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Screening on the immune-enhancing active site of Siberian solomonseal rhizome polysaccharide

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ABSTRACT

The total Siberian solomonseal rhizome polysaccharide ($SRPS_t$) and four fractional $SRPS_{30}$, $SRPS_{50}$, $SRPS_{70}$ and $SRPS_{80,}$ were extracted by one-step or stepwise ethanol precipitation method. In test in vitro, their effects on chicken peripheral lymphocytes proliferation were compared. The result showed that $SRPS_{70}$ and $SRPS_{50}$ presented better effect. In test in vivo, 14-day-old chickens were injected, respectively, with $SRPS_{50}$, $SRPS_{70}$ and $SRPS_t$, at the first vaccination of ND vaccine, once a day for three successive days. On days 7, 14, 21 and 28 after the first vaccination, the peripheral lymphocytes proliferation and antibody titer were determined. The result indicated that lymphocyte proliferation and antibody titers in $SRPS_{50}$ group at all time points were the highest, significantly higher than those of non-adjuvant control group and most of another two SRPSs groups. These results confirmed that $SRPS_{50}$ possessed the strongest action and was the immune-enhancing active site of SRPS.

1. Introduction

Polysaccharide is a kind of natural macromolecular compound of aldose and (or) ketose linked by glycosidic bond widely existing in plants, animals and microbial tissues. Polysaccharide has low toxicity and a variety of biological activity. In recent years, the action mechanism of polysaccharide has been investigated. The results show that polysaccharide can regulate the organism's system and function from several aspects, such as immunoenhancement, anti-virus, anti-oxidation, anti-tumor, and lowering blood glucose. The immunoenhancement is the most important action of polysaccharide and has become a hot issue of polysaccharide investigation (Guo, Zhang, Yan, & Tong, 2008).

Further research found that some polysaccharides had different active sites. Chen et al. compared the antioxidant and antibacterial action of intracellular and extracellular polysaccharide of *cordyceps militaris* obtained, respectively, by stepwise ethanol precipitation of 30%, 50% and 70% ethanol concentration in turn. The result showed that the antioxidant activity of extracellular polysaccharide by 70% ethanol precipitation and the antibacterial action of intracellular polysaccharide by 70% ethanol precipitation were the best (Chen, Zhu, Shao, & Fan, 2008). The effect on scavenging superoxide free radical of bulbil polysaccharide by 30% ethanol precipitation increased along with the increase of polysaccharide concentration, by 60% ethanol precipitation, decreased (Qin, 2008).

In general, the bigger molecular mass of polysaccharides, the larger volume of polysaccharides and the more detrimental play of biological activity. For instance, after schizophan was degraded by ultrasonic method, its molecular weight decreased and clinical effect improved significantly (Kengo, Wataru, Takemasa, Kawabata, & Misaki, 1981). Alban et al. investigated the relationship between molecular weight and anticoagulated blood activity of sulfated curdlan and found that both sides presented a dumbbell-shape curve (Alban, & Franz, 2000). Yang et al. found that the smaller molecular weight of chitosan, the more free amino in the molecule, and the greater fungal-inhibiting activity (SaitÔ et al., 1991).

Siberian solomonseal rhizome (SSR) is the dried rhizome of *Polygonatum sibiricum* Red., a famous traditional herbal medicine with tonification and commonly used in the treatment of pulmonasthenia, dry cough, dizziness, asthenia of splenogastroyin, dry mouth, poor appetite and so on. Siberian solomonseal rhizome polysaccharide (SRPS) is the main active ingredient of SSR. Its chemical structure and molecular weight had been reported

Abbreviations: SSR, Siberian solomonseal rhizome; SRPS, Siberian solomonseal rhizome polysaccharide; CEF, chicken embryo fibroblast; HI, hemagglutination inhibition; NDV, Newcastle disease virus; ND, Newcastle disease; SPF, specified-pathogens free; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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(Liu, Dong, Dong, Fang, & Ding, 2007; Zhang, Gereltu, Zhaorigetu, & Narisu, 2005). The polysaccharides with these chemical structure and molecular weight possessed the action of immune enhancement, anti-inflammatory, anti-virus and so on (Liu, Zheng, Dong, & Fang, 2006).

