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Optimization of pine nut (*Pinus koraiensis*) meal protein peptides on immunocompetence in innate and adaptive immunity response aspects

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ABSTRACT

Pine nut (*Pinus koraiensis*) meal protein peptides (PNMPP), a kind of Korean pine seed oil by-products, were often considered as waste disposal in food industry. In the present study, we found that 3–10 kDa PNMPP could enhance immunity of ICR mice at 100 mg·(kg·d)^{−1} both in innate and in adaptive immunity. In innate immunity, PNMPP of 3–10 kDa at 100 mg·(kg·d)^{−1} could enhance the abdominal macrophage phagocytosis of mice at 30.22% and the phagocytosis index of carbon clearance significantly ($P < .05$). In addition, we found out that 3–10 kDa PNMPP at low dose could significantly enhance cytokine interferon- γ , interleukin-2, interleukin-4, and interleukin-6 ($P < .05$). Meanwhile, in adaptive immunity, PNMPP could enhance the median hemolytic concentration of mice. These findings suggested that 100 mg·(kg·d)^{−1}, 3–10 kDa PNMPP could increase the function of immunity.

Abbreviations: ANOVA: analysis of variance; BSA: bull serum albumin; ConA: concanavalin A; CTX: cyclophosphamide; DW: deionized water; FCM: flow cytometry; IFN- γ : interferon- γ ; IL-2: interleukin-2; IL-4: interleukin-4; IL-6: interleukin-6; MW: molecular weight; MPM: mouse peritoneal macrophages; OD: optical density; PBS: phosphate buffer solution; PNMPP: pine nut (*Pinus koraiensis*) meal protein peptides; SA: salicylic acid buffer; SRBC: sheep red blood cells; HC₅₀: median hemolytic concentration

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Pine nut (*Pinus koraiensis*) meal protein peptides (PNMPP); immunity; carbon clearance; abdominal macrophage phagocytosis; cytokine

1. Introduction

The seed of Korean pine, *Pinus koraiensis*, has been used as an edible fruit for a long time because it contains essential nutrients, such as amino acids, fatty acids, and vitamins. Great progress has been made in pine nut oil field. Liu (Liu & Xu, 2012) applied steam distillation to extract valuable essential oils, and pine nut oil improves atherosclerotic markers and can be used as a functional ingredient in the food industry to promote cardiovascular health (Kang, Kim, Kim, & Choe, 2015). However, pine nut oil by-product has not been fully used. Young (Kim, Kim, Han, Park, & Watanabe, 2014) elucidated the pyrolysis characteristics of waste pine nut (*P. koraiensis*) shell, while pine nut (*P. koraiensis*)

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meal protein peptides (PNMPP) were often considered as a waste. To obtain peptides from proteins, the enzymatic hydrolysis of protein has proven to be the commonly used method, which has attracted more attention in the last decade (Wang et al., 2014). In a recent study, it was verified that protein peptide could enhance memory (Jia et al., 2014) and also had functions such as enhancing the antioxidant (Torres-Fuentes, Contreras, Recio, Alaiz, & Vioque, 2015), immunomodulatory (Huang, Chen, Chen, Hong, & Chen, 2010), anti-fatigue functions (Li, Liu, Wu, & Chen, 2012) and that it has no genetic toxicity. These facts have promoted the production and consumption of protein peptides, which has resulted in an increasing interest in the functional activity of peptides. As a traditional food, the functional development of pine nut is an irresistible trend. Since the immunity of food-borne functional peptides has not been discussed yet, we investigated the immunity of PNMPP in this research.

Currently, immunosuppressive drugs have been widely used to control undesired immune responses, such as autoimmune diseases, allergies, and allograft rejection. FK506, cyclophosphamide (CTX), and prednisone are typical immunosuppressive drugs that have been used in the clinic for many years (Zhang, Qin, & Sun, 2006). In the present study, we used CTX as an immunosuppressor to build an immunosuppressor model and focused on T lymphocytes and investigated the immunocompetence effect of PNMPP on mouse T cells both in innate and in adaptive immunity response aspects.

