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Localization of Paclitaxel in Suspension Culture of *Taxus chinensis*

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Abstract The localization of paclitaxel was investigated in suspension culture cells of *Taxus chinensis*. Over 93% of the cell-associated paclitaxel were detected throughout the entire culture period. Intracellular localization of paclitaxel over the culture time was analyzed further by cell fractionation for days 21 and 42. Paclitaxel contents in intracellular organelles were decreased at day 42, while the content in the cell wall fraction was increased at day 42 compared to the value for day 21. The localization of paclitaxel in the cell wall was confirmed by using the immunocytochemical method with the aid of a confocal laser scanning microscope.

Key words: Cell fractionation, confocal laser scanning microscope, localization, paclitaxel, *Taxus chinensis*

Paclitaxel, a diterpene alkaloid isolated from yew (*Taxus*) species, is known to be effective in cancer treatment by inhibiting depolymerization of microtubules during the mitosis of cells [11, 13]. Since paclitaxel can bind to the microtubules of various organisms including mammalian, bacteria, and higher plants [6], it is thus assumed that paclitaxel-producing cells have a self-protection mechanism against the toxicity of paclitaxel. In general, plant cells use two types of methods to protect themselves from the endogenously produced toxic compounds, which are intracytoplasmic storage in vacuoles or plastids and extracytoplasmic storage in the cell wall [8]. In a case of haploid-derived suspension cell cultures of *Taxus brevifolia*, paclitaxel was localized on the cell surface and secreted from the cells [3]. However, characteristics related to paclitaxel distribution were different depending on the cell line and culture conditions [4, 7, 9, 15, 17]. In addition, it is important to get a full understanding of the storage mechanism and location of paclitaxel to elucidate the biosynthetic pathway, to improve the production, and to develop an extraction process.

In this study, the distribution of paclitaxel was investigated by analyzing the content of intra- and extracellular paclitaxel in a culture suspension of *Taxus chinensis*, and the intracellular localization of paclitaxel was examined by cell fractionation. We further elucidated the storage site of paclitaxel through an immunolabeling method and using a confocal laser scanning microscope.

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Materials and Methods

Plant Cell Culture

Taxus chinensis cells were grown under darkness at 24± 1°C on a gyratory shaker (New Brunswick Scientific Co., G-10, U.S.A.) at 150 rpm. The liquid medium contained an inorganic salt formulation of Gamborg [5], 30 g/l of sucrose, 10 μM of naphthalene acetic acid (NAA), 0.2 μM of 6-benzylaminopurine (BA), 1 g/l of casein hydrolysate, and 1 g/l of 2-[N-morpholino]ethanesulfonic acid (MES). The cells were subcultured every two weeks by adding 50 ml of inoculum into 150 ml of fresh medium in 500-ml long neck flasks. Cultures maintained in 500-ml flask in the late exponential phase were used as seed cells. Flasks were capped with KimKap closures and taped with a Micropore surgical tape (3M, 1530-1, U.S.A.).

In order to prolong paclitaxel production, sucrose was used as the sugar initially, and maltose of 10 and 20 g/l was added to the cultures at day 7 and 21, respectively [2]. As an elicitor for paclitaxel production, 4 μM of silver nitrate was added at the beginning of the culture [2]. Dry cell weights were measured as indicated in a previous report [1]. All reagents were purchased from Sigma (St. Louis,

U.S.A.) unless otherwise stated. All data represent the average of triplicate values.

Paclitaxel Analysis

Cultures (both cell and supernatant or filtered supernatant), 2 ml, were mixed with 0.1 ml of propyl *p*-hydroxybenzoate, 0.2 ml of 1% (w/v) cetylpyridinium chloride, and 2.5 ml of methyl-*t*-butyl ether (Fluka, Swiss) by shaking at 200 rpm for 24 h. After being extracted, 2 ml of the supernatant was loaded on a 3-ml LC-NH_2 SPE column with normal phase packings and eluted with a mixture of methyl-*t*-butyl ether and methanol (85:15, v/v). The eluent of 1 ml, was dried in a vacuum evaporator, and the residue redissolved with 100 µl of methanol was used for the quantitative analysis of paclitaxel.

Quantification of paclitaxel was performed with a high performance liquid chromatography (HPLC) system (Hewlett-Packard Series 1100, Germany) by using a Curosil-PFP-5 µM column (Phenomenex, 4.6×250 mm, U.S.A.). The elution was performed by gradient with a mixture of acetonitrile and water at 65:35 (v/v) to 35:65 within 30 min. Flow rate was 1.0 ml/min, and the absorbance at 227 nm was measured. Authentic paclitaxel was purchased from Sigma (St. Louis, U.S.A.).

