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BIOTRANSFORMATION OF TETRAHYDROBERBERINE TO BERBERINE BY ENZYMES PREPARED FROM CULTURED *COPTIS JAPONICA* CELLS

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Key Word Index—*Coptis japonica*; Ranunculaceae; biotransformation; cultured cells; stereospecificity; alkaloid; berberine; tetrahydroberberine.

Abstract—Crude enzymes were extracted from cultured *Coptis japonica* cells producing large amounts of berberine. One of the enantiomers of tetrahydroberberine, (*S*)-(−)-tetrahydroberberine, was dehydrogenated to berberine by the enzyme system.

Some lines of suspension cultured cells of *Coptis japonica* produce large amounts of berberine (8–9% of the dry cell weight) in a short period (3 weeks) compared to the intact plant (4–7% of the dry plant weight) [1]. In general, it takes 5–6 years to produce *Coptis* roots, the raw material for berberine production. Cultured cells having such a high berberine productivity provide us with a suitable source of enzymes to study the biosynthesis of this alkaloid.

Tetrahydroberberine, believed to be an intermediate of berberine biosynthesis [2] (Fig. 1), was dehydrogenated to berberine by the cell-free system prepared from the cultured cells of the high potency cell line (64T-33). The reaction mixture, containing chemically synthesized tetrahydroberberine and the cell-free extract, was incubated for 60 min at 30°. The product was identified by HPLC. The product had the same retention time as authentic berberine. A linear dependence of the berberine formed on the amount of enzyme solution added was observed under the experimental conditions employed. Both berberine and tetrahydroberberine were quantitatively analysed by HPLC, and it was found that 231 nmol of the reactant formed 234 nmol of the product, which shows that 1 mol of tetrahydroberberine is converted to 1 mol of berberine [3]. Products other than berberine were not detected by HPLC. When chemically synthesized tetrahydroberberine, which is supposed to have the (*R,S*)-configuration, was used as substrate, the maximum conversion of 50% was obtained (Fig. 2). This result suggests the possibility that the enzyme contained in the cell-free extract has a stereospecificity and is active on only one of the stereoisomers. After optical resolution of (*R,S*)-(±)-tetrahydroberberine [4], (*R*)-(+)- and (*S*)-(−)-tetrahydroberberine were individually treated by the enzyme solution. Only the (*S*)-(−)-tetrahydroberberine was completely converted to berberine; its stereoisomer remained unchanged [5] (Fig. 2).

The addition of NAD^+ and $NADP^+$ (1.67 mM) to the reaction mixture did not result in a higher reaction rate or a higher berberine yield. Hydrogen peroxide could not be

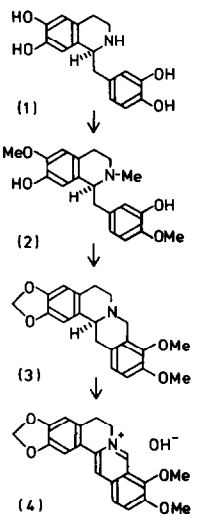

Fig. 1. Biosynthesis of berberine. (1) (*S*)-(−)-Norlaudanosoline; (2) (*S*)-(+)-reticuline, (3) (*S*)-(−)-tetrahydroberberine; (4) berberine.

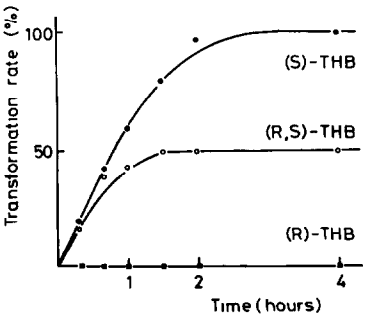

Fig. 2. Stereospecificity in the enzymatic transformation system prepared from cultured *Coptis japonica* cells. (●) (*S*)-(−)-Tetrahydroberberine (THB); (■) (*R*)-(+)-tetrahydroberberine; (○) (*R,S*)-(±)-tetrahydroberberine.

detected in the reaction mixture by the peroxidase-4-aminoantipyrine–2,4-dichlorophenol system [6]. An absolute oxygen requirement of the enzymatic reaction was demonstrated in that there was a decrease in dissolved oxygen in the reaction mixture of the solution as determined by an oxygen electrode (HANSATECH).

Tetrahydropalmatine, a structural analogue of tetrahydroberberine, was not converted to palmatine by this enzyme system. From these results, we concluded that tetrahydroberberine was converted to berberine enzymatically. In this reaction, four hydrogen atoms removed from tetrahydroberberine seemed to be accepted by O_2 to form two water molecules. As in many oxidoreductase reactions, two hydrogen atoms or two electrons are removed from the substrate, so dihydroberberine might be an intermediate in the reaction though it was not detected in the reaction mixture by HPLC. If the formation of berberine from tetrahydroberberine is carried out by two sequential enzyme reactions, the second step proceeds very rapidly compared to the first step. Another possibility is that both steps are carried out on the same enzyme molecule without dissociation of the intermediate from the enzyme molecule.

