Examination of immunogenic properties of recombinant antigens based on p22 protein from African swine fever virus

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Abstract

Introduction: The single member of the Asfarviridae family is African swine fever virus (ASFV). This double-stranded DNA virus infects wild and farmed swine and loses the pig industry large sums of money. An inner envelope, capsid, and outer envelope are parts of the ASFV particle containing structural proteins playing different roles in the process of infection or host immune defence evasion. When expressed by the baculovirus system, the p22 protein from the inner envelope was found to induce partial protection against a virulent virus strain. This study aimed to express a part of this protein in a different system and evaluate its immunogenicity.

Material and Methods: We designed two proteins, the extracellular (C terminal) part of the p22 protein (p22Ct) and its fusion with the heat-labile enterotoxin B subunit from Escherichia coli (LTB-p22Ct), which is supposed to be a potent enhancer of the immune response. Both proteins were produced in the E. coli expression system and subsequently used for mice immunisation to analyse their safety and immunogenicity. Results: The protein fused with LTB did not show the expected adjuvant properties and did not prove safe, because abscess formation was observed after immunisation. In contrast, immunisation with the p22Ct protein alone induced a higher antibody titre but caused no adverse symptoms. Conclusion: These results show the high potential of the p22Ct region as an immunogenic protein for ASFV serological detection purposes.

Keywords: African swine fever virus, heat-labile enterotoxin B subunit, immune response, p22 protein.

Introduction

African swine fever virus (ASFV) is a large double-stranded DNA virus belonging to the Asfarviridae family that causes enormous economic loss in the pig industry by killing farmed animals with a near-100% fatality rate (19). The vectors of this virus are soft ticks from the genus Ornithodoros (10) and wild Suidae in Africa. However, the primary route of transmission is contact between domestic pigs and wild boars, especially in regions with a mild climate where Ornithodoros ticks are not present. Another route of transmission is porcine products (such as meat and meat products) and slaughter and processing waste. The structure of ASFV particles includes three envelopes protecting the core containing linear double-stranded DNA. These three envelopes are the inner envelope, capsid, and outer envelope. Each envelope consists of different structural proteins playing different roles in the process of viral infection or host immune defence evasion, which is explained in more detail in the previous work (8).

The disease caused by this virus, African swine fever, is associated with many symptoms, including high fever, lesions, and haemorrhagic skin discolouration in the host organism, or rarely is not associated with any signs (29). This peracute infection usually occurs in animals that die even before the immune system can react to the infection (25). For this reason, several vaccines have been developed, but none has been effective enough to protect pigs against a virulent strain of the virus.

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Our present study focuses on the ASFV p22 protein, which may become a subunit vaccine candidate. Typically, subunit vaccines contain proteins originating from the virus infecting the host organism. Many structural antigens from ASFV have been tested as subunit vaccines, including p22, p30, p54, p72, CD2v, and other proteins (20, 21, 24). Of these proteins, p22, p30 and CD2v were capable of inducing at least a minimal immune response (4, 12, 22). Although none of the antigens successfully protected the host against a virulent virus challenge, they could still be considered potential antigens for future vaccine development.

The p22 protein was initially believed to be part of the outer membrane (5). However, recently it has been found in the inner membrane (1). An initial experiment using a non-ionic detergent showed that the p22 protein is part of the outer envelope; however, as was learned later, the detergent could disrupt not only the outer but also the capsid envelope (2, 5). The structure of the p22 antigen consists of three subparts: a 20-amino-acid-long (1...20) N-terminal domain, a 21-amino-acid (21...41) transmembrane α-helix, and an outer 148-amino-acid (42...189) C-terminal part. Recently, it has been found that the p22 protein interacts with host proteins related to several biological processes, including virus binding, signal transduction, and cell adhesion (30). Some of these proteins participate in ribosome, spliceosome, and actin filament organisation and movement, possibly also in DNA replication, or affect phagocytosis and endocytosis (30). Even though the p22 protein can interact with many host proteins, it was shown recently not to be essential for ASFV replication or virulence (27).

