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Enigmatic Gratuitous Induction of the Covalent Flavoprotein Vanillyl-Alcohol Oxidase in *Penicillium simplicissimum*

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When *Penicillium simplicissimum* is grown on veratryl alcohol, anisyl alcohol, or 4-(methoxymethyl)phenol, an intracellular covalent flavin-containing vanillyl-alcohol oxidase is induced. The induction is highest (up to 5% of total protein) during the growth phase. In addition to vanillyl-alcohol oxidase, an intracellular catalase-peroxidase is induced. Induction of vanillyl-alcohol oxidase in *P. simplicissimum* is prevented by the addition of isoeugenol to veratryl alcohol-containing media, but growth is unaffected. The inhibitory effect of isoeugenol on induction is not observed when anisyl alcohol or 4-(methoxymethyl)phenol is used as the growth substrate. Based on the induction experiments and the degradation pathways for veratryl and anisyl alcohol, we propose that induction of vanillyl-alcohol oxidase is superfluous when *P. simplicissimum* is grown on these aromatic alcohols. However, the enzyme plays an essential role in the degradation of the methyl ether of *p*-cresol, 4-(methoxymethyl)phenol.

Since the evolution of plants, microorganisms must have evolved which are able to degrade plant material to complete the Earth's carbon cycle. The only organisms capable of completely degrading wood, including the aromatic polymer lignin, are wood rot fungi. That these basidiomycetes have existed for a very long time is confirmed by the findings of their fossilized remains (8, 10). Since the degradation of lignin leads to formation of a broad spectrum of aromatic compounds, other microorganisms have developed ways to use these compounds. Several penicillia, normally soil inhabitants, degrade lignin-related aromatic compounds (2, 12, 16, 20) and even lignin to some extent (18).

Penicillium simplicissimum CBS 170.90 originally was isolated on veratryl alcohol, a central metabolite in lignin degradation (2). This fungus is able to grow on a variety of aromatic compounds as the sole carbon and energy source. Further study revealed that during growth on veratryl alcohol, a flavin-dependent aromatic alcohol oxidase is induced. This enzyme catalyzes the conversion of vanillyl alcohol to vanillin with the simultaneous production of hydrogen peroxide (3). Parallel with the induction of vanillyl-alcohol oxidase, a catalase-peroxidase is induced; this enzyme has been purified and characterized (6). Vanillyl-alcohol oxidase is a homooctamer with each subunit containing a covalently bound flavin adenine dinucleotide (22). The enzyme is most active at basic pH and consequently shows little resemblance to known fungal aryl-alcohol oxidases (22). Recently, we reported that vanillyl-alcohol oxidase is also active with aromatic amines, 4-allylphenols, 4-alkylphenols, and 4-(methoxymethyl)phenols (5, 7). The enzymatic oxidation products can be of use in the food industry because alkylphenol derivatives and aromatic aldehydes like vanillin are known flavors (4, 21). However, since the enzyme is induced in relatively large quantities and is apparently not involved in the degradation of veratryl alcohol (2), its physiological role remains obscure.

In this work, we report on the specific induction of vanillyl-alcohol oxidase by growth of *P. simplicissimum* on veratryl alcohol, anisyl alcohol, and 4-(methoxymethyl)phenol. The degradation pathways of these aromatic compounds were studied and are discussed in relation to the physiological function of vanillyl-alcohol oxidase.

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MATERIALS AND METHODS

Organism and media. All experiments were performed with *P. simplicissimum* CBS 170.90. The liquid medium (pH 5.4) contained (per liter) Na_2HPO_4, 0.65 g; KH_2PO_4, 2.5 g; NH_4Cl, 2.0 g; $(NH_4)_2SO_4$, 0.10 g; $MgCl_2 \cdot 6H_2O$, 0.075 g; 50% (wt/vol) poly(acrylic acid-co-maleic acid) (PAMA; molecular weight 3,000), 1.0 g; and 0.2 ml of a trace elements solution as described by Vishniac and Santer (24). Cells were grown in 500 ml of medium in 2-liter Erlenmeyer flasks. The flasks were incubated at 30°C with shaking, and the mycelial pellets were harvested with a cheesecloth. Carbon sources were added at 0.1% (wt/vol) (aromatic compounds) or 1.0% (wt/vol) (nonaromatic compounds). When grown on nonaromatic compounds and aromatic acids, the cells were harvested 2 days after inoculation. In all other growth experiments, the cells were harvested 4 days after inoculation. For collecting samples during growth on veratryl alcohol (0.1%), a 20-liter fermentor (15-liter working volume; 30°C; 50 rpm; air flow, 0.4 liter/min) was used which was inoculated with five mycelial pellets of glucose-grown cells. The cells were harvested regularly by withdrawing 500 ml of the culture.

