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OBTAINING AND DETERMINATION OF IMMUNOGENIC PROPERTIES OF TRX-PD-1 RECOMBINANT PROTEIN

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ABSTRACT

Monoclonal antibodies against programmed cell death receptor PD-1 are of particular interest for immunotherapy and blocking control points for tumor development. The PD-1 T-cell receptor is a regulator capable of inhibiting or completely suppressing the immune response. One of the important stages of obtaining monoclonal antibody is the immunization of BALB/c mice, the scheme of which depends on the nature of the antigen and its immunogenicity.

The gene of PD-1 was synthesized by a two-step polymerase chain reaction using the Phusion High-Fidelity DNA Polymerase. The resulting construction based on the pET32 vector was transformed by electroporation into the *E. coli* BL21 expression strain, which resulted in the *E. coli* strain BL21/pET32/Trx-PD-1. Molecular weight of the protein Trx-PD-1 was 34 kDa. Western blot demonstrated presence of a hexahistidine tag in a protein with a molecular mass of 34 kDa. The highest antibody titers were observed in mice immunized with recombinant protein at a concentration of 100 μg/ml. Western blot revealed a specific reaction of PD-1 protein without thioredoxin with sera from immunized mice.

The resulting construct pET32/PD-1 provided a high level of expression of the recombinant Trx-PD-1. The recombinant Trx-PD-1 induced high titers of antibody and stimulated B-lymphocytes.

Keywords: PD-1, extracellular domain, prokaryotic expression, recombinant protein, antibodies.

INTRODUCTION

Recently, for immunotherapy aimed at blocking the control points of tumor development, monoclonal antibodies against programmed cell death receptor PD-1 are of particular interest. The PD-1 receptor of T cells is a regulator capable of inhibiting or completely suppressing the immune response [1, 2]. In this regard, the PD-1 receptor signaling pathway plays a key role in the regulation of immunity directed against fungal and viral infections. Studies on mice that are defective in CD4 T-cells and infected with virus showed that the blockade of PD-1 and PD-L1-inhibitory pathway had a beneficial effect on defective CD8-T cells, restoring their proliferation and expression of cytokines. CD8-T cells acquired the ability to destroy infected cells, thereby reducing viral load [3, 4].

With the advent of hybridoma technology in 1978, monoclonal antibodies are widely used in various branches of science and practice, including oncology. The interest in hybridoma technology and monoclonal antibodies in the field of oncology is associated with the possibility

of obtaining funds that can influence various functions of oncogenic cells. The effect of monoclonal antibodies on various functions of cells is carried out by specific binding of antibodies to oncological cells proteins and their complexes. At present, 50 different antitumor agents based on monoclonal antibodies are represented on the world pharmaceutical market. According to some authors, trastuzumab, a monoclonal antibody to human epidermal growth factor (HER2) and several other new HER2 inhibitors have increased the lifespan of patients with HER2 + breast cancer (BC). Moreover, monoclonal antibodies to HER2 increased the 5-year survival rate of patients with early-stage breast cancer to 90-95%. Before the advent of monoclonal antibodies to HER2, breast cancer at any stage was considered a malignant neoplasm with an unfavorable prognosis for the course of the disease [5].

One of the important stages of hybridoma technology is the immunization of BALB/c mice. The significance of this stage lies in the fact that upon receipt of a hybrid eukaryotic cell, activated B-lymphocytes and the myeloma cell line are used. [6]. The purpose of the immunization process is to increase the proportion of B lymphocytes producing antibodies of a given specificity and translate these cells into a functional state in which they are able to fuse and form antibody-forming hybrid cells. The immunization scheme is highly dependent on the nature of the antigen and its immunogenicity.

Among many systems that are most attractive and affordable for the production of a heterologous protein is Gram-negative bacterium *Escherichia coli*. The advantages of rapid growth in an inexpensive nutrient medium, well-characterized genetics and the presence of a large number of cloning vectors for *E. coli* suggest efficient production of recombinant proteins. However, despite extensive knowledge on the genetics and molecular biology of *E. coli*, not every gene can be efficiently expressed with a high level of production of functional eukaryotic proteins. This is due to the unique and subtle structural features of the gene sequence expressed, the stability and translational efficiency of mRNA, protein folding, degradation of the protein by proteases of host cells and the use of protein codons toxic to the host cells [7].

