Isolation of Polysaccharides from *Perna viridis* and Their Antioxidant Activities in High-fat Diet-fed Mice

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Abstract: Previously, the *Perna viridis* polysaccharides (PVPs) were demonstrated to have a remarkable hypolipidemic effect in a mouse model of hyperlipidemia. Herein, the PVPs were purified and analyzed, and their antioxidant effects were evaluated in a mouse model of hyperlipidemia. Two main fractions P1 and P2 were separated from PVPs by DEAE-52 and Sephadex G-200 column chromatography. Chemical analysis revealed that P1 and P2 were acidic polysaccharides with sulfate groups, and their relative molecular weights (MWs) were 6.89 $\times 10^5$ Da and 2.87 $\times 10^5$ Da, respectively. The content ratios of glucosamine, uronic acid, and galactosamine for P1 and P2 were 3.7:2.6:1 and 2.8:1.6:1, respectively. High-fat diet-fed mice (a diet supplemented with 2% cholesterol) were intragastrically administered low dose [50mg/(kg d)], medium dose [100mg/(kg d)], and high dose of PVPs [200mg/(kg d)]. The results showed that the medium dose and high dose of PVPs could significantly enhance the total antioxidant capacity (T-AOC), as well as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) activities and reduce the malondialdehyde (MDA) content in serum, the liver, and the brain of the hyperlipidemic mouse model ($p < 0.01$) or ($p < 0.05$). The results demonstrate that PVP can exert a hypolipidemic effect by activating the activities of antioxidase *in vivo* and by reducing the level of peroxidation in hyperlipidemic mice.

Key words: polysaccharide; *Perna viridis*; peroxidation; hyperlipidemia, antioxidase activity

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翡翠贻贝多糖的分离及其对高脂饮食小鼠抗氧化活性的影响

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摘要：已有的研究表明：翡翠贻贝粗多糖（PVP）对高脂模型小鼠具有显著的降低血脂作用，本文对翡翠贻贝粗多糖进行了纯化与分析，并研究了其对高脂模型小鼠体内抗氧化作用的影响。PVP经DEAE-52与Sephadex柱层析分离得到两个主要级分P1和P2。经化学分析，P1和P2均为有硫酸基的酸性多糖，相对分子质量分别为6.89×10^5 Da和2.87×10^5 Da。氨基葡萄糖：糖醛酸：氨基半乳糖的百分比例分别为：3.7:2.6:1、2.8:1.6:1。以翡翠贻贝粗多糖低[50mg/(kg d)]、中[100mg/(kg d)]、高[200mg/(kg d)]剂量灌喂高脂饮食小鼠（日常饮食中添加2%胆固醇）。结果显示：PVP中、高剂量组能显著增强高脂模型小鼠血清、肝脏和大脑中的T-AOC、GSH-Px, SOD和CAT活性，且降低MDA含量($p < 0.05$或$p < 0.01$)。研究表明PVP可能通过激活高脂小鼠体内抗氧化酶活性、降低体内过氧化水平而起到降低血脂作用。

关键词：多糖；翡翠贻贝；过氧化；高血脂；抗氧化酶活性

Hyperlipidemia has been incriminated as a contributory factor of atherosclerosis. Serum lipids are associated with obesity, heart disease, stroke, and kidney failure. Epidemiological studies have demonstrated strong causal relationship between serum lipid level and hyperlipidemia[1]. The decrease of serum high density lipoprotein cholesterol (HDL-C), and the increase of low density lipoprotein cholesterol (LDL-C), and triglyceride (TG) have been considered to be significant risk factors for hyperlipidemia[2]. LDL-C is the major carriers of cholesterol toward tissues having atherogenic potential; whereas, HDL-C carries cholesterol from peripheral tissues to the liver[3]. Therefore, HDL-C gives protection against many cardiac problems and obesity.

