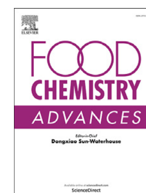
Food Chemistry Advances 2 (2023) 100297

Contents lists available at ScienceDirect

Food Chemistry Advances

journal homepage: www.elsevier.com/locate/focha



Antioxidant activities of lupin (*Lupinus angustifolius*) protein hydrolysates and their potential for nutraceutical and functional foods

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ARTICLE INFO

Keywords:
Lupin proteins
Enzymatic hydrolysis
Peptides
Antioxidant activity

ABSTRACT

Bioactive peptides are the compounds used as medicines, nutraceuticals and food preservatives. In this study, lupin protein hydrolysates were produced by enzymatic hydrolysis using pepsin, pancreatin and flavourzyme and antioxidative activities of the hydrolysates were measured. Protein was isoelectrically isolated from the lupin seed flour and enzymatically hydrolysed. The hydrolysates were ultrafiltered using molecular weight cut-off (MWCO) membranes. Fractions with masses of <2 kDa, 2–3 kDa, 3–5 kDa and 5–10 kDa were separated from the hydrolysates obtained at different hydrolysis times and then subjected to further fractionation by Size Exclusion Chromatography. Radical scavenging activities against DPPH˙, ABTS˙+, OH˙ and Fe^{2+} chelating abilities of these peptide fractions were measured. The best iron chelating, OH$^{\bullet}$, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$scavenging activities (IC_{50}) were 30 ± 5.3, 40 ± 3.9, 60 ± 3.9 and 90 ± 8.2 µg/mL respectively. Pancreatin and Flavourzyme have produced more fractions with best scavenging activities after 3–4 h of hydrolysis (IC_{50} values in the range of 40–110 µg/mL). Generally Size Exclusion purified fractions displayed superior activities than the parent fractions. Several lupin peptide fractions showed comparable antioxidant capacities to those derived from soybean protein and displayed superior activities when compared with chickpea derived peptide fractions. Therefore, lupin protein hydrolysates are a potential source for nutraceuticals and functional foods.

1. Introduction

The Australian Sweet Lupin or Narrow leaf Lupin (*Lupinus angustifolius*) belongs to the family *Leguminosae*. Lupin is grown throughout the world, though mainly in Europe, Mediterranean regions, Americas and in Australia. Numerous species of lupins exist, but only four of them are largely cultivated worldwide due to their low alkaloid content. The most commonly cultivated species are *Lupinus angustifolius* (blue lupin or narrow-leafed lupin), *Lupinus albus* (white lupin), *Lupinus luteus* (yellow lupin) and *Lupinus mutabilis* (pearl lupin). Most of the lupin produced is used as livestock feed and in the aquaculture industry. Only a small percentage of global production is consumed as human food (Lawrance, 2007). Possible allergenicity of lupin protein may be the important factor for limited human consumption. Lupin seeds are rich in protein (approximately 40% of its weight), possess high fiber content (about 28%) and are low in carbohydrates and fat. Due to its unique nutritional composition, it is an important dietary supplement and a good source of hypocholesterolemic, antioxidant, antimicrobial and anti-inflammatory agents (Lima-Cabello et al., 2018). It has also been reported that a Lupin-enriched diet is helpful in controlling blood pressure, type-2 diabetes, cardiovascular diseases, obesity, reduces the risk of certain types of cancer (Belski, 2012) and improves digestive health.

Lupin protein consists of two major constituents, namely, Albumins and Globulins that are present in the cotyledon tissue of the seeds. According to the electrophoretic mobility of lupin globulins, they can be separated into four fractions, namely, α-, β-, γ- and δ-conglutins. The conglutin-γ fraction of Lupin protein has been identified as a strong hypoglycemic agent in an animal model (Cabello-Hurtado et al., 2016). Lupin protein exhibits beneficial functional and nutraceutical properties when incorporated into food products such as bread, pasta, biscuits, soups, and salad dressings (Aiello et al., 2020; Jayasena et al., 2010).

Abundant literature demonstrates that extremely beneficial bioactive peptides can be produced from protein isolated from various foods (Udenigwe & Aluko, 2012; Wada & Lönnerdal, 2014). The hydrolysed fragments of protein molecules (peptides) that are produced by either gastrointestinal digestion or controlled enzymatic hydrolysis possess improved nutraceutical and bioactive properties over the parent protein. Peptides obtained from milk, cheese, eggs, meat and fish proteins are widely studied and have been shown to possess antioxidant, antimicrobial, antihypertensive and other bioactive properties (Meira et al., 2012; Pritchard, Phillips & Kailasapathy, 2010). Bioactive peptides derived from plant proteins such as legumes, soybean, chickpeas and lentils have been of much interest in the literature (Kamran & Reddy, 2018; Yust et al., 2012).

