

A NOVEL BUCKWHEAT PROTEIN WITH A BENEFICIAL EFFECT IN ATHEROSCLEROSIS WAS PURIFIED FROM *FAGOPYRUM TATARICUM* (L.) GAERTN.

XIAOLI ZHOU, LIN HUANG, WEN TANG, YIMING ZHOU, QING WANG and ZONGJIE LI

Shanghai Institute of Technology, Shanghai 201418, China

Abstract - Buckwheat seeds contain many kinds of functional compounds that are of benefit to patients with cardiovascular disease. In this research, a water-soluble buckwheat protein was isolated and purified through a DEAE-Sepharose anion exchange column and Sephadex G-75 gel chromatography. The isolated buckwheat protein fractions exhibited hypocholesterolemic activity in a HepG2 cell model and demonstrated prominent bile acid salt-binding activity in an *in vitro* assay. The antioxidative activity of protein fractions with hypolipidemic effects was detected in a free radical scavenging experiment. The buckwheat protein fraction with the most obvious hypolipidemic activity and free radical scavenging activity was named as WSBWP. Its molecular weight was estimated by SDS-PAGE electrophoresis to be 38 kDa. It could become a potential candidate in the treatment of atherosclerosis.

Key words: Buckwheat protein, atherosclerosis, hypocholesterolemic activity, bile acid salt binding activity, free radical scavenging activity

INTRODUCTION

Buckwheat is a crop that is resistant to adverse growing conditions and pathogens. It contains many kinds of functional compounds, such as proteins, flavonoids, dietary fiber, inositol, phytosterols and other rare components. Buckwheat extractions exhibit beneficial effects in atherosclerosis, cancer, diabetes, hypertension, hyperlipidemia, obesity, and other human disorders (Li and Zhang, 2001).

Atherosclerosis is a cardiovascular disease induced by hyperlipidemia, hypertension, diabetes mellitus, and cigarette smoking (Maggi et al., 1994). High levels of serum LDL provide enough substrates for the lipoperoxidation caused by reactive oxygen species (ROS) such as O_2^- , OH^\bullet , H_2O_2 etc. Oxidative modification of LDL (oxLDL) enhances the binding capacity of macrophages and the damaging of vessel elasticity, adding to the formation of foam cells

and fatty streaks (Murakami et al., 2002; Lin et al., 2008).

Increased cholesterol intake can cause an increase in blood cholesterol level, and lead to the upregulation of LDL and oxLDL (Peng et al., 2009). Therefore, the disruption of cholesterol is essential in retarding the progression of atherosclerosis. The hypocholesterolemic effects of buckwheat protein have already been described in animal experiments, and the functional fraction possessing cholesterol-binding capacity was certified to be an insoluble buckwheat protein (BWP) (Kayashita et al.; 1995; Metzger et al., 2007). The hypocholesterolemic mechanism of BWP was subsequently proved to be associated with the low digestibility of buckwheat protein (Kayashita et al., 1997). Buckwheat has a well-balanced amino acid composition and is rich in arginine, lysine and glycine (Pomeranz, 1983). Hence, these amino acids could also play an impor-

tant role in cholesterol-lowering activity (Katan et al., 1982).

In this work, we examined the beneficial effect of BWP in atherosclerosis. We investigated its cholesterol-lowering capability and antioxidant potential.

MATERIALS AND METHODS

Extraction of buckwheat protein (BWP)

Flour prepared from buckwheat sprouts was suspended in phosphate buffer (pH 6.5, 10mmol/L) and stirred for 2 h at room temperature. The mixture was centrifuged (5000 r/min) for 30 min at 4°C. The supernatant was collected and precipitated by saturated ammonium sulfate (Guo et al., 2007).

