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BIOTRANSFORMATION OF DIGITOXIGENIN BY CULTURED STROPHANTHUS HYBRID CELLS

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Key Word Index—Strophanthus gratus, S. amboensis; Apocynaceae; hybrid cells; electrofusion; biotransformation; digitoxigenin; $17\beta H$ -periplogenin β -D-glucoside; isomerization of 17β -lactone ring; glucosylation.

Abstract—Hybrid cells between Strophanthus gratus and S. amboensis were obtained by electrofusion and confirmed to be hybrids through isozyme and RFLP analyses. Because a new and hybrid compound, $17\beta H$ -periplogenin β -D-glucoside, was isolated as a biotransformation product of digitoxigenin by the hybrid cells, isomerization of 17β -lactone ring on S. gratus and glucosylation on S. amboensis were demonstrated simultaneously as the biotransformation abilities in the hybrid cells. Moreover, the productivity of the hybrid compound was increased by raising the sucrose concentration. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Although *Strophanthus* plants produce cardiac glycosides, their cultured cells do not produce them *de novo*. We have investigated the species specific biological transformations of digitoxigenin (1), a precursor of cardiac glycosides, by suspension cultures of some *Strophanthus* species [1–4], and shown, for example, 1β -, 4β -hydroxylation and isomerization of the 17β -lactone ring by *S. gratus* cells, glucosylation by *S. amboensis* cells and 16β -hydroxylation by *S. intermedius*.

Cell fusion as a means of producing new heredity resources and of obtaining useful metabolites [5, 6] has recently made rapid progress. The hybrid cells obtained from parents having different biotransformation capabilities might be expected to produce new biotransformation products. In this paper, we have produced hybrid cells between *S. gratus* and *S. amboensis* by electrofusion and studied the biotransformation of **1** by fused cells.

RESULTS AND DISCUSSION

Culture of fused protoplasts

White cell lines of *S. gratus* (Sg) and yellowish green cell lines of *S. amboensis* (Sa) grown in the light were

used as the parent lines for the production of heterokaryons. The difference between the two cell lines is that the cells of the green line contains chloroplasts. The diameters of the Sg protoplasts were about 50 μ m and those of the Sa protoplasts were about 20 μ m. Sg protoplasts were more susceptible to damage by electrofusion than Sa protoplasts. We, therefore, attempted to identify the fusion conditions that would not damage Sg protoplasts but that would at the same time yield high heterokaryon fusion rates. The optimum condition for electrofusion was found to be 1.0 kV cm⁻¹ DC pulse of 30 μ s duration. The frequencies of protoplast fusion were counted by microscopic observation. The frequency of heterokaryon formation was estimated to be 5.5% of total protoplasts. The frequencies of homokaryon formation were 8.7% (Sg) and 2.2% (Sa). Hence, a method of selecting the heterokaryons from the homokaryons and infused protoplasts was needed. We first tried to cultivate Sg and Sa protoplasts in a variety of media based on Murashige and Skoog's (MS) [7] and 8p medium [8]. The highest frequency of cells undergoing division was obtained for Sg protoplasts on 1/2MS (twice diluted) medium (Fig. 1). No dividing cells were obtained from Sa protoplasts on this mode. We, therefore, selected 1/2MS medium for culturing protoplasts after fusion treatment. As the colour of Sg cells was white, while Sa cells were yellowish green when grown in the light,

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Fig. 1. Division frequency in a week old culture of S. gratus (Sg) and S. amboensis (Sa) protoplasts.

any yellowish green cells growing from the fusiontreated protoplasts in 1/2MS medium would be putative somatic hybrids. After 7–9 weeks, small colonies from fusion-treated protoplasts were observed in 1/2MS medium, and they grew faster than untreated protoplasts. On transferring to MS solid medium under continuous illumination, many cell lines between white and green were obtained 1–2 months later.

Isozyme analysis

Some of the cell lines obtained from fusion-treated protoplasts were homogenized and isozyme analyses were performed. The electrophoresis bands of acid phosphatase and leucine aminopeptidase in most of the cell lines examined were combinations of both parents' bands, and these cell lines were designated as hybrids. However, the intensity of bands differed cell by cell (data not shown). Because of the lack of distinction of the bands, some cell lines could not be assigned. Therefore, we used RFLP analysis to determine whether these cell lines were hybrids or not.

