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Foot and Mouth Disease Virus Polyepitope Protein Produced in Bacteria and Plants Induces Protective Immunity in Guinea Pigs

E. P. Andrianova[1], S. R. Krementsugskaia[2], N. N. Lugovskaia[2], T. K. Mayorova[2], V. V. Borisov[2], M. A. Eldarov[1], N. V. Ravin[1], A. S. Folimonov[1]*, and K. G. Skryabin[1]

[1]Bioengineering Center, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7-1, 117312 Moscow, Russia; fax: (499) 135-0571; E-mail: folimonov@biengi.ac.ru
[2]Federal State Institution "Federal Center for Animal Health", Yuryevets, 600901 Vladimir, Russia; fax: (492) 226-1573; E-mail: borisov2@arriah.ru

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Abstract—The goal of this project was to develop an alternative foot and mouth disease (FMD) vaccine candidate based on a recombinant protein consisting of efficient viral epitopes. A recombinant gene was designed that encodes B-cell epitopes of proteins VP1 and VP4 and T-cell epitopes of proteins 2C and 3D. The polyepitope protein (H-PE) was produced in *E. coli* bacteria or in *N. benthamiana* plants using a phytovirus expression system. The methods of extraction and purification of H-PE proteins from bacteria and plants were developed. Immunization of guinea pigs with the purified H-PE proteins induced an efficient immune response against foot and mouth disease virus (FMDV) serotype O/Taiwan/99 and protection against the disease. The polyepitope protein H-PE can be used as a basis for developing a new recombinant vaccine against FMD.

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Key words: FMDV, vaccine, transient expression in plants, polyepitope protein

Livestock farming of many countries worldwide suffers great economic losses as a result of infective episodes of foot and mouth disease (FMD). Inactivated virus vaccines have been used for FMD prevention since the beginning of the 20th century. Although immunization with inactivated virus preparations is rather effective, such vaccines have a number of substantial drawbacks. The most serious of them are the problems of biological safety associated with the risk of live virus leak during vaccine production and control, possible residual infectivity of biological preparations, the difficulty of reliable diagnostic differentiation between vaccinated and diseased animals, the necessity for special laboratories where such preparations are obtained, and insufficiently high rate of their production in case of infection caused by new viruses with antigenic distinctions.

Because of these drawbacks attempts have been made to develop alternative methods of immunization against FMD—using as a vaccine fragments of structural and nonstructural viral proteins, e.g. structural protein VP1 [1, 2] or empty viral capsids [3-11], and creating attenuated viruses, e.g. with a deleted region of capsid protein VP1 [12] or proteinase L^{pro} [13, 14].

One method for alternative immunization might be a vaccine based on recombinant proteins formed by a set of effective epitopes of the FMD virus within a single polypeptide chain. Vaccination with such polyepitope proteins containing a combination of well-studied B- and T-cell viral epitopes may cause effective immune response in an animal and contribute to its more effective protection [15].

Plants are now promising systems of protein production, which are of great interest for biotechnological companies. The advantages of using plants for protein production are the low final cost and biosafety of products due to the absence of pathogens common for plants and animals. Recombinant proteins in plants may be obtained by genet-

Abbreviations: $CCID_{50}$, cell culture infectious dose 50%; FMD, foot and mouth disease; IEA, immunoenzyme assay; MNR, microneutralization reaction.

* To whom correspondence should be addressed.

ic transformation or transient expression with viral vectors [16-18]. Creation of a transgenic plant and, consequently, production of a protein from this plant requires considerable time and cost. The level of expression of target proteins by transgenic plants is usually low, determining the high cost of products due to the difficulty of their purification [19, 20]. Protein production by means of a phytoviral system of transient expression in plants has a number of advantages, first of all the higher (tens and hundreds of times) level of expression, biosafety, and short time required for creation and testing of an expression system.

Modern viral systems allow expression in plants within a few days of target proteins containing in some cases 20-30% of the total amount of soluble protein, corresponding to 1-2 g per kg of plant biomass [21]. Plant virus-based vectors are now used for obtaining various vaccine proteins in plants [22-25].

The goals of this study were to create a recombinant anti-FMD vaccine based on a polyepitope protein including effective epitopes of the FMD virus and to develop the methods of its production in bacteria and plants.

