Investigation of Kluyveromyces marxianus as a novel host for large-scale production of porcine parvovirus virus-like particles

Deqiang Yang
Fudan University

Lei Chen
Fudan University

Jinkun Duan
Fudan University

Yao Yu
Fudan University

Jungang Zhou (zhoujg@fudan.edu.cn)
Fudan University

Hong Lu
Fudan University

Research

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Abstract

Background: Porcine Parvovirus (PPV) is a Parovirinae virus that can cause embryonic and fetal loss and death and mummification in affected fetal pigs. Unlike conventional vaccines, virus-like particles (VLPs) inherit the natural structure of their authentic virions and highly immunostimulatory that can induce strong humoral immune and T cell responses with no risk of pathogenicity due to incomplete attenuation or inactivation. Production PPV VLPs is still a challenge using the traditional expression platforms due to the low yield and culture cost. Kluyveromyces marxianus is a safe and fast-growing eukaryote that can yield high biomass in low-cost cultures. In this study, we investigated the production and downstream processes of PPV VLPs in K. marxianus, and the potential for effective stand-alone vaccines.

Results: After optimization according to the codon bias of K.marxianus, the VP2 protein from Kresse strain was highly expressed. In 5L fermentator, the yield of PPV VLPs reached 2.5 g/L, qualified by HPLC, using a defined mineral medium after 48h fermentation. Two strategies, cation exchange chromatography coupled with Sephacryl® S-500 HR chromatography for the supernatants of pH adjusted cell lysates and anion exchange chromatography followed by cross-flow diafiltration for the precipitate respectively, were established to purify the intracellular PPV VLPs. The purity PPV VLPs reached about 95%, and total recovery was more than 60%. Sera from the PPV VLPs immunized mice showed high titers of IgG antibody and hemagglutination inhibitions on both swine and guinea pig erythrocytes. Spleen lymphocyte proliferation and cytokines detection suggested the PPV VLPs produced by K.marxianus can provoke the cellular immune and humoral immunity responses.

Conclusion: This is the highest production of recombinant PPV VLPs achieved to date. The superiorities, safe, high production, short lead time, and low cost, make K. marxianus a greatly competitive platform in bioproduction of PPV VLPs vaccine.

Background

Paroviruses are small, non-enveloped DNA viruses belonging to the Paroviridae taxonomic family and are divided into two subfamilies: the Parovirinae and Densovirinae that infects vertebrates and arthropods respectively [1]. In Parovirinae subfamily, paroviruses of the genera Protoparvovirus, Bocaparvovirus, Copiparvovirus and Teraparovirus, were also designated as porcine parovirus types 1 through 6 [2]. Classic porcine parovirus (porcine parovirus type 1, PPV1), newly named Ungulate protoparvovirus 1, is a member of the genus Protoparvovirus that was first isolated from a contaminant pig kidney culture in 1965 in Germany [3]. In the past two decades, with the development of molecular biology techniques, six new serotypes of parovirus have been clinically discovered in pigs and designated as Porcine parovirus (PPV) 2 to PPV7[4]. PPV7 was first discovered in the USA in 2016 as a new serotype of porcine parovirus in the rectal swabs of healthy adult pigs by the metagenomic sequencing technology [5].
PPV was a major causative agent of the swine reproductive failures traditionally summarized as the stillbirth, mummification, embryonic death, and infertility (SMEDI) syndrome[6]. The incidence and severity of symptoms in sows infected with PPV virus depending on the strain virulence and the time of gestation at which infection occurred [7]. PPV virus can replicate and be shed from infected sows with no clinical symptoms, but transplacental infections usually result in death and mummification of the fetus before 70 days of gestation, in which fetuses have not developed antibodies to eliminate the virus and survive the infection. The constant infection of PPV in the herd and the inherent high mutation rate gave impetus to the emerging of new mutated strains [8, 9]. Based on the pathogenicity, the PPV1 virus has four clinical genotypes: the NADL-8 strain, initially isolated from a dead fetus, can cross the placental barrier and causes viremia that leads to fatal infection of nonimmunocompetent fetuses[10]; Another genotype of PPV1 strains, such as the Kresse and IAF-A54 strains, is high virulent strains that associated with dermatitis disease, and displays an increased virulence and kills immunocompetent fetuses[11, 12]; The IAF-A83 strain, the third pathogenic genotype of PPV1s, causes enteric diseases[13]; Finally, the NADL-2 strain, currently used as an attenuated vaccine, is the nonpathogenic genotype of PPV1s, which cannot cross the placenta as efficiently as highly pathogenic strains in experimental infections, and causes only limited viremia[14, 15]. All isolates of PPV1 virus are found to be antigenically similar, and only a single serotype is recognized[6]. Vaccination against porcine parvovirus can’t prevent the virus infection and shedding, but it protects swine from SMEDI diseases[7].

