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Degradation of catechin by *Bradyrhizobium japonicum*

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Abstract

Rhizobia utilize phenolic substances as sole carbon source. *Bradyrhizobium japonicum* utilizes catechin, a unit of condensed tannin as carbon source. To establish the degradative pathway of catechin, the products of catechin degradation were isolated by paper chromatography and TLC and identified by HPLC, UV, IR and NMR spectra. *B. japonicum* cleaves catechin through catechin oxygenase. Phloroglucinolcarboxylic acid and protocatechuic acid were identified as the initial products of degradation. Phloroglucinolcarboxylic acid is further decarboxylated to phloroglucinol which is dehydroxylated to resorcinol. Resorcinol is hydroxylated to hydroxyquinol. Protocatechuic acid and hydroxyquinol undergo intradiol cleavage through protocatechuate 3,4-dioxygenase and hydroxyquinol 1,2-dioxygenase to form β-carboxy *cis*, *cis*-muconic acid and maleylacetate respectively. The enzymes of catechin degradative pathway are inducible. Estimation of all the enzymes involved in the catabolism of catechin reveals the existence of a catechin degradative pathway in *B. japonicum*.

Introduction

Plant tannins are considered recalcitrant to microorganisms. Tannins bind with proteins and polysaccharides and inhibit microbial growth. However, utilization of tannins such as gallotannin, chestnut tannin, myrobalan tannin, gallic acid, tannic acid, catechin and wattle tannin by microorganisms has been reported (Mahadevan & Sivaswamy 1985, Waheeta & Mahadevan 1994).

Plant phenolics of the groups flavonones, flavones, flavonols, isoflavonoids and chalcones are released during legume seed germination or from roots into the rhizosphere. These flavonoids act as signal molecules to initiate the symbiotic relationship between legume and rhizobia (Long 1989). Flavonoids interact with the *nod*D protein in rhizobia and induce the transcription of other *nod* genes.

Phenolic substances also serve as chemoattractants for rhizobia and bradyrhizobia (Parke et al. 1985). Utilization of phenolic compounds by different species of rhizobia has been reported (Parke & Ornston 1984). The capacity of rhizobia to utilize and degrade aromatic substances has been reviewed (Waheeta & Mahadevan 1994). It has been known that catechin, a unit of condensed tannin was degraded by *Bradyrhizobium japonicum* (Waheeta et al. 1984) and a *Rhizobium* strain from *Leucaena leucocephala* (Gajendran & Mahadevan 1988). In this paper, we report on the existence of catechin degradative pathway in *Bradyrhizobium japonicum*.

Materials and methods

Bacterial culture

B. japonicum was grown on synthetic medium (Waheeta et al. 1984) containing KH_2PO_4 - 1.0 g, Na_2HPO_4 - 2.36 g, NaCl - 1.0 g, NH_4Cl - 1.0 g, Na-glutamate - 0.1 g, $MgCl_2$ - 0.1 g, $CaCl_2$ - 0.13 g, $FeSO_4$ - 0.005 g and mannitol - 10.0 g per liter, pH 7.0. Mannitol was substituted with aromatic substances which were filter sterilized using Millipore filter of 0.45μ pore size and

Table 1. Identification of intermediates of catechin degradation

Aromatic substance	Rf value							UV λ max	HPLC retention time (min)
	Paper chromatography				TLC				
	Acetic acid 15%	Benzene: Acetic acid: water 10:7:3	Formic acid 2%	Benzene: Dioxan: Acetic acid 60:36:4	Benzene: Ethanol: Formic acid 85:15:1	Benzene: Dioxan: Acetic acid 90:25:4	Benzene: Ethyl acetate: Formic acid 85:15:1		
Catechin	0.58	0.01	–	–	0.23	0.09	0.02	280	7.28
Protocatechuic acid	0.66	0.11	0.56	–	0.43	0.25	0.05	265,293	7.70
Phloroglucinol-carboxylic acid	0.68	0.08	–	0.11	0.32	0.17	0.04	2.58	5.51
Phloroglucinol	0.69	–	–	0.47	–	0.18	0.05	268	7.41
Resorcinol	0.75	–	0.73	0.77	–	0.52	0.25	276	7.90
Hydroxyquinol	0.44	–	0.66	0.55	–	0.27	0.05	285	7.82

– Not determined.

amended aseptically to a final concentration of 1 or 2 mm.

