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Biocatalyzed synthesis of testosterone

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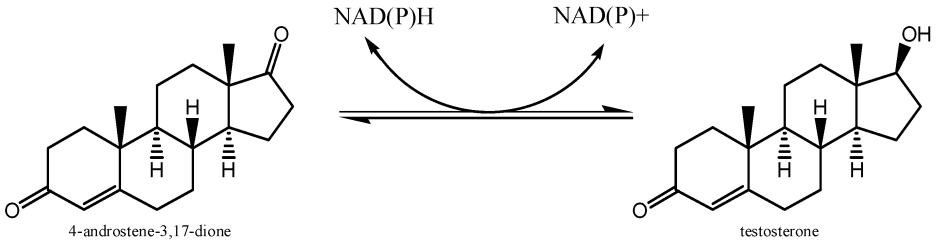
We have recently developed and submitted for a patent a new industrial process for the production of testosterone making use of biocatalysis. In particular, a non commercial enzyme was proposed to overcome a critical steps in the organic synthesis of the hormone: the regio- and stereo-specific conversion of 4-androstene-3,17-dione to testosterone.



4-androstene-3,17-dione testosterone

Design of the process was based on a strategy starting with the identification of the enzyme by crossed bioinformatics and literature screening: the chosen candidate was the 17β-hydrosteroid dehydrogenase type 5 from mouse. The coding sequence for the enzyme was cloned in a vector suitable for its expression, in fusion with an His-tag, in *E.coli*. Small scale preparations of the recombinant enzyme were initially set up and activity tests on the desired substrate were performed, using the co-factor NADPH or NADH. The small scale purifications allowed verification of the product obtained in terms of regio- and stereo-selectivity and yield, evaluation of thermal stability of the enzyme, kinetic analysis of the reaction and choice of an adequate partial organic mixture to work with.

A protocol for purification of the enzyme from medium scale cultures was then drawn and performed. The amount of enzyme obtained from these preparations, in combination with the commercially available D-glucose dehydrogenase required for the recycling of the cofactor NADH, allowed the conversion of milligrams of androstendione. Conditions for higher scale synthesis of testosterone are going to be developed.

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1. Introduction

Several steroid drugs are widely used as diuretic, contraceptive, progestational, anti-inflammatory, anabolic, anti-androgenic, and anticancer agents. Due to their chemical complexity, often these compounds are synthesized starting from natural ones, as phytosterols, that can be obtained from soybeans, conifers and rape seed. These natural raw sources are used for the production of androstenedione and androsta-1,4-dien-3,17-dione, which represent the basic substrates for the subsequent chemical and biotechnological production of steroid drugs (Schmid et al., 2001). A single step biocatalyzed transformation of androst-4-ene-3,17-dione (AD) to testosterone (TS) is a promising alternative to the chemical synthesis, performed in four steps from AD, fitting both environmental and economic requirements.
Microbiological transformation of steroids has long been used in industrial synthesis (Sedlaczek and Smith, 1988). The formation of 17β-hydroxy- from 17-oxo-steroids was reported for a wide variety of substrates and microorganisms, where putative 17β-hydroxysteroid dehydrogenases (17βHSD, E.C. number: 1.1.1.51) were responsible for the steroid transformations in endogenous metabolic pathways (Donova et al., 2005). Alternatively, 17β-hydroxy-steroids appeared as side reaction product of keto-reducing cellular enzymes. In an early report, the low water soluble AD was shown to be partially transformed to TS in the culture broth of the yeast *Saccharomyces cereviseae* (Singer et al., 1991). Zorko and collaborators studied a 17βHSD from the fungus *Cochliobolus lunatus* that catalizes the transformation of AD to TS (Zorko et al., 2000), while a different group showed that in a two solvent system, the reverse reaction can be obtained using a crude extract from *Pseudomonas testosteroni* (Cremonesi et al. 1973).
Starting from cited papers and other selected works from the literature (Rheault and Charbonneau, 1999, Deyashiki et al. 1995), we argued that the type 5 mouse 17βHSD could represent a good candidate for developing a new biocatalytic system for the conversion of AD to TS. In this work we cloned, expressed and purified this enzyme in recombinant form, setting the appropriate condition for milligram scale production of TS, using commercially available enzymes for the recycling of the cofactor NADPH. Furthermore, we tested the recombinant enzyme in different conditions to find out the best ones for its industrial application.

