Identification, characterisation and expression of early biosynthetic genes from Artemisia annua

Rydén, Anna-Margareta

Chapter 6

Role of peroxygenase in artemisinin production

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Abstract

Artemisinin is an antimalarial drug which is produced by the medicinal plant *Artemisia annua* L. (*A. annua*). The yields are notoriously low (0,2-0,8% dry weight), and therefore several approaches to increase the production are being explored. One strategy is focused on elucidating the biosynthetic pathway of artemisinin. By understanding the biosynthesis of the drug, and by isolating the responsible genes, it will be possible to produce it in heterologous hosts. Currently, the genes involved in the production of the early intermediates are known: production of artemisinic acid and dihydroartemisinic acid in yeast has been reported. These intermediates can be chemically converted to artemisinin. However, it is desirable to directly obtain artemisinin as end product in heterologous production platforms. In order to satisfy this condition, an understanding of what happens with the the intermediates artemisinic acid and dihydroartemisinic acid is needed. It is not known how these intermediates are turned into artemisinin *in planta.* We here investigate the enzymatic involvement in formation of late intermediates in the artemisinin biosynthetic pathway.

Introduction

Artemisinin is produced in small quantities (0.01-2% dry weight (Zhang et al., 2008)) in the plant *Artemisia annua* L. (*A. annua*) and has received great attention due to its antimalarial property. Research aimed at elucidating the biosynthetic route of the compound, has resulted in the isolation of four genes from the plant thus far (Bertea et al., 2006; Bouwmeester et al., 1999; Mercke et al., 2000; Rydén et al., 2010; Rydén et al., 2009; Wallaart et al., 1999; Zhang et al., 2008). The biosynthetic pathway of artemisinin has been clarified to the level of dihydroartemisinic acid (figure 1) (Bouwmeester et al., 1999; Mercke et al., 2000; Ro et al., 2006; Wallaart et al., 2001). The first committed precursor, amorpha-4,11-diene, is formed by cyclization of farnesyl diphosphate by amorphadiene synthase. Amorpha-4,11-diene is consecutively oxidized to artemisinic alcohol, artemisinic aldehyde and artemisinic acid by the cytochrome P450 Cyp71av1. Artemisinic aldehyde is also serving as a substrate for a carbon double bond reductase, Dbr2, which reduces the exocyclic carbon double bond, thereby forming dihydroartemisinic aldehyde. Dihydroartemisinic aldehyde is further oxidized by Cyp71av1 to dihydroartemisinic acid. Dihydroartemisinic acid, in contrast to artemisinic acid, is thought to be the immediate precursor to artemisinin. This is based on the finding that plants containing high levels of artemisinin also contain high levels of dihydroartemisinic acid, while plant with low levels of artemisinin display low levels of dihydroartemisinic acid and high levels of artemisinic acid. Currently, it is not clear whether the conversion of dihydroartemisinic acid to artemisinin occurs enzymatically or spontaneously *in planta*. Evidence supporting both claims has been presented. Recently, the conversion of dihydroartemisinic acid to artemisinin in dead leaves of *A. annua* has been reported (Lommen et al., 2007). However, the same laboratory also shows that dihydroartemisinic acid is converted to an unknown intermediate and

