Published in final edited form as:

Food Chem. 2010 February 1; 118(3): 582–588. doi:10.1016/j.foodchem.2009.05.024.

Fractionation and evaluation of radical-scavenging peptides from *in vitro* digests of buckwheat protein [★]

Yuanyuan Ma^a, Youling L. Xiong^{a,*}, Jianjun Zhai^b, Haining Zhu^b, and Thomas Dziubla^c

^a Department of Animal and Food Sciences, University of Kentucky, Lexington, KY 40546, USA

^b Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY 40506, USA

^c Department of Chemical and Materials Engineering, University of Kentucky, Lexington, KY 40506, USA

Abstract

Buckwheat protein (BWP) isolate was subjected to a two-stage *in vitro* digestion (1 h pepsin followed by 2 h pancreatin at 37 °C). The antioxidant potential of the BWP digests was compared by assessing their capacity to scavenge 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS^{•+}) and hydroxyl (•OH) radicals. The 2-h pancreatin digest, which demonstrated the strongest activity against both radicals, was subjected to Sephadex G-25 gel filtration. Of the six fractions collected, fractions IV (456 Da) and VI (362 Da) showed the highest ABTS^{•+} scavenging activity and were 23–27% superior to mixed BWP digest ($P < 0.05$). Fraction VI was most effective in neutralizing •OH and was 86 and 24% more efficient ($P < 0.05$) than mixed BWP digest and fraction IV, respectively. LC-MS/MS identified Trp-Pro-Leu, Val-Pro-Trp, and Val-Phe-Pro-Trp (IV), Pro-Trp (V) and tryptophan (VI) to be the prominent peptides/amino acid in these fractions.

Keywords

Buckwheat protein; *In vitro* digestion; Free radical scavenging; Gel filtration; Tandem mass spectrometry

1. Introduction

The gastrointestinal (GI) tract is one of the most vulnerable tissues inside the human body to oxidative attack by reactive oxygen species (ROS). Oxidative stress is believed to be one important cause of GI inflammation, ulcer, and colitis (Blau, Rubinstein, Bass, Singaram, & Kohen, 1999). The upper GI mucosa, itself a natural defense layer, is constantly exposed to ROS derived from endogenous as well as exogenous sources, i.e., foods, which can contain high amounts of unsaturated lipids, prooxidant transition metal ions and even directly, free radicals. For example, a diet containing iron and ascorbic acid in the presence of unsaturated fatty acids predisposed the GI lining to hydroxyl radical (•OH) mediated injury, which can lead to colitis (Carrier, Aghdassi, Platt, Cullen, & Allard, 2001). Moreover, it has been demonstrated

[★]Approved for publication as journal article number 09-07-004 by the Director of Kentucky Agricultural Experiment Station.

*Corresponding author. Tel.: +01859 257 3822, fax: +1 859 257 5318. ylxiong@uky.edu (Y.L. Xiong).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

that $\cdot\text{OH}$ can form in the gastric juice, and the radical generation is implicated in GI mucosa damage and ensuing ulcer (Nalini, Ramakrishna, Mohanty, & Balasubramanian, 1992). Hence, identifying potential antioxidants that may help neutralize radicals, particularly $\cdot\text{OH}$, thereby protecting the GI system, is of great importance.

There has been growing interest in recent years to produce bioactive peptides that can exert radical scavenging activity. Carnosine, a naturally occurring dipeptide rich in muscle foods, is a classical example of peptides that can act as a strong radical scavenger and inhibit ROS-initiated lipid oxidation (Boldyrev & Johnson, 2002). Most reported antioxidants are derived from common food protein sources using commercial enzymes. For example, canola protein hydrolysate prepared using Flavourzyme was shown to be antioxidative and can enhance water-holding capacity in cooked pork meat (Cumby, Zhong, Nacz, & Shahidi, 2008). Hydrolyzed animal proteins, e.g., gelatin hydrolysate from Alaska polluck skin (Kim, Kim, Byun, Nam, Joo, & Shahidi, 2001), also show antioxidant activity in food model systems.

