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Justicidin B 7-hydroxylase, a cytochrome P450 monooxygenase from cell cultures of *Linum perenne* Himmelszelt involved in the biosynthesis of diphyllin

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Abstract

Cell suspension cultures of *Linum perenne* L. Himmelszelt accumulate justicidin B as the main component together with glycosides of 7-hydroxyjusticidin B (diphyllin). A hypothetical biosynthetic pathway for these compounds is suggested. Justicidin B 7-hydroxylase (JusB7H) catalyzes the last step in the biosynthesis of diphyllin by introducing a hydroxyl group in position 7 of justicidin B. This enzyme was characterized from a microsomal fraction prepared from a *Linum perenne* Himmelszelt suspension culture for the first time. The hydroxylase activity was strongly inhibited by cytochrome *c* as well as other cytochrome P450 inhibitors like clotrimazole indicating the involvement of a cytochrome P450-dependent monooxygenase. JusB7H has a pH optimum of 7.4 and a temperature optimum of 26 °C. Justicidin B was the only substrate accepted by JusB7H with an apparent K_m of 3.9 ± 1.3 μM. NADPH is predominantly accepted as the electron donor, but NADH was a weak co-substrate. A synergistic effect of NADPH and NADH was not observed. The apparent K_m for NADPH is 102 ± 10 μM.

Keywords: *Linum perenne*; Linaceae; Lignan; Cytochrome P450; Justicidin B; Diphyllin; Justicidin B 7-hydroxylase

1. Introduction

Lignans, phenolic metabolites widespread in the plant kingdom, are derived by C8–C8′ oxidative dimerization of phenylpropanoids such as caffeyl, coniferyl or sinapyl alcohol (Moss, 2000). Further cyclisation and modifications of the dimers lead to a high structural diversity in this class of compounds. One can divide the lignans into different structural groups like aryltetralin [podophyllotoxin (**16**)] or arylnaphthalene type lignans [justicidins and diphyllin (**9**)] (Fuss, 2003).

Lignans have been of major interest since the early days of medical research as they possess a great variety of biological and pharmacological activities. In the last 15 years more than 120 lignans were reported to have anti-inflammatory, antimicrobial, immunosuppressive, anticancer and antioxidative activity (Saleem et al., 2005). Diphyllin (**9**) derivatives are putative remedies for topical chronic inflammatory disorders such as dermatitis and psoriasis while an acetylapioside derivative of diphyllin is a 5-lipoxygenase inhibitor (Prieto et al., 2002).

Abbreviations: JusB7H, justicidin B 7-hydroxylase; *L. perenne* H, *Linum perenne* Himmelszelt; PLR, pinoresinol–lariciresinol reductase.

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Plant species of different genera like *Haplophyllum* (Puricelli et al., 2002) and *Justicia* (Chen et al., 1996) accumulate arylnaphthalene lignans such as justicidin B (**8**) and diphyllin (**9**). Recently, we have reported on the accumulation of justicidin B (**8**) and glycosides of 7-hydroxyjusticidin B (diphyllin diglycosides) in cell suspension and hairy root cultures of *Linum perenne* H (Hemmati et al., 2007).

The biosynthesis of lignans starts with the coupling of two molecules of *E*-coniferyl alcohol (**1**) with the help of an auxilliary dirigent protein to give pinoresinol (**2**) which was shown for *Forsythia* species (Davin et al., 1990)

