Virus Research 302 (2021) 198467

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Oral administration of *Saccharomyces cerevisiae* displaying VP28-VP24 confers protection against white spot syndrome virus in shrimp

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ARTICLE INFO

Keywords:
WSSV
EBY100/pYD1-VP28-VP24
Oral administration
Protection

ABSTRACT

White spot syndrome virus (WSSV) is the major pathogen that leads to severe mortalities in cultured shrimp worldwide. The envelope proteins VP28 and VP24 of WSSV are considered potential vaccine candidate antigens. In this study, we utilized a *Saccharomyces cerevisiae* (*S. cerevisiae*) surface display system to demonstrate the feasibility of this platform for developing a vaccine candidate against WSSV. EBY100/pYD1-VP28-VP24 was generated, and the fusion protein VP28-VP24 was present on the surface of *S. cerevisiae*. *Penaeus vannamei* (*P. vannamei*) was used as an animal model. Oral administration of EBY100/pYD1-VP28-VP24 could induce significant activities of immune-related enzymes such as superoxide dismutase (SOD) and phenoloxidase (PO). Importantly, WSSV challenge indicated that oral administration of EBY100/pYD1-VP28-VP24 could confer 100% protection with a corresponding decrease in the viral load. The collective results strongly highlight the potential of a *S. cerevisiae*-based oral vaccine as an efficient control strategy for combating WSSV infection in shrimp aquaculture.

1. Introduction

White spot syndrome virus (WSSV) is one of the most threatening infectious pathogens to shrimp aquaculture worldwide and causes significant losses in shrimp yields (Lightner and Redman, 1998). WSSV is classified into a new virus family, *Nimaviridae*, under the genus *Whispovirus* (www.ncbi.nih.gov/ICTVdb/Ictv/index.htm) and infects a broad range of decapod crustaceans, including shrimp, crab, lobster and crayfish (Sánchez-Paz, 2010). Additionally, WSSV-infected shrimp succumb to death within 2 to 5 days of the onset of clinical symptoms (Syed and Kwang, 2015). To date, approximately 30 proteins are located in the WSSV envelope (Li et al., 2007; Tsai et al., 2004, Tsai et al., 2006; Xie et al., 2006), of which VP28, VP26, VP24, VP19 and VP15 are five known major structural proteins and serve as potential anti-WSSV agents. Although the interaction mechanism between WSSV and shrimp cells is not well defined, increasing evidence indicates that VP28 and VP24 are involved in viral infection, assembly and budding processes (Li et al., 2015) and are used as potential targets for the development of WSSV vaccine candidates.

It is well recognized that invertebrates lack true adaptive immunity and depend solely on innate defense systems to protect against pathogens (Hoffmann and Reichhart, 2002). However, the existence of a quasi-immune response provides an alternative strategy for vaccinating shrimp against WSSV infection (Venegas et al., 2000). Different anti-WSSV vaccination strategies, including inactivated WSSV vaccines (Namikoshi et al., 2004; Singh et al., 2005), subunit vaccines (Jha et al., 2006; Nguyen et al., 2014; Taju et al., 2018; Thomas et al., 2014; Witteveldt et al., 2004), DNA vaccines (Ning et al., 2009; Rajeshkumar et al., 2009; Rout et al., 2007) and dsRNA vaccines (Kim et al., 2007; Mejía-Ruíz et al., 2011; Nilsen et al., 2017; Rattanarojpong et al., 2016; Robalino et al., 2004; Sarathi et al., 2008; Yang et al., 2020). Viral vectored vaccines (Rattanarojpong et al., 2016; Syed and Kwang, 2011, 2015), have been explored to protect shrimp. In most WSSV vaccines, VP28 is the most important target for vaccine design since it is a major envelope protein of WSSV and acts as an attachment protein, binding the virus to shrimp cells and helping it enter the cytoplasm (Yi et al., 2004). Furthermore, VP24 is another attractive target for the development of WSSV vaccines, since VP24 is the only protein amgong the infectome proteins confirmed to be able to interact with polymeric immunoglobulin receptor-like protein (MjpIgR) of host, which can mediate WSSV infection (Niu et al., 2019). However, there has been no attempt to investigate the protective efficacy induced by the fusion antigen protein VP28-VP24 by oral administration in shrimp.

The *S. cerevisiae* surface display system has been widely engineered

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https://doi.org/10.1016/j.virusres.2021.198467
Received 17 February 2021; Received in revised form 22 May 2021; Accepted 24 May 2021
Available online 29 May 2021

to express biocatalysts, functional proteins, antibodies, and combinatorial protein libraries (Kondo and Ueda, 2004; Kuroda and Ueda, 2011; Tanaka et al., 2012). It is also evident that the *S. cerevisiae* surface display system allows the development of oral or edible vaccines, such as bacterial oral vaccines (Shibasaki and Ueda 2016; Cen et al., 2021) and viral oral vaccines (Patterson et al., 2012; Patterson et al., 2015; Lei et al., 2020). Furthermore, genetically engineered *Pichia pastoris* (*P. pastoris*) can display mutated *Penaeus monodon* Rab7 (mPmRab7) and partial VP28 (pVP28) modified by adding the C-terminal half of α-agglutinin as an N-terminal tag (Ananphongmanee et al., 2015). Because *S. cerevisiae* does not contain selective drug resistance markers, viral antigens present on the surface of *S. cerevisiae* are an attractive approach to developing oral or edible vaccines and promote oral vaccine development and application.