PPV is a negative, single-stranded DNA virus with a genome about 5000 nucleotides, and its mature viron is icosahedral symmetric particles with approximately 25 nm in diameter. The genome of PPV contains two large non-overlapping Open Reading Frames (ORFs), the 5'-end ORF, encoding a non-structural protein, and the 3'-end ORF, encoding three structural proteins VP1, VP2 and VP3 [16]. VP1 and VP2 were translated from alternatively spliced mRNA of the 3’-end ORF, while VP3 is a proteolytic product from VP2 by cleaving its 20 N-terminal residues in endosomes [17–19]. PPV capsids consist of 60 equivalent copies of heterotrimer comprised of three structural proteins, VP1, VP2 and VP3[20]. The VP2 protein is closely related to the virus-host range and antigenicity, and is generally considered the major immunoprotective antigen of PPV vaccines since it contains most of the B-cell epitopes critical to elicit neutralizing antibodies [21, 22].

The VP2 proteins can spontaneously self-assemble into virus-like particles (VLPs), mimicking the morphology of pathogenic virus and maintaining an identical hemagglutination activity [23]. The PPV VLPs is a safe and practical alternative to inactivated infectious viruses as a vaccine that can provoke a strong protective immune response without the risk of diseases and offers a ready platform for facilitating recognition, uptake, and processing by the immune system [24]. Up to date, PPV VLPs has been produced in the common hosts such as insect-baculovirus, *Saccharomyces cerevisiae, Pichia pastoris* and *Escherichia coli*, and among which the highest expression level was in *P. pastoris*, with a yield of 595.76 mg/L [25–28]. In the current study, we have sought to achieve high production of the PPV VLPs using a nonconventional yeast *Kluyveromyces marxianus*, an aerobic, Crabtree negative and homothallic hemiascomycetous yeast species that can generate energy from both respiratory metabolism and ethanol fermentation[29–31]. *K. marxianus* has been developed for biotechnological
applications, such as the production of enzymes, production of bioingredients, an anticholesterolemic agent and a host for expression of heterologous proteins, mainly due to its thermostolerance, high growth rate, broader substrate spectrum and generally recognized as safe (GRAS)[32–34]. Using *K. marxianus* as host, we achieved a 2.5 g/L VLPs of the Kresse strain in a 5 L fermentor. This production is much higher than previous reports. The PPV VLPs produced in *K. marxianus* elicited high titers of IgG antibody and hemagglutination inhibition antibody and therefore can be useful for the development of anti-PPV veterinary vaccines.

**Materials And Methods**

**Strains and plasmids**

Laboratory strain *K. marxianus* Fim-1ΔURA3 is a uracil auxotroph strain originated from *K. marxianus* Fim-1 deposited in China General Microbiological Culture Collection Center (CGMCC No.10621 [29]. The vector pUKDN115 was constructed from the pUKD-S-PIT plasmid by replacing the fragment containing an α factor signal peptide sequence and human interferon α-2a gene with a multiple cloning site (MCS) [35].

**Construction of the recombinant strain**

According to the *K. marxianus* codon preference, the VP2 gene of the Kresse strain (GenBank U44978.1) was optimized and synthesized by Genewiz Biotechnology Co., Ltd (Suzhou, China). The optimized sequence was deposited in the NCBI GenBank database under the accession number MT932328. The synthetic VP2 gene was amplified with Phanta® Super Fidelity DNA Polymerase (Vazyme, Nanjing, China) using the following oligonucleotide primers (underlining indicates the homologous sequences from pUKD-N115 vector): 5'-TTTTTTTTTT ATGAGCGAAA ACGTGGAGC-3') and 5'- AGCTTGCGGC CTTAACTAGT CTAGTACAAC TTTCTTGGG −3'. The amplicon was ligated with the *EcoR* I and *Hind* III linearized pUKDN115 by Gibson assembly [36], and directly transformed into the FIM-1 ΔURA3 strain according to the Lithium acetate transformation method [37]. Transformants formed on the SD plates (0.67% YNB, 2% glucose, 2% agar) were verified by PCR using the primers ATGAGCGAAA ACGTGGAGC-3 and CTAGTACAAC TTTCTTGGG −3', and the positive clone was designated to the KM-PPV-VP2 strain.

**Expression and identification of the VP2 Protein in K. marxianus**

Fresh clones of KM-PPV-VP2 was inoculated in 50 mL YD medium (2% yeast extract, 4% glucose) and cultured at 30 °C, 220 r/min for 72 h. One milliliter of yeast cells harvested by centrifugation was washed with 1 ml PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) twice, and then suspended in 500 µl lysate buffer (50 mM HEPS, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-Deoxycholate, pH 7.5). Approximately 400 µl of glass beads (G8772, Sigma-Aldrich, Missouri, USA) was added to disrupt cells on a Bead-beater (FastPrep-24, MP, California, USA) at 6 m/s for 2 min. Cell lysates were centrifuged at 12000 rpm, 4 °C for 20 min, and the supernatant was used for SDS-PAGE and Western blotting assays. In Western blotting, an anti-PPV VP2 polyclonal antibody and a goat anti-mouse
IgG alkaline phosphatase-conjugate (074-1806, KPL, USA) was used as the primary and secondary antibody respectively.

