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Insoluble Fraction of Buckwheat (*Fagopyrum esculentum* Moench) Protein Possessing Cholesterol-Binding Properties That Reduce Micelle Cholesterol Solubility and Uptake by Caco-2 Cells

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Buckwheat (*Fagopyrum esculentum* Moench) protein (BWP) exhibits hypocholesterolemic activity in several animal models by increasing fecal excretion of neutral and acidic sterols. In the current study, the ability of BWP to disrupt micelle cholesterol solubility by sequestration of cholesterol was investigated. When BWP (0.2%) was incubated with cholesterol and micelle lipid components prior to micelle formation, cholesterol solubility was reduced 40%. In contrast, cholesterol solubility was not decreased when BWP (0.2%) was incubated after micelle formation and incorporation of soluble cholesterol. Buckwheat flour, from which BWP was derived, had no significant effect on cholesterol solubility. Cholesterol uptake in Caco-2 cells from micelles made in the presence of BWP (0.2%) was reduced by 47, 36, 35, and 33% when compared with buckwheat flour, bovine serum albumin, casein, and gelatin, respectively. Reduction in cholesterol uptake in Caco-2 cells was dose-dependent, with maximum reductions at 0.1−0.4% BWP. In cholesterol-binding experiments, 83% of the cholesterol was associated with an insoluble BWP fraction, indicating strong cholesterol-binding capacity that disrupts solubility and uptake by Caco-2 cells.

KEYWORDS: Buckwheat (*Fagopyrum esculentum* Moench); BWP; micelles; cholesterol; Caco-2 cells

INTRODUCTION

There is growing interest in functional foods to improve health, especially those that support the prevention of cardiovascular disease (*1*). Dietary bioactive proteins are a functional food component that slow the progression of atherosclerosis (*2*−*4*). Benefits of bioactive proteins are evident in soy proteins' effect on serum cholesterol (*5*, *6*). Common buckwheat (*Fagopyrum esculentum* Moench) is gaining popularity as a novel ingredient in functional food formulation (*7*−*10*). Evidence for the benefit of buckwheat protein (BWP) in modulating cardiovascular disease risks by decreasing serum cholesterol and increasing fecal steroid excretion is apparent in several animal models (*11*−*13*). Animal feeding studies with BWP show decreased plasma cholesterol by as much as 31% (*12*−*14*). The most prevalent hypothesis for this lipid-lowering mechanism of BWP is based on its relatively insoluble nature and lipid-binding potential (*11*−*13*, *15*).

The potential importance of dietary interventions in modulating serum cholesterol, a risk factor for atherosclerosis, is realized when one considers the large pool of cholesterol in the lumen of the gut. This pool is under homeostatic control and may be modified to increase excretion, thereby reducing net absorption. The mass of cholesterol present in the gut lumen of humans ranges from 300 to 500 mg of dietary cholesterol, from 800 to 1200 mg of biliary derived cholesterol, and from 250 to 400 mg from intestinal epithelial cell turnover per day (*16*). The solubility of this pool of cholesterol depends on incorporation into micelles for presentation and uptake in the luminal brush border membrane of intestinal epithelium. Disruption of cholesterol incorporation in micelles by decreasing cholesterol solubility, a mechanism of plant phytosterols (*17*−*19*) and some proteins (*20*−*22*), is an effective means of reducing net cholesterol absorption, resulting in improved serum lipids.

To understand the importance of functional foods in the prevention of chronic disease, the mechanisms of action in the body should be elucidated. The serum-modulating effects of BWP on lipid metabolism stem from an interference with sterol absorption. This interference is a result of several factors including decreased digestibility of BWP, cholesterol-binding properties (*11*), and enhanced bile acid excretion (*12*, *13*). The purpose of this study was to understand and quantify the cholesterol-binding potential of BWP that limits cholesterol incorporation into micelles destined for eventual uptake.

MATERIALS AND METHODS

Chemical Reagents. Radiolabeled [1,2-^{3}H(N)]cholesterol (40 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St.