In addition, atherosclerosis is accompanied with the

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production of free radicals by endothelial and vascular smooth muscles. These processes initiate free radicals involved in atherogenesis through several important enzyme systems including xanthine oxidase, nicotinamide adenine dinucleotide phosphate, oxidases, and nitric oxide synthase[2]. The hypercholesterolemic state leads to an increase in free radical production; hence, elevating lipid peroxides. Oxidized lipids can elicit a wide variety of biological responses, which could contribute to atherosclerotic lesion development[4]. There is a relationship between hyperlipidemia (hypercholesterolemia) and the high level of reactive oxygen species (ROS) [5]. A relationship also exists between an increase in the production of ROS and rising activities of antioxidative defense systems such as superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), and thiol groups[6]. The conventional therapeutic modalities are available for hyperlipidemia mainly includes lipid lowering drugs like atorvastatin, lovastatin, and fibrates. Eventhough, these synthetic drugs are effective, but cause adverse effects[7]. Therefore, the study mainly focused on biologically active components from natural materials, to reduce the adverse effects of these drugs without impacting the physiological functions. Recently, many polysaccharides with antihyperlipidemic activity have been discovered in various food materials such as *Apostichopus japonicus*, *Ulva pertusa*, *Ulva lactuca*, and chitosan and chitosan derivatives[8-11]. Generally, many polysaccharides from natural materials have antioxidative and antihyperlipidemic activities, and can be developed as novel potential hypolipidemic agents.

Perna viridis is an important cultivated aquatic species in China. It has been reported that polysaccharides isolated from this oyster have antitumor and immunomodulating activity, antihyperlipidemic, and antioxidative activity *in vitro* [12-14]. In recent studies, other antioxidant polysaccharides which have been found in abalone gonad, *Stichopus variegates* Semper, *Ulva pertusa*, and sea cucumber, *Metriatyla scabra* showed antihyperlipidemic effects[15-17]. It could be hypothesized; hence, a combination of antioxidant and antihyperlipidemic activities might have the potential to attenuate the progression of cardiovascular disease and atherosclerosis. For this reason, the effect of *Perna viridis* polysaccharide (PVP) was evaluated on lipid peroxidation in cholesterol-supplemented diet mice.

1 Materials and Methods

1.1 Materials

Perna viridis was purchased from Zhanjiang Dongfeng aquatic product market (Zhanjiang, China). The shell was removed and the whole viscera were freezed at -18 ℃ for later use.

DEAE-52 cellulose and Sephadex G-200 were obtained from Whatman, standard glucans (Dextran T-2000, T-500, T-70, T-40 and T-10) from Pharmacia company(Sweden). Other reagents were all of Analytical Grade.

1.2 Experimental animals

Male Kunming mice of specific pathogen free (SPF) level, 4-week-old and weighing 18 to 22 g were obtained from Medical Experimental Animal Center of Guangdong Province, accredited by China National Accreditation Service National Laboratory in 2008. They were also maintained under standard conditions of humidity (40 to 60%), temperature (20 to 22 ℃), and a 12 hour light and darkness cycle. They were allowed free access to water and fed with ad libitum standard feed (SPF feeds, Guangdong, China) on a standard pellet diet (GB14924.3-2001, mouse feed standard, China). The pellet diet consisted of water (8.22%), crude protein (21.54%), crude fat (4.67%), crude fiber (3.5%), and ash (5.43%). Cholesterol-supplemented feed was made by adding cholesterol (2%) and cholate (0.4%) to standard diet. Investigation on animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The study protocol was approved by Guangdong Ocean University Ethics Committee.

1.3 Isolation and purification of polysaccharides

The whole viscera of *Perna viridis* was hydrolyzed by an enzyme mixture of two protease Trypsin:Subtilisin (2:5). The hydrolysate was decolored with carbon and diatomaceous earth, and then precipitated by ethanol at the total content of 75% (*V/V*). Then, the precipitate was washed by ethanol and acetone alternated for three times. Once the precipitate was decolored, removed proteins, ultrafiltrated and freeze-dried successively, a crude

polysaccharide (PVP) was obtained.

PVP was sequentially purified by chromatography on DEAE-52 cellulose and Sephadex G-200 gel according to the reported methods with slight modifications. Briefly, the solution of PVP (5 mL, 15 mg/mL) was applied to a column (1.6 cm×60 cm) of DEAE-52 cellulose. Then, the column was stepwise eluted with 0.5M sodium acetic acid and 1.5M sodium chloride solutions at a flow rate of 12 mL/h. The obtained elutes (3 mL/tube) were collected automatically and the carbohydrates were detected by the phenol-sulfuric acid method[18] .As a result, two fractions of polysaccharides P1 and P2 were obtained (Fig. 1). Each fraction was collected, concentrated, dialyzed, and further purified through a column (1.6 cm×60 cm) of Sephadex G-200 gel using distilled water as an eluent at 12 mL/h and collected 3 mL/tube.