Isolation and purification of BWP

Tartary buckwheat protein extract was applied to a DEAE-Sepharose Fast Flow anion exchange column (1.6 cm×50 cm) at a flow rate of 1.0 mL/min, and eluted with NaCl in phosphate buffer (Guo et al., 2007; Wang et al., 2004). The collected fractions were tested for hypolipidemic activity. The active peaks were further purified by Sephadex G-75 column (1.6 cm×50 cm), eluted at a flow rate of 0.4 mL/min with phosphate buffer (pH 6.5, 10 mmol/L).

Effects of BWP on the concentrations of intracellular cholesterol in HepG2 cells

The hypolipidemic activity of BWP was tested by examining the concentrations of intracellular cholesterol in human hepatocellular carcinoma HepG2 cell line. The hypercholesterolemic HepG2 cell model was induced by incubation in a cholesterol-rich medium culture containing 1% BSA and 20µg/mL cholesterol. After culture in a cholesterol-rich medium for 24 h, intracellular cholesterol concentrations were measured to insure the hypercholesterolemic cell model was successfully established. HepG2 cells were treated with different concentrations of BWP and cultured for another 24 h. The cells were washed and collected to detect intracellular concentrations

of cholesterol (Zhang et al., 2008).

In vitro bile acid salt binding activity of BWP

Sodium cholate, sodium deoxycholate and sodium taurocholate were chosen to determine the bile acid salts binding activity of BWP. BWP samples were added to 10 mL of bile salt solution, containing 6 mL of 45% sulfuric acid solution and 1mL of 0.3% furfural solution. After 30 min incubation at 65°C in a water bath, the reaction mixtures were centrifuged at 5000 rpm/min for 20 min. The supernatant was analyzed at 620 nm using an ultraviolet spectrophotometer.

Free radical scavenging activity of BWP

The scavenging of DPPH was done according to the method described by Cotelle et al. (1996). Absorbance at 517 nm of the blank control (A₀) and sample (A_s) were assayed, and the scavenging rate was calculated from the following formula: scavenging rate (%)=(A₀ - A_s)/A₀×100%.

The scavenging activity of hydroxyl (OH•) was carried out as described previously (Beauchamp and Fridovich, 1971; Ma et al., 2010). The emission wavelength of the control (A₀) and the sample (A_s) was determined at 510 nm, and the clearance rate was calculated from the following equation:

$$\text{clearance rate (\%)}=(A_0 - A_s)/A_0 \times 100\%.$$

The scavenging property of the superoxide anion (O²⁻•) was carried out following the modified procedure of Liu (2007). The absorbance of the control (A₀) and the sample (A_s) were detected at 420 nm, and scavenging activity was calculated as follows:

$$\text{scavenging activity (\%)}=(A_0 - A_s)/A_0 \times 100\%.$$

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the

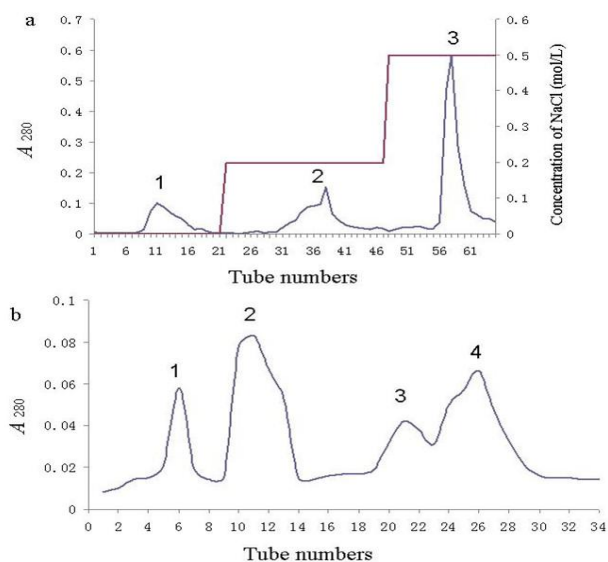


Fig. 1. Isolation and purification of BWP. (a) Buckwheat protein extraction was purified on a DEAE-Sepharose Fast Flow anion exchange column (1.6 cm×50 cm), at a flow rate of 1.0 mL/min. (b) The elution was collected and further purified on a Sephadex G-75 column (1.6 cm×50 cm), eluted at a flow rate of 0.4 mL/min with phosphate buffer (pH6.5, 10 mmol/L).