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https://doi.org/10.1016/j.focha.2023.100297
Received 2 December 2022; Received in revised form 1 May 2023; Accepted 1 May 2023
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Importantly, it has been observed that the excellent health benefits of lupin protein and fiber are not fully exploited. Research into this area is expected to be of great benefit to farmers and also to the nutraceutical/food industry. This paper focusses on lupin protein and its enzymatic hydrolysates. In humans prolonged oxidative stress leads to the occurrence of serious diseases including diabetes, cardiovascular diseases, cancer, neurological and many other diseases (Li et al., 2008). Certain phytochemicals obtained from lupin seeds (such as polyphenols) have been shown to exhibit antioxidant activities (Siger et al., 2012). However, to the best of our knowledge, there is limited literature involving either studies on biological activities of peptides derived from lupin protein or improvement of nutraceutical value (Chelliah et al., 2021; Garmidolova et al., 2022; Lammi et al., 2019; Yoshie-Stark & Waesche, 2004). Hence, they are important candidates for further detailed studies.

The objectives of this study were to hydrolyse lupin protein with different enzymes and determine antioxidant activities of resulting peptide fractions. Pepsin, pancreatin and flavourzyme were used for lupin protein hydrolysis. The hydrolysates were ultrafiltered using 10 kDa, 5 kDa, 3 kDa and 2 kDa molecular weight cut-off (MWCO) membranes to separate peptide fractions according to their molecular masses. The antioxidant activities of each of the 48 fractions thus produced were measured by $DPPH^{\cdot}$, $ABTS^{\cdot+}$ and $OH^{\cdot}$ radical scavenging and Fe^{2+} chelating assays. The most active fractions were analysed by LC-MS/MS to determine their primary structures.

2. Materials and methods

2.1. Materials

Pepsin (EC 3.4.23.1), pancreatin (EC 233- 468- 9) and Flavourzyme (EC 232–752–2) were purchased from Novozymes Australia Pty. Ltd., North Rocks, NSW, Australia. All other chemicals were of analytical grade and purchased from Sigma–Aldrich, Castle Hill, NSW, Australia.

2.2. Extraction of lupin protein

2.2.1. Sample preparation

Australian Sweet Lupin *(Lupinus angustifolius)* flour was obtained from Curtin University, Western Australia and was prepared from dehulled seeds. The flour was defatted by constant stirring with 2-propanol (1:4 w/v) for 4 h at room temperature (25 °C) and left overnight before separation of the flour and the solvent containing oil through filter paper. This process was repeated twice for complete defatting and the flour was air-dried in a fume hood (Karaca et al., 2011).

2.2.2. Isoelectric precipitation

Lupin protein was isolated by alkaline water extraction and isoelectric precipitation by the method of Sironi et al. (2005)) with some modifications. After defatting, Lupin flour was suspended in distilled water (1:10 w/v) and the pH of the suspension was adjusted to 9.0 using 1 *M* NaOH. The suspension was stirred for 1 hour at room temperature (25 °C) and centrifuged at 10,000 g for 30 min. In order to isolate the maximum yield of protein, the extraction process was repeated twice on the residue. The supernatants were collected and acidified to *p*H 4.5, the isoelectric point of the protein, using 1 *M* HCl. The precipitate was recovered by centrifugation at 10,000 g for 30 min, and the residue was neutralized by 1.0 *M* NaOH to *p*H 7 and washed with distilled water, several times. The neutralized precipitate was freeze dried and stored in a −20 °C freezer until further studies (Sironi et al., 2005).

2.3. Proximate analyses

Proximate composition of Lupin flour and defatted protein isolate were analysed by AOAC official methods 925.09 (moisture), 923.03 (ash), 920.39 (crude fat). Protein was estimated by Bradford Dye-Binding method. Protein solution was prepared by dissolving 1 mg of lupin protein powder in 1 ml of water. Carbohydrate content was calculated by difference from 100%.

2.4. Enzymatic hydrolysis of lupin protein

Lupin protein isolates were digested with pepsin (enzyme/ substrate ratio = 1:200) at 37 °C at *p*H 2 for 1, 2, 3 and 4 h. The digested samples were heated at 80 °C for 15–20 min to stop the enzymatic reaction. Similar procedure was followed for pancreatin digestion at *p*H 7 (Yoshie-Stark & Waesche, 2004).

For the digestion with flavourzyme the conditions were; enzyme/ substrate ratio of 1: 10, *p*H 8 and 50 °C. Samples were collected at hourly intervals for 4 h (Barbana et al., 2011). Digested samples were lyophilized and stored at 4 °C for further use.

The degree of hydrolysis was calculated by determination of free amino groups based on their reaction with 2,4,6-trinitrobenzenesulphonic acid (TNBS). The numbers of total amino acid groups were determined from fully hydrolysed samples treated with 6 *N* HCl at 110 °C for 20 h. The absorbance was measured at 340 nm using BMG LABTECH's ultra-fast UV/Vis spectrometer/ microplate reader to calculate the degree of hydrolysis of each sample (Salampessy et al., 2010).

2.5. Ultrafiltration of peptide fractions

Enzymatically hydrolysed peptide samples were subjected to 10 kDa, 5 kDa, 3 kDa and 2 kDa MWCO membrane (Vivaspin, Sartorius, Germany) to separate peptide fractions of different molecular weights. These fractions were further analyzed for their antioxidant activities.