The mouse peritoneal macrophages (MPM) belong to a kind of mononuclear macrophage of mice immune cells, which is the most active biological effect in body cells and has the function of consuming and digesting the pathogens. At present, there are still many researchers using the experiment of red blood cells to evaluate the function of phagocytes (Ichinose, Hara, Sawada, & Maeno, 1992). Although this method has been used for years, there are still some limitations. The combining of fluorescent microsphere technique and flow cytometry (FCM) detecting method to measure the function of phagocytes has been used more in many fields (Xiong et al., 2013), which have the characteristics of being convenient, fast, and efficient. Fluorescent microsphere technology, combined with FCM detecting methods, was used in this study to evaluate whether PNMPP has an effect on the MPM's ability of fluorescent microsphere or not.

It is known that cytokine, mainly including lymphocyte cytokine, monocyte cytokine and other cells cytokine, plays an important role in the immune response, such as the regulation of the body's inflammatory response, cell proliferation, and differentiation. Meanwhile, it is beneficial for anti-infective, anti-tumor effect, and to regulate immunity *in vivo*. Guan (Guan et al., 2011) chose cytokine TNF- α , IL-1 β , and interleukin-6 (IL-6) as targets to investigate anti-inflammatory effects on cytokine production by LPS-stimulated RAW 264.7 macrophages *in vitro*. Thus, in our study, cytokine levels were used as the target to evaluate the immune activities of PNMPP as well. To further investigate the effect of PNMPP on adaptive immunity *in vitro* (Liu, Wang, & Zhao, 2010), we selected the median hemolytic concentration (HC₅₀) of mice as an index (Chingat, Delgadob, Salazar, & Soto, 2015) to compare with the carbon clearance index.

As people living standards improving, nutritional and functional food attract wider attention, therefore, development of functional food has become an inevitable trend for the future food industry. Since the efficiency of PNMPP was increased in this study, it could be forecasted that there was a huge practical application and market development value of PNMPP.

2. Materials and methods

2.1. Chemicals

Concanavalin A (ConA), Drabkin's Reagent, and CTX were purchased from Sigma-Aldrich (St Louis, MO). Interleukin-2 (IL-2), interferon- γ (IFN- γ), interleukin-4 (IL-4), and IL-6 enzyme-linked immunosorbent assay kits were purchased from BioLegend (San Diego, CA). Roswell Park Memorial Institute-1640 (RPMI-1640) medium, fetal bovine sera were obtained from Invitrogen-Gibco (Grand Island, NY).

2.2. Reagents preparation

PNMPP was provided by the Laboratory of Nutrition and Functional Food, Jilin University (Changchun, China). The preparation process for PNMPP is shown in [Figure 1](#) (Liu, Luo, & Li, 2011). The sheep red blood cells (SRBC) were shaken at a single direction for 10 min to do de-fibering, washed by deionized water (DW) for three times, and then centrifuged at 1500 r/min for 10 min. DW was added to obtain 2% red blood cell, kept at 4°C before use. The blood sample was collected from cavia procellus, the serum was separated and SRBC was added into it (1:5, V/V). After preserving at 4°C for 0.5 h, the supernatant was absorbed and kept at -70°C. The alexin was diluted with salicylic acid buffer (SA; 1:8, V/V) when used. Ten milliliters of bull serum albumin (BSA) was added into 200 mL Hank's solution to get 5% BSA Hank's solution; 0.5 g BSA was dissolved in 50 mL phosphate buffer solution (PBS) to obtain 1% BSA solution; 100 μ L of fluorescent microspheres was added into 10 mL 1% BSA solution to get regulated liquid fluorescent microspheres and the mixture was kept at 37°C for 30 min and then treated by ultrasonic for 5 min.

2.3. Animal group design

Adult male ICR mice weighing 20 ± 2 g were purchased from Jilin University Experimental Animal Center (Changchun, Jilin, China) and observed under general condition (temperature $22 \pm 2^\circ\text{C}$, relative humidity $30 \pm 10\%$, 12 h light and dark cycle) for five days during the quarantine and acclimation period to confirm that there were no abnormalities. Mice were randomly divided into eight groups (10 animals per group) and were daily gavage-fed different molecular weights (MWs) of PNMPP and DW for 28 days. During intragastric administration, an immunocompromised mouse model was built by CTX. The mice were intraperitoneally injected immunosuppressants on the 22nd and 23rd days ([Table 1](#)). All of the procedures were carried out in strict accordance with the guidelines from the Care and Use of Laboratory Animals published by the US National Institutes of Health (Lu, Huang, Hu, Wang, & Guan, 2014).

