Cloning and Expression of Human Neuron-Specific Enolase cDNA in *Escherichia coli*

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Translated from *Byulleten' Eksperimental"noi Biologii i Meditsiny*, Vol. 152, No. 8, pp. 166-169, August, 2011
Original article submitted February 14, 2010

cDNA fragment encoding neuron-specific enolase was amplified from the cDNA library of human brain. Then the fragment was cloned for expression in *E. coli* using the vector pET28-a. High level of neuron-specific enolase expression was confirmed by SDS-PAAG electrophoresis and immunochemical identity by immunoblot analysis. The constructed producer strain is the cheapest source of neuron-specific enolase suitable for the use in diagnostic applications.

Key Words: *neuron-specific enolase*; *recombinant E. coli; central nervous system*

Enolase is key glycolytic enzyme specifically catalyzing transformation of 2-phosphoglycerate into phosphoenolpyruvate in all mammalian cells. During embryogenesis, only cells of the nervous system begin to synthesize specific γ-homodimer of enolase which is expressed in CNS throughout the ontogeny [2]. Neuron-specific enolase (NSE) differs by some parameters of enzyme activity from non-neuronal enolase. These differences are directly related to peculiarities of neuronal glycolytic pathway, clearly indicating the growing importance of glycolysis in neuronal activity. The role of NSE metabolism significantly increases during pathological processes in the brain and in particular during neuronal hypoxia prevailing in the pathogenesis of brain ischemic damages [1].

CNS tissue damage, in turn, leads to neuronal death and impairs resistance of cell membranes. In this case, NSE leaks first into the intercellular space and then into patient's blood. This explains the phenomenon of significant increase in NSE concentration in biological fluids [3,8].

Quantitative analysis of NSE in the cerebrospinal fluid and blood of the patient has long been the method of choice in the laboratory evaluation of the severity of CNS neurodegeneration. It is widely used in clinical and laboratory practice for the diagnosis, prognosis, and evaluation of treatment efficacy for a wide range of nervous and mental disorders [1].

Considerable amounts of standard preparations of the test antigen are needed for the development of quantitative immunochemical methods of NSE assay (enzyme immunoassay, immunochemical, immunoblotting, *etc*.) [1]. Obtaining the native immunochemically pure preparation from biological substrates is long and laborious. On the other hand, the preparations of neurospecific proteins derived from native mammalian brain are virtually impossible to standardize for the use in clinical laboratory test systems, and that has an impact on their quality.

The aim of our study was to obtain an effective producer of human recombinant NSE on the basis of *E. coli* BL21(DE3) strain and pET-28a cloning vector (Novagen) for use as standard antigen for the development of next generation immunoassays.

MATERIALS AND METHODS

Bacterial strains of *E. coli* XL-1Blue (Stratagene) and BL21(DE3) were cultured in Luria-Bertani medium

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0007-4888/11/1522-0206

with 30 mg/ml kanamycin. Transformation was carried out by electroporation or by calcium method [7].

We used a library of complementary DNA (cDNA) of human brain (Invitrogen). PCR amplification of NSE cDNA fragment was carried out with primers NSE-f (5'-ATGTCCATAGAGAAGATCTGG-3'), NSE-r (5'-TCACAGCACACTGGGATTAC-3') and DNA polymerase (Taq; Company SibEnzyme Ltd) with the following program: 25 cycles of 94°C for 30 sec, 61°C for 40 sec, 72°C for 2 min; 72°C for 10 min. The resulting product was reamplified in the next round using primers rHCE-f (GCGCCATATGTCCATAGAGAAGAT) and rHCE-r (GCGAATTCAGGAATCACAGCACACT). All the genetic engineering manipulations were carried out according to standard protocols [6]. The resulting PCR product was treated with restriction endonucleases EcoRI and FauNDI (Company SibEnzym). Vector pET-28a was treated with the same enzymes, and then dephosphorylated by adding alkaline phosphatase CIAP (Company SibEnzym). The reaction products were separated by preparative electrophoresis in 1.5% agarose gel and isolated using silica-spin microcolumns (Helicon) in accordance with manufacturer's recommendations. The eluted fragments were ligated together with bacteriophage T4 DNA ligase (Company SibEnzym). The preparation obtained was used for electrotransformation of competent cells *E. coli* XL-1Blue. Clones containing recombinant plasmids were selected by PCR with primers NSE-s (5'-GTGAAGGAAGCCATCGACAA-3'), T7term (5I'-GCTAGTTATTGCTCAGCGG-3') and using the following program: 30 cycles of 94°C for 15 sec, 61°C for 30 sec, 72°C for 40 sec.

Plasmid DNA was isolated from 5 ml overnight culture using a kit for release of plasmid DNA (Invitrogen) in accordance with manufacturer's recommendations. Selected plasmids were cleaved with restriction endonucleases FauNDI% and EcoRI (Company SibEnzym).

