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Protective immunity induced by the vaccination of recombinant *Proteus mirabilis* OmpA expressed in *Pichia pastoris*

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ABSTRACT

Proteus mirabilis (*P. mirabilis*) is a zoonotic pathogen that has recently presented a rising infection rate in the poultry industry. To develop an effective vaccine to protect chickens against *P. mirabilis* infection, OmpA, one of the major outer membrane proteins of *P. mirabilis*, was expressed in *Pichia pastoris*. The concentration of the expressed recombinant OmpA protein reached 8.0 μg/mL after induction for 96 h with 1.0% methanol in the culture. In addition, OmpA protein was confirmed by SDS–PAGE and Western blot analysis using the antibody against *Escherichia coli*-expressed OmpA protein. Taishan *Pinus massoniana* pollen polysaccharide, a known plant-derived adjuvant, was mixed into the recombinant OmpA protein to prepare the OmpA subunit vaccine. We then subcutaneously inoculated this vaccine into chickens to examine the immunoprotective effects. ELISA analysis indicated that an excellent antibody response against OmpA was elicited in the vaccinated chickens. Moreover, a high protection rate of 80.0% was observed in the vaccinated group, which was subsequently challenged with *P. mirabilis*. The results suggest that the eukaryotic *P. mirabilis* OmpA was an ideal candidate protein for developing an effective subunit vaccine against *P. mirabilis* infection.

Introduction

Proteus mirabilis (*P. mirabilis*) is an acute opportunistic pathogen that widely exists in nature [1]. *P. mirabilis* can infect chickens at various ages, and causes certain characteristic clinical symptoms, such as diarrhea, paralysis of the limbs, sepsis, and encephalomalacia. This pathogen can spread through congenital and contact infection [2]. These features facilitate the outbreak of the disease, especially in low-age chickens. At present, *P. mirabilis* has a rising infection rate and has brought a significant negative impact to the poultry industry; however, few vaccines are available to prevent *P. mirabilis* infection [3,4]. Therefore, an effective vaccine against *P. mirabilis* should be developed.

Vaccines based on outer membrane proteins (OMPs)[2] have been demonstrated to possess excellent immunogenicity and stimulate both humoral and cellular immunity [5]. According to our previous study, OMPs can also serve as protective antigens against *P. mirabilis* challenge [6]. In numerous Gram-negative bacteria, OmpA represents the major portion of OMPs. Studies have shown that OmpA possesses a multitude of functions and properties, including adherence to and invasion of host cells, serum resistance, and immune evasion [7–9]. A previous study showed that *Klebsiella pneumoniae* OmpA binds to a wide range of immune effector cells [10]. OmpA proteins in *Escherichia coli* and *Neisseria gonorrhoeae* have been shown to be involved in phagocytosis and activation of macrophages and dendritic cells [9,11]. In *E. coli*, OmpA also contributes to serum resistance by binding to C4b-binding protein, leading to a decrease in serum killing [8]. OmpA has been considered a new pathogen-associated molecular pattern because of its inherent conservative property among *Enterobacteriaceae*, as well as its important functions in bacterial survival and virulence [12]. Korn et al. reported that OmpA of *P. mirabilis* (a 39 kDa protein) is a highly immunoreactive protein at least in mouse, as indicated by the strong stimulation on the proliferation of B lymphocytes [13]. Based on these findings, OmpA has great potential as an effective antigen component to protect animals against *P. mirabilis* infection. Thus, we attempted to investigate the protective immunity of *P. mirabilis* OmpA in chickens.

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[2] *Abbreviations used:* TPPPS, Taishan *Pinus massoniana* pollen polysaccharide; OmpA, outer membrane protein A; BMGY, buffered glycerol-complex medium; BMMY, buffered methanol-complex medium; OD, optical density; HRP, horse-radish peroxidase; PBS, phosphate buffered saline.

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Several studies have reported that recombinant subunit vaccines can achieve a more effective immune response by including an adjuvant, which augments the adaptive immune response and generates effective immunological memory [14,15]. Taishan *Pinus massoniana* pollen polysaccharide (TPPPS), a novel plant-derived immuno-enhancer and adjuvant, has been studied in our laboratory since 2003. TPPPS can strengthen the immune response and enhance the effects of the subunit vaccine significantly [16,17]. In the present study, we aimed to investigate the synergistic effects of recombinant *P. mirabilis* OmpA mixed with TPPPS.

Recombinant OmpA from *P. mirabilis* was produced in *Pichia pastoris*, and applied TPPPS as the adjuvant for preparing the *P. mirabilis* OmpA subunit vaccine. After inoculation into chicken, this subunit vaccine mixed with TPPPS showed strong antibody response and a high rate of protection against *P. mirabilis* challenge.

Materials and methods

Strains, plasmids, and media

P. mirabilis strain Q1, the bacterial strain used for this study, was identified from chickens and previously preserved by the Animal Biotechnology and Disease Control and Prevention Laboratory of Shandong Agriculture University. *E. coli* DH5α, *P. pastoris*, and the plasmid pPIC9 were provided by Invitrogen (Carlsbad, CA, USA). Luria–Bertani (LB) broth, LB agar, yeast extract–peptone–dextrose agar, and yeast nitrogen base (YNB) without amino acids were purchased from Solarbio (Beijing, China). All yeast culture media were prepared according to the manufacturers' guidelines.

