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Antioxidant properties of Australian canola meal protein hydrolysates

Adeola M. Alashi [a,b,d,*], Christopher L. Blanchard [a,c], Rodney J. Mailer [a], Samson O. Agboola [a,b], A. John Mawson [a,b], Rong He [d], Abraham Girgih [d], Rotimi E. Aluko [d]

[a] E. H. Graham Centre for Agricultural Innovation, Charles Sturt University, Locked Bag 588, Wagga, NSW 2678, Australia
[b] School of Agricultural and Wine Sciences, Charles Sturt University, Locked Bag 588, Wagga, NSW 2678, Australia
[c] School of Biomedical Sciences, Charles Sturt University, Locked Bag 588, Wagga, NSW 2678, Australia
[d] Department of Human Nutritional Sciences, The Richardson Center for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg R3T 2N2, Canada

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ABSTRACT

Antioxidant activities of canola protein hydrolysates (CPHs) and peptide fractions prepared using five proteases and ultrafiltration membranes (1, 3, 5, and 10 kDa) were investigated. CPHs had similar and adequate quantities of essential amino acids. The effective concentration that scavenged 50% (EC_{50}) of the $ABTS^{\cdot+}$ was greatest for the <1 kDa pancreatin fraction at 10.1 μg/ml. CPHs and peptide fractions scavenged $DPPH^{\cdot+}$ with most of the EC_{50} values being <1.0 mg/ml. Scavenging of superoxide radical was generally weak, except for the <1 kDa pepsin peptide fraction that had a value of 51%. All CPHs inhibited linoleic acid oxidation with greater efficiency observed for pepsin hydrolysates. The oxygen radical absorbance capacity of Alcalase, chymotrypsin and pepsin hydrolysates was found to be better than that of glutathione (GSH) ($p < 0.05$). These results show that CPHs have the potential to be used as bioactive ingredients in the formulation of functional foods against oxidative stress.

1. Introduction

Adding value to under-utilised crops in order to optimise their use for human consumption has become popular in recent times. Australian production of canola, an oilseed of the *Brassica* family (*Brassica napus* and *Brassica campestris*) has been on the increase and reached an estimated 3.13 million tons in 2012. Although canola meal obtained after oil extraction is the second largest animal feed meal produced after soybean meal (AOF Crop Report, 2012; USDA, 2010), its current use is mostly limited to the animal and aquaculture feed industries. However, canola meal could be used for the manufacture of products with higher value-added advantages when compared to its use as an animal feed ingredient. Some previous studies have focused on utilisation of canola protein for production of bioactive peptides (BAPs) with various health/nutritional functionalities with the aims of improving human health or to supply nutritional benefits (Cumby, Zhong, Naczk, & Shahidi, 2008; Marczak et al., 2003). BAPs can be released from the parent protein's primary structure by various methods such as fermentation and enzyme catalysed proteolysis (He et al., 2012; Udenigwe & Aluko, 2012). Enzymatic hydrolysis of proteins is a simple and inexpensive method to convert a protein into free amino acids and short-chain peptides. The peptide products are much more water soluble than the original protein yet their amino acid profile could remain essentially unchanged or may be enhanced in some fractions (Cumby et al., 2008).

Antioxidants are substances that in small quantities are able to retard the oxidation of easily oxidised materials such as unsaturated fats whilst preventing the excessive accumulation of free radicals or reactive oxygen species (ROS). Free radicals are generated by the physiological processes that occur naturally in the body and by external sources such as excessive exposure to sunlight or smoking and have been linked to several degenerative diseases (Udenigwe & Aluko, 2012). Though these physiological processes are in themselves not harmful, excess free radical production beyond the body's ability to cope with them can lead to immune system impairment. Oxidative stress has also been linked to cardiovascular diseases, hypertension, cancer, and other ailments (Paravicini & Touyz, 2008). Peptide antioxidants have simpler structures than their parent proteins. This confers greater stability in different situations (e.g. heat, and exposure to proteases), they have no hazardous immunoreactions and often exhibit enhanced nutritional and functional properties in addition to their antioxidant activity (Xie, Huang, Xu, & Jin, 2008).

* Corresponding author at: School of Agricultural and Wine Sciences, Charles Sturt University, Locked Bag 588, Wagga, NSW 2678, Australia. Tel./fax: +61 269332539.
E-mail address: aalashi@csu.edu.au (A.M. Alashi).

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In this study, canola protein hydrolysates obtained by enzymatic hydrolysis using five proteases (Alcalase, chymotrypsin, pepsin, trypsin and pancreatin) were evaluated for scavenging of free radicals and ROS. The aim was to elucidate the antioxidant properties of enzymatic protein hydrolysates of Australian grown canola using various *in vitro* tests and also to determine the effect of peptide size on the measured parameters.

2. Materials and methods

2.1. Materials

Canola meal was a gift from the Cootamundra Oil Seed Pty, NSW, Australia, Alcalase 2.4L, pepsin, chymotrypsin, trypsin, DPPH (2,2 diphenyl-1 picrylhydrazyl radical), ABTS [2,2′-azinobis (3-ethyl-benzothiazoline-6-sulphonate)], AAPH [2,2′-azobis(2-methylpropionamidine)] dihydrochloride, and reduced glutathione (GSH) were purchased from Sigma Aldrich (Sigma Chemicals, St. Louis, MO). Ultrafiltration membranes with 1, 3, 5 and 10 kDa molecular weight cut-off (MWCO) sizes were purchased from Fisher Scientific (Oakville, ON, Canada).

