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PRODUCTION OF POLYCLONAL ANTIBODIES AGAINST THE DJ-1 PROTEIN ASSOCIATED WITH PARKINSON'S DISEASE

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ABSTRACT

Parkinson's disease is a slowly progressive chronic neurodegenerative disease with a global prevalence estimated at 0.3% and rising sharply with age to over 3% in people over 80 years of age. Estimates of the incidence of Parkinson's disease worldwide range from 5 to 35 new cases per 100,000 people per year. The disorder is defined by the presence of classic motor signs, including the characteristic presence of bradykinesia in all patients, tremor in most, and rigidity. One of the proteins associated with Parkinson's disease is DJ-1, also known as Parkinson disease protein 7 (PARK7). Studies have shown that DJ-1 is an antioxidant protein that plays a key role in counteracting oxidative stress in dopamine neurons. In the framework of the studies carried out in Escherichia coli BL21(DE3)-RIPL strain, the recombinant rDJ-1 protein was obtained in an amount of 3.5 mg. By immunizing a rabbit, polyclonal anti-rDJ-1 antibodies were isolated, which, in enzyme immunoassay and Western blotting, showed high sensitivity to the DJ-1 protein at a dilution of up to 1: 512,000. The resulting polyclonal antibodies showed the required informativeness when studying the expression of DJ-1 in the cell lines HEK293 (wild type) and HEK293park- (with knocked-out park gene). The resulting antibodies can be used in studies to assess the expression level of DJ-1 in immunological tests to detect and quantify the target protein: flow cytometry, ELISA, Western blotting, immunofluorescence, immunohistochemistry, immunoelectrophoresis.

Key words: Parkinson, recombinant protein, antibody.

Introduction

Parkinson's disease is a slowly progressive chronic neurodegenerative neurological disease that is more common in older people [1]. Estimates of the incidence of Parkinson's disease worldwide range from 5 to 35 new cases per 100,000 people per year [2]. The age of onset of the disease ranges from the third decade of life to old age. Global prevalence, conservatively estimated at 0.3%, rises sharply with age to over 3% in people over 80 years of age [3]. The disorder is defined by the presence of classic motor signs, including the characteristic presence of bradykinesia in all patients, tremor in most, and rigidity [4]. These motor symptoms are often preceded by non-motor manifestations such as olfactory dysfunction (in about 90% of cases), constipation, REM sleep behavior disorder, depression and/or anxiety [5].

Signs of Parkinson's disease include loss of neurons in certain areas of Substantia nigra found in the midbrain area and extensive accumulation of an intracellular protein, α-synuclein. Although neither the loss of pigmented dopaminergic neurons in Substantia nigra [6] nor the accumulation of α-synuclein in neurons are specific for Parkinson's disease, these two major neuropathologies are specific for the definitive diagnosis of idiopathic Parkinson's disease. Intraneuronal protein aggregates composed primarily of α-synuclein are found in all patients with Parkinson's disease. The existence of point mutations and multiplication of the SNCA gene (coding for α-synuclein) that cause hereditary forms of Parkinson's disease strongly supports the notion that α-synuclein plays a key role in Parkinson's disease. [7].

