ISSN 1027-5495. Functional Materials, 26, No.2 (2019), p. 325-331.
doi:https://doi.org/10.15407/fm26.02.325 © 2019 — STC "Institute for Single Crystals"

Reseach on the antioxidant activity of glycoproteins extracted from Perna Viridis (GPP)

***Shuji Liu*[1,2,3], *Zhiyu Liu*[2,3], *Yongchang Su*[2,3], *Jingna Wu*[2,3], *Min Xu*[2,3], *Yin Wang*[2,3], *Chen Bei*[2,3], *Kun Qiao*[2,3], *Jinquan Chen*[1]**

[1]College of Food Science, Fujian Agriculture and Forestry University, 350002 Fuzhou, Fujian Province, China
[2]Fisheries Reasearch Instiute of Fujian, National Research and Development Center for Marine Fish Processing (Xiamen), Key Laboratory of Cultivation and High-value Utilization of Marine Organisms in Fujian Province, 361013 Xiamen, Fujian Province, China
[3]Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources, 361013 Xiamen, Fujian Province, China

Received April 25, 2019

GPP (glycoproteins extracted from Perna Viridis), was a natural product extracted from mussel. This study was designed to evaluate both the antioxidant and free radical scavenging activities and protection against H_2O_2-induced oxidative damage of GPP. L02 cells were exposed to 100 μM H_2O_2 with or without GPP at different concentrations. Cell viabilities were monitored by MTT-test, MDA production in culture medium, ROS level, SOD and GSH activities were detected. The *in vitro* experiments demonstrated that GPP exhibited good capacity of scavenging hydroxyl radicals and superoxide anions with activities the IC_{50} value of 3.38, 3.36 mg/mL respectively. GPP could significantly increase the activity of SOD and GSH, and reduce the level of MDA and ROS in the culture medium, and protect the cells against H_2O_2-induced stress to offer vital cytoprotection against the oxidative damage induced by H_2O_2 in L02 cells. These findings suggested that GPP as a natural production is a potenti al source of natural antioxidants and some extent validates its medicinal potential.

Keywords: GPP, antioxidant activity, free radical scavenging H_2O_2-induced oxidative stress.

Данное исследование посвящено оценке как антиоксидантной, так и антирадикальной активности GPP (комплекс гликопротеинов, извлеченных из Perna Viridis — натуральный продукт, извлеченный из мидий), а также его протекторные свойства в условиях H_2O_2 — индуцированного окислительного повреждения клеток. Клетки L02 подвергали воздействию 100 мкМ H_2O_2 в отсутствии или присутствии GPP в различных концентрациях. Жизнеспособность клеток контролировали с помощью MTT-теста, оценивали образование MDA, определяли уровни ROS и GSH, измеряли активность SOD. Эксперименты *in vitro* показали, что GPP проявляет хорошую способность нейтрализовать гидроксильные и супероксид-анион радикалы с активностью IC_{50}, равной 3,38, 3,36 мг/мл соответственно. GPP может значительно повысить активность SOD и уровень GSH, а также уменьшить образование MDA и ROS и защитить клетки от H_2O_2-индуцированного стресса, обеспечивая цитопротекцию против окислительного повреждения, вызванного H_2O_2 в клетках L02. Эти данные свидетельствуют о том, что натуральный продукт GPP является потенциальным источником природных антиоксидантов и в некоторой степени подтверждает его лекарственный потенциал.

Дослідження антиоксидантної активності гликопротеинов, виділених з Perna Viridis (GPP). *Shuji Liu, Zhiyu Liu, Yongchang Su, Jingna Wu, Min Xu,Yin Wang, Chen Bei, Kun Qiao, Jinquan Chen*