Separation of intermediates

Formation of intermediates was analyzed both from spent culture and replacement culture in sodium phosphate buffer, 100 mM, pH 7.0. Phenolics were extracted with diethyl ether or ethyl acetate. The residue was dissolved in acetone and analyzed by paper chromatography using 15% acetic acid or 2% formic acid or benzene:acetic acid:water (10:7:3 - upper phase) or benzene:dioxane:acetic acid (60:36:4) and thin layer chromatography using benzene:ethanol:formic acid (85:15:1) or benzene:dioxane:acetic acid (90:25:4). Phenolics were detected by spraying diazotized *p*-nitroaniline (Gayon 1972) or potassium ferricyanide – ferric chloride reagent (Cartroux et al. 1969) or ferric chloride reagent (Raju et al. 1983) and compared with authentic compounds. Identical, unsprayed spots were eluted in ethanol, their UV absorption maxima were measured in a Beckman DU6 spectrophotometer and compared with authentic compounds.

High-performance liquid chromatographic (HLPC) analysis

Catechin and its intermediates were analyzed in a LKB HPLC system with a Lichrosorb RP18 reversed phase column (4 x 250 nm, 5 μm) and acetonitrile - 2% acetic acid in water (70/30 v/v) as the mobile phase at a flow rate of 0.5 ml/min. The sample was dissolved in the same solvent, 20 μl was injected into the column. The compounds were detected at 280 nm.

Infra red (IR) spectroscopic analysis

Sample, 2 mg was ground with 200 mg of powdered spectral grade potassium bromide and pressed to form a pellet of 13 mm size. Analysis of the intermediates of catechin degradation was carried out in a Perkin-Elmer 598 infra red spectrometer.

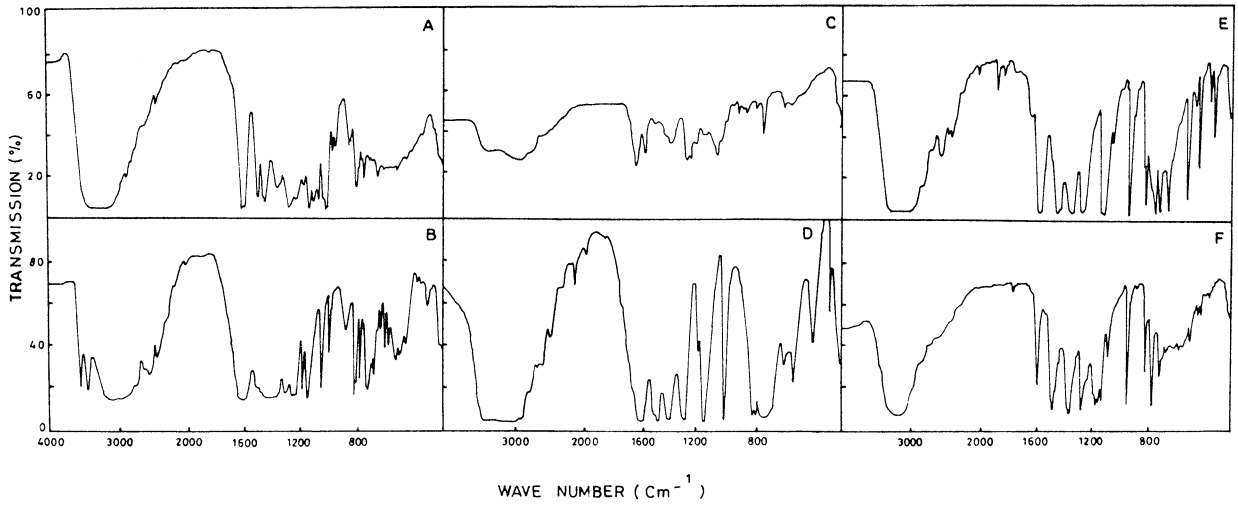
Nuclear magnetic resonance (NMR) spectral analysis

The sample, 15 to 50 mg was dissolved in 0.4 ml of deuterated acetone and the spectrum was taken in Varian EM 390, 90 MHZ proton nuclear magnetic resonance spectrometer with a 5 mm OD NMR tube. TMS was used as internal standard. Peak formation was checked in a range of 0-20 ppm and peaks observed in 0-10 ppm region were recorded.

