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ENHANCING SOLUBLE EXPRESSION AND ONE-STEP PURIFICATION OF AFRICAN SWINE FEVER VIRUS P30 PROTEIN IN *E. COLI* USING A GST-SNAC DUAL-TAG SYSTEM

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ABSTRACT

African Swine Fever (ASF), a highly contagious and deadly disease of swine caused by the African Swine Fever virus (ASFV), leads to significant economic losses due to the lack of effective vaccines or treatments. The ASFV-p30 antigen, an early-expressed and highly immunogenic protein, has significant potential for early detection; however, its structural instability presents challenges for research and recombinant production. In this study, a dual-tag strategy combining Glutathione S-transferase (GST) and sequence-specific nickel-assisted cleavage (SNAC) was used to improve the solubility and purification efficiency of ASFV-p30 in *E. coli*. The GST tag facilitated proper protein folding, while the SNAC sequence enabled Ni^{2+} chemical cleavage, allowing non-enzymatic GST removal. This approach optimizes purification, reduces processing time, and offers a promising strategy for ASF research and diagnostic applications.

Keywords: p30, dual-tag system, SNAC tag, GST tag, African Swine Fever virus (ASFV)

INTRODUCTION

African Swine Fever (ASF) is a highly contagious and deadly viral swine disease listed by the World Organization for Animal Health (OIE) as a notifiable animal disease. ASF is characterized by its rapid spread and can reach a 100% mortality rate in pigs, impacting the global livestock economy **(Dixon *et al*., 2020)**. The disease is caused by African Swine Fever virus (ASFV), and transmission occurs through direct exposure to infected domestic or wild boar, pork products (fresh pork, bacon) containing the virus, or an organism competent for ASFV, such as the *Ornithodoros* genus of soft ticks **(Gaudreault *et al*., 2020)**. Following a report in 2023, ASFV was reported in 50 countries, affecting over 953,000 domestic pigs and 28,000 wild boars **(Wang *et al*., 2023)**. The outbreak caused severe economic losses worldwide **(Nguyen-Thi *et al*., 2021)**.

The African swine fever virus (ASFV) is a DNA arbovirus belonging to the *Asfarviridae* family. Its virion has a complex multilayer structure, including a nucleoid containing the viral genome, which encodes 150–200 proteins involved in infection, immune evasion, and host regulation **(Alejo *et al*., 2018; Gallardo *et al*., 2015; Hooper *et al*., 2024; Njau *et al*., 2021)**. Among them, the highly immunogenic viral phosphoprotein p30 is produced in the early stages of infection and plays a crucial role in ASFV infection **(Petrovan *et al*., 2019; Yu *et al*., 2021)**. It is encoded by the *CP204L* gene, with a molecular weight of approximately 30 kDa, and its sequence is highly conserved across different ASFV genotypes. Moreover, ASFV-p30 expression is typically detected two to four hours post-infection and maintained throughout the infection process due to the necessity for cell interactions and viral replication in the host cell **(X. Chen *et al*., 2022; Jia *et al*., 2017)**. Antibodies against ASFV-p30 appear in pigs around eight days post-infection. These characteristics make ASFV-p30 an ideal target antigen for serological detection of infection via immunoassays, point-of-care testing (POC), and vaccine production techniques **(Hübner *et al*., 2018)**. However, ASFV antigens containing p30 are classified as a biological substance in category B, with a high potential for outbreaks **(Beltran-Alcrudo *et al*., 2017)**. For biosafety, the recombinant ASFV-p30 protein has been synthesized for research purposes. Bacterial expression systems in *E. coli* are widely used due to their low cost, high-level production, simplicity, and speed. However, non-homologous sequences, over-expression, and a lack of a post-translational modification system in *E. coli* tend to form insoluble inclusion bodies (IBs). Moreover, instability in the ASFV-p30 structure is also a notable contributing factor **(Petrovan *et al*., 2019; Zhang *et al*., 2021; Zhao *et al*., 2022; Zhou *et al*., 2023)**.

