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Original research paper

Cloning, expression and purification of recombinant AKR1D1 for therapeutic applications

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ABSTRACT

AKR1D1, a key enzyme in the aldo-keto reductase superfamily, plays a dual role in both steroid metabolism and bile acid synthesis by catalyzing the NADPH-dependent reduction of carbon-carbon double bonds, specifically converting 3-ketosteroid hormones into 5β-steroids. Positioned at the critical intersection of steroid hormone and bile acid metabolism, AKR1D1 has the potential to profoundly influence metabolic homeostasis and drug metabolism. Despite its importance, the enzyme's therapeutic implications and role in drug metabolism remain underexplored. This study presents an optimized methodology for the cloning, expression, and purification of AKR1D1 using an *Escherichia coli* expression system. We identified optimal conditions for ligation and precise DNA sequencing, emphasizing the need for lower DNA concentrations and higher purity. Protein expression was evaluated in *E. coli* strains BL21 and Rosetta, with the highest yields achieved under extended incubation at 25 °C with controlled IPTG concentrations. Using freshly transformed cells was essential for maintaining consistent protein expression. The enzyme's activity was confirmed using a spectrofluorometric assay, demonstrating efficient reduction of testosterone to 5β-DHT. This optimized methodology facilitates

the production of AKR1D1 with high specific activity, establishing a valuable platform for future research. It enables a deeper investigation into AKR1D1's contributions to drug metabolism and its therapeutic potential.

Keywords: AKR1D1, drug metabolism, *Escherichia coli* expression system, protein purification, recombinant protein

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INTRODUCTION

Human 5β-reductases are part of the aldo-keto reductase (AKR) superfamily, specifically the 1D subfamily (1). This ancient enzyme family is primarily known for its role in catalyzing carbonyl oxidoreduction reactions. Uniquely, AKR1D1 stands out within this group due to its ability to catalyze the reduction of carbon-carbon double bonds, a function distinct from other members of the AKR superfamily (2, 3). Predominantly expressed in the liver, AKR1D1 plays a critical role in bile acid synthesis and steroid catabolism (4–6). The enzyme's activity introduces an A/B *cis* ring-junction in bile acids, conferring a hydrophobic surface that significantly enhances their emulsifying properties (7, 8). Deficiencies in AKR1D1 can impair bile acid synthesis, leading to a spectrum of liver diseases ranging from mild to severe, including progressive cholestasis, neonatal hepatitis, and potentially life-threatening liver disease in later childhood (9).

Despite AKR1D1's central role in regulating glucocorticoid and bile acid availability, its contribution to metabolic homeostasis and therapeutic contexts remains poorly understood (10). AKR1D1 converts steroid hormones into their 5β-conformation, rendering them inactive relative to their parent compounds while imparting unique physiological and pharmacological properties. For instance, 5β-androstanes, devoid of androgenic effects, are potent stimulators of erythropoiesis and may serve as alternatives to testosterone for treating anemia (11). Similarly, 5β-pregnanes exhibit activities such as stimulating heme synthesis and neuromodulatory effects in the central nervous system, all while lacking progestogenic activity (12–15). These 5β-steroids and their metabolites also serve as ligands for orphan nuclear receptors, such as the farnesoid X receptor (FXR) and pregnane X receptor (PXR), influencing the hepatic metabolism of both endogenous and exogenous compounds (16, 17) emphasizing the importance of AKR1D1 in drug interactions.

Research into AKR1D1 has expanded beyond its endogenous functions to explore its interactions with xenobiotics, including both steroidal and non-steroidal compounds. Studies on substrate specificity indicate that AKR1D1 is actively involved in the metabolism of hormonal contraceptives and steroids used in hormone replacement therapy (18). Anabolic-androgenic steroids (AAS), which are often misused at supra-physiological doses, have been identified as inhibitors of AKR1D1, potentially contributing to hepatotoxic effects (19). Conversely, attempts to identify non-steroidal drug inhibitors have so far been unsuccessful (18).