1.4 Characterization of P1 and P2

The carbohydrate contents in P1 and P2 were determined by phenol-sulfuric acid method. The protein content was determined by the method described by Bradford (1976) using bovine serum albumin as the standard[19]. The content of uronic acid was determined per the method of Blumenkrantz and Asboe-Hansen (1973) using D-glucuronic acid as the standard[20] . The content of sulfate radical was determined according to the previous literature (1971)[21]. Glycosaminoglycan content was determined by Alcian Blue colorimetric. Glucosamine and galactosamine were measured by a modified Elson-Morgan reaction(1964)[22] .

1.5 Gel-permeation chromatography (GPC) analysis

The homogeneity and molecular weight of polysaccharides was determined on the Agilent 1100 HPLC system (Agilent technologies, US) equipped with Sugar KS-804 column (8 mm×300 mm), and a refractive index detector at 50 ℃. The sample solution (20μL, 1 mg/mL) was injected in each run with distilled water as the mobile phase at 1 mL/min.

1.6 Fourier transform infrared (FTIR) spectroscopy

Individually P1 and P2 (1 mg) was mixed with 100 mg of potassium bromide (KBr). After KBr pellet, the IR spectra was recorded using FTS-175C IR spectrometer (Bio-Rad Win) in the range of 400 to 4000 cm^{-1}.

1.7 Animal groups and experiment design

Male SPF Kunming mice were employed in this study. Seventy-two mice were fed with a standard diet for 7 days to acclimatize to animal facilities, and were weighed, and equally divided into six groups, each group consisting of 12 animals. Group (1) was normal control; Group (2) served as hyperlipidemic control; Group (3) was administered with the standard drug (atorvastatin, 5 mg/kg) as positive control; and Groups (4), (5), and (6) received PVP at a dose of 50, 100, and 200 mg/kg body weight, respectively. After the period of acclimation, Group (1) animals continued to receive the standard feed, and animals in other five groups were fed with a cholesterol-supplemented diet for 28 days. At the same time, the Group (3) was administered atorvastatin and the Groups (4), (5), and (6) were given different doses of PVP orally.

1.8 Determination of MDA, SOD, CAT, GSH-Px, and TAOC

At the end of the experimental period, the mice were fasted for 18 h prior to blood withdrawal. The blood was collected from the eyeballs and centrifuged at 4000 r/min at 4 ℃ for 10 min to afford the serums. The liver and brain were removed rapidly, washed, and homogenized in ice-cold physiological saline to prepare 10% (*m/V*) homogenate. Then, the homogenate was centrifuged at 4000 r/min at 4 ℃ for 10 min to remove cellular debris, and the supernatant was collected for analysis.

The biochemical carried out per the instructions of kits purchased fro assays werem Nanjing Jiancheng Bioengineering Institute, China. Briefly, SOD activity was measured through the inhibition of hydroxylamine oxidation by the superoxide radicals generated in the xanthine–xanthine oxidase system. GSH-Px activity was measured on the basis of the reactions of GSH and 5, 5-dithiobis-(2-nitrobenzoic acid). CAT activity was determined by measuring the absorbance of the yellow hydrogen peroxide (H_2O_2) and ammonium molybdate complex at 405 nm. TAOC was measured by Ferric reducing ability of plasma assay. All the above activities were expressed as unit per milliliter (U/mL) in serum or unit per milligram of protein (U/mg) in liver and brain. In addition, MDA level was measured by the thiobarbituric acid reactive substances (TBARS) method and expressed as nmol per milliliter (nmol/mL) in serum or nmol per milligram of protein (nmol/mg) in liver and brain. The protein content in liver and brain supernatant was determined by the Bradford method using bovine serum

albumin as the standard.

1.9 Statistical analysis

All values were presented as means±standard deviation (SD) of triplicate samples. Data were analyzed by Student's t-test. Differences with $P<0.05$ and $P<0.01$ were considered to be statistically significant.

2 Results and Discussion

2.1 Purification and characterization

The PVP was first separated through an anion-exchange column of DEAE-52 cellulose. Three independent elution peaks (F1, F2, and F3) were obtained (Fig.1a). The F1 and F3 fractions were collected, concentrated, dialyzed, reconcentrated, and loaded onto a column of Sephadex G-200, respectively. The column was eluted with distilled water and the resulting eluent was collected. Fig.1b-c showed each fraction generated one single elution peak affording P1 and P2, respectively.

