Bioconversion of Artemisinin to its Nonperoxidic Derivative Deoxyartemisinin through Suspension Cultures of *Withania somnifera* Dunal

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Z. Naturforsch. **65c**, 607–612 (2010); received January 9/April 1, 2010

Biotransformation of artemisinin was investigated with two different cell lines of suspension cultures of *Withania somnifera*. Both cell lines exhibited potential to transform artemisinin into its nonperoxidic analogue, deoxyartemisinin, by eliminating the peroxo bridge of artemisinin. The enzyme involved in the reaction is assumed to be artemisinin peroxidase, and its activity in extracts of *W. somnifera* leaves was detected. Thus, the non-native cell-free extract of *W. somnifera* and suspension culture-mediated bioconversion can be a promising tool for further manipulation of pharmaceutical compounds.

Key words: Artemisinin, Deoxyartemisinin, *Withania somnifera*

Introduction

The sesquiterpenes are the largest class of terpenoid compounds and common constituents of plant essential oils. Many sesquiterpenes and their alcohol, aldehyde and ketone derivatives are biologically active or precursors of metabolites with biological functions, while others have desirable fragrance, flavouring, and medicinal properties. For example, the phytoalexin capsidiol is derived from the sesquiterpene 5-*epi*-aristolochene, and amorpha-4,11-diene is the precursor of the potent antimalarial compound artemisinin (Chunzhao *et al.*, 2006). Artemisinin (artemisin), a sesquiterpene lactone having an endoperoxide bridge and obtained from the Chinese medicinal plant *Artemisia annua* L., became a promising antimalarial drug. It also shows antibacterial activity. It is a quadricycle compound that means the basic structure incorporates four rings. One of the rings has a peroxo linkage as part of the ring, *i.e.* linkage of two oxygen atoms (–O–O–) between two points on one of the rings. Artemisinin possesses 15 carbon atoms that are derived from a combination of three isoprene units. It has several features which made this antimalarial drug more popular, safe and a potent alternative to the conventional antimalarial drugs quinine and chloroquine. Nevertheless artemisinin has some limitations, like toxicity and water insolubility. Research suggested a likelihood way to modify sesquiterpene lactones generating more useful analogues (Avery *et al.*, 1999; Posner *et al.*, 1999). However, the structural complexity of artemisinin hampers its chemical modification but, on the other hand, there are several reports on its biological transformation by means of microorganisms (Fiaux de Medeiros *et al.*, 2002; Lee *et al.*, 1989; Zhan *et al.*, 2002a, b; Ziffer *et al.*, 1992). All these biotransformations of artemisinin usually lead to processes like hydroxylation and reduction of methyl, methyne and methylene groups, respectively, and hydration reactions with breakdown of heterocyclic rings of the sesquiterpene lactone. Microbial biotransformation of artemisinin, particularly by fungi and their enzymes, have been well attended, but there are very few reports on bioconversion of this potentially important antimalarial drug using plant cell culture systems (Kawamoto *et al.*, 1998; Han *et al.*, 2003). Plant systems, in contrast to the microbial system, produce a more limited range of enzymes and undifferentiated plant cells have longer doubling times than microbial cells. In addition, the desired enzymes are often produced in minute quantities. Despite these drawbacks, the plant kingdom contains some unique enzymes, which produce a

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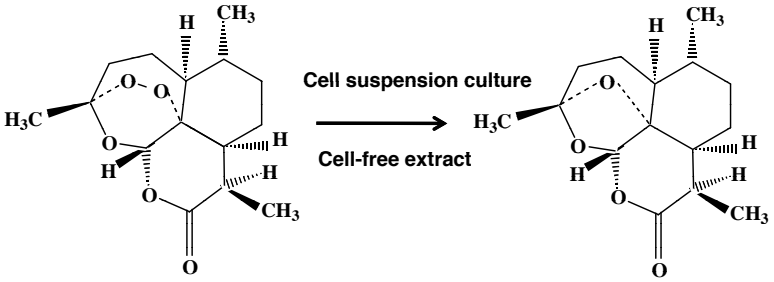

Fig. 1. Schematic representation of conversion of artemisinin to deoxyartemisinin.

