Cloning and Expression of Rat Brain Acetylcholinesterase Enzyme in *Escherichia coli*

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ÖZET: In this study, the gene region of rat (*Rattus norvegicus*) brain acetylcholinesterase enzyme was amplified in PCR with designed forward and reverse primers and it was ligated into a pET-SUMO vector under suitable conditions. This recombinant vector was transformed to competent Escherichia coli cells and it was grown in liquid LB medium including kanamycin. Colony PCR was performed from growing colony and PCR products were checked with agarose gel electrophoresis. The correct colonies were grown in a liquid medium for plasmid isolation. After plasmid isolation, these recombinant constructs were used for whether the gene inserts properly with cross-PCR. After determining the accuracy of the plasmid, recombinant vectors were transferred into the *E. coli* BL21 (DE3) cells to perform protein production. Cells were grown in IPTG induced larger media for hours. Enzyme activity and SDS-PAGE analysis were performed from homogenate for each treatment samples.

Keywords: *Rattus norvegicus*, Acetylcholinesterase, Cloning, pET-SUMO vector, *E. Coli*

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INTRODUCTION

Acetylcholine is an ester with important biological effects. The strong pharmacological effect of acetylcholine was determined in 1906 (Nachmansohhn 1952). Acetylcholine, whose effects on heart tissue were revealed for the first time by Henry Hallett Dale in 1914, is a neuromodulator that plays a role in the central and peripheral nervous system. Choline is synthesized from acetyl CoA and choline by the enzyme acetyltransferase. The enzyme acetylcholinesterase (AChE), which is abundant in the synaptic space, breaks down free acetylcholine into choline and acetate metabolites (Nirogi et al. 2010).

Acetylcholinesterase hydrolyzes acetylcholine, a neurotransmitter in cholinergic synapses. Acetylcholine released into the synaptic space binds to its receptors in the postsynaptic membrane (Lester 1977). AChE in the same region terminates signal transmission by hydrolyzing acetylcholine (Whittaker 1990). AChE with very high catalytic activity breaks down 25,000 acetylcholine molecules per second (Quinn 1987). The released choline is taken up again by the presynaptic nerve. Choline combines with acetyl CoA catalyzed by cholineacetyltransferase to form acetylcholine again (Kim et al. 2006).

Hydrolysis of acetylcholine is regulated by cholinesterase inhibitors. There are two types of cholinesterase enzymes. These include acetylcholinesterase and butyrylcholinesterase. Substances that inhibit the enzyme acetylcholinesterase are defined as cholinesterase inhibitors or anticholinesterases (Colovic et al. 2013). Cholinesterase inhibitors that inhibit hydrolysis of acetylcholinesterase enzyme contribute to central and peripheral cholinergic function and strengthen this function (Alaşehirli 2005).

Since pathogenesis and pathophysiology of Alzheimer's disease can not be fully clarified, there is no adequate and complete preventive treatment for today (Golde 2003). Acetylcholinesterase inhibitors (AChEI) are the most effective treatment agents proven (McGleenon et al. 1999). AChEIs prevent the destruction of acetylcholine by cholinesterase and increase the amount of acetylcholine in neuronal synapse and prolong the biochemical and functional effect of acetylcholine in the brain (Snowdon et al. 1997).

In this study; In this study, rat AChE gene was cloned into pET-SUMO vector and acetylcholinesterase enzyme, a product of this gene, was produced recombinantly in *E. coli* cells and the activity and molecular weight of the produced enzyme were determined.. In the following stages, it is thought that AChE enzyme purification and characterization can be carried out to prevent drug studies.

MATERIALS AND METHODS

Total RNA Isolation and cDNA Production

Total RNA was isolated rat brain by using Méndez's method (Méndez et al. 2011). The obtained products were checked by agarose gel electrophoresis and NanoDrop. cDNA library was performed according to the reverse transcriptase method. For this process, First-Strand cDNA Synthesis Kit (Invitrogen, USA) and Oligodt primers were used.

