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Expression and purification of human alpha-7 nicotinic acetylcholine receptor extracellular domain in *Pichia pastoris*

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ABSTRACT: Human α-7 nicotinic acetylcholine receptor (α-7 nAChR) is an ion channel that plays a significant function in neuronal communication and development. Defects of α-7 nAChR could cause plethora of neurologic and psychiatric impairments such as schizophrenia, Alzheimer's, and inflammatory disorders. Hence, α-7 nAChR is an interesting candidate for drug development targeting neurological and inflammatory diseases. Therefore, the ability to obtain significant amount of the α-7 nAChR extracellular domain is crucial for further biochemical and structural investigations. We constructed a *Pichia pastoris* yeast strain that secretes the α-7 nAChR extracellular domain. The recombinant protein was hyperglycosylated and could be deglycosylated with Endoglycosidase H treatment. Cross-linking experiments suggested that the recombinant protein could form oligomeric states, consistent with the known property of the native α-7 nAChR. The optimized expression yield was 4.9 mg/l.

KEYWORDS: alpha-7 nicotinic acetylcholine receptor, extracellular domain expression, *Pichia pastoris*

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are groups of cone-shaped pentameric ligand-gated ion channels found in membranes of a variety of cell types, particularly in the neuron (Fig. 1A) [1]. Their functions are to influx the cations from outside of the postsynaptic cell as neuronal transmission. The distribution of nAChRs was in both the central nervous system (CNS) and peripheral nervous system (PNS) of humans [2] as well as in several electric species such as eel, fish, and electric ray [3]. The nAChRs of electric rays were often extracted and used as the model in mechanistic studies to elucidate neuronal transmission [4] and inhibition by various animal toxins [5]. Although the nAChRs from electric rays seem to serve as a good representative model for research for decades, there are still significant differences between the sequence and the structure of nAChRs from electric rays and humans, which could lead to remarkable functional differences. For example, human homopentameric α-7 nAChRs (Fig. 1B) can be activated despite lower affinity towards neurotransmitters [6, 7]. It should be noted that the pentamerization of nAChR in humans can be classified into two types, homopentamer and heteropentamer. Therefore, the differences could be due to the diversity of subunit types of nAChR presents in humans [8], which could assemble into different species of pentamers depending on the differential expression in different cell types. For example, heteropentamer of α-3 and β-4 subunits are distributed in PNS [9], whereas homopentamer of α-7 subunits is distributed in CNS [2]. Thus, the characterization of nAChR from electric species might not offer realistic insights on nAChRs mechanism in humans.

Alpha nAChRs are well-known receptor responsible for neuronal transmission in the CNS of humans and is one of the main targets for neurotoxins [10–12]. The structure of alpha nAChRs comprises 3 parts: extracellular domain (ECD), transmembrane domain (TMD), and intracellular domain (ICD) as shown in Fig. 1A. The ECD of α-2 and α-9 nAChR had been cloned and expressed in *Pichia pastoris* [13, 14]. Since *P. pastoris* is a single cell eukaryote with a lower glycosylation level than *Saccharomyces cerevisiae* [15], the recombinantly expressed proteins in *P. pastoris*

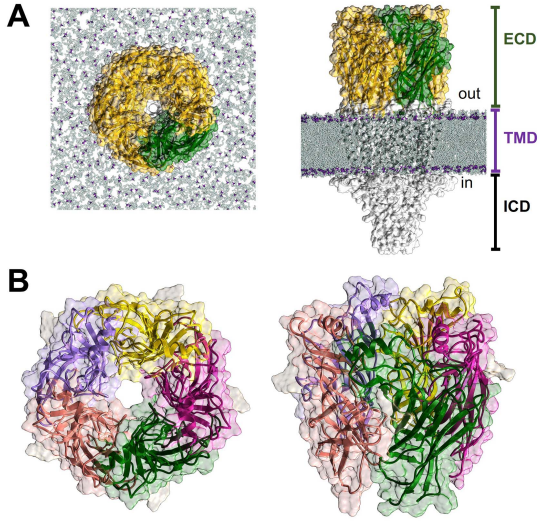

Fig. 1 Schematic representation of α-7 nicotinic acetylcholine receptor (nAChR) and its extracellular domain. (A) The pentameric α-7 nAChR consists of three distinct domains: extracellular domain (ECD) (gold, ECD; green; monomer), transmembrane domain (TMD), and intracellular domain (ICD). (B) Cartoon representation of ECD of pentameric α-7 nAChR.