AKR1D1's involvement in drug metabolism has been further elucidated by identifying the enzyme as a crucial trans-regulator of the CYP enzyme network (20). Our previous research has also demonstrated that AKR1D1 significantly influences the pharmacokinetic profile and therapeutic efficacy of drugs like clopidogrel (21) and ibuprofen (22). Given its significant roles, this study aims to develop a method for synthesizing recombinant AKR1D1 to evaluate its contribution to drug metabolism.

Recently, Penning (23) published a method for the expression, purification, and characterization of AKR1D1. Here we present an alternative method for high-yield expression of the enzyme, the methodology of which is described in this study.

EXPERIMENTAL

Cloning, expression and purification of AKR1D1

Constructing of expression plasmid (pET28b+AKR1D1). – The preparation of recombinant *AKR1D1* was performed using the *Escherichia coli* expression system as described in Sambrook's laboratory manual (24). The *AKR1D1* coding sequence (Origene, Germany) was amplified *via* PCR using forward and reverse primers (GeneriBiotech, Czech Republic). The vector pET28b+ containing a N-terminal histidine tag, was isolated from *E. coli* (Promega, USA) using the Plasmid Plus Midi Kit (Qiagen, Germany).

The coding sequence and vector were restricted using *NdeI* and *XhoI* [New England Biolabs (NEB), USA] by incubating at 37 °C overnight at 300 rpm. The restriction enzymes were inactivated at 65 °C for 20 minutes. The restriction products were purified using the QIAEX II Gel Extraction Kit (Qiagen), and concentrations were measured with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). A ligation reaction was performed at 16 °C overnight using T4 DNA ligase (NEB, USA) with three samples differing

in concentration and purity (260/280; 260/230) of the PCR-restricted products: (*i*) 10 ng μL^{-1} and 2.07/1.7; (*ii*) 6.8 ng μL^{-1} and 1.52/1.42; (*iii*) 39.5 ng μL^{-1} and 1.32/0.75.

Competent *E. coli* HB101 cells were prepared by calcium chloride treatment (Sigma-Aldrich, USA) and incubation on ice. These cells were transformed with the ligated products *via* heat shock (0 – 42 °C) and plated on agar dishes containing 50 µmol L^{-1} kanamycin (Sigma-Aldrich). Plasmids were isolated from 60 colonies using the QIAGEN Plasmid Plus Midi Kit. Verification of the pET28+*AKR1D1* plasmid was performed using restriction digestion with endonucleases (*NdeI, XhoI, EcoR, PvuI*) (NEB, Germany). The restricted products were visualised on a 0.8 % agarose gel using a TBE buffer, run at 90V until the dye front migrated approximately two-thirds of the gel length, and subsequently stained with GelRed (Biotium, USA). Sequencing analysis confirmed that the *AKR1D1* gene was cloned in-frame, with no frame shifts or premature stop codons, ensuring correct translation of the intended protein sequence.

Expression of recombinant AKR1D1. – E. coli BL21 (DE3) (Promega) and Rosetta™ (DE3) Competent Cells - Novagen (Merck, Germany) containing the recombinant expression plasmid pET28b+*AKR1D1* were grown to an A600 of 0.6–0.8 in LB medium containing 50 µg mL^{-1} kanamycin at 37 °C. Protein expression was induced by adding different concentrations of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich) at 0.1, 1, and 2 mmol L^{-1}. Incubation was carried out at 25 °C and 37 °C for 4 and 24 hours. After induction, cells were lysed using BugBuster® Protein Extraction Reagent (Merck, Germany), and the resulting fractions, including the pellet and supernatant, were analyzed for protein content using SDS-PAGE.

Purification of recombinant AKR1D1. – Following induction and lysis with BugBuster®, the supernatant (cytosolic fraction) was treated with imidazole and centrifuged. The protein was isolated from the supernatant using Ni-NTA Fast Start Kit (Qiagen) and NGC™ Discover™ 100 Pro system (Bio Rad, USA). Ultracentrifugation was performed with an Amicon Ultra-4 column (Ultracel-10k filter) (Merck) and 0.2 mol L^{-1} potassium phosphate buffer (pH 7.4) to achieve buffer exchange and concentration. The recombinant protein was visualized using SDS-PAGE. Protein concentration was determined by the Bradford method using bovine serum albumin (BSA) (Sigma-Aldrich) as a standard.

