Carbohydrate Polymers 89 (2012) 1117–1122

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Immuno-enhancing activity of sulfated *Auricularia auricula* polysaccharides

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ARTICLE INFO

Article history:
Received 16 December 2011
Received in revised form 22 March 2012
Accepted 24 March 2012
Available online 2 April 2012

Keywords:
Auricularia auricula polysaccharides
Sulfation modification
Lymphocyte proliferation
Antibody titer

ABSTRACT

The crude total *Auricularia auricula* polysaccharide (AAP_{ct}) was extracted by water decoction and ethanol precipitation, protein was removed to obtain total *A. auricula* polysaccharide (AAP_t), then was graded into AAP_1 and AAP_2 through column chromatography. $sAAP_t$, $sAAP_1$ and $sAAP_2$ were prepared by chlorosulfonic acid-pyridine method. In vitro test, the effects of $sAAP_t$, $sAAP_1$, $sAAP_2$, AAP_t, AAP_1 and AAP_2, on chicken peripheral lymphocytes proliferation were compared. The results showed that $sAAP_t$ and $sAAP_1$ demonstrated better effect. In vivo test, 14-day-old chickens were injected respectively with $sAAP_t$, $sAAP_1$, AAP_t and AAP_1 at the first vaccination of ND vaccine, once a day for three days. On days 7, 14, 21 and 28 after the first vaccination, the peripheral lymphocytes proliferation and antibody titer were determined. The results indicated that $sAAP_t$ possessed the best efficacy and would be expected to be used as a component of a new-type immunopotentiator.

1. Introduction

Auricularia auricula is a precious colloid fungus used as medicine and food in China. *A. auricula* polysaccharide (AAP) is an important active component of *A. auricula*. The fruit body of *A. auricula* contains rich polysaccharides that consists of a backbone D-glucose residue with various chains of β-1,3-branch residues, such as mannose, glucose, xylose and glucuronic acid, and which is composed of a main chain of (1 → 4)-linked D-glucopyranosyl with branching points at O-6 of (1 → 6)-linked D-glucopyranosyl residues (Ma, Wang, & Zhang, 2008; Ma, Wang, Zhang, Zhang, & Ding, 2010; Zhang, Wang, Yang, et al. 2011). In recent years, AAP was found to have many biological activities, such as antioxidant activity, anticoagulant, anti-tumor, hypolipidemic activity, lowering blood glucose and so on (Brown & Williams, 2009; Fan, Zhang, Yu, & Ma, 2007; Luo et al., 2009; Misaki, Kakuta, Sasaki, Tanaka, & Miyaji, 1981; Song & Du, 2012; Yang et al., 2011; Yoon et al., 2003; Zhang, Wang, Zhang, & Wang, 2011). Increasing attention has therefore been paid to its exploitation and utilization.

In present research, the crude total AAP (AAP_{ct}) was extracted by water decoction and ethanol precipitation method, then purified in turn by removing proteins to obtain total AAP (AAP_t). Column chromatography was used to grade AAP_t into AAP_1 and AAP_2. Three sulfated AAP (sAAP): ($sAAP_t$, $sAAP_1$ and $sAAP_2$), were prepared by chlorosulfonic acid–pyridine method. The effects of $sAAP_t$, $sAAP_1$ and $sAAP_2$ and three non-sulfated AAP_t, AAP_1 and AAP_2 on peripheral lymphocytes proliferation in vitro and in vivo, and their effects on serum HI antibody titer in the chickens vaccinated with ND vaccine were determined. The purpose of this research was to evaluate the immune-enhancing capacity of AAP following sulfation modification, select the best sulfate polysaccharide and offer theoretical evidence for development of a new-type immunopotentiator.

2. Material and methods

2.1. The A. auricula and regents

The black fungus was produced in northeast of China and purchased from a Carrefour supermarket in Nanjing city, Jiangsu province, PR China.

Chlorosulfonic acid (CSA) was purchased from Shanghai Lingfeng Chemical Reagent Ltd., Pyridine (Pyr) was purchased from Shanghai Hengxin Chemicals Ltd. The DEAE-Sephadex A-25 and Sephadex-G200 were purchased from Shanghai Lingfeng Chemical Reagent Ltd. Dimethyl sulfoxide (DMSO, No. 090601) was

Abbreviations: AAP, *Auricularia auricula* polysaccharide; sAAP, sulfated AAP; CEF, chicken embryo fibroblast; ConA, concanavalin A; CSA, chlorosulfonic acid; Pyr, pyridine; HI, hemagglutination inhibition; PHA, phytohemagglutinin; NDV, Newcastle disease virus; ND, Newcastle disease; CMF-PBS, calcium and magnesium-free phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; NA, no adjuvant; BC, blank control.