**Preparation of the anti-PPV VP2 polyclonal antibody**

The native VP2 gene of the Kresse strain was cloned into the pET-28a(+) vector within the Sac I and Nto I sites (Novagen, Madison, USA), generating the pET-28a/VP2 plasmid. After transformation into E. coli BL21(DE3), VP2 protein was induced by 0.2 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and purified by Ni-NTA (Ni Smart Beads, Smart-lifesciences, Changzhou, China) affinity basically as previously described [38]. Six weeks old Balb/C mice, purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd, were immunized with 20 µg of the purified VP2 antigen mixed with an equal volume of Freund’s adjuvant. After 35 days post-immunization (dpi), sera of the immunized mice were separated as a primary antibody for Western blotting described above.

**Transmission Electronic Microscopy (TEM)**

TEM scan of PPV VLPs was performed on a JEM-2100 Electron Microscope (JEOL Tokyo, Japan) according to Bucarey et al.[39]. Briefly, samples were spotted onto carbon-coated copper grids. After adsorption at room temperature for 5 min, the copper grids were dried with filter and negatively stained with 3% of phosphotungstic acid (PTA). The grids were examined at an accelerating voltage of 120 kV.

**High cell-density fermentation**

High cell-density fermentation was conducted in a 5 L fermentor (BXBIO, Shanghai, China) as described recently[29]. The KM-PPV-VP2 strain was inoculated in 200 mL YD medium, grown at 30 °C, 220 r/min for 18 h, and then transferred into the fermentor containing 2L defined mineral medium. During the fermentation, the dissolved oxygen was maintained above 10%, and the temperature was controlled at 30 °C. The pH was controlled automatically at 5.5 with ammonium hydroxide. At given intervals, 10 ml of culture was harvested to determine the cell density (OD_{600} nm) and wet cell weight (WCW) In SDS-PAGE analysis of the PPV VLPs production, cell samples were diluted 1:10 with PBS buffer before disruption using the glass bead disruption described above. VLPs quantification was performed on an Agilent Series 1100 System (Agilent, Waldbronn, Germany) with a TSKgel G4000 SWXL column (300 mm x 7.8 mm i.d.) (Tosoh Bioscience, Stuttgart, Germany) coupled with a TSKgel SWXL guard column (40.0 mm x 6.0 mm i.d.) (Tosoh Bioscience) as previously described [40].

**Screen of the ion-exchange chromatography (IEX) media**

The KM-PPV-VP2 cells were collected by centrifugation at 5,000 rpm for 10 min, followed by washing with deionized water twice. The rinsed cells were then suspended with PBS buffer pH7.4 or 20 mM Tris-HCl buffer 8.0 corresponding to the cation or anion resins respectively. Cell lysates were prepared by high-pressure homogenization on a JN-02C Homogenizer (JNBIO, Guangzhou, China) under a condition of 1500 bar, 4 °C for 2 times, followed by centrifugation at 10,000 rpm, 4 °C for 30 min. The screen of the IEX media was performed on the Poly-Prep®Chromatography Columns (Bio-Rad, Hercules, CA, USA) packaged 2 ml of different cation or anion resins listed in Table 1 according to the described previously [41]. Note that, in case of cation resins, the pH of cell lysates disrupted with PBS buffer should be
adjusted to pH 4.0 with acetic acid before clarification by centrifugation. After washing with 5 volumes of 20 mM acetate/Tris-HCl buffer pH 4.0, the bound proteins were eluted by 5 mL of PBS containing 1M NaCl and analyzed by SDS-PAGE.

Table 1
Ion exchange resins used in this work

| Properties | Resins          | Binding conditions       | Manufacturers   |
|------------|-----------------|--------------------------|-----------------|
| Cation     | Capto S ImpAct  | 20 mM NaAc-HAc pH4.0     | GE healthcare   |
|            | Nuvia S         |                          | Bio-Rad         |
|            | SP Bestarose FF |                          | Bestchrom       |
|            | Capto MMC       |                          | GE healthcare   |
|            | SP HP           |                          | Bestchrom       |
|            | CM Sepharose FF |                          | GE healthcare   |
|            | POROS HS        |                          | Life Science    |
|            | CaptoSP ImpRes  |                          | GE healthcare   |
|            | Nuvia cPrime    |                          | Bio-Rad         |
| Anion      | Capto Q XP      | 20 mM Tris-HCl pH8.0     | GE healthcare   |
|            | Capto Q         |                          | GE healthcare   |
|            | Q Bestarose FF  |                          | Bestchrom       |