The gene encoding the p22 protein was initially discovered on the left end of the BA71 genome and was named then based on the K177 open reading frame (ORF) (5, 11), but it was later renamed KP177R and termed the early membrane protein. The KP177R encoding gene was also found on the right end of the Malawi LIL120/1 genome (28). Vydelingum et al. (28) suspected that it could be a consequence of evolution by duplications, deletions or sequence transposition from one end of the genome to the other. The KP177R gene is also known among the genes coding for structural antigens as one of the most conserved ASFV genes (17). It was also found that the I10L ORF encodes a protein somewhat similar to p22, of which the nucleotide sequence has 40% homology with the p22 protein from BA71V (7). The I10L ORFs of the Benin97/1 and ORT88/3 isolates have 100% homology, which shows this gene’s conservation throughout the isolates identified thus far (6). However, it is still not known whether the protein expressed by this gene is an analogue in the viral structure to the p22 protein (6).

The heat-labile E. coli enterotoxin (LT) is a heterohexameric protein that promotes cell adherence of E. coli to the intestinal epithelial cells mainly because of the ADP-ribosylation activity of the A subunit (LTA) (14). The B subunit (LTB) has immunomodulatory properties as it binds the GM1 ganglioside on the surface of the immune cells and preferentially induces the apoptosis of CD8+ T-cells via the rapid loss of mitochondrial membrane potential and cell viability (26). Multiple uses have been made of LTB as a potent and robust adjuvant, either individually or fused to other proteins (9, 13).

As the p22 protein takes part in virus binding (30), it deserves further attention for its possible recognition by the immune system. We therefore decided to prepare a recombinant protein based on the outer C-terminal part of p22 and examine its immunogenicity in mice.

Material and Methods

Strains, vectors and growth conditions. The E. coli One Shot TOP 10 strain was used as the cloning host, and the E. coli BL21 Star strain was used for protein overproduction (both strains were from Thermo Fisher Scientific, Brno, Czech Republic). The One Shot TOP 10 and BL21 Star strains were grown under vigorous orbital shaking (4.5 × g) at 28°C and 37°C, respectively, in Luria-Bertani (LB) medium. For the cloning and expression of the genes, pMA-RQ (Thermo Fisher Scientific) and pET28b(+) (Merck, Darmstadt, Germany) were used, respectively.

Construct design and cloning. Constructs were designed, for the first protein containing the part of the KP177R gene (GenBank accession number MK333183) coding for the C-terminal globular segment (amino acids 42–189) of the p22 protein (p22Ct) and for the second protein containing the same gene part fused at the 3' end with the gene coding for the LTB (GenBank accession number M17873) (LTB p22Ct). In addition, a 10× His-tag coding sequence was attached to the 3’ end of each construct for encoded protein purification and detection purposes. The designed gene sequences, named p22Ct and LTB-p22Ct, were codon-optimised for production in E. coli, synthetically prepared using a commercial service (Thermo Fisher Scientific) and cloned into the pET28b(+) vector at the NcoI restriction enzyme site. For colony screening, His-tag forward (5'-CCATCAACTCAGCTTCCTTTC-3′) and T7 terminator reverse (5'-AGCCCAAATCAGCTTCCCTTTC-3′) primers were used. After cloning, the constructs within pET28b(+) were verified using a commercial sequencing service (SEQme, Dobříš, Czech Republic). The confirmed clones pET28b(+)-p22Ct and pET28b(+)-LTB-p22Ct were used for the recombinant protein production.