Enzyme assays

Cell-free enzymes were prepared by ultrasonication as previously described (Hopper & Mahadevan 1991).

Catechin oxygenase, protocatechuate 3,4-dioxygenase and hydroxyquinol 1,2-dioxygenase: Activity



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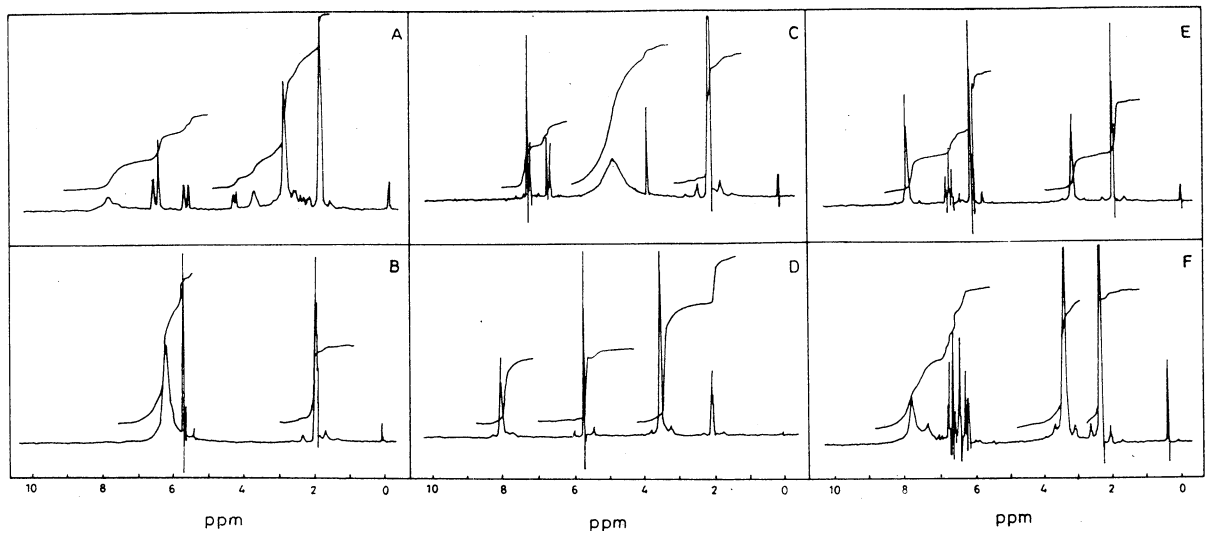

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Figure 1. Infra red spectra and Nuclear magnetic resonance spectra of catechin and purified metabolites of catechin degradative pathway. I. Infra red spectra, II. Nuclear magnetic resonance spectra. (A) Catechin; (B) Phloroglucinolcarboxylic acid; (C) Protocatechuic acid; (D) Phloroglucinol; (E) Resorcinol; (F) Hydroxyquinol.

of these enzymes was measured as described earlier (Hopper & Mahadevan 1991).

Phloroglucinol dehydroxylase and Resorcinol hydroxylase: The reaction mixture contained 10 μ mole of phloroglucinol or resorcinol (0.1 ml), 100 μ mole of $NADPH_2$ (0.1ml), 0.5 ml enzyme source and 2.3 ml of phosphate buffer, 50 mM, pH 7.0. Oxidation of $NADPH_2$ was measured as decrease in O.D. at 340 nm. One unit of activity is the amount of enzyme that catalyzes the oxidation of 1 μ mole of $NADPH_2$/min.

