Δ^5-3β-Hydroxysteroid Dehydrogenase (3βHSD) from *Digitalis lanata*. Heterologous Expression and Characterisation of the Recombinant Enzyme*

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Abstract

During the biosynthesis of cardiac glycosides, Δ^5-3β-hydroxysteroid dehydrogenase (3βHSD, EC 1.1.1.51) converts pregnenolone (5-pregnen-3β-ol-20-one) to isoprogesterone (5-pregnene-3,20-dione). A 3βHSD gene was isolated from leaves of *Digitalis lanata*. It consisted of 870 nucleotides containing a 90 nucleotide long intron. A full-length cDNA clone that encodes 3βHSD was isolated by RT-PCR from the same source. A *Sph* I/*Kpn* I 3βHSD cDNA was cloned into the pQE30 vector and then transferred into *E. coli* strain M15[pREP4]. 3βHSD cDNA was functionally expressed as a His-tagged fusion protein (pQ3βHSD) composed of 273 amino acids (calculated molecular mass 28,561 Da). pQ3βHSD was purified by metal chelate affinity chromatography on Ni-NTA. Pregnenolone and other 3β-hydroxypregnanes but not cholesterol were 3β-oxidised by pQ3βHSD when NAD was used as the co-substrate. Testosterone (4-androsten-17β-ol-3-one) was converted to 4-androstene-3,17-dione indicating that the pQ3βHSD has also 17β-dehydrogenase activity. pQ3βHSD was able to reduce 3-keto steroids to their corresponding 3β-hydroxy derivatives when NADH was used as the co-substrate. For comparison, 3βHSD genes were isolated and sequenced from another 6 species of the genus *Digitalis*. Gene structure and the deduced 3βHSD proteins share a high degree of similarity.

Key words
Δ^5-3β-Hydroxysteroid dehydrogenase · Δ^5-3-ketosteroid isomerase · *Digitalis lanata* · cardenolide biosynthesis · gene expression · Plantaginaceae · pregnenolone · progesterone

Supporting information available online at
http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Cardenolides, used for treatment of cardiac insufficiency in humans, are still isolated from plants, the most important source being *Digitalis lanata* leaves. Cardenolides are supposed to be formed from cholesterol or other phytosterols (see [1] for a comprehensive review). The first step in the putative biosynthetic route being studied extensively is the one facilitating the formation of progesterone (4-pregnene-3,20-dione) from pregnenolone (5-pregnen-3β-ol-20-one) in a two-step reaction (see Fig. **4** below) [1], [2]. 3β-Hydroxysteroid dehydrogenase (3βHSD; EC 1.1.1.51) converts pregnenolone into isoprogesterone (5-pregnene-3,20-dione) which is then isomerised to progesterone. 3βHSD has been demonstrated in *Digitalis* cell cultures and

* The nucleotide sequences reported in this paper have been submitted to GenBank™ Data Base with the corresponding accession numbers: DQ466890; AY844960; AY789449-453; AY844959.

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Dedication
Dedicated to Professor A. Wilhelm Alfermann on the occasion of his 65th birthday

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Received March 7, 2007 · **Revised** April 24, 2007 · **Accepted** May 4, 2007

Bibliography
Planta Med 2007; 73: 704–710 © Georg Thieme Verlag KG Stuttgart · New York
DOI 10.1055/s-2007-981537 · Published online June 12, 2007
ISSN 0032-0943

plants [3] and was isolated and purified from a *D. lanata* cell culture [4]. Lindemann et al. [5] amplified and sequenced a 700 bp PCR fragment coding for 233 amino acids and the same group published the sequence for a full-length 3βHSD cDNA in GenBank (Acc.-Nr. AJ345026).

3βHSDs are involved in steroid biosynthesis in animal tissues and steroid transformation in microorganisms. In animals, 3βHSD and ketosteroid isomerase activities reside in one single protein. Several membrane-bound isoenzymes have been reported from mammals, all being products of the 3βHSD gene family [6]. On the other hand, 3β/17β-hydroxysteroid dehydrogenase (3β/17βHSD), a soluble protein in *Commamonas testosteroni,* catalyses the reversible reduction/dehydrogenation of the oxo/β-hydroxy groups at positions C-3 and C-17 of steroid compounds but does not exhibit Δ^5-3-ketosteroid isomerase (3-oxosteroid Δ^5-Δ^4-isomerase, KSI; EC 5.3.3.1) activity (see [7] for review). The 3βHSD from *D. lanata* shares high homology with prokaryotic hydroxysteroid dehydrogenases lacking isomerase activity.

We here cloned 3βHSD genes from several *Digitalis* species, functionally expressed a 3βHSD cDNA isolated from *D. lanata* and characterised the recombinant protein.

