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Full Length Research Paper

Adjuvant effect of a novel water-soluble polysaccharide isolated from the stem of *Physalis alkekengi* L. var. *francheti (Mast.) Makino*

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Water-soluble polysacchride (WSP) was isolated and purified from the mature stem of *Physalis alkekengi L. var. francheti (Mast.) Makino*, which is a traditional Chinese medicine herb. Gas chromatography (GC) analysis indicated that WSP was an acid heteropolysaccharide, which was composed of Rha, Ara, Gal, Glc and GalA in molar ratio of 2.6 : 1.0 : 6.5 : 3.5 : 2.4. More importantly, adjuvant activity of WSP was evaluated in ICR mice in present study. Male ICR mice were immunized subcutaneously with ovalbumin (OVA) alone, OVA/WSP, OVA/QuilA, and saline two times at 14-day interval. The mice were sacrificed two weeks after the last immunization to analyze antibody titers in serum. It was observed that WSP significantly enhanced OVA-specific antibody titers (IgG, IgG1, IgG2b) in serum when compared with OVA-injected mice (P<0.05 or P<0.01). Thus, WSP could be considered as a promising adjuvant eliciting both Th1 and Th2 responses to improve the efficacy of vaccine.

Key words: Herbal medicine, *Physalis alkekengi* L, polysaccharide, adjuvant.

INTRODUCTION

A body of evidence showed that numerous bioactive polysaccharides isolated from herbal plants have immunomodulation and anti-cancer effects. Previous studies have shown that polysaccharides can evoke stronger humoral and cell-mediated immune responses (Chen et al., 2010; Liu et al., 2010; Ragupathi et al., 2008; Yang et al., 2008; Zhang et al., 2007). Thus, the polysaccharides from medicinal herbs are becoming an attractive material as pharmaceutical products and may provide an opportunity to develop a new adjuvant of vaccine. *Physalis alkekengi* L. var. *francheti (Mast.) Makino* (*P. alkekengi*) is widely distributed in Europe and Asia including Russia, China, Japan, etc. It is well known that *P. alkekengi* is an edible and medicinal plant in oriental countries, especially as a traditional Chinese herbal plant. The broad use of this plant in popular medicine includes anti-inflammatory, anti-cold, anti-cough and anti-fungal activities. Some active components from *P. alkekengi* such as physalin, alkaloids, and flavone have been investigated (Basey and Woolley, 1973; Helvaci, et al., 2010; Kang et al., 2011; Vessal et al., 1996). We have previously reported adjuvant and hypoglycemic activities of polysaccharide isolated from fruits of *P. alkekengi* (Tong et al., 2008; Li et al, 2011). However, no specific studies on the immunologic enhancement of the water-soluble polysaccharide (designated WSP below), which was isolated from the stem of *P. alkekengi*, have been carried out. We therefore specifically focused on evaluating the adjuvant effect of WSP. It was observed WSP could significantly enhance antibody titers against OVA in IgG, IgG1, IgG2b and might be used as an adjuvant.

P. alkekengi is in high demands in China because it has numerous ethnopharmacological properties. Cultivated *P. alkekengi* has gained popularity as wild supply alone cannot meet such high demands. The stem of *P. alkekengi* is often ignored and wasted because the fruit

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Abbreviations: WSP, Water-soluble polysaccharide; **GC,** gas chromatography; **OVA,** ovalbumin; **CP,** crude polysaccharide; **HPLC,** high performance liquid chromatography; **FID,** flame-ionization detector; **PBS,** phosphate-buffered saline; **TMB,** 3,3',5,5'-tetramethylbenzidine; **OD,** optical density; **DNA,** deoxyribonucleic acid.

with sepal is perceived as the only part needed for medicine. Thus, the present study may provide basis for newer and more efficient use of *P. alkekengi*.

MATERIALS AND METHODS

Experimental animals

Five weeks-old males ICR mice (Grade II, body weight 20 ± 2 g) used for experiment were breed at the School of Public Health, Jilin University, China (certificate no. SCXK-(JI) 2007-0003, Changchun, China). The mice were acclimatized for a period of 2-3 days before using for experiment. Before and during the experiment the mice were fed with standard laboratory diet, given tap water and maintained under a constant 12 h light and dark cycle at 21-23°C.

Materials and chemicals

The mature stem of *P. alkekengi* L. var. *francheti (Mast.) Makino*. was collected in Shuangyang district, Chanchun city, Jilin province. Ovalbumin (OVA), Ureahydrogen Peroxide Addition compound, and QuilA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Goat anti-mouse IgG1-HRP and IgG2b-HRP were from Southern Biotechnology Associate Inc (Birmingham, AL, USA); goat anti-mouse IgG-HRP was purchased from Beijing Zhongshan Golden Bridge Biotechnology Co.LTD (China). QAE Sephadex A-25 was purchased from Amersham Pharmacia Co. (Sweden). All other chemicals were of grade AR.