MATERIALS AND METHODS

Media, reagents, enzymes, bacterial strains, and oligonucleotides. Bacteria were grown on Petri dishes with LB agar or in flasks with LB broth [26] at 37°C (*Escherichia coli*, strains JM109 and Rosetta-2 (DE3)) and at 28°C (*Agrobacterium tumefaciens*, strain EHA105). The media contained the appropriate antibiotics: ampicillin (50 μg/ml), kanamycin (50 μg/ml), chloramphenicol (30 μg/ml), gentamycin (25 μg/ml), and rifampicin (15 μg/ml).

A sequence of six histidine amino acid residues was added to the synthetic gene sequence using the following oligonucleotides: m13f (CGCCAGGGTTTTCCCAGTCACGAC), M13R (CAGGAAACAGCTATGAC), and pHisPE (ACCTTGGGCGCGCCCATATGCATCATCACCATCACCATATAATCAATAACTATTATATG).

Synthetic gene construction. The nucleotide DNA sequence of a recombinant gene encoding the protein consisting of FMD viral epitopes was created using the amino acid sequences of the known B-cell epitopes of structural proteins (VP4 (21-40) [27], VP1 (135-160) [28], and (200-213) [29]) and T-cell epitopes of nonstructural proteins of the FMD virus serotype O/Taiwan/99 (2C (68-76) [30], 3D (1-115), and (421-460) [31]) (Fig. 1a). The epitopes were divided by "flexible" glycine-rich linkers G4S2 to avoid potential problems of protein folding. For enhancing the efficiency of recombinant protein expression, the codon composition of the coding DNA sequence was optimized for expression in plants of the genus *Nicotiana*. The gene was designed with exclusion of the possibility of the known sites of mRNA splicing, which could be recognized in plant cells, restriction sites, and DNA methylation sites. For gene cloning into the expression vectors, the sequences of restriction sites *Asc*I and *Nde*I were added to the 5′-end and the sequences of sites *Xho*I and *Xma*I were added to the 3′-end of this DNA. The *EcoR*I site sequence was added between the sequences encoding the B- and T-cell epitopes (Fig. 1b). The DNA of this gene was synthesized by Evrogen (Russia) and obtained as a component of plasmid pGEM-T.

Producer vectors used for protein production. The protein was obtained in *E. coli* using plasmid pET-23a(+) (Novagen, USA). Expression vector pET-23a(+)-H-PE was constructed in two steps. In the first step, an amino acid sequence of six histidine residues was added to the N-end of such chimeric sequence using the PCR method and primers m13f, pHisPE, and M13R for potential isolation of the protein. The resulting sequence was cut out of the PCR product and then cloned into plasmid pET-23a(+) at the *Nde*I and *Xho*I restriction sites.

The potato X-virus based vector pA7248-AMV was used for protein production in plants [32]. The viral vector pA7248-AMV-H-PE was obtained by cloning the protein-encoding sequence into plasmid pA7248-AMV by the *Asc*I and *Xma*I restriction sites. The vector pA7248-AMV-GUS was obtained by cloning the β-glucuronidase encoding sequence into plasmid pA7248-AMV by the *Asc*I and *Xho*I restriction sites.

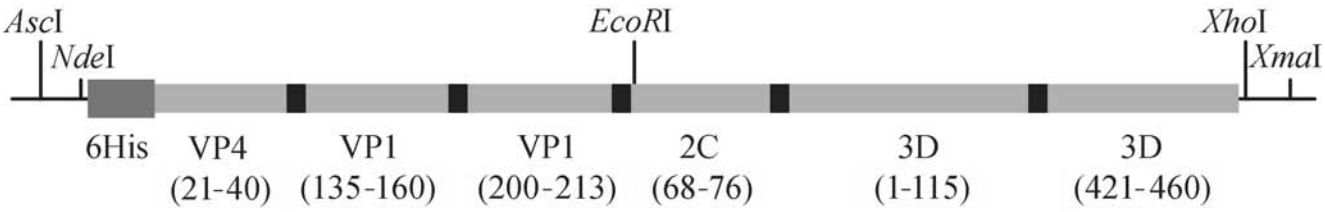
For enhancing the efficiency of target protein transient expression, the plants were agroinoculated with a mixture of cultures transformed by the vector with the target gene and by the vector producing the RNA silencing suppressor: HCPro turnip mosaic virus cloned in a modified plasmid pCambia2301. In this plasmid the nucleotide T-DNA sequence flanked by the regions necessary for its transfer into the nucleus was replaced by the recognition site for restrictase *Asc*I, the 35S-promoter sequence, the recognition site for restrictase *Pme*I, the sequence of the open reading frame encoding the suppressor protein, the recognition sites for restrictases *Pst*I and *Stu*I, and the sequences of NOS and 35S terminators of transcription. The resulting construction was designated as pCCaHCPro.

