PURIFICATION AND IMMUNE ACTIVITY OF SMALL-MOLECULE POLYSACCHARIDES FROM *GRIFOLA FRONDOSA*

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ABSTRACT

A water-soluble crude polysaccharide preparation (GFP) was obtained from *Grifola frondosa* fruit bodies by hot water (100 °C) extraction, followed by ethanol (75%) precipitation. Purified polysaccharides, GFP75-2D was obtained from the crude material by successive fractionation using DEAE-Sepharose Fast Flow ion exchange chromatography and Sephacryl S gel filtration chromatography. GFP75-2D mainly contained fucose, galactose, and mannose by UV spectroscopy scan. Moreover it showed the activities to stimulate macrophages and enhanced the release of NO from RAW264.7 macrophages. Furthermore, the concentration of Interleukin-1β (IL-1β) and TNF-α were significantly stimulated after exposed to GFP75-2D whereas modest increase was detected in IL-6.

Keywords: *Grifola frondosa*; Polysaccharide; Purification; Immune activity; Macrophage

INTRODUCTION

Maitake (*Grifola frondosa*) is a polypore mushroom that grows in clusters at the base of trees in temperate forests. The fungus is native to many provinces such as Hebei, Zhejiang, Sichuan, Jilin and Fujian in China. Great attentions have been paid to artificial cultivation of it for over 30 years in Zhejiang and Hebei province due to its delicious tastes and unique aroma. It is also prized as a medicinal mushroom rich in a variety of active substances. *Grifola frondosa* polysaccharides, main active ingredients, have been identified to have significant effects in anti-tumor [1], anti-hypertensive [2], anti-diabetic [3], immunity-improving [4], as well as many other pharmacological activities.

In this study, crude polysaccharides were purified, and then their activities were tested. In addition, structures of purified polysaccharides were analyzed using chromatographic and spectroscopic methods. Further tests were also implemented to evaluate the immune activity of these polysaccharides.

MATERIALS AND METHODS

Maitake (fruit bodies) was obtained from Zhejiang Province, China. Bacterial lipopolysaccharide (LPS) from Sigma; double-antibody (penicillin, streptomycin) from Amresco Inc., DMEM medium, RPMI-1640 medium, fetal bovine serum (FBS) for the Gibco products; ELISA kit is the product of Beijing Zheng Si Bo Ltd., and other reagents were analytical grade domestically. RAW264.7 macrophage cell line was purchased from cells institute of Chinese Academy.

Purification of small-molecule polysaccharides from GFP. Crude polysaccharides were obtained from *Grifola frondosa* by hot water extraction and ethanol precipitation, and were sequentially purified by ion exchange chromatography on DEAE-Sepharose Fast Flow column and gel permeation chromatography on Sephacryl S column, and the fraction GFP75-2D was obtained.

Identification to initial structure of GFP75-2D. Qualitative detection of sugar was determined with phenol - sulfuric acid assay [6]. GFP75-2D was prepared to1mg/mL solution for the 200 ~ 400nm UV scanning.

Analysis of monosaccharide composition. To 2mg of GFP75-2D, 3ml 2M trifluoroacetic acid (TFA) were added, and hydrolyzed for 3h at 110 °C, and after addition of methanol, the residual TFA was evaporated in vacuum. The sample was dissolved with 1mL pure water for ion chromatography analysis. The chromatographic system was: Dionex ICS2500, column for the Carbon Pac PA-20; flowing phase, A phase: deionized water, B phase: 0.25M NaOH, C phase: 1M NaAc; flow rate 0.45mL/min; elution program shown in Table 1.

Table 1: Ion chromatographic elution procedure

Time Minutes	A phase, deionized water %	B phase, 0.25M NaOH %	C phase, 1M NaAc; %
0	99.2	0.8	0
30.0	99.2	0.8	0
40.0	79.2	0.8	20
40.1	20.0	80.0	0
60.0	99.2	0.8	0

Measurement of nitric oxide (NO). Production of nitric oxide (NO) was estimated by measuring nitrite levels by the Griess reaction [5]. To each well of a 96-well microplate, 180 µl of the macrophage suspension (1×106 cells/ml) and 20 µl of various test substances were added. After incubation 37 °C in a 5% CO_2 atmosphere for 48 h, 100 µl of cell-free supernatants was mixed with 50 µl of Griess reagent (1% sulfanilamide,0.1% naphthylethyylene-diamine dihydrochloride, 2.5% phosphoricacid) and incubated for 10 min at room temperature. The optical densities (O.D.) of samples were measured at 543 nm. The nitrite concentration was determined with a standard curve of linear sodium nitrite from 0.1 to 100 µM.