Extraction, purification of polysaccharide

0.5 kg of *P. alkekengi* dry stem was extracted with distilled water (3 L) at 100°C for three times and 3 h for each time. The whole extract was filtered and centrifuged to remove water-insoluble fractions. The supernatant was precipitated with 3 volumes of ethanol at 4°C overnight after concentration by evaporation at 45°C under reduced pressure. The crude polysaccharide was recovered by centrifugation, and dried at 45°C under reduced pressure after washing successively with ethanol and ether. The polysaccharide (5 g) was dissolved in 100 ml distilled water and frozen at -20°C, thawed and centrifuged at 10,000 rpm for 20 min to remove insoluble materials. Crude polysaccharide (CP) was precipitated with 75% ethanol. CP was deproteinated by a combination of proteinase and Sevag method (Tong et al., 2008). CP was further purified on a QAE Sephadex A-25 column (3.0 × 80 cm) eluted with 0.15 mol/L NaCl at a flow rate of 0.5 ml/min, and the main polysaccharide fraction (Q-WSP) was collected. Q-WSP was further purified by High Performance Liquid Chromatography (HPLC). HPLC (Shimadzu, Japan) was equipped with a TSK-GEL G3000 PW column (21.5×600 mm) and a RID-10A Refractive Index Detector. HPLC was performed with 0.15 mol/L NaCl as the mobile phase at 5 ml/min and 25°C. WSP recovered from HPLC was dialyzed and lyophilized for further experiment. The endotoxin level in WSP solution was less than 0.5 EU (endotoxin unit)/ml. The solution of WSP was sterilized by 0.22 μm millipore filter for all animal experiments.

Phytochemical test and monosaccharide composition analysis of WSP

The concentration of polysaccharide was measured by the phenol-sulfuric acid method using D-glucose as the standard (Dubois et al., 1956).The monosaccharide of WSP was analyzed by GC. Polysaccharide was hydrolyzed and acetylated according to Johnes and Albersheim (1972). Briefly, WSP (10 mg) was hydrolyzed with 2 M TFA (2 mL) at 120°C for 2 h, and the excess acid was completely removed by co-distillation with ethanol. The hydrolyzed product was reduced with KBH_4 (30 mg), followed by neutralization with dilute acetic acid and evaporated at 45°C after adding 1mg myo-inositol and 0.1 M Na_2CO_3(1 mL) at 30°C with stirring for 45 min. The residue was concentrated by adding methanol. The reduced products (alditols) were added with 1:1 pyridine–propylamine at 55°C with stirring for 30 min, and acetylated with 1:1 pyridine–acetic anhydride in a boiling water bath for 1 h. The acetylated products were analyzed by GC, identified and estimated with myo-inositol as the internal standard. GC was performed on a Vavian 3400 (Hewlett-Packard Component, USA) equipped with DM-2330 capillary column (30 m × 0.32 mm × 0.2 μm) and flame-ionization detector (FID). The column temperature was kept at 120°C for 2 min, and increased to 250°C (maintained for 3 min) at a rate of 8°C /min. The injector and detector heater temperature were 250 and 300°C, respectively. The rate of N_2 carrier gas was 1.2 ml/min.

Immunization and collection of blood samples

In order to detect the immunologic enhancement of WSP on the mice, the male ICR mice (5 mice per group) were immunized subcutaneously with OVA (5 mg/kg) alone, or with OVA (5 mg/kg) containing QuilA (0.5 mg/kg) (OVA/QuilA) or WSP (10 mg/kg) (OVA/WSP) twice at 14-day interval. Mice were treated with saline as a control group. OVA was dissolved in saline for injection. The mice were sacrificed 2 weeks after the second immunization. The mice serum was collected to detect the titer of OVA-specific antibody.