Phloroglucinolcarboxylic acid decarboxylase: Decarboxylase was assayed by manometry using Warburg's manometer. The reaction mixture contained 5 μ mole of substrate, 0.5 ml (taken in the side arm of the flask and tipped into the main chamber to start the reaction), 0.5 ml enzyme and 2.0 ml of phosphate

Table 2. Activity of enzymes involved in catechin degradation in *Bradyrhizobium japonicum*

Enzyme	Specific activity*
Catechin oxygenase	1.362
Phloroglucinolcarboxylic acid decarboxylase	0.605
Phloroglucinol dehydroxylase	0.740
Resorcinol hydroxylase	0.892
Hydroxyquinol 1,2-dioxygenase	1.388
Protocatechuate 3,4-dioxygenase	1.150

* μ mole of substrate disappeared/min/mg protein.

buffer, 0.5 M, pH 6.0. One unit of enzyme activity was measured as the yield of 1 μ mole of CO_2/min at 30 °C.

In the control, enzyme was replaced with heat killed enzyme. Specific activity of the enzymes was calculated as units/mg protein. Protein was measured using bovine serum albumin as the standard (Lowry et al. 1951).

Results

Identification of intermediates

Isolation of phenolics from catechin grown *Bradyrhizobium japonicum* revealed the presence of 4-5 substances. From 24 h old cultures, two substances were isolated and identified as phloroglucinolcarboxylic acid and protocatechuic acid. Cultures older than 48 h showed 2 or 3 more substances, identified as phloroglucinol, resorcinol and hydroxyquinol.

To check the sequential formation of these substances, phenolics were separated from replacement cultures amended with individual intermediates. Phloroglucinolcarboxylic acid grown culture exhibited the presence of phloroglucinol, resorcinol and hydroxyquinol. Resorcinol and hydroxyquinol were isolated from phloroglucinol grown culture. Resorcinol replacement culture exhibited only hydroxyquinol. No phenolic intermediate was isolated from hydroxyquinol and protocatechuic acid replacement cultures indicating that they were cleaved directly.

To confirm the formation of these intermediates during the catabolism of catechin, the samples were analyzed by HPLC, IR and NMR spectra. The Rf values, λ max and retention time in HPLC of isolated compounds corresponded with those of authentic samples (Table 1). Infra red and nuclear magnetic resonance (Figure 1) spectral analyses of catechin and purified metabolites of catechin also confirmed the formation of these substances.

Enzymes of the pathway

The enzymes of catechin catabolic pathway – catechin oxygenase, phloroglucinolcarboxylic acid decarboxylase, phloroglucinol dehydroxylase, resorcinol hydroxylase, hydroxyquinol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase were inducible. Activity of these enzymes exhibited by catechin grown cells is presented in Table 2. Mannitol grown cells did not express activity of any of these enzymes.

Catechin oxygenase from catechin grown cells degraded catechin without any lag (Figure 2A). Protocatechuic acid was also degraded without any lag (Figure 2B). Cleavage of protocatechuic acid resulted in the formation of β-carboxy *cis*, *cis*-muconic acid. Hydroxyquinol was cleaved with a slight lag (Figure 2C). Cleavage of hydroxyquinol resulted in the formation of maleylacetate.

Discussion

Rhizobia have the capacity to utilize phenolic substances. *B. japonicum* utilized catechin, a unit of condensed tannin as sole carbon source (Hopper & Mahadevan 1991). Catechin was also utilized by rhizobia such as *R. japonicum, R. trifolii* and *Rhizobium* sp. (Muthukumar et al. 1982). Other microbes like *Aspergillus* spp., *Streptomyces* sp., *Fusarium* sp., and *Pseudomonas* spp. have been reported to utilize catechin as sole carbon source (Mahadevan & Sivaswamy 1985).

Our results clearly show the cleavage of catechin into phloroglucinolcarboxylic acid and protocatechuic acid by *B. japonicum*. Formation of these two compounds from catechin was also observed in *Aspergillus flavus* (Chandra et al. 1969). Protocatechuic acid was isolated from catechin grown *Rhizobium* sp. (Muthukumar et al. 1982). *B. japonicum* decarboxylated phloroglucinolcarboxylic acid directly to phloroglucinol. Phloroglucinol was dehydroxylated to resorcinol which was also reported earlier in *Pseudomonas* sp. (Blackwood et al. 1970). Phloroglucinol was converted by an entirely different pathway in *Fusarium solani* to pyrogallol which was further metabolized via the meta pathway to pyruvate

