С применением описанной технологии удалось получить очищенные препараты рекомбинантных белков с высокой концентрацией. Выход очищенного протеина со 100 мл культуры *E. coli* составил 1,5 мг для pK205R и 2 мг для pB602L (рис. 3, треки 2 и 4).

На основе полученных рекомбинантных антигенов планируется разработать непрямой вариант ИФА для выявления антител к вирусу АЧС в сыворотках крови свиней.

ВЫВОДЫ

Проведено молекулярное клонирование генов K205R и B602L вируса АЧС. Получены клоны *E. coli*, экспрессирующие рекомбинантные белки pK205R и pB602. Отработаны условия экспрессии и очистки, обеспечивающие высокий выход очищенных препаратов рекомбинантных белков.

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CLONING AND EXPRESSION OF AFRICAN SWINE FEVER VIRUS K205R AND B602L GENES IN *E. COLI*

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SUMMARY

A molecular cloning of African swine fever virus k205r and b602l genes in *E. coli* was carried out. The expression and purification conditions ensuring high yield of recombinant proteins were optimized. Dissolved recombinant proteins were purified by metal-chelate affinity chromatography using Ni-NTA-agarose («Qiagen»). Recombinant antigens are biologically safe, easier to prepare and ensure higher ELISA specificity. The purified protein yield from 100 ml of *E. coli* culture was 1,5 mg for pK205R and 2 mg for pB602L.

Key words: African swine fever virus, pK205R and pB602L recombinant proteins, expression.

INTRODUCTION

African swine fever (ASF) is a viral disease of pigs characterized by fever, skin cyanosis and extensive hemorrhages in internal organs. ASF is also characterized by various forms; from a peracute and acute with 100% mortality to a chronic form.

ASF is endemic in Africa, Sardinia (Italy) and starting from 2007 in Transcaucasia and the Russian Federation. Economic losses caused by African swine fever are associated with both measures taken to eradicate the disease and restrictions in international trade and worth tens of millions of U.S. dollars.

ASF agent is a large enveloped virus from *Asfaviridae* family, *Asfivirus* genus. ASF virus contains a double-stranded DNA genome of 170 to 192 kbp which codes up to 150 proteins. At least 28 out of them are structural [3].

Due to the fact that no effective and safe vaccines against ASF have been developed the disease is controlled by its timely diagnosis and outbreak eradication.

ASF laboratory diagnosis is based on the detection of the disease agent or antibodies in blood and organs of infected animals. Direct virus detection techniques (isolation in cell culture, direct IFA, PCR) are of choice for the diagnosis of peracute and acute ASF forms. Serological methods are preferred for the diagnosis of subacute and chronic forms of the disease; detection of ASFV antibodies is the major tool to detect animals infected with low virulent strains [2].

The World Organization for Animal Health recommends enzyme-linked immunosorbent assay as a technique of the first choice for ASF serological diagnosis. In

the OIE-recommended ELISA (hereinafter OIE-ELISA) semipurified proteins of ASF virus grown in MS cell culture are used as an antigen [6]. The preparation of such an antigen is hard to be standardized and associated with biological risks. Besides, notwithstanding high sensitivity of the OIE-ELISA, its specificity is rather low [4].

In this context intensive research aimed at preparation and use of recombinant antigens in ASF serological diagnosis is carried out now. Recombinant antigens are biologically safe, easier to prepare and ensure higher ELISA specificity. So far the use of several recombinant proteins like p54 [7, 8], p30 [9, 11], pp62 [5], p10, p72, pA104R, pC44L, pCP312R [12] in ASF serological diagnosis have been reported. In 2009 C. Gallardo et al. demonstrated that pK205R and pB602L recombinant proteins are promising antigens for ASF serological diagnosis. ELISA using these recombinant antigens was highly sensitive and specific for ASFV antibody detection in porcine sera [10].

The target of the work done was the obtaining of pK205R and pB602L recombinant proteins by expression in *E. coli*; and optimization of their expression and purification conditions.

MATERIALS AND METHODS

Viruses. ASFV Orenburg-2008 isolate was used for molecular cloning of K205R и B602L genes.

Viral DNA purification was carried out using 6M guanidinium thiocyanate and GF/F fibrous glass filters.

PCR. PCR mixture was made; it contained 5 µl of 10 × PCR buffer, 3 mM Mg^{2+}, 0,2 мМ dNTPs, 2 units of Taq DNA polymerase, 20 picomoles of each primer, 5 µl of DNA solution and water up to 50 µl final volume. The reaction was run using Mastercycler PCR machine (Eppendorf, Germany). The program included three minute initial denaturation at 94°C and 35 PCR cycles: 30 second denaturation at 94°C, 30 second primer annealing at 55°C and 40 second elongation at 72°C. PCR products were analyzed using electrophoresis in 2% agarose gel containing 0,001% ethidium bromide at 50 мА.

Molecular cloning of K205R and B602L genes was carried out in accordance with standard methods [1].