Quantification of interleukin-1ß (IL-1ß), interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α). Production of the cytokines IL-1ß, IL-6 and TNF-α by RAW264.7 macrophages were quantified by ELISA according to the manufacturer's instructions. RAW264.7 macrophages were adjusted to a concentration of 1×10^5 cells/ml. To each well of a 96-well microplate, 180 µl of the cell suspension and 20 µl of various test substances were added. After incubation at 37 °C

in a 5% CO_2 atmosphere for 24 h, 50 µl of cell-free supernatant from each well was used for the IL1 ß, IL-6 and TNF- α ELISA-assay.

RESULTS

Crude GFP75 fractions by ion chromatography. Six components including a component of aqueous phase and five salt phase components, named GFP75-1, GFP75-2, GFP75-3, GFP75-4, GFP75-5 and GFP75-6 respectively，were obtained by DEAE FF column chromatography(see Fig.1). Among the six components, GFP75-2, possessing immune activity and being light-colored, was ready for the further separation.

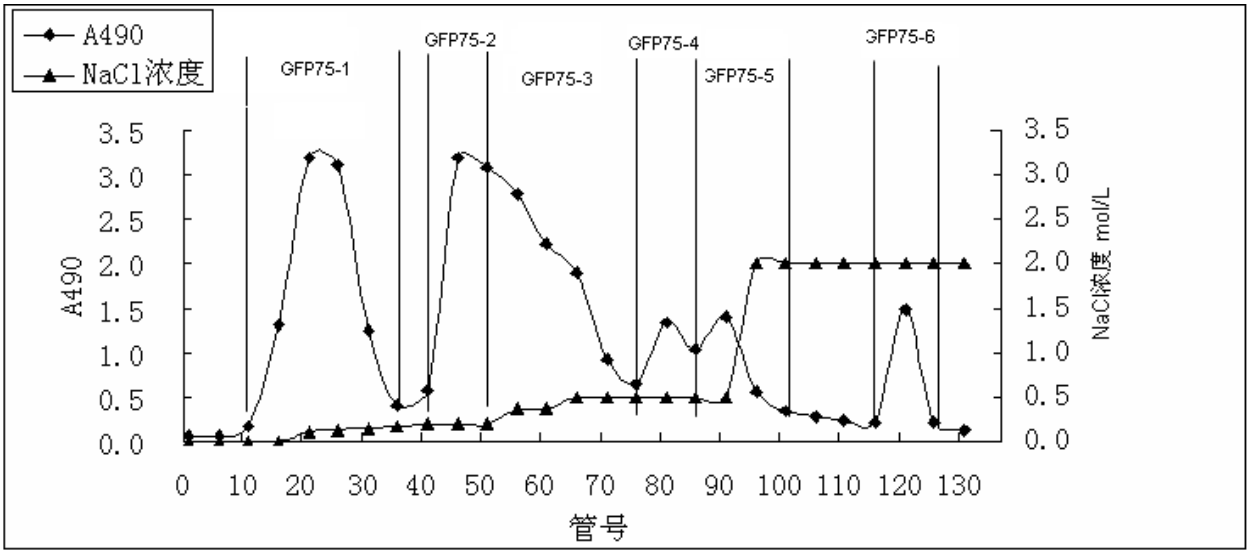


Figure 1: DEAE-Sepharose Fast Flow column chromatography of fraction GFP75

Gel column chromatography of eluted fractions of GFP75-2. Four components, named GFP75-2A, GFP75-2B, GFP75-2C and GFP75-2D, were obtained as fractions of GFP75-2 separated by Sephacryl S-400 (see Fig. 2). Parts of continued collection of small molecular weight were further separated by Sephacryl S-100 to achieve GFP75-2C and GFP75-2D of complete separation (see Fig. 3). We obtained two symmetrical single peaks. GFP75-2D was tested as a single symmetrical peak (Figure 4). Its molecular weight was about 2600Da by HPLC.