2. Materials and Methods

2.1 Materials

Steroids, cofactors, His-select® nickel affinity gel (IMAC) as well as salts and substrates were obtained from Sigma-Aldrich (Milano, Italy). Glucose and formate dehydrogenases were obtained from Codexis (Redwood City, U.S.A).

2.2 Cloning

Cloning procedures were designed according to Sambrook and Russell (2001). The cDNA coding for mouse type V 17βHSD was cloned in the pET28a plasmid (Novagen®) in two forms, in order to obtain two recombinant enzymes, i.e. with and without a N-terminal histidine tag (His-tag). The cDNA fragment containing the entire coding region (Akr1c6, GeneID: 83702) was purchased from a commercial mouse cDNA library (ImaGenes GmbH, Berlin, Germany) cloned into the multi-cloning site of pCMVsport 6 plasmid. This was used as template in two separate PCR (polimerase chain amplification) reactions with primers (*i*) 5'-TCTTCTCAGTTGGTGGGCTG-3' (forward) and T7 promoter (reverse), and (*ii*) 5'-GCTGAGAACATATGGATTCTAAG

-3' (forward, introducing a NdeI restriction site) and T7 promoter. Obtained bands were cut with NcoI plus NotI and NdeI plus NotI respectively, purified from agarose gel and ligated into the pET28a plasmid cut by the required couple of enzymes. After transformation and following screening, inserts of recombinant plasmids were completely sequenced (BMR Genomics, Padua, Italy) to verify absence of undesired mutations eventually introduced by Taq polimerase. The N-terminus of the translated his-tagged 17βHSD resulted the following: MetGlySerSerHisHisHisHisHisHis-SerSerGlyLeuValProArgGlySerHis, with an aminoacid spacer containing the recognition site for thrombin (underlined) preceding the starting Met of the enzyme.

2.3 Expression and purification of the recombinant mouse type V 17βHSDs

Chemically competent BL21 *E.coli* cells were prepared and transformed using the obtained recombinant pET28a plasmids containing sequences for native and His-tagged mouse type V 17βHSDs. Transformed cells were grown in Erlenmeyer flasks, shaking at 200 rpm, in LB medium at 37°C. Protein expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG). Inducing condition were optimised for highest yield, setting cell density, IPTG concentration, temperature of induction and time of harvest after induction. Cells were collected by centrifugation at 6000 g for 10 minutes at 4°C. Pellets were suspended in 20mM phosphate, 500 mM NaCl, 5 mM imidazole buffer, pH 7.4, and cells were broken by sonication. Samples were centrifuged at 12000 g for 10 minutes at 4°C and soluble fractions were analysed for protein contents and enzymes activity. The His-tagged protein was further purified by IMAC chromatography; column was washed with the sonication buffer till absorbance at 280 nm was significantly reduced. Bound protein was eluted by increasing imidazole content (up to 500 mM) in the buffer. Elution was constantly monitored by reading absorbance at 280 nm and harvested in fractions. Highest concentrated fractions were collected and stored in aliquots at -20°C in 18% glycerol.

2.4 Enzyme assay and synthesis of TS from AD

Reactions were performed in cuvette (1 cm optical length), by adding the purified enzymes to a freshly prepared saturated solution of AD (100 µl final volume) at 25°C in 100 mM phosphate buffer, pH 7.2, 0.2 mM NADPH (or NADH). Reactions were followed by measuring absorbance at 340 nm, wavelength useful to monitor consumption of the reduced form of the cofactor (ε_{340} 6220 $M^{-1}cm^{-1}$), using a Agilent 8453 UV-visible spectrophotometer. Rate of NADPH (or NADH) oxidation after enzyme addition was used to estimate activity of the enzyme (1 enzymatic unit corresponding to the reduction of 1 µMole of cofactor in 1 minute). For milligram scale conversion of AD, the reagent mixture (7 mL) was reacted for different periods at pH 6.5, under constant stirring and temperature. 0.15 units of the purified His-tagged recombinant 17βHSD were used in 50 mM phosphate buffer containing 7% (v/v) organic solvent, 8.57% (w/v) glucose, 1% (w/v) AD, 0.043% (w/v) NAD or NADP, 68 units of glucose dehydrogenase. TS produced in different conditions was evaluated by HPLC analysis after methylene chloride extraction, followed by filtration and evaporation.

