Biotechnol. Bioprocess Eng. **1999**, 4, 32-35

Increased Production of Digoxin by Digitoxin Biotransformation Using Cyclodextrin Polymer in *Digitalis lanata* Cell Cultures

Jong-Eun Lee, Sang-Yoon Lee, and Dong-Il Kim*

Department of Biological Engineering, Inha University, Inchon 402-751, Korea

Addition of β-cyclodextrin (β-CD) polymer during the biotransformation of digitoxin into digoxin using cell suspension cultures of *Digitalis lanata* enhanced the conversion yield. Digitoxin showed better adsorption to CD polymer compared to digoxin, so that the optimization of addition time was found to be necessary. In the case of adding CD polymer 24 hours after the feeding of substrate digitoxin, the highest digoxin production could be achieved. At this period, digitoxin was almost consumed by cells and productivity was proportionally enhanced according as the amount of substrate was increased. Immobilization of CD polymer did not promote the biotransformation. When 3.33 g/L of CD polymer was added, 90% and 50% of digitoxin and digoxin was adsorbed respectively. Thus selective inclusion complex formation could be expected. Adsorption rate was found to be rapid and saturation was obtained within 10 hours of contact.

Key words: cyclodextrin polymer, *Digitalis lanata*, digitoxin, digoxin, biotransformation, plant cell culture

INTRODUCTION

Plant cell culture has the potential to make a significant impact on the production of useful metabolites for pharmaceuticals and various other uses [1]. It can be used for *de novo* production of phytochemicals as well as recombinant proteins. In addition, plant cells in culture can be used as an enzyme source in the biotransformation of organic chemicals into more valuable structures. It is especially valuable in the case of chemicals obtained from plant with stereospecificity and complex structures that hamper chemical synthesis. Reactions which are restricted only to plant cells and which produce economically valuable products can be commercially interesting with biotechnological relevance. One of the examples thoroughly studied for many years is the biotransformation of digitoxin into digoxin by *Digitalis lanata* cultures [2,3]. Digitoxin and its 12β-hydroxylated product, digoxin, are cardiac glycosides (cardenolides) used in the treatment of certain cardiac diseases [4]. Digoxin has better pharmaceutical properties. That is the reason why plant cell culture has been used for the biotransformation of digitoxin. Their chemical structures and derivatives such as glucosylated cardenolides were shown in the previous report [5].

Schematic diagram of digitoxin biotransformation is shown in Fig. 1. Two enzymatic conversion steps are involved. One is 12β-hydroxylation (step 1) and the other is 16′-O-glucosylation (step 2). In order to produce digoxin effectively, step 2 reaction should be minimized not to produce unwanted side products such as purpureaglycoside A and deacetyllanatoside C. Interesting fact is that both glucosylated side products are accumulated in the vacuoles, although digitoxin and digoxin can permeate cell membrane. Therefore, if we can remove digoxin selectively from the medium as soon as it is produced, its productivity can be enhanced theoretically. Any forms of integrated bioprocessing (extractive bioconversion) composed of cell culture and bioseparation *in situ* can be applied for this process. Extractive bioconversion is the concept of increasing the productivity of performance of biotechnological processes by the continuous removal of a product from the site of its production [6]. *In situ* adsorption with digoxin-selective resins was already found to be successful in enhancing digoxin production [5]. *In situ* extraction may also be used if we can find optimum two phase system including not only organic-aqueous two phase but also aqueous two-phase system.

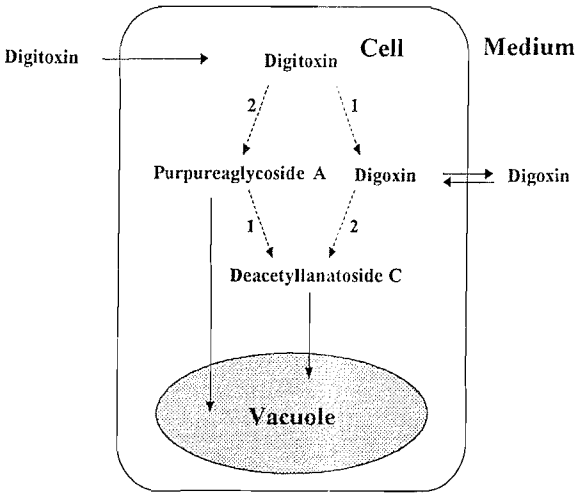

Fig. 1. Schematic diagram of digitoxin biotransformation in *Digitalis lanata* plant cell cultures: →, traffic; ⇢, enzyme reaction; 1, 12β-hydroxylase; 2, 16′-O-glucosyltransferase.