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http://dx.doi.org/10.1016/j.carbpol.2012.03.082

produced by Kemiou Institute of Chemical Engineering in Tianjin. Lymphocyte separation medium (No. 090818) was manufactured by Shanghai Huajing Biology Inc. All other chemicals used were analytical grade.

RPMI-1640 (GIBCO) with the supplement of 100 IU mL^{-1} benzylpenicillin, 100 IU mL^{-1} streptomycin and 10% fetal bovine serum was used for washing and re-suspending cells, diluting mitogen and culturing the cells. Phytohemagglutinin (PHA, Sigma, No. L8754), as a T-cell mitogen, was dissolved into 0.1 mg mL^{-1} with RPMI-1640. Hanks' solution was used for diluting blood. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, American Co.) was dissolved into 5 mg mL^{-1} with calcium and magnesium-free (CMF) phosphate-buffered saline (PBS, pH 7.4). Sodium heparin was dissolved into 5 mg mL^{-1} with PBS. These reagents were filtered through a 0.22 μm millipore membrane filter. PHA and sodium heparin solution were stored at −20 °C, MTT solution was kept at 4 °C in a dark bottle, RPMI-1640 were stored at 4 °C.

ND vaccine (La Sota strain, No. 091288) was purchased from Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Science.

2.2. Preparation of sAAP

2.2.1. Extraction of AAP

1000 g of *A. auricula* powder was decocted with 30-fold water three times for 1.0 h per time (Shi, Yue, Xian, & Zhang, 2009), and condensed into decoction corresponding to 1 g material medica per milliliter. The crude total AAP (AAP_{ct}) was extracted by one-step ethanol precipitation and 95% ethanol was added into the above-mentioned decoction to make ethanol concentration reach to 80% (v/v) (Chen, Zhou, & Yin, 2003; Wang & Li, 2010). After 24 h, the precipitation was dried at 60 °C and 37.5 g AAP_{ct} was obtained. Its polysaccharide content was 35.28% determined by phenol-sulfuric acid method (Li & Wang, 2008; Yu, Yang, Liu, & Liu, 2009)

2.2.2. Purification of AAP

The proteins in AAP_{ct} were removed by trichloroacetic acid method (Fan, Zhang, & Xia, 2004; Guo, Yan, Zhang, & Wu, 2001; Wang, Peng, Huang, & Tian, 2001). Briefly, 11 g AAP_{ct} was dissolved in 110 mL of distilled water on 60 °C water bath. The pH of the solution was regulated to 7 with 10% NaOH solution, 3% trichloroacetic acid added up to 7.5% (v/v), placed for 4 h at 4 °C, then centrifuged for 20 min at 3000 rpm. Ethanol was added to the supernatant up to 80% (v/v), placed for 24 h and centrifuged. The precipitation was dissolved with deionized water, the tannin was removed through polyamide adsorption column (Zheng, 2000), then dialyzed in dialysis sack against tap water for 24 h and distilled water for 48 h and lyophilized to obtain the pure AAP_t, with the polysaccharide content of 60.92%. Further fractionation was performed using column chromatography (Zhang, Wang, & Zhang, et al. 2011). The solution of l% AAP_t was added into a column (1.6 cm × 60 cm) of DEAE Sephadex A-25 cellulose column chromatography. Fractions were prepared in stepwise elution with increased ionic strength of NaCl (0.1, 0.2, and 0.3 mol/L^{-1}) at a flow rate of 0.5 mL/min. Two elution peaks were collected and further purified respectively by gel filtration chromatography on a Sephadex-G200 column (1.6 cm × 60 cm). The major fractions obtained were concentrated and dialyzed against deionized water with dialysis sack, concentrated and lyophilized to obtain two purified AAPs coded as AAP_1 and AAP_2. Their polysaccharide contents were 25.97% and 12.57%, respectively.

2.2.3. Sulfation modification of AAP

Three sAAPs ($sAAP_t$, $sAAP_1$ and $sAAP_2$) were prepared by chlorosulfonic acid-pyridine method with the same modified conditions as the reference (Li, 2006; Li & Wang, 2008): the ratio of chlorosulfonic acid to pyridine of 5:1, reaction temperature of 50 °C and reaction time of 1.5 h. Their polysaccharide contents were 23.74%, 12.60% and 10.14%, respectively, and their degrees of substitution (DS) determined by Antonopoulos' method (Dodgson & Price, 1962; Zhang, Wang, Yang, et al. 2011) were 0.22, 1.46, and 1.19, respectively.