Material and Methods

Chemicals

Most of the steroids used here were obtained from commercial sources (e.g., Steraloids Inc.; Newport, RI, USA). Isoprogesterone (5-pregnene-3,20-dione) was synthesised following the procedure advised by Djerassi et al. [8] with minor modifications and purified by preparative TLC.

Plant material

Plant seeds were obtained from the Genbank of the Institute for Plant Genetics and Research on Cultivated Plants in Gatersleben, Germany and were grown under standard greenhouse conditions. Leaves from *D. lanata* Ehrh. and several other species, namely *D. purpurea, D. ferruginea, D. grandiflora, D. parviflora, D. mariana* and *D. thapsi* were included in this study.

Suspension cultures of the *D. lanata* cell line K3OHD [9] were grown in 300-mL Erlenmeyer flasks kept in the dark at 24 °C on gyratory shakers (110 rpm). Cells were sub-cultured every 7 d by inoculating 10 g cells (wet weight) into 100 mL of fresh growth medium [10].

DNA/RNA extraction

Fresh plant tissues were ground to a fine powder in liquid nitrogen using a mortar and pestle. Genomic DNA as well as total RNA extraction was carried out with E.Z.N.A. Plant DNA or RNA Mini Kits, respectively (Peqlab Biotechnologie GmbH; Erlangen, Germany). Messenger RNA was isolated by Oligotex direct mRNA Kit (Qiagen GmbH; Hilden, Germany). All molecular methods were performed according to [11].

PCR DNA amplification

Polymerase chain reaction amplifications were performed according to [12]. Gene-specific primers for PCR amplification were generated based on the submitted sequence for 3βHSD from *D. lanata* (Acc.-Nr. AJ345026). The full-length clones were amplified using the two synthetic oligonucleotide primers (Eurogentec GmbH; Köln, Germany) 5′-ATG TCG TCA AAG CCA AGG T-3′ (HSDdir) and 3′-CTA ACG CAC GAC GGT GAA G-5′ (HSDrev).

Each reaction vessel (50 μL total volume) contained 2.5 units SAWADY Taq-DNA-Polymerase (Peqlab Biotechnologie GmbH), 1x 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. 31 cycles of 3 min denaturation at 95 °C each, followed by 1 min annealing at 56 °C and 2 min extension at 72 °C. An additional 5 min extension at 72 °C was added to complete the amplification. PCR products were analysed using 1 % agarose gel electrophoresis in a TAE buffer system. DNA was stained with ethidium bromide and made visible under UV_{365nm} light. SmartLadder (Eurogentec GmbH) was used for size determination, producing a pattern of 14 equally spaced bands ranging from 200 up to 10 000 bp. Usually three clones each were sequenced from both sites for complete consensus sequence (MWG Biotech AG; Martinsried, Germany).

RT-PCR was performed with a Titan One Tube RT-PCR System (Roche Diagnostics GmbH; Mannheim, Germany) using RNA and mRNA for the preparation of templates.

In silico analysis

After sequencing, all data have been analysed by different software packages provided by the European Bioinformatics Institute (http://www.ebi.ac.uk). Searching and sequence analysis were performed with BLAST software in the GenBank Data Base. The nucleic acid as well as the translated (TRANSEQ) amino acid sequences were aligned by ClustalW (http://www.ebi.ac.uk/clustalw/index.html).

Heterologous expression

The open reading frame of the full-length 3βHSD cDNA was amplified by PCR using the primers 5′-TATAGCATGCATGC ATG TCG TCA AAG CCA AGG T-3′ (HSDsphdir) and 5′-TATAGGTAC CTA ACG CAC GAC GGT GAA G-3′(HSDkpnrev) to introduce *Sph*I and *Kpn*I restriction sites, respectively. The amplified fragment was cloned into the pQE30 vector system (Qiagen) for over-expression in *E. coli* strain M15[pREP4]. 1.0 mM IPTG (isopropyl-β-D-thiogalactoside) was added to the medium to induce 3βHSD formation. Cultivation continued at 37 °C for 5 h. 3βHSD cDNA was expressed as a His-tagged fusion protein containing the following peptide at its N-terminus: MRSGSHHHHHHGSAC. The recombinant 3βHSD (termed pQ3βHSD) was isolated following the supplier's protocol (QIAexpressionist; Qiagen). The Ni-NTA-matrix (nickel-nitrilotriacetic acid coupled to Superflow resin) was washed extensively with 20 mM imidazole buffer before elution with 250 mM imidazole buffer. Protein analysis on SDS-PAGE was performed as reported earlier [13], [14]. Protein was quantified using the method described by Bradford [15].

