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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.
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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*

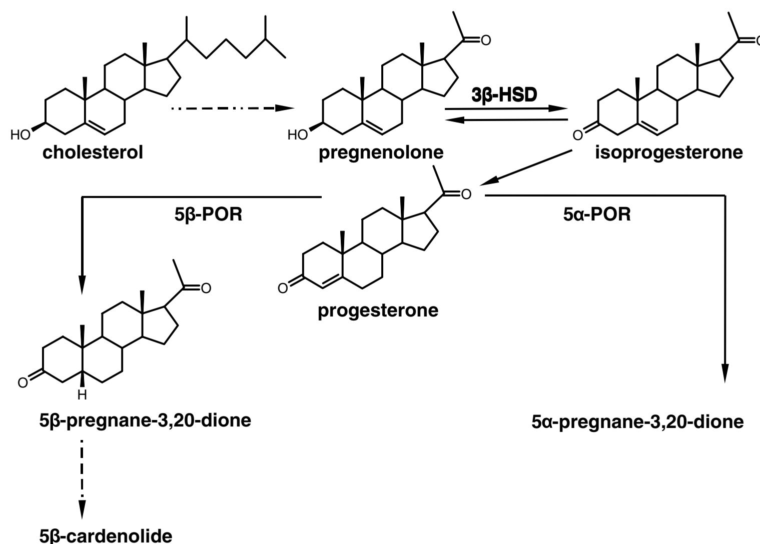

Fig. 1. Early steps in cardenolide biosynthesis.

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