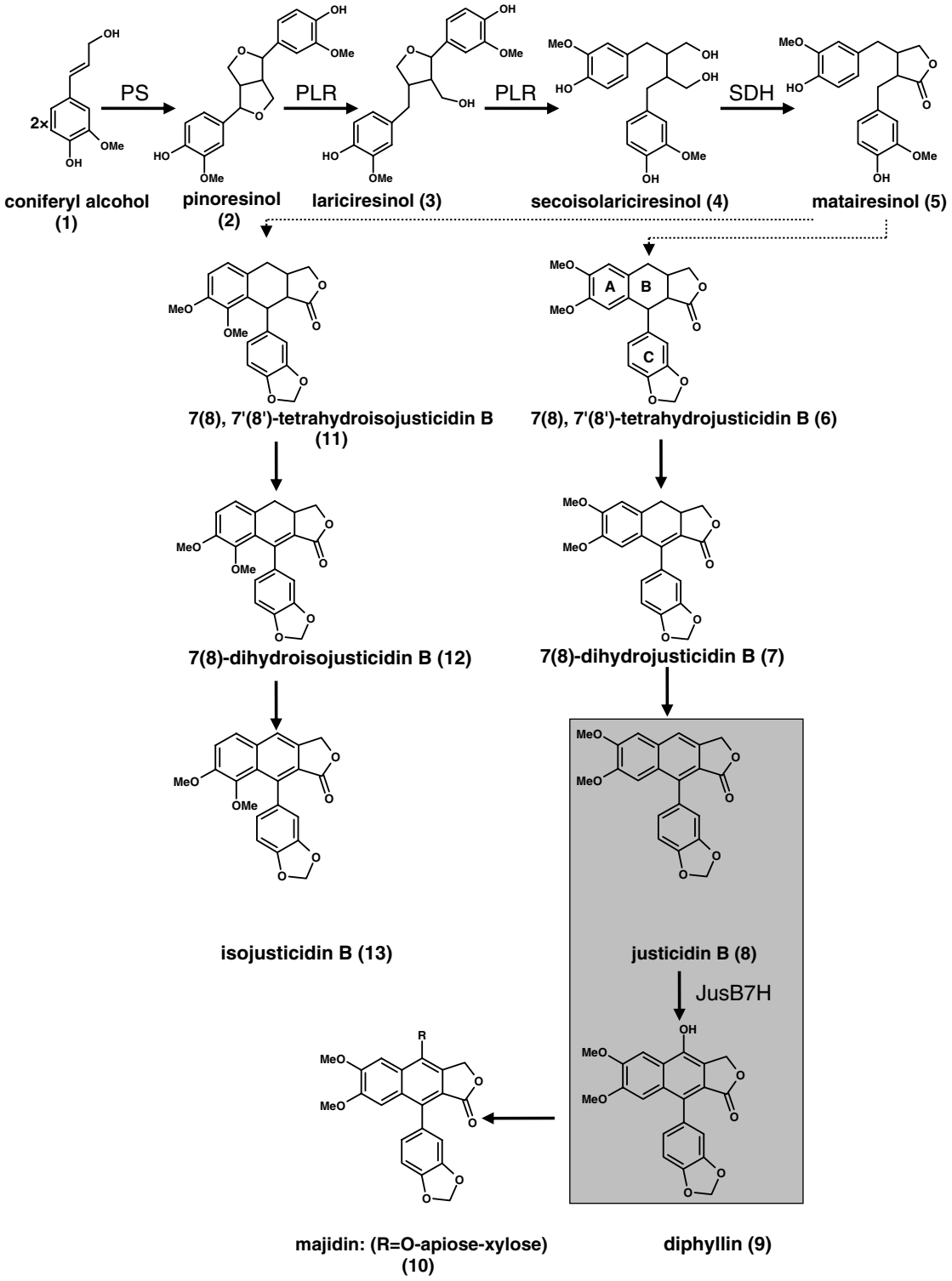

Fig. 1. Hypothetical biosynthetic pathway leading to diphyllin (**9**) and isojusticidin B (**13**). PS: pinoresinol synthase, PLR: pinoresinol–lariciresinol reductase, SDH: secoisolariciresinol dehydrogenase, JusB7H: justicidin B 7-hydroxylase.

(Fig. 1). Pinoresinol (**2**) is reduced via lariciresinol (**3**) to secoisolariciresinol (**4**) by a bifunctional enzyme pinoresinol–lariciresinol reductase (PLR) (Dinkova-Kostova et al., 1996; Fujita et al., 1999; von Heimendahl et al., 2005). Secoisolariciresinol (**4**) is converted to matairesinol (**5**) via $NADP^+$ dependent dehydrogenation as in *Forsythia intermedia* (Xia et al., 2001). Recently, we have cloned a cDNA encoding a (+)-pinoresinol/(−)-lariciresinol reductase (PLR-Lp1) from cell cultures of *L. perenne* H (Hemmati et al., 2007). The involvement of PLR-Lp1 in the early steps of arylnaphthalene lignan biosynthesis was proven by an RNAi approach. This was the first attempt to understand the biosynthesis of arylnaphthalene lignans in this species. Later steps in the biosynthesis of these compounds most probably starting from matairesinol (**5**) are poorly understood. Schmidt and Vößing (2006) reported on the occurrence of 7(8)-dihydroisojusticidin B (**12**) as possible intermediate in the biosynthesis of isojusticidin B (**13**) in the aerial parts of *L. perenne* L. Together with justicidin B (**8**) and diphyllin diglycosides (Hemmati et al., 2007), cell cultures of *L. perenne* H accumulate trace amounts of isojusticidin B (**13**), a compound which was previously found in a cell culture of *Linum austriacum* (Mohagheghzadeh et al., 2002).