Δ^5-3β-Hydroxysteroid dehydrogenase (3βHSD) assay

The solution containing pQ3βHSD was passed through PD-10 columns (Amersham Biosciences Europe GmbH; Freiburg, Germany) for buffer exchange (20 mM NaP_i buffer, pH 7.8) and the protein eluate was diluted if necessary. The standard assay contained in a final volume of 1 mL: 0.2 mg protein, 0.3 μmol pregnane and 1.0 μmol NAD in 20 mM NaP_i buffer, pH 7.8. To check the reverse reaction the same buffer was used. The assay then contained in a final volume of 1 mL: 0.2 mg protein, 2 mM NAD, 2 μmol glucose 6-phosphate and 6.4 nkat glucose 6-phosphate dehydrogenase. The mixture was pre-incubated for 10 min at 30 °C prior to the addition of 0.3 μmol pregnane. After the addition of the respective substrate, samples were incubated at 50 °C and 550 rpm for 1 h (Eppendorf Thermomixer 5437, Eppendorf; Hamburg, Germany). Heat-inactivated samples served as controls. The reaction was terminated by adding 1 mL dichloromethane. After vortexing for 30 s, the organic phase containing the pregnanes was removed, evaporated and the residue was dissolved in 50 μL ethanol. This solution was qualitatively analysed using thin layer chromatography (TLC). The samples and the respective standards were spotted on TLC plates (silica gel 60, Merck; Darmstadt, Germany), and subsequently developed with dichloromethane: ethyl acetate (8 : 2). Pregnane spots were visualised with anisaldehyde reagent [16].

In addition, pregnanes were analysed by HPLC according to [3] using a 1525 Binary HPLC Pump equipped with a 2487 Dual λ Absorbance Detector (Waters; Eschborn, Germany). 20 μL were applied on a Symmetry column (C_{18}, 5 μM, 4.6 × 150 mm) and the pregnanes eluted with a step gradient of H_2O (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 nm and 240 nm and identified by their retention times by comparison with authentic pregnanes. The retention time was for progesterone 15.8 min, for pregnenolone 16.4 min and for isoprogesterone 16.8 min. For quantitative analyses an internal standard, 3-keto-Δ^4-19-*O*-acetylstrophanthidol, was added to the assay prior to dichloromethane extraction followed by HPLC analysis.

Enzyme properties

Determination of pH optimum: The effect of the buffer pH on the enzyme activity was examined with the following buffers: 20 mM NaPi (Buffer I) at pH 6.0 to 7.5, Tris-HCl (pH 7.1 to 8.9) and 20 mM glycine-NaOH (pH 8.6 to 10.5). The enzyme was dissolved in the respective buffer after passing through PD-10 columns (Amersham Biosciences Europe GmbH) for buffer exchange.

Determination of temperature optimum: Incubations were carried out at temperatures ranging from 20 to 70 °C. The protein extracts (Buffer I, pH 7.8) were pre-incubated at the respective incubation temperature for 5 min.

Kinetic constants: These were determined for all substrates in both reaction directions at pH 7.8 and at 50 °C using the standard enzyme assay containing 0.1 μmol of NAD or 0.2 μmol NAD and an NADH-regenerating system to check forward or reverse reaction, respectively.

Dehydrogenase reaction (NAD as co-substrate): Substrate concentration range 0.04 – 1.00 mM; concentrations for determination of kinetic constants for co-substrate: 0.1 – 2.0 mM NAD.

Reductase reaction (NADH as co-substrate): Substrate concentration range 0.2 – 2.5 mM; concentrations for determination of kinetic constants of co-substrate: 0.5 – 5.0 mM NADH.

Isoelectric point (pI) of pQ3bHSD: This was determined by isoelectric focusing on Criterion™ Gels IEF 3 – 9/Criterion™ Cell (BioRad; München, Germany).

Supporting information

PCR amplification of 3βHSD genes from genomic DNA and alignment of deduced amino acid sequences for 3βHSD and other related enzymes of the dehydrogenase family from different plant species are available online as Supporting Information and at http://www.biologie.uni-erlangen.de/pharmbiol/Herletal3bHSD.pdf.

Results and Discussion

Oligonucleotide primers from peptide fragments of the 3βHSD isolated from *D. lanata* leaves [4] have been deduced by Lindemann et al. [5]. The same authors amplified and sequenced a 700-nucleotide cDNA fragment for a putative 3βHSD. Based on their reports and the submitted GenBank entry for a full length 3βHSD cDNA (AJ345026, 780 nucleotides, 259 amino acids), we generated primers for PCR amplification and RT-PCR. Leaves of *Digitalis lanata* were used for genomic DNA and cDNA preparation. The genomic clone for *D. lanata* (DQ466890) was obtained using two 19 nucleotide synthetic primers (HSDdir and HSDrev). The sequenced 3βHSD gene consisted of 870 nucleotides containing a 90 nucleotides long intron (18 – 107). Genomic sequences for 3βHSD were isolated from additional six *Digitalis* species, namely *D. purpurea, D. ferruginea, D. grandiflora, D. parviflora, D. mariana, D. thapsi*, and analysed. All PCR products were found to be of a similar size; they did not differ significantly from each other (Fig. **1S**, Supporting information). All genomic 3βHSD gene sequences contained a 90 – 96 bp intron at the 5′ end of the gene. The deduced protein sequences coded by these genes are aligned in Fig. **1**. The sequence identity within the genus is very high, about 94 % on the nucleotide level and 94 – 98 % on the amino acid level.

