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Optimization of selenylation conditions for Chinese angelica polysaccharide based on immune-enhancing activity

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ABSTRACT

Chinese angelica polysaccharide (CAP) was extracted by water decoction and ethanol precipitation, purified through eliminating protein by Sevage method and column chromatography of Sephadex G-200, then selenizingly modified by nitric acid–sodium selenite method according to $L_9(3^4)$ orthogonal design of three-factors, the usage amount of sodium selenite, reaction temperature and reaction time, at three level to obtain nine selenizing CAPs, $sCAP_1$–$sCAP_9$. Their effects on chicken peripheral lymphocytes proliferation in vitro were compared by MTT assay taking the non-modified CAP as control. The results showed that selenylation modification could significantly enhance the immune-enhancing activity of CAP, $sCAP_2$ presented best effect and the optimal modification conditions were 200 mg of sodium selenite for 500 mg of CAP, the reaction temperature of 70 °C and the reaction time of 6 h.

1. Introduction

Chinese angelica (CA) is a well-known Chinese herbal medicine used for the treatment of various diseases as a tonic medicine for thousands of years, especially for modulate the immune system, prevent platelet aggregation and act as an antioxidant (Kuang et al., 2006; Liu, Dong, Wu, Luo, & Yu, 2003; Shen et al., 2005; Yang, Jia, Meng, Wu, & Mei, 2006; Ye et al., 2001). It is reported that Chinese angelica polysaccharide (CAP) is the main effective ingredient of CA and has the actions of enhancing immunity, antivirus, anti-tumor, lowering blood glucose, antioxidant and so on (Han et al., 1998; Jeon, Han, Ahn, & Kim, 1999; Yonei, 1987).

Selenium is an essential microelement for vital movement. It is a key constituent of selenoproteins, e.g. glutathione peroxidases (Beckett & Arthur, 2005), and acts as a very important antioxidant in many types of cells (Mukherjee, Anbazhagan, Roy, Ghosh, & Chatterjee, 1998). It is known that organic selenium can be betterly absorbed and has less toxicity as compared with inorganic selenium (Rayman, 2000). Selenium polysaccharide, including natural selenium polysaccharides extracted from plants or synthesized derivatives with selenium and polysaccharide, belongs to organic selenium compound and possesses more or stronger biological activities in comparison with selenium-free polysaccharide, such as the immunomodulation, hypoglycaemic, hypolipidemic, antitumor and antibacterial effects and so on (Fan et al., 2006). Therefore, the selenylation modification becomes a hot spot in polysaccharides research field.

It is reported that there are many selenylation modification methods for polysaccharide such as Selenium oxidate–pyridine method (Gong, 1997; Wang et al., 2009a; Wang, Deng, Wan, Zuo, & Li, 2009b), nitric acid–sodium selenite (HNO_3–Na_2SeO_3) method (Yang, Huang, Jiang, Zhu, & Han, 2010), acetic acid–sodium selenite method (Liang, Ma, Zhao, Xu, & Sun, 2011) and so on. Among these methods, HNO_3–Na_2SeO_3 method is commonly used since the reaction conditions are simple, production is fast and selenium content of modifier is higher. The main factors affected HNO_3–Na_2SeO_3 method including the usage amount of sodium selenite, reaction temperature and reaction time (Li, Miu, & Liu, 2001).

In this research CAP was extracted by water decoction and ethanol precipitation, purified through eliminating protein by Sevage method and column chromatography of Sephadex G-200, then selenizingly modified by HNO_3–Na_2SeO_3 method according to $L_9(3^4)$ orthogonal design of three-factors, the amount of sodium selenite (Na_2SeO_3), reaction temperature and reaction time each at three level, to obtain nine selenizing CAPs, $sCAP_1$–$sCAP_9$. Their effects on chicken peripheral lymphocytes proliferation in vitro

Abbreviations: CA, Chinese angelica; CAP, Chinese angelica polysaccharide; sCAP, selenizing Chinese angelica polysaccharide; HNO_3, nitric acid; Na_2SeO_3, sodium selenite; HCl, hydrochloric acid; $HClO_4$, perchloric acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PHA, phytohemagglutinin; CMF, calcium and magnesium-free; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide.