2.6. Size exclusion chromatography of ultrafiltered fractions

Selected MWCO peptide fractions with high antioxidant activities were further fractionated using size-exclusion chromatography (SEC). Each of the MWCO fractions (5 mg/ml) was prepared in 0.01 *M* sodium phosphate buffer and fractionated by size exclusion chromatography using HiLoad 16/600 Superdex 30 prep grade column equilibrated with phosphate buffer and eluted with phosphate buffer at a flow rate of 0.5 mL/min. The peptide elution profile was determined by Lowry's method (Zhang et al., 2012). The relevant fractions were collected, pooled, and concentrated by freeze-drying.

2.7. Determination of antioxidant activity

2.7.1. $DPPH^{\bullet}$ radical scavenging assay

The 1,1 –diphenyl-2-picryl hydrazyl ($DPPH^{\bullet}$) free radical scavenging assay was conducted using the method of Blois (1958) with some modifications. Each peptide fraction (50 μL at 1 mg/mL) was added to a 150 μL of 62.5 μM DPPH. After 30 min of incubation, the absorbance of the reaction mixtures was measured at 517 nm using a multiwell plate reader. Ascorbic acid (vitamin C) was used as a positive control. The blank consisted of distilled water instead of peptide sample (Blois, 1958; Zhang et al., 2012). The scavenging activity was calculated by the following equation:

$$DPPH\ scavenging\ activity\ (\%) = \left(A_{blank} - A_{sample}/A_{blank}\right)\ X\ 100$$

Where A_{blank} is the absorbance of reaction mixture without the sample and A_{sample} is the absorbance of reaction mixture with the sample. All measurements were performed in triplicate.

The IC_{50} values (the concentration required for 50% inhibition) were calculated from concentration dependent study of radical scavenging abilities of lupin derived peptide fractions.

2.7.2. $ABTS^{\bullet+}$ radical scavenging assay

The $ABTS^{\bullet+}$ radical scavenging assay was carried out by the method of Re et al. (1999) with few modifications. A 7 mM ABTS solution was prepared with water and reacted with potassium persulphate at a molar ratio of 1:0.5 with the final concentration of 2.45 m*M*. The mixture was allowed to stand in the dark at room temperature for at least 12 h to generate ($ABTS^{\bullet+}$) radical cations. The $ABTS^{\bullet+}$ solution was further diluted with Phosphate buffer solution (0.1 M, pH 7.4) until the absorbance reached 0.70 (±0.02) at 734 nm and equilibrated at 30 °C to observe the reaction with antioxidant compounds. Add 20 µl (1 mg/mL) of each dilution to 200 µl of $ABTS^{\bullet+}$ solution (A734 nm = 0.700 ± 0.020) in each well of a multiwell plate. The absorbance reading was taken at 30 °C after 6 min of initial mixing. Water was replaced with 20 µl of antioxidant samples to measure the blank. All determinations were carried out in triplicate (Re et al., 1999). The percentage inhibition of absorbance at 734 nm is calculated by the following equation:

$$ABTS\ scavenging\ activity\ (\%) = \left(A_{blank} - A_{sample}/A_{blank}\right)\ X\ 100$$

Where A_{blank} is the absorbance of reaction mixture without the sample and A_{sample} is the absorbance of reaction mixture with the sample. All measurements were performed in triplicate.

The IC_{50} values (the concentration required for 50% inhibition) were calculated from concentration dependant study of $ABTS^{\bullet+}$ radical scavenging abilities of lupin derived peptide fractions.

2.7.3. Hydroxyl radical ($OH^{\bullet}$) scavenging assay

The hydroxyl radical scavenging assay was performed by using the method of de Avellar et al. (2004) with some modifications. 1,10-phenanthroline of concentration 0.75 mmol/L was prepared in phosphate buffer (0.1 *M*, *p*H 7.4) and $FeSO_4$ was prepared with water at the same concentration (0.75 mmol/L). Each reagent (50 µL) was mixed with 50 µL of peptide fractions (1 mg/ml) in a multiwall plate and 50 µL of 0.01% aqueous H_2O_2 was added to initiate the reaction. The mixture was incubated at 37 °C for 60 min, and then the absorbance was measured at 536 nm. The blank absorbance was measured without H_2O_2 and peptide fraction and control was measured without peptide (de Avellar et al., 2004). The hydroxyl radical scavenging activity was calculated by using the equation:

$$OH\ scavenging\ activity\ (\%) = \left(A_{sample} - A_{control}/A_{blank} - A_{control}\right)\ X\ 100$$

Where A_{blank} is the absorbance of blank solution containing 1,10-phenanthroline and $FeSO_4$, $A_{control}$ is the absorbance of control solution containing 1,10-phenanthroline, $FeSO_4$ and H_2O_2, and A_{sample} is the absorbance of reaction mixture with the sample. All measurements were performed in triplicate.

The IC_{50} values (the concentration required for 50% inhibition) were calculated from concentration dependant study of $OH^{\bullet}$ radical scavenging abilities of lupin derived peptide fractions.