Дане дослідження присвячено оцінці як антиоксидантної, так і антирадикальної активності GPP (комплекс гликопротеінів, добутих з Pernaviridis — натуральний препарат, добутий з мідій), а також його протекторних властивостей в умовах H_2O_2 — індукованого окислювального пошкодження клітин. Клітини L02 піддавали дії 100 мкМ H_2O_2 у відсутності або присутності GPP у різних концентраціях. Життєздатність клітин контролювали за допомогою МТТ-тесту, оцінювали освіту MDA, визначали рівні ROS і GSH, вимірювали активність SOD. Експерименти *in vitro* показали, що GPP проявляє хорошу здатність нейтралізувати гідроксильні і супероксид-аніон радикали з активністю IC50, що дорівнює 3,38, 3,36 мг/мл відповідно. GPP може значно підвищити активність SOD і рівень GSH, а також зменшити утворення MDA і ROS і захистити клітини від H_2O_2-індукованого стресу, забезпечуючи цитопротекцію проти окисного пошкодження, викликаного H_2O_2 у клітинах L02. Ці дані свідчать про те, що натуральний продукт GPP є потенційним джерелом природних антиоксидантів і в деякій мірі підтверджує його лікарський потенціал.

1. Introduction

Oxidation is closely related to the life and health. The free radicals containing unpaired electrons such as hydroxyl free radicals, superoxide anion are produced in physiological metabolic processes of body. But when the excessive free radicals accumulated in the body and can't be cleared in time, they will lead to the reaction of biological macromolecules such as nucleic acid, protein, producing a large number of oxide, affecting normal cells and energy metabolism, thus accelerate the aging of the human body, or cause cardiovascular disease, cancer, etc. [1]. Reactive oxygen species (ROS) includes various radicals, hydrogen peroxide (H_2O_2), reactive hydroxyl (·OH), superoxide anion (O^{2-}), nitric oxide (NO), and peroxyl radicals (ROO). However, recent research suggested that excessive amounts of ROS and oxidative stress also in liver damage. Antioxidants defense system from oxidative damage by scavenging the generated excessive free radicals through chelating, reacting with free radicals, quenching singlet oxygen, and catalytic metals. Nowadays, the antioxidants in use are either derived from synthesis or natural origin. Considering the carcinogenic side-effects, people prefer natural origin antioxidants to synthetic antioxidants. Therefore, more and more new natural origin are investigated and researched to extract antioxidants.

Perna Viridis (Asian green mussel) is an edible marine bivalve that is mainly found in the south of the East China Sea, South China Sea, and the Taiwan Straits. *P. Viridis* is abundant and delicious, and is of high nutritional and medicinal value [2]. Previous studies have shown that mussel extracts have anti-inflammatory, anti-tumor, antihypertensive [3], anti-coagulation [4], anti-viral, and anti-bacterial effects [5, 6]. The anti-bacterial ingredients from common mussels are shown to be related to lipopolysaccharide (LPS) and other polysaccharides [5, 7].

Glycoproteins in general are proteins covalently modified with oligosaccharides, and play key roles in a variety of biological processes contributing to diverse functions, such as anticancer [8], immunological enhancement [9], antimicrobial, cell adhesion, maintenance of protein conformation and stability [10], and anti-hypertension activities [11]. These properties make glycoprotein one of the current hot areas of research [12].

In this study, GPP, extracted glycoproteins from *P. viridis* was designed to evaluate the antioxidant activity by detecting the two free radical scavenging activities including hydroxyl radical and superoxide anion *in vitro*, and investigated their antioxidant capacities on H_2O_2-induced stress L02 cells. Taken together, our research findings are promising for development of *P. viridis* as food resources and healthy diet.

2. Materials and methods

2.1. Cells and reagents

Human Hepatocyte Cell Line L02 was purchased from SUN YAT-SEN University. PRMI1640, trypsin and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY). 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (St. Louis, MO). MDA, SOD, GSH, ROS assay kits were acquired from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemi-

cals and reagents were of the highest grade commercially available.

2.2 Preparation of GPP

According to the method described by Liu et al [13], the flesh of *P. viridis* was homogenized and mixed with 0.2 M NaCl in a 1:1 ratio, and then extracted for 60 min at 62°C. The extract was cooled to room temperature, filtered through three layers of gauze, and separated by passing through a ceramic membrane and hyperfiltration-membrane that intercepted 5000 Da molecules. After that purification was performed by using chromatography through a DEAE-52 column and a Sephadex G150 column, successively. Then it was dialyzed and frozen-dried to obtain the powder of GPP. The powder contained a sugar content of 35.0 % and a protein content of 56.7 %.