Protein expression in *E. coli* cells by the method of autoinduction. The method of autoinduction [33] with modifications was used for efficient expression of the protein.

Transformed Rosetta-2 cells were incubated for 12 h at 37°C on LB agar with 1% glucose. Cell colonies were transferred into the ZYP-0.8G medium; the cultures were grown in a shaker for 7 h at 37°C; 5 μl of cell culture was inoculated into the ZYP-5052 medium and grown in the shaker at 21, 28, or 37°C for 12-48 h.

Transformation of agrobacteria and agroinfiltration of plants were carried out by the standard methods [34]. For transformation in an ice bath, 0.5 μg of plasmid DNA was

a



b

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H-PE      GG CGC GCC CAT ATG CAT CAT CAC CAT CAC CAT ATA ATC AAT
Amino acids              M   H   H   H   H   H   H   I   I   N
AAC TAT TAT ATG CAG CAA TAC CAG AAC AGC ATG GAC ACA CAA CTC GGG
 N   Y   Y   M   Q   Q   Y   Q   N   S   M   D   T   Q   L   G
GAT GGA GGT GGC GGT AGC TCA AAA TAT GGT GAA TCA CCC GTA ACT AAT
D   G   G   G   G   S   S   K   Y   G   E   S   P   V   T   N
GTT AGG GGC GAT CTA CAA GTA CTA GCT CAG AAA GCT GCT CGA ACA TTA
V   R   G   D   L   Q   V   L   A   Q   K   A   A   R   T   L
GGT GGG GGT GGT TCC TCC AGG CAC AAA CAA AAG ATT GTT GCT CCA GTT
G   G   G   G   S   S   R   H   K   Q   K   I   V   A   P   V
AAG CAA CTG CTA GGG GGC GGT GGA AGT TCT GAA TTC GAA TGG CTG GAC
K   Q   L   L   G   G   G   G   S   S   E   F   E   W   L   D
AAC GCA AGA CAG GCT GGA GGG GGA GGA TCT AGT GGC TTG ATT GTT GAC
N   A   R   Q   A   G   G   G   G   S   S   G   L   I   V   D
ACA CGT GAT GTT GAG GAA AGA GTA CAC GTC ATG CGA AAG ACT AAG CTA
T   R   D   V   E   E   R   V   H   V   M   R   K   T   K   L
GCA CCT ACC GTT GCT CAC GGA GTA TTT AAC CCT GAG TTT GGA CCA GCT
A   P   T   V   A   H   G   V   F   N   P   E   F   G   P   A
GCT CTC TCC AAT AAA GAT CCT AGA TTG AAT GAA GGT GTC GTT TTG GAT
A   L   S   N   K   D   P   R   L   N   E   G   V   V   L   D
GAA GCT ATC TTT TCA AAA CAC AAA GGA AAC ACT AAG ATG TCT GAA GAA
E   A   I   F   S   K   H   K   G   N   T   K   M   S   E   E
GAT AAA GCT CTT TTT CGT CGA TGT GCT GCA GAT TAC GCC TCA AGA TTG
D   K   A   L   F   R   R   C   A   A   D   Y   A   S   R   L
CAT AGC GTA CTA GGC ACA GCC AAC GCT CCT CTT TCT ACT TAC GAA GCT
H   S   V   L   G   T   A   N   A   P   L   S   T   Y   E   A
ATT AAG GGG GTC GAT GGC CTT GAT GCA ATG GAG CCT GAT ACT GGC GGT
I   K   G   V   D   G   L   D   A   M   E   P   D   T   G   G
GGG GGA TCA AGT CAG GAA AAG TTA ATT TCA GTT GCT GGT TTG GCC ATA
G   G   S   S   Q   E   K   L   I   S   V   A   G   L   A   I
CAC TCT GGC CCA GAT GAA TAT CGT AGA CTG TTT GAA CCT TTT CAA GGT
H   S   G   P   D   E   Y   R   R   L   F   E   P   F   Q   G
TTG TTT GAG ATC CCC TCA TAT AGG AGC CTC TAT CTT TAA TAG CTC GAG
L   F   E   I   P   S   Y   R   S   L   Y   L
CCC GGG
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Fig. 1. Sequence encoding recombinant H-PE protein. a) Scheme of sequence encoding H-PE protein; glycine-rich linkers are in black. b) Sequence of synthetic H-PE gene optimized for expression in plants. Restriction sites are underlined; glycine-rich linkers are in gray; six histidine residues are in black.

added to the competent cells. The cells were kept for 5 min at 37°C and then, after adding 500 μl of LB medium, grown for 4 h at 28°C under continuous stirring. The cells were inoculated on LB agar and incubated at 28°C for 3-4 days.