Glutathione S-transferase (GST) is a 26 kDa protein naturally present in eukaryotic cells. GST derived from the parasitic helminth *Schistosoma japonicum* is widely used as a fusion tag in recombinant protein expression systems and is one of the earliest strategies developed to enhance protein solubility. It acts as a chaperone to support target protein folding and has a high affinity for glutathione. The purification of GST-fusion proteins is typically achieved through affinity chromatography utilizing glutathione. The rapid folding of the GST tag can promote the folding of the fused recombinant protein **(Harper *et al*., 2011; Shendge *et al*., 2022)**. Therefore, this property was exploited in the present study to enhance the solubility and stability of ASFV-p30 in the *E. coli* expression system. However, a major limitation of the GST tag is its large molecular size; consequently, it is usually removed by enzymatic cleavage with site-specific proteases to avoid affecting target protein applications. Using proteases to remove GST may reintroduce instability and compromise the integrity of the recombinant protein, influencing its structure or function **(Cole *et al*., 2007)**. Moreover, enzymes can be extremely costly, particularly when producing proteins on a large scale.

A non-enzymatic approach to the cleavage of fusion tails is chemical cleavage. A sequence-specific nickel-assisted cleavage (SNAC) tag is a short peptide chain that was developed as a chemically cleavable linker. It enables the removal of the fusion target protein by the Ni^{2+} ion under biocompatible cleavage conditions with high efficiency **(Dang *et al*., 2019)**. This method is appealing because it leaves only a small Gly residue at the C-terminus and eliminates the need for enzyme purification. Unlike the use of proteases, which requires an additional step to remove the protease after cleavage of the affinity tag, ion removal is not required. This tag offers great potential for application in recombinant protein purification with low cost and technical simplicity **(Huang *et al*., 2020)**.

Nowadays, the absence of an effective vaccine or treatment for ASF emphasizes the urgency of advancing diagnostic research, screening methods, and treatment development. In this study, a dual-tag expression strategy for the ASFV-p30 protein, combining the solubility-enhancing GST tag and a chemically cleavable SNAC linker, was developed in the *Escherichia coli* expression system. Unlike fusion tag systems commonly used to improve recombinant protein solubility, such as MBP, GST, SUMO, or Halo tags, where tag removal typically requires site-specific proteases, the present design enables efficient GST removal through Ni^{2+}-

mediated cleavage of the SNAC sequence in physiological conditions **(Dang *et al*., 2019; Kosobokova *et al*., 2016)**. This approach not only enhances soluble expression but also enables simplified, enzyme-free tag removal and reduces purification time, providing a novel and promising solution not only for ASF but also for poorly soluble antigens.

MATERIAL AND METHODS

Bacterial strains, plasmids, and swine serum samples

E. coli DH5α [F− endA1 hsdR17 (rk−/mk−) supE44 thi λ-recA1 gyrA96 ΔlacU169 (ϕ80 lacZ ΔM15)] and *E. coli* BL21 (DE3) (F+ ompT hsdSB (rB-mB-) gal dcm (DE3) strains were used for cloning and expression of protein. Cells were cultured in Luria–Bertani (LB) medium supplemented with 100 µg/mL of ampicillin for selection. The ASFV-p30 coding sequence was obtained from the NCBI database and optimized for expression in *E. coli*. The *ASFV-p30* gene was amplified from the G-block template (Twist Biosciences, USA) by polymerase chain reaction (PCR) with gene-specific primers (1172F Bgl/1173R Not) containing SNAC sequence at the 5' end and overlapping recognition regions corresponding to the cloning sites on the pGEX-5X-1 plasmid. The PCR gene products were analyzed in agarose gel electrophoresis (1.5%). The pGEX-5X-1 plasmid was used to construct the SNAC-p30-ASFV expression vector controlled by the T7 promoter induced by IPTG, and carrying a GST-tag sequence. The antiserum (kindly provided by National Veterinary Joint Stock Company) was obtained from 7–8-week-old domestic pigs experimentally infected with a local ASFV strain, and the presence of p30-ASFV-specific antibodies was confirmed using the Ingenasa kit.