2.4. Carbon clearance test in mice

The mice were weighed and injected the India ink (diluted 3–4 times) via the tail vein (0.01 mL/g body weight). Blood samples were drawn from retro orbital vein at intervals

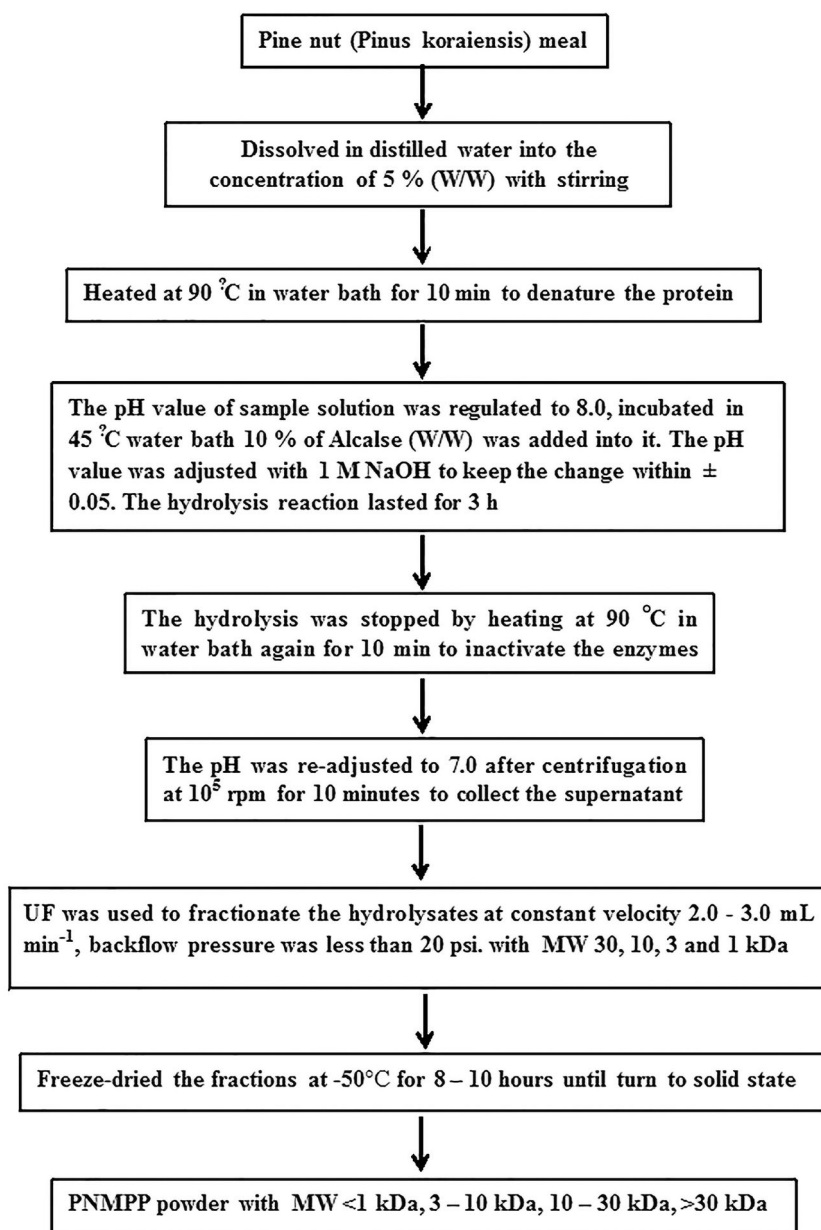


Figure 1. Peptide preparation.

of 2 and 10 min. A 20 μL of blood sample was mixed with 2 mL 0.1% Na_2CO_3 . The optical density (OD) was measured at 600 nm on a microplate reader; Na_2CO_3 solution was used as the blank control. The mice were sacrificed to obtain the liver and spleen, dry the organ surface with filter paper, and were weighed. The Phagocytic index indicates the ability of mice to carbon clearance. The Phagocytic index is calculated by Equations (1) and (2), where OD_1 and OD_2 are optical densities of blood sample at 2 and 10 min, t_1 is 2 min

Table 1. Animal groups design.