Transformed agrobacteria were inoculated into LB medium with 10 mM MES and grown for 12 h on a shaker at 28°C. The cells were precipitated at 4000 rpm for 5 min; the precipitate was resuspended in the inducing buffer containing 10 mM $MgSO_4$ and 10 mM MES. Cell suspension was prepared in the inducing buffer with optical absorption (A_{600}) 0.4. On addition of acetosyringone solution to the final concentration of 150 μM, the cell suspension was kept at room temperature for 3 h. The suspensions of agrobacterial cells transformed by the construction encoding the target gene (pA7248-AMV-H-PE or pA7248-AMV-GUS) and the construction of RNA silencing suppressor (HCPro) were mixed and introduced using a syringe without a needle into the intercellular space of plant leaves.

Isolation and purification of recombinant bacterial protein. The bacterial protein with six amino acid residues was isolated under denaturing conditions by affinity chromatography on Ni-NTA Sepharose (Promega, USA) in a Qiagen column (Germany) using modified procedures 10 and 17 from The QIA*expressionist* Manual (Qiagen).

Isolation and purification of plant recombinant protein. The plant protein with six amino acid residues was isolated under denaturing conditions on Ni-NTA Sepharose (Promega) in a Qiagen column by the procedure described for bacterial protein isolation with the modification that the weighed portion of fresh leaves of the plant expressing the recombinant protein was pounded to homogeneous powder in liquid nitrogen. Western blot analysis of the proteins was carried out by the standard method [35].

Immunization of laboratory animals with H-PE protein preparations. For preliminary assessment of immunogenicity of the H-PE protein obtained in bacteria, eight guinea pigs were immunized by a single intramuscular injection into hind limbs of oil emulsion containing 300 μg of the H-PE protein in the inoculation volume. Immunization substance was prepared from 30 parts of the water phase containing the H-PE protein in 4 M urea, 10 mM Tris, pH 8.0, and 70 parts of oil adjuvant Montanide ISA 70 (Seppic, France). A second group of eight guinea pigs did not receive the protein. For assessment of immunogenicity of the H-PE proteins, oil emulsion was introduced into three groups of guinea pigs (eight animals in each) intramuscularly into hind limbs with different doses of the protein: 350, 120, and 40 μg for the first, second, and third group, respectively. The fourth group (the control) included eight guinea pigs not receiving the protein.

Infection of guinea pigs with FMD virus. In 21 or 28 days after immunization, the groups of guinea pigs were subjected to control infection with FMD virus O/Taiwan/99 adapted for these animals, homologous to the vaccine strain, intradermally into the plantar surface of hind limbs at a dose exceeding 10,000-fold the dose that caused the death of 50% of culture cells (0.2 cm^3). Infection results were recorded after 7 days and assessed by generalization of the FMD process. Formation of secondary aphthae on the forelimbs, where the virus was not introduced, was considered as generalization. The vaccine control was considered as valid if no less than seven animals out of eight not vaccinated guinea pigs developed the generalized form. Reliability of immunization results was assessed by the binomial test with the level of significance $\alpha = 0.005$.

Indirect method of immunoenzyme assay (IEA). Indirect IEA was performed by the standard scheme with some modifications. The FMD virus antigens (strains O/Primorsky/00, O/Manisa, and O/Taiwan/99) were obtained from the suspension of infected passaged PGSK-30 culture during precipitation of inactivated viral particles by 8% polyethyleneglycol solution (6 kDa) with addition of NaCl to the final concentration of 0.9% and treatment with chloroform followed by antigen purification and concentration by ultracentrifugation through 20% sucrose solution. Preparations of the FMD virus antigen were analyzed in 12-15% polyacrylamide gel [36].

