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# Antigenic and immunogenic properties of a chimera of two immunodominant African swine fever virus proteins

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**Summary.** A chimera of the two immunodominant African swine fever (ASF) virus proteins p54 and p30 was constructed by insertion of the gene *CP204L* into a *Not* I restriction site of *E183L* gene. The resulting chimeric protein p54/30, expressed by a recombinant baculovirus in insect cells and in *Trichoplusia ni* larvae, retained antigenic determinants present in both proteins and reacted in Western blot with a collection of sera from inapparent ASF virus carrier pigs. Remarkably, pigs immunized with the chimeric protein developed neutralizing antibodies and survived the challenge with a virulent African swine fever virus, presenting a reduction of about two logs in maximum viremia titers with respect to control pigs. In conclusion, this study revealed that the constructed chimeric protein may have utility as a serological diagnostic reagent and for further immunological studies that may provide new insights on mechanisms of protective immunity to ASFV.

## Introduction

African swine fever (ASF) virus is an icosahedral cytoplasmic deoxyvirus that infects domestic pigs and soft ticks of the *Ornithodoros* genus. This virus is presently the sole member of *Asfarviridae* family [6], which produces different forms of a disease, ranging from highly lethal to subclinical [5, 15]. The lack of a commercially available vaccine makes diagnostic procedures the only methodology that can help to plan the complete eradication of the disease in affected countries. In most cases ASF disease is diagnosed by the detection of antibody, due to the presence of strains of reduced virulence [3, 11].

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Serological diagnostic tests for ASF based on the use of recombinant proteins have been previously described [1, 2, 14]. The use of recombinant proteins as reagents in the serological tests provide many advantages when compared to antigen production based on the obtantion of antigens from infected cell extracts. These recombinant antigens provide simpler interpretation of the tests, reduce false positive reactions produced by cellular culture compounds that contaminate the antigens [7], avoid the use of potentially dangerous live virus in antigen production, and allow for a better standardisation in antigen production. ELISA and immunoblotting tests have been adapted for this disease using recombinant antigenic virus proteins p30 and p54 respectively [1, 2, 14].

Studies about the immune response against this virus have demonstrated that neutralizing antibodies are induced in ASF convalescent pigs [9, 18, 19]. These antibodies are able to neutralize the virus before and after binding to susceptible cells, inhibiting both virus attachment and internalization [9]. Antibodies to protein p54 inhibit a first step of the viral infection cycle related to virus attachment, while antibodies to protein p30 are implicated in the inhibition of virus internalization [9]. Both proteins are involved in two distinct steps of virus attachment and both contribute to the antibody-mediated protective immune response [10].

In the present work, a chimera of the two immunodominant ASF virus proteins p54 and p30 was constructed by insertion of the gene *CP204L*, coding for p30, into a central region of the *E183L* gene which accepts foreign sequences without affecting the function of the coded p54 protein. The resulting chimeric protein, expressed in baculovirus, retained the antigenic and immunogenic properties of both proteins. This feature make the p54/30 chimera an interesting tool for diagnosis and for further immunological studies about the protective immune response against ASF virus.

## Materials and methods

### *Virus, sera and larvae*

The recombinant baculovirus Bacp30 and Bacp54, expressing the ASF virus proteins p30 and p54 respectively [14], were used to infect Sf9 insect cells or *Trichoplusia ni* (cabbage looper) larvae.

Nine field pig sera previously diagnosed as ASF positive sera by ELISA and collected between 1991 and 1994, were obtained from innaparent carrier pigs from Southwest geographic areas of Spain (enzootic areas during these years). Two additional sera from pigs oronasally inoculated with $10^5$ 50% tissue culture infectious doses ($TCID_{50}$) of the attenuated virus E75 $CV_1$-4 and collected on day 20 postinoculation, were also used as positive ASF control sera in Western blot. Three normal pig sera were pooled and used as negative controls.

### *Cell culture and larval growth conditions*

Sf9 cells, grown as a monolayer in 75 $cm^2$ tissue culture flasks, were inoculated with virus stocks by co-incubating virus and Sf9 cells in culture medium (lacking foetal bovine serum, FBS) for 1 h at 27 °C. The inoculum was aspirated and replaced with medium supplemented with 10% FBS. The infected cells were then cultured at 27 °C and collected for assays when cytopathic effect was evident (around 72 h postinfection).