### Plasmid construction and cloning of AChE

For recombinant production of AChE protein, the coding sequence of rat AChE (NM_172009) was cloned into the vector (pET-SUMO). The rat AChE gene sequence (NM_172009) was amplified with designed forward and reverse primer with PCR, sequence information of the primers is given in Table 1. Afterwards, AChE gene was amplified by applying the PCR parameters given in Table 2. PCR products were checked by agarose gel electrophoresis and PCR product was purified from agarose gel. This sequence was cloned into the pET-SUMO vector (Figure 1).

Table 1. Primer sequences used in amplification of the rat AChE gene sequence.

Primer	5'-3'
Forward	ATGAGGCCTCCCTGGTATCC
Reverse	TCACAGGTCTGAGCAGCGT

Table 2. PCR parameters applied in amplification of AChE gene

Steps	Temperature (°C) / Time / Cycle
Pre-denaturation	94 / 4 min / 1
Denaturation	94 / 90 s / 30
Annealing	60 / 40 s / 30
Extension	72 / 100 s / 30
Final extension	72 / 10 min / 1
End of the reaction	4 / - /-
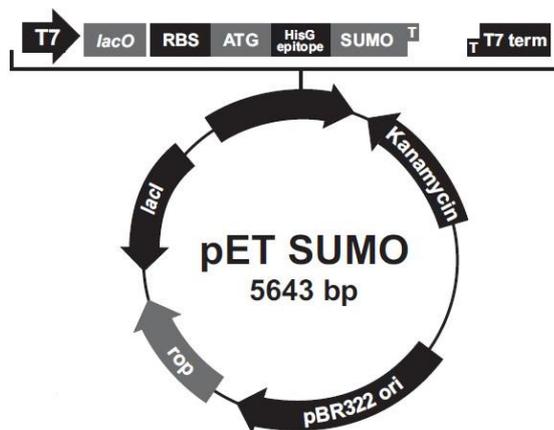
Figure 1. pET-SUMO vector map

Recombinant protein expression

Obtained recombinant vector was transformed to component *E. coli* One Shot Mach-T1^R cells and ıt was incubated for 1 night at 37°C in LB medium (1% tryptone, 0.5% NaCl, 2% agar and 0.5% yeast) containing 50 µg/mL kanamycin. Colony PCR was performed to determine the colonies carrying the recombinant vector. Four combinations of vector-specific and gene-specific primers (gene-gene, vector-vector, gene forward-vector reverse, and gene reverse-vector forward) were used to determine whether the vector was an insert. Plasmid isolation was performed.

Isolated plasmids were transferred to *E. coli* BL21(DE3) cells and grown in LB plates to perform protein production. Transfer procedure was performed by classical heat shock method. A single colony was taken from the agar plate and inoculated in 10 mL of LB medium and left in an incubator at 37°C and 200 rpm for 1 night to prepare the preculture.

To obtain the recombinant protein, 1 mL of preculture was added to 200 mL of 50 µg/mL antibiotic and 1% glucose including LB medium. When OD600=0.6 measured in the spectrophotometer, IPTG was added to the medium and incubated at 200 rpm and 37°C.

Protein purification and pilot expression of AChE

After IPTG induction, cells were centrifuged at 3000 rpm for 5 minutes to determine whether protein was produced. Cell pellets were resuspended in 1 mL of lysis buffer. Next, the cells was sonicated and lysed by a freeze-thaw cycle (3 times) with liquid nitrogen (-196°C) and a 42°C water bath. Then

the cell lysate was centrifuged at 10.000 rpm for 1 min. The supernatant was taken into a new tube and stored at -20°C.

Produced Rat AChE protein concentration was detected by the Bradford method (Bradford 1976) using Bovine Serum Albumin (BSA). The absorbance was measured in the spectrophotometer at 595 nm. AChE enzyme activity was determined by Ellman's method (Ellman et al., 1961). Time dependent expression level (0-7 s) and molecular weight of the produced recombinant protein were analysed by SDS-PAGE (12%) method.