2.7.4. Iron (Fe^{2+}) chelating activity

Ferrous ion-chelating activity was determined according to the method of Meira et al. (2012) with some modifications. 1 m*M* $FeCl_2$ (25 µL) solution was added to 250 µL hydrolysate samples (2 mg/mL). The reaction was initiated by the addition of 5 m*M* ferrozine (25 µL) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Water (250 µL) was used instead of sample as blank. The absorbance of the solution was then measured spectrophotometrically at 562 nm. EDTA was used as a positive control. All tests and analyses were carried out in triplicate.

The percentage of inhibition of ferrozine - Fe^{2+} complex formation was given in the formula:

$$Fe^{2+} - Chelating\ activity\ (\%) = \left(A_{blank} - A_{sample}/A_{blank}\right)\ X\ 100$$

Where A_{blank} is the absorbance of reaction mixture without the sample and A_{sample} is the absorbance of reaction mixture with the sample. The IC_{50} values were calculated from concentration dependent study.

2.8. Mass spectrometry of active fractions

Solutions of the peptides in PBS were diluted to 20 µg/mL in 0.1% aqueous formic acid and placed in chromatography vials stored at 4 °C. Peptides were separated by reverse phase liquid chromatography, using a Waters nanoAcquity UPLC fitted with a nanoAcquity Symmetry C18 trapping column (5 µm, 180 µm x 20 mm) and nanoAcquity C18 BEH analytical column (1.7 µm, 100 µm x 100 mm). The analytical column was thermostated to 35 °C. Solvent A consisted of 0.1% formic acid in ultrapure water (Milli-Q, Merck/Millipore) and solvent B consisted of 0.1% formic acid in LC-MS grade acetonitrile (Burdick and Jackson). Sample solution aliquots of 3 µL were withdrawn and trapping/desalting was accomplished over 3 min at a flow rate of 5 µL/min of 1% solvent B. Separation of the peptides was accomplished using a 60 min run with a solvent flow rate of 0.4 µL/min using the following linear gradient method: 0 min, 1% B; 1 min, 1% B; 31 min, 50% B; 33 min, 85% B, and 37 min, 1% B.

Mass spectrometry was accomplished using a Waters Xevo QToF-MS instrument, fitted with a nanospray source (10 µm ID emitter tip) and working in positive ion mode. The capillary voltage was 2.3 kV and the cone voltage 25 V. The ion block was held at 70 °C, cone gas 10 L/h, nanoflow gas 0.5 bar, purge gas 100 L/h (all N_2). Mass accuracy was obtained with a parallel lockmass experiment, in which infusion at 0.5 µL/min of leucine encephalin (200 ng/mL) in 50% aqueous acetonitrile with 0.1% formic acid was performed. Three types of data-dependent acquisition (DDA) methods were employed:

(1) *m/z* 350–3000, threshold 50 counts/s, top 3 ions for 3 s, charge states +2 to +4, collision energy ramp 15–25 V for *m/z* 350, and 30–40 V for *m/z* 3000
(2) *m/z* 100–350, threshold 50 counts/s, top 3 ions for 3 s, charge states +2 to +4, collisions energy ramp 15–25 V for 100, and 30–40 V for 350
(3) *m/z* 350–1500, threshold 50 counts, top 2 ions for 5 s, 1+ charge state, collision energy ramp 15–25 V for 350 and 30–40 V for 1500

Ion chromatograms were analysed using MassLynx version 4.1 software (Waters). Raw data files were converted to .pkl format using ProteinLynx Global Server (Waters) and searched against the SwissProt database using Mascot. *De novo* determination of peptide amino acid sequences was performed using Peaks version 7 software (Bioinformatics Solutions).

2.9. Statistical analysis

Statistical analysis of the results was carried out using SPSS (Version 22, IBM SPSS, Chicago, IL, USA) and Microsoft Excel. The triplicate data were expressed as mean±standard deviation values. The group mean was compared using a two-way analysis of variance and Duncan's multiple range tests. The results were found to be statistically significant as p-values were <0.05.

3. Results and discussion

3.1. Proximate composition

The flour and protein isolate from lupin pulse showed significant variation on proximate composition. The lupin flour showed protein content of 42.56%, Ash (3.56%), fat (6.51%) and carbohydrates content of 38.25%. The lupin protein isolates extracted by isoelectrically with protein content increased to 95% and other nutrient constituents such as fat, ash and carbohydrate contents was reduced to 0.35%, 0.03% and 2.43%, respectively (Table S1). Lqari et al. (2002) reported 87.4% and 33.8% protein in *Lupinus angustifolius* protein isolate and flour respectively. Rodriguez-Ambriz et al. (2005) also reported similar results with 93.2% and 55.3% protein content in *Lupinus campestris* protein isolate and flour respectively.

3.2. Degree of hydrolysis

Enzymatic hydrolysis has been carried out to efficiently produce lupin protein hydrolysates and to test their biological activities. Three enzymes (pepsin, pancreatin and flavourzyme) were used to produce bioactive peptides in this research. The results of degree of hydrolysis of lupin protein with these enzymes are presented in Table 1. The highest degree of hydrolysis was obtained after 4 h hydrolysis with flavourzyme (78.5%) which is followed by pancreatin after 4 h hydrolysis (77%). The degree of hydrolysis was the lowest (68.88%) for pepsin after the same hydrolysis period of 4 h. These findings are consistent with the literature (Barbana et al., 2011).

