Effect of chloromethane on veratryl alcohol and lignin peroxidase production by the fungus *Phanerochaete chrysosporium*

DAVID B. HARPER,^{1,2*} JOHN A. BUSWELL³ and JAMES T. KENNEDY²

¹ Department of Food and Agricultural Chemistry, The Queen's University of Belfast, Newforge Lane, Belfast BT9 5PX, UK

² Food and Agricultural Chemistry Research Division, Department of Agriculture for Northern Ireland, Newforge Lane, Belfast BT9 5PX, UK

³ Department of Biology, Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

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Biosynthesis of veratryl alcohol, a secondary metabolite considered to be an important component of the lignin-degrading system in the fungus *Phanerochaete chrysosporium* Burds INA-12, was initiated up to 36 h earlier in fungal cultures supplemented with 0.6 and 1.25 mM-CH₃Cl compared with unsupplemented mycelia. Peak concentrations of the idiolyte were also about 70% higher in the presence of 0.6 mM-CH₃Cl, although peak levels elicited by 0.2, 0.4 and 1.25 mM-CH₃Cl were lower than in unsupplemented cultures. Advanced initiation of veratryl alcohol biosynthesis in the presence of CH₃Cl was reflected in the earlier appearance of lignin peroxidase (LiP) activity. This effect was most noticeable in cultures supplemented with 0.4 and 0.6 mM-CH₃Cl, where LiP was evident up to 24 h before detection in unsupplemented cultures. However, peak levels of LiP produced by CH₃Cl-augmented mycelia were always less – below 40% in the case of 1.25 mM-CH₃Cl – than those recorded in cultures without supplementation. These effects may be explained by restricted metabolic availability of CH₃Cl as methyl donor for veratryl alcohol biosynthesis in the early stages of fungal growth.

Introduction

Chloromethane (CH₃Cl) is a gaseous natural product derived from methionine and released as a secondary metabolite by mycelia of many species of the Hymenochaetae, a widely distributed family of wood-rotting bracket fungi (Harper, 1985; Harper & Kennedy, 1986; Harper & Hamilton, 1988; Harper *et al.*, 1988). Surprisingly, CH₃Cl also appears to have a primary metabolic role in these species, acting as a methyl donor in the methylation of aromatic compounds such as benzoic and furoic acids (Harper *et al.*, 1989; McNally *et al.*, 1990). A CH₃Cl-utilizing phenol methylating system has also been demonstrated in *Phellinus pomaceus* (Harper *et al.*, 1989; McNally & Harper, 1991).

Recently, CH₃Cl was shown to be a methyl donor in the biosynthesis of veratryl (3,4-dimethoxybenzyl) alcohol in species of white-rot fungi belonging to other families such as the Corticiaceae and Polyporaceae, none of which apparently emit detectable quantities of CH₃Cl at any stage of growth (Harper *et al.*, 1990). Thus *Phanerochaete chrysosporium*, *Coriolus versicolor* and

Phlebia radiata incorporated high levels of C²H₃ into the 3- and 4-*O*-methyl groups of veratryl alcohol when grown in the presence of C²H₃Cl. This observation led to the suggestion that a tightly channelled multi-enzyme system in which CH₃Cl biosynthesis is closely coupled to CH₃Cl utilization is present in such fungi, allowing CH₃Cl to be used as methyl donor without any significant release of the compound to the external environment (Harper *et al.*, 1990).

In *P. chrysosporium*, veratryl alcohol is a secondary metabolite (Lundquist & Kirk, 1978) synthesized *de novo* via a pathway involving phenylalanine, 3,4-dimethoxycinnamyl alcohol and veratryl glycerol (Shimada *et al.*, 1981). Veratryl alcohol has been reported to increase the level of ligninolytic enzymes in *Phanerochaete chrysosporium* (Faison & Kirk, 1985; Faison *et al.*, 1986; Leisola *et al.*, 1984), and is a substrate for lignin peroxidase (LiP) which is a major component of the fungal ligninolytic system (Buswell & Odier, 1987; Kirk & Farrell, 1987; Tien, 1987). Veratryl alcohol also stimulates the oxidation of monomethoxylated aromatic compounds, such as anisyl alcohol, by LiP (Harvey *et al.*, 1986). Monomethoxylated aromatics are normally poor substrates for the enzyme and it has been postulated that the idiolyte may

