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Molecular cloning and heterologous expression of progesterone 5β-reductase from *Digitalis lanata* Ehrh. ☆

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Dedicated to the memory of Prof. Dr. Ernst Reinhard.

Abstract

A full-length cDNA clone that encodes progesterone 5β-reductase (5β-POR) was isolated from *Digitalis lanata* leaves. The reading frame of the 5β-POR gene is 1170 nucleotides corresponding to 389 amino acids. For expression, a *Sph*1/*Sal*1 5β-POR fragment was cloned into the pQE vector and was transformed into *Escherichia coli* strain M15[pREP4]. The recombinant gene was functionally expressed and the recombinant enzyme was characterized. The K_m and v_{max} values for the putative natural substrate progesterone were calculated to be 0.120 mM and 45 nkat mg^{-1} protein, respectively. Only 5β-pregnane-3,20-dione but not its α-isomer was formed when progesterone was used as the substrate. Kinetic constants for cortisol, cortexone, 4-androstene-3,17-dione and NADPH were also determined. The molecular organization of the 5β-POR gene in *D. lanata* was determined by Southern blot analysis. The 5β-POR is highly conserved within the genus *Digitalis* and the respective genes and proteins share considerable homology to putative progesterone reductases from other plant species.

Keywords: *Digitalis lanata*; Plantaginaceae; Veronicaceae; Progesterone 5β-reductase (5β-POR); Cardenolide biosynthesis; Over-expression

1. Introduction

Leaves of *Digitalis* plants are still the major source for the isolation of cardenolides that are used to treat cardiac insufficiency in humans. Cardenolides are characterized by a steroid nucleus with its four rings connected *cis–trans–cis*, having a 14β-hydroxy group and an unsaturated five-membered lactone ring at C-17β. Typically, sugar side chains of variable length are attached at position C-3 of the cardenolide genins. Through studies using radiolabelled precursors, the putative biosynthetic pathway was basically deduced, but it is not yet fully understood on a biochemical level (Kreis et al., 1998).

Taking cholesterol as the starting point, about 20 enzymes which probably affect the formation of cardenolides have been identified and characterized in *Digitalis* (Lindemann and Luckner, 1997; Kreis et al., 1998). But only some of them have been purified, including the progesterone 5β-reductase (5β-POR), a key enzyme of cardenolide biosynthesis catalysing the conversion of progesterone to 5β-pregnane-3,20-dione. The enzyme has been partially sequenced (Gärtner et al., 1994). To find a possible route for manipulating cardenolide biosynthesis in plants, a more detailed knowledge of the enzymes and genes involved in cardenolide formation is necessary for studying the regulation and engineering of

Abbreviations: 3β-HSD, Δ^5-3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-ketosteroid isomerase; 5β-POR, progesterone 5β-reductase; RT-PCR, reverse transcriptase polymerase chain reaction; t_R, relative retention time.

☆ The nucleotide sequences reported in this paper have been submitted to GenBank™ Data Base with the corresponding Accession Numbers AY574950/AY585867.

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the cardenolide pathway (Kreis et al., 1998; Eisenbeiß et al., 1999; Luckner and Wichtl, 2000).

So far, molecular data from *Digitalis* are available only for a few house keeping genes (tRNA-Leu, 18S ribosomal RNA) and several enzymes like aldo-keto reductase (Gavidia et al., 2002), acyl-CoA-binding protein (Metzner et al., 2000), cyclophilins (Scholze et al., 1999; Küllertz et al., 1999), for review see Luckner and Wichtl (2000). Cardenolide specific genes are described for: cardenolide-16′-*O*-glucohydrolase (Schöninger et al., 1998; Framm et al., 2000), lanatoside-15′-*O*-acetylesterase (Kandzia et al., 1998), and Δ^5-3β-hydroxysteroid dehydrogenase (Finsterbusch et al., 1999; Lindemann et al., 2000). The gene for progesterone 5β-reductase of *D. obscura* (*Dop5βr*; AJ555127) was reported by Roca-Perez et al. (2004). The *p5βr* gene from *D. purpurea* was cloned and a partial genomic clone from *D. obscura* has been used to analyse the cardenolide production in 10 natural populations under seasonal aspects. We here report for the first time the cloning and heterologous functional expression of 5β-POR from leaves of *Digitalis lanata* Ehrh. and the biochemical characterization of the recombinant enzyme.