3.3. Antioxidant activities

3.3.1. DPPH˙ radical scavenging assay

The free radical, 1,1-diphenyl-2-picryl hydrazyl (DPPH˙), is a relatively stable species that has been widely used to determine radical scavenging activity of natural compounds. DPPH˙ is stable in methanol and shows maximum absorbance at 517 nm. The purple-coloured DPPH˙ radical is an oxidant and is reduced by an antioxidant to the pale-yellow DPPH˙ species, resulting in decolourisation and decrease in absorbance. The decrease in absorbance is proportional to the radical scavenging potential of the sample/ peptide fraction (Girgih et al., 2011). The results of IC_{50} values of DPPH˙ radical scavenging activities of MWCO fractions of lupin protein hydrolysates are presented in Table 1. Significant DPPH$^{\bullet}$ scavenging activities are observed for the hydrolysates produced by the three enzymes studied. Overall, the <2 kDa fractions displayed lower IC_{50} value for all the enzymes. Lowest IC_{50} values (larger scavenging activities) were observed for the fractions isolated from 4 h, <2 kDa flavourzyme hydrolysates (Table 1). Amongst the three enzymes studied, flavourzyme has produced better results for all the molecular weight fractions (Table 1). This observation is consistent with the fact that the degree of hydrolysis was high with this commercial enzyme. A previous study on pea protein hydrolysates by flavourzyme exhibited 11% scavenging activity at 1 mg/mL (Pownall et al., 2010). The results presented above demonstrate that lupin hydrolysates display much higher activities at the same concentration.

Amongst all the enzymes studied, a fewer number of pepsin hydrolysates showed good scavenging activity. As can be seen from Table 1, pepsin hydrolysed fractions; 3–5 kDa, 3 h and <2 kDa, 4 h showed significant activity (IC_{50} value 100 and 115 μg/mL respectively) at high concentrations (1 mg/mL). The pepsin hydrolysates all <2 kDa fractions showed better antioxidant activity (IC_{50} ranged between 115 and 160 μg/mL; 33–45% scavenging activities) when compared to the larger molecular weight fractions obtained with the same enzyme (Table 1). The same MWCO fractions (<2 kDa) from flavourzyme hydrolysates also showed higher antioxidant activities (IC_{50}: 60–130 μg/mL) indicating that lower molecular weight correlates with antioxidant capacity. However, a number of larger molecular weight fractions obtained from pancreatin hydrolysates also displayed significant antioxidant activities (Table 1) indicating the contribution from the nature of amino acid residues and peptide structures to antioxidant activity in addition to molecular weights.

Generally, the size exclusion fractionation produced peptides with superior activities (Table S2) relative to mixtures. The radical scavenging activities of SE purified fractions are inversely proportional to their size (Fig. 5A). As can be seen from this figure, the smallest molecular weight fraction (P4) showed highest activity.

3.3.2. ABTS$^{\bullet+}$ radical scavenging assay

ABTS$^{\bullet+}$ is the pre-formed radical mono-cation of 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) which is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of hydrogen atom donating antioxidants. The results (IC_{50} values) of ABTS$^{\cdot+}$radical scavenging abilities of lupin protein hydrolysates are

Table 1
IC_{50} values of lupin protein hydrolysates MWCO fractions (μg/mL) with respect to four radicals tested ($p \le 0.05$).