In the present study, a *S. cerevisiae*-based vaccine (EBY100/pYD1-VP28-VP24) was generated in which the fusion protein VP28-VP24 was co-displayed on the surface of *S. cerevisiae*, and its protective efficacy was evaluated in *Penaeus vannamei* by oral administration. Consequently, EBY100/pYD1-VP28-VP24 represents a promising preventative approach for shrimp culture against WSSV infection that can be used in the field.

2. Materials and methods

2.1. Molecular construction of EBY100/pYD1-VP28-VP24

The VP28 gene (GenBank accession No. FJ756456.1; 609 bp) was amplified by PCR using specific primers: F-1: 5′-CTAGCTAGCGATCTTTCTTTCACTCTTT-3′ and R-1 5′-AGAACCACCACCACCAGAACCACCACCACCCTCGGTCTCAGTGCCAGAGTA-3′, which contain the *Nhe*I restriction site sequence in the F-1 primer and a Gly/Ser linker sequence (45 bp) in the R-1 primer. Meanwhile, the VP24 gene (GenBank accession No. DQ681068.1; 627 bp) was amplified by PCR based on the following primers: F-2: 5′-GGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTCACATGTGGGGGGTTT-3′ and R-2: 5′-CCGGAATTCTTATTTTTCCCCAACCTT-3′, which include the Gly/Ser linker sequence (45 bp) in the F-2 primer and the *Eco*RI restriction site sequence in the R-2 primer. The VP28 gene and VP24 gene were used as PCR templates after ligation by the T4 DNA ligase, and the F-1 and R-2 primers were used as PCR primers. The resulting VP28-VP24 gene was cloned in frame into the pYD1 plasmid with the endogenous Aga2p signal peptide sequence and then transformed into competent *E. coli* DH5α cells. Further, pYD1-VP28-VP24 was electroporated into competent *S. cerevisiae* EBY100 cells after being linearized. EBY100/pYD1-VP28-VP24 was screened on selective minimal dextrose plates (0.67% yeast nitrogen base (YNB) without amino acids, 2% glucose, 0.01% leucine, 2% agar, and 1 M sorbitol), cultured in YNB-CAA-Glu (0.67% YNB, 0.5% casamino acids, 2% glucose) media and induced in YNB-CAA-Gal (0.67% YNB, 0.5% casamino acids, 2% galactose) media. The EBY100/pYD1 served as a negative control for all subsequent tests.

2.2. Western blot analysis

EBY100/pYD1-VP28-VP24 pellets cells equal to an OD_{600nm} of 2 (1 $OD_{600} \approx 10^7$ cells) were mixed at a 1:1 ratio with sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis sample buffer containing 20% β-mercaptoethanol and heated to 98 °C for 10 min. SDS-PAGE was performed on 12% precast gels (Bio-Rad, CA, USA). Subsequently, proteins were transferred to 0.45 μm nitrocellulose membranes (Bio-Rad, CA, USA). The membranes were blocked for 2 h at room temperature in PBS with 5% nonfat milk and 0.05% Tween 20. The membrane was incubated with a polyclonal rabbit anti-VP28 antibody (1:500) or anti-VP24 antibody (1:500) (Sangon, Shanghai, China) as the primary antibody. After horseradish peroxidase (HRP)-labeled secondary antibody staining using anti-rabbit IgG antibody (1:5000) (R&D Systems, USA), the blots were imaged using the West Pico Chemiluminescent Substrate (Bio-Rad, USA) and a ChemiDoc XRS System (Bio-Rad, CA, USA).

2.3. Immunofluorescence assay and flow cytometric analysis

To detect the VP28-VP24 display on the surface of *S. cerevisiae* EBY100, 2 OD_{600nm} of EBY100/pYD1-VP28-VP24 pellets were collected at 72 h post-induction and incubated with a polyclonal rabbit anti-VP28 antibody (1:500) or anti-VP24 antibody (1:500) at 4 °C for 1 h. After washing with sterile PBS, the pellets were incubated with FITC-conjugated goat anti-rabbit IgG antibody (1:5000) at 4 °C for 45 min. The FITC-labeled pellets were resuspended in 505 μL of sterile PBS. Five microliters of the FITC-labeled pellets was examined under an inverted phase contrast fluorescence microscope (Leika, Germany), and the remaining 500 μL was subjected to flow cytometric analysis using a BD FACSAira III (BD Bioscience, San Jose, CA, USA).