Cyclodextrins (CDs) are cyclic oligosaccharides compo-

* Corresponding author
Tel: +82-32-860-7515 Fax: +82-32-875-0827
e-mail: kimdi@inha.ac.kr

sed of 6, 7, or 8 α-1,4-linked D-glucose molecules [7]. They are usually named as α-, β-, or γ-CD, respectively. Their molecular structures are doughnut shaped with a hydrophobic shell and a more hydrophobic cavity. For that reason, CDs are water-soluble and able to form inclusion complexes by accommodating hydrophobic guest molecules within the cavity [8]. Since they are biocompatible and enhance solubilization of organic compounds, it is expected that they can be used to increase the solubility of hydrophobic plant secondary metabolites produced by cell suspension cultures in aqueous media. Formation of inclusion complexes may reduce toxicity of the product which results in enhancing the productivity. Stimulatory effects of CD in biotransformation were reported using microorganisms [8,9]. In addition to the utilization of various CDs, CD polymers can also be used. CD polymers can be prepared with various cross-linking agents such as epichlorohydrin, diisocyanatohexane, phenyl isocyanate, and divinylbenzene [10]. Polymeric forms of CD are insoluble in water so that they can be recycled and function as specific adsorbents. Therefore, it may be possible to use CD polymer as one of adsorbents *in situ* with cell cultivation. Various kinds of CD polymers have been used in the removal of bitter components from fruit juices [11] and decaffeination process [12].

In this study, β-CD polymer was used to enhance the production of digoxin from digitoxin biotransformation in *Digitalis lanata* cell suspension cultures. Optimization of feeding condition of β-CD polymer was performed. Feeding effects and adsorption characteristics were also investigated.

MATERIALS AND METHODS

Cell Lines and Media

The *Digitalis lanata* cell line K3OHD was provided by Dr. 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 not added. Suspension cells were grown in 100-mL Erlenmeyer flasks with 50 mL of medium on a gyratory shaker at 150 rpm under dark conditions. Temperature was kept at 25℃ and the cells were subcultured in 10 days intervals.

Biotransformation

For the production of digoxin from digitoxin, 8% (w/v) glucose solution without any other nutrient at pH 5.5 was used as a production medium [3]. Cells in the late exponential growth phase in growth medium were used as inoculum in 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 and the biotransformation was carried out at 25℃. All the experiments were performed in duplicate. The results reported were obtained from the average of results from duplicate cultures and the deviation was not so significant.

Utilization of Cyclodextrin Polymer

β-CD polymer was obtained from Aldrich Chemical Co. (U.S.A.). In addition to free CD polymer, immobilized CD polymer in Superseal teabag paper was used to enhance recyclability. 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. For the investigation of adsorption kinetics of digitoxin and digoxin on CD polymer, stock solution of digitoxin and digoxin was added to make 200 mg/L into a flask with 30 mL of medium containing 1.67 and 3.33 g/L of CD polymer. Supernatants were removed at certain time intervals and the concentrations of both cardenolides were determined.

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. Supernatant after the centrifugation 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 HPLC system (Model 910, Young-In Scientific Co., Seoul, Korea) with UV detector. Curosil G column (4.6 $\times$ 250 mm, 6 μm, Phenomenex Inc., USA) was used for the analysis. The mobile phase was a mixture of acetonitrile and water (35 : 65, v/v). 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

Effects of Cyclodextrin Polymer Concentration and Addition Time

In order to use CD polymer in biotransformation effectively, optimization of addition time and concentration is necessary since addition of CD polymer to the bioconversion medium introduces a physicochemical interaction with both substrate and product. When free CDs were used, molar ratio of CD to substrate influenced microbial biotransformation [13]. In the case of using polymeric resins as *in situ* adsorbents, the best addition time was found to be the moment when the digoxin level reached maximum while digitoxin was almost consumed [5]. Fig. 2(a) shows the effect of β-CD polymer concentration on the production of digoxin. CD polymers were added at the beginning of biotransformation. Addition of CD polymers at 3.33 g/L lowered digoxin production considerably, while a slight increase was noticed at 1.67 g/L. Since the polymers were added at the beginning, they can adsorb the substrate, digitoxin. Signifi-