Here we suggest a biosynthetic pathway leading to the arylnaphthalene lignans diphyllin (**9**), its glycosides and isojusticidin B (**13**). The detection of the enzyme justicidin B 7-hydroxylase (JusB7H) which catalyzes the reaction from justicidin B (**8**) to diphyllin (**9**) in *L. perenne* H suspension cultures confirms one of the last steps of our hypothetical biosynthetic pathway leading to diphyllin glycosides. We here describe the properties of the JusB7H, a cytochrome P450-dependent monooxygenase.

2. Results and discussion

2.1. A hypothetical biosynthetic pathway leading to diphyllin, its glycosides and isojusticidin B

The biosynthesis of lignans starts with the coupling of two molecules of coniferyl alcohol (**1**) to give pinoresinol (**2**) which is further converted via lariciresinol (**3**) to secoisolariciresinol (**4**) by PLR. We could show that a PLR is really involved in the formation of justicidin B (**8**) and diphyllin diglycosides in *L. perenne* Himmelszelt (Hemmati et al., 2007). Secoisolariciresinol dehydrogenase catalyzes the formation of matairesinol (**5**) from secoisolariciresnol (**4**). Nothing was known about the further steps leading to the arylnaphthalene lignans when Schmidt and Vößing (2006) detected 7(8)-dihydroisojusticidin B (**12**) in the aerial parts of *L. perenne* L. These findings give rise to a hypothetical pathway leading to diphyllin diglycosides like majidin (**10**) and isojusticidin B (**13**) (Fig. 1). We suggest that matairesinol (**5**) is converted via cyclisation (formation of ring B) and further derivatization in ring C to 7(8),7′(8′)-tetrahydroisojusticidin B (**11**) and to 7(8),7′(8′)-tetrahydrojusticodin B (**6**) The latter are converted by two dehydrogenation steps to 7(8)-dihydroisojusticidin B (**12**), respectively 7(8)-dihydrojusticidin B (**7**), and then isojusticidin B (**13**) and justicidin B (**8**). Justicidin B (**8**) can be hydroxylated at position 7 to form diphyllin (**9**). As the cell cultures of *L. perenne* H accumulate not diphyllin (**9**) but diphyllin diglycosides, glycosyltransferases are responsible for the formation of majidin (**10**).

2.2. Identification of JusB7H from L. perenne H as a cytochrome P450-dependent monooxygenase

Justicidin B (**8**) and glycosides of 7-hydroxyjusticidin B [diphyllin (**9**)] are the main detectable compounds in cell cultures of *L. perenne* H (Hemmati et al., 2007). The introduction of the 7-hydroxyl group into justicidin B (**8**) leading to diphyllin (**9**) is an aromatic hydroxylation catalyzed by JusB7H (Fig. 1). Diphyllin (**9**) has been identified as the enzyme product by HPLC-SPE-^{1}H NMR spectroscopy based on the following data. (1) The shorter retention time on the reversed phase HPLC column of the product (R_t 28.5 min) compared to the substrate justicidin B (**8**) (R_t 37.3 min) was consistent with the higher polarity of a hydroxylated product. (2) The UV spectrum recorded by the PDA detector during the HPLC-SPE-NMR run was identical with that of an authentic standard. (3) The ^{1}H NMR spectrum of the enzyme product matched that of the authentic standard but differed from that of the substrate in the missing signal of H-7 and a downfield shift of H-6 [δ 7.37 in the spectrum of justicidin B (**8**) versus δ 7.56 in diphyllin (**9**)].

The hydroxylation required NADPH as electron donor. The enzyme system was localized in the microsomal fractions after $MgCl_2$-precipitation and centrifugation at 48,000g. Assays with the supernatant containing soluble enzymes did not result in any hydroxylation reaction. The requirement for NADPH and the localization of the enzyme in the so called microsomal fraction were hints that JusB7H might be a cytochrome P450-dependent monooxygenase. Subcellular localization in the microsomal fraction is typical of this enzyme class, which is thought to reside mostly in the endoplasmic reticulum (Ro et al., 2001). Further data sustaining this indication, was verified by the strong inhibition of JusB7H by cytochrome *c*, an electron acceptor competing for electrons transferred from a NADPH:cytochrome P450 reductase to cytochrome P450 (Ortiz de Montellano and Correia, 1995). JusB7H activity was severely inhibited by a very low concentration of cytochrome *c*. 50% inhibition of JusB7H was achieved by 0.76 μM cytochrome *c* (Fig. 2). This value is comparable to concentrations necessary to inhibit other cytochrome P450 monooxygenases. 1.6 μM cytochrome *c* leads to a 50% inhibition of DOP6H of *L. album* (Federolf et al., 2007). An inhibition of 90% for DOP6H of *L. flavum* was observed at a concentration of 2.5 μM cytochrome *c* (Molog et al., 2001). N-substituted imidazols like clotrimazole were effective inhibitors for JusB7H.