Extra- and Intracellular Paclitaxel Measurement

For carrying out the extracellular paclitaxel analysis, the culture broth was filtered through a 0.45-µm filter (Sartorius Minisart, Germany) and 2 ml of the filtrate were extracted and analyzed by the HPLC method as described above. Extracellular paclitaxel contents in the culture broth were calculated according to the filtrate volume except for the cells in each sample. Intracellular paclitaxel contents were obtained by subtracting the amounts of extracellular paclitaxel from those of the total cell culture broth.

Cell Fractionation

Cell fractionation was performed according to the method described by Shimakura *et al.* [10]. Cells were harvested by being filtered through Whatman No. 541 filter paper. As shown in Fig. 1, harvested cells (30 g fresh weight) were homogenized with a pestle and mortar in 10 mM of Tris-HCl buffer (pH 7.4, 60 ml) containing 0.25 M of sucrose, 1.5 mM of $CaCl_2$, 2 mM of dithiothreitol, and 0.2 mM of phenylmethylsulfonyl fluoride. The homogenate was filtered through a nylon screen (40 µm mesh). The residue was washed with the same buffer and distilled water to obtain a crude cell wall fraction. The filtrate was combined with the washings and centrifuged at 1,000 ×g for 10 min to remove large organelles such as nuclei, plastids, and starch grains in the sediment. The supernatant was recentrifuged at 10,000 ×g for 15 min to select endoplasmic reticulum (ER) and microsomes among others. To achieve further purification of starch grains, the 1,000 ×g pellet was suspended in 85% (v/v) Percoll (Pharmacia, Sweden) solution containing the same components as the homogenization buffer and centrifuged at 6,000 ×g for 20 min to yield sedimentary starch grains and floatable organelles such as plastid and nuclei.

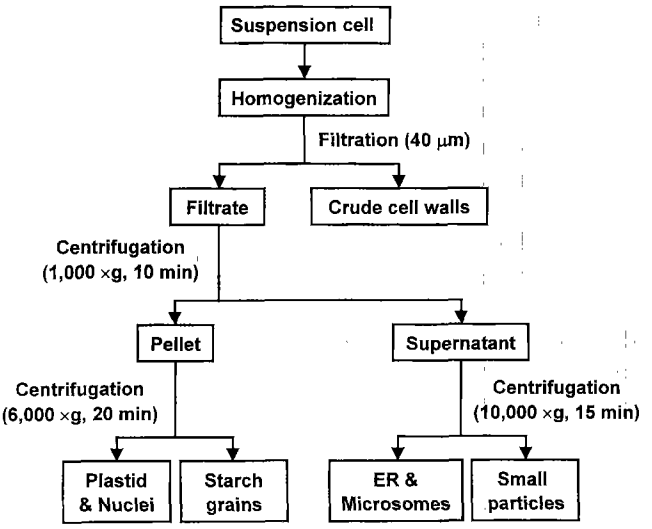

Fig. 1. Cell fractionation method for investigation of the intracellular distribution of paclitaxel in a cell suspension of *Taxus chinensis*.

Immunofluorescence Labeling

Cells cultured for 35 days were rinsed three times with a pH 7.0 phosphate-buffered saline (PBS) and treated with 2% (w/v) pectinase (Fluka, 76290, Switzerland) and 2% (w/v) cellulase (Fluka, 22179, Switzerland) solution to improve the permeability of the cell wall. Blocking solution composed of 1% (v/v) bovine serum albumin, 10% (v/v) goat serum, and PBS solution were added to the cells and incubated in a gyratory shaker for 1 h at room temperature. Mouse anti-paclitaxel antibody (Hawaii Biotechnology, TA-12, U.S.A.) was added at 1:200 dilution ratio as a primary antibody and incubated for 1 h at 50 rpm at room temperature by using a gyratory shaker. Cells were rinsed three times with PBS, and the supernatant was removed. Blocking solution was added to make a total volume of 2.5 ml, and the cells were incubated for 10 min by using the methods described above. Goat anti-mouse IgG-Alexa™ 488 conjugate (Molecular Probes Inc., A-11001, U.S.A.) was then added at 1:5,000 dilution ratio to the cells. After 1 h incubation, the cells were rinsed 4 times with PBS solution and they were fixed by adding 1% (v/v) formaldehyde and incubating further for 30 min.

Epifluorescence and Confocal Laser Scanning Microscopy

Zeiss fluorescence filter set 48 77 09 (excitation, 450–490 nm; emission, >420 nm) was used to detect fluorescence labeling. Images were collected on a Leitz DM IRBE confocal laser scanning microscope. The fluorescent images

were collected by using the confocal photomultiplier tubes (PMT) as 1,054×1,054 pixels digital images and stored in a computer for further analysis. Filter sets were adjusted to rhodamin and FITC (fluorescein isothiocyanate) channel to obtain separate images, one pseudocolored red and the other green, which were then overlayed. The confocal was set to acquire z sections through the cells at 0.8 μm intervals by using a calibrated motor drive that was attached to the fine focus of the microscope. The z sections totaled 50–70 individual optical slices depending on the dimension of the cells. To obtain three-dimensional images, the Volume Works (Molecular Dynamic, U.S.A.) computer program was used.