The concentrated and purified FMD virus antigen was adsorbed in a 96-well MaxiSorp polystyrene plate (Nunc, Denmark) in carbonate-bicarbonate buffer (pH 9.6), 1-2 mg of protein per well. The regions of wells not bound to the antigen were blocked with BSA-TBST buffer (0.02 M Tris-HCl, 0.15 M NaCl, 0.05% Tween-20, 1% BSA). Samples were applied to the plate in FS-TBST buffer (0.02 M Tris-HCl, 0.15 M NaCl, 0.05% Tween-20, 5% fetal serum) by the method of double serial dilutions and incubated for 30 min at 37°C. After four-fold washing of the plate with the TBST buffer (0.02 M Tris-HCl, 0.15 M NaCl, 0.05% Tween-20), 50 μl of antispecies immunoperoxidase conjugate (MedGamal Branch, Gamaleya Research Institute of Epidemiological Medicine, Russian Academy of Medical Sciences) diluted in FS-TBST (1 : 1000) was introduced into each well and again incubated for 30 min at 37°C. Then the plate was washed once more and stained with ABTS substrate mixture (MP Biomedicals, USA). After 10-15 min, the reaction was stopped with 1% SDS. The extent of reaction was recorded in a multichannel spectrophotometer by optical absorption (A) measurement at 405 nm. The final dilution, when A of a well was less than or equal to the double mean A value of the negative control, was considered as the titer of the serum.

The commercial kit O FMDV Ab PrioCHECK (The Netherlands) was used as a reference test for revealing the antibodies against the type O FMD virus. The reaction was conducted in accordance with the manufacturer's instructions and considered to be positive at PI ≥ 50%.

Microneutralization reaction (MNR). This reaction was performed on 96-well plates (Corning Costar, USA) in passaged cultures of pig kidney IB-RS-2 cells against 100 $CCID_{50}$ culture FMD virus of the O/Taiwan/99 type. Before the reaction, the blood serum of the guinea pigs was diluted 1 : 4 with Eagle's maintenance medium and inactivated for 30 min at 56°C to removal nonspecific inhibitors. The sera were titrated by a double-step method beginning with the 1 : 16 dilution, supplemented with an equal volume of the FMD virus working dosage, and kept for 1 h at 37°C. Then the cell culture suspension with cell concentration of $0.8 \cdot 10^6$ per cm^3 was introduced into wells of the plates. The plates were kept for 48 h in a CO_2-incubator at 5% CO_2 and 37°C. The reaction was monitored under an inverted microscope; the serum titer was calculated by Kerber's method. The limiting dilution of the serum when the infectious effect of the virus of 100 $CCID_{50}$ was neutralized in 50% infected culture of IB-RS-2 cells was considered as a virus-neutralizing titer of antibodies of the tested serum. The sera with activity of antibodies in the dilution 1 : 45 and more were considered as positive.

RESULTS AND DISCUSSION

H-PE protein production in *E. coli* cells. For obtaining the polyepitope protein in *E. coli* cells, the H-PE gene sequence was cloned in plasmid pET-23a(+). The resulting plasmid (pET-23a(+)-H-PE) was used for transformation of the strain *E. coli* Rosetta-2 with the plasmid pRARE2 allowing the effective translation of rare codons. The H-PE protein was expressed by the method of autoinduction.

At the incubation temperature of 37°C, the level of protein expression in the Rosetta-2 bacterial culture transformed by plasmid pET-23a(+)-H-PE was quite high (~80% of the total cell proteins) (Fig. 2a).

The solubility of bacterial protein H-PE was tested using cell cultures grown at 28 and 21°C. It was shown that H-PE produced in bacteria is insoluble under physiological conditions (data not shown).

Isolation and purification of the H-PE protein from *E. coli* cells. The H-PE protein produced in *E. coli* cells was isolated by chromatography on Ni-NTA Sepharose under denaturing conditions. Eluate fractions with the maximum protein content were dialyzed against 4 M urea and 10 mM Tris-HCl solution (pH 8.0). The H-PE protein was isolated from 65 mg of cell precipitate in an amount of ~1 mg with a rather high level of purification (~95%) (Fig. 2b). The H-PE protein preparation contained low molecular weight proteins, most likely the products of H-PE degradation or premature termination of the translation occurring in culture cells at the stage of saturation. Purified protein preparations were used to obtain a mouse polyclonal antiserum.