Group number	Intragastric administration	CTX dose	Molecular weight	Intragastric administration dose
1	DW (Negative control)			0.2 g/kg/d
2	DW (Positive control)	0.04 g/kg/d		0.2 g/kg/d
3	PNMPP	0.04 g/kg/d	>30 kDa	0.2 g/kg/d
4	PNMPP	0.04 g/kg/d	3–10 kDa	0.1 g/kg/d
5	PNMPP	0.04 g/kg/d	3–10 kDa	0.2 g/kg/d
6	PNMPP	0.04 g/kg/d	3–10 kDa	0.4 g/kg/d
7	PNMPP	0.04 g/kg/d	1–3 kDa	0.2 g/kg/d
8	PNMPP	0.04 g/kg/d	<1 kDa	0.2 g/kg/d

and t_2 is 10 min, respectively.

$$K = \frac{\lg OD_1 - \lg OD_2}{t_2 - t_1} \quad (1)$$

$$\text{Index of phagocytosis } \alpha = \frac{\text{Body weight}}{\text{Liver weight} + \text{spleen weight}} \times \sqrt[3]{K} \quad (2)$$

2.5. Hemolysis assays

The mice were intraperitoneally injected 2% (V/V) SRBC (0.2 mL). After four days, blood samples were collected from the eyes of the mice, stewing for 1 h until serum dissolved. Serum was then prepared by centrifugation at 1500 r/min for 5 min. A 10 μ L serum sample was diluted by 1 mL SA (diluted 5 times). The reaction sample was obtained by 100 μ L diluted serum sample added 50 μ L SRBC (10%) and 100 μ L diluted complement. The blank control sample was obtained by 100 μ L SA added 50 μ L SRBC (10%) and 100 μ L diluted complement. The samples were stored at 37°C for 30 min in the water bath and then were centrifuged at 1500 r/min for 5 min. The supernatant of 50 μ L was collected in another 96-well plates (Costar, Washington), mixed with 150 μ L Drabkin's Reagent. Set median hemolytic hole: 12.5 μ L SRBC (10%, V/V) + 187.5 μ L Drabkin's Reagent, mixed by micro oscillator and stewed for 10 min, the OD₅₄₀ was measured on a microplate reader. Compared with the control group, a sample group of HC₅₀ was significantly higher ($P < .05$) and it could be considered that the experimental results were positive. The HC₅₀ was calculated by Equation (3):

$$HC_{50} = \frac{OD_{540} \text{ of sample}}{\text{Half hemolysis } OD_{540} \text{ of SRBC}} \times \text{dilution ratio} \quad (3)$$

2.6. Macrophage phagocytosis rate of fluorescent microsphere by FCM

Each mouse was injected 0.2 mL 2% SRBC intraperitoneally four days before the experiment. The mice were sacrificed by cervical dislocation. Each mouse was given 3 mL 5% BSA Hank's solution by intraperitoneal injection. Then, each abdomen of mice was rubbed gently to make the peritoneal macrophages to flow out fully. Then, the abdomen was cut by a slot and the peritoneal macrophages lotion was drawn by syringe. After filtering the lotion, the concentration of the cell number was turned to 4×10^5 to 6×10^5 cells mL⁻¹. Then, the peritoneal macrophages lotion was drawn 1 mL in six-well culture plate and added to regulate liquid fluorescent microspheres (1×10^7 cells-plate⁻¹). The plate was put in a 5% CO₂ cell incubator at 37°C for 2 h. After

incubation, the cells were washed by PBS twice. A volume of 0.3 mL of PBS was added to each well and the adherent cells were scraped off. The phagocytic ability was reflected by the phagocytic rate (%) as in Equation (4):

$$\text{Phagocytic rate(\%)} = \frac{\text{Number of macrophages in fluorescent microspheres}}{\text{All the macrophages}} \times 100\% \tag{4}$$

If there were significant *differences* between the experimental group and the control group, the result could be judged as positive.