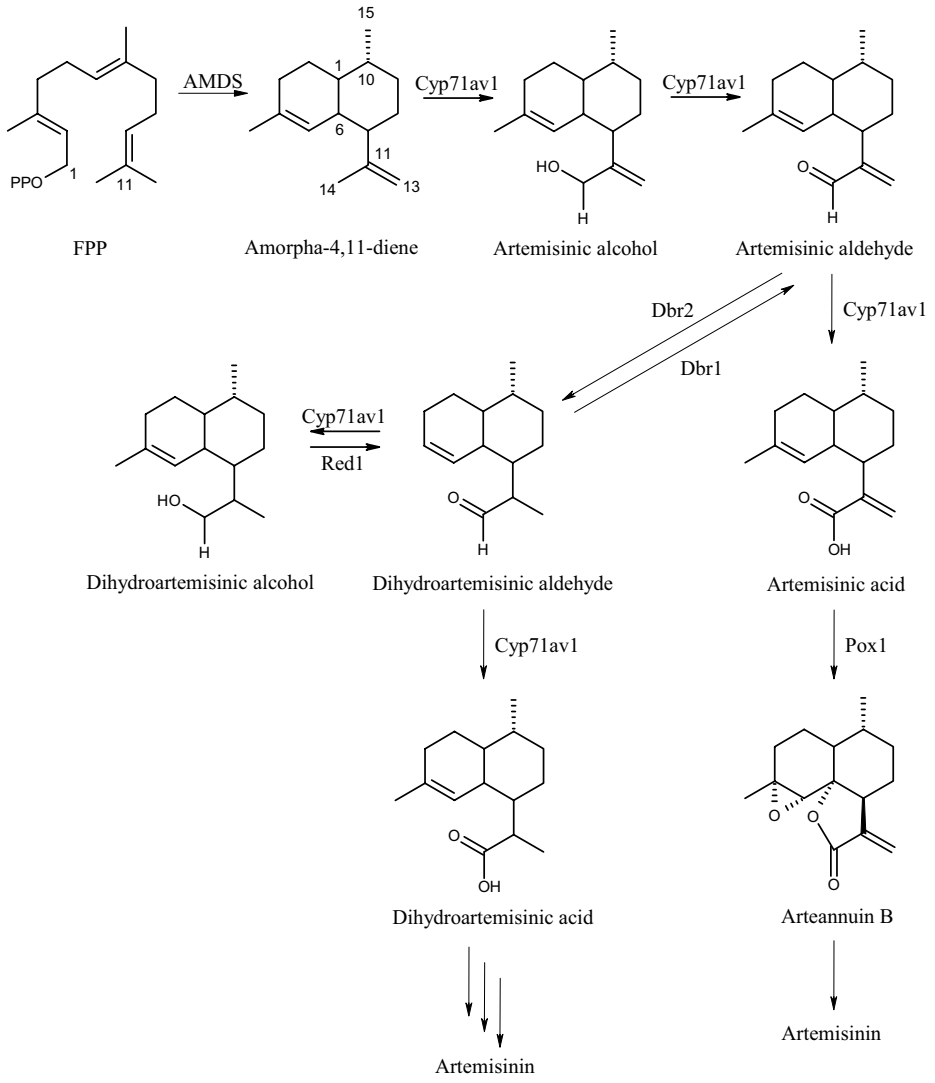

Figure 1. Proposed biosynthesis of artemisinin. The identities of Dbr1 and Pox1 are not fully determined, but are supported by biochemical evidences reported in this chapter. Enzymatic conversion of arteannuin B has been reported (Dhingra et al., 2001).

thereafter into artemisinin in living as well as dead tissue *in planta* (Lommen et al., 2006). This unknown intermediate is postulated to be dihydroartemisinic acid hydrogen peroxide and can be formed non-enzymatically (Brown et al., 2004; Sy, 2002; Wallaart et al., 1999; Wallaart et al., 1999), as well as possibly enzymatically. The involvement of reactive oxygen species, such as hydrogen peroxide, in artemisinin production has been pointed out by adding salicylic acid to *A. annua* plants (Pu et al., 2009) and DMSO to *A. annua* shoot cultures (Mannan et al., 2010). There are also evidences that pin point artemisinic acid as the precursor of artemisinin. A link between artemisinic acid, arteannuin B and artemisinin has been established in hairy-root cultures (Weathers et al., 1997) and enzymatic conversion of arteannuin B has been presented (Dhingra et al., 2001), although the identity of the enzyme involved remains obscure.

Results and Discussion

Enzyme conversion assays

In order to evaluate a possible enzymatic conversion of either artemisinic acid or dihydroartemisinic acid to artemisinin, leaves and flowers of *A. annua* were collected and crude protein isolations were obtained from the fresh plant material. These protein solutions were tested with the substrates dihydroartemisinic aldehyde, artemisinic acid and dihydroartemisinic acid while adding the cofactor NADPH (figure 2) or hydrogen peroxide (figure 3 and figure 4).

As shown in figure 2, the production of dihydroartemisinic alcohol from the substrate dihydroartemisinic aldehyde by Red1 (figure 1) (Rydén et al., 2010) is dominant, as can be expected with NADPH added as cofactor, but there is also a significant production of artemisinic acid and traces of artemisinin. Artemisinic acid is

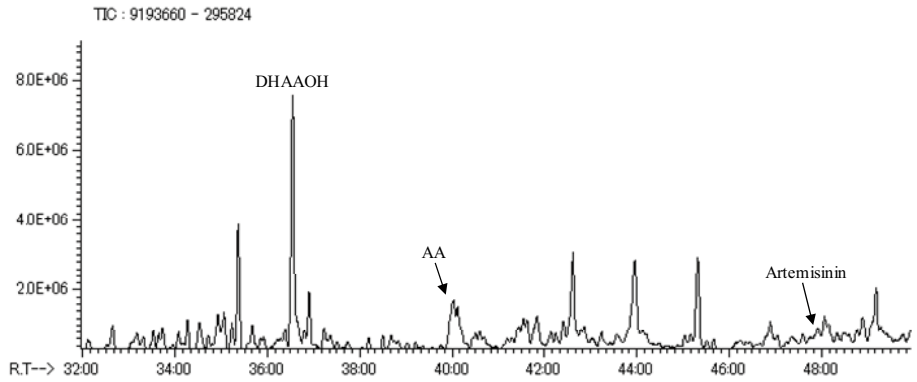

Figure 2. GC chromatogram of assay with crude *A. annua* protein isolation, dihydroartemisinic aldehyde and NADPH.

