Cloning, expression, and characterization of human brain acetylcholinesterase in *Escherichia coli* using a SUMO fusion tag

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Abstract: The molecular structure of acetylcholinesterase (AChE) attracts interest because of its versatility and significant role in the cholinergic system. The main purpose of the present study was to clone a full-length cDNA sequence of human brain acetylcholinesterase (hAChE) into pET SUMO vector and express it successfully. The integrity of the constructed plasmid was confirmed by cross PCR. This recombinant construct was expressed in *Escherichia coli* BL21 (DE-3). In this work, we produced hexahistidine (6xHis) tagged fusion protein by isopropyl β-D-1-thiogalactopyranoside (IPTG) induction and purified using nickel (Ni²⁺) affinity chromatography. Using anti-His antibody, we detected ~90 kDa fusion protein. The expression of the hAChE gene in a microbial host resulted in good biological activity. Using the Ellman method, the recombinant AChE exhibited activity with optima at pH 9.0 glycine–NaOH buffer and room temperature. Kinetic parameters, Kₘ and Vₑₘₚ, were determined as 0.63 and 0.69, respectively.

Key words: Acetylcholinesterase, cloning, recombinant protein, enzyme characterization

1. Introduction

Characteristic of cholinergic synapses is termination of synaptic transmission by neurotransmitter hydrolysis (Zimmerman and Soreq, 2006). Acetylcholinesterase (AChE) is present at all cholinergic synapses and exerts an essential role in cholinergic transmission by swiftly hydrolyzing the acetylcholine that is a key component of cholinergic signaling. This function of enzyme depends on two main features: its extraordinary processing speed and its specific localization in synaptic clefts (Bernard et al., 2011; Lee et al., 2012).

Recent studies suggest that termination of transmission is just one of the many roles of AChE. In addition to its cholinesterase activity, the enzyme is also involved in apoptosis, microtubule formation, inflammation, stress response, and cell proliferation/differentiation (Layer and Willbold, 1995; Small et al., 1996; Soreq and Seidman, 2001; Pick et al., 2006; Shaked et al., 2009). The enzyme can be found in multiple forms in various developmental stages, tissues, or cell types such as erythrocytes, nerve endings, human fibroblasts, osteoblasts, spleen, lung, rat kidney, and leukocytes (Inkson et al., 2004; Patocka et al., 2004; Thiermann et al., 2007) but essentially exists in most regions of the central and peripheral nervous system (Tripathi and Srivastava, 2008; Shaked et al., 2009; Silverman et al., 2014). This distinct localization ability of enzyme may occur because diverse AChE molecular forms are generated by sequential regulation of AChE pre-mRNA (Li et al., 1993; Chan et al., 1999; Shapira et al., 2000). These versatile features and functions of the enzyme, especially in cholinergic traffic, also make it a potential multitarget for Alzheimer disease (AD) treatment candidate drugs (Du and Carlier, 2004; Colovic et al., 2013).

Current therapeutic strategies for AD focus on increasing acetylcholine (ACh) bioavailability at the synapse. Therefore, revealing enzyme response against therapeutic agents that alter AChE activity is extremely important for orienting treatment strategies (Singh et al., 2013). Nowadays, protein-based therapeutics are approved for clinical use and many new products are launched each year because of their significant advantages such as enhanced efficacy, greater safety, and reduced immunogenicity (Carter, 2011). As well as the clinical use, the production of a protein by recombinant methods is quite important in terms of clarification of the structural and biochemical properties of the enzyme (Kawai et al., 2003).

Proteins have many different structures in their physiochemical properties. Thus, production of a protein is quite an arduous task. In the case of use of prokaryotes as a host organism, fusion partners can help to overcome these obstacles and can be used an efficient approach.
to improve the yield of targeted proteins expressed in *E. coli* (Esposito and Chatterjee, 2006; Hayashi and Kojima, 2010; Zhou et al., 2015). It well known that many small molecules such as ubiquitin exert chemical chaperoning effects on fused proteins (Malakhov et al., 2004). As a result of this action, attachment of SUMO (small ubiquitin-like modifier) or ubiquitin to the N-terminus of a partner protein, recombinant fusion proteins are dramatically increased (Panavas et al., 2009; Wang et al., 2010). SUMO is a modifier protein consisting of approximately a hundred amino acids and is reversible attached to target proteins and absent in prokaryotes (Seeler and Dejean, 2003; Johnson, 2004). This attachment changes the stability, function, and/or localization as a consequence of changing of the protein structure (Wang et al., 2008; Panavas et al., 2009).

