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Antioxidant and Bile Acid Binding Activity of Buckwheat Protein in Vitro Digests

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The objective of the study was to assess the antioxidant and bile acid removing potential of buckwheat protein (BWP) during a two-stage in vitro digestion (1 h of pepsin followed by 2 h of pancreatin). Antioxidant activity of the digests was analyzed by determining: (1) Fe^{2+} chelation, (2) reducing power, (3) 2,2′-azinobis (3-ethylbenzothiszoline-6-sulfonic acid) ($ABTS^{+\bullet}$) radical scavenging capacity, and (4) TBARS formation in a liposome system. The initial pepsin digestion decreased the BWP antioxidant activity; however, subsequent pancreatin digestion fully recovered the reducing power and increased ($P < 0.05$) the ability to chelate Fe^{2+} (45%), scavenge $ABTS^{+\bullet}$ (87%), and curtail lipid peroxidation (45%) when compared with intact BWP. The final BWP digest exhibited a 67% increase ($P < 0.05$) in cholic acid binding capability over that of the nondigested BWP control but was comparable to the control in binding chenodeoxycholic and deoxycholic acids. Digestion-resistant peptides were largely responsible for bile acid elimination.

KEYWORDS: Buckwheat protein; in vitro digestion; antioxidant; bile acid binding

INTRODUCTION

Buckwheat protein (BWP) has attracted much attention due to its purported health benefits, notably reducing the serum cholesterol level (*1*), suppressing gallstone and tumors (*2*, *3*), and inhibiting angiotensin I-converting enzyme (*4*). The cholesterol-lowering effect associated with buckwheat consumption is attributed to bile acid binding. Bile acids are biological products synthesized from cholesterol and account for the majority of cholesterol utilization in the human body. Thus, depletion of bile acids from its circulation would promote cholesterol conversion into additional bile acids, thereby leading to significant reductions of liver and serum LDL cholesterol levels (*5*). Moreover, secondary bile acids are bacterial derivatives of bile acids occurring in the lumen of large bowel and contribute to carcinogenesis of colorectal cancer (*6*). Binding bile acids would promote the excretion of carcinogenic secondary bile acids and neutralize their toxicity by preventing their active site from interacting with the colonic epithelium.

Despite the evidence of health-promoting effects from animal studies, and the general belief that the bioactivities are imparted by the specific peptides, the release of such active peptides from BWP during the course of food digestion is not well understood. On the basis of soy protein digestion studies, certain soy polypeptides that resisted gastrointestinal (GI) proteases that could bind bile acids and increase their excretion (*7*, *8*) appeared to be responsible for the hypocholesterolic effect of BWP. Compared to soy protein, buckwheat protein exhibited greater potential in promoting bile acid excretion and lowering the plasma cholesterol level in experimental rats (*2*). However, there has not been research to determine the bile acid binding activity of in vitro digests and the specific peptides from BWP.

Furthermore, as with the hydrolytical products of most other dietary proteins, BWP digests are expected to contain antioxidant activity to further contribute to its bioactivity in the GI and possibly even in the cell if absorbed. Many enzyme-hydrolyzed proteins (peptides) are capable of scavenging radicals and, therefore, inhibit lipid oxidation in food systems as well as in the GI tract. Reported proteins that are sources of such antioxidant peptides include soy protein (*9*), whey protein (*10*, *11*), casein (*12*), corn protein (*13*, *14*), and potato protein (*15*, *16*). As free radical-mediated lipid peroxidation is a significant cause of gastric mucosal injury and gastric ulcers (*17*), an elucidation of the antioxidant activity of BWP GI digests would assist with the development of dietary strategies to minimize oxidative stress.

The main purpose of this study was to understand the dynamic bioactivity changes of BWP as it moves through the upper digestive tract. The specific objectives were to elucidate the antioxidant activity of BWP in vitro digests and to determine their bile acid binding activity. Digests of BWP obtained from different stages of in vitro pepsin−pancreatin sequential digestion were characterized and evaluated for their specific activities.

MATERIALS AND METHODS

Extraction of Buckwheat Protein (BWP). Low-fat buckwheat flour was purchased from Bulkfoods.com (Toledo, OH). The product specification sheet from the supplier indicated 3.6% fat, 71.4% total carbohydrate, and 25% protein. Before protein extraction, the flour was stirred with hexane (1:1 w/v,

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four changes) for 48 h to remove residual fat. After vacuum evaporation of residual hexane, the dried, defatted flour powder was subjected to the process of protein extraction according to the method of Tomotake (*18*) with some modifications. Defatted buckwheat flour (1 kg) was manually dispersed into 10 L of deionized water, and the pH was adjusted to 8.0 using 1 N NaOH. After stirring with a propeller (~50 rpm) at 4 °C for 2 h, the suspension was centrifuged at 5000*g* for 20 min. The supernatant (protein extract) was decanted and adjusted to pH 4.5 using 1 N HCl to isoelectrically precipitate protein. The protein precipitate was washed twice with deionized water, neutralized with 0.1 N NaOH, and lyophilized using a Dura-Top MP freeze-dryer (FTS Systems Inc., Stone Ride, NY). The freeze-dried BWP powder contained 81.5% protein, as determined by the Vario Max CN nitrogen analyzer (Elementar Analysensysteme GmbH, Hanau, Germany), and less than 0.5% lipid, as measured by the Soxhlet method using petroleum ether as solvent (*19*). The BWP powder was placed in Ziplock bags and stored at −20 °C before use.