One of the proteins associated with Parkinson's disease is DJ-1, also known as Parkinson disease protein 7 (PARK7). The PARK7 gene encodes a protein of the C56 peptidase family, has 8 exons and is located on the 1p36.23 chromosome region. Deglycase DJ-1 is 20 kDa, consists of 189 amino acids with seven β-strands and nine α-helices, and is present as a dimer. Under oxidative conditions, DJ-1 inhibits α-synuclein aggregation due to its chaperone activity [8, 9], functioning as a redox chaperone and as a sensor of oxidative stress. Accordingly, DJ-1 is able to protect neurons from oxidative stress and cell death [9]. In parallel, the DJ-1 protein acts as a positive regulator of androgen receptor-dependent transcription. DJ-1 is expressed in both retinal neurons and mammalian retinal pigment epithelium, where it plays a neuroprotective role against oxidative stress under both physiological and pathological conditions [10, 11]. Mutations in DJ-1 (PARK7) cause an early onset of autosomal recessive Parkinson's disease [12] and are associated with increased cellular oxidative stress [13, 14]. DJ-1 mutations are known to be associated with recessive forms of familial parkinsonism. Several studies have suggested that DJ-1 is an antioxidant protein that plays a key role in counteracting oxidative stress in dopamine neurons [15]. Confirmation of the important role of DJ-1 in the development of Parkinson's disease is that the absence of DJ-1 can cause motor dysfunction even in the absence of nigral neurodegeneration [16, 17]. However, due to the limited data on pathology, it is still difficult to draw a conclusion about the influence of the expression level of DJ-1 and its modification on the human neurological state. Several DJ-1 defi-

cient mouse models have been developed. Studies have shown that the DJ-1 knockout mouse model is hypersensitive to the effects of MPTP and rotenone and the mouse locomotor model remains unchanged even in the absence of DJ-1. However, a clear motor deficit was observed in DJ-1 knockout mice after neurotoxin administration compared to an intact mouse model, indicating that DJ-1 still plays a neuroprotective role in the nervous system [18], leading researchers to conclude the importance of expression level of the DJ-1 protein.

The aim of this work was to obtain polyclonal antibodies against the DJ-1 protein. To achieve this goal, it was necessary to obtain a recombinant analog of the human DJ-1 protein in *Escherichia coli* cells, immunize the rabbit, isolate anti-rDJ-1 polyclonal antibodies and evaluate their sensitivity and specificity in ELISA and Western blotting.

Materials and methods

Nutrient media, strains, vectors

For the selection of transformant clones, agar medium based on Lennox Broth was used: 1% trypton, 0.5% yeast extract, 0.5% NaCl, 1.5% agar. SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 20 mM MgSO4, 20 mM glucose (pH7.5) was used to incubate the transformed cells. Terrific Broth medium was used to cultivate the strain. (TB), having the following composition: 1.2% tryptone, 2.4% yeast extract, 0.5% glycerin, 170 mM KH2PO4, 720 mM K2HPO4.The concentration of ampicillin in the nutrient medium was 200 μg/ml.The pPARK7 vector was used as a plasmid., and the host strain was BL21(DE3)-RIPL.

BL21(DE3)-RIPL strain transformation and gene expression

The 100 ng of plasmid DNA was added to 50 μl of a cell suspension of competent cells and electroporated using a MicroPulser (BioRad, USA) under the following modes: voltage — 2.5 kV, resistance — 200 Ω, capacitance — 25 μF. After electroporation, the cells were diluted with SOC medium in a ratio of 1:20, incubated in a shaker at 37°C for 1 hour. Then the culture was seeded on a solid nutrient medium with ampicillin and kept for 16 hours at 37°C.

A single transformant colony was cultured in TB medium at 37°C with ampicillin in a volume of 1 L. In the middle of the logarithmic growth phase at OD600 = 0.6, 0.3 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added and incubated for 16 hours at 18 °C. Cells were collected by centrifugation at 6000×g, 4 °C for 7 minutes.

Chromatographic Purification of Recombinant DJ-1 Protein

Cells were suspended in buffer (20 mM Tris-HCl pH 8.0 and 500 mM NaCl) and lysed on a UP200S ultrasonicator pulsed at 24 kHz (10 pulses, 10 seconds/pulse) on ice. The lysate was clarified by centrifugation at 40000×g, 60 min, 4°C. The clarified lysate was applied to a Ni-NTA agarose column (Invitrogen). The column was washed with 20 mM Tris-HCl pH 8.0 buffer, 500 mM NaCl, 20 mM imidazole. The DJ-1 protein was eluted with 150–500 mM imidazole in 20 mM Tris-HCl pH 8.0, 500 mM NaCl. The eluates were analyzed by SDS-PAGE according to the Laemmli method [19]. Purified recombinant DJ-1 was dialyzed against PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4 pH 7.4) for 16 hours with buffer changes. The protein concentration was measured by the Bradford method [20].