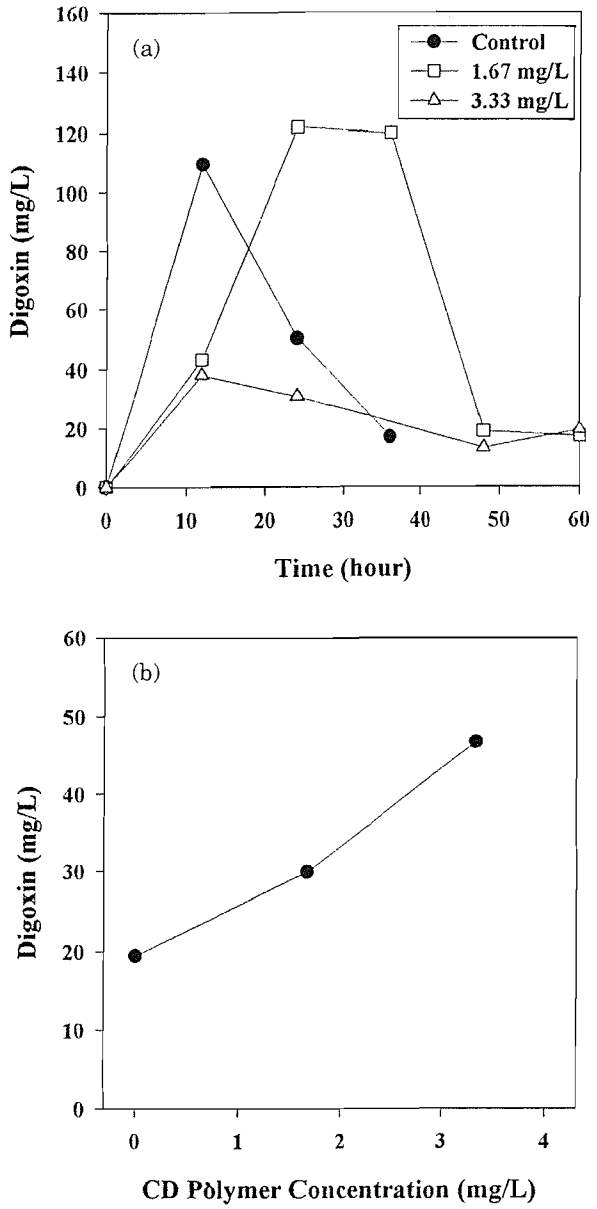

Fig. 2. Effects of CD polymer concentration and addition time on digitoxin biotransformation. CD polymer was added at the beginning of biotransformation (a) and 24 hours after the start of biotransformation (b) respectively.

cant reduction of digitoxin biotransformation at 3.33 g/L may be originated from digitoxin adsorption. Uptake of unused substrate digitoxin may not be possible if it is adsorbed. However, as shown in Fig. 2(b), when CD polymer was added 24 hours after the onset of biotransformation, presence of CD polymer enhanced digoxin production regardless of their concentration. Higher concentration at 3.33 g/L supported better results. The reason for this may be that digitoxin was taken up by the plant cells and some amount of digoxin was produced during initial 24 hours. Therefore, produced digoxin was adsorbed on CD polymer, which prevented the intermediate product from glucosylation and increased the level of digoxin in the culture medium. It is known that the longer the presence of adsorbent in the culture, the greater the effect of *in situ* adsorption. Thus, the length of contact time should also be considered.

