Expression and purification of African Swine Fever virus p72 trimers as subunit vaccine candidate

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ABSTRACT

In an effort to control and eventually eliminate African Swine Fever (ASF), the development of vaccines has long been sought. Protein p72, the major capsid protein of African Swine Fever Virus (ASFV), is considered as a promising candidate for subunit vaccine development. The high-resolution cryo-EM structure of p72 was reported recently, demonstrated that p72 molecules could form a thermostable trimer with the aid of B602L. However, the development of ASFV p72 subunit vaccine was still largely hindered, due to limitations in the expression of the antigen in sufficient yield and quality.

Here we successfully co-expressed p72 and B602L in the Saccharomyces cerevisiae system, and the system had produced a high yield p72 trimer at harvest time. A downstream process, an affinity gravity-flow chromatography (strep-tag system) followed by FPLC size exclusion chromatography were performanced under pH 8.5. In order to figure out how intracellular pH effect p72 trimers' conformation, the prepared p72 trimer was then exposured to different buffers with pH values ranging from 3~8.5. The result indicated that the low pH of lysosome and endosome may cause p72 trimer shrink or depolymerize.

Keywords: African Swine Fever Virus, p72, Vaccine, Saccharomyces cerevisiae

1. Introduction

African swine fever (ASF) caused by the African swine fever virus (ASFV), is a highly contagious and lethal disease of domestic or wild pigs[1-3]. The progress of ASFV outbreaks in China, began in the summer of 2018 and haven't been under control so far, have already caused huge economic loss[4]. Thus, the development of effective vaccines or treatment against ASFV infection is urgently needed.

ASFV is the only member of the Asfarviridae family. Prior studies showed that the ASFV virion has a complex structure with multiple membrane and protein layers[5, 6]. The major capsid protein p72, one of the key protective antigens, form homo-trimers and assemble the outmost icosahedral protein shell[6, 7]. The structure of p72 trimer was determined by Liu, Q., et al[7], their result also revealed that p72 molecules can be assembled to form a thermostable trimer when B602L was co-expressed with p72; but the structure of B602L was remain unknown.

However, efforts to produce effective subunit vaccines based on prior work upon p72 are obstructed by the limitation of low yield. Liu, Q., et al[7] have successfully co-expressed p72 and B602L in mammalian in vivo expression systems, HEK293F cells. Here, we offer the process development of p72 trimer in the Saccharomyces cerevisiae system. The Saccharomyces cerevisiae system has demonstrated both high yield potential as well as the additional benefit of post-translational modifications, and also been used for the commercial production of

heterologous proteins[8, 9]. While the previous study reported on research and discovery aspects of p72 trimer[7], characterization from manufacturing and quality control view-points was not addressed. In this study, we report a reproducible expression and purification process of massive p72 trimer by co-expressed p72 and B602L in Saccharomyces cerevisiae system.

Moreover, the massive production of p72 trimer makes it much easier to study the intracellular conformational changes of ASFV outer shell. Considering p72 is the most dominant structural component of the virion, the p72 trimer must adopt a conformational change in cell to release the gene out of the shell. Here, we treated the prepared p72 trimer with low pH buffer, mimic the acid environment of lysosome and endosome (pH 3-5). The pH changes introduced a peak shift in FPLC size exclusion chromatography, likely to verify our conjecture.

2. Materials and methods

Expression and purification of P72 trimers. The constructed expression vector carrying gene encoding p72 (with strep-tag) and B602L (with myc-tag) was transformed to competent cells to give new expression strain. Transformants were selected on plates containing SC-URA-LEU medium after incubation at 30 °C for 2–3 days. The new strain was grown in shake flask containing 50 mL SC-URA-LEU, at 30 °C overnight. Then the overnight cultures of cells were inoculated in 1 L YPD with 2% glucose. The culture was harvested by centrifugation at 6000 rpm for 10 min when the O.D.600 were about 2.5 and 3.5. Cells were resuspending in 50mL of Buffer W (Buffer W was prepared according to IBA's manual of Strep-Tactin XT, pH8.5), followed with cell disruption by utilizing homogenizing machine. Collect the supernatant of cell disruption product after centrifugating at 17000 rpm for 90 min, and load all supernatant onto a Strep-Tactin XT gravity-flow column (IBA), perform affinity chromatography as IBA's manual described. The collected eluents were analyzed with SDS-PAGE and final pool was selected based on purity. The final pool was concentrated to > 5 mg/mL using Merck-Millipore 3O kDa, 15 mL concentrator.