Construction of pGEX-SNAC-p30-ASFV

The pGEX-5X-1 plasmid was digested with *Bam*HI and *Not*I restriction enzymes (Thermo Scientific) to generate linear fragments containing complementary overlaps with the PCR-amplified gene product. Subsequently, the linearized plasmid was mixed with the PCR product, and the expression vector was assembled following the recombinase-free cloning method (RFC) **(Vo-Nguyen *et al*., 2022)**. The mixture was transformed into a competent *E. coli* DH5*α* by the heat shock method. The transformants were screened on LB agar plates containing ampicillin. Recombinant clones were then selected and verified by colony PCR with specific primers (1172F Bgl/1173R Not). All designed primers are listed in Table 1. Plasmids pGEX-SNAC-p30-ASFV isolated from PCR-confirmed positive colonies were validated by Sanger sequencing.

Table 1 Primer sequences were used in this study.

Primer	Sequence
1172F Bgl	CAAAATCGGATCTGATCGAAGGTCGTGAGATCTCCGG CGGTAGTCATCATTGGGGATCCGATTTTATTCTGAAC
1173R Not	AGGCAGATCGTCAGTCAGTCACGATGCGGCCGCGCTA GCTCAGTGGTGGTGGTGGTG
pGEX5	ACCCAATGTGCCTGGATGCGTTCCC
pGEX3	CTCCGGGAGCTGCATGTGTCAGAGG

Expression and purification of GST-SNAC-p30-ASFV protein

The recombinant pGEX-SNAC-p30-ASFV plasmid was transformed into *E. coli* BL21(DE3) using the same procedure as for *E. coli* DH5*α*. The recombinant GST-SNAC-p30-ASFV protein was induced with 0.1 mM IPTG at 16 °C for 18 hours. Harvested cells containing pGEX-SNAC-p30-ASFV were lysed by sonication (30%, 3 mins) in PBS buffer (pH 7.4) to obtain proteins in total, soluble, and insoluble fractions. A negative control with *E. coli* BL21(DE3)/pGEX-5X-1 was included. Recombinant protein expression was analyzed by SDS-PAGE on a 15% gel and visualized with Coomassie Brilliant Blue staining, while Gel Analyzer software was used to quantify the proportion of GST-SNAC-p30-ASFV protein in the soluble fraction. Plasmid pGEX-5X-1 allowed recombinant proteins to be expressed as GST-tag proteins. Soluble lysates were then purified using a GSTrap™ 4B 1 ml column (Cytiva) on an ÄKTA™ system FPLC, with reduced glutathione (pH 8.0) used to elute the GST-tagged target proteins. The purified proteins were. subsequently analyzed by SDS-PAGE with silver staining.

SNAC cleavage and collection of purified soluble recombinant ASFV-p30 protein

To remove the GST tag from the purified GST-SNAC-p30-ASFV protein, cleavage was performed through the SNAC sequence. The purified protein was first exchanged into cleavage buffer containing 0.1 M HEPES, 0.1 M acetone oxime, and 0.1 M NaCl (pH 8.2), after which 0.1 mM $NiCl_2$ was added. The mixture was vortexed well and incubated at 22 °C for 16 hours. Following incubation, Ni^{2+} ions. and other buffer components were removed by dialysis, as described by **Dang *et al*. (2019)**. Additionally, Western blot analysis with an anti-GST HRP conjugate antibody (Cytiva, 1:30,000 dilution) confirmed the presence of both the GST-SNAC-p30-ASFV fusion protein and the cleaved products generated by Ni^{2+} treatment. The cleavage efficiency of Ni^{2+} was quantified using Gel Analyzer software, according to the following formula:

$$\frac{Raw\ volume\ before\ cleavage\ -Raw\ volume\ after\ cleavage}{Raw\ volume\ before\ cleavage} \times 100$$

Evaluation of the interaction between recombinant ASFV-p30 protein and anti-p30-ASFV antibody

