Purification and Properties of Benzyl Alcohol Oxidase from Botrytis cinerea

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A benzyl alcohol oxidase (BAO) was purified to homogeneity from Botrytis cinerea. The enzyme was found to have a molecular mass of 214 kDa with a trimeric structure, and optimal pH and temperature of 5.0 and 30°C, respectively. The enzyme activity was not sensitive to metal ions or to metal ion chelators, while thiol blocking reagents strongly inhibited BAO activity. Sulfur dioxide irreversibly inhibited the enzyme activity and the inhibitory effect of ethanol was weak and reversible. Benzyl alcohol was the most effective alcohol substrate for BAO. Para or meta monosubstituted benzyl alcohol with methyl or methoxy groups were good substrates. BAO also oxidized cinnamyl alcohol, furfuryl alcohol, and some terpenic alcohols with an alkenyl group near the reactive carbonyl. Secondary alcohol, methanol and phenol were not substrates. Product inhibition studies suggested that benzoaldehyde and benzyl alcohol were bound at different places to the active site. O₂ was the only electron acceptor identified and Botrytis cinerea benzyl alcohol oxidase was classified as EC 1.1.3.7 according to stoichiometrical studies. We discuss the metabolic role of BAO in the Botrytis cinerea-grape host-parasite relationship.

There is evidence that the bitter almond taste in wines is related to a benzyl alcohol oxidase (BAO) supplied by the vintage. This wine BAO has been purified and found to be a trimeric protein having sulphydryl groups that are required for its activity. We have reported that a mycelial and an extracellular BAO were synthesised by Botrytis cinerea. It was shown that this mycelial enzyme is actually parietal and partially released without much modification in the medium (unpublished data). Aromatic alcohol oxidases have been detected in mycelial crude extracts of Botrytis cinerea. Farmer found an extracellular aromatic alcohol oxidase in the growth medium of the wood-rotting basidiomycete Polystictus versicolor. A veratryl alcohol oxidase has also been purified from the growth medium of the lignin-degrading basidiomycete Pleurotus sajor-caju.

Aromatic alcohol dehydrogenases have also been reported. A benzyl alcohol dehydrogenase has been isolated from Acinetobacter calcoaceticus and from Pseudomonas sp. An enzyme considered to be a vanillyl alcohol dehydrogenase has been purified from Rhodopseudomonas acidophila. Jaeger described an intracellular conferyl alcohol dehydrogenase from Rhodococcus erythropolis. An aromatic alcohol dehydrogenase has also been detected in potato tubers and a cinnamyl alcohol dehydrogenase from Phascolus vulgaris has been reported.

We recently postulated that a wine benzyl alcohol oxidase was brought by Botrytis cinerea on grapes. The present study investigated the purification and characterization of benzyl alcohol oxidase from Botrytis cinerea. Moreover, we compared wine BAO and Botrytis cinerea BAO and the results are related to the metabolism of aromatic compounds by Botrytis cinerea.

Materials and Methods

Source of the organism. Botrytis cinerea isolated from grapes was kindly provided by M. Rascol, Laboratoire de Mycologie, Faculté de Pharmacie, Montpellier, France.

Growth of the organism. Botrytis cinerea was grown on sporulating medium (malt 1%, agar 1.5%) for 15 days. Conidiospores were harvested with 0.01% Tween 80. Growth medium (Yeast Nitrogen Base 0.67%, galactose 2%, Yeast Extract 1%, 50 mM KH₂PO₄) was sown with a conidiospore suspension at 10⁶ cells/ml. Cultures were grown at 28°C in 1-liter flasks containing 200 ml of medium with shaking, until maximum BAO activity was reached (exponential stage—72H).

BAO extraction. Mycelium was removed by centrifugation at 30,000 x g for 10 min. The mycelium was washed 3 times with 3 volumes of 0.2 M phosphate citrate buffer (pH 5), homogenized in an Ultraturrax, and disintegrated in a Branson sonifier 250, for 10 min, at 0°C. The supernatant was taken as the crude extract.

