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Secretory expression, purification and functional characterization of 17β-hydroxysteroid dehydrogenase type 1 from mammalian HEK293T cells

Jiong Chen [a, *, 1], Wei Feng [a, 1], Yue Zhao [b]

[a] Department of Forensic Biology, Henan University of Science and Technology, Luoyang 471023, China
[b] CITIC Heavy Industries Co., Ltd, Luoyang 471003, China

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ABSTRACT

17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) mainly catalyzes the reduction of estrone into estradiol. The enzymatic conversion is a critical step in estradiol accumulation in breast tissue, which is a valuable prognosis index of breast cancer disease. However, the source of 17β-HSD1 for inhibitor design is limited. In this study, the fragment encoding human 17β-HSD1 was successfully cloned and expressed in human embryonic kidney (HEK) 293T mammalian cells. The recombinant protein was purified by immobilized metal ion affinity chromatography yielding above 17 mg of purified 17β-HSD1 protein per liter of cell culture, with a specific activity of 8.54 μmoL/min/mg of protein for conversion of estradiol into estrone, with NAD^+ as cofactor at pH 9.2. Enzyme characterization studies revealed that the protein has estrogenic activity and the K_m value for estrone is about 20 nM. The recombinant protein purified from transfected HEK293T cells had higher specific activity compared to that of the enzyme purified directly from placenta. The present data show that the mammalian cell expression system can provide active 17β-HSD1 which is functionally identical to its natural counterpart and easy to purify in qualities suitable for its structure-function study.

1. Introduction

Breast cancer is the most common cancer among women, carrying a lifetime risk of 10% in western populations [1]. Most breast cancers are estrogen-dependent, and it has been shown that the most potent estrogen, estradiol (E_2), is a well-known factor responsible for the stimulation and proliferation of breast cancer [2,3]. The binding of E_2 with estrogen receptor forms a complex which interacts with specific sites on the genome and affects transcription of neighboring genes [4]. Therefore, the inhibition of E_2 synthesis is a valuable approach for treating estrogen-dependent breast cancer. Two principal pathways are involved in E_2 synthesis in breast cancer, namely the 'aromatase pathway' which transforms androgens into estrogens and the 'sulfatase pathway' which converts estrone sulfate (E_1S) into estrone (E_1). The last step of E_2 formation is catalyzed by 17β-hydroxysteroid dehydrogenases (17β-HSD), and 14 members of 17β-HSD have been identified until now [5]. Most 17β-HSDs belong to the short-chain dehydrogenase/reductase (SDR) family, with the exception of type 5, which is an aldo–keto reductase (AKR) [6–10]. Despite their name that implies that they catalyze redox reactions at the C_{17} position of the steroidal nucleus, 17β-HSD members demonstrate differences in nucleotide cofactor and substrate specificities, subcellular compartmentalization and tissue specific expression patterns [11–13]. The most estrogenic 17β-HSD is 17β-HSD type 1 (17β-HSD1), which plays an important role in the interconversion of active and inactive forms of estrogens by the NAD(P) (H)- linked oxidoreductive transfer of a hydride to and from the 17-position of steroid molecules. In this manner, 17β-HSD1 can catalyze E_1 activation and E_2 inactivation and hence regulate the occupancy of estrogen receptors, and thus the proliferative effects of estrogens [14–16].

17β-HSD1 consists of 327 amino acids with a subunit mass of about 35 kDa [17]. It is a homodimer that is highly expressed in placenta, ovaries and breast cancer tissue [18–20]. Significantly greater 17β-HSD1 activity was detected in breast cancer tissue of postmenopausal than premenopausal patients, as are the E_2/E_1

* Corresponding author. Department of Forensic Biology, Henan University of Science and Technology, Kaiyuan Street 263, Luoyang 471023, China.
E-mail address: cj11091@haust.edu.cn (J. Chen).
[1] These authors contributed equally to this work.

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ratio [21]. 17β-HSD1 overexpression has been reported for the more aggressive hormone-dependent breast cancers that show poor prognosis and short recurrence times [22]. Therefore, this enzyme plays a significant role in the regulation of estrogen exposure and estrogen-dependent growth of breast cancer tissue. 17β-HSD1 inhibitors could be a new approach for the treatment of estrogen receptor positive breast cancer.

The structure–function relationship of human 17β-HSD1 has been investigated by purifying this enzyme from human placenta, or Spodoptera frugiperda (Sf9) insect cells, or *Escherichia coli* (*E. coli*) cell expression systems [23–25]. However, these methods are expensive and time-consuming. Bacterial expression systems offer considerable advantages over eukaryotic systems in terms of the ease of scale-up, but the specific activity was low maybe for the reason of posttranslational modification. In the present study, we established an efficient method to produce and characterize recombinant 17β-HSD1 from HEK293T mammalian cells for the study of its biological action and mechanistic roles. We found that optimizing the expression time and growth conditions resulted in good yields of the recombinant protein that could be purified to homogeneity in native, functional form. The mammalian cell expression system can provide 17β-HSD1 with high activity in large quantities for crystallography study and also facilitate the future production of mutant forms which will be used to understand how the enzyme is able to exert its activity at diametrically opposite positions on the steroid substrates.