Sequencing was performed using primers T7prom (5'-TAATACGACTCACTATAGGG-3') and T7term, with BigDye Terminator Kit (Amersham) and automatic sequencer ABI 3100 Genetic Analyzer (Company PINNY).

E. coli BL21(DE3) strain was transformed with a plasmid pT7NSE-1 and inoculated into 50 ml medium LB containing 30 μg/ml kanamycin. Then the optical density of the culture at 550 nm reached 1, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM. After onset of induction, the cells were incubated for 3 h at 37°C with continuous shaking (200 rpm), and 1 ml aliquots were taken after 30 min and 1, 2, and 3 h. Aliquots of the culture were centrifuged for 3 min at 13,000*g* in a microcentrifuge. The sediments were resuspended in 100 μl lysing buffer (50 mM Tris HCl, pH 6.8, 100 mM β-mercaptoethanol, 1% sodium dodecyl, 0.0025% bromophenol blue, 10% glycerol). After heating at 100°C for 3 min, the samples were centrifuged again, and 15 μl supernatant was applied onto 12.5% SDS-PAGE gel. Protein bands were transferred to a nitrocellulose membrane by using a Bio-Rad blotter in 0.05 M Tris-glycine buffer with 20% methanol at a current of 0.8-1.0 mA/cm^2 for 2-3 h. After transfer, nitrocellulose membrane was washed with distilled water and stained with 0.1% Ponso C (Pancreac) assessing transfer efficiency. Then immunochemical visualization was performed using NSE with mouse monoclonal antibodies against NSE (Abcam). After incubation with primary antibody (1 h), the membranes were washed 3 times with phosphate-buffered saline, and biotinylated anti-mouse horse IgG (1:200; Vector Labs) in 1% normal horse serum were added. Then the membranes were washed again in phosphate-buffered saline and visualized with NSE substrate mixture (0.025% diaminobenzidine in 0.01% solution of H_2O_2). The membranes were washed in distilled water, air-dried, and scanned.

The obtained producer strain was used for isolation and purification of recombinant NSE product. Preparation and purification of the protein was performed by metal chelate chromatography. To this end, 2 ml suspension of the producer stock strain was added to 100 ml of broth medium (LB, 30 μg/ml kanamycin) and incubated on a rotary shaker incubator (37°C, 200 rpm) till the optical density OD_{600} reached 0.7. Then IPTG was added to the suspension to a final concentration of 1 mM and continued to incubate under the same conditions for another 4 h. At the end of each incubation, the suspension was centrifuged for 30 min at 3500*g* at 4°C to precipitate the cells. The sediment was subjected to 3-fold freeze-thawing cycles, then lysed in buffer A (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris Cl, pH 8.0) at the rate of 5 ml per 1 g of wet precipitate for 45 min on a rotary shaker (200 rpm, 20°C). The obtained product was passed twice through a syringe with a 0.2 mm needle to reduce viscosity and centrifuged at 13,000*g* and 4°C for 30 min. Agarose equilibrated with buffer A Ni-NTA was added to the removed supernatant and incubated with moderate shaking for 30 min at 20°C. The suspension was then transferred to a column, the carrier was washed with 10-fold volume of buffer B (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris Cl, pH 6.3). The product was eluted in buffer C (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris Cl, pH 4.5) in a volume equal to 2 volumes of column; 0.5 ml eluate samples were taken. The product was stored at -20°C. The purity of the obtained product was assessed by SDS-PAGE electrophoresis. Immunochemical characterization was performed by immunodiffusion and immunoblotting.

RESULTS

cDNA library of normal human brain was used as a source of cDNA. The sequences of primers were designed on the basis of previously determined sequences of human NSE mRNA [6]. NSE cDNA fragment was amplified in two rounds of PCR using internal (nested) primers containing synthetic recognition sites for restriction endonucleases NdeI and EcoRI. When rendering the reaction products in 1.5% agarose gel, the product was detected with the expected molecular weight of 1300 b.p.

The obtained PCR product was cloned into the vector pET-28a carrying the promoter and terminator of T7 bacteriophage, as well as efficient translation initiation site. Ten transformants selected at random were tested for the presence of the insertions. If amplified, all tested colonies gave the product of expected molecular mass. Among them, six clones were randomly selected to isolate plasmid DNA for subsequent restriction analysis and determination of the nucleotide sequence of insertions.

Plasmid from the clone containing insertion with intact nucleotide sequence, was used to transform the producing strain BL21(DE3), carrying the gene for the RNA polymerase of T7 bacteriophage under the control of the lac-promoter.