likely produced by a combination of $NADP^+$ and an unknown carbon double bond reductase (Dbr1). $NADP^+$ is produced by the action of Red1, which consumes NADPH and produces $NADP^+$ and dihydroartemisinic alcohol. The substrate dihydroartemisinic aldehyde is oxidized to artemisinic aldehyde by Dbr1 while regenerating NADPH. Based on a published biochemical analysis of a known carbon double bond reductase from *A. annua*, Dbr2, it is likely that Dbr1 is different from Dbr2 (Zhang et al., 2008). The authors showed that Dbr2 converts artemisinic aldehyde to dihydroartemisinic aldehyde, but the reverse reaction was not reported. Artemisinic aldehyde, produced by Dbr1, is further oxidized to artemisinic acid by Cyp71av1. The steps between artemisinic acid and artemisinin are not clear.

The artemisinin intermediates artemisinic acid and dihydroartemisinic acid were exposed to crude *A. annua* protein isolations and hydrogen peroxide, to determine if the conversion to artemisinin is enzyme dependent or spontaneous (figure 3 and figure 4). Several cofactors such as KI and $FeCl_2$ and other metal ions were investigated for a possible involvement in conversion of the acids to artemisinin, but neither enzymatic nor spontaneous

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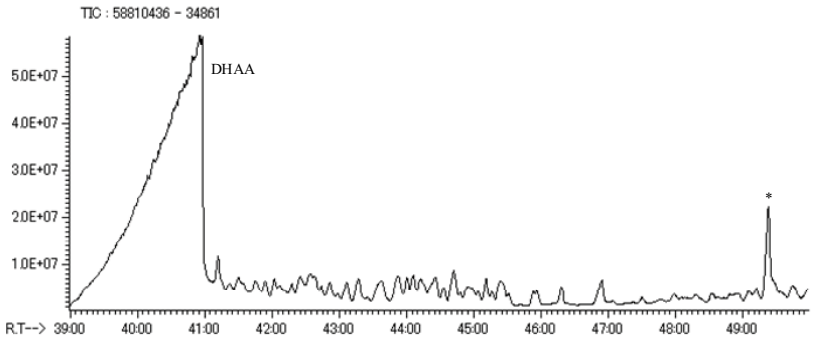
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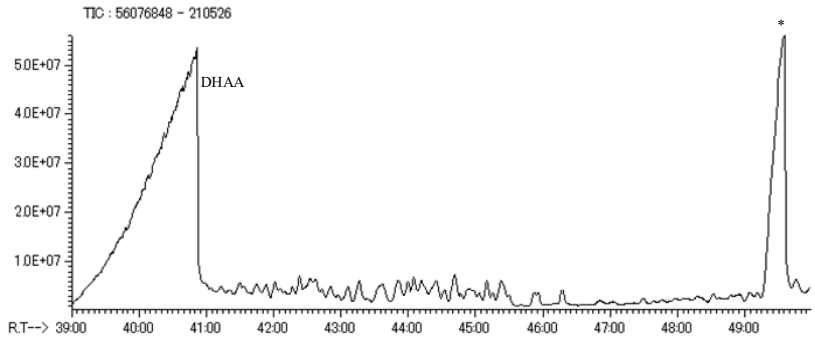
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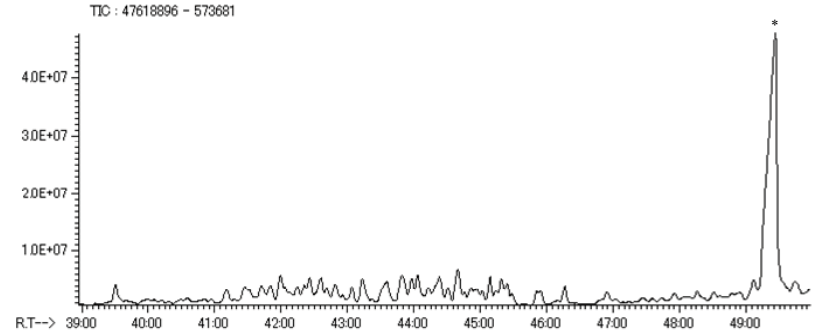
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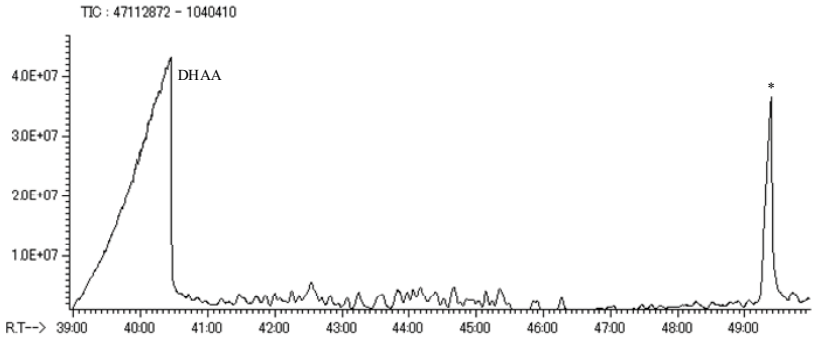
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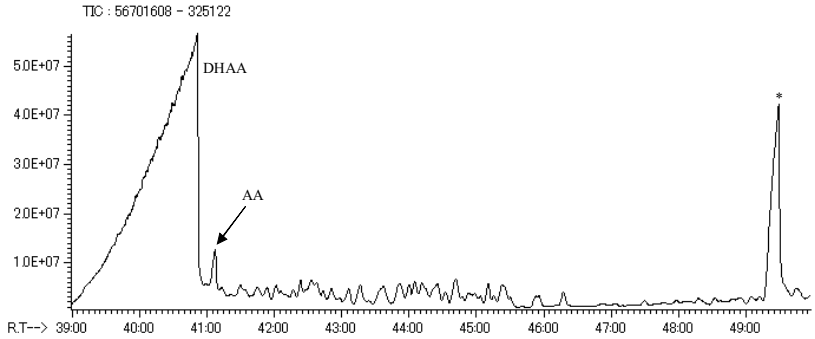
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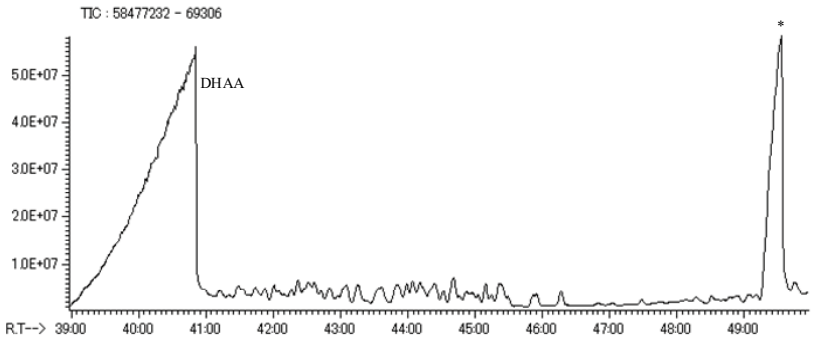
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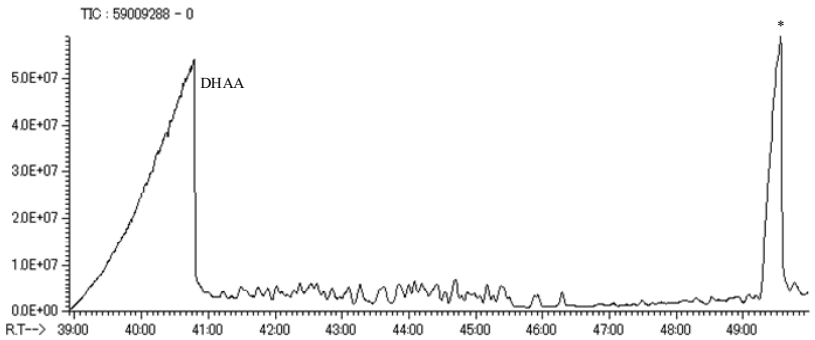

Figure 3. Enzyme assays with crude protein isolations from *A. annua* flowers and leaves and dihydroartemisinic acid as substrate. AA – artemisinic acid, DHAA – dihydroartemisinic acid, * – unknown. The artemisinic acid in figure 3e is a small impurity of the substrate.