2. Materials and methods

2.1. Materials

HEK293T cells were from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM high glucose), fetal bovine serum (FBS), non-essential amino-acids and L-glutamine were products of GIBCO (Rockville, USA). Cell culture flasks and polystyrene roller bottles (2 L, 2125 cm^2) were purchased from Greiner Bio-One (Germany). Protease inhibitor cocktail tablet was from Roche (Germany). Linear polyethylenimine (PEI, 25K) was from Polysciences (USA). *E. coli* host strain DH5α were purchased from Beyotime (Guangzhou, China). DNA Gel Extraction kit and Plasmid Mini-prep kit were from TIANGEN (Beijing, China). T4 DNA ligase and restriction enzymes were products of Takara (Dalian, China). The tryptone and yeast extract were from Oxoid (Basingstoke, UK). The Ni-NTA resin was product of Qiagen (Germany). BCA Protein Assay kit was purchased from Pierce (Rockford, USA). Amicon Ultra centrifugal filter was purchased from EMD Millipore (Darmstadt, Germany). Other chemicals, except where indicated, were purchased from Sigma-Aldrich (Saint Louis, USA).

2.2. Construction of recombinant plasmid

The plasmid containing the coding region of human 17β-HSD1 cDNA was purchased from GeneCopoeia Inc. (Guangzhou, China). It was constructed in a pDONR™ vector. The pHLsec plasmid was a gift from Dr. Radu Aricescu, Cancer Research UK Receptor Structure, University of Oxford, England. Polymerase chain reaction (PCR) primers were designed to amplify the fragment encoding the human full-length 17β-HSD1 (984bp). The sequences of the primers were as follows: forward, 5'-cggaattcatggcccgcaccgt (underlined, EcoR I site), reverse, 5'-ccgctcgagtcactgcggggcggccggaggat (underlined, Xho I site). After amplification with the recombinant pDONR™ vector containing 17β-HSD1 full-length cDNA as a template, the PCR product of 17β-HSD1 fragment was digested with EcoR I and Xho I, and cloned into the pHLsec vector predigested with EcoR I and Xho I to construct pHLsec-17βHSD1. The construct was expected to express a fusion protein containing a C-terminal His tag. All constructs were confirmed by sequencing. Plasmid DNA was propagated in DH5α *E. coli* cells and purified using TIANGEN EndoFree Maxi Plasmid Kit according to the manufacturer's protocol.

2.3. Cell culture

We used adherent HEK293T cells for recombinant protein expression. This cell line was maintained in DMEM high glucose supplemented with 4 mM L-glutamine, non-essential amino-acids and 10% FBS. Cells are normally grown in 75 cm^2 cell culture flasks in a humidified 310 K incubator with 5% CO_2. Cells were inoculated at a density of 4×10^6 cells per flask and passaged twice a week. Large-scale cultures for protein production are performed in expanded-surface polystyrene roller bottles (2125 cm^2). These roller bottles do not have a gas-permeable cap; therefore, it is important to flow CO_2 gas into each bottle for 20–30 s before tightening the cap.

2.4. Transient expression of recombinant human full-length 17β-HSD1 in mammalian cells

HEK293T cells were maintained at 37 °C with 5% CO_2 in DMEM supplemented with 10% FBS, non-essential amino-acids and 4 mM L-glutamine. One day before transfection, HEK293T cells were seeded at a density of 4×10^5 cells per well in a 6-well plate. Transient expression was performed with plasmids (pHLsec and pHLsec-17βHSD1) by VigoFect reagent (Vigorous, Beijing, China) according to the manufacturer's instructions. The secretion of 17β-HSD1 in the supernatants was analysed at different times post-transfection by in vitro enzyme activity assay. Before the assay, 2 ml supertant in each well was collected and concentrated to about 120 μl with Amicon Ultra 0.5 ml centrifugal filters MWCO 10 kDa (Millipore, USA) to enrich 17β-HSD1 for enzyme activity assay. Large-scale cultures for protein production were performed in DMEM medium supplemented with 2% FBS, non-essential amino-acids and 4 mM L-glutamine in 2 L expanded-surface polystyrene roller bottles (250 ml culture volume) at 40 rpm on a cellnest roller in a humidified 37 °C and 5% CO_2 atmosphere. One day before transfection, HEK293T cells were seeded at a density of 1.5×10^6 cells/ml in four roller bottles with 250 ml media each. Cells were transfected with pHLsec-17βHSD1 plasmid and PEI. DNA-PEI complexes were prepared as previously reported [26]. Briefly, 0.6 mg plasmid was mixed with 0.9 ml PEI stock (1 mg/ml) PEI at a ratio of 1:1.5 (w: w). Plasmid DNA and PEI were diluted in separate aliquots of serum-free media and incubated for 5 min at room temperature (RT). Both aliquots were mixed together, incubated for 10 min at RT and then added to the cells. After 3 days, the cell density reached to about 6×10^8 cells per bottle with viability above 80%. Then the culture supernatant was harvested by centrifuging at 6000 rpm for 20 min at 4 °C and prepared for purification.

2.5. Purification of recombinant human 17β-HSD1

The 1 L supernatant was centrifuged at 1000 rpm and filtered with 0.22 mm filters. For the enrichement of target protein, the supertant was concentrated by Amicon Stirred Ultrafiltration Cell Model 8400 with 10KDa MWCO membrane (Millipore, USA) and added to 60 ml of buffer A (50 mM Tris-Cl, 300 mM NaCl, 5 mM imidazole, pH 8.0). 17β-HSD1 protein was then captured on an Ni-NTA affinity resin by passing the concentrated supernatant over two 12.5 ml resin packed in 26 mm wide column at a flow rate of 1 ml/min. The Ni-NTA column was pre-equilibrated with 5 column