Preparation of cell extracts and protoplasts. Cell extracts were prepared by three 10-s sonications of washed cell suspensions (0.5 ml of 50 mM potassium phosphate [pH 7.0]) with cooling intervals of 50 s. Cell debris was removed by centrifugation. Protoplasts were prepared by the method of Witteveen et al. (26).

Analytical methods. Veratryl alcohol (1.0 g) was purified on a silica column (2.5 by 19 cm). After the column was washed with 100 ml of petroleum ether (boiling point, 60 to 80°C), veratryl alcohol was collected by elution with 100 ml of methanol. Gas chromatography-mass spectrometry analysis showed that this fraction was of high purity (>99.5%). The protein content of cell extracts was determined by the method of Lowry et al. (13). High-pressure liquid chromatography (HPLC) analysis was performed at room temperature on a Hewlett-Packard 1040-1050 series HPLC system with a Chromspher C_{18} (100 by 4.6 mm) column (Chrompack). The eluent contained methanol, water, and acetic acid (33:66:1). For detection, a Waters 996 diode array detector was used. Stock solutions of a broad range of aromatic compounds (1.0 mM) were separately analyzed for their retention times and spectral characteristics, enabling identification of the formed degradation products (retention times [minutes]: veratryl alcohol, 2.65; veratraldehyde, 4.94; veratrate, 4.58; anisyl alcohol, 3.60; anisaldehyde, 7.27; anisate, 7.99; 4-(methoxymethyl)phenol, 3.40; 4-hydroxybenzaldehyde, 2.64; 4-hydroxybenzoate, 2.25; 3,4-dihydroxybenzoate, 1.61; vanillin, 2.98 and 4-hydroxybenzylalcohol, 1.64).

Biochemical analysis. The vanillyl-alcohol oxidase activity was assayed at 30°C and pH 10.0 with vanillyl alcohol as the aromatic substrate (3). Catalase and peroxidase were assayed as described previously (6). For peroxidase activity, 2,6-dimethoxyphenol was used as the electron donor. Glucose-6-phosphate dehydrogenase was assayed by the method of Bruinenberg et al. (1). Protocatechuate 3,4-dioxygenase activity was measured spectrophotometrically at 290 nm (9). Enzyme units are expressed as micromoles of substrate converted per minute.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried

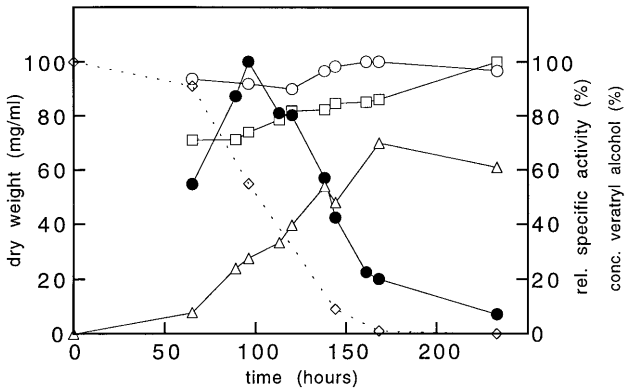


FIG. 1. Growth of *P. simplicissimum* on veratryl alcohol (0.1%, wt/vol) and some enzyme activities. Cells were grown in a 20-liter fermentor as described in Materials and Methods. Symbols: △, dry weight; ◇, veratryl alcohol; ○, glucose-6-phosphate dehydrogenase; ●, vanillyl-alcohol oxidase; □, catalase.

out in slab gels, essentially as described by Laemmli (11). For protein staining, Coomassie brilliant blue R-250 was used. For fluorescence detection of vanillyl-alcohol oxidase, the gel was incubated in 5% acetic acid solution for 5 min after electrophoresis. Upon illumination with UV light, vanillyl-alcohol oxidase is visible because of the fluorescence of the covalently bound flavin (3). To obtain sufficient fluorescence signal for photographing, five times the amount of protein was loaded compared to that in the protein-stained gels.