Insect larvae were obtained from a laboratory rearing of *Trichoplusia ni*. The eggs were put into larvae developmental cages, containing artificial insect diet [12]. The eggs hatched and about 90% grew to the appropriate size (3 cm in length, 120–150 mg) in 10–14 days at $22 \pm 1\,^{\circ}C$ in a climatic chamber. Fourth instar larvae were sedated by incubation on ice for 15 min and then injected near the proleg (forward along the body cavity) with 20 μl of medium containing different infectious doses of recombinant baculoviruses. Larvae were maintained during infection at 22 °C or 30 °C. When larvae became pale, swollen and lethargic (72–96 h after infection), they were harvested and immediately frozen at −70 °C.

### *Transfection and selection of baculovirus recombinants expressing the chimeric ASF virus protein p54/30*

DNA amplification of the *CP204L* gene from the BA71V ASF virus strain was carried out by PCR using the following primers (i) 5′-GCGCGGCCGCGATGGATTTATTTTAAATAT-3′ (5′ primer) and (ii) 5′-GCGCGCGGCCGCAAACATTAAATGTAGGTGAG-3′ (3′ primer), containing the recognition enzyme sequence for *Not*I. The PCR product was digested with the restriction enzyme and cloned into pGEM54 plasmid containing the *E183L* gene previously digested with the same enzyme to generate the chimeric gene *183/204*. The cloned chimeric gene was sequenced to check for possible sequence changes introduced by the PCR amplification. Then, *183/204* chimeric gene was subcloned in *Bam* HI into the plasmid pBacPAK8 (Clontech) generating the recombinant plasmid pBac183/204. Recombinant baculovirus clones were constructed and selected as described [14].

### *Cell culture and larva sample preparation*

Recombinant proteins were solubilized from baculovirus-infected cells by using an hypothonic treatment with 25 mM $NaHCO_3$ pH 8.3. Cell debris were clarified by centrifugation and a 1:10 volume of 10×PBS was added. Infected larvae were homogenised on ice with a tissue disrupter in presence of 0.75 ml/larva of extraction buffer (2.5 mM dithiothreitol, 0.01% Triton X-100, 1 mM phenylmethylsulfonyl fluoride). The resulting crude extract was centrifuged at $12{,}000 \times \mathbf{g}$ for 30 min. In the case that oxidation of supernatants were evident (discoloration to brown or black) 10 mM β-mercaptoethanol was added. Lipids were discarded from larvae extracts and centrifuged again.

### *Immunoblotting assay*

Proteins from infected cells or crude larvae extracts previously quantified were resolved in 17% acrylamide gels and transferred to a nitrocellulose filter. The portion of the filter containing the proteins with molecular weights between 20 and 70 K was cut and divided into 4.0 mm wide strips. Filters were incubated with 2% nonfat dry milk as blocking solution for 1 h and reacted with test sera diluted at 1:40 for 1 h. The presence of immunocomplexes were detected using peroxidase-labeled protein A and 4-Chloronaphtol as substrate.

### *Vaccination and challenge procedures*

Two large white pigs (3 months old) were immunized with the recombinant chimeric p54/30 protein obtained from $5 \times 10^6$ baculovirus-infected cells, containing about 100 μg of recombinant protein per dose. Pigs received five doses of antigen administered intramuscularly in the presence of Freund's adjuvant (complete in the first inoculation and incomplete in the other inoculations). One unvaccinated pig and other immunized with $10^8$ Sf9 cells were used as control pigs.

All pigs were challenged intramuscularly with $5\times10^2$ $TCID_{50}$ of the highly virulent virus isolate E75. This dose represents a challenge of between 50 and 500 $LD_{100}$ [13]. Blood samples were collected before and at different times after virus challenge for viremia determination. The virus was titrated in porcine alveolar macrophages by detection of infected cells using specific antibodies [17]. Titers were calculated and expressed as $TCID_{50}$/ml.

The neutralization activity of the sera was measured in pig macrophage cell cultures essentially as described previously [8, 9], and expressed as the maximum serum dilution that neutralized 50% of virus infectivity. The neutralization data were obtained from three independent experiments and referred to a nonimmune serum.