For in vitro test, three sulfated $sAAP_t$, $sAAP_1$ and $sAAP_2$ and three non-sulfated AAP_t, AAP_1 and AAP_2 were diluted into 1 mg mL^{-1} with PBS. For in vivo test, AAP_t, AAP_1, $sAAP_t$ and $sAAP_1$ were into 4 mg mL^{-1} diluted with deionized water. The diluted preparations were sterilized by pasteurization and detected for endotoxin by pyrogen tests (Veterinary Pharmacopoeia commission of the People's Republic of China, 2000). When the endotoxin amount was up to the standard of Chinese Veterinary Pharmacopoeia (less than 0.5 EU mL^{-1}), they were stored at 4 °C for the test (Kong, Hu, Rui, Wang, & Li, 2004).

2.2.4. Infrared spectroscopy analysis

An FT-IR spectroscopy analysis was used to investigate the vibrations of molecules and polar bonds between the different atoms. It is possible to analyze structures of polysaccharides such as monosaccharide types, glucosidic bonds, and functional groups using an FT-IR spectroscopy (Yang & Zhang, 2009; Zhang, Wang, & Yang, et al. 2011). 1.5–2 mg of AAP or sAAP dried and finely ground were mixed with 100 mg of KBr and compressed into pellets. Then the FT-IR spectra was recorded in a wavenumber range of 4000–400 cm^{-1} with a Nicolet 200 Magna-IR spectrometer (Nicolet Instrument Corp). In order to obtain accurate FT-IR spectra, 200 scans were collected. The major peaks (intensity and wavenumber) were found using OMNIC software (Nicolet Instruments Corp.). The spectra of the polysaccharides were calculated using the OMNIC software (Li & Wang, 2008; Wu et al., 2010; Yang et al., 2011).

2.3. In vitro test

Safe concentrations of three sAAPs and three non-sulfated AAPs for chicken embryo fibroblast (CEF) were measured by the MTT assay (Tang, Hu, Zhang, & Song, 1998). The result showed that the A_{570} values of AAP_t at 250 μg mL^{-1}, AAP_1 at 15.63 μg mL^{-1}, AAP_2 and $sAAP_t$ at 3.91 μg mL^{-1}, $sAAP_1$ and $sAAP_2$ at 7.81 μg mL^{-1} group were not significantly lower than those of corresponding cells control group. Therefore these concentrations could be considered as their maximal safe concentrations. In order to make the comparison at the same level, their maximal safe concentrations were supposed to be as 3.91 μg mL^{-1}.

Three $sAAP_s$ and three AAP_s were dissolved with RPMI-1640 into five concentrations from 3.91 to 0.244 μg mL^{-1} based on above-mentioned determination result of safety concentration. The chicken blood samples (5 mL) were collected from the heart and transferred immediately into aseptic capped tubes with sodium heparin, diluted with an equal volume of Hanks' solution and carefully added on the surface of lymphocytes separation medium. After 20 min of centrifugation at 2000 rpm, a white cloud-like lymphocytes band was collected and washed twice with RPMI 1640 media without fetal bovine serum. The resulting pellet was re-suspended into 2.5×10^6 mL^{-1} with RPMI 1640 media and divided into two parts, one part was added with PHA (final concentration reaching to 10 μg mL^{-1}), and respectively inoculated into 96-well culture plates, 100 μL per well. Then, in polysaccharide groups the six polysaccharides at series of concentrations were added, in the cell and PHA control groups, RPMI 1640 media and PHA respectively, 100 mL per well, four wells for each concentration.

