Author(s): Agboola, S. ; Alashi, A.M. ; Aluko, R. ; Blanchard, C.L. ; Girgih, A. ; Mailer, R.J. ; Mawson, A.J. ; Rong, H.

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Antioxidant properties of Australian canola meal protein hydrolysates

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

a. *E. H. Graham Centre for Agricultural Innovation,*

b. *School of Agricultural and Wine Sciences, Charles Sturt University and*

c. *School of Biomedical Sciences, Charles Sturt University, Locked Bag 588, Wagga Wagga, NSW 2678, Australia*

d. *Department of Human Nutritional Sciences and*

The Richardson center for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg Canada, R3T 2N2

[*] Corresponding Author:

Adeola M. Alashi

School of Agricultural and Wine Sciences, Charles Sturt University Locked Bag 588, Wagga Wagga, NSW 2678, Australia.

Email: aalashi@csu.edu.au; Telephone/Facsimile: +61269332539

Abstract

Antioxidant activities of canola protein hydrolysates (CPHs) and peptide fractions prepared using five proteases and ultrafiltration membranes (1, 3, 5, & 10 kDa) were investigated. CPHs had similar and adequate quantities of essential amino acids. The effective concentration that scavenged 50% (EC_{50}) of the $ABTS^{\bullet+}$ was greatest for the <1 kDa pancreatin fraction at 10.1 µg/ml. CPHs and peptide fractions scavenged $DPPH^{\bullet+}$ 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.

Key words: Canola; antioxidant properties, protein hydrolysates, membrane ultrafiltration, radical scavenging activities, trolox equivalent antioxidant capacity (TEAC)

1. Introduction

Adding value to under-utilized crops in order to optimize 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 utilization 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 (Marczak et al., 2003; Cumby, Zhong, Naczk, & Shahidi, 2008). BAPs can be released from the parent protein's primary structure by various methods such as fermentation and enzyme catalyzed 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 oxidized 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 et al., 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)

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-sulfonate)], AAPH [2,2'-azobis(2-mthylpropionamidine)] 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 18ºC 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 -20ºC until required for further analysis.

2.2.2. Enzymatic hydrolysis of CPI

CPI was hydrolyzed 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 60ºC), chymotrypsin (pH 8.0 and 37ºC); pepsin (pH 3.0 and 37ºC); trypsin (pH 8.0 and 37°C) and pancreatin (pH 8.0 and 40ºC). 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 85ºC for 15 min. The resulting CPHs were lyophilized and stored at -20ºC 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 lyophilized 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 lyophilized and stored at -20^{o}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, Bieber, & 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, & Aluko (2011) using an 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 color (734 nm). The reaction was carried out according to a previously described method (Arts, Sebastiaan Dallinga, Voss, Haenen, & Bast, 2004) with slight modifications. Briefly, $ABTS^{\bullet+}$ 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 hours to generate the ABTS radical cation ($ABTS^{\bullet+}$**)**. For the analysis, the $ABTS^{\bullet+}$ stock was diluted using PBS buffer and equilibrated at 30^{o}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 $ABTS^{\bullet+}$ 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 $ABTS^{\bullet+}$ scavenged was calculated using the following equation:

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

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}, $ABTS^{\bullet+}$) was calculated for each sample by non-linear regression from a plot of percentage $ABTS^{\bullet+}$ 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:

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

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

The effective concentration that scavenged 50% of the free radicals ($EC_{50,}$ DPPH) was calculated for each sample by non-linear regression from a plot of % DPPH activity versus sample concentration (mg/mL).

2.4.3. Superoxide radical scavenging activity (SRSA)

The method described by Xie et al. (2008) was used to determine SRSA. Samples (1 mg/mL final concentration) were each dissolved in 50 mM Tris-HCl buffer, pH 8.3 containing 1 mM EDTA and 80 µL was transferred into a clear bottom microplate well; 80 µL of buffer was added to the blank well. This was followed by addition of 40 µL 1.5 mM pyrogallol (dissolved in 10 mM HCl) into each well in the dark and the change in the rate of reaction was measured immediately at room temperature over a period of 4 min using a spectrophotometer at a wavelength of 420 nm. The superoxide scavenging activity was calculated using the following equation:

$$\text{Superoxide scavenging activity (\%)} = \left(\frac{(\Delta A/min)b - (\Delta A/min)s}{(\Delta A/\text{min})b}\right) * 100$$

Where *b* and *s* are blank and sample, respectively.

2.4.4. Inhibition of linoleic acid oxidation

Linoleic acid oxidation was measured using the method described by He, Girgih, Malomo, Ju, & Aluko (2013). Samples (at final concentrations of 1 mg/mL) were dissolved in 1.5 mL of 0.1 M sodium phosphate buffer, pH 7.0. A 1 mL aliquot of 50 mM linoleic acid (dissolved in 95% ethanol) was added to the samples and blank (buffer). The mixtures were incubated at 60^{o}C under dark conditions for 7 days. The degree of color development was measured as follows at intervals of 24 h. A 100 µL aliquot of the assay mixture above was transferred into a reaction tube to which 4.7 mL of 75% (v/v) ethanol, 100 µl of 30% (w/v) ammonium thiocyanate and 100 µL of 0.02 M ferric chloride dissolved in1 M HCl was added. After shaking and incubating at room temperature for 3 min, 200 µL was transferred into a clear-

bottom 96-well plate and the absorbance measured at 500 nm using a spectrophotometer. An increase in absorbance value implied an increase in the level of linoleic acid oxidation. The percentage inhibition of linoleic acid was calculated using the following equation:

$$\text{Inhibition of linoleic acid (\%)} = \left(\frac{1 - As}{Ab}\right) \times 100$$