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Louis, MO). Micelle lipid components, delipidated bovine serum albumin (99% fatty acid free), casein, gelatin, β-sitosterol, 5α-cholestane, fatty acid methyl ester mix, isooctane, and other solvents were purchased from Sigma Chemical Co. (St. Louis, MO). Bio-Safe II was obtained from Research Products International (Mt. Prospect, IL). Tri-Sil and boron trifluoride were purchased from Pierce (Rockford, IL) for phytosterol and fatty acid analysis, respectively. Amino acid standards and derivatization reagents *o*-phthalaldehyde (OPA) and fluorenylmethyl chloroformate (FMOC) were purchased from Agilent Technologies (Santa Clara, CA).

Preparation of Buckwheat Protein. A crude BWP was prepared from buckwheat flour (BWF) according to previously published methods with slight modification (*23*). Briefly, 5 g of BWF (Standard Process, Inc., Palmyra, WI) derived from whole seed (*Fagopyrum esculentum* Moench) using a classifier mill (Prater-Sterling, Bolingbrook, IL) was suspended in 40 mL of distilled water. The pH of the suspension was adjusted to 8.0 with 0.01 N NaOH and mixed with a Polytron (Kinematica, Lucerne, Switzerland) mechanical homogenizer for 3 min. The slurry was separated into supernatant and pellet by continuous centrifugation for 20 min at 7500*g*. The supernatant was adjusted to pH 4.5 with 0.01 N HCl to precipitate proteins isoelectrically. The precipitated proteins were centrifuged for 20 min at 7500*g* to recover the protein pellet, followed by a 10 mL wash with distilled water. The washed pellets were freeze-dried (Virtis, Gardiner, NY) to prepare BWP.

Chemical Analysis. Protein content of BWF, BWP, and the insoluble BWP fraction, as described in experiments below, was determined according to the Association of Official Analytical Chemists (*24*) using the Kjeldahl method. Amino acid analysis was performed by an Agilent 1100 HPLC by derivatization with OPA and FMOC followed by ultraviolet and fluorescent detection according to Agilent publication 5980-1193EN. Crude ash was determined by gravimetric analysis in a 550 °C oven. Crude fat was determined by gravimetric analysis after 2:1 chloroform/methanol extraction. Neutral detergent fiber was determined by an Ankom^{200} fiber analyzer (Macedon, NY). Fatty acid and phytosterol analyses were performed by an Agilent 6890 N GC-FID and 5975 mass selective detector on DB-Wax, and HP-5MS capillary columns, respectively (*25*). Determination of the major phenolic rutin was quantified by HPLC as previously described (*26*, *27*).

Cholesterol Solubility in Mixed Micelles. The effects of BWP on cholesterol solubility in mixed micelles was measured according to the method of Ikeda et al. (*28*) with the following modifications. Mixed micelle lipid components [2 mM monoolein, 4 mM oleic acid, 2 mM egg phosphatidylcholine, 0.2 mM cholesterol, and 20 nM [^{3}H]-cholesterol (40 Ci/mmol)] were prepared in 2:1 chloroformm/methanol and dried under nitrogen. BWP or BWF (0.2%) and 6 mM taurocholate were added in phosphate-buffered saline (PBS) and sonicated for 1 h. Following sonication, the micelles were filtered using a Whatman GD/X (0.2 μm) filter, and the eluent containing soluble cholesterol was measured by liquid scintillation counting (LS6500 Beckman Coulter, Fullerton, CA). Nonsoluble cholesterol remains crystalline in nature and adheres to the surface of the hydrophilic filter membrane. Experiments involving post-addition of protein were prepared by adding the protein to the mixed micelles after the formation and incorporation of cholesterol and sonication for an additional 1 h prior to filtration. The results were reported as nanomoles of soluble cholesterol.

Caco-2 Cell Culture Experiments. Colon adenocarcinoma (Caco-2) cells from American Type Culture Collection (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, nonessential amino acids (1%), and 2 mM glutamine. Cells were cultured at 37 °C in a humidified incubator with 5% CO_2. Cells were plated in 24-well plates at a density of 1×10^5 cells/cm^2 for uptake experiments. Cells were used in transport experiments 12−16 days after reaching confluence.