The putative 3βHSD genes sequenced here have all been submitted to GenBank (Acc.-Nr. DQ466890, AY789449-453, AY844959-960). Aligned with genes of other plant species available in public databases, *Digitalis* putative 3βHSD genes display considerable similarities with putative alcohol dehydrogenase genes obtained from *Pisum sativum, Arabidopsis thaliana* and *Oryza sativa*. They are also very similar to isopiperitenol dehydrogenase derived from *Mentha x piperita* [17] and secoisolariciresinol dehydrogenase from *Forsythia x intermedia* [18], both genes involved in the formation of plant secondary metabolites. Deduced *Digitalis lanata* 3βHSD shares 47 % and 49 % identity with the proteins of *Mentha piperita* and *Forsythia intermedia,* respectively (Fig. **2S**, Supporting information).

Since the genomic sequences of all *Digitalis* 3βHSD genes analysed here contained an intron, RT-PCR amplification was used

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D.lan     MSSKPRLEGKVAIITGAASGIGEETARLFVEHGASVVVADVQDELGRQVVASVNSDDKIS 60
D.tha     MSSKPRLEGKVAIITGAASGIGEEAARLFVEHGASVVVADVQDELGRQVVASVNSDDKIS 60
D.par     MSSKPRLEGKVAIITGAASGIGEEAARLFVEHGASVVVADVQDELGRQVVASVNSDDKIS 60
D.gra     MSSKPRLEGKVVIITGAASGIGEEAARLFVEHGASVVVADVQDELGHQVVASVNSDDKIS 60
D.fer     MSSKPRLDGKVAIITGAASGIGEEAARLFVEHGASVVVADVQDELGRQVVASVNSDDKIS 60
D.pur     MSSKPRLDGKVAIITGAASGIGEEAARLFVEHGASVVVADVQDELGRQVVASVNSDDKIS 60
D.mar     MSSKPRLDGKVAIITGAASGIGEEAARLFVEHGASVVVADVQDELGRQVVASVNSDDKIS 60
          *******:***.************:********************:**************

D.lan     YYHCDVRDEKQVAATVRYAVEKYGRLDIMLSNAGVFGALMTNVIDLDMVDFENVLATNVR 120
D.tha     YYHCDVRDEKQVAATVRYAVEKYGRLDVMMSNAGVFGALMTNVIDLDMVDFENVLATNVR 120
D.par     YYHCDVRDEKQVAATVRYAVEKYGRLDVMMSNAGVFGALMTNVIDLDMVDFENVLATNVR 120
D.gra     YHHCDVRDEKQVAATVRYAVEKYGRLDVMMSNAGVFGALMTNVIDLDMVDFENVLATNVR 120
D.fer     YHHCDVRDEKQVEATVRYAVEKYGRLDVMVSNAGVFGALMTTVIDLDMVDFENVLATNVR 120
D.pur     YHHCDVRDEKQVEATVRYAVEKYGRLDVMVSNAGVFGALMTTVIDLDMVDFENVLATNVR 120
D.mar     YHHCDVRDEKQVEATVRYAVEKYGRLDVMVSNAGVFGALMTTVIDLDMVDFENVLATNVR 120
          *:********** ***************:*:***********.******************

D.lan     GVANTIKHAARAMVEGKVKGSIICTASVSASLGGMGPPAYTASKHAVLGLVKGACAELGV 180
D.tha     GVANTIKHAARAMVEGKVKGSIICTASVSASLGGMGPPAYTASKHAVLGLVKGACAELGV 180
D.par     GVANTIKHAARAMVEGKVKGSIICTASVSASLGGMGPPAYTASKHAVLGLVKAACAELGV 180
D.gra     GVANTIKHAARAMVEGKVKGSIICTASVSASLGGMGPPAYTASKHAVLGLVKAACAELGV 180
D.fer     GVANTIKHAARAMVEGNVKGSIICTASVSASLGGMGPPAYTASKHAVLGLVKAACAELGV 180
D.pur     GVANTIKHAARAMVEGNVKGSIICTASVSASLGGMGPPAYTASKHAVLGLVKAACAELGV 180
D.mar     GVANTIKHAARAMVEGNVKGSIICTASVSASLGGMGPPAYTASKHAVLGLVKAACAELGV 180
          ****************:************************************.*******