- **a.** Crude protein extract with dihydroartemisinic acid as substrate and hydrogen peroxide.
- **b.** Crude protein extract with dihydroartemisinic acid as substrate.
- **c.** Crude protein extract with no substrates.
- **d.** Boiled crude protein extract with dihydroartemisinic acid as substrate and hydrogen peroxide.
- **e.** Boiled crude protein extract with dihydroartemisinic acid as substrate.
- **f.** Assay buffer with dihydroartemisinic acid and hydrogen peroxide.
- **g.** Assay buffer with dihydroartemisinic acid.

activity could be detected using these conditions (data not shown). Figure 3 shows the results when crude protein isolations from *A. annua* leaves and flowers were tested with dihydroartemisinic acid and hydrogen peroxide as substrates. It is evident, that no enzymatic conversion occurs and also that there is no spontaneous conversion of dihydroartemisinic acid to artemisinin or a related intermediate. It is possible that other cofactors or cosubstrates will initiate a

conversion of dihydroartemisinic acid. However, it is unlikely that dihydroartemisinic acid is converted to artemisinic acid and then to artemisinin (Bertea et al., 2005). The fate of dihydroartemisinic acid remains obscure.

On the other hand, the artemisinin intermediate artemisinic acid, is converted to arteannuin B (figure 4). This has been shown in *A. annua* hairy root cultures, but the responsible enzyme could not be identified (Arsenault et al., 2010). Arteannuin B has been shown to be enzymatically converted to artemisinin (Dhingra et al., 2001). Our finding point to a cytosolic protein, Pox1, which is dependent on hydrogen peroxide for production of arteannuin B from artemisinic acid. Pox1 is likely a protein that is involved in protection against reactive oxygen species and is probably overexpressed in stress situations in the plant.

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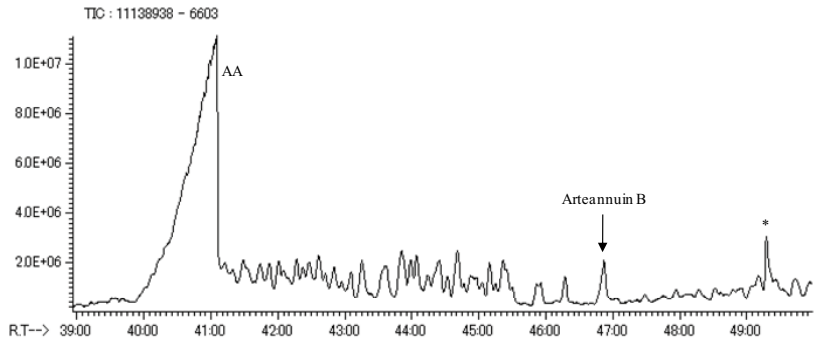