Proteins in raw and processed foods can possess antioxidant peptide sequences and structural domains; the active fragments are released during the GI digestion process. Reported high-efficiency radical scavenging peptides released through *in vitro* pepsin and pancreatin digestion include those from casein (Hernandez-Ledesma, Amigo, Ramos, & Recio, 2004), maize zein (Zhu, Chen, Tang, & Xiong, 2008), oyster protein (*Crassostrea gigas*) (Qian, Jung, Byun, & Kim, 2008), and mussel protein (*Mytilus coruscus*) (Jung et al., 2007).

Buckwheat, a traditional grain widely considered as a functional food source, has gained its fame due to published studies that linked its proteins to various health benefits, e.g., cholesterol reduction (Kayashita, Shimaoka, Nakajoh, Yamazaki, & Norihisa, 1997), tumor inhibition (Liu et al., 2001), and hypotension regulation (Ma, Bae, Lee, & Yang, 2006). Because many of the health promoting functions are inherently related to the radical scavenging activity of peptides from the protein digests, it is hypothesized that hydrolysis of buckwheat protein can release the peptide fragments capable of stabilizing ROS and inhibiting lipid oxidation. A preliminary study supported this hypothesis (Ma & Xiong, 2009). However, the specific peptides or peptide fractions responsible for the antioxidant functions have not been elucidated.

In the present study, the ability of mixed as well as individual fractions of *in vitro* pepsin-pancreatin sequential digests of buckwheat protein to stabilize $\cdot\text{OH}$ and $\text{ABTS}^{+\cdot}$ radicals was investigated. The objective was to identify the most effective antioxidant peptide fraction(s) from buckwheat *in vitro* digests. Initially, the digest with the highest radical scavenging capacity was fractionated by means of gel filtration. The ability to stabilize hydroxyl radical by each post-column fraction was subsequently examined, and the prominent peptides in active fractions were sequenced by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2. Materials and Methods

2.1. Extraction of buckwheat protein (BWP)

Low-fat buckwheat flour was purchased from Bulkfoods.com (Toledo, OH, USA). 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, 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, Shimaoka, Kayashita, Nakajoh, and Kato (2002) 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 M 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 M HCl to isoelectrically precipitate protein. The protein

precipitate was washed with deionized water two times and then neutralized with 0.1 M NaOH before lyophilization. Freeze dried BWP powder was stored at -20°C before use.

2.2. Preparation of protein digests

BWP *in vitro* digests were prepared according to the method of Lo and Li-Chan (2005). The suspension of BWP (5%, w/v) in nanopure deionized water was adjusted pH 2.0 with 1 M HCl, followed by the addition of pepsin (4%, w/w, protein basis). The mixture was incubated 1 h in a shaking water bath set 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), the pH was adjusted to 7.5 with 1 M NaOH. The digestion was restarted and 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 $\rightarrow$ pancreatin sequential digestion, adjusted to neutrality (pH 7.0) with 1 M NaOH/HCl, and heated at 96°C for 5 min to inactivate the enzymes. Each aliquot was freeze dried and kept at -20°C before use.

2.3. Gel filtration

Preliminary results showed that the two-stage *in vitro* digestion yielded a high radical scavenging activity in the final BWP digest (i.e., 180 min total digestion time). Therefore, this digest, referred to as “D_{180min}”, was subjected to peptide fractionation using a low-pressure size exclusion chromatography with a 2.6 cm (dia.) $\times$ 70 cm (length) Sephadex G-25 fine column (Pharmacia XK 26/70, Piscataway, NJ, USA).

A 2 mg/mL of D_{180min} solution, prepared from lyophilized powder by dissolving in the elution buffer (0.02 M phosphate, pH 7.4), was clarified and sterilized through a 25-mm syringe filter with a 0.22 μm membrane (Fisher Scientific, Pittsburgh, PA). The purified solution (10 mL) was loaded to the Sephadex column and eluted in a 4°C cold room with the elution buffer at a 0.9 mL/min flow rate. Peptide fractions were collected using an automated fraction collector, and the absorbance (215 nm) of the eluents was measured. In order to collect enough peptides for antioxidant assays, a total of 23 chromatographic runs were conducted. The corresponding peptide fractions from the 23 replicates were pooled and lyophilized. Freeze dried fractions were stored at -20°C for further analysis.