Radicals	Enzymes	Pepsin Hydrolysates				Pancreatin Hydrolysates				Flavourzyme Hydrolysate			
	Time (h)	1	2	3	4	1	2	3	4	1	2	3	4
Degree of Hydrolysis		31.11±2.4^{a}	48.15±3.3^{b}	61.48±2.3^{c}	68.88±4.7^{d}	14.82±1.3^{a}	19.26±0.7^{b}	38.52±1.4^{c}	77.03±3.8^{d}	65.92±4.6^{a}	72.59±5.1^{b}	74.81±6.2^{b}	78.52±5.2^{c}
MWCO Fractions (kDa)													
DPPH$^{\bullet}$	5–10	300±15.5^{cC}	120±13.5^{aA}	145±5.0^{bB}	120±8.6^{aA}	90±20.3^{abA}	115±17.3^{bA}	75±13.5^{Aa}	80±12.4^{aA}	100±14.3^{aA}	110±19.0^{abA}	95±14.3^{aA}	120±3.2^{bC}
	3–5	550±32.1^{dD}	500±13.2^{cD}	100±22.4^{aA}	150±13.6^{bB}	150±12.3^{Cb}	110±14.6^{bA}	90±10.5^{aB}	100±9.8^{abB}	150±11.3^{bB}	180±15.2^{cC}	110±15.1^{aB}	160±7.5^{bD}
	2–3	220±18.5^{aB}	200±14.1^{aC}	225±18.9^{aC}	530±17.0^{bC}	150±21.6^{cB}	120±12.5^{aB}	230±25.0^{dC}	70±13.7^{aA}	160±12.3^{cB}	180±10.6^{cC}	80±8.5^{aA}	100±4.7^{bB}
	< 2	140±17.2^{aA}	160±12.3^{bB}	130±15.1^{aB}	115±10.6^{aA}	190±17.2^{bC}	150±20.1^{aB}	250±23.2^{cC}	170±8.9^{abC}	90±9.3^{bA}	130±10.2^{cB}	120±12.9^{cB}	60±3.9^{aA}
ABTS$^{\bullet+}$	5–10	260±9.7^{bB}	340±13.9^{dB}	220±16.5^{aA}	310±13.5^{cB}	350±21.5^{bC}	480±13.9^{cD}	140±19.4^{aB}	330±18.6^{bC}	450±20.3^{dD}	230±18.3^{bB}	340±20.4^{cC}	190±9.5^{aA}
	3–5	610±29.2^{cD}	520±16.9^{aC}	650±28.5^{bB}	530±14.4^{aC}	120±12.3^{bA}	430±16.5^{cC}	85±5.3^{aA}	410±15.3^{cD}	200±17.6^{aB}	240±14.5^{bB}	280±13.5^{cB}	450±15.6^{dC}
	2–3	570±25.0^{bC}	690±36.5^{dD}	650±15.7^{cB}	290±14.9^{aB}	350±25.3^{dC}	90±8.2^{aA}	150±9.3^{cB}	110±7.9^{bA}	170±14.5^{aA}	160±11.3^{aA}	165±5.6^{aA}	190±8.3^{bA}
	< 2	180±12.1^{aA}	280±20.4^{cA}	220±7.5b^{A}	190±11.9^{aA}	180±20.2^{aB}	290±16.2^{cB}	230±10.0^{bC}	190±5.3^{aB}	285±21.5^{aC}	390±21.5^{cC}	400±15.3^{dD}	340±15.5^{bB}
OH$^{\bullet}$	5–10	180±12.0^{cB}	120±8.5^{aA}	150±9.3^{bB}	210±11.2^{dC}	150±15.6^{bA}	60±3.5^{aA}	70±8.9^{aB}	390±14.3^{cD}	90±6.5^{aA}	160±9.6^{bC}	150±11.5^{bB}	85±15.5^{aB}
	3–5	280±20.1^{bC}	520±16.4^{cD}	600±23.5^{dD}	160±6.3^{aB}	210±15.5^{aC}	320±14.3^{bD}	400±16.5^{cD}	200±12.8^{aC}	160±4.6^{bB}	130±7.5^{aB}	200±14.6^{cC}	165±18.4^{bC}
	2–3	150±9.5^{aA}	210±6.5^{bB}	350±13.5^{dC}	300±24.0^{cD}	210±12.3^{cC}	120±11.3^{bB}	50±3.7^{aA}	55±3.6^{aA}	100±5.5^{cA}	110±6.3^{cA}	60±7.6^{bA}	40±3.9^{aA}
	< 2	150±6.3^{aA}	360±21.3^{bC}	120±9.6^{aA}	140±11.3^{aA}	190±7.3^{cB}	260±13.6^{dC}	120±9.5^{aC}	160±16.8^{bB}	230±14.7^{bC}	220±10.5^{bD}	60±6.3^{aA}	70±6.4^{aB}
Fe^{2+}	5–10	320±18.3^{aD}	630±17.4^{cC}	620±15.9^{cC}	390±17.3^{bB}	200±18.2^{bA}	280±21.4^{cC}	580±19.5^{dD}	120±11.1^{aA}	620±24.1^{cD}	520±18.4^{bD}	400±15.6^{aC}	380±13.7^{aB}
	3–5	80±12.2^{bA}	30±7.3^{aA}	35±7.4^{aA}	45±8.3^{aA}	440±23.1^{cC}	310±21.5^{aD}	520±17.2^{dC}	360±12.5^{bB}	210±19.3^{aB}	250±15.6^{bB}	220±16.3^{aA}	200±18.5^{aA}
	2–3	220±15.3^{bB}	41±8.2^{aA}	43±6.7^{aA}	38±5.3^{aA}	600±31.3^{cD}	200±19.8^{bcB}	180±12.3^{bA}	120±7.5^{aA}	480±22.6^{cC}	360±25.3^{aC}	520±21.7^{dD}	420±20.1^{bC}
	< 2	260±15.0^{bC}	380±17.1^{cB}	160±12.3^{aB}	500±22.7^{dC}	260±8.4^{bB}	140±9.3^{aA}	340±15.3^{cB}	360±17.7^{cB}	160±11.3^{bA}	85±9.9^{aA}	300±13.2^{cB}	360±24.5^{dB}

Values were expressed as the average of triplicates ± Standard deviation.
Different superscript letters (A-D) in the column and (a-d) in the row indicate a significant difference at ($p \le 0.05$) according to Duncan's Multiple Range Test.