The plates were incubated at 39.5 °C in a humid atmosphere of 5% CO_2, then according to MTT assay the cellular A_{570} values were determined as the index of peripheral T lymphocytes proliferation,

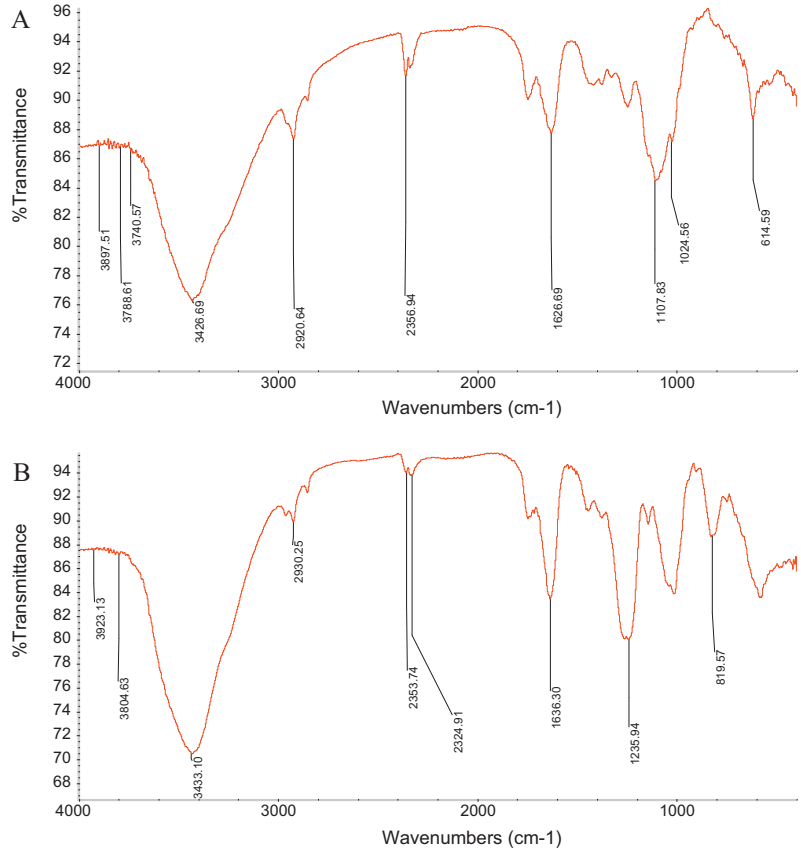

Fig. 1. Infrared (FT-IR) spectra of AAP_t (A) and $sAAP_t$ (B).

and the cellular proliferation rate was calculated to compare the strength of lymphocytes proliferation according to the equation: Proliferation rate (%) = ($\bar{A}_{570}$ value of polysaccharide group − $\bar{A}_{570}$ value of cell or PHA control group)/$\bar{A}_{570}$ value of cell or PHA control group) × 100% (the $\bar{A}$ was the average value of five concentration groups of polysaccharide or four wells of control group) (Abula et al., 2011; Tang et al., 1998; Verma et al., 2010; Yu, Jiang, Yan, & Zhu, 2004).

2.4. In vivo test

2.4.1. Experimental animals

One-day-old White Roman chickens (male), purchased from Tangquan Poultry Farm, were housed in wire cages (80 cm × 100 cm) in air-conditioned rooms at 37 °C with the light period of 24 h at the beginning of pretrial period. The temperature was gradually reduced to the room temperature and the light time to 12 h per day, which were kept constant in the subsequent days. The chickens were fed with the commercial starter diet provided by the feed factory of Jiangsu Academy of Agricultural Science.

2.4.2. Experimental design

One hundred and eighty chickens were randomly divided into six groups, sAAPt, sAAP1, AAPt, AAP1, no adjuvant (NA) and blank control (BC) group. At 14 days old, their average maternal antibody titer was 3.2 log 2, the chickens except blank control group were vaccinated with Newcastle disease vaccine and the exercise repeated on the 28 day old. At the same time of the first vaccination, the chickens in groups 1–4 were intramuscularly injected respectively with 0.5 mL of corresponding polysaccharides, in NA and BC group, with 0.5 mL of physiological saline, once a day for three successive days. On days 7 (D_7), 14 (D_{14}), 21 (D_{21}) and 28 (D_{28}) after the first vaccination, four chickens were sampled randomly from each group for determination of peripheral lymphocytes proliferation by MTT assay and six chickens were sampled randomly from each group for examination of serum hemagglutination inhibition (HI) antibody titer by micro-method (Abula et al., 2011; Tang et al., 1998; Thekisoe, Mbati, & Bisschop, 2004).

