Pergamon

PII: S0031-9422(96)00627-9

Phytochemistry, Vol. 44, No. 5, pp. 865–867, 1997

0031-9422/97 $17.00+0.00

CYTOCHROME-P450-DEPENDENT FORMATION OF α-HYDROXYACETOVANILLONE FROM ACETOVANILLONE IN *SOLANUM KHASIANUM*

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(*Received* 12 *July* 1996)

Key Word Index—*Solanum khasianum*; Solanaceae; cell culture; cytochrome P450; α-hydroxyacetovanillone.

Abstract—Microsomal preparations from heterotrophic cell cultures of *Solanum khasianum* catalyse the hydroxylation of the α-methyl group of acetovanillone. The reaction requires both O_2 and NADPH. The enzyme is inhibited by CO (blue light reversible) and various cytochrome P450 inhibitors, indicating that the acetovanillone α-hydroxylase is a cytochrome-P450-dependent monooxygenase. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Acetovanillone (AV) and acetosyringone as well as their α-hydroxyl derivatives are known as wound-inducible compounds, which activate the virulence genes of *Agrobacterium tumefaciens*, especially in species within the Solanaceae [1]. In photomixotrophic cell cultures of *Solanum khasianum*, α-hydroxyacetovanillone (HO-AV) has been identified as a major compound accumulating in the cell culture medium, both constitutively and upon elicitation [2].

Several hydroxylation reactions of terminal methyl groups in plants, e.g. hydroxylations in lipid or terpenoid metabolism, have been characterized as cytochrome P450 dependent [3, 4]. Therefore we examined the hydroxylation of the α-C of acetovanillone (Fig. 1) with regard to cytochrome P450 dependency. The characterization of the responsible enzyme activity is described.

RESULTS AND DISCUSSION

The hydroxylation of AV was initially measured with the microsomal membrane fractions of photomixotrophic *S. khasianum* cell cultures, where HO-AV accumulates in the cell culture medium [2]. Incubation of these microsomes with AV and NADPH led to the formation of a new compound, which was identified as HO-AV on the basis of UV spectra and co-chromatography with an authentic standard. However, the product yield was very low (near the detection limit), which severely hampered the further characterization of the enzyme. These low enzyme activities (*ca* 1–2 pkat mg^{-1} protein) were observed both in control cultures and in cultures elicited with yeast elicitor for 16 hr. The detection of cytochrome-P450-dependent enzyme activities in green tissues has also proved to be difficult in chickpea [5]. In general, little is known about P450 activities in green plant tissue, because most studies were performed with dark-grown plant material. The low hydroxylation activity in the green *S. khasianum* cultures suggests that lipophilic material from chloroplasts interferes with the microsomal enzyme and, in fact, the green colour of the isolated microsomal pellet suggests contamination of the microsomes with chloroplast membrane fragments. On the other hand, the cinnamic acid 4-hydroxylase (CA4H), which was measured as a microsomal marker enzyme, showed high activity (*ca* 40 pkat mg^{-1} protein) in the microsomes from the photomixotrophic cultures. Furthermore, feeding of the photomixotrophic cell cultures with AV led to a high accumulation of HO-AV in the culture medium. Four hours after substrate application, almost 70% of the AV was hydroxylated to HO-AV, and after 8 hr it was completely hydroxylated.

In order to improve the product yields in the enzyme assays, microsomes of heterotrophic cell cultures were used, which also accumulate HO-AV in the culture medium. In fact, the use of these microsomes reliably yielded more product, so that the heterotrophic cultures were chosen for further characterization of the

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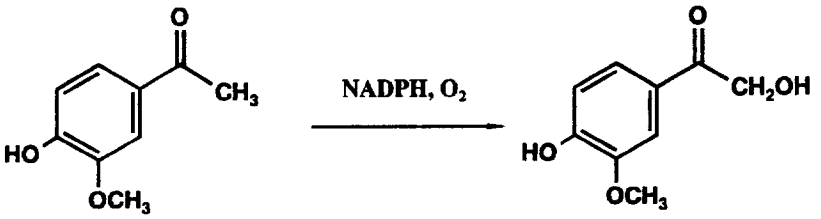

AV **HO-AV**

Fig. 1. Formation of α-hydroxyacetovanillone (HO-AV) from acetovanillone (AV).

hydroxylase. There was no difference in enzyme activities between elicited and non-elicited cultures (5–10 pkat mg^{-1} protein). NADPH is essential for the hydroxylation, whereas various other tested cofactors (NADH, NAD^+, $NADP^+$, FAD) had no effect. Simultaneous addition of NADPH (0.8 mM) and NADH (0.8 mM) showed no synergistic effects, in contrast to various other cytochrome P450 enzymes [6]. In our first experiments the microsomes were isolated by centrifugation at 100 000 *g* for 1 hr, but nearly the same activities were obtained by centrifugation at only 30 000 *g*. No hydroxylation activity could be measured in the soluble protein fraction (30 000 *g* supernatant), whereas in the fraction pelleting at 5000 *g* still 8% of the microsomal activity (pelleting at 30 000 *g*) could be detected. This fractionation behaviour has also been shown for other cytochrome P450 enzymes [7]. In these assays the CA4H was always successfully measured as a marker enzyme for the microsomal fraction.

