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Scientific paper

Expression and Purification of Active Human 17β-Hydroxysteroid Dehydrogenase Type 1 from *Escherichia coli*

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Abstract

Breast cancer cell growth is often dependent on the presence of steroidal hormones. The 17β-hydroxysteroid dehydrogenase type 1 isoform (17βHSD1) catalyzes NADPH-dependent conversion of estrone to estradiol, a more potent estrogen, and represents potential drug target for breast cancer treatment. To provide active enzyme for inhibitor screening, 17βHSD1 is usually expressed in insect or mammalian cells, or isolated from human placenta. In the present study we describe a simple protocol for expression and purification of active human 17βHSD1 from *Escherichia coli* BL21(DE3) cells. Soluble human 17βHSD1 was expressed using a pET28a(+)-based plasmid, which encodes a hexahistidine tag fused to the N-terminus of the protein, and purified by nickel affinity chromatography. The enzyme activity of purified 17βHSD1 was verified by three methods: thin-layer chromatography, an alkali assay and a spectroscopic assay. These non-radioactive enzyme assays require only standard laboratory equipment, and can be used for screening compounds that modulate 17βHSD1 activity.

Keywords: Steroid hormone; 17β-Hydroxysteroid dehydrogenase; 17βHSD1; breast cancer; alkali assay; TLC

1. Introduction

Endogenous steroid hormones are derived from a cholesterol precursor by a series of successive, coordinated enzymatic reactions in a biosynthetic pathway known as steroidogenesis.[1] Steroidogenic enzymes can be classified into two groups: cytochrome P450 (CYP450) enzymes and steroid dehydrogenases.[2] 17β-Hydroxysteroid dehydrogenases (17HSDs) catalyze the last step in the biosynthesis of the active forms of androgen and estrogen hormones[2], by stereospecific hydrogenation at the C17β position, while in the opposing direction oxidation results in inactivation of these C18 and C19 steroids.[3] In mammalian cells, 17HSDs regulate ligand availability for steroid receptors, acting as a molecular "switch" responsible for catalyzing conversion between the active and inactive forms of steroid hormones.[4] These multifunctional enzymes are characterized by different cofactors (NAD(P)H), substrates (e.g. steroid hormones, bile acids, fatty acids) and tissue expression specificities.[5]

The 17β-Hydroxysteroid dehydrogenase type 1 isoform (17βHSD1) catalyzes: 1) NAD(P)H-dependent reduction of estrone to estradiol, a more potent estrogen, predominantly in the breast, ovaries and placenta[4,5]; 2) conversion of dehydroepiandrosterone (DHEA) to androstenediol; and 3) inactivation of dihydrotestosterone.[6] Mutations and changes in intracellular NAD(P)(H) cofactor abundance and redox state affect the equilibrium ratio

of E2 to E1 catalyzed by 17βHSD1.[7] Imbalances in steroid biosynthesis and signaling are often associated with the development of endocrine disorders, as well as hormone-sensitive cancers.[8] Due to urbanization, changes in lifestyle and increased exposure to endocrine disruptors, the incidence of hormone-dependent malignancies, such as breast and prostate cancer is rising and continues to represent a global health problem.[9] Compared to normal breast tissue, expression of 17βHSD1 is higher in tumor cells[6] and is associated with poor prognosis.[10] Thus, inhibition of this steroid-converting enzyme and reduction of estrogen levels could potentially be a promising treatment strategy. The pentose-phosphate pathway is active in metastatic breast cancers, potentially leading to increased E2 synthesis through the action of 17βHSD1. Therefore, exploring inhibition of interconnected signaling pathways, such as glucose metabolism, could also reduce estrogen levels and become approach for treating estrogen-dependent diseases.[7] Possibly due to its roles in the regulation of intracellular estradiol levels and involvement in breast carcinogenesis and other estrogen-dependent diseases, 17βHSD1 was the first human steroidogenic enzyme to be studied by protein X-ray crystallography. Ghosh et al. reported the X-ray structure of human 17βHSD1 (PDB ID: 1BHS), enabling rational structure-based design of selective 17βHSD1 inhibitors.[11,12] Inhibitors of 17βHSD1 based on steroidal (especially estrone derivatives) [13], nonsteroidal and flavonoid scaffolds have been identified, but have not yet been clinically approved. [10]

