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A simple method for enhancing paclitaxel release from *Taxus canadensis* cell suspension cultures utilizing cell wall digesting enzymes

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Abstract Paclitaxel storage in *Taxus* suspension cell cultures was studied through the simple use of cell wall digesting enzymes. The application of cellulase (1%) and pectolyase (0.1%) to *Taxus canadensis* suspension cultures induced a significant increase in the paclitaxel present in the extracellular medium while maintaining membrane integrity, suggesting that paclitaxel is stored in the cell wall. The addition of cell wall digesting enzymes to a cell culture bioprocess may be an effective way of enhancing paclitaxel release to the extracellular medium and hence simplify product recovery.

Keywords Paclitaxel · Storage · Cell wall · Protoplast

Introduction

Paclitaxel, a secondary metabolite produced by the yew tree, has been approved for the treatment of breast, ovarian and lung cancers as well as the AIDS-related Kaposi's sarcoma. Although paclitaxel production via plant cell culture has been extensively studied (Fett-Neto et al. 1992; Wickremesinhe and Arteca 1994; Ketchum et al. 1995, 1999; Srinivasan et al. 1995; Mirjalili and Linden 1996; Yukimune et al. 1996; Dong and Zhong 2001; Cusido et al. 2002), the majority of these studies focused on optimization of paclitaxel production through an analysis of the biosynthetic pathway, with little attention given to storage and transport. Some *Taxus* cell lines have been demonstrated to release as little as 7–10% of the total paclitaxel produced to the extracellular medium (Wickremesinhe and Arteca 1994; Pestchanker et al. 1996; Choi et al. 2001). In order to design an optimal production process for paclitaxel, it is essential that the majority of paclitaxel be released into the extracellular medium. Therefore, it is important to determine how paclitaxel is released and where paclitaxel is stored within the cell. We have demonstrated that vesicular trafficking is involved in the release of paclitaxel and that calcium can alter significantly paclitaxel distribution (data not shown). In this paper, we report our findings on paclitaxel storage and suggest a simple strategy for enhancing its release to the extracellular medium.

The majority of secondary metabolites are hydrophilic and, therefore, the main storage compartment associated with the cell is the aqueous environment of the vacuole. However, hydrophobic secondary metabolites typically accumulate in membranes, vesicles, dead cells or extracellular sites such as the cell wall (Guern et al. 1984). Paclitaxel is hydrophobic and essentially insoluble in aqueous solutions (including cellular cytoplasm). By means of immunofluorescence, Durzan and Ventimiglia (1994) observed that taxanes (including paclitaxel) bind to cell surfaces of *T. brevifolia* suspension cultures. Russin et al. (1995), using transmission electron microscopy (TEM) in conjunction with labeling, found that paclitaxel is localized in the cell walls of the phloem, vascular cambium and xylem of *T. cuspidata* plant tissue. However, in both of these investigations, sample processing was difficult due to the hydrophobic nature of paclitaxel, which complicated the interpretation of the data. Using immunocytochemical methods and cell fractionation, Choi et al. (2001) showed that paclitaxel concentrates in the cell walls of *T. chinensis* suspension

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cultures. There have been only a few reports on the preparation of protoplasts from *Taxus* cells (Aoyagi et al. 2002; Luo et al. 1999). We report herein on studies performed using cell wall digesting enzymes to determine if paclitaxel is stored in the cell wall of *Taxus* suspension cells.

Materials and methods

The C93AD (*Taxus canadensis*) cell line was developed by the US Plant Soil and Nutrition Laboratory (Ithaca, N.Y.) by Dr. Donna Gibson. Suspensions were subcultured every 2 weeks into fresh medium consisting of Gamborg's B5 basal salts (Gamborg et al. 1968) with 20 g/l sucrose, supplemented with 2.7 μM α-naphthalene acetic acid (NAA), 0.1 μM benzyladenine (BA), 2.5 mM glutamine, 62.5 mg/l ascorbic acid and 62.5 mg/l citric acid. Glutamine, ascorbic acid and citric acid were filter-sterilized and added post-autoclaving. Cell cultures were maintained in glass shake flasks (either 125 ml or 250 ml) capped with Bellco (Vineland, N.J.) foam closures. Ten milliliter aliquots of 14 day-old cultures were transferred into 40 ml of fresh medium with at least 2 ml of packed cell volume transferred to each new flask. The cultures were kept at 23–25°C on gyratory shakers (20 rpm) in the dark.