2.7. Determination of cytokine levels in vivo

In order to obtain the splenocytes suspension of mice, the mouse spleen was removed and placed in 5 mL Hank's solution containing plate in a sterile environment, the splenocytes suspension was filtered through a 200 mesh screen, and washed in Hank's solution for three times, and then centrifuged at 1000 r/min for 5 min. Splenocytes collected from the ICR male mice under aseptic conditions were mechanically scraped and plated at a density of 5×10^9 cells mL⁻¹ on 24-well plates containing 2 mL of RPMI 1640 complete medium added ConA (7.5 mg L⁻¹). The plate was incubated at 37°C with 5% CO₂ for 36 h. The splenocytes supernatants were centrifuged at 3000 r/min for 10 min until assayed for cytokines. Before determination, the ELISA kit was kept at room temperature for 20 min (Wróblewska et al., 2004). The concentrations of cytokine IFN-γ, IL-2, IL-4, IL-6 in the supernatants of splenocyte culture were measured by ELISA using commercially available reagents according to the instructions of the manufacturer (Dingguo Biological Technology Co., Beijing, China), and the OD₄₅₀ value was measured by a microplate reader. Based on the standard concentration (Table 2), draw the standard curve which index was OD450 value. The concentration of the samples was calculated according to the standard curve (Lu et al., 2011).

2.8. Statistical analysis

Analysis of variance (ANOVA) was performed with SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). All experiments were performed in triplicate and data are expressed as mean ± standard deviation. Statistical analyses were performed using least significant difference and regression analysis. The significance of the regression coefficients was evaluated using an F-test. The fit quality of the polynomial model equation was expressed by R². The regression coefficients were used for statistical calculation to generate the regression models. Significant differences were determined at 95% confidence intervals.

Table 2. Standard curve of cytokine level.

	S0	S1	S2	S3	S4	S5
IL-2	0	20	40	80	160	320
IL-4	0	10	20	40	80	160
IL-6	0	7.5	15	30	60	120
IFN-γ	0	50	100	200	400	800

3. Results and discussion

3.1. Carbon clearance index

Carbon particles were injected into the blood via the tail vein and were carried to the liver, spleen by blood circulation, and then were removed by macrophage of the organs. Within a certain concentration range, the carbon clearance rate can reflect the phagocytosis of phagocytes, in other words, the body's immune ability. As Figure 2 shows, compared with the control group, whose value was $5.549 \pm 0.29\%$, the phagocytosis index of carbon clearance was related to the MWs and could be enhanced significantly when administered at the doses of 1–3 kDa and 3–10 kDa on mice ($P < .05$). The highest activity group was at the dose 3–10 kDa, and the highest value was $7.57 \pm 0.28\%$. In addition, the phagocytosis index of carbon clearance on mice was significantly reduced at a dose of <1 kDa, and the value was $4.897 \pm 0.20\%$ ($P < .05$).

3.2. HC_{50} index

SRBC, as an antigen, stimulates the mice-secreted specific hemolysin. The amount of antibodies reflects the state of the body's immune function and the specific immune body level; the level was expressed as HC_{50} . The result of hemolysis is shown in Figure 2, compared with the negative and positive control groups; after giving the mice PNMPP for four weeks, the content of serum hemolysin was increased significantly ($P < .05$). The highest mice serum hemolysin was 3–10 kDa low-dose group and the value was 79.19 ± 0.90 . Thus, PNMPP could enhance the immunity of mice and 3–10 kDa PNMPP was the optimal composition.

3.3. Macrophage phagocytosis rate of fluorescent microsphere

The results are shown in Table 3 and Figure 3. Compared with the negative and positive groups, the macrophage phagocytosis fluorescent microspheres rate of the experimental group was significantly higher, except >30 kDa ($P < .05$). Figure 3 shows the amount of

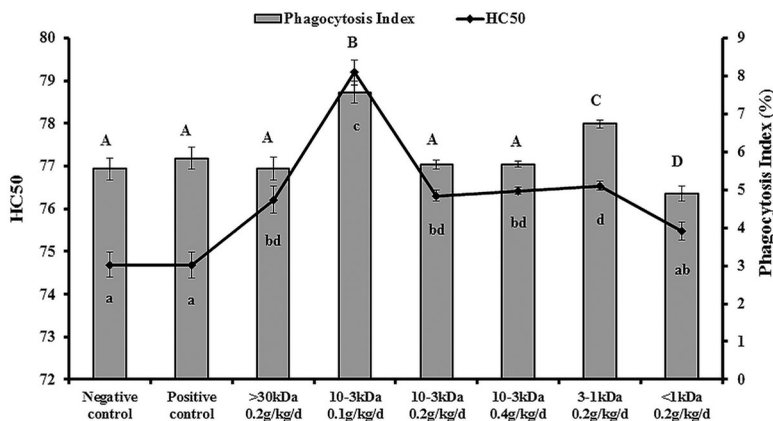


Figure 2. Result of carbon clearance and HC_{50} .