All SDS-PAGE protein separations were performed using a 4 % stacking gel and a 12.5 % separation gel in Tris-Glycine SDS running buffer. Electrophoresis was conducted at a constant

voltage of 120V for 45 minutes, followed by staining with Coomassie Brilliant Blue. Precision Plus Protein™ All Blue Prestained Protein Standards (Bio-Rad) were used as molecular mass markers.

Standard spectrofluorometric assay

A continuous fluorometric assay monitored the reduction of testosterone to 5β-DHT by measuring the decrease in NADPH emission at 460 nm (excitation at 340 nm) on an Infinite M200 Tecan fluorescence spectrophotometer (Tecan, Switzerland). Standard assay conditions included 10 µmol L^{-1} testosterone, 15 µmol L^{-1} NADPH, 4 % acetonitrile in 100 mmol L^{-1} potassium phosphate buffer (pH 6.0) in a final volume of 100 µL. Following a 10-minute incubation at 37 °C, reactions were initiated by adding the substrate and monitored at 37 °C for 45 minutes.

RESULTS AND DISCUSSION

Cloning, expression, and purification of AKR1D1

The enzyme AKR1D1 was successfully cloned, expressed, and purified under the described experimental conditions. Fig. 1 shows the isolated enzyme band at 37 kDa.

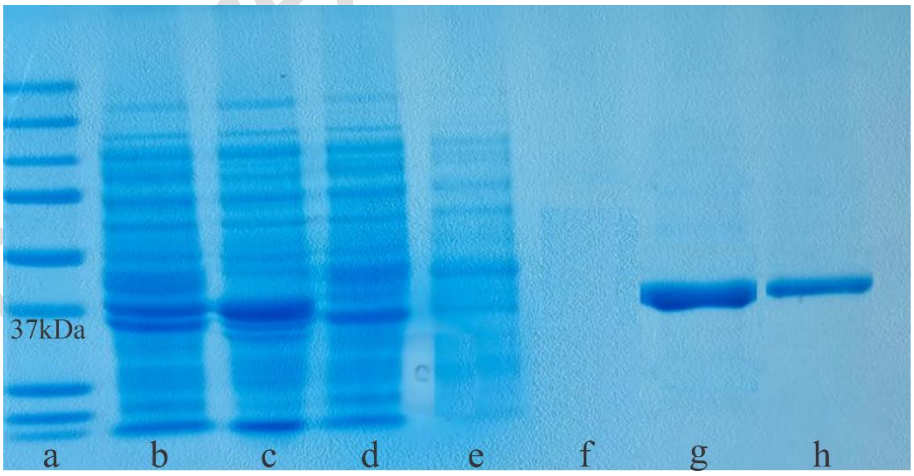


Fig. 1. SDS-PAGE analysis of purified AKR1D1 protein: the figure shows each stage of enzyme isolation from *E. coli* BL21: a) molecular mass ladder, b) cytosolic fraction, c) pellet fraction, d) flow-through, e) first wash, f) second wash, g) first elution, h) second elution.

Constructing of recombinant plasmid

The pET vector series was selected due to its efficient T7 promoter system, which can be easily induced using IPTG, and its medium copy number, designed to minimize the metabolic burden on the host cell. Specifically, the pET-26b (+) vector includes an *N*-terminal pelB signal sequence for periplasmic protein expression and a 6x histidine tag to facilitate protein purification. While any His-tag, whether at the N- or C-terminus, can potentially alter the protein's native folding, stability, and activity in both *in vitro* and *in vivo* environments, we selected an *N*-terminal His-tag to reduce the potential for interference with *AKR1D1*'s functional regions, as placing tags closer to critical domains could impact protein activity (25, 26). Studies have shown that C-terminal tagging may increase susceptibility to proteolytic degradation in *E. coli* (27), potentially impacting yield and stability, whereas N-terminal tagging generally supports more consistent recovery of functional protein (28). Although the *N*-terminal placement was chosen to minimize interference, it remains possible that the tag could affect AKR1D1's function in certain applications. *AKR1D1* cDNA was successfully subcloned into a pET-16b vector, as described by Penning (23).