Purification of the PPV VLPs

To purify the PPV VLPs by IEX chromatography, yeast cells were suspended in PBS buffer pH7.4 and disrupted by high-pressure homogenization. The pH value of cell lysates was subsequently adjusted to pH 4.0. After centrifugation at 10,000 rpm, 4 °C for 30 min, the supernatant of pH adjusted cell lysate was loaded onto an XK 50/30 column (GE Healthcare) packed with 400 ml of Capto S ImpAct resin. Binding VLPs were eluted with 20 mM sodium acetate buffer containing 500 mM NaCl. The precipitate of the pH adjusted cell lysate was redissolved in an equal volume of 20 mM Tris-HCl buffer pH 8.0. By centrifugation, the clarified supernatant was loaded onto an XK 50/30 column packed with 400 ml of Capto Q XP resin. After elution by 20 mM Tris-HCl buffer pH 8.0 plus 500 mM NaCl, fractions were diafiltered with 10 volumes of PBS on ÄKTA flux (GE Healthcare, USA) equipped with a 750 kDa column (11-0005-50, GE healthcare). For further polishing purification of PPV VLPs was performed on the AKTA Purifier 100 (GE Healthcare, USA) equipped with a HiPrep™ 26/60 Sephacryl® S-500 HR column (GE Healthcare). About 4 ml IEX purified sample was injected and eluted with PBS at a rate of 0.5 ml/min. Protein concentration was measured by the BCA Protein Assay Kit (23250, Thermo Fisher Scientific).
**Vaccination of mice with PPV VLPs**

The purified VLPs was diluted with PBS buffer and emulsified with MONTANIDE™ Gel 01 adjuvant (Seppic, Paris, France) at the rate of 10%, giving a final antigen concentration of 240 µg/ml. Fifteen of 6-week old female SPF Balb/c mice were randomly divided into 3 groups (n = 5). The mice were subcutaneously injected with 20 µg, 40 µg PPV VLPs, and 250 µl of PBS as a control, respectively. Blood samples were collected from cheek each week for 49 dpi and then incubated in 37 °C for 1 h. by centrifugation at 3,000 rpm for 4 min, and sera were separated and stored in small aliquots at -20 °C.

**Antibodies detection by enzyme-linked immunosorbsorbent assay (ELISA)**

The 96-well Costar Assay Plate (Corning, NewYork, USA) were coated with the Ni-NTA affinity-purified VP2 protein as previously described [39]. ELISA detection of the titers of anti-PPV IgG in mouse sera was performed as described previously by Duan et al [41].

**Serum hemagglutination inhibition (HI) antibody assay**

The HI antibody titers of serum samples were determined in U-bottom 96-well plates according to the standard method as described previously [42]. Briefly, serum samples were inactivated at 56 °C for 30 min. Before the test, non-specific inhibitors of hemagglutinin in the serum samples were removed by treating with 25% kaolin and 3% porcine erythrocytes. The treated serum was serially diluted 1:4 with PBS buffer, and each 25 µl of serum diluent was mixed with an equal volume of 40 µg/ml purified PPV VLPs. The plate was incubated for 1 h at 37 °C, and then 50 µL of 1% porcine erythrocyte was added per well. Finally, the plates were incubated at room temperature for 40 min to calculate the HI titer, the reciprocal of the highest dilution inhibited the hemagglutinin completely.

**Spleen lymphocyte proliferation and Cytokine detection assay**

At 42 dpi, three mice from the groups injected with 20 µg PPV VLPs and PBS were euthanized to separate the spleen lymphocytes with a mouse lymphocyte separation medium (Dakewe, Beijing, China). The separated spleen cells were cultivated in 96-well plates containing 100 µl of 1640 culture medium (Thermo Fisher Scientific, Illinois, USA) at a concentration of 3 × 10^6 cells/ml. After adding 0.2 µg Concanavalin A (Sigma, MO, USA), the plates were incubated at 37 °C for 48 h. The proliferative responses were detected using the Cell Titer 96® AQueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA), and the stimulation index (SI) was calculated as the ratio of the stimulated sample divided to unstimulated control at OD_{490 nm}. Cytokines secreted by the spleen cells were measured using the Cytometric Bead Array (CBA) Mouse Th1/Th2 Cytokine Kit (Becton Dickinson Biosciences, San Jose, CA, USA).