The chemical compositions of PVP, P1, and P2 were given in Table 1. Hence, the obtained PVP containing GAG (45.36%), protein (18.12%), sulfate (6.89%), and uronic acid (7.89%). P1 and P2 had less protein than PVP, which means the protein can be partly removed from PVP. The ratio of glucosamine, uronic acid and galactosamine were 3.7:2.6:1 in P1 which contained 75.56% total GAG. The ratio of glucosamine, uronic acid and galactosamine were 2.8:1.6:1 in P2 which contained 69.78% total GAG. Sulfuric radical content in P1 and P2 were 19.65% and 21.56%, respectively.

The GPC profile (Fig.2) showed single and symmetrically sharp peaks, indicating both P1 and P2 were homogeneous polysaccharides with an average molecular weight of 6.89×10^5 Da and 2.87×10^5 Da, respectively.

Table 1 Chemical compositions and contents of PVP, P1 and P2 (%)

	PVP	P1	P2
Protein	18.12	10.75	8.45
Glycosaminoglycan	45.36	75.56	69.78
Sulfuric radical	6.89	19.65	21.56
Uronic acids	7.89	19.85	16.56
Glucosamine	15.65	28.00	29.11
Galactosamine	3.71	7.67	10.56

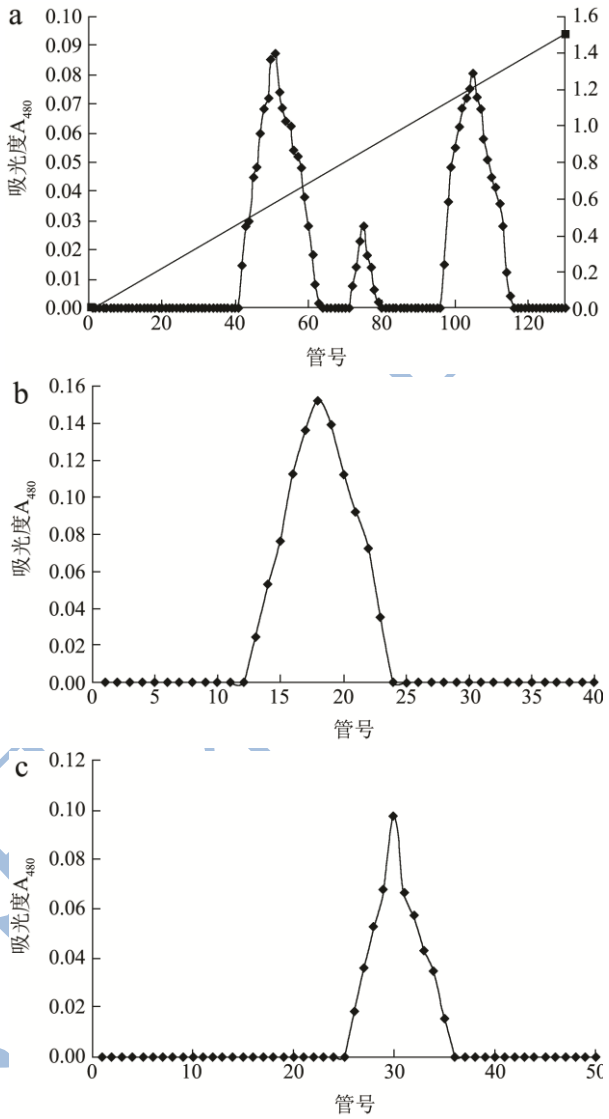

Fig.1 Chromatographic purification of polysaccharides from *Perna viridis*.

Note: a. DEAE-52 cellulose chromatogram of PVP (fraction1: tube 42-62, fraction 3: tube 97-115), using 0.5M sodium acetic acid and 1.5M sodium chloride solution as eluant at 12 ml/h and collecting 3.0ml per tube; b and c. Sephadex G-200 chromatogram of fraction 1 and 3(P1 and P2), using distilled water as eluant at 12 ml/h and collecting 3.0ml per tube.

