Acta Botanica Sinica
植 物 学 报 2004, **46** (11): 1338-1346 http://www.chineseplantscience.com

Molecular Cloning of a Classical Plant Peroxidase from *Artemisia annua* and Its Effect on the Biosynthesis of Artemisinin *In Vitro*

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Abstract: A full-length cDNA (*apod1*) encoding a peroxidase was isolated from *Artemisia annua* L. using rapid amplification of cDNA end (RACE) strategy. The peroxidase activity of recombinant protein (APOD1) expressed in *E. coli* BL21 (DE3) pLysS cells was about 1.8-fold higher with guaiacol than ascorbate, which indicated that APOD1 was a plant classical peroxidase (class **III** peroxidase). The deduced amino acids of *apod1* had 42.0% homology to the peroxidase from *Lupinus albus*, 36.2% to *Armoracia rusticana*, 38.9% to *Triticum aestivum*, 33.6% to *Nicotiana tabacum* and 32.8% to *Lycopersicon esculentum*, respectively. Northern blotting analysis showed that *apod1* was expressed in the roots, stems and leaves of *A. annua*. APOD1 favored the bioconversion of artemisinic acid to artemisinin in the cell-free extracts of *A. annua* indirectly but no oxidization with artemisinic acid as the only substrate.

Key words: *Artemisia annua* ; artemisinic acid; artemisinin; peroxidase

Artemisinin is a secondary metabolite that has been found to have strong antimalarial activity with little or no side effects (Klayman, 1985). The relatively low yield of artemisinin in *Artemisia annua* is a serious limitation to the commercialization of the drug (Woerdenbag *et al*., 1994). The enhanced production of artemisinin, therefore, is highly desirable. Due to the complexity and high cost in chemical synthesis (Schmid and Hofheinz, 1983), the biosynthesis of artemisinin is still potential for the production of artemisinin, one of approaches is to synthesize this drug through bioconversion of less complex and more easily accessible precursors, such as arteannuin B and artemisinic acid, employing cell-free system of the plant itself. Using cell-free system from *A. annua*, artemisinic acid and arteannuin B were converted into artemisinin (Nair and Basile, 1992; 1993; Dhingra and Narasu, 2001). Moreover, Sangwan *et al*. (1993) reported that an included horseradish peroxidase greatly enhanced the bioconversion of artemisinic acid and arteannuin B into artemisinin in cell-free systems. Horseradish peroxidases are assigned to class **III** plant peroxidases, which can oxidize a wide range of organic substrates at the expense of H_2O_2 and be involved in several primary and secondary metabolic processes such as lignin polymerization (Christensen *et al*., 1998) and melanin synthesis (Gesualdo *et al*., 1997), so it is reasonable for horseradish peroxidase (class **III** peroxidase) to play a positive role in the bioconversion of artemisinic acid into artemisinin.

In the present study, bearing an attempt to find such an enzyme involved in the bioconversion of artemisinic acid into artemisinin, we isolated a class **III** peroxidase from *A. annua* of high artemisinin strain 001 and studied its effect on the bioconversion from artemisinic acid to artemisinin in the cell-free system from *A. annua*. To our knowledge, it is the first report about the isolation of a class **III** peroxidase from *A. annua*.

1 Materials and Methods

1.1 Plant materials and growth conditions

Artemisia annua L. of high artemisinin yielding strain 001 was collected from Sichuan Province, China, and was grown in the greenhouse at 25 ℃ under a light cycle of 16 h light (*ca*. 3 000 lx) and 8 h dark.

1.2 Total RNA extraction and cDNA synthesis

Total RNA was isolated from young leaves of *A. annua* using SV total RNA isolation system (Promega) according to the manufacturer's instructions. Before reverse transcription, RNA was treated with DNase **I** to remove residual DNA. Single-strand cDNA was synthesized using reverse transcript kit (TaKaRa) according to the manufacturer's instructions with oligo dT-adaptor primer (5'-$(T)_{18}$GATTTCTGTCCGACGAC-3').