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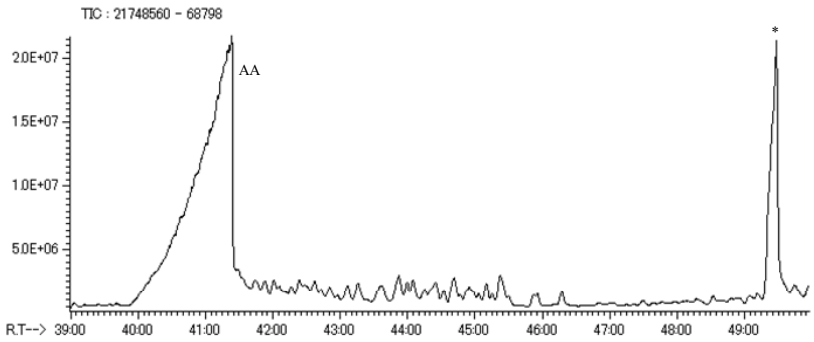
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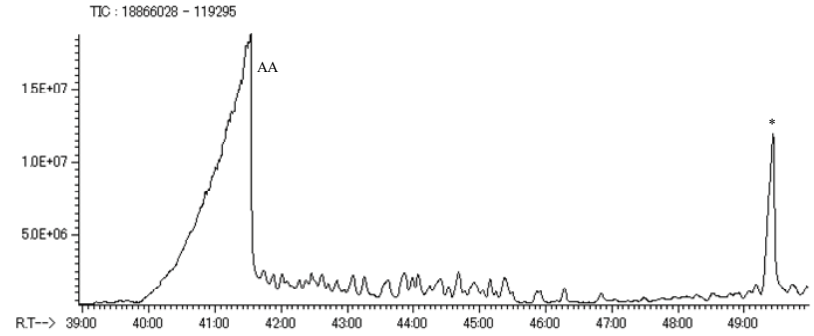
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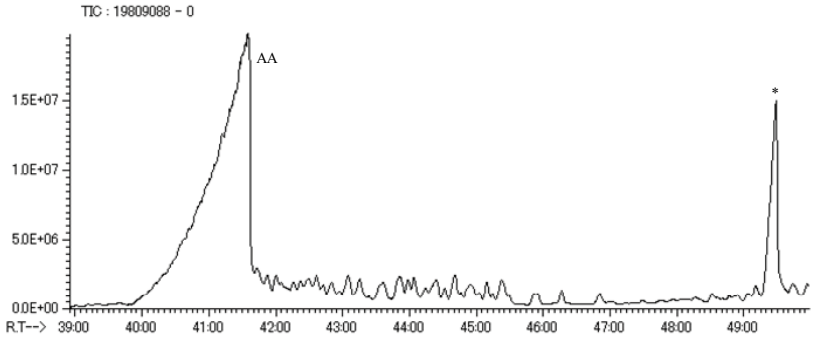
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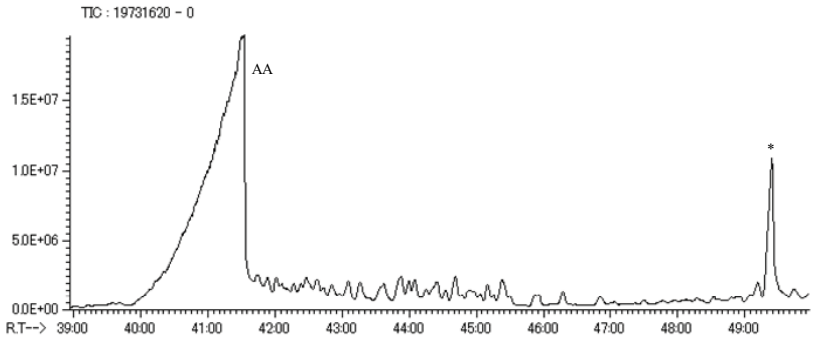

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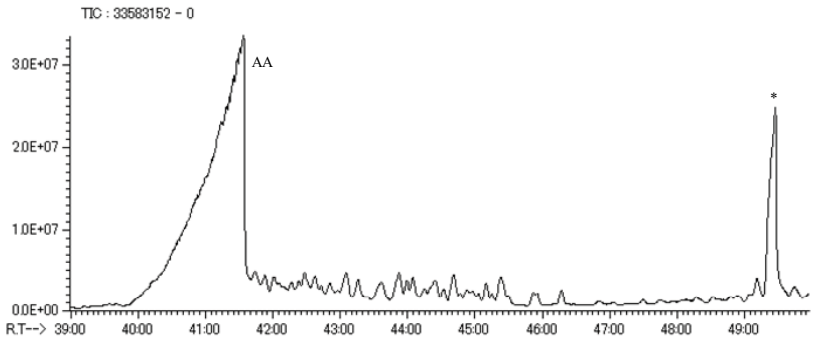

Figure 4. Enzyme assays with crude protein isolations from *A. annua* flowers and leaves and artemisinic acid as substrate. AA – artemisinic acid, * – unknown.

- **a.** Crude protein extract with artemisinic acid as substrate and hydrogen peroxide.
- **b.** Crude protein extract with artemisinic acid as substrate.
- **c.** Boiled crude protein extract with artemisinic acid as substrate and hydrogen peroxide.
- **d.** Boiled crude protein extract with artemisinic acid as substrate.
- **e.** Assay buffer with artemisinic acid and hydrogen peroxide.
- **f.** Assay buffer with artemisinic acid.

Protein pull down assay

In an effort to isolate Pox1 from *A. annua*, a protein pull down approach was taken (figure 5). This method is a one-step purification protocol and significantly speeds up the protein purification process compared to the traditional chemical biology approach (Kanoh et al., 2006; Kanoh et al., 2005). The method relies on the substrate molecules being randomly covalently attached to small beads. These beads are used to pull down proteins that interact with the substrate, thus facilitating purification and concentration of the target protein in one step. The sample is eluted from the beads using detergent and the sample is thereafter analyzed on an SDS-PAGE gel. The protein bands that appear from the eluted sample are cut out from the gel and analyzed with mass spectrometry and/or *de novo* sequencing. As our sample is originating from *A. annua*, simple mass spectrometry is not sufficient, as there is no protein fragment database to compare the results with. Therefore, it is necessary to perform *de novo* protein sequencing on the excized proteins.

