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Water-soluble polysaccharide isolated with alkali from the stem of *Physalis alkekengi* L.: Structural characterization and immunologic enhancement in DNA vaccine

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ABSTRACT

A water-soluble polysaccharide (WSPA) was isolated with alkali and purified from the mature stem of *Physalis alkekengi* L. WSPA (M_w = 31 kDa) was an acid heteropolysaccharide, which consisted of Rha, Ara, Xyl, Gal, Glc and GalA in ratio of 1.0:2.5:0.8:2.7:4.4:1.4. The results from structural analysis indicated backbone and branches of WSPA were composed of (1 → 3)-linked Glc, (1 → 3)-linked Gal, (1 → 2)-linked Xyl, (1 → 2)-linked Ara, and (1 → 2)-linked Rha residues. However, GalA was distributed only in the backbone of WSPA. All branches of WSPA were at O-2 of (1 → 6)-linked Gal and terminated with Glc. More importantly, it was found that WSPA significantly enhanced specific antibody IgG response with higher titers of IgG1 as well as IgG2b ($p < 0.05$) in mice immunized with DNA vaccine. Therefore, WSPA can be considered as a potential adjuvant candidate in DNA vaccine.

1. Introduction

Physalis alkekengi L. var. *francheti (Mast.) Makino (P. alkekengi)* is widely distributed in Europe and Asia. It is an edible and medicinal plant in East Asia, and especially it is used as a traditional Chinese herbal plant. *P. alkekengi* is in high demands in China because it has numerous ethnopharmacological properties such as anti-inflammatory, anti-cold, anti-cough and anti-fungal activities (Kang, Kwon, & Choi, 2011). 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 with sepal is perceived as the only part needed for medicine. Thus, our lab has been focusing on polysaccharide purified from the mature stem of *P. alkekengi*. We previously reported polysaccharide, which is isolated with hot water from the mature stem of *P. alkekengi*, can enhance both humoral immunity and cellular immunity in OVA-immunized mice (Shang, Zhang, Han, & Wang, 2011).

Systemic candidiasis arises as a result of a normal commensal fungus when the normal microflora balance is disrupted in the host (LeibundGut-Landmann, Wüthrich, & Hohl, 2012; Magliani et al., 2012; Vecchiarelli, Pericolini, Gabrielli, & Pietrella, 2012). Over the past two decades, morbidity and mortality of systemic candidal infection has increased significantly even in the presence of antifungal therapy (Gudlaugsson et al., 2003). Previous findings indicated that fungal heat shock protein 90 (HSP90) plays a key role in protection against systemic *Candida albicans* (*C. albicans*) infection (Burnie & Matthews, 2003, 2004; Matthews & Burnie, 1996, 2001, 2005). Consequently, we previously constructed the DNA vaccine (pD-HSP90C) containing epitope C (LKVIRK) of *C. albicans* HSP90 against systemic candidiasis, and found pD-HSP90C can evoke production of specific antibody against epitope C (LKVIRK) in mice (Yang, Han, Zhao, & Wang, 2014). However, pD-HSP90C is poorly immunogenic, despite the fact that they can induce humoral and cellular immune responses. Therefore, we investigated adjuvant effect of WSPA in DNA vaccine pD-HSP90C.

In present study, we detected the structure of the polysaccharide (designated WSPA below) isolated with alkali from the mature stem of *P. alkekengi*, and evaluated the adjuvant effect in DNA vaccine. The results showed that WSPA (M_w = 31 kDa) consisted of Rha, Ara, Xyl, Gal, Glc and GalA in ratio of 1.0:2.5:0.8:2.7:4.4:1.4. More importantly, it was shown that WSPA could significantly enhance specific-antibody titers against epitope C (LKVIRK) in IgG, IgG1, IgG2b, and may be used as an adjuvant.