2.5 Other Analytical methods

SDS-page electrophoresis in 12% polyacrylamimide slab gels was performed according to J. Sambrook 2001. HPLC analysis was carried out using C-18 reverse phase column (Zorbax Eclipse XDB-C18, HPLC 1100 series, Agilent).

3. Results and Discussion

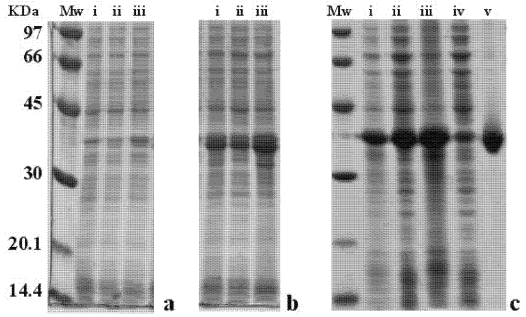

Figure 1. SDS-PAGE analysis of protein contents of E.coli cells transformed with an empty PET28a plasmid (a) or with recombinant plasmids for expression of native (b) and His-tagged (c) forms of type V mouse 17βHSD: total proteins extract (i), soluble and (ii), insoluble fraction (iii), flow through (iv) and eluted fraction (v).

The type V 17βHSD enzyme from mouse was cloned in *E. coli* cells using the pET28a plasmid system to obtain an inducible expression of the enzyme in its native or His-tagged form. The tag position at the N-terminus of the protein was chosen after considering the high homology (75% identity) with of human type V 17βHSD. Observation of the known structure of the latter enzyme (Qiu et al. 2004) suggested that a putative tail at the C-terminus could have modified substrates accessibility to the active site. After cloning and induction of transformed BL21 cells, the enzyme was expressed at high level in all condition tested, both in its native (37KDa) and in the His-tagged (HT, 39,2 KDa) form. Recombinant products represented the most abundant proteins in the cells and their expression levels increased in a time-depending manner after induction. In order to test the activity of expressed enzymes before purification, 100 ml of cell culture were induced by 0.1mM IPTG at 37°C. After 2h, cells were harvested and a cell extract was obtained (Fig. 1). This was tested for conversion of AD to TS in the assay performed as described in Materials and Methods. NADPH consumption in the reaction was compared with that of a control reaction, in which added extract derived from cells transformed with an empty pET28a plasmid (Fig 2a).

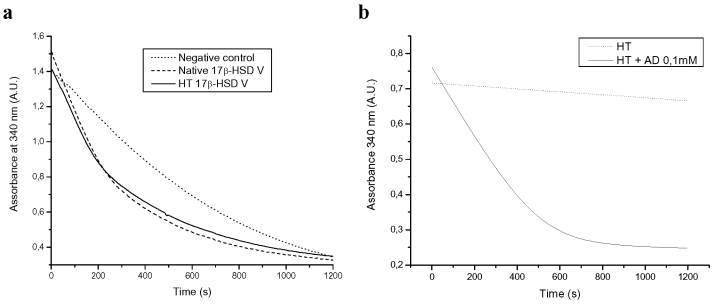

Figure 2. Enzyme activity tests performed as described. (a) NADPH consumption by 20 µl of total soluble fraction obtained from induced E. coli cells transformed by empty or recombinant plasmids. (b) NADPH consumption by 5 µl of purified His-tagged (HT) 17βHSD.