RESULTS AND DISCUSSION

Qualitative Determination of Isolated RNA by Agarose Gel Electrophoresis and cDNA Library

Total RNA isolation from rat brain tissue was performed according to the manufacturer's protocol and qualitatively determined by agarose gel electrophoresis (Figure 2) and rat cDNA library was performed (Figure 3).
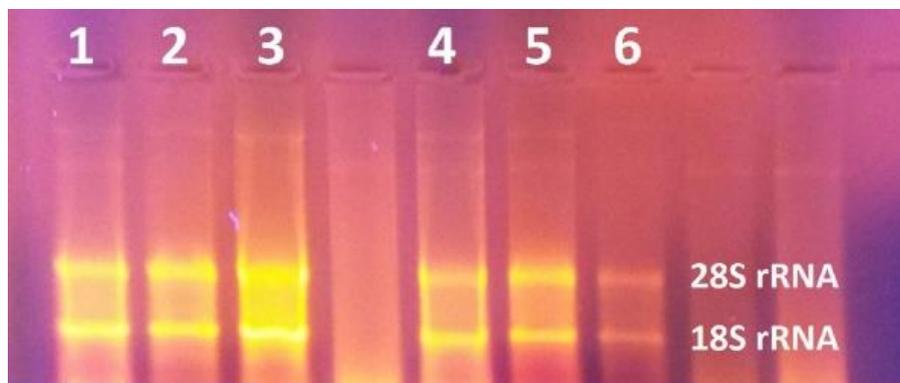

Figure 2. Gel image of total RNA from rat brain tissue (1, 2, 3, 4, 5, 6 gel RNA samples loaded with 18S and 28S rRNA bands)
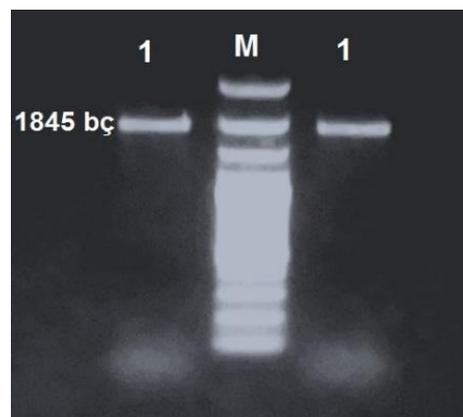

Figure 3. The gene region obtained by PCR from the cDNA library. (1: PCR sample loaded on gel, M: Marker)

Cloning of ACHE into pET-SUMO vector

The rat AChE sequence was ligated and cloned into the pET-SUMO vector. Correct placement of the gene in the pET-SUMO vector was confirmed by colony and cross PCR (Figure 3, Figure 4 and Figure 5).
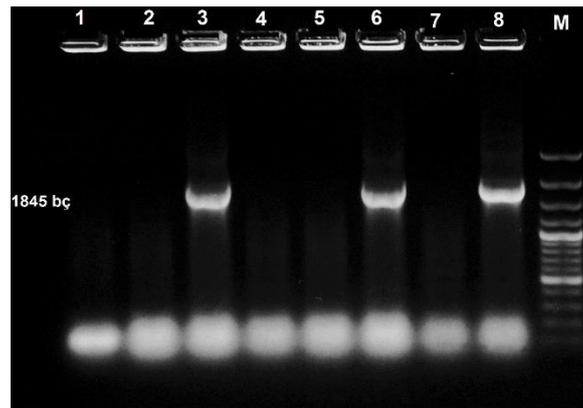
Figure 3. Gel image of bands obtained from colony PCR with primer of target gene. (M: Marker, 1, 2, 3, 4, 5, 6, 7, 8: Colonies enumerated for PCR, 3rd, 6th and 8th colonies were evaluated as positive because they gave bands in the 1845 bp)
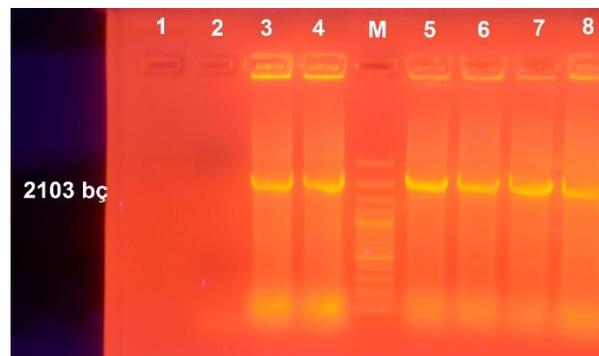
Figure 4. Gel image of bands obtained by colony PCR with vector primer. (M: Marker, 1, 2, 3, 4, 5, 6, 7, 8: Numbered colonies for PCR, 3rd, 4th, 5th, 6th and 8th colonies were positive because they gave band in 2103 bp region. we evaluated)
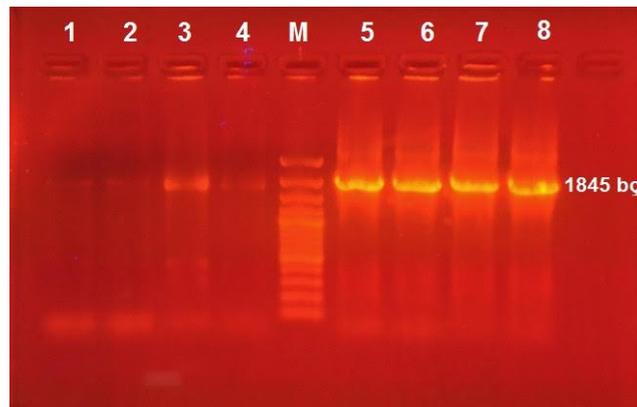
Figure 5. Gel image obtained by cross-PCR from plasmid