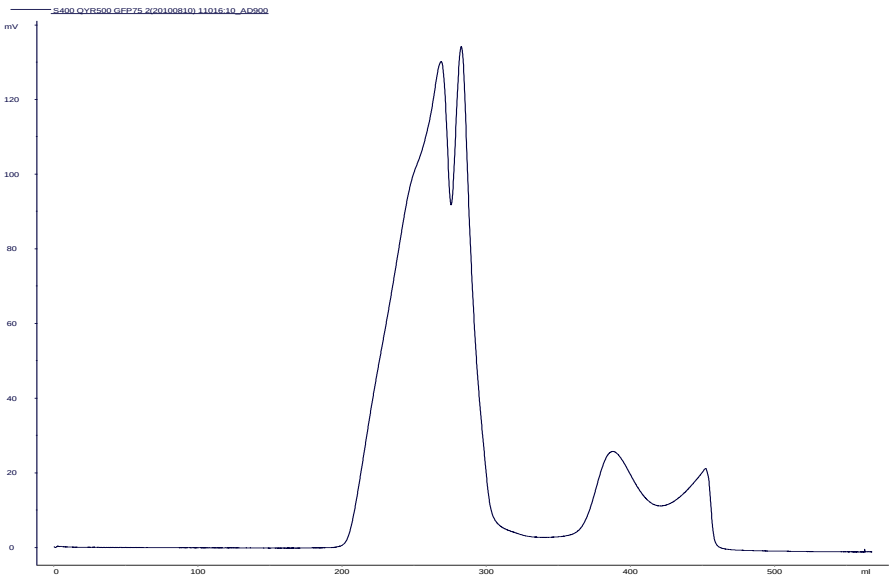


Figure 2: Sephacryl S-400 column (26×100) chromatography of fraction GFP75-2

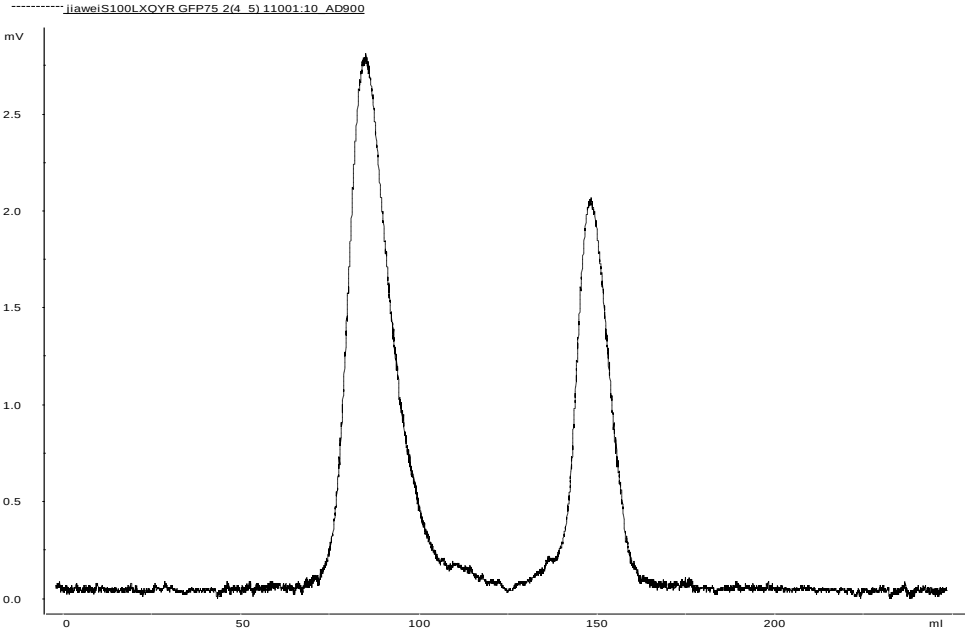


Figure 3: Sephacryl S-100 column (16×100) chromatography of low molecular weight fraction GFP75-2

