Vanillic acid metabolism by *Sporotrichum pulverulentum*: evidence for demethoxylation before ring-cleavage

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**Abstract.** In order to determine the influence of glucose on vanillate metabolism and how vanillic acid is prepared for ring-cleavage by the white-rot fungus *Sporotrichum pulverulentum* Nov., vanillate metabolism was studied using three different glucose-nitrogen media. When vanillate was added to cultures of *S. pulverulentum* oxidative decarboxylation occurred rapidly but was repressed by glucose. After the initial decarboxylation, methanol was detected as an early metabolic product appearing before $^{14}$CO$_2$ release from $^{14}$C-ring-labelled vanillate. Methanol was also formed from methoxyhydroquinone in cultures of *S. pulverulentum*, and in *vitro* from vanillic acid on addition of purified laccase and peroxidase.

It is proposed that the main pathway for vanillic acid degradation by *S. pulverulentum* involves decarboxylation to methoxyhydroquinone (MHQ). Demethoxylation of MHQ to a demethylated quinone is followed by enzymic reduction to give the tri-hydroxylated compound hydroxyquinol, which then undergoes ring-cleavage. The structure and fate of the ring-cleavage product maleylacetate has been clarified earlier in our laboratory.

**Key words:** Vanillic acid – *Sporotrichum pulverulentum* – Decarboxylation – Demethoxylation – Ring-cleavage – Laccase – Peroxidase – $^{14}$CO$_2$ evolution

The resistance of lignin to chemical and microbial attack greatly influences both the use of wood as a source of fibres and as a source of carbohydrates. Thus, research into the microbial metabolism of lignin has expanded considerably in recent years (Crawford 1981). However, the complex molecular structure of lignin makes these investigations difficult. For this reason less complex lignin model substances have been widely used (Higuchi 1981). Vanillic acid has been identified as a prominent intermediate in spruce wood decayed by *Phanerochaete chrysosporium* (Chen et al. 1982). Ishikawa et al. (1963) reported that vanillic acid was formed when the white-rot fungus *Fomes fomentarius* was cultivated on veratrylglycerol-$\beta$-guaiacylether, which represents a major type of intermonomer linkage in lignin. Vanillic acid has also been reported to be a metabolite when white-rot fungi were grown on the lignin model compound ferulic acid (Ishikawa et al. 1963; Nishida and Fukuzumi 1978; Gupta et al. 1981).

The metabolic pathways for vanillic acid degradation by the white-rot fungus *Sporotrichum pulverulentum* have been investigated by Ander et al. (1980a). It was shown that vanillic acid was both decarboxylated to methoxyhydroquinone and reduced to vanillin and vanillyl alcohol. Decarboxylation was catalyzed by the enzyme vanillate oxidase which has been purified and partially characterized (Buswell et al. 1979). It was further suggested that the aromatic ring-cleavage by *S. pulverulentum* required the benzene nucleus to contain three hydroxyl groups in a 1,2,4-configuration (Buswell and Eriksson 1979). However, the mechanism by which the third hydroxyl group was inserted in the ring remained unclear. This has now been clarified, and in this paper we also describe the dependence of vanillic acid metabolism on different glucose and nitrogen concentrations. In another paper (Ander et al. 1983) the physiological requirements for degradation of lignin and lignin-related monomers and one dimer have been compared.

**Materials and methods**

**Organisms.** *Sporotrichum pulverulentum* Nov. (ATCC 32629). This strain is the anamorph of *Phanerochaete chrysosporium* Burds. (Johnsrud, unpublished results). *P. chrysosporium* Burds. (ME-446, ATCC 35451) was used for comparison in some experiments.

**Chemicals and labelled substances.** Carboxyl-labelled vanillic acid [$^{14}$C-COOH-vanillate] (1.2 x 10$^6$ dpm/mg), methoxy-labelled vanillic acid [$^{14}$C-OCH$_3$-vanillate] (1.1 x 10$^6$ dpm/mg) and [$^{14}$C-U-ring]-labelled vanillic acid [$^{14}$C-ring vanillate] (2.38 x 10$^6$ dpm/mg) were the gifts of Dr. Konrad Haider. Inst. für Pflanzenernährung und Bodenkunde, Bundesforschungsanstalt für Landwirtschaft, Braunschweig, FRG. The $^{14}$C-labelled methanol and formaldehyde were purchased from New England Nuclear, Boston, MA, USA. Stock solutions of both were prepared so as to contain 5.55 x 10$^6$ dpm/ml. Reagent grade vanillic acid was obtained from US Biochem. Corp., Cleveland, OH, USA, methoxyhydroquinone from Ega-Chemie, Steinheim/Albuch, FRG, and 2,2-dimethylsuccinic acid from Fluka AG, Buchs, Switzerland.

**Cultivation.** The medium used was the dimethylsuccinate (DMS) medium described by Ander et al. (1980a) and Fenn and Kirk (1979). Trace elements were according to Ander and
Eriksson (1976) and in all cultivations 0.1% Difco yeast extract was used. Glucose and nitrogen concentrations were varied as follows: LH medium contained 0.25% glucose and 20.6 mM nitrogen, HH medium contained 1.0% glucose and 20.6 mM nitrogen and HL medium contained 1.0% glucose and 2.6 mM nitrogen = 0.10 g/1 asparagine - H2O and 0.05 g/1 NH4NO3. To maintain the pH, 30 mM dimethylsucinate was used. The media were adjusted to pH 4.2 with NaOH, filter-sterilized and inoculated with 1 g/1 of S. pulverulentum per 10 ml culture medium in 125 ml flasks. Labelled vanillic acids, methanol or formaldehyde were added simultaneously to give ca. 30,000 dpm per culture. Unlabelled vanillic acid or methoxyhydroquinone were added separately as indicated in the text. All cultures were incubated stationary at 39°C.