2.4. Quantification of VP28-VP24 expressed on the surface of S. cerevisiae

Quantification of EBY100/pYD1-VP28-VP24 expressing the VP28-VP24 protein was measured by enzyme-link immunosorbent assay (ELISA). Briefly, 10 OD_{600nm}/mL EBY100/pYD1-VP28-VP24 pellets were resuspended in 100 μL of a polyclonal rabbit anti-VP28 antibody (GeneTex, USA) or a polyclonal rabbit anti-VP24 antibody (Cusabio, China) (0, 10, 15, 20, 25, 30, 35, 40, 129 45, 50, 55, or 60 μg/mL) in PBS containing 2% BSA. After incubation at 4 °C for 2 h, 1 mg/mL horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody was added and incubated at room temperature for 1 h. Last, 100 μL of the HRP substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was reacted with the cells for 25 min in the dark, and 100 μL of 2 mol/L H_2SO_4 was added to stop the reaction. The OD_{450nm} value of the supernatant was measured using a microplate reader (BioTek, USA). EBY100/pYD1 was used as a negative control.

2.5. Competitive ELISA to verify specific binding of EBY100/pYD1-VP28-VP24

Sandwich ELISA was performed by using 2 OD_{600nm} of EBY100/pYD1-VP28-VP24 expressing VP28-VP24 protein and bound to purified PmRab7 (0, 10^1, 10^2, 10^3, 5×10^3, 10^4, 2×10^4 and 5×10^4 ng/mL) (Sino Biological, China), which PmRab7 is a VP28-binding protein involved in WSSV infection in shrimp (Sritunyalucksana et al., 2006). Then, cells were washed three times with PBST and further incubated with polyclonal rabbit PmRab7 primary antibody at room temperature for 1 h. Subsequent steps were the same as those described for the ELISA assay above.

2.6. Vaccine preparation, shrimps and oral administration

EBY100/pYD1-VP28-VP24 cells were collected at 72 h post-induction and then inactivated at 60 °C for 1 h. The concentration was adjusted to 0.5 OD_{600nm}/μL.

Inactivated EBY100/pYD1-VP28-VP24 was mixed with a commercial shrimp diet (feed pellets) and then further coated with cod liver oil. The optimized mixing ratio of EBY100/pYD1-VP28-VP24 and feed pellets was 1:40 (μg/μg) (Thomas et al., 2014). The ratio of cod liver oil to feed pellets was 20 μL/g. The same volume of EBY100/pYD1 or PBS was coated and used as a control. The feed pellets were then stocked for a maximum of 6 days at 4 °C until further use.

P. vannamei (approximately 0.3 grams) was supplied by Fujian Yuhai Aquatic Production Co., Ltd. (China) and divided into three experimental groups ($n = 110$ per group). Each group was housed in two separate 200 L fiber reinforced plastic (FRP) tanks under constant aeration, and the seawater quality was maintained through 10% water

exchange daily. Shrimps were acclimatized in the laboratory for 7 days under the following conditions: 28 ± 1 °C, pH 8.4, DO ≥ 4 mg/L, and 30‰ salinity. Each experimental tank was closed with FRP sheets to avoid cross contamination through aerosols.

For oral administration, shrimps were orally administered with EBY100/pYD1-VP28-VP24-coated feed pellets at a rate of 5% of the body weight for 7 consecutive days. The same volume of EBY100/pYD1-coated feed pellets or coated PBS feed pellets were used as a control.

All animal studies complied with the Guidelines for the Use and Care of Experimental Animals and were approved by the Animal Committee of the Institute of Southwest Jiaotong University.

2.7. Superoxide dismutase (SOD) and phenoloxidase (PO) activities

The hepatopancreas of shrimp (n = 10 per group) were used to measure the activity of SOD and PO enzyme activities at days 2, 4 and 6 after the last oral administration using the method described previously (Chen et al., 2017). In brief, approximately 50 mg of hepatopancreas was collected and homogenized with 400 μL of sterile phosphate buffer. The supernatant was isolated after centrifugation at 8000 × g for 10 min at 4 °C and then determined using a commercial assay kit (R&D Systems, USA) by measuring the enzyme activities at 490 nm, Protein concentration in samples was determined using the BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. Each enzymatic assay was performed in triplicate. The SOD and PO activities were expressed as U/mg protein, respectively.

2.8. Virus challenge

On the eighth day after the final oral administration, the shrimps (n = 100 per group) were immersed in sea water containing a dilution of 1:10,000 WSSV stock solution at a concentration of 2 × 10^7 copies/mL for 2 h and then immersed in fresh seawater without WSSV. The oral administration experiments were repeated three times. The cumulative mortality of shrimp in each group was recorded for 14 days after WSSV challenge. Furthermore, gill tissues from dead and live shrimps were analyzed by immunohistochemistry assay at day 7 post WSSV challenge.