variety of chemicals. Chemical synthesis of some of these phytochemical compounds is extremely complicated and costly. Hence, in spite of having disadvantage biotransformations using plant cells and isolated enzymes have enormous potential for production of pharmaceuticals. So, we studied the biotransformation of artemisinin through a plant cell suspension culture. We report here for the first time the biotransformation of artemisinin to its main derivative deoxyartemisinin by losing its peroxo bridge using a cell suspension culture of the medicinally important plant *Withania somnifera* (Fig. 1). The optimized *W. somnifera* plant extracts were also utilized to establish that this type of transformations is due to the peroxidase catalytic activity found in the plant.

Material and Methods

Cell suspension culture

Cell suspension culture lines of *W. somnifera* were obtained from previously maintained cell suspension cultures, raised from friable callus (Sabir *et al.*, 2008). These cell lines were maintained in Murashige and Skoog (1962) medium supplemented with 2,4-D (3 mg/l), kinetin (0.5 mg/l), and 3% (w/v) sucrose. The pH value of the medium was adjusted to 5.6 prior to autoclaving. The medium was sterilized by autoclaving at 121 °C and a pressure of 103 kPa, and sub-cultured every 3 weeks. These cultures were kept on a gyrorotatory shaker of 100 rpm speed and at (25 ± 1) °C.

Substrate feeding to culture

Pure artemisinin (25 mg/750 μl) was dissolved in absolute ethanol, and was added to 250 ml fully grown suspension culture of stage between middle log and early stationary phase at a final concentration of 350 μM. Cells without any addition and with equal volume of only alcohol were referred as cell control, and only maintenance media with substrate were media control. All these cultures were incubated for different times on a gyrorotatory shaker at 100 rpm and the above-mentioned culture conditions.

Harvesting of metabolite and products

After 3, 6, 12, 20 and 40 d of incubation, cell cultures were harvested and extracted thrice in equal volumes of diethyl ether, and allowed to evaporate. The dried residue was dissolved in absolute ethanol (0.5 ml) for analysis of artemisinin and its biotransformed derivatives.

Preparation of cell-free extract

Cell-free extract was prepared from the leaves of *W. somnifera* by grinding in liquid nitrogen followed by the addition of sodium phosphate buffer (pH 7.0) and 10% PVPP (polyvinyl pyrrolidone). The homogenate was centrifuged at 10,000 × *g*, 4 °C, for 30 min. The resultant cell-free supernatant was collected and subjected to peroxidase-based enzyme assay and partial purification.

Ammonium sulfate precipitation

The crude cell-free extract was precipitated with different fractions (0–20% and 20–65%) of ammonium sulfate, and the enzyme activity was assayed in the precipitate obtained at both fractionation steps.

Peroxidase assay

The cell-free extract and ammonium sulfate-fractionated precipitate were used for enzymatic assays. The reaction mixture contained cell-free extract, artemisinin (5 mM) as substrate and 0.1 M

sodium phosphate buffer of different pH values (5.5, 6.5, 7.5, and 8.5). Different categories of control reactions, such as enzyme control (E) lacking enzyme, substrate control (S), heat-killed enzyme (HK), and zero time (ZT), along with experimental (Ex) were also set up. All these reaction mixtures were incubated at 32 °C for 2 h and overnight. Reactions were stopped by adding diethyl ether. After vigorous shaking of the reaction mixture, the upper layer was taken out and allowed to completely evaporate. The dried residue was dissolved in a known volume of absolute ethanol for further analysis.

Thin-layer chromatography (TLC)

TLC was performed on pre-coated silica gel plates (Merck). The solvent system was *n*-hexane/ethyl acetate (70:30). The compounds separated on TLC plates were visualized by anisaldehyde reagent.