Cholesterol Uptake from Naturally Derived Micelles. Natural micelle solutions for cholesterol uptake experiments were prepared by diluting a pool of pig bile ($n = 4$) derived from a local abattoir with DMEM as previously described (*29*). The pooled bile solutions were analyzed for cholesterol (Thermo Electron, Waltham, MA), bile acids (*30*), and phospholipids (Wako, Richmond, VA). Total protein content of bile was determined by the BCA protein assay (Pierce). The pooled samples were aliquoted and frozen at −80 °C until use.

In experiments with naturally derived micelles, radiolabeled cholesterol (0.1 μCi/mL) was dissolved in 2:1 chloroform/methanol and dried in a glass vial under nitrogen. BWP was added to the vial followed by pig bile diluted with phenol red-free DMEM to achieve a final concentration of 500 μM cholesterol. The sample was sonicated for 1 h prior to the treatment of cells. Prior to uptake experiments, Caco-2 cells were washed three times with serum-free DMEM. Following incubation with micelles for 90 min at 37 °C, the cells were washed three times in PBS containing 1% (w/v) taurocholate to remove nonspecifically bound cholesterol. Cell monolayers were solubilized in radioimmunoprecipitation (RIPA) buffer, and an aliquot was removed and mixed with Bio-Safe II for liquid scintillation counting. Data are expressed as picomoles of cholesterol per milligram of cell protein as determined by the Bradford assay (Bio-Rad, Hercules, CA). Experiments describing the post-addition of protein were performed by first sonicating cholesterol with diluted bile. Protein was added with further sonication for an hour prior to use in cell culture.

Cholesterol Binding of BWP. To determine cholesterol binding, protein or cellulose (0.2%, w/v) was incubated with 10 μM cold cholesterol and 0.01 μCi/mL radiolabeled cholesterol dissolved in PBS to a final ethanol concentration of 2% (v/v). The samples were refrigerated (4 °C) for 30 min and mixed by vortex every 10 min. After 30 min, an aliquot was counted to determine the specific activity (DPM/nmol). Samples were centrifuged for 5 min at 13000 rpm, and an aliquot of the supernatant was counted by liquid scintillation. The percent cholesterol remaining as an insoluble pellet was determined as the difference between total cholesterol and the percent recovered in the supernatant. To determine bound cholesterol, an aliquot of the supernatant was centrifuged through a protein desalting spin column by size exclusion (Pierce) to remove free cholesterol from the flow-through eluent. Bound cholesterol in the flow through was determined by liquid scintillation. Results were reported as percent cholesterol recovered in nanomoles.

Statistical Analysis. Data from each assay are means ± standard error of the mean (SEM). The statistical difference between treatments was determined by analysis in GraphPad Prism (San Diego, CA) by one-way analysis of variance (ANOVA) and Bonferroni's multiple-comparison test with $p < 0.05$ considered to be significant. Statistical differences between amino acids in BWP and the insoluble BWP were determined by Student's *t* test.

RESULTS

Characterization of BWP. Isolation of crude BWP is a relatively simple method based upon acid precipitation. The percent recovery of BWP from whole flour derived from buckwheat seed is relatively low, ∼2−3% (w/w) of the starting mass. Buckwheat protein isolated through the precipitation method was enriched approximately 4-fold from 14% in the BWF to 64% in the BWP (**Table 1**). Other measured BWP constituents include a crude lipid fraction of approximately 15%, ash 13%, non-fiber carbohydrate 5%, fiber 3%, and water 1%. Amino acid analysis results were comparable with previously determined values (*14*, *15*). Fatty acid analysis revealed a similar profile for both flour and BWP with oleic (C18:1), palmitic (C16:0), and stearic acid (C18:0) comprising the predominant fatty acids. Rutin and β-sitosterol, the main flavonoid and phytosterol, were significantly less in the BWP (707 vs 299 and 489 vs 39 μg/g, respectively).

The insoluble fraction of BWP was found to have a protein composition similar to that of crude BWP with the exception of the higher proportion of cystine (34.5 vs 27.3 mg/g of N, $p < 0.01$) less crude fat, and fiber (w/w). The insoluble fraction of BWP also contained less rutin (299 vs 125 ppm) and higher amounts of β-sitosterol (39 vs 125 ppm) (**Table 1**).