In present research, total SRPS ($SRPS_t$) was extracted by one-step ethanol precipitation and four fractional SRPSs, $SRPS_{30}$, $SRPS_{50}$, $SRPS_{70}$ and $SRPS_{80}$, were extracted by stepwise ethanol precipitation methods. The effects of five SRPSs on peripheral lymphocytes proliferation in vitro and in vivo and serum HI anti-body titer in the chickens vaccinated with ND vaccine were determined. The purpose of this research is to track the immune-enhancing active site of SRPS and to offer the theoretical basis for development of new-type polysaccharide immunopotentiator.

2. Materials and methods

2.1. Extraction and purification of SRPS

SSR decoction pieces was bought from Zhangjiagang City Green Chinese Medicine Decoction Pieces Co., Ltd, License No. Su Y20060235. SRPS was extracted by water decoction and ethanol precipitation (Xia, Xie, Huang, Li, & Zhang, 2006). 500 g SSR was decocted twice with water after soaked and reflowed with 80% ethanol on 80 °C of water bath and filtered. The filter liquor was incorporated and condensed into 500 mL after centrifugation at 2500 rpm. 200 mL of drug liquor was used to extract total SRPS ($SRPS_t$) by one-step precipitation method adding ethanol up to 80% of working concentration (v/v), other 300 mL, four fractional SRPSs, $SRPS_{30}$, $SRPS_{50}$, $SRPS_{70}$ and $SRPS_{80}$, by stepwise precipitation, added ethanol up to 30%, 50%, 70% and 80% in turn.

The proteins in SRPSs were removed by trichloroacetic acid method according to the reference (Guo, Yan, Zhang, & Wu, 2001; Wang, Peng, Huang, & Tian, 2001; Zhu, & Tong, 2005). Briefly, 14 g polysaccharides was completely dissolved in 140 mL of distilled water on 60 °C water bath, regulated pH into 7 with 10% NaOH solution, added with 3% trichloroacetic acid up to 7.5% (v/v), placed for 4 h at 4 °C then centrifuged 20 min at 3000 rpm. The supernatant was added with ethanol up to 80% (v/v), placed for 24 h and centrifuged. The precipitation was dissolved with deionized water, the tannin and pigment were removed, respectively, through polyamide adsorption column (Zheng, 1998) and hydrogen dioxide method (Yang, Wu, & Tang, 2008), then dialyzed in dialysis sack against tap water for 48 h and distilled water for 24 h, and lyophilized to obtain the pure SRPS.

The carbohydrate contents of five SRPSs were measured by phenol-vitriol method (Yu, Yang, Liu, Liu, & Ma, 2009). The polysaccharide contents (%) of $SRPS_t$, $SRPS_{30}$, $SRPS_{50}$, $SRPS_{70}$ and $SRPS_{80}$ were 35.14%, 52.43%, 79.43%, 65.57% and 35.14%, respectively. They were dissolved into 5 mg mL^{-1} with distilled water according the content, sterilized by pasteurization and detected for endotoxin by pyrogen tests. 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.

2.2. Reagents and vaccine

RPMI-1640 (Gibco), supplemented with benzylpenicillin 100 IU mL^{-1}, streptomycin 100 IU mL^{-1} and 5% fetal bovine serum, was used as nutritive medium. For maintenance medium (MM), the serum concentration was reduced to 2% and was used as washing and re-suspending the cells, diluting the mitogen and cultivating the cells. Phytohemagglutinin (PHA, Sigma), as the T-cell mitogen, was dissolved into 0.5 mg mL^{-1} with RPMI-1640. Sodium heparin was dissolved into 2 mg mL^{-1} with PBS. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco Co.) was dissolved into 5 mg mL^{-1} with calcium and magnesium free (CMF) phosphate-buffered saline (PBS, pH 7.4). These reagents were filtered through a 0.22 μm syringe filter. PHA and sodium heparin solution were stored at −20 °C, MTT solution at 4 °C in dark bottle. Lymphocytes separation medium (Ficoll–Hypaque, ρ: 1.077 ± 0.002, no. 001220) was the product of Shanghai Hengxin Chemicals Ltd. Dimethyl sulfoxide (DMSO) was produced by Shanghai Lingfeng Chemical Reagent Ltd.

ND vaccine (Lasota strain, no. 081328) was offered by Nanjing Tianbang Bio-industry Co., Ltd.