Rabbit Immunization and Isolation of Anti-rDJ-1 Polyclonal Antibodies

A 7-month-old male rabbit was used for immunization. Prior to immunization, 1 ml of blood was taken from a rabbit. The first rabbit immunization was performed with a 1:1 mixture of DJ-1 protein with complete Freund's adjuvant (total volume 1 ml). The protein concentration for the first immunization was 300 μg/ml. For the second immunization, the protein was mixed with incomplete Freund's adjuvant, also in a ratio of 1:1. In subsequent immunizations, the protein concentration was 150 μg/ml. The antigen was injected subcutaneously at several points along the spine. The interval between immunizations was 7 days.

Isolation of immunoglobulins was carried out by salting out in ammonium sulfate. Ammonium sulfate was added to the serum to obtain a 20% saturated solution and incubated at 4 °C on a magnetic stirrer for 3 hours. Next, the solution was centrifuged (10000×g, 20 min), the resulting supernatant was transferred to a new flask and adjusted with ammonium sulfate to a concentration of 45%. The mixture was incubated under the same conditions for 16 hours. Desalting was performed using dialysis against PBS (pH 7.2).

Enzyme linked immunosorbent assay (ELISA)

For ELISA, a 96-well plate (Nunc-Immuno Plate) was used. Protein DJ-1 was applied to the plate at a concentration of 1 μg/ml, the plate was incubated at 4°C for 16 hours. Next, the plate was washed three times with PBS buffer with 0.1% Tween-20 (PBST). Plate blocking was performed with 1% skim milk for one hour at 37°C. After repeated washing with PBST buffer, serum containing antibodies against the DJ-1 protein was added in various dilutions: 1:100, 1:80 in duplicate. Next, a binary titration was performed with 100 μl. Incubation was carried out for one hour at 37 °C. After incubation, the plate was washed with PBST buffer and anti-species (secondary) antibodies conjugated with horseradish peroxidase (anti-Rabbit) at a dilution of 1:10,000 were added and incubated for an hour at 37°C. Reactions were stopped by adding an equal volume of 0.1 N sulfuric acid. The results were detected with teramethylbenzidine (TMB) (10 mg per 10 ml citrate-phosphate buffer, pH 5.0 and 100 μl 3% H_2O_2) at 490 nm.

Western blotting

HEK293 and HEK293park- cells were grown in DMEM supplemented with 10% serum for 5 days. Cells were collected by standard trypsinolysis and the num-

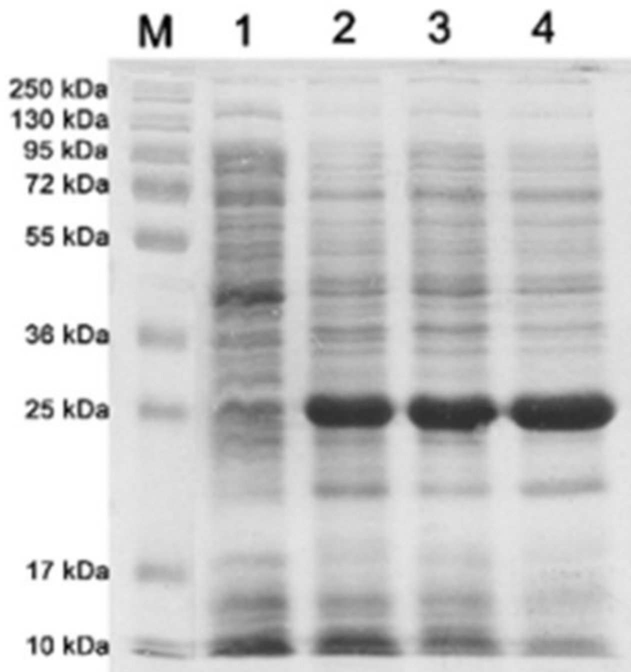
ber of collected cells was evaluated. After cell sedimentation, 1 ml of lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 1% Triton X-100, 0.1% SDS) was added to the pellet, suspended and homogenized with ultrasound. SDS-PAGE was performed on two lysates, recombinant DJ-1 was used as a positive control in different amounts: 6.5 µg, 3.9 µg, 2.6 µg and 1.3 µg.