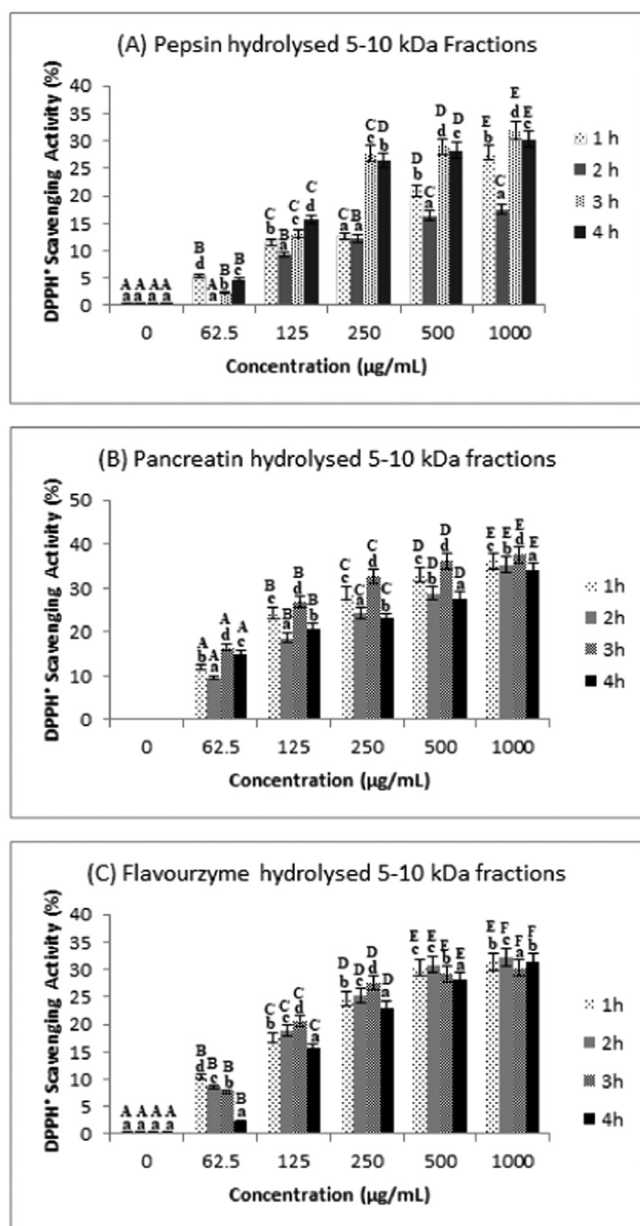

Fig. 1. DPPH• Scavenging Activity of lupin protein hydrolysed by different enzymes: (A) Pepsin hydrolysed 5–10 kDa fractions; (B) Pancreatin hydrolysed 5–10 kDa fractions; (C) Flavourzyme hydrolysed 5–10 kDa fractions.
(Note: Different letters on the graph (A-F) and (a-d) indicates significant differences (p≤ 0.05), with respect to concentration and hydrolysis time respectively, according to Duncan's Multiple Range Test).

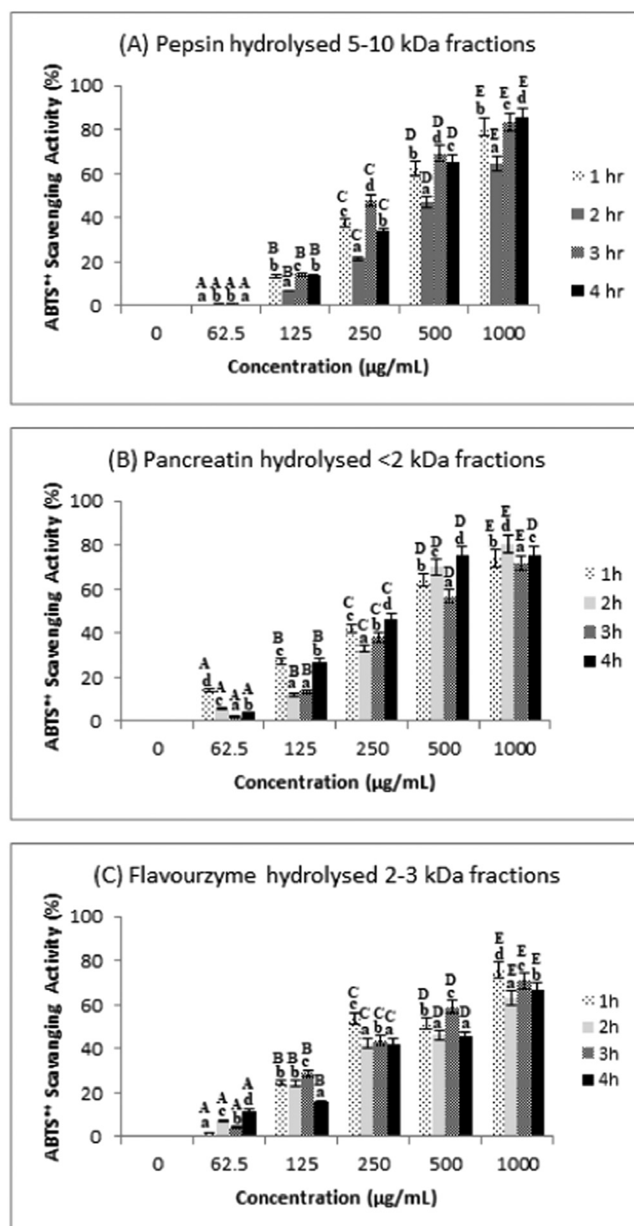

Fig. 2. $ABTS^{\bullet+}$Scavenging Activity of lupin protein hydrolysed by different enzymes: (A) Pepsin hydrolysed <2 kDa fractions; (B) Pancreatin hydrolysed <2 kDa fractions; (C) Flavourzyme hydrolysed 2–3 kDa fractions.
(Note: Different letters on the graph (A-F) and (a-d) indicates significant differences (*p*≤ 0.05), with respect to concentration and hydrolysis time respectively, according to Duncan's Multiple Range Test).