Our group is interested in the synthesis and characterization of steroidal inhibitors of steroidogenic enzymes, as well as steroidal ligands of hormone-receptors for use against hormone-dependent cancers or other disorders. As part of this effort, here we report a simple protocol to obtain active human 17βHSD1 for screening steroidal derivatives as potential inhibitors. Previous studies have relied on heterologous expression of 17βHSD1 in insect or mammalian cells[13,14]; or purification of endogenous 17βHSD1 enzyme from the cytosolic fraction of human placenta.[15,16] However, human placenta is difficult to obtain while insect and mammalian expression systems require special equipment and are considerably more challenging and expensive than bacterial expression systems. To date, only one previous study reported the expression and purification of human 17βHSD1 from *E. coli*. [17] In that study, the coding sequence for 17βHSD1 was cloned into a pET28a(+) plasmid and expression in *E. coli* was induced during low temperature (13°C) incubation by addition of 0.25 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG). Unfortunately, the majority of the expressed 17βHSD1 protein was located in the insoluble fraction, and attempts to refold the protein from inclusion bodies yielded inactive, misfolded enzyme.[17] However, recombinant 17βHSD1 isolated from the soluble fraction was demonstrated to be folded by circular dichroism spectroscopy and was shown to be able to catalyze oxidation of estradiol with a pH optimum of 9.3.[17] Mass-spectrometric and radiometric detection are generally used for measuring the enzyme activity of 17βHSDs, with the first method being recommended as more sensitive and accurate.[18] However, this powerful analytical method requires specialized equipment and radioassays are based on the use of tritium-labeled substrates, which limits their application.

In the present study, we optimize the methods originally reported in Chang et al. to obtain soluble 17βHSD1 from this pET28a(+)-17βHSD1 plasmid using induction at 23°C and purification by nickel affinity chromatography. Reduction of estrone to estradiol by recombinant human 17βHSD1 is shown using thin-layer chromatography (TLC). The activity of 17βHSD1 was also measured using an alkali and fluorometric assay, which represent alternative, non-radioactive approaches to measure 17HSD activity that are inexpensive and require only standard laboratory equipment. The methods described in the present study should facilitate production of active human 17βHSD1 from *E. coli* and provide a set of simple *in vitro* screening tools for identification of novel 17βHSD1 inhibitors.

2. Experimental

2. 1. Materials

The plasmid vector pET28a(+)-17bHSD1 was a generous gift from Dr. Chi-Ching Hwang, Department of Biochemistry, Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Taiwan.[17] The host *Escherichia coli* strain BL21(DE3) was obtained from Novagen (Merck KGaA, Germany). All chemicals were of analytical grade and were used without further purification. Components for the preparation of LB media were obtained from Torlak Institute (Belgrade, Serbia). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was obtained from Fisher Scientific (ThermoFisher Scientific). Lysozyme, tris-hydrochloride and mono- and dibasic potassium phosphate were purchased from Sigma-Aldrich. Kanamycin sulfate, imidazole and NADPH were purchased from Carl Roth. All organic solvents used were from Lach-Ner. HisTrap HP columns were obtained from Cytiva and TLC plates (Silica gel 60 F_{254}) from Merck.

2. 2. Expression and Purification of 17βHSD1

A pET28a(+) plasmid containing the coding sequence for human 17b-HSD1 (pET28a-17bHSD1) was used to transform chemically competent *Escherichia coli* BL21(DE3) cells using a calcium chloride heat shock method.[19] Plasmid DNA (pET28a(+)-17bHSD1) was purified according to the manufacturer's instructions using a QIAprep Spin Miniprep Kit (QIAGEN), yielding approximately 80 ng/μl of plasmid. Purified plasmid was verified by restriction digestion using *Eco*RI (40,000 U/μl) according to the manufacturer's instructions (Sigma)