BAO purification. All procedures were done at +4°C. During purification, dithiothreitol (1 mM) and glycerol (20% v/v) were required to stabilize the enzyme activity.

Ammonium sulfate fractionation. Ammonium sulfate was added to 60% saturation to the crude extract obtained from the mycelium. The precipitate was centrifuged and discarded. Ammonium sulfate was added to the supernatant to 80% saturation. The precipitate was removed by centrifugation and dissolved in a small amount of buffer A (40 mM Tris-HCl buffer (pH 7.6), containing 1 mM dithiothreitol (DTT) and 20% v/v glycerol).

Diaphragation. The extract was diaphragated and concentrated on a Diaflo ultrafilter YM10 (cut-off of 10,000) with buffer A at 0°C.

Ion exchange chromatography. The extract was put on a Q Sepharose Fast Flow column (1 x 10 cm) pre-equilibrated with buffer A. The column was washed with buffer A until the absorbance of the effluent decreased to approximately 0.1, at 280 nm. Buffer A was then changed to buffer B (buffer A, with 0.1 M NaCl) to eliminate proteins. Buffer B was replaced with a gradient mixer containing buffer B and buffer C (buffer A, with 0.3 M NaCl). The gradient was replaced by buffer D (buffer A, with 1 M NaCl) to wash the column. The flow rate was 90 ml/hr. Fractions...
containing enzymatic activity were pooled and concentrated with Diaflo Ultrafilters YM10 to obtain an extract.

**Gel filtration column chromatography.** The concentrated extract was put on a Sephacryl S300 column (1 x 100 cm) preequilibrated with buffer A. The flow rate was 5 ml·hr⁻¹. The peak fractions containing most of the enzyme activity were pooled.

**Enzyme assay.** Three different methods were used to measure the BAO oxidation reaction:

\[
\text{benzyl alcohol} + O_2 \rightarrow \text{benzaldehyde} + H_2O_2
\]

**Monitoring of O₂ consumption.** A reaction mixture (1.5 ml), containing 0.2 M phosphate citrate buffer at pH 5, 10 mM benzyl alcohol, and a sample of enzyme were placed in a cell. The activity was measured at 30°C by estimation of oxygen consumption using a Clark electrode. The blank assay was done under the same conditions with thermally denatured enzyme.

**Monitoring of H₂O₂ formation.** The enzyme was assayed by measuring the color alterations produced by H₂O₂ in a paired reaction, done as follows: a sample of enzyme was added to 1 ml of reagent containing 0.2 M phosphate citrate buffer (pH 5), 10 U·l⁻¹ horseradish peroxidase (Sigma), 50 mg·l⁻¹ o-dianisidine and 10 mM alcohol substrate. Color intensification was monitored at 480 nm on a Kontron spectrophotometer using a blank containing the reaction mixture with no alcohol substrate.

**Monitoring of benzaldehyde formation.** The assay mixture (1 ml) contained 0.2 M phosphate citrate buffer (pH 5) and 10 mM benzyl alcohol. The reaction was started by the addition of a sample of enzyme extract. A blank assay was prepared under the same conditions with thermally denatured enzyme. Benzaldehyde formation was monitored with a Varian 5000 HPLC, with a Merck Lichrosorb RP8 column, elution with acetonitrile/water (55%–65% v/v), at a flow rate of 1 ml·min⁻¹ and using 4-dimethylaminobenzaldehyde as an internal standard.

One unit of enzyme was defined as the amount of enzyme catalyzing the transformation of 1 μmol of substrate per minute in our assay conditions at 30°C.

**Protein concentration.** Proteins were measured by the procedure of Bradford, using bovine serum albumin as the standard. During the purification procedure, the absorbance at 280 nm was measured with a Kontron spectrophotometer.