Received 9 Jul. 2004 Accepted 21 Sept. 2004
Supported by the National Natural Science Foundation of China (39870911).
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1.3 cDNA cloning using the rapid amplification of cDNA ends (RACE) strategy

1.3.1 3'-RACE Comparison of plant classical peroxidases isolated from *Armoracia rusticana* (Fujiyama *et al*., 1990), *Triticum aestivum* (Baga *et al*., 1995), *Nicotiana tabacum* (Diaz *et al*., 1993) and *Lycopersicon esculentum* (Roberts and Kolattukudy, 1989) revealed highly conserved region RLHFHDCFVDG and VSCADILAIA. Degenerated primer F_1 (5'-CTT/A/G/CCAC/TTTCCAT/CGACTGCTT-3') and F_2 (5'-GTT/ATCTTGTGCT/AGATATT/A/GT/CTT/AGCT/CGC-3') was designed. The first round of PCR was performed in a 25-μL total volume containing 1 μL of reverse transcript product, 0.2 μmol/L oligo dT-adaptor primer, 0.2 μmol/L F_1 primer, 2 U *pfu* DNA polymerase (Promega), 0.2 mmol/L dNTP in 1 × reaction buffer (20 mmol/L Tris-HCl, pH 8.8, 10 mmol/L KCl, 10 mmol/L $(NH_4)_2SO_4$, 2 mmol/L $MgSO_4$, 0.1 mg/mL BSA and 0.1% Triton X-100). Cycling conditions were set as follows: one cycle of pre-denaturation at 94 ℃ for 2 min and then at 94 ℃ for 40 s, at 50 ℃ for 40 s and at 72 ℃ for 2 min for a total of 32 cycles. One μL first PCR product was used as template and 0.2 μmol/L F_2 primer and adaptor primer were used for the second round of PCR. The second PCR program was the same as the first one but the annealing temperature was set at 55 ℃. The PCR product was cloned into a pGEM-T vector (Promega) according to the manufacturer's instructions and sequenced with a DNA sequencing system (ABI model 377A, Applied Biosystems, Perkin-Elmer).

1.3.2 5'-RACE The 5' rapid amplification of cDNA end was performed with gene-specific primers designed against partial sequences obtained through 3'-RACE. Primer sequences were as follows: GSP-RT 5'-TGGGTCAACGTTCTTTGGGCAC-3', GSP_1 5'-CATGTCAGCTTGGGTCAATCC-3', GSP_2 5'-GCAGGTTTCCACCAACGCTAG-3'. First strand cDNA was synthesized using Super Reverse Transcriptase with GSP-RT primer under the recommended conditions. Hybrid DNA-RNA was digested with RNase H. The first-strand cDNA was then purified by QIAquick PCR purification (Qiagen) and ethanol precipitation. Then polyA tailing of the first-strand cDNA was performed with terminal transferase from Promega. The first PCR was carried out in a 25-μL total volume containing 1 μL of the above linked cDNA, 0.2 μmol/L GSP_1 primer, 0.2 μmol/L oligo dT-adaptor primer, 0.2 mmol/L dNTP, 2.5 U *Taq* polymerase in 1 × reaction buffer. The PCR was cycled once of 2 min denaturation at 94 ℃ and then at 94 ℃ for 40 s, at 52 ℃ for 40 s and at 72 ℃ for 1 min for a total of 30 cycles. One μL of the first PCR product was used as a template in the second PCR. The second PCR was performed in a 25-μL total volume containing 1 μL of the first PCR product, 0.2 μmol/L GSP_2 primer, 0.2 μmol/L adaptor primer, 0.2 mmol/L dNTP, 2.5 U *Taq* polymerase. The PCR cycled condition was the same as the first PCR except the annealing temperature set at 57 ℃. The resultant PCR product was cloned into pGEM-T vector and sequenced.

1.4 Comparison of peroxidase protein sequences

Several plant classic peroxidase sequences were aligned with the deduced amino acid sequences of APOD1 using CLUSTAL W program (Thompson *et al*., 1994).

1.5 Tissue expression analysis of *apod1* using Northern blotting

Total RNA was isolated from different tissues (roots, stems, leaves) of *A. annua* using SV total RNA isolation system. Five μg of RNA samples were fractionated on formaldehyde agarose gels, transferred to a Hybond N^+ membrane, and hybridized to the *apod1* cDNA clone using primer-a-gene labeling system (Promega). Hybridization was carried out overnight at 65 ℃. Membrane was rinsed briefly in 2× SSC, 0.1% SDS, twice for 5 min each in 1×SSC, 0.1% SDS. They were then exposed to X-film.