Abbreviations: WSPA, polysaccharide isolated with alkali from the mature stem of *P. alkekengi*; HSP90, heat shock protein 90; pD-HSP90C, a recombinant plasmid containing epitope C (LKVIRK) from HSP90 of *C. albicans*; rP-HSP90C, a recombinant protein containing epitope C (LKVIRK) from HSP90 of *C. albicans*.

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2. Materials and methods

2.1. Materials and chemicals

The mature stems of *P. alkekengi* were collected in Shuangyang district, Chanchun city, Jilin province. 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.

2.2. Isolation and purification of WSPA

In order to remove water-extractable and water-soluble polysaccharide, 500 g of *P. alkekengi* dry stems was extracted with distilled water at 100 °C for 6 times and 3 h for each time; the whole extract was filtered and centrifuged to collect the stems. The stems then extracted with 0.1 mol/L NaOH at 100 °C for 3 times and 3 h for each time; the whole extract was filtered and centrifuged to collect the supernatant. The collected supernatant was added HCl to adjust pH = 7. The supernatant was precipitated with 75% (v/v) ethanol at 4 °C overnight after concentration. The crude polysaccharide was recovered by centrifugation, and dried after washing successively with ethanol (95%) and ether respectively. The polysaccharide (5%, w/v) was frozen at −20 °C, thawed and centrifuged at 10,000 rpm for 20 min to remove insoluble materials. Crude polysaccharide (CP) was precipitated with 95% ethanol. CP was deproteinated by a combination of proteinase and Sevag method. Briefly, CP was added proteinase, dialyzed in distilled water at 37 °C for 48 h, and the concentrated aqueous solution was treated by Sevag reagent (1-butanol:chloroform = 1:4) with oscillation; the supernatant solution was recovered after centrifugation, and retreated with Sevag reagent until there was no free protein.

CP was further purified on a QAE Sephadex A-25 column (3.0 cm × 80 cm) by gradient elution with 0.1–4 mol/L NaCl after eluting with 0.1 mol/LNaCl. The main polysaccharide fraction (Q-WSPA) was collected from gradient elution. To obtain WSPA, Q-WSPA was further purified by High Performance Liquid Chromatography (HPLC). HPLC (Shimadzu, Japan) was equipped with a TSK-GEL G3000 PW_{XL} column (21.5 (ID) × 600 (L) mm) and a RID-10A Refractive Index Detector. HPLC was performed with 0.15 mol/L NaCl as the mobile phase at 0.5 mL/min and 40 °C. WSPA recovered from HPLC was dialyzed and lyophilized for further experiment. The endotoxin level in WSPA solution was less than 0.5 EU (endotoxin unit)/mL. The solution of WSPA was sterilized by 0.22 μm millipore filter for all animal experiments.

2.3. Homogeneity and molecular weight

The homogeneity and molecular weight of WSPA were evaluated by HPLC (Shimadzu, Japan) equipped with a TSK-GEL G3000PW_{XL} column (78 (ID) × 300 mm (L)) and a RID-10A Refractive Index Detector. HPLC was performed with 20 μL WSPA (5 mg/mL) dissolved in distilled water, and 0.2 mol/L NaCl was used as the mobile phase at 0.6 mL/min and 35 °C. The columns were calibrated with T-series Dextran (T-200, T-80, T-40, T-20, and T-10) as standards.

2.4. Analysis of monosaccharide composition

Gas chromatography (GC) was used for identification and quantification of the monosaccharide compositions according to methods from Johnes and Albersheim (1972) and previously reported by Tong, Liang, and Wang (2008) Briefly, WSPA (10 mg) was hydrolyzed with 2 mol/L TFA at 120 °C for 3 h, and the hydrolyzed product was converted into the alditol acetates. 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 30 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.

