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Orthogonal test design for optimization of the extraction of polysaccharides from *Phascolosoma esulenta* and evaluation of its immunity activity

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Abstract

Yield of polysaccharides from *Phascolosoma esulenta* obtained by phosphate buffer extraction through an orthogonal experiment ($L_9(3)^4$) were investigated to get the best extraction conditions. The results showed that extraction temperature, ratio of phosphate buffer to raw material, extraction time, and ratio of trypsinase to raw material were the main four variables that influenced the yields of extracts. The highest yield was obtained when extraction temperature, ratio of phosphate buffer to raw material, extraction time and ratio of trypsinase to raw material were 40 °C, 2, 5.5 h and 1.6, respectively. The immunity-stimulating method showed that polysaccharides from *P. esulenta* could significantly raise liver, spleen and thymus index of mice and enhance Con A-stimulated mouse spleen cells proliferation. These results indicate that polysaccharides from *P. esulenta* had significantly higher immunity-stimulating activities.

Keywords: Polysaccharides from *Phascolosoma esulenta*; Orthogonal experiment; Immunity; Mice; Extraction

1. Introduction

Phascolosoma esulenta is one of famous seafoods produced in china. This sipunculan has inconspicuous, finger-like, unbranched filiform tentacles (at the anterior end of the introvert) (Ivanova et al., 2003). The introvert (extensible anterior end) has dark blotches and transverse streaks, with 15–25 rings of small hooks near its anterior end. Skin is rough due to conical papillae that are largest at the posterior region of the trunk (Ip, Chew, Peng, & Lim, 1992). Except for its usage in food, *P. esulenta* is still a natural nutrient and a important traditional Chinese medicine which may be especially useful for nourishing the kidneys, nourishing the YIN, and moisturizing the lungs (Peng & Lei, 2007). It can still improve immunity activity and fight tiredness (Jiang, Sheng, Jia, Chu, & Li, 2004). Therefore, it is assumed to be a traditional Chinese herbal supplement FREE from additives, chemicals or preservatives.

The polysaccharides isolated from natural plants, animals and fungi have been used in traditional Chinese medicine (TCM) as anti-tumor, antioxidant and immunomodulating agent (Cao et al., 2006; Kim, Oh, & Park, 2003; Zhang, Cheung, Chiu, Wong, & Ooi, 2006; Kosaraju, D'ath & Lawrence, 2006; Xie et al., 2006; Zhu & Lin, 2006). They also exhibit liver protective, hypoglycemic and platelet aggregation-inhibiting activities (Don, King, Chiu, & Peng, 2006; Park, Lai, & Kim, 2004). The active constituents responsible for each of these activities have been qualitatively described (Gan, Zhang, Yang, & Xu, 2004; Han et al., 2003; Ebringerová et al., 2008). Modern medicine has proven that the extract of *P. esulenta* contains a large amount of biological active substances, such as protein and microelement (Jiang et al., 2004). Previous studies have shown that the extract exhibit the anti-tumor activity, antioxidant, anti-aging, anti-fatigue and are able to stimulate the milk secretion of mice (Jiang et al., 2004; Shen,

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Jiang, & Jia, 2003). Of particular significance among these function components is its polysaccharides. Previous studies have shown that the polysaccharide components of *P. esulenta* exhibit the strong immunity-stimulating activity and are able to stimulate the expression of CD4 and T-cell counts (Peng & Lei, 2007). In the present experiment, we optimized extraction parameters of polysaccharides from *P. esulenta* by employing an orthogonal $L_9(3)^4$ test design and evaluated its immunity activity.

2. Experimental

2.1. Reagents

Ethanol, chloroform, bluestone, phenol, *n*-butanol, potassium bromide and sulfuric acid were obtained from the Guangzhou Reagent Co. (Guangzhou, China). Albumen bovine, Con A, and 3-(4,5)-dimethylthiahiazo(-*z*-y1)-3,5-di-phenytetrazoliumromide (MTT) were purchased from Sigma Chemical Company (St. Louis, MO). RPMI 1640 Culture medium was purchased from Nikken Bio Medical Laboratory (Kyoto, Japan). Calf serum was purchased from Huamei Bioengineering Company of Shanghai (ShangHai, China). All other solvents and chemicals were analytical grade and purchased from NanJing JianChen Chemical Ltd., Co, (NanJing, China).

The *P. esulenta* was purchased from a local market (Taizhou, China).