The FT-IR spectrum of P1 and P2 were shown in Fig. 3. The strong and wide absorption band of about 3200 to 3600 cm^{-1}, 2800 to 3000 cm^{-1}, and 1640 to 1650 cm^{-1} corresponded to O-H, C-H, and C-O stretching vibrations, respectively. The bands approximately in the region of 3397, 2931, and 1643 cm^{-1} in P1 and 3433, 1647cm^{-1} in P2 were characteristic absorptions of polysaccharides. Peaks

of about 900 to 1200 cm^{-1} were due to C-O-C stretching vibrations of sugar ring and C-O bending vibrations. The strong absorptions at 1643 and 1647 cm^{-1} corresponding to the secondary-CONH group implied the existence of proteins. There are -NH(1569cm^{-1} , 1558 cm^{-1}), and -O-SO_3^- group (1237cm^{-1} , 1233 cm^{-1}) which are the characteristics of glycosaminoglycan. 932 and 843 cm^{-1} absorption in P1 and P2 were due to β-glucosidic bonds and α-glucosidic bonds, respectively. The IR spectrum shows a band beside 1235 cm^{-1} related to the >S=O stretching vibration, which confirms the presence of sulphate group.

2.2 Effect of PVP on the antioxidative defense

PVP have shown the antihyperlipidemic and antioxidative activity *in vitro* [13~14]. There may be an important relationship between the antihyperlipidemic effect and the antioxidant activity of PVP. PVP could decrease the contents of serum total cholesterol (TC), TG, and LDL-C; and increase the contents of serum HDL-C and NO, reduce the contents of liver TC and TG, and enhance the activities of serum lecithin cholesterol acyltransferase, and the activities of human pancreatic lipase, and hepatic lipase in the serum and liver.

Effects of PVP on activities of SOD, CAT, GSH-Px, and levels of MDA, and TAOC in serum, liver and brain in cholesterol-supplemented diet mice were shown in Table 2,3,4. Oxidative stress and lipid peroxidation result from an imbalance between the cellular antioxidant defense systems, and the production of free radicals, and ROS and could lead to the development of various metabolic diseases such as atherosclerosis [24] .

Atherosclerosis is a leading cause of cerebral infarction, especially the arterothrombtic cerebral infarction. The TBARS concentration has long been used as an indicator of the lipid peroxidation process and oxidative stress in laboratory animals. Cholesterol-supplemented diet group shows a significant increase of 43%, 85%, and 44% in serum, liver, and brain TBARS level, respectively.

Several antioxidant enzymes including SOD and GSH-Px are important buffers in the interception, and degradation of superoxide anions, and H_2O_2. SOD is a critical enzyme that protects cells from damage, and its activity indirectly reflects its antioxidant ability. GSH-Px is an important enzyme to scavenge ROS , the products of oxidation, and thus protects the body from lipid peroxidation. Noticeably, significant decreases ($P<0.01$) of antioxidant enzyme activities (SOD, GSH-Px, and CAT) in serum, liver, brain and TAOC in serum and liver were observed between the treatments of normal and model control group, respectively.

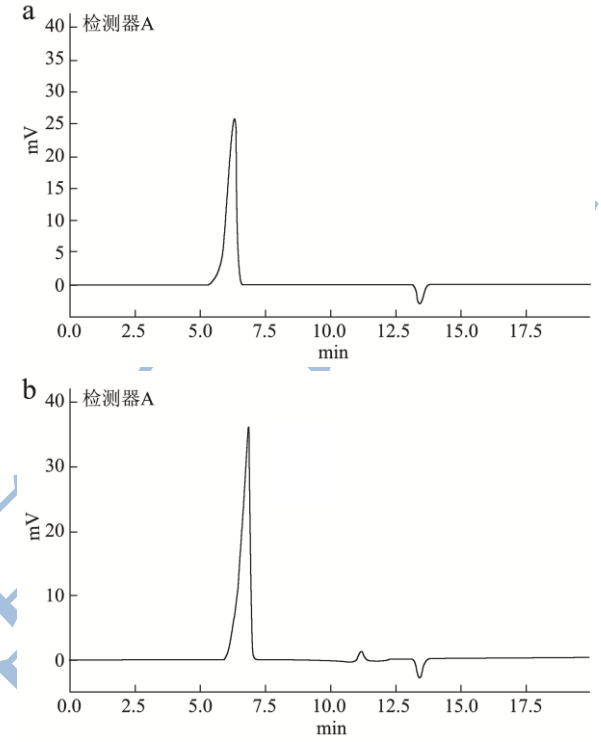

Fig.2 HPGPC profile of P1(a) and P2(b)