H-PE protein production in *N. benthamiana* plants. For H-PE protein production in cells of *N. benthamiana*, the encoding sequence was cloned in the viral vector pA7248-AMV. The resulting plasmid (pA7248-AMV-H-

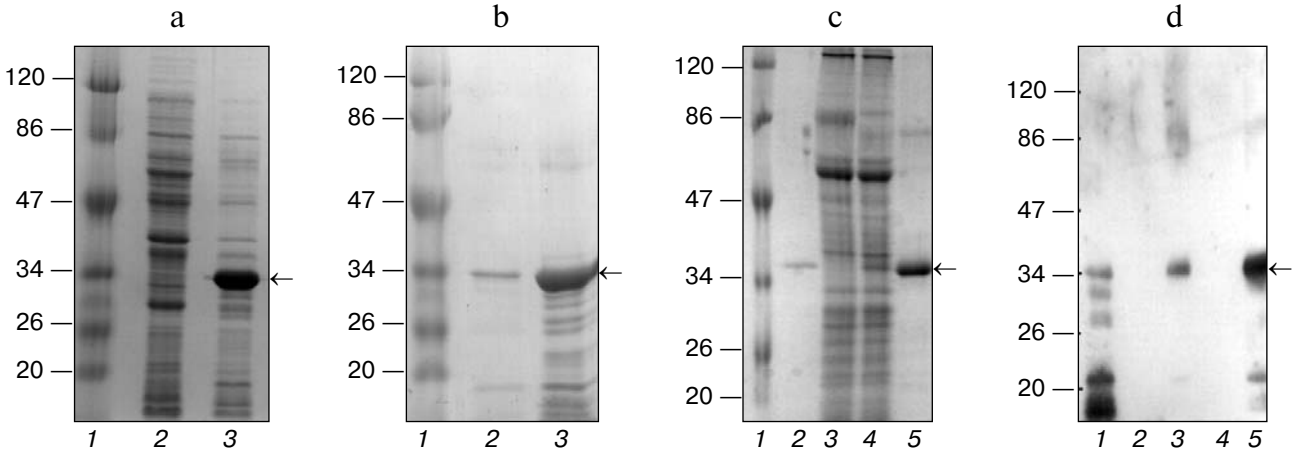

Fig. 2. Preparation of H-PE protein. a) Expression of H-PE protein in *E. coli*: *1*) marker; *2*, *3*) protein of culture cells not transformed and transformed by expression plasmid pET-23a(+)-H-PE. b) Isolation and purification of bacterial H-PE protein: *1*) marker; *2*) protein of culture cells transformed by plasmid pET-23a(+)-H-PE (~0.5 μg of H-PE protein); *3*) purified H-PE protein. c) Expression of H-PE protein in *N. benthamiana* plants: *1*) marker; *2*) protein of *E. coli* culture cells transformed by plasmid pET-23a(+)-H-PE (~0.5 μg of H-PE protein); *3*, *4*) a tissue sample of a leaf agroinoculated with a mixture of culture cells transformed by pA7248-AMV-GUS, HCPro, and pA7248-AMV-H-PE, HCPro; *5*) purified H-PE protein from plants. d) Western blot analysis of H-PE protein preparations with the serum of mice immunized with H-PE from *E. coli*: *1*) protein of *E. coli* culture cells transformed by plasmid pET-23a(+)-H-PE (~5 ng H-PE protein); *2*) protein of *E. coli* culture cells not transformed by plasmid pET-23a(+)-H-PE; *3*, *4*) tissue sample of leaf agroinoculated with mixture of cell cultures transformed by pA7248-AMV-H-PE, HCPro (~5 ng of H-PE protein) and pA7248-AMV-GUS, HCPro; *5*) purified H-PE protein from plants (~25 ng).

PE) was used for transformation of *A. tumefaciens* strain EHA105.

Cells of the plants agroinoculated with pA7248-AMV-H-PE could express a protein with electrophoretic mobility like in the H-PE protein expressed in bacteria (Fig. 2c). The tissue of the same leaf infiltrated with the culture of agrobacteria transformed by the pA7248-AMV-GUS construction was used as a negative control. The share of the H-PE protein in the plant was 0.7-1% of total protein content in leaf tissue.