Generation of antibodies. Polyclonal antibodies directed against vanillyl-alcohol oxidase and catalase-peroxidase from *P. simplicissimum* were generated and purified as described previously (6). By using dot blots, the detection limit of both antibodies was estimated to be less than 10 pg. For detection of vanillyl-alcohol oxidase and catalase-peroxidase on Western blots, an alkaline phosphatase-based immunoassay was used. For molecular weight estimation, prestained SDS-polyacrylamide gel electrophoresis standard proteins were used (Bio-Rad).

Chemicals. Aromatic compounds were from Aldrich except for vanillin and vanillyl alcohol, which were from Janssen Chimica. All other chemicals were of commercially available analytical grade.

RESULTS

Growth of *P. simplicissimum* on veratryl alcohol. It was found in a previous study that *P. simplicissimum* is able to grow on veratryl alcohol as the sole carbon and energy source (2). During growth on this aromatic alcohol, vanillyl-alcohol oxidase is induced (3). Because the commercially available veratryl alcohol contains some minor aromatic impurities (4%), we decided to purify it to exclude any effect of these aromatic impurities on the induction pattern. However, when the fungus was grown on highly purified veratryl alcohol, the same level of induction of vanillyl-alcohol oxidase was found. Fig. 1 shows that the vanillyl-alcohol oxidase activity was highest during growth. When all the veratryl alcohol was depleted, the enzyme activity level dropped significantly. Other measured enzyme activities, glucose-6-phosphate dehydrogenase and catalase, were present at a nearly constant level. As *P. simplicissimum* contains two different types of catalases, an atypical periplasmic catalase and a catalase-peroxidase (6), no conclusions can be drawn with respect to the time-dependent induction of each of these hydroperoxidases.

Subcellular localization. Protoplasts of *P. simplicissimum* cells were prepared to determine the subcellular location of vanillyl-alcohol oxidase. Preliminary results had indicated that both vanillyl-alcohol oxidase and catalase-peroxidase were not extracellular or cell wall localized (6). The measured activities of protoplast and cell extracts confirmed that both vanillyl-alcohol oxidase and catalase-peroxidase are intracellular enzymes (Table 1). The apparent increase in the peroxidase activity in protoplasts from veratryl alcohol-grown cells is caused by loss of the competing periplasmic catalase activity (6). When the fungus was grown on glucose, no vanillyl-alcohol oxidase activity was found (Table 1). Immunoblot detection confirmed this finding (see below). Extracts of glucose-grown cells contained periplasmic catalase activity but only minor amounts of catalase-peroxidase activity. This indicates that this enzyme is also induced by growth on veratryl alcohol. Western blot analysis confirmed the presence of some catalase-peroxidase in glucose-grown cells (data not shown).

Induction of vanillyl-alcohol oxidase. To study the induction of vanillyl-alcohol oxidase in more detail, *P. simplicissimum* CBS 170.90 was grown on several aromatic compounds [phenylalanine, benzoate, 4-hydroxybenzoate, 3-hydroxybenzoate, vanillic acid, ferulic acid, caffeic acid, veratrate, veratryl alcohol, veratraldehyde, anisyl alcohol, vanillyl alcohol, homovanillyl alcohol, vanillyl amine, vanillin, phenol, catechol, *p*-cresol, and 4-(methoxymethyl)phenol] and nonaromatic compounds (glucose, fructose, sucrose, oleate, and acetate). The fungus did not grow on 4-methoxyphenol, eugenol, isoeugenol, 4-ethylphenol, or 4-hydroxy-3-methoxypropylphenol under the conditions used in this study. Growth on the vanillyl-alcohol oxidase substrates vanillyl alcohol and vanillyl amine did not result in any detectable vanillyl-alcohol oxidase activity. Activity could be detected only in extracts of cells grown on veratryl alcohol, anisyl alcohol or 4-(methoxymethyl)phenol. As can be seen from Fig. 2, the amount of enzyme induced by growth on these aromatic compounds is relatively large (Fig. 2, lanes B, D, and G). Purification of vanillyl-alcohol oxidase from anisyl alcohol-grown cells revealed that up to 5% of the total protein may consist of vanillyl-alcohol oxidase (data not shown). This value is even higher than the value originally reported for veratryl alcohol-grown cells (3).