**Molecular mass.** The molecular mass of the native enzyme was measured by gel filtration chromatography (Sephacryl S300). The column (1 x 100 cm) was equilibrated with buffer A and calibrated with standard proteins: thyroglobulin (670 kD), catalase (232 kD), bovine serum albumin (66 kD), and chymotrypsinogen A (25 kD). The flow rate was 5 ml·hr⁻¹, and the void volume was established with blue dextran.

The molecular mass of the enzyme under denaturing conditions was estimated by SDS PAGE gel electrophoresis (12%), by the method of Laemmli. Each standard protein and sample was incubated in Laemmli buffer at 90°C for 5 min. Electrophoresis was done for 2 hr with a constant current of 30 mA. Coomassie blue R250 was used for protein staining. The standard proteins were myosin (200 kD), β-galactosidase (116 kD), phosphorylase b (97.4 kD), bovine serum albumin (66 kD), and ovalbumin (42.7 kD).

### Results

#### BAO purification

The BAO purification procedures using ion exchange and gel filtration chromatographies are summarized in Table I. The enzyme was purified 177-fold with a yield of 50%.

The purified enzyme was homogeneous as seen by denaturating polyacrylamide gel electrophoresis (Fig. 1).

**Molecular mass determination**

The subunit molecular mass of BAO, estimated by SDS PAGE, was 70 ± 3 kD (Fig. 1). The molecular mass of the native enzyme estimated by the method of Andrews was 214 ± 5 kD. Comparison of the subunit and native molecular mass suggested that the enzyme is trimeric.

#### Effects of pH and temperature on enzyme activity

The effects of pH on enzyme activity were studied with the following 0.2 M buffers: citrate phosphate (pH 2.6 to 5.6) and phosphate (pH 6 to 8). We obtained an optimal pH of 5 for the BAO oxidation reaction.

The enzyme was most active at 30°C. Activation and inactivation energy values were estimated at 61 kJ·mol⁻¹ and 117 kJ·mol⁻¹, respectively.

#### Effects of metal ions and enzyme inhibitor on BAO activity

The effects of metal ions on enzyme activity were tested with Cu²⁺, Zn²⁺, Fe²⁺, Mg²⁺, and Mn²⁺ at the same concentration (1 mm). No significant effect was observed under our assay conditions.

BAO was assayed in the presence of the following metal ion chelators: o-phenanthroline and EDTA both at 1, 5, and 10 mM. The enzyme activity was unaffected by metal ion chelators, thus suggesting that BAO does not require metal ions for activity.

The enzyme activity was strongly inhibited by the sulfhydryl reagent p-chloromercuribenzoate (pCMB), with

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<th>Total protein (mg)</th>
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</table>

### Table 1. Purification of Benzyl Alcohol Oxidase (BAO) of Botrytis cinerea

![Fig. 1. Electrophoresis of Benzyl Alcohol Oxidase of Botrytis cinerea.](image-url)
$I_{50}$ at a concentration of 7.3 $\mu M$ and total inhibition at 12 $\mu M$. This indicates that sulfhydryl groups in the proteins are essential for enzyme activity.

Inhibition by ethanol and sulfur dioxide, which are two classical compounds in wine, on enzyme activity was also investigated. To find if the inhibition by these two compounds was reversible or irreversible, the variation of $1/(\text{inhibition rate})$ versus $1/(\text{inhibitor concentration})$ was

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**Fig. 2. Inhibitory Effect of Ethanol on Benzyl Alcohol Oxidase Activity.**
The enzyme assays were done in 0.2 M citrate phosphate buffer (pH 5), 10 mM benzyl alcohol at 30°C. A: Plot of relative activity versus inhibitor (ethanol). B: Plot of $V_0/(V_0 - V)$ versus $1/(\text{ethanol})$. $V_0$, initial rate without inhibitor; $V$, initial rate in the presence of inhibitor.

