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Enzyme-assisted extraction of paclitaxel and related taxanes from needles of *Taxus chinensis*

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ABSTRACT

A simple and efficient enzyme-assisted extraction procedure was developed and optimized for the extraction of paclitaxel and related compounds, namely 7-xyl-10-deacetylpaclitaxel, 10-deacetylpaclitaxel, cephalomannine and 7-*epi*-10-deaceylpaclitaxel from the needles of *Taxus chinensis*. The method was evaluated based on parameters such as enzyme type, pH and the concentration of the enzyme solution, incubation time and temperature. Statistical treatment of the results revealed that the selected parameters were all significant. Optimum conditions would be as follows: the needles were treated with 1 mg/mL cellulase solution, ratio of liquid to solid was 10:1 (mL/g), and incubated at pH 4 for 24 h at 40 °C. Under the optimum conditions, the yields of paclitaxel, 7-xyl-10-deacetylpaclitaxel, 10-deacetylpaclitaxel, cephalomannine and 7-*epi*-10-deaceylpaclitaxel were 1.78-fold, 1.26-fold, 2.46-fold, 3.01-fold and 2.25-fold of those in the control, respectively. In addition, the effect of enzyme on the structural changes of plant cells was observed by scanning electron microscopy (SEM). Enzyme-assisted extraction was proved to be environment-friendly and economical, and could be used in natural product extraction in large scale.

1. Introduction

Paclitaxel (Taxol®), a diterpene with unique chemical structure and pharmacological effect, has been used for the treatment of a variety of human solid tumours, including leukemia, certain ovarian, breast, head, neck, skin and non-small cell lung tumours [1–4]. It exerts the anti-cancer activity by inhibiting the depolymerization of microtubule [5]. The cellular target of paclitaxel's action is tubulin, a protein capable of undergoing polymerization to produce microtubules [6,7]. Paclitaxel induces the formation of stable microtubules, which leads to blockage of the cells in the late G_2/M phase of the cell cycle and inhibition of cell division [8].

Paclitaxel has been called by the U.S. National Cancer Institute (NCI), the most important anti-cancer agent for the past 15 years. However, using *Taxus* plant as the primary source of paclitaxel for research and clinical purposes is not environmentally sustainable because the yield of paclitaxel from bark is only about 0.01–0.06% [5,9–15], and bark stripping leads to the destruction of scarce plant resource. Shortage in supply and increasing demands for clinical use necessitate the investigation for alternative sources. At present, lots of methods have been developed to resolve this problem, such as tissue-culture processes, searching for other plant sources, semi-synthesis from 10-DAB III and other taxanes such as 10-deacetylpaclitaxel (10-DAT), cephalomannine, 7-*epi*-10-deacetylpaclitaxel (7-*epi*-10-DAT), and 7-xyl-10-deacetylpaclitaxel (7-xyl-10-DAT) (Fig. 1), etc. [3,16–20]. So far, the extraction of paclitaxel and other taxanes from *Taxus* species has still been the main method because of the lower production cost and easier operation.

Although many reports about extracting taxanes by using refluxing, ultrasonic, soxhlet and percolation methods have been published [9,21–23], some disadvantages still exist such as long extraction time, large organic solvent consumption, and low efficiency. Therefore, high efficient extraction method of taxanes represents a hot spot in *Taxus* species research.

Enzyme-assisted extraction is a method applied to the study of secondary metabolites releasing from biogenic materials. It possesses the advantage of environmental-friendship, high efficiency and easy operation process. It has been represented as an alternative way for natural product extraction.

Some reports confirmed that a significant amount of paclitaxel is associated with the cell surfaces [24,25] and cell walls of the phloem, vascular cambium and xylem [26] in *Taxus* plant cells. Hydrolytic enzymes including cellulase, beta-glucosidase and pectinase, which are commonly used in extraction [27,28,29], can

Abbreviations: 7-xyl-10-DAT, 7-xyl-10-deacetylpaclitaxel; 10-DAT, 10-deacetylpaclitaxel; 7-*epi*-10-DAT, 7-*epi*-10-deaceylpaclitaxel; SEM, scanning electron microscopy.

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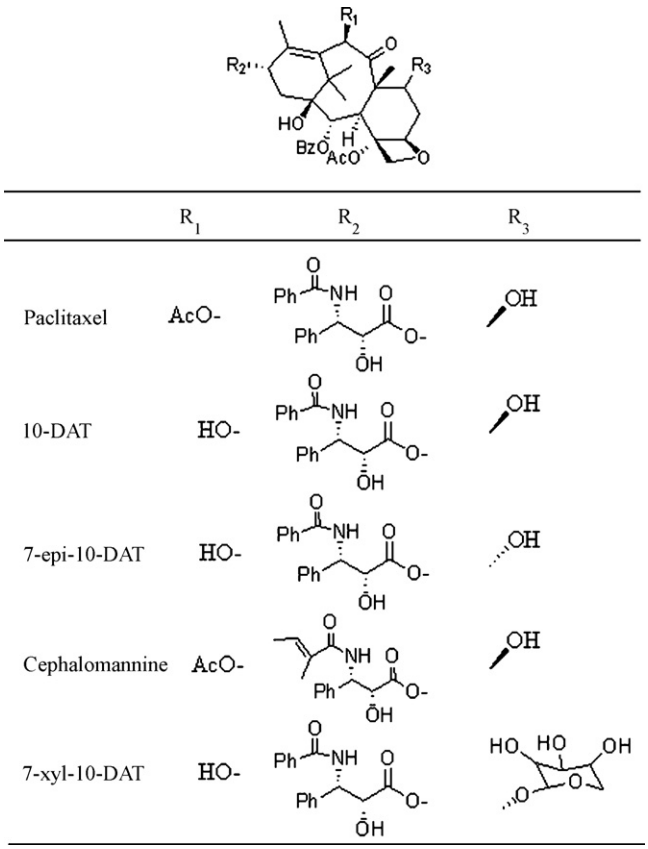