D.lan     HGIRVNSVAPYGVATPMPCSAYGMTPSQMEEANNSRANLKGVVLKAKHVAEAALFLASDE 240
D.tha     HGIRVNSVAPYGVATPMPCSAYGMTPSQMEEANNSRANLKGVVLKAKHVAEAALFLASDE 240
D.par     HGIRVNSVAPYGVATPMPCSAYGMTPSQMEDANNSRANLKGVVLKAKHVAEAALFLASDE 240
D.gra     HGIRVNSVAPYGVATPMPCSAYGMTPSQMEDANSSRANLKGVVLKAKHVAEAALFLASDE 240
D.fer     HGIRVNSVAPYGVATPMPCSAYGMTPSQMEDANCSRANLKGVVLKAKHVAEAALFLASDE 240
D.pur     HGIRVNSVAPYGVATPMPCSAYGMTPSQMEDANCSRANLKGVVLKAKHVAEAALFLASDE 240
D.mar     HGIRVNSVAAYGVATPMPCSAYGMTGSQMEEANNSRANLKGVVLKAKHVAEAALFLASDE 240
          *********.*************** ****:** *************************

D.lan     SAYVSGQNLAVDGGFTVVR 259
D.tha     SAYVSGQNLAVDGGFTVVR 259
D.par     SAYVSGQNLAVDGGFTVVR 259
D.gra     SAYVSGQNLAVDGGFTVVR 259
D.fer     SAYVSGQNLAVDGGFTVVR 259
D.pur     SAYVSGQNLAVDGGFTVVR 259
D.mar     SAYVSGQNLAVDGGFTVVR 259
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Fig. **1** Alignment of deduced 3βHSD amino acid sequences from different *Digitalis* species. *D. pur-Digitalis purpurea; D. fer-D. ferruginea; D. tha-D. thapsi; D. lan-D. lanata; D. par-D. parviflora; D. gra-D. grandiflora; D. mar-D. mariana*. Symbols: "*****" means that the residues in that column are identical in all sequences in the alignment, "**:**" means that conserved substitutions have been observed and "**.**" means that semi-conserved substitutions are observed.

to synthesise 3βHSD cDNA for subsequent cloning into the expression vector pQE. The 3βHSD cDNA isolated from *D. lanata* contained an open reading frame of 780 nucleotides corresponding to 259 amino acids (calculated molecular mass 26,857 Da). The primers HSDsphdir and HSDkpnrev (see Material and Methods) were used to amplify 3βHSD cDNA and integrate it into the expression vector. Finally, 3βHSD cDNA was expressed as a His-tagged fusion protein composed of 273 amino acids (calculated molecular mass 28,561 Da).

After IPTG induction the pQ3βHSD gene was expressed in *E. coli*. The protein was purified by metal chelate affinity chromatography on Ni-NTA. After purification the recombinant 3βHSD appeared as a homogenous band of 30 kDa (Fig. **2**). As a control M15[pREP4] cells were transformed with an empty pQE30 vector. The protein extracts prepared from bacteria containing only the vector did not display an induced 30 kDa protein band although they contained traces of other proteins (Fig. **2**, lanes 6 and 7).

The pQ3βHSD was most active at a pH of 8.5 (20 mM Tris-HCl) and a temperature of 55 °C. The isoelectric point (pI) of recombinant His-tagged 3βHSD, as determined by isoelectric focusing (IEF), was 6.5; the theoretical value was calculated to be 6.86 (http://www.expasy.ch/tools/pi_tool.html).

With a view to further characterise recombinant His-tagged 3βHSD, we tested several steroids as substrates. It was found that pQ3βHSD converts pregnenolone to isoprogesterone in the presence of NAD. Under standard incubation conditions pregnenolone was mainly transformed into isoprogesterone together with variable amounts of progesterone. As a result of non-enzymatic isomerisation, prolonged exposition yielded increasing amounts of progesterone even if the enzyme reaction was terminated by boiling.

In addition to pregnenolone, assumedly the biosynthetic precursor of cardenolides, several other steroids with a 3β-hydroxy group were tested as substrates for the pQ3βHSD. 5β-Pregnan-3β-ol-20-one, 5α-pregnan-3β-ol-20-one, 21-hydroxypregnenolone (C_{21}-steroids) and 5-androsten-3β-ol-17-one (a C_{17}-steroid) were all well accepted, whereas cholesterol was not. For many microorganisms, the enzyme responsible for the modification of 3β-hydroxy-5-ene to 3-keto-4-ene belongs to the oxidases ("cholesterol oxidase", ChO; EC 1.1.3.6) which accept C_{17}, C_{21} as well as C_{27} 3β-steroids. Since cholesterol was not accepted by the pQ3βHSD it is assumed to be unrelated to these oxidases.

Testosterone (4-androsten-17β-ol-3-one), a C_{17}-steroid with a 3-carbonyl group and a 17β-hydroxy group, was converted to 4-androstene-3,17-dione. This indicates that pQ3βHSD also possesses 17β-dehydrogenase activity.