Preparation of Protein Digests. BWP in vitro digests were prepared according to the method of Lo and Li-Chan (*20*). The suspension of BWP (5%, w/v) in double deionized water was adjusted pH 2.0 with 1 N HCl, followed by the addition of pepsin (4%, w/w, protein basis) (Sigma-Aldrich, Inc., St. Louis, MO). The mixture was incubated for 1 h in a shaking water bath at 37 °C to allow pepsin digestion. Subsequently, the pH was adjusted to 5.3 using 0.9 M $NaHCO_3$. After the addition of pancreatin (4% w/w, protein basis) (Sigma-Aldrich, Inc., St. Louis, MO), the pH was adjusted to 7.5 with 1 N NaOH. Digestion was continued in the 37 °C shaking water bath for another 2 h. Aliquots of hydrolysates were removed at 0, 30, 60, 90, 120, and 180 min during the pepsin → pancreatin sequential digestion, adjusted to neutrality (pH 7.0) with 1 N NaOH/HCl, and heated in a 96 °C water bath for 5 min to inactivate the enzymes. Each aliquot was freeze-dried, transferred to Ziplock bags, and stored at −20 °C before use. The protein content of freeze-dried digests was measured using a Vario Max CN nitrogen analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

Degree of Hydrolysis (DH). The DH was determined by the 2,4,6-trinitrobenzenesulfonic acid (TNBS; Sigma-Aldrich, Inc., St. Louis, MO) method of Lo and Li-Chan (*20*). Aliquots of 1-mL BWP hydrolysate samples removed at 0, 30, 60, 90, 120, and 180 min were mixed with 1 mL of 24% trichloroacetic acid. Each solution was centrifuged at 8000*g* for 10 min, and 0.2 mL of the supernatant was mixed with 2 mL of 0.05 M sodium borate buffer (pH 9.2). After the addition of 1 mL of 4 mM TNBS, the solution was kept in a 25 °C water bath with a lid (to prevent light) for 30 min. Subsequently, 1 mL of 2 M sodium phosphate (NaH_2PO_4) containing 18 mM sodium sulfite (Na_2SO_3) (mixture pH 4.4) was added to terminate the reaction. The absorbance was read at 420 nm, and the amino acid concentration (*h*) was determined from the leucine standard curve. The DH was calculated according to the following equation: DH (%) = $(h_s/h_t) \times 100\%$, where h_t represents the total free amino concentration, which was measured by complete hydrolysis of BWP with 6 N HCl at 110 °C for 22 h, and h_s represents free amino concentration of hydrolyzed BWP samples.

Protein Solubility. Protein solubility was determined by the method of Kong and Xiong (*13*) with some modifications. Freeze-dried BWP hydrolysate powders were suspended (~6 mg/mL) in 0.01 M Na_2HPO_3 (pH 7.0) by continuous stirring for 1 h at room temperature. After centrifuging at 8000*g* for 10 min, the supernatant was collected. Protein concentrations in the supernatant and in the original suspension were measured using the Biuret method (*21*). Protein solubility was expressed as the percentage of supernatant protein concentration divided by that in the original suspension, then multiplying by 100.

Electrophoresis. Intact BWP and its digests were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (*13*). Protein samples were dissolved in 0.01 M Na_2HPO_3 (pH 7.0) to make 1 mg/mL protein solutions and mixed (1:1, v/v) with the SDS−PAGE sample buffer (4% SDS, 20% glycerol, and 0.125 M Tris-HCl, pH 6.8) with or without 10% β-mercaptoethanol (βME). The mixed solution was boiled at 100 °C for 3 min and then centrifuged at 1800*g* for 10 min to remove insoluble particles. Prepared samples (0.5 μg/μL protein) were loaded on a 3% polyacrylamide stacking gel (20 μL/well) and then separated in a 15% polyacrylamide resolving gel together with a protein molecular weight (MW) marker (Bio-Rad laboratories, Hercules, CA). The MWs of sample protein bands were estimated from the regression line built in the plot with protein migration distance (R_f) as the *x*-axis and log MW as the *y*-axis.

Metal Ion Chelation. The ability of BWP digests to chelate transition metal ion Fe^{2+} was determined according to Wu et al. (*22*). Sample solutions (1 mL) were mixed with 0.1 mL of 2 mM $FeCl_2$ (final concentration in assay solution, 0.04 mM) and 3.7 mL of deionized water by vortexing. After 3 min, the reaction was stopped by 0.2 mL of 5 mM ferrozine (Sigma-Aldrich, Inc., St. Louis, MO), and the absorbance of the solution was measured at 562 nm. The chelation ability of each solution was calculated by the following equation: chelation ability (%) = $[(\text{blank } A_{562} - \text{sample } A_{562})/\text{blank } A_{562}] \times 100\%$.

Reducing Power. The reducing power of BWP digests was measured according to the method of Li et al. (*23*). Digest sample solutions (2 mL) were mixed with an equal volume (2 mL) of 0.2 M phosphate buffer (pH 6.6) and 2 mL of 1% potassium ferricyanide. The mixtures were incubated in a 50 °C water bath for 20 min, after which 2 mL of 10% trichloroacetic acid was added, followed by centrifugation at 6500*g* for 10 min. An aliquot of 2 mL of the supernatant was diluted with 2 mL of deionized water and then mixed with 0.4 mL of 0.1% $FeCl_3$ (final concentration in assay solution, 0.0091% or 1.6 mM). After 10 min of reaction, the absorbance at 700 nm was determined. An increased color density reflected an increased accumulation of Fe^{2+} and, therefore, indicated a stronger ferric reducing power.

Radical Scavenging Activity (RSA). ABTS radical scavenging ability was determined by the decolorization assay (*24*). $ABTS^{+\bullet}$ was generated by a mixed solution of 7 mM ABTS (Sigma-Aldrich, Inc., St. Louis, MO) and 2.45 mM potassium persulfate. After 12−16 h reaction, a dense green-blue colored solution with excessive accumulation of $ABTS^{+\bullet}$ was diluted with 0.2 M phosphate buffer (pH 7.4) to the absorbance level of 0.7 ± 0.02 at 734 nm. The RSA of protein samples was then determined by adding 10 μL samples into 990 μL of diluted $ABTS^{+\bullet}$ solution. The absorbance of the 1 mL mixture solution was recorded at 1, 2, 5, and 10 min during the reaction. The extent of decolorization represented the magnitude of scavenging ability and was calculated from a standard curve obtained from Trolox standards with concentrations of 50, 100, 250, 500, and 1000 μM. Trolox equivalent antioxidant capacity (TEAC, μM) was used to express RSA.