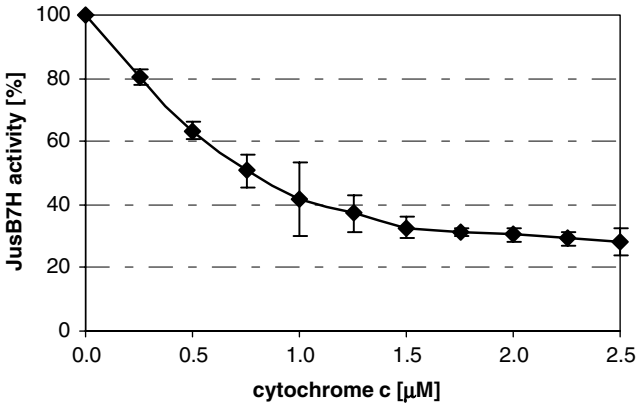

Fig. 2. Inhibition of JusB7H from suspension cultures of *Linum perenne* H by cytochrome *c* (100% activity corresponds to 0.20 ± 0.01 µkat kg^{-1}). Values are mean ± SD of two independent assays each conducted in duplicate.

0.56 mM clotrimazole or 1 mM tetcyclacis, another known inhibitor of cytochrome-P450-dependent monooxygenases, led to a 50% inhibition of JusB7H activity. The activity of DOP6H from *L. album* and tabersonine 16-hydroxylase from *Catharanthus roseus* is inhibited to 50% by 11 µM and 50 µM clotrimazole, and 11 µM and 0.1 mM tetcyclacis, respectively (Federolf et al., 2007; St-Pierre and De Luca, 1995).

2.3. Basic characteristics of JusB7H

The basic characteristics of JusB7H are typical of cytochrome P450 monooxygenases. No activity was observed when either cosubstrate (NADPH) was omitted or after heat inactivation of the microsomal preparation. Maximal JusB7H activities were measured at a reaction pH around 7.4 (0.1 M Tris-HCl buffer), a value corresponding to the cytoplasmic pH. Half maximal specific activities were measured at pH 7.0 and 8.4. The optimal reaction temperature was 26 °C. However, considerable JusB7H activities were observed at 0 °C and 40 °C with 36% and 52% of the activity at 26 °C, respectively, indicating a rather broad temperature window with high enzyme activity. The enzyme activity under standard reaction conditions was linear up to 700 µg microsomal protein per assay and up to an incubation time of 75 min.

2.4. Substrate specificity of JusB7H

JusB7H catalyzes the formation of diphyllin (**9**) from justicidin B (**8**). Most cytochrome P450 enzymes from plants exhibit a narrow substrate specificity (Donaldson and Luster, 1991; Sandermann, 1992). Structurally related substances were not converted to the related products hydroxylated either at position 6 or 7 with microsomal preparations of *L. perenne* H shown by comparison to authentic standards of possible reaction products. If no standards were available the absence of new additional peaks in addition to the not altered peak area of the substrate in the HPLC chromatograms of the reaction mixtures indicated the lack of any conversion of the substrate. In detail, the substances tested were matairesinol (**5**), yatein (**14**), deoxypodophyllotoxin (**15**), podophyllotoxin (**16**), β-peltatin A-methylether (**17**), 7(8),7′(8′)-dehydrodeoxypodophyllotoxin (**18**), 7(8),7′(8′)-dehydropodophyllotoxin (**19**), 5′-demethyljusticidin B (**20**) (Fig. 3). Microsomal preparations from two cell cultures of *L. album* accumulating either podophyllotoxin (**16**) (line PT) or 6-methoxypodophyllotoxin (line 6M) (Federolf et al., 2007) did not catalyze the hydroxylation of justicidin B (**8**). These data indicate the high substrate specificities of the enzymes found in the microsomal preparations.