2.3. Determination of test in vitro

Firstly the safe concentrations of five SRPSs for chicken embryo fibroblast (CEF) were measured by the MTT assay. The result showed that the A_{570} values of $SRPS_{30}$ and $SRPS_{70}$ at 62.5 μg mL^{-1}, $SRPS_{50}$ at 31.25 μg mL^{-1}, $SRPS_{80}$ at 39.063 μg mL^{-1} and $SRPS_t$ at 156.25 μg mL^{-1} group were not significantly lower than that of corresponding cells control group. Therefore these concentrations could be considered as their maximal safe concentration. In order to make the comparison at the same level, their maximal safe concentrations were supposed as 31.25 μg mL^{-1}.

Five SRPSs were dissolved with RPMI-1640 media from 31.25 μg mL^{-1} to 1.953 μg mL^{-1}. Blood samples (5 mL per chicken) were collected from heart of chicken and were immediately transferred into aseptic capped tubes with sodium heparin, diluted with equal volume of Hanks' solution and carefully layered on the surface of lymphocyte separation medium. After 20 min centrifugation at 800 × g, a white cloud-like lymphocytes band was collected and washed twice with RPMI1640 media without fetal bovine serum. The resulting pellet was re-suspended into 2.5×10^6 mL^{-1} with RPMI 1640 media, divided into two parts. One part was added with PHA, and, respectively, incubated into 96-well culture plates, 100 μL per well. Then in polysaccharide groups the five SRPSs at series of concentrations were added, in cell control group and PHA control group, RPMI 1640 media and PHA, respectively, 100 μL per well, four wells each concentration. The final concentration of PHA reached to 10 μg mL^{-1}. The plates were incubated at 39.5 °C in a humid atmosphere of 5% CO_2 (CO_2 incubator, American Revco Company). After 44 h of the incubation period, 30 μL of MTT (5 μg mL^{-1}) was added into each well, and the plates were continued to incubate for 4 h. 100 μL of DMSO was added into each well to dissolve the formazan crystals. The plates were shaken for 5 min to dissolve the crystals completely. The absorbance of cell in each well was measured by microliter enzyme-linked immunosorbent assay reader (DG-3022, East China Vacuum Tube Manufacturer) at a wave length of 570 nm (A_{570} value) as the index of peripheral T lymphocytes proliferation. In order to compare the strength of lymphocyte proliferation, the lymphocytes proliferation rate was calculated according to the equation (Yu, Jiang, Yan, & Zhu, 2004): lymphocyte 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 $\bar{A}_{570}$ value was the average value of five concentration groups of polysaccharide or four wells of control group).

2.4. Determination of test in vivo

Two hundred and fifty 14-day-old White Roman chickens (male), purchased from Tangquan Poultry Farm and the average ND-HI titer of maternal antibody was 3.3 $\log_2$, were randomly averagely divided into five groups. The chickens except blank control (BC) group were vaccinated with Newcastle disease vaccine and repeated vaccination at 28 days old. At the same time of the first vaccination, the chickens in three polysaccharide groups were intramuscularly injected, respectively, with 0.5 mL (2.5 mg)of

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

Concentrations ($\mu g\,mL^{-1}$)	$SRPS_{30}$	$SRPS_{50}$	$SRPS_{70}$	$SRPS_{80}$	$SRPS_t$
31.25	0.240 ± 0.001^{a}	0.253 ± 0.002^{a}	0.271 ± 0.009^{a}	0.207 ± 0.004^{c}	0.216 ± 0.003^{ab}
15.625	0.183 ± 0.005^{c}	0.246 ± 0.004^{ab}	0.243 ± 0.003^{b}	0.229 ± 0.005^{ab}	0.220 ± 0.002^{a}
7.813	0.197 ± 0.003^{b}	0.236 ± 0.003^{bc}	0.242 ± 0.009^{b}	0.216 ± 0.002^{bc}	0.203 ± 0.002^{bc}
3.906	0.198 ± 0.006^{b}	0.224 ± 0.002^{d}	0.252 ± 0.006^{ab}	0.231 ± 0.007^{a}	0.204 ± 0.003^{bc}
1.953	0.200 ± 0.001^{b}	0.231 ± 0.002^{cd}	0.248 ± 0.005^{b}	0.227 ± 0.001^{ab}	0.203 ± 0.006^{bc}
Cell control	0.189 ± 0.004^{bc}	0.184 ± 0.002^{e}	0.183 ± 0.002^{c}	0.193 ± 0.002^{d}	0.193 ± 0.002^{c}

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

$SRPS_{50}$, $SRPS_{70}$ and $SRPS_t$, in vaccination control (VC) 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 the determination of peripheral lymphocytes proliferation by MTT assay (A_{570} values and lymphocytes proliferation rate), lymphocyte proliferation rate (%) = ($\bar{A}_{570}$ value of polysaccharide group − $\bar{A}_{570}$ value of VC group)/$\bar{A}_{570}$ value of VC group × 100% (the $\bar{A}_{570}$ value was the average value of four time points). At the same time points, six chickens were sampled randomly from each group for examination of serum hemagglutination inhibition (HI) antibody titer by micro-method.