During the construction of the insert, successful ligation was achieved with lower concentrations of the coding sequence (10 ng μL^{-1} and 6.8 ng μL^{-1}) and higher purity levels (2.07/1.7 and 1.52/1.42, resp.), compared to a sample with a higher concentration (39.5 ng μL^{-1}) and lower purity (1.32/0.75). Sequencing analysis indicated that only the 10 ng μL^{-1} sample contained the correct DNA sequence, whereas the 6.8 ng μL^{-1} sample exhibited point mutations, rendering it unsuitable for further experiments.

The correct insert was confirmed using gel electrophoresis by comparing the sizes of the empty vector (without the coding sequence) and the recombinant plasmid, as well as by treating the insert with restrictive endonucleases and visualizing the resulting fragments (Fig. 2).

Fig. 2. Agarose gel electrophoresis of the expression vector treated with endonucleases. Lanes are as follows: a) DNA ladder, b, c, d) vector digested with *EcoR* and *PvuI*, e, f) vector digested with *NdeI* and *XhoI*.

Protein expression

Protein expression in E. coli *strains*. – *Escherichia coli* has become the most widely used expression system for producing stably folded, globular proteins. It is a preferred host organism due to its fast growth kinetics, ability to reach high cell densities, ease of transformation with exogenous DNA, and low-cost maintenance using complex media derived from inexpensive components (29, 30). AKR1D1 has previously been expressed successfully in a combination of BL21 *E. coli* strains C41(DE3) and C43(DE3), which have mutations that enhance their ability to express toxic proteins (23).

Initial expression conditions and optimization. – BL21 cells were first made competent and subsequently transformed with the recombinant plasmid. Initial expression conditions involved induction with 1 mmol L^{-1} IPTG at 37 °C for 4 hours with shaking at 180 rpm. Samples were taken at hourly intervals and analyzed *via* PAGE. These initial expression conditions were chosen based on established in-house methods for synthesizing AKR1C enzymes, which share a high degree of structural similarity with AKR1D1 (18). However, there

was no significant difference in protein expression levels between the zero and fourth hour, and most of the protein was insoluble, not present in the supernatant.

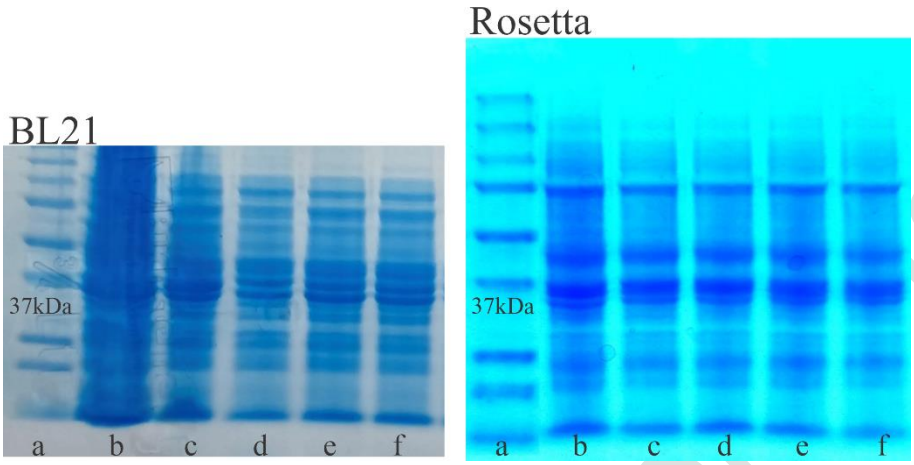


Fig. 3

SDS-PAGE analysis of protein content in *E. coli* BL21 and Rosetta cells after treatment with BugBuster. Lanes are as follows: a) molecular weight ladder, b) pellet fraction, c) 4 hours post-IPTG induction, d) 1 hour post-IPTG induction, e) 2 hours post-IPTG induction, f) 3 hours post-IPTG induction.

To address the formation of inclusion bodies, a common issue in *E. coli* potentially due to incorrect protein folding, the temperature was reduced to 25 °C, and the induction time was extended to 24 hours. This adjustment led to an increase in protein yield and a higher proportion of soluble protein, though the ratio of soluble to insoluble protein remained lower than desired. Another approach involved lowering the IPTG concentration to 0.1 mmol L^{-1} to slow the folding process, but this resulted in a significantly reduced protein yield.