2. Results and discussion

2.1. PCR amplification and cloning

The early steps of cardenolide biosynthesis are usually described as outlined in Fig. 1 (for review, see Kreis et al., 1998). We here focussed on the crucial step leading to 5β-configured pregnanes supposed to be the direct precursors of *Digitalis* cardenolides.

Initial experiments were carried out using degenerated oligo nucleotide primers derived from the peptide sequences of progesterone 5β-reductase from *D. purpurea* (Gärtner et al., 1990, 1994) taking Kazusa's codon usage system into account (www.kazusa.or.jp). The resulting fragments showed high sequence homology to the genomic clone of *Dop5βr* gene of *D. obscura* and to the progesterone 5β-reductase (*p5βr*) from *D. purpurea*. After submission of the sequence for *p5βr* gene (AJ310673; Roca-Perez et al., 2004) the results were confirmed by PCR amplification with distinct primers. The 5β-POR was amplified by RT-PCR from cDNA prepared from *D. lanata*, *D. purpurea* and *D. obscura*. DNA fragments of nearly identical length were also obtained when genomic DNA of *D. purpurea* was used as template. After subcloning of the PCR fragments into the TOPO cloning vector system the nucleotide sequence was elucidated by MWG® Biotech AG (Ebersberg, Germany).

We here isolated a full-length cDNA clone that encodes a progesterone 5β-POR from leaves of *D. lanata*. An identical match was observed between the deduced and directly determined amino acid sequence of the progesterone 5β-reductase peptides from *D. purpurea* (Gärtner et al., 1994). RT-PCR using RNA and/or mRNA from mature leaves resulted in one single DNA fragment of the appropriate size. The DNA fragments and the nucleotide sequences obtained from *D. lanata*, *D. purpurea* and *D. obscura* did not differ in size (Fig. 2A). PCR amplification with genomic DNA as a template resulted in a fragment of 1247 bp, slightly different in size from the cDNA fragments (Fig. 2A, Lane 4). Actually, the sequence of the genomic clone contained a small intron as obtained after sequencing data analysis.

2.2. Alignments

Several authors proposed that 5β-POR has a key function in cardenolide biosynthesis (Gärtner et al., 1990; Lindemann and Luckner, 1997) producing the required 5β-configured pregnane intermediates leading to the various cardenolide genins. Fig. 3 shows the alignment of deduced 5β-POR protein sequences for *D. lanata*

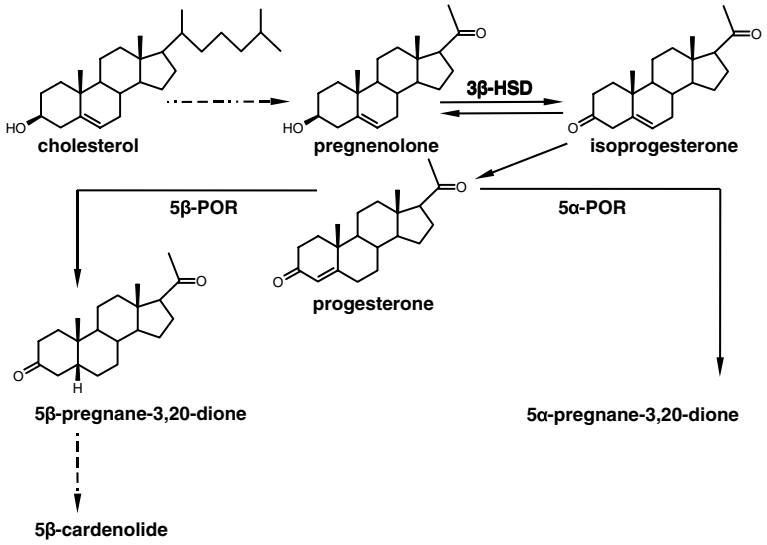

Fig. 1. Early steps in cardenolide biosynthesis.