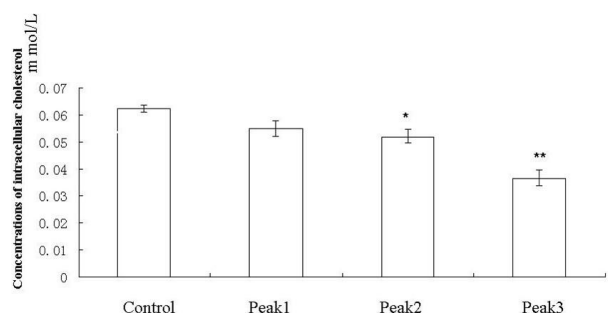


Fig. 2. Hypocholesterolemic activity of the three peaks filtered through DEAE-Sepharose anion exchange column. * indicates $P < 0.05$, ** indicates $P < 0.01$. All data given are the mean of at least three independent experiments.

discontinuous system (12% separating/4% stacking gel). Molecular weights of protein subunits were calculated using the following markers: phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit actin (43.0 kDa), bovine carbonic anhydrase (31.0 kDa), trypsin inhibitor (20.1 kDa), and hen egg white lysozyme (14.4 kDa).

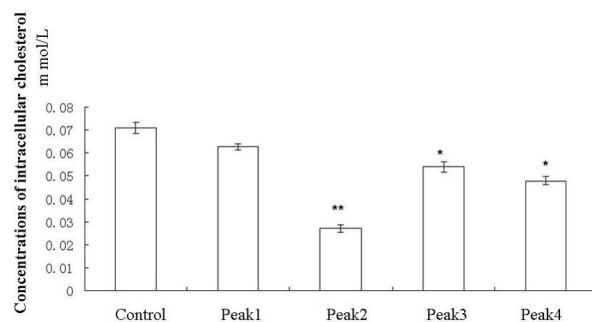


Fig. 3. Hypocholesterolemic activity of four peaks isolated by Sephadex G-75 gel chromatography. * indicates $P < 0.05$, ** indicates $P < 0.01$. All data given are the mean of at least three independent experiments.

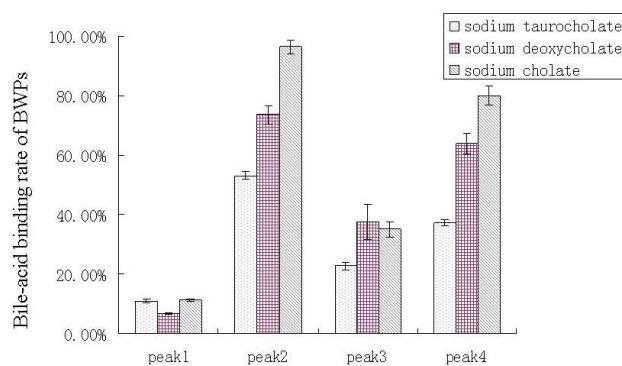


Fig. 4. The bile acid-binding activity of four peaks isolated by Sephadex G-75 gel chromatography. All data given are the mean of at least three independent experiments.

Statistical analysis

Statistical analysis of the data was carried out by SPSS 17.0 statistical software. The level of significance was set at $P < 0.05$.