**Results**

**Expression of PPV VP2 in *K. marxianus***
The VP2 gene of the Kresse strain was used to express in *K. marxianus*, since this PPV strain displayed an increased virulence compared with the other virulent strains and kill immunocompetent fetuses[12]. The native VP2 coding sequence was redesigned for expression in *K. marxianus* according to the codon usage bias of *K. marxianus*, and inserted directly upstream of the *K. marxianus* inulinase promoter of the pUKDN115 vector (Fig. 1a). The resulting vector pUKDN115-VP2 was transformed into *K. marxianus* Fim-1 *ura3Δ* strain, generating the recombinant strain KM-PPV-VP2. For detection of the expression of VP2 protein, the KM-PPV-VP2 strain was cultured in YD medium for 72 h and cell lysates were subjected to SDS-PAGE and Western blotting respectively. Compared with Fim-1 *ura3Δ* harbouring empty pUKDN115 vector the KM-PPV-VP2 cell lysate contained a 65 kDa band. This band was consistent with the theoretical molecular weight of VP2 protein (Fig. 1c) and further confirmed by Western Blotting with anti-PPV VP2 polyclonal antibody (Fig. 1d).

Previously studies showed that the recombinant VP2 expressed in insect, eukaryotic or prokaryotic cells can form virus-like particles (VLPs)[23, 25–28]. To test whether the VP2 protein was spontaneously assembled into VLPs in *K. marxianus*, the cell lysates of KM-PPV-VP2 was scanned by TEM and results demonstrated the VLPs was formed in *K. marxianus*, with a diameter of approximately 20 nm (Fig. 1b).

**High production of PPV VLPs in fed-batch fermentation**

Scale-up production of VP2 protein was conducted in a 5 L reactor using a defined chemical medium. Throughout the fermentation, glucose was fed at a limited rate to maintain the dissolved oxygen above 10% and achieve a high cell density of the KM-PPV-VP2 strain. After 48 hour fermentation, the cell density (OD$_{600}$ nm) reached 560, with a dry biomass more than 120 g/L (Fig. 2a). At certain time points, cells were disrupted to detect the VP2 protein expression by SDS-PAGE. As shown in Fig. 2b, the VP2 began to accumulate intracellularly at 18 h, and the yield at 48 hours, quantified via SE-HPLC using the purified VLPs as a standard, was approximately 2.5 g/L.

**Screening IEX media to capture the PPV VLPs from cell lysates**

To purify the PPV VLPs by ion-exchange chromatography, pH effects on its solubility was investigated before the IEX media screening. The soluble PPV VLPs in disruption process of KM-PPV cells by high-pressure homogenization was analyzed with three buffers ranging from pH 4.0 to 8.0. As shown in Fig. 3a, the PPV VLPs protein were found in the precipitate when using 20 mM acetate buffer pH4.0, but it was soluble in both PBS buffer pH7.4 and 20 mM Tris-HCl buffer pH8.0 (Fig. 3a). Therefore, to obtain soluble PPV VLPs and facilitate downstream purification steps, PBS buffer pH 7.4 was used to disrupt the KM-PPV cells. Subsequently, the KM-PPV cell lysates disrupted in PBS buffer pH 7.4 were pH adjusted to 5.3, 4.8, 4.3, and 4.0 with 100 mM acetic acid respectively. SDS-PAGE results indicated low pH decreased the solubility of PPV VLPs significantly, with approximately 50% remained in precipitate at pH4.0 (Fig. 3b).

Besides pH values, twelve cation or anion resins were also investigated for the potential in the purification of PPV VLPs (Table 1). The experimental results were shown in Fig. 4. Among the cation resins, the Capto
S, Nuvia S, and POROS HS resins showed the best performances to capture the PPV VLPs, with few observed in the flowthrough fractions, but the SP HP, Nuvia cPrime, SP Bestarose FF, Capto MMC, Capto SP ImpRes, and Capto Q XP resins shared relatively weak affinities. For the cation resins CMFF and the anion resins, Capto Q, and Q Bestarose FF, most PPV VLPs were observed in the flowthroughs, hence, these resins were unsuitable for the ion-exchange chromatography. Thus, we choose the Capto S ImpAct resin for cation exchange chromatography due to its high binding capacity and good selectivity.

The supernatants with different pHs described above were assessed for the binding affinity of the strong cation resin Capto S ImpAct for the PPV VLPs. These data are presented in Fig. 5. Overall PPV VLPs were bound to the Capto S ImpAct resin at pHs range of 4.0 to 5.3, but the impurities at pH 4.0 were relatively less compared to other pHs.