Molecular weight (MW) distribution of the individual peptide fractions was estimated from a MW calibration curve generated from the elution volume of the following standards (Sigma Chemical Co., St. Louis, MO) that were chromatographed separately in the Sephadex G-25 column under the same condition as described above: cytochrome C (12327 Da), aprotinin (6512 Da), bacitracin (1423 Da), and tetrapeptide GGYR (452 Da). The elution volume (mL) of blue dextran was used to establish the void volume of the column. Data were fitted in the exponential decay model (modified single with 3 parameters) of the SigmaPlot Ver. 9 software (Systat Software, Inc., Chicago, IL, USA), which yielded the following equation:

$$\text{LogMW} = 2.3429e^{\left(\frac{34.8528}{\text{Vol} - 79.1592}\right)}$$

2.4. Radical-scavenging activity (RSA)

The RSA of BWP *in vitro* digests and the peptide fractions of the final digest (D_{180min}) was evaluated using $\cdot\text{OH}$ and $\text{ABTS}^{+\cdot}$ systems. The $\cdot\text{OH}$ assay involved the inhibition of radical formation rather than scavenging radicals that are already produced (i.e., pre-existed), while the $\text{ABTS}^{+\cdot}$ scavenging assay was carried out by using pre-generated cationic radicals. Furthermore, $\text{ABTS}^{+\cdot}$, a synthetic radical species, is much larger in size than $\cdot\text{OH}$, which is known to be most reactive of all the reactive oxygen species in food systems. Therefore, the

analysis of RSA of BWP digests in the two different radical generation systems may lead to a better understanding of RSA of BWB peptides than would the individual assay systems.

2.4.1. $\cdot\text{OH}$ scavenging—The $\cdot\text{OH}$ scavenging activity measurement was carried out according to the method of Moore, Yin, and Yu (2006). Briefly, 30 μL samples were each mixed with 170 μL of 9.28×10^{-8} M fluorescein in a 96-well polystyrene plate (Fisher Scientific, Pittsburgh, PA, USA), followed by the addition of 40 μL of 0.1999 M H_2O_2 and 60 μL FeCl_3 . The mixed solution was immediately transferred to a Cary Eclipse fluorescence spectrophotometer equipped with a microplate reader (Varian, Victoria, Australia). The measurement with 0.1 s reading time per well and 1 min per plate was conducted with a 485 nm excitation wavelength and a 535 nm emission wavelength for 3 h to obtain the fluorescein decay curve. The $\cdot\text{OH}$ scavenging capacity was expressed as trolox equivalent (μM), which was determined from the regression equation built on a series of trolox standards (20, 40, 80, and 100 μM). The concentration of the standards was set as the x-axis and the net area under the decay curve was set as the y-axis. The calculation of the area under curve (AUC) is shown below, where f represents the fluorescence value at a particular time during the decay:

$$\text{AUC} = 0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \dots + f_{i-1}/f_0 + 0.5 (f_i/f_0)$$

2.4.2. ABTS $^{\cdot+}$ scavenging—The ABTS $^{\cdot+}$ scavenging ability was determined by the decolorization assay (Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999). Briefly, ABTS $^{\cdot+}$ was generated by a mixed solution of 7 mM ABTS and 2.45 mM potassium persulphate. After 12–16 h reaction, a dense green-blue colored solution with excessive accumulation of ABTS $^{\cdot+}$ radical 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 was then determined by mixing 10 μL samples (2 mg/mL protein) and 990 μL diluted ABTS $^{\cdot+}$ solution for digestion aliquots (0, 30, 60, 90, 120 and 180 min) assay, and 100 μL samples (0.189 mg/mL protein) into 900 μL of diluted ABTS $^{\cdot+}$ solution for post-column fractions and final digest mixture ($D_{180\text{min}}$) for comparison. The absorbance of 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 generated with 50, 100, 250, 500, and 1000 μM of trolox. Trolox equivalent antioxidant capacity (TEAC) was used to express RSA.