2.9. Quantification of WSSV by real-time PCR

The WSSV viral loads in shrimp were determined by real-time quantitative PCR (RT-qPCR). Briefly, gill tissues were collected at 3, 5 and 7 days post-infection (dpi). Viral DNA was isolated from 50 mg of gill tissue by using a DNA tissue kit (TaKaRa, Japan) according to the manufacturer's instructions. Primers (forward primer: 5′-GATCTTTCTTTCACTCTTT-3′ and reverse primer: 5′-TCGGTCTCAGTGCCAGAGTA-3′) were used to amplify a 609-bp fragment from the VP28 gene of WSSV. The PCR cycle parameters were an initial denaturation at 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, annealing for 30 s, extension for 1 min at 72 °C and a final extension at 72 °C for 10 min. The PCR of each sample was carried out in triplicate. A standard curve was obtained using serial dilutions of plasmid pUC57-vp28 and used to quantify the WSSV genomic copy number. The geometric mean of viral genomic copies per reaction was calculated for each group after setting the results for the negative samples to one copy per sample.

2.10. Statistical analysis

The data are presented as the mean ± standard deviation (SD) from three independent experiments and were analyzed using one-way analysis of variance (ANOVA) with post hoc multiple comparison analysis. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, USA). The Kaplan-Meier method was used to determine the survival rate after the WSSV challenge. $p <$ 0.05 was considered statistically significant.

3. Results

3.1. Construction and characterization of EBY100/pYD1-VP28-VP24

We generated a *S. cerevisiae*-based vaccine in which the fusion gene VP28-VP24 was fused with Aga2 via a Gly-Ser linker, in which Aga2 was the subunit of the a-agglutinin receptor of the yeast surface display plasmid pYD1. Then, Aga2 was bound to Aga1 of yeast through two disulfide bonds to stabilize the surface display of the fusion protein VP28-VP24 (Fig. 1a). Expression of the fusion protein VP28-VP24 was confirmed by Western blot analysis. As expected, a specific band, as shown in Fig. 1b, that corresponds to the size of the fusion protein VP28-VP24, whose molecular weight is approximately 61 kDa and consists of Aga2 (10 kDa), VP28 (27.5 kDa), the Gly-Ser linker (1.5 kDa) and VP24 (22 kDa). In contrast, no positive signal was detected in the EBY100/pYD1 control.

Further, immunofluorescence assays and flow cytometric analysis were used to visualize the expression location of the fusion protein VP28-VP24. Compared with the EBY100/pYD1 control, EBY100/pYD1-VP28-VP24 appeared to be strongly positive by direct labeling with the primary antibody in both the immunofluorescence assay and flow cytometric analysis (Fig. 1c and d, right panel).

Collectively, the results indicate the presence of the fusion protein VP28-VP24 expressed on the surface of yeast.

3.2. Quantification of the surface-expressed the VP28-VP24 protein by ELISA

As shown in Fig. 1e, the optical densities of 10 OD_{600nm}/mL EBY100/pYD1-VP28-VP24 was relatively stable at concentrations of 50 μg/mL for the polyclonal anti-VP28 antibody and polyclonal anti-VP24 antibody, respectively. This result suggested that 1 OD_{600nm} of EBY100/pYD1-VP28-VP24 expressed approximately 5 μg of VP28-VP24 protein.

3.3. Specific binding of EBY100/pYD1-VP28-VP24 confirmed by competitive ELISA in vitro

When 1 OD_{600nm} of EBY100/pYD1-VP28-VP24 expressing 5 μg of VP28-VP24 protein were pre-incubated with various concentrations of PmRab7 before exposure to polyclonal anti-Rab7 antibody, saturation binding was observed between 5 × 10^3 to 5 × 10^4 ng/mL (Fig. 1f). By contrast, EBY100/pYD1 did not bind PmRab7. The results indicated that specificity of the interaction between EBY100/pYD1-VP28-VP24 and its binding partner PmRab7.

3.4. SOD and PO activities induced by EBY100/pYD1-VP28-VP24

The SOD and PO activities were determined in homogenized tissues of shrimp fed EBY100/pYD1-VP28-VP24. As shown in Fig. 2a, the levels of SOD activities achieved at days 4 and 6 were significantly increased and were 67.8 ± 4.6 at day 5 and 56.8 ± 3.7 at day 7 compared with those of shrimp fed PBS or EBY100/pYD1 (the control groups). Similarly, the levels of PO activities were very significant in shrimps orally administered with EBY100/pYD1-VP28-VP24, with PO activities of 2.12 ± 0.22 at day 4 and 1.76 ± 0.066 at day 6. These results strongly demonstrate that shrimp administered EBY100/pYD1-VP28-VP24 orally can induce high levels of SOD and PO activities.

