Interference of Peptone and Tyrosine with the Lignin Peroxidase Assay

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The N-unregulated white rot fungus *Bjerkandera* sp. strain BOS55 was cultured in 1 liter of peptone-yeast extract medium to produce lignin peroxidase (LiP). During the LiP assay, the oxidation of veratryl alcohol to veratraldehyde was inhibited due to tyrosine present in the peptone and the yeast extract.

The N-unregulated white rot fungus *Bjerkandera* sp. strain BOS55 produces several extracellular enzymes that cause extensive degradation of lignin. Among these is lignin peroxidase (LiP), which uses veratryl alcohol (VA) as both a cofactor and a substrate. VA can participate in the catalytic cycle of LiP, which involves two successive one-electron oxidations of VA. Veratraldehyde (VAD) is formed predominantly through a one-electron-oxidized intermediate, the veratryl cation radical ($VA^{\cdot+}$) (5, 6). The $VA^{\cdot+}$ can oxidize other compounds, such as phenols (2, 3, 7), and is consequently reduced back to VA.

VA is commonly added to cultures of white rot fungi to increase LiP activities, but in the case of *Bjerkandera* spp., LiP is produced at high levels only when these fungi are cultured on a high-nitrogen medium and not under nitrogen-limiting conditions. The best results are obtained with mycological peptone and yeast extract (4, 9).

In this study, strain BOS55 was cultured on 1 liter of peptone-yeast extract medium to obtain large amounts of LiP. We observed that the LiP activity could be greatly enhanced by precipitating the LiP proteins with $(NH_4)_2SO_4$. Since this indicated that medium components interfered with the LiP assay, we decided to determine which components in the culture broth were responsible for this effect.

BOS55 was grown on standard peptone-yeast extract medium minus manganese (8, 9). Polyurethane foam cubes that were completely covered with the fungus were used as an inoculum. This inoculum was prepared in a Petri dish containing 15 ml of growth medium (without 2,2-dimethylsuccinate and VA) and five polyurethane foam cubes (length, 1 cm; width, 1 cm; height, 1 cm; $\rho = 35 \text{ kg/m}^3$). One foam cube was used per culture.

BOS55 was grown statically in the dark at 30°C in 1 liter of medium in a (5-liter) Erlenmeyer flask that contained a magnetic stirring bar. Passive aeration was assured by using a beaker which was put upside down on top of the Erlenmeyer flask instead of using cotton wool plugs. Before sampling, the culture fluid was stirred; a 10-ml sample was then removed and stored at –20°C. This storage procedure did not affect LiP activity.

LiP proteins in the crude samples were precipitated by addition of $(NH_4)_2SO_4$ (80%, 0°C), followed by centrifugation (10,000 $\times g$, 15 min, 0°C). The pellet was dissolved in twice the

original volume with demineralized water. LiP activity was measured in this sample and compared to that in a sample to which the $(NH_4)_2SO_4$ was not added.

The LiP activity then was measured spectrophotometrically ($\lambda = 310 \text{ nm}$; 30°C) by monitoring the production of VAD ($\epsilon = 9,300 \text{ M}^{-1} \text{ cm}^{-1}$ [11]). The reaction mixture (0.5 ml) contained 50 mM tartrate (pH 3.0), 2 mM VA, and a 50- μl LiP sample. The LiP activity was corrected for interferences prior to the addition of H_2O_2 (0.5 mM [10]), which was used to initiate the LiP assay. The LiP activity is expressed in units per liter (1 U forms 1 μmol of VAD $\cdot \text{min}^{-1}$).

The effect of peptone, L-tyrosine, and L-phenylalanine (Acros, Geel, Belgium) on the LiP activity was studied by using a partially purified preparation of LiP from *Phanerochaete chrysosporium* (100,000 U/liter), obtained from Tienzyme, Inc. (State College, Pa.). In the assay mixture, this LiP was diluted 4,000 and 8,000 times. Also, a purified LiP preparation from *Bjerkandera* sp. strain BOS55 was used for these experiments and for recording the visible (VIS) spectra of the heme region (146 U of LiP/liter, 0.44 mM L-tyrosine, 2 mM VA, 0.5 mM H_2O_2).