Measurement of OVA-specific antibody

OVA-specific antibodies (IgG, IgG1 and IgG2b) in serum of the immunized mice were evaluated by an indirect ELISA according to the methods of Yang et al. (2005). Briefly, microtiter plates (Nunc) were coated with 100 μl OVA (50 mg/L) in 0.05 mol/L carbonate-bicarbonate buffer pH 9.6 for 24 h at 4°C. The wells were washed three times with phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20, and blocked with PBS containing 5% skim milk powder at 37°C for 1 h. After washing with PBS containing 0.05% (v/v) Tween 20 three times, 100 μl of a series of diluted serum from immunized mice or PBS containing 5% skim milk powder as control were added to the triplicate wells. The plates were then incubated for 1 h at 37°C, followed by washing with PBS containing 0.05% (v/v) Tween 20. Aliquots of 100 μl of goat anti-mouse IgG-HRP, IgG1-HRP and IgG2b-HRP (diluted 1:5000 with PBS containing 5% skim milk powder respectively) were added to each plate. The plates were further incubated for 1 h at 37°C Substrate 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well after washing with PBS, and the plate was incubated for 15 min at room temperature. Reaction was terminated by adding 50 μl of 2 mol/L H_2SO_4 to each well and optical density (OD) was detected at 450/630 nm with ELISA reader (Model 680, Bio-Rad, USA). Antibody titer was expressed by Log_2 value of the highest dilution of serum.

Satistical analysis

All results were expressed as mean ± SD. Data were analyzed by standard *t*-test. P values less than 0.05 were considered statistically significant.

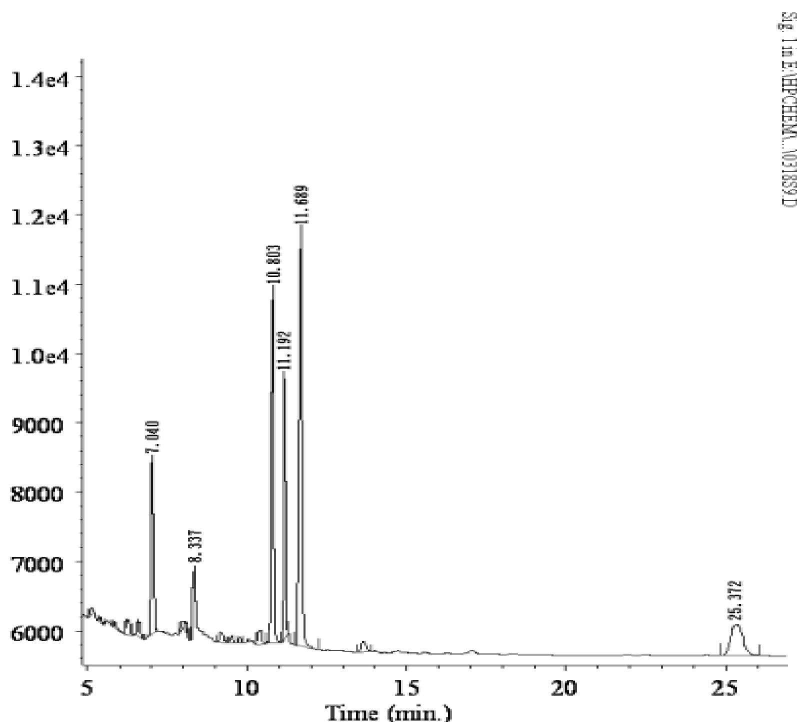

Figure 1. GC profile of WSP. Peaks from left to right: Rha, Ara, Gal, Glc, Intrenal standard, GalA.

RESULTS

Phytochemical test and monosaccharide composition analysis of WSP

Results from phenol-sulfuric acid assay showed that WSP contained 96.5% carbohydrate. GC analysis indicated that WSP was composed of Rha, Ara, Gal, Glc, GalA with a relative molar ratio of 2.6 : 1.0 : 6.5 : 3.5 : 2.4 (Figure 1).

OVA- specific antibody response evoked by WSP

Anti-sera of the immunized mice were collected two weeks after the last immunization to analyze OVA-specific antibody titers (IgG, IgG1 and IgG2b) by indirect ELISA. Data shown in Figure 2 demonstrated that IgG antibody titers in OVA-immunized mice were enhanced significantly by WSP and QuilA (P<0.05). Moreover, considerable enhancements of IgG1 and IgG2b antibody titers in groups OVA/WSP and OVA/QuilA were detected when compared with group OVA (P<0.05, or P<0.01). The data indicated clearly that WSP evoked a stronger antibody response in OVA-immunized mice.

DISCUSSION

New vaccines such as recombinant proteins and deoxyribonucleic acid (DNA) have relatively poor immunogenicity when compared with live attenuated and whole inactivated organisms. Although a large number of adjuvants in the scientific literature has been reported, alum is the only adjuvant approved for human use in USA (Garapati et al., 2009; Leroux-Roels et al., 2010; Mbow et al., 2010). Although alum adjuvant is powerful inducer of Th2 response, however, it poorly induces Th1 response producing IFN-γ, TNF-α, and other cytokines. Therefore, development of novel adjuvants, which are capable of strongly eliciting both humoral and cellular immune responses (Th1 and Th2), are necessary to maximize the efficacy of new or available vaccines (Khajuria et al., 2007; Kukhetpitakwong et al., 2006; Livingston et al., 1994; Liu et al., 2008).