Expression of recombinant proteins. The clones pET28b(+)-p22Ct and pET28b(+)-LTB-p22Ct were transformed into chemically competent E. coli BL21 Star cells by heat shock. Positive clones were inoculated into LB medium and grown overnight at 37°C. A small-scale expression of LTB-p22Ct and p22Ct proteins in 5 mL of the E. coli BL21 Star cell cultures was performed using the pET28b(+) vector. The expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG), and the cultures were grown for 4 h at 37°C.
A large-scale expression was performed to produce higher protein quantities for purification and further experiments. An aliquot of 20 mL of the culture was inoculated into 1 L of the LB medium and allowed to grow at 37°C with shaking until the optical density (OD600) reached 0.7–0.8. Recombinant protein production was induced by adding IPTG at a final concentration of 0.2 mM. The culture was grown at 28°C for 16 h and then collected by centrifugation (8,000 × g, 10 min, 20°C). Bacterial pellets were either used immediately for protein purification or stored at −20°C.

**Purification of p22Ct and LTB recombinant proteins.** Bacterial pellets were resuspended in an extraction buffer (25 mM Tris-HCl pH 9.0, 300 mM NaCl, 2% glycerol, 0.1% Triton X-100, 15 mM imidazole). Lysozyme (1 mg/mL) and phenylmethylsulfonyl fluoride (1 mM final concentration) were added to the cell suspension for better lysis and inhibition of proteases. The resuspended cells were disrupted by ultrasonic homogenisation (3 × 10 min, 6 s pulse and 9 s pause periods) or French press. Cell debris was removed by centrifugation at 14,000 × g for 15 min, and then the supernatant was vacuum filtered through a 0.4 µm filter. The final supernatant, representing the soluble fraction, was used further for affinity purification.

Nickel-nitriilotriacetic acid (Ni-NTA) Agarose (Qiagen, Hilden, Germany) was pre-equilibrated with 25 mM Tris-HCl pH 9.0, containing 300 mM NaCl, 2% glycerol, 0.1% Triton X-100, and 15 mM imidazole. The soluble fraction was loaded twice onto the matrix to increase the binding of the desired proteins. Unbound proteins were washed out with the same buffer, and further elution was performed with buffers containing 30, 40, 50 mM, and finally 300 mM imidazole. The eluted protein samples were concentrated and buffer-exchanged for the buffer without imidazole using Amicon 10,000 Mw centrifugal filters (Thermo Fisher Scientific). Subsequently, the samples were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. The purified proteins were stored at −80°C. Protein concentrations were determined by Bradford reagent (Bio-Rad, Hercules, CA, USA) and band density on SDS-PAGE gels using ImageLab software (Bio-Rad) with bovine serum albumin (BSA) as the standard.

**Gel electrophoresis and Western blot analysis.** Whole-cell extract samples were prepared by centrifuging 1 mL of the cell cultures at 16,000 × g; the pellets were then resuspended in 50 µL of extraction buffer (25 mM Tris-HCl pH 9.0, 300 mM NaCl, 2% glycerol, 0.1% Triton X-100) and mixed with a sample buffer (62.5 mM Tris-HCl pH 8.0, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue) at a volume ratio of 1:2. Alternatively, the purified protein samples were mixed with the sample buffer at a ratio of 1:1. All samples were heated for 5 min at 100°C, spun down, and separated under reducing conditions using vertical 4% stacking and 15% separating SDS-PAGE gels. The electrophoresis was run in 0.25 mM Tris-glycine buffer at pH 8.8 and 80 V for 15 min and then at 150 V until the sample dye reached the end of the gel. The gels were stained with 0.1% Coomassie Brilliant Blue G250 and unstained using 40% methanol and 10% acetic acid. SDS-PAGE was also used to assess protein mobility under non-reducing conditions. In that case, the sample buffer did not contain 2-mercaptoethanol, and the sample was not heated.

For Western blot analysis, the electrophoresed proteins were transferred to a polyvinylidene difluoride membrane using a wet transfer apparatus (Bio-Rad) for 16 h at a constant voltage of 20 V. The membrane was rinsed twice with 25 mM Tris-HCl buffer at pH 8.0 containing 0.9% NaCl (Tris-buffered saline – TBS), blocked with 5% non-fat milk in TBS containing 0.05% Tween 20 for 90 min, and then incubated with a 1:500 diluted His-tag primary antibody (MA1-21315; Thermo Fisher Scientific) for 90 min. Immunodetection was performed for 90 min with a 1:4000 diluted murine immunoglobulin G kappa secondary antibody conjugated to horseradish peroxidase (sc-516102; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an enhanced chemiluminescent substrate (ECL kit, Bio-Rad). As a molecular mass marker, the Novex Sharp Pre-Stained Protein Standard (Thermo Fisher Scientific) was used.