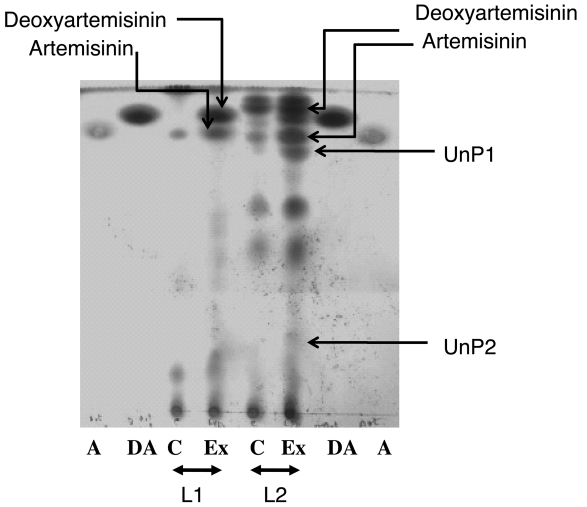

Fig. 2. Thin-layer chromatogram of bioconversion of artemisinin by two suspension culture cell lines of *Withania somnifera* (L1 and L2). Lanes A and DA, authentic artemisinin and deoxyartemisinin, respectively; lanes C, control; lanes Ex, experimental incubations with suspension lines L1 and L2.

Results and Discussion

Biotransformation of artemisinin through suspension culture

For the bioconversion of artemisinin two cell lines (L1 and L2) of *W. somnifera* were selected. The thin-layer chromatogram (Fig. 2) shows that cell line L1 has very few compounds and it converts artemisinin into only deoxyartemisinin, while on the other hand cell line L2 has two other unknown compounds beside deoxyartemisinin, one was just below the substrate artemisinin [unknown product 1 (UnP1)] and the other [unknown product 2 (UnP2)] was at lower Rf value. Different types of cell culture lines of *W. somnifera* attributed to different types of enzymes present therein, which leads to diverse profiles of product formation that are specific to the cell lines. These endogenous and bioconversion compounds were visible on TLC plates from both the experimental and controls of the cell lines L1 and L2 (Fig. 2).

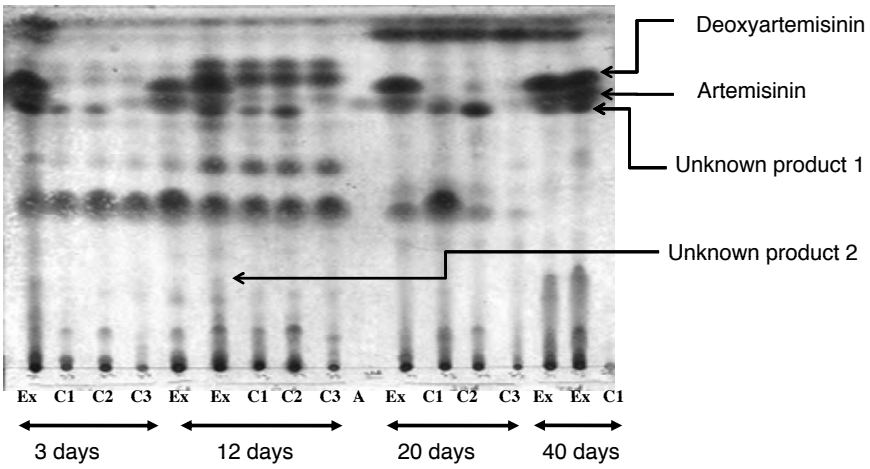

Fig. 3. Thin-layer chromatogram of the time course of biotransformation of artemisinin using suspension culture cell lines of *Withania somnifera.* Lanes Ex, experimental; lanes C1, only alcohol control cells; lanes C2, control cells; lanes C3, media control; lane A, authentic artemisinin.

Single-dose feeding with artemisinin of the cell cultures of *W. somnifera* resulted in production of three types of different compounds. In order to explore the ability of cells to transform artemisinin into its derivatives, different time-course incubations were carried out for maximum conversion. Fig. 3 shows the formation of product within different time intervals. After 3 days of feeding to the cells, conversion of artemisinin into deoxyartemisinin started and continued until 40 days of incubation, while the concentration of product was highest after 12 days of incubation. Two unknown compounds were also formed in incubated cells; UnP1 was just below to the substrate artemisinin and UnP2 was at a very low Rf value. UnP1 started to form at highest concentration after 3 days of incubation and its accumulation sustained till 12 days of incubation, whileUnP2 was produced in traces after a 12-days incubation period.