RESULTS

Tartary buckwheat soluble protein extraction was passed through a DEAE-Sepharose anion exchange filtration column. Three separated peaks were obtained (Fig. 1a). The isolated peaks were examined for hypolipidemic activity. The fraction contained in the third peak exhibited the highest activity. The third peak was further separated on a Sephadex G-75 column (Fig. 1b). Four separated peaks were collect-

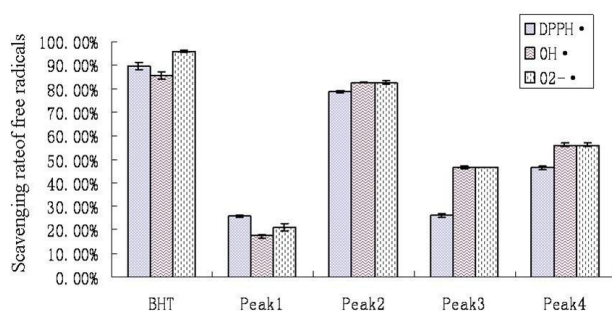


Fig. 5. Free radical scavenging activity of four peaks isolated by Sephadex G-75 gel chromatography. BHT was used as positive control. All data given are the mean of at least three independent experiments.

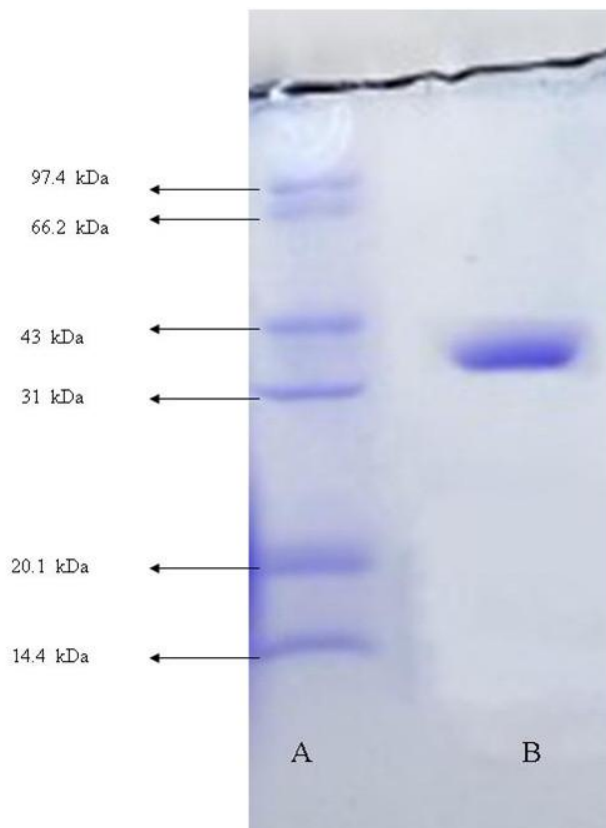


Fig. 6. SDS-PAGE gel electrophoresis. A - molecular weight marker; B - WSBWP

ed. Hypolipidemic activity detection revealed that the fraction contained in peak 2 revealed a stronger hypolipidemic activity than the other peaks.

The contents in peaks 2 and 3 exhibited hypocholesterolemic activity; the contents in peak 3 lowered the level of intracellular cholesterol ($P < 0.01$) the most (Fig. 2). Therefore, fraction 3 was collected, concentrated and further purified on a Sephadex G-75 column and four peaks were obtained. Of the four peaks filtered by the G-75 column, peaks 2, 3 and 4 all exhibited cholesterol-lowering properties (Fig. 3). Peak 2 demonstrated a prominent hypocholesterolemic activity ($P < 0.01$) and was designated as the water-soluble buckwheat protein (WSBWP).

As shown in Fig. 4, the binding rate of sodium cholate by BWPs was much higher than the binding rate of sodium taurocholate and sodium deoxycholate. Moreover, peak 2 filtered from the G-75 chromatography column demonstrated the most obvious bile acid-binding activity.

The antioxidative activity of BWPs was measured by scavenging DPPH•, OH• and O₂•- free radicals. BWPs possessed a free radical scavenging activity (Fig. 5). Comparing the antioxidative activities of the four peaks filtered through the G-75 column, the fraction of peak 2 demonstrated the highest scavenging rate.