Table 1. Chemical Characterization of BWF, BWP, and Insoluble BWP

	BWF	BWP	BWP insoluble
		% (w/w)	
protein[a]	13.7 ± 1.2	63.5 ± 1.0	62.9 ± 0.6
fat	3.5 ± 0.1	15.4 ± 0.2	11.5 ± 0.2
NDF[b]	24.6 ± 4.2	3.4 ± 0.7	0.8 ± 0.2
ash	4.4 ± 2.2	12.7 ± 2.1	10.0 ± 1.3
NFC[c]	53.8	5.0	14.8
rutin (μg/g)	707.1 ± 18.6	299.3 ± 36.8	124.5 ± 28.6
β-sitosterol (μg/g)	488.5 ± 50.7	38.7 ± 0.6	124.9 ± 27.5
		mg/g of N	
amino acid analysis[d]			
Asp	1.2	30.8	30.5
Glu	2.7	69.3	58.5
Ser	0.8	19.1	16.8
His	0.4	8.8	8.1
Arg	1.3	39.6	33.2
Ala	0.7	14.9	14.6
Tyr	0.2	10.4	10.3
Cy2	1.5	27.3	34.5[e]
Pro	0.1	3.8	4.0
Gly	0.9	20.7	16.9
Thr	0.5	11.7	12.3
Val	0.9	26.5	25.2
Met	0.0	6.6	6.0
Phe	0.7	17.5	18.7
Ile	0.4	13.7	14.3
Leu	1.2	31.5	30.0
Lys	1.0	22.8	18.8
total	14.4	375.1	352.5

[a] Protein (N × 6.25). [b] NDF, neutral detergent fiber. [c] NFC, non fiber carbohydrate (NFC = 100 − crude protein − crude fat − NDF − ash). [d] Values are means of triplicate analyses. [e] Statistical significance from BWP ($p < 0.01$ by Student's t test).

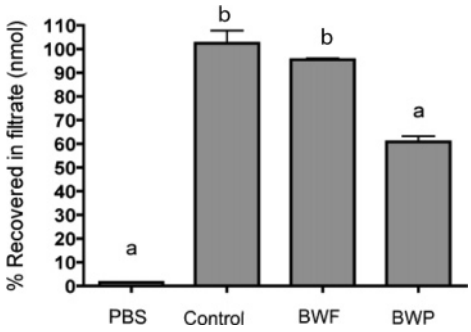

Figure 1. Micelle cholesterol solubility measured in PBS or mixed micelles. Mixed micelles prepared in the presence of BWP indicated reduced cholesterol solubility. Data are expressed as the percent recovery of cholesterol in nanomoles as the mean ± standard error ($n = 3$). Treatments consist of control (no protein), BWF (0.2%), and BWP (0.2%). Letters above the bars (a, b) indicate significant difference ($p < 0.001$) from control (no protein) and BWF, as determined by ANOVA, Bonferroni's multiple-comparison test.

Cholesterol Solubility in Micelles Is Decreased by Pretreatment with BWP. The solubility of cholesterol was determined after micelles had been made in the presence of BWP. The filtered eluent is indicative of soluble cholesterol, which is 100% soluble in lipid micelles prepared in the absence of protein, as indicated by the control treatment (**Figure 1**). The solubility of cholesterol in PBS without micelle lipid components was poor (<2%) due to the insolubility of cholesterol in PBS. The addition of BWF was not significantly different from control, whereas BWP significantly reduced cholesterol solubility by approximately 40% as compared to control ($p < 0.001$).