Consequently, it might alter interactions between DNA and other DNA-bound protein complexes, causing the transcription factors accessibility to genetic material (Eilebrecht et al., 2010). Because of SUMO conjugation consequences that vary from substrate to substrate, functional mechanisms of this attachment are not fully resolved at the molecular level (Johnson, 2004). To date, SUMO fusion tags were successfully used in various difficult-to-express proteins’ production with improved yield and solubility (Malakhov et al., 2004; Zuo et al., 2005; Marblestone et al., 2006). In the present work, we introduced the use of pET-SUMO recombinant plasmid to express AChE. We optimized the conditions required for AChE expression and obtained the enzyme with high activity after simple purification. Beyond that, the AChE was characterized biochemically. This study provides a promising method for the simple, swift, and effective production of recombinant human acetylcholinesterase for use in drug development and in understanding and unravelling biochemical conduct against therapeutics.

### 2. Materials and methods

#### 2.1. Bacterial strains, plasmids, medium and reagents

*E. coli* One Shot Mach1-T1*R* chemically competent cells were used for the transformation of vectors and *E. coli* BL21 (DE3) One Shot was used as a host strain for protein expression. The pET SUMO vector (Invitrogen) was used for T-A cloning and protein expression. All other analytical grade chemicals were obtained from Sigma (Germany).

#### 2.2. Plasmid construction and cloning of AChE

Adult human brain cDNA was purchased from Invitrogen (USA). In order to produce recombinant protein in prokaryote expression systems, the coding sequence of human AChE (GenBank accession number M55040.1) was cloned into the pET SUMO expression vector (Invitrogen). The coding sequence of the target gene was amplified by PCR using the forward primer 5'-ATGAGGCCCCGCCAGTGTCT-3' and the reverse primer 5'-TCACAGGCTCTGACCGCATCC-3'. The PCR protocol was as follows: after the first denaturation step at 94 °C for 4 min, followed by 35 cycles of 2.5 min at 94 °C, 30 s at 66 °C, and 2 min at 72 °C a final extension for 5 min at 72 °C was added at the end of this reaction. Using a GeneJET Gel Extraction Kit (Thermo Scientific), 1845 bp PCR product was purified from agarose gel. This fragment was cloned into the pET SUMO vector (Figure 1). All steps were done according to the manufacturer’s instructions.

#### 2.3. Recombinant protein expression

The generated construct encoded a 645 amino acid recombinant protein. The N-terminal of its sequence contained a 6xHis tag. Component *E. coli* One Shot Mach1-T1*R* cells treated with ligation mixture were grown overnight at 37 °C in LB plates (1% tryptone, 0.5% NaCl, 0.5% yeast extract, and 2% agar) containing 50 µg/mL kanamycin. The presence of the DNA insert was determined by screening colonies using colony PCR. To determine the insert, four combinations (gene–gene, vector–vector, gene forward–vector reverse, and gene reverse–vector forward) of vector specific and insert specific primers were used. Plasmid purification was performed from desired clones using a GeneJET Plasmid Miniprep Kit (Thermo).

Plasmids were transformed into *E. coli* BL21(DE3) using the classic heat shock method and grown in LB plates. Preculture was prepared as follows: a single colony from the agar plate was inoculated into 10 mL of LB medium and grown at 37 °C and 170 rpm overnight.

For production of 6xHis fusion protein, 1 mL of the preculture was added to 200 mL of 50 µg/mL kanamycin and 1% glucose containing LB medium. IPTG was added when OD$_{600}$ reached approximately 0.4–0.6 and then the medium was incubated at 180 rpm and 37 °C.

![Figure 1. Schematic overview depicting the construction of the prokaryotic expression vector pET-SUMO.](image-url)
2.4. Protein purification

After induction, cells were harvested by centrifugation at 3000 for 5 min. Cell pellets were re-suspended in 1 mL of lysis buffer (50 mM potassium phosphate, pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, and 10 mM imidazole). Cell suspension was disrupted again by three freeze–thaw cycles using liquid nitrogen and a 42 °C water bath after the sonication step. The lysate was centrifuged at 12,000 rpm for 1 min. Supernatant was transferred to a fresh tube. Protein was concentrated to 30-fold and recovered with an Amicon Filter (Merck Millipore). Using a ProBond Ni-NTA resin column (Invitrogen) the filtrate obtained from cell homogenate was purified. For purification soluble concentrated filtrate was incubated with Ni–NTA resin (2 mL) equilibrated in native binding buffer and allowed to bind to the Probond affinity column for 30–60 min at room temperature. All untagged proteins were allowed to pass through the column using pH 8 wash buffer (50 mM NaH₂PO₄, 20 mM imidazole, and 0.5 M NaCl). Using pH 8 elution buffer (0.5 M NaCl, 250 mM imidazole, and 50 mM NaH₂PO₄) the bound proteins were recovered from the column. This fraction was dialyzed twice against a buffer (20 mM Na₂HPO₄, pH 8) at 4 °C for 2 h. The final sample was stored ice cold and then used for Bradford protein quantification, western blot, SDS-PAGE analysis, and enzyme activity assays.