2.4.3. Serum HI antibody assay

Blood samples (1.0 mL per chick) were drawn into Eppendorf tubes from the brachial vein of chicken and allowed to clot at 37 °C for 2 h. The serum was separated by centrifugation and stored at −20 °C for HI antibody assay. Briefly, after the serum was inactivated at 56 °C for 30 min, twofold serial dilution were made in a 96-well V-shaped bottom microtiter plate containing 50 μL of CMF-PBS in each well, then 50 μL of NDV antigen (4 HA units) was added into all the wells except for the last row which was the control. Serum dilutions ranged from 1:2 to 1:2048. The plate was incubated at 37 °C for 20 min, then 50 μL of 1% rooster erythrocytes suspension was added to each well and continued to incubate for 30 min. A positive serum, a negative serums, erythrocytes and antigens were also included as controls. The highest dilution of serum which caused complete inhibition was considered as the endpoint. The geometric mean titer was expressed as reciprocal $\log_2$ values of the highest dilution that displayed HI (Abula et al., 2011; Barta, Barta, & Pierson, 1992; Thekisoe et al., 2004).

2.5. Statistical analysis

Data are expressed as means ± SD. Duncan's multiple range tests was used to determine the difference among groups. Significant differences were considered at $P < 0.05$.

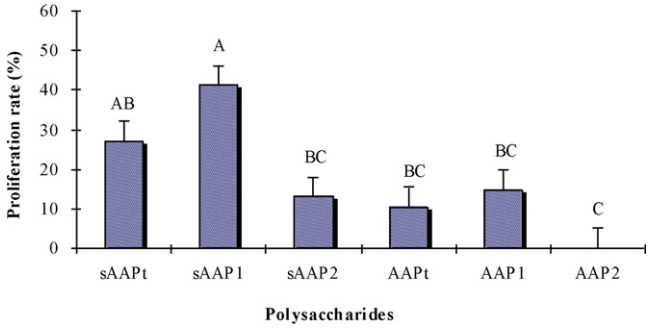

Fig. 2. The lymphocyte proliferation rate of every group in single stimulation of AAPs. Bars marked without the same letters (A–C) differ significantly ($P < 0.05$).

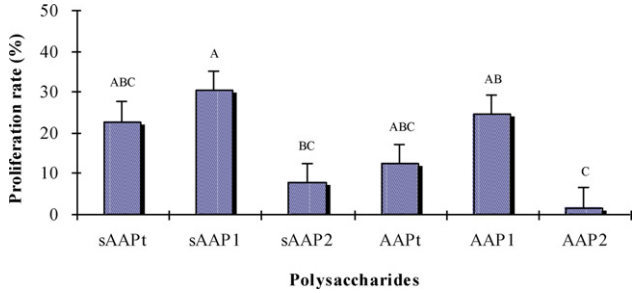

Fig. 3. The lymphocyte proliferation rate of every group in synergistical stimulation of AAPs with PHA. Bars marked without the same letters (A–C) differ significantly ($P < 0.05$).

3. Results

3.1. The Infrared spectroscopy characteristic of sAAP

The FT-IR spectra of AAP_t and $sAAP_t$ in the ranges of 4000 to 400 cm^{-1} are illustrated in Fig. 1(A and B). In the spectra of AAP_t and $sAAP_t$, the band in the region of 3600–3200 cm^{-1} corresponds to the hydroxyl stretching vibration and in the region of 3000–2800 cm^{-1} the band corresponds to a weak C—H stretching vibration. The bands attributed to C—O—C stretching vibrations appeared at about 1400–1000 cm^{-1}. This amalgamation indicates that the AAP_t and $sAAP_t$ were polysaccharides. In comparison with the spectrogram of AAP_t, the FT-IR spectroscopy of $sAAP_t$ showed two characteristic absorption bands, one at 1235 cm^{-1} describing an asymmetrical S=O stretching vibration and the other at 819 cm^{-1} representing a symmetrical C—O—S vibration associated with a C—O—SO_3 group, which signifies that $sAAP_t$ were successfully sulfated.

3.2. The lymphocyte proliferation in single stimulation of sAAP

The A_{570} values of every group are listed in Table 1. The A_{570} values of $sAAP_t$ and $sAAP_1$ at 1.953-0.244 μg mL^{-1} groups, $sAAP_2$ at 0.244 μg mL^{-1} group and AAP_t and AAP_2 at 0.488 μg mL^{-1} groups were significantly higher than those of corresponding cell control group ($P < 0.05$).

The lymphocyte proliferation rates of every group are illustrated in Fig. 2. During the single stimulation, the lymphocyte proliferation rate of $sAAP_1$ group was the highest (41.25%) as compared to those of AAP_1 (14.75%), $sAAP_2$ (12.92%), AAP_t (10.49%) and AAP_2 (0.15%) groups ($P < 0.05$).