Pilot expression of AChE

The protein quainty of the samples taken from the samples induced by IPTG at the 1st, 2nd, 4th, 6th and 7th hours are indicated in Table 3. Accordingly, samples induced with IPTG at 6 and 7 hours were determined to contain approximately 2.7 fold more protein than samples not induced by IPTG. Then cells were harvested and cell lysate was detected by SDSPAGE. With SDS-PAGE analysis, the presence of approximately 80 kDA of product was detected in the gel. (Figure 6).

Table 3. Activity measurement results of acetylcholinesterase enzyme

Time of Samples (h)	IPTG + (unit)	IPTG − (unit)
0	16	12
2	27	16
4	44	22
6	72	26
7	68	24
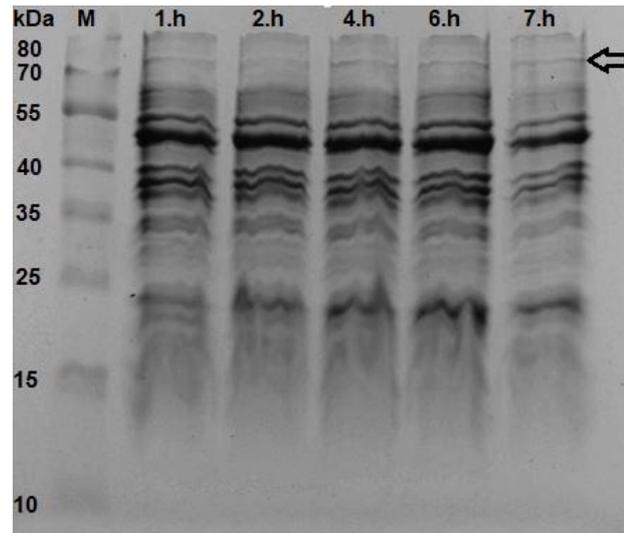
Figure 6. SDS-PAGE analysis of cell homogenate obtained after lysis (M: Marker, kDa: Kilodalton, 1.h, 2.h, 4.h, 6.h, 7.h: When samples were taken)

In this study, it was aimed to clone acetylcholinesterase (AChE) enzyme from rat brain tissue and express it using a prokaryotic host. In this context, the region encoding the enzyme was isolated from the cDNA library of the relevant tissue using specific primers. The resulting gene region was transferred to a vector for cloning purposes. This recombinant vector was then transferred to a suitable expression host to produce the enzyme.

AChE plays a biological role in neurotransmission at synapses at the cholinergic junction by rapidly hydrolyzing its substrate acetylcholine to acetate and choline (Soreq and Seidman, 2001). In addition, non-cholinergic roles of cholinesterase such as cell adhesion, neurite outgrowth, and apoptosis have been reported in vertebrates and invertebrates (Park et al., 2004; Zhang and Greenberg, 2012; He et al., 2012; Lu et al.,2012).

Acetylcholinesterase enzyme was obtained recombinantly from different species such as human (Velan et al. 1991), rat (Legay et al. 1993; Coussen et al. 1995) and mouse (Vellom et al. 1993) for different purposes. The main objectives of the studies are structural analysis (Kronman et al. 1992), post-translational arrangements that occur during the maturation of the protein (Coussen et al. 1995), and the explanation of the substrate relationship of AChE, which is different from other cholinesterases (Vellom et al. 1993). It was on the clarification of tasks and their reasons. In these studies, the enzyme was successfully produced in many different hosts, from mammalian cell lines (Lazar et al. 1993) to plants (Yamamoto et al. 2009).