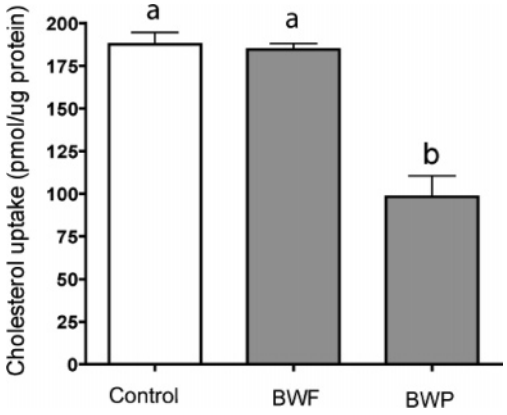

Figure 2. Determination of cholesterol uptake in Caco-2 cells with control (no protein) and pretreatment of micelles with BWF and BWP, 0.2% (w/v). Data are reported as picomoles of cholesterol per microgram of protein and plotted as mean ± SEM ($n = 4$). Statistical analysis was performed by ANOVA, Bonferroni's multiple-comparison test. Letters above the bars (a, b) indicate significant difference from control and BWF ($p < 0.001$).

The solubility of cholesterol in micelles was determined by the post-addition of BWP. The post-addition of BWP negated the decreased cholesterol solubility seen previously. Loss of BWP activity by post-addition at concentrations of 0.05, 0.1, and 0.4% was not significantly different from the control. The highest concentration tested (0.4%), which showed no significant effect, was 2-fold higher than the effective concentration used in **Figure 1** to decrease micelle cholesterol. The average percent recovery of cholesterol in the assay was 94.1 ± 6.7%.

Cholesterol Uptake from Naturally Derived Micelles Made in the Presence of BWP Is Decreased in Caco-2 Cells. In experiments with Caco-2 cells, cholesterol uptake was highly dependent upon micelle cholesterol solubility. Mixed micelles were prepared over a concentration gradient of 0−10 mM taurocholate, an essential component of micelle formation and cholesterol solubility (*31*). An increase in cholesterol uptake throughout the concentration gradient of taurocholate was indicative of a 10-fold change (data not shown). In experiments with naturally derived micelles, cholesterol uptake was as much as 10 times the amount of artificially prepared mixed micelles (data not shown). Addition of BWF (0.2%) had no effect on cholesterol uptake, whereas BWP significantly reduced uptake by 55.5 ± 3.9 and 53.2 ± 3.4% compared to control (no protein) and BWF, respectively (**Figure 2**).

BWP Is More Effective than BSA, Gelatin, and Casein in Decreasing Cholesterol Uptake in Caco-2 Cells. Cholesterol uptake from micelles made in the presence of BWP and other proteins including BSA, gelatin, and casein was determined (**Figure 3**). Protein was added at a final concentration of 0.2% (w/v), and naturally derived micelles were formed. BWP decreased cholesterol uptake by 32.0 ± 5.3% ($p < 0.001$) when compared to BSA, casein, and gelatin. No difference was found between BSA, gelatin, and casein treatments.

Cholesterol Uptake from Micelles Made in the Presence of BWP (0−0.4%) in Caco-2 Cells Is Dose Responsive. In a gradient experiment, Caco-2 cells were incubated with micelles made in the presence of increasing amounts of protein (0−0.4%, w/v). The concentration of BWP was inversely proportional to cholesterol uptake (**Figure 4**). A maximal decrease in cholesterol uptake occurred at 0.1% and persisted throughout the range tested. Further experiments with BWP in Caco-2 experiments were routinely performed at 0.2%.

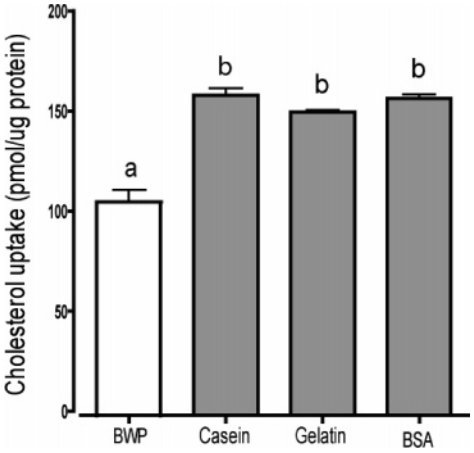

Figure 3. Cholesterol uptake in Caco-2 cells from natural micelles made in the presence of proteins including bovine serum albumin (BSA), casein, and gelatin. Treatments were performed at 0.2% (w/v), plotted as mean ± standard error ($n = 4$). Statistical analysis was performed by ANOVA, Bonferroni's multiple-comparison test. Letters (a, b) indicate significant difference from BSA, casein, and gelatin ($p < 0.001$).