3.3. The lymphocyte proliferation in synergistical stimulation of sAAP with PHA

The A_{570} values of every group are listed in Table 2. The A_{570} values of $sAAP_1$ at 1.953–0.244 μg mL^{-1}, $sAAP_t$ and $sAAP_2$ at 0.244 μg mL^{-1} groups were significantly higher than those of corresponding PHA control group ($P < 0.05$).

The lymphocyte proliferation rates of every group were illustrated in Fig. 3. During synergistical stimulation of AAP and sAAP with PHA, the lymphocyte proliferation rate of $sAAP_1$ group (30.31%) was the highest and significantly higher than those of AAP_2 (1.68%) and $sAAP_2$ (12.34%) groups ($P < 0.05$). The rates of the following groups were: AAP_1 (24.42%), $sAAP_t$ (22.66%), AAP_t (12.37%) and $sAAP_2$ (7.69%). The lymphocyte proliferation rate of AAP_2 group was the lowest (1.68%).

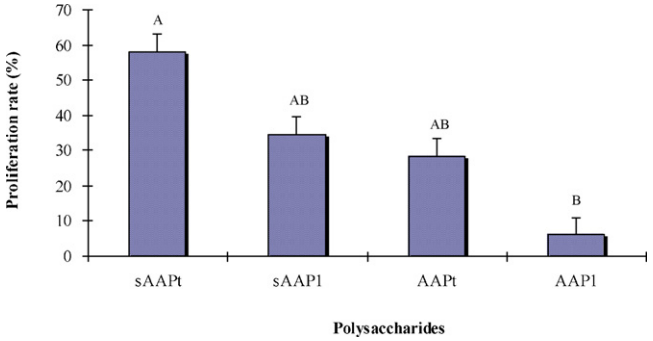

Fig. 4. The lymphocyte proliferation rate of every group in the in vivo test. Bars marked without the same letters (A and B) differ significantly ($P < 0.05$).

3.4. The changes of lymphocyte proliferation in the in vivo test

Changes of lymphocyte proliferations of every group are listed in Table 3. The A_{570} values of all AAP groups at all time points after the first vaccination were higher than NA group. On D_7, the A_{570} values in $sAAP_t$ groups were the highest. On D_{14}, the A_{570} value in $sAAP_t$ group was significantly higher than those in NA and AAP_1 groups ($P < 0.05$). On D_{21}, the A_{570} value in $sAAP_1$ group was the highest and significantly higher than those in NA and AAP_1 groups ($P < 0.05$). On D_{28}, the A_{570} value in $sAAP_t$ group was the highest and significantly higher than those in NA, AAP_t and AAP_1 groups ($P < 0.05$).

The lymphocyte proliferation rates of every group are illustrated in Fig. 4. The proliferation rate of $sAAP_t$ group (58.19%) was the highest, the next was $sAAP_1$ group (34.54%) and both higher than those of AAP_t (28.29%) and AAP_1 group (6.01%).

3.5. The changes of serum antibody titer

The antibody titers of every group are listed in Table 4. At all time points after the first vaccination, the serum antibody titers in four polysaccharide groups were higher than those in NA group ($P < 0.05$). $sAAP_t$ at four time points, $sAAP_1$ on D_7, D_{21} and D_{28}, AAP_t group on D_7 were significantly higher than those in NA group ($P < 0.05$). The serum antibody titers in $sAAP_t$ on D_7, D_{14} and D_{28} and in $sAAP_1$ group on D_{21} were the highest, $sAAP_t$ and $sAAP_1$ group on D_{21} and D_{28} were significantly higher than those in AAP_t and AAP_1 group, respectively.

4. Conclusion

The lymphocyte proliferation is an index to evaluate cellular immunity. The lymphocytes proliferation rate reflects the intensity of cellular immunity (Barta et al., 1992; Thekisoe et al., 2004). The results of in vitro test showed that during single stimulation,

Table 1
The lymphocyte proliferation of every group in single stimulation of AAPs (A_{570} value).