3.5. Protection conferred by EBY100/pYD1-VP28-VP24

On the eighth day after the final administration, the shrimp were challenged with WSSV to determine the protection efficacy. As shown in Fig. 3a, a significantly higher cumulative mortality of 50% was observed in the PBS- or EBY100/pYD1- administered group at day 3 and day 5 and reached 100% at day 7 and day 8 after WSSV challenge, whereas the shrimps orally administered with EBY100/pYD1-VP28-VP24 showed

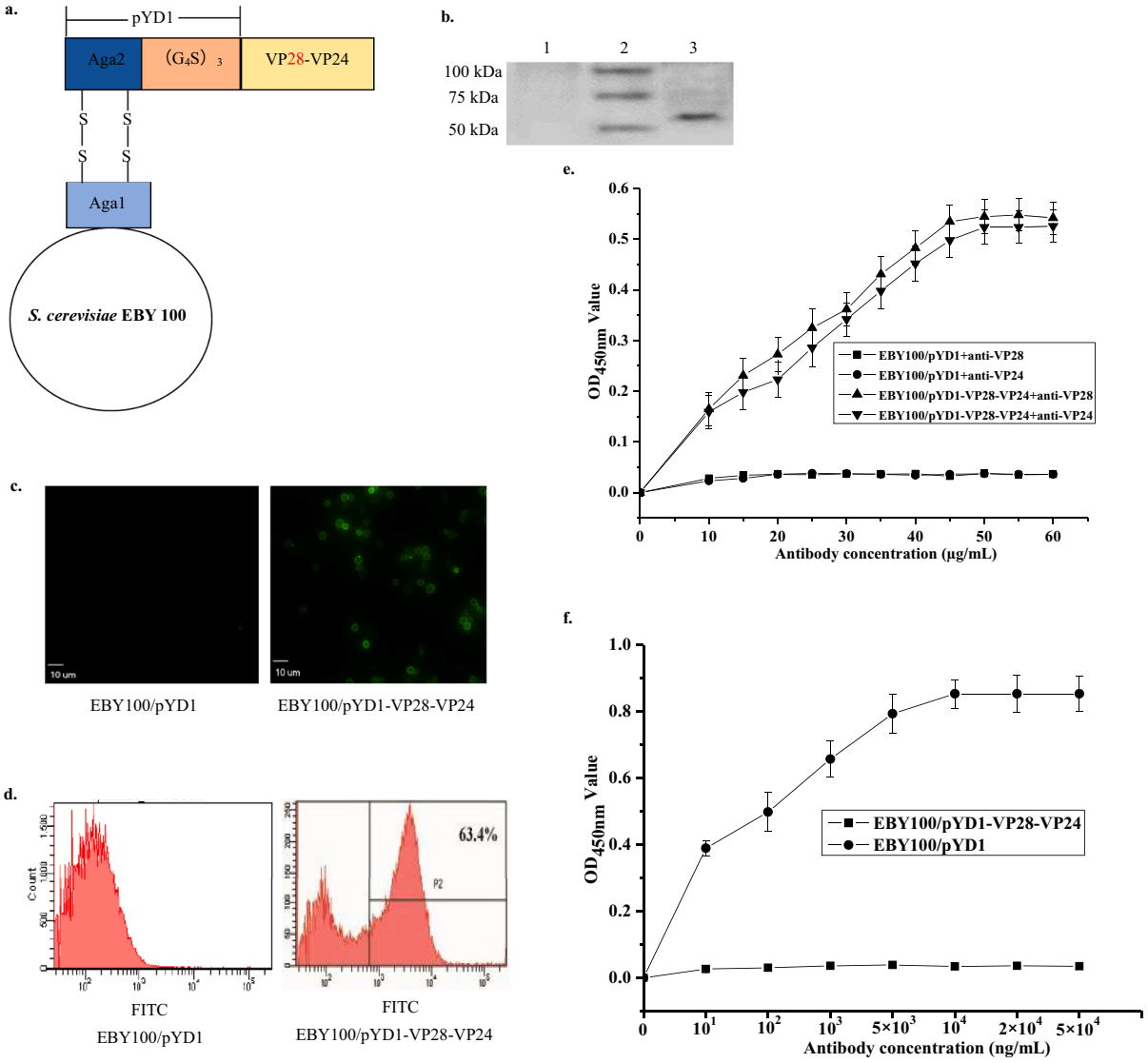

Fig. 1. Schematic diagram and characterization of the fusion protein VP28-VP24 present on the surface of *S. cerevisiae*. **(a)** Schematic model of EBY100/pYD1-VP28-VP24. Aga1 is the yeast a-agglutinin receptor and is then covalently ligated with the secreted Aga2 of pYD1 through two disulfide bonds. A Gly-Ser linker is inserted between Aga2 and the fusion protein VP28-VP24 for stabilization. **(b)** Western blot analysis. Lane 1: EBY100/pYD1; lane 2: Western blot marker (Precision Plus Protein™, Bio-Rad); lane 3: EBY100/pYD1-VP28-VP24. **(c)** Immunofluorescence assay. Negative control EBY100/pYD1 (left) and EBY100/pYD1-VP28-VP24 (right). Magnification: 400 × . **(d)** Flow cytometric analysis. Negative control EBY100/pYD1 (left) and EBY100/pYD1-VP28-VP24 (right). (In total, 10,000 cells were counted). **(e)** Quantification of the surface-expressed the VP28-VP24 protein by ELISA. The values were obtained from three independent experiments. Bar = mean ± SD. (f) Competitive ELISA to verify specific binding of EBY100/pYD1-VP28-VP24. The values were obtained from three independent experiments. Bar = mean ± SD.