2.4.3. LC-MS-MS—Gel filtration fractions (5 μL each) that exhibited strong radical scavenging activity were subjected to LC-MS/MS analysis using a QSTAR XL quadrupole time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA, USA) coupled with a nano-flow HPLC system (Eksigent Technologies, Dublin, CA, USA) through a nano-electrospray ionization source (Protana, Toronto, Canada) (Lu & Zhu, 2005). The samples were injected by an autosampler, desalted on a trap column (300 μm i.d. $\times$ 5 mm length, LC Packings, Sunnyvale, CA, USA), and subsequently separated by reverse phase C18 column (75 μm i.d. $\times$ 150 mm length, Vydac, Columbia, MD, USA) at a flow rate of 200 nL/min. The HPLC gradient was linear from 5% to 80% mobile phase B in 55 min using mobile phase A (H_2O , 0.1% formic acid) and mobile phase B (80% acetonitrile, 0.1% formic acid).

Peptides eluted out of the reverse phase column were analyzed online by mass spectrometry (MS) and selected peptides were subjected to tandem mass spectrometry (MS/MS) sequencing. The automated data acquisition using information-dependent mode was performed on QSTAR XL under control by Analyst QS software (Applied Biosystems, Foster City, CA, USA). Each cycle typically consisted of one 1-sec MS survey scan from 150 to 1200 (m/z) and two 2-sec MS/MS scans of singly, doubly and triply charged species with mass range of 100 to 1200 (m/z). The spectra were interpreted using the *de novo* peptide sequencing module of the Analyst QS software.

2.5. Statistical analysis

The study employed a randomized complete block design with replication as the block. There were a minimum of three replications. Each analysis was done 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.

3. Results and Discussion

3.1. RSA of BWP in vitro digests

In the $\cdot\text{OH}$ scavenging test, the potential of an antioxidant to inhibit $\cdot\text{OH}$ formation or to stabilize the radical was indicated by the rate of fluorescence decay of fluorescein (Moore et al., 2006). All BWP *in vitro* digest samples showed a slower fluorescence decay than non-hydrolyzed BWP (data not shown), indicating that hydrolysis improved $\cdot\text{OH}$ scavenging activity of BWP. Despite some variations, there was an overall trend that the $\cdot\text{OH}$ scavenging activity increased with digestion (Fig. 1). In the Fenton reaction ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^-$), the impact of $\cdot\text{OH}$ on susceptible compounds could be restrained by either Fe^{2+} chelation or $\cdot\text{OH}$ stabilization or both. Hence, the strong $\cdot\text{OH}$ elimination activity of BWP digests, notably that of $\text{D}_{180\text{min}}$, can be attributed to the removal of free Fe^{2+} prooxidant and stabilization of radicals through hydrogen or electron donation. The first mechanism was a postulation on the basis of the $\cdot\text{OH}$ assay, which can be supported by the strong iron chelation ability of 2 h pancreatin digests (i.e., $\text{D}_{180\text{min}}$) (Ma and Xiong, 2009).

The $\text{ABTS}^{+\cdot}$ radical scavenging activity assay also demonstrated a positive and more consistent rise in the scavenging capacity with digestion time, culminating at 2 h of pancreatin treatment (i.e., end of the total 180 min *in vitro* digestion) (Fig. 2). In the $\text{ABTS}^{+\cdot}$ method, the antioxidant activity was measured exclusively by the ability of an antioxidant to act as a hydrogen or electron donor to neutralize preformed $\text{ABTS}^{+\cdot}$ radicals (Re et al., 1999). However, in the $\cdot\text{OH}$ method, a test antioxidant is placed in a radical generation system where the antioxidant capacity was expressed both as inhibition of the radical initiation and elimination of formed radicals (Moore et al., 2006). The remarkable similarity in the results of $\cdot\text{OH}$ and $\text{ABTS}^{+\cdot}$ assays on different digests suggested that inhibition of radical initiation was probably not a critical factor determining the efficacy of BWP digests as antioxidants.