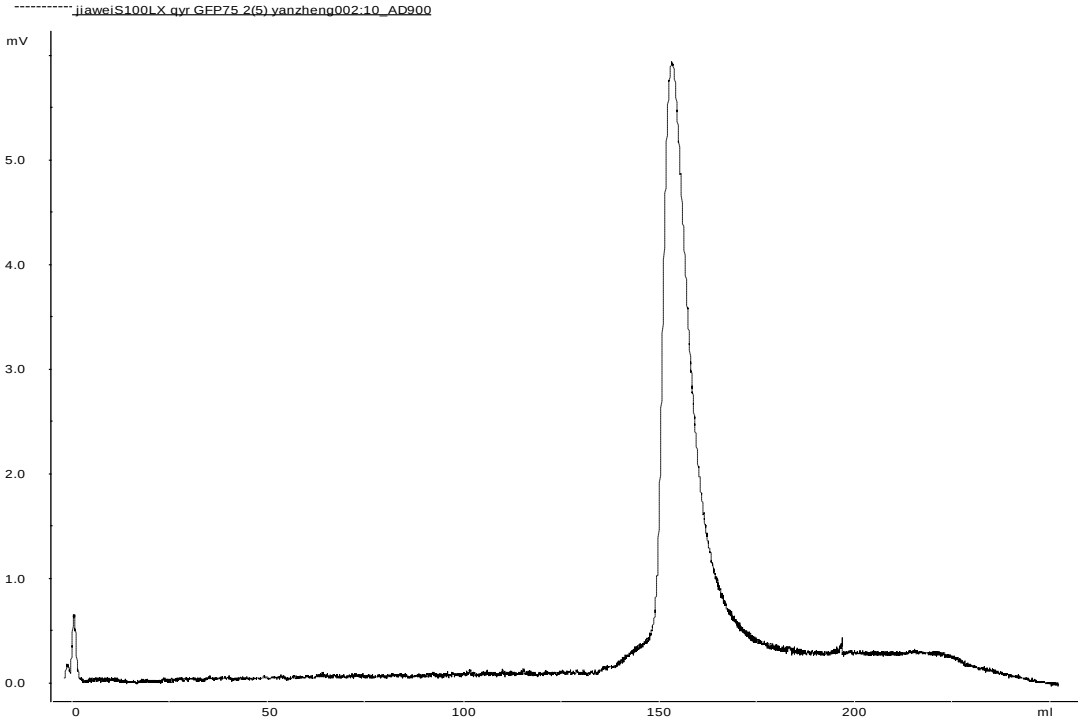


Figure 4: Sephacryl S-100column (16×100) chromatography of fraction GFP75-2D

Preliminary identification of the structure. GFP75-2D showed a characteristic color reaction of sugar by the phenol - sulfuric acid assay. There was no characteristic absorption peak in the vicinity of 280 nm and 254 nm, indicating no proteins and nucleic acids. 200 ~ 400nm in the UV scan shows that there was no-phenolic components in GFP75-2D (Fig. 5).

