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Efficient *in vitro* refolding and functional characterization of recombinant human liver carboxylesterase (CES1) expressed in *E. coli*

Usa Boonyuen [a], Kamoltip Promnares [b], Suwapat Junkree [c], Nichloas P.J. Day [d,e], Mallika Imwong [a,*]

[a] Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand
[b] Department of Molecular Biotechnology and Bioinformatics, Faculty of Science, Prince of Songkla University, Hat Yai, Songkla 90112, Thailand
[c] Central Equipment Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand
[d] Mahidol Oxford Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand
[e] Centre for Tropical Medicine, Nuffield Department of Medicine, Churchill Hospital, Oxford, United Kingdom

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ABSTRACT

Human liver carboxylesterase 1 (CES1) plays a critical role in the hydrolysis of various ester- and amide-containing molecules, including active metabolites, drugs and prodrugs. However, it has been problematic to express recombinant CES1 in bacterial expression systems due to low solubility, with the CES1 protein being mainly expressed in inclusion bodies, accompanied by insufficient purity issues. In this study, we report an efficient *in vitro* method for refolding recombinant CES1 from inclusion bodies. A one-step purification with an immobilized-metal affinity column was utilized to purify His-tagged recombinant CES1. Conveniently, both denaturant and imidazole can be removed while the enzyme is refolded via buffer exchange, a dilution method. We show that the refolding of recombinant CES1 was successful in Tris–HCl at pH 7.5 containing a combination of 1% glycerol and 2 mM β-mercaptoethanol, whereas a mixture of other additives (trehalose, sorbitol and sucrose) and β-mercaptoethanol failed to recover a functional protein. His-tagged recombinant CES1 retains its biological activity after refolding and can be used directly without removing the fusion tag. Altogether, our results provide an alternative method for obtaining a substantial amount of functionally active protein, which is advantageous for further investigations such as structural and functional studies.

Introduction

Human liver carboxylesterase (CES1, EC number: 3.1.1.1, 3.1.1.56), an enzyme responsible for the hydrolysis of ester- and amide-containing molecules, is mainly expressed in the liver where it is crucial for the processing of active metabolites (heroin and cocaine) and is also involved in trans-esterification reactions [1–4]. In addition, CES1 is known to play an important role in the hydrolysis of several classes of drugs and the activation of prodrugs, including angiotensin-converting enzyme (ACE)[1] inhibitors and the anti-influenza agent oseltamivir [5–10], also known as Tamiflu, a recommended treatment by the World Health Organization (WHO). Oseltamivir is a prodrug in the form of oseltamivir phosphate, which requires conversion into its active carboxylate form by CES1. Two known CES1 mutations, G143E and p.D260fs, have been shown to impair the function of the enzyme with regard to the activation of oseltamivir, indicating the significant role of CES1 in oseltamivir metabolism [8,9].

Extensive studies have been carried out to investigate the role of CES1 in oseltamivir metabolism in terms of pharmacokinetics, structure and function. Accordingly, different expression systems, purification processes and enzymatic assays have been established, with most previous works employing conventional cell-culture techniques to produce a functional enzyme. Mammalian cell lines, COS7 and Flp-In-293 with the pCDNA5/FRT/V5-His TOPOTA expression system, have frequently been utilized for the production of CES1. Wild-type (WT) as well as mutant proteins have been

* Corresponding author.
E-mail addresses: usa.boo@mahidol.ac.th (U. Boonyuen), kamoltip.p@psu.ac.th, kpromnares@gmail.com (K. Promnares), suwapat.jun@mahidol.ac.th (S. Junkree), nickd@tropmedres.ac (N.P.J. Day), noi@tropmedres.ac (M. Imwong).

[1] *Abbreviations used:* CES1, carboxylesterase 1; ACE, angiotensin-converting enzyme; WHO, World Health Organization; WT, wild-type; pNPA, p-nitrophenyl acetate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CD, circular dichroism; pNP, p-nitrophenol; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; GFP, green fluorescent protein; DTT, dithiothreitol; SNP, single-nucleotide polymorphism; IPTG, isopropyl-β-D-thiogalactopyranoside.

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expressed and investigated with regard to hydrolysis, metabolism and activation of several compounds mediated by CES1, such as p-nitrophenyl acetate (pNPA), oseltamivir, methylphenidate, trandolapril and clopidogrel and its metabolite [6–10]. Insect cells, Sf9 and Sf21, are also preferred for expressing CES1, which is accomplished using a baculovirus expression system [2,3,11,12]. In addition to insect cell culture, there was a report in which *Trichoplusia ni* whole-insect larvae were used as a production source of CES1 recombinant protein [13]. Finally, yeast expression systems involving *Saccharomyces cerevisiae* and *Pichia pastoris* are also used for the expression of CES1 [14]. Although eukaryotic expression systems are suitable for expressing eukaryotic proteins, especially when post-translational modifications are needed, they are relatively complex, expensive and require long time periods to obtain the desired protein compared to bacterial expression systems. Furthermore, eukaryotic expression systems typically yield only small to moderate amounts of protein.