Western blotting was performed according to [21] using a PVDF membrane and 5% skimmed milk powder as a blocking agent. Incubation with polyclonal anti-rDJ-1 (1:10,000 dilution) was performed at room temperature for 1 hour. The membrane was washed with TBS buffer with 0.1% Tween-20. Anti-species (anti-Rabbit) antibodies diluted 1:20,000 were used as secondary antibodies. The chemiluminescence reaction was detected with the Chemiluminescense Detection kit for HRP (Biological Industries).

Results and discussion

Expression of the *park7* gene and purification of the recombinant protein DJ-1

The *park7* gene encoding the DJ-1 protein was cloned into the pPARK7 vector. In this vector, the *park7* gene is built under the control of the tac promoter, which is activated when IPTG is added to the medium. In the open reading frame, the resulting DJ-1 protein has 189 amino acid residues and carries the 6His-tag histidine tag from the N-terminus. The calculated mass of the recombinant DJ-1 protein is 20 kDa, the pI value is 3.32. The antibiotic resistance marker of the pPARK7 vector is ampicillin. Ampicillin-resistant clones were obtained by transformation of competent cells of the *Escherichia coli* BL21(DE3)-RIPL strain with the pPARK7 vector.

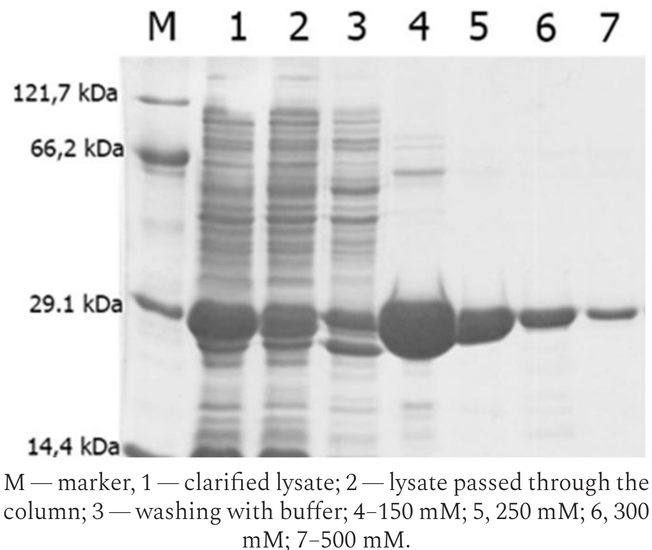


M — marker; 1 — without induction; 2–2 hours of induction; 3–4 hours of induction; 4–16 hours of induction

Figure 1 — SDS-PAGE electrophoresis of lysate samples after induction of recombinant strain Escherichia coli BL21(DE3)/pPARK7

Verification of the expression of the *park7* gene showed that the recombinant strain, when IPTG was added, effectively expressed the gene throughout the entire induction stage. Figure 1 shows the results of electrophoretic separation of lysates of the recombinant strain before and after induced activation of the tac promoter.

As can be seen from Figure 1, when IPTG is added, a linear accumulation of the DJ-1 recombinant protein is observed in the cells of the strain. Additionally, a high level of expression of the *park7* gene in a heterologous environment can be noted. The high expression level of the *park7* gene and the accumulation of the recombinant DJ-1 protein in a water-soluble form in the cytosolic fraction of the lysate facilitate protein purification. Figure 2 shows the results of the purification of the recombinant DJ-1 protein using metal affinity chromatography on a column with a Ni-NTA sorbent.