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**Fig. 3. Inhibitory Effect of SO$_2$ on Benzyl Alcohol Oxidase Activity.**
The enzyme assays were done in 0.2 M citrate phosphate buffer (pH 5), 10 mM benzyl alcohol at 30°C. A: Plot of relative activity versus inhibitor (SO$_2$). B: Plot of $V_0/(V_0 - V)$ versus $1/(\text{SO}_2)$. $V_0$, initial rate without inhibitor; $V$, initial rate in the presence of inhibitor.
plotted. In such a graphic representation, a plot with a straight line which, by extrapolation, intercepts the \( Y \) axis at unit one indicates a reversible inhibition and when the straight line intercepts the \( Y \) axis at a point less than unit one, this indicates an irreversible inhibition. A weak inhibition was observed for ethanol with \( I_{50} \) at a concentration of 1 M and \( V_0(V_0 - V) \) versus 1/ethanol produced a straight line which, by extrapolation, intercepted the \( Y \) axis at unit one, indicating a reversible inhibition (Fig. 2A and 2B). In a similar study, sulfur dioxide irreversibly inhibited BAO activity (the intercept of the \( Y \) axis was less than one unit, with \( I_{50} \) at a concentration of 0.7 mM (Fig. 3A and 3B).

**Electron acceptor**

The specificity of the enzyme for various electron acceptors was tested with NAD\(^+\), NADP\(^+\) (340 nm), phenazine methosulfate (PMS) with nitro blue tetrazolium (NBT-535 nm), or with dichlorophenol indophenol (DCIP-600 nm) and cytochrome c (550 nm), at a concentration of 100 \( \mu \)M. None of the five electron acceptors tested were used by BAO. \( \text{O}_2 \) was the only electron acceptor identified. Without \( \text{O}_2 \) (under \( \text{N}_2 \)) no BAO activity was detected.

**Substrate specificity**

An extensive variety of aromatic and aliphatic alcohols were tested as potential BAO substrates. Table II shows the relative activity of BAO with the tested compounds. A comparison of the kinetic parameters of the best substrates is shown in Table III. It is generally established that the higher the catalytic efficiency (\( V_m/K_m \)), the closer the structure of the substrate transition state intermediate is to the active site of the enzyme.\(^{18}\)

Compounds with a reactive carbinal “\( \alpha \beta \) alkenyl” group were substrates (nerol and geraniol). When the alkenyl group was associated with an aromatic structure, catalytic efficiency greatly increased (benzyl alcohol, cinnamyl alcohol, and furfural). The presence and the position of an alkenyl group near the reactive carbinal and its conjugation with an aromatic ring appeared to be essential chemical conditions for a good catalysis with BAO.

However, phenyl-2-ethanol, having a reactive carbinal “\( \beta \) alkenyl” group and phenyl-3-propanol, having a reactive carbinal “\( \gamma \) alkenyl” group were very poor substrates.

The tested aliphatic alcohols, which have no alkenyl groups, were not substrates and BAO was totally inactive on methanol.

Secondary alcohols, such as phenyl-1-ethanol and phenyl-1-propanol, were not substrates.

BAO did not catalyze the oxidation of phenol and cyclohexanol.

BAO had the highest catalytic efficiency with benzyl alcohol, and the acceptability of some substrates seemed to depend on the nature and position of the substituent group on the aromatic ring. Introduction of ortho substituted groups strongly inhibited BAO. This could have been due to steric effects involved in binding of the substrate to the enzyme and suggests that the active site may have a cleft structure.\(^{18}\)

Benzyl alcohol substrates in para or meta positions were good substrates for the enzyme. The electron-withdrawing properties of the substituent groups of the aromatic ring may also have been involved in controlling accessibility of particular substrates. Indeed, the best substrates for BAO were benzyl alcohol with methyl substituents, while methoxy substituents were poorer substrates and hydroxyl substituents were bad substrates.

**Product inhibition studies**

To obtain some information on the BAO mechanism, product inhibition studies were done.

The effects of benzaldehyde on the oxidation of benzyl alcohol were first studied. As shown in Fig. 4A, benzaldehyde appears to be a true non-competitive inhibitor of benzyl alcohol oxidase and the inhibition constant \( K_i \) obtained from the secondary plot shown in Fig. 4B, was
46 mM. These results could suggest that benzaldehyde and benzyl alcohol were bound to the active site at different places.