JusB7H shows a high affinity towards justicidin B (**8**): the apparent K_m value for this substrate determined by Hanes plots (data not shown) and Lineweaver–Burk plots was 3.9 ± 1.3 µM with a saturation concentration of 50 µM (Fig. 4). The low apparent K_m value for justicidin B (**8**) which shows a high enzyme substrate specificity is

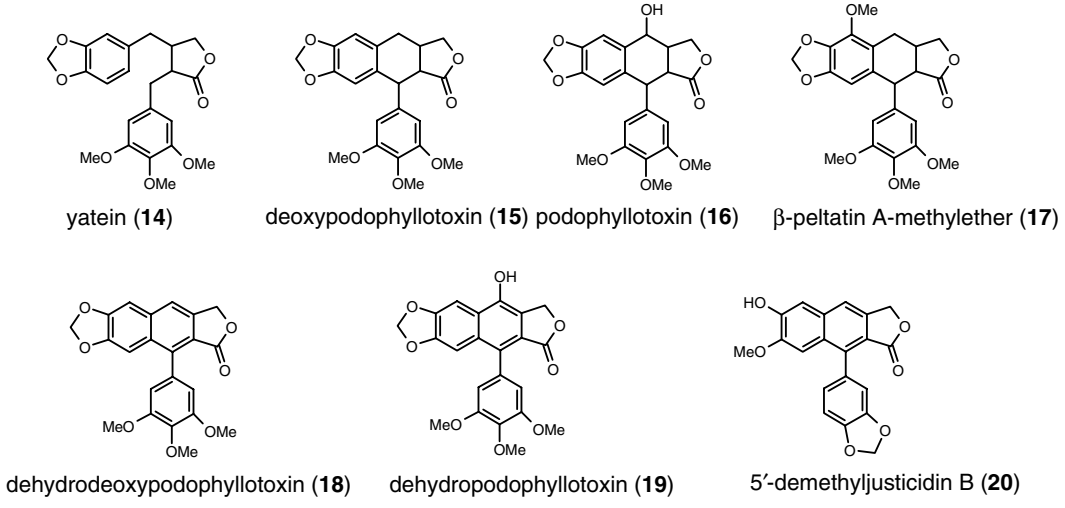

Fig. 3. Compounds with structures similar to justicidin B (**8**) used in JusB7H assays.

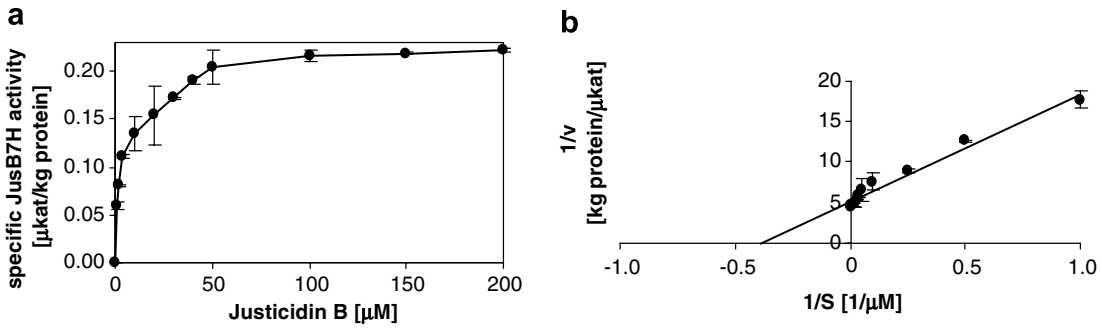

Fig. 4. (a) Substrate saturation curve of JusB7H from cell cultures of *L. perenne* H for justicidin B (**8**). (b) The corresponding Lineweaver–Burk diagram. The apparent K_m was determined to be 3.9 ± 1.3 µM. Kinetic constants are mean ± SD of three independent assay series each carried out in duplicate.

also found with other cytochrome P450 enzymes like the low apparent K_m for geranylhydroquinone 3″-hydroxylase from *Lithospermum erythrorhizon* with a value of 1.5 µM for geranylhydroquinone (Yamamoto et al., 2000). The apparent K_m for cinnamic acid is in the range 2–30 µM for most cinnamic acid 4-hydroxylases (Werck-Reichhardt, 1995).

JusB7H is strictly dependent on NADPH as an electron donor. The apparent K_m for NADPH was 102 ± 9.8 µM, as shown by Lineweaver–Burk diagrams (Fig. 5). The higher apparent K_m for NADPH in the JusB7H assay is also observed for other cytochrome P450 enzymes. The cinnamic acid 4-hydroxylase from *Anthoceros agrestis* shows an apparent K_m value of around 60 µM for NADPH (Petersen, 2003). The deoxypodophyllotoxin-6-hydroxylases from *Linum nodiflorum* and *L. album* show apparent K_m values of around 55 µM and 41 µM for NADPH, respectively (Kuhlmann, 2004; Federolf et al., 2007).