**Purification of PPV VLPs**

Based on the pH effects and resin screen findings, two strategies, including the IEX chromatography and Sephacryl® S-500 gel filtration, were set out to purify the PPV VLPs expressed by K. marxianus. To facilitate the cation exchange purification of the PPV VLPs, the KM-PPV-VP2 cell lysates were adjusted to pH 4.0 and the supernatant was separated for the Capto S ImpAct resin purification. About 250 mL elution fraction containing 2.73 mg/mL PPV VLPs antigen were obtained from 1 L fed-batch fermentation culture (Fig. 6a). The purity of PPV VLPs in the IEX elution fraction reached about 64% that was detected by an HPLC method. Thereafter, elution collection was subject to a polishing step, Sephacryl® S-500 gel filtration, to remove trace impurities. After two-step purification, the purity of PPV VLPs reached above 95%, with the recovery of the PPV VLPs is 30%. To confirm the conformation of the purified PPV VLPs, samples collected at the polishing step were examined by TEM (Fig. 7b), and the result showed that the purified VLPs remained its original shape.

However, the pH adjustment KM-PPV-VP2 cell lysates resulted that approximately half of PPV VLPs was remained in the precipitate. To recover this part of antigen, we introduced an anion exchange purification process using Capto Q XP resin. An equal volume of 20 mM Tris-HCl buffer pH8.0 to the cell lysates was used to dissolve the PPV VLPs in the precipitate. After centrifugation again, the supernatant was loaded onto an XK 50/30 column packaged Capto Q XP resin. As a result, most of PPV VLPs were captured by the anion resins, but the purity of elution analyzed by HPLC was lower than 10% (Fig. 7). HPLC analysis also revealed the main impurities were small molecules, whose molecular weights were far smaller than PPV VLPs. Thus, the elution of anion exchange purification was diafiltrated by a crow-flow mode with a 750 kDa column, and the purity of PPV VLPs reached higher than 95%(Fig. 7). By the combination of anion and cation exchange chromatography, the total recovery of PPV VLPs increased to more than 60%.

**Antibody response in the mice**

To investigate the immunogenicity of recombinant PPV VLPs prepared by K. marxianus, different doses of the purified PPV VLPs antigen emulsified with MONTANIDE™ Gel 01 adjuvant were subcutaneously injected into SPF Balb/c mice. After 14 dpi, mice sera were separated to measure the PPV specific antibodies by ELISA assay every 7 days. As shown in Fig. 8a, PPV-specific antibody was detected in the
vaccinated mice sera after 14 dpi. In both immunized groups, the antibody levels increased with the days post-immunization. However, the PPV-specific antibody in mice immunized 40 µg antigen was significantly higher than those of 20 µg, indicating that mice immunized with 40 µg of PPV VLPs could effectively produce high-level IgG antibodies.

In the hemagglutination assays, the PPV VLPs related to Kresse strain showed strong hemagglutination activity on both porcine and guinea pig erythrocytes. The hemagglutination inhibition activities of antibody in immunized mice sera were then conducted with 40 µg/ml purified VLPs and serially diluted porcine/guinea pig erythrocytes. After just immunized for 14 dpi, the 2log titers of HI reached around 6 for the PPV specific antibodies, while the mice sera injected with PBS could not inhibit the aggregation of PPV VLPs on pig blood cells (Fig. 8b). After 28 dpi, HI titers in nearly all mice rose to values about 8.

**Spleen lymphocyte proliferation and cytokine detection**

The spleen lymphocytes isolated from mice after 42 dpi showed promptly response to conA stimulation in vitro proliferation and consequently giving a higher SI than the negative control group (Fig. 8c), indicating that PPV VLPs act as strong immunogen to provoke the cellular immune responses. At the same time, to investigate the induction of humoral immunity spleen lymphocytes we opted to detect the levels of cytokines IL-2, IL-4, IL-5 and TNF–α in spleen lymphocytes cultures after stimulation for 72 h. On average, all the tested cytokines, including IL-2, IL-4, IL-5 and TNF, were higher in immunized spleen lymphocyte cultures than those of PBS groups(Fig. 8d). These results suggested that PPV VLPs produced by *K. marxianus* could induce humoral immunity as well.

**Discussion**

The commercially available PPV vaccines are inactivated whole-virus and live-attenuated vaccines. Irrespective of safety risks such as incomplete inactivation and reversion to virulence, albeit small probability, there are intrinsic disadvantages involved with the administration of inactivated or attenuated vaccines that it hampered to discriminate the differentiation of infected from vaccinated animals (DIVA) in seropositive animals[43]. Intrigued by the inherently safe vaccine and useful epitope carrier nanoparticles[44], we developed *K. marxianus* as a new platform for the production of PPV VLPs. Attribute to high growth rate and facile high biomass, the recombinant *K. marxianus* strain produced 2.5 g/L PPV VLPs after just a 48 h fermentation. As a comparison, the expression level of VLPs in *Pichia pastoris*, *E. coli*, *S. cerevisiae* is 595.76 mg/L [28], 15 mg/L [38], and 8–9 mg/L respectively [45]. Moreover, *K. marxianus* has a notable advantage over other strains on the production of VLPs that there is no additional inducement during its fermentation since glucose serves as the carbon resources to support cell growth as well as an inducer for the inulinase promoter to obtain high expression of heterologous proteins [29].