To reduce the toxicity of eukaryotic proteins for *E. coli* and transfer the protein into a soluble form, thioredoxin is the most widely used protein. The possibility of expressing a large number of human recombinant proteins to study their physiological role and mechanisms of action has been studied. For example, recombinant hCTRP2 was well soluble and actively expressed using strain BL21-codon plus (DE3) and the expression plasmid pET32 [8]. To produce recombinant polypeptide-38 (PACAP38), adenylate cyclase activator of carp pituitary, the gene was cloned into the pET32a (+) vector to express the thioredoxin (Trx)-PACAP fusion protein in *E. coli* BL21 (DE3). The resulting level of expression of thioredoxin PACAP reached 36% of the total protein, and more than 85% of the recombinant protein existed in soluble form. The purified Trx-PACAP protein specifically inhibited the proliferation of T98G human glioblastoma cells. Inhibition of proliferation was completely blocked by antibodies against PACAP [9].

Materials and methods

Bacterial strains, plasmids, and antibodies. We used *E. coli* DH5α, BL21 (DE3) (Novagen, USA) strains, the pGEM-TEasy plasmid vector (Promega, USA), pET32 (Novagen, USA) and pET32 (Invitrogen). *E. coli* cells were grown in LB medium. For Western blot, mouse anti-His-tag monoclonal antibodies and anti-species conjugate were used.

Obtaining a strain of microorganism producing recombinant Trx-PD-1 protein. Gene synthesis was performed by a two-round polymerase chain reaction using Phusion High-Fidelity DNA Polymerase polymerase (Thermo Fisher Scientific). The synthesized gene was cloned into the expression plasmid pET32. Oligonucleotides presented in table 1 were used for gene synthesis.

Table 1. Oligonucleotides for the synthesis of recombinant PD-1 protein.

№	Name	Nucleotide sequence	Length
1	PD1 1	ggatccgaattcccatgggactggatagc	29
2	PD1 2	agcgccggagaaaaggtcggcgggttccacgggcgatccgggctatccagtcccatggga	60
3	PD1 3	acctttctccggcgctgctggtggtgaccgaaggcgataacgcgacgtttacctgcagc	60
4	PD1 4	gcgataccagttcagcacaaagctttcgctggtgttgctaaagctgcaggtaaacgtcgc	60
5	PD1 5	tgtgctgaactggtatcgcatgagcccgagcaaccagaccgataaactggcggcgtttcc	60
6	PD1 6	aacgcggaagcggcaatcctgacccggctggctgcgatcttccggaaacgccgccagttt	60
7	PD1 7	tgccgcttccgcgttacccagctgccgaacggccgcgattttcatatgagcgtggttcgt	60
8	PD1 8	cgcaccgcacagataggtgccgctatcgttgcggcgcgcacgaaccacgctcatatgaaa	60
9	PD1 9	cctatctgtgcggtgcgattagcctggcgccgaaagcgcagattaaagaaagcctgcgtg	60
10	PD1 10	tcggcacttccgcacgacgttccgtcacgcgcagttcggcacgcaggctttctttaatct	60
11	PD1 11	cgtgcggaagtgccgaccgcgcatccgagcccgtctccgcgcccggcgggccagttccag	60
12	PD1 12	agaagcttctcgagttaatgatgatgatgatgatgctggaactggcccgcc	51
13	PD1 start	ggatccgaattcccatgggact	22
14	PD1 stop	agaagcttctcgagttaatg	20

The transformation of BL21 (DE3) competent cells with the pET-32/rPD-1 plasmid vectors was performed by electroporation using MicroPulser (BioRad). Single colonies of transformants were cultured in LB broth with an antibiotic. In the middle of the logarithmic growth phase of the bacterial mass (OD600 = 0.6), an inducer, isopropyl-β-D-1-galactopyranoside (IPTG), was introduced at a final concentration of 0.1 mM and incubated for 16 hours. The collection of cells was carried out by centrifugation at + 4°C, 6000 g for 7 minutes.