Expression of recombinant proteins. E. *coli* were cultivated in an orbital shaker at 150 rpm and 37°C. IPTG inducer (Promega, USA) was added into cell culture in its log phase. Expression level and recombinant protein size were determined by electrophoresis in 12% polyacrylamide gel.

RESULTS AND DISCUSSION

K205R and B602L genes were amplified by PCR using Russian ASFV isolate. Primers containing Bam HI and Hind III restriction sites were used in the reaction. The amplicones were treated with restriction endonucleases and then cloned in expression plasmid vector under T5 promoter. Recombinant plasmid physical maps are shown in Figures 1 and 2.

As a result of transformation of JM109 *E. coli* competent cells by recombinant plasmids clones expressing ASFV pK205R and pB602L recombinant proteins were obtained. Molecular mass of recombinant proteins was consistent with the estimated one (Fig. 3, Bands 1 and 3).

In order to increase the level of recombinant protein accumulation in *E. coli* cells the experiments aimed at the optimization of expression conditions were carried out. The optimization was done in relation to two parameters: inducer (IPTG) concentration and expression duration.

The influence of inducer concentration on expression level was studied within the range of 0,01 to 2 мМ. Protein expression level was estimated visually using the electrophoregram. The accumulation of pK205R and pB602L recombinant proteins was maximal at IPTG concentration of 0,5 мМ, and was not changed by the further inducer concentration increase.

The results obtained enabled to establish that IPTG concentration of 0,5 мМ was sufficient for the expression of pK205R and pB602L recombinant proteins; and all subsequent experiments were carried out using the said inducer concentration.

When the optimal inducer concentration was established the kinetics of protein expression was studied. For this purpose the aliquots of cell culture were taken 0,25; 0,5; 1; 2; 4 and 18 hours post induction and were studied in 12% polyacrylamide gel. It was found that proteins were relatively stable, because even after 18 hours of expression no proteolysis was observed. pK205R and pB602L accumulation was maximal 4 hours post induction and it remained the same afterwards, that's why the abovementioned time period was used for the subsequent experiments.

The next stage of the research was devoted to the optimization of pK205R and pB602L purification and concentration. There were six histidine residues at N-terminus of the proteins. This enabled to purify pK205R and pB602L by metal-chelate affinity chromatography. Due to the fact that recombinant proteins accumulated in *E. coli* cells were insoluble they were purified under denaturing conditions.

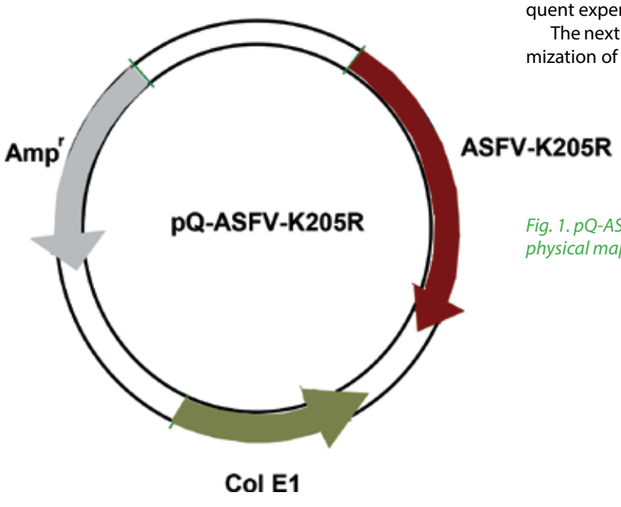

Fig. 1. pQ-ASFV-K205R recombinant plasmid physical map

When using 8M urea as a denaturant just an insignificant part of recombinant proteins was dissolved; the rest of them precipitated with cell debris during centrifugation. pK205R and pB602L were dissolved much better when using 6M guanidinium chloride than urea. Hence 6M guanidinium chloride was included into the buffer for cell lysis and washing.

Dissolved recombinant proteins were purified by metal-chelate affinity chromatography using Ni-NTA-agarose («Qiagen»). The conditions recommended by the adsorbent manufacturer for recombinant protein elution turned out to be inapplicable because proteins could not be eluted from the adsorbent under conventional conditions, i.e. low pH levels and 0,2M imidazole. Only when imidazole concentration in the elution buffer was increased up to 0,4M the most part of the proteins eluted. The further increase in imidazole concentration in the elution buffer did not influence the yield of proteins in the eluate. Thus the use of 0,4M imidazole solution was optimal for protein elution.