Table 3. The effects of PNMPP on MPM fluorescent microspheres.

Group		Gated %						Phagocytic rate %	
		Total	M1	M2	M3	M4	M5	Phagocyte	Standard error
Negative control		100	84.19	2.27	0.87	0.51	0.33	15.81 ^a	1.68
Positive control		100	78.42	6.63	3.42	1.74	0.84	21.58 ^{a,b}	3.0225
>30 kDa	Moderate dose	100	84.87	7.82	2.91	1.05	0.31	15.13 ^a	1.335
10-3 kDa	Low dose	100	63.99	11.42	6.15	3.46	1.89	36.01 ^c	5.795
10-3 kDa	Moderate dose	100	76.2	10.69	5.39	1.96	0.87	23.8 ^b	3.205
10-3 kDa	High dose	100	75.29	8.37	4.22	1.83	0.84	24.71 ^b	3.52
3-1 kDa	Moderate dose	100	73.42	8.38	4.04	1.85	0.98	26.58 ^b	4.4625
<1 kDa	Moderate dose	100	72.67	11.17	5.26	2.08	0.87	27.33 ^b	4.645

Note: The error bars indicate the SD of three replicates. The same letter means that the variance of two samples is not significant ($P > 0.05$). The different letters mean that variance of two samples are significant ($P > 0.05$).

fluorescent microspheres measured by FCM; x-coordinate expresses the pulse height and the y-coordinate expresses the number of fluorescent microspheres. The number of fluorescent microspheres was the sum of M1 to M5, which could be approximately expressed as M1. The experiment measured 1×10^4 phagocytes marked with fluorescent. The less gated the number was, the larger the macrophage phagocytosis rate was, and the better immune activity was 3–10 kDa PPMNP; $100 \text{ mg} \cdot (\text{kg} \cdot \text{d})^{-1}$ had the strongest function that could enhance the abdominal macrophage phagocytosis at a value of $36.01 \pm 5.79\%$.

3.4. Effect of PNMPP on cytokine secretion in vivo

3.4.1. Standard curve of cytokine level

The results of standard curve are shown in Figure 4. The parameters of the equation were obtained by Excel (Windows Software, USA). The fit of the curve was checked by the coefficient of determination R^2 , which was calculated to be 0.99. The higher the value of R^2 is, the better the curves are adapted to the responses. The following quadratic model explains the standard curve of different cytokine levels (IL-2, IL-4, IL-6, IFN- γ).

3.4.2. Effect of PNMPP on cytokine secretion in vivo

The concentrations of cytokines in the culture supernatant of ConA-induced splenocytes were determined by ELISA. The results are shown in Figure 4. Compared with the negative and positive groups, different MWs PNMPP had a different reaching influence on cytokine levels, especially on IL-2 and IFN- γ . The cytokine level on IL-2 was higher significantly in 3–10 kDa low-dose PPMNP ($P < .05$) whose value was $274.048 \pm 5.26\%$, which indicated that the components could enhance the release of IL-2. As for cytokine IL-4, it was found that at the low and moderate dose in the MWs of 3–10 kDa, the concentrations of cytokines were significantly higher compared to the control groups as well ($P < .05$), and the value was $213.588 \pm 4.49\%$. When it came to IL-6, IFN- γ , the cytokines levels were enhanced significantly at 3–10 kDa low dose ($P < .05$), and the values were $158.025 \pm 2.4\%$ and $777.95 \pm 5.18\%$. The results indicated that PNMPP could enhance the cytokine levels of IL-2, IL-4, IL-6, and IFN- γ and the cytokine secretion was related to the MWs, and the most significant ($P < .05$) dose was in the MWs of 3–10 kDa at $100 \text{ mg} \cdot (\text{kg} \cdot \text{d})^{-1}$.