Construction of recombinant expression vector pPIC9–OmpA

To amplify OmpA genes, a pair of primers (P1: 5′-CGGAATTCATGAAAAAGACAGCTATCGC-3′, P2: 5′-TTGCGGCCGCTTAGTGATGATGATGATGATGTTGACCAGGTTGAACAAC-3′) was designed according to the OmpA gene sequence in *P. mirabilis* (GenbankType: RefSeq(Nucleotide) NC_010554.1). The PCR products were digested with EcoR I and Not I, and cloned into the pPIC9 vector, which was digested with the same endonucleases. The recombinant plasmids were named pPIC9–OmpA, transformed into *E. coli* DH5α, verified by restriction digestion, and sequenced (Shanghai Sunny).

Transformation of P. pastoris and selection of transformants

The constructed plasmids pPIC9–OmpA were linearized with Sac I at 37 °C for 2 h, and transformed into competent *P. pastoris* cells by lithium chloride methods according to the instructions of Invitrogen Company [18]. Transformants were selected on MD plates and colony-purified by re-streaking on fresh selection plates. Correct genomic integration at the *Pichia* AOX1 promoter locus was confirmed by PCR using the universal AOX1 primers (p3: 5′-GACTGGTTCCAATTGACAAGC-3′, p4: 5′-GCAAATGGCATTCTGACATCC-3′). Methanol utilization plus (Mut+) phenotype was checked by comparing the growth rates of the selected yeast transformants on MD and MM plates.

Expression of P. mirabilis OmpA protein in P. pastoris

Induction of the expression of OmpA in *P. pastoris* was performed as described by the manufacturer's instructions (Invitrogen). The transformed yeast cells were cultured in 50 mL of BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% YNB, 4×10^{-5}% biotin, and 1% glycerol) medium at 28 °C with vigorous shaking (250 rpm) to OD600 of 2–6. The cells were harvested by centrifugation and re-suspended in 250 mL of BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% YNB, 4×10^{-5}% biotin, and 0.5% methanol) medium to an OD600 of 1 for induction under similar growth conditions. Methanol (100%) was added every 24 h up to a final concentration of 1.0% to ensure continuous induction, and the culture medium (1.0 mL) was sampled daily to determine the optimal induction time for maximum display by 12% gel SDS–PAGE.

Western blot of the expressed OmpA

Protein obtained at 96 h was electrophoresed on 12% SDS–polyacrylamide gels and transferred to nitrocellulose by electroblotting. The membrane was blocked with 10% skim milk diluted in PBS buffer, and incubated with rabbit polyclonal antibody against *E. coli* BL21-expressed OmpA at room temperature for 1 and 2 h. After washing for three times with TBST (1 × Tris-buffered saline (TBS) + 0.05% Tween 20), the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (TRANS, Beijing, China) at room temperature for 2 h. Protein bands were revealed using an EasySee® Western Blot Kit (TRANS, Beijing, China).

Purification of recombinant P. mirabilis OmpA

Pichia clone was grown in 200 mL of BMGY medium, cultivated in 1000 mL of BMMY medium under optimized culture conditions, and prepared for purification by a Proteinlso™ Ni-NTA Resin kit (TRANS, Beijing, China) according to the manufacturer's recommendation. The protein was isolated from the culture supernatant by precipitation with ammonium sulfate at 50–80% saturation, and dissolved in 50 mL of washing buffer (20 mM Na_2HPO4, 0.5 M NaCl, and 20 mM imidazole, pH 7.4). The supernatant was then filtered through a 0.45 μm filter and applied to the Proteinlso™ Ni-NTA resin column, which was equilibrated with washing buffer. After elution of proteins lacking the His tag using washing buffer, the recombinant protein was eluted from the resin using elution buffer (20 mM Na_2HPO4, 0.5 M NaCl, and 0.5 M imidazole, pH 7.4). The eluted fractions were collected and analyzed by SDS–PAGE and Western blot. Purity was estimated by analysis of SDS–PAGE gel image with GeneTools software from SynGene (Bio-Rad, USA). Protein concentration was determined by an Easy II Protein Quantitative Kit (BCA) (TRANS, Beijing, China).

Preparation of adjuvants and recombinant subunit vaccine

TPPPS (net content = 72.20%) was provided by our laboratory and prepared according to a certain proportion based on our previous experiments [16]. TPPPS was diluted to 800 μg/mL (net content) with deionized water, sterilized by filtering, and stored at 4 °C until use. Freund's adjuvant was prepared as previously described [19].

TPPPS was diluted and mixed with OmpA of *P. mirabilis* at the ratio of 1:1, reaching a final concentration of 400 μg/mL, with OmpA at doses of 50, 100, and 150 μg/mL. Freund's adjuvant and OmpA were mixed in equal volumes to obtain final OmpA doses of 50, 100, and 150 μg/mL. Stability and sterility tests were performed after the vaccines were prepared.

Immunization and artificial challenge of the chickens

A total of 480 one-day-old SPF chickens were randomly divided into four groups (n = 120 per group), and allowed to acclimatize for 3 d before the start of the experiments. Groups I–IV were injected subcutaneously with 0.2 mL of TPPPS–OmpA vaccine, F-OmpA