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High-yield expression and purification of the transmembrane region of ion channel-forming amyloid-β protein for NMR structural studies

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ABSTRACT

Numerous studies of APP in the literature to date have focused on its processing at the plasma membrane, its membrane-bound oligomeric state, and the calcium-permeable ion channel formation of non-fibrillar Aβ in the cell membrane. Despite these studies, little is known structurally about the transmembrane region of APP beyond a theoretical model. This is due to challenges in the expression, purification, and sample preparation of eukaryotic membrane proteins. To determine the three-dimensional structures of the intact transmembrane domain from human APP (hAPP-TM) and to elucidate the structure and formation mechanisms of the hAPP channel, the isotopically labeled protein must be produced and purified in sufficient quantity. Here, we describe a procedure whereby the hAPP-TM peptide, comprising residues 692–723 of hAPP, was successfully expressed and purified sufficiently to perform NMR analysis. To increase expression levels of the target protein, we designed a construct containing two tandem repeats of the target gene. The fusion protein was expressed in the form of inclusion bodies, purified on immobilized nickel affinity chromatography, and chemically cleaved by cyanogen bromide. Final purification of hAPP-TM was achieved by preparative reversed-phase high performance liquid chromatography (HPLC). The final yields of purified hAPP-TM were around 5 mg/l of M9 minimal media.

1. Introduction

Membrane proteins (MPs) constitute about 30% of all proteins in eukaryotic cells [1]. Many are involved in major disease processes in the body, and are therefore primary targets for current or future pharmaceutical applications [2]. Although it is necessary to determine the structures of MPs to understand their biological functions and to design novel drug therapies, few eukaryotic MP structures are known today because they are more difficult to express and handle than soluble proteins. Although the production of many globular proteins by chemical peptide synthesis is now a routine procedure [3–5], the synthesis of MPs is limited to small quantities that are just enough to achieve NMR structural studies. In contrast, heterologous expression of proteins in *Escherichia coli* has been proven to be the most cost-effective and safe method of recombinant protein production, providing the basis for a variety of methods to understand protein structure and activity [6–8]. However, there is no single system to consistently produce high quantities of MPs in *E. coli* due to their low levels of expression, poor intrinsic solubility and inherent toxicity. Thus, each MP requires designing a vector construct suitable for high-level expression, screening for the *E. coli* strain best suited to expression, and optimizing the conditions for expression and purification. In this paper, we demonstrate such optimization for the expression and purification of APP-TM.

One of the hallmarks of Alzheimer's disease (AD) is the accumulation of amyloid plaques between neuronal cells in the brain. These plaques are primarily composed of Aβ, an approximately 40 amino acid peptide [9,10], which is formed as a protein fragment proteolytically snipped from APP. The APP is an integral transmembrane protein which has been suggested to play a central role in the pathogenesis of Alzheimer's disease. To date, most investigators studying the underlying cause of AD have focused on cell biology approaches and the plaque-forming properties of Aβ. This focus has led to the amyloid cascade hypothesis, currently the predominant model for the underlying cause of the disease. However, the precise mechanism of its toxic effect is not yet fully understood, and a number of evidence have led us and others to seriously question the validity of this hypothesis. For example, globular and non-fibrillar Aβ proteins are continuously produced during normal cellular metabolism, and both are also present in Alzheimer tissues [11–13]. Additionally, small, non-fibrillar Aβ protein alone is sufficient to cause cellular degradation [14]. An alternate mechanism for Aβ protein toxicity suggests that