Comparison of expression in Rosetta(DE3) cells. – Competent Rosetta(DE3) cells were transformed according to the manufacturer's instructions and subjected to the same initial conditions as BL21 cells. As with BL21, Rosetta cells exhibited similar limitations at 37 °C for 4 hours (Fig. 3a,b). Optimal conditions for Rosetta were also found to be 25 °C for 24 hours. Unlike BL21, Rosetta cells produced comparable protein yields with both 0.1 mmol L^{-1} and 1 mmol L^{-1} IPTG, indicating a more robust response to varying inductor concentrations (Fig. 4).

a) b)

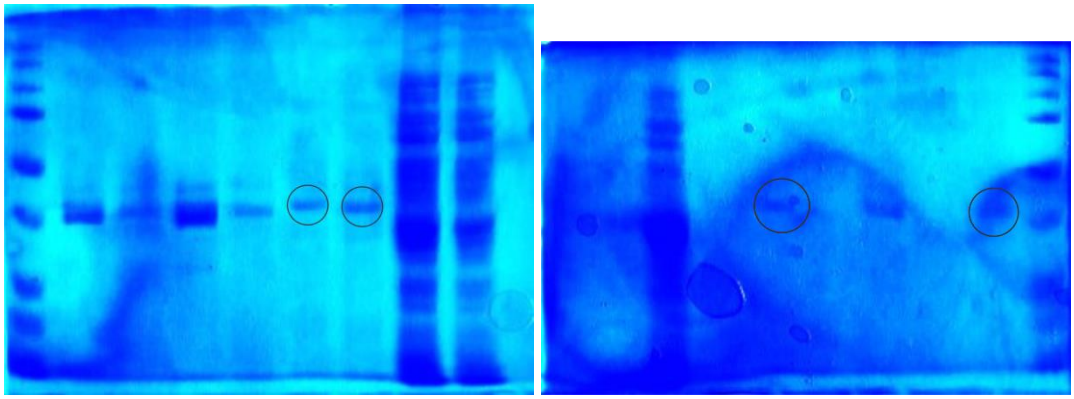


Fig. 4. SDS-PAGE analysis of isolated enzyme from BL21 cells with varying IPTG concentrations. The circled bands represent isolated enzyme expressed with: a) 1 mol L^{-1} IPTG, b) 0.1 mol L^{-1} IPTG.

Despite the use of an appropriate secretion vector to facilitate protein translocation to the periplasm and the optimization of growth and induction conditions (31), the formation of inclusion bodies persisted in both expression hosts. This issue suggests that further strategies are required to enhance the yield of soluble protein in the cytosolic fraction.

Impact of transformation method and protein stability. – Significant differences in protein expression were observed based on the transformation method used. Cultures where each flask was individually transformed with the recombinant plasmid yielded significantly higher protein levels compared to those prepared from a single batch of transformed cells. Additionally, repeated IPTG induction followed by centrifugation led to reduced protein synthesis, highlighting the need to optimize induction protocols.

One notable limitation was the inability to detect protein expression when using previously transformed and stored *E. coli* cells at –20 °C. This finding underscores the importance of using freshly transformed cells *in situ* to achieve successful protein expression.

Protein purification

The protein was purified using two approaches: Immobilized metal affinity chromatography (IMAC) and Fast protein liquid chromatography (FPLC). IMAC is a widely adopted technique for purifying recombinant proteins that contain a 6× histidine tag. In our study, Ni-NTA resin was utilized for purification under native conditions, following the manufacturer's protocol. While this method is efficient and provides a straightforward approach to histidine-tagged protein isolation, its limited capacity allows for the purification of only 250 mL of *E. coli* culture per column. This limitation can be a challenge for proteins with lower

expression levels, which require larger culture volumes to achieve adequate yields. From the 250 mL culture used in our experiments, we successfully purified 5.6 mg of AKR1D1 protein.