Natural products have been a source of compounds with pharmacological activities. Polysaccharides, which are isolated from mushrooms, fungi, yeast, algae, lichens and plants, have attracted significant attention because of their immunomodulatory and antitumor effects (Schepetkin and Quinn, 2006). More importantly, previous studies have shown that polysaccharides can evoke stronger humoral and cell-mediated immune responses (Chen et al., 2010; Liu et al., 2010). It is well known QuilA, a mixture of partially purified saponins extracted from the bark of *Quillaja saponaria* Molina, can stimulate Th1 immune response and the production of CTLs. Unfortunately, high toxicity and the undesirable haemolytic effect of QuilA limit its use in human vaccines

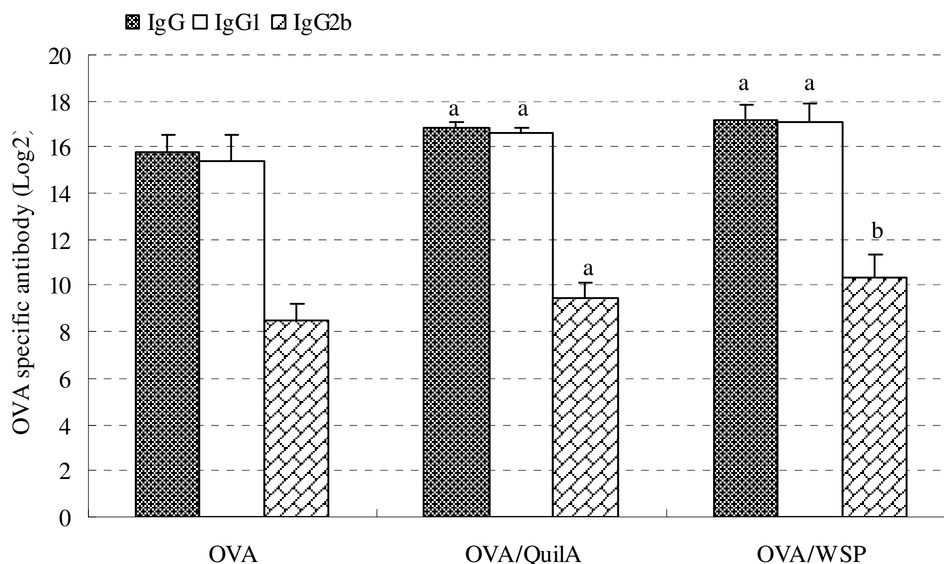

Figure 2. Effect of WSP on OVA-specific IgG, IgG1, IgG2b antibody titers in OVA-immunized mice. The values were present as mean ± SD. [a] P <0.05, [b] P<0.01 *vs* group OVA.

(Kensil et al., 1995; Oda et al., 2000; Marciani et al., 2001). However, most polysaccharides from herbal plants are typically less immunogenic, non-toxic and biodegradable; they are an unlimited natural resource and low cost to manufacture (Petrovsky, 2006). Hence, the use of herbal immuno-modulators may be helpful in overcoming the limitation of Alum or QuilA, and herbal adjuvant can be one of the ideal candidates.

In present study, WSP was purified from the stem of *P. alkekengi* L. by QAE Sephadex A-25 and HPLC. Phytochemical test revealed WSP contained 96.5% carbohydrate. GC analysis indicated that WSP was an acid heteropolysaccharide and composed of Rha, Ara, Gal, Glc, GalA with a relative molar ratio of 2.6: 1.0: 6.5: 3.5: 2.4 (Figure 1).

It is well known that in Th1 response mediating T cell immunity associated with serum antibody responses the predominant isotypes are IgG2a, IgG2b and IgG3, while in Th2 response associated with humoral immunity the predominant isotypes are IgG1 and IgA (Livingston et al., 1994). We therefore detected antibody titers in IgG, IgG1, IgG2b to evaluate Th1 and Th2 response evoked by WSP in ICR mice. To detect the adjuvant effect of WSP, male ICR mice were immunized subcutaneously with OVA alone or OVA containing either WSP (10 mg/kg) or QuilA (positive control) (0.5 mg/kg) twice at 14-day interval. The mice were dissected two weeks after the last immunization to analyze antibody titers in serum. It was observed that WSP not only substantially enhanced OVA-specific IgG level in OVA-immunized mice (P<0.05), but also increased OVA-specific IgG1 and IgG2b antibody titers (P<0.05 or P<0.01) (shown in Figure.2). This finding suggested WSP at suitable dose was effective on Th1 and Th2 response, which was as effective as that of QuilA. Thus, WSP may have a potential as adjuvant in vaccine.

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