Inhibition of Lipid Peroxidation. Liposomes were prepared from soybean phosphatidylcholine (Sigma-Aldrich, Inc., St. Louis, MO) by sonicating phosphatidylcholine (0.2 mg/mL) in 0.12 M KCl and 5 mM histidine buffer (pH 6.8) in a 4 °C walk-in cooler for 45 min with a Model 300 Branson SonifierSonic Dismembrator (Fisher Scientific, Fair Lawn, NJ). To evaluate the antioxidant activity of BWP hydrolysates, 1 mL of samples (2 mg/mL) was mixed with 5 mL of liposome suspension. Lipid peroxidation was initiated by adding 0.1 mL of 50 mM $FeCl_3$ and 0.1 mL of 10 mM ascorbic acid. The mixture was incubated in a 37 °C water bath for 2 h. Deionized water (1 mL) or 0.01% BHA (1 mL) in place of BWP sample solutions were used as controls.

The degree of lipid peroxidation and its inhibition was determined by the thiobarbituric acid-reactive substances (TBARS) method as outlined by Wang and Xiong (*15*). TBARS values were expressed as: TBARS (mg/L) = $(A_{532}/V_s) \times 9.48$,

where A_{532} is the absorbance (532 nm) of the assay solution, V_s is the volume of liposome oxidation sample solution (1 mL), and 9.48 is a constant derived from the dilution factor and the molar extinction coefficient (152,000 M^{-1} cm^{-1}) of the red, TBA reaction product.

Bile Acid Binding. Freeze-dried buckwheat protein powders (0, 60, 120, and 180 min in vitro digestion) were dissolved in 0.1 M sodium phosphate buffer (pH 6.3), which simulated the physiological pH of the duodenum. The soluble part of each solution was collected through centrifugation at 8000*g* for 10 min. Both soluble supernatants and the whole protein suspensions (mixture) were subjected to the bile acid binding assay according to the method of Yoshie-Stark and Wasche (*25*) with slight modifications. After adjusting the protein concentration to 2 mg/mL in phosphate buffer (pH 6.3), 0.5 mL of protein solutions was individually mixed with 4.5 mL of 2 mM bile acid solution to obtain a bile concentration within the physiological range of 1.5−7 mM. Mixed solutions were incubated at 37 °C in a shaking water bath for 2 h, then centrifuged at 26890*g* for 10 min, and supernatants collected. Sediments were mixed into 5 mL of 0.1 M sodium phosphate buffer (pH 6.3), mixed well with a vortex, and centrifuged. The combined supernatants from the two centrifugations were stored at −20 °C before use.

Individual bile acid concentrations of test solutions and reagent blanks were determined using an assay kit (Kit 450, Trinity Biotech, Berkeley Heights, NJ) consisting of Reagent A (2.5 mM nicotinamide adenine dinucleotide, 0.61 mM nitroblue tetrazolium chromaphore, and diaphorase, pH 7.0) and Reagent B (3α-hydroxysteoid dehydrogenase). Briefly, 4 mL of Reagent A was mixed with 1 mL of Reagent B to prepare the test reagent; a blank reagent was prepared by mixing 4 mL of Reagent A with 1 mL of deionized water. Control and BWP samples (0.2 mL) were both mixed with 0.5 mL of test reagent and 0.5 mL of blank reagent. The mixtures were incubated at 37 °C for 5 min, and the absorbance was measured at 530 nm. A standard curve, from which unbound (free) bile acid concentrations in BWP-treated samples were determined, was generated from bile acids at concentrations of 25, 50, 100, 150, and 200 μM. The percentage of bound bile acid was calculated as: bound (%) = $[(C_c - C_s)/C_c] \times 100\%$, where C_c and C_s represent bile acid concentrations in the control and in samples, respectively.

Statistical Analysis. The study employed a randomized complete block design with replication as the block. There were a minimum of three replications on different days for antioxidant analyses and two replications for bile acid binding assays. Each analysis was performed in duplicate. Data were subjected to analysis of variance using the general linear model's procedure of the Statistix software 9.0 (Analytical Software, Tallahassee, FL). When a treatment effect was found significant, Tukey HSD all-pairwise multiple comparisons were performed to identify significant differences between individual means.

RESULTS AND DISCUSSION

Digestion. As expected, the degree of hydrolysis of BWP increased during in vitro digestion (**Figure 1**). However, digestion by pepsin appeared to be limited because after 60 min, the DH reached only 6.9%. Subsequent pancreatin digestion rapidly improved DH, which reached 34% within 30 min (or 90 min total digestion time). The DH continued to rise with pancreatin digestion, reaching 52% at the end of 2 h. Pancreatin is a mixed enzyme system, in which the principal proteases trypsin, chymotrypsin, elastase, and carboxypeptidase functioned collectively to cleave peptide bonds.

The in vitro digestion brought about a complex change in protein solubility. Rather than showing an increase, the solubility was lowered ($P < 0.05$) after 30 min of pepsin digestion (**Figure 1**). It can be suggested that by clipping or severing peptides bonds located on the BWP surface, pepsin hydrolysis resulted in an increased exposure of hydrophobic amino acid side chain groups, thereby promoting hydrophobic association and insolubilization of proteins. The results supported a previous finding that limited hydrolysis reduced the solubility and promoted aggregation and insolubilzation of soy proteins (*26*). With pancreatin, BWP solubility was enhanced probably by the generation of low-molecular-weight peptides where charges (i.e., $-NH_3^+$ and $-COO^-$) from peptide cleavage became a dominant force. Charged groups present in short peptides would promote electrostatic repulsion and enhance protein−water interactions.