and 1% agarose gel electrophoresis. Chemically competent *Escherichia coli* BL21(DE3) cells that had been stored at –80 °C were thawed on wet ice in 1.5 mL microcentrifuge tubes, and 2 µl of plasmid DNA was gently mixed with 90 µl of thawed cells and incubated on ice for 40 min. Cells were then transferred into a 42 °C water bath (heat-shock) for 45 seconds, followed by recovery on ice for 2 minutes. Afterwards, 900 µl of LB media was added to the transformation mixture, and cells were gently mixed for 1 hour at 37 °C. Cells were then spread onto LB agar plates containing kanamycin (50 µg/mL) and incubated overnight at 37 °C. The following day, a single colony was picked, inoculated into 10 mL of fresh LB media containing kanamycin (50 µg/mL) and incubated at 37 °C overnight in a Biosan orbital shaker-incubator ES-20/60. An aliquot of bacterial cells was then transferred into 1 L of fresh media (1:100) and grown at 37 °C to an optical density at 600 nm (OD_{600}) of approximately 0.4. Protein expression was then induced by addition of IPTG at a final concentration of 0.25 mM and incubation was continued at room temperature (23 °C) for 18 hours. Cells were collected by centrifugation at 5000 × g for 10 minutes at room temperature and the supernatant was discarded. The resulting cell pellet was resuspended in 20 mL of 20 mM TrisHCl pH 7.95, 0.3 M NaCl, 5 mM imidazole. Lysozyme was added at a final concentration of 1 mg/mL and the cell suspension was frozen in an ethanol-dry ice bath and stored at –80 °C. Cell lysis was done using a combination of lysozyme treatment, three freeze-thaw cycles and sonication using a Soniprep 150 sonicator set at 50% amplitude and 30 seconds pulse settings. A single freeze-thaw cycle consists of freezing cells in an ethanol-dry ice bath followed by thawing in a 37 °C water bath. Cells were then sonicated at a frequency of 14 kHz. Seven sonication steps of 30 seconds each were performed, coupled with a recovery interval on ice of 30 seconds between each sonication step. Following sonication, lysed cells were clarified by centrifugation at 12000 × g for 45 minutes at 4 °C, to separate the soluble and insoluble fractions. His-tagged 17βHSD1 was then purified from the soluble fraction by nickel-affinity chromatography using a 1 mL HisTrap HP column (Cytiva) connected to peristaltic pump Pharmacia LKB P-1 following the manufacturer's instructions with slight modifications. A 1 mL HisTrap HP column was equilibrated with 10 column volumes (CV) of binding buffer (20 mM TrisHCl pH 7.95, 0.5 M NaCl) and the soluble fraction was applied. The column was then washed with 10 CVs of binding buffer, and then with 5 CVs of binding buffer plus 20 mM imidazole. 17βHSD1 protein was eluted with 5 CVs of elution buffer (20 mM TrisHCl pH 7.95, 0.5 M NaCl, 400 mM imidazole pH 8.3). Eluted protein was further desalted and excess of residual imidazole was removed by size-exclusion chromatography column packed with Bio-Gel P-10 (Bio-Rad, exclusion limit 20 000 daltons). Protein containing fractions were pooled and buffer was exchanged into 20 mM TrisHCl pH 7.95, 0.1 M NaCl. Samples of fractions from all expression and purification steps were collected and analyzed by SDS-PAGE using a 12% (w/v) polyacrylamide gel. Concentration of total protein was measured by the Bradford method.[20]

2. 3. Enzyme Assay Coupled with TLC Detection

Enzyme activity assays for 17βHSD1 were carried out immediately after purification, because we observed a substantial loss of enzyme activity after storage of the enzyme at –80 °C. Steroid substrates were freshly dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM. Estrone at a final concentration 178 µM was incubated at 37 °C for 90 min in a 560 µl assay mixture containing 50 µl of purified 17βHSD1 enzyme (70 µg), NADPH (final concentration 178 µM) and 100 mM potassium phosphate buffer pH 6.0. In control reactions, 17βHSD1 enzyme was omitted. The reaction was stopped by adding 500 µl of methylene chloride and steroid reaction products were extracted by vortexing for 5 minutes. Phase separation was performed by centrifugation at low speed. The lower organic layer was carefully aspirated and evaporated to dryness at room temperature. Dry residue was then dissolved in 50 µl of methylene chloride and spotted on a 60 F_{254} TLC Silica gel plate. TLC plates were developed using a toluene and ethyl acetate (2:1) solvent system as a mobile phase. After drying, spots were sprayed with 50% H_2SO_4 and heated at ~ 120 °C before images of the plate were captured in Biometra gel imaging system BDAdigital. Concentration of estradiol formed in the reaction was obtained from standard curve after densitometric analysis and specific enzyme activity was expressed as nmol E2 formed min^{-1} mg^{-1}.