2.2. Preparation of polysaccharides from Phascolosoma esulenta

Phascolosoma esulenta (20 g) were homogenized in a blender. The result material was then extracted with 0.3 mol/L, pH 8.0, phosphate buffer containing a appropriate ratio of trypsinase at 40 °C for a given time. Then, protein in the extract was removed by sevag method. The extract was neutralized with acetic acid (HOAc), precipitated with the addition of 70% ethanol and separated by centrifugation (15 min, 9000 rpm, 25 °C) to obtain crude extract. The crude extract was then dialyzed against tap water, deionized with mixed ion exchange resins and dried under reduced pressure to give desire product.

2.3. Optimization of polysaccharides extraction

An orthogonal $L_9(3)^4$ test design in the extraction mode was used for optimization the extraction conditions. In this study, extraction was accomplished with 100 ml volume phosphate buffer. Nine extractions were carried out at extraction temperature of 40, 45 and 50 °C, ratio of phosphate buffer to raw material 1.7, 2 and 2.3, extraction time 4.5, 5, 5.5 and ratio of trypsinase to raw material 1.6, 1.8, 2 on the basis of the single-factor test. Table 1 shows the experimental conditions for the extraction of polysaccharides from *P. esulenta*. The extract was collected and evaporated to dryness at 60 °C under reduced pressure, and weighted.

Table 1
Factors and levels for orthogonal test

Variable	Level		
	1	2	3
A, extraction temperature	40	45	50
B, ratio of phosphate buffer to raw material	1.7	2	2.3
C, extraction time	4.5	5	5.5
D, ratio of trypsinase to raw material	1.6	1.8	2

2.4. Animal experiment

Forty kunming mice, weighing 30–45 g, were used in these experiments. They were obtained from (Shanghai SLAC Laboratory Animal Co. Ltd). These mice were randomly divided into 4 groups (10 in each group)—control group, the three experiment groups of low, middle and high dose of polysaccharides. All animal use procedures were according to the Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee. The three experiment groups of mice orally received doses of 3.0, 6.0, 9.0 mg/kg bw of polysaccharides from *P. esulenta*. Control group of mice orally received same volume of saline. All mice were allows to be free access to water and food. Mice were killed after the last medicine administration and their livers, spleens and thymus were rapidly removed, weighed and then frozen immediately at −4 °C.

2.5. Mitogen-induced proliferation of spleen cells and colorimetric MTT assay

Whole spleen cells were harvested from kunming male mice (2 months old), suspended in RPMI-1640 medium containing 10% FCS (fetal calf serum), and centrifuged to remove the supernatant (Wang et al., 2002). The collected precipitated cells were first suspended in 1 mL of RBC lysis buffer (8% NH_4Cl), then 14 mL more of the same lysis buffer were added to destroy red blood cells. After 1 min, the solution was diluted with 15 mL RPMI-1640 medium to stop the reaction, centrifuged to collect the cells, and adjusted the cell final concentration to 1×10^7 cells/mL with RPMI-1640 medium. Concanavalin A (Con A, final concn.: 1 μg/mL) was added to the resulting mixture. The cells were incubated with or without polysaccharides from *P. esulenta* in 96-well ELISA plates at 37 °C with 5% CO_2 for 44 h. The cell proliferation was measured based on the MTT assay.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) in phosphate buffered saline (PBS) at 5 mg/mL (25 μL) was added to each well, and plates were incubated at 37 °C for 4 h. Acid-isopropanol (100 μL of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. The

plates were read on a Microplate Reader for Elisa (GF-M2000), using a test wavelength of 570 nm, a reference wavelength of 620 nm. Plates were normally read within 1 h after the addition of isopropanol.

3. Results and discussion

3.1. Effect of extraction temperature on extraction yield of polysaccharides from Phascolosoma esulenta

In this work, the efficiencies of different extraction temperature on extraction yield of polysaccharides from *P. esulenta* were investigated, and the results are listed in Fig. 1. Firstly, the other extraction conditions of polysaccharides from *P. esulenta,* e.g. ratio of phosphate buffer to raw material, extraction time and ratio of trypsinase to raw material, were fixed at 2, 5 h, 2, respectively and extraction temperature was just changed. As be shown in Fig. 1, extraction yield of polysaccharides from *P. esulenta* continued to increase with the increasing extraction temperature and reached the peak value (2.1%) when extraction temperature was 45 °C. The extraction yield of polysaccharides from *P. esulenta* started to decrease after extraction temperature exceeds 45 °C.

