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Enhanced Production of Digoxin by Digitoxin Biotransformation Using *In Situ* Adsorption in *Digitalis lanata* Cell Cultures

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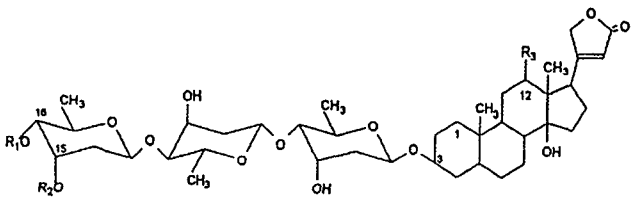
Abstract For the enhanced production of a cardiac glycoside, digoxin, using *in situ* adsorption by biotransformation from digitoxin in plant cell suspension cultures, selection of proper resins was attempted and the culture conditions were optimized. Among various kinds of resins tested, Amberlite XAD-8 was found to be the best for digoxin production in considering adsorption characteristics as well as the effect on cell growth. Adequate time for resin addition was determined to be 36 h from the beginning of biotransformation and the presence of resins should be as short as possible to increase the productivity. In addition, to prevent the cells from direct contact with resin particles, immobilized systems were designed and examined. Immobilization further improved the advantages of *in situ* adsorption. It was confirmed that the increase of the contact area for mass transfer was an important factor in utilizing an immobilized system to enhance digoxin production.

Key words: *Digitalis lanata*, biotransformation, digitoxin, digoxin, plant cell culture, *in situ* adsorption

Biotransformation by plant cell cultures is a technique of utilizing enzymes in plant cells for the conversion of any organic chemicals into more valuable structures. In comparison to the well-known biotransformation capabilities of microorganisms, including steroid bioconversion, application of plant cell cultures for biotransformation has been limited [15]. However, it is very important to use plant cells in culture for the production of useful compounds by exploiting biotransformation reactions [13]. It is useful especially in the case of phytochemicals with streospecificity and complex structure that hamper chemical synthesis [2]. Reactions which are restricted only to plant cells and which produce chemicals of high economic value can be of commercial interest and biotechnological relevance. One such example is the biotransformation of digitoxin into digoxin by *Digitalis lanata* cultures [1, 5]. Cardiac glycosides (cardenolides) such as digitoxin and digoxin are important pharmaceuticals in the treatment of a certain cardiac disease [11].

Cardenolides consist of a steroid nucleus and a sugar side-chain of variable length. Basic structures are shown in Fig. 1. Among them, digoxin is the most commonly used cardenolide due to its better pharmaceutical properties. Many researchers have studied ways to make better use of this compound, including extensive studies on biotransformation by *Digitalis cell* cultures [7, 8, 9, 14]. The main reaction is the 12β-hydroxylation, which converts digitoxin to digoxin by 12β-hydroxylase. Once the side reaction, i.e. 16'-O-glucosylation, proceeds by uridine diphosphate glucose (UDP-glucose): digitoxin 16'-O-glucosyltransferase, unwanted products such as purpureaglycoside A and deacetyllanatoside C are synthesized from digitoxin and digoxin, respectively.

Although digitoxin and digoxin can permeate cell membrane, their glucosylated forms are accumulated in the vacuoles [6]. Therefore, it is desirable to adsorb digoxin in the medium selectively by using proper



	R_1	R_2	R_3
Digitoxin	H	H	H
Digoxin	H	H	OH
Purpureaglycoside A	Glucosyl	H	H
Deacetyllanatoside C	Glucosyl	H	OH
Lanatoside A	Glucosyl	CH_3	H
Lanatoside C	Glucosyl	CH_3	OH
α-acetyldigitoxin	H	CH_3	H
α-acetyldigoxin	H	CH_3	OH

Fig. 1. Chemical structures of digitoxin and its derivatives.