Recombinant therapeutics can be obtained using microbial systems, animal and plant hosts. Microbial systems are one step ahead at this point compared to other intermediaries, as they have the capacity to produce fast and high-yield products in addition to their low cost table. In addition, the

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microbial systems do not have a contagious viral concern and are accepted by the authorities as the most suitable hosts recommended for this purpose (Picanco-Castro et al. 2013).

SUMO proteins, like ubiquitins, are synthesized in a precursor form before being conjugated to the target protein. The main difference between these two groups is that while ubiquitins are targeted to a protein for its degradation, cellular processes such as nuclear transport, transcriptional regulation and protein stability occur on the SUMO-targeted protein (Boggio et al. 2004). Producing efficient and soluble recombinant protein, especially in low-level hosts such as *E. coli*, is one of the most important problems encountered (Wang et al. 2010). To overcome this problem, approaches such as the use of fusion proteins, promoter alteration and chaperone co-expression are preferred (Thomas and Baneyx 1997; Zhang et al. 1998).

Fusion proteins not only ensure the expression and proper folding of the target protein, but also protect the related protein from degradation, facilitate purification and detection. For this reason, it has started to be preferred quite frequently in recombinant production techniques. The most commonly used fusion proteins are maltosebinding protein (MBP) (Pryor and Leiting 1997), NUS A (De Marco et al. 2004), glutathione S-transferase (GST), ubiquitin (Ub), thioredoxin (TRX) (LaVallie et al. . 1993) and SUMO (Catanzariti et al. 2004).

Some recent studies have shown that acetylcholinesterase may be a marker and regulator for apoptosis (Park et al. 2004; Ye et al. 2015). Therefore, conclusions can be drawn that AChE may be a promising tumor suppressor (Lu et al. 2013).

Neurodegenerative diseases are a type of neurological diseases, and they generally manifest themselves with the loss of function of neurons over time and ultimately the death of the cell. Today, the causes and pathological mechanisms of the formation of these diseases have not yet been fully explained (Lu et al. 2013). Many neurodegenerative diseases are characterized by disturbances in the cholinergic system. For this reason, inhibition of cholinergic system components or keeping the amount of molecules targeted by these components at an optimum level is seen as the most common and most likely approach for the treatment of related diseases (Holzgrabe et al. 2007). However, since these types of diseases are generally progressive, current treatment strategies can only slow down the progression seen in the pathogenesis of the disease (Dorronsoro et al. 2003).

The acetylcholinesterase enzyme is a very sensitive enzyme that can lose its activity quickly. With the help of manipulative techniques such as directed mutation generation methods, it is possible to produce more useful products both in the industrial and therapeutic fields, which can maintain the initial activity for a longer time by increasing the stability of the enzyme with the changes made on the active site.

It has been proven in the literature that bacterial production systems are very suitable models for the production of recombinant products. However, the biggest disadvantage of these systems is that they cannot produce after a certain point because the growth medium is not sufficient after a certain level. Today, specialized systems called bioreactor/fermentor are used, especially for microbial models. With the mentioned systems, the production of microbial biomass, enzymes, metabolites and recombinant products can be done on larger scales. Considering that the amount of product and productivity are obtained from a very small volume in our study, it is thought that better results can be obtained when the production is done by using this type of intermediaries.

CONCLUSION

In summary, *Rattus norvegicus* AChE gene cloning and expression have been performed, which is of extremly importance to enable further studies on the characterization of AChE enzyme. Today, developing technology allows the design and construction of novel proteins by modification of the amino acid sequence *in vitro* using recombinant DNA technology. In this way, it is thought that research can be done about how mutations can be made in vitro in the active part of AChE enzyme and how enzyme activity can change. Whereby about the molecular structure of AChE enzyme shown as targets for drug design and function can be made more extensive information. We believe that obtaining this data may allow the design of selective inhibitors against this enzyme, which has an important place in the cholinergic junction.

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Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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