As to yeast cells, there is no additional treatment to remove the endotoxin, but other downstream operations, such as disrupting the rigid wall of the yeast cells to release VLPs, clarification of cell lysates, and purification processes, are the main factors that significantly increased the production costs overall.
In previous studies, the PPV VLPs were commonly purified by sucrose or cesium chloride gradient centrifugation, which are not conducive to large-scale production [25, 47]. Aiming to create a simple and low-cost purification process, we assessed the ion-exchange chromatography for purification of PPV VLPs comprehensively. Due to the low isoelectric point, yeast cell lysates should be acidified to cationized PPV VLPs and to facilitate resin binding. Simultaneously, the cell lysate turbidity was reduced and most of the impurities were settled. However, about 50% of VLPs were precipitated during this process. As an alternative strategy to elevate the recovery, we dissolved the precipitated VLPs by 20 mM Tris-HCl buffer pH 8.0, in which PPV VLPs are highly soluble, and PPV VLPs were then purified by anion exchange chromatography coupled with a cross-flow diafiltration. Due to first round of clarification, most of the negatively charged competitors including nucleic acid, polysaccharides, and small molecules in the yeast cell lysate were removed, which can decrease the binding efficiency of Capto Q XP resins with PPV VLPs (Figs. 4 and 6). The purified PPV VLPs from precipitate gave a purity of 95% that was comparable to the gel-filtration purification. By this means, less than 40% of PPV VLPs were lost after these purification processes. Our finding provides a new guideline for PPV VLPs purification from intracellular yeast cells.

Currently, most of the inactivated or attenuated PPV vaccines originated from the Cluster A strain NADL-2. The Kresse strain isolated in the USA in 1985 is the most serious type of PPV-1, isolated in the USA in 1985, that can kill the immunocompetent fetuses [13]. These two strains have a high identity both in sequence and genomic organization but have quite different pathogenicities [18]. Compared with the NADL-2 strain, three substitutions in VP1/VP2 protein structurally located on the surface of the capsid that may contribute to the highly virulent of PPV Kresse strain [48]. A recent study revealed the genotypes of porcine paroviruses isolated in South Korea in 2018 was more identical to the ancient Kresse strain. This re-evolution to its original strains may increase the epidemiologic risk of PPV [49]. Additionally, the spread of PPV-27a, a unique virulent cluster D strain that has a unique immunological feature, raised the concerns about the efficacy of the currently used inactivated and attenuated vaccines. A recent report revealed that a “Kresse-like” K22 PPV strain-based vaccine showed stronger protection than the commercial NADL-2 based vaccines in a PPV-27a strain challenge [50]. Enlightened by these, we set out to express the VP2 capsid protein of Kresse strain to form VLPs in our K. marxianus expression platform. Another, recombinant VLPs can be easy changed to desired genotype based on a well-established expression platform in a shorter time.

After immunization of mice, high titers of specific IgG antibodies were induced at doses of 20–40 µg per mouse PPV VLPs IgG. Sera of immunized showed high hemagglutination inhibition, a serologic index to the specific antibody of a virus, against either porcine or guinea pig erythrocytes [51]. It seems VLPs produced in K. marxianus maintain the natural morphology of pathogenic virus. Previous studies had observed that PPV virus can agglutinate chicken, guinea pig, mouse, human, monkey, rat, and cat erythrocytes [15], but the cause of hemagglutination to swine erythrocyte by the Kresse strain VLPs remains to be tested. Except for the antibodies, the mouse spleen cell growth experiment further confirmed that this vaccine has a good immune effect. In the cytokine detection test, this vaccine was shown not only to cause humoral immunity but also to induce cellular immunity. These results suggest the PPV VLPs produced in this study can be used to protect swine from porcine parovirus diseases.
Abbreviations

PPV: porcine parvovirus; SMEDI: stillbirth, mummification, embryonic death, and infertility; ORFs: Open Reading Frames; VLPs: virus-like particles; MCS: multiple cloning sites; IPTG: isopropyl-β-d-thiogalactopyranoside; dpi: days post-immunization; PTA: phosphotungstic acid; IEX: ion exchange chromatography; DIVA: differentiation of infected from vaccinated animals.

Declarations

Authors’ contributions

DY, LC and JZ designed and performed the experiments, analyzed the data and wrote the manuscript. HL and JZ devised the project and supervised the work. JD performed the electron microscopy. YY constructed the plasmid and performed the codon optimization of the VP2 gene. All authors provided critical feedback and helped shape the research, analysis and manuscript. All authors read and approved the final manuscript.