1.6 *Escherichia coli* expression of *apod1* cDNA

The ORF of *apod1* was amplified using a sense primer (5'-GGACATATGGGTCGTATAATTGTTTTTC-3') harboring *Nde* Ⅰ site (underlined) and an antisense primer (5'-GCAGTCGACTCAATGCAGCAAATTACAA-3') with *Sal* Ⅰ site (underlined). The PCR product was introduced into the expression vector pET-30a through the *Nde* Ⅰ and *Sal* Ⅰ sites and designated as pET-Apod1. The construct was then transferred into *E. coli* strain BL21 ((DE3) pLysS). *E. coli* cells harboring pET-Apod1 or pET-30a were inoculated in LB medium containing 50 μg/mL kanamicin and 34 μg/mL chloramphenicol. They were induced with 0.5 mmol/L IPTG (isopropyl-ß-*D*-thiogalactopyra-noside) when they reached an A_{600} of 0.5 and grown for further 12 h at 25 ℃. The cells were then harvested by centrifugation at 5 000 *g* for 10 min. For total protein extraction, the collected cells were resuspended in 1 × SDS-polyacrylamide gel sample buffer and boiled for 10 min. For soluble protein preparation, they were resuspended in buffer A (50 mmol/L potassium phosphate, pH 7.0) and disrupted by sonication. The cell lysates were centrifuged at 12 000*g* for 10 min at 4 ℃ and the supernate was obtained as soluble protein fractions.

Ammonium sulphate fractionation was applied to the supernantant of the expressed enzyme induced from bacterial culture harboring pET-Apod1. The precipitations of 40% – 65% ammonium sulphate saturation was collected, resuspended in 10 mL buffer A and dialysed against the same

buffer overnight. The dialysate was used as the crude enzyme expressed in pET-Apod1 for the activity assay. The soluble protein expressed in pET-30a served as the control for the enzyme activity assay.

The protein fractions obtained as above were used for SDS-PAGE analysis.

1.7 APOD1 enzyme activity assay

The APOD1 activity with ascorbate as the substrate was determined in a reaction mixture containing 50 mmol/L potassium phosphate (pH 7.0), 1 mmol/L ascorbate, 0.5 mmol/L H_2O_2 and 100 μg expressed crude protein in a final volume of 3 mL. The reaction was initiated with the addition of H_2O_2. Oxidation of ascorbate was followed by the decrease of absorbance at A_{290} (De = 2.8 mmol·L^{-1}·cm^{-1}) due to ascorbate oxidation. Oxidation of guaiacol was measured using the same reaction mixture but with 20 μg crude enzyme added, ascorbate replaced by 20 mmol/L guaiacol. The peroxidase activity with guaiacol as the substrate was assayed by the increase in A_{470} (De = 26.6 mmol·L^{-1}·cm^{-1}) (Mamuka *et al*., 1997). Protein concentration was determined using the Bradford protein assay procedure with BSA as a standard.

1.8 Effect of APOD1 on the bioconversion of artemisinic acid to artemisinin *in vitro*

1.8.1 Preparation of cell-free extracts Cell free extracts were prepared as described by Sangwan *et al.* (1993) with little modification. All the procedures of cell-free extract preparation were performed at 4 ℃. Immature leaves (10 g) of *A. annua* were homogenized with 100 mL of cold 100 mmol/L potassium phosphate (pH 7.0) containing 2 mmol/L DTT, 5 mmol/L ascorbate, 3 mmol/L EDTA, 0.2 mol/L sucrose, 4 mmol/L $MgCl_2$ and 2% PVP. The homogenate was filtered through four layers cheesecloth and then the collected filtrate was centrifuged at 20 000*g* for 10 min. The supernatant was used for the cell-free extracts.

1.8.2 Incubation systems System A consisted of 100 μg artemisinic acid, 100 μg crude enzyme induced from pET-30a, 0.1 mmol/L NADPH, 4 mmol/L $FeCl_3$, 2.5 mmol/L 2-oxoglutarate and 2 mmol/L H_2O_2 in 10 mL cell-free extracts. System B contained the same reaction mixtures as system A but with 100 μg crude enzyme induced from pET-30a replaced by that from pET-apod1. System C included all the same constitutes as system A except that the cell-free extracts had been ultrafiltrated as follows. System D included the same reaction components as system B but in ultrafiltrated cell-free extracts. System E contained 100 μg artemisinic acid, 100 μg crude enzyme induced from pET-apod1, 1 mmol/L NADPH, 4 mmol/L $FeCl_2$, 2.5 mmol/L 2-oxoglutarate, 2 mmol/L H_2O_2 in 100 mmol/L potassium phosphate (pH 7.0).

In systems C and D, cell-free extracts were ultrafiltrated through a filter with 10 kD cut off to remove low molecular weight secondary metabolites. The procedures of ultrafiltration of the cell-free extracts were according to the methods described by Nair and Basile (1993).

The incubation was carried out in stoppered glass tubes at 30 ℃ for 180 min in a water bath. After incubation, the reactions were quenched with 1 mL $CHCl_3$:EtOH (1:1) mixture and were extracted by hexane. Hexane was removed and the residue was dissolved in methanol for HPLC analysis.