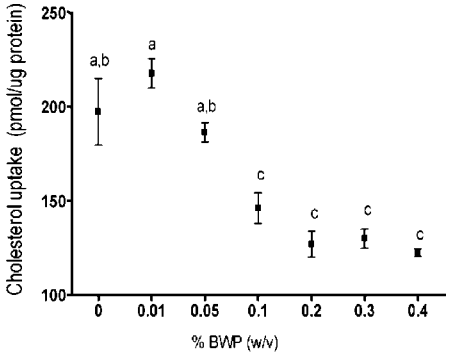

Figure 4. Cholesterol uptake in Caco-2 cells performed over a BWP gradient (0–0.4%, $n = 3$). A maximal decrease in cholesterol uptake occurred over the range of 0.1–0.4% (w/v). Statistical analysis was performed by ANOVA, Bonferroni's multiple-comparison test. Letter designations indicate significance at the $p < 0.05$ level.

Micelles Made by the Post-addition of BWP Failed To Decrease Cholesterol Uptake in Caco-2 Cells. Similar to findings in cholesterol solubility experiments, there were differential effects of pre- and post-addition of BWP in cell culture uptake studies. In experiments with BWP added after the incorporation of cholesterol into micelles, BWP failed to reduce cholesterol uptake by Caco-2 cells. The presence of BWP (0.2%) added prior to micelle formation in naturally derived micelles decreased cholesterol uptake by 25% compared to control (BSA) and 32% in comparison with the post-addition of BWP ($p < 0.001$). The BSA treatment and the post-addition of BWP were not significantly different (**Figure 5**).

Cholesterol-Binding Potential by BWP Is Largely Due to an Insoluble Fraction. The cholesterol-binding potential of various proteins including casein, BSA, and BWP was compared to control (no protein) and the insoluble polysaccharide cellulose. BWP in solution is not entirely soluble, leaving a large percent of insoluble BWP, which pellets after centrifugation. Cellulose was used to model the maximum binding potential that may occur due to mass trapping of cholesterol by insoluble material. The difference between the mass of cholesterol in the supernatant and the total amount added was expressed as the cholesterol in the pellet. A statistically significant portion of cholesterol of approximately (83.4 ± 5.7%, $p < 0.001$) was

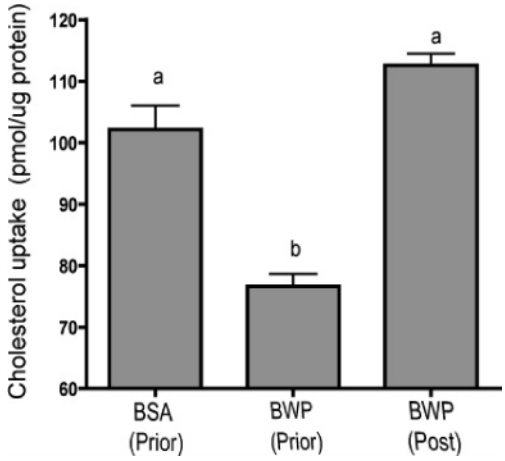

Figure 5. Cholesterol uptake in Caco-2 cells determined after the preparation of micelles in the presence of BWP both pre- and post-micelle formation (see Materials and Methods). Treatments were performed at 0.2% (w/v) ($n = 4$). Statistical analysis was performed by ANOVA, Bonferroni's multiple-comparison test. Letters above the bars (a, b) indicate statistical difference from BSA and BWP post ($p < 0.001$).