The enzymes cellulase and pectolyase have been shown to digest the cell walls of *Taxus* suspension cells (data in Results section) and hence were used to probe paclitaxel localization in the cell wall. Cellulase from *Trichoderma viride* was purchased from Sigma Chemical Company (cat. no. C-1794, St. Louis, Mo.). Pectolyase Y-23 from *Aspergillus japonicus* was purchased from ICN Biomedicals (cat. no. 320951). All additional materials utilized in these experiments were obtained from Sigma Chemical Company. The final concentration of the protoplast solution when combined with the cell suspension consisted of 1% cellulase, 0.1% pectolyase and 0.5 M mannitol. A 15 ml aliquot of concentrated protoplast solution was added to 5 ml of the suspension culture 14 days after subculture for both unelicited and methyl jasmonate-elicited (100 μM) *Taxus canadensis* (C93AD) cell cultures. Methyl jasmonate (MJ) (Bedoukian Research, Danbury, Conn.) was diluted in an ethanol:water (12:13) solution for use and added at 100 μM 8 days after subculturing. Flasks were kept under normal culturing conditions (gyratory shakers at 120 rpm in the dark, 23–25°C), and samples were taken at various time intervals for viability staining and paclitaxel analysis.

The viability of cells was determined using fluorescein diacetate (FDA) stain in which 20 μl of a 0.5 mg/ml FDA solution (in acetone) was added to 1 ml of suspended cells. After approximately 10 min, cell samples were viewed under the fluorescent microscope. Cells that were viable fluoresced bright green under UV light and were scored as viable cells.

Paclitaxel was identified and quantified with high performance liquid chromatography (HPLC) analysis by a comparison of retention time and absorption spectra with authentic standards provided by either Sigma Chemical Company or Hauser Chemical Research (Boulder, Colo.). New standard curves were developed for each run and used to quantify paclitaxel in each sample. The samples were separated on a Metachem (5 μm, 250×4.6 mm) Taxsil column with disposable guard cartridge (Metachem Technologies, no. 0335-CS). The mobile phase was acetonitrile:water (1.9:2.1), 1 ml/min flow rate, and detection was with UV at 228 nm and photodiode array scans of each peak from 200–300 nm. Instrumentation consisted of a Waters model 717 Plus autosampler, model 510 pump and model 996 photodiode array detector. Data acquisition, processing and instrument control was accomplished with Millennium (Waters 1994) version 2.15 software.

Results and discussion

We have developed a protoplast procedure for *Taxus* suspension cells that results in high viability and yield for all *Taxus* cell lines. Figure 1 shows both protoplasts and undigested cells of *T. canadensis* cell line C93AD (similar results were obtained with several different *Taxus* species; data not shown). Corresponding fluorescence micrographs taken after FDA staining are given for each and indicate the high viability obtained. Experiments (with sampling every 0.5–1 h) showed that the best yield

