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Taishan *Pinus massoniana* pollen polysaccharides promote immune responses of recombinant *Bordetella avium* ompA in BALB/c mice

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ABSTRACT

To study the effects of Taishan *Pinus massoniana* pollen polysaccharides (TPPPS) on *Bordetella avium* outer membrane protein A (ompA) recombinant protein vaccine, ompA was expressed, confirmed by Western blotting and mixed with TPPPS. Female BALB/c mice were randomly divided into six groups (I–VI). Groups I, II, and III were treated with TPPPS–ompA at doses of 200, 400, and 800 mg/ml, respectively. Groups IV, V, and VI were treated with Freund's adjuvant–ompA, pure ompA, and physiological saline, respectively. On days 3, 7, 14, 21, 28, 35, 42, and 49 after the first vaccination, antibody titers, interleukin-2 (IL-2) levels, peripheral blood CD4+ and CD8+ levels, and T lymphocyte proliferation rates in peripheral blood, as well as secreting-type immunoglobulin A (SIgA) levels in the duodenum, were measured. The antibody titers against ompA, IL-2, T lymphocyte proliferation rate, CD4+, and CD8+ in Group II were significantly ($P < 0.05$) higher than those in other groups. However, little difference in SIgA content was observed among Groups I, II, and IV. These results indicated that TPPPS strengthened humoral and cellular immune response against recombinant ompA vaccine and 400 mg/ml TPPPS showed significance ($P < 0.05$) compared with Freund's adjuvant. Therefore, TPPPS can be developed into an adjuvant for recombinant protein vaccines or plant-derived medicine for animal husbandry.

1. Introduction

Bordetella avium infection continues to be an economic issue in the poultry industry in China, particularly in the turkey industry. *B. avium* is the causative agent of bordetellosis, a disease that lowers hatchability, decreases the laying rate, and causes upper respiratory tract symptoms often in adult chicken and leads to serious secondary infections [1]. In 2009, Harrington et al. isolated *B. avium* from patients with respiratory disease and found that *B. avium* was a rare opportunistic human pathogen [2]. *B. avium* has a rising infection rate and has brought significant negative impact to the poultry industry. Antibiotic treatments and live-attenuated vaccines have proven ineffective in controlling this pathogen within commercial poultry flocks [3].

Widely used in subunit vaccine research, prokaryotic expression systems are characterized by low production cost, high production yield, and great convenience [4]. However, proteins expressed by these systems have defects like protein misfolding and poor immunogenicity. Finding an efficacious adjuvant can help solve the problem. Adjuvants that have been explored include insoluble colloidal aluminum salts, oil emulsion adjuvants, antigen microcysts, molecular adjuvants, and cytokines. Although hundreds of adjuvants have been proposed over the last few decades, most have failed to perform well due to such limitations as lack of efficacy, systemic toxicity, and prohibitive cost [5].

The discovery of novel plant compounds that modulate the immune system has become an increasingly important area of research, particularly in the search for new-generation vaccine adjuvants [6]. Immune responses following infection or vaccination can be influenced by plant polysaccharides [7]. Many polysaccharides, such as *Astragalus* polysaccharides (ASP) [8], *Panax ginseng* polysaccharides [9], and *Ganoderma lucidum* polysaccharide F3 [10], have immunopotentiating function and adjuvant efficacy. Several of these polysaccharides (e.g., ASPs) have been developed into commercial immunostimulants and adjuvants and thus significantly influence the control of immunosuppressive diseases among poultry in China. In our laboratory, we have explored *Aloe vera* [11], propolis [12], Taishan *Robinia pseudoacacia* [13], and the Taishan *Firmiana simplex* flower [14] and found that they have different levels of immune enhancement and adjuvant function. Taishan *Pinus massoniana* pollen polysaccharides have been studied in our laboratory since 2003 and have been found to be an effective adjuvant for inactivated vaccines and have a generally higher efficiency than ASP [15].