RESULTS AND DISCUSSION

Paclitaxel Distribution in Cultures

To characterize the pattern of paclitaxel distribution, intracellular and extracellular paclitaxel contents were investigated. In our cultures, over 93% of the paclitaxel exhibited cell-associated characteristics throughout the culture period (Fig. 2). Paclitaxel production showed a maximum level of 26.3 mg/l at day 35, and decreased slightly thereafter. Dry cell weight increased steadily from day 10 to day 42, thanks to the intermittent feeding of maltose. Paclitaxel in an extracellular medium decreased slightly from day 21 to 42, where the levels ranged from 6.1 to 2.9%, which differed from the other report where the cell-associated paclitaxel was always found to be less than 10% of the total paclitaxel in suspension culture of *Taxus cuspidata* [7]. A maximum release of 66% paclitaxel in the suspension culture of *Taxus cuspidata* was also reported [4]. Seki *et al.* [9] reported that 70–80% of produced paclitaxel was released into the medium in the culture suspension of *Taxus cuspidata*. On the contrary, the amount of secreted paclitaxel was less than 25% of the total paclitaxel produced in the suspension culture of *Taxus chinensis* cells [15]. Wickremesinhe and Arteca [17] also reported that the levels of paclitaxel found in the culture media were usually less than 10% of the total amount found in the cell suspension cultures of *Taxus* × *media cv. Hichsii*. It was noted that the distribution of paclitaxel in the culture differed according to the *Taxus* species. Since over 93% of paclitaxel was found in the cells, further research was mainly focused on the localization of the cell-associated paclitaxel.

Paclitaxel Localization by Cell Fractionation

Several subcellular organelles were fractionated by using differential centrifugation. To confirm that there is a possibility of contamination among the cell components during cell fractionation, the quantities of cellulose – a major component of cell wall – in each fraction were determined [14], but no cross contaminations were detected (data not shown). Figure 3 shows the distribution of paclitaxel in various cellular fractions in *Taxus chinensis* cells. Paclitaxel content in each organelle was determined at days 21 and 42. At day 21, 42.2% of paclitaxel was localized in crude cell wall, and its level increased to 54.8% at day 42. About 34.9% of paclitaxel was located in plastid or nuclei at day 21, and its level decreased to 25.3% at day 42. In starch grains, 4.8% of

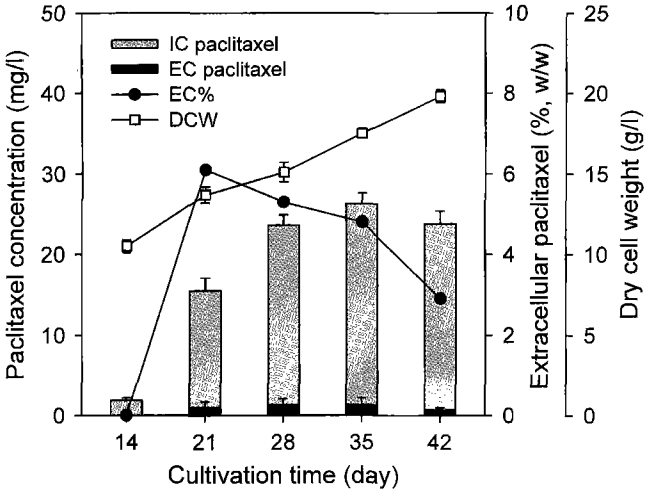

Fig. 2. Time course distribution of paclitaxel in a suspension culture of *Taxus chinensis*.
IC paclitaxel, intracellular paclitaxel; EC paclitaxel, extracellular paclitaxel; EC%, relative percentage of extracellular paclitaxel=concentration of the extracellular paclitaxel (mg/l)/concentration of total paclitaxel (mg/l)×100; DCW, dry cell weight. Bars represent the standard deviations of three replications.