Purification of recombinant Trx-PD-1 protein. Cell lysis was carried out using a UP200S ultrasonic disintegrator at a frequency of 24 kHz in a pulsating mode (10 pulses, 10 s/pulse) on ice in a buffer (20 mM NaCl, 20 mM Hepes pH-7.5). Protein purification was performed by metal-chelate chromatography on Ni2 + ions using a HisTrapTM HP 1 mL column (GE Healthcare). The equilibration and loading of the lysate were performed according to the manufacturer's protocol. A stepwise imidazole gradient with an initial buffer (500 mM NaCl, 20 mM Hepes pH-7.5, 20 mM imidazole) and a final buffer (500 mM NaCl, 20 mM Hepes pH-7.5, 500 mM imidazole) was used to search for the eluting concentration of imidazole. A liquid chromatograph for fast purification of FPLC proteins (Fast protein liquid chromatography, AKTA) was used in the work. Protein detection in fractions was performed at a wavelength of 260 nm. The protein concentration in the lysate and fractions was determined by Bradford using bovine serum albumin as a standard [10].

Western blot. Electrophoresis of rabies virus antigen was performed on an 11% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS) according to the U.K. Laemmli method on the apparatus for vertical electrophoresis (BioRad, USA) [11]. Electrophoretic transfer of antigens from the gel to the nitrocellulose membrane was performed using an immunoblotting device (BioRad, USA) according to the method of P.K. Towbin [12].

For the immunochemical manifestation of specific antigens, the nitrocellulose membrane was first incubated in 1% BSA solution overnight at 4°C. Then it was washed three times in buffer (137 mM NaCl, 10 mM Na_2HPO4, pH-7.4) and buffer (137 mM NaCl, 10 mM Na2HPO4, pH-7.4, Tween-20) and kept for 1.5 hours at 37°C in a solution of monoclonal antibodies purified from ascites fluid, at a dilution of 1: 100 in a buffer (137 mM NaCl, 10 mM Na2HPO4, pH-7.4, Tween-20). After that, the carrier was washed again and incubated in a working dilution of anti-peptide antibodies labeled with peroxidase for 1 hour at 37°C, and the washing procedure was repeated. The substrate solution was prepared immediately before use as follows: 0.01 g of 4-chloro-naphthol (Sigma, USA), dissolved in 2 mL of ethanol, mixed with 18 mL of buffer (137 mM NaCl, 10 mM Na2HPO4, pH-7.4) and 0.01 mL of 3% hydrogen peroxide was added.

Immunization of mice. Mice on the first day of immunization were injected intraperitoneally with 100 µg of antigen in 0.1 mL of Freund's incomplete adjuvant (Gibco, USA). On days 7, 11, 12, 13 of immunization, animals were injected with 100 µg of antigen in phosphate-buffered saline, pH 7.2-7.4. Three days after the last immunization, the serum of the immunized mice was tested for the presence of antibodies to the recombinant Trx-PD-1 protein by enzyme immunoassay.

ELISA. The immunological plate was immobilized by diluting the antigen at a concentration of 10 µg/mL. The antigen was diluted in pH-9.5 bicarbonate buffer and incubated at + 4°C for 12 hours. After incubation, the plate was washed 4 times with a solution of the phosphate-saline buffer with Tween-20, pH-7.2. The free surface of the plate was filled with a 10% solution of skimmed milk in phosphate-saline buffer + tween-20, pH-7.2. The plate with blocking solution was incubated at 37°C for 1 hour and washed as above.

Prepared dilutions of the studied and control sera were added to the wells of an immunological plate in a volume of 0.1 mL. Wells E12 and H12 were left without serum as conjugate control. The plate was incubated for 1 hour at 37°C and the washing procedure was repeated. A working dilution of the anti-species conjugate in a volume of 0.1 mL was added to all wells of the plate, except the well H12 (blank), and incubated for 1 hour at 37°C. A working dilution of the conjugate was prepared by adding to 10 mL of phosphate-saline buffer + tween-20, pH-7.2, 1 µL of Anti-Bovine IgG conjugate (whole molecule) − Peroxidase antibody produced in rabbit (Sigma, USA). The plate was washed 3 times with phosphate-saline buffer + tween-20, pH-7.2 and 3 times with distilled water. After washing, 0.1 mL solution of tetramethylbenzidine (TMB) was added. The plate was incubated in a dark place at room temperature (20-22°C) for 15 minutes. The reaction was stopped by adding 0.1 mL of 2 M sulfuric acid. The results of the reaction were taken into account on a spectrophotometer at a wavelength of 492 nm.

RESULTS

Obtaining a strain of the microorganism producing recombinant Trx-PD-1 protein. To obtain a recombinant Trx-PD-1 protein, the gene was synthesized by a two-step polymerase chain reaction. The synthesized gene with a length of 500 nucleotides was cloned into pET32 expression plasmid at the NcoI and XhoI restriction sites. As a result, the genetic construction of the gene carrying PD-1 receptor fragment shown in Figure 1 was obtained. Analysis of the nucleotide sequence of the gene showed no mutational changes.