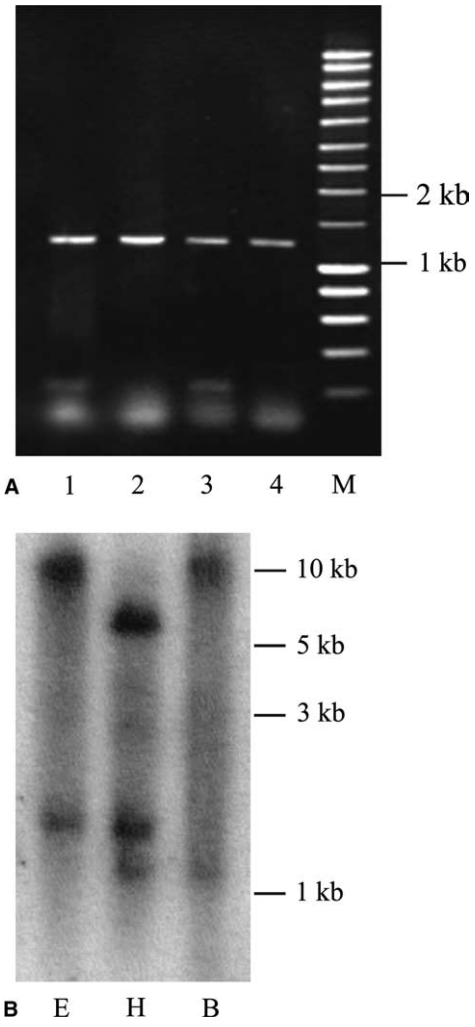

Fig. 2. (A) PCR amplification of 5β-POR from *D. lanata*; (Lane 1) DNA fragment of genomic DNA from *D. lanata*; (Lane 2) cDNA fragment from RNA of *D. obscura* after RT-PCR; (Lane 3) cDNA fragment from RNA of *D. purpurea* after RT-PCR; (Lane 4) cDNA fragment from RNA of *D. lanata* after RT-PCR; (M) DNA-Marker SmartLadder (200–10,000 bp). (B) Southern blot of *D. lanata* DNA digested with *Eco*R1 (E), *Hin*dIII (H) and *Bam*H1 (B) hybridized with ^{32}P-dGTP labelled 5β-POR cDNA.

(AY574950), *D. purpurea* (AY585868) and the *Dop5βr* gene of *D. obscura* (AJ555127). The sequences of the deduced 5β-POR gene products are 95–99% identical. A high degree of homology was also seen when the nucleotide sequence of the cDNA was analysed *in silico* and compared with the reports for *Dop5βr* of *D. obscura* (Roca-Perez et al., 2004) and *Dop5βr* of *D. purpurea* (AJ310673) (Fig. 3). Hence, it seems as if the 5β-POR genes are highly conserved within the genus *Digitalis*. Recently, Bräuchler et al. (2004) proposed a phylogenetic cladogramme of the genus *Digitalis* on the basis of ITS- and *trn*L-F sequences and found *D. lanata* and *D. obscura* more closely related to each other than to *D. purpurea* which is supported by our data.

The deduced 5β-POR protein sequences were found similar to those of *Oryza sativa* (about 58%), *Populus tremuloides* (about 64%) and to the *Arabidopsis thaliana* wound-induced gene AWI (Yang et al., 1997). For the competing 5α-POR (Kreis et al., 1998), several conserved functional protein domains (LEGFXAF, LIHX, IPFV) were described by Thigpen and Russell (1992) and Bhattacharyya et al. (1999). None of these domains could be found in the 5β-PORs isolated so far. On the other hand, the two peptide fragments sequenced by Gärtner et al. (1994) were identified in all 5β-POR products sequenced so far (Roca-Perez et al., 2004; AY585865-68, AY750898, AY574950, AY738710-12).

2.3. Southern blot analysis

The molecular organization of the 5β-POR from *D. lanata* was determined by Southern blot analysis of genomic DNA (Sambrook et al., 1989), digested with the restriction endonucleases *Eco*R1, *Bam*H1 and *Hin*dIII (Fig. 2B). The full-length cDNA clone of 1170 bp was ^{32}P-dGTP labelled and used as a probe for hybridization. Under high stringency conditions two bands were detected for cuts by *Eco*R1 and *Bam*H1 restrictases, while three bands were found for *Hin*dIII, as outlined in Fig. 2B. These results indicate that 5β-POR from *D. lanata* consists of almost 1–2 genes, as the size of the genomic clone of the 5β-POR is known (1250 bp, AY585867).