would likely be correctly folded with post-translational modification that might resemble those found in humans [16]. Moreover, the yeast expression system is more cost-efficient for scaling up than insect or mammalian cell expression systems. However, the expression of most alpha nAChRs in *P. pastoris* did not often result in pentameric state [17] . The success of homopentameric assembly was found in α-2 nAChR crystallization.

Here, we aimed to heterologously produce the ECD of human α-7 nAChR in *P. pastoris* as the ECD constitutes the binding sites for not only the animal toxins (antagonists) but also the neurotransmitters (agonists). The DNA sequence of human α-7 nAChR ECD added with polyhistidine tag was integrated into the host genome via homologous recombination. The concentration of 0.15 M NaCl presented in buffered complex glycerol medium (BMGY), and buffered complex methanol medium (BMMY) resulted in enhanced expression levels. To our surprise, despite the fact that the expressed human α-7 nAChR ECD was heavily glycosylated, the recombinant α-7 nAChR likely produced as oligomers as judged by cross-linking experiments.

MATERIALS AND METHODS

Chemicals, enzymes and vectors

The materials were purchased from different companies: pGEM-T Easy vector system from Promega, USA; pcDNA3.1-CHRNA7 from Addgene, USA; Plasmid extraction kit from Macherey-Nagel, Germany; Agar Bacteriological Grade and peptone from Titan Biotech, India; D-Biotin and yeast nitrogen base from Himedia, India; Protein ladder from Enzmart, Thailand; PCR buffer, DNA ladder, dNTPs, T4 DNA Ligase, and Taq polymerase from Vivantis, Malaysia; Sodium dodecyl sulfate, α-methyl glucopyranoside, and zeocin antibiotic drug from Sigma-Aldrich, USA; restriction enzymes from New England BioLabs, USA; and Gel extraction kit from QIAgen, USA.

Construction of human α-7 nAChR ECD *P. pastoris* expression plasmid

The forward and reverse primers (5′-ACGTCTCAGATC ATGGCGAGTTCC-3′ and 5′-GCGGTCTAGATAGAGTGT CCTG-3′) were designed by using pcDNA3.1-CHRNA7 as the template. Both primers included restriction sites for *BsmB*I and *Xba*I, respectively. PCR amplification was conducted on the human α-7 nAChR ECD gene for 35 cycles started with an initial denaturation at 94 °C for 2 min. Each cycle composes denaturation at 94 °C for 30 s, annealing at 59.5 °C for 30 s, and extension at 72 °C for 30 s. The final extension was conducted at 72 °C for 7 min. Then, the PCR products were separated on 1% low electroendosmosis (LE) agarose gel electrophoresis at 120 V in the Tris/Borate/EDTA (TBE) buffer. The amplified human α-7 nAChR ECD gene was excised from agarose gel followed by extraction using QIAquick® Gel Extraction Kit.

The amplified human α-7 nAChR ECD gene was subcloned into the pGEM-T Easy vector by TA cloning. The constructed plasmid was transformed into *Escherichia coli* DH5-α competent cells (prepared by calcium chloride procedure) via a heat-shock protocol. Single colonies were selected from the Luria-Bertani (LB) agar plate with 100 μg/ml ampicillin to further culture and extract the plasmid. The extracted plasmids were digested with *BsmB*I at 55 °C before incubating with *Xba*I at 37 °C. Restriction fragments with a correct size were ligated with digested pPICZα-B using T4 DNA ligase. The ligation was conducted at 16 °C for 30 min before inactivating at 65 °C for 15 min. The constructed pPICZα-B plasmid with inserted human α-7 nAChR ECD gene was transformed into *E. coli* DH5-α competent cells and selected on low salt LB (NaCl 0.5 g/100 ml) agar with 50 μg/ml zeocin. Single colonies were picked and cultured at 37 °C in low salt LB broth with zeocin, then plasmid extraction was performed. The DNA sequences of the extracted plasmids were confirmed by DNA sequencing at U2Bio sequencing service, Thailand.