2.5. Protein analysis

The protein concentration was determined by Bradford assay (Bradford, 1976) using bovine serum albumin as the standard protein. Absorbance was read at 595 nm. Assessment of AChE activity was performed using Ellman’s assay (Ellman et al., 1961). Time dependent expression level, purity, and molecular mass of the recombinant protein were determined by SDS-PAGE (12%). The exact size of fusion protein was determined by western blot analysis using Anti-HisG Antibody (Invitrogen). Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control.

2.6. Characterization of purified enzyme

Effect of pH on enzyme activity was measured using the following various buffers: sodium phosphate (pH 5.5–8), Tris-HCl (pH 7.5–9) and glycine–NaOH (pH 9–10.5). To determine optimal pH and buffer concentration for ionic strength, the enzyme activity was measured using different concentrations (ranging from 50 mM to 1 M) of glycine–NaOH buffer. Enzyme activity was assayed at various temperatures from 4 °C to 90 °C for determining optimum temperature. In order to determine K_M, and V_max values, acetylcholine iodide was used as a substrate with different concentrations.

3. Results

3.1. Construction and validation of recombinant pET-SUMO-hrAChE vector

Recombinant vector was constructed as described in the methods. After amplification and purification (Figure 2) of the target protein gene sequence, full-length hAChE was ligated and cloned into pET-SUMO vector. Colony and cross PCR results (Figures 3A and 3B) showed that the gene inserted correctly into pET-SUMO vector. The length of amplified products was consistent with theoretical expectations (Table 1).

3.2. Expression of rAChE

The hAChE protein was expressed in *E. coli* as a His-tagged protein. For the production of recombinant protein, a single clone of *E. coli* BL21 (DE3) harboring the vector was used. After IPTG induction, cells were harvested by three cycles of rapid freeze (liquid nitrogen) and thaw (42 °C water bath). Cell lysate was analyzed by SDS-PAGE. Approximately 90 kDa fusion protein appeared as expected (data not shown). According to this result, optimal expression conditions were adopted as 37 °C for 7 hours by 1 mM IPTG (Figure 4).

3.3. Purification and identification of target protein

Purified and dialyzed hAChE was firstly determined by the Bradford assay. Bradford results (Table 2) show that IPTG induction and concentration experiments significantly affected protein yielding. For verification of the target protein, western blot analysis was performed using an Anti-HisG antibody. 6xHis tag fusion protein with ~90 kDa molecular weight was recognized (Figure 5).

3.4. Determination of kinetic constants

The recombinant purified AChE was utilized to determine the kinetic characteristics of the enzyme. The optimum pH for recombinant enzyme activity was observed in glycine–NaOH (pH 9) buffer. Activity was observed at the maximum rate at this pH (Figure 6). In order to determine the effect of ionic strength on the enzyme activity, measurements were performed using different concentrations (between 0.05 and 1.5 M) of glycine–NaOH buffer. Maximum enzyme activity was observed in 1 M (pH 9) glycine–NaOH buffer (Figure 7).

In order to determine the optimal temperature for maximum rhAChE activity efficiency, enzyme activity was measured between 0 and 90 °C. According to the results, room temperature was considered the optimum temperature for enzymatic activity. However, enzyme activity decreased drastically at excessively high and low temperatures. Activity completely ceased at 90 °C (Figure 8). K_M and V_max were determined by Lineweaver–Burk analysis using acetylcholine iodide (Figure 9). Kinetic constants were calculated by assaying enzyme activity.
as previously described by varying the concentrations of substrate from 0.05 to 0.35 mM. Obtained results for $K_M$ and $V_{\text{max}}$ were 0.63 mM and 0.69 EU/mL, respectively.