2.2. Sample preparation

2.2.1. Extraction of canola protein isolate (CPI)

Defatted canola meal (20 g) was suspended in 200 ml of 0.1 M NaOH pH 12.0 and extracted by stirring for 1 h at room temperature and then centrifuged at 18 °C and 3000*g* for 10 min. Two additional extractions of the residue from the centrifugation process were carried out with the same volume of 0.1 M NaOH. The supernatants were pooled, adjusted to the isoelectric point (pH 4.0) using 0.1 M HCl solution, centrifuged (3000*g* for 10 min) and the precipitate recovered. The precipitate was washed with distilled water, adjusted to pH 7.0 using 0.1 M NaOH, freeze-dried as the CPI and stored at −20 °C until required for further analysis.

2.2.2. Enzymatic hydrolysis of CPI

CPI was hydrolysed in batches using five food grade enzymes at an enzyme substrate ratio of 1:20 for all the enzymes, to obtain canola protein hydrolysates (CPHs) after 4 h of incubation. The following hydrolysis conditions were used: Alcalase (pH 8.0 and 60 °C), chymotrypsin (pH 8.0 and 37 °C); pepsin (pH 3.0 and 37 °C); trypsin (pH 8.0 and 37 °C) and pancreatin (pH 8.0 and 40 °C). The pH was maintained for each hydrolysis process using either 1 M NaOH or 1 M HCl as appropriate, while the temperature was maintained using a thermostat. After the 4 h digestion period, the enzymes were inactivated by heating and holding at 85 °C for 15 min. The resulting CPHs were lyophilised and stored at −20 °C until required for further analysis.

2.2.3. Membrane fractionation

The CPHs were fractionated using an ultrafiltration stirred cell Amicon® 8400 (Millipore Corp., Billerica, MA, USA). The molecular cut-off weights (MWCO) of the membranes used sequentially were 1, 3, 5 and 10 kDa, respectively. Thus, permeate from the 1 kDa membrane (<1 kDa) concentration step was collected and lyophilised while the retentate was passed though the 3 kDa; the permeate (1–3 kDa) was collected and the retentate passed through the 5 kDa membrane to again collect the permeate (3–5 kDa). Finally the retentate from the 5 kDa membrane was passed through the 10 kDa membrane to collect the permeate (5–10 kDa), while the retentate was discarded. All permeates were lyophilised and stored at −20 °C until required for further analysis. Percentage protein contents of the CPI, CPHs and membrane ultrafiltration permeates were determined using the modified Lowry method (Markwell, Haas, Biebar, & Tolbert, 1978).

2.3. Determination of the amino acid composition

The amino acid profile of each sample was determined according to the established methods described by Girgih, Udenigwe, and Aluko (2011) using a HPLC system after hydrolysis with 6 M HCl. The cysteine and methionine contents were determined after performic acid oxidation while the tryptophan content was determined after alkaline hydrolysis.

2.4. Determination of antioxidant properties

Five separate estimates of antioxidant performance were made for each sample based on their protein contents as follows.

2.4.1. ABTS radical scavenging activity

This assay is based on the percentage inhibition of the peroxidation of the ABTS radical, which is observed as a discoloration of a blue green colour (734 nm). The reaction was carried out according to a previously described method (Arts, Sebastiaan Dallinga, Voss, Haenen, & Bast, 2004) with slight modifications. Briefly, $ABTS^{\cdot+}$ was prepared by dissolving 7 mM ABTS and 2.45 mM potassium persulphate in phosphate buffered saline (PBS), pH 7.4 and allowing this to stand in the dark for 16 h to generate the ABTS radical cation ($ABTS^{\cdot+}$). For the analysis, the $ABTS^{\cdot+}$ stock was diluted using PBS buffer and equilibrated at 30 °C to an absorbance of 0.7 ± 0.02 at 734 nm using a Helios λ thermo spectrophotometer (Electron Corporation Helios Gamma, England). Trolox was dissolved in 80% ethanol. The antioxidant capacity was measured by mixing 200 μL of samples with 2 mL of $ABTS^{\cdot+}$ solution and the decline in absorbance was observed for 5 min. Appropriate blanks were run for each sample and the radical scavenging capacity was compared to that of Trolox (6.25–200 μM) and results were expressed as mM Trolox equivalent (TE) per gram of sample on protein equivalent basis. The percentage $ABTS^{\cdot+}$ scavenged was calculated using the following equation:

$$\text{Percentage } ABTS^{\cdot+} \text{scavenged} = \frac{A_i - A_f}{A_i} \times 100,$$

where A_i and A_f are initial and final absorbance of the sample, respectively.

The effective concentration that scavenged 50% of the free radicals (EC_{50}, $ABTS^{\cdot+}$) was calculated for each sample by non-linear regression from a plot of percentage $ABTS^{\cdot+}$ scavenged versus sample concentration (μg/ml).

2.4.2. DPPH radical scavenging activity

The scavenging activity of CPH and its fractions against the DPPH radical was determined using the method described by Girgih et al. (2011) with slight modifications for a 96-well flat bottom plate. Samples were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 containing 1% (v/v) Triton-X. DPPH was dissolved in methanol to a final concentration of 100 μM. A 100 μL aliquot of each sample was mixed with 100 μL of the DPPH radical solution in a 96-well plate to final concentrations of 0.2–1 mg/mL and incubated at room temperature in the dark for 30 min. The buffer was used in the blank assay while GSH served as the positive control. Absorbance was measured at 517 nm using a spectrophotometer and the percentage DPPH radical scavenging activity was determined using the following equation:

$$\text{DPPH radical scavenging activity } (\%) = \left(\frac{Ab - As}{Ab}\right) \times 100,$$

where *Ab* and *As*, are absorbance of the blank and sample, respectively.