The obtained transformant was grown in broth culture to the mean log-phase and induced by adding IPTG. Aliquots taken after 30 min and 1, 2, and 3 h were analyzed by SDS-PAGE electrophoresis. After adding IPTG, aliquots accumulatied the protein with the expected molecular mass (47 kDa; Fig. 1, *a*).

The purity of obtained NSE preparation was more than 95%. The average yield was 2-3 mg per 100 ml of induced culture. Immunochemical identity of the recombinant NSE preparation was confirmed by Western blot analysis with monoclonal anti-NSE-antibodies. The NSE preparation was visualized in the zone corresponding to the molecular weight of native NSE (Fig. 1, *b*).

Thus, the cloning of the PCR product into the vector pET-28a yielded *E. coli* strain capable to express recombinant NSE immunochemically identical to the preparation derived from brain tissue by previously described methods [2].

Quantitative ELISA of NSE in biological fluids are firmly established in clinical laboratory practice as a reliable diagnosing and monitoring method which can readily assess the effectiveness of therapies for various CNS diseases [4].

However, in the development of standardized tests in most cases still NSE preparations, derived from brain tissue by methods preparative protein chemistry, are used. Such preparations can not be completely standardized. That does not allow to compare the results of NSE quantitative analysis, obtained using different test systems.

Wide-scale introduction of recombinant DNA technology enables us to obtain a standardized antigen, which can be used for obtaining EIA components. We have previously described a method of solid-phase ELISA of glial fibrillary acidic protein (GFAP) on the basis of recombinant protein and antibodies obtained by immunization with recombinant GFAP [5].

In this study we used a similar approach to produce recombinant NSE. The technique obtains high-

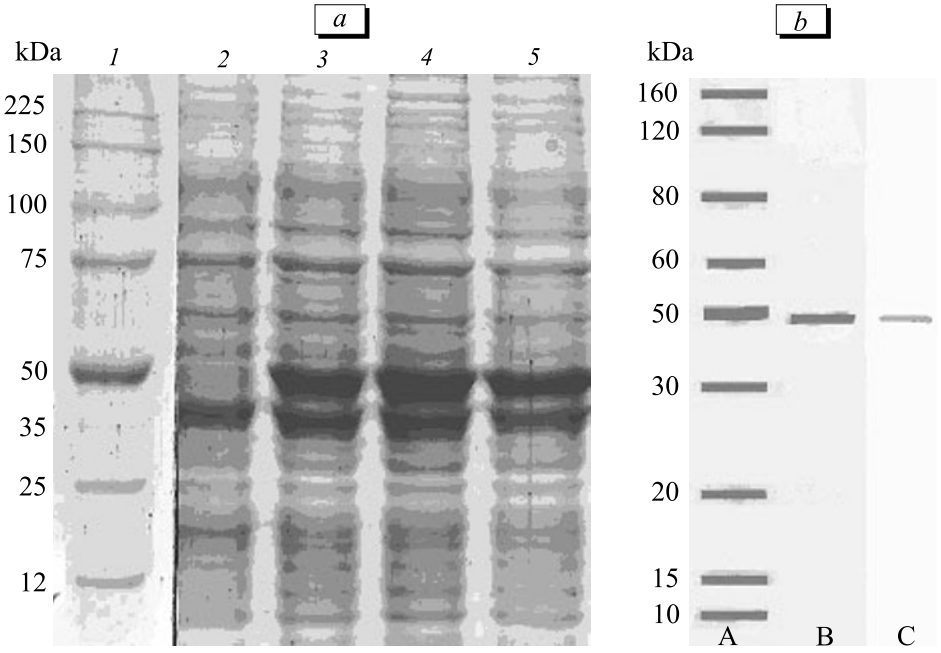

Fig. 1. Electrophoresis of total protein from NSE clone-producer (*a*) and Western blot analysis of protein preparation from NSE producer clone with monoclonal anti-NSE-antibodies (*b*). *1*) non-induced culture; *2-5*) culture 0.5, 1, 2 and 3 h after induction, respectively. A: protein markers of molecular aggregations; B: electrophoresis of the purified NSE preparation in 7.5% polyacrylamide; C: immunochemical NSE visualization with monoclonal anti-NSE-antibodies.

level expression of the target NSE cDNA after induction of the promoter. Cloning in vector yields a fusion protein containing additional N-terminal part consisting of hexahistidine motif and a site recognized by thrombin. This property, in turn, opens up the prospects for rapid and efficient purification of the target protein in one step by metal chelate affinity chromatography.

Thus, the developed technology of obtaining highly purified recombinant NSE provides new opportunutues for producing components of ELISA test systems. Introduction of the test system with standardized components undoubtedly enhance its specificity, reliability, and reproducibility. That will significantly improve the quality of diagnosis of diseases characterized by elimination of NSE into blood and cerebrospinal fluid.

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