Enzyme assay for artemisinin peroxidase from cell-free extract of W. somnifera

The biotransformed product deoxyartemisinin may form after breakage of the peroxo bridge in the artemisinin structure. Therefore, an enzymatic study was carried out to interpret the enzyme involved in conversion of artemisinin into deoxyartemisinin. Artemisinin peroxidase was assayed at different pH values, ranging from pH 5.5 to pH 8.5 of sodium phosphate buffer, for 2 h of incubation. Only reactions performed at pH 5.5 and pH 6.5 exhibited of the substrate artemisinin (Fig. 4a). On the other hand there were no spots visible in reactions performed at pH 7.5 and pH 8.5 (Fig. 4a). When these reaction mixtures were allowed to run overnight, conversion of the substrate artemisinin into the product deoxyartemisinin could be detected (Fig. 4b).

The precipitated ammonium sulfate fraction (20–65%) was also checked for enzyme activity. The salt-precipitated protein did improve the product formation (Fig. 5). At pH 6.5 the crude extract showed a new product just above deoxyartemisinin produced from the reaction fed with artemisinin (Fig. 6). Such biotransformations have been reported in the literature. Using a cell-free system from *Artemisia annua*, artemisinic acid and arteannuin B were converted to artemisinin (Nair and Basile, 1992, 1993; Dhingra and Narasu, 2001). Moreover, Sangwan *et al.* (1993) reported that an horseradish peroxidase included in the cell-free system greatly enhanced the bioconversion of artemisinic acid and arteannuin B into artemisinin. This is the first report on bioconversion of artemisinin into its derivative deoxyartemisinin through a plant cell-free extract other than from *A. annua.*

Bioconversion of artemisinin mainly leads to hydroxylation at diverse positions of artemisinin, *i.e.* to hydroxyartemisinin. Derivatization of artemisinin through suspension culture of *W. somnifera* leads to the loss of the peroxo group and formation of deoxyartemisinin, like certain strains of *Staphylococcus aureus* (Srivastava *et al.*, 2009). Such conversions have also been reported previously from the fungus *Penicillium chrysogenum* (Lee *et al.*, 1989).

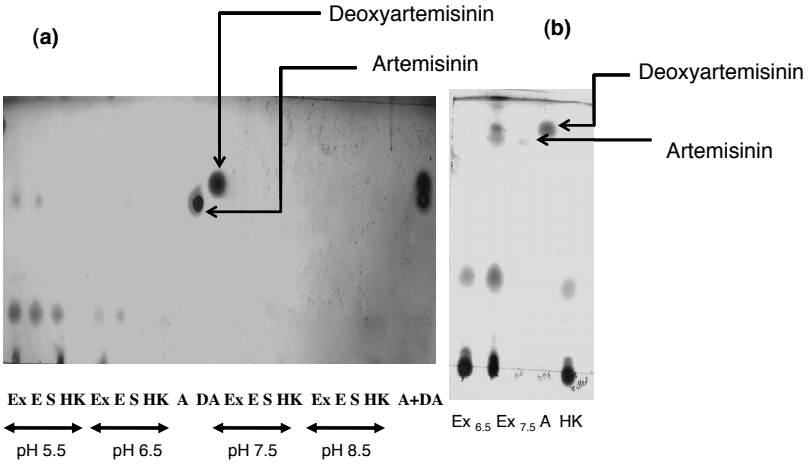

Fig. 4. (a) Thin-layer chromatogram of enzymatic conversion of artemisinin at different pH values, incubated for 2 h. Lanes Ex, experimental; lanes E, enzyme control; lanes S, substrate control; lanes HK, heat-killed enzyme control; lane A, authentic artemisinin; lane DA, deoxyartemisinin. (b) Thin-layer chromatogram of enzymatic conversion of artemisinin at different pH values, incubated overnight. Lane $Ex_{6.5}$, experimental at pH 6.5; lane $Ex_{7.5}$, experimental at pH 7.5; lane A, authentic artemisinin; lane HK, heat-killed enzyme control.