1.8.3 Determination of artemisinin and artemisinic acid by HPLC Artemisinin content was determined according to the method described by Zhao and Zeng (1985). For the determination of artemisinic acid content, the methanol soluble portions of the hexane extracts were directly used for HPLC analysis under the following conditions: C18 column (150 × 3.9 mm), mobile phase of 1% TFA in water: acetonitrile (30:70) at a flow rate of 1 mL/min, UV detector at 210 nm.

2 Results

2.1 cDNA cloning of *apod1*

The nucleotide sequences of the full-length cDNA encoding a peroxidase were obtained using RACE strategy (GenBank accession No. AY208699) and designated as *apod1*. The cDNA of 1 182 bp contained an open reading frame (ORF) of 987 bp, an 36 bp-5' UTR (untranslated region) and 160 bp-3' UTR. The deduced amino acid sequence of the full-length peroxidase of *A. annua* is shown in Fig.1.

2.2 Amino acid sequence similarity

The deduced amino acid sequence of *apod1* showed variable similarity to other plant classic peroxidases. An alignment of six peroxidases including APOD1, has been carried out (Fig.2), APOD1 showed 42.0% similarity to *Lupinus albus* (Price *et al*., 2003), 36.2% to *Armoracia rusticana* (Fujiyama *et al*., 1990), 38.9% to *Triticum aestivum* (Baga *et al*., 1995), 33.6% to *Nicotiana tabacum* (Diaz *et al*., 1993) and 32.8% to *Lycopersicon esculentum* (Roberts and Kolattududy, 1989), respectively. Several highly conserved regions of classical peroxidases were found in APOD1. The first one (Phe-His-Asp-Cys-Phe-Val) belonged to the acid/base catalysis region and included the functional histidine residue (Dominique *et al*., 1990). The second one (Val-Ser-Cys-Ala-Asp) presented in all the six peroxidases. The third conserved domain (Ala-Leu-Ser-Gly-