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Table 1
The modification conditions, yields and contents of selenium and carbohydrate of sCAPs.

sCAPs	A Na_2SeO_3 (mg)	B Temperature (°C)	C Time (h)	Yeild (%)	Selenium content (mg g^{-1})	Carbohydrate content (%)
$sCAP_1$	200	50	6	18.00	6.99	37.2
$sCAP_2$	200	70	8	36.80	12.98	50.9
$sCAP_3$	200	90	10	32.28	12.33	23.5
$sCAP_4$	300	50	6	27.64	11.99	42.8
$sCAP_5$	300	70	8	23.72	10.50	42.6
$sCAP_6$	300	90	10	40.16	10.66	57.2
$sCAP_7$	400	50	6	32.18	9.56	44.5
$sCAP_8$	400	70	8	37.08	6.41	63.2
$sCAP_9$	400	90	10	42.76	7.98	34.7

were compared by MTT assay taking the non-modified CAP as control. The aim of this study is to explore the probability of selenylation modification to improve the immune-enhancing activity of CAP, choice out the best sCAP and optimal modification condition, and offer theoretical evidence for the development of new-type immunopotentiator.

2. Materials and methods

2.1. CA and reagents

Chinese angelica (CA) bought from Nanjing Dahua Pharmacy of Jiangsu province was the product of Fengyuan Chinese traditional medicine company in Tongling, Anhui province, China.

Nitric acid (HNO_3) was the product of Shanghai Lingfeng Chemical Reagent Ltd. Sodium selenite bought from Shanghai Lingfeng Chemical Reagent Ltd. Sodium selenite was dissolved into 0.05 g mL^{-1} with ultrapure water. Standard selenium stored solution at 100 μg mL^{-1} supplied by National Standard Substance Research Center was accurately diluted into 1 μg mL^{-1} of standard selenium solution. Hydrochloric acid (HCl) was the product of Nanjing Chemical Reagent Ltd. Perchloric acid ($HClO_4$) was the product of Tianjin Xinyuan Chemical Reagent Ltd.

RPMI-1640 (Gibco) supplemented with benzylpenicillin 100 IU mL^{-1}, streptomycin 100 IU mL^{-1} and 10% fetal bovine serum, was used for washing and re-suspending the cells, diluting the mitogen and culturing the cells. Hanks' solution, pH was adjusted to 7.4 with 5.6% sodium bicarbonate solution, supplemented with benzylpenicillin 100 IU mL^{-1} and streptomycin 100 IU mL^{-1}. Lymphocytes Separation Medium (Ficoll–Hypaque, *p*: 1.077 ± 0.002, No. 20110923) was the product of Shanghai Hengxin Chemicals Ltd. Phytohemagglutinin (PHA, Sigma), as the T-cell mitogen, was dissolved into 0.5 mg mL^{-1} with RPMI-1640. The 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, pH7.4). 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 bottles. Dimethylsulfoxide (DMSO) was the product of Shanghai Lingfeng Chemical Reagent Ltd., No. 060902.

2.2. Extraction and purification of CAP

Dried CA (1000 g) was crushed into 0.3–1 cm^3 small block, soaked 12 h with 2000 mL of 95% ethanol, reflowed for 1 h twice in water bath of 80 °C. After aired 12 h, the drug was decocted with 20-fold volume water 3 times each for 30 min. The physic liquor was filtrated through two-layers gauze, concentrated into 1000 mL, centrifugated at 2500 rpm for 20 min and added with 95% ethanol up to 90% of concentration (v/v), after standing 24 h, dried by vacuum freeze-drying machine (Model LGJ-25, Dongxing Machinery Industry Co., Ltd. Shamen City). The precipitation was lyophilized to get crude CAP.

The crude CAP was eliminated protein by Sevage method (Staub, 1965) and dissolved into 0.05 g mL^{-1} with distilled water, added into a chromatographic column of Sephadex G-200 (2 cm × 100 cm) and eluted with distilled water. The flow rate was maintained at 12 mL h^{-1}, the eluent was collected by automatic fraction collector, 4 mL per tube, and measured for polysaccharide by the phenol–sulfuric acid method. The elution curve was drawn (one peak). The eluents contained polysaccharides were merged and lyophilized to get one purified CAP. Its carbohydrate content was 92.7% determined by the phenol–sulfuric acid method (Li & Wang, 2008; Yu, Yang, Liu, & Liu, 2009).

2.3. Selenylation modification of CAP

The HNO_3–Na_2SeO_3 method was applied (Yang et al., 2010).