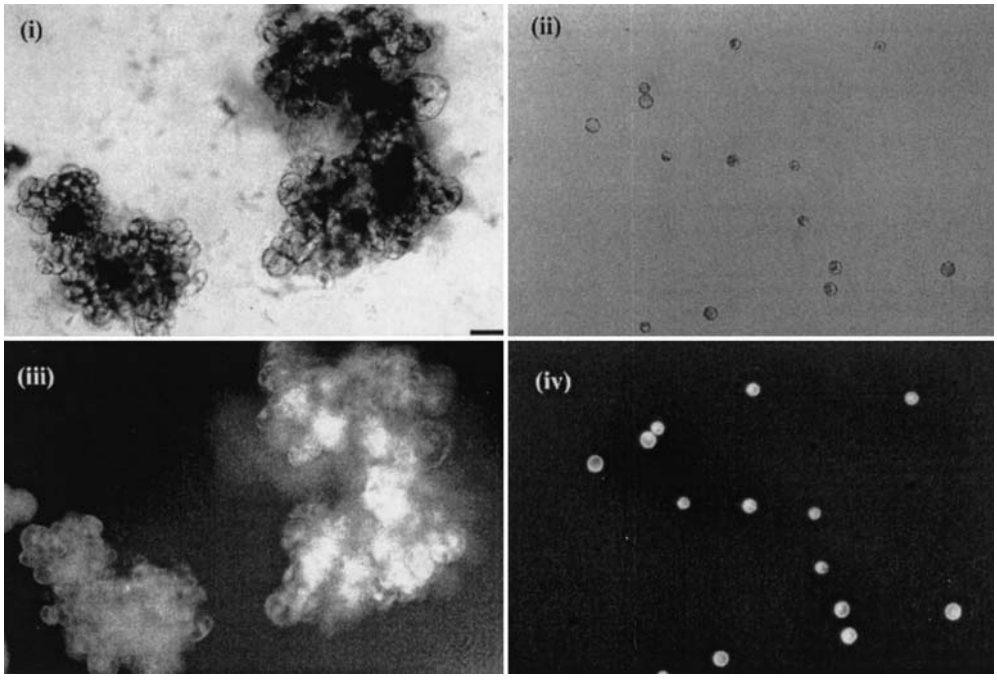

Fig. 1i–iv Comparison of both undigested cells and protoplasts by means of viability staining (FDA). Magnification: ×100. All pictures show the C93AD cell line (*Taxus canadensis*). **i** Unprotoplasted cells (*bar*:100 μm), **ii** protoplasts after digestion, **iii** corresponding picture of (**i**) with viability staining, **iv** corresponding picture of (**ii**) with viability staining

Table 1 Paclitaxel concentrations in cultures treated with cell wall digesting enzymes. Cell wall digesting enzymes were added 14 days after subculture to *Taxus canadensis* (C93AD) cell cultures

Time point (min)	Unelicited extracellular paclitaxel (mg/l)	Methyl jasmonate-elicited* extracellular paclitaxel (mg/l)
0	2.0±0.3[a]	22.9±1.3[c]
5	6.5±0.4[b]	52.7±9.2[d]
30	5.4±0.6[b]	38.0±4.7[d]
60	6.1±0.1[b]	40.2±6.9[d]

*Selected cultures were elicited with 100 μM methyl jasmonate on day 8. Reported paclitaxel concentrations represent averages (±standard deviations) between two replicate flasks. Means with different letters are significantly different based on Tukey's multiple comparison test with a minimum family error rate of 0.05

of viable cells was obtained after 4 h of digestion, where 1.0×10^6 protoplasts were isolated per gram of cell mass (fresh weight). At this time, viability was measured to be approximately 96%. Extended digestion only served to decrease the yield, as viability dropped to less than 40% at 8 h. Using a similar protoplast solution, Aoyagi et al. (2002) achieved comparable yields. These same investigators achieved even higher yields [up to 6.4×10^6 protoplasts per gram cell mass (fresh weight)] by varying the protoplast solution (e.g., utilizing Sumizyme AC, addition of citric acid, and application of degassing treatments). As a yield of 1.0×10^6 is sufficient for our work, additional complexity in the protoplast solution was not necessary. Other investigators have suggested that protoplast stability may be affected by the proteases present, leading to decreased viability via lysis (Jayshankar et al. 1993; Thomas and Katterman 1984), while pectin lyase (included in pectolyase Y-23) has been shown to have a deleterious effect upon plant cells (Ishii 1988). Viability counting was done on over 200 protoplasts at each time point.