**Immunoblot assay with ASFV-positive pig sera.** The membrane prepared as above was cut into strips, each containing one protein sample. Individual strips were blocked with 2% BSA in 10 mM phosphate-buffered saline (PBS) at pH 7.3 and incubated for 1 h while being shaken with 1:100 diluted ASFV-positive pig serum (serum 1 was sourced from the European Union Reference Laboratory for African Swine Fever, Valdeolmos, Spain and sera 2–4 were positive sera from wild boars provided by the National Veterinary Research Institute, Puławy, Poland). The strips were rinsed with PBS containing 0.1% Tween 20 (T-PBS) and 2% BSA and incubated for 1 h with 1:30,000 diluted rabbit Anti-Pig IgG (whole molecule)– Peroxidase secondary antibody (A5670; Merck). After washing three times for 5 min with T-PBS containing 2% BSA, the signals of bound antibodies were developed using 3,3’5,5’-Tetramethylbenzidine (TMB) Liquid Substrate System for Membranes (T0565; Merck) and evaluated using ImageLab software.

**Mice immunisation.** General immunogenicity of the prepared recombinant proteins was assessed by mice immunisation. Six-week-old female BALB/c mice (Envigo, Indianapolis, IN, USA), three mice in each group, were vaccinated subcutaneously dorsally in the neck region every two weeks, receiving three doses in total. A single vaccination dose (prepared immediately before application) was composed of a mixture of the p22Ct or LTB-p22Ct recombinant protein (10 µg) and 10% aluminium hydrogel adjuvant (InvivoGen, Toulouse, France) in 0.15 mL PBS. The control mice
group was vaccinated with the adjuvant alone. Mice were bled on day 42, two weeks after the last vaccination, and the presence of antigen-specific antibodies in the sera was analysed by ELISA.

Serological test and titre measurement. Nunc MaxiSorp ELISA plates (Thermo Fisher Scientific) were coated overnight with 4 µg/well of the p22Ct or LTB-p22Ct recombinant protein dissolved in 0.05 M carbonate-bicarbonate buffer at pH 9.6. The wells were then rinsed with PBS and blocked with 2% BSA in PBS at 37°C for 60 min. Aliquots of 100 µL of mice sera diluted 1:50 in T-PBS with 2% BSA were added to antigen-coated wells and incubated for 60 min at 37°C. Subsequently, the plates were washed three times with T-PBS, and 100 µL of 1:30,000 diluted rabbit Anti-Mouse IgG (whole molecule)–Peroxidase (A9044; Merck) in T-PBS containing 2% BSA was added to each well. The plates were then incubated for 60 min at 37°C, washed with T-PBS containing 2% BSA, and 100 µL per well of the TMB-Complete substrate (TestLine Clinical Diagnostics, Brno, Czech Republic) was added. Colour development was stopped with 1 M H₂SO₄, and the optical density was read at 450 nm using an Infinite M200 PRO multimode plate reader (Tecan, Männedorf, Switzerland). The serum titre was determined as the dilution ratio of the test serum with an absorbance value equal to the average absorbance of the negative mouse serum from the control group.

Statistical analysis. Antibody titre differences between the two recombinant protein groups (p22Ct and LTB-p22Ct) analysed by ELISA conducted on blood samples were statistically analysed using one-way analysis of variance. The titre results of p22Ct and LTB-p22Ct were compared with those of the corresponding control group, and a P-value of 0.05 or lower was considered statistically significant. Statistical analysis was conducted using Microsoft Excel.