Degradation and accumulation of aromatic metabolites by whole cells. The degradation pathway of veratryl alcohol by *P. simplicissimum* was already established in an earlier report (2). To study the steps used by this fungus to degrade anisyl alcohol and 4-(methoxymethyl)phenol, a similar approach was used. Intact mycelia were incubated with the above-mentioned phenols, and the accumulation of aromatic degradation products was analyzed by HPLC (Fig. 3). As a control, we analyzed the accumulation during veratryl alcohol degradation, which showed the same degradation products as described previously (2). As can be seen from Fig. 3, anisyl alcohol was readily degraded via two initial oxidation steps into the acid form, which was subsequently demethylated. After that, 4-hydroxybenzoate was hydroxylated to give 3,4-dihydroxybenzoate. Like the veratryl alcohol-grown cells (2), anisyl alcohol- and 4-(methoxymethyl)phenol-grown cells contained protocatechuate 3,4-dioxygenase activity, catalyzing intradiol ring

TABLE 1. Specific enzyme activities in extracts of mycelium and protoplasts of *P. simplicissimum* cells grown on veratryl alcohol and glucose

Source of extract	Activity of[a]:			
	G6PDH (mU/mg)	VAO (mU/mg)	CAT (U/mg)	PER (U/mg)
Veratryl alcohol-grown cells				
Mycelium	360	81	154	0.22
Protoplast	386	78	93	0.34
Glucose-grown cells				
Mycelium	790	0	38	<0.05
Protoplast	690	0	<1	<0.05

[a] G6PDH, glucose-6-phosphate dehydrogenase; VAO, vanillyl-alcohol oxidase; CAT, catalase; PER, peroxidase.

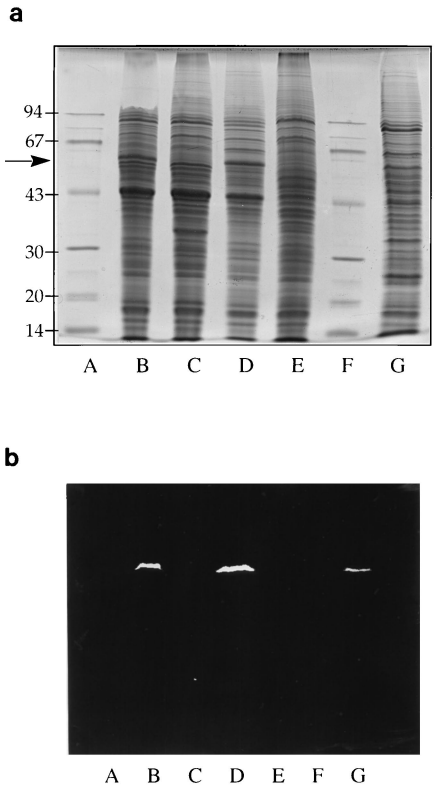


FIG. 2. SDS-PAGE of extracts of *P. simplicissimum* cells grown on different substrates. Lanes: A and F, marker proteins (from top to bottom, phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20 kDa; and α-lactalbumin, 14 kDa); B to E and G, extracts of cells grown on veratryl alcohol (B), veratryl alcohol plus isoeugenol (0.01%) (C), anisyl alcohol (D), glucose (E), and 4-(methoxymethyl)phenol (G). (a) Protein stained; (b) fluorescence detection. The presence of vanillyl-alcohol oxidase (~65 kDa) is indicated by an arrow in panel a and was confirmed by fluorescence detection of a similarly loaded gel.