In an experiment aimed at increasing LiP production on a 1-liter scale, *Bjerkandera* sp. strain BOS55 was cultured on peptone-yeast medium (8, 9). During the course of the experiment, LiP in the extracellular culture fluid was assayed by measuring the formation of VAD from VA. However, unlike assays performed with semipurified preparations of LiP, the formation of VAD showed a biphasic pattern. Figure 1 shows a typical example of the LiP assay with the extracellular fluid collected on day 14 (line b). There is an initial slow increase in the extinction at 310 nm, lasting about 70 s in the first period, followed by a second period in which the increase in extinction at 310 nm was significantly greater. This slope of the line in the second period was used to calculate the LiP activity. However, when the LiP in the extracellular culture fluid was precipitated with 80% $(NH_4)_2SO_4$ and resuspended, the LiP assay (line a) typically showed no lag phase and the rate of VAD formation was significantly higher than the maximal rate observed with the crude sample. From these observations we concluded that compounds in the culture fluid partially inhibited LiP during the assay.

To study this effect as a function of culture age, both the crude extracellular culture fluid and the $(NH_4)_2SO_4$ precipitate were assayed over a period of 30 days. Figure 2A shows that LiP activity in the precipitated samples first appeared on day 8, reaching a maximum activity of approximately 600 U/liter on day 14, after which it remained constant until the end of the experiment (day 30). In contrast, in the crude samples no

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FIG. 1. Typical VAD formation patterns observed during the LiP assay with resuspended 80% $(\text{NH}_4)_2\text{SO}_4$ protein precipitate (line a) and with extracellular culture fluid sampled on day 14 (line b) of a 1-liter culture of *Bjerkandera* sp. strain BOS55. Line b shows the biphasic character consisting of phase I, the lag phase, and phase II, the slope of which was used to calculate the LiP activity. E_{310} , extinction at 310 nm.

activity could be measured before day 12. From day 14 to day 18, LiP was inactivated to some extent. From day 20 onward, the LiP activities in the crude samples and in the treated samples were comparable. This suggests that medium components were responsible for the interference with the LiP assay.

Figure 2B shows the duration of the lag phase as a function of cultivation time. In the untreated samples, long lag phases were observed between day 14 and day 18, and these were proportional to the observed losses of LiP activity. During this period the precipitated samples showed a constant LiP activity and no lag phase.

Several medium components, such as 2,2-dimethylsuccinate

FIG. 2. (A) LiP activity measured directly in the extracellular culture fluid of *Bjerkandera* sp. strain BOS55 (○) and in the resuspended 80% $(\text{NH}_4)_2\text{SO}_4$ protein precipitate (●) versus culture time. (B) Lag phases observed during the LiP assay of the extracellular culture fluid (○) and the resuspended 80% $(\text{NH}_4)_2\text{SO}_4$ protein precipitate (●). The measurements are averages of three parallel cultures. Error bars, standard errors of the means.

FIG. 3. The effect of peptone on LiP from *P. chrysosporium* (27.4 [■] and 13.7 [●] U/liter of assay mixture). The open circles and squares show the lag phases as observed during the LiP assay.

and the BIII mineral solution (10), were tested, but they did not inhibit LiP, leaving peptone and yeast extract as the most logical components in the medium to study. For these studies, a commercially available, partially purified LiP preparation from *P. chrysosporium* was used. The same experiments were also carried out with a purified LiP preparation from *Bjerkandera* sp. strain BOS55. The results (not shown) were the same as those obtained with LiP from *P. chrysosporium*.

Both yeast extract and peptone were found to inhibit LiP, but yeast extract inhibited LiP to a much smaller extent. Furthermore, it occurs only as a minor medium component (1 g/liter) compared to peptone (5 g/liter), and for this reason we decided to study the inhibition by peptone in more detail.

The effect of peptone at different concentrations on the activity of the semipurified LiP was tested at two enzyme concentrations in the assay mixture, 13.7 and 27.4 U/liter. Figure 3 shows that complete inhibition occurred at 39 and 78 mg of peptone per liter for the low and high LiP concentrations, respectively. An increase in LiP inhibition was associated with an increase in lag phase duration, which is similar to the pattern observed in the fungal culture (Fig. 2).