3.2. Effect of ratio of phosphate buffer to raw material on extraction yield of polysaccharides from Phascolosoma esulenta

In this work, the effect of ratio of phosphate buffer to raw material on extraction yield of polysaccharides from *P. esulenta* was investigated, and the results are listed in Fig. 2. Firstly, the other extraction conditions of polysaccharides from *P. esulenta,* e.g. extraction temperature, extraction time and ratio of trypsinase to raw material, were fixed at 45 °C, 5 h, 2, respectively and ratio of phosphate buffer to raw material was just changed. As be shown in Fig. 2, extraction yield of polysaccharides from *P. esulenta* continued to increase with the increasing ratio of phosphate buffer to raw material and reached the peak value (1.97%) when ratio of phosphate buffer to raw material was 2. The extraction yield of polysaccharides from *P. esulenta* started to decrease after ratio of phosphate buffer to raw material exceeds 2.

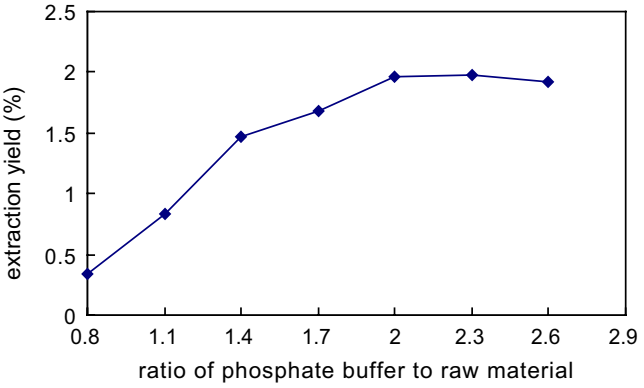

Fig. 2. Effect of ratio of phosphate buffer to raw material on extraction yield of polysaccharides from *Phascolosoma esulenta*.

3.3. Effect of extraction time on extraction yield of polysaccharides from Phascolosoma esulenta

The effect of extraction time on extraction yield of polysaccharides from *P. esulenta* is shown in Fig. 3. Firstly, extraction time was set at 3.5, 4, 4.5, 5, 5.5, 6 h while other extraction parameters were given as followings: extraction temperature 45 °C, ratio of phosphate buffer to raw material 2 and ratio of trypsinase to raw material 2. It could be found that with increasing extraction time from 3 to 5 h, the extraction yield of polysaccharides from *P. esulenta* increased from low to high till at 5 h to maximum, and then dropped at 5 h (see Fig. 3).

3.4. Effect of ratio of trypsinase to raw material on extraction yield of polysaccharides from Phascolosoma esulenta

The effect of ratio of trypsinase to raw material on extraction yield of polysaccharides from *P. esulenta* is

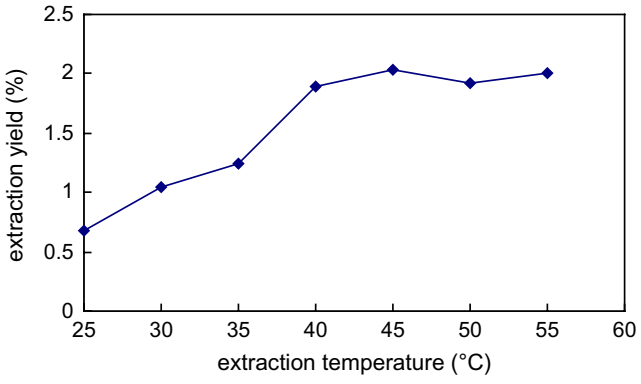

Fig. 1. Effect of different extraction temperature on extraction yield of polysaccharides from *Phascolosoma esulenta*.

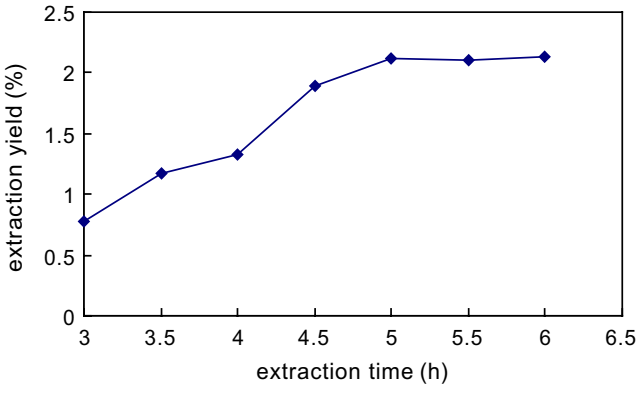

Fig. 3. Effect of extraction time on extraction yield of polysaccharides from *Phascolosoma esulenta*.