Addition of $NADP^+$ alone did not lead to a hydroxylation reaction. 0.5 mM NADH could only sustain a very low hydroxylation activity of 0.015 ± 0.001 µkat/kg protein in comparison to 0.17 ± 0.02 µkat/kg protein with 0.25 mM NADPH (Fig. 6). For some cytochrome P450-dependent reactions, simultaneous addition of NADPH and NADH lead to a synergistic effect of these two reducing equivalents (Clemens et al., 1993; Gerardy and Zenk, 1993). This phenomenon is explained by the participation

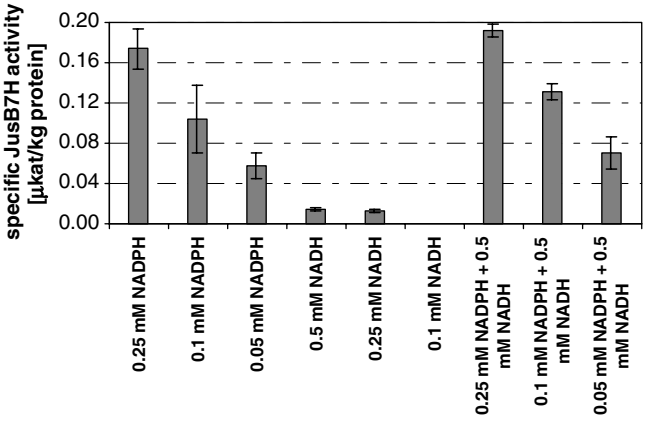

Fig. 6. Activity of JusB7H with different electron donors. Values are mean ± SD of two independent assays each conducted in duplicate.

of a NADPH:cytochrome P450 reductase as well as cytochrome b_5 in the electron transfer to the cytochrome P450 monooxygenases (De Vetten et al., 1999). Simultaneous addition of 0.05–0.25 mM NADPH together with 0.5 mM NADH resulted only in additive activities but the so called synergistic effect was not observed (Fig. 6). The synergistic effect was neither observed for some other cytochrome P450 enzymes like deoxypodophyllotoxin 6-hydroxylase in *L. flavum* (Molog et al., 2001).

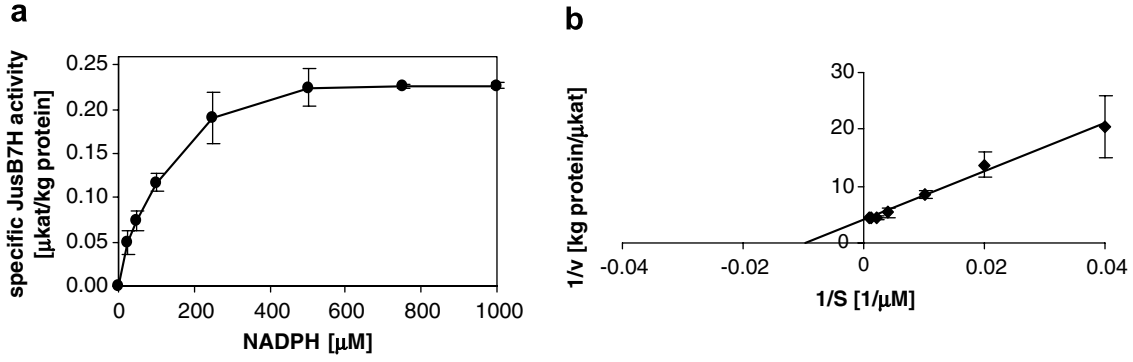

Fig. 5. (a) Substrate saturation curve of JusB7H from cell cultures of *L. perenne* H for NADPH. (b) The corresponding Lineweaver–Burk diagram. The apparent K_m was determined to be 102 ± 9.8 µM. Kinetic constants are mean ± SD of three independent assay series each carried out in duplicate.

NADPH: cytochrome P450 reductase is known to contain FAD and FMN as prosthetic groups. These flavine nucleotides might be partially lost during enzyme preparation and purification (Petersen, 1997). Addition of 5 μM FAD did not lead to an enhancement of JusB7H activity, on the contrary activities were about 28% lower. This might indicate that FAD is tightly bound to the reductase and not lost during the preparation of microsomes.