Since paclitaxel is a hydrophobic metabolite, we tested the possibility that it is localized in the cell wall using the protoplasting procedure described above. Studies were performed on both untreated cell cultures and MJ-elicited cell cultures. MJ is a potent elicitor of paclitaxel biosynthesis (Ketchum et al. 1999; Mirjalili and Linden 1996; Yukimune et al. 1996; Dong and Zhong 2001) and is likely to be used in any large-scale cell culture processes that are developed for paclitaxel production. In the experiments reported here, MJ induced an eightfold increase in total paclitaxel production (from 7.0 mg/l to 55.4 mg/l), thereby demonstrating the applicability of this method for both moderate and high paclitaxel production systems.

Cellulase (1%) and pectolyase (0.1%) were added to *T. canadensis* (C93AD) cell cultures, and paclitaxel levels were measured via HPLC after 0, 5, 30, and 60 min. Viability was also checked via FDA staining at each of the time points. After 5 min, extracellular paclitaxel levels increased from 2.0 mg/l to 6.5 mg/l in the unelicited cell cultures and from 22.9 mg/l to 52.7 mg/l in MJ-elicited cell cultures, with no further statistically significant increases at later time points (see Table 1). Viability in the cell wall digesting enzyme-treated cultures was the same as in the control cultures (approx. 90%), indicating that the enhancement of paclitaxel release was not due to compromised membranes. Untreated cultures were also sampled to determine the total paclitaxel content (both cell-associated and extracellular). Cell-associated refers to paclitaxel found intracellularly—between the cell wall and the plasma membrane or associated with the cell wall matrix. Total paclitaxel values were 7.0 mg/l and 55.4 mg/l in unelicited and MJ-elicited cell cultures, respectively, indicating that more than 90% of the total paclitaxel was recovered in the extracellular medium following treatment with the enzymes and demonstrating that most of the cell-associated paclitaxel was either localized to the cell wall matrix or to the space between the cell wall and the plasma membrane.

These data suggest that paclitaxel accumulates in the cell wall, a result that is consistent with other reported observations (Durzan and Ventimiglia 1994; Russin et al. 1995; Choi et al. 2001; Aoyagi et al. 2002). Using cellular fractionation, Choi et al. (2001) demonstrated that most of the cell-associated paclitaxel (between 42.2% and 54.8%) in *T. chinensis* cultures was localized to the cell wall fraction. Note that these numbers do not include paclitaxel that may be found between the cell wall and the cell membrane. Aoyagi et al. (2002) used a similar protoplast procedure as the one reported in this paper to demonstrate that 30–35% of the total cell-associated paclitaxel for *T. cuspidata* cell cultures could be localized to either the cell wall or the space between the cell wall and the plasma membrane. The discrepancies in specific percentages observed in this paper and other recent publications (Choi et al. 2001; Aoyagi et al. 2002) may be related to differences in cell species or lines tested. It is important to note, however, that all reports confirm that a significant amount of paclitaxel is associated with the cell wall in *Taxus* systems.

This simple method may be applicable to the localization of other hydrophobic secondary metabolites in suspension cultures. The addition of cell wall digesting enzymes to cell cultures where secondary metabolites are stored in the cell wall is additionally a simple and effective way of enhancing release into the extracellular medium, and studies are currently underway to optimize this protocol for paclitaxel production via plant cell suspension culture. This method has been demonstrated to be applicable for both unelicited and MJ-elicited cultures, which is critical as optimal cell culture processes for paclitaxel supply are likely to involve the use of MJ elicitation. Studies are underway to further localize paclitaxel accumulation to either the cell wall matrix, the extracellular space between the cell wall and the plasma membrane or in defined pockets in the cell wall, as is the case for tetrahydrocannabinol accumulation in *Cannabis* glandular trichomes (Kim and Mahlberg 1997). This "fine" localization may be very important for the in vitro production of paclitaxel because it then may not be

necessary to completely remove the cell wall to induce secretion. This is the first report on the potential use of cell wall digesting enzymes in the enhancement of secretion of secondary metabolites in bioprocesses involving plant cell cultures.

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