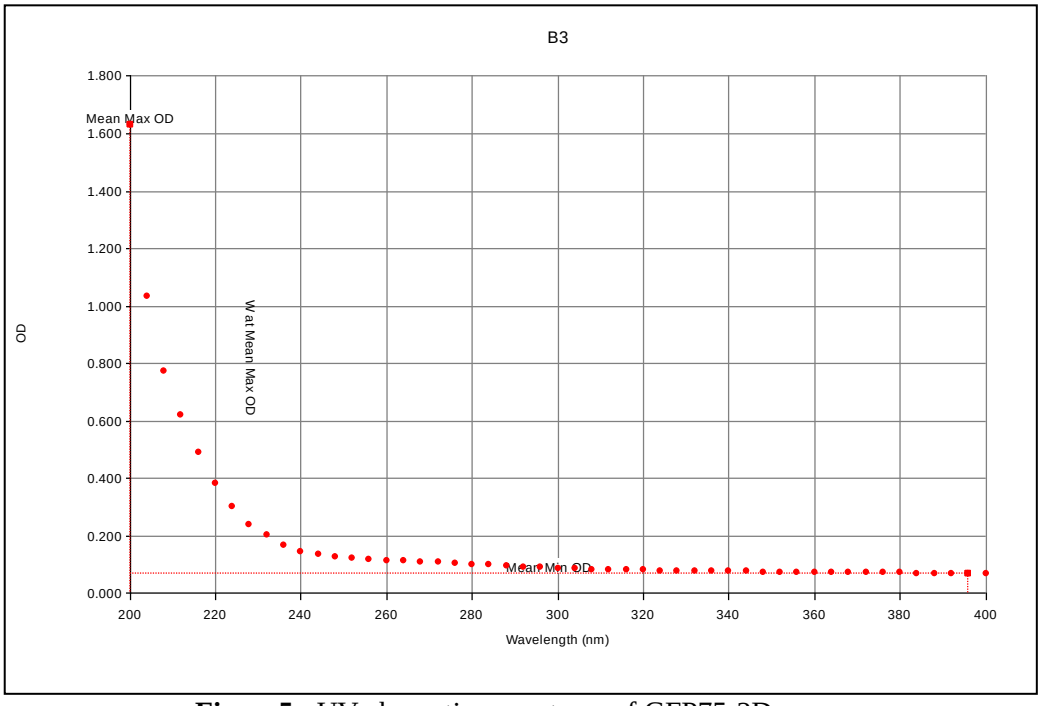


Figure5: UV absorption spectrum of GFP75-2D

Six peaks were displayed in GFP75-2D ion chromatogram results (Fig. 6). We could draw that GFP75-2D contains mainly fucose, rhamnose, galactose, glucose and mannose with the molar ratio of 1:1.3:3.6:4.0:4.5 by reference to standard single-sugar control standard.

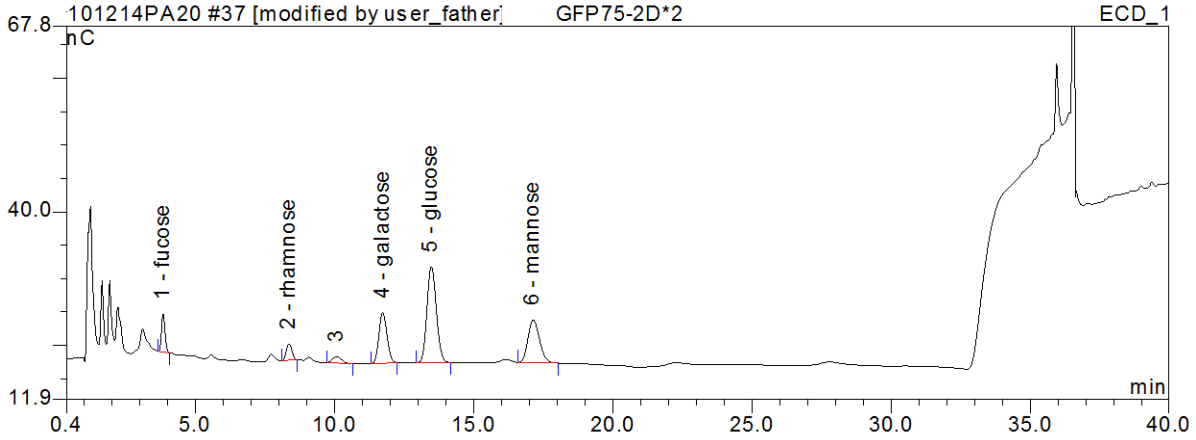


Figure 6: Ion chromatogram of the monosaccharide in GFP75-2D

NO production of the macrophage cell line RAW264.7 was stimulated with eluted fractions by ion-exchange chromatography. The water phase elution fractions couldn't stimulate macrophages to produce NO (Fig.7). Relatively, salt elution fractions could stimulate macrophages to produce NO. The results indicated that immune activity of GFP75 obtained by ion chromatography was strengthened. The salt phase elution fractions, GFP75-2 and GFP75-3 showed higher immune activity than the others. In addition, these two fractions contained more active ingredients due to gradually increased immune activity with higher sample concentration. GFP75-2 possessing good immune activity and light color was further purified.