The SDS−PAGE patterns of BWP samples are shown in **Figure 2**. The electrophoretic results of intact BWP under both nonreducing and reducing conditions were in general agreement with those reported by in the literature (*27*), and the albumin made up the main BWP fraction. Band I, observed under nonreducing conditions, appeared to consist of several unresolved polypeptides with estimated MWs of 56 to 69 kDa. They were attributed to albumin and globulin polypeptides (*27*, *28*). These BWP polypeptides were linked by disulfides because they were readily dissociated into several lower MW components under reducing conditions (with βME). Band II, with an estimated molecular weight of 41 kDa, belonged to albumin (*28*) but was resistant to βME, indicating the lack of disulfide bonds. Under reducing conditions, a salient new band (IV) emerged. This band, with an estimated MW of 23 kDa, matched the basic polypeptide from buckwheat globulin (*27*).

Consistent with the DH analysis, protein bands were diminished by enzymatic digestion, especially when subjected to pancreatin where most protein bands vanished. The pepsin digests of BWP consisted mostly of relatively large peptides (the dark smear below 14 kDa), while the pancreatin digests were largely devoid of them (**Figure 2**). Bands I and II were susceptible to enzyme digestion, while band III with a MW of around 14 kDa was resistant to pepsin digestion but disappeared upon incubation with pancreatin. This protein might be the major buckwheat allergen associated with immediate hypersentivity reactions in patients with corresponding allergies (*29*). After 2 h of pancreatin digestion, essentially all of the polypeptide bands disappeared with the exception of some 14 kDa, disulfide-linked remnants that remained faintly visible but were reduced to smaller peptide fractions when treated with βME. These disulfide remnants might later act as growth-promoting factors for bifidobacteria, a physiologically positive probiotic (*30*).

Metal Ion Chelation. An enzyme-dependent metal ion chelation property was manifested by the BWP digests. Pepsin digestion (30 and 60 min) slightly lowered the ferrous (Fe^{2+}) chelation ability when compared with that of nonhydrolyzed BWP (**Figure 3A**), probably due to the disruption of the inherent iron-binding structure. Pepsin digestion would alter the native conformation of intact protein, thereby facilitating further digestion by pancreatin. However, this modification in protein structure seemed to lead to the elimination of iron-binding sites in BWP and the consequent decrease in iron chelation activity.

The ferrous chelation activity was regained and surpassed that of nondigested BWP by 45% ($P < 0.05$) following 30 min of pancreatin digestion. Zhu et al. (*14*) have also reported that pepsin-digested zein had a decreased metal

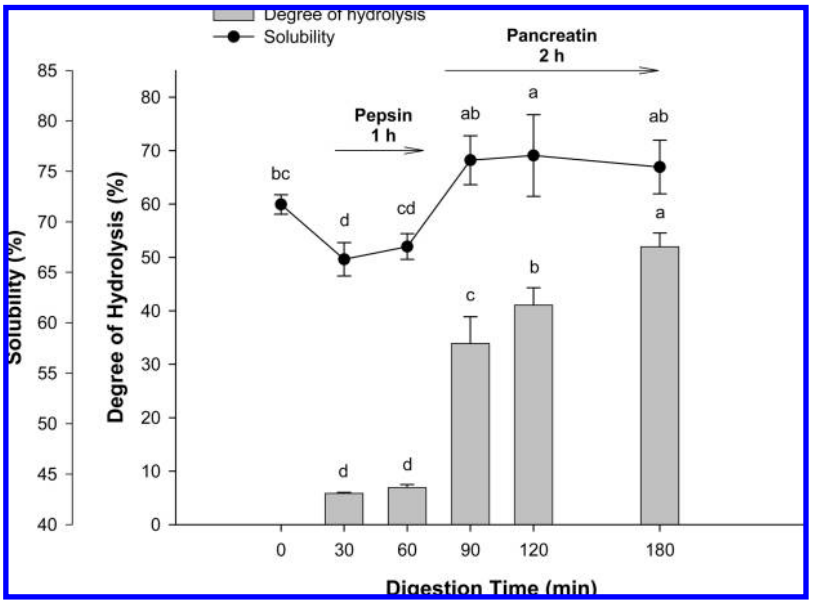

Figure 1. Degree of hydrolysis and solubility of in vitro digests of buckwheat protein. Means (n = 3) without a common letter differ significantly (P < 0.05).

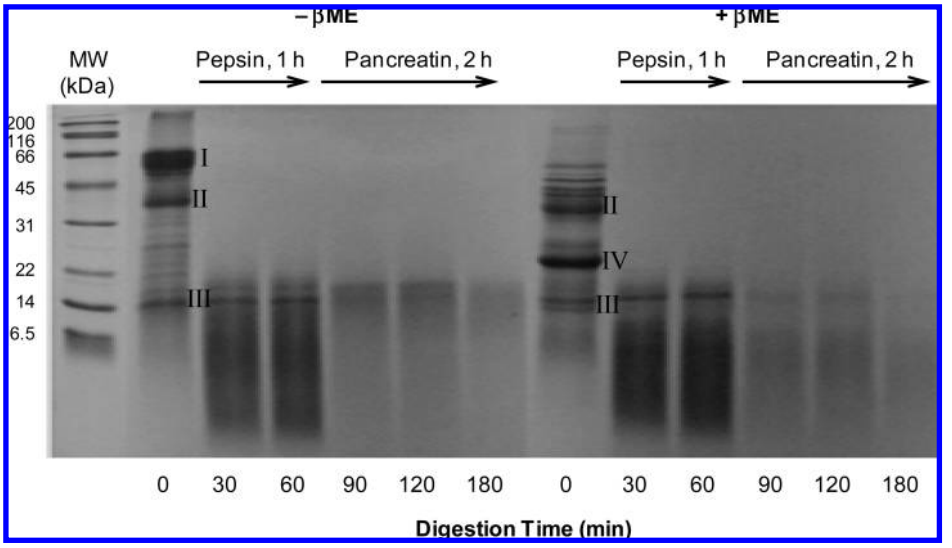

Figure 2. SDS−PAGE of intact (nonhydrolyzed, 0 min) and in vitro digested buckwheat protein (pepsin, 30 and 60 min; pancreatin, 90, 120, and 180 min).