The enzyme activity found in the yellow cell lines (156T-1, 64T-33) producing more than 250 times higher berberine per dried cell mass had more than 100 times higher enzyme activity compared to a white cell line having low berberine productivity. These results suggest that the enzyme plays an important role in berberine biosynthesis, but it is not the only rate-determining step of the production (Table 1).

At the final step of the berberine biosynthesis, (*S*)-(−)-tetrahydroberberine is stereospecifically dehydrogenated to berberine as proved in this study. The (*S*)-configuration at the asymmetric carbon of the intermediates seems to be retained throughout the berberine synthetic pathway. (*S*)-(−)-Norlaudanosoline was reported to be specifically synthesized from dopamine and 3,4-dihydroxyphenylacetaldehyde by *Eschschlotzia tenuifolia* [7]. (*S*)-(+)-Reticuline was effectively incorporated through (*S*)-(−)-scoulerine into berberine [2, 8, 9]. In spite of the above facts, an intermediate having (*R*)-configuration, (*R*)-(−)-reticuline, plays a role in morphine alkaloid biosynthesis [10, 11].

EXPERIMENTAL

Cultured cell material. The original cultured *Coptis japonica* cells were induced from small fragments of rootlets of *Coptis japonica* Makino var dissecta (Yatabe) Nakai (Japanese name, Seribaworen). Cloning was carried out with small cell aggregates. Fine cell aggregates were collected by filtration, and plated in Petri dishes with Linsmaier–Skoog medium [12]. The appended letter 'T' means the cell line obtained from medium with tyrosine. After cloning and selection, cell lines producing large or moderate amounts of berberine were obtained [13–15]. These cell lines were cultured on Linsmaier–Skoog suspension medium, containing 10^{-5} M NAA and 10^{-8} M BA at 26°, on a rotary shaker at 100 rpm in the dark.

Substrate synthesis. Berberine was reduced to tetrahydroberberine by $NaBH_4$ in MeOH–H_2O (10:1) according to the method reported previously [16]. The product was purified by repetitive recrystallization from HOAc [17]. (Found: C, 60.32, H, 6.00; N, 3.55; Cl, 9.47. Calc. for $C_{20}H_{21}NO_4 \cdot HCl \cdot H_2O$: C, 60.99, H, 6.09; N, 3.56; Cl, 9.02%.) UV $\lambda_{max}^{H_2O}$ nm: 282. GC/MS 70 eV, *m/z* (rel. int.): 339 $[M]^+$ (63), 308 $[M-OMe]^+$ (10), 174 (31), 164 (100), 149 (79). (*R,S*)-(±)-Tetrahydroberberine was optically resolved by (+),(−)-di-*p*-toluoyl tartaric acid according to the method of ref. [3]. (*R*)-(+)-Tetrahydroberberine had mp 134° (lit. 132°), $[\alpha]_D^{28}$ +302° (*c* 0.52; $CHCl_3$) (lit. +299°) (*S*)-(−)-Tetrahydroberberine had mp 135–136° (lit. 133–134°), $[\alpha]_D^{28}$ −303° (*c* 0.53; $CHCl_3$) (lit. −299°).

Enzyme preparation. The crude enzyme soln was obtained by the following procedure, all performed at 0–4°. Wet cultured cells (30 g) were homogenized with 30 ml 100 mM phosphate buffer, pH 7.0, containing 5 mM dithioerythritol, in a pre-chilled mortar with quartz sand. After removal of the debris in the resulting suspension by centrifugation, protein precipitated at 0.9 saturation of $(NH_4)_2SO_4$ was collected by centrifugation and resuspended in a minimal vol. of 15 mM Tris–HCl buffer, pH 7.5. The soln was subjected to dialysis against the same buffer. The supernatant, after centrifugation of the above soln at 20000 *g* for 20 min, was used as crude enzyme soln.

Enzyme assay. The incubation mixture (1.5 ml) contained 0.5 ml enzyme soln, 635 nmol tetrahydroberberine in 5 mM Tris–HCl buffer, pH 7.5, containing 10% DMF. The mixture was incubated at 30° for 60 min and at the end of the incubation period it was kept in a boiling water bath for 3 min. After removal of the resulting precipitate by centrifugation, the increase of absorbance at 425 nm was measured. The supernatant was analysed by HPLC (Waters) equipped with μBondapak C_{18}, using the solvent system MeCN–H_2O–HOAc (40:60:1) containing 5 mM octanesulfonate, flow rate 1 ml/min. *A* 280 nm was measured for identification of the product and assay of the decrease of the substrate.

Table 1. Relation between tetrahydroberberine oxidase activity and berberine content in each cell line of *Coptis japonica*

Cell line	THB oxidase activity (μkat/kg protein)	Berberine content (dry %)
Non-selected	10.9	0.64
81T-105T	0.12	0.02
64T-33	22.9	5.41
156T-1	15.6	9.11

THB, Tetrahydroberberine.

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