## Results

### *Construction, selection, and characterization of a recombinant baculovirus expressing the chimeric protein p54/30*

To generate the chimeric p54/30 protein, we took advantage of the occurrence of dispensable repetitions in the gene *E183L* [4, 16], to introduce the gene *CP204L* into the coding sequence for three alanine residues which provides for a Not I restriction endonuclease recognition site. Following this approach, we generated the transfer vector pBac183/204 to obtain the recombinant baculovirus expressing the chimeric protein p54/30. Figure 1 shows the general outline for the generation of the recombinant baculovirus Bac54/30. Six virus clones were selected from the cotransfection and purified in three consecutive plaque assays in Sf9 cells. The purified viruses were titrated and used for infecting the insect cells at a multiplicity of infection of 5. At 48 h postinfection the cells were harvested and subjected to Western blot analysis using anti-ASF virus serum to select the highest expressing clone. A single reactive band of about 56 kDa was evident in all clones (Fig. 1), presenting some of them differences in the intensity of the band (data not shown).

One of the highest expressing clone was analyzed by Western blot using monospecific antisera against p54 or p30 protein, and both sera recognized the chimeric protein (Fig. 2A). The specificity of the reaction was confirmed by using extracts from cells infected with Wild-type baculovirus and reacted with specific antibodies, as well as with Bac54/30-infected cell lysates reacted with control negative serum. Recombinant protein was detected only associated to the cells at 48 h postinfection and more than 65% of the recombinant p54/30 protein was easily solubilized from baculovirus-infected cells by using an hypothonic treatment with 25 mM $NaHCO_3$ pH 8.3.

Attempting to express this chimeric protein using the recombinant baculovirus in *Trichoplusia ni* insect as an inexpensive medium for accumulation of large quantities of recombinant protein, we infected fourth instar larvae with the Bac54/30 virus with $1.8\times10^5$ infectious doses in 20 μl of PBS. The kinetic of recombinant protein expression was studied showing maximum expression levels at 72–96 h post-infection (Fig. 2B). A single reactive band with identical mobility of the recombinant chimeric protein expressed in baculovirus-infected cells was detected in larvae extracts.

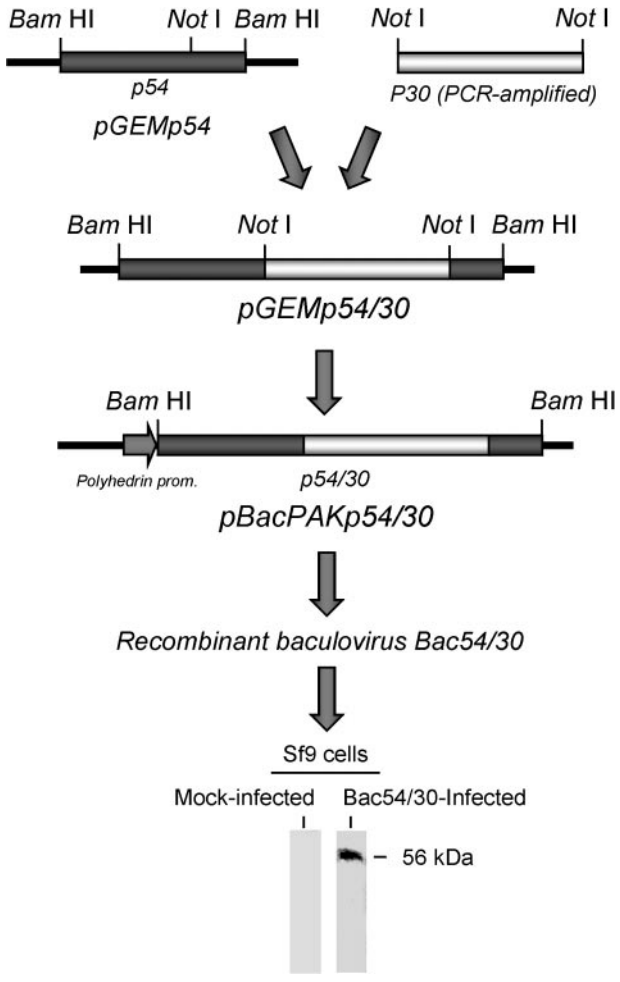


**Fig. 1.** General outline for the generation of the chimeric p54/30 polypeptide and its expression in baculovirus

To analyze the influence of temperature on recombinant p54/30 production during infection of larvae with Bac54/30, infected larvae were maintained at 24 °C or 30 °C during infection. As a result of this experiment, we showed that infected larvae at 30 °C rendered four times more recombinant protein than larvae maintained at 24 °C (Fig. 2C). In these optimal infection conditions, we estimated from the data shown in Fig. 2D that a single infected larva accumulates an amount of recombinant chimeric protein similar to that obtained from $7.2 \times 10^6$ infected Sf9 cells.