2.3.1. Design of modification condition

Three factors respectively at three levels, the usage amount of sodium selenite at 200, 300 and 400 mg for 500 mg of CAP (A), the reaction temperature at 50, 70 and 90 °C (B) and the reaction time for 6, 8 and 10 h (C), were selected (Li et al., 2001). Nine modification conditions were designed according to orthogonal test as L_9 (3^4) (Table 1).

2.3.2. Selenylation reaction

CAP of 4.5 g was divide equally into 9 portions, respectively added into the three-necked flask filled with 50 mL of 5% HNO_3 solution stirring to make CAP completely dissolve, Then the sodium selenite solution was added and stirring reaction was performed at definitive temperature and duration designed in Table 1. After the reaction finished, the mixture was cooled to room temperature, adjusted pH to 5–6 with saturated sodium carbonate solution, dialyzed in dialysis sack with 1 kDa ultrafiltration membrane against tap water and sampled for determination of sodium selenium every 6 h by ascorbic acid method (Li et al., 2001). The dialysis was stopped till no sodium selenium was detected. The polysaccharide solutions were concentrated and lyophilized by vacuum freeze-drying machine. Nine selenizing CAPs, named $sCAP_1$–$sCAP_9$, were obtained.

2.4. Identification of sCAPs

The contents of selenium and carbohydrate and FT-IR spectra of sCAPs were tested. The carbohydrate contents were determined by phenol–sulfuric acid method.

2.4.1. Assay of selenium content

The atomic fluorescence spectrometry was used for determination of selenium content (Gao, Qin, & Huang, 2006) by atomic fluorescence spectrometer (Model AFS-930, Beijing Jitian

instrument Co., Ltd.). The working conditions of the spectrometer were as follows: the negative high voltage was 270 V, lamp current was 80 mA, the atomization temperature was 200 °C, the height of atomization gas was 8 mm, the discharges of carrier gas and shield gas flow were 400 mL min^{-1} and 800 mL min^{-1} respectively, the injection volume was 1 mL, the readings was peak area, reading time was 7 s, the delay time was 1.5 s. 100 mL of standardry selenium solution and 5% HCl solution as diluent were linked to the atomic fluorescence spectrometer. The concentrations of standard curve were set at 0, 4, 8, 12, 16 and 20 ng mL^{-1} which were automatically diluted and the fluorescence intensity were detected by the spectrometer. The standard curve was drawn taking the selenium mass concentration as abscissa and fluorescence intensity as vertical axis.

20 mg of sCAP weighed accurately was dissolved in 10 mL of ultrapure water, 0.5 mL of sCAP solution was accurately measured and added into triangular flask with cork, 10 mL of $HClO_4$–HNO_3 (v/v, 1:1) mixed acid solution was added to digest for 12 h at 4 °C, then to heat at 100 °C replenishing the mixed acid solution timely. When sCAP solution become clear, colorless and accompanied by white smoke, it was concentrated to about 2 mL, cooled to room temperature, and diluted accurately into 25 mL with 5% HCl solution in which 1 mL of the solution was accurately measured and diluted into 100 mL with 5% HCl solution as sample solution. The blank sample solution was prepared by the same method. The fluorescence intensities of the sample solution were detected by the spectrometer. The selenium contents were calculated according to the standard curve.

2.4.2. Infrared spectroscopy analysis

The FT-IR spectra of sCAPs and CAP in a wavenumber range of 4000–400 cm^{-1} were recorded by KBr pellets method with a Nicolet 200 Magna-IR spectrometer (Nicolet Instrument Corp).

2.5. Assay of peripheral lymphocytes proliferation

The effects of nine sCAPS on chicken peripheral lymphocytes proliferation in vitro were determined by MTT assay (Wang et al., 2005). $sCAP_1$–$sCAP_9$ were diluted with RPMI-1640 twofold serially from 100 μg mL^{-1} to 0.097 μg mL^{-1}, CAP, from 500 μg mL^{-1} to 0.463 μg mL^{-1}, total 11 concentrations. Blood samples (5 mL) were collected from 35-day-old non-vaccinated White Roman chickens (male) (provided by Tangquan Poultry Farm) and transferred immediately into aseptic capped tubes with sodium heparin, then diluted with equal volume of Hanks' solution and carefully layered on the surface of lymphocytes separation medium. After 20 min of centrifuged 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 to 2.5×10^6 mL^{-1} with RPMI-1640 media, and incubated into 96-well culture plates, 100 μL per well. Then, in polysaccharide groups the ten polysaccharides at series of concentrations were added, 100 μL per well, four wells each concentration. The plates were incubated at 38.5 °C in a humid atmosphere of 5% CO_2. After incubation for 44 h, 20 μL of MTT (5 μg mL^{-1}) was added into each well, and continued to incubate for 4 h. The plates were shaken for 5 min to dissolve the crystals completely. The absorbance of cells in each well was measured by microliter enzyme-linked immunosorbent assay reader (Model DG-3022, East China Vacuum Tube Manufacturer) at a wave length of 570 nm (A_{570} value) (Talorete, Bouaziz, Sayadi, & Isoda, 2006; Wang et al., 2005). When the A_{570} values of polysaccharide group were not significantly lower than that of the cells control group, it indicated that the polysaccharides had no cytotoxicity, the corresponding concentrations were considered as its maximal safe concentration for lymphocytes. The experiment showed that the maximal safe concentrations of all sCAPs and CAP were 125–1.563 μg mL^{-1}. Their safe concentrations were supposed as 1.563 μg mL^{-1} in order to make the comparison at the same level.