2.5. Partial acid hydrolysis

Partial acid hydrolysis was performed according to the method described by Sun et al., 2011). WSPA (75 mg) was hydrolyzed with 0.05 mol/L TFA (5 mL) at 95 °C until amount of precipitate remained stable. The hydrolyzed WSPA was centrifugated to recover the precipitate ($WSPA_1$), and the supernatant was dialyzed against distilled water for 48 h after removing the acid by addition of ethanol repeatedly. Ethanol was added to the solution in the dialysis sack after dialysis, and the precipitate and supernatant designated as $WSPA_2$ and $WSPA_3$ respectively were recovered after centrifugation. The fraction out of dialysis sack (WSPA4) was collected by precipitation with ethanol. All fractions ($WSPA_1$, $WSPA_2$, $WSPA_3$, and WSPA4) were dried for GC analysis (Wang, Luo, & Liang, 2004).

2.6. Periodate oxidation and smith degradation

Periodate oxidation and Smith degradation of polysaccharide was carried out as described by Liu and Bhatia (Bhatia, Gupta, & Soni, 2014; Liu & Sun, 2011). WSPA (25 mg) dissolved in 12.5 mL of distilled water were mixed with 12.5 mL of 30 mM $NaIO_4$, and the mixture was kept in darkness at room temperature. 0.1 mL aliquots were withdrawn from the mixture at 4 h interval, diluted (250 times) with distilled water, and optical density (OD) was detected at 223 nm (Dixon & Lipkin, 1954). Ethylene glycol (2 mL) was added to terminate the periodate oxidation reaction when the OD value remained stable. The periodate-oxdized product (2 mL) was used to assess the amount of formic acid by titration with 0.00513 mol/L sodium hydroxide, and the rest was extensively dialyzed against tap water and distilled water for 24 h, respectively. The content inside the dialysis sack was concentrated and reduced with $NaBH_4$, adjusted pH = 7 with 50% acetic acid, and then dialyzed. One-third of the solution mentioned above was freeze-dried and fully hydrolyzed for GC analysis; others were added to the same volume of 1 mol/L sulfuric acid, kept for 40 h at 25 °C, adjusted pH 6.0 with $BaSO_4$, and filtered for analysis by Smith degradation. The filtrate was dialyzed (molecular weight cut-off of 3 kDa), and the content out of dialysis sack was desiccated for GC analysis; the content inside the dialysis sack was precipitated with ethanol, the supernatant and precipitate were also dried out for GC analysis after centrifugation.

2.7. Carboxyl-group reduction

Periodate oxidation and smith degradation was carried out based on the methods of Tong et al. (2008). WSPA (30 mg) dissolved in 27.5 mL of distilled water was mixed with 750 mg of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulphonate (CMC) and incubated at room temperature for 3 h at pH 4.75 adjusted by adding 0.01 mol/L HCl constantly. 2.0 mol/L $NaBH_4$ (15 mL) was added slowly during the next 2 h, and pH of the mixture was maintained at 7.0 with adding 4.0 mol/L HCl. Then, the reaction mixture was dialyzed against distilled water. The dialyzate was lyophilized, and carboxyl-group reduced product was recovered and analyzed by GC–MS.

2.8. Methylation analysis

WSPA (20 mg) and reduced WSPA (20 mg) were methylated twice according to the method of Luo (2014). The methylated products were extracted by chloroform. No absorption peak in the region 3600–3300 cm^{-1} was detected by IR spectrum and this indicated that it was completely methylated. The product was hydrolyzed with formic acid and 2 mol/L TFA, and excess acid was evaporated by co-distillation with distilled water. The hydrolyzed product was reduced with $NaBH_4$ for 24 h, and acetylated with acetic anhydride–pyridine (1:1) at 100 °C for 2 h. The alditol acetates of the methylated sugars were analyzed by GC–MS.

2.9. Preparation of recombinant DNA vaccine

Our lab has constructed DNA vaccine pD-HSP90C which was a recombinant plasmid containing epitope C (LKVIRK) from HSP90 of *C. albicans*. It was found that pD-HSP90C can evoke production of specific antibody against epitope C (LKVIRK) in mice (Yang et al., 2014). Briefly, DNA vaccine pD-HSP90C and pcDNA3.1 were transformed to *E. coli.* DH5α, and purified for vaccination with EndoFree Plasmid Mega kit (Tiangen, China).