### *Use of baculovirus-expressed p54/30 chimeric protein in the serological diagnosis of ASF*

A panel of 5 field pig sera from naturally infected pigs (innaparent carriers) from enzootic areas of Spain in past years were reacted in Western blot with

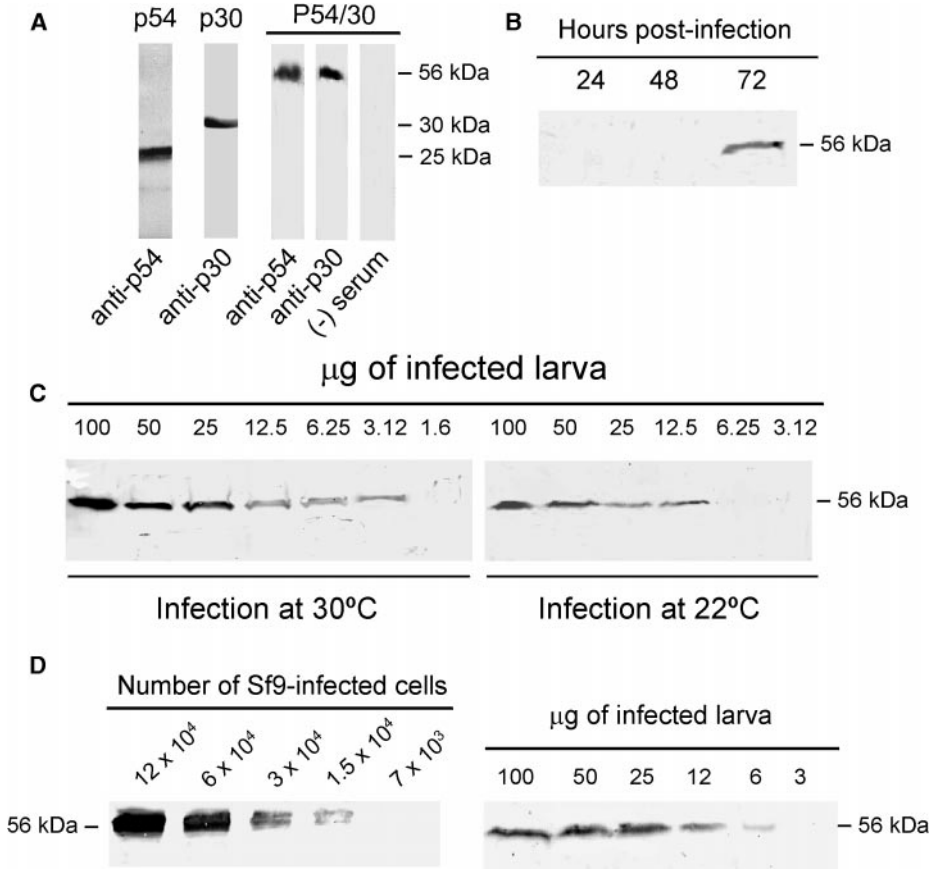


**Fig. 2.** Expression of the ASF virus proteins p54, p30 and the chimeric p54/30 protein in larvae of *Trichoplusia ni*. **A** Analysis by Western blot of Sf9 cells infected with recombinant baculoviruses expressing p54, p30 and the chimeric p54/30 proteins, reacted with sera against p54 and p30 proteins. **B** Kinetics of recombinant chimeric p54/30 protein expression in larvae inoculated with $1.5 \times 10^6$ infectious doses. Larvae protein extracts were analysed at different times after infection by Western blot with an anti ASF virus serum, using 50 μg of total larva protein per strip. **C** Comparative analysis of chimeric p54/30 protein production in larvae maintained during infection at 22 or 30 °C. **D** Comparative yield production analysis of recombinant p54/30 protein expression in larvae at 72–96 h post infection in relation to the amount of the same protein expressed in infected Sf9 cells

p54, p30 and the chimeric p54/30 proteins expressed in larvae. Additionally, 3 sera from pigs infected with an attenuated ASF virus were also analyzed using these antigens. All sera reacted with the three recombinant proteins (Fig. 3). This data and the fact that antibodies against p54 and p30 proteins reacted with the chimeric protein (Fig. 2A), suggest the preservation in the chimeric protein of antigenic determinants contained in both ASF virus proteins which are recognized by infected pigs. No protein from uninfected larvae reacted with the sera.