Results

The p22 protein of ASFV contains a short N-terminal domain, a single membrane-spanning helix and an outer large C-terminal domain. Using the Phyre2 protein fold recognition server (15), we predicted by comparing the amino acid sequence of the C-terminal domain of p22 with other known proteins from the database that the separate domain should fold into a stable tertiary structure (Fig. 1).

Fig. 1. Predicted protein structure of the p22 C terminal protein (amino acids 42–189) by the Phyre2 protein fold recognition server (15). The colours progress as in a rainbow from the N- to the C-terminus.

Two DNA constructs were prepared, p22Ct coding for the C-terminal globular fragment of the p22 protein shown in Fig. 1 and LTB-p22Ct coding for the N-terminal fusion of that fragment with the heat-labile B-subunit of enterotoxin. We also prepared variants with a 4× Myc tag, but they were not used for further study. The p22Ct and LTB-p22Ct gene constructs were then cloned into the pET28b(+) expression vector (Fig. 2).

Fig. 2. DNA construct design of p22 C terminal (p22Ct) (left) and heat-labile enterotoxin B subunit fused with p22 C terminal (LTB-p22Ct) (right) sequences, cloned into the pET28b(+) expression vector. The p22Ct gene is coloured ruby red, the LTB coding sequence is orange, the pink colour represents the coding sequence for a 10× His-tag, yellow sections are the origins of replication, pale green the kanamycin resistance gene KanR and blue the multicloning site (MCS).
Fig. 3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis of crude extracts from *E. coli* BL21 Star cells expressing p22 C terminal (p22Ct) and heat-labile enterotoxin B subunit fused with p22 C terminal (LTB-p22Ct). Lanes 1, 3, 5, and 7 show the 20 µL samples from non-induced cells, while lanes 2, 4, 6, and 8 represent the cells after induction with 0.2 mM isopropyl β-D-1-thiogalactopyranoside. Bands corresponding to the p22Ct (lanes 2 and 4) and LTB-p22Ct (lanes 6 and 8) proteins are shown in red boxes.

Fig. 4. Expression and purification of p22 C terminal (p22Ct) (A–C) and heat-labile enterotoxin B subunit fused with p22 C terminal (LTB-p22Ct) (D–F) recombinant proteins in *E. coli* BL21 analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blot. A – band 1: cells harbouring empty pET28b(+) as control (after induction); band 2: cells harbouring pET28b(+)–p22Ct before induction; band 3: soluble protein fraction; band 4: cells after induction (each sample 20 µL); band 5: washout fraction (50 mM imidazole) from nickel-nitrilotriacetic agarose (20 µL); bands 6–9: p22Ct eluted by 300 mM imidazole (samples of 5, 10, 4, and 0.5 µg, respectively); B – protein staining; C – Western blot with the anti-His-tag antibody of the purified p22Ct protein (11 µg); D – band 1: pET28b(+) cells after induction; band 2: pET28b(+)–LTB-p22Ct cells before induction; band 3: soluble protein fraction; band 4: cells after induction (each 20 µL); band 5: washout fraction; bands 6–9: eluted LTB-p22Ct protein (4, 3, 2 and 0 µg); E – protein staining; F – Western blot of purified LTB-p22Ct (16 µg); M – protein molecular mass marker.
First, a small-scale expression in 5 mL cultures was performed. Gel electrophoresis showed that the expression level of the LTB-p22Ct fusion protein was much lower than that of the p22Ct protein (Fig. 3), while still being detectable.

Secondly, a large-scale expression was performed under the same conditions in 1 L cultures (Fig. 3). The larger-scale expression yields of p22Ct and LTB-p22Ct in 1 L cultures, estimated with BSA as a standard, were 4.9 and 3.8 mg per L of culture.

In the subsequent immunoblot testing of purified p22Ct and LTB-p22Ct recombinant proteins for their ability to interact with serum antibodies (IgG) from ASFV infected pigs, both proteins showed positive reactions with the four sera used as primary antibodies (Fig. 5).