Artemisinic acid was chosen as bate on the beads for a protein pull down assay based on the biochemical conversion data presented in this report (figure 4), which point to enzymatic conversion of artemisinic acid to arteannuin B, and literature supporting enzymatic conversion of arteannuin B to artemisinin (Dhingra et al., 2001). As shown in figure 5, three unique protein bands in the eluate can identified, whereof two are approximately the size of 50 kDa and a smaller protein of approximately 25 kDa. The sequencing of these bands is currently in process. After obtaing sequence fragments of the proteins and comparing them to the literature, the cDNA of the Pox1 candidate genes will be cloned from our trichome cDNA library (Bertea et al., 2006). These genes will thereafter be heterologously expressed in *E. coli*, purified and biochemically characterized. This work is in progress through a collaboration with Yokohama City University in Japan.

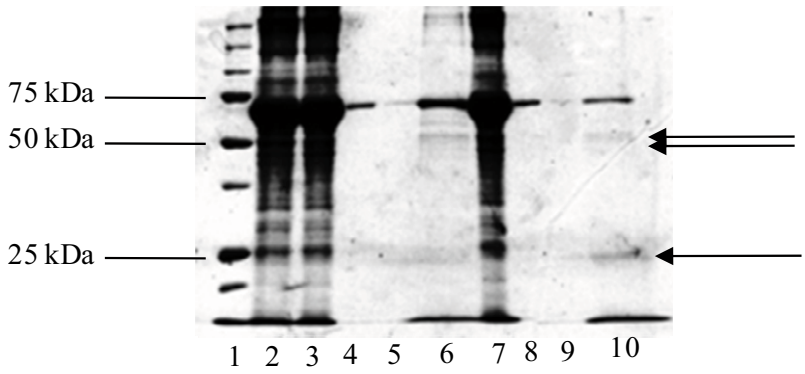

Figure 5. 10% SDS-PAGE gel of protein pull down assay using artemisinic acid as capture molecule on the beads. Lanes were loaded with samples which were treated as described below.

1. Protein ladder marker.
2. Desalted crude protein isolation with bovine serum albumin.
3. Desalted crude protein isolation with bovine serum albumine, supernatant from pre-clearing with control beads.
4. Desalted crude protein isolation with bovine serum albumine, wash 1, control beads.
5. Desalted crude protein isolation with bovine serum albumine, wash 2, control beads.
6. Desalted crude protein isolation with bovine serum albumine, elution, control beads.
7. Desalted crude protein isolation with bovine serum albumine, supernatant from pull down, beads with covalently attached artemisinic acid.
8. Desalted crude protein isolation with bovine serum albumine, wash 1, control beads, beads with covalently attached artemisinic acid.
9. Desalted crude protein isolation with bovine serum albumine, wash 2, control beads, beads with covalently attached artemisinic acid.
10. Desalted crude protein isolation with bovine serum albumine, elution, beads with covalently attached artemisinic acid.

If the protein fragments reveal interesiting candidates to the Pox1 enzyme, the chosen protein pull-down approach shows good potential for reducing the workload normally associated with chemical biology (Zhang et al., 2008).

Materials and Methods

Chemicals

Neat artemisinic acid, dihydroartemisinic acid and dihydroartemisinic aldehyde were synthesized as described by Bertea et al. (2005). Neat arteannuin B and artemisinin was isolated as described by Wallaart et al. (2000). Other chemicals were purchased from Oriental yeast Co. Tokyo, Japan or Sigma-Aldrich, USA. Beads used for the protein pull down assay were a gift from Hiroyuki Osada, RIKEN, Japan.

Plant material

Plants, originally identified by Wallaart et al., (1999) were grown for collection of flowers, leaf and root material using seeds obtained from University of Groningen. Seeds are deposited in the herbarium De Kruidhof in Buitenpost, the Netherlands, under registration number GR001. The *A. annua* line belongs to the high artemisinin chemotype and has the characteristic pattern of a high dihydroartemisinic acid to artemisinic acid ratio. Seeds were sown in potting compost and grown in climatrons with the conditions 21/18°C (16/8 h). Plants were watered as necessary without addition of fertilizer. When the plants started to flower, flowers and leaves were collected in four categories. Plant material was kept on ice during handling, then frozen in liquid nitrogen and stored at -80 °C.