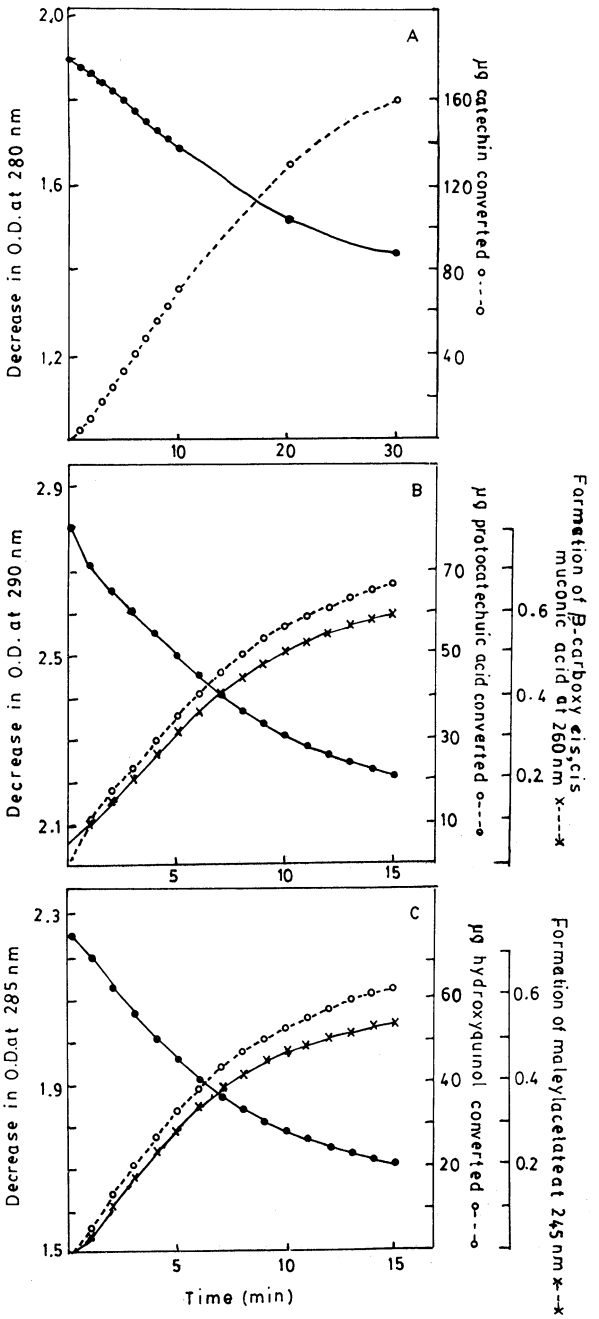

Figure 2. Oxygenases involved in catechin degradation in *Bradyrhizobium japonicum*. (A) Cleavage of catechin by catechin oxygenase; (B) Cleavage of protocatechuic acid by protocatechuate 3,4-dioxygenase; (C) Cleavage of hydroxyquinol by hydroxyquinol 1,2-dioxygenase.