2.10. DNA vaccine Immunization and electroporation in vivo

The male ICR mice (6 mice per group) were immunized with either pD-HSP90 (10 μg/mice) alone or in presence of WSPA (5, 10, 20 μg/mice) twice at a 14-day interval. PBS and pcDNA3.1 (10 μg/mice) were used as controls. The mice were immunized intramuscularly in tibialis anterior muscle followed by electroporation with two-needle array electrodes (BTX, San Diego, CA) (Widera et al., 2000). Electroporation parameters in vivo were 20 V/mm distance between the electrodes; 50-ms pulse length; 6 pulses with reversal of polarity after 3 pulses, at 1, given by a BTX 820 square were genetor. The mice were sacrificed two weeks after the second immunization, and the immunized mice sera were collected to detect the specific antibody against epitope C (LKVIRK) by ELISA and Western blot.

2.11. Preparation of recombinant protein rP-HSP90C

We previously constructed the recombinant protein rP-HSP90C (M_w = 34 Da) containing epitope C (LKVIRK) from HSP90 of *C. albicans*. It was found that recombinant protein rP-HSP90C can be an antigen to analyze specific antibody against epitope C (LKVIRK) due to its antigenicity (Yang et al., 2014). In present study, the recombinant protein rP-HSP90C was used as an antigen for analysis of specific antibody against epitope C (LKVIRK) by ELISA and Western blot. Briefly, rP-HSP90C was expressed in *E. coli* BL21 (DE3) by pET28a-HSP90C under the control of the T7 promoter and lac operator. rP-HSP90C was purified by NiNTA agarose column (Qiagen).

2.12. Western blot

The recombinant protein rP-HSP90C was run SDS-PAGE on 13% polyacrylamide gel. Then, it was transferred onto nitrocellulose membrane in buffer (25 mM Tris, 192 mM glycin, 20% methanol, pH 8.3) after electrophoresis, and was later blocked at 4 °C overnight with buffer (150 mM NaCl, 10 mM Tris, 3 (w/v)% skim milk, 0.05% Tween 20, pH 7.5). The nitrocellulose membrane with transferred proteins was incubated with sera from immunized mice, patient sera, and healthy sera at 37 °C for 2 h after being washed three times in buffer (150 mM NaCl, 10 mM Tris–HCl, 0.05% Tween 20, pH 7.5). All sera were diluted to 1/40 when used. After being washed, the nitrocellulose membrane was incubated with peroxidase-conjugted goat anti-mice or goat anti-human HRP-IgG (Vector Laboratories Inc., USA) and stained with 3-amino-9-ethylcarbozole (AEC) to detect conjugated protein bands (Yang et al., 2014).

The human sera mentioned above were from the First Clinical Hospital of Jilin University, Changhun, China. Permissions of using sera were obtained from The Regional Ethical Review Board of Jilin University and Northeast Normal University. Written consents were obtained from patients infected with systemic candidiasis and healthy people.

2.13. Measurement of specific antibody in sera of immunized mice

rP-HSP90C-specific antibodies (IgG, IgG1, and IgG2b) in sera of the immunized mice were evaluated by an indirect ELISA based on the methods of Yang et al. (2005). The indirect ELISA is used primarily to determine the antibody titers in the serum of an immunized animal. Briefly, microtiter plates (Nunc) were coated with 100 μL rP-HSP90C (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 the wells with PBS containing 0.05% (v/v) Tween 20 three more times, 100 μL of a series of diluted sera from immunized mice or PBS containing 5% skim milk powder were added to the triplicate wells as control. The plates were then incubated for 1 h at 37 °C, followed by being washed 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 being washed with PBST, 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 (Molecular devices, spectra MAX190, USA). The end point titer was used in this study. Antibody titer was expressed by Log_2 value of the highest dilution of serum that gave an absorbance value which exceeded an optical density of 0.05 and that was twofold greater than that of a matched dilution of sera from PBS-injected mice (Yang et al., 2014).