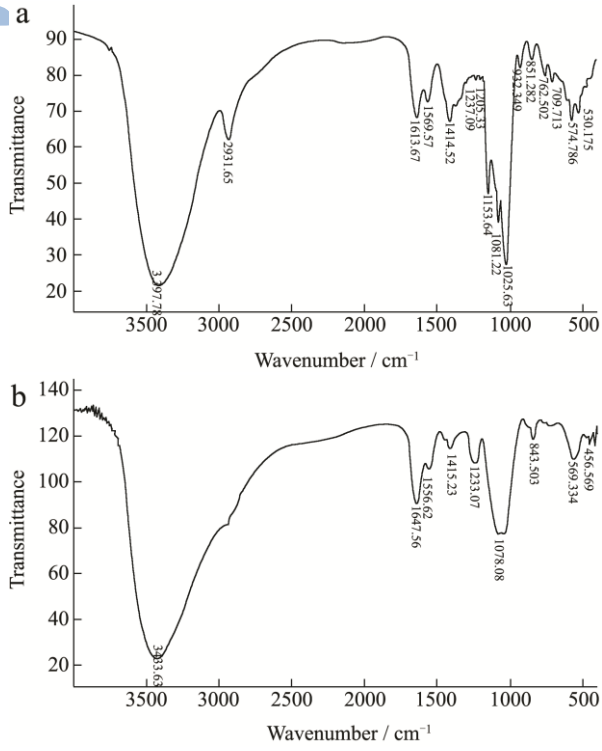

Fig.3 HPGPC profile of P1(a) and P2(b)

A long term high cholesterol diet could cause TG and cholesterol to synthesize faster than its transporting rate and to be accumulated in the liver cells, resulting in fatty liver development. Fatty liver formation has been related to the oxidative stress and lipid peroxidation in liver. Lipid peroxidation in liver interferes with liver lipid metabolism causing lipid deposition, which again increases lipid peroxidation, damaging the liver cells; and hence, leading to the significant increase of liver MDA content, and the significant reduction of the liver SOD activity [23-24]. Compared to model control group, PVP and atorvastatin treatments inhibited significantly (P<0.05) the formation of MDA, and raised the activities of antioxidant enzymes, and the level of TAOC in a dose-dependent manner. Whereas, 200 mg/kg PVP was administered to cholesterol-supplemented diet mice, the activities of SOD, GSH-Px, CAT, and TAOC level in serum were increased by 12%, 15%, 20%, and 18%, respectively; and the MDA was decreased by 28%. Similarly, when 200 mg/kg PVP was administered to cholesterol-supplemented diet mice, the activities of SOD, GSH-Px, CAT, and TAOC level in liver were increased by 10%, 37%, 10%, and 46%, respectively; and the MDA was decreased by 38% (Table 3).

Table 2 Effects of PVP on the activity of SOD, CAT and GSH-P_X and levels of MDA and TAOC in serum of mice

Group	SOD/(U/mL)	CAT/(U/mL)	GSH-P_X/(U/mL)	MDA/(nmol/mL)	TAOC/(U/mL)
Normal control	197.42±8.26	12.22±1.29	752.98±59.21	12.35±0.94	6.96±0.49
Model control	168.58±12.87**	9.54±0.90**	606.69±57.79**	17.71±0.80**	5.64±0.32**
Atorvastatin	186.39±13.58$^{\#\#}$	10.67±0.85$^{\#\#}$	705.71±49.26$^{\#\#}$	13.16±0.95$^{\#\#}$	6.58±0.64$^{\#\#}$
PVP(50 mg/kg)	179.46±10.20$^{\#}$	10.23±0.90	647.59±74.55	13.92±1.38$^{\#\#}$	6.25±0.39$^{\#}$
PVP(100 mg/kg)	184.00±9.00$^{\#\#}$	11.12±1.26$^{\#\#}$	682.43±41.68$^{\#}$	13.57±0.67$^{\#\#}$	6.42±0.24$^{\#\#}$
PVP(200 mg/kg)	189.16±6.10$^{\#\#}$	11.41±0.81$^{\#\#}$	695.57±66.95$^{\#\#}$	12.81±1.10$^{\#\#}$	6.63±0.62$^{\#\#}$

Note: The data represents the mean±SD, n=12 for each group.$^{\#}$ p <0.05 compared with model control group.$^{\#\#}$ p <0.01 compared with model control group.** p<0.01 compared with normal control group.