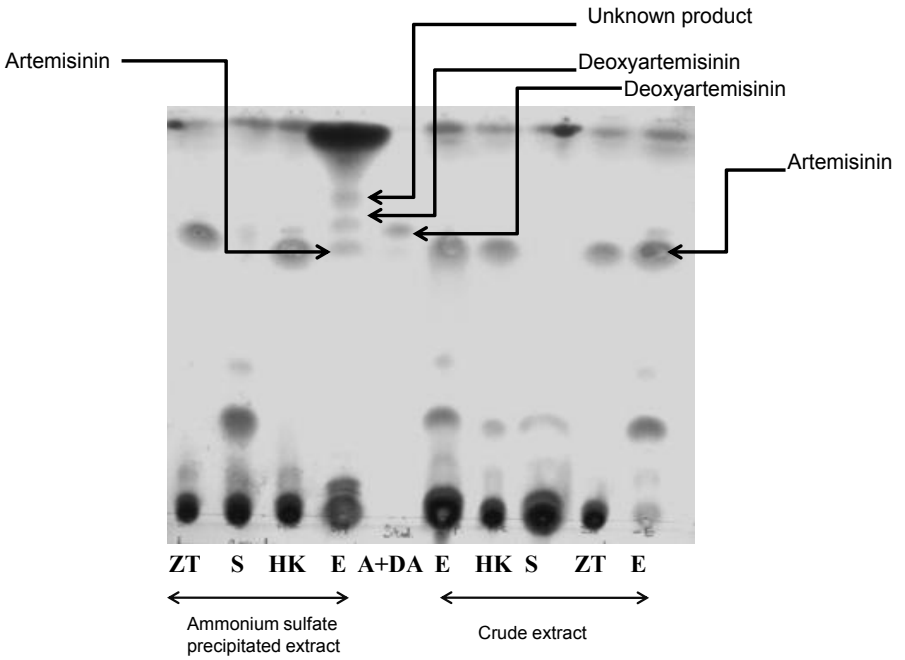

Fig. 5. Thin-layer chromatogram of enzymatic activity in ammonium sulfate-purified and crude fractions of *Withania somnifera*. Lanes ZT, zero time control; lanes S, substrate control; lanes HK, heat-killed enzyme control; lanes E, enzyme control; lane A+DA, authentic artemisinin and deoxyartemisinin.

Thus, the cell suspension culture of the medicinally important plant *W. somnifera* has the capability to transform the very important antimalarial drug artemisinin into its non-peroxidic analogue deoxyartemisinin; additionally, other interesting new compounds were detected from bioconversion, which can be promising for further pharmaceutical purposes.

Consequently, a multistep route catalyzed by cell culture often generates metabolites which help to establish biosynthetic pathways (Berlin *et al.*, 1989) and lead to favoured reactions for the compounds which are extremely complicated in structure and costly in synthesis. For this reason, biotransformations using plant cells and isolated enzymes have a vast prospective for innovation of pharmaceuticals.

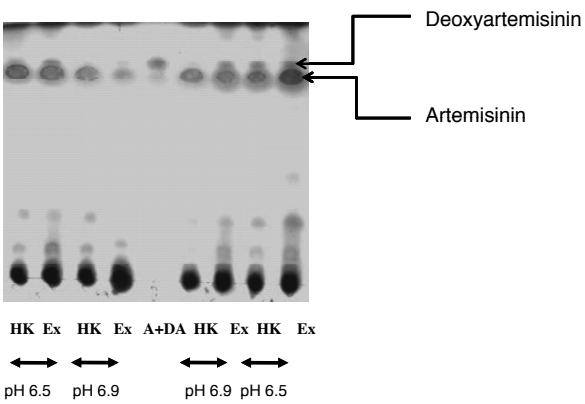

Fig. 6. Thin-layer chromatogram of enzymatic activity in crude and ammonium sulfate-purified fractions at pH 6.5 and pH 6.9. Lanes HK, heat-killed enzyme control; lanes Ex, experimental; lane A+DA, authentic artemisinin and deoxyartemisinin.

Acknowledgements

The authors are thankful to Network project NWP-09 funded by CSIR, Government of India, for financial support, and to the Director, CIMAP for the facilities and encouragement. F. S. is also thankful to CSIR, New Delhi for a Senior Research Fellowship grant.

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