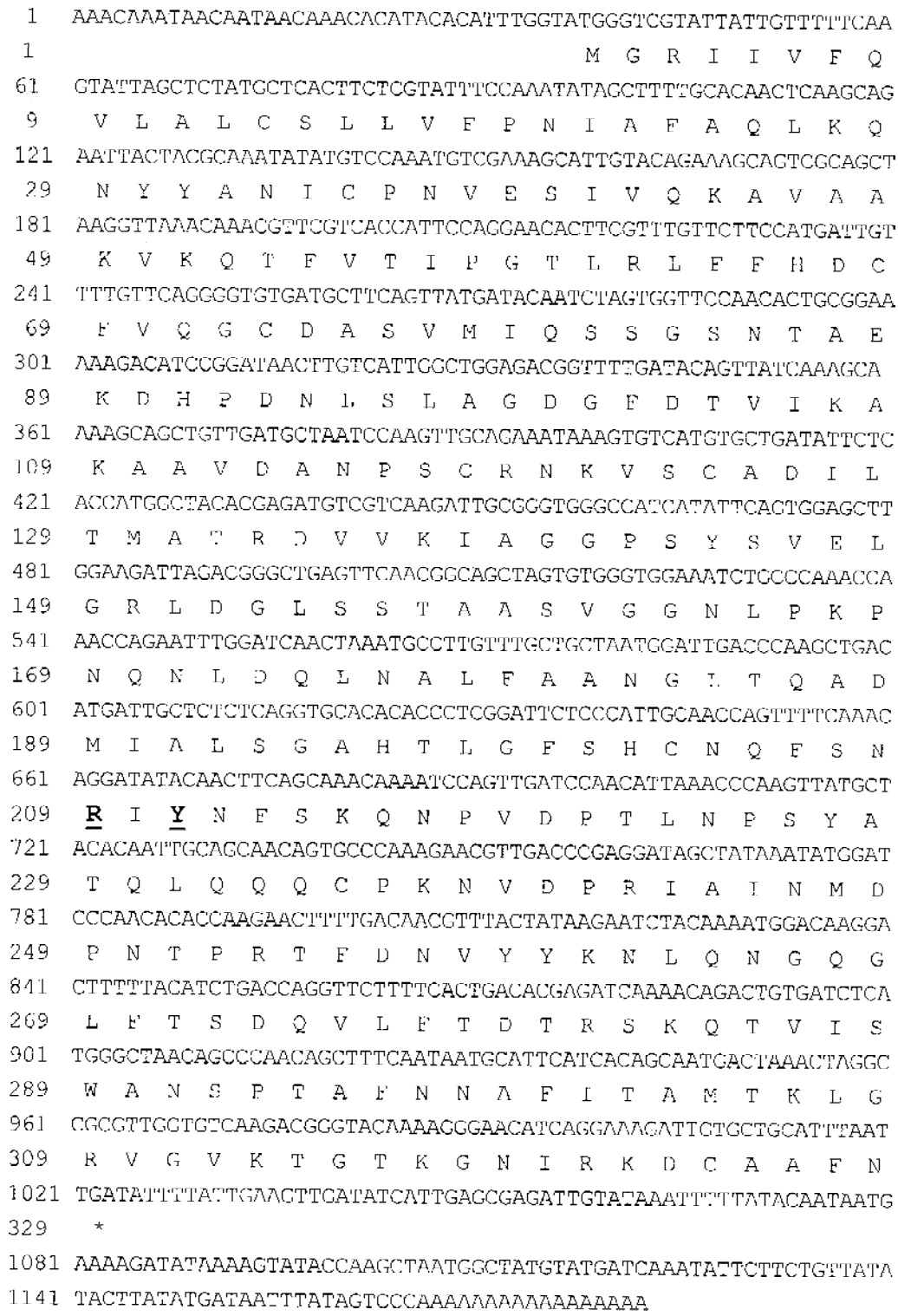

Fig.1. *apod1* cDNA sequence and its deduced amino acid sequence. The bold residues underlined represented the binding sites of substrate (Fujiyama *et al*., 1988). *, stop codon.

Ala-His-Thr) included the histidine residue involved in the fifth ligand of heme (Dominique *et al*., 1990). The Arg^{221} and Tyr^{223} were putative binding sites of the substrate (Fujiyama *et al*., 1988) (Fig.2).

2.3 Tissue expression analysis of *apod1* using Northern blotting

Northern blotting analysis demonstrated that *apod1* was expressed in roots, stems and leaves of *A. annua* (Fig.3).