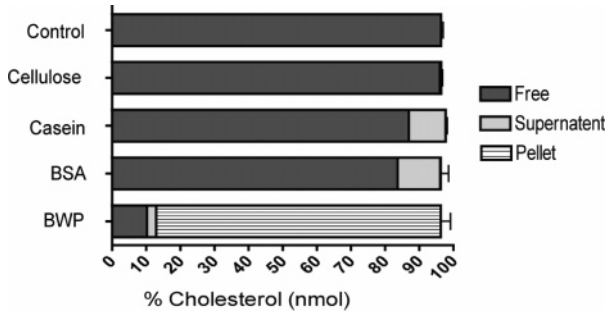

Figure 6. Cholesterol binding capacity in the absence (control) or presence of various proteins (0.2%) by incubation with 10 μM cholesterol, 0.01 μCi/mL [^{3}H]cholesterol at 4 °C in PBS with 2% (v/v) ethanol for 30 min. Cholesterol associated with the pellet was determined as the difference between the total cholesterol added and the amount remaining in the supernatant after centrifugation. Protein-bound cholesterol in the supernatant was determined after elution through a desalting column to remove free cholesterol. Free cholesterol was determined as the difference between the total cholesterol and the amount eluted from the desalting column. Values are reported as the percent nanomoles recovered ($n = 4$).

associated with the BWP pellet, whereas no detectable cholesterol was associated with BSA, casein, or cellulose (**Figure 6**).

The determination of protein-bound cholesterol in the supernatant was accessed after a desalting column procedure to remove free cholesterol. An aliquot of the supernatant after centrifugation was desalted over a centrifuge spin column. Protein-bound cholesterol eluted first, leaving free cholesterol in the spin column resin. A relatively small amount of bound cholesterol was found in the supernatant of BWP (2.6 ± 1.7%) as compared with BSA (12.5 ± 0.5%) and casein (10.7 ± 0.9%). There was essentially no bound cholesterol in the supernatant of either control (no protein) or cellulose (**Figure 6**).

DISCUSSION

The discovery of novel food ingredients capable of modifying risk factors for cardiovascular disease is important in providing steps toward prevention. Functional food ingredients such as phytosterols provide clinically relevant reductions, that is, 20−25% in serum lipids (*32*). Mechanistically, one of the ways phytosterols work is by reducing the solubility of cholesterol

in micelles (*33*), thereby reducing net cholesterol absorption. Here for the first time evidence shows that the cholesterol-binding properties of a crude buckwheat protein fraction prevented cholesterol solubility in micelles, limiting net cholesterol uptake.

Previous animal experiments indicated that the serum lipid modulating activity of BWP was dependent on crude BWP as compared to protein composed of either high or low molecular weight fractions. Fractions composed of a protein digest or a BWP lipid extract were also less bioactive than the crude BWP (*11*). Hence, efforts in this study were focused on crude BWP.

In the preparation of BWP, the percent recovery was low, ~2%. Recent reports of a high-protein BWF with similar hypocholesterolemic activity had a slightly higher yield (5%) (*14*). The final protein composition of the current extract (63%) was similar to that of previously published reports of 65% protein (*15*). Protein composition was enriched by >4-fold by alkali extraction. Subsequent extractions of the flour may improve yields. The in vitro analytical techniques used in the current experiment allow for quick determination of any bioactivity that may remain after subsequent extractions to improve protein yield.

Dietary protein sources reduce cholesterol solubility in micelles. Results from the cholesterol solubility assay demonstrated a reduction in cholesterol that was specific to BWP as opposed to flour. Reductions in cholesterol micelle solubility of 40% were greater than reductions seen with other reported protein sources such as casein (5%), ovomucin (24%), and spirulina concentrate (30%). Furthermore, the effective concentration of protein was lower (0.2% w/v) than that from other reported protein sources (0.5%) capable of reducing micelle cholesterol solubility (*21*, *22*).

The effectiveness of BWP in reducing the solubility of cholesterol in micelles was negated when BWP was added after the formation of micelles. This was attributed to an interaction of the BWP with cholesterol prior to incorporation into the micelle. This interaction was preventative in forming micelles composed of soluble cholesterol and would suggest that BWP forms a complex with cholesterol that is unable to be incorporated into the micelle. Due to the insoluble nature of BWP in an aqueous media, even after sonication, it is hypothesized that BWP has a net overall hydrophobic charge. This hydrophobic charge is likely to assimilate with other nonpolar molecules such as cholesterol. Recent experiments in our laboratory were performed that indicated no significant interaction of BWP with a more polar micelle component, the bile acid taurocholate. However, due to in vivo evidence of increased fecal acidic steroids BWP may have some affinity for other bile acids not tested in our experiments. Determinations of BWP solubility indicate <60% over the pH range of 2−10 (*15*), similar to current observations. The demonstrated nonpolar and insoluble nature of BWP makes its physical presence in the lumen of the gut advantageous in associating with nonpolar cholesterol and preventing absorption due to insolubility.