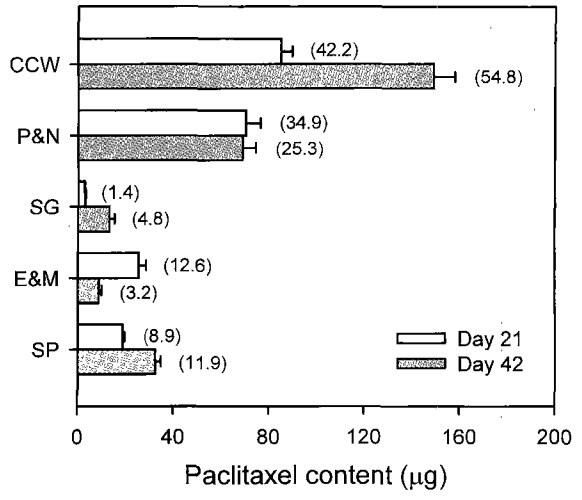

Fig. 3. Distribution of paclitaxel in various cellular fractions of the cultured cells of *Taxus chinensis* at days 21 and 42.
CCW, crude cell walls; P&N, plastids and nuclei; SG, starch grains; E&M, ER and microsomes; SP, smaller particles (supernatant fraction). Number in the bracket means the relative percentage (w/w) of paclitaxel content in each fraction compared to the total paclitaxel content. Bars represent the standard deviations of three replications.

paclitaxel was detected at day 42, whereas 1.4% was detected at day 21. In endoplasmic reticulum (ER) or microsomes, paclitaxel was 12.6% at day 21, and its portion greatly decreased to as little as 3.2% at day 42. In a supernatant fraction containing smaller particles, the paclitaxel content increased slightly to 2.0% at day 42 compared to that at day 21. According to these results, we found that the paclitaxel in the plastid and ER fractions decreased, while that in the cell wall increased from days 21 to 42. In many cases, the sites of biosynthesis and accumulation of secondary metabolites are separated in the cell. Some of the enzymes related to paclitaxel biosynthesis were known to be located in the plastids, like many terpenoids [18], and the biosynthetic steps were known to occur in the plastids [12]. The increased paclitaxel content in a cell wall fraction at day 42 suggested that paclitaxel could be transferred to the cell wall in order to remove the toxic effect of paclitaxel itself.

Localization of Paclitaxel through Immunolabeling Methods Using Confocal Laser Scanning Microscopy

The immunocytochemical method is an efficient tool to detect the location of target material. In this experiment, the concentrations of primary and secondary antibodies were optimized to obtain concrete results. The type of probes that enable the emission of fluorescence also plays an important role. IgG-Alexa™ 488 conjugate was used as a probe instead of the normally used FITC, because auto-fluorescence of the cell was too strong to discriminate against real signal from the background when the FITC was used. Two percent (w/v) of both cellulase and pectinase was recognized as the optimal concentrations of the enzymes, allowing the permeation of the antibodies to take place without damaging the cell (data not shown).

Confocal laser scanning microscopy has some advantages over the conventional fluorescence microscopy in offering a greater resolution and a three-dimensional image without any physical proof of the sample specimen, and this is the reason why this type of microscope is used for the intracellular localization of secondary metabolism [16]. Figure 4 shows the representative micrographs of the *Taxus chinensis* suspension cell which were obtained by using both conventional (Fig. 4a) and confocal laser scanning microscopes (Fig. 4b). In Fig. 4b, fluorescence of paclitaxels is in green, and the auto-fluorescence of cells is in red. Intensity of fluorescence of paclitaxel in the cytoplasm was weaker than that in the cell wall. According to these observations, our cell line seemed to entrap a large amount of paclitaxel on the cell wall compartment. In this experiment, we used the cells which were 35 days old. As shown in Fig. 2, the dry cell weight was increased from day 35 to day 42. Therefore, the cells at day 35 in this experiment seemed to have enough activity for the cell division to take place, and it showed that the storage of paclitaxel in the cell wall was not a result of senescence of the cells.

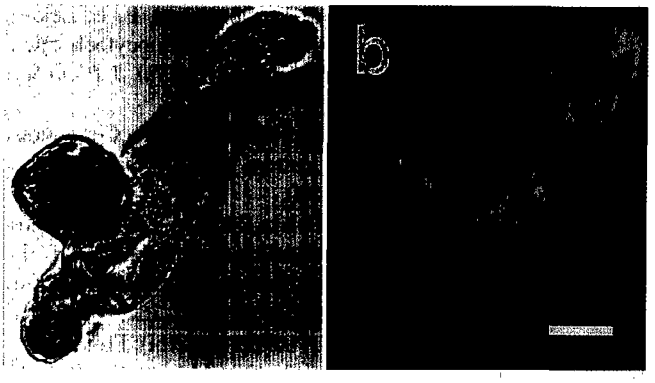

Fig. 4. Micrographs of the representative suspension cell of *Taxus chinensis* at day 35.
(a) Micrograph of the cell obtained by using a conventional microscope; (b) Three-dimensional micrograph of the cell obtained by using a confocal laser scanning microscope. Green parts indicate trace of paclitaxel in the cell. Bar represents 20 μm.

In conclusion, most of the paclitaxel in our cell line existed in the intracellular region, and the majority of paclitaxel was deposited to the cell wall. These results explained how the cells of *Taxus chinensis*, which produced high concentration of paclitaxel, could survive under such toxic conditions without releasing paclitaxel from these cells.

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