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

2.4.5. Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed according to the method described by Girgih, Udenigwe, Hasan, Gill, & Aluko, (2013), with slight modifications. Briefly, 120 µL fluorescein (0.008 µM) and 20 µL sample (50 µg/mL) or Trolox (6.25-50 µM) dissolved in 75 mM phosphate buffer, pH 7.4) were transferred into 96-well microplate wells and incubated at 37ºC for 20 min in a spectrofluorimeter (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA). After adding 60 µL of AAPH (150 mM) to the microplate, the fluorescence was recorded under constant shaking at 1 min intervals for 60 min at excitation and emission wavelengths of 485 and 530 nm, respectively (slit widths 9 nm). Fluorescence measurements were normalized to the curve of the blank (no antioxidant). From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as follows:

$$\text{AUC} = 1 + \sum_{i=1}^{i=60} f_i / f_0$$

Where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. The net AUC of the sample was calculated by subtracting the AUC of the blank. Regression equations obtained from the net values of Trolox were used to calculate the ORAC value for each sample. Final ORAC values for all the samples were expressed as micromoles of Trolox equivalent (TE) per g of their protein equivalent.

2.5. Statistical analysis

Experiments were performed in triplicates, based on the protein equivalent of each sample and the results are expressed as means ± standard deviation. Results were subjected to one way analysis of variance (ANOVA) using the SAS® system for Windows® V8(TS M1). Statistical significance of differences between samples were accepted at ($p<0.05$) using the Duncan's multiple range test.

3. Results and discussion

3.1. Protein content and yield of membrane fractions

The percentage proteins in CPI used in this study was 76.2 %. The protein content and yield (i.e. the amount of protein recovered from the fractionation process for each membrane filtration) of CPHs and fractions are reported in Table 1. The protein content was found to increase with increasing fraction size up to 3-5 kDa, with a significant decrease ($p<0.05$) noticed for the 5-10 kDa fractions for all the enzyme types used except for pepsin where there was no significant difference between the 3-5 and 5-10 kDa fractions. This trend was however reversed for the protein yield, as lower yields were obtained as the MW increased, however, 1-3 kDa had higher protein yields than the <1 kDa fractions except for Alcalase. This is expected, as there was a strong positive correlation between all the protein contents and the yields for all the fractions. A significantly higher difference ($p<0.05$) was also observed between the protein content of the 1-3 kDa fractions for these enzymes when compared to the <1 kDa fractions. All the hydrolysates had protein contents ≥50% with pepsin hydrolysates being the lowest at 58.9%, while chymotrypsin and trypsin had the highest at 85.7 and 85.5%, respectively. These values are lower than those obtained from peanut protein hydrolysates using Alcalase after extended prolonged period of hydrolysis, but higher than that recorded for rapeseed 3 h Alcalase hydrolysates (Jamdar et al., 2010; Mäkinen, Johannson, Gerd, Pihlava, & Pihlanto, 2012). The >50% yields obtained indicate

that most of the proteins were susceptible to enzymatic hydrolysis and could be converted into peptide products, which would be beneficial economically for industrial purposes. Percentage yield is a parameter that can also be used to determine the efficiency of the hydrolysis process (Girgih et al., 2011). Pepsin and pancreatin hydrolysates had the lowest yields of 55.0 and 55.2% respectively. This is probably due to the nature of the enzymes and the condition of hydrolysis since pancreatin is a mixture of endo- and exoproteases, which probably led to high levels of free amino acids that were not measured by the method used for protein content determination. Pepsin is an endoprotease and is most efficient at cleaving bonds involving the aromatic amino acids phenylalanine, tryptophan and tyrosine; this specificity could have limited the rate of canola protein hydrolysis by pepsin and hence lowered the hydrolysate yield. The percentage yield of protein hydrolysates is not commonly reported in literature; therefore adequate comparison could not be made. In this study, percentage yield for the <1 kDa fractions were higher than those obtained for flaxseed <1 kDa fractions (Udenigwe, Lin, Hou, & Aluko, 2009) except for the pepsin hydrolysed fraction, even though the protein contents obtained were higher. The results show that canola proteins were better hydrolysed by the enzymes when compared to flaxseed protein. High yield has also been attributed to both efficiency of the hydrolysis process and economic viability for commercialization purposes (Girgih et al., 2011).

3.2. Amino acid composition of CPI and CPHs

The composition of the amino acids in protein hydrolysates has been shown to play an important role with respect to their antioxidant activities (Udenigwe & Aluko, 2011; Zhao et al., 2012). For example, hydrophobic amino acids (HAA) act as antioxidants by increasing the solubility of peptides in lipids and thereby facilitating better interaction with the free radicals that cause oxidative damage (Rajapakse, Mendis, Jung, Je, & Kim, 2005). Table 2,

shows the amino acid composition of canola protein isolate and its enzymatic hydrolysates. The HAA comprised about 40% of total amino acids for all the hydrolysates, which is similar to the values obtained for rapeseed protein hydrolysates (Zhang, Wang, & Xu, 2008; Pan, Jiang, & Pan, 2009). Cysteine was found to be the limiting amino acid in all the samples. This is typical of legume seed proteins which are usually deficient in sulfur-containing amino acids (Moure, Sineiro, Domínguez, & Parajó, 2006). In contrast, the high levels of lysine and leucine observed in the canola protein hydrolysates could enhance scavenging of superoxide radicals (Udenigwe et al., 2011). Threonine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine and lysine values were also found to be higher than the FAO/WHO recommendations for animal proteins (Table 2), indicating that the canola proteins are of high nutritional quality and may be used as a protein source in human diet. Overall, the amino acid composition of the protein hydrolysates reflected those of the CPI, which suggests that the protein hydrolysis process did not have a negative effect on the amino acid composition of the hydrolysates.