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adsorbents as soon as it is produced. This kind of *in situ* adsorption has been used successfully for the efficient production of various phytochemicals in plant cell culture systems [12, 16]. Depending upon the nature of the products, a neutral styrene divinylbenzene resin (XAD-4), a neutral polycarboxylic ester resin (XAD-7), and activated charcoal were used as adsorbents [3, 4, 10]. However, there has been no report on the utilization of *in situ* adsorption in any biotransformation by plant cell cultures. Enhanced production of the target compound digoxin can be expected by *in situ* removal during the biotransformation because of the prevention of further reaction into glucosylated byproduct. Due to the close similarity in chemical structures of digitoxin and digoxin, selective adsorption of digoxin is not easy. Hence, it is important to choose a highly selective resin as well as to optimize when the resin will be added and how long the presence of resins should be maintained.

In this study, various resins were evaluated for use in the increased production of digoxin from digitoxin by biotransformation with *in situ* adsorption using *Digitalis lanata* cell suspension cultures. For the efficient use of selected resins, optimizations of addition time and the length of contact time were investigated. Moreover, effects of the types of resin, such as free powder and immobilized forms, on the production of digoxin were also investigated.

MATERIALS AND METHODS

Plant Cell Culture and Culture Medium

The *Digitalis lanata* cell line K3OHD was kindly provided by Dr. Wolfgang Kreis (Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany) and had been maintained in modified Murashige and Skoog medium containing 340 mg/l KH_2PO_4, 4 mg/l glycine, and 30 g/l glucose. Growth regulator was omitted. The suspension cultures were grown in 100-ml Erlenmeyer flasks with 50 ml of medium on a gyratory shaker at 150 rpm under dark conditions. The temperature was kept at 25°C and the cells were subcultured in 10 day intervals.

Biotransformation

For the production of digoxin from digitoxin, 8% (w/v) glucose solution at pH 5.5 without any other nutrient was used as a production medium [9]. Cells in the late exponential growth phase in growth medium were used as inoculum in the production phase. In order to avoid heterogeneity of the inoculum, all the cells from different flasks were collected in a large flask and mixed well by shaking. The cells were filtered through Whatman No. 1 filter paper on a Buchner funnel under slight vacuum and washed with fresh production medium. Eight gram fresh cells were inoculated into a 100-ml Erlenmeyer flask containing 30 ml of production medium. Digitoxin was supplied aseptically using stock solution at 30 g/l in dimethyl sulfoxide (DMSO) and the biotransformation was carried out at 25°C. All the experiments were performed in duplicate. The results reported were obtained from the average value from duplicate cultures and the deviation was not significant.

Biotransformation with *In Situ* Adsorption

Several kinds of resins were tested to select the best one for adsorption of digoxin. There were various nonionic exchange resins (Amberlite XAD-4, XAD-7, XAD-8), acidic cation exchange resins (Dowex 50 W, Amberlite IRC-50, IRC-200), basic anion exchange resins (Dowex 1 IX4-50, Amberlite IRA-93, IRA-400), and a mixed bed resin (TMD-8). All the resins were obtained from Sigma Chemical Co. (U.S.A.). For *in situ* adsorption of the product, the resins were added into the culture as free powder or in immobilized forms. Immobilized resins were utilized to prevent the cells from direct contact with adsorbents and they were prepared by one of the following methods, Ca-alginate gel immobilization, entrapment in Superseal teabag paper (Crompton, U.S.A.), or packing in a glass tube with two permeable ends (teabag paper). The average diameter of the Ca-alginate gel bead was 4 mm. The sizes of teabag paper and glass tube were 28 mm × 28 mm and 10 mm × 30 mm, respectively. Superseal is a plain paper consisting of a selected blend of cellulose and thermoplastic fibers and is used as a heat-sealing teabag because it has excellent particle retention and a high degree of wet strength. The amount of resins used in all experiments was the same at 5% (w/v) and all the resins were pretreated with methanol by soaking for 24 h and washed with several volumes of distilled water.