2.5. Serum HI antibody assay

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 as the controls. 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 serum, erythrocytes and antigens were also included as controls. The highest dilution of serum 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 (Thekisoe, Mbati, & Bisschop, 2004).

2.6. 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 $P < 0.05$.

3. Results

3.1. Experiment in vitro

3.1.1. The lymphocytes proliferation changes in single stimulation

The A_{570} values of every group are listed in Table 1. The A_{570} values of $SRPS_{50}$, $SRPS_{70}$ and $SRPS_{80}$ at 31.25–1.953 $\mu g\,mL^{-1}$ groups, $SRPS_{30}$ at 31.25 $\mu g\ mL^{-1}$, and $SRPS_t$ at 31.25–15.625 $\mu g\,mL^{-1}$ were significantly larger than those of corresponding cell control group ($P < 0.05$).

The lymphocyte proliferation rates of every group are illustrated in Fig. 1. During the single stimulation, the lymphocyte proliferation rate of $SRPS_{70}$ group was the highest (37.51%), the following was in $SRPS_{50}$ group (29.71%), and in these two groups were significantly higher than those of other three groups ($P < 0.05$) and there was no significant difference between two groups ($P < 0.05$). The lymphocyte proliferation rate of $SRPS_t$ was the lowest.

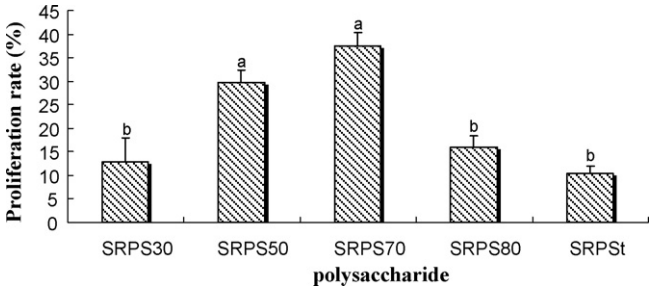

Fig. 1. The lymphocyte proliferation rate of every group in single stimulation. Bar marked without the same superscripts differ significantly ($P < 0.05$).

3.1.2. The lymphocytes proliferation changes in synergistical stimulation with PHA

The A_{570} values of every group are listed in Table 2. The A_{570} values of $SRPS_{30}$ and $SRPS_{70}$ at 31.25 $\mu g\,mL^{-1}$, $SRPS_{50}$ at 31.25–1.953 $\mu g\,mL^{-1}$ and $SRPS_{80}$ at 1.953 $\mu g\,mL^{-1}$ groups were significantly larger than those of corresponding PHA control group ($P < 0.05$). The A_{570} values between every concentration of $SRPS_t$ group and corresponding PHA control group have no significant difference ($P > 0.05$).

The lymphocyte proliferation rates of every group were illustrated in Fig. 2. During synergistical stimulation of polysaccharide with PHA, the lymphocyte proliferation rate of $SRPS_{50}$ group was the highest (13.40%) and significantly higher than those of other four groups ($P < 0.05$). The lymphocyte proliferation rate of $SRPS_t$ group was the lowest.

3.2. Experiment in vivo

3.2.1. The lymphocytes proliferation changes

The changes of lymphocytes proliferation of every group are listed in Table 3. On day 7 after the first vaccination (D_7), there were no significant differences between the A_{570} values of all fractional polysaccharide groups and VC group ($P > 0.05$). On D_{14}, the A_{570} value in $SRPS_{50}$ group was the largest, significantly larger than those in other four groups ($P < 0.05$), the following was $SRPS_t$ group and significantly larger than those in other three groups ($P < 0.05$). On D_{21}, the A_{570} value in $SRPS_{50}$ group was the largest, the follow-