3.3. ABTS radical scavenging activity

The $ABTS^{\bullet+}$ radical scavenging activity, expressed as the trolox equivalent antioxidant capacity (TEAC), measures the antioxidant activity of a substance as compared to the standard trolox (Samaranayaka & Li-Chan, 2011). This assay is widely used to analyze phenolic compounds from plant extracts and other phytochemicals but only to a lesser extent used for peptides from plant proteins. Fig. 1 shows that the EC_{50} (μg/mL) for peptide scavenging of $ABTS^{\bullet+}$ was significantly ($p<0.05$) lower (<25 μg/mL) when compared to that of CPI (85.1 μg/mL). However, glutathione reduced (GSH) had the lowest ($p<0.05$) value at 1.6 μg/mL. Therefore, the CPHs have superior $ABTS^{\bullet+}$ scavenging ability when compared to the unhydrolyzed CPI. The 4-6 fold difference between the unhydrolyzed and hydrolyzed

samples is an indication that antioxidant peptides were released from CPI during enzyme digestion and the peptides are able to donate hydrogen atoms for $ABTS^{\bullet+}$ reduction. Alcalase and chymotrypsin hydrolysates were generally found to perform better as $ABTS^{\bullet+}$ scavengers with lower EC_{50} values than pepsin, trypsin and pancreatin hydrolysates. However, the pancreatin <1 kDa permeate peptides fraction with an EC_{50} value of 10.1 μg/mL was the most effective ($p<0.05$) $ABTS^{\bullet+}$ scavenger among the fractions. The <1 and 1- 3 kDa permeate peptides from Alcalase and chymotrypsin hydrolysates also had low $ABTS^{\bullet+}$ scavenging values (11.2-14.4 μg/mL). The EC_{50} values obtained for the Alcalase hydrolysate are lower than that reported for Alcalase hydrolysed amaranth protein (22.4 μg/mL), and generally, CPHs and fractions are also better than the other hydrolysates reported for amaranth protein (Tironi & Ãnon, 2010). The $ABTS^{\bullet+}$ EC_{50} values obtained in this study, are also lower than those reported for cocoa seed proteins (Preza et al., 2010). Generally for each CPH, the smaller-sized peptides (<3 kDa) had significantly ($p<0.05$) lower EC_{50} values and hence stronger potency as $ABTS^{\bullet+}$ scavengers when compared to the larger-sized peptides (>3 kDa).

3.4 DPPH radical scavenging activity

The EC_{50} values for DPPH radical scavenging activity of CPH and its membrane-separated fractions (Fig. 2a) show that Alcalase and its derived fractions scavenged the radical to a 50% inhibition at a range of 0.5-0.9 mg/mL for all the hydrolysate and fractions. The DPPH EC_{50} values obtained for the Alcalase hydrolysates in this work are better than the IC_{50} values obtained for Alcalase hydrolyzed *Ruditapes philippinarum* (marine bivalve molluscs) peptides but similar to the chymotrypsin hydrolysed fractions (Kim et al., 2013). Pepsin hydrolysed <1 kDa fraction had the lowest EC_{50} values amongst all the samples. GSH was used as a positive control, and had an EC_{50} value of 0.18 mg/mL in this study. The

scavenging activity of CPH and its fractions reveals that the peptides were able to scavenge effectively the DPPH radical. DPPH radical is a stable free radical that shows maximal absorbance at 517 nm in methanol. When DPPH encounters a proton-donating substance (H^+), the radical is scavenged and the rich purple color of the DPPH is reduced to a pale yellow color, apparently because the DPPH radical accepts an electron to become a stable diamagnetic molecule (Liu, Kong, Xiong, & Xia, 2010). The fractions analysed had a significantly lower EC_{50} value ($p<0.05$) than their parent hydrolysates with all the enzymes used except for pepsin hydrolysates which had a higher scavenging activity than its 3-5 and 5-10 kDa fractions. This indicates that that the lower molecular weight (LMW) peptides had a greater DPPH scavenging activity. LMW peptides have been shown to exhibit higher antioxidant activity, as recorded for both hemp protein fractions and barley hordein hydrolysates (Bamdad, Wu, & Chen, 2011; Girgih et al., 2011). Although, the effect was varied amongst the fractions, depending on the enzyme, chymotrypsin, pepsin and trypsin recorded lower EC_{50} values for <1 kDa fractions while Alcalase and pancreatin showed the lowest values in the 5-10 kDa fractions. The scavenging activity, was also found to be concentration dependent (data not shown) which is in agreement with similar studies for canola, rapeseed and soybeans hydrolysates (Cumby et al., 2008; Yoshie-Stark, Wada, & Wäsche, 2008). The EC_{50} values for Alcalase were found to be similar to those reported for rapeseed protein hydrolysates and fractions (Pan et al., 2009; He et al., 2013). Generally, the CPH samples recorded better EC_{50} values than those for hempseed protein hydrolysates (Tang, Wang, & Yang, 2009).