The release of 14CO2 from triplicate cultures were counted after absorption in NaOH as described by Ander et al. (1980a). For each point of measurement correction was made for evaporation of unmetabolized methanol or formaldehyde into the NaOH. Radioactivity was measured in a Packard model 3255 Tri Carb Liquid Scintillation Spectrometer (Ander et al. 1980a).

Methanol and formaldehyde determination

1. Chromotropic acid method. About 40 ml mycelium-free culture solution was distilled in a nitrogen atmosphere at 125°C. The distillate (slightly less than 40 ml) was condensed in 10 ml ice-cold water and diluted to exactly 50 ml. Methanol and formaldehyde in the distillate were determined according to Adler and Hernestam (1955). In this method methanol is oxidized to formaldehyde by KMnO4. The total amount of formaldehyde including that originally present, was determined from absorbance values at 570 nm after addition of chromotropic acid. The yield of the distillation was calculated to be 85%. The original amount of formaldehyde in the distillate was estimated by omitting KMnO4.

2. Gas chromatography method. GC was performed using a Varian 1200, with flame ionization detector and a 2 ml column packed with Porapak-QS 80 – 100 mesh. Operating conditions: Injection 225°C, detection 225°C and oven temperature 120°C, flow-rate of carrier gas N2 ca. 60 ml/min. Under these conditions, methanol and formaldehyde gave the same retention time. If no formaldehyde was detected by the chromatographic method then methanol in the distillate could be estimated.

3. Radioactivity counting method. 25 µl methoxyl-labelled VA (1 x 105 dpm per flask) was added to the LH medium after growth of S. pulverulentum for 24 h. After a further 24 and 41 h, the amount of volatiles radioactive compounds in the medium was determined by distillation of culture solutions from two or three flasks. The distillate (< 7 ml) was trapped in 3 ml ice-cold water and diluted to 10 ml. The radioactivity of 1 ml distillate was counted as described above. The amount of radioactivity in the distillate was calculated as % of added 14C and represents % conversion to methanol. Bubbling a culture solution containing labelled methanol plus unreacted O14CH3-VA with N2 for 30 min at 19°C did not give significant radioactivity in the distillate (37 cpm). Distillation of 2.1 x 105 dpm O14CH3-VA in sterile LH medium at 125°C gave 42 cpm. Incubation of VA or MHQ in sterile medium at 39°C for 4 days did not give rise to methanol.

Treatment of methoxyl-labelled vanillic acid with laccase and peroxidase

a) To 40 ml 0.1 M DMS-buffer, pH 4.2, were added 25 µl methoxyl-labelled VA (1 x 105 dpm) and 0.1 ml purified laccase (diluted 1:10, obtained from B. Reinhammar, Chalmers Institute of Technology, Gothenburg, Sweden (cf. Fährnaeus and Reinhammar 1967)). This corresponds to 0.42 laccase units/ml as determined with syringaldazine (Ander and Eriksson 1977) using the same DMS-buffer as above. The mixture was incubated with shaking for 2 h at 28°C, and then directly distilled and counted for 14C as under point 3 above.

b) To 40 ml 0.1 M DMS-buffer, pH 4.2, were added 25 µl methoxyl-labelled VA, 0.5 ml H2O2 (2 ml perhydrol 30% + 100 ml water) and 0.5 ml peroxidase (Sigma) (6 mg/30 ml). This corresponds to 0.035 peroxidase units/ml as determined with syringaldazine (Ander and Eriksson 1977) and DMS-buffer. According to Sigma the above used amount of peroxidase give 33 pyrogallol units at pH 6.0. After incubation for 2 h as above the resultant mixture was distilled and measured for 14C. The experiments under a) and b) were also performed in the presence of 2.4 mM unlabelled VA.

Inhibition of methanol formation by catalase

To 3 days old cultures of S. pulverulentum on LH and HL media was added 39,556 units of catalase (Millipore Corp. Freehold, NJ, USA) in 2.0 ml water. Control cultures received only 2.0 ml sterile water or boiled catalase. After incubation for 5 min to destroy hydrogen peroxide, 20 µl O14CH3-VA was added and formation of methanol was measured by the radioactivity counting method after 4 h incubation.

Results

Release of 14CO2 from labelled vanillic acids

Vanillic acids, 14C-labelled in the carboxyl-, ring- and methoxyl-carbons were used as substrates for Sporothrichum pulverulentum in the three different glucose-nitrogen media HH, HL and LH (see Materials and methods). The evolution of 14CO2 from the carboxyl and ring carbons in these media is shown in Fig. 1. In LH medium the carboxyl group was metabolized very rapidly, and the 14CO2 peak appeared 1.5 days after inoculation. The metabolism of the carboxyl group in HL medium was also rapid but the 14CO2 peak appeared somewhat later. In HH medium the rate of 14CO2 release decreased and two peaks appeared. It is evident from Fig. 1 that the metabolism of ring-carbons to 14CO2 is very similar to the metabolism of the carboxyl group except for a slight time lag. Thus, the most rapid 14CO2 evolution took place in LH medium and although rapid was slightly delayed in HL medium. In HH medium two peaks were also observed.

Very different patterns of 14CO2 release were obtained from methoxyl-labelled VA. As shown in Fig. 2A, the 14CO2 peaks with LH, HH and HL media appeared after 4, 7 and 9 days, respectively. It is clear that glucose repressed the metabolism of the methoxyl group to 14CO2.

Metabolism of methanol and formaldehyde

Methanol and formaldehyde are two possible products of demethoxylation (Trojanowski et al. 1966; Ishihara and