Cloning, expression and purification of recombinant human α-7 nAChR ECD in *P. pastoris*

The plasmid was linearized by *Sac*I restriction enzyme before introducing *P. pastoris* X33 competent cells by electroporation as described in the Invitrogen Easy

Select™ *Pichia* expression kit manual. Briefly, the *P. pastoris* X33 competent cell was prepared by treating the cultured cell with cold water and a cold 1 M sorbitol solution. The voltage of 2.0 kV was applied through a single pulse during electroporation. The electroporated cells were then grown on Yeast Extract Peptone Dextrose Medium with Sorbitol (YPDS) agar with 100 g/ml zeocin for colony selection. The agar plates were incubated for three days at 30 °C. The single colony formed on the plates was inoculated into 25 ml of BMGY media with 0.15 M NaCl and grew at 30 °C in a shaking incubator (200 rpm) until cells were in log-phase growth, approximately 17 h. The cells were harvested by centrifugation at 3000 × *g* for 5 min at 25 °C. After decanting the BMGY medium, the cell pellet was resuspended in 200 ml of BMMY with 0.15 M NaCl to induce the protein expression and subsequently transferred to a 1 l flask with a sterile gauze cover. The culture continued to grow in the shaking incubator for the next 72 h. A sample of 1 ml culture was collected every 24 h before the addition of methanol to 0.5% final concentration was performed (induction maintenance). At the end of 72 h of culture, the supernatant containing secreted human α-7 nAChR ECD protein was separated from the cells by centrifugation at 4500 × *g* for 15 min at 4 °C.

Coomassie-stained SDS-PAGE and Western blot were used to analyze protein expression. Primary and secondary antibodies used in Western blot were anti-his tag and goat anti-mouse antibody, respectively. A secondary antibody was conjugated to horseradish peroxidase, and diaminobenzidine (DAB) was used as the substrate for the enzyme activity detection. The glycosylation in protein was investigated by treating the supernatant with Endo H enzyme before Western blot analysis. The supernatant from the colony with the highest protein expression was purified by affinity chromatography using Co^{2+}-IMAC resin. Imidazole was added to the collected supernatant to reach 5 mM final concentration before equilibration with Co^{2+}-IMAC. The resin beads were then washed with sodium phosphate buffer pH 7.4 containing 10 mM imidazole before being eluted by sodium phosphate buffer pH 7.4 containing 250 mM imidazole. The protein concentration after purification was measured by the Bradford protein assay.

Cloning, expression, and purification of maltose-binding protein-fused Endoglycosidase H (MBP-Endo H)

The gene encoding for *Streptomyces plicatus* Endoglycosidase H (Endo) H with a Tobacco Etch Virus protease cleavage site at the N terminus was synthesized (Integrated DNA Technologies, Singapore) as three DNA fragments (Table 1). The fragments were assembled using Gibson Assembly Master Mix (New England Biolabs). The assembled product was then amplified using the primers 5′-ATGGGCGGCCGCAGCGGCGAAAACCTGTACTTCCAGG GCGTGGC-3′ and 5′-CGACGGATCCTCATTACGGCGTA CGAACTGCTTCGCTACCGTACAG-3′, and then cloned into the *Not*I and *Bam*HI sites of pMAL c5x (New England Biolabs). Expression from the construct should yield Endo H with the maltose-binding protein fused at the N-terminus (MBP-Endo H). The sequence was verified with DNA sequencing.

For MBP-Endo H expression, the expression plasmid was transformed into Tuner (DE3). The bacteria were grown in Terrific Broth at 37 °C until OD600 reached 0.6. The temperature was then lowered to 30 °C and protein expression was induced with 0.1 mM IPTG for 6 h. The bacterial cells were then pelleted and frozen at −80 °C until purification.