Analysis of Cardenolide

The total methanolic extract of the suspension culture was obtained by adding the same amount of methanol as that of the culture broth and sonicating for 20 min. After centrifugation, the supernatant was used for the determination of cardenolides. When resins were used for *in situ* adsorption, they were separated from the culture broth and adsorbed cardenolides were extracted with methanol. Filtered samples were injected into an HPLC system (Model 910, Young-In Scientific Co., Korea) with a UV detector. A Curosil G column (4.6 mm × 250 mm, 6 μm, Phenomenex Inc., U.S.A.) was used for the analysis. The mobile phase was a mixture of acetonitrile and water (35:65, v/v). The flow rate was 1 ml/min and measuring wavelength was 220 nm. Standard cardenolides for HPLC analysis was purchased from Roth (Germany) and the solvent was obtained from Fisher Scientific (U.S.A.).

RESULTS AND DISCUSSION

Selection of Resins as Solid Adsorbents

Biotransformation efficiency can be increased by *in situ* recovery using the appropriate resin with a higher selectivity for the product. However, most of the resins with high affinity to digoxin may adsorb digitoxin almost to the same extent because the chemical structures of both cardenolides are quite similar. Uptake of unused substrate digitoxin may not be possible if it is adsorbed. Therefore, a comparative study was performed to choose a resin which can be used for *in situ* adsorption experiments. Various kinds of resins were selected such as nonionic, acidic, basic, and mixed type resins. Cardenolide stock solution (30 g/l in DMSO) was added into a flask containing 5% (w/v) resins to make the final concentration at 100 mg/l. As shown in Figs. 2 and 3, adsorption of both digitoxin and digoxin on nonionic exchange resins was proven to be the best for adsorption. Among those nonionic resins, XAD-7 and XAD-8 show the fastest adsorption rate to reach the highest level. Most of cardenolides supplied were adsorbed within 2 h. However, in terms of the adsorption capacity, XAD-8 was much better than XAD-7. In other studies on the selective adsorption on plant products, XAD-7 was commonly used [12, 16]. In ajmalicine production from *Catharanthus roseus* cell cultures, XAD-7 greatly enhanced the release of ajmalicine with 40% increase in total productivity [3]. The adsorption and desorption of sanguinarine from aqueous solution was most effective with XAD-7 [16]. However, XAD-4 resin was selected as the adsorbent for caffeine production from *Coffea arabica* cells because it displayed a higher capacity for adsorbed caffeine than XAD-7 [10].

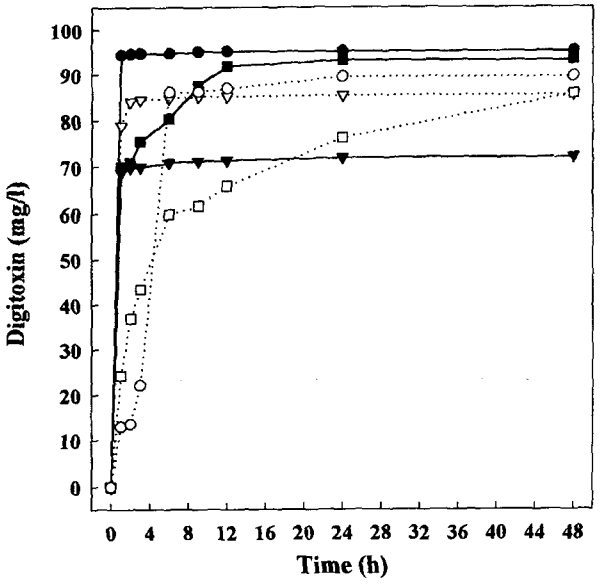

Fig. 2. Adsorption kinetics of digitoxin on nonionic exchange resins (●, XAD-8; ■, XAD-4; ▼, XAD-7) and acidic cation exchange resins (○, IRC-200; □, Dowex 50W; ▽, IRC-50).