2.4. Over-expression

Over-expression of 5β-POR was achieved in *Escherichia coli* after IPTG (0.1 mM) induction at low temperature (4 °C). The enzyme was purified by Ni-NTA batch fractionation under native conditions and analysed by SDS–PAGE (Fig. 4). As a control, the bacterial host cells were transformed with an empty pQEX vector. The Ni-NTA-purified *r*5β-POR was analysed by SDS–PAGE where a strong band at 40 kDa indicated that the recombinant protein has the same size as the 5β-POR isolated from *D. purpurea* leaves by Gärtner et al. (1994). Traces of other proteins (less than 3% of overall protein) could also be detected in the SDS–PAGE but these were also found in the control extracts of the bacteria containing the pQEX vector only. Since Janknecht et al. (1991) stated that proteins containing neighbouring histidins are not common in bacteria we suggest that these "contaminating" proteins remained bound to the column material due to the low stringency condition (20 mM imidazole) chosen in the washing step.

2.5. Function of r5β-POR

The 5β-POR gene over-expressed in *E. coli* yielded an enzymatically active protein. The Ni-NTA-purified protein was checked for 5β-POR activity using the assay described earlier (Stuhlemmer and Kreis, 1996). A typical experiment is shown in Fig. 5A. TLC analysis revealed the formation of one single product, namely 5β-pregnane-3,20-dione, when progesterone was used as the substrate (Fig. 5, Lane 3). 5α-Pregnane-3,20-dione, the 5α-isomer of the pregnane-3,20-dione was not formed. This fact is supported by the different R_f-values and the different colours of the two

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MSWWWAGAIGAAKKRLEEDDAQPKHSSVALIVGVTGIIGNSLAEILPLADTPGGPWKVYG
MSWWWAGAIGAAKKKLEEDDAPPKHSSVALIVGVTGIIGNSLAEILPLADTPGGPWKVYG
MSWWWAGAIGAAKKKLEEDDAPPKHSSVALIVGVTGIIGNSLAEILPLADTPGGPWKVYG
************** ****** ************************************
VARRTRPAWHEDNPINYVQCDISDPDDSQAKLSPLTDVTHVFYVTWANRSTEQENCEANS
VARRTRPAWHEDNPINYVQCDISDPDDSQAKLSPLTDVTHVFYVTWANRSTEQENCEANS
VARRTRPAWHEDNPINYIQCDISDPDDSLAKLSPLTDVTHVFYVTWANRSTEPENCEANS
***************** ********** ********************** *******
KMFRNVLDAVIPNCPNLKHISLQTGRKHYMGPFESYGKIESHDPPYTEDLPRLKYMNFYY
KMFRNVLDAVIPNCPNLKHISLQTGRKHYMGPFESYGKIESHDPPYTEDLPRLKYMNFYY
KMFRNVLDAVIPNCPNLKHISLQTGRKHYMGPFESYGKIESHDPPYTEDMPRLKYINFYY
************************************************ ***** ****
DLEDIMLEEVEKKEGLTWSVHRPGNIFGFSPYSMMNLVGTLCVYAAICKHEGKVLRFTGC
DLEDIMLKEVEKKEGLTWSVHRPGNIFGFSPYSMMNLVGTLCVYAAICKHEGKVLRFTGC
DLEDIMLEEVEKKEGLTWSVHRPGNIFGFSPYSMMNLVGTLCVYAAICKHEGKVLRFPGC
******* ************************************************* **
KAAWDGYSDCSDADLIAEHHIWAAVDPYAKNEAFNVSNGDVFKWKHFWKVLAEQFGVGCG
KAAWDGYSDCSDADLIAEHHIWAAVDPYAKNEAFNVSNGDVFKWKHFWKVLAEQFGVECG
KAAWDGYSDCSDADLIAEHHIWAAVDPYAKNEAFNVSNGDVFKWKHFWKVLAEQFGVECG
********************************************************* **
EYEEGVDLKLQDLMKGKEPVWEEIVRENGLTPTKLKDVGIWWFGDVILGNECFLDSMNKS
EYEEGEDLKLQDLMKGKEPVWEEIVRGNGLTPTKLKDVGIWWFGDVILGNECFLDSMNKS
EYGEGVDLKLQDLMKGKEAVWEEIVRENGLTPTKLKDIGIWWFGDVILGNECFLDSMNKS
** ** ************ ******* ********** **********************
KEHGFLGFRNSKNAFISWIDKAKAYKIVP AY574950 D. lanata
KEHGFLGFRNSKNAFISWIDKAKAYKIVP AJ555127 D. obscura
KEHGFLGFRNSKNAFISWIDKAKAYKIVP AJ310673 D. purpurea
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Fig. 3. Alignment of deduced 5β-POR proteins from different sources: AY574950 – *D. lanata*; AJ555127 – *D. obscura*; AJ310673 – *D. purpurea*. Differences are bold.