2.3. Superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity of GPP was investigated by the method of Y.Li et al. [14] with some modifications, using spectrophotometric detecting the inhibition of pyrogallol auto-oxidation. In the experiment, O2–. anions were generated in 3.5 mL of tris-HCl-EDTA buffer (0.1 M, pH 8.0) containing 0.5 mL of pyrogallol solution (10 mM), and 1.0 mL of different concentrations of GPP. The optical density (OD) of releases chromphoric products while pyrogallol auto-oxidationwas measured at 320 nm. The capacity of scavenging the anion radical that was determined as the percentage of inhibiting pyrogallol auto-oxidation, was calculated from OD in the presence or absence of pyrogallol and GPP.

2.4. Hydroxyl radical-scavenging activity

The scavenging activity for hydroxyl radical was measured according to the modified method of Y.Li et al. [14]. The HO· radicals were generated from H_2O_2 and $FeSO_4$, and detected by their ability to hydroxylate salicylate. Both 0.5 mL of salicylic acid-anhydrous ethanol (9.0 mM), 0.5 mL of $FeSO_4$ (9.0 mM) were mixed thoroughly. Then H_2O_2 (8.8 mM) and 1.0 mL different concentrations of GPP were added. The mixture was incubated at 37 °C for 30 min, and the absorbance of the hydroxylate salicylate complex was measured at 510 nm. The inhibition percentage of hydroxyl radical was calculated by following equation:

$$\text{Hydroxyl radical–scavenging activity}\,(\%) = [(A_s - A_1)/(A_0 - A_1)] \cdot 100, \quad (1)$$

where A_s, absorbance of the sample; A_1, absorbance of control solution containing Salicylic acid-anhydrous ethanol, $FeSO_4$ and H_2O_2; A_0, absorbance of blank solution containing salicylic acid-anhydrous ethanol and $FeSO_4$.

2.4 Cell culture

Liver cell LO2 were cultured in a humidified atmospheric incubator in 5 % CO_2 at 37°C in PRMI1640 with 100 units/mL penicillin, 100 μg/mL streptomycin and 10 % FBS.

2.5 MTT assay

Cell viability was determined by MTT assay as described in [15]. Cells were seeded at a density of $2 \cdot 10^5$ cells/well in 96-well plates, and cultured for 24 h. Then the cells were treated with serial concentrations of GPP (0, 1, 5, 10, 25, 50, 100, 200, 400, 800 μg/mL) followed by incubating 10 μL MTT working solution (5 mg/mL) at 37°C for 4 h. After 4 h incubation, the formazan crystals were dissolved in 150 μL dimethyl sulfoxide (DMSO). The absorbance at 570 nm A570 was measured by (Tecan). The percentage of cell viability was calculated according to the following equation:

$$Cellviability\,(\%) = . \quad [A570(sample)/A570(control)] \cdot 100\% \quad (2)$$

To induce oxidative stress, a series of concentrations of H_2O_2 ranging from 50 to 400 μM were employed to replace GPP described above to establish optimal effective H_2O_2 concentration.

2.6. Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS was measured according to a previously published method [16]. L02 cells were cultured in 24-well plates at a density of $2 \cdot 10^5$ cells/well for 24 h incubation. After that the cells were recovered in serum-free PRMI1640 medium for 6 h following by the cells were subjected to GPP (50, 100, 200 μg/mL) for 24 h. The cells culture were centrifuged at 1000 g for 8 min to collect cells and washed twice with phosphate buffer saline (PBS). The washed cells were resuspended in PBS and incubated with DCFH-DA

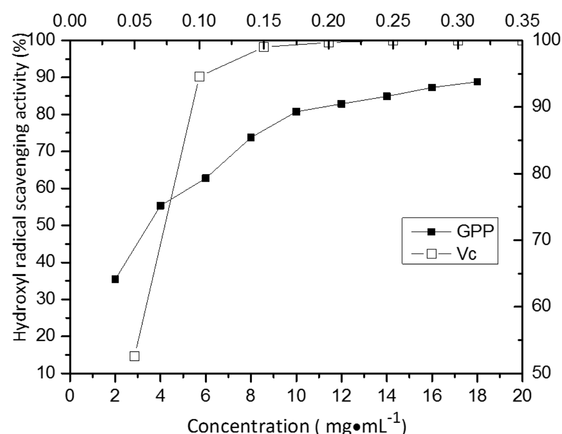

Fig. 1. The scavenging ability of GPP on hydroxyl radicals.