For MBP-Endo H purification, the bacterial pellet was re-suspended in 20 mM Tris pH 7.5, 200 mM NaCl, and 1 mM EDTA (Endo H column buffer) supplemented with 1 mM PMSF and 30 mg/ml lysozyme. The mixture was sonicated and centrifuged to remove cellular debris. The supernatant was then applied to an amylose resin (New England Biolabs) column. The column was washed extensively with the Endo H column buffer. The bound MBP-Endo H protein was eluted with the Endo H column buffer supplemented with 1 M α-methyl glucopyranoside. The eluted MBP-Endo H was dialyzed against 20 mM Tris pH 7.5, 50 mM NaCl, and 1 mM EDTA and concentrated to 615.3 μM or 45.3 mg/ml before flash-frozen and storage at −80 °C. The molecular weight of MBP-Endo H is 73.6 kDa with the extinction coefficient at 280 nm of 102 680 $M^{-1}cm^{-1}$ (1 mg/ml = 1.395 absorbance unit at 280 nm). The enzyme was catalytically active in the presence of the MBP tag, and the tag was not removed. For enzymatic deglycosylation under native and denaturing conditions, the same protocol for commercial Endo H was followed (New England Biolabs).

RESULTS AND DISCUSSION

Construction of pPICZα-B plasmid with human α-7 nAChR ECD gene

The human α-7 nAChR ECD gene (G23 to Y232) was amplified from pcDNA3.1-CHRNA7 by PCR. After performing agarose gel electrophoresis of the PCR products, a band with the correct size above 600 bp was shown in Fig. 2A. The desired product was extracted from the gel and subcloned into a pGEM-TEAZY vector. After plasmid extraction and double digestion with *BsmB*I and *Xba*I endonucleases, the gene was cloned into a modified pPICZaB with a His tag after the signal peptide and upstream of the restriction sites. The recombinant plasmid was then transformed into competent DH5-alpha cells and subsequently grew on LB/zeocin agar. Double digestion with the same endonucleases used in cloning was performed to identify

Table 1 DNA fragments for assembly into the Endo H gene.

Fragment	Sequence (the overlapping regions are noted with italicized and underlined alphabets)
Endo H 1	GGCGAAAACCTGTACTTCCAGGGCAGTGGCGCGCCGGCTCCGGTCAAGCAAGGGCCGACTTCTGTGGCTTATGTCGA GGTGAACAATAATAGCATGCTGAATGTGGGTAAGTATACTTTAGCGGACGGCGGCGGTAACGCCTTCGATGTTGCGGTA ATTTTCGCCGCTAATATCAACTATGATACCGGTACGAAAACAGCCTACCTCCATTTTAATGAGAACGTCCAGCGCGTCCT CGACAATGCCGTGACACAGATCCGTCCGCTGCAGCAACAAGGTATTAAGGTGCTGCTGAGTGTTTTAGGAAACCACCA GGGA*GCCGGGTTCGCTAATTTCCCCTCACAGCAG*
Endo H 2	*GCCGGGTTCGCTAATTTCCCCTCACAGCAG*GCCGCCTCGGCGTTTGCCAAACAGTTGTCTGATGCTGTTGCTAAGTAT GGCCTCGACGGCGTCGATTTTGACGATGAGTATGCGGAGTATGGCAACAATGGGACCGCCCAGCCGAATGACTCGTC GTTTGTACATCTCGTGACAGCGTTACGTGCGAATATGCCCGACAAAATCATTTCACTTTACAATATCGGTCCGGCGGCAT CGCGTCTGTCCTATGGTGGCGT*TGATGTTAGTGACAAATTTGACTATGCGTGG*
Endo H 3	*TGATGTTAGTGACAAATTTGACTATGCGTGG*AATCCATATTACGGCACATGGCAGGTTCCGGGAATTGCGCTGCCGAAAG CCCAGTTAAGCCCAGCCGCGGTCGAAATCGGCCGTACGTCCCGCAGCACAGTCGCGGATCTCGCCCGCCGTACCGTG GATGAAGGGTACGGTGTTTATCTGACATATAATCTGGACGGGGGTGACCGTACCGCGGATGTCAGCGCTTTTACCCGTG AACTGTACGGTAGCGAAGCAGTTCGTACGCCG