isomers on TLC (Fig. 5A, Lane 4–5). The results have been further confirmed by GC analyses which proofed that only the 5β-isomer but not the 5α-isomer of pregnane-3,20-dione

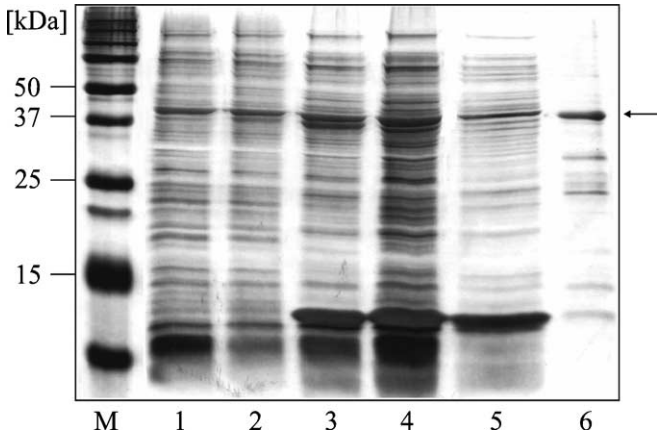

Fig. 4. Expression of recombinant 5β-POR in *E. coli* analyzed on SDS–PAGE (12%). M – molecular weight marker BioRad (München); (1) bacterial homogenate not induced by IPTG; (2) bacterial homogenate induced by 0.1 mM IPTG and cultivated at 4 °C for 96 h; (3) cell lysate before Ni-NTA column; (4) flow through fraction from Ni-NTA column; (5) wash fraction; (6) imidazole eluate from Ni-NTA column (recombinant protein arrowed).

was produced (Fig. 5B). The minor compounds seen in Fig. 5B/III at $t_R = 28.30$ and $t_R = 27.94$ do not represent pregnane isomers as could be deduced from the MS spectra. Interestingly, 5β-pregnane-3,20-dione, the product of the enzyme reaction was not converted to other products as it was seen in partially purified enzyme preparations from *D. lanata* leaves (Kreis et al., 1998), indicating that those extracts contained other pregnane-modifying enzymes and that *r*5β-POR catalyses only the 5β-reduction of progesterone. It should be stressed that 5β-POR or homologous genes have not been found in bacteria as yet. Actually, 5β-POR activity could not be detected in protein extracts from bacteria containing the pQEX vector only.

2.6. Substrate preferences

The substrate preferences and kinetic properties of *r*5β-POR were investigated using an HPLC method for product identification and quantification. Besides the putative natural substrate progesterone other steroid substrates were tested. Relative activities as well as K_m- and v_{max}-values were calculated and are shown in Table 1 and Fig. 6. The *r*5β-POR did not only accept progesterone but also testosterone, 4-androstene-3,17-dione, cortisol and cortisone.