Concentration ($\mu g\,mL^{-1}$)	$sAAP_t$	$sAAP_1$	$sAAP_2$	AAP_t	AAP_1	AAP_2
3.906	0.148 ± 0.018^{bc}	0.226 ± 0.010^{bc}	0.217 ± 0.016^{ab}	0.112 ± 0.019^{c}	0.132 ± 0.005^{b}	0.116 ± 0.017^{b}
1.953	0.160 ± 0.017^{b}	0.264 ± 0.005^{ab}	0.190 ± 0.016^{b}	0.124 ± 0.014^{bc}	0.098 ± 0.007^{a}	0.122 ± 0.026^{b}
0.977	0.162 ± 0.016^{b}	0.292 ± 0.023^{a}	0.211 ± 0.040^{b}	0.132 ± 0.021^{bc}	0.159 ± 0.013^{a}	0.137 ± 0.021^{b}
0.488	0.170 ± 0.014^{b}	0.300 ± 0.016^{a}	0.207 ± 0.023^{b}	0.165 ± 0.019^{a}	0.154 ± 0.010^{a}	0.176 ± 0.027^{a}
0.244	0.212 ± 0.019^{a}	0.274 ± 0.014^{a}	0.259 ± 0.028^{a}	0.143 ± 0.011^{ab}	0.157 ± 0.012^{a}	0.120 ± 0.019^{b}
Cell control	0.134 ± 0.019^{c}	0.192 ± 0.017^{c}	0.192 ± 0.017^{b}	0.122 ± 0.022^{bc}	0.122 ± 0.022^{ab}	0.134 ± 0.019^{b}

Column data marked without the same superscripts (a–c) differ significantly ($P < 0.05$).

Table 2
The lymphocyte proliferation of every group in synergistical stimulation of AAPs with PHA (A_{570} value).

Concentration ($\mu g\,mL^{-1}$)	$sAAP_t$	$sAAP_1$	$sAAP_2$	AAP_t	AAP_1	AAP_2
3.906	0.153 ± 0.021^{b}	0.268 ± 0.025^{bc}	0.218 ± 0.022^{c}	0.139 ± 0.018^{b}	0.157 ± 0.011^{b}	0.113 ± 0.036^{b}
1.953	0.165 ± 0.020^{b}	0.303 ± 0.033^{ab}	0.233 ± 0.027^{bc}	0.161 ± 0.021^{ab}	0.207 ± 0.022^{a}	0.190 ± 0.035^{a}
0.977	0.167 ± 0.019^{b}	0.317 ± 0.038^{ab}	0.224 ± 0.019^{bc}	0.175 ± 0.012^{ab}	0.213 ± 0.013^{a}	0.141 ± 0.026^{ab}
0.488	0.175 ± 0.017^{b}	0.324 ± 0.028^{a}	0.263 ± 0.021^{ab}	0.185 ± 0.012^{ab}	0.205 ± 0.009^{a}	0.144 ± 0.021^{ab}
0.244	0.217 ± 0.022^{a}	0.280 ± 0.023^{ab}	0.295 ± 0.023^{a}	0.205 ± 0.032^{a}	0.183 ± 0.021^{ab}	0.139 ± 0.024^{ab}
PHA control	0.143 ± 0.027^{b}	0.229 ± 0.019^{c}	0.229 ± 0.019^{bc}	0.154 ± 0.015^{ab}	0.154 ± 0.015^{b}	0.143 ± 0.027^{ab}

Column data marked without the same superscripts (a–c) differ significantly ($P < 0.05$).

Table 3
The lymphocyte proliferations of every group in test in vivo (A_{570} value).

Groups	D_7	D_{14}	D_{21}	D_{28}
$sAAP_t$	0.219 ± 0.041^{a}	0.239 ± 0.016^{a}	0.211 ± 0.002^{ab}	0.330 ± 0.028^{a}
$sAAP_1$	0.161 ± 0.040^{ab}	0.194 ± 0.013^{ab}	0.243 ± 0.019^{a}	0.263 ± 0.049^{ab}
AAP_t	0.184 ± 0.064^{ab}	0.174 ± 0.030^{ab}	0.209 ± 0.007^{ab}	0.236 ± 0.007^{bc}
AAP_1	0.120 ± 0.037^{ab}	0.157 ± 0.024^{bc}	0.178 ± 0.010^{b}	0.231 ± 0.020^{bc}
NA	0.111 ± 0.016^{ab}	0.155 ± 0.011^{bc}	0.171 ± 0.019^{bc}	0.209 ± 0.008^{bc}
BC	0.098 ± 0.013^{b}	0.099 ± 0.010^{c}	0.139 ± 0.010^{c}	0.182 ± 0.006^{c}

Column data marked without the same letters (a–c) differ significantly ($P < 0.05$).

Table 4
The variation of antibody titer of every group in test in vivo (Log_2).