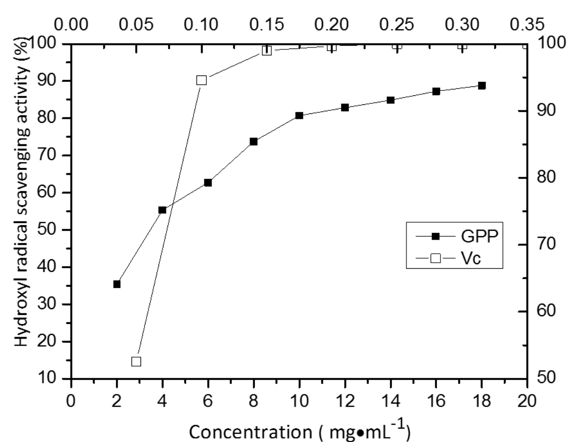

Fig. 2. The scavenging ability of GPP on superoxide anion radicals.

(10 μM) for 40 min at 37°C and then exposed to H_2O_2 (100 μM) for 5 min to stimulate the ROS generation and subsequently washed with PBS three times. The formation of DCF wa s detected by using the Elx 808 Universal Microplate Reader (BIO-TEK, INC) at an emission wavelength of 525 nm and excitation wavelength of 485 nm.

2.7. Determination of SOD, GSH, and MDA activities

The activities of SOD, GSH, MDA, AST, and ALT were measured according to previous studies [17,18]. Briefly, L02 cells were cultured in 96-well plates ($2 \cdot 10^5$ cells/well) for 24 h incubation. After that the cells were treated with different concentrations of GPP (50, 100, 200 μg/mL) for 24 h, cells treated without GPP as control group, following by the cells were challenged with H_2O_2 (100 μM) to induce oxidative stress, for 4 h at 37°C. Then the cell culture medium was harvested and centrifuged at 1500×g for 10 min at 4°C. The supernatant was collected to detected enzyme activity. According to the manufacturer's instructions, the SOD, GSH and MDA levels were measured by their commercially available kits respectively.

2.8. Statistical analysis

All data were expressed as a mean±SD, and analyzed by one-way ANOVA analysis of variance using the Dunnet test. A value of $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Hydroxyl radical-scavenging activity

Hydroxyl radical is the most reactive free radical and can cause strand damages in DNA leading to mutagenesis, cytotoxicity and lipid peroxidation [19]. The hydroxyl radial-scavenging abilities of different concentration of GPP were presented in Fig. 1. The GPP exhibited potent or moderative activity in a concentrations dependent manner.

The fitting curve equations of hydroxyl radical scavenging by GPP was as follow: $y = 51.292\ \mathrm{Ln}(x) - 12.439$, $R^2 = 0.9851$, with the IC_{50} value of 3.38 mg/mL. Ascorbic acid as a positive control, with an IC_{50} value of 0.3 mg/mL was highly effective on hydrogen radical scavenging.

3.2. Superoxide radical-scavenging activity

Superoxide anions are a precursor to active free radicals which would react with biological macromolecules and thereby causing tissue damage [20]. They take an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical in living system. Superoxide anion scavenging of the different concentrations of GPP is presented in Fig. 2. The higher concentration of glycoprotein was, the stronger the ability of scavenging superoxide anion was. The fitting curve equations of hydroxyl radical scavenging by GPP was as follow: $y = 24.786\ \mathrm{Ln}(x) + 19.996$, $R^2 = 0.9857$, with the IC_{50} value of 3.36 mg/mL. Also the ascorbic acid as a positive control was highly effective on hydrogen radical scavenging.

Results showed that GPP has good antioxidant and free radicals (hydroxyl radical and superoxide anion) scavenging activities and can be a potential source of natural antioxidant.