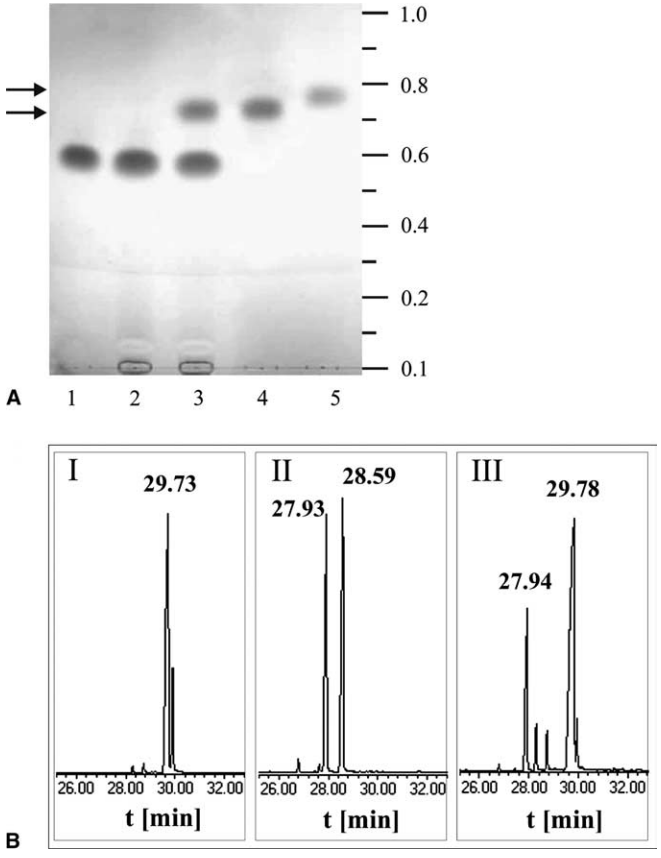

Fig. 5. (A) TLC analysis of *r*5β-POR activity. (1) progesterone; (2) inactivated assay by heat treatment (10 min, 100 °C); (3) purified Ni-NTA 5β-POR (+IPTG, 96 h at 4 °C); (4) 5β-pregnane-3,20-dione; (5) 5α-pregnane-3,20-dione. Ordinate – R_f-values. (B) GC analysis of *r*5β-POR activity. I: progesterone, $t_R = 29.73$ min; II: 5β-pregnane-3,20-dione, $t_R = 27.93$ min; 5α-pregnane-3,20-dione, $t_R = 28.59$ min; III: enzyme assay, progesterone, $t_R = 29.78$ min and 5β-pregnane-3,20-dione, $t_R = 27.94$ min.

Other substrates, such as pregnenolone, 21-OH-pregenenolone and isoprogesterone were not accepted by *r*5β-POR. NADPH is the only co-substrate and cannot be replaced by NADH (data not shown). The K_m-value for progesterone was 120 μM, for NADPH it was 8 μM. The K_m-values determined for the enzyme isolated from *D. purpurea* leaves (Gärtner et al., 1994), which were similar or nearly identical, are shown in Table 1 for comparison.

From the data obtained in the experiments described above two important conclusions can be drawn. (1) Essential structural elements for substrates of *r*5β-POR are the carbonyl group at C-3 and the Δ^4-double bond in conjugation to it. (2) Less important are the side chain at C-17 and the substitution pattern of the steroid ring system in its periphery.

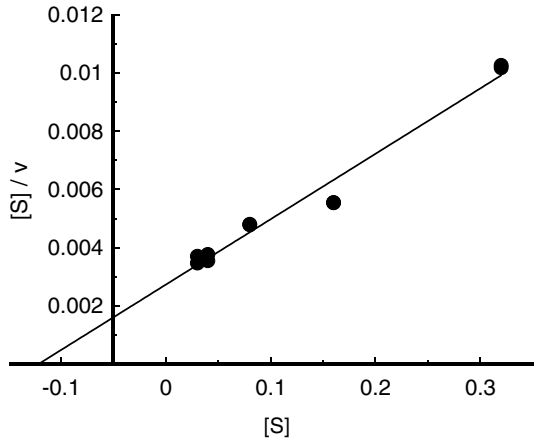

Fig. 6. Graphical determination (Hanes plot) of the K_m-value of *r*5β-POR for progesterone (120 μM).

2.7. Biochemical characterization of r5β-POR

Using progesterone as a substrate, pH and temperature dependencies of the purified enzyme were investigated. Recombinant 5β-POR activity was optimal at pH 7.8. The enzyme worked best at around 40 °C. The pI of the *r*5β-POR as determined by isoelectric focusing (IEF) was 6.5.

