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A polysaccharide purified from Radix Adenophorae promotes cell activation and pro-inflammatory cytokine production in murine RAW264.7 macrophages

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[ABSTRACT] Radix Adenophorae, a traditional Chinese medicine, has been reported to have a variety of biological functions. In the present study, a polysaccharide component, Radix Adenophorae Polysaccharide (RAPS), was purified from Radix Adenophorae by decoloring with ADS-7 macroporous adsorption resin, DEAE-52 cellulose ion-exchange chromatography, and Sephacryl S-300HR gel chromatography, with the purity of 98.3% and a molecular weight of 1.8×10^4 Da. The cell viability assay and microscopic examination revealed that RAPS promoted the proliferation and activation of macrophages. At 400 μg·mL^{-1}, RAPS stimulated RAW264.7 cell proliferation by 1.91-fold compared with the control. Meanwhile, RAPS significantly increased the secretion of pro-inflammatory cytokines (TNF-*α* and IL-6) in a dose-dependent manner in the supernatant of RAW264.7 cell culture as determined by ELISA. At 400 μg·mL^{-1}, the production of TNF-*α* was 20.8-fold higher than that of the control. Simultaneously, the production of nitric oxide (NO) and the expression of inducible nitric oxide synthase (iNOS) were increased in RAW264.7 cells incubated with RAPS, as measured by Griess assay and Western blot analysis. The NO production of cells treated with RAPS (400 μg·mL^{-1}) reached 15.8 μmol·L^{-1}, which was 30.4-fold higher than that of the control (0.53 μmol·L^{-1}). These data suggested that RAPS may enhance the immune function and protect against exogenous pathogens by activating macrophages.

[KEY WORDS] Polysaccharide; RAPS; Purification; Macrophage activation; Pro-inflammation

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Introduction

Radix Adenophorae, a traditional Chinese medicine (TCM) named Nan Sha Shen, is the dry root of *Adenophora tetraphylla* (Thunb.) Fisch. or *Adenophora stricta* Miq. [1]. It is reported that Radix Adenophorae has many functions, such as strengthening the phagocytosis of giant cells, regulating cellular and humoral immunity, enhancing cardiac function and relieving pain and cough, according to the TCM theory [2]. Previous studies have found that polysaccharides are widely used in the immunotherapy due to their effective treatment, low toxicity, and limited side effects [3]. Some of these polysaccharides have been reported to have antitumor [4-5], antiviral [6], antioxidant [7], anti-inflammatory [8], and immunomodulatory [9] activities. These polysaccharides are generally derived from microorganisms, plants and animals. Polysaccharides purified from medicinal plants exert therapeutic properties by regulating innate immunity and macrophage function [10-11]. A previous study has shown that crude polysaccharides exist in Radix Adenophorae [12], from which we have purified the polysaccharide component Radix Adenophorae Polysaccharide (RAPS). However, the immunomodulatory activity of RAPS has not been studied.

Macrophages play a vital role in the induction and regulation of the specific immune response because of its

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strong phagocytic and antigen presentation ability [13]. These functions depend on the secretion of pro-inflammatory cytokines, including tumor necrosis factor (TNF-α), interleukin-6 (IL-6), and nitric oxide (NO) [14-15]. TNF-α is an important pro-inflammatory cytokine and immunomodulatory molecule, which participates in the host defense response. Biological functions of IL-6 include anti-bacterial, anti-viral, and anti-tumor activities. NO is an inflammatory mediator that is produced by the activation of inducible nitric oxide synthase (iNOS) from L-arginine in macrophages [16]. Therefore, the regulation of inflammatory cytokines, which are released from activated macrophages, is an important approach for the treatment of various inflammatory disorders [17].

In the present study, we successfully purified the polysaccharide component RAPS from Radix Adenophorae with a purity of 98.3%. Meanwhile, we are the first to discover that RAPS could effectively activate macrophages. We used murine RAW264.7 macrophages to explore the mechanisms of action for RAPS by assaying the proliferation and pro-inflammatory cytokine production of the cells. Our data suggested that RAPS may have the ability to enhance the immunity against exogenous pathogens by activating macrophages.