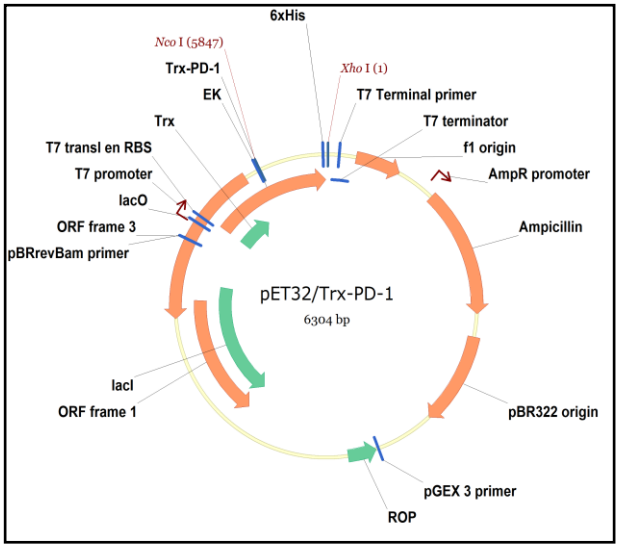
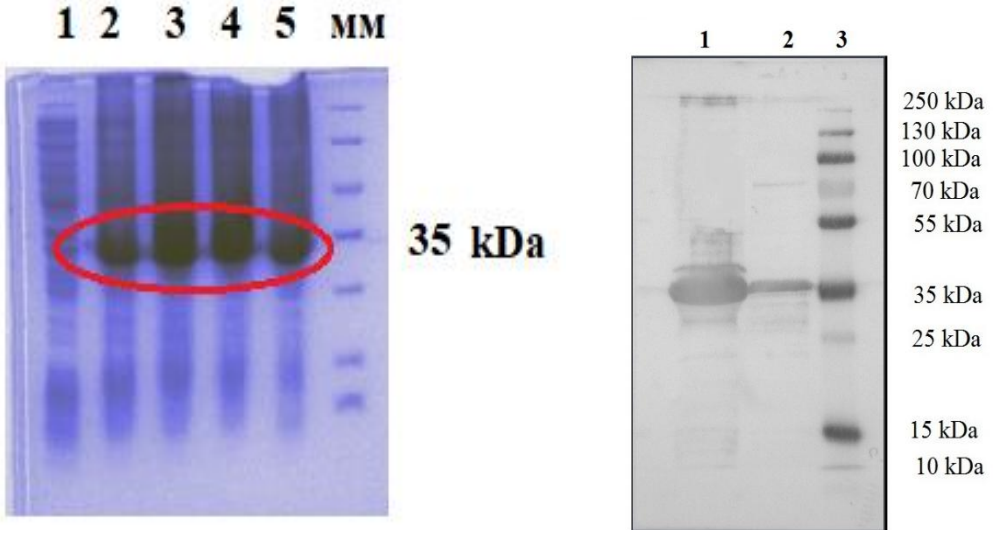


Fig. 1. Plasmid map of construct pET32/Trx-PD-1

The resulting structure was transformed by electroporation into expression strain *E. coli* BL21, which resulted in the *E. coli* strain BL21/pET32/Trx-PD-1. To determining expression activity, the cells were cultured in LB medium with the addition of 0.2 mM IPTG and sampling at various time points. Selected samples were sonicated and analyzed by polyacrylamide gel electrophoresis. SDS-PAGE analysis showed expression of the recombinant protein after induction of IPTG in 2 hours (Figure 2, lane 2). The molecular mass of the protein was 34 kDa, which is consistent with the theoretical value of rPD-1. Over expression of protein occurred after 4 hours of incubation with IPTG and did not change during 12 hours of incubation (Figure 2, lane 3-5). The protein was mainly expressed in inclusion bodies since after centrifugation the protein was detected only in the sediment (data not shown). Additionally, the obtained samples were examined by Western blot using anti-His-tag monoclonal antibodies (Figure 2B).



(A) Lane 1 - cell culture without IPTG; Lane 2 - 2 hours incubation with IPTG; Lane 3 - 4 hours incubation with IPTG; Laine 4 - 6 hours incubation with IPTG; Lane 5 - 12 hours incubation with IPTG; MM - molecular markers, (B) Lane 1–6 hours of incubation with IPTG; Lane 2 - without IPTG; Lane 3 - molecular markers.