The molecular weight of the WSBWP was established by SDS-PAGE electrophoresis to be 38 kDa (Fig. 6).

DISCUSSION

Diets containing buckwheat products lower the risk of developing high cholesterol and high blood pressure, and reduce the progress of atherosclerosis cardiovascular disease. Buckwheat protein has been proved to be one of the dietary factors of benefit to cholesterol metabolism (Kayashita et al., 1995). Moreover, buckwheat protein products regulate plasma cholesterol better than that of soy protein (Tomotake et al., 2000).

However, previous hypolipidemic activity studies on animal models mainly focused on the mixture of the complex buckwheat protein product (BWP), lip-

id, dietary fiber, starch, water (Kayashita et al., 1997). Recently, Zuo et al. (2010) compared the hypolipidemic effects of various tartary buckwheat proteins (including albumin, globulin and glutelin) in hyperlipidemic mice. In this research, buckwheat protein extraction was isolated by a DEAE-Sepharose anion exchange column and Sephadex G-75 gel filtration, and the hypolipidemic activity of separated buckwheat protein extractions was respectively studied by *in vitro* methods.

Data demonstrated that peaks 2, 3 and 4 from the Sephadex G-75 column possessed hypocholesterolemic activity ($P < 0.05$), and peak 2 (named WSBWP) showed the most obvious hypocholesterolemic activity ($P < 0.01$). Since bile acids play an important role in enterohepatic circulation cholesterol metabolism (Chen et al., 2008), the binding activity with sodium taurocholate, sodium deoxycholate and sodium cholate of BWP fractions was respectively detected. Similar to the hypocholesterolemic effects, peak 2 (WSBWP) revealed the most prominent binding activity; in particular, the binding rate of sodium cholate reached 96.4% (Fig. 4).

Hyperlipidemia, hypertension and diabetes mellitus are all risk factors inducing the deterioration of atherosclerosis. Blood cholesterol transported by LDL can be oxidized by reactive oxygen species (ROS) such as O_2^- , OH^\bullet and H_2O_2 . The formation of oxidative LDL (oxLDL) aggravates the emergence of fibrous plaque and atheromatous plaque in artery atherosclerosis. Therefore, the antioxidative activity of BWP fractions were tested for scavenging free radicals (DPPH $^\bullet$, OH^\bullet and O_2^-). According to their hypolipidemic activity, WSBWP exhibited the most obvious scavenging ability.

CONCLUSION

A new hypolipidemic buckwheat protein designated WSBWP was isolated from tartary buckwheat. Its molecular weight was identified by SDS-PAGE electrophoresis to be 38 kDa. Due to its significantly hypocholesterolemic activity, bile acid-binding activity and antioxidative activity, this protein has

the potential to be applied in atherosclerosis therapy. However, its clinical effects need to be studied further.

Acknowledgments - This work was supported by the Chinese National Natural Science Foundation Grant No. 31071527.