ion chelation ability, which was recovered by pancreatin treatment. The result can be attributed to the generation of active peptides and peptide fractions. A number of previous studies have demonstrated a positive linkage between metal ion sequestering capacity and enzymatic production of short peptides, such as peptides derived from soy (*9*) and chicken (*22*). The enhanced ferrous ion chelation can result from increased exposures of functional amino acid residues. The strong Fe^{2+} and Cu^{2+} binding activity exhibited by carnosine (an endogenous dipeptide in muscle tissue) has been attributed to the histidine residue (*31*). According to Chen et al. (*9*), the metal ion chelation ability of peptides was positively associated with the number of histidines, especially the presence of N-terminal histidine. Newly generated carboxyl groups from peptide cleavage would also contribute to Fe^{2+} binding by BWP digests. In addition to the specific active amino acid side chain groups, the structure−function relationship for metal binding by peptides has been studied. According to Ducan et al. (*32*), a cage structure in metallothionein could exclude the surrounding water and increase the concentration of cupric ion, thereby allowing segments of the cysteinal protein to bind more Cu^{2+} compared to that in a loose structure.

Reducing Power. During the reducing process where the ferric ion (Fe^{3+}) is converted to the ferrous ion, Fe^{3+} acts as the electron acceptor. Thus, reducing power is indicative of the antioxidant capacity of converting radicals (electron accepters) to stable products by electron donation (*33*). An increased reducing power following proteolytic hydrolysis is attributed to the exposure of proton/electron donors of specific side-chain groups. However, pepsin digestion actually decreased the reducing potential of BWP (**Figure 3B**). It is plausible that limited peptide cleavage by pepsin promoted hydrophobic association of peptides and, therefore, lowered the availability of potential electron donors. This premise can be supported by the parallel drop in protein solubility (**Figure 1**).

The reducing power was quickly recovered upon ensuing pancreatin digestion, suggesting an increased exposure of specific hydrogen donor amino acid residues. The trypsin present in pancreatin specifically cleaves at the carboxylic side of lysine and arginine. The cleavage could remove

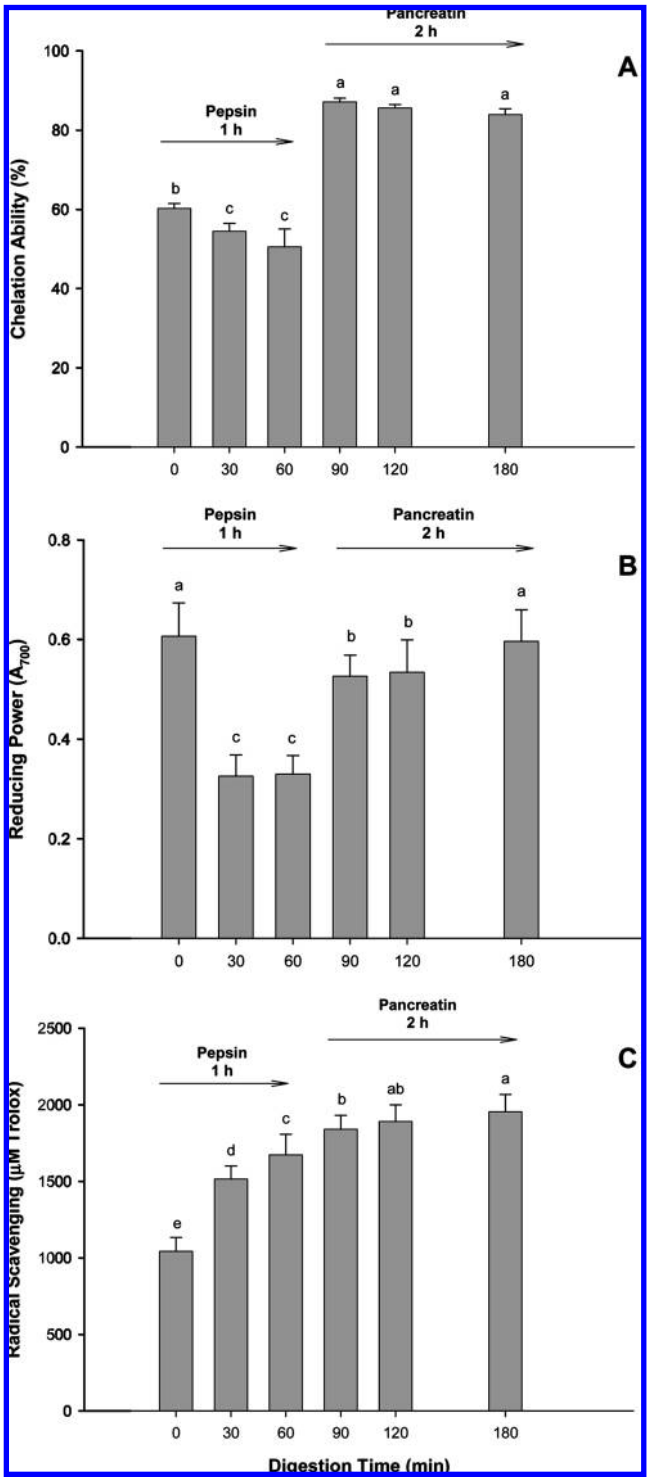

Figure 3. Iron (Fe^{2+})-chelation ability (**A**), reducing power (**B**), and $ABTS^{+\bullet}$ scavenging activity (5 min reaction time) (**C**) of in vitro digests of buckwheat protein. Means (n = 3) without a common letter differ significantly ($P < 0.05$).

structural hindrance, thereby increasing the potential of the ε- and the guanidine amine groups as hydrogen donors, especially when buckwheat has very high levels of lysine and arginine (*34*). Certainly, the extra amino groups generated from peptide bond cleavages, including those in free amino acids produced during digestion, would contribute as a major source of protons and electrons. However, pepsin prefers the C-terminal of aromatic amino acids such as phenylalanine and tryptophan. Hence, peptide scission by pepsin would enable a limited production of protons and electrons.