3.2. Peptide fractionation

Six peptide fractions were obtained by the Sephadex G-25 size exclusion gel filtration (Fig. 3). Based on the regression equation between elution volume and MW of each standard, the estimated mean MWs of these fractions were 3611 (I), 960 (II), 529 (III), 456 (IV), 365 (V), and 362 Da (VI). Assuming an average MW of 135 Da for amino acids, fraction I would be a peptide mixture consisting predominantly of those with 26 amino acid residues, and fractions II, III, IV and V or VI would have a preponderance of heptamer, tetrameric, trimeric, or dimeric peptides, respectively. Fraction VI would also contain free amino acids. Based on the nitrogen analysis of the freeze dried eluent powders, these fractions accounted for 23.7% (I), 60.2% (II), 10.5% (III), 3.2% (IV), 1.4% (V), and 1.0% (VI) of the total eluent protein.

The incomplete separation of fractions I–IV suggested that they each contained peptides with various sizes, some of which were overlapping in neighboring fractions. A relatively broad distribution of molecular weight masses in hydrolyzed proteins is commonly associated with gel filtration (Adler-Nissen, 1986). The tailing parts of the eluents (fractions V and VI) would consist of mixed short peptides and free amino acids (Zhu et al., 2008). Thus, a more robust separation system (e.g., a high performance liquid chromatography) that enables a precise MW

distribution measurement is desirable for better elucidating the relationship of peptide MW distribution with antioxidant activity.

3.3. RSA of peptide fractions

The $\cdot\text{OH}$ and $\text{ABTS}^{+\cdot}$ scavenging activities of various gel filtration fractions of the final BWP *in vitro* digest ($D_{180\text{min}}$) are shown in Fig. 4 ($\cdot\text{OH}$ scavenging capacity vs. fractions) and Fig. 5 ($\text{ABTS}^{+\cdot}$ scavenging capacity vs. fractions). Fraction VI exhibited the strongest $\cdot\text{OH}$ scavenging activity, which was 86% greater ($P < 0.05$) than the mixed BWP final digest ($D_{180\text{min}}$). Fractions IV and VI showed the highest potential in scavenging $\text{ABTS}^{+\cdot}$, and the activity was enhanced by an average of 24.5% ($P < 0.05$) compared to mixed final digest.

Although the $\text{ABTS}^{+\cdot}$ scavenging activity of fractions IV and VI indicated no significant differences, fraction VI showed a particularly strong $\cdot\text{OH}$ elimination activity and was 24% more effective than fraction IV. These results suggested that fractions IV and VI were primary contributors to the radical scavenging capacity of the final BWP *in vitro* digest. The weaker antioxidant activity of the final mixed digest compared to the individual fractions IV and V can be explained by the dilution effect of relatively ineffective larger peptides. Fractions I (3611 Da), II (960 Da) and III (529 Da), which were present in the mixed final digest ($D_{180\text{min}}$), all displayed extremely low activity against $\text{ABTS}^{+\cdot}$ and $\cdot\text{OH}$. Yet, they made up the bulk of the total proteins/peptides in the mixed final digest.

3.4. Peptide sequence

Gel filtration fractions IV, V and VI, which exhibited relatively strong radical scavenging activity as indicated above, were subjected to individual peptide separation and sequence identification. The extracted ion chromatograms (XIC) and the tandem MS/MS spectra of the prominent peptides in these fractions are shown in Fig. 6. There were three prominent peptides in fraction IV, their XICs with the m/z values of 415.22, 401.2 and 548.27 are displayed in panels (A), (C) and (E), respectively. Their MS/MS spectra are shown in panels (B), (D) and (F). *De novo* sequencing determined the sequences of the peptides as Trp-Pro-Leu (B), Val-Pro-Trp (D) and Val-Phe-Pro-Trp (F), respectively. Fraction V produced relatively lower total signals in the LC-MS/MS analysis. A peptide with the m/z value of 302.14 was evident (panel G) and the MS/MS spectrum (panel H) supported the sequence of dipeptide Pro-Trp. Fraction VI showed a dominant XIC peak with elution time from 10 to 25 min (m/z at 205.12, panel I). The MS/MS spectrum displayed a characteristic pattern of fragments (m/z values of 118, 146 and 188) of the amino acid tryptophan as previously reported (Yamada, Miyazaki, Shibata, Hara, & Tsuchiya, 2008). Tryptophan was also detected in fractions V and IV with lower abundance (data not shown). The decreasing sizes of the prominent peptides in fractions IV, V and VI were consistent with the estimated molecular weights based on the gel filtration fractionation (Fig. 3).