Abbreviation: LiP, lignin peroxidase.

act as a one-electron redox mediator in their oxidation and in lignin depolymerization (Harvey *et al.*, 1986; Schoemaker *et al.*, 1985). Other authors have proposed that veratryl alcohol counteracts inactivation of LiP by preventing the formation of lignin peroxidase Compound III (LiP III), an oxidized enzymic intermediate which is irreversibly inactivated in the presence of excess H_2O_2 , and by converting LiP III back to the native enzyme (Haemmerli *et al.*, 1986; Tonon & Odier, 1988; Valli *et al.*, 1990).

Harper *et al.* (1990) noted that veratryl alcohol biosynthesis during growth of *P. chrysosporium* appeared to be initiated earlier in the presence of 0.5 mM- CH_3Cl . Additionally, LiP activity in the culture medium was apparently enhanced under these conditions. However, these changes seemed critically dependent on CH_3Cl concentration and the nature of the relationship involved was not investigated systematically by these authors. The central role assigned to veratryl alcohol in lignin degradation renders the biosynthesis of the compound and its regulation of crucial importance. Consequently, in this paper we report a detailed examination of the effect of a range of CH_3Cl concentrations on veratryl alcohol production and LiP activity at stages during growth of *P. chrysosporium* in an attempt to determine whether the metabolic availability of CH_3Cl controls the onset of ligninolytic activity in cultures by reason of its effect on veratryl alcohol biosynthesis.

Methods

Organism and maintenance medium. *Phanerochaete chrysosporium* Burds INA-12 (CNCM I-398), previously utilized in investigations by Buswell *et al.* (1984) and Harper *et al.* (1990), was employed in the study.

Chemicals. Chloromethane was obtained from BDH and 3,4,5-trimethoxybenzyl alcohol from Aldrich.

Culture medium and culture conditions. *P. chrysosporium* cultures were grown without agitation at 37 °C in 100 ml conical flasks in medium (10 ml) containing (g l⁻¹): KH_2PO_4 (0.2), $MgSO_4 \cdot 7H_2O$ (0.05), NaCl (0.59), $CaCl_2 \cdot 2H_2O$ (0.013), thiamin (0.0025), Difco yeast extract (0.01), glycerol (10), L-asparagine monohydrate (1.0), NH_4NO_3 (0.5) and 2,2-dimethylsuccinic acid (1.46). The medium was supplemented with 1 ml of trace element solution (Ander & Eriksson, 1976) and the pH adjusted to 5.0 before filter sterilization. After inoculation of the medium with 5×10^5 conidia l⁻¹, prepared as described by Eriksson & Johnsrud (1983), flasks were flushed with 100% O_2 for 2 min before being sealed with a polytetrafluoroethylene (PTFE)-coated rubber stopper. When the growth medium required supplementation with CH_3Cl the following technique was used. From the PTFE-coated rubber stopper was suspended an aluminium clamp in which a Durham tube was fitted. Immediately before sealing the flasks, various volumes of aqueous 60 mM- CH_3Cl were pipetted into the tubes so as to establish a range of concentrations (0.2, 0.4, 0.6 and 1.25 mM) in the culture media of the flasks after equilibration of the gaseous and aqueous phases (Harper *et al.*, 1990).

Cultures (five replicates) grown in the presence of each CH_3Cl concentration and also in the absence of the compound were harvested after periods of incubation ranging from 40–211 h. Mycelial dry weight, veratryl alcohol concentration and LiP activity in the culture supernatant are recorded as the mean of the five replicates. The entire experiment was repeated four times with similar results as regards the effects of CH_3Cl on veratryl alcohol production and LiP activity although the absolute concentration of veratryl alcohol and LiP activity in the fungal culture medium tended to vary from experiment to experiment. The results given in the paper are of one representative experiment.