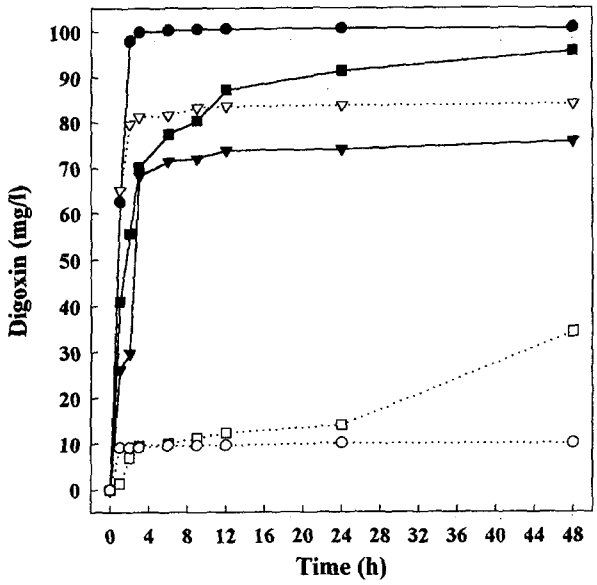

Fig. 3. Adsorption kinetics of digoxin on nonionic exchange resins (●, XAD-8; ■, XAD-4; ▼, XAD-7) and acidic cation exchange resins (○, IRC-200; □, Dowex 50W; ▽, IRC-50).

Most of the studies on the selective adsorption of plant products were devoted to the nonionic resins. In the case of digitoxin and digoxin, the adsorption rate and capacity of Amberlite IRC-50, an acidic cation exchange resin, were higher than those of XAD-7. Adsorption of digoxin on the other resins including Dowex 1 IX4-50, IRA-93, IRA-400, and TMD-8 was found to be quite low (data not shown). Therefore, XAD-7, XAD-8 and IRC-50 were selected for the following study.

Effect of Resins on Plant Cell Growth

The resins were added to cell suspension cultures and the effects on growth were examined. Five days after inoculation with 3 kinds of resins, dry cell weight was measured for comparison. The results are shown in Fig. 4. It was clear that the addition of resins inhibited cell growth significantly. The inhibition by IRC-50 was the most evident and XAD-7 was the next. On that account, IRC-50 was not used for further studies. The reason for the repression of cell growth is usually explained by the removal of some essential components in the medium and the reduction of their availability to the cells [16]. Unlike these two resins, XAD-8 was less inhibitory.

Optimization of Resin Addition Time and Length of Contact Time

Since none of the resins tested showed any selectivity on digoxin, optimization of addition time was thought to be

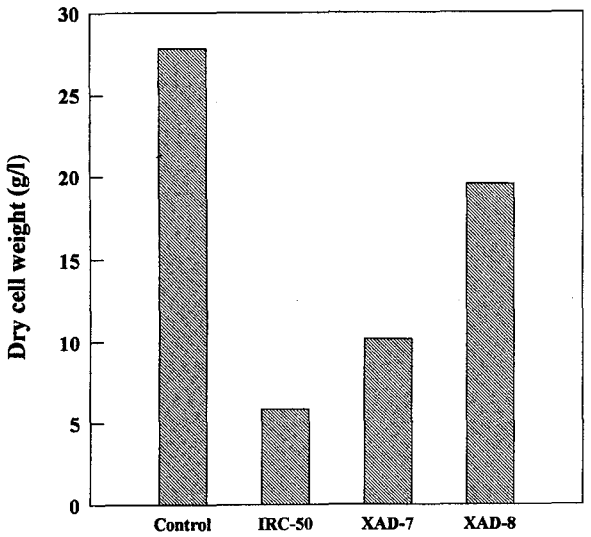

Fig. 4. Effect of IRC-50, XAD-7, and XAD-8 resins on *Digitalis lanata* cell growth.

necessary to enhance the efficiency of biotransformation. The best time for resin addition might be the moment when the digoxin level reached maximum while digitoxin was almost used up. To decide the optimum time for addition, XAD-8 resin was added at various times after the onset of biotransformation with 200 mg/l of digitoxin. Biotransformation was performed for 48 h against each case and digoxin levels are shown in Fig. 5. When the resins were added at 36 h, digoxin level was the highest. This result corresponded with the time course of digitoxin biotransformation without the resins as shown