fission. Incubation of 4-(methoxymethyl)phenol-grown cells with 4-(methoxymethyl)phenol resulted in a rapid accumulation of 4-hydroxybenzaldehyde. Because demethylation of 4-(methoxymethyl)phenol is efficiently catalyzed by vanillyl-alcohol oxidase (5), this suggests that vanillyl-alcohol oxidase is involved in the initial degradation of 4-(methoxymethyl)phenol. By analogy to the degradation of veratryl alcohol and anisyl alcohol, 4-hydroxybenzaldehyde was oxidized to 4-hydroxybenzoate and converted to 3,4-dihydroxybenzoate to enter the β-ketoadipate pathway (19) (Fig. 4). An identical accumulation pattern of products for the degradation of 4-(methoxymethyl)phenol was found when anisyl alcohol-grown cells were used (Fig. 3).

Effect of isoeugenol on induction of vanillyl-alcohol oxidase. A series of induction experiments were performed in which the fungus was grown in the absence or presence of isoeugenol. Isoeugenol is a strong competitive inhibitor for vanillyl-alcohol oxidase (5). These experiments resulted in some unexpected and interesting observations. When *P. simplicissimum* was grown on anisyl alcohol (0.1%) or 4-(methoxymethyl)phenol (0.1%) in the presence of isoeugenol (0.01%), no effect was found on the induction of vanillyl-alcohol oxidase or growth rate of the fungus. However, when *P. simplicissimum* was grown in veratryl alcohol-containing media to which isoeugenol (0.01%) was added, no vanillyl-alcohol oxidase activity could be detected. Furthermore, SDS-polyacrylamide gel electrophoresis revealed that expression of the enzyme (65 kDa) was totally suppressed (Fig. 2, lane C). Only when antibodies were used could some vanillyl-alcohol oxidase be detected (Fig. 5). Apart from the band corresponding to native vanillyl-alcohol oxidase, some other minor bands were detected with antibodies. The smaller bands most probably reflect proteolytic degradation products of the enzyme because these fragments were also fluorescent. The larger bands may be cross-reactive proteins or precursor proteins of vanillyl-alcohol oxidase, and they have been observed previously (3). Although isoeugenol has a drastic effect on the induction of vanillyl-alcohol oxidase, its presence did not affect fungal growth, and the protein pattern of cell extracts was quite similar to the protein pattern of extracts of veratryl alcohol-grown cells in the absence of isoeugenol (Fig. 2, lane C). Except for the disappearance of vanillyl-alcohol oxidase, only one major difference could be clearly seen: the appearance of a protein band around 35 kDa. Similar effects were observed when lower isoeugenol concentrations (0.005 and 0.001%) were used.

DISCUSSION

In this study, the degradation of anisyl alcohol and 4-(methoxymethyl)phenol by *P. simplicissimum* was investigated. Like veratryl alcohol, these aromatic compounds induce high levels of the covalent flavoprotein vanillyl-alcohol oxidase, while with a range of other growth substrates, no induction occurs.