These results were in concert with the general finding that short peptides with 2–10 amino acids exert greater antioxidant potential and other bioactive properties than their parent native proteins or large polypeptides (Kitts & Weiler, 2003). Through peptide bond cleavage, hydrolysis allows the release of active peptides capable of sequestering oxygen radicals, chelating prooxidant metal ions, and inhibiting lipid peroxidation in food systems (Elias, Kellerby, & Decker, 2008). For example, an oligopeptide with the sequence of His-Gly-Pro-Leu-Gly-Pro-Leu (797 Da), which was purified from fish skin hydrolysate, showed strong activity against ROS as well as linoleic acid peroxidation (Mendis, Rajapakse, & Kim, 2005). Two antioxidant short peptides, with the sequences of His-Val-Thr-Glu-Glu and Pro-Val-Pro-Ala-Glu-Gly-Val, were identified from chicken essence (Wu, Pan, Chang, & Shiau, 2005). Proline, as well as leucine, were found to play an important role in the antioxidant activity of peptides derived from soy protein, e.g., Leu-Leu-Pro-His-His (Chen, Muramoto, Yamauchi,

& Nokihara, 1996). In our study, proline was present in all four prominent peptides identified in BWP digest fractions that showed strong antioxidant activity. In addition, valine was also present in two of four peptides. Zhu et al. (2008) reported that the peptide fractions rich in di-, tri-, and tetrapeptides from the zein *in vitro* digest (1–8 mg/mL protein) had comparable or stronger antioxidant activity than that of 0.1 mg/mL ascorbic acid or BHA. These peptides were superior to nonhydrolyzed zein.

The dominant existence of tryptophan in fraction VI suggested that tryptophan was a potent antioxidant in BWP digests, consistent with previous findings (Christen, Peterhans, & Stocker, 1990; Elias, McClements, & Decker, 2005). It is noteworthy that tryptophan was also present in all four peptides with significant abundances in the three strong radical scavenging fractions (IV, V, VI), further supporting its antioxidant property. Furthermore, because short peptides are smaller molecules than intact proteins, at an equal weight concentration basis, the peptides' molar concentration would be significantly greater than that of intact proteins. This difference would also contribute to the higher antioxidant activity of BWP peptides.

In conclusion, free radical scavenging activity of BWP was accentuated by *in vitro* digestion, especially after 2 h pancreatin digestion following the 1 h pepsin treatment. On an equal weight concentration basis, fractions enriched with di-, tri- and tetrameric peptides containing tryptophan and proline exhibited the strongest radical scavenging activity. These short peptides are implicated in the protection of the upper digestive tract of humans from oxidative stresses and may partially explain why dietary BWP promotes the health of the GI system.

Acknowledgments

This research was supported by a CSREES/USDA NRI grant to Y.L.X. (2008-35503-18790). The Proteomics Core directed by H.Z. is in part supported by the NIH/NCRR Center of Biomedical Research Excellence in the Molecular Basis of Human Disease (P20-RR020171) and NIH/NIEHS Superfund Basic Research Program (P42-ES007380). The NIH Shared Instrumentation Grant S10RR023684 (to H.Z.) is acknowledged.