The H-PE protein was isolated and purified from plants by affinity chromatography on Ni-NTA Sepharose. The H-PE protein was isolated from 120 g of fresh plant tissue in the amount of ~8 mg (equivalent to 67 mg protein from 1 kg plant tissue). The correspondence of the H-PE protein produced in plants to the protein produced in bacteria was verified by Western-blot analysis with plant tissue samples taken from the zone infiltrated by the agrobacterial culture transformed by plasmid pA7248-AMV-H-PE and plasmid pA7248-AMV-GUS (negative control), the total protein of *E. coli* cells, and the preparation of the H-PE protein isolated from plants (Fig. 2d). Interaction of the mouse serum antibodies to the bacterial protein H-PE with the protein accumulated in plants and its purified preparation and the similar electrophoretic mobility of these proteins are evidence of their mutual correspondence and probable absence of posttranslational modifications of the H-PE protein obtained in plants. The antiserum to protein H-PE was highly specific because there was almost no interaction between the antibodies and the host proteins of bacterial and plant cells.

Preliminary assessment of immunogenicity of bacterial protein H-PE. Blood samples were taken from eight immunized and two control guinea pigs 21 days after vaccination. The guinea pig blood sera were tested by indirect IEA with the FMD virus antigens O/Primorsky/00 and O/Manisa (test systems of the Federal State Institution, All-Russian Research Animal Protection Institute) in the reference test system with the O FMDV Ab PrioCHECK kit and MNR (Table 1).

Virus antigens interacting with antibodies were analyzed by Western blot with the antigens O/Primorsky/00 and O/Manisa (Fig. 3a). Antibodies of the sera of immunized animals interacted mainly with the structural protein VP1 of FMD virus serotype O.

Single introduction of recombinant bacterial protein into guinea pigs (300 μg) induced in animals the formation of virus-neutralizing antibodies to FMD virus type O/Taiwan/99 revealed by MNR, by indirect IEA with FMD virus antigen types O/Primorsky/00 and O/Manisa, and by IEA with O FMDV Ab PrioCHECK.

In 28 days after immunization, guinea pigs were exposed to control infection with FMD virus O/Taiwan/99 adapted to these animals. Generalization of FMD infection 7 days after the infection was absent in six out of seven immunized animals but present in seven control guinea pigs.

The data of the preliminary assessment of immunogenicity of bacterial protein as a component of emulsion vaccine show that it is able to induce immune response in laboratory animals at a dose of 300 μg. The results of MNR and IEA suggested that the dose of introduced protein may be reduced. It was also necessary to test the plant polyepitope protein.

Study of immunogenic and protective activity of bacterial and plant H-PE protein in guinea pigs. The minimal dose of the H-PE protein inducing protection against the virus was determined as follows: guinea pigs were immunized with vaccines containing different quantities of bacterial or plant protein (300, 120, and 40 μg). In 17 days after immunization, blood sera taken from the animals immunized with bacterial protein were tested by MNR. Blood samples were also taken from guinea pigs immunized with the plant protein and tested by indirect IEA with FMD virus antigen O/Taiwan/99 (test system of All-Russian Research Animal Protection Institute) and with the O FMDV Ab PrioCHECK kit (Table 2). In 21 days

Table 1. Results of the study of blood sera of guinea pigs in IEA and MNR for presence of antibodies to FMD virus type O

Group of animals	IEA			MNR with the FMD virus O/Taiwan/99
	All-Russian Research Animal Protection Institute (FMD virus antigen O/Primorsky/00)	All-Russian Research Animal Protection Institute (FMD virus antigen O/Manisa)	O FMDV Ab PrioCHECK PI, % ($PI_{pos} \geqslant 50\%$)	
Immunized with H-PE protein	1 : 860 ± 360	<1 : 200	72.5 ± 5.5	>1 : 64
Not immunized	<1 : 200	<1 : 200	<10	<1 : 16
Immunized with antigen of swine vesicular disease virus (negative control)	<1 : 200	<1 : 200	<10	–

Note: PI_{pos}, percentage of inhibition (positive); –, not investigated.