2. 4. Alkali Assay

A modified alkali assay that was previously reported for testing the activity of human aromatase[21] was used to evaluate 17βHSD1 activity. This assay is based on the formation of a fluorescence product between a strong alkali and $NADP^+$ produced during NADPH-dependent reduction of substrate. Initially, a reaction mixture containing 70 µg of protein, 40 µM estrone and 200 µM NADPH in up to 500 µl 100 mM potassium phosphate buffer pH 6.0 was incubated at 37 °C for 90 minutes. Upon completion of the reaction, 100 µl of product mixture was transferred to a 96-well microplate (Greiner Bio-One MICROLON) and mixed with 80 µl of 0.3 M HCl at room temperature for 15 minutes in order to eliminate excess unoxidized NADPH. In the next step, 80 µl of this mixture was transferred into a new well and 270 µl of 10 M NaOH was added. Fluorescence intensity was measured at the beginning of the reaction (0 min) using a Fluoroskan Ascent FL with excitation and emission wavelengths of 340 and 460 nm, respectively. To prevent degradation of the resulting fluorescent alkali product, incubation was carried out in the dark at 30 °C for 2 hours. Finally, 10

mM imidazole was added and fluorescence intensity was measured again (120 min) under identical conditions. Parallel control experiments were conducted with 17βHSD1 enzyme that was previously denatured by boiling at 100 °C for 10 min. All experiments were performed in duplicate. Results are expressed as the mean value of the change in fluorescence intensity ($\Delta F = F_{120'} - F_{0'}$). Concentration of NADPH consumed in the reaction was obtained from standard curve and specific enzyme activity was expressed as nmol NADPH consumed min^{-1} mg^{-1}.

2. 5. Fluorimetric NADPH Assay

Because 17βHSD1 is an NADPH-dependent enzyme, reduction of estrone by 17βHSD1 was also assayed indirectly by measuring the decrease in NADPH fluorescence at excitation/emission wavelengths of 340/460 nm using a Fluoroskan Ascent FL fluorimeter. Reaction mixtures contained 67 μM estrone and 167 μM NADPH in 100 mM potassium phosphate buffer pH 6.0. Reactions were initiated with the addition of 50 μl of 17βHSD1 containing fraction (70 μg total protein by Bradford assay). Substrates were freshly dissolved in DMSO and the concentration of DMSO solvent did not exceed 1% in any well. Kinetic measurement of NADPH consumption was performed in a 96-well microplate (Greiner Bio-One MICROLON) at 37 °C and reactions were monitored at 15 seconds-intervals for 20 minutes. A control, blank assay was conducted without 17βHSD1 enzyme to assess non-enzymatic NADPH consumption. Fluorescence *vs*. time data was plotted in Origin Pro8 after slope normalization.

3. Results and Discussion

In humans, the 17βHSD1 isoform is mainly responsible for the final step of estrogen biosynthesis in pre-menopausal women, and is therefore considered to be a potential therapeutic target for estrogen-sensitive cancers and other hormone-dependent disorders.[22] Previous studies have shown that human placental microsomes are rich source of 17βHSD1 for experiments[15], and that Sf9 insect cell expression systems can provide active 17βHSD1 enzyme in high yield.[13,23] However, experiments using human placental tissue require special ethical approval, while heterologous expression of proteins in insect cell systems is technically more challenging and more expensive than use of bacterial expression systems.

Few published reports have described use of human 17βHSD1 expressed from *Escherichia coli*.[10] In the present study we expressed and purified human 17βHSD1 from *Escherichia coli* and optimized three simple non-radioactive assays for *in vitro* measurement of enzyme activity. Expression of 17βHSD1 was induced by addition of 0.25 mM IPTG in mid-logarithmic phase of growth of BL21(DE3) *Escherichia coli* cells transformed with pET28a(+) 17b-HSD1 plasmid. After induction with IPTG and incubation of cells at 23 °C for 18 h, a new band corresponding to ~ 40 kDa was detected by SDS-PAGE gel electrophoresis, consistent with the predicted molecular weight of 39.2 kDa for His-tagged 17βHSD1 (Figure 1, Panel A).

Following induction, cells were lysed by three freeze-thaw cycles and sonication, and the resulting cell lysate was clarified by centrifugation. Comparison of the soluble and insoluble fractions shows that a portion of the expressed 17βHSD1 protein appeared to be localized in the soluble fraction (Figure 1 Panel A). This soluble fraction was applied to a 1 mL HisTrap HP column equilibrated in binding buffer (20 mM TrisHCl pH 7.95, 0.5 M NaCl), washed once in binding buffer, and again in binding buffer plus 20 mM imidazole. Protein was eluted in binding buffer plus 400 mM imidazole (Figure 1 panel B). As measured by Bradford assay, a final yield of 1.4 mg/mL total protein was obtained from 1 L culture of *E. coli* cells, which was sufficient for enzymatic activity studies. Using densitometric analysis of SDS-PAGE gel to quantify protein bands it was determined that 17βHSD1 protein amounts to 10% of the total protein content.