In this study, TPPPS was used as an adjuvant for recombinant protein vaccine, which was created with the *Escherichia coli* expression system to express *B. avium* ompA which has been shown to elicit higher levels

Abbreviations: TPPPS, Taishan *Pinus massoniana* pollen polysaccharides; *B.avium* ompA, *Bordetella avium* outer membrane protein A; ASP, *Astragalus* polysaccharides; OD, optical density; SIgA, secreting-type immunoglobulin A.

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of antibodies than other *B. avium* outer membrane proteins and could serve as a protective antigen [16]. This study aims to detect how adjuvant TPPPS influences the humoral, cellular, and mucosal immunity responses of mice against recombinant ompA and discuss the possibility of developing TPPPS as a commercial adjuvant for recombinant protein vaccine.

2. Materials and methods

2.1. Growth and maintenance of bacterial cultures

B. avium LL, isolated in our laboratory in 2008 (Shandong, China) was used in this experiment. *E. coli* strains DH5a and BL21 (DE3) were purchased from Takara (Dalian, China). *B. avium* LL and *E. coli* BL21and DH5α were routinely grown and maintained on lysogeny broth (LB) agar and broth or with 10 μg/ml of ampicillin.

2.2. PCR amplification, cloning, and sequencing

To amplify ompA (ompApp 1–597, NCBI GeneBank accession number: M96550.1), primers were used to produce a 597 bp fragment:

[ompA F: 5′-GCGAATTCCATGAACAAACCCTCCAAA-3′ (1416012–1416031 bp); ompA R: 5′-CCGCTCGAGTTACTTGCGGCTACCGACGATT-3′ (1416576–1416596 bp)].

Gel electrophoresis was conducted through routine procedures to confirm the PCR products [17]. PCR products of correct size were extracted with a commercial DNA extraction kit (Omega, USA), cloned into the pMD18-T vector (Takara, Dalian, China), and sequenced by Genescript (Nanjing, China).

2.3. Expression, purification, and identification of ompA

Gene ompA were cloned into pET32a + (Takara, China), and the recombinant plasmids were transformed into *E. coli* BL21 competent cells. *E. coli* BL21 with ompA–pET32a + was incubated in LB at 37 °C until its OD600 reached 0.4–0.6, and isopropyl-β-D-thiogalactopyranoside (Omega, USA) was then added to the culture. After incubation at 25 °C for 8 h, bacteria were harvested at 4 °C, 2000 rpm/min for 30 min.

Recombinant ompA was purified through His-tag according to the method used by Asgarian-Omran (2012) [18]. The ompA was identified by SDS-PAGE and Western blotting [19].

2.3.1. Preparation of TPPPS and other adjuvants

TPPPS was prepared according to our previous experiment [15]: Taishan *P. massoniana* pollen collected from the Mountain Tai region, whose pollen exines were broken by the Ultra-Micro Pulverizer (Taian Zhengxin Science and Technology), were separately packed with filter paper, and placed in a Soxhlet extractor. Pollen fat was extracted with ethyl ether. The fat-free pollen was then mixed with de-ionized water at a volume ratio of 1:15, and 0.3% pepsin (Solarbio, China) was then added. The polysaccharide was fully dissolved after 2 h-incubation at 50 °C and 8 h-incubation at 85 °C. *P. massoniana* pollen suspension after incubation was precipitated at 4 °C overnight, with the sediment removed. Supernatant fluid was filtered through a filter net, centrifuged (at 10,000 rpm/min for 10 min), and concentrated under reduced pressure (to preserve the polysaccharide structure) with a rotary evaporator. Protein was removed twice from the concentrated solution with Sevage reagent (chloroform: n-butanol = 5:1). Finally, the polysaccharide was precipitated with absolute ethyl alcohol four times as much as the polysaccharide (by volume). TPPPS was obtained after freeze drying.

The polysaccharide contents were detected according to the anthrone–sulfuric acid method used by Leyva [20]. TPPPS was diluted with de-ionized water, sterilized by filtration, and stored at 4 °C. Freund's adjuvant was prepared as previously described [21].