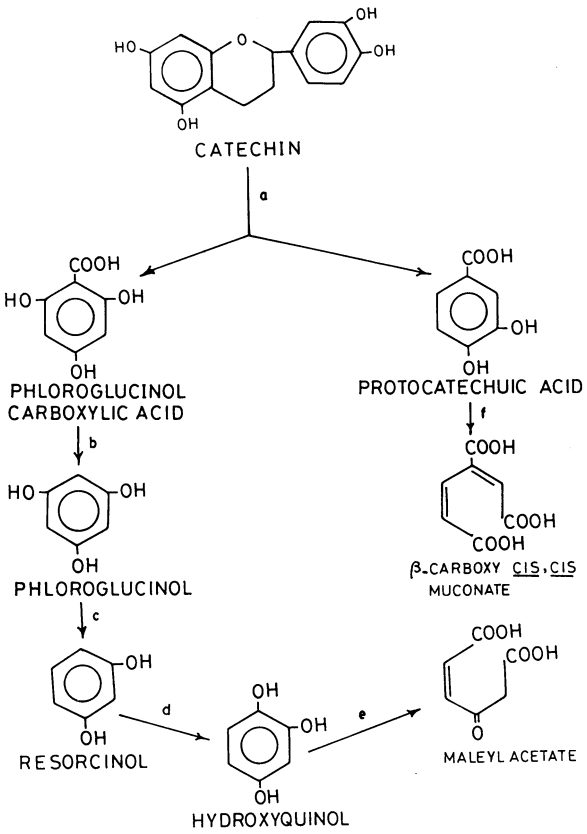

Figure 3. Proposed pathway for catechin degradation by *Bradyrhizobium japonicum*. (a) Catechin oxygenase; (b) Phloroglucinolcarboxylic acid decarboxylase; (c) Phloroglucinol dehydroxylase; (d) Resorcinol hydroxylase; (e) Hydroxyquinol 1,2-dioxygenase; (f) Protocatechuate 3,4-dioxygenase.

(Walker & Taylor 1983). Conversion of resorcinol to hydroxyquinol was observed with *Pseudomonas* sp. (Larway & Evans 1965; Chapman & Ribbons 1976). But *Azotobacter vinelandii* converted it to pyrogallol (Groseclose & Ribbons 1981). Protocatechuic acid was utilized as sole carbon source by almost all species of rhizobia (Hussien et al. 1974, Muthukumar et al. 1982, Parke & Ornston 1984). Formation of β-carboxy *cis,cis*-muconic acid and maleylacetate from protocatechuic acid and hydroxyquinol by *B. japonicum* (Figure 2a,b,c) indicated that these compounds were cleaved through *ortho* pathway enzymes protocatechuate 3,4-dioxygenase and hydroxyquinol 1,2-dioxygenase respectively. The enzymes of catechin degradative pathway are inducible (Table 2). This was indicated by mannitol grown cells which did not exhibit activity of any of these enzymes. Based on the formation of intermediates and induction of enzymes,

the degradative pathway of catechin is established in *B. japonicum*. The pathway for degradation of catechin by *B. japonicum* is represented in Figure 3. *Pseudomonas solanacearum* also degraded catechin through a similar pathway except that protocatechuic acid was converted to catechol at higher concentrations (Boominathan & Mahadevan 1987). Catechol and wattle tannin were also utilized as sole carbon source by *B. japonicum* (data not shown) indicating that rhizobia can be tested for their efficacy to cleave recalcitrant molecules present as pollutants in soils.

There were other reports on degradation of flavonoids by rhizobia. Quercetin, a major flavonoid constituent in roots and nodules of *Lotus* species was catabolized by rhizobia to phloroglucinol and protocatechuic acid (Rao et al. 1991). Recently, catabolism of *nod* gene inducing flavonoids by rhizobia, yielding conserved A- and B- ring products was reported by Rao & Cooper (1994). Incubation of rhizobia with flavonoids yielded phloroglucinol or resorcinol as A-ring products and a wide range of B-ring derivatives like *p*-coumaric acid, *p*-hydroxybenzoic acid, protocatechuic acid, phenylacetic acid and caffeic acid.

R. meliloti exhibited positive chemotaxis to luteolin, a specific nodulation inducer of *nod*ABC from seed exudate (Caetano-Anolles et al. 1988) and to 4′,7-dihydroxyflavone, 4′,7-dihydroxyflavonone and 4,4′-dihydroxy-2-methoxychalcone from root exudate (Dharmatilake & Bauer 1992). Though isoflavonoids, the nodulation inducers in bradyrhizobia, are not good chemoattrctants, the bradyrhizobia strongly responded towards β-ketoadipic acid (Parke et al. 1985) and hydroxycinnamic acids (Kape et al. 1991). The organic acids succinate, malonate, malate, fumarate, α-ketoglutarate, pyruvate and the amino acids glutamate, aspartate were also identified as important attractants for *B. japonicum* (Barbour et al. 1991).

Thus the products of degradation of nodulation inducers or other aromatic compounds or the presence of β-ketoadipic acid may serve as chemoattractants and will strongly attract bradyrhizobia to the vicinity where aromatic degradation has been carried out even by other microorganisms. Presence of dicarboxylic acids might strongly attract bradyrhizobia and also enhance degradation of phenolic substances.

Acknowledgement

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