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Progesterone 5β-reductase of *Erysimum crepidifolium*: cDNA cloning, expression in *Escherichia coli*, and reduction of enones with the recombinant protein

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ABSTRACT

Erysimum is a genus of the Brassicaceae family closely related to the genus *Arabidopsis*. Several *Erysimum* species accumulate 5β-cardenolides. Progesterone 5β-reductases (P5βRs) first described in *Digitalis* species are thought to be involved in 5β-cardenolide biosynthesis. P5βRs belong to the dehydrogenase/reductase super-family of proteins. A full length cDNA clone encoding a P5βR was isolated from *Erysimum crepidifolium* leaves by 5′/3′ RACE-PCR (termed *Ec*P5βR). Subsequently, the *P5βR* cDNAs of another nine *Erysimum* species were amplified by RT-PCR using 5′ and 3′ end primers deduced from the Ec*P5βR* cDNA. The Ec*P5βR* cDNA is 1170 bp long and encodes for 389 amino acids. The Ec*P5βR* cDNA was ligated into the vector pQE 30 UA and the recombinant His-tagged protein (termed r*Ec*P5βR) was over-expressed in *Escherichia coli* and purified by Ni-chelate affinity chromatography. Kinetic constants were determined for progesterone, 2-cyclohexen-1-one, isophorone, and NADPH. The by far highest specificity constant ($k_{cat}\,K_M^{-1}$) was estimated for 2-cyclohexen-1-one indicating that this monocyclic enone may be more related to the natural substrate of the enzyme than progesterone. The atomic structure of r*Ec*P5βR was modelled using the crystal structure of P5βR from *Digitalis lanata* 2V6G as the template. All sequence motifs specific for SDRs as well as the NFYYxxED motif typical for P5βR-like enzymes were present and the protein sequence fitted into the template smoothly.

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1. Introduction

The genus *Erysimum* belongs to the Brassicaceae family and comprises ca. 200 species spread all over the world. Rollins (1993) reported nineteen species to occur in North America. A much larger number of *Erysimum* species are found in Central Europe. Isolated species (e.g., *Erysimum nuratense*) were documented in Central Asia (Makarevich et al., 1994). Several hybrids (wallflowers) have been created and introduced as ornamental plants.

Herbal preparations of *Erysimum* species are used in folk medicine for treating cardiac diseases, oedema and dyspepsia in humans (Zhu, 1989). Most of the therapeutic effects are probably due to the occurrence of cardiac glycosides. More than 50 different cardiac glycosides have been isolated from various *Erysimum* species (Nagata et al., 1957; Gmelin and Bredenberg, 1966; Makarevich et al., 1976, 1994; Lei et al., 1998). They are all members of the cardenolides, i.e., a group of C_{23}-steroids having a butenolide ring attached to C-17. Cardenolides are scattered throughout several angiosperm orders (Kreis and Müller-Uri, 2010) and *Erysimum* is one of few genera in the Brassicales known to contain cardenolides. Depending on the annealing of the rings A and B (Fig. 1) a distinction is drawn between 5α-, 5β-, Δ4- and Δ5-cardenolides. Cardenolides are potent inhibitors of Na^+/K^+-ATPase (Erdmann et al., 1986) and all cardenolides used in the therapy of cardiac insufficiency in humans belong to the 5β-cardenolides. When investigating host plant discrimination within crucifers and the feeding responses of leaf beetles, Nielsen (1978) found that 5β-hydroxy-cardenolides, abundant in *Erysimum*, are more potent feeding inhibitors than 5α-hydroxy-cardenolides. Moreover, 5β-cardenolides represent promising candidates for targeted cancer chemotherapy (Newman et al., 2008).

Cardenolides are derived from mevalonic acid via phytosterol and pregnane intermediates and their biosynthesis was mainly studied in *Digitalis* (Kreis and Müller-Uri, 2010; Herl et al., 2007). Progesterone 5β-reductase (P5βR) was proposed to be a key enzyme in the formation of 5β-cardenolides in *Digitalis* (Gärtner et al., 1990; Fig. 1). Only recently, Pérez-Bermúdez et al. (2010)

Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; P5βR, progesterone 5β-reductase; 5′/3′ RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription polymerase chain reaction; SDR, short-chain dehydrogenase/reductase; St5βR, steroid 5β-reductase.

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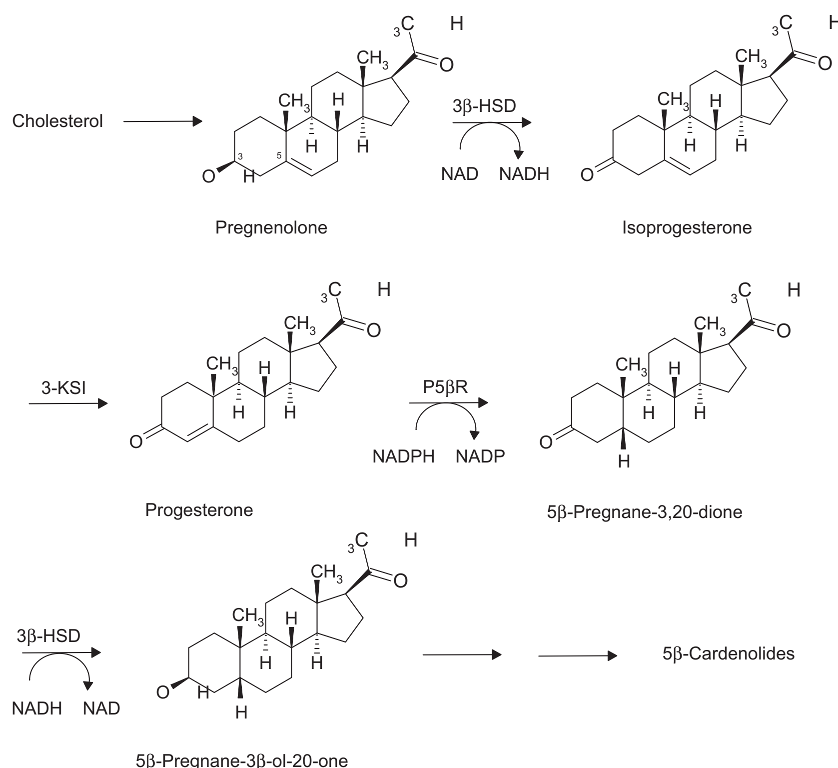