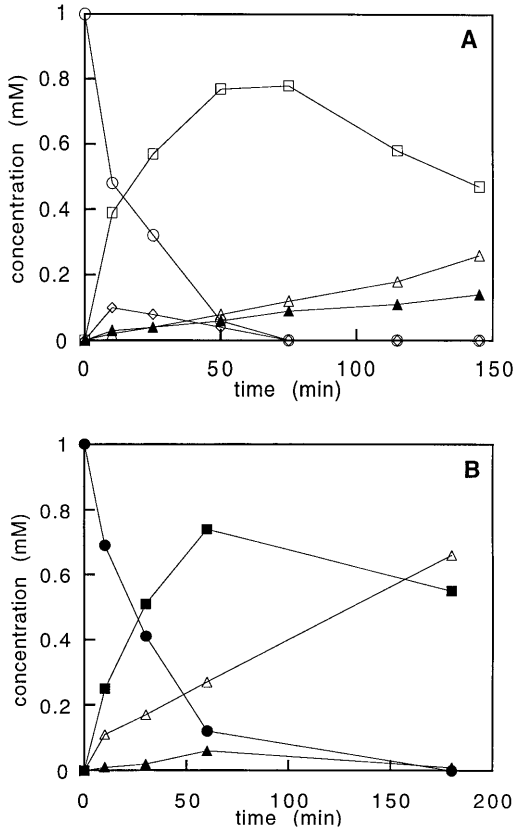


FIG. 3. Consumption of anisyl alcohol (A) and 4-(methoxymethyl)phenol (B) and transient accumulation of degradation products. Incubation mixtures (50 ml) contained anisyl alcohol-grown washed cells (5 g [wet weight]) and 1 mM anisyl alcohol or 4-(methoxymethyl)phenol. Symbols: ○, anisyl alcohol; ◇, anisaldehyde; □, anisate; △, 4-hydroxybenzoate; ▲, 3,4-dihydroxybenzoate; ●, 4-(methoxymethyl)phenol; ■, 4-hydroxybenzaldehyde.

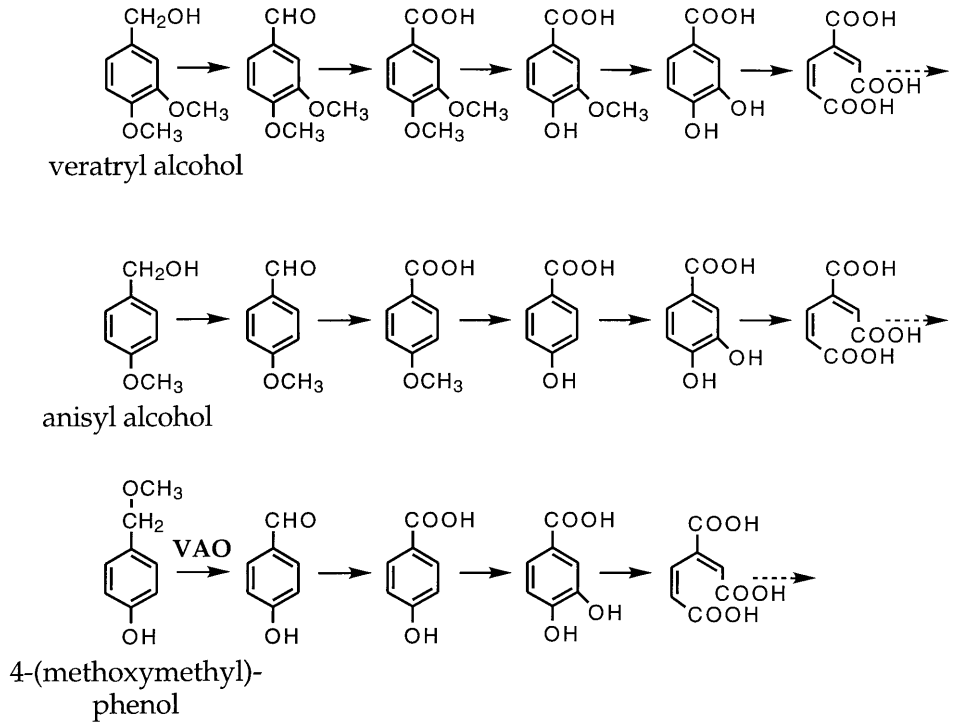
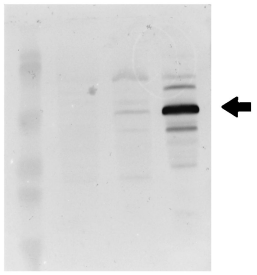


FIG. 4. Degradation pathways of veratryl alcohol (2), anisyl alcohol, and 4-(methoxymethyl)phenol by *P. simplicissimum* CBS 170.90.

Growth on metabolites of the above-mentioned aromatics or growth on the vanillyl-alcohol oxidase substrates vanillyl alcohol and vanillyl amine did not result in vanillyl-alcohol oxidase induction. Although the induction seems to be very strictly regulated, no rationale was found for the induction of vanillyl-alcohol oxidase by the two aromatic alcohols. The degradation pathways for veratryl alcohol and anisyl alcohol do not involve any step which can be catalyzed by vanillyl-alcohol oxidase, suggesting that the enzyme is superfluously induced. The nonfunctional presence of vanillyl-alcohol oxidase in *P. simplicissimum* is supported by the growth experiments with isoeugenol. The latter compound is a very potent inhibitor of vanillyl-alcohol oxidase activity (5) and is also known for its antifungal activity (17). Although isoeugenol is not degraded by *P. simplicissimum* and does not influence fungal growth on veratryl alcohol, it strongly suppresses the induction of vanillyl-alcohol oxidase. This drastic effect is not seen when *P. simplicissimum* is grown on anisyl alcohol or 4-(methoxymethyl)phenol as the sole carbon source. It is rather uncommon that an enzyme inhibitor causes the suppression of the respective enzyme. Isoeugenol may therefore compete on a genetic level with the inducer veratryl alcohol, whereas anisyl alcohol and 4-(methoxymethyl)phenol might have a higher affinity in triggering the expression of vanillyl-alcohol oxidase. To obtain further insight into the regulation of the vanillyl-alcohol oxidase gene, we are presently cloning the respective gene.