RFLP analysis

We randomly selected some yellowish green cell lines (B2-18, C14, 18, E5 and F8) from the many cell lines obtained from fusion-treated protoplasts. When the DNA extracted from the cell was digested with NheI, all of the cell lines had a combination of parents' DNA fragments hybridized with the probe (Fig. 2). The results indicate that they are all hybrids. When the DNA was digested with EcoRI, BamHI, XbaI, HindIII, EcoRV, XhoI, SpeI, BlnI, CpoI, NotI and Sse83871, there were no particular fragments due to parents' DNA (data not shown).

Biotransformation of digitoxigenin (I) by hybrid cells

Experiment 1: after incubation of digitoxigenin (1) (120 mg) with F8 cells (262 g fr. wt) for 15 days, periplogenin β -D-glucoside (7) (65 mg; yield 37%, the main product) and compound 8 (7 mg; vield 4%) were isolated as the biotransformation products. At the same time, $17\beta H$ -periplogenin (3), 3-epi- $17\beta H$ -periplogenin (4), 3-epiperiplogenin (5) and digitoxigenin β -D-glucoside (6) were identified with authentic samples by TLC and HPLC. The HR-FAB-mass spectrum of 8 exhibited a peak due to $[M + Na]^+$ at m/z 575.2880 $(C_{29}H_{44}O_{10}Na)$. In the ¹H NMR spectrum of 8, signals at δ 4.43 (1H, br s, $W_{1/2} = 8$ Hz, H-3 α), 3.30 (1H, dd, $J = 9.5, 9.5 \text{ Hz}, \text{H}-17\beta$) and 1.06 (3H, s, H₃-18) were observed for the aglycone part and signals at δ 4.96 $(1H, d, J = 8 Hz, H^{-1'}), 3.93 (1H, dd, J = 8, 8 Hz, H^{-1})$ 2'), 4.28 (1H, dd, J = 12, 5 Hz, H-6a') and 4.47 (1H, dd, J = 12, 2 Hz, H-6b') for the sugar part. In the ¹³C NMR spectrum, the data for the aglycone part were similar to those of $17\beta H$ -periplogenin (3) [1], except for C-2 to C-4, and the data for the sugar part corresponded to the data for sugar part of periplogenin β -D-glucoside (7) [9]. From these spectral data, the structure of 8 was elucidated as $17\beta H$ -periplogenin β -D-glucoside. It is a new compound which is unique to the hybrid cells.

Experiment 2: after incubation of 1 with hybrid cell lines (F8, B18 and C14), the parent cells (Sg and Sa, separately) and a mixed culture of Sg and Sa cells, the chloroform and chloroform-isopropyl alcohol (3:2) fractions were analysed by HPLC (Table 1). Both 7 and 8 were detected in three hybrid cell lines (7 > 8: by comparison of peak area on HPLC) but only 7 was detected in Sa cells and the mixed culture of Sg and Sa cells. On the other hand, in agreement with previous studies [1, 2], neither 7 nor 8 was detected in Sg cells.

Therefore, the isomerization of the 17β -lactone ring brought about by Sg cells and the glucosylation by Sa



Fig. 2. Hybridization of labelled pRR217 to Nhe I digests of DNA from S. gratus (Sg), S. amboensis (Sa) and the cells developed from fusion treated protoplasts between Sg and Sa,

Table 1. Biotransformation products of digitoxigenin (1) byS. gratus (Sg), S. amboensis (Sa) and their hybrid cells (F8,B18 and C14 cell lines)

Products	Sg	Sa	Sg + Sa	F8	B 18	C14
3	*		*	*	*	
4	*		*			
5		*	*	*	*	
6		*	*	*	*	*
7		**	*	**	**	**
8				*	*	*

*,** Identified with authentic samples (*<**: peak area on HPLC).

cells occurred simultaneously in the hybrid cells. The possible biotransformation pathway of 1 by the hybrid cells is shown in Scheme 1. Although 3–5 were not detected in the C14 cell line, it seems that this cell line has higher activity of glucosylation (1 to 6, 2 to 7 and 3 to 8) than of epimerization of 3β -hydroxyl to 3α -hydroxyl (2 to 5 and 3 to 4) compared to F8 and B18 cell lines.