When all parameters were optimized, the following procedure of pK205R and pB602L recombinant protein expression and purification was adopted. 1 ml of *E. coli* recombinant clone cell culture was added into a flask with 100 ml of LB medium containing 100 mg/ml of ampicillin and was incubated at 170 rpm, +37°C. IPTG was added into cell culture at OD_{600}=0,5 density up to 0,5 мМ final concentration and was incubated for 4 hours more under the same conditions. *E. coli* cell culture was transferred into centrifuge beakers and centrifuged for 15 minutes at 5000 rpm. Supernatant was removed; the precipitate was resuspended in 5 ml of lysis/washing buffer (6M guanidinium chloride, 0,1M NaH_2PO_4, 0,01M Tris-HCl, pH 8,0) and mixed for 30 minutes. The lysate was centrifuged for 5 minutes at 12000 g. The precipitate was removed and 1 ml of adsorbent (Ni-NTA agarose) was added to the supernatant and then was mixed for 15 minutes; after that the suspension was centrifuged for 1 minute at 12000 g. The supernatant was removed; then it was washed twice in 10 ml of lysis/washing buffer. For the purpose of recombinant protein elution 1 ml of elution solution (0,4M imidazole, 0,1M NaH_2PO_4, 0,01 мМ Tris-HCl, pH 8,0) was added into the precipitate, mixed for 1 minute and then centrifuged for 1 minute at 12000 g. The supernatant contained purified recombinant proteins. To evaluate the level of recombinant protein purification and concentration electrophoresis in 12% polyacrylamide gel by Laemmli's method was performed.

Using the described technique purified recombinant proteins at high concentration were prepared. The purified protein yield from 100 ml of *E. coli* culture was 1,5 mg for pK205R and 2 mg for pB602L (Fig. 3, Bands 2 and 4).

Based on the obtained recombinant antigens it is planned to develop indirect ELISA for the detection of ASFV antibodies in porcine sera.

CONCLUSIONS

Molecular cloning of ASFV K205R and B602L genes was carried out. *E. coli* clones expressing pK205R and pB602 recombinant proteins were obtained. The expression and purification conditions were optimized which enabled to get high yield of purified recombinant proteins.

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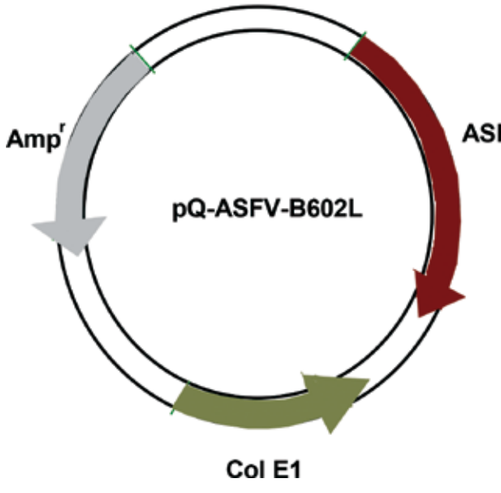

Fig. 2. pQ-ASFV-B602L recombinant plasmid physical map

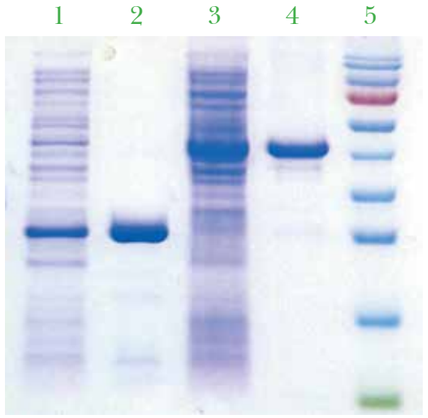

Fig. 3. ASFV pK205R and pB602L recombinant protein expression in E. coli, testing in 12% polyacrylamide gel

1 – pK205R recombinant protein expression in E. coli;
2 – pK205R recombinant protein purified product;
3 – pB602L recombinant protein expression in E. coli;
4 – pK205R recombinant protein purified product;
5 – protein molecular mass marker (170, 130, 95, 72, 55, 43, 34, 26, 17, 10 kDa).

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ИЗУЧЕНИЕ ИММУНОБИОЛОГИЧЕСКИХ СВОЙСТВ ВАКЦИНЫ ПРОТИВ АДЕНОВИРУСНОЙ ИНФЕКЦИИ, ИЗГОТОВЛЕННОЙ НА ОСНОВЕ АДЕНОВИРУСА ПТИЦ 2 СЕРОТИПА

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РЕЗЮМЕ

В работе представлены данные по изучению иммуногенной активности инактивированной вакцины, изготовленной на основе изолята аденовируса птиц FAdV-2. Показана динамика накопления антител в крови цыплят. Также продемонстрирована высокая устойчивость иммунизированных цыплят к заражению гомологичным вирусом (FAdV-2) и отсутствие такой защиты к заражению гетерологичным вирусом (FAdV-4).

Ключевые слова: аденовирус птиц, инактивированная вакцина, антиген, специфические антитела, иммуногенность.

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STUDY OF IMMUNOBIOLOGICAL PROPERTIES OF AVIAN ADENOVIRUS SEROTYPE 2 – BASED VACCINE AGAINST ADENOVIRUS INFECTION

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SUMMARY

The data on studying immunogenic activity of the inactivated vaccine produced on the basis of avian adenovirus isolate FAdV-2 are demonstrated in the paper. The dynamics of antibody accumulation in chicken blood is shown. The high resistance of immunized chicks to homologous virus (FAdV-2) infection and lack of protection against heterologous virus (FAdV-4) infection are also demonstrated.

Key words: avian adenovirus, inactivated vaccine, antigen, specific antibodies, immunogenicity.