2.14. Statistical analysis

Data were expressed as mean ± SD. One-way ANOVA (SPSS software) was used in analyzing all the other data. *P* value of less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Isolation, purification and structural analysis of WSPA

The yield of the total crude water-soluble polysaccharide extracted with alkali from the stem of *P. alkekengi* was 3.4% of the dry material. The contents of carbohydrate and proteins in crude polysaccharide were 19%, 9% respectively. After crude polysaccharide was deproteinated by a combination of proteinase and Sevag method, the main polysaccharide fraction Q-WSPA was collected from crude polysaccharide purified on a QAE Sephadex A-25. To obtain WSPA, Q-WSPA was further purified by HPLC.

WSPA had a negative response to Bradford test, and no absorption at 280 or 260 nm in the UV spectrum further indicated the absence of protein and nucleic acid (Liu & Sun, 2011). HPLC analysis demonstrated molecular weight of WSPA (31 kDa) and its

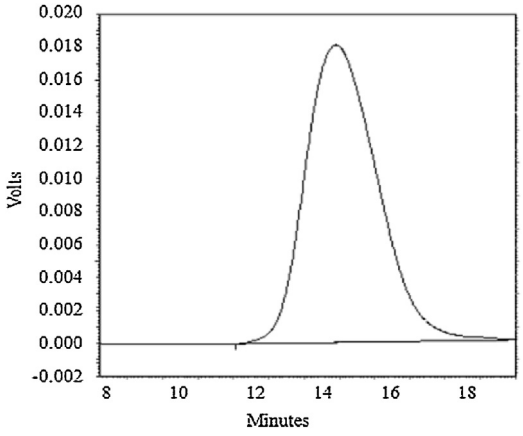

Fig. 1. HPLC Profile of WSPA.

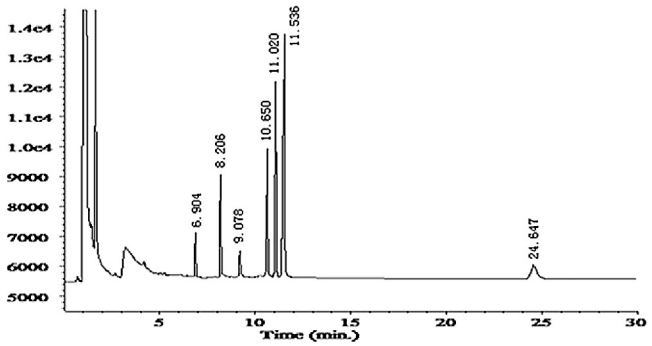

Fig. 2. GC profile of WSPA. Peaks from left to right: Rha, Ara, Xyl, Gal, Glc, Internal Standard, and GalA.

homogeneity (Fig. 1). The total carbohydrate of WSPA was 96.0% determined by phenol–sulfuric acid assay. Analysis of GC indicated that WSPA was an acid heteropolysaccharide, which consists of Rha, Ara, Xyl, Gal, Glc and GalA in ratio of 1.0:2.5:0.8:2.7:4.4:1.4 (Fig. 2).

A typical major broad stretching peak around 3424.06 cm^{-1} in IR spectrum of WSPA (Fig. 3) revealed the hydroxyl group, and a weak band at 2934.25 cm^{-1} showed the C–H stretching vibration. The broad band at 1631.64 cm^{-1} was due to the bound water. The band at 862.76 cm^{-1} indicated there were α-configurations in WSPA (Sun et al., 2010).