Caco-2 cell experiments were supportive of the mechanism suggested by the micelle cholesterol solubility experiments. In the progression of events during the digestion process, cholesterol is present from dietary, biliary, and endogenous sources. Dietary sources of cholesterol are present in an emulsion state with triglycerides and phospholipids, in the mixed milieu of macronutrients in the diet. This milieu, otherwise known as chyme, is further mixed with biliary cholesterol, which is only sparingly soluble in bile salt micelles (*34*). Absorption of the combined pool of dietary and biliary cholesterol is dependent on digestion of the lipid emulsion by pancreatic lipases prior to incorporation into micelles (*35*). Without incorporation of cholesterol into lipid micelles, cholesterol cannot be absorbed (*36*, *37*). BWP is known to have low digestibility and to be capable of increasing fecal dry weight output (*11*). This makes the presence of BWP in the mix of dietary and biliary cholesterol important in the physiologic design of the above experiments. We incubated BWP, representative of a dietary protein source, with free cholesterol likely to originate from the diet or biliary secretions. In a physiologic scenario, this interaction occurs prior to micelle formation and entry into the lumen of the gut. In the less physiologic situation of incubating BWP with micelles already preloaded with cholesterol, there was no reduction in cholesterol uptake.

In cell culture experiments performed, all proteins tested had some effect on cholesterol uptake as compared to treatments with no protein. Average reductions in cholesterol uptake due to other protein sources such as BSA, casein, and gelatin were 20% when compared to BWP reduction of 44%. BSA and casein were shown to have an affinity for cholesterol in the soluble protein fraction. Due to this affinity, cholesterol−protein conjugates may be unable to incorporate into micelles. Alternatively, more soluble proteins may preferentially incorporate into micelles and limit cholesterol solubility. A noticeable difference in the proteins tested was the degree of solubility. BSA was entirely soluble, whereas casein and gelatin were only slightly more soluble than BWP in micelle solutions. In addition, it was the insoluble fraction of BWP that had the greatest cholesterol-binding ability. BWP contains several proteins with molecular mass ranges between casein (~23 kDa) and BSA (~66 kDa), and on a per weight basis in experiments (0.2%) BWP represented less total protein due to a crude protein composition of only 58%.

On a per weight basis the concentration of β-sitosterol in the insoluble BWP fraction was greater than the crude BWP. Phytosterols are mechanistically capable of displacing cholesterol from micelles and may also increase cholesterol ABC efflux transporters (*38*), both means of decreasing net cholesterol absorption. However, BWF had a much greater concentration of β-sitosterol and correspondingly less activity, indicating the phytosterols are not solely responsible. This observation is in agreement with other findings of loss of biological activity with a BWP lipid fraction (*11*). In addition, the increase in the amino acid cystine, an oxidation product of cysteine, is interesting because cysteine has been described to play a role in cholesterol-binding domains of both the low-density lipoprotein (LDL) receptor family (*39*) and the cholesterol transporter Niemann−Pick protein NPC2 (*40*). Further analysis is needed to determine if cysteine might play a role in BWP's cholesterol-binding abilities.

Findings from this study demonstrate a novel mechanism for the hypocholesterolemic effect of BWP. The cholesterol-binding properties of a BWP are likely to offer practical usage in functional foods intended for improving cardiovascular risk factors. Future experiments with BWP should focus on kinetic parameters of cholesterol binding as well as competition with other micelle lipid components in an effort to further explore mechanistic questions in reducing micelle cholesterol solubility.

ABBREVIATIONS USED

BWP, buckwheat protein; BWF, buckwheat flour; BSA, bovine serum albumin; Caco-2, colon adenocarcinoma; DMEM, Dulbecco's Modified Eagle's Medium.

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