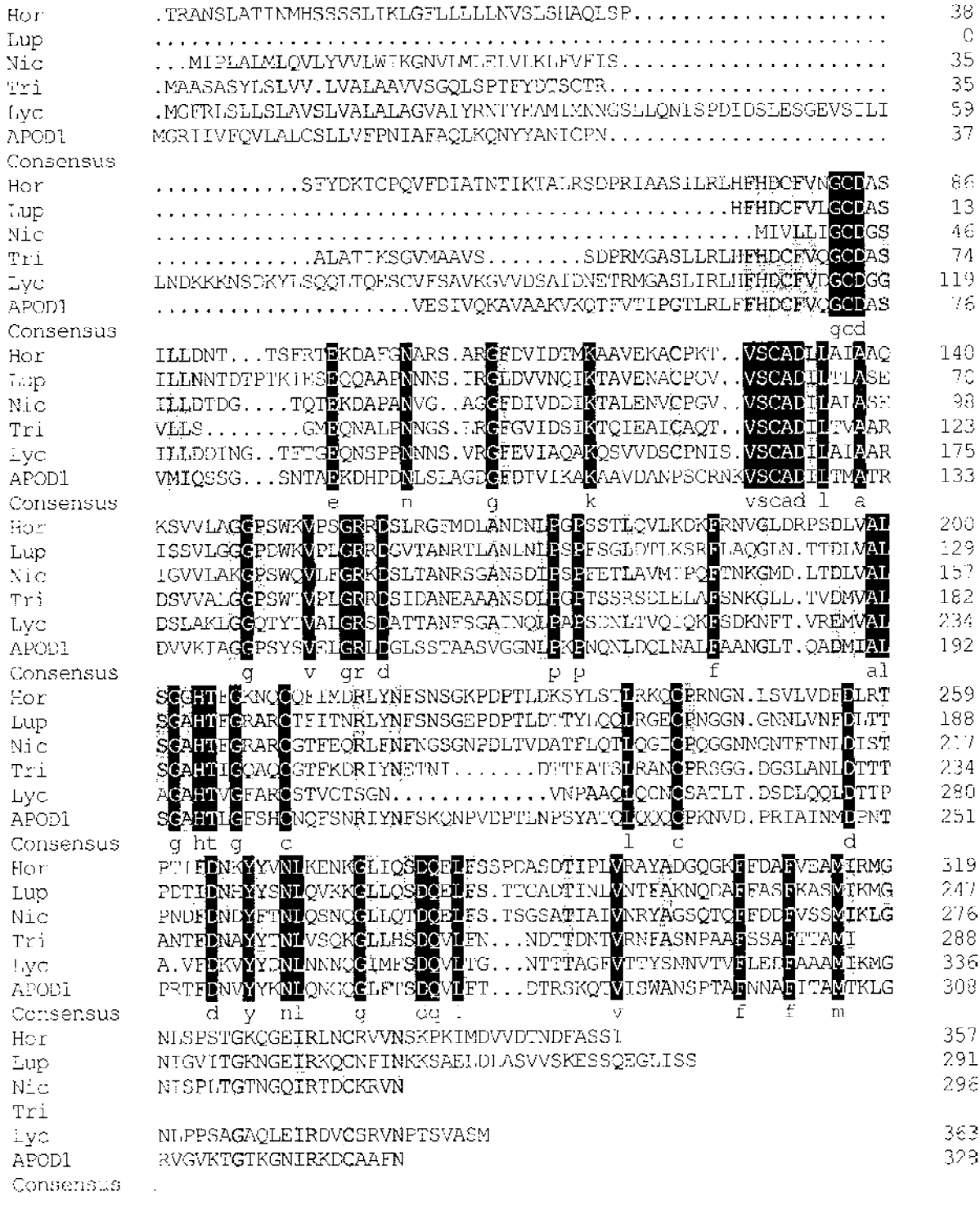

Fig.2. Alignment of the amino acid sequence of APOD1 with other classical plant peroxidases. APOD1, a putive peroxidase from *Artemisia annua*; Hor, horseradish peroxidase from *Armoracia rusticana*; Lup, a peroxidase from *Lupinus albus*; Lyc, an anionic peroxidase from *Lycopersicon esculentum*; Nic, tobacco anionic peroxidase from *Nicotiana tabacum*; Tri, a peroxidase from *Triticum aestivum*.

2.4 *apod1* expression in *E. coli*

The vector pET-Apod1 containing *apod1* mature protein was transferred into *E. coli* BL21 ((DE3) pLysS) cells and the protein was induced by addition of 0.5 mmol/L IPTG for 12 h at 25 ℃. SDS-PAGE result showed that the recombinant polypeptide was successfully induced in this *E. coli* expression and soluble induced APOD1 was also found distinctly in the supernatant. The concentration of soluble APOD1 increased with the precipitation by 40% – 65% saturation of ammonium sulphate (Fig.4).

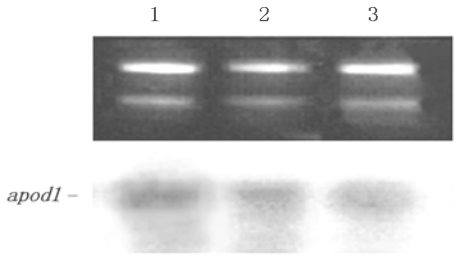

Fig.3. Tissue expression analysis of *apod1* by Northern blotting analysis. Lane 1, leaves; lane 2, stems; lane 3, roots.

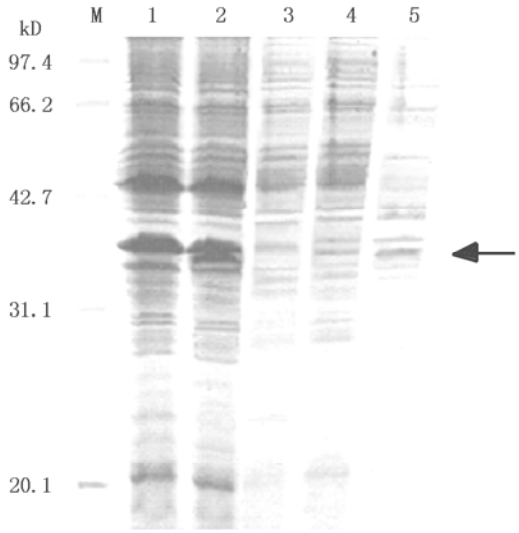

Fig.4. SDS-PAGE analysis of APOD1 expression *in vitro*. M, molecular weight marker; lane 1, total protein expressed in pET-30a; lane 2, total protein expressed in pET-Apod1; lane 3, soluble fraction expressed in pET-30a; lane 4, soluble fraction expressed in pET-Apod1; lane 5, 40%-65% $(NH4)_2SO_4$ precipitation of the soluble protein expressed in pET-Apod1. Arrows indicate the induced expression of APOD1.

2.5 Enzyme activity analysis of the expressed APOD1 protein

The expressed protein activities with guaiacol and ascorbate as the substrates were assayed. One of the ways of distinguishing ascorbate peroxidases from classical ones was to compare the relative activity for guaiacol and ascorbate oxidation. The relative specific activities differences for these two donors by APOD1 were striking. APOD1 catalyzed the oxidation of guaiacol at about 1.8-fold higher rate than that of ascorbate (Fig.5). Based on the comparison of protein sequences and the activity analysis, APOD1 was believed as a classical plant peroxidase.