Experiment 3: after incubation of 1 (410 mg) with F8 cells (586 g fr. wt) for 18 or 19 days in a medium containing a higher concentration of sucrose (60 g 1^{-1}), 7 (50 mg; yield 8.3%) and 8 (98 mg; yield 16.2%) were isolated as crystals. Periplogenin (2) and 3–6 were identified with authentic samples by HPLC. The

greater production of **8** relative to **7** observed in this culture is due to unknown causes.

EXPERIMENTAL

General. ¹H and ¹³C NMR: Varian XL-400; EI-MS and positive FAB-MS: Jeol JMS-DX300; HR-FAB-MS: Jeol JMS-AX505.

Protoplast isolation. Callus cells induced from leaves of Strophanthus gratus [1] and S. amboensis [2] were used as parents. In order to select hybrid cells, the white cell line of S. gratus (Sg) and the yellowish green cell line of S. amboensis (Sa) grown in the light were used. Protoplasts were prepd from cell suspensions of Sg and Sa at 4–9 days after inoculation into MS medium containing sucrose 30 g l⁻¹, 1 mg l⁻¹, 2, 4-D and 0.1 mg l⁻¹ kinetin (DK medium). After incubating the cells in enzyme soln containing 0.6 M mannitol, 1% Cellulase Onozuka RS (Yakult Honsya Co., LTD), 0.1% Pectolyase Y23 (Seishinseiyaku Co., LTD) for 4 hr at 30°, the protoplasts were isolated by centrifugation in 20% sucrose.

Protoplast fusion and culture. The freshly isolated species of protoplasts (Sa and Sg) were mixed $(5 \times 10^4$ protoplasts ml⁻¹ each) and fused in a fusion chamber with 1.0 kV cm⁻¹ DC pulses of 30 μ s duration using a somatic hybridizer (Shimazu Co., Ltd.). After 10 min, the treated cells were collected by centrifugation and cultured at 10⁴ cells ml⁻¹ in 1/2MS medium containing 0.6 M mannitol, 10 g l⁻¹ sucrose, 1 mg l⁻¹ 2, 4-D and 0.1 mg l⁻¹ kinetin in the dark at 30°. When the cell colonies grew to *ca* 0.5–1 mm in diameter (7–



Scheme 1. Biotransformation of digitoxigenin (1) by S. gratus (Sg), S. amboensis (sa) and their hybrid cells.

9 weeks after electrofusion), they were transferred to DK solid medium containing gellan gum 2 g l^{-1} . The cultures were grown under continuous illumination (10 000 lux) using a fluorescent lamp at 30⁺.

Iosenzyme analysis. Cells were homogenized with 0.1 M Na-Pi buffer (pH 7.0) at 4°. After centrifugation, isoenzymes in the supernatant were analysed by native PAGE (1 mm thick gel, 7.5% acrylamide). The gels were stained to detect acid phosphatase [10] and leucine aminopeptidase [11].

RFLP analysis. DNA was extracted from the cells according to the method of ref. [12]. The DNA was digested with restriction enzyme. Restriction fragments were sepd by electrophoresis in agarose gels. Southern blotting to nylon membrane (Hybond-N, Amersham) was carried out using the VacuGene vacuum blotting system (Pharmacia LKB). After drying, the membrane efficiently held DNA by crosslinking with a UV transilluminator. The labelling of DNA, hybridization and detection were performed using the DNA Labelling and Detection Kit Non-radioactive from Boehringer. Plasmid pRR217, containing ribosomal DNA from rice in the EcoRI cloning site of pBR325, was used as a probe [13]. The plasmid pRR217 was generously provided by Dr K. Oono.

Biotransformation of digitoxigenin (1) by hybrid cells. Digitoxigenin (1) (30–50 mg) suspended with Tween 80 (5%) was added to each flask (250 ml DK liquid medium $1 1^{-1}$ flask) and then the cells (*ca* 30–

70 g fr. wt per flask) from 4-week-old static cultures were incubated at 25° under light in a shaker (90 strokes min⁻¹) for 15–19 days. After incubation, CHCl₃ and CHCl₃–*iso*PrOH (3:2) extracts of the cells and the medium were prepared and examined on TLC with visualization using Kedde's reagent and 10% H₂SO₄. After purification using silica gel CC and pre. HPLC (Senshu Pak. PEGASIL ODS, solvent 60% MeOH in H₂O, flow-rate: 3 ml min⁻¹), the biotransformation products were isolated or identified with authentic samples by TLC and HPLC.