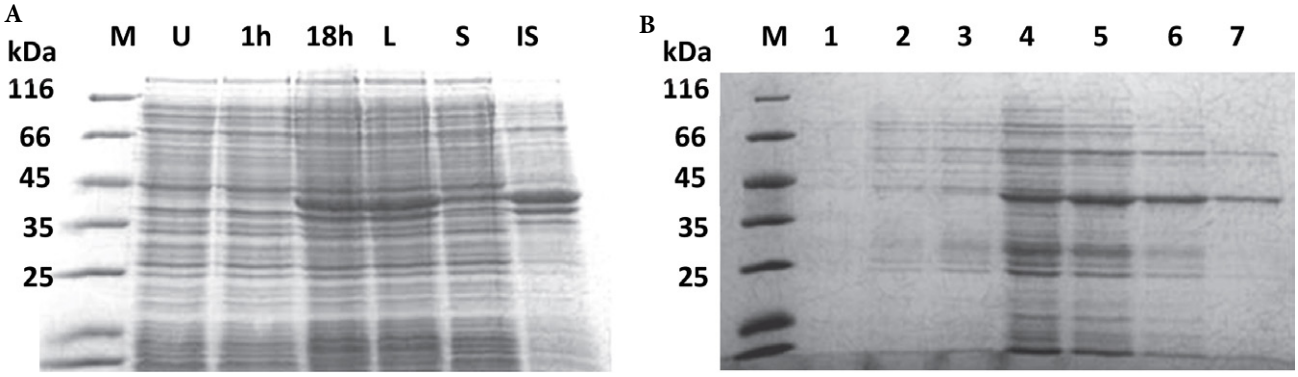

Figure 1. Analysis of the expression and purification of human His-tagged 17βHSD1 by SDS-PAGE electrophoresis. Panel A) Induction of expression of human 17βHSD1 in BL21(DE3) *E. coli* cells with 0.25 mM IPTG followed by 18h incubation at room temperature (23 °C): M-molecular weight marker; U – uninduced cell control; 1h – protein expression 1 hour after IPTG induction at room temperature; 18h – protein expression 18 hours after IPTG induction at room temperature; L – lysate; S – soluble fraction; IS – insoluble fraction. B) M-marker (ROTI®Mark STANDARD); wash fractions (lanes 1, 2) and elution fractions (lanes 3–7) from a 1 mL HisTrap HP nickel column.

In agreement with Chang et al., in our hands the majority of 17βHSD1 was also localized to the insoluble fraction in inclusion bodies. Chang et al were not able to refold and recover functional, catalytically active 17βHSD1 following solubilization of these inclusion bodies using 6 M urea.[17] In addition to refolding, yield of soluble protein can sometimes be improved by use of different host *Escherichia coli* strain and/or plasmid vector combination.[24] Mottinelli et al obtained active human 17βHSD1 from a different plasmid vector (pQE30) and different BL21 *Escherichia coli* host (BL21-CodonPlus DE3 RIL), using a 2 hour induction period at 37 °C and 0.5 mM IPTG.[10] Resulting cells were immediately harvested by centrifugation, resuspended in phosphate buffered saline and lysed by sonication before direct use in enzyme activity assays. Because cell homogenates were not clarified and no further purification steps were employed, the level of 17βHSD1 protein in the soluble fraction following this protocol remains unknown. Interestingly, although not explicitly mentioned by the authors, it appears that in Mottinelli et al. the whole cells and resulting cell homogenate were never frozen before measurement of 17βHSD1 activity. Considering that we observed significant loss of 17βHSD1 activity upon storage of purified protein at –80 °C, consistent with observations also reported by Chang et al[17], it is possible that freezing itself could be detrimental to 17βHSD1 activity. It may be possible to improve upon the results of the present study by optimizing or eliminating freezing steps during expression and purification of human 17βHSD1.

