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Modification of lily polysaccharide by selenylation and the immune-enhancing activity

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Phosphate-buffered saline (PubChem CID: 24978514)
Phytohemagglutinin (PubChem CID: 45480564)
Dimethylsulfoxide (PubChem CID: 679)
Potassium bromide (PubChem CID: 253877)
Sodium selenite (PubChem CID: 24934)
Nitric acid (PubChem CID: 944)
Hydrochloric acid (PubChem CID: 313)
Ascorbic acid (PubChem CID: 54670067)
Heparin sodium (PubChem CID: 22833565)

ABSTRACT

Lily polysaccharide (LP) was extracted, purified and selenizingly modified by HNO_3–Na_2SeO_3 method according to $L_9(3^4)$ orthogonal design. Nine selenizing LPs, sLP_1–sLP_9, were obtained and their immune-enhancing activities were compared taking unmodified LP as control. The results in vitro test showed that sLP_6 presented the strongest activity in promoting lymphocytes proliferation in single and synergetic with PHA, and the relative expression level of IL-2, IL-6 and IFN-γ mRNA of chicken peripheral lymphocytes. The results in vivo test showed that sLP_6 could promote lymphocytes proliferation and enhance the serum antibody titers and serum IL-2, IL-6, IFN-γ contents more significantly than LP in chickens vaccinated with Newcastle Disease (ND) vaccine. These results indicate that polysaccharide selenizing can significantly enhance the immune-enhancing activity of LP and the optimal modification conditions are 400 mg of Na_2SeO_3 per 500 mg of LP, the reaction temperature of 70 °C and the reaction time of 6 h.

1. Introduction

Lily is the dry fleshly scale leaf of *Lilum lancifolium* Thunb., *Lilium brownii* F. E. Brown var. *viridulum* Baker or *Lilum pumilum* DC. and a commonly used Chinese herbal medicine. It possesses the action of nourishing yin (tonifying blood and body fluid), moisturizing lung and easing mental anxiety (Veterinary Pharmacopoeia Commission of the People's Republic of China, 2010). Lily mainly contains polysaccharide, phospholipid, saponins, alkaloid, amino acid and variety of microelement in which the content of polysaccharide is the highest (Qiu et al., 2004). Modern medical researches have shown that lily polysaccharide has the actions of enhancing immunity, lowering blood glucose, antioxidant, anticancer and so on (Li, Li, Liu, & Li, 2009; Yang, Sun, & Fang, 2002).

It was reported that lily polysaccharide possessed 350.5 kDa of average molecular weight, and consisted of glucose, mannose and galactose in a molar ratio of 19:10:1, in which β-Glu(1→4) and α-Man(1→3) alternately formed the main chain in a approximate ratio of 2:1 with β-Glu(1→) as its reducing end and one α-Gal(1→6) branched with one α-Man(1,3) at its 6-O site existed

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in average 30 monosaccharides (Chen, Zhang, Zhu, Yangqiu, & Han, 2014; Gao, 2008; Zhang et al., 2009).

Selenium is a necessary microelement of organisms and has varied biological functions such as antioxidant, hypoglycemic, hypolipidemic, anti-tumor and so on (Xie & Zhang, 2002). In the nature selenium exists in two forms, the inorganic and the organic. As compared with inorganic selenium, organic selenium that exists usually in the form of selenium protein or selenium polysaccharide is characterized by higher biological activity, lower toxicity and easier to be absorbed (Xu, 1994). Selenium polysaccharide can exert the efficacy of selenium and polysaccharide, its activity is higher than that of selenium or polysaccharide (Liu, He, & Chen, 2003). Generally natural selenium polysaccharide exists in plants or microorganisms, but its content is lower even though the plant grown in high selenium area, so that the requirement is far from being met. Evidence showed that selenizing lily polysaccharide could be prepared by employing sodium selenite which hydrolyze generation of H_2SeO_3 to react with 6 site —CH_3OH of glucose and/or mannose in esterification reaction under the catalysis of nitric acid-barium chloride (HNO_3–$BaCl_2$) (Zhang et al., 2009).

In this research, the lily polysaccharide was extracted, purified and modified in selenylation by nitric acid-sodium selenite method (Qin et al., 2013) according to L_9 (3^4) orthogonal design to obtain nine selenizing LPs (sLPs), sLP_1–sLP_9. Their effects on chicken peripheral lymphocytes proliferation in vitro were compared taking unmodified LP as control. Then one selenizing polysaccharide with strongest activity was picked out and intramuscularly injected into 14-day-old chickens vaccinated with ND vaccine. Its effects on peripheral lymphocytes proliferation, serum HI antibody and contents of IL-2, IL-6 and IFN-γ were compared with unmodified LP. The aim of this research is to explore whether the selenylation modification can further enhance the immune activity of LP, screen out the best sLP and modification conditions and provide experiment basis for development of new polysaccharide immunopotentiator.

2. Materials and methods

2.1. Lily and reagents

Lily bought from Nanjing Jinling Pharmacy was product of Haozhou Shuanghua Chinese Herbal Drugs Factory, Anhui Province, China.

Sodium selenite (lot no. 2309B511) was bought from Sangon Biotech. Hydrochloric acid (GR, lot no. 14021230071) and nitric acid (GR, lot no. 14021230071) were the product of Nanjing Chemical Reagent Co., Ltd. Potassium bromide (SP, lot no. F20111017) and ascorbic acid (AR, lot no. 20130801) was the product of Sinopharm Chemical Reagent Co., Ltd. Se element standard solution (GSB 04-1751-2004, standard values 1000 μg mL^{-1}) was provided by National Center of Analysis and Testing for Nonferrous Metals and Electronic Materials.