3.5. Superoxide radical scavenging activity

Superoxide is generated in biological systems during the normal catalytic function of a number of enzymes and during the oxidation of hemoglobin. It is also useful for testing the

antioxidant activity of hydrophilic and lipophilic compounds (Moure, Domínguez, & Parajó, 2006a). The low cysteine value (Table 2) could have a positive effect because a previous work has shown that sulfur-containing amino acids contribute negatively to superoxide scavenging properties of protein hydrolysates (Udenigwe et al., 2011). Canola protein hydrolysates and their membrane-separated fractions showed different SRSA based on the different proteases used at 1 mg/mL final concentration (Fig. 2b). Alcalase, chymotrypsin and their membrane-separated fractions all had less than 30% activity, except for pepsin <1 kDa fraction with a SRSA value of 51.3%, which is close to that of GSH (59.5%). Trypsin and pancreatin hydrolysates and most of their derived fractions had significantly higher SRSA values than those exhibited by the corresponding Alcalase, chymotrypsin and pepsin samples. Ultrafiltration recovered soy protein fractions, as well as Alcalase-hydrolyzed rapeseed and soy protein hydrolysates, had higher SRSA values than those values obtained in this study (Moure et al., 2006a; Pan et al., 2009).

3.6. Inhibition of linoleic acid oxidation

Lipid peroxidation is an oxidative chain reaction in which one lipid molecule after another becomes oxidized to the maximum possible extent so as to form lipid peroxides (Cai, 2005). The role of antioxidants during lipid peroxidation is to reduce the peroxyl radical to the hydro-peroxide before it can propagate the radical chain (Schneider, 2009). It was observed (Fig. 3) that over the 7 days incubation period, GSH was able to inhibit the oxidation of linoleic acid consistently as revealed by the negligible peroxide formation from the 5th to 7th day and a mean inhibition level of 98%. The inhibition effect of peptic hydrolysate was closest to that of GSH with 82% inhibition when compared to that of the uninhibited (blank) reaction. In contrast, the uninhibited reaction had two days of absorbance increases, which indicates peroxidation of linoleic acid before absorbance gradually decreased, though the

values were still higher than those of inhibited reactions. This has been attributed to the instability and decomposition of hydrogen peroxide to other secondary oxidation products, which lead to a decrease in the absorbance, after prolonged incubation (Chen, Zhao, Zhao, Cong, & Bao, 2007). The decrease in absorbance observed due to the formation of the blood red color from ferric thiocyanate, is obtained after ferrous sulphate and the oxidized products reacts to form ferric sulphate. Other hydrolysates such as pancreatin, trypsin and Alcalase were also very effective inhibitors with >50% inhibition of linoleic acid oxidation over the course of 7 days, showing that they were able to reduce the peroxyl radical to hydroperoxides. The samples generally exhibited a significantly higher percentage inhibition ($p<0.05$) on day 2 when compared to day 5 (Fig 3 insert), except for GSH where no difference was found between percentage inhibition on day 2 and 5. This may be attributed to the gradual loss in their ability to adequately reduce the linoleic peroxyl radical with continuous incubation, due to the slow formation of peroxidation of linoleic acid in the system. However, CPHs showed better linoleic acid oxidation inhibition when compared to peanut protein hydrolysates, which only showed inhibition activity at higher concentrations (Chen et al., 2007). The unhydrolyzed CPI and chymotrypsin hydrolysate were the least effective as shown by their <50% level of inhibition after day 5 of incubation (Fig 3). These results are comparable to those of previously reported studies from hempseed and rapeseed protein hydrolysates, which also showed effective inhibition of linoleic acid oxidation (Girgih et al., 2011; He, Girgih, Malomo, Ju, & Aluko, 2013). After day 5 of incubation the percentage linoleic inhibition reported in this work (Fig 3 insert) were 55-98% for GSH, and the hydrolysates, which are higher than the values (15-53% inhibition) reported for sardinella by-product protein hydrolysates during a similar incubation period (Bougatef et al., 2010).

3.7. Oxygen Radical Absorbance Capacity

To successfully measure the antioxidant capacity of any antioxidant sample, it is important to use more than one method, taking into account the various modes of actions of antioxidants and the conditions of the measuring system (Samaranayaka et al., 2011). In this study, ORAC values of CPHs and membrane-separated fractions were expressed as μM Trolox equivalent per gram of protein (Fig. 4). This assay follows a hydrogen atom transfer (HAT) mechanism (the H of the peptides neutralizes the radicals formed), therefore measuring the capacity of the antioxidant (peptide sample) to break the chain reactions of thermally generated peroxyl radical from AAPH (Silva, Souza, Rogez, Rees, & Larondelle, 2007). All the samples tested exhibited ORAC values which ranged from 159 to >2200 μM TE/g protein, representing a 14-fold variation amongst the samples tested. The fractions that exhibited the highest ORAC values with no significant difference ($p<0.05$) were Alcalase <1 kDa and 1-3 kDa. chymotrypsin <1 kDa and pepsin 3-5 kDa fractions exhibited slightly lower values in the range 2000-2200 μM TE/g with no significant difference. These values are however higher than those obtained for defatted peanut hydrolysates and its fractions (1160-952 TE/g) (Zheng et al., 2012). The ORAC values obtained for Alcalase and chymotrypsin hydrolysates, their <1 and 1-3 kDa fractions and pepsin hydrolysed <1 and 3 kDa fractions are higher than the value obtained for GSH which is an important antioxidant *in vivo*. Trypsin hydrolysate and membrane fractions showed the lowest ORAC values ranging from 160 to 930 μM TE/g, followed by pancreatin hydrolysates and its fractions (340 to 1490 μM TE/g). The ORAC values obtained for trypsin and pancreatin hydrolysates are however comparable to those obtained for milk peptides (63-1489 μM TE/g) that were prepared by hydrolysis with microbial proteases (Hogan, Zhang, Li, Wang, & Zhou, 2009). Alcalase, chymotrypsin, pepsin and their fraction also showed higher ORAC values than those reported for salmon protein hydrolysates and its RP-HPLC fractions (Girgih et al., 2013). The results clearly show that canola protein hydrolysates have a high potential to disrupt reactions that involve peroxyl

radicals, which potentially has physiological relevance for health promotion or prevention of free radical-induced chronic diseases.

