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DETERMINATION OF CARDENOLIDES IN HAIRY ROOT CULTURES OF *DIGITALIS LANATA* BY ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT.—A competitive ELISA technique using digoxin-specific antibody has been developed to determine digoxin and its related compounds in hairy root cultures of *Digitalis lanata*. The ELISA could detect 0.2–2 nM digoxin and closely related cardenolides. Hairy roots cultured in the dark accumulated very small amounts of cardenolides (0.02–0.07 μg/g dry wt), while the content of cardenolides in green hairy roots cultured in the light was increased maximally 600-fold (16.5 μg/g dry wt) compared to those in the dark.

Digitalis lanata Ehrh. (Scrophulariaceae), a biennial plant, is pharmaceutically important as a source of the cardenolides digoxin, lanatoside C, and deslanoside, which are commonly used in the treatment of heart diseases. Tissue cultures of this plant have been investigated by many researchers (1,2) for clonal propagation (3–5) and the production (6–8) and biotransformation of cardenolides (9–12). In order to estimate the productivity of a large number of clones, it is desirable to develop a simple assay technique to measure trace amount of the compounds in a small amount of sample. Radioimmunoassay (RIA) (13) and hplc (14,15) have been carried out for the quantitative analysis of cardenolides. However RIA cannot be used in a usual laboratory in Japan, and hplc requires much time for analysis. Therefore, we tried to establish the enzyme-linked immunosorbent assay (ELISA) for cardenolides to investigate the production of cardenolides by hairy root culture of *D. lanata*.

RESULTS AND DISCUSSION

ELISA FOR DIGOXIN.—The cross-reactivity of the digoxin-specific monoclonal antibody (DIG 64.2B.5) used in this study is summarized in Table 1. This antibody has a high affinity for digoxin and its related compounds, digoxigenin, lanatoside C, and deslanoside, which have the same aglycone as digoxin. It showed less reactivity with gitoxin and almost no reactivity with other cardenolides. This might be due to the different aglycone structure. A typical standard curve of the ELISA for digoxin is shown in Figure 1. It indicates that our competitive ELISA can detect 0.2–2 nM digoxin in a sample. In a preliminary experiment, an RIA for digoxin using the same antibody demonstrated almost the same detectable range. In order to verify our ELISA, the cardenolide contents in leaves of plants cultivated in the field or in hydroponic facilities were quantitatively analyzed by both hplc and ELISA (Figure 2). The content of digoxin equivalents in the leaves by ELISA correlated well to the total content of digoxin, deslanoside, and lanatoside C as analyzed by hplc. This indicates that the digoxin equivalents obtained by ELISA represent the total amount of digoxigenin glycosides. We then tried to estimate the production of cardenolides in hairy roots by ELISA.

PRODUCTION OF CARDENOLIDES IN HAIRY ROOT CULTURES.—Five hairy root clones were established by infection of *D. lanata* leaf segments with *Agrobacterium rhizogenes* strain A4. The hairy roots could be maintained in phytohormone-free (HF)

TABLE 1. Cross-reactivity of Anti-digoxin Antibody in ELISA.

Inhibitor	Relative IC_{50} value[a]
digoxin	1.0
digoxigenin	1.2
lanatoside C	1.2
deslanoside	1.8
gitoxin	46
digitoxin	2800
digitalin	16000
digitoxigenin	23000
gitoxigenin	>540,000
digitonin	>6,000,000
ouabain	>6,000,000
digitoxose	>120,000,000

[a]Concentration required to give 50% inhibition relative to digoxin. Concentration of digoxin was set at 1.0.

half strength Murashige-Skoog (½ MS) liquid medium, whereas non-transformed root cultures induced from shoot culture in vitro could not be maintained on HF ½ MS medium. The growth rate and cardenolide production in hairy roots are shown in Table 2. Hagimori *et al.* (6) and Diettrich *et al.* (16) demonstrated that undifferentiated calli and root-forming calli cultured in the light did not become green. In our experiments, the hairy roots cultured under light turned green and grew better than those kept in the dark. These phenomena were also observed in hairy roots of *Bidens suphureus* and *Tagetes*