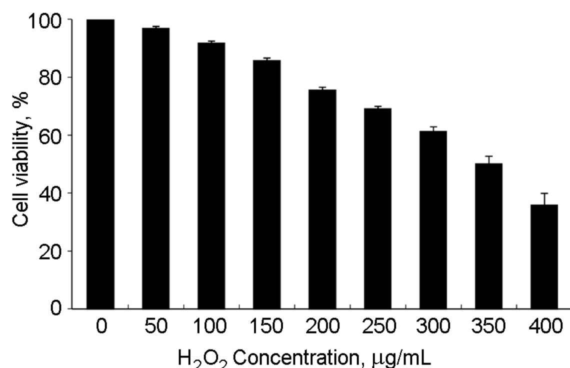

Fig. 3. Effect of different concentrations of H_2O_2 on the L02 cell viability.

3.3. Effects of different concentrations of H_2O_2 on cell proliferation

H_2O_2-induced oxidative stress model was established to determine the compatible dose of H_2O_2 in the experiment. As the data shown in Fig. 3, with the increase concentration of H_2O_2, the damage to L02 cells became more serious. When the concentration of H_2O_2 was 100 μM, the proliferation of L02 cells was decreased to 91.7 % ($P < 0.05$). And the survival rate of L02 cells was lower than 85 % when the concentration of H_2O_2 more than 150 μM. Therefore, 100 μM H_2O_2 was selected to establish the H_2O_2-induced oxidative stress model for subsequent experiments.

3.4. Cell viability

Increasing concentrations of GPP lowered cell viability (Fig. 4). When adding 1–200 g/mL of GPP, the cells viability maintained above 99%. With the concentration of GPP increased to 800 g/mL, the cells viability gradually decreased but remained above 90 %. The results indicated that GPP had nontoxic effect on L02 cells. The dose of GPP was determined to be 50, 100, 200 g/mL.

3.5. Effects of GPP on ROS in H_2O_2-induced oxidative stress cells

ROS is a series of reactive oxygen species produced in the aerobic metabolism of cell. ROS can regulate cell growth and apoptosis, while it has cytotoxic effects. ROS, as a common indicator, is used to evaluate the influence of foreign substances on the body. H_2O_2 is a kind of ROS closely related to oxidative stress in cell. So the excessive H_2O_2 can cause oxidative damage and stimulate cells to produce more ROS. As can be seen from Fig. 5, 100 M H_2O_2 induced

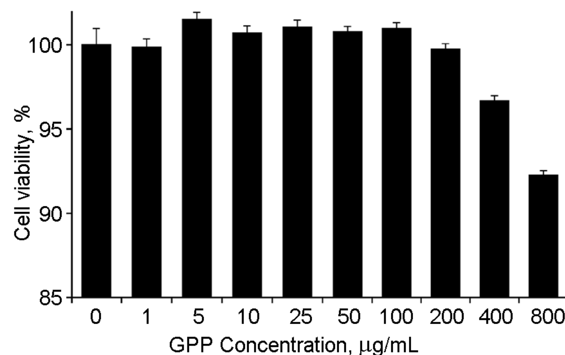

Fig. 4. Effect of GPP on the L02 cell viability.

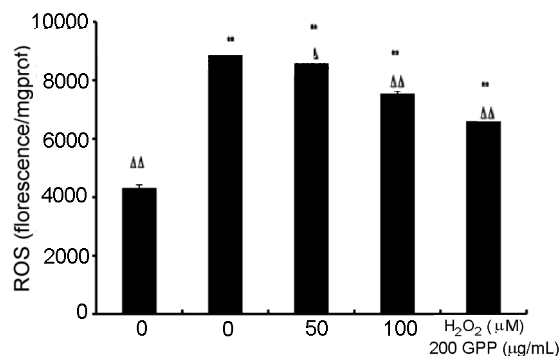

Fig. 5. The effect of GPP on the intracellular ROS production ("Δ" compared with the group of 100 μM H_2O_2 + 0 μg/mL GPP, $^{\Delta}P < 0.05$, $^{\Delta\Delta}P$ <<|0.01; "*" compared with the group of 0 μM H_2O_2 + 0 μg/mL GPP, $^{*}P < 0.05$, $^{**}P < 0.01$).

the dramatically increased of ROS (1-folds). The pretreatment of the cells with different concentrations (50, 100, 200 μg/mL) of GPP inhibited the production of ROS by 6.36 %, 17.73 % and 28.00 %, respectively ($P < 0.05$). Thus, GPP can inhibit the production of ROS in H_2O_2-induced cells, showing a dose-effect manner.