4.0. Conclusions

In this study, the scavenging of free radicals was mostly dependent on the peptide size of the CPHs with LMW peptides being more effective. Antioxidant properties of the CPHs were not influenced by amino acid composition, which suggests greater influence of specific sequences of amino acids on the peptide chains. The peptides showed a high potential to act as antioxidants, based on their effectiveness against various free radicals. The antioxidant activities of < 1 kDa pepsin hydrolysed fractions for $DPPH^{\bullet+}$, and superoxide scavenging activities, followed the same trend as the antioxidant activity exhibited by GSH. Overall, the pepsin <1 kDa peptide fraction seem to have the best antioxidant activity and should be subjected to future studies that will identify the amino acid sequence. This would be beneficial in the formulation of functional foods and nutraceutical that can reduce radical chain reactions or inhibit the reactive oxidants that initiate oxidative stress. They can also be used in the food manufacturing industry to prevent the onset of rancidity in foods containing fats.

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List of figures

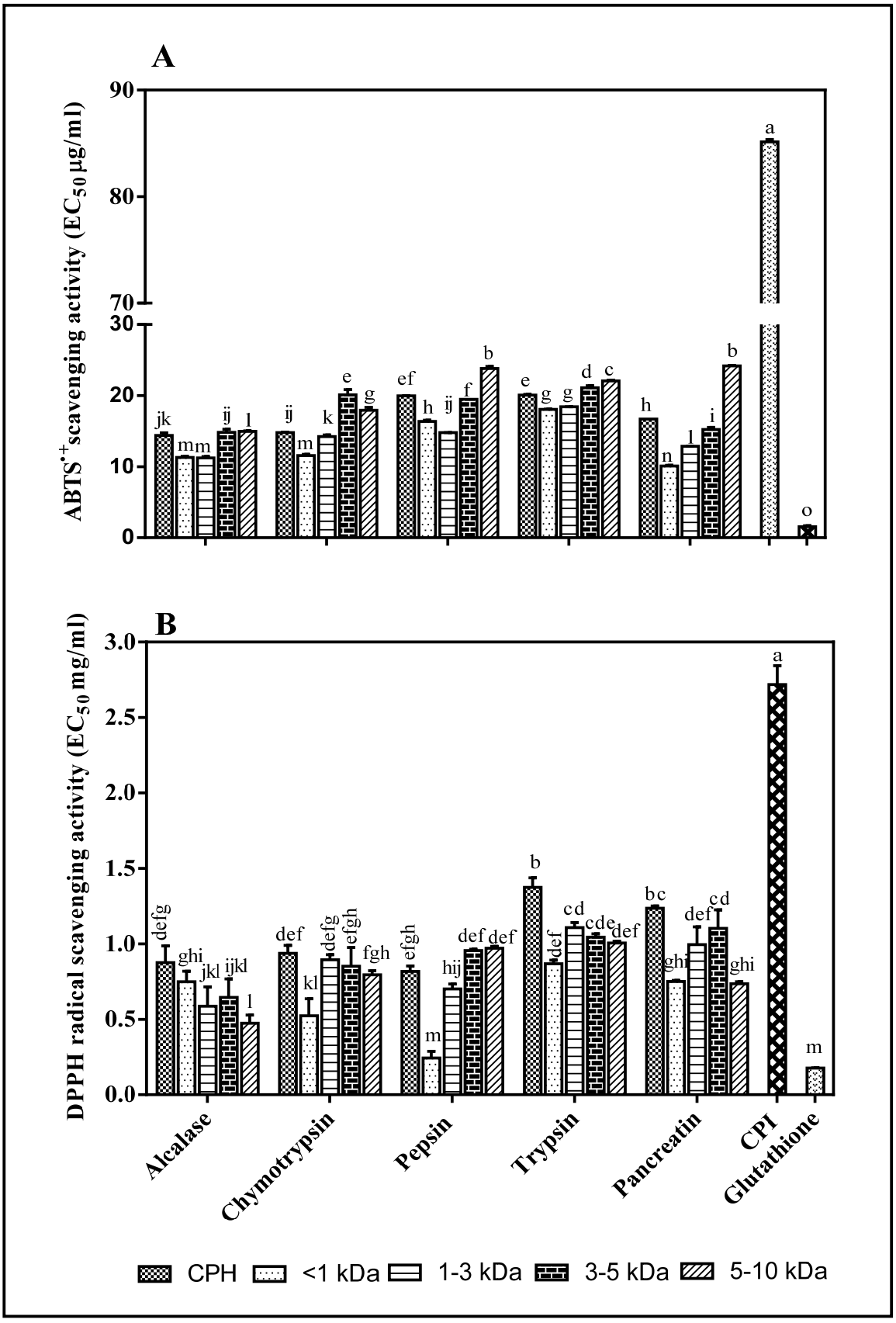

A
ABTS•+ scavenging activity (EC50 μg/ml)
90
80
70
30
20
10
0
jk mm ij l ij m k e g ef h ij f b e g g d c h n l i b a o
B
DPPH radical scavenging activity (EC50 mg/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
defg ghi jkl ijkl l def kl defg efgh fgh efgh m hij def def b def cd cde def bc ghi def cd ghi a m
Alcalase
Chymotrypsin
Pepsin
Trypsin
Pancreatin
CPI
Glutathione
CPH
<1 kDa
1-3 kDa
3-5 kDa
5-10 kDa

