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Establishment of *Camellia sinensis* cell culture with high peroxidase activity and oxidative coupling reaction of dibenzylbutanolides

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Abstract—We succeeded in establishment of *Camellia sinensis* cell culture with high peroxidase activity. When dibenzylbutanolides (**1a–c**) were reacted with *C. sinensis* cell culture, peroxidase-catalyzed oxidative coupling of **1a–c** to cyclic products (**2a–c**) proceeded quantitatively in the absence of foreign hydrogen peroxide as cofactor.

The development of enzymes for oxidation reaction aimed at green chemistry is very important. Horseradish peroxidase (HRP) is a commercially available metalloporphyrin enzyme and has been established as an effective biocatalyst for organic and inorganic oxidation reactions by using hydrogen peroxide or organic hydroperoxides.[1] Kutney et al. reported HRP-catalyzed carbon–carbon bond formation with phenolic systems: enzyme-catalyzed ring closured reaction of dibenzylbutanolides suitable for biotransformation to lignans as potential intermediates for the synthesis of etoposide which is now used clinically as an antitumor agent.[2] However, the addition of H_2O_2 to the reaction mixture brought about red–brown darkening of the solvent to decrease chemical yield. From these points of view, we have investigated plant cell cultures in which cell wall peroxidases rapidly metabolize a huge amount of H_2O_2 produced by the addition of foreign substrates. Recently, we have found *Camellia sinensis* cell culture, an efficient source of peroxidase (POD) enzymes. In this work, we would like to report establishment of *C. sinensis* cell culture with high POD activity and the oxidative coupling of dibenzylbutanolides (**1a–c**) by its culture.

We carried out an examination to establish *C. sinensis* callus culture providing us high POD activity. The calli derived from cotyledons of 'Sayamakaori' seeds expressed the highest POD activity per g calli. When the calli were cultured on MS medium[3] with 10 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), POD activity was assayed to be raised by 2∼3 times. Next, we assayed POD activities of *C. sinensis*, *Daucus carota*, *Catharanthus roseus* and *Nicotiana tabacum* suspension cell cultures with purpurogallin assay.[4] The POD activity was determined by combining 5% pyrogallol (2 mL), freshly-prepared 0.5% hydrogen peroxide (1 mL), water (14 mL), 0.1 M pH 6.4 phosphate buffer (2 mL) and enzyme sample (1 mL), recording absorbance at 420 nm and calculating the number of units present from the standard curve for purpurogallin. POD activities of various plant cell cultures are shown in Table 1. The highest level of POD activity was found in 12-day-old *C. sinensis* cell culture (entry 1). The cells of *C. sinensis* cell culture excreted POD into the broth during growth (entry 2).

Table 1. The POD activity in the plant cell culture (±S.D.)

Entry	Plant cell culture		POD U/mL
1	*C. sinensis*	Cell	15.5±0.32
2	*C. sinensis*	Broth	12.4±0.50
3	*D. carota*	Cell	8.0±0.20
4	*D. carota*	Broth	1.6±0.15
5	*N. tabacum*	Cell	9.6±0.32
6	*N. tabacum*	Broth	7.9±0.26
7	*C. roseus*	Cell	0.5±0.10
8	*C. roseus*	Broth	0.1±0.05

Keywords: oxidation; coupling reaction; catalysts; enzymes and enzyme reaction.

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When the previously synthesized racemic dibenzylbutanolides **1a**–**c**[5] were subjected to *C. sinensis* plant cell culture, racemic cyclic products **2a**–**c**[5] were given quantitatively in the absence of foreign hydrogen peroxide as cofactor as shown in Table 2. The oxidative coupling reaction was performed by two methods, that is: [A] with freely suspended plant cell culture in the stationary phase after 12 days of incubation, [B] with immobilized plant cell culture. In the case of *C. sinensis*, **1a**–**c** were quantitatively cyclized to the desired (*trans* relationship of the lactone ring) ring-closed product (**2a**–**c**) not only with method [A], but also with [B] in the absence of foreign hydrogen peroxide as cofactor (entries 2, 3, 10, 14). Next, we attempted the repetitive use of immobilized *C. sinensis* cell culture (ICSC). After three consecutive reuses, the biocatalyst still maintained the POD activities to afford **2a**–**c** quantitatively (entries 4, 5, 11, 12, 15, 16). To our surprise, we succeeded in the biotransformation of **1a**–**c** to **2a**–**c** with ICSC in hexane containing no water (entries 6, 13, 17). In this case, all of the substrates were incorporated in the immobilized cell immediately on adding. This method has the advantage of extracting with a small amount of an organic solvent and is a very good system for water-insoluble substrates. Studies are now in progress to establish enantioselective oxidation with *C. sinensis* cell culture.