Group	D_7	D_{14}	D_{21}	D_{28}
$sAAP_t$	6.0 ± 0.408^{a}	6.8 ± 0.601^{a}	6.7 ± 0.558^{a}	7.0 ± 0.365^{a}
$sAAP_1$	5.8 ± 0.250^{ab}	6.3 ± 0.211^{ab}	7.2 ± 0.401^{a}	6.2 ± 0.307^{a}
AAP_t	5.3 ± 0.479^{ab}	5.7 ± 0.494^{ab}	5.3 ± 0.211^{b}	5.0 ± 0.258^{b}
AAP_1	4.5 ± 0.646^{bc}	5.5 ± 0.671^{ab}	5.0 ± 0.258^{b}	5.2 ± 0.401^{b}
NA	3.8 ± 0.250^{c}	5.0 ± 0.408^{b}	5.2 ± 0.374^{b}	4.6 ± 0.400^{b}
BC	2.3 ± 0.250^{d}	2.2 ± 0.200^{c}	2.0 ± 0.000^{c}	2.4 ± 0.245^{c}

Column data marked without the same superscripts (a–d) differ significantly ($P < 0.05$).

the A_{570} values of $sAAP_t$ and $sAAP_1$ groups at 1.953–0.244 $\mu g\,mL^{-1}$ and $sAAP_2$ group at 0.244 $\mu g\,mL^{-1}$ were significantly higher than those of cell control group, the proliferation rate of $sAAP_1$ group was the highest, the second was $sAAP_t$ group, both significantly or numerically higher than that of AAP_1 or AAP_t groups. During synergistic stimulation, the A_{570} values of $sAAP_1$ group at 1.953–0.244 $\mu g\,mL^{-1}$, $sAAP_t$ and $sAAP_2$ groups at 0.244 $\mu g\,mL^{-1}$ were significantly higher than those of PHA control group and the lymphocyte proliferation rate of $sAAP_1$ group was the highest. This confirms that $sAAP_1$ and $sAAP_t$ could significantly enhance cellular immunity singly or synergistically with PHA, and sulfation modification could improve the immune-enhancing activity of AAP.

The results of in vivo test demonstrated that the A_{570} values in $sAAP_t$ group on D_{14} and D_{28}, $sAAP_1$ group on D_{21} were significantly higher than those in NA group, which showed that they could significantly promote the cellular immune response of ND vaccine. The A_{570} values in $sAAP_t$ on D_7, D_{14} and D_{28}, $sAAP_1$ group on D_{21} were the highest, in $sAAP_1$ group on D_{21} and in $sAAP_t$ group on D_{28} were significantly higher than that in non-sulfated AAP_1 and AAP_t group, respectively. The proliferation rate of $sAAP_t$ was the highest, the second was $sAAP_1$ group, both were respectively higher than those of AAP_t and AAP_1 groups, which indicated that sulfation modification could improve the immune-enhancing activity of AAP. It was also proved that through sulfation modification, AAP could significantly enhance the function of immune competent cells in vitro and in vivo (Li, 2006; Zhang, Wang, & Yang, et al. 2011).

The antibody level in poultry is the marker reflected humoral immune function in bird species (Qiu, Hu, Cui, Zhang, & Wang, 2007). The experimental results demonstrated that the antibody titers in $sAAP_t$ group at all time points after the first vaccination, $sAAP_1$ group on D_7, D_{21} and D_{28} and AAP_t group on D_7 were significantly higher than that of NA group, which implied that they could enhance the humoral immunity response of ND vaccine. The antibody titers in $sAAP_t$ group on D_7, D_{14} and D_{28} and $sAAP_1$ group on D_{21} were the highest, especially on D_{21} and D_{28} in $sAAP_t$ and $sAAP_1$ group were significantly higher than those in non-sulfated AAP_t and AAP_1 group, respectively, which indicated that sulfation modification could raise the humoral immune-enhancing activities of AAP. Our previous research results also confirmed that the immune-enhancing efficacy of sulfated polysaccharides were much better than those of non-modified polysaccharides (Ma, Guo, Wang, Hu, & Shen, 2010).

The results of in vitro and in vivo test confirmed that sulfation modification could enhance the immune-enhancing activity of AAP, $sAAP_t$ presented the strongest activity in clinical experiment and would be expected as a candidate component of new-type immunopotentiator.

Acknowledgments

The project was supported by National Natural Science Foundation of China (30871887), National Science & Technology Pillar Program of China (2011BAD 34B02) and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions. We are grateful to all other staff in the Institute of Traditional Chinese Veterinary Medicine of Nanjing Agricultural University for their assistance in the experiments.

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