Fig. 1

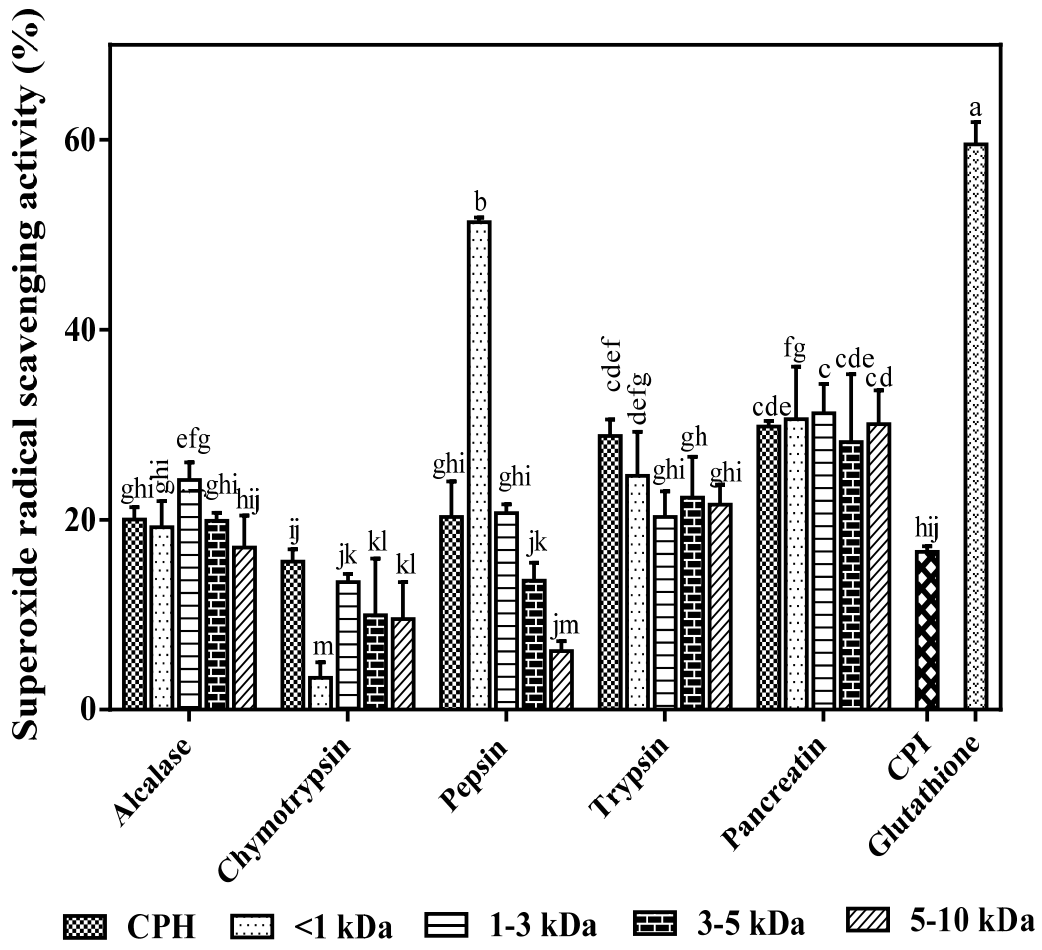
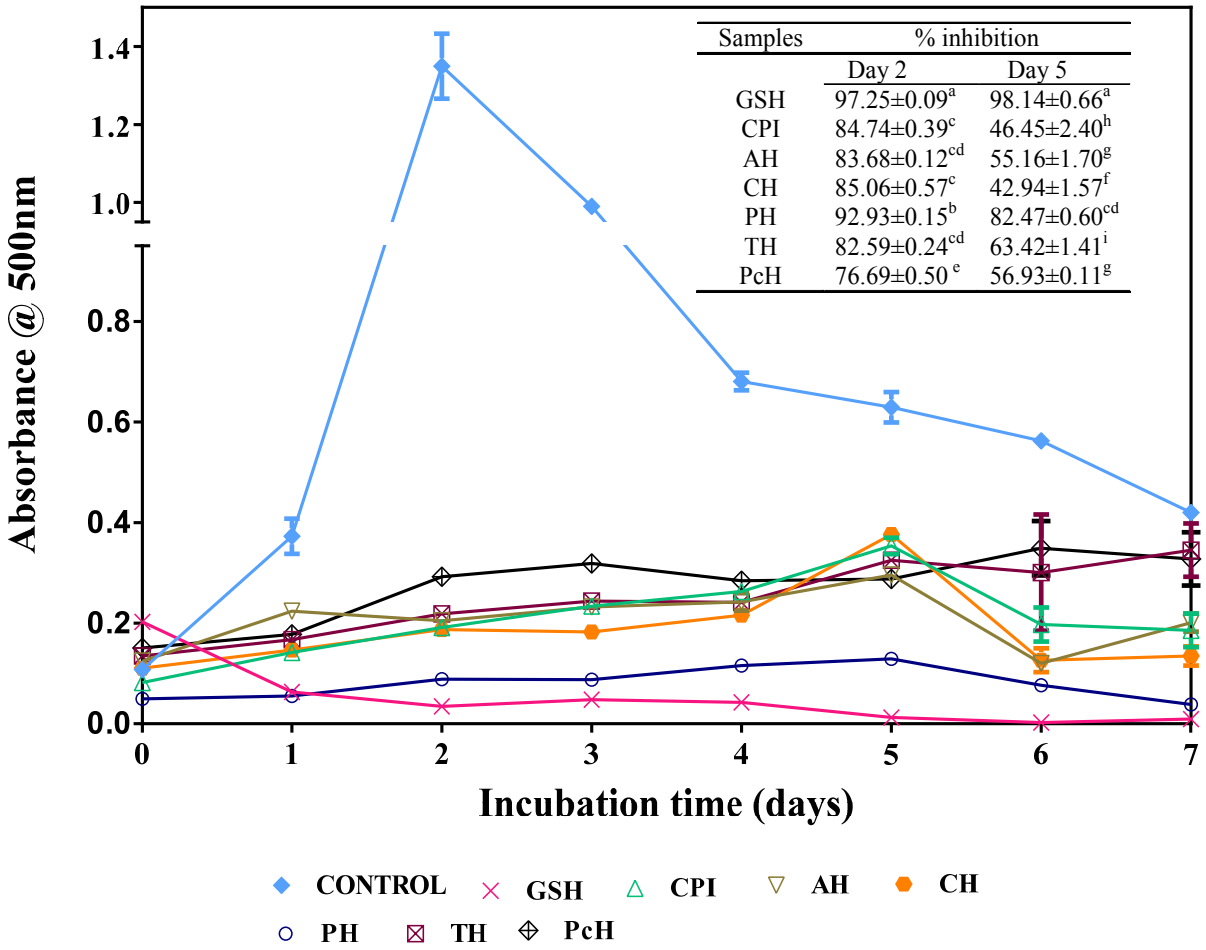