The role of cysteine, a strong electron donor, would be minimal because most cysteine residues are in the disulfide bond form. Cysteine is a limiting amino acid in buckwheat mostly located in the albumin fraction (0.12 to 0.16 g/100 g protein) (*35*). Choi and Ma (*28*) also reported deficiency of free cysteine in buckwheat globulin. It is noteworthy that the metal (Fe^{2+}) chelation activity of BWP hydrolysate would not significantly influence the Fe^{3+} reducing test; the Fe^{2+} binding capacity was considerably weak (with 0.04 mM Fe^{2+}) when compared to the BWP's reducing potential (with 1.6 mM Fe^{2+}). Therefore, the reducing power assay did not seem to be confounded by the metal binding activity of hydrolyzed BWP.

Radical Scavenging Activity (RSA). The $ABTS^{+\bullet}$ scavenging assay is a colorimetric method in which the rate of decoloration, representing the elimination of $ABTS^{+\bullet}$, was expressed by the trolox equivalent antioxidant capacity (TEAC). The rate of decoloration essentially reached a plateau after a reaction time of 5 min (result not shown). Therefore, the TEAC values shown at 5 min were used as a comparison index in the subsequent evaluation of different treatments.

The TEAC value of BWP increased steadily during in vitro digestion. The improvement in the $ABTS^{+\bullet}$ scavenging activity ranged from 45% (30 min with pepsin) to 88% (2 h with pancreatin) ($P < 0.05$) when compared with the nonhydrolyzed BWP control (**Figure 3C**). This digestion−radical quenching relationship differed from that for iron reducing activity, which dropped markedly following pepsin digestion and then recovered by the subsequent pancreatin treatment. Therefore, a different mechanism appeared to be involved in $ABTS^{+\bullet}$ scavenging when compared with reducing activity. The consistent increase in RSA indicated a potential role of buckwheat protein digests in alleviating radical-mediated gastric mucosal injury and gastric ulcers.

Effect of Dosage on Antioxidant Activity. The final pepsin−pancreatin digest of BWP showed the strongest antioxidant reducing and radical scavenging activity; therefore, it was used to test the hydrolysate concentration effect on antioxidant efficacy. As shown in **Figure 4**, the antioxidant activity increased with protein concentration. Although a plateau was reached for Fe^{2+} chelation, the $ABTS^{+\bullet}$ scavenging and reducing power continued to exhibit dose-dependent improvement in the 0−3 mg/mL protein concentration range. The metal ion chelation test is based on the principle that residual free metal ions (unbound by protein hydrolysate) react with ferrozine to develop a purplish-pink chromatophore. The metal binding by the BWP hydrolysate seemed to be so efficient that it essentially chelated all free ferrous ions at the 1 mg/mL protein concentration level. This was readily visualized because reaction solutions with 2 mg/mL BWP hydrolysate in the metal ion chelation assay were basically colorless.

Inhibition of TBARS Formation. To validate the antioxidant activity of BWP hydrolysate, samples from the pepsin−pancreatin two-stage digestion were added to an oxidizing liposome solution, and the inhibition of lipid oxidation (TBARS) was monitored. As shown in **Figure 5A**, pepsin-treated BWP promoted oxidation, but subsequent pancreatin digestion inhibited TBARS production ($P < 0.05$). At the concentration of 2 mg/mL (0.2%), the final BWP digest (2 h pancreatin) was comparable to 0.01% BHA ($P < 0.01$) in suppressing lipid oxidation.

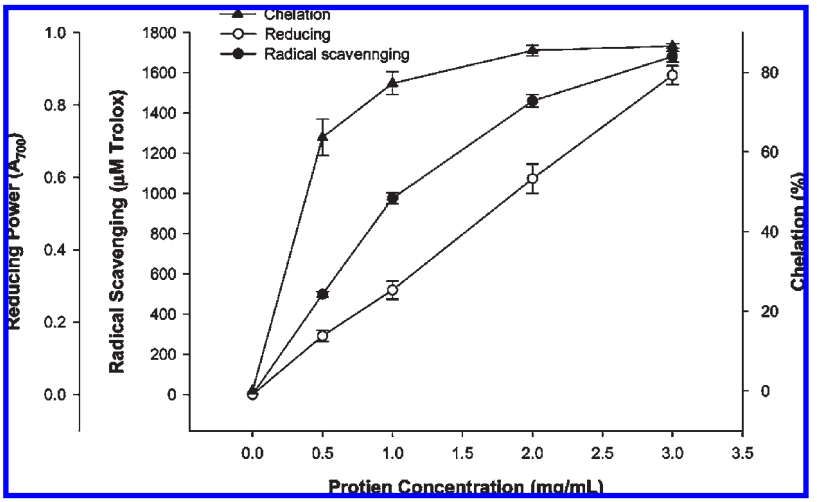

Figure 4. Effect of protein concentration on the chelation, reducing, and radical scavenging activities of the final in vitro digests (180 min digestion) of buckwheat protein ($n = 3$).