An approach of the inhibition effect of $H_2O_2$ on BAO activity showed that a complex inhibitory mechanism was involved.

**Discussion**

The previously reported properties of wine BAO,\(^2\)

are close to those of the BAO in *Botrytis cinerea*, thus suggesting that it is probably the same enzyme.

The inhibition effects of wine compounds, such as sulfur dioxide and ethanol, on *Botrytis cinerea* BAO led to the same conclusions as those obtained for wine BAO. The two enzymes also showed the same sensitivity to thiol-blocking reagents and metal ion chelators. The inhibition of aromatic alcohol oxidoreductases by thiol-blocking reagents is a common feature.\(^{10,11,18}\) The BAO mechanism could
involve a reactive thiol group in the active site of the enzyme. MacKintosh\textsuperscript{18} also suggested that, since \textit{p}-chloromercuri-ribenzoate contains a benzene ring, its potency might also be an active site controlled phenomenon. The insensitivity of BAO to inhibition by metal ion chelators is another property it shares with other aromatic alcohol oxidoreductases.\textsuperscript{11,14--20} BAO therefore does not require metal ions for its activity.

The substrate specificity of BAO showed that benzyl alcohol had the best catalytic efficiency of all compounds tested. Compounds with a reactive carbinol \textit{"\textalpha;" linked} group conjugated with an aromatic ring were excellent substrates, thus showing the importance of electron delocalization for good catalysis with BAO. The best substituents also seemed to be methyl groups, in \textit{para} or \textit{meta} position, while substituents in the \textit{ortho} position hindered binding, probably because of steric effects.

Product inhibition studies showed that benzaldehyde was a true non-competitive inhibitor of benzyl alcohol oxidation, suggesting that they are bound in different positions on the active site. Molina\textsuperscript{21} reached the same conclusions concerning ethanol and acetalddehyde binding to grape alcohol dehydrogenase. O\textsubscript{2} was the only electron acceptor identified and it was essential for BAO activity. BAO showed an aryl alcohol oxidase enzymatic reaction stoichiometry. It should therefore be classified as EC 1.1.3.7, which requires SH groups but not metal ions for its activity.

Identification of the physiological role of the enzyme depends upon its physiological substrate. Benzyl alcohol is widely distributed in plants, usually as an ester.\textsuperscript{12} Cinnamyl and coniferyl alcohols are precursors of lignin synthesis.\textsuperscript{13} Many plants respond to microbial attack by deposition of lignin and other wall-bound phenolic materials at the point of attack and this may occur in peripheral tissues, which in uninfected plants do not contain lignin.\textsuperscript{13,22} Moreover, bean cinnamyl alcohol dehydrogenase, an enzyme of the phenylpropanoid reductive metabolism specific for lignin is another reported plant response.\textsuperscript{13} This enzyme catalyzes the reduction of cinnamaldehyde to cinnamyl alcohol.

The BAO catalyzed the oxidation of aromatic alcohols to the corresponding aldehyde with the production of \textit{H}_2\textit{O}_2. Bratt\textsuperscript{41} reported that the \textit{H}_2\textit{O}_2 produced by \textit{Botrytis cinerea} was involved in degradation of flax stem lignin, with possible associated laccase activity. Muheim\textsuperscript{19} postulated that an oxidative attack by laccase, peroxidase, and active oxygen on the lignin polymer, with the intervention of an arylic alcohol dehydrogenase, was the predominant reaction for lignin degradation by \textit{Phanerochaete chrysosporium}. Bourdessens\textsuperscript{7} pointed out the possible involvement of a veratryl alcohol oxidase of \textit{Pleurotus sajor-caju} in lignin biodegradation, in combination with laccase. Hence, BAO of \textit{Botrytis cinerea} could play a role in the attack on grapes, as a response to a microbial-induced plant defense metabolism.

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\textbf{References}