M — marker, 1 — clarified lysate; 2 — lysate passed through the column; 3 — washing with buffer; 4–150 mM; 5, 250 mM; 6, 300 mM; 7–500 mM.

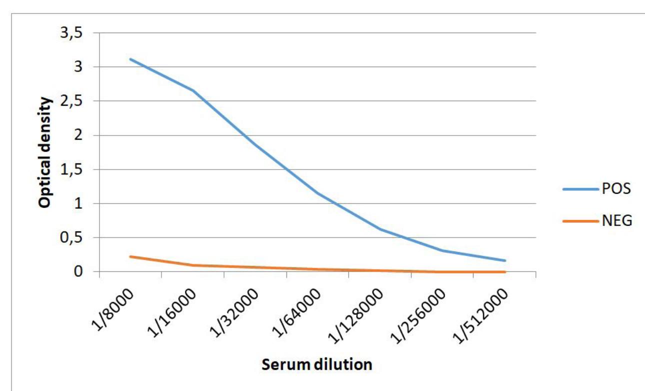
Figure 2 — Results of chromatographic purification of protein DJ-1

As can be seen from Figure 2, recombinant DJ-1 began to elute at a concentration of 150 mM. Measurement of the protein concentration in the pooled fractions showed that the yield of the purified recombinant DJ-1 protein is 3.5 mg per 1 liter of the induced culture of the BL21(DE3)-RIPL/pPARK7 strain, which is a high indicator.

Rabbit immunization and production of anti-DJ-1 polyclonal antibodies

Previously, the optimal amount of injected antigen was determined and the optimal rabbit immunization scheme was established [22]. Therefore, the concentration of purified and dialyzed recombinant DJ-1 protein was adjusted to 0.6 mg/ml by adding PBS. The selection of control serum makes it possible to determine the titer of specific antibodies formed after immunization. The sevenfold immunization carried out within 1 month contributed to the development of an appropriate immune response. A total of 6 dilutions of antiserum from 1:8000 to 1:512000 were performed with an equal amount of DJ-1 recombinant protein. The antibody titer

is defined as the maximum dilution of serum at which the ratio of optical density 490 (OD490) (OD490 serum after immunization/OD490 serum before immunization) is greater than 2:1. The antibody titer was shown to be ~1:512,000 (Figure 3).



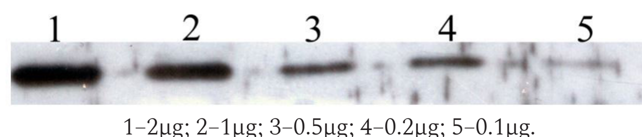
POS — positive serum, NEG — negative serum

Figure — 3 Analysis of the antibody titer in the serum of an immunized rabbit by ELISA

Checking the antibody titer using ELISA showed that the end point of the titration corresponds to a dilution of 1: 512,000 (Figure 3). The specificity of polyclonal antibodies was determined using different proteins: GFP (green fluorescent protein), RBD (receptor binding domain of SARS-Cov-2), and lysozyme. The choice of GFP and RBD is justified by the fact that all of them, like DJ-1, were obtained recombinantly in *Escherichia coli* by plasmid expression. Using ELISA, it was found that the obtained antibodies have a high specificity for the DJ-1 protein and do not interact with other proteins.

Since the titer of antibodies in the serum of the immunized rabbit to rDJ-1 after 7 immunizations was high, immunoglobulins were isolated by salting out with ammonium sulfate.

To test the sensitivity of the obtained anti-rDJ-1 antibodies, Western blotting was performed against the recombinant DJ-1 protein in the following amounts: 2 µg, 1 µg, 500 ng, 200 ng, and 100 ng (Figure 4). As can be seen from the results of Western blotting, antibodies at a dilution of 1:10,000 detect up to 0.1 µg of the DJ-1 protein.