In order to elucidate the cardenolide pathway further, the recombinant 5β-POR will now be subjected to crystallization studies with a view to understand its mode of action and substrate discrimination.

3. Experimental

3.1. Plant material

Seeds of *D. lanata* Ehrh. were obtained from the Genbank of the Institute for Plant Genetics and Research on Cultivated Plants in Gatersleben (Germany) and grown under standard greenhouse conditions. For comparison, *D. purpurea* L. and *D. obscura* L. emend. Pau were included in this study.

Table 1
Substrate specificity of the *r*5β-POR

Substrate	K_m (mM)	v_{max} (nkat/mg)	v_{max}/K_m	Efficiency (%)
Progesterone	0.120 ± 0.048 (0.037[a])	45.0 ± 7.9	375.0	100
Cortisol	0.291 ± 0.102	81.2 ± 16.5	279.0	133
4-Androstene-3,17-dion	0.228 ± 0.069	18.5 ± 1.6	81.1	47
Cortexone	1.597 ± 0.275	63.3 ± 6.7	39.6	33
NADPH	0.008 ± 0.002 (0.007[a])	31.1 ± 2.0	3887.5	–

Relative activities are calculated on the basis of progesterone (=100%) as the substrate. For the determination of substrate specificities the standard enzyme assays for 5β-POR were used.

[a] K_m-value for progesterone (37 μM) and for NADP (7 μM) for the plant enzyme (*D. purpurea* shoot cultures) were taken from Gärtner et al. (1994).

3.2. DNA/RNA extraction

The plant tissues were ground to a fine powder in liquid nitrogen using mortar and pestle. Genomic DNA and total RNA extraction was carried out with E.Z.N.A.® Plant DNA and RNA Mini Kits, respectively (Peqlab, Biotechnologie GmbH, Erlangen, Germany). Messenger RNA was isolated using Oligotex™ direct mRNA Kit (QIAGEN GmbH, Hilden, Germany). All molecular biology methods were performed according to Sambrook et al. (1989).

3.3. PCR DNA amplification

Polymerase chain reaction amplifications were performed according to Williams and Tsang (1991). Each reaction (50 μl total volume) contained 2.5 U SAWADY Taq-DNA-Polymerase (Peqlab, Biotechnologie GmbH, Erlangen, Germany), 1× reaction buffer S, 0.5 mM $MgCl_2$, 0.5 mM of each dNTP, 2 μM of primers and 0.2 μg of genomic DNA. A Personal Cycler 20 (Biometra GmbH, Göttingen, Germany) was used for amplification according to the supplier's recommendation. Thirty-one cycles of 3 min denaturation at 95 °C, followed by 1 min annealing at 56 °C and 2 min extension at 72 °C. Finally, a 5 min extension at 72 °C was added to complete the amplification. PCR products were analyzed by 1% agarose gel electrophoresis in TAE buffer system. Gels were stained with ethidium bromide and visualized by illumination at UV_{365}. For size determination SmartLadder (Eurogentec GmbH, Köln, Germany) was used, producing a pattern of 14 regularly spaced bands ranging from 200 to 10,000 bp. RT-PCR was performed by Titan™ One Tube RT-PCR System (Roche Diagnostics GmbH, Mannheim, Germany) using RNA or mRNA as a template. All constructed plasmids were sequenced to confirm their identities (MWG AG, Ebersberg, Germany).

3.4. Subcloning and sequencing of PCR products

Bands of the expected size (1170 bp) were extracted with QIAEX II Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) and ligated into pCR 2.1-TOPO vector for subsequent transformation in *E. coli* strain TOP10 (Invitrogen, Karlsruhe, Germany). Transformed cell colonies were selected on ampicillin containing plates. Plasmid isolation was carried out using the E.Z.N.A.® Plasmid Miniprep Kit (Peqlab GmbH, Erlangen, Germany) prior to nucleotide sequence determination (MWG AG, Ebersberg, Germany). To confirm their identities the sequences were determined from both ends of cDNA using forward and reverse primers from the pCR 2.1-TOPO vector.

3.5. Southern blot hybridization

In Southern blot analysis 10 μg genomic DNA were digested with *Eco*R1, *Bam*H1, and *Hin*dIII. The nylon membrane was hybridized with ^{32}P-dGTP labelled cDNA for 5β-POR from *D. lanata* and washed under high stringency conditions (Sambrook et al., 1989). DNA bands were visualized by phosphor imaging using Fujifilm BAS-1500.