Determination of veratryl alcohol. Culture supernatant (10 ml) after addition of 3,4,5-trimethoxybenzyl alcohol (0.2 ml of a 500 g ml⁻¹ solution in acetone) as an internal standard was extracted with chloroform (3 × 15 ml) and the bulked extract was washed with saturated NaCl solution (10 ml). The extract was dried over anhydrous $MgSO_4$ and evaporated to 0.5 ml under reduced pressure. Finally the solution was made up to 5 ml with diethyl ether.

The extracts were analysed by gas chromatography-mass spectrometry using the conditions described by Harper *et al.* (1990). Veratryl alcohol was quantified by comparison of the ion current at *m/e* 168 at the retention time of veratryl alcohol with a calibration graph prepared using solutions containing known concentrations of the compound. Ion current at *m/e* 198 produced by trimethoxybenzyl alcohol, the internal standard, was also monitored so as to allow correction for recovery.

LiP assay. The activity of LiP in culture supernatants was determined by measuring the increase in absorbance at 310 nm due to oxidation of veratryl alcohol to veratraldehyde in the presence of H_2O_2 (Tien & Kirk, 1984). In the assay, supernatant (1 ml) was incubated with 4 mM-veratryl alcohol and 0.27 mM- H_2O_2 in 60 mM-sodium tartrate buffer, pH 3.0, at 37 °C. Activities are expressed as nmol veratraldehyde formed min⁻¹ (ml culture medium)⁻¹.

Assessment of growth. Mycelial growth was measured at various stages of incubation as the weight of mycelia harvested by filtration and dried at 100 °C.

Results and Discussion

Mycelial growth, veratryl alcohol concentration and LiP activity in the culture medium were measured at stages during growth of *P. chrysosporium* at a range of concentrations between 0 and 1.25 mM- CH_3Cl . For the sake of clarity and to aid interpretation, a comparison of the effects of only two CH_3Cl concentrations on these parameters is shown in Fig. 1(a, b, c).

Although a slightly higher growth yield was observed in the presence of 0.6 mM- CH_3Cl ($P < 0.05$ for final growth weight in the presence of 0.6 mM- CH_3Cl compared with that in the absence of the compound), CH_3Cl had little effect on the general pattern of fungal growth (Fig. 1a). However, even this small difference in final growth weight became statistically insignificant when the concentration of CH_3Cl was increased to 1.25 mM. Nevertheless, it is clear that veratryl alcohol production (Fig. 1b) was initiated about 36 h earlier in mycelia grown in the presence of both 0.6 and 1.25 mM- CH_3Cl than in mycelia cultured in unsupplemented media. With

Fig. 1. Effects of 0.6 and 1.25 mM-CH₃Cl on (a) growth, (b) veratryl alcohol concentration and (c) LiP activity during growth of *P. chrysosporium* INA-12. Cultures of *P. chrysosporium* INA-12 were grown at 37 °C in 100 ml conical flasks containing glycerol high nitrogen medium (10 ml) under 100% O₂ in the presence of 0, 0.6 and 1.25 mM-CH₃Cl. At intervals during growth, the contents of five replicate flasks of each treatment were harvested individually for measurement of mycelial dry weight, veratryl alcohol concentration and LiP activity. ○, No CH₃Cl; ●, 0.6 mM-CH₃Cl; ▲, 1.25 mM-CH₃Cl.

0.6 mM-CH₃Cl, this enhanced production was maintained at all stages of growth up to 163 h ($P < 0.05$ for comparison of veratryl alcohol concentration in the presence of 0.6 mM-CH₃Cl with that in its absence after incubation periods of 90, 115 and 163 h, and $P < 0.1$ for incubation period 66 h). A maximum veratryl alcohol concentration of 1.2 μmol per culture was attained at 163 h, considerably in excess of the maximum of 0.7 μmol per culture achieved in the absence of CH₃Cl. Despite the earlier induction of veratryl alcohol biosynthesis manifested by cultures grown in the presence of 1.25 mM-CH₃Cl ($P < 0.01$ after 66 h incubation), the increased concentration of the compound relative to that in the unsupplemented medium did not persist in the later stages of growth; indeed, the maximum level of veratryl alcohol observed was significantly less than that in the unsupplemented medium ($P < 0.1$ after 163 h incubation).