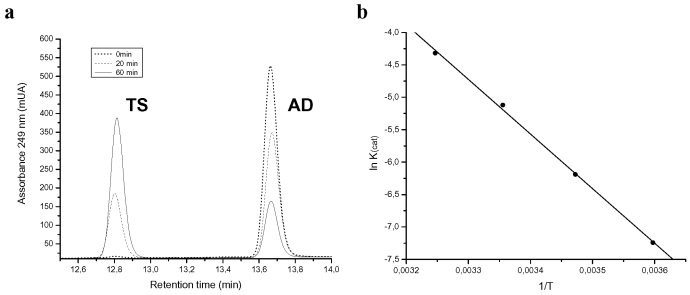

Figure 3. Characterization of the purified His-tagged form of type V mouse 17βHSD: (a) enzymatic conversion of AD (initial solution ~0,1 mM, NADPH 10 mM) to TS, time-course HPLC analysis; (b) temperature depending Arrhenius plot of enzymatic activity.

We measured faster consumption of cofactor in the presence of expressed recombinants enzymes than in their absence, indicating that enzymes were expressed in active forms. Moreover, no significant difference was observed in the rate of NADPH consumption between native recombinant form and His-tagged form of mouse type V 17β-HSD. In the reaction set up as negative control the oxidation of NADPH was ascribed to the presence of different, endogenous dehydrogenases in *E. coli* cell extracts.
Further purification of the His-tagged (HT) 17β-HSD by IMAC chromatography, allowed easy removal of the spurious dehydrogenases activities and brought, in a single step, to a satisfactory level of protein purification, as evinced by SDS-page analysis (Fig. 1). Different preparations of the enzymes were carried out, starting from bacterial cultures in the scale of 1 to 6 liters: average yield of purified enzyme was estimated in 75 mg/liter of culture (Bradford quantification) and enzyme activity was typically higher than 6 mU/mg (Fig. 2b).
Furthermore, the regio- and stereo- selectivity of the enzyme was evaluated by time course HPLC analysis the conversion of from AD to TS; no other side-product was detected, testifying high selectivity in the reduction of the 17 keto group of AD (Fig. 3a). A series of experiments was then performed to check the activity of the purified enzyme in different working conditions, in order to set best temperature, cofactor and solution composition for the scaling up of the reaction. These experiments clearly enlightened the biocathalytic efficacy of the recombinant enzyme. Rate of catalysis resulted strongly dependent from temperature (Fig.3b) and activity slightly reduced by addition of small organic fraction. The enzyme accepted both NADPH or NADH as cofactor and could catalyse the reverse reaction.

Table 1. Conversions of AD to TS obtained using different co-solvent andcofactor with the purified enzyme.

Cofactor	Co-solvent	Temp. (°C)	Time (h)	Conversion %
NADP+[NAD+]	-	25	90 [70]	49 [85]
NADP+	acetonitrile	30	44	95
NADP+	DMF	30	44	52
NADP+	DMSO	25	41	90
NADP+ [NAD+]	methanol	25	41 [70]	98 [88]
NADP+ [NAD+]	ethanol	25	41 [70]	97 [61]
NADP+ [NAD+]	isopropanol	25	41 [70]	97 [98]
NADP+ [NAD+]	*ter*-butanol	25	41 [70]	92 [98]

Following these preliminary tests a series of synthesis of TS from AD in the milligram scale was performed, by using two phase systems, due to the low solubility of steroids in water. Table 1 summarizes different conditions tested and respective values of conversion obtained. Comparable results were obtained independently from system for cofactor recycling used, either formate or glucose dehydrogenase.
The chemical synthesis of pharmaceutical drugs often needs several steps, especially when regio- and stereo-specific modifications of molecules are required, and implies the utilization of hazardous and hardly disposable compounds. This results in increase of costs, decrease of yields and burden of pollutants with high environmental impact. Biocathalysis certainly helps in overcoming such limitations of industrial chemical synthesis, but its application is currently restricted by the limited number of commercially available useful enzymes. We have used recombinant DNA technology to produce a new enzyme that could be used for the transformation of AD to TS in a single step. Moreover, we have demonstrated its adaptability, selectivity and industrial applicability. The newly designed process, patent pending (Fogal et al. 2009), is going to be developed.

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