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FORMATION OF CONIFERYL ALCOHOL FROM FERULIC ACID BY THE WHITE ROT FUNGUS *TRAMETES*

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Abstract—The white rot fungus, *Trametes* sp., was cultivated in a medium containing ferulic acid, glucose and ethanol under aerobic conditions in submerged culture. The ferulic acid was transformed into coniferyl alcohol, coniferylaldehyde, dihydroconiferyl alcohol, vanillic acid, vanillyl alcohol, 2-methoxyhydroquinone and 2-methoxyquinone during 48–120 hr of cultivation. The amount of coniferyl alcohol in the culture reached a maximum after 90 hr with *ca* 40% of the initial amount of ferulic acid. Cinnamic acid, *p*-methoxycinnamic acid, 3,4-dimethoxycinnamic acid, *p*-coumaric acid and sinapic acid were also transformed into the corresponding alcohols, benzoic acids and benzyl alcohols in the fungus culture.

INTRODUCTION

While studying the metabolic products and intermediates from cinnamic acid derivatives in relation to the degradation of lignin and decolourization of kraft pulp waste liquor by white rot fungi, an unexpected high yield of coniferyl alcohol was formed from ferulic acid in the culture of the fungus, *Trametes* sp. [1]. The white rot fungus is known to reduce veratric acid to veratraldehyde [2–5]. It also converts ferulic acid to vanillic acid [6], with the formation of traces of coniferylaldehyde [5]. Polymerization also occurs, due to laccase activity [7] and in the case of coniferyl alcohol, quinone methide radicals are produced with the formation of lignin [8]. The retention of coniferyl alcohol in the culture is therefore unexpected.

Ferulic acid is somewhat inhibitory to mycelial growth of the fungi at high concentration [9] though it acts as a growth-promoting substance for the wood-rot fungus at low concentration [10]. Therefore the culture conditions in a ferulic acid-containing medium were first investigated. The additional carbon sources, glucose and ethanol, which were effective for the reduction of the black brown colour of kraft pulp waste liquor during the cultivation of the white rot fungus [1], were found to be necessary for the metabolism of ferulic acid.

RESULTS

Responses of fungal cells to ferulic acid and culture conditions

The white rot fungi hardly grew in the medium containing ferulic acid which was sterilized by autoclaving. Autoclaving resulted in a coloured culture solution, the colour presumably being inhibitory. The medium was therefore prepared by adding the ferulic acid solution through a membrane filter to the sterilized basal medium. Even so, a high concentration of ferulic acid (over 1/100 M) was inhibitory for its further metabolism by the fungus. Even with 1/100 M ferulic acid in the culture, only a small amount of coniferyl alcohol was produced after a long incubation in the presence of a co-substrate, ethanol and/or glucose. In the absence of co-substrates, vanillic acid was detectable as the result of a slight degradation of ferulic acid.

Consumption of ferulic acid in the culture in the presence and absence of co-substrates at three concentrations (1/50, 1/100 and 1/300 M ferulic acid) is shown in Table 1. Ferulic acid was consumed comparatively fast at 1/300 M concentration in the presence of 1% glucose or ethanol, and the best culture condition was 1/300 M ferulic acid with 0.5% glucose and 0.5% ethanol in a medium with an initial pH 5.5. The following experiments were carried out under these conditions.

Identification of metabolic products

The mycelial pellets of the fungus, *Trametes* sp., were inoculated into 100 ml of the medium described above, and incubated with shaking at 26.5°. Periodically during cultivation, the medium was scanned from 200 to 400 nm for changes in the pattern of absorption. When the absorption maximum at 345 nm decreased to about half of the initial absorption, the culture filtrate was extracted exhaustively with chloroform, after acidification and satn with salt. The metabolic products formed were coniferyl alcohol, coniferylaldehyde, dihydroconiferyl alcohol, vanillic acid, vanillyl alcohol, 2-methoxyhydroquinone and 2-methoxyquinone. A large scale (6 l.) cultivation of the fungus gave the same results.

Changes in the amounts of metabolites in the course of cultivation

The fate of ferulic acid and its metabolic intermediates were followed through cultivation in 2 l. of medium using a mini-fermenter. A small amount (50 ml) of the culture was periodically analysed by GLC, after trimethyl silylation. Coniferyl alcohol appeared after 48 hr of cultivation and reached a maximum concentration with approximately 40% yield at 90 hr cultivation. After

Table 1. Culture conditions for metabolism of ferulic acid by *Trametes*

Substrate [ferulic acid] (M)	Co-substrates concn (%) glucose	ethanol	Cultivation time (hr)	Residual ferulic acid (%)*	Coniferylalcohol formed (%)*	Vanillic acid formed (%)*
1/100	1.0	0.0	72	96	—	+
			160	38	9	16
1/100	0.0	1.0	72	95	—	+
			160	81	—	5
1/100	0.5	0.5	72	100	—	—
			160	0	36	18
1/100	0.0	0.0	72	99	—	—
			160	94	—	+
1/100†	0.5	0.5	72	100	—	—
			160	86	3	2
1/50	0.5	0.5	72	100	—	—
			160	100		
1/300	1.0	0.0	50	28	7	17
			73	4	1	27
1/300	0.0	1.0	50	63	+	12
			73	0	+	42
1/300	0.5	0.5	50	13	22	62
			73	0	+	32
1/300	0.0	0.0	50	77	—	8
			73	65	—	18
1/300†	0.5	0.5	50	78	—	2
			73	72	3	7

*% was calculated by residual ferulic acid (M)/initial ferulic acid (M) or coniferyl alcohol formed (M)/initial ferulic acid (M) × 100%.