Experiment 1: 1 (120 mg: 30 mg \times 4), F8 cells (262 g fresh wt), and 15 days incubation. Compounds 7 (65 mg; yield 37%) and 8 (7 mg; yield 4%) were isolated and 3–6 were identified.

Experiment 2: 1 (90 mg: 30 mg × 3) and F8 cells (173 g fresh wt); 1 (60 mg: 30 mg × 2) and B16 cells (106 g fresh wt); 1 (60 mg: 30 mg × 2) and C14 cells (87 g fresh wt); 1 (90 mg: 30 mg × 3) and Sg cells (178 g fresh wt); 1 (90 mg: 30 mg × 3) and Sg cells (133 g fresh wt); 1 (90 mg: 30 mg × 3) and Sg cells (87 g fresh wt)+Sa cells (81 g fresh wt), 17 days incubation in each case. These CHCl₃-*iso*PrOH (3:2) frs were analysed by HPLC, especially 7 and 8 (R_i 10.2 min and 8.4 min: solvent 60% MeOH in H₂O). Both 7 and 8 (7 > 8) were detected in three hybrid cell lines, only 7 was detected in Sa cells and the mixed culture of Sg and Sa cells, neither 7 nor 8 was detected in Sg cells.

Table 2. Biotransformation products of digitoxigenin (1)
formed by S. gratus (Sg), S. amboensis (Sa) and their hybrid
cells (F8, B18 and C14 cell lines)

Products	Sg	Sa	$Sg + Sa^+$	F8	B18	C14
2	*		*	*	*	*
3	*		*	*	*	
4	*		*			
5		*	*	*	*	
6		*	*	*	*	*
7		**	*	**	**	**
8				*	*	*

⁺ Co-culture of Sg and Sa in same flask.

*,** Identified with authentic samples (*<**: peak area on HPLC).

Experiment 3: 1 (410 mg: 40 mg × 4, 50 mg × 2, 50 mg × 3) and F8 cells (586 g fresh wt: 255, 142 and 189 g) were incubated for either 18 or 19 days in DK liquid medium containing sucrose (60 g 1^{-1}). Compound 7 (50 mg; yield 8.3%) and 8 (98 mg; yield 16.2%) were isolated and 2–6 were identified by HPLC (17.9, 16, 9.9, 11.6 and 13.2 min).

 $17\beta H$ -Periplogenin β -D-glucoside (8). Amorphous solid. HR-FAB-MS: $C_{29}H_{44}O_{10} + Na$ required 575.2832, $[M + Na]^+$ at m/z 575.2880; EI-MS m/z (rel. int.): 552 [M]⁺ (5), 354 (69), 336 (52), 318 (100), 300 (40), 201 (43); ¹H NMR (400 MHz, C₅D₅N): δ 1.00 (3H, s, H₃-19), 1.06 (3H, s, H₃-18), 3.30 (1H, dd, $J = 9.5, 9.5 \text{ Hz}, \text{H-}17\beta$, 3.93 (1H, dd, J = 8, 8 Hz, H-2'), 4.28 (1H, dd, J = 12, 5 Hz, H-6a'), 4.43 (1H, br s, $W_{1/2} = 8$ Hz, H-3 α), 4.47 (1H, dd, J = 12, 2 Hz, H-6b'), 4.69 (1H, br d, J = 18 Hz, H-21a), 4.85 (1H, brd, J = 18 Hz, H-21b), 4.96 (1H, br d, J = 8 Hz, H-1'), 5.99 (1H, br s, H-22); ¹³C NMR (100 MHz, C₅D₅N): δ 26.3 (t, C-1), 26.9 (t, C-2), 74.1 (d, C-3), 34.1 (t, C-4), 73.4 (s, C-5), 36.5 (t, C-6), 24.4 (t, C-7), 40.9 (d, C-8), 39.5 (d, C-9), 41.3 (s, C-10), 21.2 (t, C-11), 31.2 (t, C-12), 49.4 (s, C-13), 85.4 (s, C-14), 31.8 (t, C-15), 25.1 (t, C-16), 49.0 (d, C-17), 18.7 (q, C-18), 17.5 (q, C-19), 173.0 (s, C-20), 74.3 (t, C-21), 116.8 (d, C-22), 174.4 (s, C-23), 101.6 (d, C-1'), 75.4 (d, C-2'), 79.0 (d, C-3'), 71.9 (d, C-4'), 79.1 (d, C-5'), 62.9 (t, C-6').

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