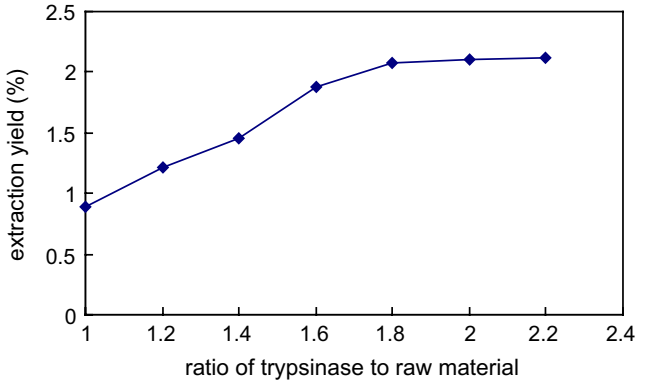

Fig. 4. Effect of ratio of trypsinase to raw material on extraction yield of polysaccharides from *Phascolosoma esulenta*.

shown in Fig. 4. Firstly, ratio of trypsinase to raw material was set at 1, 1.2, 1.4, 1.6, 1.8, 2 and 2.2 while other extraction parameters were given as followings: extraction temperature 45 °C, ratio of phosphate buffer to raw material 2 and extraction time 5 h. There was similar trend as above for varying ratio of trypsinase to raw material on extraction yield of polysaccharides from *P. esulenta*. With increasing the ratio of trypsinase to raw material from 1 to 2, the extraction yield of polysaccharides from *P. esulenta* first increased and then dropped. The maximum was 2.11% when the ratio of trypsinase to raw material was 2 (see Fig. 4).

3.5. Optimization of the extraction parameters of polysaccharides from Phascolosoma esulenta

The first step in the extraction parameters of polysaccharides from *P. esulenta* is to optimize the operating conditions to obtain an efficient extraction of the target compounds and avoid the co-extraction of undesired compounds such as fatty acids and their esters. Since various parameters potentially affect the extraction process, the optimization of the experimental conditions is a critical step in the development of a solvent extraction method. In fact, the extraction temperature, extraction time, ratio of phosphate buffer to raw material and ratio of trypsinase to raw material are generally considered to be the most important factors. Optimization of the suitable extraction conditions in the polysaccharides extraction can be carried out by using an experimental design. In the present study, all selected factors were examined using an orthogonal $L_9(3)^4$ test design. The total evaluation index was used to analysis by statistical method. The results of orthogonal test and extreme difference analysis are presented in Table 2. The analysis of variance was performed by statistical software SPSS 12.0 and the result is listed in Table 2. The extract obtained from each test in the polysaccharides extraction was weighted and quantitatively analyzed and then the extraction yields of the crude extract and each compound were calculated. The results of experiments presented in Table 2 indicated that the maximum extraction yield of the crude extract was 2.18%. However, we cannot select the best extraction conditions only based on these outcomes in Table 2, and a further orthogonal analysis was warranted. Thus, the *K*, *k* and *R* values were calculated and listed in Table 2. As seen from Table 2, we can find that the influence to the mean extraction yields of the compounds decreases in the order: C > A > D > B according to the *R* values. The extraction time was found to be the most important determinant of the yield. In other words, the maximum yield of the polysaccharides was obtained when extraction temperature, ratio of phosphate buffer to raw material, extraction time and ratio of trypsinase to raw material were 40 °C, 2, 5.5 h and 1.6, respectively.

Table 2
Analysis of $L_9(3)^4$ test results

No.	*A*, extraction temperature (°C)	*B*, ratio of phosphate buffer to raw material	*C*, extraction time (h)	*D*, ratio of trypsinase to raw material	Extraction yield (%)
1	1	1	3	2	2.04
2	2	1	1	1	1.54
3	3	1	2	3	1.79
4	1	2	2	1	2.18
5	2	2	3	3	1.98
6	3	2	1	2	1.24
7	1	3	1	3	1.57
8	2	3	2	2	1.81
9	3	3	3	1	1.89
*K*1	5.79	5.37	4.35	5.61	
*K*2	5.33	5.4	5.78	5.09	
*K*3	4.92	5.27	5.91	5.34	
R	0.87	0.13	1.56	0.52	

R refers to the result of extreme analysis.

3.6. Effect of the polysaccharides from Phascolosoma esulenta on liver, spleen and thymus index of mice

Some species of *P. esulenta* have reported to exhibit obvious immunity activity. For example, Peng and Lei (2007) reported that polysaccharide extracted from *Sipunculus nudus* L. are capable of withstanding spleen and thymus shrinkage by induced cyclophosphamide and obviously protect against leukocytopenia. Jiang et al. (2004) reported that the extract of *Sipunculidae* contains 55. 3% ss, 18 kinds of amino acids and 8 kinds of trace elements. It markedly enhanced the weight index of thymus and spleen.