3.6. In silico analysis

After sequencing, all data were analyzed by different software packages (European Bioinformatics Institute). For searching and sequence analysis BLAST® search of the GenBank™ data base was carried out. The nucleic acid as well as the translated (TRANSEQ®) amino acid sequences were aligned using ClustalW (EBI Services) (http://www.ebi.ac.uk/clustalw/index.html).

3.7. Over-expression of 5β-POR in E. coli

The 5β-POR gene was cloned as a *Sph*1/*Sal*1 fragment into pQEX vector system (QIAGEN, Hilden Germany) for over-expression in *E. coli* host strain M15[pREP4]. For optimal expression the cells were grown to $OD_{600} \approx 0.5$, isopropyl-β-D-thiogalactoside (0.1 mM IPTG) was added to the medium and the cells were then further cultivated at 4 °C for 96 h for better uptake and increase of the solubility. Recombinant 5β-POR was isolated in native form according to the manufacturer's manual (QIAexpressionist™, Hilden, Germany). The Ni-NTA matrix was washed with 20 mM imidazole buffer before elution by 250 mM imidazole buffer. Protein analysis on SDS–PAGE was performed as reported earlier (Müller-Uri and Reva, 2000).

3.8. Enzyme assay for 5β-POR

To check 5β-POR activity, the method described by Stuhlemmer and Kreis (1996) was used with minor modifications. The assay contained in a final volume of 1000 μl: 945 (0.2 mg/ml) purified protein fraction, 6.4 mM $NADP^+$, 32.1 mM glucose-6-phosphate, 42 nkat glucose-6-phosphate-dehydrogenase and 0.3 mM progesterone. Heat-inactivated (10 min, 100 °C) samples served as controls. The mixtures were kept in 2-ml Eppendorf tubes and incubated at 30 °C and 550 rpm for 3 h prior to extraction, using 1000 μl dichloromethane. The organic phase was evaporated and the residue was dissolved in 50 μl ethanol. This solution was analysed by thin layer chromatography. The plates were developed with dichloromethane:ethyl acetate (8:2). Pregnane spots were visualized with anisaldehyde reagent (Jork, 1990).

3.9. HPLC analysis

An HPLC method (modified after Seidel et al., 1990) was applied to confirm the results from thin layer chromatography and to determine kinetic constants. The residue obtained after extraction and evaporation (see above) was dissolved in 50 μl methanol. The analysis was performed on a Waters 1525 Binary HPLC Pump system with a Waters 2487 Dual λ Absorbance Detector. The separation of substrates and products was achieved on a Symmetry® column

(C_{18}, 5 μM, 4.6 × 150 mm). The compounds were eluted with a step gradient composed of double-distilled water (solvent A) and acetonitrile (solvent B): start (25%B), 10 min (65%B), 18 min (100%B), 21 min (100%B), 23 min (25%B), 25 min (25%B). Pregnanes were detected at 205 and 240 nm and identified and quantified on the basis of authentic compounds and external standards, respectively.

3.10. GC–MS analysis

For GC analysis the evaporated fraction from the assay was dissolved in 100 μl dichloromethane. All fractions were analysed by GC Hewlett-Packard HP 6890 MSD Type 5972 A using helium and a fused capillary column HP Optima 5 (30 m × 25 mm × 0.25 μm). A flow rate of 1 ml/min was applied. The temperature programme used included a 150 °C initial step for 4 min, temperature shift up to 280 °C with 5 °C/min, followed by 10 min at 280 °C. Relative retention time t_R is given with respect to ($t_R = 1$).

3.11. Enzyme properties

The isoelectric point (pI) of *r*5β-POR was determined by isoelectric focusing on Criterion™ Gels IEF 3–9/Criterion™Cell (BioRad, München). The incubation time was 30 min. The temperature optimum and the energy of activation were determined at pH 7.8; otherwise the above conditions were applied. The K_m- and v_{max}-values of the *r*5β-POR for the different substrates were determined in the presence of about 100 μg/ml, 0.3 μg substrate and an incubation time of 30 min.

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