Superoxide radical scavenging activity (%)
0
20
40
60
ghi
hi
efg
ghi
hij
ij
m
jk
kl
kl
ghi
b
ghi
jk
jm
cdef
defg
ghi
gh
ghi
cde
fg
c
cde
cd
hij
a
Alcalase
Chymotrypsin
Pepsin
Trypsin
Pancreatin
CPI
Glutathione
CPH
<1 kDa
1-3 kDa
3-5 kDa
5-10 kDa

Fig. 2



Samples	% inhibition	
	Day 2	Day 5
GSH	97.25±0.09^{a}	98.14±0.66^{a}
CPI	84.74±0.39^{c}	46.45±2.40^{h}
AH	83.68±0.12^{cd}	55.16±1.70^{g}
CH	85.06±0.57^{c}	42.94±1.57^{f}
PH	92.93±0.15^{b}	82.47±0.60^{cd}
TH	82.59±0.24^{cd}	63.42±1.41^{i}
PcH	76.69±0.50^{e}	56.93±0.11^{g}

Fig. 3

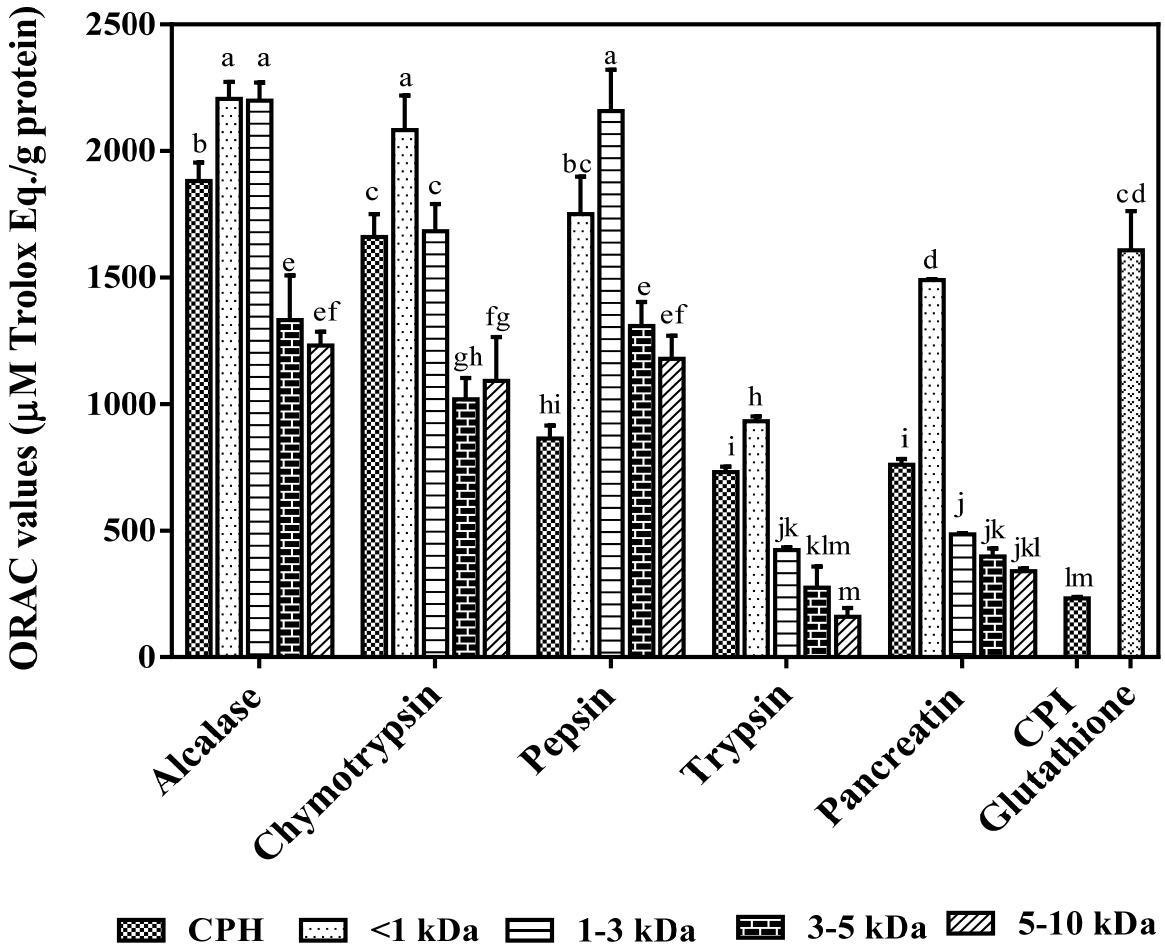

ORAC values (µM Trolox Eq./g protein)
2500
2000
1500
1000
500
0
b
a a
e
ef
c
a
c
gh
fg
hi
bc
a
e
ef
i
h
jk
klm
m
i
d
j
jk
jkl
lm
cd
Alcalase
Chymotrypsin
Pepsin
Trypsin
Pancreatin
CPI
Glutathione
CPH
<1 kDa
1-3 kDa
3-5 kDa
5-10 kDa