SDS-PAGE. For in-process analysis, samples were diluted with 5 x Loading sample buffer, heated at 95 °C for 10 min and loaded on10% SDS-PAGE gels. Gels were run at 140 V for 60min in l x Tris-Glycine running buffer and stained with Stain solution.

Western blot. Following SDS-PAGE, proteins were transferred onto 0.2 pm nitrocellulose membrane (Pall) and blocked in 5% skim milk in TBS containing 0.05% Tween 20 (PBST) at 4 °C overnight. Primary antibody at a 1:5000 dilution of strep antibody or myc antibody in 2% skim milk in TBST, was added and incubated for one hour at room temperature. Membranes were washed with TBST (3x for 10 min each) and secondary antibody, 1:5000 dilution of goat anti mouse IgG-HRP in 2% skim milk (PBST), was added and incubated at room temperature for one hour. Membranes were then again washed with TBST (3 x for 10 min each) and developed using TMB (3,3,5,5'-Tetramethylbenzidine, Sigma Aldrich) and hydrogen peroxide.

Protein concentration. The BCA assay was performed on microtiter plates for both in process and final product analysis to determine the protein concentration of p72.

SE (Size Exclusion)-FPLC. SE-FPLC analysis of purified p72 was performed on a FPLC size exclusion chromatography column (GE healthcare, Superdex 200, 16/600) operated at column temperature of 4 °C on an AKTA system. The mobile phase consisted of 150 mM sodium chloride and 20 mM TRIS (pH 8.5)/20 mM citric sodium (pH 5/pH 3) with a flow rate of 1 mL/min. The final pool was concentrated to > 5 mg/mL using Merck-Millipore 3O kDa, 15 mL concentrator.

Negative-stain electron microscopy. Following SE-FPLC, the p72 trimer sample were imaged by negative-stain transmission electron microscopy (TEM). The negative-stain EM was

performed by School of Medicine, Tsinghua University.

Mass spectrum analysis. Following SDS-PAGE, the bands corresponding to p72 and B602L were separately collected, MS was performed by Mass Spectrum Laboratory, College of biological science, China Agricultural University.

Determination of the critical value of pH inducing deformation. To determine how intracellular pH effect the conformation of p72 trimers, the concentrated samples (2-3 mg/ml) were subsequently diluted by buffers with different pH (1M TRIS/HEPES/citric sodium, pH 8.5/ 8.0/ 7.5/ 7.0/ 6.5/ 6.0/ 5.5/ 5.0/ 4.5/ 4.0/ 3.5/ 3.0), then analyzed by SDS-PAGE.

3. Result

Expression and purification of P72 trimers. Recombinant p72 trimer was successfully produced in Saccharomyces cerevisiae system and purified by Strep-Tactin affinity chromatography. The expression of p72 was verified by western blot analysis (not shown), each band was detected using anti-strep antibody and anti-myc antibody as described in Materials and Methods.

Purified protein samples were analyzed by 10% SDS-PAGE, and gels were stained with Coomassie Blue (Fig. 1). P72 and B602L bands were located at the expected molecular mass, approximate 73 kDa and 68 kDa. Although P72 bands were still detected in the tenth fractions, B602L bands were fading away from the fifth washing fraction. The size of bands indicated that p72 has about three times as much protein content as B602L.

Bands corresponding to p72 and B602L were then analyzed by MS. The results of intact mass determination were consistent with the theoretical mass of the protein. The protein sequence coverage are both above 52%: first thirty-eight N-terminal amino acids (MASGGAFCLIANDGKADKIILAQDLLNSRISNIKNVNK) of p72 and twenty-two N-terminal amino acids (AEFNIDELLKNVLEDPSTEISEETLK) of B602L determined by MS were consistent with the sequences reported earlier.

To verify the polymerization state of p72, SE-FPLC was employed under pH 8.5. A typical SE-FPLC profile is shown in Fig. 2, shows p72 (red line) as a trimer (approximately 260kDa) and absence of significant degradation. The peak fractions were gathered and concentrated up to 5 mg/ml, the total purified protein content was determined up to 10mg per liter of culture media. The negative-stain EM image of the purified p72 trimer sample is shown in Fig. 3.

Determination of the critical value of pH inducing deformation. Concentrated p72 trimers were diluted in the buffer of pH 5 and pH 3 (mimic the acid environment of endosome and lysosome). The SE-FPLC was subsequently performed again. The result (Fig. 2) showed that both the peak of pH 5 (green line) and pH 3 (blue line) were shifted backward comparing to the peak of ph8.5, the shifted peaks were corresponding to ~180kda and 100kda. This result indicated the p72 trimers may shrink or depolymerized into homodimer (two molecules of p72) or heterodimer (p72 and B602L, one molecule for each） under pH 5, while under pH3 p72 trimers may depolymerized into monomers.