As an alternative to expression in eukaryotic cells, bacteria are among the most extensively hosts used for protein production because expressing proteins in bacterial systems is inexpensive and easy to handle and large amounts of protein are achievable [15,16]. A previous attempt to express CES1 in bacteria failed to produce the functional protein because CES1 was mainly expressed in inclusion bodies [14]. Indeed, this is a major problem with expressing human proteins in bacteria, as the proteins are misfolded and aggregate, preventing the production of a functional eukaryotic protein [17–20]. As a result, various methods have been established to overcome insolubility issues: fusion tags and growth temperature, auto-induction and purification conditions have all been optimized to improve the solubility of proteins expressed in *Escherichia coli* [21–23]. Moreover, various types of folding aids are employed in bacterial systems. *E. coli* molecular chaperones, e.g., GroES and GroEL, have shown to promote protein folding when co-expressed with human aromatase and aldehyde dehydrogenase enzymes [24,25]. Additionally, it has been proven that including some chemical chaperones, e.g., polyols, amino acids and polyethylene glycol, in the growth medium is beneficial for producing soluble and functional proteins [26–28]. However, when the aforementioned methods are unsuccessful and the protein is still mainly expressed as inclusion bodies, efficient protocols for refolding proteins is, therefore, necessary to produce biologically active proteins, which will be advantageous for structural and functional studies. Many effective protocols for refolding proteins from inclusion bodies have been established and proven to successfully produce active enzymes. The refolding of proteins can mainly be achieved in two different ways: dialysis and dilution. During the refolding process, chemical chaperons or additives are usually added to assist in protein conformation, and additives such as trehalose, glycerol, sorbitol, arginine and sucrose have been reported to be advantageous for the refolding step [17–19,29–33].

In the present study, we report the heterologous expression of CES1 in *E. coli*, in which it was mainly expressed in inclusion bodies. Thereafter, different refolding conditions were applied to retain the catalytically active enzyme. In addition to WT CES1, three natural variants (S76N, D204E and A270S) were expressed, purified, refolded and characterized.

Materials and methods

Plasmid constructs and site-directed mutagenesis

In this study, we investigated the expression, purification and refolding of CES1 isoform a (NCBI Reference Sequence: NP_001020366.1), which is one and two amino acids longer than isoforms b and c, respectively. Therefore, the numbering of amino acid residues is herein shifted by one residue compared to previous reports. The CES1 gene was amplified from human liver cDNA (BD Biosciences) using Platinum Taq DNA polymerase (Life Technologies) with a forward primer GGATCCGAATTCCATCCGTCCTCGC and a reverse primer GGTGCTCGAGTCACAGCTCTATGTG. The amplified region was designed to start at amino acid 20, omitting the first 19 amino acids of the signal peptide to prevent complications of post-translational modification. Two restriction enzyme sites were introduced, *Eco*RI and *Xho*I, in the forward and reverse primers, respectively (underlined). PCR products were gel purified and digested with *Eco*RI and *Xho*I (New England Biolabs). The digested amplicon (encoding 20–568 residues) was cloned into the pET28a expression vector with an N-terminal His-tagged and transformed into the *E. coli* expression host BL21 (DE3). Three constructs of CES1 natural mutations, S76N, D204E and A270S, were generated using a site-directed mutagenesis kit (Agilent Technologies) with specific primers. All constructs were verified by bidirectional DNA sequencing and restriction digestion to confirm that the desired plasmids were obtained.

Expression of recombinant CES1

A single colony of BL21 (DE3) harboring the recombinant plasmid was inoculated in 5 ml of LB medium containing 50 μg/ml kanamycin and incubated at 37 °C with 250 rpm shaking overnight. The fresh overnight cultures were inoculated in LB medium in the presence of 50 μg/ml kanamycin at a dilution of 1:100 and grown at 25 °C with 250 rpm shaking until the absorbance at 600 nm (OD_{600}) reached 0.3. Then, the cultures were induced with isopropyl-β-D-thiogalactopyranoside (IPTG, Merck) at a final concentration of 0.5 mM and were further incubated at 25 °C with 200 rpm shaking for 4 h before being harvested by centrifugation at 1000×*g* for 10 min.

Purification of recombinant CES1

The CES1 protein was purified under denaturing condition due to its main expression in the insoluble fraction. Cell pellets were resuspended in lysis buffer (20 mM sodium phosphate, pH 7.4, 300 mM NaCl, 10 mM imidazole and 2 mM β-mercaptoethanol) and disrupted by sonication. The cell lysate was centrifuged at 20,000×*g* for 45 min at 4 °C, and the supernatant was removed. To reduce protein interference, the pellet was washed with 2 M urea in lysis buffer by incubating on ice for 15 min and then subjected to centrifugation at 20,000×*g* for 45 min at 4 °C. The inclusion bodies were solubilized in buffer A (lysis buffer containing 8 M urea) by incubating the solution on ice for 1 h. The solubilized solution was centrifuged for 1 h at 20,000×*g* and 4 °C. The supernatant was collected and incubated with pre-equilibrated TALON Metal Affinity Resin (BD Biosciences) in buffer A at 4 °C for at least 1 h. The unbound proteins were removed with wash buffer (20 mM sodium phosphate, pH 7.4, 300 mM NaCl, 8 M urea, 20 mM imidazole and 2 mM β-mercaptoethanol; 4 times, 6 ml each). The elution of CES1 was accomplished with elute buffer (20 mM sodium phosphate, pH 7.4, 300 mM NaCl, 8 M urea and 250 mM imidazole, 2 mM β-mercaptoethanol; for 5 times, 4 ml each).

In vitro refolding of recombinant CES1 using the dilution method

In the refolding step, urea (protein denaturant) and imidazole were gradually removed via dilution. This procedure was performed while the protein was refolded by buffer exchange with 50 mM Tris–HCl, pH 7.5, containing different combinations of additives and reducing agents (Table 1) using Amicon Ultra centrifugal filter devices (Millipore). The protein solution was mixed with