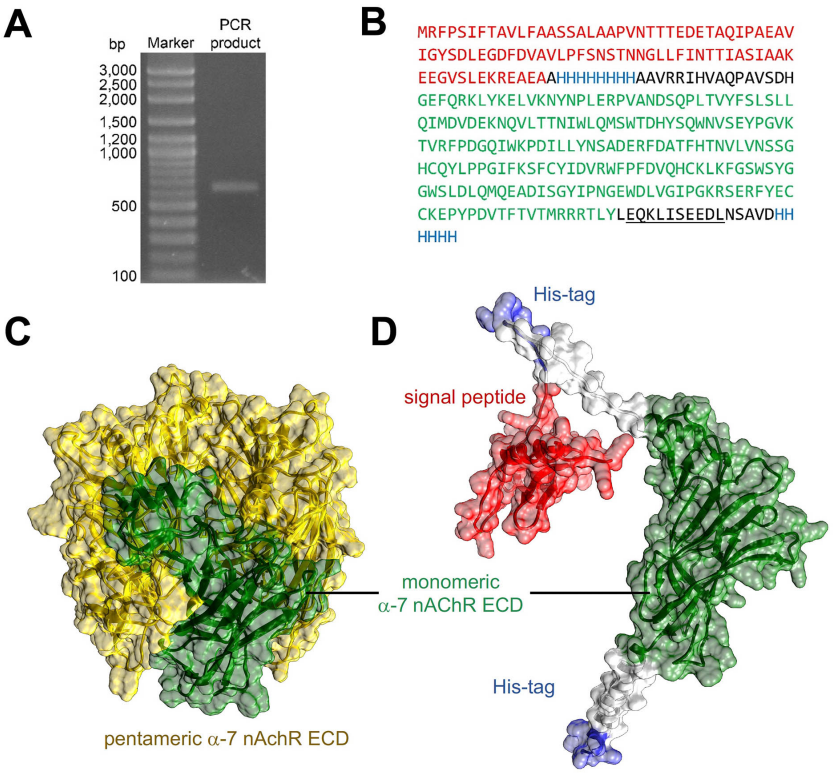

Fig. 2 Construction of recombinant α-7 nAChR ECD. (A) Separation of the human α-7 nAChR ECD gene PCR products by 1% LE agarose gel electrophoresis at 120 V. (B) Amino acid sequence of the human α-7 nAChR ECD expression construct (red, signal sequence; blue, his-tag; black, linker sequence; green, α-7 nAChR ECD). (C) Structure of pentameric human α-7 nAChR ECD (gold, oligomer; green, monomer). (D) AlphaFold v2.0 predicted monomeric structure of the human α-7 nAChR ECD expression construct (red, signal sequence; blue, his-tag; black, linker sequence; green, α-7 nAChR ECD).

positive clones. DNA sequencing confirmed the correct insertion of the human α-7 nAChR ECD gene (Fig. 2B). The mutations at L87S and S149F were noted as PCR errors. These mutations are on the surface of the protein away from the ligand binding site, thus they likely do not affect α-7 nAChR ECD function.

Expression and purification of recombinant human α-7 nAChR ECD

The constructed pPICZα-B plasmid with the human α-7 nAChR ECD gene was introduced into *P. pastoris* via electroporation and successfully integrated into the