The majority of 17βHSD1 was located in the 3rd elution fraction (lane 5). Pool of fractions 4–6 was immediately applied to a desalting size-exclusion column and new pooled eluates with the highest protein content were used as a source of enzyme for *in vitro* assays. Since we observed loss of 17βHSD1 activity after freezing, all enzyme assays were performed immediately after purification. Cold inactivation of 17HSDs from human placenta was previously reported and protective effects of addition of glycerol, substrate and cofactor were investigated by Jarabak et al.[25] We did not notice that freezing in the presence of glycerol improved enzyme stability after storage at –80 °C.

Published procedures for measuring 17HSD activity usually rely on detection of radioactivity[10,13,16] carrying health risks and requiring use of labeled probes and expensive antibodies[26]. With this in mind, to overcome the hazards of handling radioisotopes, reduce costs and simplify experimental design, we optimized alternative non-radioactive screening assays for the characterization of 17βHSD1 modulators. Although Chang et al reported optimal enzymatic activity at a relatively high pH value of 9.3 in the reaction mixture and a temperature optimum of 25 °C[17]; we performed our activity measurements at pH 6.0 and temperature (37 °C), similarly to Motinelli et al[10] and reflecting physiological conditions.

High performance liquid chromatography (HPLC) is considered to be an analytical procedure of choice for the separation and quantification of steroid products of enzymatic reactions catalyzed by 17HSDs or other steroidogenic enyzmes.[10,13,27] Taking into consideration the relatively high costs of HPLC for preliminary screening, we coupled our enzymatic assay with a more inexpensive and fast TLC detection method. As shown in TLC chromatograms (Figure 2.A and B.) conversion of estrone (E1) to estradiol (E2) was catalyzed by addition of purified 17βHSD1 protein. Confirmation was based on the identical mobility of reaction product (R) and reference standard, estradiol (E2). Moreover, in control reactions in the absence of 17βHSD1 enzyme, no conversion of estrone to estradiol was observed by TLC. Results from TLC also suggest that freezing affects the stability and activity of recombinant 17βHSD1: complete loss of 17βHSD1 activity was observed after storage at –80 °C for several days (Figure 2.C). In order to quantify zones from TLC chromatograms, programs for densitometric image analysis, such as Image J have been used.[28–30] Formation of 17β-hydroxysteroids was previously analyzed by TLC only in the context of biotransformation studies, where steroid molecules undergo metabolic transformations over different time intervals.[31,32] Specific enzyme activity of 17βHSD1 was determined by measuring formation of estradiol. Densitometric analysis of TLC plates and quantification of the amount of product formed was performed using open-source ImageJ software. TLC chromatogram of estradiol at different con-

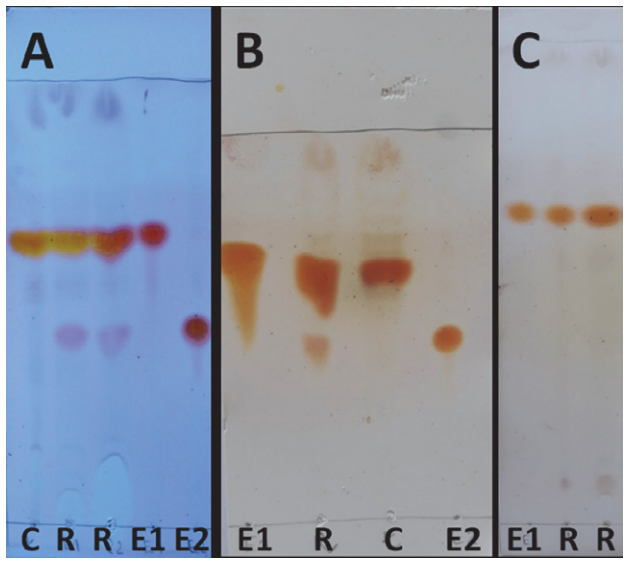

Figure 2. TLC chromatograms of reactions catalyzed by addition of human 17βHSD1 immediately after purification (A and B) or after freezing the enzyme at –80 °C for several days (C). Reaction mixtures consisted of 178 μM estrone, 70 μg of an enzyme, 178 μM NADPH and 100 mM potassium phosphate buffer (pH 6.0). After incubation at 37 °C for 90 minutes steroid metabolites were extracted by vortexing with methylene chloride. Toluene and ethyl acetate (2:1) solvent were used for development and spots were visualized by spraying with 50% H_2SO_4 and heating. Some of the samples were spotted in duplicate. R – Product of enzyme reaction; C – control in the absence of an enzyme; E1 – reference standard estrone; E2 – reference standard estradiol.

centration range and standard curve of peak areas from TLC-densitometry vs. concentrations of estradiol (25, 50, 75, 100 and 125 μM) are provided in Supplemental file 1. (Figure S1 and Figure S2). Specific enzyme activity of 17βHSD1 was 10 nmol min^{-1} mg^{-1}.