For a typical experiment, we used suspension-cultured cells which had originally been isolated from *C. sinensis*, *N. tabacum*, *D. carota* and *C. roseus* as described in our previous papers.[6–16] ICSC were prepared according to the following procedure. Freely suspended *C. sinensis* (4.6 g cells and 20 mL broth) and 60 mL B5 medium[17] in the stationary phase after 8 days of incubation was mixed with 5% sodium alginate solution (80 mL). The resultant mixture was dropped into a 0.6% $CaCl_2$ solution (1000 mL) and rinsed with water to give ICSC. ICSC (including 7 g cells and 30 mL broth) was added to freshly prepared B5 medium (80 mL per flask) and was shaken on a rotary shaker (110 rpm) in the dark at 25°C. A substrate (35 mg) was added to the freely suspended plant cell cultures (19 g cells and 80 mL broth), ICSC cells (including 7 g cells and 30 mL broth) in 80 mL freshly B5 medium or ICSC cells (including 7 g cells and 30 mL broth) in 20 mL hexane. After regular intervals of incubation, the incubation mixture was filtered, the filtered cells or immobilized cells were washed with CH_2Cl_2, and the filtrates were combined. The combined mixture was extracted with CH_2Cl_2. The organic layer was dried over anhydrous $MgSO_4$. In the case of reaction in hexane, homogenated cells were extracted with CH_2Cl_2. Butanolides **1a** and **1b** were synthesized by the synthetic route of Kutney.[18,19] Butanolide **1c** was synthesized by the same synthetic route of Kutney.[19]

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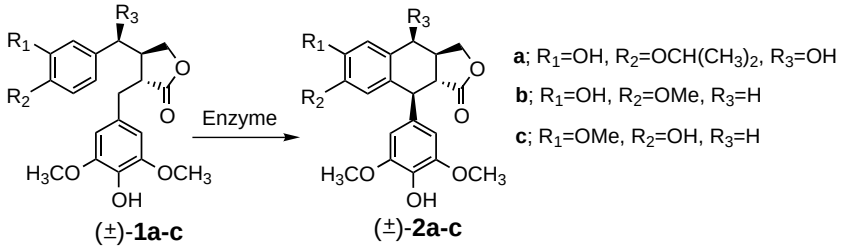
Table 2. Biotransformation of dibenzylbutanolide (**1a**–**c**) to cyclic product (**2a**–**c**) by peroxidase enzymes



Entry	Substr.	Enzyme	Method	Time (h)	Solvent	Product **2a**–**c** (%)	Recovery **1a**–**c** (%)
1	**1a**	HRP(H_2O_2)		0.5		18	15
2	**1a**	*C. sinensis*	A	6	B5 medium	Quant.	0
3	**1a**	*C. sinensis*	B	24	B5 medium	Quant.	0
4	**1a**	(Second reuse)	B	24	B5 medium	Quant.	0
5	**1a**	(Third reuse)	B	24	B5 medium	Quant.	0
6	**1a**	*C. sinensis*	B	24	Hexane	52	24
7	**1a**	*C. roseus*	A	168	B5 medium	37	23
8	**1a**	*N. tabacum*	A	168	MS medium	14	66
9	**1a**	*D. carota*	A	168	MS medium	17	83
10	**1b**	*C. sinensis*	B	24	B5 medium	Quant.	0
11	**1b**	(Second reuse)	B	24	B5 medium	Quant.	0
12	**1b**	(Third reuse)	B	24	B5 medium	Quant.	0
13	**1b**	*C. sinensis*	B	24	Hexane	48	26
14	**1c**	*C. sinensis*	B	1	B5 medium	Quant.	0
15	**1c**	(Second reuse)	B	1	B5 medium	Quant.	0
16	**1c**	(Third reuse)	B	1	B5 medium	Quant.	0
17	**1c**	*C. sinensis*	B	1	Hexane	52	17

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5. **1c**: FAB-MS m/z 388 (M^+), HRMS (EI) calcd for $C_{21}H_{24}O_7$ 388.1522, found 388.1507. Mp 35∼40°C. ^1H NMR ($CDCl_3$) δ: 2.40–2.72 (4H, m, H(2), H(3), H(7″)), 2.90 (1H, dd, H(7′), $J=5.3$, 10.9 Hz), 3.78–3.87 (10H, m, -OCH_3, H (4)), 4.17 (1H, dd, H(4), $J=7.3$, 3.2 Hz), 6.25–6.90 (7H, m, H(2″), H(6″), H(2′), H(3′), H(6′), OH (4′), OH (3′)).
   2c: FAB-MS m/z 387 $(M+H)^+$. HRMS (EI) calcd for $C_{21}H_{22}O_7$ 386.1366, found 386.1366. ^1H NMR ($CDCl_3$) δ: 2.27 (1H, dd, H(2), $J=15$, 12 Hz), 2.60 (1H, m, H(3)), 2.80 (1H, dd, H(4), $J=13.9$, 5 Hz), 3.10 (1H, dd, H(4), $J=14$, 5 Hz), 3.83 (3H, s, -OCH_3 (6)), 3.88 (6H, s, -OCH_3 (3′, 5′)), 3.98 (1H, dd, H(11), $J=11$, 7 Hz), 4.05 (1H, brd, H(1), $J=11$ Hz), 4.25 (1H, dd, H(11), $J=11$, 7 Hz), 5.53 (2H, brs, OH(4′), OH(7)), 6.63, 6.84, 7.26 (1H, each, s, s, s, H(5), H(8), H(2′), H(6′)).
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