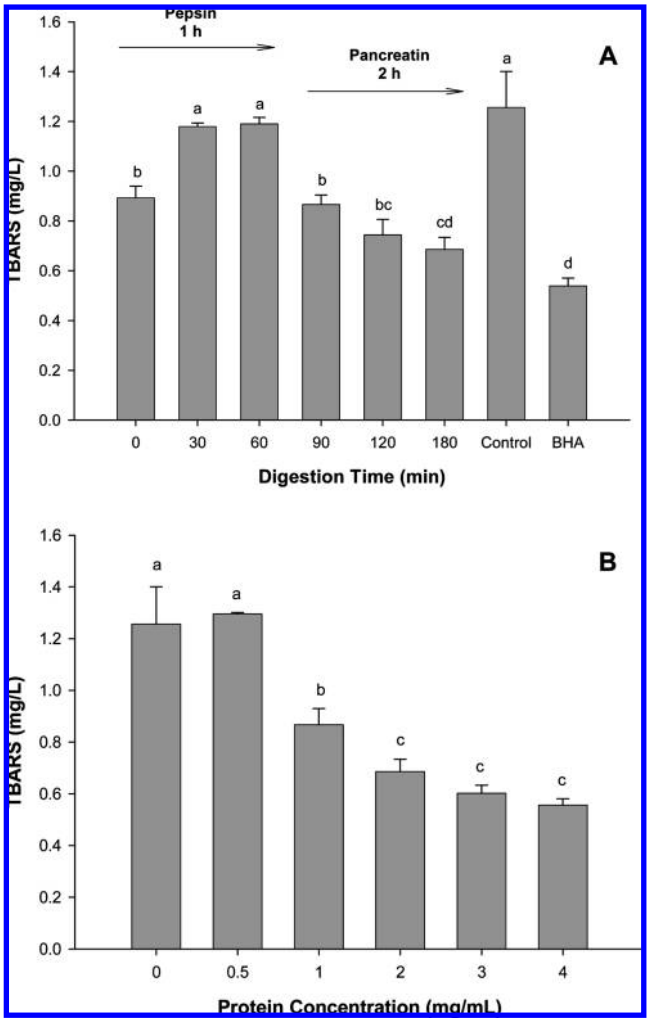

Figure 5. Inhibition of TBARS formation in the Fe^{2+}/ascorbate-oxidized liposome by in vitro digests of buckwheat protein prepared at different digestion times (**A**) or by various concentrations of the final protein digest (180 min digestion) (**B**). Means ($n = 3$) without a common letter differ significantly ($P < 0.05$).

It was possible that the decreased iron binding by pepsin cleavage, presumably due to structural changes in BWP, facilitated $^{\bullet}OH$ production in the Fenton-type Fe^{3+}-ascorbate reaction in the abundance of liposome phospholipids. Unlike $ABTS^{+\bullet}$, the hydroxyl radical is small, extremely reactive, and can be more efficiently stabilized by small-sized peptides (*36*). Therefore, a higher amount of malonaldehyde was detected in liposomes mixed with pepsin digests that contained relatively large peptides. Furthermore, the relatively large molecular size of pepsin digests compared to small peptide fractions from pancreatin digestion might also limit their motility and thus decrease their efficiency to prevent the initiation of lipid peroxidation.

The overall inhibition of lipid oxidation by the enzymatic digests appeared to be related to their chelation and reducing properties. Linear regression plots showed significant correlations ($P < 0.05$) of TBARS reduction with the chelation ability ($r = 0.88$) and reducing power ($r = 0.91$) of the final BWP digest (not shown). Thus, the removal of the prooxidant iron initiator by pancreatin BWP digests contributed, at least in part, to the overall inhibition of lipid oxidation catalyzed by the iron redox cycling. The result also suggested that by acting as a proton donor, the pancrease BWP digests prevented the decomposition of lipid peroxide to secondary products.

Consistent with the results from the individual metal ion chelation, proton donation, and radical scavenging assays, the inhibition of TBARS formation in the liposome system by the final in vitro BWP digest (2 h with pancreatin) was positively related to the dosage of the protein digest (**Figure 5B**). Notwithstanding, the lack of further decrease in the TBARS concentration when 2 mg/mL or higher amounts of the pancreatin digest were added indicated that the inhibition of lipid peroxidation had reached a maximum level that was close to the threshold or limit of malonaldehyde detection in the TBA color reaction.

Bile Acid Binding. Both intact and digested BWP were capable of binding cholic acid (**Figure 6A**). Pepsin digestion (60 min) produced a numerical reduction in cholic acid binding, but the effect was nonsignificant. While the supernatant of BWP showed a trend of continuing decline in cholic acid binding during in vitro digestion, mixed BWP samples exhibited steady increases in cholic acid binding upon pancreatin digestion with the final mixed digest (180 min) displaying a two-third gain over that of the nondigested control ($P < 0.05$). Cleavages of peptide bonds by trypsin, chymotrypsin, elastase, carboxypeptidase, and probably also some minor proteases present in pancreatin would lead to the release of peptides that interacted with cholic acid.

