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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.

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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

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