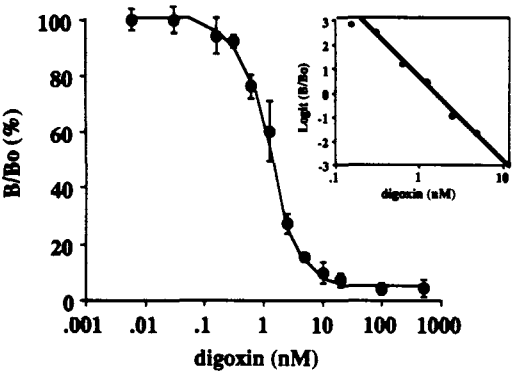

FIGURE 1. Standard curve of the ELISA for digoxin. B/Bo is the percentage of binding in the presence of digoxin. Bo is 100% binding in the absence of digoxin. Data shown are the means of six replicates. Bars represent the standard deviations of the means. The inserted figure gives a linearized Logit-log plot, with use of the same data. Logit (B/Bo) indicates ln [(B/Bo)/(100 − B/Bo)].

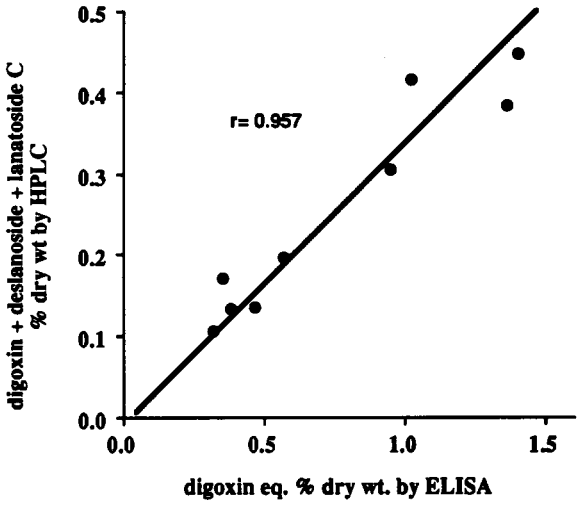

FIGURE 2. Correlation of cardenolide contents between ELISA and hplc. The correlation coefficient (r) was calculated from fitting a straight line by least squares.

patula (17,18). These cultures, grown under light, became green and showed a consistently higher growth rate than those in the dark. The roots cultured in the dark accumulated only small amounts of cardenolides (0.02–0.07 digoxin equivalents μg/g dry wt), and light significantly promoted the production of cardenolides (0.12–16.47 digoxin equivalents μg/g dry wt). Callus and embryogenic cell cultures have been tried to obtain cardenolides efficiently (1,2). Hagimori *et al.* (6) and Diettrich *et al.* (16) investigated the relationship between morphogenetic capacity and ability for cardenolide formation. They reported that undifferentiated or root-forming calli either accumulated insignificant amounts of cardenolides or they lost their ability for cardenolide formation, while shoot-forming calli accumulated 4 μg/g fresh wt cardenolides (6) and somatic embryos accumulated 1000 μg/g dry wt cardenolides (16). Their results suggested that cardenolides might be synthesized in the aerial parts of plants. We also measured the cardenolide contents of shoots in vitro, and it was 266.6 digoxin equivalents μg/g

TABLE 2. Growth and Cardenolide Production in Hairy Root Cultures.[a]

Clone	Condition	Growth rate (fresh wt)	Digoxin equivalents μg/g dry wt
4	16 h light	38.8	11.56
	dark	38.7	0.02
5	16 h light	4.4	0.12
	dark	4.0	0.07
8	16 h light	19.5	16.47
	dark	14.3	0.04
9	16 h light	32.5	3.34
	dark	16.3	0.03
10	16 h light	65.9	0.23
	dark	30.5	0.05

[a]Roots were cultured in ½ MS liquid medium at 25° for 1 month on a rotary shaker (100 rpm).

dry wt. The roots of clone 4 and 8 cultured in the light, which showed the best production of the five clones, accumulated lower amounts of cardenolides than did shoots. However, our study here proves that hairy roots have the ability to produce cardenolides because the roots have been subcultured over 1 year and maintained root characteristics in the light. It is noteworthy that the roots in the dark accumulated very small amounts of cardenolides, 0.02–0.07 μg/g dry wt, whereas the light significantly enhanced the accumulation of cardenolides (0.1–16.5 μg/g dry wt), up to 600-fold (clone 4, Table 2). Further experiments will be required to investigate the relationship between culture conditions and biosynthesis of cardenolides in order to improve their production in the hairy roots.