Crude protein isolation from A. annua

2 g Polyvinylpolypyrrolidone (PVPP) was soaked and gently mixed over night at 4°C in 50 ml buffer A (50 mM potassium phosphate buffer pH 7.5, 20% v/v glycerol, 2.5 mM EDTA pH 8.0, 0.2% BSA, pH 7.5). The slurry was pelleted and the buffer exchanged for 50 ml buffer B (50 mM potassium phosphate buffer pH 7.5, 20% v/v glycerol, 50 mM ascorbic acid, 50 mM natriummetabisulfiet, 5 mM DTT, 2.5 mM EDTA pH 8.0, 0.2% BSA, pH 7.5) supplemented with ½ a tablet of protease cocktail inhibitors (Roche). Plant material was chilled with liquid nitrogen and ground to a fine powder using a mortar and pestle. Thereafter 2 g powder was suspended in 15 ml buffer/PVPP slurry. The mixture was gently shaken for 10 min at 4°C and filtered through a fine nylon mesh to separate the fluid from PVPP and plant particles. The filtrate was centrifuged at 20.000 g for 20 min at 4°C and was thereafter de-salted on a PD-10 column with buffer B by following the instructions of the manufacturer (GE-Healthcare).

Enzyme conversion assays

Oxidoreductase assay

To 1 ml desalted crude protein isolation, 2.5 µl 10 mM dihydroartemisinic aldehyde was added as substrate and 1.7 mg NADPH as cofactor. The mixture was incubated 25°C for 4 hours. Thereafter the reaction was stopped on ice and 250 µl $NaCl_{aq}$ was added. The mixture was repeatedly extracted with 1 ml hexane three times, and the organic phases were collected in one glass vial. The extract was carefully dried with a gentle nitrogen gas flow while cooled on ice. The solid remains in the vial were resuspended in 100 µl hexane and thereafter analyzed with GC-MS. Peaks were identified using authentic standards.

Peroxygenase assay

All boiled control samples were heated to 99°C for 10 min and cooled down on wet ice for 5 min before further processing. To 1 ml desalted crude protein isolation, 50 µl 2 mg/mL artemisinic acid or dihydroartemisinic acid was added per sample. To each sample, 11 µl 3% hydrogenperoxide was added and thereafter gently mixed followed by 30 min incubation at 25°C. The mixture was incubated for 4 hours and during this time, every 30 min 11 µl 3% hydrogenperoxide was added and the sample gently mixed. A total of 8 cycles with hydrogenperoxide replenishment was thus obtained. The reaction was stopped on ice and 250 µl $NaCl_{aq}$ was added. The mixture was repeatedly extracted with 1 ml hexane three times, and the organic phases were collected in one glass vial. The extract was carefully dried with a gentle nitrogen gas flow while cooled on ice. The solid remains in the vial were resuspended in 100 µl hexane and thereafter analyzed with GC-MS. Peaks were identified using authentic standards.

Protein pull down assay

A 100 µl aliquot from the *A. annua* crude desalted protein isolation (described above) was taken as a control sample for analysis with SDS-PAGE. The beads used for the pull down assay were rinsed 3 times with ice-cold binding buffer (20 mM potassium phosphate buffer pH 7.5, 20% v/v glycerol, 150 mM KCl, final pH 7.5) to remove the storage buffer which contains sodium azide. 1 ml crude protein extract was pre-cleared by adding 50 µl rinsed control beads (100 µl 50% v/v slurry, pelleted and supernatant discarded). The mixture was incubated for 1 hour at 4°C with gentle shaking. Thereafter the mixture was pelleted and the pre-cleared supernatant transferred to 50 µl rinsed substrate beads. The mixture was incubated over night with gentle shaking for 6 hours at 4°C. Thereafter the sample was centrifuged and the supernatant saved in a microcentrifuge tube. The pellet was washed two times with 1 ml

ice-cold binding buffer and to the final pellet was added 30 µl SDS-PAGE sample buffer (40% v/v glycerol, 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol). The mixture was vigorously mixed and heated for 10 min at 99°C followed by a centrifugation in a bench top centrifuge at 12000 rpm for 10 min. The eluate was collected and analyzed on a 10% SDS-PAGE gel. 20 µl aliquots from the saved supernatants were analyzed on the same gel.

GC-MS

GC-MS analyses were performed on an Agilent 6890 series gas chromatograph system coupled to a JEOL JMS-SUN 200 mass selective detector in electron impact mode (70 eV) equipped with a guard column (1 m x 0.25 mm i.d., GL-Sciences) and a HP-5MS analytical column (30 m x 0.25 mm i.d., 0.25 µm film). The temperature program was set to an initial temperature of 60°C followed by a first temperature gradient of 3°C min^{-1} to 220°C and then a second gradient of 30°C min^{-1} to 300°C with an endpoint hold of 4 min. Helium flow was set to 0.7 ml min-1, detector temperature and injector temperature to 250°C using 500 scans min^{-1} with a scan range of 40-350 m/z.

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