Therefore, to determine effect of the polysaccharides from *P. esulenta* on liver, spleen and thymus index of mice, we quantified the liver, spleen and thymus index of mice. In control group, liver index was 2.89, while the index was 2.92 in low dose of polysaccharides treatment group, a slight increase ($P > .05$), indicating that low dose of polysaccharides administration didn't significantly affect liver index of mice; In middle and high dose of polysaccharides treatment groups, a significant increase ($P < .05$) could be

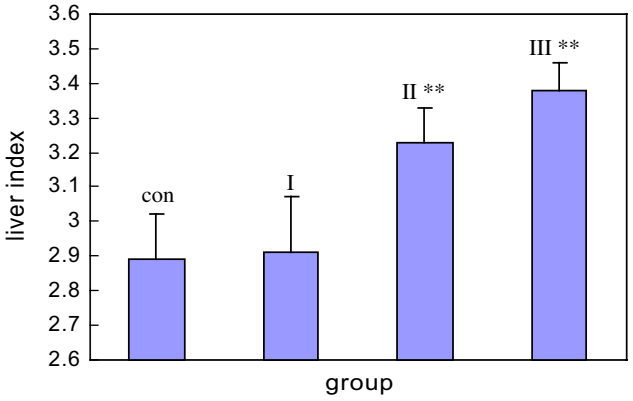

Fig. 5. Effect of the polysaccharides from *Phascolosoma esulenta* on liver index $^{**}P < .01$.

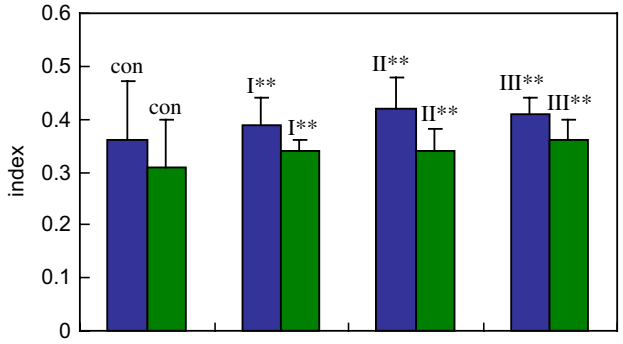

Fig. 6. Effect of the polysaccharides from *Phascolosoma esulenta* on spleen and thymus index. Spleen index; thymus index; ** $P < .01$. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

observed in comparison with control group, indicating that middle and high dose of polysaccharides administration significantly affect liver index of mice (Fig. 5).

In control group, spleen and thymus index of mice were 0.36 and 0.31, respectively, while a significant increase of spleen and thymus index of mice ($P < .05$) could be observed in all polysaccharides treatment groups (groups I, II, III) compared with control group, indicating that low, middle and high dose of polysaccharides administration significantly affect spleen and thymus index of mice (Fig. 6). These results suggested that administration of polysaccharides from *P. esulenta* could stimulate the immunity activity of mice, which supported Peng and Lei's (2007) and Jiang, Sheng, Jia, Chu, & Li's work (2004).

3.7. Effect of the polysaccharides from Phascolosoma esulenta on Con A-stimulated mouse spleen cells proliferation

The colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for cell proliferation was then carried out to evaluate Con A-stimulated mouse spleen cells in the presence of various concentrations of the polysaccharides from *Phascolosoma esulenta*. In comparison with the control experiment (without treatment of samples), the cell proliferation activity was significantly enhanced with polysaccharides from *P. esulenta* in a dose-dependent pattern ($P < .01$) (Fig. 7).

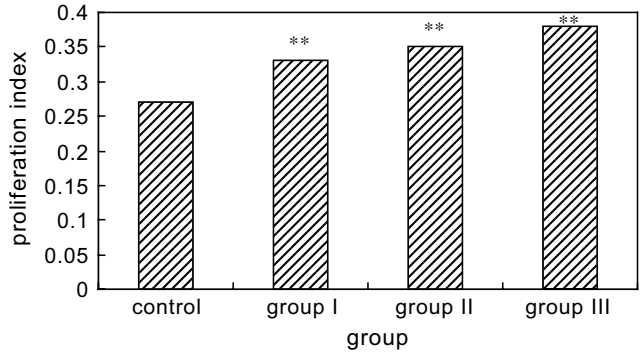

Fig. 7. Con A-stimulated mouse spleen cells proliferation in the presence of various concentrations of the polysaccharides from *Phascolosoma esulenta* was evaluated by the colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) $^{**}P < .01$.

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