A modified dot blot assay was used to evaluate the interaction between recombinant p30 protein and specific ASFV-p30 antibody present in positive swine serum. The purified GST-SNAC-p30-ASFV protein was spotted on a nitrocellulose membrane (dots 8 – 12). The membranes were then blocked with 1% skim milk at room temperature for 1 h. As a negative control, purified GST from the pGEX-5X-1 plasmid was applied to dots 1 – 5. Dots 6 and 7 were served as positive controls to confirm successful protein coating on the nitrocellulose membrane. For the primary antibody step, five AFSV-positive swine serum samples containing anti-p30 antibodies were diluted 1:200 (50 µL. each) and applied. In contrast, PBS buffer (1.42 g Na_2HPO_4; 0.24 g KH_2PO_4; 8 g NaCl; 0.2 g KCl; prepared in 1 L, pH 7.4) was applied to dots 6 and 7. After incubating for 1 h at room temperature, the membranes were washed five times with PBS-T. Subsequently, secondary antibodies were added: HRP-labeled goat anti-swine IgG (Santa Cruz, 1:10,000 dilution in PBS-T) and anti-GST HRP-conjugated antibody (Cytiva, 1:10,000 dilution in PBS-T) for dots 6 and 7. These were incubated for 1 h at room temperature, followed by five PBS-T washes. Finally, the TMB substrate was added to visualize the binding signal.

RESULTS AND DISCUSSION

Amplification of the *SNAC-p30-ASFV* gene and cloning of the pGEX-*SNAC-p30-ASFV* vector

The *SNAC-p30-ASFV* gene was amplified by PCR using gene-specific primers, producing a band of approximately 700 bp, consistent with the expected size of the target gene (Fig. 2B). Colony PCR with the same primers confirmed the presence of the insert in recombinant clones. Sequencing of positive clones further verified sequence homology with the *ASFV-p30* gene from GenBank. These results demonstrated that the pGEX-*SNAC-p30-ASFV* expression vector was successfully constructed.

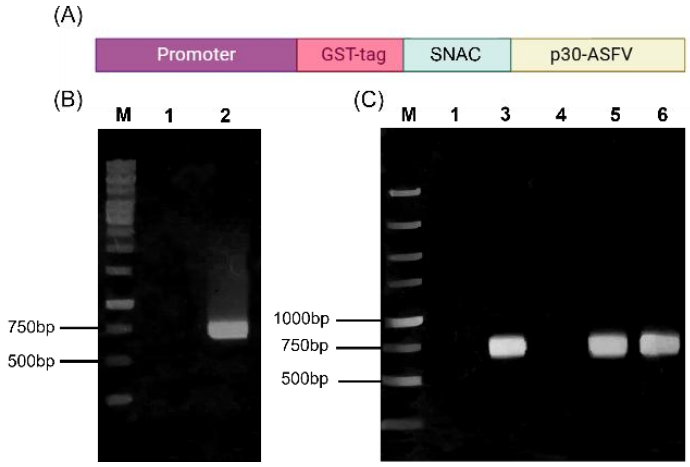

Figure 1 Schematic representation of the gene location on the vector (A), PCR amplification of the *SNAC-p30-ASFV gene* (B), and colony PCR result with gene-specific primers (C). M, 1kb DNA ladder; 1, negative control; 2-3, PCR products of the *SNAC-p30-ASFV* gene; 4-6, colony PCR of transformants using primers 1172F Bgl/1173R Not.

Expression and purification of the recombinant GST-SNAC-p30-ASFV protein

The pGEX-*SNAC-p30-ASFV* vector was transformed into *E. coli* BL21(DE3) cells for recombinant protein expression. A positive clone was induced by IPTG, and protein expression was analyzed by SDS-PAGE (15% gel) with Coomassie Blue staining, which revealed a strong band at approximately 50 kDa (Fig. 3A; lanes 2-4), corresponding to the predicted sizes of GST-SNAC-p30-ASFV protein. No expression was observed in the negative control (Fig. 3A; lane 1). Approximately 32% of the GST-SNAC-p30-ASFV protein was pressent in the soluble fraction of the *E. coli* expression system.