2.3.2. Preparation of vaccines

The concentration of expressed ompA was determined with a Bradford protein assay kit (Takara, China) and mixed with TPPPS at a ratio of 1:1, reaching final values of 50, 100, and 150 μg/ml, with TPPPS at doses of 200, 400, and 800 mg/ml respectively. Freund's adjuvant and ompA were mixed in equal volumes to reach a final ompA dose of 50, 100, and 150 μg/ml. Stability and sterility tests were performed after the vaccines were prepared.

2.3.3. Animals and immunity

Female BALB/c mice aged four to five weeks and each weighing around 20 g were randomly placed in 18 cages, divided into six groups, and vaccinated three times. Groups I, II, and III were immunized with TPPPS–ompA vaccine on days 3, 18, and 33, respectively. The final ompA concentrations were 50 μg/ml at the first vaccination, 100 μg/ml at the second, and 150 μg/ml at the third. The TPPPS concentrations were 200, 400, and 800 mg/ml for groups I, II, and III, respectively. Group IV was treated with Freund's adjuvant (F)–ompA at concentrations of 50, 100, and 150 μg/ml at the first, second, and third vaccinations, respectively. Group V was similarly treated, except with pure ompA. Group VI was the blank control group, which was injected with equivalent physiological saline. Blood and duodenum were sampled on days 3, 7, 14, 21, 28, 35, 42, and 49 after vaccination. All experiment animals were treated according to the International Experimental Animal Welfare Regulations.

2.3.4. Serum antibody titers and IL-2 detection

Serum antibody titers were detected through indirect enzyme-linked immunosorbent assay (ELISA) [22]. Serum IL-2 was detected according to the protocol of the mouse IL-2 ELISA kit (Nangjing Jiancheng, China).

2.3.5. Peripheral blood T lymphocyte proliferation assay

Aseptic blood (0.05 ml) was collected with anticoagulant tubes through the eye orbit. Lymphocytes were separated [23], counted, and compounded into an RPMI-1640 medium with 10% fetal bovine serum with cell density adjusted to 1×10^6 cells/ml. The solution obtained was then moved into 96-well plates (Corning Costar) at 100 μl/well, and ConA (final concentration = 20 mg/ml) was then added. The negative-control well was simultaneously prepared. After 48 h cultivation, cells with and without ConA were mixed into the suspension. The suspension was then transferred to centrifuge tubes for centrifugation and washed twice with phosphate buffered saline (PBS). Finally, lymphocytes from each group were suspended in 1 ml PBS and adjusted to 1×10^6 cells per tube, and 50 μl of propidium iodide dye (Sigma, USA) was added to the solution. The lymphocytes were incubated in a lucifugal environment for 30 min and washed with PBS again [24]. Upon irradiation of the laser generated by flow cytometry (Guaga Easy Cyte Mini) at a wavelength of 488 nm, lymphocyte percentages at the S phase in both the experimental and control tubes were measured. The percentage at the S phase (SPF) of the tubes containing ConA represented the degree of T lymphocyte proliferation. SPF was calculated as follows:

$$\mathrm{SPF} = \frac{S}{(G_0 + G_1) + S + (G_2 + M)} \times 100\%.$$

2.3.6. Peripheral blood CD4 + and CD8 + detection

Fresh anticoagulant (0.5 ml)–venous blood (anticoagulant/blood = 1:1) mixed with 1 ml PBS was carefully added to a 4 ml lymphocyte separation medium (P8620-200, Solarbio, China) and centrifuged at 2000 rpm for 15 min. Lymphocytes were harvested and washed twice with PBS, and 10 μl CD4 + dye (fluorescein isothiocyanate anti-mouse CD4, clone GK1.5 Cat: 100406, Lot: B149566, BioLegend, USA) and 10 μl CD8 + dye (phycoerythrin anti-mouse CD8, clone 53-6.7, Cat: 100708,