Recombinant 17βHSD1 activity was also evaluated by an alkali assay, which was initially developed to test modulators of aromatase activity.[21] This alkali assay is based on fluorimetric detection of an alkali product formed after exposure of $NADP^+$ to a strong alkali, where fluorescence intensity is directly proportional to enzyme activity. NAD(P)H-linked enzymatic activity of 17βHSD1 results in release of $NADP^+$ during reduction of estrone. A potential drawback of this assay appears to be the light sensitivity of the alkali product, which is overcome by adding a stabilizing agent, imidazole.[33] To our knowledge, this is the first report describing use of an optimized alkali assay to study the catalytic properties of purified recombinant 17βHSD1. Tsotsou et al used a similar assay to identify novel CYP450 enzymes substrates in high throughput format using whole *Escherichia coli* cells, confirming that the assay is substrate independent.[34] Using the alkali assay, we detected a 9-fold increase in fluorescence intensity in reactions conducted in the presence of active 17βHSD1 enzyme versus heat-treated, denatured enzyme (Figure 3). Our results are in accordance with previous findings, where the fluorescence intensity measured by the alkali assay in the presence of active aromatase was approximately 10-fold higher than in the presence of heat-inactivated aromatase enzyme.[21] Specific enzyme activity of 17βHSD1 was determined by measuring the consumption of NADPH. Standard curve of fluorescence intensity of alkali product vs. NADPH concentrations (2.5, 25, 62.5, 125, 250 μM) for alkali assay is provided in Supplemental file 1. (Figure S3). Specific enzyme activity of 17βHSD1 was 12 nmol min^{-1} mg^{-1}.

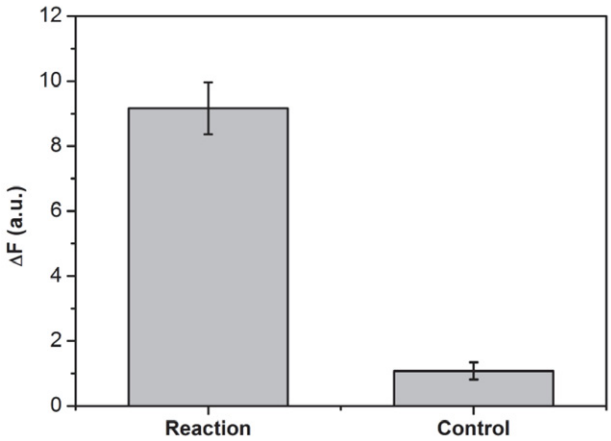

Figure 3. Measurement of the activity of recombinant human 17βHSD1 by alkali assay. A reaction mixture containing 40 μM estrone, 200 μM NADPH and 70 μg of recombinant 17βHSD1 in 100 mM potassium phosphate buffer (pH 6.0) was incubated at 37 °C for 90 min. Formation of $NADP^+$ was monitored by measuring fluorescence of alkali product using an excitation wavelength of 340 nm and emission wavelength of 460 nm. All experiments were performed in duplicate. Results are expressed as the mean difference in fluorescence signal ΔF ($F_{120'} - F_{0'}$) 120 minutes after addition of strong alkali. Control experiments were conducted in the presence of heat-inactivated, denatured enzyme. Histograms were plotted in Origin Pro8 after slope normalization.

The activity of recombinant human 17βHSD1 was also measured by monitoring NADPH consumption using fluorescence spectroscopy. Fluorimetric and spectrophotometric assays for measuring formation or consumption of cofactor in NAD(P)(H)-dependent reactions are routinely used to determine the activity of many oxidoreductases.[35,36] Fluorescence *vs.* time data for reactions catalyzed by 17βHSD1 and control reactions conducted in the absence of enzyme is shown in Figure 4. A decrease in fluorescence intensity over time was observed for reactions conducted in the presence of recombinant human 17βHSD1, corresponding to a loss of NADPH. In contrast, no fluorescence changes were observed in control reactions conducted in the absence of enzyme (Figure 4).