$sCAP_1$–$sCAP_9$ and CAP were diluted with RPMI-1640 twofold serially from 1.563 μg mL^{-1} to 0.098 μg mL^{-1} total 5 concentrations. The preparation of lymphocytes as above, the resulting pellet was re-suspended into 2.5×10^6 mL^{-1} with RPMI-1640 media. The solution was divided into two parts, one part was added with PHA (the final concentration reaching to 10 μg mL^{-1}), and respectively incubated into 96-well culture plates, 100 μL per well. Then, in polysaccharide groups the ten polysaccharides 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. At the same time, cell control group (CC, only adding RPMI-1640 media), PHA control group (adding RPMI-1640 media and PHA) and blank group (no cell) were designed. The cellular A_{570} values were determined by above-mentioned method as the index of lymphocytes proliferation. Meanwhile the cellular proliferation rate was calculated to compare the strength of lymphocytes proliferation according to the equation (Lu, 2008; Yu, Jiang, Yan, & Zhu, 2005): Proliferation rate (%) = $(\bar{A}_{\text{polysaccharide group}} - \bar{A}_{\text{control group}})/\bar{A}_{\text{control group}}) \times 100\%$ ($\bar{A}$ was a average value of five concentration groups of polysaccharide or four wells of cell or PHA control group).

2.6. Statistical analysis

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

3. Results

3.1. The yields and contents of selenium and carbohydrate of sCAPs

The modification conditions, yields and contents of selenium and carbohydrate of sCAPs are listed in Table 1. The yield of $sCAP_9$ was the highest, up to 42.76%, and next were $sCAP_6$, $sCAP_8$ and $sCAP_2$. The highest selenium content was $sCAP_2$ (12.98%), and next were $sCAP_3$, $sCAP_4$ and $sCAP_6$. The highest carbohydrate content was $sCAP_8$ (63.2%), and next were $sCAP_6$, $sCAP_2$ and $sCAP_7$.

3.2. The infrared spectroscopy characteristic of sCAP

The FT-IR spectra of CAP and sCAPs in the ranges of 4000–400 cm^{-1} are illustrated in Fig. 1. In the spectra of CAP and sCAP, the band in the region of 3600–3200 cm^{-1} corresponds to the hydroxyl stretching vibration. The bands attributed to C—O—C stretching vibrations appeared at about 1400–1000 cm^{-1} and the bands attributed to C=O stretching vibrations appeared at 1620 cm^{-1}. This amalgamation indicates that the CAP and sCAP were polysaccharides (Fig. 1A and B). As compared with the spectrogram of CAP, the FT-IR spectroscopy of sCAPs presented one characteristic absorption bands at 669 cm^{-1} describing an asymmetrical Se—O—C stretching vibration (Fig. 1B), which signified that sCAP was successfully modified in selenylation.

3.3. The lymphocytes proliferation changes in single stimulation of polysaccharide

The A_{570} values of every group are listed in Table 2. The A_{570} values of $sCAP_1$–$sCAP_4$, $sCAP_6$ and $sCAP_8$ at 1.563–0.098 μg mL^{-1}, CAP_5 at 0.781–0.098 μg mL^{-1}, $sCAP_7$ at 0.391 and 0.098 μg mL^{-1}, $sCAP_9$ at 1.563–0.391 μg mL^{-1} and 0.098 μg mL^{-1} and CAP at