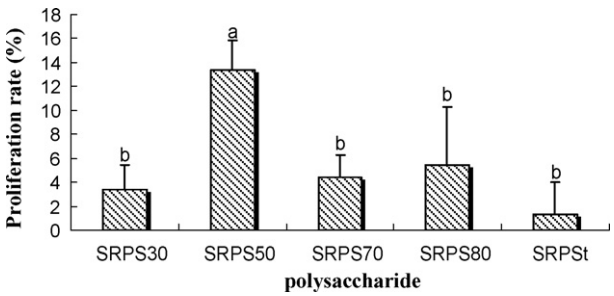

Fig. 2. The lymphocyte proliferation rate of every group in synergistical stimulation with PHA. Bar marked without the same superscripts differ significantly ($P < 0.05$).

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

Concentrations ($\mu g\,mL^{-1}$)	$SRPS_{30}$	$SRPS_{50}$	$SRPS_{70}$	$SRPS_{80}$	$SRPS_t$
31.25	0.230 ± 0.005^a	0.242 ± 0.005^a	0.242 ± 0.012^a	0.208 ± 0.001^c	0.232 ± 0.014^a
15.625	0.212 ± 0.001^b	0.243 ± 0.009^a	0.217 ± 0.002^b	0.211 ± 0.004^{bc}	0.225 ± 0.011^a
7.813	0.217 ± 0.010^{ab}	0.240 ± 0.003^a	0.212 ± 0.004^b	$0.220 \pm \pm 0.006^b$	0.203 ± 0.004^a
3.906	0.208 ± 0.005^b	0.238 ± 0.006^a	0.218 ± 0.001^b	0.218 ± 0.002^{bc}	0.214 ± 0.003^a
1.953	0.204 ± 0.004^b	0.245 ± 0.003^a	0.220 ± 0.001^b	0.252 ± 0.004^a	0.204 ± 0.010^a
PHA control	0.206 ± 0.001^b	0.206 ± 0.001^b	0.209 ± 0.001^b	0.209 ± 0.001^{bc}	0.213 ± 0.007^a

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

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

Groups	D_7	D_{14}	D_{21}	D_{28}
$SRPS_{50}$	0.341 ± 0.013^a	0.554 ± 0.004^a	0.348 ± 0.003^a	0.470 ± 0.006^a
$SRPS_{70}$	0.356 ± 0.002^a	0.349 ± 0.007^c	0.341 ± 0.003^a	0.367 ± 0.007^b
$SRPS_t$	0.307 ± 0.006^b	0.397 ± 0.018^b	0.304 ± 0.005^b	0.334 ± 0.008^c
VC	0.363 ± 0.010^a	0.354 ± 0.002^c	0.313 ± 0.005^b	0.304 ± 0.006^d
BC	0.278 ± 0.006^c	0.288 ± 0.012^d	0.283 ± 0.002^c	0.265 ± 0.007^e

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

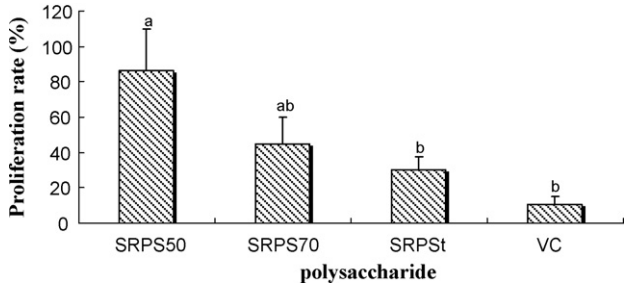

Fig. 3. The lymphocyte proliferation rate of every group in test in vivo. Bar marked without the same superscripts differ significantly ($P < 0.05$).

ing was $SRPS_{70}$ group and in these two groups were significantly larger than those in other three groups ($P < 0.05$). On D_{28}, the A_{570} value in $SRPS_{50}$ group was the largest and significantly larger than those in other four groups ($P < 0.05$), the following was $SRPS_{70}$ and $SRPS_t$ group, they were significantly larger than that in VC groups ($P < 0.05$).

The lymphocyte proliferation rates of every group are illustrated in Fig. 3. The proliferation rate of $SRPS_{50}$ group was the highest (86.63%) and significantly higher than those of VC and $SRPS_t$ groups ($P < 0.05$). The lymphocyte proliferation rate of VC group was the lowest, the following was $SRPS_t$ group.