Fig. 2. SDS-PAGE electrophoresis (A) and Western blot (B) of recombinant Trx-PD-1 protein.

The results of Western blot demonstrated the presence of a hexahistidine tag in a protein with a molecular mass of 34 kDa, which corresponds to the predicted molecular mass of recombinant Trx-PD-1 protein.

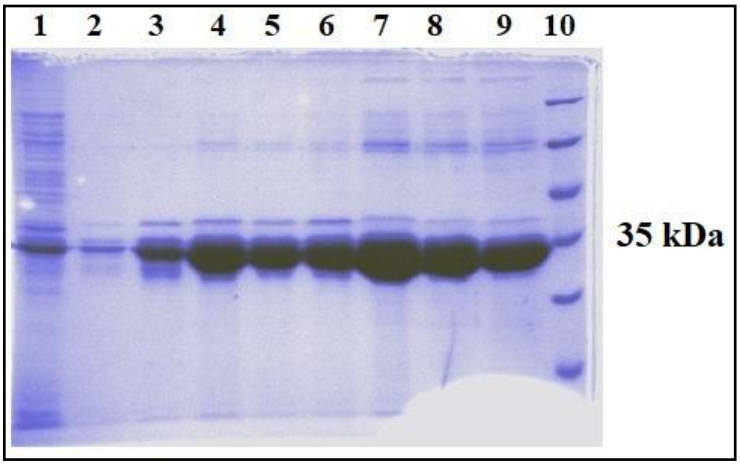
Isolation and purification of recombinant Trx-PD-1 protein. To optimize an isolation and purification of recombinant Trx-PD-1 protein, the cell culture was cultivated under the following conditions: inducer concentrations of 0.1 mM, 0.25 mM, 0.5 mM, 1 mM; temperatures of nutrient medium 25°C and 37°C. The results of the experiment were evaluated by the relative content of recombinant proteins in the insoluble fraction of the lysates. The obtained data are presented in table 2.

Table 2. Relative amounts of recombinant proteins (%) in insoluble fractions of lysates of producer strains under various conditions of expression induction

Concentration of IPTG		0.1 mM	0.2 mM	0.5 mM	1 mM
pET32/Trx-PD-1	25°C	50%	50%	30%	10%
	37°C	8%	8%	n/d	n/d
Note : n/d - not detected					

As follows from the above data, for all strains-producers selected for further work, the maximum production of recombinant protein in the soluble fraction was observed under the following cultivation conditions: inducer concentration 0.2 mM, cultivation temperature after induction of expression 37°C.

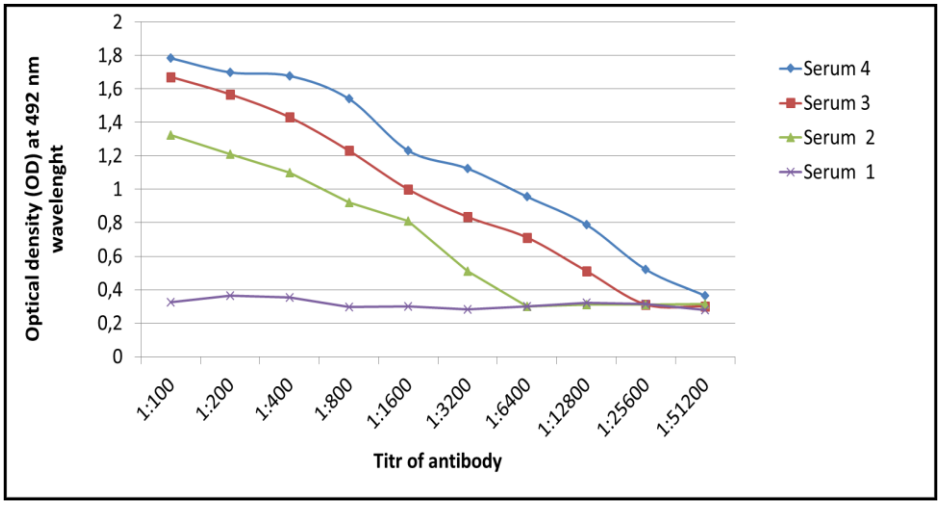
As a result of testing parameters for isolation and purification of recombinant protein, purified preparations of antigens were obtained. Purification of recombinant protein was performed by nickel-sepharose-based metal chelate chromatography. To elute the protein f buffer containing 0.2 mM imidazole was used. The electrophoretic analysis showed the homogeneity of the purified preparations of recombinant Trx-PD-1 protein (Figure 3).