Materials and Methods

Materials

Radix Adenophorae was purchased from Tongrentang Pharmacy (Beijing, China), which was produced in Guizhou of China to extract the polysaccharide. ADS-7 macroporous adsorption resin was purchased from Cangzhou Bon Adsorber Technology Co., Ltd. (Cangzhou, Hebei, China). DEAE-52 cellulose and Sephacryl S-300 HR were purchased from Whatman (Maidstone, United Kingdom). Dextran series with varying molecular weights of 5 000, 12 000, 25 000, 50 000, 80 000, 150 000, 270 000 and 410 000 Da were purchased from Amersham Biosciences (Uppsala, Sweden). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Chemical Laboratories (Kumamoto, Japan). Dulbecco's modified Eagle's medium-high glucose (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Vienna, NY, USA). Streptomycin, penicillin, and Griess reagent were purchased from Beyotime Biotechnology (Nantong, Jiangsu, China). IL-6 and TNF-α mouse sandwich-based enzyme-linked immunosorbent assay (ELISA) kits were purchased from Multi Sciences (Hangzhou, Zhejiang, China). Anti-iNOS and anti-β-actin antibodies were purchased from SAB (College Park, MD, USA).

Isolation and purification of polysaccharide

Dried crushed Radix Adenophorae (100 g) was extracted thrice with hot distilled water (*W*/*V*, 1 : 10) at 90 °C (for 3 h each). After filtration using gauze, the aqueous extracts were combined and concentrated into 200 mL at 50 °C in a vacuum. Subsequently, the proteins in the solution were removed using the Sevag method five times and the supernatant was precipitated with a four-fold volume of ethanol (80% final concentration; *V*:*V*) under vigorous stirring at 4 °C overnight. The precipitate was obtained through filtration and dried under reduced pressure after washing with ethanol and acetone to collect the crude polysaccharide (13.9 g, 13.9%).

The crude polysaccharide (1.0 g) was dissolved in 25 mL of distilled water and centrifuged at 8000 r·min^{-1} for 10 min. For decoloration, the supernatant was applied onto an ADS-7 macroporous adsorption column (2.6 cm × 30 cm) that was eluted with deionized water at a flow rate of 3 mL·min^{-1}. The main fraction was collected and concentrated into 20 mL by ultrafiltration (0.05 MPa). The retentate was purified with a cellulose DEAE-52 column (4.5 cm × 30 cm) that was eluted with deionized water and in a gradient of 0−1.0 mol·L^{-1} NaCl at a flow rate of 1 mL·min^{-1}. The first peak eluted with deionized water was named RAPS and the second one obtained by gradient NaCl elution was called RAPS-S. A Sephacryl S-300 HR gel-filtration column (1.6 cm × 120 cm) was used to further purify RAPS using distilled water as an eluent at a flow rate of 0.3 mL·min^{-1} after the concentration into 10 mL through ultrafiltration. The eluates were dialyzed and lyophilized to obtain white fluffy pure polysaccharide RAPS (191.2 mg, Fig. 1).

Homogeneity and molecular weight

The purity and molecular weight of RAPS were determined by high-performance gel permeation chromatography using the Agilent 1100 HPLC system (G1311A tetra-pump, Agilent Technologies Inc., PaloAlto, CA, USA), equipped with Shodex SB-805 and Shodex SB-802 columns (7.8 mm × 300 mm) purchased from Shodex (Tokyo, Japan) and a G1362A refractive index detector (RID). The polysaccharides were dissolved in distilled water (10 mg·mL^{-1}) and passed through a 0.22-μm filter. 20 μL of the sample was injected onto the HPLC column that was eluted with distilled water at 1.0 mL·min^{-1}. The HPLC system was pre-calibrated with T-series Dextran standards.

The carbohydrate content was determined by phenol-sulfuric acid method using glucose as the standard [18]. The protein content was estimated by Bradford method [19]. The content of uronic acids was measured by the m-hydroxydiphenyl method using galacturonic acid (GalA) as the standard [20].

Cell culture, cell proliferation assay, and microscopic observation

The murine macrophage cell line RAW264.7, purchased from ATCC (Manassas, VA, USA), was maintained in DMEM containing 10% heat-inactivated FBS, 100 U·mL^{-1} of penicillin and 100 μg·mL^{-1} of streptomycin at 37 °C under a humidified atmosphere with 5% CO_2 and 95% air.

The cytotoxic effects of RAPS on RAW264.7 cells were assayed using the CCK-8 method [21]. Briefly, adherent RAW264.7 cells in 96-well plates (5 × 10^4 cells/well) were