Fig. 1. The structures of paclitaxel and its analogues (structure is drawn using ChemOffice 2004).

interact on cell wall, break down its structural integrity so as to increase the releasing of paclitaxel and its analogues notably. Cellulase, pectinase and beta-glucosidase can hydrolyze cellulose, pectin and beta-1,4 glucosidic linkages in glucosides, respectively [30]. Enzyme-assisted extraction of paclitaxel and related taxanes from needles of *Taxus chinensis* has not been reported.

The main aim of the present study is to examine and optimize the process of enzyme-assisted extraction of paclitaxel and related taxanes from the needles of *T. chinensis*. For this purpose, the selection of enzyme type, pH and the concertration of enzyme solution, incubation time and temprature were studied, in order to obtain high yields of above natural products economically and environment-friendly

2. Experimental

2.1. Plant materials

In this paper, *T. chinensis* was selected as plant material, which grows faster and has bigger biomass than other species in China. Fresh twigs of *T. chinensis* were collected in July 2007 from the Botanical Garden of the Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, Harbin, China, and identified by Professor Nie Shaoquan from the same Key Laboratory. The samples were separated into needle and stem fractions, the former was dried and grounded.

2.2. Chemicals and reagents

Paclitaxel and cephalomannine standards were obtained from Sigma (St. Louis, MO, USA). 7-xyl-10-DAT, 10-DAT and 7-*epi*-10-DAT were purchased from Shanghai Jinhe Bio-Technology Co., Ltd (Shanghai, China). For stock solutions, the reference compounds of these taxanes were dissolved in acetonitrile to obtain a concentration of 50 μg/mL, respectively. For the optimization of the separation of taxanes using chromatography, a mixed solution with 10 μg/mL for each compound was prepared in acetonitrile. Cellulase (EC 1.1.1.27, ≥1000 U/mg) and beta-glucosidase (EC 3.2.1.21, ≥47 U/mg) were purchased from Shanghai WeeBeyond Scientific & Trade Co., Ltd., pectinase (EC 3.2.1.15, 1.41 U/mg) was purchased from Fluka Chemical Co.

Acetonitrile of chromatographic grade was obtained from J & K Chemical Ltd. (China). Ethanol and methanol of analytical grade were purchased from Beijing Chemical Reagents Co. (Beijing, China) and double-distilled water was used in all experiments. All solvents prepared for HPLC were filtered through a membrane filter (0.45 μm pore size) and degassed under ultrasonic condition before use.

2.3. Apparatus and chromatographic conditions

HPLC analysis was performed in a Jasco LC system (Jasco Company, Japan) equipped with a Jasco PU-1580 intelligent HPLC pump and coupled to Jasco UV-1575 intelligent UV–vis Detector, as well as Millennium 32 system software. Chromatographic separation was performed on a Curosil-PFP column (5 μm, 4.6 mm × 250 mm, Phenomenex, Torrance, CA, USA) equipped with an Analytical KJ0-4282 C_{18} guard cartridge system (Phenomenex, Torrance, CA, USA). The mobile phase consisted of acetonitrile (A) and double-distilled water (B). Baseline separation of paclitaxel and other taxanes was achieved by an isocratic elution which contains 37% A. Effluents were monitored at 234 nm UV wavelength. The injection volume and the flow rate of mobile phase were 10 μL and 1 mL/min, respectively. The column was maintained at 35 °C.

2.4. Enzyme-assisted extraction and pretreatment

Cellulase, beta-glucosidase and pectinase, respectively were quantified accurately and dispersed in deionized water to obtain enzyme solutions of certain concentrations (0.5–2 mg/mL). 10 g dry needles powder was added to the enzymatic solution and adjusted to certain pH (3.5–7.0) with 0.1 M HCl solution and shaken on a flat-bed orbital shaker for a period of time (12–30 h) at certain temprature (20–50 °C). After the treatment was over, the extract was filtered. The residue was collected into conical flask and 100 mL 80% ethanol was added. Then the conical flask was put into ultrasonic bath (Kunshan Ultrasonic Instrument Co., Ltd. China) and was extracted for 30 min at 25 °C and 100% power. The residue was then filtered, these processes were repeated twice. Filtrate collected was concentrated in vacuo (55 °C) in a rotary evaporator. The syrup was reconstituted with methanol into 5 mL and degreased with 5 mL 50% methanol which could remove waxes and nonpolar constituents contained in the needle samples largely. After 12 h, the sample solution was then centrifuged and filtered through a membrane filter (0.45 μm pore size) before analyzed by HPLC. All the experiments were performed in triplicate and all tests of significance were done with a probability value (p-value) of $\alpha < 0.05$.

2.5. Scanning electron microscopy

In order to examine the effect of enzyme on the structural changes of the plant cells, the *T. chinensis* leaf samples before and after enzyme treatment were scanned by *Scanning electron microscopy* (Quanta-200). The samples were fixed on adhesive tape and then sputtered with gold, and examined under high vacuum condition at an voltage of 15.0 kV (20 μm, 1000× magnification).