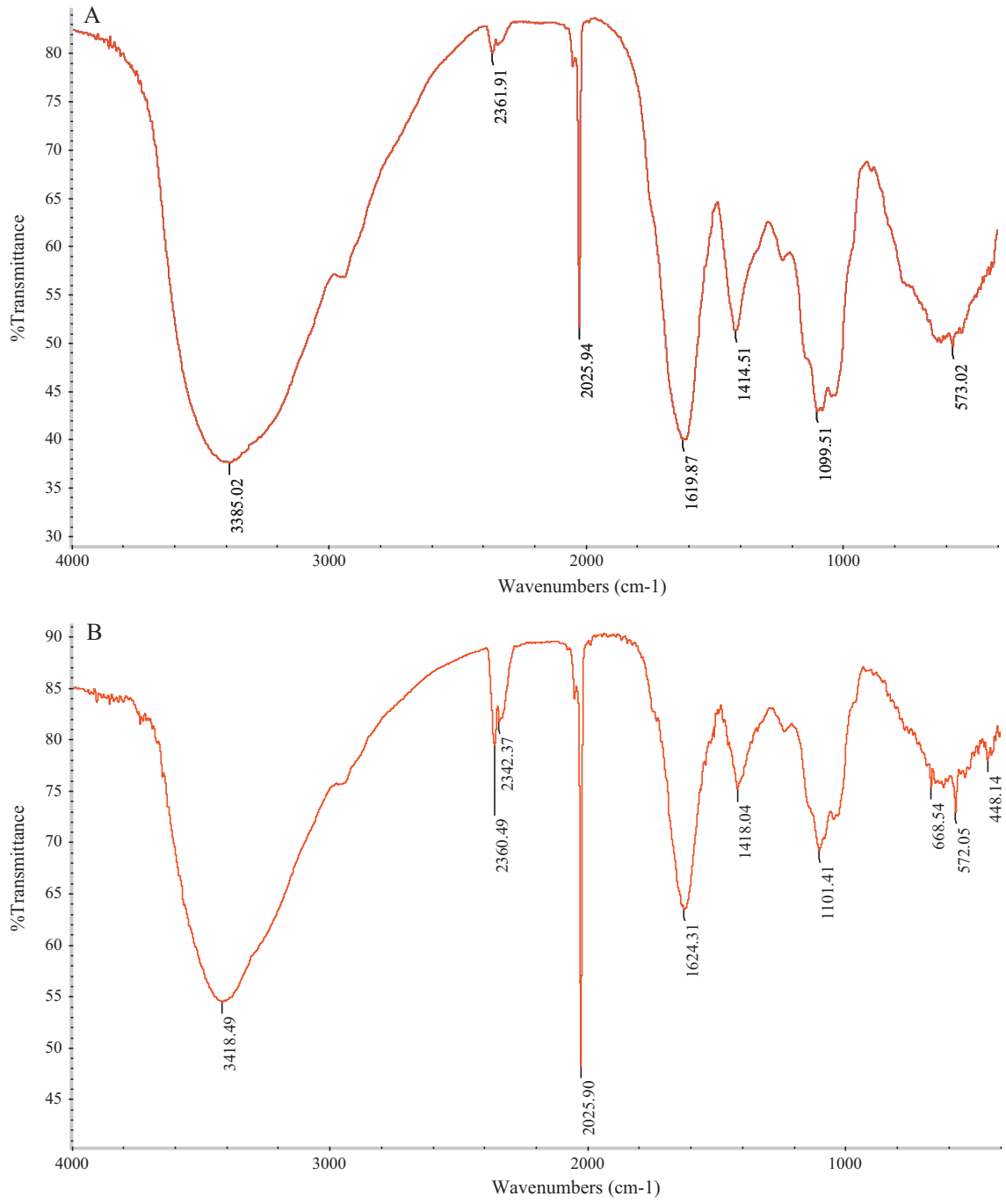

Fig. 1. Infrared spectrum of CAP and selenylated CAP A. CAP; B. sCAP.

0.781–0.391 μg mL^{-1} groups were significantly larger than that of the corresponding cell control group ($P < 0.05$).

The lymphocytes proliferation rates of every group are illustrated in Fig. 2. During the single stimulation of polysaccharide, the proliferation rate of $sCAP_2$ group was the highest (29.90%) and the following was $sCAP_6$ group (24.61%) and $sCAP_8$ group (22.88%), they were significantly higher than that of CAP group (5.26%) ($P < 0.05$).