Moreover, pQ3βHSD was able to catalyse the reduction of 3-ketosteroids when NADH was used as co-substrate. Pregnane-3,20-diones without a Δ^4- or Δ^5-double bond like 5β-pregnane-3,20-dione and 5α-pregnane-3,20-dione were accepted. The 5β-configurated dione yielded two products, namely 5β-pregnan-

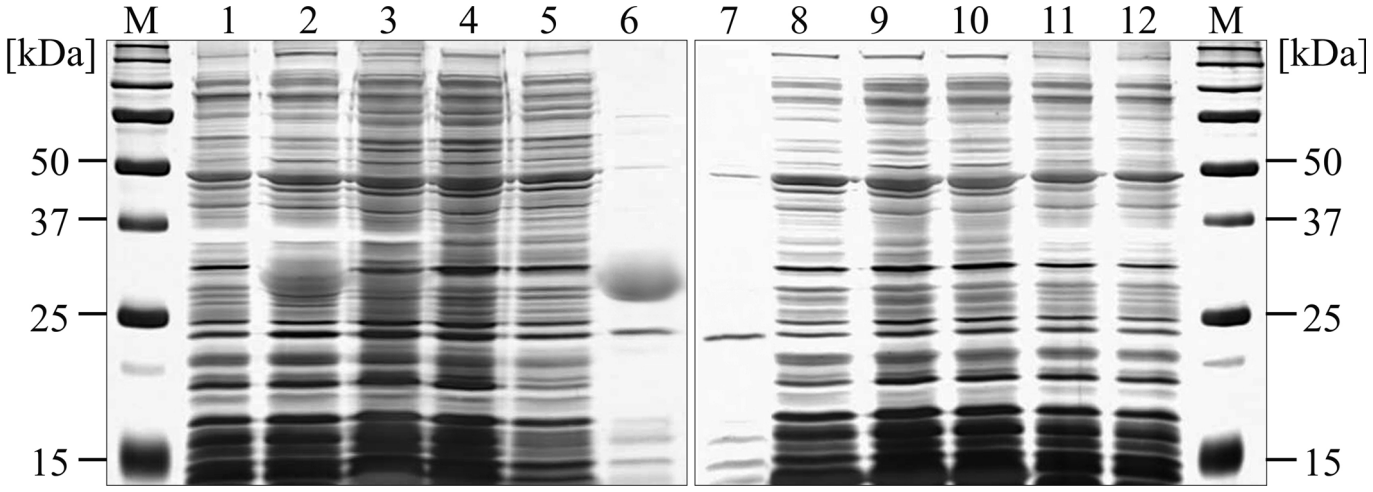

Fig. **2** SDS-PAGE analysis of the heterologously expressed pQ3βHSD. Marker proteins (lane M); lanes 1 – 6: protein from cells with pQ3βHSD, lanes 7 – 12: protein from control cells containing the empty pQE expression vector only. No IPTG added (lane 1); IPTG-induced cells cultivated for 5 h at 37 °C (lane 2); Cell lysate before Ni-NTA column (lane 3); column flow-through (lane 4); column wash fraction (lane 5); imidazole (200 mM) eluate with pQ3β-HSD band at ca. 29 kDa (lane 6); Imidazole eluate of control (lane 7); wash fraction of the Ni-NTA column (lane 8); column flow-through (lane 9); bacterial lysate (lane 10); IPTG-induced cells cultivated at 37 °C for 5 hours (lane 11); No IPTG added (lane 12).

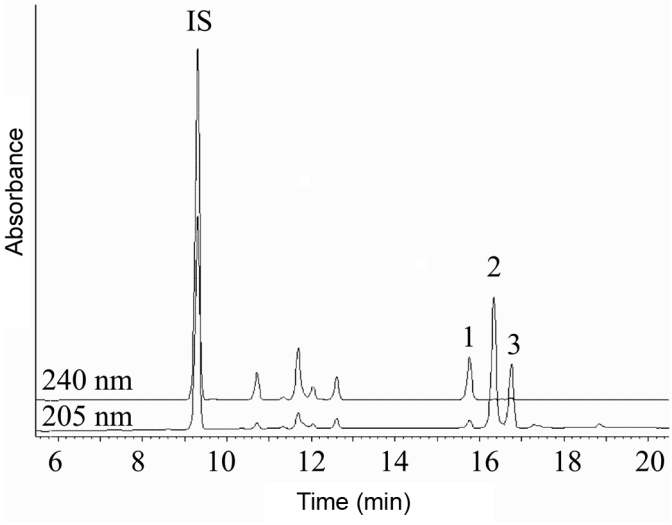

Fig. **3** pQ3βHSD activity measured by HPLC analysis: 3-keto-Δ^4-19-*O*-acetyl-strophanthidol (IS, internal standard); progesterone (**1**); pregnenolone (**2**); isoprogesterone (**3**).