The soluble fraction of GST-SNAC-p30-ASFV from cell lysate supernatants was purified by affinity chromatography using GSTrap™ 4B (1 ml). The soluble GST

fusion protein was loaded onto the column, washed with binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), and eluted with 10 mM reduced glutathione. Supernatant samples from each purification step were collected and analyzed by SDS-PAGE with silver staining. The results showed that the recombinant GST-SNAC-p30-ASFV protein was obtained with high purity (~90%) (Fig. 3B).

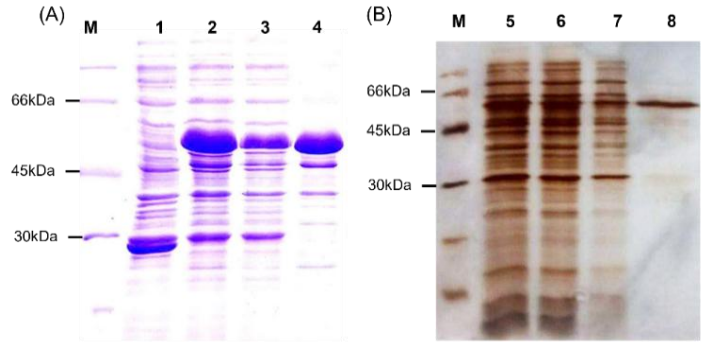

Figure 3 Expression and purification of the GST-SNAC-p30-ASFV protein analyzed by SDS-PAGE with Coomassie Brilliant Blue staining (A) and by silver staining (B). M, Low molecular weight protein marker; 1, *E. coli* BL21(DE3)/pGEX-5X-1 (negative control); 2-4, *E.coli* BL21(DE3)/pGEX-*SNAC-p30-ASFV*; 2, total fraction; 3, soluble fraction; 4, insoluble fraction; 5, pre-purified GST-SNAC-p30-ASFV sample; 6, flow-through; 7, washing; 8, purified GST-SNAC-p30-ASFV protein.

SNAC cleavage and evaluation of the interaction between recombinant ASFV-p30 protein and anti-p30-ASFV antibody

The GST tag was removed from the purified GST-SNAC-p30-ASFV protein using the SNAC cleavage strategy. SDS-PAGE analysis revealed two bands, approximately 26 kDa and 30 kDa, corresponding to the expected sizes of the GST tag and ASFV-p30 protein, respectively. These results indicate that the SNAC tag was efficiently cleaved, achieving more than 50% completion (Fig. 4B). Densitometric analysis using Gel Analyzer software (version 23.1.1) showed that the proportion of ASFV-p30 was approximately equivalent to that of GST in the digested sample. Western blotting with an anti-GST HRP-conjugated antibody confirmed that the GST-ASFV-p30-SNAC fusion protein was successfully purified and cleaved (Fig. 4B).

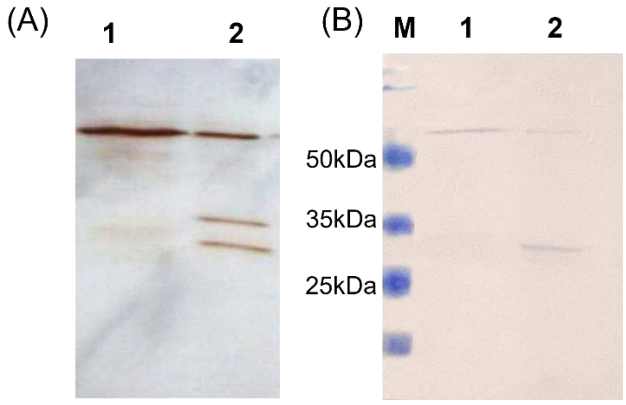

Figure 4 Ni^{2+} cleavage of GST-SNAC-p30-ASFV protein analyzed by silver-stained SDS-PAGE gel (A) and by Western blot with anti-GST antibody (B). M, Prestained protein marker; 1, GST-SNAC-p30-ASFV protein before cleavage; 2, GST-SNAC-p30-ASFV protein after cleavage.