3.4. The lymphocytes proliferation changes in synergistical stimulation of polysaccharide with PHA

The A_{570} values of every group are listed in Table 3. The A_{570} values of $sCAP_2$–$sCAP_6$–$sCAP_9$ and CAP at 1.563–0.098 μg mL^{-1}, $sCAP_1$

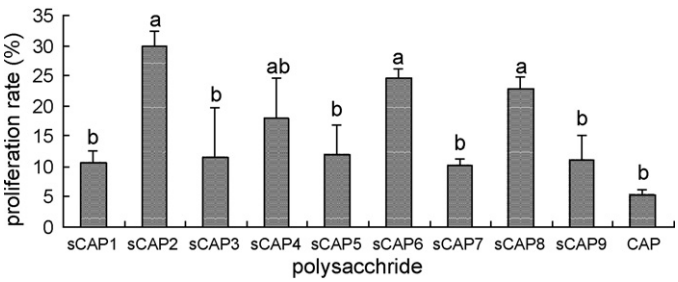

Fig. 2. The lymphocytes proliferation rate of every group in single stimulation of polysaccharide Bars marked without the same letters (a–b) differ significantly ($P < 0.05$).

Table 2
The lymphocytes proliferation changes of every group in single stimulation of polysaccharide (A_{570} value).

Concentration ($\mu g\,mL^{-1}$)	$sCAP_1$	$sCAP_2$	$sCAP_3$	$sCAP_4$	$sCAP_5$
1.563	0.275 ± 0.004^{a}	0.287 ± 0.013^{b}	0.265 ± 0.005^{bc}	0.274 ± 0.007^{b}	0.260 ± 0.002^{de}
0.781	0.270 ± 0.002^{ab}	0297 ± 0.005^{b}	0.257 ± 0.003^{c}	0.276 ± 0.009^{b}	0.272 ± 0.004^{bc}
0.391	0.257 ± 0.002^{c}	0.288 ± 0.010^{b}	0.267 ± 0.007^{b}	0.285 ± 0.007^{b}	0.280 ± 0.013^{b}
0.195	0.268 ± 0.007^{ab}	0.347 ± 0.016^{a}	0.261 ± 0.004^{bc}	0.300 ± 0.001^{a}	0.266 ± 0.004^{cd}
0.098	0.252 ± 0.005^{c}	0.353 ± 0.018^{a}	0.272 ± 0.009^{a}	0.273 ± 0.004^{b}	0.322 ± 0.008^{a}
Cell control	0.239 ± 0.012^{d}	0.242 ± 0.006^{c}	0.237 ± 0.002^{d}	0.239 ± 0.012^{c}	0.250 ± 0.010^{e}
Concentration ($\mu g\,mL^{-1}$)	$sCAP_6$	$sCAP_7$	$sCAP_8$	$sCAP_9$	CAP
1.563	0.310 ± 0.015^{a}	0.239 ± 0.010^{c}	0.309 ± 0.010^{a}	0.323 ± 0.008^{a}	0.268 ± 0.008^{bc}
0.781	0.291 ± 0.008^{b}	0.235 ± 0.005^{c}	0.316 ± 0.011^{a}	0.275 ± 0.008^{cd}	0.279 ± 0.010^{a}
0.391	0.288 ± 0.013^{b}	0.281 ± 0.005^{b}	0.308 ± 0.014^{ab}	0.283 ± 0.004^{bc}	0.278 ± 0.006^{a}
0.195	0.290 ± 0.013^{b}	0.249 ± 0.009^{c}	0.309 ± 0.010^{a}	0.273 ± 0.002^{e}	0.272 ± 0.005^{bc}
0.098	0.299 ± 0.009^{ab}	0.330 ± 0.011^{a}	0.295 ± 0.006^{b}	0.291 ± 0.009^{b}	0.272 ± 0.003^{bc}
Cell control	0.237 ± 0.002^{c}	0.242 ± 0.006^{c}	0.250 ± 0.010^{c}	0.260 ± 0.014^{e}	0.260 ± 0.014^{c}

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

Table 3
The lymphocytes proliferation changes of every group in synergistical stimulation of polysaccharides with PHA (A_{570} value).