3.2.2. *The serum antibody titer changes*

The antibody titers of every group are listed in Table 4. At all time points after the first vaccination, the ND-HI antibody titers in three polysaccharide groups were higher than that in VC group, and in $SRPS_{50}$ groups were the highest and significantly higher than those in VC and most of other two SRPS groups ($P < 0.05$). On day 14 after the first vaccination, the antibody titers in $SRPS_{70}$ and $SRPS_t$ groups were significantly higher than that in VC group ($P < 0.05$).

Table 4
The antibody titer variation of every group in test in vivo (log_2).

Groups	D_7	D_{14}	D_{21}	D_{28}
$SRPS_{50}$	5.4 ± 0.40^a	8.0 ± 0.32^a	7.4 ± 0.50^a	6.0 ± 0.32^a
$SRPS_{70}$	4.6 ± 0.40^{ab}	6.8 ± 0.20^b	5.6 ± 0.40^b	5.0 ± 0.32^b
$SRPS_t$	5.0 ± 0.32^{ab}	6.4 ± 0.24^b	6.4 ± 0.50^{ab}	4.8 ± 0.32^b
VC	4.0 ± 0.31^b	5.2 ± 0.20^c	5.6 ± 0.24^b	4.4 ± 0.24^b
BC	2.8 ± 0.20^c	2.8 ± 0.20^d	2.6 ± 0.24^c	2.4 ± 0.24^c

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

4. Discussion

Because polysaccharides with different molecular weights possess different solubilities in alcohols or ketones at different concentrations, the fractional precipitation method can be used to isolate polysaccharides according to this property (Whistler, Bushway, & Singh, 1976). In this research, after SSR decoction was added with ethyl alcohol firstly to reach to 30% of work concentration, the SRPS with the largest molecular weight was isolated, then in turn increased the ethanol concentration into 50%, 70% and 80%, the SRPSs with medium and small molecular weight (or oligosaccharides) were stepwise precipitated (Yan, Han, Jiang, & Huang, 2004). In order to compare the immunoenhancement between graded and total SRPS, $SRPS_t$ was extracted by one-step ethanol precipitation.

The lymphocyte proliferation is an important index to evaluate cellular immunity. The results of test in vitro showed that during single stimulation, the A_{570} values of $SRPS_{50}$, $SRPS_{70}$ and $SRPS_{80}$ at 31.25–1.953 $\mu g\,mL^{-1}$ and $SRPS_{30}$ and $SRPS_t$ at 31.25 $\mu g\,mL^{-1}$ groups were significantly higher than that of cell control group, which confirmed that they at those concentrations could significantly stimulate lymphocyte proliferation singly, thereby enhancing cellular immunity. The lymphocytes proliferation rate reflected the strength of cell immunity. The experimental results showed that the lymphocyte proliferation rate of $SRPS_{70}$ group was the highest, followed by $SRPS_{50}$ group, they were significantly higher than those of other three groups and there was no significant difference between two groups, while lymphocytes proliferation rate of $SRPS_t$ group was the lowest, which indicated that $SRPS_{70}$ and $SRPS_{50}$ possessed the best action, while $SRPS_t$, the weakest.

PHA and ConA can stimulate T lymphocytes to generate transformation and mitosis (Wang, Deng, Wan, Zuo, & Li, 2009). This research determined the synergistical stimulation action of SRPS and PHA. The result showed that the A_{570} values of $SRPS_{50}$ at 31.25–1.953 $\mu g\,mL^{-1}$, $SRPS_{30}$ and $SRPS_{70}$ at 31.25 $\mu g\,mL^{-1}$, and $SRPS_{80}$ at 1.953 $\mu g\,mL^{-1}$ groups were significantly larger than that of corresponding PHA control group, which indicated that they at these concentrations could significantly stimulate lymphocyte proliferation synergistically with PHA. The lymphocyte proliferation rate of $SRPS_{50}$ group was the highest and significantly higher than those of other four groups, while in $SRPS_t$ group, the A_{570} values of every concentration were not significantly higher than that of the PHA control group and lymphocyte proliferation rate was the low-

est, which confirmed that $SRPS_{50}$ possessed the strongest action, while $SRPS_t$, the weakest.