Lane 1 - washing the column; Lane 2 - 50 mM imidazole; Lane 3 - 150 mM imidazole; Lanes 4-6 - 250 mM imidazole; Lane 7-9 - 500 mM imidazole; Lane 10 - molecular markers.

Fig.3. Electrophoresis of purified fractions of recombinant Trx-PD-1 protein expressed by strain BL21/pET32/Trx-PD-1.

Immunogenic properties of recombinant Trx-PD-1 protein. Laboratory mice were immunized intraperitoneally with recombinant Trx-PD-1 protein in concentrations of 25, 50 and 100 μg/mL. As a result of immunization, the highest antibody titers were observed in mice immunized with protein at a concentration of 100 μg/mL. The antibody titer in the serum of immunized mice was 1:25600 (Figure 4). However, this dose of recombinant Trx-PD-1 protein was harmless to the mouse. Cross-reaction serum of immunized animals with heterogeneous antigens and proteins was not observed. Western blot of PD-1 protein without thioredoxin with sera from immunized animals revealed a specific reaction of antibodies with a protein having a molecular weight of 21 kDa (data not shown).



Serum 1 - not immunized mouse; Serum 2 - mouse immunized with protein at a concentration of 25 μg/mL; Serum 3 - mouse immunized with proteins at a concentration of 50 μg/mL; Serum 4 - mouse immunized with protein at a concentration of 100 μg/mL

Fig.4. ELISA of mice serum immunized with recombinant Trx-PD-1 protein.

DISCUSSION

Melanoma cells can inhibit anticancer immunity and avoid cytotoxic T-cell attacks by activating programmed cell death receptor 1 (PD-1) [13]. In this connection, monoclonal antibodies are widely used for the formation of anticancer immunity. For example, Pembrolizumab antibodies demonstrated good antitumor activity against melanoma and non-small cell lung cancer [14]. Upon receipt of monoclonal antibodies, the success of the study is associated with the presence in sufficient quantities of pure antigen preparations. The most commonly used for the expression of proteins, bacteria are the most economical in terms of cost, speed and ease of use.

Recently, gene synthesis under de novo conditions has become a powerful tool in biotechnology. The ability to synthesize genes of any desired sequence provides unlimited research possibilities. Recently, significant advances have been made in gene synthesis under de novo conditions using the polymerase chain reaction commonly used to create functional sequences from short single-stranded oligonucleotides.

As a result of the research, the expression and purification of recombinant Trx-PD-1 in the *E. coli* system were carried out. To obtain a protein, a genetic design based on the pET32 vector was carried out, oligonucleotides were synthesized under de novo conditions, and the PD-1 receptor extracellular domain was synthesized using Phusion polymerase, cloned into expression vectors and transformed into *E. coli* expression strains BL21 (DE3).

Strain producers although produced protein in fairly high quantities, the protein was in the form of Taurus inclusion and had an insoluble form. The protein from inclusion bodies was solubilized in a solution of 8 mol/L urea and subjected to refolding using the method of ion exchange chromatography on a nickel-sepharose column. This method allows to use a high initial protein concentration and effectively carry out refolding with simultaneous separation of the target product from high-molecular protein aggregates. At the same time, the necessary conditions for refolding are provided: the spatial isolation of protein molecules from each other in the pores of the sorbent and a smooth decrease in the concentration of the denaturing agent (urea). The results of the correct refolding were confirmed by studies of laboratory mice immunized with various doses of Trx-PD-1 protein. According to a study of the immunogenic properties of the TrX-PD-1 protein, it was revealed that the recombinant protein has good immunogenicity and expresses the formation of antibodies specific to the PD-1 protein without thioredoxin.

According to literature data, denatured PD-1 can be re-formed into a unique native spatial structure by refolding on a column. In addition, the re-formed PD-1 had a high binding activity with its related ligand PD-L1, suggesting that glycosylation is not required for binding to PD-L1. These results indicate that the prokaryotic expression system can be used to obtain biologically active soluble PD-1 using appropriate refolding strategies [15].