Concentration ($\mu g\,mL^{-1}$)	$sCAP_1$	$sCAP_2$	$sCAP_3$	$sCAP_4$	$sCAP_5$
1.563	0.300 ± 0.007^{d}	0.366 ± 0.005^{b}	0.357 ± 0.004^{ab}	0.300 ± 0.005^{cde}	0.312 ± 0.009^{a}
0.781	0.295 ± 0.010^{cd}	0.344 ± 0.007^{bc}	0.332 ± 0.008^{c}	0.308 ± 0.011^{bc}	0.282 ± 0.006^{c}
0.391	0.304 ± 0.018^{cd}	0.376 ± 0.006^{a}	0.360 ± 0.017^{ab}	0.305 ± 0.008^{cd}	0.301 ± 0.010^{c}
0.195	0.331 ± 0.009^{a}	0.352 ± 0.015^{b}	0.371 ± 0.004^{a}	0.328 ± 0.003^{a}	0.301 ± 0.006^{b}
0.098	0.313 ± 0.003^{ab}	0.373 ± 0.007^{a}	0.321 ± 0.007^{d}	0.317 ± 0.009^{b}	0.302 ± 0.003^{b}
PHA	0.287 ± 0.016^{d}	0.310 ± 0.008^{d}	0.312 ± 0.012^{d}	0.287 ± 0.016^{e}	0.276 ± 0.009^{c}
Cell control	0.239 ± 0.012^{e}	0.242 ± 0.006^{e}	0.237 ± 0.002^{e}	0.239 ± 0.012^{f}	0.250 ± 0.010^{d}
Concentration ($\mu g\,mL^{-1}$)	$sCAPS_6$	$sCAPS_7$	$sCAPS_8$	$sCAPS_9$	CAP
1.563	0.364 ± 0.015^{b}	0.329 ± 0.009^{d}	0.329 ± 0.014^{a}	0.321 ± 0.011^{b}	0.328 ± 0.010^{b}
0.781	0.354 ± 0.010^{b}	0.343 ± 0.008^{c}	0.319 ± 0.010^{ab}	0.337 ± 0.009^{a}	0.345 ± 0.010^{a}
0.391	0.379 ± 0.005^{a}	0.346 ± 0.006^{c}	0.323 ± 0.018^{ab}	0.322 ± 0.020^{b}	0.326 ± 0.011^{a}
0.195	0.325 ± 0.009^{c}	0.358 ± 0.011^{b}	0.314 ± 0.003^{ab}	0.327 ± 0.002^{b}	0.337 ± 0.013^{a}
0.098	0.360 ± 0.008^{a}	0.386 ± 0.004^{a}	0.307 ± 0.011^{b}	0.322 ± 0.007^{b}	0.315 ± 0.006^{c}
PHA	0.312 ± 0.012^{d}	0.310 ± 0.008^{e}	0.276 ± 0.009^{c}	0.299 ± 0.007^{c}	0.299 ± 0.007^{d}
Cell control	0.237 ± 0.002^{e}	0.242 ± 0.006^{f}	0.250 ± 0.010^{d}	0.260 ± 0.014^{d}	0.260 ± 0.014^{e}

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

at 0.195–0.098 $\mu g\,mL^{-1}$, $sCAP_3$ at 1.563–0.195 $\mu g\,mL^{-1}$, $sCAP_4$ at 0.781–0.098 $\mu g\,mL^{-1}$, $sCAP_5$ at 0.195–0.098 and 1.563 $\mu g\,mL^{-1}$ groups were significantly larger than that of the corresponding PHA control group ($P < 0.05$). The A_{570} values of $sCAP_1$–$sCAP_9$ and CAP at 1.563–0.098 $\mu g\,mL^{-1}$ groups and all PHA groups were significantly larger than that of the corresponding cell control group ($P < 0.05$).

The lymphocytes proliferation rates of every group are illustrated in Fig. 3. During synergistical stimulation of polysaccharide with PHA, the proliferation rate of $sCAP_2$ group was the highest (16.84%) and significantly higher than that of CAP group ($P < 0.05$);

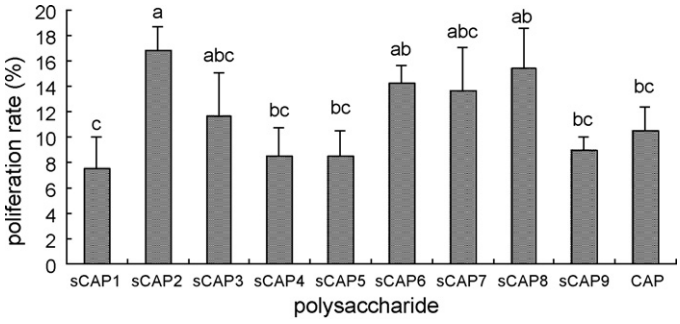

Fig. 3. The lymphocytes proliferation rate of every group in synergistical stimulation of polysaccharides with PHA Bars marked without the same letters (a–c) differ significantly ($P < 0.05$).

the following were $sCAP_8$ group (15.39%), $sCAP_6$ groups (14.22%), $sCAP_7$ group (13.66%) and $sCAP_3$ group (11.61%) and higher than that of CAP group (10.44%) ($P > 0.05$).