According to the results of test in vitro, $SRPS_{50}$ and $SRPS_{70}$ were selected and their immunoenhancements were further compared by clinical test taking $SRPS_t$ as control. The results of lymphocyte proliferation demonstrated that the A_{570} values in $SRPS_{50}$ group had always been the largest from day 14 after the first vaccination, and significantly higher than those in corresponding VC group and most of other two SRPS groups, in $SRPS_{70}$ group on D_{21} and D_{28} and in $SRPS_t$ group on D_{14} and D_{28}, significantly higher than those in corresponding VC group, which indicated that at these time points they could significantly promote lymphocyte proliferation thus improving the immune function of chickens. At the same time, the lymphocyte proliferation rate of $SRPS_{50}$ group was the highest and significantly higher than those of VC and $SRPS_t$ groups. These results suggested that $SRPS_{50}$ possessed the strongest action, while $SRPS_t$, the weakest. Luo et al. reported that fractional astragalus polysaccharides (APS) extracted with ethanol at 60% working concentration could significantly promote the splenic lymphocyte proliferation (Luo, Gu, Xu, Jiang, & Li, 2009). Huang et al. confirmed that fractional APS_{50} and APS_{60} possessed better stimulating effects on peripheral lymphocytes proliferation in chicken (Huang, 2008).

The humoral immunity is one of the major factors of organism resisting infectious diseases. The antibody level is the marker reflecting humoral immune state. The antibody determination results demonstrated that at all time points the antibody titers in three polysaccharide groups were higher than that in VC group, and in $SRPS_{50}$ group were the highest and significantly higher than those in VC and most of other two SRPS groups. The antibody titers in $SRPS_{70}$ and $SRPS_t$ group on D_{14} were significantly higher than that in VC group. This proved that they at these time points could significantly enhance humoral immune function of chickens vaccinated with Newcastle disease vaccine and $SRPS_{50}$ possessed the strongest action. The similar results were reported. For example, fractional APS_{60} could remarkably enhance serum antibody titer and IL-2 content, and extend the duration of antibody titers (Luo, Gu, Lu, Xu, & Li, 2010; Luo, Gu, Xu, & Li, 2009). Fractional *ganoderma lucidum* polysaccharides extracted with ethanol at 65% and 80% of working concentration had most obvious effects on improving the leukocyte phagocytosis and liver-protecting function in mice (Zhou, & Chen, 2007).

By above-mentioned comparison, it can be seen that $SRPS_{50}$ possesses the strongest immune-enhancing action and is the immune-enhancing active site of SRPS and would be expected as a component drug of new-type polysaccharide immunopotentiator.

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References

Alban, S., & Franz, G. (2000). Characterization of the anticoagulant actions of a semi-synthetic curdlan sulfate. *Thrombosis Research*, *99*(4), 377–388.

Chen, H., Zhu, Y., Shao, Y., & Fan, M. (2008). Study on the physiologic function of fractional cordyceps militaris polysaccharide extracted by alcohol. *Food and Fermentation Industries*, *34*(9), 16–19.

Fang, J. (1984). Isolation, purification and purity identification of polysaccharide. *Pharmaceutical Bulletin*, *19*, 622–625.

Guo, L., Yan, Z., Zhang, X., & Wu, L. (2001). Effect of trichloroacetic acid on the stability of protein structure. *Journal of the Fourth Military Medical University*, *22*(22), 2113.

Guo, X., Zhang, S., Yan, G., & Tong, J. (2008). The immuno-modulating effect and livestock production on application of Chinese herbal polysaccharides. *Poultry Science*, *2*, 44–46.

Huang, X. (2008). *Sulfated modification of astragalus polysaccharide and antivirus and enhancing immunity activity of the modifiers*. PhD thesis, Nanjing Agriculture University.

Kengo, T., Wataru, I., Takemasa, K., Kawabata, S., & Misaki, A. (1981). Ultrosonic degradation of schizophyllan, an antitumor polysaccharide produced by *schizophyllan commune* fries. *Carbohydrate Research*, *89*(1), 121–135.

Liu, L., Dong, Q., Dong, X., Fang, J., & Ding, K. (2007). Structural investigation of two neutral polysaccharides isolated from rhizome of *Polygonatum sibiricum*. *Carbohydrate Polymers*, *70*, 304–309.

Liu, L., Zheng, Y., Dong, Q., & Fang, J. (2006). Polysaccharides from *Polygonatum sibiricum* and their immunological activities. *Chinese Traditional and Herbal Drugs*, *37*(8), 1132–1134.