REFERENCES

- Beauchamp, C. and I. Fridovich (1971). Superoxide dismutase: improved assays and assay applicable to acrylamide gels. *Anal Biochem.* (44), 276-287.
- Chen, Z.Y., Jiao, R. and K.Y. Ma (2008). Cholesterol-lowering nutraceuticals and functional foods. *J Agric. Food Chem.* 56(19), 8761-73.
- Cotelle, N., Bernier, J., Catteau, J.P., Pommery, J., Wallet, J.C. and E.M. Gaydou (1996). Antioxidant properties of hydroxyflavones. *Free Radic Biol. Med.* 20, 35-43.
- Guo, X., Zhu, K., Zhang, H., and H. Yao (2007). Purification and characterization of the antitumor protein from Chinese tartary buckwheat (*Fagopyrum tataricum* Gaertn.) water-soluble extracts. *J Agric. Food Chem.* 55, 6958-6961.
- Kahlon, T. and C. Woodruff (2002). In vitro binding of bile acids by soy protein, pinto beans, black beans and wheat gluten. *Food Chemistry*, 79, 425-429.
- Katan, M., Vroomen, L. and R. Hermus (1982). Reduction of casein-induced hypercholesterolaemia and atherosclerosis in rabbits and rats by dietary glycine, arginine and alanine. *Atherosclerosis.* 43(2-3):381-391.
- Kayashita, J., Shimaoka, I., Nakajoh, M., Yamazaki, M. and N. Kato (1997). Consumption of buckwheat protein lowers plasma cholesterol and raises fecal neutral sterols in cholesterol-fed rats because of its low digestibility. *J. Nutr.* 127 (7), 1395-1400.
- Kayashita, J., Shimaoka, I. and M. Nakajoh (1995). Hypocholesterolemic effect of buckwheat protein extract in rats fed cholesterol enriched diets. *Nutrition Research.* 15, 691-698.
- Lin, L.Y., Peng, C.C., Yang, Y.L. and R.Y. Peng (2008). Optimization of bioactive compounds in buckwheat sprouts and their effect on blood cholesterol in hamsters. *J Agric. Food Chem.* 56(4), 1216-23.
- Li, S.Q. and Q.H. Zhang (2001). Advances in the development of functional foods from buckwheat. *Crit. Rev. Food Sci. Nutr.* 41, 451-464.
- Maggi, E., Chiesa, R., Melissano, G., Castellano, R. Astore, D., Grossi, A., Finardi, G. and G. Bellomo (1994). LDL oxidation in patients with severe carotid atherosclerosis. A study

- of in vitro and in vivo oxidation markers. *Arteriosclerosis, Thrombosis, and Vascular Biology*. **14**, 1892-1899.
- Ma, Y., Xiong, Y.L., Zhai, J., Zhu, H. and T. Dziubla (2010). Fractionation and evaluation of radical-scavenging peptides from in vitro digests of buckwheat protein. *Food Chem.* **118**(3), 582-588.
- Metzger, B., Barnes, D. and J. Reed (2007). Insoluble fraction of buckwheat (*Fagopyrum esculentum* Moench) protein possessing cholesterol-binding properties that reduce micelle cholesterol solubility and uptake by Caco-2 cells. *J Agric. Food Chem.* **55**(15), 6032-6038.
- Murakami, S., Kondo, Y., Toda, Y., Kitajima, H. and K. Kameo (2002). Effect of taurine on cholesterol metabolism in hamsters: Upregulation of low density lipoprotein (LDL) receptor by taurine. *Life Sci.* **70**, 2355-2366.
- Peng, C.C., Chen, K.C., Yang, Y.L., Lin, L.Y. and R.Y. Peng (2009). Aqua-culture improved buckwheat sprouts with more abundant precious nutrients and hypolipidemic activity. *Int J Food Sci Nutr.* **60** (Suppl 1), 232-245.
- Pomeranz, Y (1983). Buckwheat: structure, composition, and utilization. *Crit Rev Food Sci Nutr.* **19**(3), 213-258.
- Tomotake, H. (2000). A buckwheat protein product suppresses gallstone formation and plasma cholesterol more strongly than soy protein isolate in hamsters. *Nutr.* **130**(7), 1670-1674.
- Zhang, L., Xie, Z., Song, H., Song, K., Ye, S., Gu, X., Zhao, G., and Q. Zhang (2008). Effect of Ginkgo biloba extract (GBE50) on cholesterol metabolism in cultured cells. *Chin Pharmacol Bull.* **24**(3), 313-317.
- Zuo, G.M. and J.H. Wang (2010). Hypolipidemic and antioxidant effects of tartary buckwheat proteins in hyperlipidemic mice. *Food Science* **31**(7), 247-250.