3α-ol-20-one and 5β-pregnan-3β-ol-20-one whereas the 5α-configurated dione yielded only one product, namely 5α-pregnan-3β-ol-20-one. 4-Androstene-3,17-dione was also accepted as a substrate, but the 17-keto instead of the 3-keto function was reduced.

A clear preference for NAD (NADH for reduction) as co-substrate(s) was observed. NADP and NADPH were also accepted, but were about 3 to 5 times less effective.

HPLC was used to study the kinetic properties of pQ3βHSD and to quantify enzyme activity. A typical chromatogram of an experiment using pregnenolone as the substrate is shown in Fig. **3**. Kinetic constants for pQ3βHSD were determined with several substrates. The kinetic data calculated for pQ3βHSD were compared with those of the partially purified plant 3βHSD reported by Finsterbusch et al. [4] (Table **1**). The V_{max} values for substrates containing Δ^4- or Δ^5-double bonds differ considerably whereas other values appear to be very similar. Especially the values for pregnenolone must be regarded as "apparent" since part of the isoprogesterone formed is isomerised non-enzymatically to progesterone.

The hydroxysteroid dehydrogenases described so far belong to one of two protein families, namely aldo-keto reductases (AKR) or short-chain dehydrogenases/reductases (SDR) [19], [20]. Both families contain prokaryotic and eukaryotic members. The *Digitalis* 3βHSD is a soluble enzyme and it shares this property with members of the SDR family [21], [22]. In contrast, mammalian 3βHSDs are localised in the membranes of the smooth ER and mitochondrial cristae [23] (see [6] for a recent review). SDR type proteins may occur as homo-oligomers of subunits with molecular masses of about 30 kDa whereas 3βHSDs belonging to the AKR family have higher molecular masses. As already pointed out by Finsterbusch et al. [4] the *Digitalis* 3βHSDs probably belong to the SDR enzyme superfamily (group of hydroxysteroid dehydrogenases).

The pQ3βHSD described here exhibits 3α-, 3β- and 17β-dehydrogenase and 3/17-keto reductase activities. A 3β/17βHSD was also reported to occur in *Commomonas testosteroni*, whereas other HSDs display stricter substrate specificities (see [24] for more details).

The results obtained with pQ3βHSD have to be compared with data reported for putative hydroxysteroid oxidoreductases supposed to be involved in cardenolide metabolism [1], [4]. For example, the 3α-hydroxysteroid-5β-reductase described by Stuhlemmer et al. [25] did not accept 5α-steroids or Δ^4/Δ^5-unsaturated pregnenes, whereas 5β-steroids including digitoxigenone were converted to their respective 3α-derivatives. From inhibition studies with crude enzyme preparations it was concluded

Table **1** Substrates and products of pQ3βHSD. Kinetic constants determined here were compared with published data [4]

Substrate(s)	*Product(s)*	K_m *pQ3βHSD (μM)*[a]	V_{max} *pQ3βHSD (μkat/kg)*[a]	V_{max}/K_M
NAD		260 (32.0)	19.5 (n.d)	0.08
+ Pregnenolone	Isoprogesterone + Progesterone[b]	90 ± 9 (20)	182 ± 43 (770)[c]	2.02
+ 5β-Pregnan-3β-ol-20-one	5β-Pregnane-3,20-dione	314 ± 66 (110)	488 ± 111 (610)[c]	1.55
+ 5α-Pregnan-3β-ol-20-one	5α-Pregnane-3,20-dione	116 ± 23 (120)	206 ± 20 (690)[c]	1.78
+ 5-Androsten-3β-ol-17-one	5-Androstene-3,17-dione	642 (130)	539 (680)[c]	0.84
NADH		642 (n.d.)	4750 (n.d.)	7.40
+ 5β-Pregnane-3,20-dione	5β-Pregnan-3β-ol-20-one + 5β-Pregnan-3α-ol-20-one	1725 (n.d.)	9071 (n.d.)	5.26
+ 5α-Pregnane-3,20-dione	5α-Pregnan-3β-ol-20-one	106 ± 1 (n.d.)	428 ± 30 (n.d.)[c]	4.04

[a] Data of Finsterbusch et al. [4] in brackets; n.d. = not determined.
[b] Isoprogesterone was the main product (about 90%), see Fig. **3**.
[c] Means ± SD of at least two independent experiments.