100% survival with no mortality throughout the experimental period. Histopathological sections of gill tissue of survived shrimps were with normal nuclei compared with the moribund shrimps (Fig. 3b). These results clearly show that EBY100/pYD1-VP28-VP24 is an effective WSSV oral vaccine candidate.

3.6. Quantification of WSSV load by RT-PCR

Furthermore, WSSV-infected gill tissues from the groups of orally administered shrimps were collected for the quantification of WSSV copy numbers by RT-PCR at different days post-challenge. As shown in Fig. 3c, the WSSV copy numbers in the EBY100/pYD1-VP28-VP24 group were significantly lower than those in the PBS or EBY100/pYD1 group at day 3 post-challenge. The WSSV copy numbers in shrimps orally administered with EBY100/pYD1-VP28-VP24 dropped to 0.338 ± 0.042 copies/mg at day 5 post-challenge, and later, 100% of these shrimps survived (Fig. 3a), whereas the WSSV copy numbers in the PBS and EBY100/pYD1 control groups reached 6.46 ± 0.531 and 6.186 ± 0.0538 copies/mg at day 5 post-challenge, respectively, and all of the shrimps died at day 7 or 8 post-challenge (Fig. 3a).

4. Discussion

WSSV is one of the most infectious pathogens in the shrimp aquaculture industry worldwide, and it has already caused very large economic losses. Despite considerable research efforts on the development of the pathogenic mechanism of WSSV (Li et al., 2015; Zou et al., 2021), there are still no effective vaccines or antiviral agents available for field application. In this study, we demonstrated that an *S. cerevisia*-based oral vaccine candidate, the fusion protein VP28-VP24 of WSSV co-displaying on the surface of *S. cerevisia,* could have the potential in stimulating immunity-related activities. Importantly, 100% protection