When the serological activity of the two groups’ samples against the recombinant proteins was assayed, the antibody titre was high for the mice group immunised with p22Ct, while the samples from the
LTB-p22Ct group did not react at all in ELISA (Fig. 6). The mice allocated to the p22Ct protein group were fully immunised with three doses. However, in the case of the LTB-p22Ct group, abscesses occurred after the first dose. Due to this development, only one dose was administered.

Due to the difference in immune response induction in the mice, the oligomerisation of both recombinant proteins was examined by SDS-PAGE under non-reducing conditions (Fig. 7). As calculated by comparing the mobility of recombinant proteins with those of protein molecular mass standards, the 33 kDa LTB-p22Ct fusion most probably forms hexameric complexes of 198 kDa. In contrast, the p22Ct protein prevalently retains the monomeric form of 23 kDa.

**Discussion**

The global swine pandemic caused by the African swine fever virus has a negative impact on the economy of many countries throughout the world; therefore, the virus needs to be eliminated. Many researchers worldwide have tried to design a new effective vaccine for combating the disease caused by this virus; however, they have not been successful in protecting the pigs against virulent viral strains. A previous study showed that administration of ASFV proteins from the inner and capsid membranes could somewhat delay the onset of clinical symptoms from the time of viral challenge, but could not stop the further progress of the disease; the pigs administered the proteins eventually died. The four proteins investigated (p22, p30, p54 and p72) were expressed in a baculovirus system. The individual proteins were not purified to homogeneity but used as a mixture to immunise the pigs (20). The p22 protein chosen for our study was previously considered a potential antigen for new vaccine development (4, 22). A study using DNA prime and recombinant vaccinia virus boost (12) showed that p22 (the KP177R early membrane protein) is a potential antigen for inducing a protective immune response or can serve as an infection serological marker. However, the role of p22 in the infection process is still unknown. Recently the focus on developing an effective vaccine turned to the heat-labile enterotoxin B-subunit, LTB-p22Ct, which was expected to stimulate the immune response in the host (9, 13).

In contrast to previous studies, we selected only the large C-terminal globular part of the protein, p22Ct (amino acids 42 to 189). We also prepared its fusion with the heat-labile enterotoxin B-subunit, LTB-p22Ct, which was expected to stimulate the immune response in the host (9, 13).

After cloning the corresponding constructs into the *E. coli* BL21 pET28b(+) expression vector, the results of small-scale cultures indicated that the expression of the LTB-p22Ct protein was much lower than that of p22Ct (Fig. 3). The homologous overexpression of LTB in *E. coli* is often low and prone to forming inclusion bodies (18). However, our experiments in 1 L cultures produced comparable yields of both proteins of about 4 mg per L, corresponding roughly to 3% of the theoretical maximum yield of the protein of interest in *E. coli*. The formation of inclusion bodies was not observed.

Our results show that p22Ct, the C-terminal globular part of the p22 protein, but not the fusion protein LTB-p22Ct, can induce an immune response in mice. The exact cause of the observed difference is unclear. The most probable reason is that mice immunised with LTB-p22Ct were injected with only one dose rather than the three doses of p22Ct administered. A significant problem appeared in the form of abscess formation in the mice vaccinated with the fusion protein; therefore, these mice received only one dose of antigen. This is probably also the reason why the mice immunised with LTB-p22Ct could not produce specific antibodies against the protein. Another reason may be that LTB could become less active when produced by recombinant technology in *E. coli* (18). As described previously, LTB binds to the GM1 receptor in a pentameric association, which is essential for stimulating the immune response (16). However, the SDS-PAGE under non-reducing conditions showed that LTB-p22Ct is more likely to form a hexamer, which may be a reason for its lack of immunogenicity (Fig. 7).

Our results also show that p22Ct can produce a high antibody titre in mice, and is thereby indicated to be a highly potent and immunogenic region. It can be considered a candidate primarily in serological diagnostics for the development of specific antigen-based detection techniques such as fluorescent antibody tests, ELISA and immunoblotting (23). However, further experiments should be conducted to investigate its possible link to other immunostimulatory domains.

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