Veratryl alcohol concentrations after 90 h and 163 h for cultures incubated at each CH₃Cl concentration are compared with those for unsupplemented cultures in Fig. 2. It is evident from the 90 h values that CH₃Cl concentrations between 0.2 and 0.6 mM caused initiation of veratryl alcohol biosynthesis at an earlier stage of growth. However, this stimulation did not result in significantly higher veratryl alcohol concentrations at 163 h except in the case of 0.6 mM-CH₃Cl, where the mean veratryl alcohol level in the culture medium was 70% greater than in the unsupplemented medium.

From Fig. 1(c) and Fig. 3, it is apparent that the earlier initiation of veratryl alcohol biosynthesis in the presence of CH₃Cl is mirrored by an earlier induction of LiP activity. This effect is particularly marked in cultures supplemented with 0.4 and 0.6 mM-CH₃Cl, where LiP

Fig. 2. Effect of CH₃Cl concentration on veratryl alcohol concentration in the culture medium after 90 and 163 h growth of *P. chrysosporium* INA-12. After growth of *P. chrysosporium* INA-12 at a range of CH₃Cl concentrations and harvesting as described in Fig. 1, veratryl alcohol concentrations were determined in each of five replicate cultures as described in Methods. Values given are means $\pm$ SD. □, Veratryl alcohol concentration after 90 h growth; ■, veratryl alcohol concentration after 163 h growth.

production begins approximately 24 h ahead of that in unsupplemented cultures ($P < 0.01$ for comparison of LiP activity in the presence of 0.6 mM-CH₃Cl with that in its absence after 66 h incubation, and $P < 0.1$ after 115 h; $P < 0.05$ for comparison of LiP activity in the presence of 0.4 mM-CH₃Cl with that in its absence after 90 h incubation, and $P < 0.01$ at 115 h). Earlier production of LiP in the presence of CH₃Cl does not seem to be associated with the eventual attainment of higher maximum activity. On the contrary, peak values for LiP activity in cultures grown in the presence of CH₃Cl are somewhat less than those recorded in the unsupple-

Fig. 3. Effect of CH_3Cl concentration on LiP activity in the culture medium after 115 and 163 h growth of *P. chrysosporium* INA-12. After growth of *P. chrysosporium* INA-12 at a range of CH_3Cl concentrations and harvesting as described in Fig. 1, LiP activities were determined in each of five replicate cultures as described in Methods. Values given are means $\pm$ SD. $\square$, LiP activity after 115 h growth; $\blacksquare$, LiP activity after 163 h growth.

mented cultures. This inhibition is particularly significant in cultures grown in the presence of 1.25 mM- CH_3Cl ($P < 0.01$ after 163 and 187 h incubation and $P < 0.05$ at 211 h incubation), which showed a peak level of enzyme activity less than 30% of that of the unsupplemented cultures.

Lignin peroxidase production by *P. chrysosporium* INA-12 grown on glycerol is considerably higher than the enzyme levels reported for other more-studied strains of the fungus, including *P. chrysosporium* ME-446 (ATCC 34541) from which LiP was originally isolated. Moreover, whereas enzyme synthesis in these strains is repressed by high nitrogen, *P. chrysosporium* INA-12 produces LiP under conditions of nutrient nitrogen sufficiency. The high levels of LiP observed when glycerol serves as carbon source may be related to the relatively poor growth rate of *P. chrysosporium* INA-12 on this compound which essentially mimics the nutritional status of carbon limitation. More recently, several other strains of *P. chrysosporium* which grew poorly on glycerol were shown to produce high titres of LiP (Roch *et al.*, 1989). The triggering of secondary metabolism in *P. chrysosporium*, and hence the onset of ligninolysis and LiP synthesis, by carbon limitation is now well-documented (Keyser *et al.*, 1978; Jeffries *et al.*, 1981; Faison & Kirk, 1985).