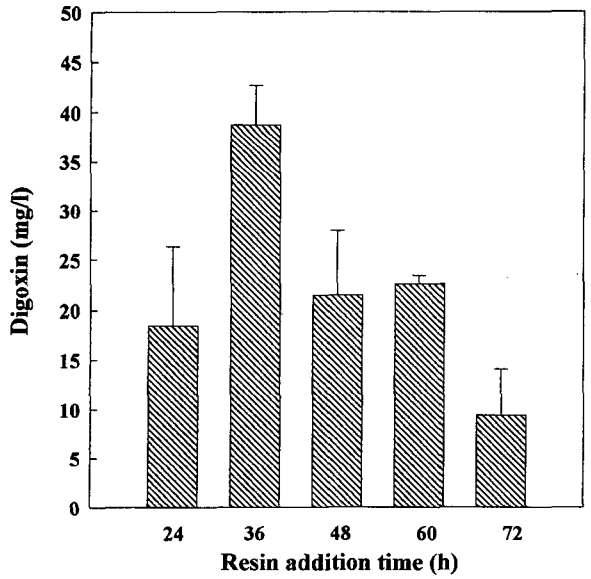

Fig. 5. Effect of XAD-8 resin addition time after the beginning of biotransformation on the production of digoxin. Biotransformation was continued for 48 h after the addition of XAD-8 resin.

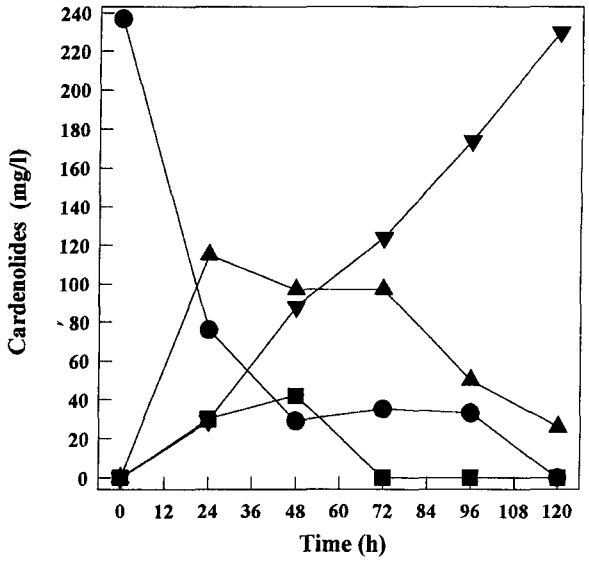

Fig. 6. Time course of biotransformation of digitoxin by *Digitalis lanata* cell suspension cultures (●, digitoxin; ■, digoxin; ▼, deacetyllanatoside C; ▲, purpureaglycoside A).

in Fig. 6. Around 36 h after the beginning of biotransformation, more than 80% of digitoxin was consumed and digoxin concentration reached a high level. After that time, glucosylation continued which decreased the digoxin yield. Accordingly, it was apparent that the optimum resin addition time was 36 h after the supply of digitoxin.

The longer the presence of resins in the culture, the greater may be the effect of *in situ* adsorption on the biotransformation. Therefore, the effect of duration of contact after the addition of resins at 36 h on digoxin yield was investigated. In the case of XAD-8 resin addition, contact time from 6 h to 30 h did not affect digoxin yield greatly. In order to reduce the overall process time, it is better to reduce the contact time if there are no big differences in digoxin yields. Consequently, when XAD-8 is used, 6 h of contact will be enough for the enhanced production of digoxin. However, when XAD-7 resin was added, the result was different. The longer the contact with XAD-7 up to 30 h, the lower the production of digoxin. This might be due to the difference in digoxin affinity between two resins. Severer growth inhibition by XAD-7, as shown in Fig. 4, could be another reason. In this sense, we can say that XAD-8 has advantages over XAD-7 for digoxin adsorption.