All the fractions $WSPA_1$ (precipitation after hydrolysis), $WSPA_2$ (precipitation in the dialysis sack), $WSPA_3$ (supernatant in the dialysis sack), and $WSPA_4$ (fraction out of dialysis sack) were recovered

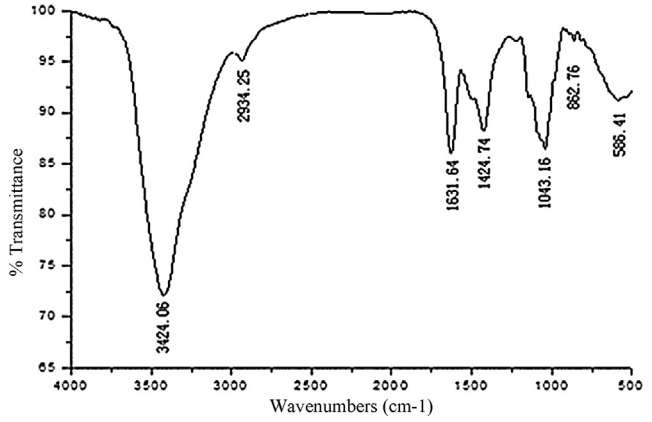

Fig. 3. IR Spectrum of WSPA.

Table 1
GC analysis for fractions from partial acid hydrolysis.

Fractions	Molar ratios					
	Ara	Xyl	Rha	Glc	Gal	GalA
WSPA1 [a]	2.8	2.2	2.7	3.9	3.5	1.0
WSPA2 [b]	–	–	–	+	–	–
WSPA3 [c]	1.7	1.0	2.4	4.3	4.2	tr
WSPA4 [d]	2.0	1.0	2.6	3.3	4.4	1.6

tr, trace.
[a] Precipitation after hydrolysis.
[b] Precipitation in the sack.
[c] Supernatant in the sack.
[d] Fraction out of sack.

after partial acid hydrolysis of WSPA, and subjected to GC analysis. Results shown in Table 1 indicated that backbone and branches of WSPA were composed of Glc, Gal, Xyl, Ara, and Rha. However, GalA was only distributed in the backbone of WSPA.

Results from periodate oxidation showed 0.882 mol periodate was consumed and 0.124 mol formic acid was produced per sugar residue, indicating the presence of monosaccharides which are 1→ linked or (1 → 6)-linked. The amount of consumed periodate (0.882 mol) was more than twice the yield of generated formic acid (0.124 mol × 2) indicating the existence of large amounts (1 → 4) or (1 → 2)-linked sugar residue.

The periodate-oxidized WSPA were fully hydrolyzed and analyzed by GC analysis (Table 2). The presence of Glc, Gal, GalA revealed some residues of Glc, Gal, GalA were (1 → 3)-linked, (1 → 2,3)-linked, (1 → 2,4)-linked, (1 → 3,4)-linked, (1 → 3,6)-linked or (1 → 2,3,4)-linked that can not be oxidized. The presence of Ara, Xyl indicated some residues of Ara, Xyl were (1 → 3)-linked, (1 → 2)-linked, (1 → 2,4)-linked, (1 → 3,5)-linked, (1 → 2,5)-linked that can not be oxidized. Gly was obtained demonstrating the presence linkages of (1 →)-linked, (1 → 2)-linked, (1 → 6)-linked, (1 → 2, 6)-linked. No erythritol was found revealing there were no linkages of (1 → 4)-linked, (1 → 4, 6)-linked. No EryA was detected indicating there were (1 → 3) or (1 → 2, 3) linkages in GalA. No Rha was observed, demonstrating that all Rha linkages can be oxidized by periodate.

The fully methylated WSPA and reduced WSPA were hydrolyzed with acid, converted into alditol acetates, and analyzed by GC–MS. The results indicated both WSPA (Table 3) and reduced WSPA were composed of seven components, including 3,5-Me_2-Ara, 3,4-Me_2-Rha, 2,3,4,6-Me_4-Glc, 3,5-Me_2-Xyl, 2,4,6-Me_3-Glc, 2,4,6-Me_3-Gal, 3,4-Me_2-Gal. The more difference in molar ratio of 2,4,6-Me_3-Gal between WSPA and reduced WSPA (Table 3) suggested linkage of GalA is (1 → 3)-linkage.

