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

(Received 15 April 1991; revised 2 August 1991; accepted 5 September 1991)

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 Hymenochaetaceae, a widely distributed family of wood-rotting bracket fungi (Harper, 1985; Harper & Kennedy, 1986; Harper & Hamilton, 1988; Harper et al., 1989). 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 Corticaceae 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
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 H2O2, 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-CH3Cl. Additionally, LiP activity in the culture medium was apparently enhanced under these conditions. However, these changes seemed critically dependent on CH3Cl 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 CH3Cl 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 CH3Cl controls the onset of lignonolytic activity in cultures by reason of its effect on veratryl alcohol biosynthesis.

**Methods**

*Organism and maintenance medium.* *Phanerochaete chrysosporium* Burds INA-12 (CNM 1-398), 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): KH2PO4 (0.2), MgSO4·7H2O (0.05), NaCl (0.59), CaCl2·2H2O (0.013), thiamin (0.0025), Difco yeast extract (0.01), glycerol (0.1), l-asparagine monohydrate (1.0), NH4NO3 (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 × 105 conidia L-1, prepared as described by Eriksson & Johnsrud (1983), the flasks were flushed with 100% O2 for 2 min before being sealed with a polytetrafluoroethylene (PTFE)-coated rubber stopper. When the growth medium required supplementation with CH3Cl the following technique was used. From the PTFE-coated rubber stopper was suspended an aluminum clam in which a Durham tube was fitted. Immediately before sealing the flasks, various volumes of aqueous 60 mM-CH3Cl 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 CH3Cl 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 CH3Cl 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 MgSO4 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 H2O2 (Tien & Kirk, 1984). In the assay, supernatant (1 ml) was incubated with 4 mM-veratryl alcohol and 0.27 mM-H2O2 in 60 mM-sodium tartrate buffer, pH 3.40, 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-CH3Cl. For the sake of clarity and to aid interpretation, a comparison of the effects of only two CH3Cl 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-CH3Cl (P < 0.05 for final growth weight in the presence of 0.6 mM-CH3Cl compared with that in the absence of the compound), CH3Cl 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 CH3Cl 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-CH3Cl than in mycelia cultured in unsupplemented media. With
Effects of chloromethane in P. chrysosporium

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. O, 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 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-
mented cultures. This inhibition is particularly significant in cultures grown in the presence of 1-25 mM CH₃Cl (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 titers 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-organisms 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 CH₃Cl 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 CH₃Cl, i.e. 1.25 mM CH₃Cl, 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 CH₃Cl concentration explains the somewhat variable results reported by Harper et al. (1990).

The broadly parallel effects of CH₃Cl 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—CO₂) (Faison & Kirk, 1985). Similar stimulatory effects on Lip levels have subsequently been observed in several other white-rot fungi (*Phlebia radiata*, *Coriolus versicolor*, *Merulius tremello-sus*, *Pleurotus ostreatus* and *Chrysosporium pruinosum*) 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.

References