†Anaerobically: rubber plug was used instead of cotton plug.

120 hr, vanillyl alcohol became the predominant product in the place of vanillic acid. 2-Methoxyhydroquinone, a further degraded metabolite appeared before the transformation of vanillic acid to vanillyl alcohol. Vanillyl alcohol accumulated as an end product, but 2-methoxyhydroquinone diminished after long incubation.

Metabolic products from other cinnamic acid derivatives

Cinnamic acid, *p*-methoxycinnamic acid, 3,4-dimethoxycinnamic acid, *p*-coumaric acid and sinapic acid were metabolized to the corresponding alcohols, benzoic acids and benzyl alcohols during the cultivation of the fungus. Metabolism of 3,4,5-trimethoxycinnamic acid gave only a trace of trimethoxycinnamyl alcohol. Caffeic acid was metabolized with exceptional rapidity by the fungus with production of protocatechuic acid.

DISCUSSION

The white rot fungus, *Trametes* sp., is able to grow in the medium containing ethanol as main carbon source (concn 2%), but not in the medium containing only ferulic acid. In the latter case, ferulic acid was degraded to vanillic acid. When ethanol was present as co-substrate with ferulic acid, it was again degraded to vanillic acid and only slightly reduced to coniferyl alcohol (Table 1). When glucose was present in the culture, ferulic acid was not only degraded to vanillic acid but also reduced to coniferyl alcohol. There were large accumulations of coniferyl alcohol in the culture in which both co-substrates, glucose and ethanol were present. This suggests that the transformation of ferulic acid to coniferyl alcohol requires not only high energy sources such as ATP and $NADP^+$ but NADPH regenerated by reducing enzyme systems, and glucose and ethanol may supply the energy sources and reducing agents by their oxidative metabolic pathway in the fungus.

EXPERIMENTAL

Species of fungus and culture method. The white rot fungus, *Trametes* sp., was isolated from the wood-rot fungi in New Guinea by Dr. K. Aoshima. The stock and adaptive cultures were maintained in a liquid basal medium containing 0.01% dioxane–lignin (100 ml) in 500 ml flasks agitated by a reciprocal shaker at a speed of 120 rpm at 26.5°. The mycelial pellet suspension of the adaptive culture which had been incubated for 3–4 days was inoculated into the basal medium containing ferulic acid and co-substrates, glucose and EtOH, and incubated as described above. Large scale incubation was carried out in 6 l medium using a jar-fermenter under the following condition: aeration—4 l./min, pres.—0.1 kg/cm², stirring—300 rpm, 27 ± 1°, and also in 2 l. medium using a mini-fermenter under aeration and stirring (300 rpm). The basal medium was composed of L-asparagine, 0.2 g; L-aspartic acid, 0.3 g; KH_2PO_4, 1.2 g; K_2HPO_4, 0.5 g; $(NH_4)_2PO_4$, 2 g; yeast extract, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g, $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$, 10 mg; $ZnSO_4 \cdot 7H_2O$, 4 mg; $MnSO_4 \cdot 6H_2O$, 5 mg; and thiamine–HCl, 3 mg in 1 l. H_2O adjusted to pH 5.5. The medium was sterilized in the flask at 120° for 15 min. Ferulic acid and the other cinnamic acid derivatives were added into the sterilized medium through a membrane filter (0.45 μ pore size, 13 or 25 mm diameter) as a soln in dil. NH_4OH.

Isolation and identification of metabolites of ferulic acid. *Trametes* sp. was cultivated in 6 l. of basal liquid medium supplied with 1/300 M ferulic acid, 0.5% glucose and 0.5% EtOH in a jar-fermenter for 3 days. The culture medium was filtrated and the filtrate and washings of residual mycelial pellets were combined. This was then acidified to pH 2, satd with NaCl and extracted with $CHCl_3$ containing 2% *iso*-AmOH three times. The extract soln was evapd to dryness. The extract was separated by LC(Si gel) with C_6H_6–MeOH–HOAc (45:8:4). The isolates were identified by comparison with authentic samples using PMR and MS.

Quantitative analysis of metabolites from ferulic acid. Trametes sp. was cultivated in 2 l. medium in a mini-fermenter. 50 ml samples were periodically removed. These samples were extracted with $CHCl_3$ in the same manner as above and dried. The extract was dissolved in 5 (or 10) ml acetonitrile and 30 μl of this soln was added to 10 μl of TMSi reagent, BSTFA, in a vial. After 0.5 hr 20 μl of the mixture was injected into a column (3 m × 3 mm) packed with 10% SE-52 at GC (TC, carrier gas H_2, flow rate 100 ml/min, temp. programmed 100–300° at 10°/min, inj. temp. and det. temp. 300°). The resultant peak of each TMSi was integrated with a digital integrator. The amount of the compound was determined using a standard calibration curve obtained from the authentic sample.

Qualitative analysis of the metabolites of cinnamic acid derivatives. Trametes sp. was cultivated in a medium supplied with 1/300 M cinnamic acid derivative, 0.5% glucose and 0.5% EtOH (100 ml) for 3–4 days on a reciprocal shaker. The culture medium was extracted with $CHCl_3$ as above. The condensed and dried extract was dissolved in a small amount of $CHCl_3$ and added to BSTFA. The mixture was analysed by GLC as above. The TMSi's were identified by comparison with the retention time of an authentic sample of the corresponding compound and also by a similar comparison of fragment patterns and M^+ in GC–MS analysis.

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