To verify that fusion with SNAC and GST did not affect the structural stability or antigenicity of the recombinant ASFV-p30, the cleaved GST-SNAC-p30-ASFV protein was analyzed by dot blot using five p30-positive swine sera, previously confirmed as positive with the Ingenasa kit. A clear colorimetric signal was observed on dots 8 - 12, which contained the recombinant ASFV-p30, but not on the negative control (Fig. 5). These results demonstrated that the recombinant ASFV-p30 protein reacted with all the positive sera for anti-ASFV antibodies, indicating strong immunoreactivity. Thus, the antigenicity of the recombinant ASFV-p30 remains unaffected by the dual-tag fusion with SNAC and GST.

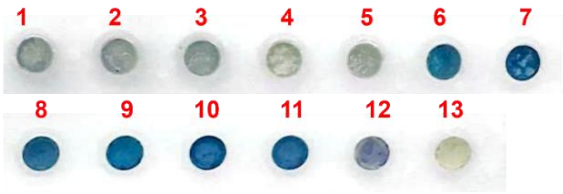

Figure 5 Interaction between recombinant ASFV-p30 protein and anti-p30-ASFV antibody analyzed by dot blot. 1-5, purified GST protein sample from the pGEX-5X-1 incubated with five p30-positive sera and detected with anti-swine IgG-HRP; 6, purified GST protein sample from the pGEX-5X-1 incubated with anti-GST HRP; 7, cleaved p30-SNAC-GST incubated with anti-GST HRP; 8-12, cleaved GST-SNAC-p30-ASFV incubated with five p30-positive sera and detected with anti-swine IgG-HRP; 13, technical negative control.

DISCUSSION

The highly immunogenic phosphoprotein p30 is an early-expressed, highly conserved viral protein that plays a crucial role in ASFV infection, making it an attractive target for both serological detection and vaccine development **(Hübner *et al*., 2018)**. Owing to its phosphoprotein nature and structural instability, ASFV-p30 expression has traditionally relied on eukaryotic systems such as insect or mammalian cells, which provide the post-translational modifications required for proper folding. However, these systems involve complex procedures, extended timelines, and relatively low yield **(H. Chen *et al*., 2024; Petrovan *et al*., 2019; Zhou *et al*., 2023)**. To improve production efficiency, bacterial expression systems have been explored; however, in prokaryotic hosts, ASFV-p30 is typically produced as insoluble inclusion bodies or requires fusion tags to enhance solubility. In certain subunit vaccine development and biopharmaceutical manufacturing, removal of fusion tags is essential to ensure product safety, preserve optimal immunogenicity, and maintain the native biological activity of the target antigen **(Köppl *et al*., 2022; Mainali *et al*., 2025)**. To address these limitations, we developed a dual-tag SNAC-GST system as a novel strategy for cost-effective and efficient protein purification. In this system, GST enhances the soluble expression of structurally unstable proteins such as ASFV-p30 in *E. coli*, while the SNAC tag enables Ni^{2+}-mediated cleavage of the fusion protein. Since its introduction by Dang (2019), the SNAC tag has shown promise for protein cleavage under physiological conditions with high biocompatibility.

The present study demonstrates enhanced soluble expression and effective tag removal. However, the cleavage product requires additional purification to eliminate residual GST and uncleaved SNAC–GST fusion proteins. Further validation is needed to optimize cleavage efficiency, as it is strongly influenced by Ni^{2+} concentration, incubation time, and temperature. Because structural stability may decrease after tag removal, appropriate low-temperature storage and buffer optimization are necessary to maintain protein integrity. Although dot blot analysis using p30-positive sera confirmed the antigenicity of the recombinant protein, this evaluation remains qualitative. Development of an ELISA will be required to determine sensitivity and specificity before advancing toward diagnostic kit applications. Overall, this study presents a feasible and practical strategy for the precise production of structurally unstable proteins in bacterial expression systems.

CONCLUSION

This study successfully constructed a recombinant vector carrying the ASFV-p30 gene using a dual-tag system (GST-SNAC-p30-ASFV). The protein was expressed in the soluble fraction and purified to high purity by affinity chromatography. Importantly, the SNAC sequence enabled efficient cleavage through Ni^{2+}-mediated while maintaining antigenic reactivity. This strategy offers an efficient approach for producing otherwise insoluble proteins, with potential applications in biological research and biotechnology.

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