EXPERIMENTAL

PRODUCTION OF ANTI-DIGOXIN ANTIBODY.—To prepare antigens, digoxin was conjugated to human serum albumin and ovalbumin according to the method of Smith *et al.* (19). BALB/c mice were immunized with digoxin-conjugated human serum albumin (Dig-HSA), and hybridomas were produced by the same procedure as described previously (20) by fusing the spleen cells of mice with P3/NS1-Ag4-1 myeloma cells. Hybridoma cells were cloned after screening by the ELISA using digoxin-conjugated ovalbumin (Dig-OVA) as a coating antigen. ELISA-positive clones were further subcloned (21), and a subclone (DIG 64. 2B. 5) was selected for this study. The culture supernatants were obtained by centrifugation (2000*g*) just before the cultures became confluent and used as antibody sources.

ELISA FOR DIGOXIN.—An ELISA plate (96 wells) was coated with 50 μl/well Dig-OVA (ca. 2 μg/ml) in $NaHCO_3$ buffer (pH 9.6) at 4° overnight. After washing with phosphate-buffered saline (pH 7.2) containing 0.5 ml/liter Tween 20 (T-PBS), the wells were blocked by phosphate-buffered saline supplemented with 1 g/liter casein (C-PBS). Fifty-μl samples serially diluted with C-PBS were added to the well before subsequent addition of 50 μl anti-digoxin antibody (diluted × 50 with C-PBS), and the plate was kept at room temperature for 30 min. After rinsing with T-PBS, anti-digoxin antibody bound to the well was subjected to the reaction with peroxidase-conjugated sheep anti-mouse IgG (BYOSIS, S.A., France, BI 3413/8249; 50 μl, diluted × 2000 with C-PBS) at room temperature for 30 min. The wells were washed thoroughly with T-PBS, and 50 μl ABTS (Kirkegaard & Perry Laboratories, Inc.) was added as a substrate. After 30 min, the activity of enzyme bound to the solid phase was measured by the absorbance at 405 nm. Standards of digoxin were included on each plate, and digoxin equivalents in samples were calculated from each standard curve.

HPLC ANALYSIS.—The leaf extracts of *D. lanata* were analyzed for their cardenolide contents using a combination of a 4.6 × 50 mm and a 4.6 × 250 mm column, both filled with Nucleosil 5 C_{18} (Macherey and Nagel, FRG) (10). The cardenolides were eluted with an MeCN/H_2O gradient (34–82% MeCN), flow rate 1.5 ml/min, column temperature 40°, uv at 220 nm.

HAIRY ROOT CULTURE.—The hairy roots of *D. lanata* were induced on leaf segments in vitro by co-culture with *A. rhizogenes* strain A4. The hairy roots, after eliminating the bacteria on HF ½ MS solid medium containing 500 mg/liter Claforan®, were maintained on the same medium without antibiotic in the dark at 25°. To examine the production of cardenolides, the hairy roots were transferred into HF ½ MS liquid medium and cultured with or without light (16 h, 5000 lux) for 1 month. Voucher specimens are deposited at the Herbarium of Breeding and Physiology Laboratory in this research station.

EXTRACTION.—*D. lanata* used for comparison study between hplc and ELISA were seed-propagated plants which were cultivated in the field for 7 months or the plants from in vitro culture which were cultivated in hydroponic facilities for 3 months. For hplc analysis, dried leaf powder (ca. 100 mg) of *D. lanata* was sonicated in 70% EtOH for 20 min, followed by addition of 1 ml 15% $Pb(AcO)_2$, and then filtered. The residue was washed twice with 2 ml H_2O and 1 ml 10% Na_2HPO_4. The filtrate was extracted with 3 ml $CHCl_3$ and 2 ml $CHCl_3$-iPrOH (3:2) × 2 (14). The extract, evaporated to dryness, was dissolved in 70% MeOH and analyzed by hplc. For the ELISA analysis, 70% EtOH extracts of dried samples were diluted with an appropriate volume of C-PBS and subjected to the ELISA.

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