The results from analysis of GC-MS were consistent with the results from partial acid hydrolysis, Periodate oxidation, Smith degradation, and monosaccharide composition analyzed by GC. The structural analysis indicated backbone and branches of WSPA were composed of (1 → 3)-linked Glc, (1 → 3)-linked Gal, (1 → 2)-linked Xyl, (1 → 2)-linked Ara, (1 → 2)-linked Rha residues. However, all of GlA was distributed in the backbone of WSPA. All branches of WSPA were at O-2 of (1 → 6)-linked Gal and terminated with Glc.

Table 2
GC analysis for periodate-oxidized products and Smith-degradation of WSPA.

Fractions	Molar ratios							
	Glycerol	Erythritol	Ara	Xyl	Rha	Glc	Gal	GalA
Full acid hydrolysis	3.7	–	1.9	1.2	–	3.3	1.0	1.5
Smith-degradation								
Out of sack	3.6	–	2.0	1.0	–	2.9	1.3	–
Supernatant in sack	–	–	1.7	1.0	–	3.1	1.5	tr
Precipitation in sack	–	–	1.9	1.0	–	2.1	1.5	1.3

Table 3
GC–MS analysis of WSPA and reduced WSPA.

Retention time (min)	Methylated sugars	Mass fragments (m/z)	Type of linkage	Molar ratio	
				WSPA	Reduced WSPA
11.810	3,5-Ara	43.45.71.87.101.129.161.189	1 → 2	2.78	2.81
13.055	3,4-Rha	43.87.89.99.129.131.189	1 → 2	1.00	1.00
13.268	2,3,4,6-Glc	43.45.71.87.101.117.129.145.161.205	1 →	1.57	1.44
14.248	3,5-Xyl	43.45.71.87.101.129.161.189.	1 → 2	0.94	0.95
14.764	2,4,6-Glc	43.45.87.101.117.129.161	1 → 3	3.41	3.52
15.052	2,4,6-Gal	43.45.87.101.117.129.161	1 → 3	1.69	3.17
16.865	3,4-Gal	43.87.99.129.189	1 → 2,6	0.73	0.84

3.2. DNA vaccine pD-HSP90C evoked production of specific antibody against epitope C (LKVIRK) in mice

In order to detect whether DNA vaccine pD-HSP90C evoked specificity of antibody against epitope C (LKVIRK), sera of the immunized mice were used to analyze the specificity of antibody against rP-HSP90C by Western blot. The sera from patients infected with systemic *C. albicans* and healthy people were used as the positive and negative controls respectively.

Western blot (Fig. 4) indicated that sera from the mice immunized with pD-HSP90C (M3), pD-HSP90C/WSPA-5 μg (M4), pD-HSP90C/WSPA-10 μg (M5), and pD-HSP90C/WSPA-20 μg (M6) recognized recombined protein rP-HSP90C, which patient's sera (P) also recognized. However, the sera of mice immunized with PBS (M1), pcDNA3.1 (M2), and healthy sera (H) did not recognize rP-HSP90C. It was clear that pD-HSP90C evoked the production of specific antibody against epitope C (LKVIRK) in mice.

3.3. WSPA enhanced specific antibody titers against rP-HSP90C in sera of mouse immunized with DNA vaccine pD-HSP90C

It has been reported that both humoral and cellular responses are fundamental to control the systemic Candida infections (Van de Veerdonk, Kullberg, & Netea, 2012). The ideal adjuvant is capable of strongly eliciting both humoral and cellular immune responses (both Th1 and Th2 responses) to maximize the efficacy of vaccines (Petrovsky, 2006; Silva, Cooper, & Petrovsky, 2004). 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 (Sun, 2006). Therefore, IgG2b and IgG1 in sera of immunized mice were detected to characterize the immune response in ICR mice immunized with DNA vaccine and WSPA.