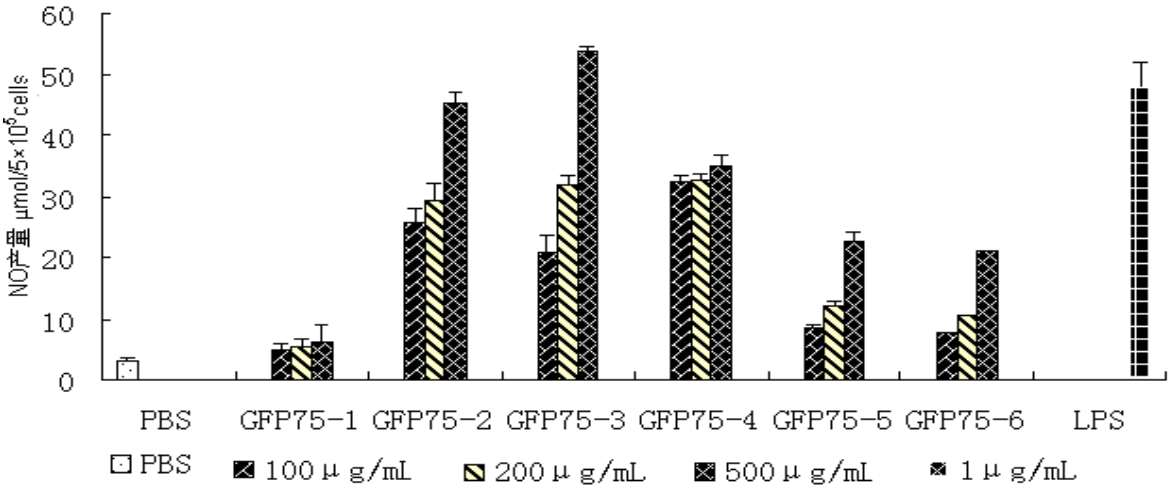


Figure7: NO production of fractions from anion exchange chromatography of GFP75

The NO production of RAW264.7 after stimulation by the purified GFP75-2D significantly increased (Fig.8). At low concentrations 10 μg/mL, GFP75-2D already showed a good stimulation. The concentration of 100 μg/mL，the activity of GFP75-2D was higher than the positive control LPS.

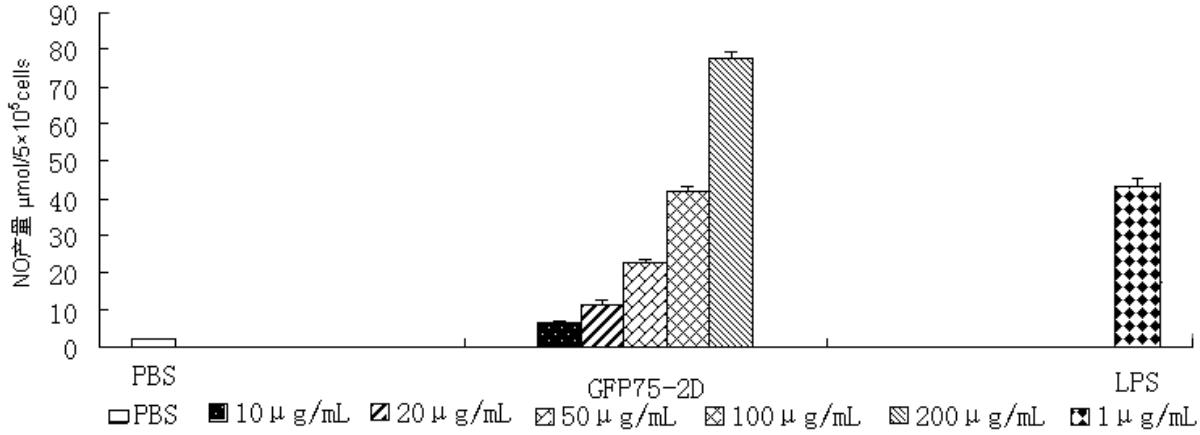


Figure8: Effect of GFP75-2D on NO release from RAW264.7 cells

Change in cytokine TNF-*α*, IL-1β, IL-6 production of RAW264.7 after stimulation by GFP75-2D *in vitro*. The release of TNF-α (Fig.9) after stimulation by GFP75-2D were significantly increased in the cell culture supernatant, at the same time, the sample stimulus group didn't release TNF-α. Tthe production of TNF-α was the highest when GFP75-2D concentration reached 100 μg/mL, slightly higher than the positive control LPS. The release of IL-1β (Fig.10) and GFP75-2D concentrations was positively correlated. When GFP75-2D concentration increased into 100 μg/mL, the release of IL-1β was the highest too, but slightly lower than the positive control LPS. The release of IL-6 (see Fig.11) and GFP75-2D concentrations were positively correlated too, and achieved the highest at 100 μg /mL, but far in the positive control LPS.