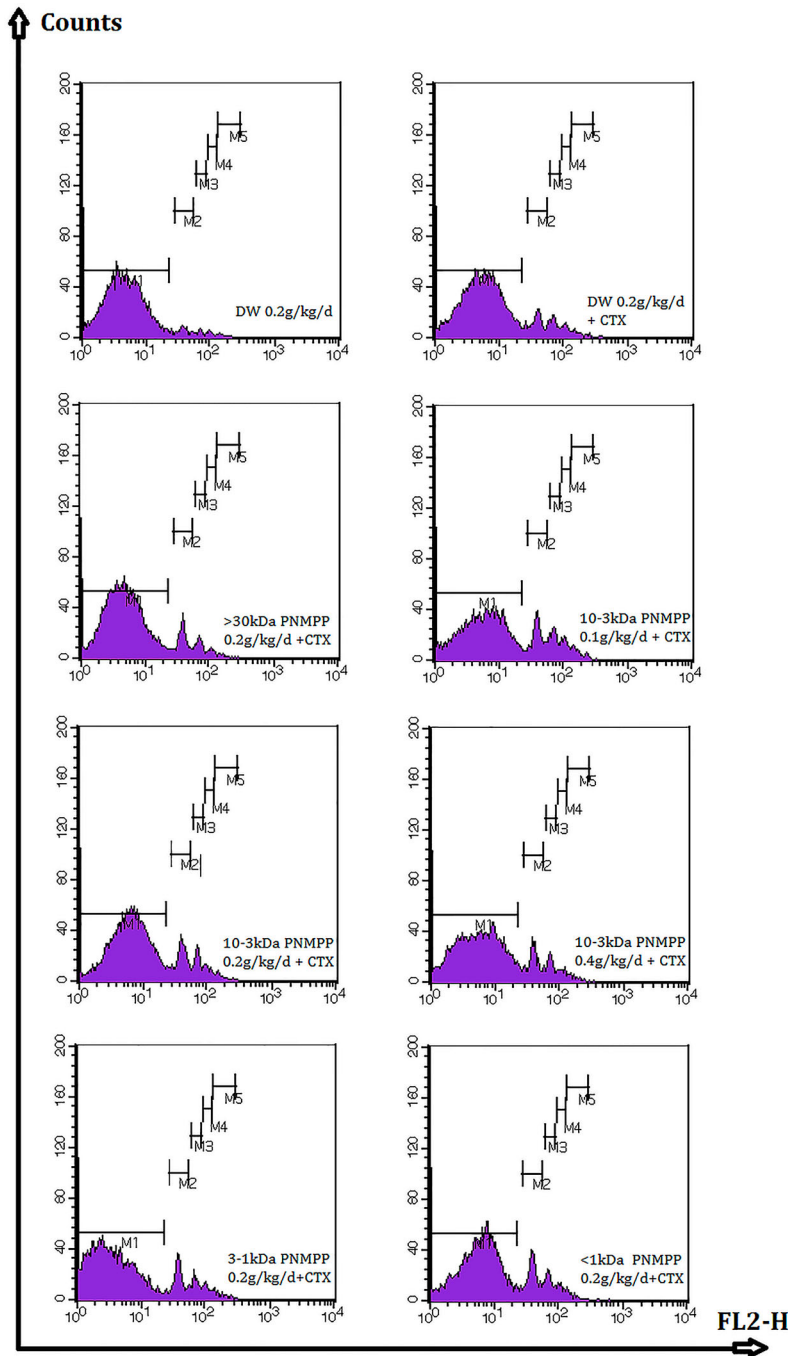


Figure 3. Macrophage phagocytosis rate of fluorescent microsphere by FCM. Macrophages were stimulated in 6-well flat-bottom plates and cultured for 2 h, collected, and the fluorescent microspheres were calculated by flow cytometry. (a) Negative Control group, (b) Positive Control group, (c) >30 kDa 0.2 g/kg/d group, (d) 3–10 kDa 0.1 g/kg/d group, (e) 3–10 kDa 0.2 g/kg/d group, (f) 3–10 kDa 0.4 g/kg/d group, (g) 1–3 kDa 0.2 g/kg/d group, (h) <1 kDa 0.2 g/kg/d group. These results were from three independent experiments and presented as means $\pm$ S.D. Significant differences with control group were designated as $P < 0.05$.

