium*.

Adducts with higher molecular weights or with more complexity in the "lignin" portion or both also might be particularly useful in some applications. Such adducts, for example, might circumvent the potential problem of intracellular degradation and utilization by a non-lignin-degrading enzyme system. Our data here show that this is clearly not a problem with *P. chrysosporium*, but it might be with some microbes.

The use of adducts such as compound I as ligninase-dependent growth substrates also provides a potential selection procedure for strain improvement based on ligninase activity. One of us (M.T.) is presently attempting to select for mutants of *P. chrysosporium* that degrade lignin during primary metabolism, i.e., N-deregulated mutants. This is being attempted with the leucine adduct, which has been

prepared in a synthesis analogous to that described here for the glycine adduct. The strategy entails initially obtaining leucine auxotrophs (1). These will be subjected to a second mutagenesis in which the survivors will be plated onto a medium containing excess NH_4^+ -nitrogen and the leucine adduct. Nitrogen-deregulated mutants, producing the ligninase under these high NH_4^+ -nitrogen conditions, will liberate leucine from the adduct and thus be able to form colonies. These nitrogen-deregulated leucine auxotrophs will then be further mutagenized to obtain nitrogen-deregulated mutants.

Although selection strategies may differ with each specific application, the use of these ligninase-dependent growth nitrogen adducts should open up new avenues for selecting and improving lignin-degrading organisms. The future development of these procedures may further our understanding of how nature degrades this abundant polymer and provide strains with enhanced industrial potential (16).

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