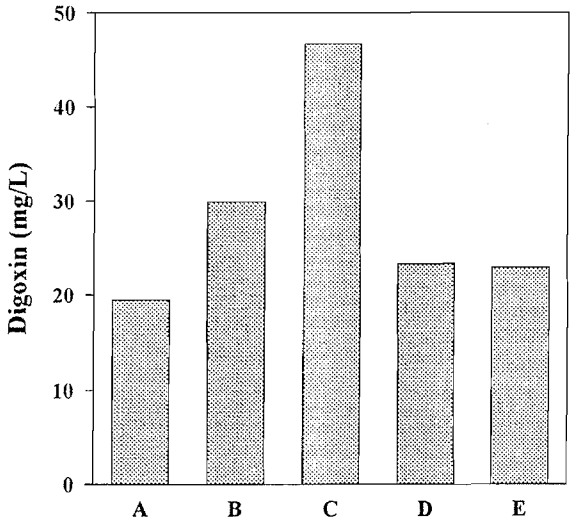

Fig. 3. Effects of CD polymer addition method on digoxin production in 100-mL flask containing 30 mL of medium: A, without CD polymer; B, 1.67 g/L free CD polymer; C, 3.33 g/L free CD polymer; D, 1.67 g/L immobilized CD polymer; E, 3.33 g/L immobilized CD polymer.

Feeding Effects

When CD polymer is used as free powder form, it is impossible to use them again. To enhance the recycled utilization of CD polymer, immobilization was considered for easier separation from the culture after the end of biotransformation. This may also help the purification of products. In addition to the advantages of easy recovery and reuse of CD polymer, it is possible to interfere direct contact between plant cells and CD polymer. It was shown that the adsorbed digoxin on polymeric resins could be desorbed and used by the cells through direct contact [5]. For the simple immobilization, entrapment in Superseal teabag paper was used. Comparison of mode CD polymer addition on digitoxin biotransformation was performed with 1.67 and 3.33 g/L of free and immobilized CD polymers. They are added at one day after the start of biotransformation and the results are summarized in Fig. 3. Although the addition of free CD polymer enhanced digoxin production significantly as already seen in Fig 2(b), immobilization reduced the enhancement. Contrary to the case of using polymeric resins as adsorbents, mass-transfer limitation may exert negative effect. Comparatively low amount of CD polymer added may be another reason. For further study, optimization of CD polymer concentration is thought to be desirable.

Adsorption Kinetics

Biotransformation efficiency can be increased by *in situ* recovery using appropriate second phase. Most of resins with high affinity to digoxin adsorbed digitoxin almost to the same extent because the chemical structures of both cardenolides are similar [5]. Depending upon the chemical structure of guest molecule, CD polymer had a greater capacity to form inclusion compounds than did CD monomer [11]. Adsorption kinetics of digitoxin and digoxin on CD polymers were studied to elucidate their characteristics and the results are shown in Fig. 4. When 1.67 g/L of CD polymers was added, 50% of both cardenolides were