Fig. 1. Putative biosynthetic pathway for 5β-cardenolides. 3β-HSD = 3β-hydroxysteroid dehydrogenase; 3-KSI = 3-ketosteroid isomerase; P5βR = progesterone 5β-reductase.

reported a stress-induced isoform of progesterone 5β-reductase (labelled P5βR2) from *D. purpurea.* P5βR and P5βR2 belong to the short chain dehydrogenase/reductases (SDR) super-family of proteins (Persson et al., 2003; Thorn et al., 2008). Genes encoding P5βR (AT4G24220, AY062451) and 3β-hydroxysteroid dehydrogenase (3β-HSD, Herl et al., 2007) (AT2G47140, NM_130282) have been reported from *Arabidopsis thaliana* and the respective enzymes heterologously expressed, thus indicating that putative cardenolide-biosynthetic enzymes are also present in crucifers (Witt, 2008; Herl et al., 2009).

Since *Erysimum* and *Arabidopsis* are close relatives (Bailey et al., 2006) we assume that *Erysimum* will turn out to be a better plant genus than *Digitalis* to study cardenolide biosynthesis on both the biochemical and molecular biology level. One may expect a high degree of sequence identities of the genes and proteins of *Arabidopsis* and *Erysimum* species and this will enable us to further elucidate the pathway and the evolution of cardenolide formation. For example, chalcone synthase and phytochrome A of *Erysimum* and *Arabidopsis* are 98% and 99% identical, respectively (BLAST search). Assuming that (1) cardenolide formation requires similar enzymatic steps in all cardenolide-producing angiosperms and that (2) some of the enzymes operating in cardenolide formation are substrate-promiscuous catalysts present in most if not all angiosperms (Bauer et al., 2010), we here aimed at the demonstration of progesterone 5β-reductase enzymes and genes in various *Erysimum* species.

2. Results and discussion

2.1. Isolation of *P5βR* cDNA from *Erysimum crepidifolium* leaves

Progesterone 5β-reductase activity was demonstrated in the soluble protein fraction prepared from *E. crepidifolium* leaves when using progesterone and NADPH as substrate and co-substrate, respectively. Formation of possible follow-up products was seen as well; indicating the presence of further pregnane-converting enzymes suggested being involved in cardenolide biosynthesis (Kreis and Müller-Uri, 2010).

After having demonstrated progesterone 5β-reductase activity *in vitro* we directly aimed at the isolation of a progesterone 5β-reductase cDNA (Ec*P5βR)* from *E. crepidifolium* leaves. We already reported the sequences of two *Erysimum* P5βRs and their heterologous expression (Bauer et al., 2010). Here, the isolation of the first full-length *Erysimum P5βR* cDNA is described. It was synthesised by 5′/3′ RACE PCR using a 170 bp DNA fragment and mRNA isolated from *E. crepidifolium* leaves. The DNA fragment used was a consensus sequence deduced from the conserved motif domains of the *A. thaliana VEP1* cDNA when compared with the respective sequences of the *P5βR* cDNAs of various *Digitalis* species. In this way the 5′ and 3′ ends of the *P5βR* gene were determined and a full-length cDNA could be created using the 5′ end and 3′ end primers Erydir1 and Eryrev1170 (Table 1). The encoded protein and its recombinant form His-tagged at the *N*-terminus (see below Section 2.2) were termed *Ec*P5βR and r*Ec*P5βR, respectively.

Subsequently, the *P5βR* genes of another nine *Erysimum* species were amplified using the 5′ end and 3′ end primers (Table 1) deduced from the *E. crepidifolium P5βR* cDNA. Actually, the cDNA sequences obtained did not differ much. All *Erysimum P5βR* cDNAs

Table 1
List of primers used for amplification and cloning of *Erysimum P5βR* genes.

Use	Term	Sequence 5′-3′	T_M (°C)
5′RACE	SIrevmotiv	TTTGGTCTGTGGATAGACCACG	66
	JMErySp2rev	ATAGACCACGTCACACTAATTTCT	66
	Revery3	ATTCTGGATCTGCAATCTCGG	62
3′RACE	SIdirmotiv	AAGCACTACCTTGGCCCTTT	60
	JM603dir	AGGCCAAACACGATCTTTGGA	62
	JM721dir	AGAAGGCTTGGGAAGGGTTCA	64
RT-PCR	Erydir1	ATGAGTTGGTGGGGGGCT	58
	Eryrev1170	TCAAGGCACGATCTTGAAAGC	62

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