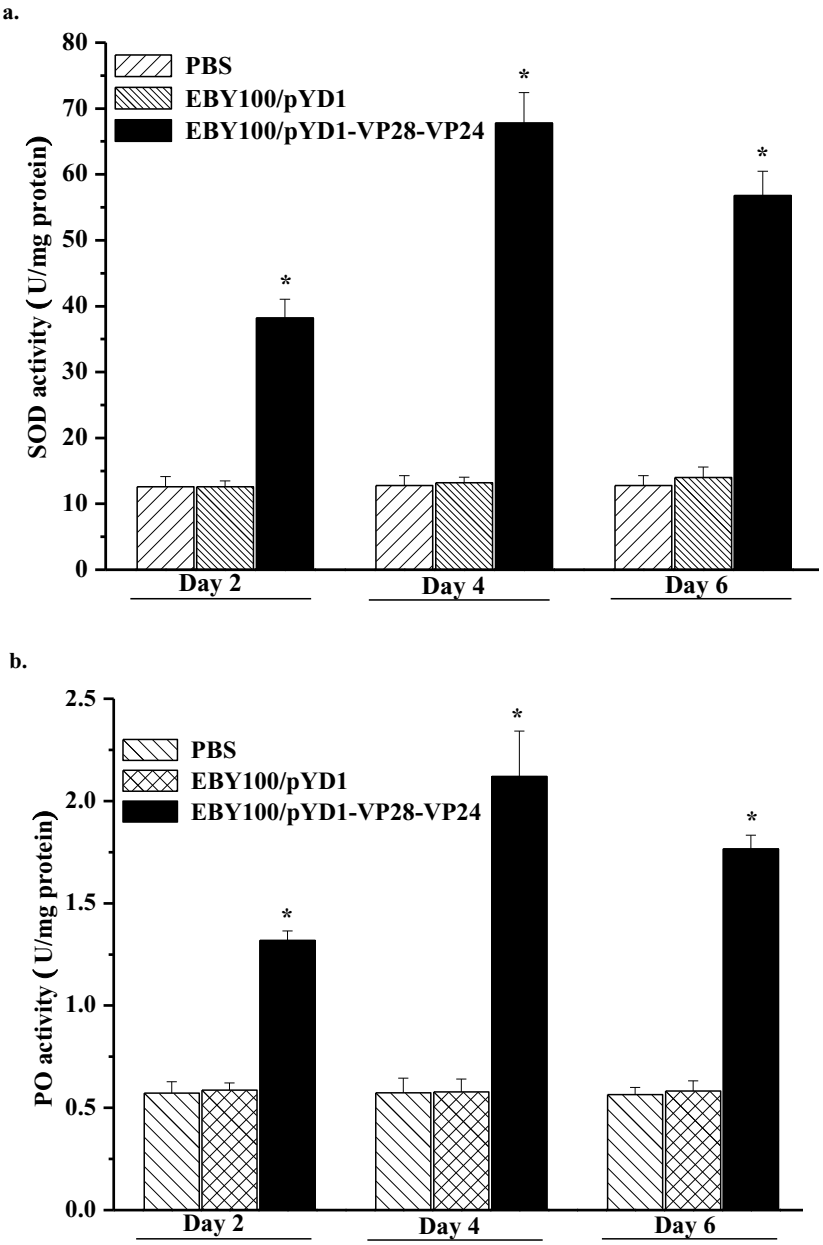

Fig. 2. Immune-related enzymes in *P. vannamei* induced by EBY100/pYD1-VP28-VP24 after the last oral administration at days 1, 4 and 6. **(a)** SOD activity. **(b)** PO activity. The data are shown as the mean ± SD. The asterisks indicate significant differences compared with the PBS- and EBY100/pYD1-administered controls. * $p < 0.05$.

was conferred in *P. vannamei* by oral administration of EBY100/pYD1-VP28-VP24.

The expression of the fusion protein VP28-VP24 in *S. cerevisiae* was analyzed by Western blotting (Fig. 1b), which is a molecular biological method to detect antibody-protein interactions in vitro. Next, immunofluorescence assays and flow cytometric analysis were used to examine the specificity of the immunofluorescence labeling system and correct localization of the fusion protein. Based on these analyses, we demonstrated that the fusion protein VP28-VP24 was present on the surface of *S. cerevisiae* (Fig. 1c, d). Furthermore, quantification of the surface-expressed the VP28-VP24 protein was measured by ELISA (Fig. 1e). Competitive ELISA implied that the structure of expressed VP28-VP24 protein on the surface of *S. cerevisiae* folded correctly and that they retained their ability to bind with partner protein PmRab7 (Fig. 1f). Together, Western blotting associated with immunofluorescence assay, flow cytometric analysis and ELISA can widely serve as a universal technology platform for qualitative and quantitative analyses of surface proteins.

It should be noted that shrimp do not have an adaptive immune response system and solely depend on innate immunity (Hoffmann and Reichhart, 2002). Therefore, generalized innate immunity plays a crucial role in shrimp defense mechanisms by activating germline-encoded pattern recognition receptor (PRR)-mediated recognition of pathogen-associated molecular patterns (PAMPs) (Syed and Kwang, 2015). Moreover, humoral immune genes, including prophenoloxidase (proPO), alpha 2-macroglobulin (α2M), crustin and PmRACK, and cell-mediated immune genes, including caspase and Rab7, are upregulated in gill tissues upon vaccination and challenge (Sudheer et al., 2015). Furthermore, the activities of immune-related enzymes such as SOD and PO can reflect the levels of innate immune