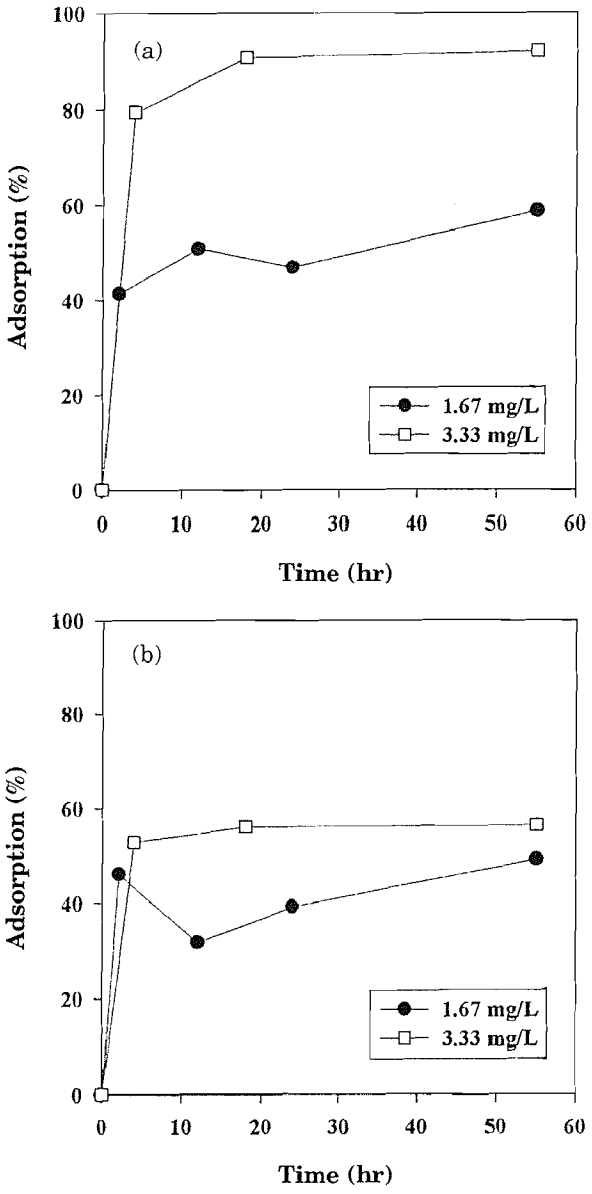

Fig. 4. Adsorption kinetics of digitoxin (a) and digoxin (b) on CD polymer at 1.67 and 3.33 g/L.

adsorbed very rapidly. However, when the amount of CD polymer was increased to 3.33 g/L, much more digitoxin was adsorbed compared to digoxin. This can explain the result seen in Fig. 2(a), where digoxin production was very low. Preferential adsorption of substrate digitoxin may inhibit the biotransformation apparently. At low concentration of CD polymer, considerable amount of digitoxin was not adsorbed and hydroxylated into digoxin.

In conclusion, adequate use of CD polymer at proper concentration enhanced the production of digoxin via biotransformation by *D. lanata* plant cell suspension cultures. By optimizing the mode of addition and their concentration, much more efficient biotransformation may be possible.

Acknowledgements This work was supported by sabbatical year program (1998) of Inha University.

REFERENCES

[1] Payne, G. F., V. Bringi, C. L. Prince, and M. L. Shuler (1991) *Plant Cell and Tissue Culture in Liquid System.* Oxford University Press. New York, NY.

[2] Jones, A. and I. A. Veliky (1981) Biotransformation of cardenolides by plant cell cultures. *Planta Med.* 42: 160-166.

[3] Kreis, W. and E. Reinhard (1992) 12 β -Hydroxylation of digitoxin by suspension-cultured *Digitalis lanata* cells: Production of digoxin in 20-litre and 300-litre air-lift bioreactors. *J. Biotechnol.* 26: 257-273.

[4] Luckner, M. and B. Diettrich (1988) Cardenolides. pp. 193-212 In: F. Constabel and I. K. Vasil (eds.) *Cell Culture and Somatic Cell Genetics.* Vol. 5. Academic Press. London, U.K.

[5] Hong, H.-J., J.-E. Lee, J.-E. Ahn, and D.-I. Kim (1998) Enhanced production of digoxin by digitoxin biotransformation using *in situ* adsorption in *Digitalis lanata* cell cultures. *J. Microbiol. Biotechnol.* 8(5): 478-483.

[6] Mattiason, B. and O. Holst (1991) Objectives for extractive bioconversion. p. 1-9. In: B. Mattiason and O. Holst (eds.) *Extractive Bioconversions.* Marcel Dekker, Inc. New York, NY.

[7] Szejtli, J. (1989) Downstream processing using cyclodextrins. *Trends Biotechnol.* 7: 170-174.

[8] Bar, R. (1989) Cyclodextrin-aided bioconversion and fermentation. *Trends Biotechnol.* 7: 2-4.

[9] Bar, R. (1989) Cyclodextrin-aided microbial transformation of aromatic aldehydes by *Saccharomyces cerevisiae. Appl. Microbiol. Biotechnol.* 31: 25-28.

[10] Shaw, P. E. and B. S. Buslig (1986) Selective removal of bitter compounds from grapefruit juice and from aqueous solution with cyclodextrin polymers and with Amberlite XAD-4. *J. Agric. Food Chem.* 34: 837-840.

[11] Shaw, P. E., J. H. Tatum, and C. W. Wilson, III (1984) Improved flavor of navel orange and grapefruit juices by removal of bitter components with β-cyclodextrin polymer. *J. Agric. Food Chem.* 32: 832-836.

[12] Yu, E. K. C. (1988) Novel decaffeination process using cyclodextrins. *Appl. Microbiol. Biotechnol.* 28: 546-552.

[13] Hesselink, P. G. M., S. van Vliet, H. de Vries, and B. Witholt (1989) Optimization of steroid side chain cleavage by *Mycobacterium* sp. in the presence of cyclodextrins. *Enzyme Microb. Technol.* 11: 398-404.