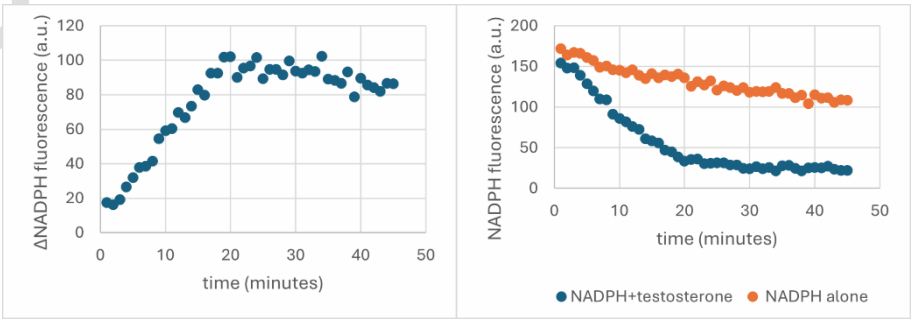
For larger-scale purification, we employed FPLC using the Next generation chromatography (NGC) system, which enabled us to process 1 L of culture efficiently. This method resulted in variable yields of 35 mg and 20 mg of protein, depending on the transformation conditions. FPLC offers several advantages, including minimal operator intervention, ease of handling, and a greater purification capacity, making it a preferable approach for scenarios involving lower expression levels of the protein.

Penning reported using Ni-Sepharose columns for IMAC, achieving a yield of approximately 56 % for AKR1D1 purification (23). For FPLC, they employed various HisTrap columns, though the specific yields were not disclosed. This indicates that both IMAC and FPLC techniques can be used for the purification of AKR1D1, with the choice of method depending on the protein's expression levels and the loading capacities of the columns.

Specific enzyme activity

The kinetic activity of the isolated enzyme was assessed using testosterone as a substrate. A continuous decrease in fluorescence of the coenzyme NADPH was observed, as reflected by the increasing delta NADPH values over time, with the most significant change occurring at the 20-minute mark of the reaction (Fig. 5a). To account for the inherent instability of NADPH, a control reaction was performed without testosterone, which exhibited a marked difference compared to the samples containing testosterone (Fig. 5b). The final specific activity was determined to be 80 nmol of testosterone reduced per minute per milligram of protein under the specified conditions.

a) b)



F i g . 5 change in NADPH fluorescence (ΔNADPH) over time in the presence of testosterone. b) comparison

Future directions and optimization strategies. – While significant progress was made in optimizing AKR1D1 expression, further enhancements are needed to boost soluble protein yield. Future efforts could focus on co-expressing molecular chaperones to aid protein folding or optimizing growth conditions to reduce inclusion body formation. Moreover, structural and kinetic studies of AKR1D1 should be integrated into future work to gain deeper insights into its interactions with drug substrates and inhibitors. These studies will help to elucidate the mechanistic basis of AKR1D1's role in modulating drug metabolism, guiding the development of therapeutic strategies that harness the enzyme's activity while minimizing potential adverse effects.

CONCLUSIONS

In this study, we developed an optimized methodology for the efficient cloning, expression, and purification of recombinant AKR1D1 using an *E. coli* expression system. By fine-tuning conditions such as incubation temperature, IPTG concentration, and utilizing *in situ* transformed cells, we successfully synthesized AKR1D1, achieving high specific activity of its active form.

Our findings underscore AKR1D1's role in steroid and bile acid metabolism, which is critical for endogenous regulatory processes. Although not within the direct scope of this study, AKR1D1's established interactions within the cytochrome P450 network suggest that it could influence drug metabolism in broader pharmacokinetic contexts. These insights position AKR1D1 as a promising area for further study, particularly regarding its potential role in drug interactions and therapeutic applications.

This study lays a solid foundation for advancing AKR1D1's role in metabolic regulation and its applications in precision medicine. Future research should focus on exploring AKR1D1's interactions with drug substrates and inhibitors to deepen our understanding of its therapeutic potential.

Supplementary materials available upon request.

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Authors contributions. – Kristina Shutevska: Investigation, Formal analysis, Writing-original draft.

Aleksandra Kapedanovska Nestorovska : Conceptualization, Formal analysis, Writing -Review & Editing, Supervision.

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