3.6. Effects of GPP on SOD, GSH and MDA in H_2O_2-induced oxidative stress cells

Superoxide dismutase (SOD) and Glutathione (GSH) play an important role in maintaining the oxidative balance of the body. And their activities can reflect the ability of scavenging free radicals. Malondialdehyde (MDA), a cytotoxic product of lipid peroxidation, can reflect the state lipid peroxidation damage for tissue [21].

SOD activity and GSH level were significantly lower ($P < 0.05$) in cells treated with 100 μM by 43.59 % and 72.93 % compared to control cells respectively (Table). SOD activity were significantly ($P < 0.05$) enhanced

Table. The effect of GPP on the content of SOD, GSH and MDA

Groups	SOD (U/mgprot)	GSH (μM/gprot)	MDA (nmol/mgprot)
0 μM H_2O_2 + 0 μg/mL GPP	48.70±1.33[d]	66.75±0.63[e]	10.29±0.17[a]
H_2O_2 + 0 μg/mL GPP	27.47±1.15[a]	18.07±0.63[a]	15.60±0.38[e]
H_2O_2 + 50 μg/mL GPP	32.97±1.87[b]	22.41±0.48[b]	14.32±0.04[d]
H_2O_2 + 100 μg/mL GPP	37.89±1.51[c]	30.28±0.77[c]	13.00±0.17[c]
H_2O_2 + 200 μg/mL GPP	45.24±2.20[d]	51.32±1.58[d]	12.23±0.09[b]

Note: Values in the same column with different superscript([a, b, c, d, e]) differ significantly ($P < 0.05$); Data expresses as Mean±SD ($N = 6$).

by 20.02 %, 37.93 % and 64.69 % in cells treated with 50, 100 and 200 μg/mL of GPP compared to H_2O_2 treated cells, respectively.

GPP was effective in restoring GSH levels in the cells that was lowed due to H_2O_2. GSH levels in cells at GPP 50, 100 and 200 μg/mL was 1.24, 1.68, 2.84 folds higher compared to H_2O_2 treated cells respectively.

As shown in Table, induced increases in MDA levels were significantly suppressed by GPP treatment ($P < 0.01$). The data presented that H_2O_2 led to dramatically increase of MDA (1.5-fold) leakage from the cytoplasm into culture medium compared to the control groups. The pretreatment of the cells with different concentrations (50, 100, 200 g/mL) of GPP significantly inhibited the leakage of MDA in culture medium.

4. Conclusions

The free radicals contain unpaired electrons, generated in the redox processes of physiochemical pathways, and there are numerous kinds of them. Excessive free radicals would threaten body in many ways [22], leading to cardiovascular diseases, diabetes, cancer, aging and some other chronic diseases. In general, antioxidants can scavenge free radicals by giving electrons through their own reduction [23]. The fact that the good capacities of scavenging hydroxyl radicals and superoxide anion of GPP conformed in this study, was similar with the result of glycoprotein extracted from oyster is of good ability of scavenging superoxide anion investigated by Huang et al [24]. Compared to research of Li et al. [25], GPP had better ability to scavenging hydroxyl radi cal than glycosaminoglycan extracted from mussel.

When a large number of antioxidant enzymes such as SOD, CAT, GSH, etc., existed in the body, which can scavenge free radicals, it can maintain the balance between the oxidation and antioxidant systems [26]. As the body encountered oxidative stress, the balance between the oxidative and antioxidant systems in the body will be disturbed, resulting in excessive accumulation of oxygen radicals such as ROS in cells. So the production of ROS in cells can directly determine the degree of damage caused by oxidative stress [27]. In this study, GPP could inhibit the production of ROS in H_2O_2-induced cells.

To summarise, the GPP exhibited free radicals scavenging activities. Also the present study suggested that GPP could significantly increase the activity of SOD and GSH, and reduce the level of MDA in the culture medium, and protect the cells against H_2O_2-induced stress to offer vital cytoprotection against the oxidative damage induced by H_2O_2 in L02 cells. These assays indicated that GPP is a significant source of natural antioxidant, which may be helpful in preventing the progress of various oxidative stresses. Therefore, further investigation is needed to research and definite the specific target and function mechanism.