References

- Adler-Nissen, J. Elsevier Applied Science. Vol. 427. New York, NY: 1986. Enzymic Hydrolysis of Food Proteins.
- Blau S, Rubinstein A, Bass P, Singaram C, Kohen R. Differences in the reducing power along the rat GI tract: Lower antioxidant capacity of the colon. *Molecular and Cellular Biochemistry* 1999;194:185–191. [PubMed: 10391139]
- Boldyrev, A.; Johnson, P. Carnosine and related compounds: antioxidant dipeptides. In: Johnson, P.; Boldyrev, A., editors. *Oxidative Stress at Molecular, Cellular and Organ Levels*. Research Signpost; Kerala, India: 2002. p. 101-114.
- Carrier J, Aghdassi E, Platt I, Cullen J, Allard JP. Effect of oral iron supplementation on oxidative stress and colonic inflammation in rats with induced colitis. *Alimentary Pharmacology and Therapeutics* 2001;15:1989–1999. [PubMed: 11736731]
- Chen HM, Muramoto K, Yamauchi F, Nokihara K. Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. *Journal of Agricultural and Food Chemistry* 1996;44:2619–2623.
- Christen S, Peterhans E, Stocker R. Antioxidant activities of some tryptophan metabolites: possible implication for inflammatory diseases. *Proceedings of the National Academy of Sciences of the United States of America* 1990;87:2506–2510. [PubMed: 2320571]
- Cumby N, Zhong Y, Naczki M, Shahidi F. Antioxidant activity and water-holding capacity of canola protein hydrolysates. *Food Chemistry* 2008;109:144–148.
- Elias RJ, McClements DJ, Decker EA. Antioxidant activity of cysteine, tryptophan, and methionine residues in continuous phase β -lactoglobulin in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry* 2005;53:10248–10253. [PubMed: 16366723]

- Elias RJ, Kellerby SS, Decker EA. Antioxidant activity of proteins and peptides. *Critical Review in Food Science and Nutrition* 2008;48:430–441.
- Hernandez-Ledesma B, Amigo L, Ramos M, Recio I. Application of high-performance liquid chromatography-tandem mass spectrometry to the identification of biologically active peptides produced by milk fermentation and simulated gastrointestinal digestion. *Journal of Chromatography – A* 2004;1049:107–14. [PubMed: 15499922]
- Jung WK, Qian ZJ, Lee SH, Choi SY, Sung NJ, Byun HG, Kim SK. Free radical scavenging activity of a novel antioxidative peptide isolated from *in vitro* gastrointestinal digests of *Mytilus coruscus*. *Journal of Medicinal Food* 2007;10:197–202. [PubMed: 17472488]
- Kayashita J, Shimaoka I, Nakajoh M, Yamazaki M, Norihisa K. Consumption of buckwheat protein lowers plasma cholesterol and raises fecal neutral sterols in cholesterol-fed Rats because of its low digestibility. *The Journal of Nutrition* 1997;127:1395–1400. [PubMed: 9202097]
- Kim SK, Kim YT, Byun HG, Nam KS, Joo DS, Shahidi F. Isolation and characterization of antioxidative peptides from gelatin hydrolysate of Alaska pollack skin. *Journal of Agricultural and Food Chemistry* 2001;49:1984–1989. [PubMed: 11308357]
- Kitts DD, Weiler K. Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design* 2003;9:1309–1323. [PubMed: 12769739]
- Liu Z, Ishikawa W, Huang X, Tomotake H, Kayashita J, Watanabe H, Kato N. A buckwheat protein product suppresses 1,2-Dimethylhydrazine-induced colon carcinogenesis in rats by reducing cell proliferation. *The Journal of Nutrition* 2001;131:1850–1853. [PubMed: 11385078]
- Lo WMY, Li-Chan ECY. Angiotensin I converting Enzyme inhibitory peptides from *in vitro* pepsin-pancreatin digestion of soy protein. *Journal of Agriculture and Food Chemistry* 2005;53:3369–3376.
- Lu X, Zhu H. Tube-gel digestion: A novel proteomic approach for high throughput analysis of membrane proteins. *Mol Cell Proteomics* 2005;4:1948–1958. [PubMed: 16150870]
- Ma MS, Bae IY, Lee HG, Yang CB. Purification and identification of angiotensin I-converting enzyme inhibitory peptide from buckwheat (*Fagopyrum esculentum* Moench). *Food Chemistry* 2006;96:36–42.
- Ma Y, Xiong YL. Antioxidant and bile acid binding activity of buckwheat protein *in vitro* digests. *Journal of Agricultural and Food Chemistry*. 2009 (In press).
- Mendis E, Rajapakse N, Kim SK. Antioxidant properties of a radical-Scavenging peptide purified from enzymatically prepared fish skin gelatin Hydrolysate. *Journal Agriculture and Food Chemistry* 2005;53:581–587.
- Moore J, Yin JJ, Yu L. Novel Fluorometric assay for hydroxyl scavenging capacity (HOSC) estimation. *Journal of Agriculture and Food Chemistry* 2006;54:617–626.
- Nalini S, Ramakrishna BS, Mohanty A, Balasubramanian KA. Hydroxyl radical formation in human gastric juice. *Journal of Gastroenterology and Hepatology* 1992;7:497–501. [PubMed: 1327262]
- Qian ZJ, Jung WK, Byun HG, Kim SK. Protective effect of an antioxidative peptide purified from gastrointestinal digests of oyster, *Crassostrea gigas* against free radical induced DNA damage. *Bioresource Technology* 2008;99:3365–3371. [PubMed: 17904358]
- Re R, Pellegrini A, Proteggente A, Pannala M, Yang C, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine* 1999;26:1231–1237. [PubMed: 10381194]
- Tomotake H, Shimaoka I, Kayashita J, Nakajoh M, Kato N. Physicochemical and Functional Properties of Buckwheat Protein Product. *Journal of Agriculture and Food Chemistry* 2002;50:2125–2129.
- Wu HC, Pan BS, Chang CL, Shiau CY. Low-molecular-weight peptides as related to antioxidant properties of chicken essence. *Journal of Food and Drug Analysis* 2005;13:176–183.
- Yamada K, Miyazaki T, Shibata T, Hara N, Tsuchiya M. Simultaneous measurement of tryptophan and related compounds by liquid chromatography/electrospray ionization tandem mass spectrometry. *Journal of Chromatography B* 2008;867:57–61.
- Zhu LJ, Chen J, Tang X, Xiong YL. Reducing, radical scavenging and chelation properties of *in vitro* digests of Alcalase-treated zein hydrolysate. *Journal of Agriculture and Food Chemistry* 2008;56:2714–2721.