CONCLUSION

This paper presents the results of the preparation of recombinant extracellular fragment of human PD-1 receptor, consisting of thioredoxin - 6His-tag - PD-1- 6His-tag and determination of its immunogenic properties. The resulting construct pET32/PD-1 provided a high level of expression of the recombinant protein Trx-PD-1. The immunogenic properties of the obtained recombinant Trx-PD-1 protein ensured the formation of sufficiently high antibody titers and stimulated B-lymphocytes.

Acknowledgments

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TRX-PD-1 РЕКОМБИНАНТТЫ АҚУЫЗЫН АЛУ ЖӘНЕ ИММУНОГЕНДІК ҚАСИЕТТЕРІН АНЫҚТАУ

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ТҮЙІН

PD-1 жасушаларының тіршілік қабілетін жоюға бағдарламаланған рецепторға қарсы моноклоналды антиденелер ісіктің дамуының бақылау нүктелерін бұғаттау және иммунотерапиясы үшін ерекше қызығушылық тудыруда. Иммундық жауапты толығымен басатын немесе ингибирлеуге қабілетті PD-1 Т-жасушалық рецепторы реттегіш болып табылады. Моноклоналды антиденелерді алудың негізгі сатыларының бірі BALB/c тышқандарын иммундеу табылады, оның жүйесі антигеннің табиғаты мен иммуногенділігіне байланысты.

PD-1 гені Phusion High-Fidelity ДНҚ-полимеразасын қолдану арқылы екі сатылы ПТР-мен синтезделді. pET32 векторына негізделіп алынған конструкцияны *E.coli* BL21 экспрессиондық штаммына электропорация арқылы трансформацияланды, нәтижесінде *E.coli* BL21/pET32/Trx-PD-1 штамы алынды. Trx-PD-1 ақуызының молекулалық салмағы 34 кДа құрды. Вестерн-блот 34 кДа молекулалық салмақтағы ақуызда гексагистидин белгісінің бар екенін көрсетті. Ең жоғарғы титрлер, рекомбинантты ақуызбен 100мкг/мл концентрациясында байқалды. Вестерн-блот арқылы иммунделген тышқандардың сарысуымен тиоредоксинсіз PD-1 ақуызының айрықша реакциясын көрсетті.

pET32/PD-1 алынған конструкциясы рекомбинантты Trx-PD-1 экспрессиясын жоғары деңгейде қамтамасыз етті. Рекомбинантты Trx-PD-1 антиденелердің жоғарғы титрін индуцирлеп және В-лимфоциттердің түзілуін ынталандырды.

Негізгі сөздер: PD-1, жасушадан тыс домен, прокариоттық экспрессия, рекомбинантты ақуыз, антиденелер.

ПОЛУЧЕНИЕ И ОПРЕДЕЛЕНИЕ ИММУНОГЕННЫХ СВОЙСТВ РЕКОМБИНАНТНОГО БЕЛКА TRX-PD-1

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АБСТРАКТ

Моноклональные антитела против рецептора запрограммированной гибели клеток PD-1 представляют особый интерес для иммунотерапии и блокирования контрольных точек развития опухоли. Т-клеточный рецептор PD-1 представляет собой регулятор, способный ингибировать или полностью подавлять иммунный ответ. Одним из важных этапов получения моноклональных антител является иммунизация мышей BALB/c, схема которых зависит от природы антигена и его иммуногенности.

Ген PD-1 был синтезирован двухступенчатой полимеразной цепной реакцией с использованием ДНК-полимеразы Phusion High-Fidelity. Полученную конструкцию, основанную на векторе pET32, трансформировали электропорацией в экспрессионный штамм *E.coli* BL21, в результате чего получали штамм *E.coli* BL21/pET32/Trx-PD-1. Молекулярная масса белка Trx-PD-1 составила 34 кДа. Вестерн-блот продемонстрировал присутствие гексагистидиновой метки в белке с молекулярной массой 34 кДа. Самые высокие титры антител наблюдались у мышей, иммунизированных рекомбинантным белком в концентрации 100 мкг / мл. Вестерн-блот выявил специфическую реакцию белка PD-1 без тиоредоксина с сыворотками иммунизированных мышей. Полученная конструкция pET32/PD-1 обеспечивала высокий уровень экспрессии рекомбинантного Trx-PD-1. Рекомбинантный Trx-PD-1 индуцировал высокие титры антител и стимулировал формирование В-лимфоцитов.

Ключевые слова: PD-1, внеклеточный домен, прокариотическая экспрессия, рекомбинантный белок, антитела.