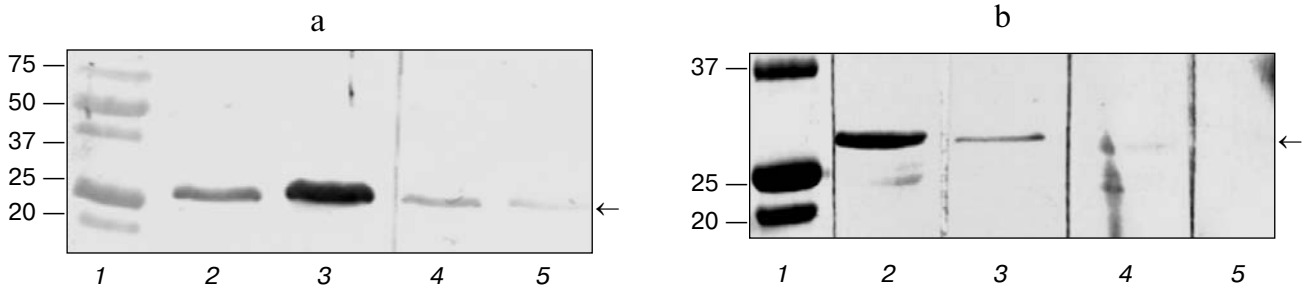

Fig. 3. Western blot analysis of FMD virus antigens. a) Western blot with sera of guinea pigs immunized with H-PE from *E. coli*: *1*) marker; *2*, *3*) antigens O/Manisa and O/Primorsky/00 treated with serum of immunized guinea pig; *4*, *5*) antigens O/Manisa and O/Primorsky/00 treated with serum of control guinea pig. b) Western blot with sera of guinea pigs immunized with H-PE from plants: *1*) marker; *2*, *3*) antigens O/Primorsky/00 and O/Taiwan/99 treated with serum of immunized guinea pig from group 2; *4*, *5*) antigens O/Primorsky/00 and O/Taiwan/99 treated with serum of immunized guinea pig from group 4.

after immunization all groups of guinea pigs were subjected to the control infection by the FMD virus O/Taiwan/99 adapted to these animals.

A single immunization of guinea pigs with a preparation dosage containing 350 and 120 μg of plant protein H-PE induced the production of antibodies, which was determined according to the O FMDV Ab PrioCHECK reference test.

The virus antigens interacting with antibodies from the blood sera of guinea pigs immunized with plant H-PE were analyzed by Western blotting with FMD virus antigens O/Taiwan/99 and O/Primorsky/00 (Fig. 3b). Immunoblotting of the blood sera of immunized animals showed the presence of antibodies to the VP1 protein of FMD virus type O.

A single immunization of guinea pigs by emulsion vaccine containing the bacterial or plant H-PE protein in immunizing dose of 350 or 120 μg, respectively, induced in the animals the formation of virus-neutralizing antibodies to FMD virus type O/Taiwan/99 revealed by MNR or IEA and Western-blot. In all animals immunized by these doses, no appearance of the secondary aphthae on fore limbs was shown at 7 days after the infection. With the level of $\alpha = 0.005$, the protection of guinea pigs immunized with 350 and 120 μg of the protein was reliable. The results of resistance of guinea pigs immunized with the bacterial protein to the control infection correlated with the level of specific antibodies revealed by the MNR of their sera.

Secondary aphthae were registered in four of eight guinea pigs in the group of animals immunized with bac-

Table 2. Results of study of immunogenic and protective activities of bacterial and plant H-PE proteins in guinea pigs

Group number	Quantity of introduced protein, μg	MNR	O FMDV Ab PrioCHECK PI, % ($PI_{pos} \geq 50\%$)	Test system of All-Russian Research Animal Protection Institute	Control infection: number of protected animals/number of experimental animals	*p*
Activity of bacterial H-PE protein						
1	350	<1 : 45	–	–	8/8	0.004
2	120	<1 : 32	–	–	8/8	0.004
3	40	<1 : 16	–	–	4/8	0.637
4	n/i	>1 : 16	–	–	0/8	n/d
Activity of plant H-PE protein						
1	350	–	78 ± 14.7	>100	8/8	0.004
2	120	–	70 ± 22.7	>100	8/8	0.004
3	40	–	58.5 ± 19.7	>50	6/8	0.145
4	n/i	–	16.3 ± 6.5	<50	0/8	n/d

Note: PI_{pos}, percentage of inhibition (positive); n/d, not determined; n/i, not introduced.

terial H-PE (40 μg) and two of eight animals immunized with plant protein. FMD symptoms did not appear in some not-immunized (control) guinea pigs.

The results of assessment of immunogenicity of bacterial and plant polyepitope H-PE proteins demonstrate that a single immunization of guinea pigs with a dose of 120 μg in combination with oil adjuvant Montanide ISA 70 can induce immune response in the animals that is detected by the methods of IEA and MNR, and it causes resistance to the control infection with the homologous adapted FMD virus type O/Taiwan/99. For investigation of the possibility of using H-PE protein as a vaccine, it is necessary to assess its immunogenicity in agricultural animals.

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