Fig. 1. Hydroxyl radical scavenging capacity of *in vitro* sequential digests of buckwheat protein. Means ($n = 3$) without a common letter differ significantly ($P < 0.05$). Sample solutions: 0.10 mg/mL protein.

Fig. 2. ABTS^{•+} scavenging capacity of *in vitro* sequential digests of buckwheat protein. Means (n = 3) without a common letter differ significantly ($P < 0.05$). Sample solutions: 0.159 mg/mL protein.

Fig. 3. Sephadex G-25 gel filtration of the final *in vitro* digest (180 min total digestion time) of buckwheat protein (n = 23), and regression plot of log MW vs. elution volume (n = 3).

Fig. 4. Hydroxyl radical scavenging capacity of gel filtration fractions of final *in vitro* digest (180 min total digestion time) of buckwheat protein. Means ($n = 3$) without a common letter differ significantly ($P < 0.05$). Sample solutions: 0.10 mg/mL protein.

Fig. 5. ABTS⁺• scavenging capacity of gel filtration fractions of the final *in vitro* digest (180 min total digestion time) of buckwheat protein. Means ($n = 3$) without a common letter differ significantly ($P < 0.05$). Sample solutions: 0.159 mg/mL protein.

Fig. 6. Extracted ion chromatograms (A, C, E, G, I) and tandem MS/MS spectra (B, D, F, H, J) of the prominent peptides present in gel filtration fractions IV (A–F), V (G; H), and VI (I; J). The peptides/amino acid were identified as Trp-Pro-Leu (B), Val-Pro-Trp (D), Val-Phe-Pro-Trp (F), Pro-Trp (H) and Trp (J).