Poor fungal growth on glycerol may also account for the results shown in Fig. 1(a, b) where production of the secondary metabolite, veratryl alcohol, apparently follows primary metabolite kinetics. Secondary metabolites are often defined as compounds produced after growth has ceased or in the absence of growth. However, many examples are documented involving other micro-organ-

isms where production of a secondary metabolite occurs during exponential phase growth, either when a relatively poor source of carbon is used for growth during batch culture or in steady state cultures in the chemostat (Demain *et al.*, 1979). The observed decrease in veratryl alcohol levels after 163 h probably reflects a high rate of turnover of this compound in the culture medium (Harper *et al.*, 1990; Leisola *et al.*, 1985). This is consistent with the key metabolic roles assigned to the metabolite (Buswell & Odier, 1987).

In summary, it would appear that 0.6 mM- CH_3Cl causes induction of both veratryl alcohol biosynthesis and LiP at an earlier stage of fungal growth than would normally be the case, although only veratryl alcohol reaches a peak value higher than that in the unsupplemented medium. Higher concentrations of CH_3Cl , i.e. 1.25 mM- CH_3Cl , whilst causing a similar advancement of veratryl alcohol and LiP production to an earlier stage of growth, lead to a marked depression in the veratryl alcohol concentrations and LiP activities achieved at later stages of growth. The critical dependence of these effects on the exact CH_3Cl concentration explains the somewhat variable results reported by Harper *et al.* (1990).

The broadly parallel effects of CH_3Cl on both veratryl alcohol biosynthesis and LiP activity are in accord with the proposal that veratryl alcohol production is closely linked with LiP activity and ligninolysis in *P. chrysosporium* (Faison & Kirk, 1985; Leisola *et al.*, 1984). Veratryl alcohol production and the appearance of ligninolysis in this fungus share several common features. Both appear at the onset of idiophase triggered by either nitrogen or carbon limitation (Keyser *et al.*, 1978; Jeffries *et al.*, 1981), are enhanced by elevated oxygen concentrations (Shimada *et al.*, 1981), and are suppressed by culture agitation (Shimada *et al.*, 1981). Gold *et al.* (1982) have also described mutant and revertant strains of *P. chrysosporium* in which lignin decomposition and veratryl alcohol biosynthesis are coupled, although this relationship apparently does not exist in other mutants (Liwicki *et al.*, 1985). Veratryl alcohol is also ranked among the most effective of several lignin substructure molecules which elicit elevated levels of LiP, although addition of exogenous idiolyte alone to growing (trophophasic) cultures of *P. chrysosporium* failed to elicit the earlier appearance of either LiP or the complete ligninolytic system (lignin $\rightarrow \text{CO}_2$) (Faison & Kirk, 1985). Similar stimulatory effects on LiP levels have subsequently been observed in several other white-rot fungi (*Phlebia radiata*, *Coriolus versicolor*, *Merulius tremellosus*, *Pleurotus ostreatus* and *Chrysosporium pruinsum*) that produce extracellular peroxidases resembling the LiP of *P. chrysosporium* (Hatakka *et al.*, 1987; Waldner *et al.*, 1986; Biswas-Hawkes *et al.*, 1987). However,

although the ligninolytic system is considered to be inducible (Ulmer *et al.*, 1984; Faison *et al.*, 1986), increases in LiP mRNA or in translation of the LiP protein have yet to be directly attributed to veratryl alcohol (Tien, 1987).

The mechanism by which CH₃Cl affects veratryl alcohol biosynthesis is unknown, but it is possible that the rate of methylation of postulated veratryl alcohol precursors such as caffeic and ferulic acids may be restricted by the metabolic availability of CH₃Cl as methyl donor in the early stages of growth so limiting veratryl alcohol biosynthesis.

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