To explain the inhibitory effect of peptone, we examined the inhibitory effect of L-tyrosine on LiP, because the pattern of inhibition showed clear similarities to those reported for other phenolic compounds (2, 3, 7).

We observed a pattern (not shown) similar to that observed in Fig. 3 and found that LiP was completely inhibited at 10 and 18 μM L-tyrosine for the low and high LiP concentrations, respectively. As a control, we tested the effect of the nonphenolic analog of L-tyrosine, L-phenylalanine. L-Phenylalanine did not inhibit LiP.

Peptone and L-tyrosine appeared to inhibit LiP by the same mechanism, as evidenced by the fact that in both cases the relationship between the decrease in LiP activity and the length of the lag phase was linear and highly correlated (Fig. 4).

To study this mechanism in more detail, we recorded VIS spectra of the LiP heme group during the assay. Figure 5 shows spectra recorded 7 min after the addition of H_2O_2 . After this time the spectra did not change substantially. The LiP heme group absorbs light with wavelengths between 380 and 430 nm (Fig. 5, line A). When LiP is incubated with just H_2O_2 , the heme peak in the VIS spectrum disappears (Fig. 5, line D). This is an indication of bleaching of the heme. For LiP in the presence of both VA and H_2O_2 , we observed a temporary shift of the heme peak to 418 nm. This indicates the formation of LiPII and/or LiPIII. After 7 min, the heme peak was back in its

FIG. 4. Relationship between the observed decrease in LiP activity (percent inactivation) and the length of lag phase. Results in the presence of peptone (triangles; $y = 0.344x + 5.095$; $R^2 = 0.9579$) and L-tyrosine (diamonds; $y = 0.334x + 3.226$; $R^2 = 0.9641$), calculated by using data from both LiP concentrations (i.e., 27.4 U/liter [solid symbols] and 13.7 U/liter [open symbols]), are shown.

original position (Fig. 5, line B). This illustrates that VA protects LiP from irreversible inactivation by H_2O_2 .

Incubation of L-tyrosine, VA, H_2O_2 , and LiP reduced the absorbance of the heme substantially (Fig. 5, line C). This is explained by irreversible bleaching of the heme by H_2O_2 , since the same thing happened in the presence of H_2O_2 only.

The mechanism of VAD production by LiP involves the formation of a VA^{+} , which is an essential intermediate. During the lag phase, VA^{+} s generated by LiP are very effectively reduced back to VA by phenolics until the latter are completely oxidized (3, 7). The oxidation of the phenolics and

subsequent reduction of the VA^{+} s are observed as a lag phase in which VAD is not produced. This mechanism explains the lag phase as observed in our experiments.

The rate-limiting step in the catalytic cycle of LiP is the reduction of LiPII back to native LiP. LiPII can easily react with H_2O_2 , forming inactive LiPIII. VA^{+} s can overcome the accumulation of LiPIII since they revert it back to active ferric LiP (1). In the presence of phenolics, however, the VA^{+} s are reduced; as a consequence of this, LiP compound III accumulates during the lag phase (3). LiPIII can react with excess H_2O_2 , resulting in an irreversible inactivation of the enzyme by bleaching of the heme (2). This theory fits well with our findings that the heme peak in the VIS spectrum disappears during incubation of LiP with either VA plus L-tyrosine or just H_2O_2 alone.

Thus, L-tyrosine present in peptone and yeast extract inhibits oxidation of VA to VAD by LiP from both *P. chrysosporium* and *Bjerkandera* sp. strain BOS55, and a secondary consequence is the inactivation of LiP by H_2O_2 , resulting in an underestimation of the LiP activity.

There is also indirect evidence that peptides containing tyrosine inhibit LiP, since the long lag phase observed at day 8 (Fig. 2B) implicates that some peptides precipitated with $(NH_4)_2SO_4$ also inhibited LiP. The observed lag phase was not due to the presence of free tyrosine, because amino acids are not precipitated by this method.

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FIG. 5. VIS spectra of LiP from *Bjerkandera* sp. strain BOS55 taken 7 min after the addition of H_2O_2 (or water). Lines: A, LiP with water only (without H_2O_2); B, LiP in the presence of VA and H_2O_2 ; C, VA plus L-tyrosine and H_2O_2 ; D, LiP with H_2O_2 only.