4. Discussion

The lymphocytes proliferation reflects the Cellular immunity state (Thekisoe, Mbati, & Bisschop, 2004), and is an important index that evaluates cellular immune function (Wang et al., 2009a,b). Assay of lymphocyte proliferation is a commonly used method for the detection of immune-enhancing activity of drugs. The A_{570} value is correlated to the number of live cells. The bigger A_{570} value is, the stronger lymphocyte proliferation is. When the A_{570} value of the polysaccharide group is significantly larger than that of control group, it indicates that the polysaccharide has significant immune-enhancing activity. The experimental results showed that during single stimulation of polysaccharide, the A_{570} values of $sCAP_1$–$sCAP_4$, $sCAP_6$ and $sCAP_8$ at five concentrations groups, $sCAP_5$ and $sCAP_9$ at four concentrations groups and $sCAP_7$ and CAP at two concentrations groups were significantly larger than that of corresponding cell control group, this indicated that these polysaccharides at these concentrations could significantly promote lymphocytes proliferation singly; During synergistical stimulation of polysaccharide with PHA, the A_{570} values of $sCAP_2$, $sCAP_6$–$sCAP_9$ and CAP at five concentrations groups, $sCAP_3$ and $sCAP_4$ at four concentrations groups, $sCAP_5$ at three concentrations

groups and $sCAP_1$ at two concentrations groups were significantly larger than that of corresponding PHA control group, this indicated that sCAPs and CAP at suitable concentrations could significantly promote T lymphocytes proliferation synergistically with PHA.

The lymphocytes proliferation rate is the indicator to compare the strength of immune-enhancing activity of polysaccharide. In this research during single stimulation of polysaccharide, the proliferation rate of $sCAP_2$ group was the highest, the following was $sCAP_6$ and $sCAP_8$ groups, they were significantly higher than that of CAP group; during synergistical stimulation of polysaccharide with PHA, the proliferation rate of $sCAP_2$ group also was the highest and significantly higher than that of CAP group, the following was $sCAP_8$, $sCAP_6$, $sCAP_7$ and $sCAP_3$ groups, they were numerically higher than that of CAP group. These results indicated that immune-enhancing activities of some sCAPs were significantly or numerically stronger than that of non-modified CAP, selenylation modification could significantly enhance the immune-enhancing activity of CAP, $sCAP_2$ presented best effect and its modification conditions could be as the optimal modification conditions of CAP for improving immune-enhancing activity, that is 200 mg of sodium selenite for 500 mg of CAP, the reaction temperature of 70 °C and the reaction time of 6 h.

It was reported that selenizing polysaccharides could significantly improve the cellular and humoral immune response in mice (Xu, Wang, Jin, & Yang, 2009). This experiment confirmed that selenylation modification could significantly enhance the immune-enhancing activity of CAP. As for the mechanisms of selenizing polysaccharides to enhance immunity, besides CAP possessing immunomodulatory action, selenium plays important role in immune response (Hoffmann & Berry, 2008). It can raise the proliferation rate of T lymphocytes and the function of B lymphocytes and increase the number of neutrophil (Hawkes, Kelley, & Taylor, 2001).

However, the immune-enhancing activity of selenizing polysaccharide is not absolutely positive correlation with its selenium content. For example, among 9 sCAPs, $sCAP_3$ had the second highest selenium content and the lowest carbohydrate content, its immune-enhancing activity was similar to that of CAP, while $sCAP_8$ had the lowest selenium content and the highest carbohydrate content, its immune-enhancing activity was significantly higher than that of CAP. $sCAP_2$ had the highest selenium content and the third highest carbohydrate content, it presented the strongest immune-enhancing activity. From this it could be seen that the immune-enhancing activity of selenizing polysaccharide depended on the synergism both carbohydrate and selenium at higher content.

In conclusion, selenylation modification can significantly enhance the immune-enhancing activity of CAP, $sCAP_2$ presented best effect and the optimal modification conditions were 200 mg of sodium selenite for 500 mg of CAP, the reaction temperature of 70 °C and the reaction time of 6 h.

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