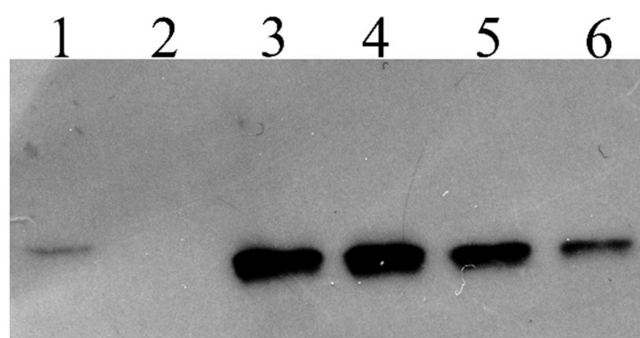


1–2µg; 2–1µg; 3–0.5µg; 4–0.2µg; 5–0.1µg.

Figure 4 — The result of testing the sensitivity of polyclonal antibodies to the recombinant protein DJ-1

The resulting antibodies were tested on lysates of the HEK293 cell line (wild type) and the HEK293park- cell line (with *park7* gene knockout). Figure 5 shows the results of this testing.

As follows from Figure 5, the resulting polyclonal antibodies interact both with the wild-type PARK7 (DJ-1) protein in the cell lysate and with the purified recombinant protein in an amount of up to 1.3 µg. In the HEK293park- cell lysate the *park7* gene expression was not observed.



1 — HEK cell lysate; 2 — lysate of HEK cells with *park7* gene knockout; 3 — rDJ-1 (6.5 µg); 4 — rDJ-1 (3.9 µg); 5 — rDJ-1 (2.6 µg); 6 — rDJ-1 (1.3 µg)

Figure 5 — The result of testing polyclonal antibodies on lysates HEK293 and HEK293park-

Thus, the obtained polyclonal antibodies can be used to detect and quantify the target protein in immunological tests such as flow cytometry, ELISA, Western blotting, immunofluorescence, immunohistochemistry, immunoelectrophoresis, and immunodiffusion tests. Anti-rDJ-1 antibodies have additional value in the use of immunoaffinity chromatography as ligands for the preparation of immunoaffinity sorbents for purification of DJ-1.

To study the role of DJ-1 in human neurodegenerative diseases, the authors obtained polyclonal and monoclonal antibodies to the human DJ-1 protein, which were used to show pathological co-localization of DJ-1 with other proteins associated with neurodegenerative diseases and a decrease in the solubility of DJ-1 in tissues [23]. This shows the demand for polyclonal antibodies anti-rDJ-1 in the study of the molecular mechanisms of Parkinson's disease.

Conclusion

One of the key proteins in the pathogenesis of Parkinson's disease is deglycase DJ-1, also known as PARK7, which plays a neuroprotective role in the human nervous system. The antioxidant function of Dj-1 affects the resistance to oxidative stress in dopamine neurons. As part of the work carried out, the DJ-1 protein gene was expressed in *Escherichia coli* cells. The recombinant DJ-1 protein was isolated and purified by chromatography at 3.5 µg. By immunizing a rabbit with recombinant DJ-1 protein, a pronounced immune response was obtained and polyclonal anti-rDJ-1 antibodies were isolated and precipitated from the serum, which showed high affinity in ELISA and Western blotting against recombinant rDJ-1. The resulting polyclonal antibodies have high sensitivity and specificity and can be used in studies of the pathogenesis of Parkinson's disease.