The initial demethylation step in the degradation of 4-(methoxymethyl)phenol by *P. simplicissimum* is catalyzed by vanillyl-alcohol oxidase. Furthermore, substrate specificity studies have revealed that this oxidase can readily convert substrates with relatively large substituents at the C-α atom (5, 7). From this, we propose that 4-(methoxymethyl)phenol and analogs of this *p*-cresol methylether (e.g., ethylether) are plausible candidates as physiological substrates for this enzyme. To our knowledge, 4-(methoxymethyl)phenol and analogous cresol ethers have never been described in the literature as being present in nature. It can, however, easily be envisaged that these phenolic compounds may be formed during the degradation of lignin, an aromatic polymer containing extensive ether bonds. This report describes for the first time a degradation pathway for 4-(methoxymethyl)phenol which involves a cleavage of an ether bond in the first step of degradation catalyzed by a flavoenzyme.

Together with the induction of vanillyl-alcohol oxidase in *P. simplicissimum*, an intracellular catalase-peroxidase is expressed. Recently, we postulated that induction of this unusual peroxidase might be coupled to the induction of vanillyl-alco-



A B C D

FIG. 5. Western blot of cell extracts (20 μg) of *P. simplicissimum* CBS 170.90 grown on glucose (lane B); veratryl alcohol plus isoeugenol (0.01%) (lane C), and veratryl alcohol (lane D), using antibodies directed against purified vanillyl-alcohol oxidase. The arrow indicates the position of vanillyl-alcohol oxidase. Lane A contains prestained marker proteins (from top to bottom: phosphorylase *b*, 107 kDa; bovine serum albumin, 76 kDa; ovalbumin, 52 kDa; carbonic anhydrase, 36.8 kDa; trypsin inhibitor, 27.2 kDa; lysozyme, 19 kDa). Note that the apparent molecular mass of the prestained marker proteins differs from that of the unstained marker proteins (cf. Fig. 2).

hol oxidase, because the latter enzyme produces hydrogen peroxide (6). However, this study shows that the induction of the catalase-peroxidase is not restricted to growth on aromatic compounds. So far, these enzymes, exhibiting both catalase and peroxidase activity, were found mainly in bacteria and show homology to the yeast cytochrome *c* peroxidase (25). Their peroxidatic activity is, however, relatively low, and their catalase activity is strongly inhibited by high concentrations of hydrogen peroxide (15), which might be the reason for their recent discovery in fungi (6). From this study, it is not clear which catalytic function of the catalase-peroxidase is operative in this fungus. Increased levels of catalase-peroxidase activity in parallel with vanillyl-alcohol oxidase induction suggests that the catalase-peroxidase plays an essential role in the intracellular elimination of hydrogen peroxide. In this respect, it is worth mentioning that the homologous yeast cytochrome *c* peroxidase plays an essential role in the detoxification of hydrogen peroxide in the yeasts *Saccharomyces cerevisiae* and *Hansenula polymorpha* (23).

It would be interesting to determine whether the catalase-peroxidase from *P. simplicissimum* is located in peroxisomes like most eucaryotic catalases or in the mitochondria like yeast cytochrome *c* peroxidase. Also, it is interesting to know if vanillyl-alcohol oxidase is located in a specific organelle. Until now, all eucaryotic enzymes containing a covalently bound flavin have been localized in mitochondria or peroxisomes (14). The localization of vanillyl-alcohol oxidase and catalase-peroxidase might therefore give valuable information about their physiological role.

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