For standardising the antigen concentration of chimeric protein in this technique we titrated the recombinant chimeric protein with a pool of infected

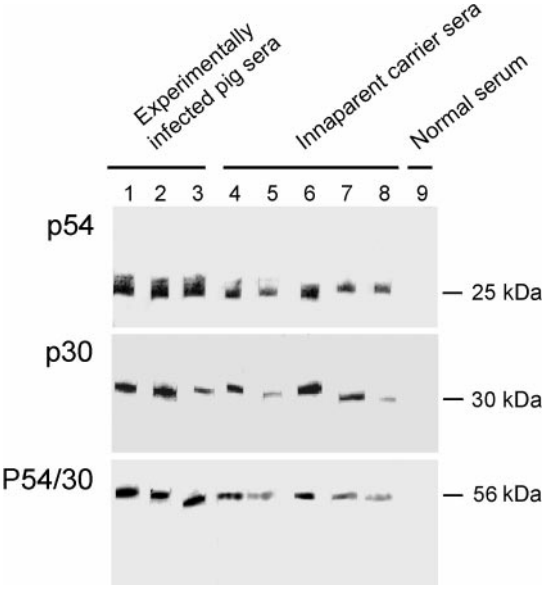


**Fig. 3.** Comparative analysis of ASF specific antibody detection by Western blot in infected pig sera using recombinant p54, p30 and the chimeric proteins (50 μg of total larva protein per strip). Sera were recovered from experimentally infected pigs with the attenuated virus E75CV$_1$-4 and from pigs from enzootic areas from Spain which were diagnosed as positive of ASF virus infection

field pig sera in Western blot (not shown). The results demonstrated that optimal reactivity of recombinant p54/30 with the positive pool of sera in Western blot was obtained using 30 μg of larvae extracts. This means that a single infected larva provides sufficient antigen to carry out at least 300 diagnostic tests by Western blot.

### *Vaccination with p54/30 chimeric protein induces a protective immune response*

Protein p54/30 is a chimera of two ASF virus proteins involved in virus-cell interactions and in virus neutralization. To analyze the immune response induced by this protein, two pigs were immunized with baculovirus-infected cells expressing the protein p54/30. Pigs were bled 15 days after the last inoculation, and a Western blot analysis showed that the antisera reacted against proteins p54 and p30 (Fig. 4A). Antisera were also analyzed in a neutralization assay in pig macrophages, showing neutralizing titers of 1:200 (Fig. 4B). These two sera at 1:5 serum dilution were able to block both virus attachment (78–82%) and internalization (75–77%), indicating that relevant epitopes in neutralization of p54 and p30 were also preserved in the chimeric protein.

To test the protective capacity of such neutralizing antibodies, at day 15 after the last immunization with recombinant protein, all pigs were challenged intramuscularly with the highly virulent virus isolate E75. Clinical signs of ASF such as fever, anorexia, lethargy, recumbency, and cyanosis were monitored daily. Control pigs dyed on postinoculation day 6, 3 days after clinical onset of the disease. In contrast, pigs immunized with the chimeric p54/30 protein presented dramatic changes in the disease course with respect to control pigs. These pigs presented a delay in the onset of clinical signs of about 4 days, surviving to lethal