In order to determine the critical value of pH inducing deformation, we add concentrated p72 trimers (2-3mg/ml) into 1M buffers of pH ranging from 3 to 8.5. The result is as below: p72 trimers deposit immediately when transfered into buffer under pH 5.5; when pH under 4, the solution would become colloid-like, the precipitant and supernatant remained unseparatable after high speed centrifugation.

The role of B602L in deformation. The supernatant from series protein solution under various pH (3~8.5) from the previous step were analyzed by SDS-PAGE (Fig. 4), to investigate

whether the B602L has playing a role in this pH dependent conformation changing. Unfortunately, though the protein concentration were decreased due to the severe deposition at low pH, the B602L band was still existed.

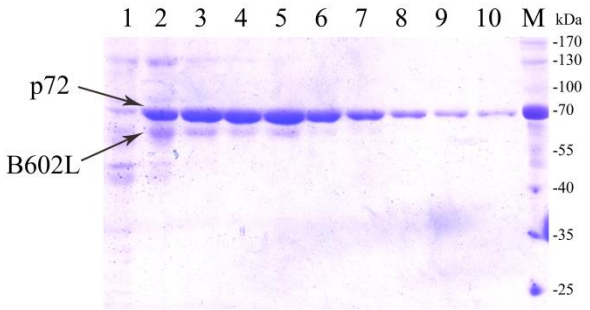

Fig. 1. SDS-PAGE analysis of purified p72 during affinity chromatography.
M: Pre stained protein marker
1-10: The first to the tenth fractions (one column volume per fraction)

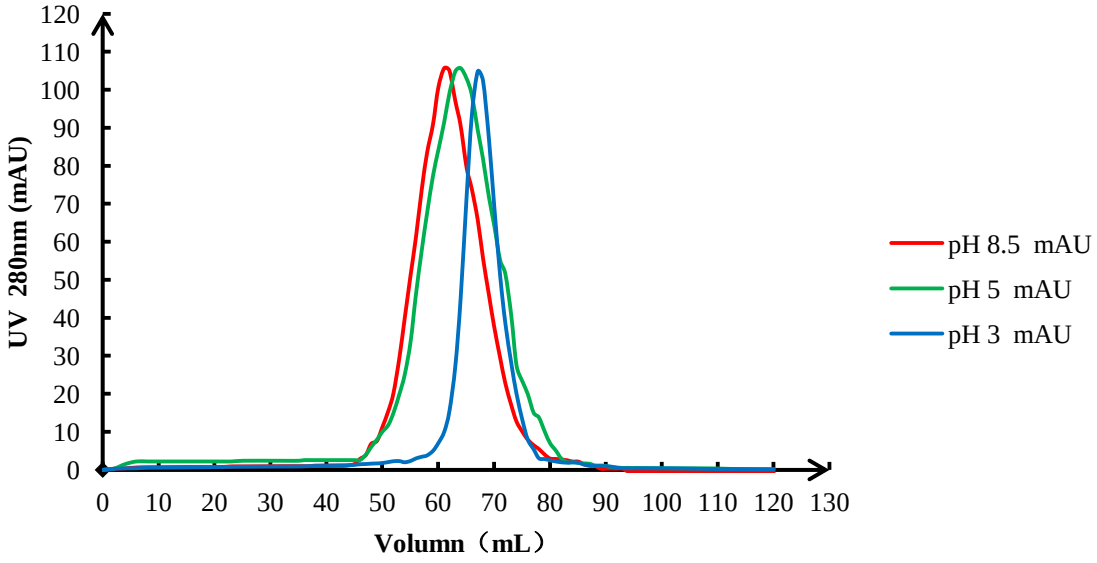

Fig. 2. Size exclusion FPLC analysis of p72 under different pH.