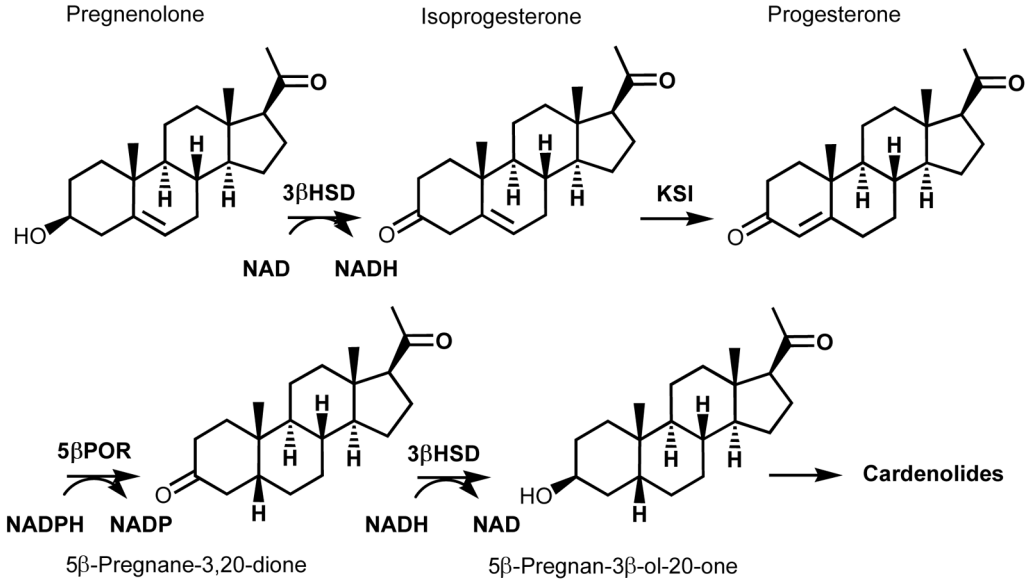

Fig. **4** Putative early steps and enzymes in cardenolide biosynthesis (3βHSD: Δ^5-3β-hydroxysteroid dehydrogenase, KSI: Δ^5-3-ketosteroid isomerase, 5βPOR: progesterone 5β-reductase.

that the formation of 3α- and 3β-hydroxy-5β-pregnanes are catalysed by two different stereospecific oxidoreductases [26]. In many aspects the r3β-HSD described here behaves like these enzymes which have never been isolated and characterized in pure form. Therefore, our results support the hypothesis of Finsterbusch et al. [4] who discussed that 3βHSD might be a multifunctional SDR type enzyme in steroid metabolism. As far as the cardenolide pathway is concerned, we presume that 3βHSD catalyses two steps in cardenolide biosynthesis, namely the dehydrogenation of pregnenolone and the reduction of 5β-pregnane-3,20-dione (Fig. **4**). The presence of 3βHSD in other species not accumulating cardenolides [3], [4] indicates that the enzyme may also be involved in other metabolic pathways. RNAi experiments may help to elucidate this issue further.

One of the crucial questions still remains, namely whether 3βHSD of *D. lanata* actually possesses KSI activity. This question has already been addressed by Finsterbusch et al. [4] but could not be answered conclusively. The authors therefore advised studies employing a recombinant form of 3βHSD. However, even when studying the recombinant enzyme, the catalytic properties of KSI and non-enzymatic isomerisation of isoprogesterone are still fundamental problems to solve this question. KSI is one of the most efficient enzymes known, with k_{cat}/K_M approaching the diffusion control limit [27]. Therefore, even trace amounts of KSI present in a 3βHSD enzyme preparation might simulate KSI side reaction. On the other hand, the non-enzymatic isomerisation of β,γ-unsaturated ketones, such as isoprogesterone or 5-androstene-3,17-dione, has been studied extensively [28]. The isomerisation itself is a multi-step reaction which might be influenced by many parameters, such as the presence of acetate or carboxylate residues and, of course, biocatalysts.

Lindemann et al. [5] already stated that 3βHSD from *D. lanata* shows sequence similarities with microbial hydroxysteroid dehydrogenases but not with animal enzymes and contains a conserved putative short chain dehydrogenase (SDR) [29] domain. Hence, it may be speculated that 3βHSD and KSI activities do not reside in one single protein in *D. lanata*. The molecular structure of the *Digitalis* 3βHSD supports the biochemical evidence since no KSI-like domains can be found in the protein sequences deduced from the published nucleotide sequences. However, although preliminary experiments indicate that isoprogesterone was isomerised only non-enzymatically and that thus progester-

one was an artefact rather than a product of pQ3βHSD catalysis, the question is still open and we intend to give experimental evidence for the assumption that KSI is not associated with HSD in the *Digitalis* 3β-HSD using the recombinant enzyme described here.

Acknowledgements

The authors thank G. Fischer for excellent technical assistance. Plants, seeds and herbal material were kindly provided by Dr. W. Welß, Botanical Garden FAU Erlangen, Prof. Dr. A. Graner, Genbank Gatersleben, Prof. G. Heubl, LMU München and Prof. V. Melzheimer, Marburg. We thank Barbara White for linguistic advice.

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