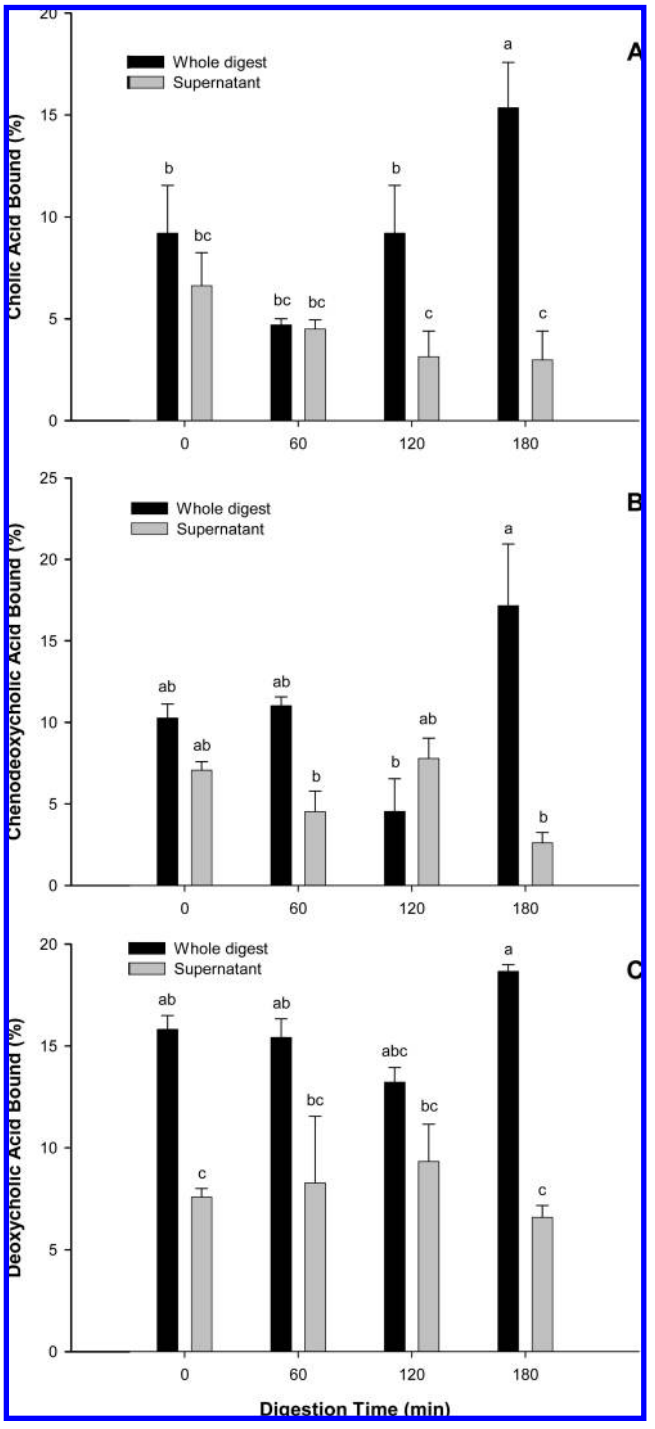

Figure 6. Binding ability of in vitro digests of buckwheat protein for cholic acid (**A**), chenodeoxycholic acid (**B**), and deoxycholic acid (**C**). Means ($n = 2$) without a common letter differ significantly ($P < 0.05$).

It is noteworthy that binding by the soluble fraction (supernatants) of the BWP pancreatin digests was weaker ($P < 0.05$) than mixed digests, confirming that the ability of the protein digests to eliminate the bile acid was largely confined to insoluble or enzyme-resistant polypeptides (*37*). In another study, Higaki et al. (*8*) showed that the majority of excreted peptides in rats' feces were in the insoluble form, while only a trace amount of soluble peptides was found.

Sugano et al. (*7*) reported that bile acid binding peptides could be produced from pepsin digestion of soy protein only under conditions that required exhaustive digestion (24 h) followed by heating (80 °C for 30 min). A long digestion process, 4 h of pepsin followed by 24 h of pancreatin, was employed by Higaki et al. (*8*) to generate resistant peptides from soy protein. Our result indicated that resistant cholic acid binding peptides could be more readily produced from buckwheat during in vitro digestion. Furthermore, Yoshie-Stark and Wasche (*25*) found a similar lack of direct association between hydrolysis and bile acid binding in lupin protein and its hydrolysates.

An irregular digestion time versus chenodeoxycholic acid binding relationship was also seen in the study (**Figure 6B**). Similar to cholic acid binding, binding of chenodeoxycholic acid by the final (180 min) mixed BWP pancreatin digest tended to be stronger than other digests ($P < 0.05$). Moreover, individual whole mixtures of BWP digests tended to have a higher binding activity compared to those of their corresponding supernatants except for the 120 min digest. Since cholic and chenodeoxycholic acid are primary bile acids, their abstraction from their recycling circulation would lead to an enhanced utilization of cholesterol to synthesize additional bile acids, resulting in hepatic clearance of cholesterol and consequent decrease in serum cholesterol concentration (*5*, *25*). For this reason, the almost identical improvements in cholic acid binding (66.8%) and chenodeoxycholic acid binding (67.2%) by the final mixed pancreatin digest (180 min) would suggest that the cholesterol lowering effect of buckwheat protein as demonstrated in previous studies was due to the binding of both primary bile acids by its in vitro digests.

Bile acid binding by BWP digests was further evaluated on deoxycholic acid, a secondary bile acid and a potential carcinogen derived from primary bile acids by bacterial flora in the lumen of the large bowel (*6*). While the deoxycholic acid binding capacity of BWP did not change during in vitro digestion, the mixed digests again showed a greater tendency to bind this bile acid derivative than their respective soluble peptide fractions (supernatants) with the difference being clearly demonstrated ($P < 0.05$) on the final (180 min) digested samples (**Figure 6C**). In general, digestion-resistant proteins are rich in hydrophobic amino acid residues and, therefore, bind bile acids strongly via hydrophobic reactions (*8*). The increased hydrophobicity of secondary bile acid due to bacterial dehydroxylation would promote hydrophobic interactions with side groups of proteins and peptide fractions. Consistent with this hydrophobic reaction theory, the mixed BWP digest samples had a greater overall binding activity ($P < 0.05$) for deoxycholic acid than for cholic and chenodeoxycholic acids. In spite of the differences, insoluble resistant polypeptides in BWP that survived in vitro digestion are expected to play a role in removing all three bile acids.

In conclusion, the antioxidant and bile acid binding experiments in this study demonstrated a complex in vitro digestion−activity relationship for in vitro digests of BWP. Yet, it was clear that the most potent antioxidant peptides were produced at the final stage of the upper GI digestion process (i.e, 2 h with pancreatin) and that the strongest bile acid binding BWP fragments, which were insoluble, were also generated toward the end of pancreatin digestion. The release of the specific active peptide sequences as well as the exposure of certain amino acid side chain groups were implicated in the elicitation of antioxidant and bile acid binding activities from BWP through in vitro digestion. The results support the central hypothesis that dietary BWP could not only protect the human digestive system against radical mediated injuries but also help maintain

serum cholesterol at a healthy level via the bile acid binding and excretion mechanism. Furthermore, because of the bile acid-removing potential, BWP digests might be beneficial in inducing the growth of bifidobacteria that were found to be inhibited by bile salts (bile acids) (*38*). Antioxidative and bile acid binding peptides produced with digestive enzymes may be utilized in health food formulations as well as in nutraceuticals suited for the prevention or treatment of certain illnesses in humans. Notwithstanding, it should be noted that this study only investigated BWP digestion in the upper digestive tract; the effect of further intestinal digestion and bacterial degradation postduodenum on the antioxidant and bile acid binding properties of BWP requires further exploration. Ultimately, laboratory animal in vivo studies must be performed to substantiate the potential health promoting functions of the specific peptides derived from BWP digestion.

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