Luo, Y., Gu, X., Lu, Z., Xu, L., & Li, Z. (2010). Effects of rating polysaccharide by alcohol in astragalus polysaccharides on the serum interleukin-2 contents in chickens. *China Animal Husbandry & Veterinary Medicine*, *37*(1), 32–34.

Luo, Y., Gu, X., Xu, L., Jiang, Q., & Li, Z. (2009). Effects of astragalus polysaccharide (APS) rating components on proliferation of spleen lymphocytes in chickens. *Journal of Traditional Chinese Veterinary Medicine*, *5*, 13–14.

Luo, Y., Gu, X., Xu, L., & Li, Z. G. (2009). The extraction of rating polysaccharides by alcohol in APS and effects on the immunity function of AI and ND vaccine in chickens. *Acta Agriculture Boreali-Occidentalis Sinica*, *18*(6), 39–42.

Qin, E. (2008). Separation and purification of bulbil polysaccharides and its antioxidation. *Modern Food Science and Technology*, *24*(11), 1100–1103.

SaitÔ, H., Yoshioka, Y., Uehara, N., Aketagawa, J., Tanaka, S., & Shibata, Y. (1991). Relationship between conformation and biological response for $(1 \rightarrow 3)$-β-D-glucans in the activation of coagulation factor G from limulus amebocyte lysate and host-mediated antitumor activity. Demonstration of single-helix conformation as a stimulant. *Carbohydrate Research*, *217*, 181–190.

Thekisoe, M. O., Mbati, P. A., & Bisschop, S. P. R. (2004). Different approaches to the vaccination of free ranging village chickens against Newcastle disease in Qwa-Qwa, South Africa. *Veterinary Microbiology*, *101*, 23–30.

Wang, Y., Deng, J., Wan, E., Zuo, Z., & Li, H. (2009). Effects of maduramicin on lymphocyte immune function and Newcastle disease immunity in broilers. *Animal Husbandry & Veterinary Medicine*, *41*(5), 71–73.

Wang, Z., Peng, Z., Huang, L., & Tian, G. (2001). Isolation, purification, physicochemical properties and biological activities of a polysaccharide SPPC-1 from *Spirulina Platensis*. *Chinese Journal of Marine Drugs*, *81*(3), 17–19, 27.

Whistler, R. L., Bushway, A. A., & Singh, P. P. (1976). Noncytotoxic antitumor polysaccharides. *Advances in Carbohydrate Chemistry & Biochemistry*, *32*, 235–244.

Xia, Q., Xie, Y., Huang, Z., Li, Z., & Zhang, P. (2006). Optimum extraction of polygonapolysaccharose from *rhizoma polygonati* by orthogona test. *China Pharmacist*, *9*(10), 905–906.

Yan, Q., Han, L., Jiang, Z., & Huang, C. (2004). Molecular mass distribution of astragalus polysaccharides. *Food Science*, *25*(8), 27–30.

Yang, D., Wu, Y., & Tang, Q. (2008). Study on methods for decolorizing polysaccharide from Cardamine enshiensis by hydrogen peroxide. *Food Science and Technology*, *33*(1), 174–181.

Yu, J., Jiang, Z., Yan, H., & Zhu, L. (2004). Effect of pachyman on cell-medicated immunity activity and anti-tumor function in chicken infected with MDV. *Chinese Journal of Veterinary Science and Technology*, *34*(11), 70–73.

Yu, W., Yang, X., Liu, W., Liu, F., & Ma, H. (2009). Assay study on content of polysaccharides in ficus carica by phenol-sulfuric acid method. *Food Science and Technology*, *34*(10), 256–258, 262.

Zhang, X., Gereltu, B., Zhaorigetu, & Narisu. (2005). Determination of relative molecular mass and composition for *Polygonatum sibiricum* polysaccharide by high performance liquid chromatography. *Chinese Journal of Chromatography*, *23*(4), 394–396.

Zheng, P. (1998). *Pharmacy of Chinese Materia Medica* (pp. 326–327). China: Medical Science and Technology Press.

Zhou, L., & Chen, Y. (2007). Isolation and activity of fractional ganoderma lucidum polysaccharide. *Journal of Huaibei Professional and Technical College*, *6*(5), 94–95.

Zhu, M., & Tong, Q. (2005). Study on removal of protein from hericium erinaceus polysaccharides. *Journal of Henan University of Science & Technology (Natural Science Edition)*, *26*(4), 25–27.