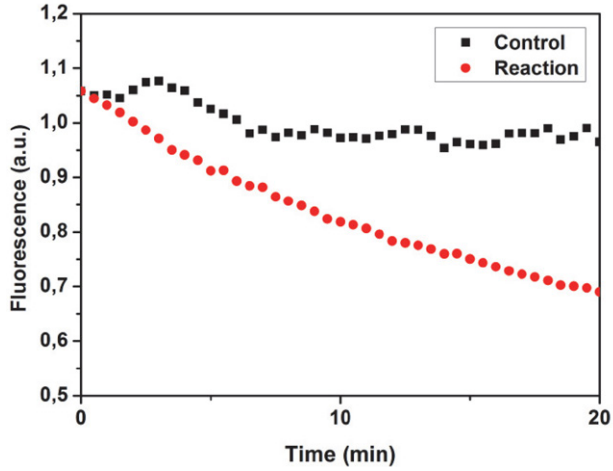

Figure 4. Reaction mixture containing 67 μM estrone, 167 μM NADPH and 70 μg of recombinant 17βHSD1 in 100 mM potassium phosphate buffer (pH 6.0) was incubated at 37 °C for 20 minutes and fluorescence intensity was recorded at 15 seconds-intervals. Reactions were initiated by addition of enzyme fraction and fluorescence was measured over time using an excitation wavelength of 340 nm and emission wavelength of 460 nm. The observed decrease in NADPH fluorescence is consistent with NADPH-dependent conversion of estrone to estradiol catalyzed by recombinant 17βHSD1. In the control reaction, enzyme was omitted. Fluorescence *vs.* time data was plotted in Origin Pro8 after slope normalization.

4. Conclusion

Reduction of estradiol levels in ovaries by inhibition of 17βHSD1 indirectly reduces estrogen-dependent activation of estrogen receptors, and represents a promising prevention or treatment strategy in premenopausal women diagnosed with estrogen-sensitive breast cancer, ovarian cancer or endometriosis. Although inhibitors of 17βHSD1 have been identified, none of these has yet reached clini-

cal trials. Here we provide a simple protocol for expression and purification of human 17βHSD1 from *E. coli*, and for the first time demonstrate 17βHSD1 activity using three independent *in vitro* assays. Catalytic conversion of estrone to estradiol was confirmed directly by detection of product formation or indirectly monitoring consumption of cofactor during reaction. While the current work represents valuable insight into the production of active human 17βHSD1 from *E. coli*, purification requires further improvement and optimization. The present study could be useful for researchers interested in preliminary *in vitro* screening of candidate compounds for 17βHSD1 inhibition using non-radioactive methods.

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Author contribution

Sofija S. Bekić: Methodology, Investigation, Software, Visualization, Writing- Original draft. Jovana J. Plavša: Methodology, Investigation, Writing- review & editing. Miha Pavšič: Methodology, Supervision, Writing- review & editing. Brigita Lenarčič: Methodology, Supervision, Writing- review & editing. Edward T. Petri: Conceptualization, Supervision, Writing- review & editing. Andjelka S. Ćelić: Conceptualization, Supervision, Writing- review & editing. All authors have read and approved the final manuscript.

Disclosure statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Povzetek

Rast celic raka dojk je pogosto odvisna od prisotnosti steroidnih hormonov. Izoforma 17β-hidroksisteroidne dehidrogenaze tipa 1 (17βHSD1) katalizira od NADPH odvisno pretvorbo estrona v estradiol, ki je močnejši estrogen, in predstavlja potencialno tarčo zdravil za zdravljenje raka dojk. Da bi zagotovili aktivni encim za presejanje inhibitorjev, se 17βHSD1 običajno izrazi v insektnih celicah ali celicah sesalcev ali pa se izolira iz človeške placente. V tej študiji opisujemo preprost protokol za izražanje in čiščenje aktivnega človeškega 17βHSD1 iz celic *Escherichia coli* BL21(DE3). Topen človeški 17βHSD1 je bil izražen z uporabo plazmida na osnovi pET28a(+), ki kodira heksahistidinsko oznako, združeno z N-koncem proteina, in prečiščen z nikljevo afinitetno kromatografijo. Encimsko aktivnost prečiščenega 17βHSD1 smo preverili s tremi metodami: tankoslojno kromatografijo, alkalnim testom in spektroskopskim testom. Ti neradioaktivni encimski testi zahtevajo le standardno laboratorijsko opremo in se lahko uporabljajo za presejanje spojin, ki modulirajo aktivnost 17βHSD1.