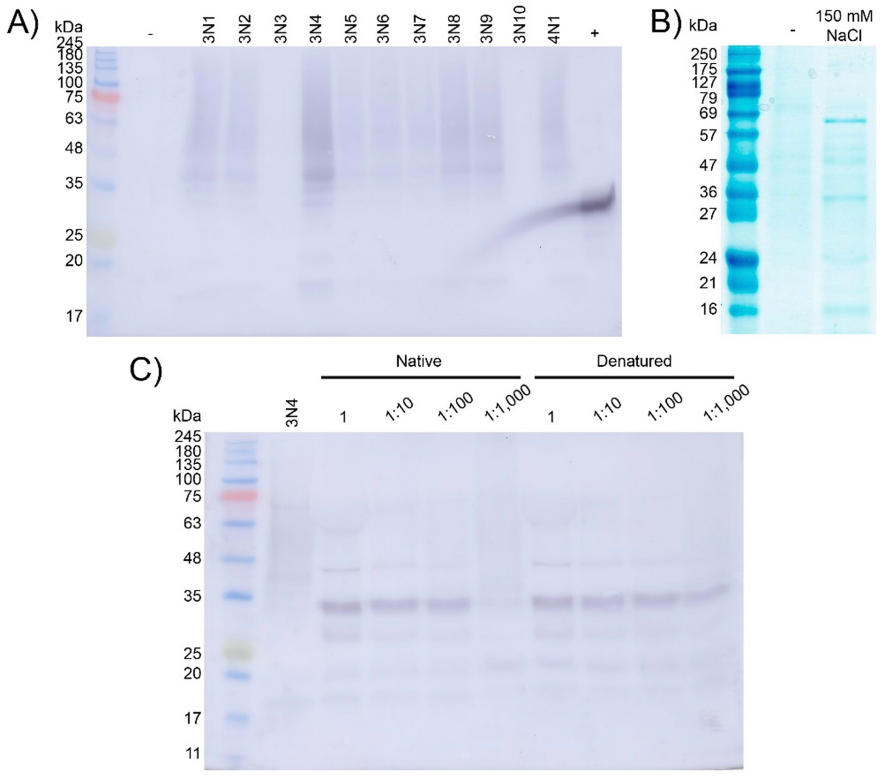

Fig. 3 Optimization and purification of α-7 nAChR ECD. (A) The Western blot probed with a mouse anti-polyHistidine (Abbkine) primary antibody and a goat anti-mouse secondary antibody. Negative (spent media from non-recombinant yeast) and positive controls (His-tagged mannose binding protein) were marked with − and +, respectively. (B) Optimization of α-7 nAChR ECD production in 3N4 clone through addition of 0.15 M NaCl into culture media. (C) Western blot of the deglycosylation of the spent media from the 3N4 clone. The first 3N4 lane is the non-digested control. Deglycosylation of the native and denatured protein was performed using various enzyme dilutions as indicated.

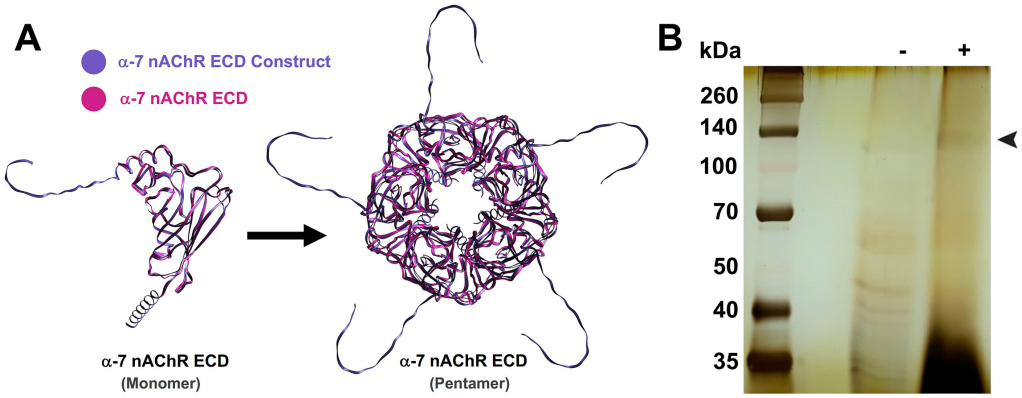

Fig. 4 Oligomerization of α-7 nAChR ECD. (A) Superimposition between α-7 nAChR ECD and signal cleaved α-7 nAChR in both monomeric and pentameric forms. The addition of his-tag and the flexible linkers do not affect the overall structure of the protein and do not hinder the oligomerization. (B) Cross-linking of the expressed protein by 0.12% glutaraldehyde addition analyzed by 10% gel SDS-PAGE and silver staining revealed a potential oligomeric band.