Heparin sodium (lot no. 425C0215) was the product of Biosharp and dissolved into 2 mg mL^{-1} with calcium and magnesium-free (CMF) phosphate-buffered saline (PBS, pH 7.4) and filtered through a 0.22 μm syringe filter. Lymphocytes separation medium (lot no. LTS0977) was the product of Tianjin Haoyang Biological Manufacture Co., Ltd. RPMI1640 (Gibco) supplemented with benzylpenicillin 100 IU mL^{-1}, streptomycin 100 IU mL^{-1} and 10% mycoplasma-free fetal bovine serum, was dissolved into 10 mg mL^{-1} and used for washing and re-suspending the cells, diluting the mitogen and culturing the cells. Mycoplasma-free fetal bovine serum (lot no. 150228) was the product of Zhejiang Tianhang Biotech Co., Ltd. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Biosharp, lot no. B0013K030100) was dissolved into 5 mg mL^{-1} with CMF PBS (pH 7.4) and filtered through a 0.22 μm syringe filter. Phytohemagglutinin (PHA, Sigma, lot no. L-8754) and heparin sodium solution were stored at −20 °C, MTT solution at 4 °C in dark bottles. Dimethylsulfoxide (DMSO) was the product of Nanjing Chemical Reagent Co., Ltd, lot no. 13081411275. Concanavalin A (ConA, lot no. L7647) was the product of Sigma, USA.

Newcastle Disease vaccine (ND vaccine, La Sota strain, No. 119087) was purchased from Nanjing Tianbang Biotechnology Co., Ltd. Chicken interferon-γ (IFN-γ) ELISA kits, chicken interleukin-2 (IL-2) ELISA kits and chicken interleukin-6 (IL-6) ELISA kits were the products of Suzhou Kaerwen Biotechnology Inc.

2.2. Extraction and purification of LP

Dried lily (1000 g) was crushed into small block, soaked with 3000 mL of 95% ethanol for 12 h, reflowed in water bath of 60 °C for 90 min. After atmospheric drying, the lily was decocted thrice, each adding 8-fold volume water and for 4 h, 2 h and 2 h in turn. The decoctions were filtrated through two-layer gauze, mixed into about 23 L, concentrated by rotary evaporator (Heizbad Hei-VAP, Heldolph, Germany) into 1000 mL, and centrifugated by centrifuge (D37520, Biofuge primo R, Thermo Fisher SCIENTIFIC) at 3000 rpm for 20 min. The supernatant was added with 5.33 L of 95% ethanol (the final concentration up to 80%, v/v) with stirring, after standing for 24 h, the precipitation was lyophilized in vacuum freeze-drying machine (Scientz-12N, Ningbo Xinzhi Biotech Co., Ltd.). 96.61 g of crude lily polysaccharide was obtained whose yield was 9.66% (Yang, Li, Li, Li, & Liu, 2005).

20 g of crude lily polysaccharide was dissolved in 200 mL distilled water, the pH was adjusted to 7.0 with 10% NaOH solution, the trichloroacetic acid (3%) was added up to 7.5% of total volume. After mixed, the solution was standed at 4 °C for 4 h, centrifugated at 3000 rpm for 20 min. The precipitation was lyophilized to get 17.31 g of protein-eliminating LP whose yield was 86.55% (Guo, Yan, Zhang, & Wu, 2001).

DEAE-Cellulose-52 (Whatman 4057200 Cat. No.C8350) was soaked in distilled water for 12 h, then pack into chromatograph column (2.6 cm × 30 cm). After the column was balanced for 48 h, 50 mg of protein-eliminating LP was dissolved into 10 mg mL^{-1} with distilled water was added into column. The elution was performed using 500 mL of distilled water and the flow rate was maintained at 1 mL min^{-1}. The eluent was collected by automatic fraction collector (Model BS-100A automatic fraction collector and HL-2B constant flow pump, Shanghai Huxi Analysis instrument Factory CO., Ltd), 5 mL per tube, total 96 tubes and detected for polysaccharide by the phenol-sulfuric acid method (Yang, Li, Li, & Liu, 2004). The elution curve was drawn (one peak, Fig. 1A). The eluents in tubes contained polysaccharides were merged and lyophilized to get 31.17 mg of purified LP whose yield was 62.34% (Ni, Li, Ren, & Mei, 2009; Zhang et al., 2013).

2.3. Modification conditions and selenylation reaction

The HNO_3–Na_2SeO_3 method was applied (Qiu et al., 2014). Nine modification conditions were designed according to L_9 (3^4) orthogonal test of three factors respectively at three levels, the Na_2SeO_3 usage amount at 200, 300 and 400 mg for 500 mg LP (A), the reaction temperature at 50, 70 and 90 °C (B) and the reaction time for 6, 8 and 10 h (C) (Table 1).

In nine conical flasks filled with 50 mL of 0.5% HNO3 solution, 500 mg of purified LP was respectively added stirring to make LP be completely dissolved. Then according to the conditions designed in Table 1, 200 mg or 300 mg or 400 mg of sodium selenite was added and the reaction was performed at 50 °C or 70 °C or 90 °C for 6 h or 8 h or 10 h, respectively. After the reaction finished, the reaction liquid was cooled to room temperature, adjusted pH