Utilization of Immobilized XAD-8 Resin

About 15% increase of digoxin production by using *in situ* adsorption was noticed when the optimized conditions were applied. However, the level of increase was lower than expected. The reason for this phenomenon was thought to originate from the free contact between cells and

resins during the course of biotransformation. Although the resins adsorbed digoxin, it could be desorbed and taken up by the cells through direct contact. It was confirmed that if digoxin was adsorbed intentionally prior to use and added into the cell culture in the adsorbed state, it was glucosylated into deacetyllanatoside C (data not shown). This means that the cells can absorb digoxin attached on the surface of resins because spontaneous desorption was negligible when the cells were not present. To prevent the cells from direct contact with resins, an immobilization technique was applied. In addition to the prevention of direct contact, utilization of immobilization provides an advantage of easy recovery of resins and helps in reuse. For this purpose, three types of immobilized XAD-8 resins were used: Ca-alginate gel immobilization, entrapment in Superseal teabag paper, and packed bed in glass tube with two permeable ends. Free XAD-8 resins were also used for comparison. Optimized resin addition time and length of contact time obtained from the above experiments were used. Biotransformation was performed in 100-ml shake flasks and the results are summarized in Fig. 7. As anticipated, digoxin production was enhanced with the addition of free resins. The highest digoxin yield was obtained when teabag paper was used for the immobilization of resins. On the other hand, contrary to expectation, digoxin yields in the Ca-alginate gel immobilized system and the packed system were lower than that with free resins. This can be explained by the mass transfer limitation of the two systems. The lower yield in the packed bed system which had the smallest mass transfer area showed the importance of the contact area for adsorption. Therefore, for more complete comparison, the same mass transfer area should have been applied in addition to the same amount of resins. The reason why the fraction of digoxin in the cells was large compared with other cases in packed beds may also be explained by the insufficient transfer of medium through the glass tube. Use of encapsulated resins by teabag paper enhanced the production of digoxin mostly because they provided not only the largest contact area among the three systems but also the proper partitioning between cells and resins.

In conclusion, it was confirmed that the application of *in situ* adsorption using adequately immobilized resins enhanced the production of digoxin by 12β-hydroxylation significantly.

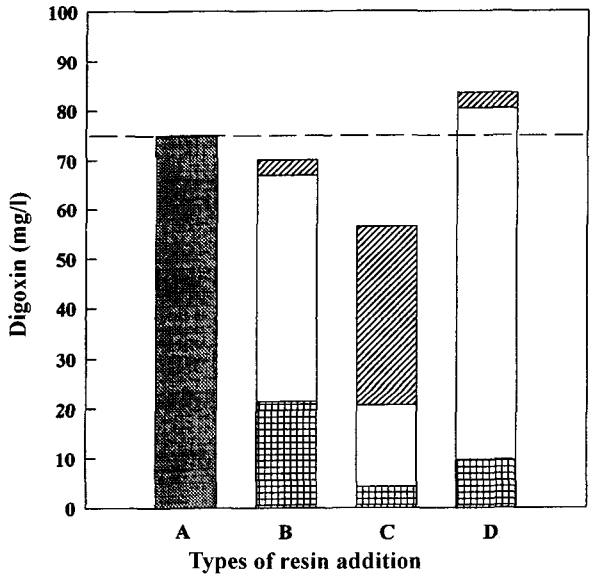

Fig. 7. Effect of various types of XAD-8 resin addition with the optimized addition time and length of contact on the production of digoxin.

A, free resin; B, Ca-alginate gel immobilized resin; C, immobilized resin in glass tube with two permeable ends; D, immobilized resin in Superseal teabag paper (▨, digoxin in the cells; □, digoxin adsorbed on the resins; ■, digoxin in the medium). When free resin was added, distribution of digoxin was not detected. A perforated line indicates the amount of digoxin produced in the control cultures without resin.

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