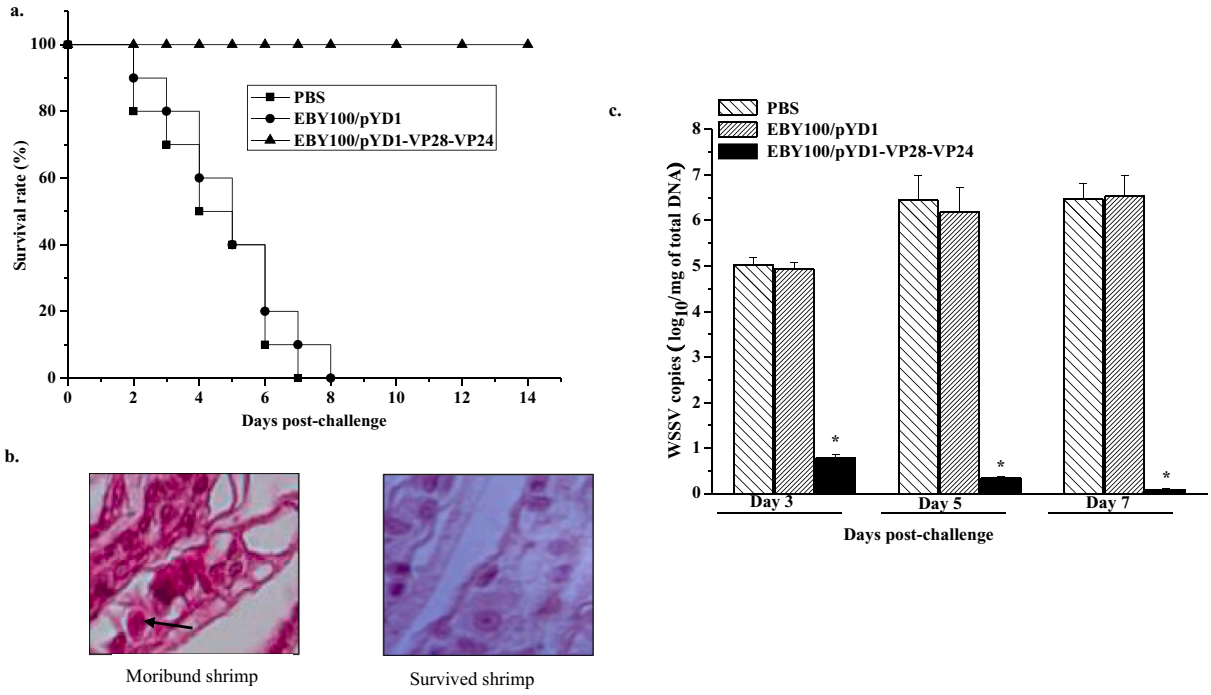

Fig. 3. The protection efficacy conferred by oral administration of EBY100/pYD1-VP28-VP24 against WSSV challenge after the last oral administration on day 8. **(a)** Survival rate. **(b)** Immunohistochemistry analyses of gill tissues from the moribund and survived shrimp at day 7 post WSSV challenge. Results from immunohistochemistry analyses are representative of three independent experiments. The arrow mark indicates hypertrophied nuclei characteristic of WSSV infection. **(c)** Quantification of WSSV copy numbers determined by RT-PCR at days 3, 5 and 7 post-challenge. The data are shown as the mean ± SD. The asterisks indicate significant differences compared with the PBS- and EBY100/pYD1-administered controls. * $p < 0.05$. (c.

response in shrimp (Nguyen et al., 2014). The present study also indicated that higher activity levels of SOD and PO induced by EBY100/pYD1-VP28-VP24 might contribute to resistance to WSSV in shrimp.

In particular, VP28 plays an important role in WSSV infection and is generally chosen for developing WSSV vaccine candidates. A WSSV vaccine candidate originating from seminal studies showed that purified recombinant VP28 (rVP28) was coated with feed pellets and administered orally to *Penaeus monodon*, resulting in approximately 77% protection (Witteveldt et al., 2004) and 30–70% protection in *Penaeus japonicus* (Caipang et al., 2008). Furthermore, the use of *B. subtilis* spores expressing a chimeric protein, CotB-VP28, could provide 65% protection in *Litopenaeus vannamei* (Nguyen et al., 2014). Another study revealed that oral vaccination with Bac-VP28, in which the VP28 gene was inserted into a baculovirus vector, showed significantly higher survival rates of 81.7% and 76.7% upon WSSV challenge at days 3 and 15 post-vaccination, respectively, whereas shrimp receiving Bac-wt or the positive control showed 100% cumulative mortality at 10 days after WSSV challenge (Syed and Kwang, 2011). It is more likely that VP28 alone may be inadequate for serving as a WSSV vaccine candidate that provides more than 90% protection. Notably, VP24 is another important envelope protein of WSSV and is also used for developing WSSV vaccine candidates (Thomas et al., 2014). Therefore, an alternative approach is the combination of VP28 and VP24, which may provide a significantly higher protection efficacy against WSSV. The survival rate of shrimp treated with constructs expressing antisense VP24 and VP28 reached 90% (Ahanger et al., 2014). Our findings demonstrate that oral administration of EBY100/pYD1-VP28-VP24 confers 100% protection with a significant decrease in the viral load in *P. vannamei*, suggesting that using the fusion protein of VP28 and VP24 is a promising strategy for designing and developing WSSV vaccine candidates based on various manufacturing technology platforms.

S. cerevisiae and *P. pastoris* cell display systems are used globally for the expression of heterologous proteins with therapeutic value against infectious diseases (Kumar and Kumar, 2019). In *P. pastoris*, both N- and C-terminus-free display systems have been established using cell wall proteins (α-agglutinin, a-agglutinin, Flo1p, Pir1p, Sed1p, and Tip1p) from *S. cerevisiae* (Kuroda and Ueda, 2013; Tanino et al., 2006; Wang et al., 2007, 2008). In addition, most *P. pastoris* surface display systems contain antibiotic selective markers, which significantly limits their applications (Harnpicharnchai et al., 2010). Therefore, *S. cerevisiae*-based vaccines have great potential application for oral administration based on their safety profile (Kumar and Kumar, 2019).

In conclusion, a novel approach was identified in which EBY100/pYD1-VP28-VP24 was used as a potential WSSV oral vaccine candidate in shrimp. The oral administration of EBY100/pYD1-VP28-VP24 could induce significant activities of immune-related enzymes and protect shrimp from WSSV challenge, as substantiated by a 100% survival rate. The results of the present study indicate the feasibility of using EBY100/pYD1-VP28-VP24 and provide insight into the development of novel field-applicable WSSV oral vaccines.

Author statement

Han Lei: Conceptualization, Methodology and Supervision. **Shuangqin Li**: Performing Experiments and Writing-Original draft preparation. **Xin Lu**: Data analysis. **Yi Ren:** Results Interpretation. All authors read and contributed to the final manuscript.

Declaration of Competing Interest

The authors declare no conflicts of interests.

Acknowledgments

This work was supported by a grant from National Natural Science

Foundation of China (no. 32070920), Sichuan Science and Technology Program (no. 2019YFN0134) and the Fundamental Research Funds for the Central Universities (no. 2682020ZT87) to H. Lei.

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