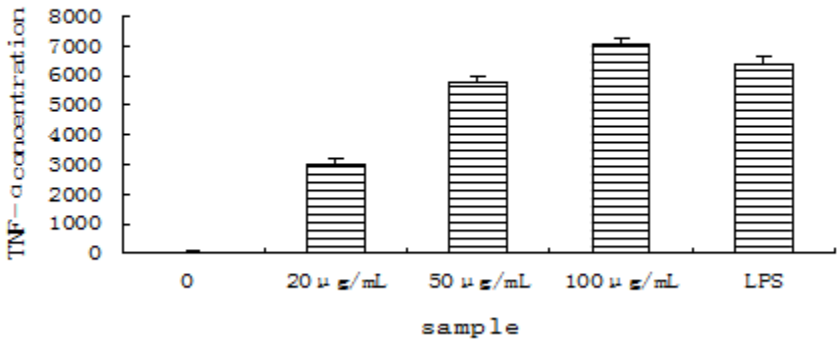
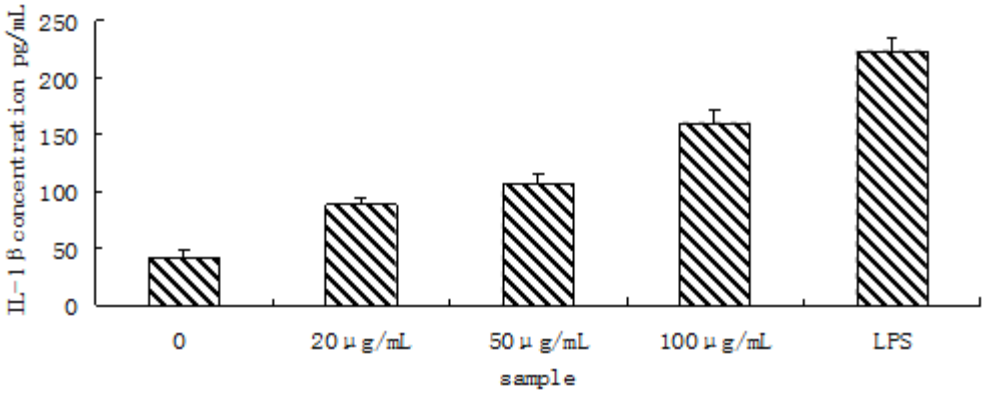


Figure 9: Effect of GFP75-2D on TNF-α production in RAW264.7



Fiure10: Effect of GFP75-2D on IL-1β production in RAW264.7

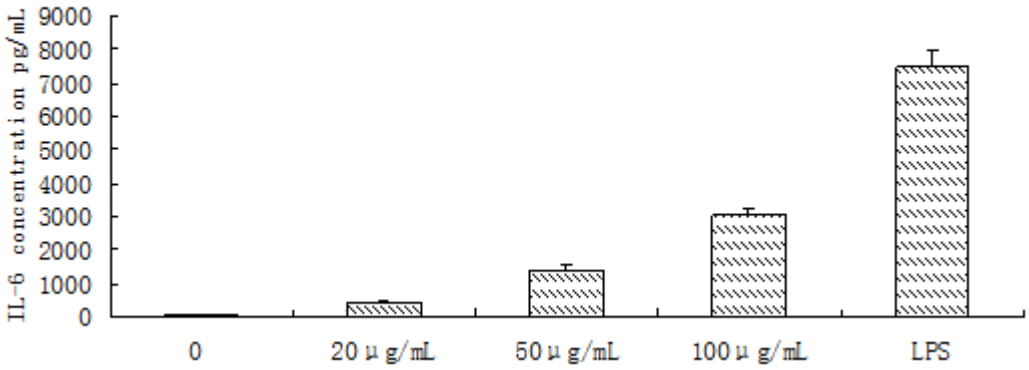


Figure 11: Effect of GFP75-2D on IL-6 production in RAW264.7

DISCUSSION

Generally we believed that the biological activity of polysaccharides depended on molecular size. The small molecular weightpolymers of sugar obtained from *Grifola frondosa* fruiting bodies in this study has shown a good immune activity, while the small molecular weight GFP and their immune activity was rarely reported. The *Grifola frondosa* polysaccharides stimulated macrophage cell line RAW264.7 into the activated state, and also made contribution to increase in cellular NO secretion, cytokine TNF-α, IL-1β and IL-6 secretion, and concentration-dependent with the purified polysaccharide GFP75-2D. This suggested that GFP enhance the body's immune system related to activation of macrophages to promote TNF-α, IL-1β and IL-6 secretion of cytokines. It also showed that GFP activated macrophages through a variety of ways to play a role in immune response. It's worth further investigating at the reason whether the existance of receptor-binding specificity of the fragments or not that lead to GFP75-2D possesses a good immune activity with low molecular weight.

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