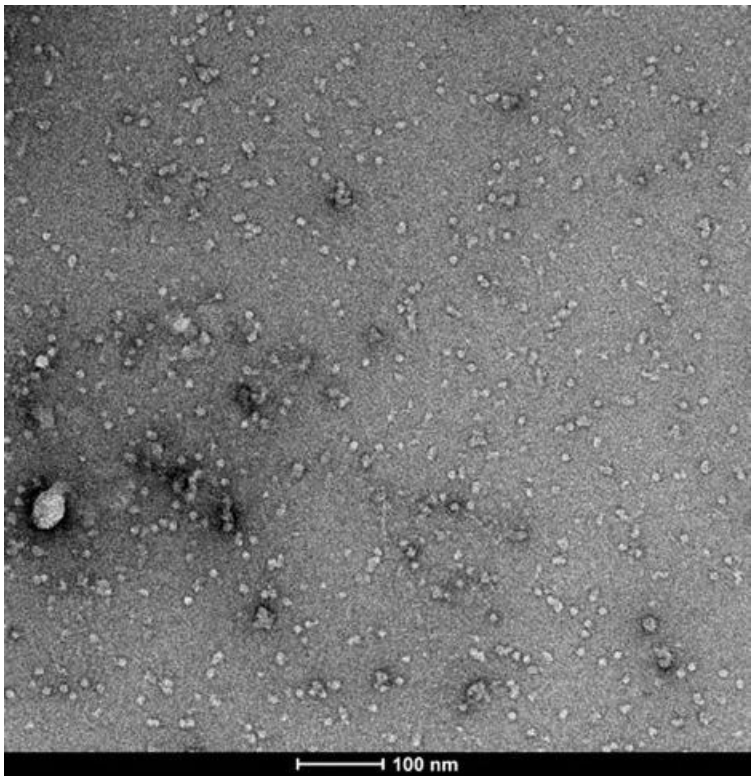

Fig. 3. The negative-stain EM result after Size exclusion FPLC analysis.

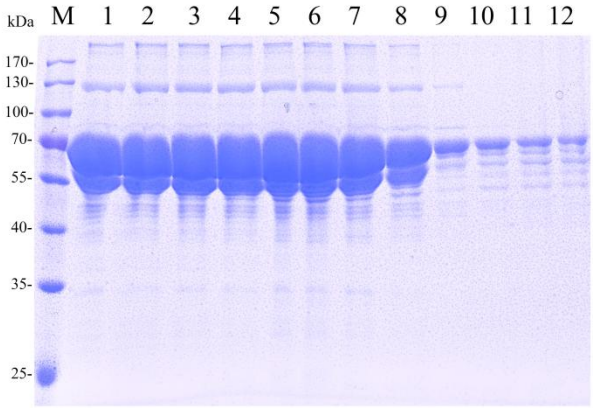

Fig. 4. SDS-PAGE of p72 under different pH.
M: Pre stained protein marker;
1: pH 8.5; 2: pH 8.0; 3: pH 7.5;
4. pH 7.0; 5: pH 6.5; 6: pH 6.0;
7: pH 5.5; 8: pH 5.0; 9: pH 4.5;
10: pH 4.0; 11: pH 3.5; 12: pH 3.0.

4. Discussion

To support the continued development of an ASFV sbunit vaccine, we report an optimized, scalable, and reproducible process to produce p72 trimers expressed in Saccharomyces cerevisiae system. A final yield of up to 10 mg/L is reported, suitable to readily produce greater

purified protein and sufficient to support immune challenge test and structure determination (EM and crystallization), the primary objective of the process developed here.

While p72 trimer is stable under pH as low as 6, studies are in progress to evaluate the conformation of p72 at even lower pH. In this study, we demonstrate that the p72 trimer would deform at pH 5.5. The SE-FPLC profile showed that the p72 trimer may deform into a trimer with tighten conformation or depolymerized into p72 homodimer or p72-B602L heterodimer under pH 5, while under ph3 p72 trimers may depolymerized into monomers with loosen conformation.

Sánchez EG et al.[10] had reviewed the endosomal pathway of ASFV: viron enters the cell by the endosomal pathway. After viral particles uptake, pH-dependent uncoating process takes place in late endosomes. It is noteworthy that, one of the earliest key aspects is the acidification of endosomes. There is a gradation in pH between the vesicles of the endolysosomal system, which progressively acidify from the early endosomes with a pH near 7 through the late endosomes and lysosomes whose pH is around 5. Such acidification is necessary for ASFV to complete its infectious cycle. Our results seem to initially explain why the viron cannot remain intact at pH below 5. However, the structure of p72 at low pH and B602L still need to be resolved to provide a more detailed explanation of this acid-pH-dependent uncoating process.

On the other hand, from the perspective of vaccine development, this pH dependent deformation could guide us to select the appropriate protein cross-linking conditions, so that the vaccine can maintain the optimal conformation at low pH.

In conclusion, we developed a scalable process to produce p72 trimer in Saccharomyces cerevisiae system. The availability of this recombinant protein in pure form should facilitate the crystallization and detailed analysis of the structure of p72 (at low pH) and B602l, as well as the further research on mechanism of acid-pH-dependent uncoating process. The availability of a large quantity of stable p72 trimer will help in development of efficient ASFV subunit vaccine.

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