genome of *P. pastoris* X33, as resulted in the growth of a single colony on the YPDS/zeocin agar plate. We picked eleven colonies to express the human α-7 nAChR ECD as a secreted protein. Protein expression was screened for using a Western blot against the hexahistidine tag (Fig. 3A). Nine colonies could express the ECD of human α-7 nAChR, which was considered as 81.82% of successful transformation in *P. pastoris* X33. Colony 3N4 provided the highest expression level. Interestingly, each lane in the Western blot result showed up as a smear. This may be caused by glycosylation that makes the change in protein mobility during SDS-PAGE. As Western blot was quite a sensitive detection method, we scaled up the expression to determine whether same smearing behavior was observed in Coomassie-stained purified protein.

To improve protein expression, we also considered salt concentration as one of the critical factors that could be optimized. BMGY and BMMY media used in the cultivation and expression of proteins in *P. pastoris* only contain a trace amount of NaCl. To further mimic the physiological concentration of NaCl in human cells, we added 0.15 M of NaCl into both BMGY and BMMY media during the expression of colony 3N4, which resulted in the highest level of expression.

Purification of recombinant human α-7 nAChR ECD

The expressed human α-7 nAChR ECD was secreted to the BMMY media. The supernatant was collected, and the cell pellet was discarded. The purification was successfully conducted by using affinity chromatography of Co^{2+}-IMAC resin. The total protein yield was 4.9 mg/l of the bacteria culture, which was about tenfold higher than the yield in the previous study [18]. This work thus demonstrates the establishment of *P. pastoris* as reliable source for the production of recombinant human α-7 nAChR ECD production, which would be valuable for the study of neuronal transmission as well as for the screening of potent drugs against neurological, psychiatric, and inflammatory disorders.

To confirm the glycosylation, the Endo H enzyme was used to deglycosylate the secreted protein (Fig. 3B). The 30 kDa band that corresponded to the molecular weight of α-7 nAChR ECD calculated from the amino acid sequence was observed. The results also demonstrated that the expressed protein was glycosylated in many patterns since various band sizes were noted. Higher molecular weight bands might contain O-linked glycan since Endo H only deglycosylated N-linked glycans [19]. Lower molecular weight bands could be partially degraded α-7 nAChR ECD, likely reaction products of yeast proteases. Notably, the presence of a major band at (30 kDa) is well correlated with the size of the monomeric α-7 nAChR ECD. It should be noted that these results were in agreement with the expression of human neuronal nicotinic receptor α4 and β2 subunits in *P. pastoris*, which were also expressed in glycosylated forms [20]. Besides *P. pastoris*, the glycosylation of recombinant α-7 nAChR was also detected in the proteins expressed in *Xenopus* oocytes [21]. Prediction of glycosylation site by NetNGlyc 1.0 Server [22] revealed only three possible N-glycosylated residues in the ECD of human α-7 nAChR: N24, N68, and N111.

The oligomerization of proteins in media was investigated by chemical cross-linking using 0.12% (v/v) glutaraldehyde. SDS-PAGE analysis of the cross-linked specimen displayed a clear shift in the band position. Clear bands at ∼140 kDa and higher could be detected (Fig. 4). This thus suggests the presence of higher order structures, presumably the oligomeric state, in the secreted media.

CONCLUSION

Recombinant human α-7 nAChR ECD was successfully expressed in high amounts as a soluble protein in *P. pastoris*. The expressed proteins were secreted into surrounding BMMY medium and thus allowing a simpler harvest and purification strategy. The recombinant protein could form oligomers as suggested by cross-linking. The glycosylation patterns were diverse and possible to be removed by the Endo H enzyme. This work thus demonstrates the potential of *P. pastoris* as a candidate host for the production of recombinant nAChRs.

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