4. Discussion
The most commonly reported expression systems are still prokaryotic or mammalian-cell based (Sørensen, 2010). Each system offers specific properties. The advantages and disadvantages of different expression systems are shown in Table 3. For achieving production of recombinant protein with high quality, there should be compatibility between the host and the desired protein (Costa et al., 2014). Today, efficient strategies for the generation of recombinantly expressed proteins are focused on obtaining production in the least possible time and at the lowest cost. Producing large amounts of recombinant proteins rapidly, efficiently, and economically often requires the use of bacterial host organisms because of their priceless characteristics such as ability to grow quickly, availability of a wide variety of mutant strains, well-known genome, and inexpensive requirements for optimal conditions (Swartz, 2001; Terpe, 2006).

Proteins are commonly used biomolecules in scientific research and in medical and industrial areas. However, it is well known that removal of these molecules from their natural environment is a very difficult and expensive process. Another important problem in this regard is the contamination risk of proteins with disease-causing agents (Houdebine, 2000; Swartz, 2001; Ma et al., 2003). Recombinantly produced proteins are very close to their natural state; thus they can have various features compared with nonrecombinants such as they can be used without triggering an immune rejection, there is no contamination risk with human or animal disease agents because they are obtained using isolated hosts, and their activities are alterable using molecular techniques (Demain and Vaishnav, 2009; Dolinska et al., 2014). Besides the advantages mentioned above there are also some restrictions. The most important hallmark or unwanted phenomenon in production of recombinant eukaryotic proteins using prokaryotic hosts is posttranslational modifications (Kamionka, 2011) such as glycosylation, proteolytic maturation, and disulfide bond formation. Glycosylation is one of the major types and common forms of protein modifications in nature (Chan et al., 2012), and can affect the biological activity, stability, and transports of proteins (Helenius and Aebi, 2001; Solá and Griebenow, 2009). Cholinesterase sequences have individual putative glycosylation signals. However, the location and number of these signals are not well conserved along the family members. Human, rat, and mouse AChEs

Figure 2. Electrophoresis of PCR products for AChE on 1% agarose gel electrophoresis.
display three glycosylation sites and these three sites are well conserved in all mammalians (Soreq et al., 1990). Previous mutagenesis studies have shown that elimination of N-glycosylation had no effect on catalytic activity and peripheral site functions of recombinant human AChE (Velan et al., 1993; Chen et al., 2011a). In contrast to catalytic activity and the assembly of AChE, glycosylation is required for membrane trafficking (Chen et al., 2011b). When considered in this regard, the use of E. coli seems to be compatible for recombinant hAChE production.

**Figure 3A.** Amplification products of recombinant pET-SUMO vector transformed cells using AChE primers (1–8: selected colonies).

**Figure 3B.** Cross PCR analysis of purified recombinant pET-SUMO vector (1, 2; vector reverse and forward primers, 3, 4; gene forward and vector reverse primers, 5, 6; gene reverse and vector forward primers, 7, 8; gene primers).
As we know, the production of native and recombinant proteins in a well-characterized, purified, and biologically active form has become a main goal for many sectors such as the drug, agricultural, and biopharmaceutical industries (Schmidt, 2004; Demain and Vaishnav, 2009). This field not only intends to generate biologically active protein but also seeks to achieve high-throughput (quality, time scale, quantity etc.) production (Sanden et al., 2003; Correa and Oppezzo, 2011). In addition, another major objective of these companies or research groups is to develop new and efficient approaches for generating large quantities of recombinantly produced proteins. To improve these parameters, it is possible to use expression partners (also known as affinity tags or fusion tags) in E. coli. Moreover, these tags can also bring additional properties such as protection from degradation, improvement of affinity for recoveries of proteins, and solubility (Costa et al., 2014). For nearly four decades, fusion tags have been widely used in protein overproduction (Majorek et al., 2014). Polyhistidine tags are especially suited for target protein production with minimal perturbation. In some cases, as we observed, the tag may interfere with enzyme activity (Freydank et al., 2008; Panek et al., 2013). However, generally produced fusion proteins are as active as wild-

### Table 1. PCR product sizes for different combinations.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Template DNA</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Recombinant plasmid</td>
<td>Vector</td>
<td>Vector</td>
<td>2103</td>
</tr>
<tr>
<td>2</td>
<td>Recombinant plasmid</td>
<td>Gene</td>
<td>Vector</td>
<td>1998</td>
</tr>
<tr>
<td>3</td>
<td>Recombinant plasmid</td>
<td>Vector</td>
<td>Gene</td>
<td>1950</td>
</tr>
<tr>
<td>4</td>
<td>Recombinant plasmid</td>
<td>Gene</td>
<td>Gene</td>
<td>1845</td>
</tr>
</tbody>
</table>