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non-fibrillar Aβ proteins can form calcium-permeable ion channels in the cell plasma membrane [15,16]. These channels may allow excessive calcium influx and disrupt normal cellular calcium homeostasis [17–20]. A number of studies investigating this alternate amyloid hypothesis have focused on APP processing at the plasma membrane [21–24], its membrane-bound oligomeric state [16], and calcium-permeable ion channel formation by non-fibrillar Aβ in the cell membrane. Although understanding the process at the membrane is important, little is known structurally about the entire transmembrane region of hAPP. Due to the previously described challenges in preparing samples of eukaryotic membrane proteins, only a theoretical structural model calculated by the CHARM program supported the hypothesis that small Aβ oligomers can form a large well-defined pore is currently available [25–28]. In this paper, we describe the recombinant production of a 39 amino acid hAPP-TM with C-terminal homoserine lactone comprising residues 692–723 of hAPP. This region contains a 24-residue intact transmembrane domain and an 8-residue periplasmic domain. The APP-TM coding sequence was cloned downstream of a 125 amino acid ketosteroid isomerase (KSI) gene and upstream of a hexa-histidine tag sequence in pET-31b(+) vector and transformed into *E. coli*. To increase the expression level of the target protein, we designed and screened a construction containing two tandem repeats of the target gene and tested several *E. coli* strains for most efficient heterologous expression.

The ^{15}N-labeled fusion protein was expressed in the form of inclusion bodies and purified by immobilized nickel affinity chromatography under denaturing conditions, which prevented aggregation during this process. Target peptide release was chemically accomplished by cyanogen bromide. Final purification of hAPP-TM was achieved by preparative reversed-phase HPLC. The final yields of purified ^{15}N isotope labeled hAPP-TM were approximately 5 mg/l of M9 minimal media. Tris–tricine polyacrylamide gel electrophoresis confirmed that we successfully produced and isolated high purity hAPP-TM, and the final sample was identified by mass spectroscopy. Proper folding of the hAPP-TM in a micellar environment was optimized and measured by heteronuclear NMR spectra recorded on a uniformly ^{15}N-labeled sample. The methods to produce hAPP-TM described here are expected to facilitate the elucidation of the amyloid channel structure and the mechanisms of its formation, critical information for developing therapeutic agents.

2. Materials and methods

2.1. Preparation of oligonucleotides

The 117-base oligonucleotide coding sequence for hAPP-TM was chemically synthesized by Integrated DNA Technologies (USA). hAPP-TM consists of approximately 25 apolar residues followed by 6 moderately polar residues of 2 glycines and 4 lysines. The two methionine residues in position 15 and 31 of hAPP-TM were mutated to isoleucine (M15I, M31I; ATG → ATC) to avoid cleavage at these positions. The sense and antisense primers were annealed by heating to 95 °C and gradually cooling to room temperature over 30 min. After annealing, both N- and C-termini were generated so that cohesive ends were compatible with AlwN I. This unique AlwN I site enables high-yield production of the target peptide by unidirectional insertion of coding sequences immediately adjacent to a methionine residue. Synthetic oligonucleotide sequences and resulting amino acid sequences are shown at the top of Fig. 1. The annealed DNAs were isolated by 2% agarose gel electrophoresis and then purified by a DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. The concentration of the purified DNA was determined spectroscopically.

2.2. Construction of expression vector

The purified DNA fragments encoding hAPP-TM were ligated into the AlwN I digested pET31b(+) vector (Novagen, USA) with Quick T4 DNA ligase (New England Biolabs, USA) at room temperature for 5 min. At the same time, hAPP-TM coding sequences were also formed as tandem repeats upon unidirectional end-to-end self-ligation interspersed with a methionine codon. In pET31b(+), monomeric or multimeric hAPP-TM coding sequence was inserted downstream of a 125 amino acid bacterial KSI gene as a fusion partner and upstream of a hexa-histidine tag sequence as shown in Fig. 1.

2.3. Screening of expression vector

To screen the constructed expression vector for tandem repeats of the hAPP-TM insert, the resulting pET31b-(hAPP-TM)$_n$ plasmids were transformed into Novablue (Novagen, USA) *E. coli* competent cells. Plasmid DNA from individual colonies was purified using a Qiaprep miniprep kit (Qiagen, Germany), and digested with XbaI and XhoI (New England Biolabs, USA). Identification of clones containing the insert DNA was confirmed by electrophoresis on a 1.5% agarose gel. The complete sequence of the inserted gene with 1–3 repeats of hAPP-TM was confirmed by direct sequencing of the plasmid DNA.