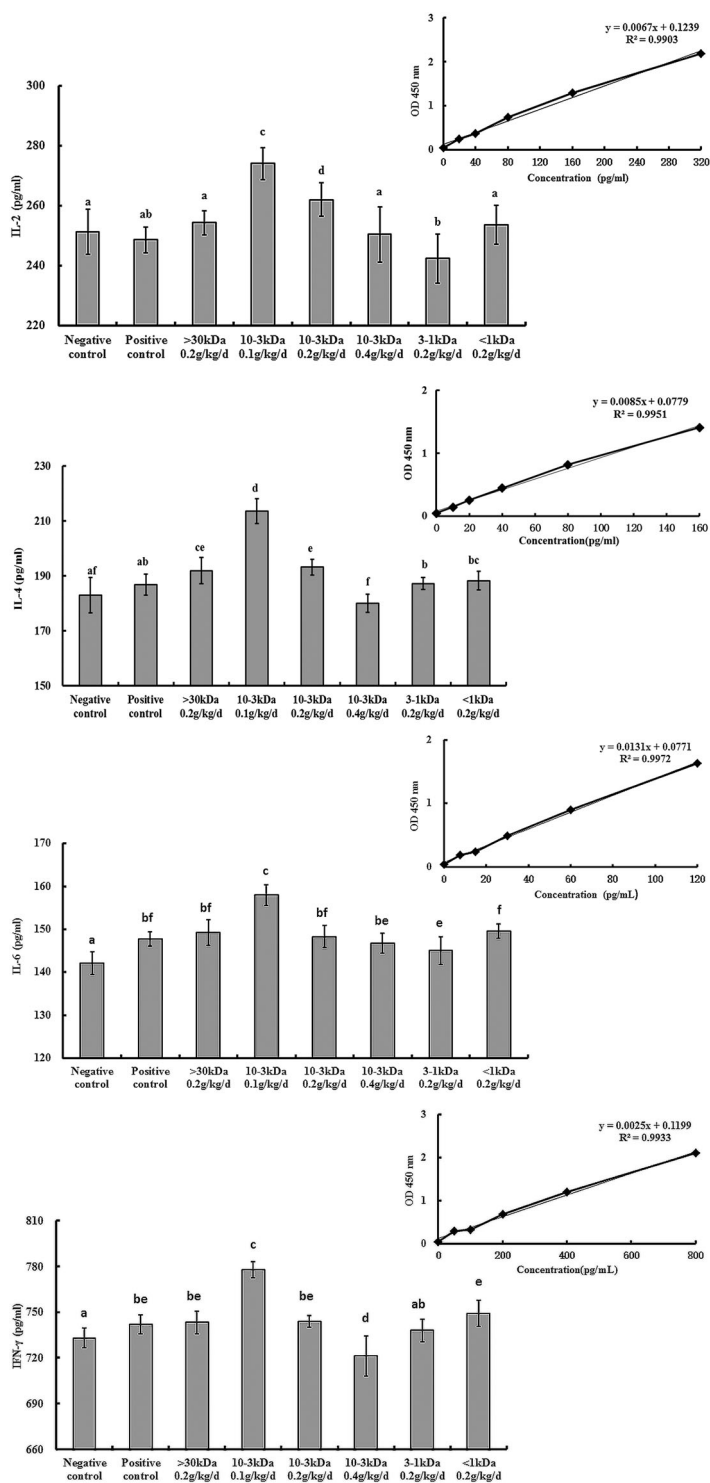


Figure 4. Effect of PNMPP on cytokine secretion *in vivo*.

4. Conclusions

In this paper, the effect of PNMPP on innate and adaptive immunity response was observed, and the optimal composition was selected. PNMPP could enhance immunity in ICR mice at $100 \text{ mg} \cdot (\text{kg} \cdot \text{d})^{-1}$ both in innate and in adaptive immunity. In the innate immunity aspect, PNMPP of 3–10 kDa could enhance the phagocytosis index of carbon clearance significantly ($P < .05$). Moreover, 3–10 kDa PPMNP low dose had the strongest function to enhance the abdominal macrophage phagocytosis of mice and cytokine release. In adaptive immunity, 3–10 kDa PNMPP at low dose could enhance the HC_{50} of mice. These findings suggested that a low dose 3–10 kDa PNMPP could increase the function of immunity. This paper provided a reasonable solution for the development of pine nut by-products, and it is meaningful to further explore its action mechanism.

Disclosure statement

No potential conflict of interest was reported by the authors.

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