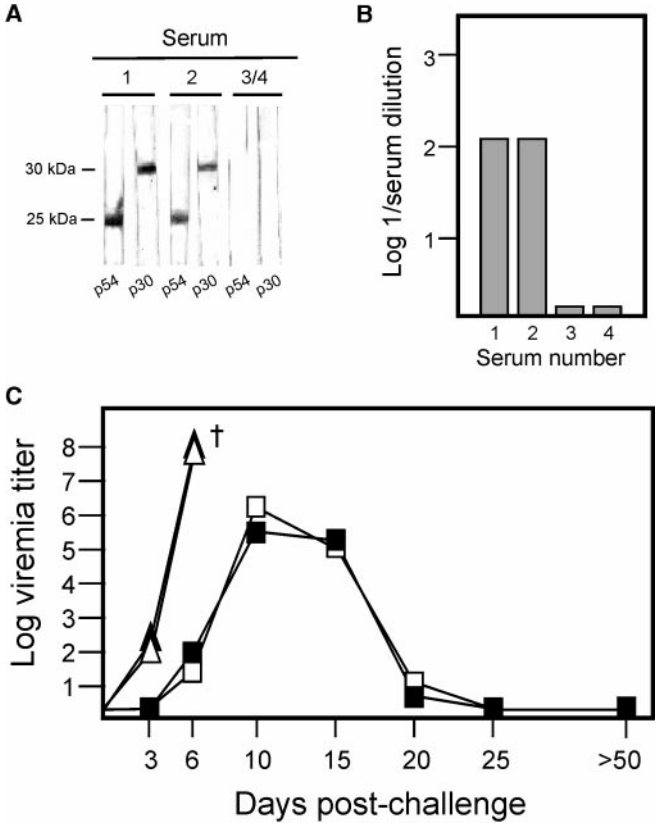


**Fig. 4.** Immunogenicity of the chimeric p54/p30 protein in immunized pigs. **A** Reactivity of sera from immunized pigs with the chimeric protein (sera 1 and 2) and a pool of sera from control pigs (serum 3/4) with recombinant p54 and p30 proteins in Western blot. **B** Neutralizing titers of sera from vaccinated (1 and 2) and control pigs (3 and 4). Results, expressed as means of three independent neutralization experiments, are indicated as logarithm of the maximum serum dilution that neutralized 50% of virus infectivity in pig macrophages. **C** Effect of vaccination procedure with the chimeric protein (pigs 1 ■ and 2 □) on viremia titers and pig survival following challenge with virulent ASF virus E75 ($5 \times 10^2$ $TCID_{50}$), compared to control pigs (pigs 3 ▲ and 4 △)

infection after a transient symptomatic period of about 2 weeks duration. Protected pigs showed a mild disease pattern with respect to control pigs, but with similar symptoms (fever, recumbency and anorexia), indicating that the protection conferred was not complete. Immunized pigs with the chimeric protein showed a reduction of about two logs in maximum viremia titers with respect to control pigs (Fig. 4C) and remained clinically normal after the symptomatic period during a postchallenge monitoring period exceeding 50 days.

To ascertain the complete elimination of the virus, protected pigs were euthanized at 55 days postchallenge inoculation, and the presence of virus in different organs (blood, spleen, kidney and mediastinal lymph nodes) was investigated by PCR using pairs of primers specific for amplification of *E183L* and

*CP204L* genes. Sampled organs were negative for ASFV in both pigs (not shown).

## Discussion

In the present work we have generated a chimeric protein by insertion of the coding sequence for protein p30 between two *Not* I restriction sites of the gene coding for protein p54, one of the sites of maximum variability in the virus genome [16]. In a previous work, we demonstrated the feasibility of introducing foreign epitopes into the ASF virus protein p54 without affecting the functionality or antigenicity of this protein [4]. The main purpose of this work was to combine in only one polypeptide antigenic and immunogenic determinants contained in two of the most antigenic and immunogenic ASF virus proteins, which are complementary in terms of diagnosis and protective immune response [10, 14]. The combination of both antigens in one should facilitate the production of diagnostic reactives or the generation of a subunit vaccine.

The above results demonstrate that the chimeric protein p54/30 is recognized by with monospecific antisera directed against both p54 and p30 proteins, suggesting that their antigenic determinants were preserved. The production of the recombinant chimeric protein was carried out in insect Sf9 cells as well as in larvae of *Trichoplusia ni*. These larvae have demonstrated to be an inexpensive and an easily processed source of antigen. In previous studies, the larval homogenates functioned in immunoblotting and ELISA techniques in a reproducibly manner without further purification of antigen. The yield of recombinant chimeric protein production in larvae is comparable to the yields obtained with other recombinant proteins produced by this system [2]. Interestingly, the temperature at which the larvae were maintained during infection was very important in terms of recombinant protein accumulation. At 30 °C larvae produced at least four times more recombinant protein than when infection progressed at 22 °C. It could be due to the optimal temperature for baculovirus replication cycle in insects. In fact, a comparative titration of virus recovered from infected larvae at both temperatures rendered differences higher than 1 log of virus titer (not shown).

Immunogenic determinants of proteins p54 and p30 seem to be also preserved in the chimeric protein since immunized pigs with protein p54/30 developed neutralizing antibodies that protect vaccinated pigs against fatal ASF disease. This protective immune response was previously achieved only when a combined immunization with p54 and p30 was carried out [10]. Proteins p54 and p30 are involved in two different steps of virus attachment and both contribute to the antibody-mediated protective immune response [10]. Our results confirmed that it is neccesary to induce antibodies that are able to block virus attachment and internalization to obtain a certain level of protection against ASF. In the present work, we have demonstrated that protection can be provided by only one polypeptide, the chimeric protein p54/30. This finding could facilitate further studies on vaccine development against this disease using recombinant proteins approach.

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