2.4. Screening of expression host cell

To get the highest yield of fusion protein, each recombinant plasmid with single, double, and triple inserts was transformed into three different *E. coli* strains. The cultures were grown in LB medium supplemented with appropriate antibiotics at 37 °C, and IPTG was added to a final concentration of 1 mM when the OD_{600} reached 0.5. The expression level of fusion protein in IPTG-induced cultures was checked by 12% tris–tricine PAGE.

2.5. Expression of the KSI-(hAPP-TM)$_2$-His$_6$ fusion protein

E. coli strain C43(DE3) was transformed with pET31b(+) vector containing the KSI-(hAPP-TM)$_2$-His$_6$ construct. For expression, a single colony was used to streak plates containing LB medium supplemented with carbenicillin (50 mg/ml) (Amresco, USA), and the plates were incubated overnight at 37 °C. A 50 ml starter culture was inoculated from a single colony and grown overnight in LB media at 37 °C while shaking. 10 ml of the fully saturated culture was then transferred to 1 l of M9 minimal medium and the culture grown at 37 °C. Uniformly ^{15}N-labeled proteins were overexpressed in M9 minimal media containing 1 g/l ^{15}N-enriched ammonium sulfate (Cambridge Isotope Lab, USA) for NMR structural studies. When the OD_{600} reached 0.5–0.6, expression of the recombinant proteins was induced by the addition of IPTG (Amresco, USA) to a final concentration of 1 mM. The culture was further grown for 14 h after induction, and the cells were pelleted by centrifugation at 6000 rpm and 4 °C for 30 min, and then frozen at −80 °C until used for further studies.

2.6. Purification of the KSI-(hAPP-TM)$_2$-His$_6$ fusion protein

100 ml of lysis buffer (20 mM Tris, 500 mM NaCl, 15% glycerol) containing 1 mg/ml lysozyme (Sigma, USA) as a lysis agent was used to resuspend the frozen cell pellet from 1 l culture. Resuspended cells were lysed by ultrasonication on ice and the soluble part removed by centrifugation at 13,200 rpm for 30 min at 4 °C. The insoluble pellet was resuspended by stirring in 90 ml Ni-NTA binding buffer (20 mM Tris, 500 mM NaCl, 5 mM imidazole, pH 8.0) containing 6 M guanidine HCl

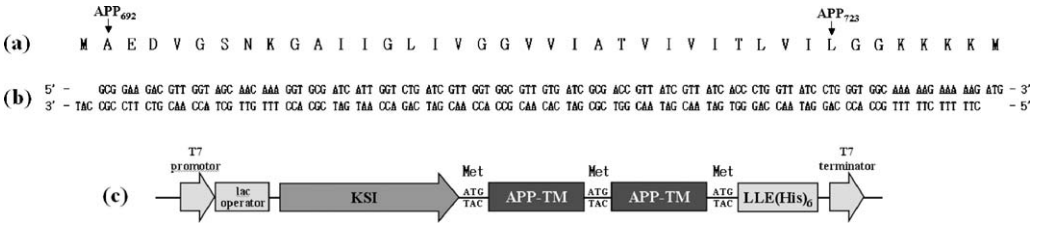

Fig. 1. (a) Amino acid sequence of the recombinant hAPP-TM peptide and (b) the corresponding nucleotide coding sequence of the synthetic hAPP-TM gene. (c) A schematic diagram of the expressed KSI-(hAPP-TM)$_2$-His$_6$ DNA construct. The ATG and its complementary codon for methionine were used in the construction of tandem repeats of the hAPP-TM. Note that methionine is a CNBr chemical cleavage site.