Table 3 Effects of PVP on the activity of SOD, CAT, and GSH-P_X and levels of MDA and TAOC in the liver of mic

Group	SOD/(U/mg protein)	CAT/(U/mg protein)	GSH-PX/(U/mg protein)	MDA/(nmol/mg protein)	TAOC/(U/mg protein)
Normal control	315.12±19.81	103.72±9.91	401.21±20.40	4.13±0.63	3.33±0.67
Model control	275.49±15.49**	88.61±3.99**	279.87±28.31**	7.65±0.96**	2.19±0.44**
Atorvastatin	302.70±19.97$^{\#\#}$	98.74±3.19$^{\#\#}$	389.41±14.85$^{\#\#}$	5.00±0.85$^{\#\#}$	3.18±0.83$^{\#\#}$
PVP(50 mg/kg)	295.56±16.14$^{\#}$	94.86±4.78$^{\#}$	347.80±15.59$^{\#\#}$	5.32±0.65$^{\#\#}$	2.56±0.60
PVP(100 mg/kg)	301.04±18.13$^{\#\#}$	95.47±4.47$^{\#}$	374.50±8.57$^{\#\#}$	5.10±0.68$^{\#\#}$	2.97±0.48$^{\#}$
PVP(200 mg/kg)	303.67±18.22$^{\#\#}$	97.81±5.91$^{\#\#}$	382.95±18.75$^{\#\#}$	4.77±0.58$^{\#\#}$	3.19±0.72$^{\#\#}$

Note: The data represents the mean±SD, n=12 for each group.$^{\#}$ p <0.05 compared with model control group.$^{\#\#}$ p <0.01 compared with model control group.** p <0.01 compared with normal control group.

Table 4 Effects of PVP on the activity of SOD, CAT, and GSH-PX and levels of MDA in the brain of mice

Group	SOD/(U/mg protein)	CAT/(U/mg protein)	GSH-PX/(U/mg protein)	MDA/(nmol/mg protein)
Normal control	205.24±8.97	13.26±0.98	79.79±6.27	5.52±0.53
Model control	171.28±4.73**	9.63±0.56**	59.43±7.41**	7.95±0.57**
Atorvastatin	193.89±8.02$^{\#\#}$	12.78±1.59$^{\#\#}$	74.12±3.95$^{\#\#}$	6.21±0.55$^{\#\#}$
PVP(50 mg/kg)	179.82±4.61	10.91±1.09$^{\#}$	64.17±4.12	6.91±0.41$^{\#\#}$
PVP(100 mg/kg)	184.15±7.72$^{\#}$	12.37±0.88$^{\#\#}$	69.81±5.65$^{\#\#}$	6.65±0.34$^{\#\#}$
PVP(200 mg/kg)	190.80±14.05$^{\#\#}$	12.75±1.76$^{\#\#}$	72.10±6.07$^{\#\#}$	6.15±0.48$^{\#\#}$

Note: The data represents the mean±SD, n=12 for each group.$^{\#}$ p <0.05 compared with model control group.$^{\#\#}$ p <0.01 compared with model control group.** p <0.01 compared with normal control group.

High-fat diet enhanced lipid peroxidation (TBARS) and protein damage (carbonyl) in cerebral cortex and hippocampus; and reduced the non-enzymatic antioxidants defenses (sulfhydryl) in cerebral cortex and cerebellum; and reduced CAT and SOD activities in all brain tissues; and enhanced NO production in all cerebral

tissues[25]. In present study, when 200 mg/kg PVP was administered to cholesterol-supplemented diet mice, the activities of SOD, GSH-Px, and CAT of brain tissue were increased by 11%, 21%, and 32%, respectively; and the MDA was decreased by 23% (Table 4). These findings suggested PVP suppressed the increased TBARS induced by a high cholesterol diet by blocking lipid peroxidation through activation of antioxidant enzymes.

3 Conclusions

Polysaccharide from *Perna viridis* can prevent the oxidative injury in mice fed with cholesterol-supplemented diet, which could promote the activities of endogenous antioxidation enzymes and reduce the level of peroxidation in the body, which may be a potential substance of natural antioxidants as a therapeutic agent for hyperlipidemia.

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