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ПАРКИНСОН АУРУЫМЕН БАЙЛАНЫСТЫ DJ-1 АҚУЫЗЫНА ҚАРСЫ ПОЛИКЛОНАЛДЫ АНТИДЕНЕЛЕРДІ АЛУ

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ТҮЙІН

Паркинсон ауруы — жаһандық таралу деңгейі 0,3%-ға бағаланған және 80 жастан асқан адамдарда жасы ұлғайған сайын 3%-дан асатын баяу үдемелі созылмалы нейродегенеративті ауру. Паркинсон ауруымен сырқаттанушылықтың бағалауы әлем бойынша жылына 100 000 адамға 5-тен 35 жаңа жағдайға дейін өзгереді. Бұзылу классикалық моторлық белгілердің болуымен анықталады, соның ішінде барлық науқастарда брадикинезияның тән болуы, көпшілігінде тремор және қаттылық. Паркинсон ауруымен байланысты ақуыздардың бірі — DJ-1, сонымен қатар Parkinson disease protein 7 (PARK7) деп аталады. Зерттеулер DJ-1 допаминдік нейрондардағы тотығу стрессіне қарсы тұруда маңызды рөл атқаратын антиоксиданттық ақуыз екенін көрсетті. Escherichia coli BL21(DE3)-RIPL штаммында жүргізілген зерттеулер аясында 3,5 мг мөлшерінде рекомбинантты rDJ-1 ақуызы алынды. Қоянды иммундау арқылы поликлональды анти-rDJ-1 антиденелері бөлініп алынды, олар иммундық ферментті талдауда және вестерн-блотингте 1: 512 000 сұйылту көрсеткішінде DJ-1 ақуызына жоғары сезімталдықты көрсетті. Алынған поликлоналды антиденелер HEK293 және HEK293park- (нокаутталған park гені бар) жасушалық желілерінде DJ-1 экспрессиясын зерттеу кезінде қажетті ақпарат мазмұнындылықты көрсетті. Алынған антиденелер нысана ақуызды анықтау және сандық анықтау үшін иммунологиялық сынақтарда DJ-1 экспрессия деңгейін бағалау үшін зерттеулерде пайдаланылуы мүмкін: ағынды цитометрия, ИФА, Вест-блотинг, иммунофлуоресценция, иммуногистохимия, иммуноэлектрофорез.

Кілтті сөздер: Паркинсон, рекомбинантты ақуыз, антидене.

ПОЛУЧЕНИЕ ПОЛИКЛОНАЛЬНЫХ АНТИТЕЛ ПРОТИВ БЕЛКА DJ-1, АССОЦИИРОВАННОГО С БОЛЕЗНЬЮ ПАРКИНСОНА

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АБСТРАКТ

Болезнь Паркинсона является медленно прогрессирующим хроническим нейродегенеративным заболеванием, глобальную распространенность, которой оценивают в 0,3% и которая резко возрастает с возрастом до более 3% среди лиц старше 80 лет. Оценки заболеваемости болезнью Паркинсона по всему миру колеблются от 5 до 35 новых случаев на 100 000 человек в год. Расстройство определяется наличием классических двигательных признаков, включая характерное наличие брадикинезии у всех пациентов, тремора у большинства и ригидности. Одним из белков, ассоциированных с болезнью Паркинсона является DJ-1, также известная как Parkinson disease protein 7 (PARK7). Проведенные исследования показали, что DJ-1 является антиоксидантным белком, играющим ключевую роль в противодействии окислительному стрессу в дофаминовых нейронах. В рамках проведенных исследований в Escherichia coli штамма BL21(DE3)-RIPL был получен рекомбинантный белок rDJ-1 в количестве 3,5 мг. Путем иммунизации кролика были выделены поликлональные антитела анти-rDJ-1, которые в иммуноферментном анализе и вестерн-блоттинге показали высокую чувствительность к белку DJ-1 в разведении до 1: 512 000. Полученные поликлональные антитела показали требуемую информативность при изучении экспрессии DJ-1 в клеточных линиях HEK293 (дикий тип) и HEK293park- (с нокутированным геном park). Полученные антитела могут быть использованы в исследованиях по оценке уровня экспрессии DJ-1 в иммунологических тестах для обнаружения и количественного определения целевого белка: проточная цитометрия, ИФА, вестерн-блоттинг, иммунофлуоресценция, иммуногистохимия, иммуноэлектрофорез.

Ключевые слова: Паркинсон, рекомбинантный белок, антитела.