3. Conclusion

Justicidin B (**8**) can be either hydroxylated at position 7 to give diphyllin (**9**) by JusB7H or at position 6. The NMR records after the enzyme assays showed the 7-hydroxylated derivative [diphyllin (**9**)] as the only product. Hydroxylation reactions are supposed to be performed by cytochrome P450 monooxygenases, dioxygenase enzymes or peroxidases. Several hydroxylases introducing OH-groups into aromatic rings have been shown to be cytochrome P450-dependent monooxygenases, e.g. cinnamic acid 4-hydroxylase (Russell and Conn, 1967; Russell, 1971), xanthone 6-hydroxylase (Schmidt et al., 2000), and deoxypodophyllotoxin 6-hydroxylase (Molog et al., 2001). But the introduction of the 7-hydroxyl group is an unclarified question for some secondary compounds like podophyllotoxin (**16**). For instance the 7-hydroxylation of the aromatic C ring of deoxypodophyllotoxin (**15**) to podophyllotoxin (**16**) by deoxypodophyllotoxin 7-hydroxylase or the 7-hydroxylation of the aliphatic C ring of β-peltatin A-methylether (**17**) to 6-methoxypodophyllotoxin by β-peltatin A-methylether 7-hydroxylase have not been characterized yet (Federolf et al., 2007).

We have demonstrated the identity of JusB7H as a cytochrome P450 monooxygenase. Since the cell cultures of *L. perenne* H accumulate diphyllin (**9**) only with disaccharides attached to the free OH-group diphyllin (**9**) has to be converted to diglycosides which are more water soluble and can be stored in the vacuole. Our data together with the cloning of a PLR from *L. perenne* H and the findings of dihydroarylnaphthalenes in the aerial part of this plant species support our hypothetical pathway leading to diphyllin glycosides.

4. Experimental

4.1. Chemicals

Justicidin B (**8**) and diphyllin (**9**) were purchased from Early Discovery Chemistry Ltd, Hove, UK. Podophyllotoxin (**16**) and 5′-demethyljusticidin B (**20**) were from Xi'an Sino Dragon Import and Export Co. Ltd., Xi'an, China and Specs and Biospecs Rijkwijk, The Netherlands, respectively. Deoxypodophyllotoxin (**15**) was synthesized from podophyllotoxin (**16**) in our laboratory (Katja Federolf, Düsseldorf, Germany, unpublished results). 7(8),7′(8′)-dehydropodophyllotoxin (**19**) and 7(8),7′(8′)-dehydrodeoxypodophyllotoxin (**18**) were gifts from A. D. Kinghorn, Ohio, USA. *β*-peltatin A-methylether (**17**) was a gift from M. Medarde, Salamanca, Spain. 7-hydroxymatairesinol (**21**) was donated by R. Sjöholm, Arbonova, Turku, Finland and *β*-peltatin (**22**) was a gift from McChesney, Oxford, MS, USA. 6-methoxypodophyllotoxin (**23**) was isolated from *Linum album* hairy roots (J. Windhövel, unpublished). NADPH was from Applichem and NADH from Biomol. Cytochrome *c* was obtained from Fluka. Clotrimazole, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from Sigma. Tetcyclacis was provided by Wilhelm Rademacher (BASF AG, Ludwigshafen).

4.2. Cell cultures

Cell suspension cultures of *L. perenne* H were established and maintained as described previously (Hemmati et al., 2007).

4.3. Preparation of microsomes

Five-day-old suspension cells of *L. perenne* H were harvested by vacuum filtration. All further steps were carried out at 0–4 °C. Cells were homogenized by using a prechilled mortar and pestle together with 0.2 g Polyclar 10, 0.17 g sea sand and 1 ml buffer [0.1 M Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM diethyldithiocarbaminate] per gram fresh weight. The homogenate was centrifuged at 8000*g* for 20 min. The supernatant was adjusted to 50 mM $MgCl_2$ and stirred on ice for 30 min. The sediment containing aggregated membranes was resuspended in buffer (as above) by a Potter and Elvehjem glass homogeniser after centrifugation at 48,000*g* for 25 min. Crude microsomal extracts for enzyme assays were obtained by passing the resuspended pellet through a preequilibrated PD10 column using the same buffer. Protein concentrations were determined according to Bradford (1976) by using bovine serum albumin (1 mg ml^{-1}) as a standard.

4.4. Enzyme assays and HPLC analysis for JusB7H

Standard assays contained 50 μM JusB (**8**), 1 mM NADPH and 0.7 mg protein in a total volume of 500 μl Tris-HCl (pH 7.5) with 1 mM dithiothreitol and 1 mM diethyldithiocarbaminate. After vigorous mixing assays were routinely incubated for 75 min at 26 °C, stopped by addition of 50 μl 6 M HCl and cooled on ice. An NADPH regenerating system consisting of 10 mM glucose-6-phosphate, 0.07 units glucose-6-phosphate dehydrogenase and 4 mM DTT were added to the assay which was incubated for 5 h for the identification of the reaction product diphyllin (**9**) by HPLC-SPE-NMR. For JusB7H in vitro inhibition experiments, 0–500 μM tetcyclacis (in DMSO) or 0–500 μM clotrimazole (in MeOH) and 0–2.5 μM cytochrome *c* in 0.1 M Tris-HCl (pH 7.5, 1 mM dithiothreitol, 1 mM