Author details

1 State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, 2005 Songhu Road, Shanghai, China, 200433

2 Shanghai Engineering Research Center of Industrial Microorganisms, 2005 Songhu Road, Shanghai, China, 200433

3 Shanghai Collaborative Innovation Center for Biomanufacturing (SCICB), East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China

Conflict of Interest

The authors declare that they have no competing interests.

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Consent for publication

Not applicable

Ethical approval
All mice experimental procedures were approved by the Animal Experiment Committee of Fudan University. All applicable international, national, and institutional guidelines for the care and use of animals were strictly followed.

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**Figures**
Figure 1

Expression and characterization of the PPV VP2 protein in K. marxianus. a Illustration of the recombinant expression plasmid pUKDN125-PPV VP2. b TEM scan of the assembled PPV VLPs in K. marxianus intracellularly. SDS-PAGE(c) and Western Blot (b) assays of the lysates of the recombinant strain after shaking for 66h. Lane M: PageRuler Prestained Protein Ladder; Lane 1: the lysate of Fim-1 Δura3 transformed with the empty plasmid pUKDN125; Lane 2: the lysate of KM-PPV-VP2.
Figure 2

High cell-density fermentation of the KM-PPV-VP2 strain. a The growth curve of KM-PPV-VP2 in a 5L fermentor. b SDS-PAGE of the VP2 protein expression in KM-PPV-VP2 cell lysates at the indicated time. Yeast cells were collected at an interval of 6h, and cell cultures were then diluted with PBS at a rate of 1:10. After disruption by high-pressure homogenization, each of 15μl cell lysates was loaded to analyze by SDS-PAGE analysis.

Figure 3

Effects of pH values on the solubility of PPV VLPs. a Detections of PPV VLPs released during cell disruption under the given pH values. b The solubility of the PPV VLPs after adjusting the KM-PPV-VP2 yeast cell lysates, disrupted with PBS buffer pH7.4, to pH values ranging from 5.3 to 4.0. Lane L: cell lysates; Lane F: flow-through fractions; Lane E: elution fractions.
Figure 4

Screening the IEX chromatography media for purification of PPV VLPs. Cation exchange resins screen was performed using the KM-PPV-VP2 cell lysates that were disrupted with PBS buffer pH 7.4 PBS and acidified to pH 4.0 with acetic acid. For anion exchange resins, samples were prepared by using 20 mM Tris-HCl as the buffer to suspend and disrupt the KM-PPV-VP2 cells. Lane F: flow-through fractions; Lane E: elution fractions.

Figure 5

pH value effects on the cation exchange purification of PPV VLPs with Capto S ImpAct resin. The KM-PPV-VP2 cell lysates disrupted with PBS buffer pH 7.4 PBS were adjusted to pH 5.3-4.0 with acetic acid.
After centrifugation at 12000rpm for 15min, 2ml supernatants were loaded onto Biorad Poly-Prep chromatography columns packaged with 1m Capto S ImpAct resin and eluted PBS pH7.4 containing 0.5M NaCl. Lane F: flow-through fractions; Lane E: elution fractions.

SDS-PAGE (a) and HPLC (b) analyses of the purified PPV VLPs remained in the pH adjusted precipitate. The precipitate was dissolved by 20mM Tris-HCl buffer. After clarification by centrifugation, supernatants were loaded onto a Capto Q XP column and PPV VLPs were eluted with 20mM Tris-HCl pH7.4 containing 0.5M NaCl. Elution fractions were ultrafiltrated with a 750kDa column in a tangential flow filtration. RS: the redissolved supernatant; F: flow through fractions; E: elution fractions; U: the ultrafiltrated VLPs.
Scale-up purification of the PPV VLPs. SDS-PAGE analysis of the purified PPV VLPs during IEX and Gel filtration. Lane M, PageRuler Prestained Protein Ladder; Lane 1, cell lysate prepared by PBS pH 7.4; lane 2, the supernatant of cell lysate adjusted to pH 4.0; Lane 3, Elution fraction of Capto S ImpAct exchange chromatography; lane, elution of Sephacryl® S-500 Gel filtration. b TEM scan of the Gel filtration purified PPV VLPs. Bar, 100nm. HPLC analysis of the supernatant pH adjusted cell lysate (c), IEX purified PPV VLPs (d), and gel filtration purified PPV VLPs (e).
Figure 8

Immunogenicity assays of the PPV VLPs vaccine. 

a The titers of anti-PPV IgG in mouse sera immunized with PPV VLPs. Mice in each group (n=5) were immunized with 20 μg and 40 μg of the PPV VLP vaccine, and PBS. 

b HI antibody titers of mouse immunized with 20 μg VLPs. Bars represent mean ± SEM (n=5). 

c Stimulation index of the immunized mouse lymphocytes. At 56 dpi, spleen lymphocytes of the immunized mice and PBS group were isolated and then were stimulated in triplicate with conA. 

d The levels of the cytokines secreted by lymphocytes at 56 dpi.