Sera of the immunized mice were used to analyze rP-HSP90C-specific antibody titers (IgG, IgG1 and IgG2b) using indirect ELISA. It was shown (Fig. 5) that IgG, IgG1, and IgG2b antibody titers in pD-HSP90C-immunized mice were significantly different compared to pcDNA3.1 ($p < 0.05$). More importantly, IgG, IgG1, and IgG2b antibody titers in pD-HSP90C-immunized mice were greatly augmented by WSPA-10 or 20 μg ($p < 0.05$). The data demonstrated

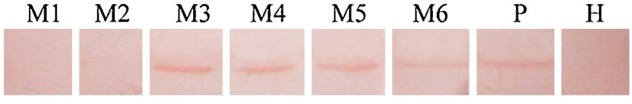

Fig. 4. Western blot analysis of recombinant protein rP-HSP90C using sera of immunized mice. rP-HSP90C was submitted to SDS-PAGE (12%), then transferred to nitrocellulose membrane and incubated with sera from the immunized mice, patient and healthy people. Sera from the mice immunized with pD-HSP90C (M3), pD-HSP90C/WSPA-5 μg (M4), pD-HSP90C/WSPA-10 μg (M5), and pD-HSP90C/WSPA-20 μg (M6) recognized rP-HSP90C. However, sera from the mice immunized with PBS (M1) and pcDNA3.1 (M2) did not show rP-HSP90C. Patient sera (P) recognized rP-HSP90C while healthy sera (H) did not recognize rP-HSP90C.

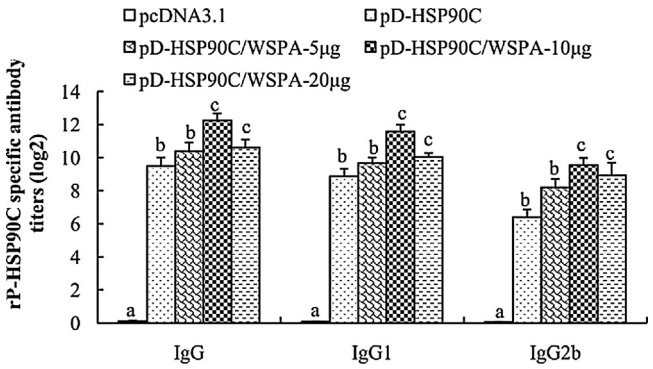

Fig. 5. Effect of WSPA on rP-HSP90C-specific antibody titer IgG, IgG1 and IgG2b in sera of mouse immunized with DNA vaccine pD-HSP90C. ICR mice (6 mice per group) were immunized intramuscularly followed by electroporation with pD-HSP90C in the absence or presence of WSPA (pcDNA3.1, and PBS used as controls) twice at a 14-day interval. The mice were sacrificed two weeks after the second immunization. The immunized mice sera were collected to detect the titer of specific antibody against rP-HSP90C by ELISA. The values were present as mean ± SD.

that WSPA at suitable dose significantly enhanced specific antibody IgG response with higher titers of IgG1 as well as IgG2b in mice immunized with pD-HSP90C. The results suggest that WSPA may be considered as a potential adjuvant candidate for DNA vaccine.

4. Conclusion

This study demonstrated that WSPA (M_w = 31 kDa) was an acid heteropolysaccharide, which consists of Rha, Ara, Xyl, Gal, Glc and GalA in ratio of 1.0:2.5:0.8:2.7:4.4:1.4. The structural analysis indicated backbone and branches of WSPA were composed of (1 → 3)-linked Glc, (1 → 3)-linked Gal, (1 → 2)-linked Xyl, (1 → 2)-linked Ara, (1 → 2)-linked Rha residues. GlA was distributed completely in the backbone of WSPA. All branches of WSPA were at O-2 of (1 → 6)-linked Gal and terminated with Glc. Preliminary pharmacological assays suggested that WSPA at suitable dose was effective on Th1 and Th2 response. Thus, WSPA may be a potential adjuvant in DNA vaccine.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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