diethyldithiocarbaminate) were added to the standard assay in variable concentrations. In control assays with the solvents alone, no effect on the JusB7H activity was observed. Stopped assays were extracted with ethyl acetate (3×500 μl). The combined ethyl acetate phases were dried under vacuum. The residues were redissolved in 80 μl methanol and subjected to HPLC analysis. Analysis and quantification of the reaction products was performed on a Hypersil Hypurity™ Elite C18 column (250 mm long, 4 mm i.d.) with an elution system employing water containing 0.01% (v/v) phosphoric acid (A) and acetonitrile (B). The following gradient was used: 0 min 25% acetonitrile, 25 min 38%, 43 min 43%, 46 min 55%, in 54 min 70%. The flow rate was 0.8 ml/min for the first 25 min, then 1 ml/min. HPLC was performed on a HPLC-PDA system from Thermoquest (Egelsbach, Germany) equipped with a Spectra System KO 6000 LP photodiode array detector, autosampler Spectra System AS1000, degasser and a pump Spectra System P2000. The monitoring wavelength was 230 nm.

4.5. Identification of diphyllin by LC-SPE-^{1}H NMR

The organic layer of the enzyme assay was separated, evaporated to dryness, redissolved in methanol, and subjected to reversed phase HPLC on a LiChrospher 100 RP-18 column (250×4 mm, particle size 5 μm) using acetonitrile: water (0.1% trifluoroacetic acid) as a mobile phase (flow rate 1.0 ml min^{-1}). The following gradient was applied: 0 min 25% MeCN, 25 min 38%, 43 min 43%, 46 min 55%, 54 min 70%. HPLC was performed on an Agilent 1100 chromatography system (quaternary pump G1311A; autosampler G1313A; J&M photodiode array detector, monitoring wavelength 230 nm and 254 nm). The HPLC system was connected to a Prospekt 2 SPE unit (Spark Holland), which was used for trapping. The post-column eluent flow was diluted with H_2O by a makeup pump before trapping the peak eluting at R_t 28.5 min on a poly(divinylbenzene) SPE cartridge (HySphere resin GP, Spark Holland). The SPE device was connected by a capillary to a Bruker Avance 500 MHz spectrometer equipped with a Cryofit™ flow insert (30 μl active volume). The cartridge containing the trapped peak was dried with a stream of nitrogen gas for 30 min. Then the compound was eluted with MeCN-d_3 and directly transferred to the NMR spectrometer for data acquisition at 300 K. A ^{1}H NMR spectrum was measured in MeCN-d_3. Justicidin B (**8**) was also trapped from the LC-SPE-NMR of the assay mixture (R_t 37.3 min) and the ^{1}H NMR was measured. In order to compare the ^{1}H NMR spectra of the product directly with that of the authentic standard, a sample of diphyllin (**9**) was run under identical LC-SPE-NMR conditions.

4.5.1. NMR data of diphyllin (*9*)

^{1}H NMR (500 MHz; MeCN-d_3): δ 7.56 (1H, *s*, H-6), 6.96 (1H, *d*, $J = 8.0$ Hz, H-5'), 6.84 (1H, *d*, $J = 1.9$ Hz, H-2'), 6.79 (1H, *dd*, $J = 8.0$, 1.9 Hz, H-6'), 7.04 (1H, *s*, H-3), 6.05 (1H, *d*, $J = 1.0$ Hz, O–CH_2–O), 6.04 (1H, *d*, $J = 1.0$ Hz, O–CH_2–O), 5.35 (2H, *s*, H-9), 3.99 (3H, *s*, OCH_3-5), 3.70 (3H, *s*, OCH_3-4).

4.5.2. NMR data of justicidin B (*8*)

^{1}H NMR (500 MHz; MeCN-d_3): δ 7.84 (1H, *s*, H-7), 7.37 (1H, *s*, H-6), 6.99 (1H, *d*, $J = 8.0$ Hz, H-5'), 6.88 (1H, *d*, $J = 1.9$ Hz, H-2'), 6.83 (1H, *dd*, $J = 8.0$, 1.9 Hz, H-6'), 7.07 (1H, *s*, H-3), 6.07 (1 H, *d*, $J = 1.0$ Hz, O–CH_2–O), 6.06 (1H, *d*, $J = 1.0$ Hz, O–CH_2–O), 5.37 (2H, *s*, H-9), 3.97 (3H, *s*, OCH_3-5), 3.71 (3H, *s*, OCH_3-4).

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