### Table 2. Yielding comparisons of recombinant protein.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Expression lysate (mL)</th>
<th>$A_{595}$</th>
<th>Yield (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal medium (uninduced)</td>
<td>100</td>
<td>0.171</td>
<td>18.6</td>
</tr>
<tr>
<td>Induced medium lysate</td>
<td>100</td>
<td>0.547</td>
<td>59.4</td>
</tr>
<tr>
<td>Induced medium concentrated filtrate</td>
<td>100</td>
<td>1.755</td>
<td>190.8</td>
</tr>
<tr>
<td>Concentrated filtrate dialysis</td>
<td>100</td>
<td>0.426</td>
<td>46.3</td>
</tr>
</tbody>
</table>
type ones (Guergova-Kuras et al., 1999; Job et al., 2002). When comparing our results with those of a previous study (Fischer et al., 1993) kinetic constants appear relatively high but product quantity obtained was better than in the mentioned paper. The observed relatively low affinity of rhAChE to acetylcholine iodide (substrate) may be a result of undesirable changes in protein structure caused by uncleaved 6xHis-SUMO tag (Terpe, 2003).

In this work, we obtained a stable recombinant human AChE that was enough to allow kinetic studies from the brain after cloning and expressing with SUMO partner. Recombinant and biologically active enzyme was removed and purified from the pellet fraction of cell lysate. As we mentioned above, the SUMO system is superior for problematic protein expression when compared with other commonly used fusion tags. Another reason for

![Figure 5](image1.png)

**Figure 5.** Western blotting and ImageJ relative intensity ratio analysis of recombinantly produced hAChE using antiHis-tag antibody. GAPDH was used as loading control. M; protein molecular size marker, Lane 1; Negative control (uninduced), Lane 2; Transformed BL21(DE3) cells 7 h after 1 mM IPTG induction.

![Figure 6](image2.png)

**Figure 6.** Optimum pH. The reaction was carried out in the following buffers: sodium phosphate (pH 5.5–8), Tris-HCl (pH 7.5–9), and glycine–NaOH (pH 9–10.5).
Figure 7. Optimum ionic strength. The reaction was carried out in different concentrations between 0.1 and 1 M of glycine–NaOH buffer.

Figure 8. Optimum temperature. The activity was assayed in 1 M glycine–NaOH buffer (pH 9).

Figure 9. $K_m$ was calculated by slope rate of the Lineweaver–Burk double-reciprocal plot representing reciprocals of initial recombinant enzyme velocity versus reciprocals of different ATC concentrations.
preferring this technique is the substrate specificity of SUMO protease. Specific N-terminus amino acids are necessary for biological activity, half-life, and stability of numerous therapeutic proteins. This protease recognizes a specific site that acts as the target for cleavage and does not cleave erroneously.

In summary, advantageous features of this fusion system, which has advanced beyond the others, such as using a single vector, rapid cloning of any gene without restriction digestion, high-level expression, affinity tagging methodology, and tag removal make it useful. Taken together, our findings suggest that His tag SUMO expression system is a rapid and efficient way to produce human AChE and is useful for further studies in order to observe the effect of some chemicals and/or biological components on enzyme activity or structural modifications.

**Table 3.** Comparison of different expression hosts for protein production (Ma et al., 2003).

<table>
<thead>
<tr>
<th>System</th>
<th>Cost</th>
<th>Production time</th>
<th>Scale-up capacity</th>
<th>Product quality</th>
<th>Contamination risks</th>
<th>Storage cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Low</td>
<td>short</td>
<td>High</td>
<td>Low</td>
<td>Endotoxins</td>
<td>Moderate</td>
</tr>
<tr>
<td>Yeast</td>
<td>Medium</td>
<td>Medium</td>
<td>Medium</td>
<td>Medium</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>Mammalian cell</td>
<td>High</td>
<td>Long</td>
<td>Very low</td>
<td>Very high</td>
<td>Viruses, prions, and oncogenic DNA</td>
<td>Expensive</td>
</tr>
<tr>
<td>Transgenic animals</td>
<td>High</td>
<td>Very long</td>
<td>Low</td>
<td>Very high</td>
<td>Viruses, prions, and oncogenic DNA</td>
<td>Expensive</td>
</tr>
<tr>
<td>Plant cell</td>
<td>Medium</td>
<td>Medium</td>
<td>Medium</td>
<td>High</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>Transgenic plants</td>
<td>Very low</td>
<td>Long</td>
<td>Very high</td>
<td>High</td>
<td>Low</td>
<td>Inexpensive</td>
</tr>
</tbody>
</table>

References


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