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Fractionation and evaluation of radical scavenging peptides from *in vitro* digests of buckwheat protein ☆

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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-ethylbenzothiszoline-6-sulphonic acid) ($ABTS^{+\cdot}$) 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 $ABTS^{+\cdot}$ scavenging activity and were 23–27% superior to mixed BWP digest ($P < 0.05$). Fraction VI was most effective in neutralising ·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.

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 defence 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 that ·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 neutralise radicals, particularly ·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, Naczk, & Shahidi, 2008). Hydrolysed animal proteins, e.g., gelatin hydrolysate from Alaska polluck skin (Kim et al., 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), tumour 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 hypothesised that hydrolysis of buckwheat protein can release the peptide fragments capable of

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stabilising 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 stabilise ·OH and $ABTS^{+\cdot}$ 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 stabilise 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 neutralised with 0.1 M NaOH before lyophilisation. 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 → 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 "$D_{180\ min}$", was subjected to peptide fractionation using a low-pressure size exclusion chromatography with a 2.6 cm (dia.) × 70 cm (length) Sephadex G-25 fine column (Pharmacia XK 26/70, Piscataway, NJ, USA).

A 2 mg/mL of $D_{180\ min}$ solution, prepared from lyophilised 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 lyophilised. 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 evolution 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 three parameters) of the SigmaPlot Ver. 9 software (Systat Software Inc., Chicago, IL, USA), which yielded the following equation:

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

2.4. Radical scavenging activity (RSA)

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

The ·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 ·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:

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

2.4.2. $ABTS^{+\cdot}$ scavenging

The $ABTS^{+\cdot}$ scavenging ability was determined by the decolorization assay (Re et al., 1999). Briefly, $ABTS^{+\cdot}$ was generated by a mixed solution of 7 mM ABTS and 2.45 mM potassium persulphate. After