2.6 Effect of APOD1 on the content of artemisinic acid and artemisinin in the cell free extracts from *A. annua*

The effect of APOD1 on the contents of artemisinic acid and artemisinin in the cell free systems was studied. The

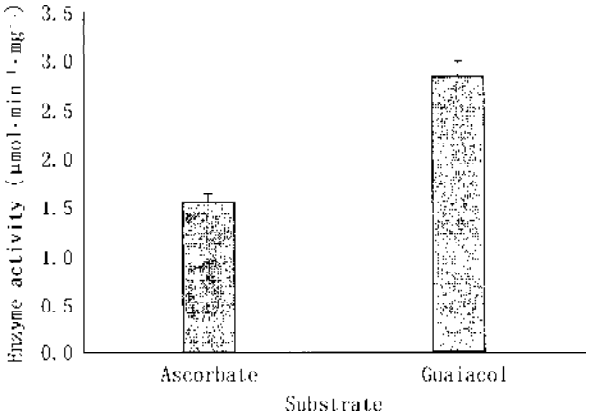

Fig.5. Enzyme activities of APOD1 with ascorbate and guaiacol as the substrates. The value was determined at three independent replicates.

quantifications of artemisinic acid and artemisinin were analyzed by HPLC. The retention time for artemisinic acid and artemisinin were between 5.2 – 5.3 and 7.2 – 7.3 min respectively under the analysis conditions (Fig.6). Compared with that in system A, the content of artemisinic acid decreased by about 30% while the content of artemisinin increased by about 50% when the expressed APOD1 was added into the cell-free extracts of *A. annua* in system B. However, when the cell-free extracts were ultrafiltrated to remove small molecular weight metabolites, no significant differences of the contents of artemisinic acid and artemisinin occurred between systems C and D. This result indicated that the positive effect of APOD1 on the bioconversion of artemisinic acid to artemisinin disappeared when small molecular weight metabolites were removed from the cell-free extracts (Table 1). Consequently, we suspected that APOD1 just affected the bioconversion of artemisinic acid to artemisinin indirectly. Moreover, when APOD1 was added to the system E in which artemisinic acid was the only substrate, the analysis by HPLC indicated that no any transformatic products were detected in system E.

3 Discussion

Attempts to produce artemisinin were made by means of biotechnology and chemical synthesis (Gelder *et al*., 1997). Due to low artemisinin content in *A. annua*, several gene engineering means were employed to improve the production of artemisinin (Chen *et al*., 1999; 2000). However, most attention was paid to the pathway before the formation of artemisinic acid. Since the level of artemisinic acid was more higher than that of artemisinin (Roth and Acton, 1987), the “bottle neck” of artemisinin biosynthesis should present in the pathway from artemisinic acid to artemisinin.

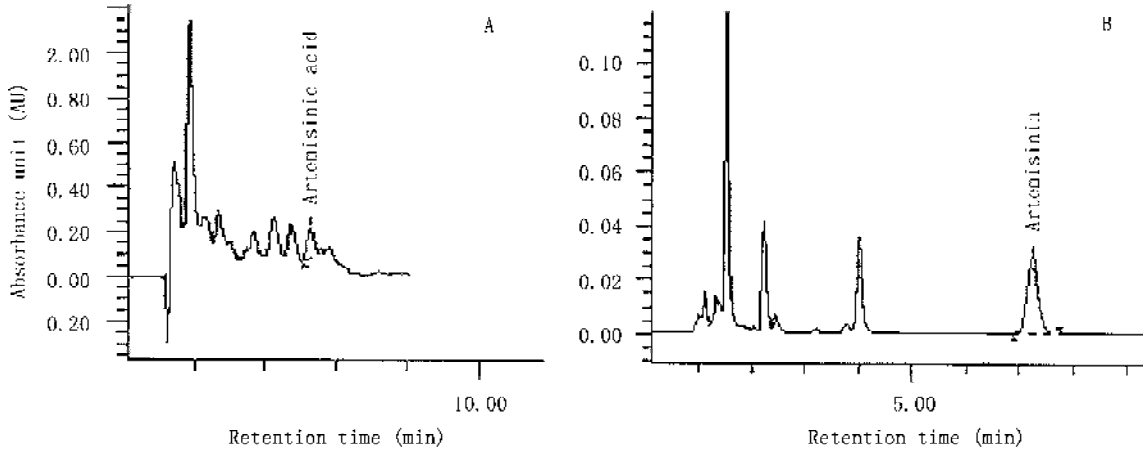

Fig.6. Spectrometric HPLC of artemisinic acid and artemisinin in cell-free extracts. **A.** HPLC profile for artemisinic acid detection. **B.** HPLC profile for artemisinin detection.