Further characterization of the AV α-hydroxylase showed an optimum pH of 7.5 and a temperature optimum of 30°. However, at this temperature the product yield was linear for only 20 min, whereas at 25° the linear period lasted for 45 min. Therefore, all incubations were performed at 25° for 45 min because of the higher product yield. The reaction proceeded linearly for up to 200 μg protein per assay (0.5 ml) and it was strictly dependent on the presence of O_2. Removal of O_2 from the assay by the O_2 consuming glucose/glucose oxidase/catalase system [8] led to a complete loss of enzyme activity. The apparent K_M value for AV as substrate is 3.5 μM and for NADPH it is 70 μM. Incubation of the microsomes with acetosyringone led to the formation of α-hydroxyacetosyringone, whereas with 4-hydroxyacetophenone, 3,4-dihydroxyacetophenone and 3-methoxyacetophenone, respectively, as substrates no hydroxylation products were detected, suggesting that both the 3-methoxyl and 4-hydroxyl groups at the phenyl ring are essential for the enzyme activity.

The NADPH and O_2 dependence of the reaction as well as its localization in the microsomal fraction suggest that the hydroxylation is cytochrome P450 dependent. Inhibition studies were performed with CO and several P450 inhibitors to confirm this suggestion. The blue light reversible inhibition by CO is regarded as the most specific reaction to demonstrate the involvement of cytochrome P450 monooxygenases [9]. The AV hydroxylation was strongly inhibited upon incubation in a CO/O_2 (9:1) atmosphere in the dark (Table 1). This inhibition could be partly reversed by illumination of the enzyme assay with blue light. In addition, the IC_{50} values obtained with various P450 inhibitors also support the suggestion (Table 2). The most effective inhibitors were juglone and plumbagin. Cytochrome c also acts as a very potent inhibitor in contrast to the two tested triazoles, which were much less effective.

All these data clearly demonstrate that the hydroxylation of the α-methyl group of AV is catalysed by a cytochrome-P450-dependent monooxygenase. Furthermore, the *S. khasianum* cell culture may be regarded as a versatile system for further studies of this hydroxylase.

Table 1. Effect of CO and blue light on the acetovanillone α-hydroxylase activity*

	Enzyme activity (%)	
Assay conditions	Dark	Blue light (450 nm)
Air	100	100
N_2/O_2 (9:1)	83	79
CO/O_2 (9:1)	12	52

*Standard assay conditions; 100% = 7.1 pkat mg^{-1} protein (dark) and 6.6 pkat mg^{-1} protein (blue light).

Table 2. Concentration (μM) of cytochrome P450 inhibitors that cause 50% inhibition of the enzyme activity

Inhibitor	IC_{50} (μM)
Cytochrome c	5
Tetcyclacis	44
Ketoconazol	420
Juglone	1
Plumbagin	0.8

EXPERIMENTAL

Cell cultures. Photomixotrophic cell cultures were grown as described previously [2]. Heterotrophic cell cultures were established by transfer of these cultures into darkness and cultivated like the photomixotrophic cultures except that the culture medium contained 3% sucrose. Elicitation of the cultures was performed as described in [2].

Preparation of microsomes. Microsomal suspensions were prepared as described for chickpea cultures [10] with the following alterations. The homogenized cells were first centrifuged at 5000 *g* for 15 min. The microsomes were then obtained by centrifugation of the supernatant at 30 000 *g* for 1 hr. The resulting microsomal pellet was resuspended in 0.05 M Tris/HCl buffer (pH 7.5) containing 0.4 M sucrose, 1 mM EDTA and 1 mM DTE.

Enzyme assays. 0.05 mM Tris/HCl (pH 7.5) with 0.4 M sucrose, substrate (100 μM) and NADPH (0.8 mM) were incubated with microsomes (100–200 μg protein) in a total vol. of 0.5 ml for 45 min at 25°. The reaction was stopped by the addition of 20 μl 6 M HCl and extracted twice with 600 μl EtOAc. The combined EtOAc phases were evapd to dryness and the extracted compounds were redissolved in small vols of MeOH for HPLC analyses. HPLC was conducted on an RP18 column (4 × 125 mm, 5 μm). The solvents were an MeCN azeotrope and H_2O. Substrate and product were separated by a linear MeCN azeotrope gradient from 10% to 20% in 5 min and from 20% to 25% in 15 min and detected with a photodiode array detector. Microsomal protein was determined by the method in ref. [11] using BSA as the standard.

Feeding experiments. 1 μmol AV in 500 μl MeOH was added to non-elicited photomixotrophic cell cultures. As a control 500 μl MeOH was added to a parallel cell culture. After 0, 4 and 8 hr 5 ml culture medium was extracted and analysed as described in [2].

Acknowledgements—Financial support by Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie, Bonn and Fonds der Chemischen Industrie is gratefully acknowledged.

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