Fig.4

Table 1 Protein content (PC) and yield of canola protein hydrolysates and peptide fractions obtained from ultrafiltration membrane separation*

Proteases	Hydrolysates		<1 kDa		1-3 kDa		3-5 kDa		5-10 kDa	
	PC (%)[1]	Yield (%)[2]	PC (%)[1]	Yield (%)[2]	PC (%)[1]	Yield (%)[2]	PC (%)[1]	Yield (%)[2]	PC (%)[1]	Yield (%)[2]
Alcalase	75.80±0.36^{b}	66.75±2.46^{c}	71.71±2.23^{b}	30.06±0.47^{a}	57.88±2.27^{d}	15.16±0.75^{e}	82.63±0.92^{d}	8.53±0.20^{e}	78.13±2.47^{de}	3.99±1.01^{c}
Chymotrypsin	85.72±0.35^{a}	71.08±0.51^{b}	80.25±2.29^{a}	18.68±0.17^{cd}	91.58±1.50^{ab}	28.58±1.00^{a}	95.66±2.67^{ab}	12.15±1.03^{bc}	76.92±3.08^{e}	7.82±0.63^{b}
Pepsin	58.98±0.12^{d}	55.07±0.22^{d}	26.45±3.52^{e}	7.85±0.53^{e}	65.11±2.73^{c}	17.29±1.03^{d}	94.32±1.80^{b}	14.71±0.71^{a}	94.27±3.01^{a}	10.70±1.46^{a}
Trypsin	85.47±0.27^{a}	77.37±6.12^{a}	68.84±2.79^{c}	20.66±0.74^{bc}	96.86±2.21^{a}	21.92±0.35^{c}	97.79±1.29^{a}	13.58±0.88^{ab}	90.01±0.57^{b}	7.73±1.07^{b}
Pancreatin	66.54±0.34^{c}	55.15±2.54^{d}	50.10±1.70^{d}	19.63±0.89^{c}	92.32±1.29^{ab}	24.51±0.72^{b}	88.92±1.93^{c}	10.79±0.20^{d}	82.88±0.53^{c}	7.52±0.83^{b}

*Mean ± standard deviation (n=3); column values with different alphabets are significantly different ($p < 0.05$)

[1]Weight of protein in a hydrolysate expressed as a ratio of the weight of protein in the starting material (canola protein isolate)

[2]Weight of protein in a fraction expressed as a ratio of the weight of protein in the respective hydrolysate

Table 2. Percentage amino acid profile of canola protein isolate and hydrolysates

Amino acids	CPI	AH	CH	PH	TH	PcH	FAO/WHO, (1991)
Asx	9.7	10.0	10.1	10.3	9.9	9.8	
Threonine	5.2	5.1	5.4	5.0	5.2	5.2	3.4
Serine	5.3	5.2	5.5	5.0	5.4	5.3	
Glx	15.7	16.3	15.6	16.8	15.7	15.6	
Proline	5.7	5.6	5.4	5.9	5.7	5.8	
Glycine	5.7	5.6	5.7	6.0	5.8	5.8	
Alanine	4.8	4.8	4.8	4.7	4.8	4.8	
Cysteine	0.7	0.8	0.9	0.6	0.8	0.6	
Valine	5.8	56	5.8	5.5	5.7	5.7	3.5
Methionine	1.9	1.8	1. 8	1.6	1.7	1.9	
Isoleucine	4.3	4.2	4.1	4.1	4.2	4.4	2.8
Leucine	7.9	8.0	8.1	7.5	8.4	8.3	6.6
Tyrosine	3.9	4.0	4.0	3.8	3.8	4.0	1.1
Phenylalanine	5.0	4.8	4.8	4.5	4.9	4.9	6.3*
Histidine	3.6	3.5	3.3	3.5	3.4	3.3	1.9
Lysine	5.4	5.5	5.7	5.7	5.6	5.5	5.8
Arginine	7.9	7.6	7.5	8.1	7.9	7.7	
Tryptophan	0.9	0.8	1.1	0.8	0.9	1.0	
AAA	9.8	9.6	9.8	9.1	9.6	9.8	
BCAA	18.0	17.8	18.0	17.1	18.3	18.5	
HAA	40.8	40.3	40.7	39.1	40.8	41.5	
PCAA	16.9	16.6	16.4	17.3	16.9	16.6	
NCAA	25.4	26.3	25.7	27.1	25.6	25.4	
SCAA	2.6	2.5	2. 7	2.2	2.5	2. 7	

CPI–Canola protein isolates, AH–Alcalase hydrolysate, CH–Chymotrypsin hydrolysate, PH–Pepsin hydrolysate, TH–Trypsin hydrolysates, PcH–Pancreatin hydrolysates

Aromatic amino acids (AAA) = phenylalanine, tryptophan and tyrosine; Branch chain amino acids = leucine, isoleucine and valine; Combined total of hydrophobic amino acids (HAA) = alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine; positively charged amino acids (PCAA) = arginine, histidine, lysine; negatively charged amino acids (NCAA) = asx (asparagine + aspartic acid) and glx (glutamine + glutamic acid); Sulphur containing amino acids (SCAA) = methionine and cysteine; * Phenylalanine + Tyrosine