Acknowledgements. In this paper, the research was sponsored by Fujian provincial public basic scientific research, China] under Grant [2014R1003-9]; Major science and technology projects in Fujian province, China] under Grant [2014NZ0001-1]; Research Project of Province-owned Public Scientific Research Institutes in Fujian Province [2015R1003-9]. We would like to thank Professor Yutian Pan (Engineering Technological Center of Mushroom Industry, Minnan Normal University, 363000, Zhangzhou, Fujian Province, China) for his help in extracting and analyzing GPP.

References

1. Chen Hui Yin, Yen Gow Chin, *Food Chem.*, **101**, 686 (2007).
2. U.Grienke, J.Silke, *Food Chem.*, **142**, 48 (2014).

3. H.Zhu, M.Geng, H.Guan, *J.Ocean UnivQindao*, **30**, 463 (2000).
4. P.D.Scotti, S.C.Dearing, D.R.Greenwood, R.D.Newcomb, *Comp Biochem. Physiol. B*, **128**, 767 (2001).
5. M.Charle, S.Chernysh, H.Philippe et al., *J. Biol. Chem.*, **271**, 21808 (1996).
6. B.Hernototh, *Fish Shellfish Immunol.*, **4**, 25 (2003).
7. B.Xu, P.Hagglund, H.Stalbrand, J.Janson, *J. Biotechn.*, **92**, 267 (2001).
8. K.Veena, P.Shanthi, P,Sachdanandam, *Biol. Pharm. Bull.*, **29**, 565 (2006).
9. M.A.Mesaik, N.Dastagir, N.Uddin et al., *J. Agric Food Chem.*, **63**, 177 (2015).
10. C.M.D'avila-Levy, F.M.Araujo, A.B.Vermlho et al., *Microbiol Lett.*, **231**, 171 (2004).
11. S.M.Rafiquzzaman, J.M.Lee, R.Ahmed et al., *Int. J. Food Sci. Tech.*. **50**, 143 (2015).
12. M.Wang, H.Ma, C.Tian et al., *J. Func. Foods*, **35**, 315 (2017).
13. S.J.Liu, Y.T.Pan, Z.Y.Liu et al., *J. Southwest China Normal University (Natural Science Edition)*, **42**, 45 (2017).
14. Yanhong Li, Bo Jiang, Tao Zhang et al., *Food Chem*, **106**, 444 (2008).
15. Y.E.Lihua, SHI Zhe, Liu Huixue et al., *J. Rare Earth*, **29**, 178 (2011).
16. Chao Zhu, Yuchen Dong, Haile Liu et al., *Biomed. Pharmacotherapy*, **88**, 124 (2017).
17. Ce Shi, Xiangrong Chen, Zuojia Liu et al., *Biomed. Pharmacotherapy*, **85**, 740 (2017).
18. J.G.Wu, Y.J.Kan, Y.B.Wu et al., *Pharm. Biol.*, **54**, 919 (2016).
19. A.E.Mohammad, S.Ali, *Food Chem Toxicology*, **48**, 846 (2010).
20. M.Rajesh, S.A.Sreenivas, C.Avijit, *Free Radicals Antioxidants*, **1**, 87 (2011).
21. B.Jamila, T.Helene, D.Mickael et al., *Biomed. Pharmacotherapy*, **89**, 490 (2017).
22. M.Gasperlin, M.Gosenca, *Expert Opin Drug Deliv.*, **8**, 905 (2011).
23. Huang Dejiang, OuBoxin, *Agric Food Chem.*, **53**, 1841 (2005).
24. Huang Lai Zhen, Zhong Min, Hu Xueqiong et al., *J. Guangdong Ocean University*, **31**, 95 (2011).
25. Li Mengjie, Fan Xiuping, Wu Hongmian et al., *Modern Food Sci..Technol.*, **27**, 759 (2011).
26. R.Giardino, G.Giavaresi, M.Fini et al., *Artificial Cells, Blood Substitutes, and Immobilization Biotechnology*, **30**, 189 (2002).
27. S.Kaur, H.P.Singh, D.R.Batish et al., *Biochen. Syst. Ecol*, **44**, 390 (2012).