Table 1 Contents of artemisinin and artemisinic acid extracted from different incubation systems (10 mL) detected by HPLC

Content	Incubation systems				
(mg)	A	B	C	D	E
Artemisinic acid	0.452 0[a] ± 0.004 5[b]	0.318 0 ± 0.005 0	0.066 0 ± 0.003 0	0.069 0 ± 0.004 0	0.094 0 ± 0.003 7
Artemisinin	0.032 0 ± 0.005 3	0.063 0 ± 0.002 1	0.013 0 ± 0.001 5	0.011 0 ± 0.003 5	ND

ND, not detected; a, each value represents three independent determinations; b, standard error (*SE*).

Attempts to affect the bioconversion of artemisinic acid into artemisinin hold a great potential to improve artemisinin biosynthesis remarkably. However, there were not any reports about the isolation of key enzymes involved in the pathway from artemisinic acid to artemisinin, so attempts to improve the bioconversion from artemisinic acid into artemisinin by gene engineering means were a challenge. Horseradish peroxidase, as a classical plant peroxidase, was reported to improve the bioconversion of artemisinic acid into artemisinin greatly *in vitro* (Sangwan *et al*., 1993). Classical plant peroxidases can oxidize many substrates and were reported to be involved in the biosynthesis of many metabolites such as lignin and melanin, so it seemed probable that classical plant peroxidases had a positive role in the biosynthesis of artemisinin. Using rapid amplification of cDNA ends (RACE) strategy, we successfully isolated a peroxidase (APOD1) from *A. annua*. By comparing the amino acids of APOD1 with those of other plant classical plant peroxidases, it was found that several conserved regions of plant classical peroxidases occurred in APOD1. Enzyme comparative activities of APOD1 with ascorbate and guaiacol as the substrates revealed that APOD1 catalyzed the oxidation of guaiacol at about 1.8-fold higher rate than ascorbate. Based on the amino acid sequence similarities and peroxidation activities, it was concluded that APOD1 was a classical plant peroxidase.

The effect of APOD1 on the bioconversion of artemisinic acid to artemisinin in the cell-free systems of *A. annua* was investigated. The results indicated that including APOD1 into the cell-free extracts of *A. annua* favored the accumulation of artemisinin *in vitro*. However, when small weight molecular metabolites were removed from the cell-free extracts, the positive effect of APOD1 on the biosynthesis of artemisinin disappeared (in system D). Moreover, APOD1 had no oxidization activity with artemisinic acid as an only substrate (in system E). These results indicated that APOD1 might interact with small weight molecular metabolites and indirectly increased the accumulation of artemisinin in cell-free extracts. Since the cadinene skeleton of artemisinin is polyoxygenated and the formation of dihydroartemisinic acid hydroperoxide, a direct precursor of artemisinin, was singlet oxygen-dependent, the inclusion of singlet oxygen in the culture medium should facilitate the accumulation of dihydroartemisinic acid hydroperoxide which then can be very easily oxidized to artemisinin (Wallaart *et al*., 1999; 2000). Singlet oxygen can be formed in enzymatic catalysis (Wallaart *et al*., 1999). It had been found that singlet oxygen presented in the enzyme-H_2O_2 mixture (Sangwan *et al*., 1993). APOD1, as a plant classical peroxidase, may oxidize other substrates with H_2O_2 in cell-free systems of *A. annua*, introduce singlet oxygen into the reaction mixture and consequently activate the accumulation of artemisinin,

Moreover, Farouk *et al.* (1986) reported that artemisinic acid could be converted into arteannuin B by singlet oxygen. Therefore, it appeared that the singlet oxygen generated in the cell-free system including APOD1-H_2O_2 mixture resulted in a decrease of artemisinic acid level and an increase of artemisinin level. Surely further experiment should be carried out to elucidate the mechanism of the effect of APOD1 included in the cell-free extracts on the content variations of artemisinic acid and artemisinin. In the present study, it was indicated that APOD1 just had an indirect effect on the bioconversion of artemisinic acid to artemisinin in cell-free systems of *A. annua*.

Acknowledgements: We sincerely thank Prof. LI Ying from Pharmacy Institute, The Chinese Academy of Sciences for supporting artemisinic acid authentic sample.

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(Managing editor: WANG Wei)