presented in Table 1. These results show that the MWCO peptide fractions from lupin generally display larger IC_{50} values towards $ABTS^{\cdot+}$ radicals than towards the other radicals studied ($DPPH^{\cdot}$ and $OH^{\cdot}$ radicals). However, the percentage scavenging activities of the fractions towards $ABTS^{\cdot+}$ radicals has been much superior compared to the other two radicals (Fig. 2A–C). From these figures it can be seen that the percentage scavenging activities of the chosen fraction are consistently larger, at all concentrations, towards $ABTS^{\cdot+}$ rather than the other two radicals. This was indeed the trend observed for all MWCO fractions.

Pepsin hydrolysed 5–10 kDa and <2 kDa fractions displayed excellent $ABTS^{\cdot+}$ scavenging activities. The highest activities (~80% at 1000 µg/mL) were observed for 5–10 kDa fractions obtained after 1 h, 3 h and 4 h hydrolysis (Fig 2A).

Pancreatin produced the best activities for <2 kDa fractions (Fig 3B) followed by 5–10 kDa fractions. Overall, the <2 kDa fractions performed consistently well at medium to high concentrations. Lower IC_{50} values (larger scavenging activities) were observed for the fractions isolated from 3 h (3–5 kDa) pancreatin hydrolysates (Table 1). Flavourzyme has produced consistently better results for most the molecular weight fractions. This observation is consistent with the fact that the degree of hydrolysis was high with this commercial enzyme. The 2–3 kDa fractions (Fig. 2C) showed the highest activities. A majority of the fractions isolated from pancreatin and flavourzyme hydrolysates displayed significant IC_{50} values. Three of the pancreatin hydrolysed fractions showed best scavenging abilities with IC_{50} values in the range of 85–110 µg/mL (Table 1). The least number of fractions produced by pepsin showed

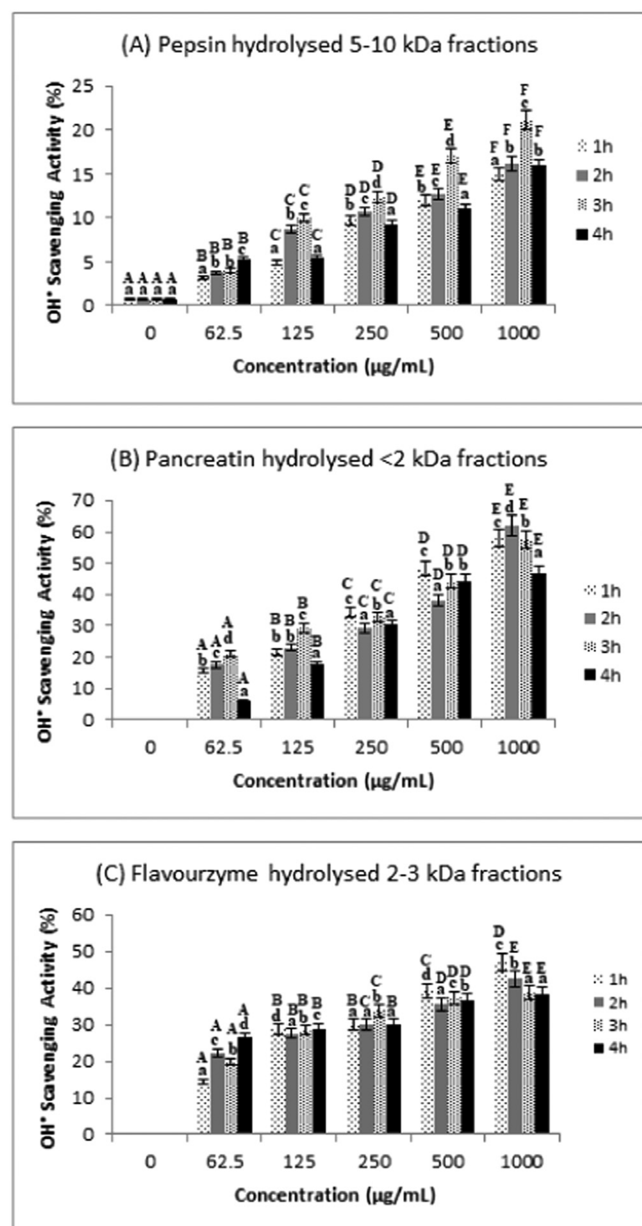

Fig. 3. OH• Scavenging Activity of lupin protein hydrolysed by different enzymes: (A) Pepsin hydrolysed 5–10 kDa fractions; (B) Pancreatin hydrolysed <2 kDa fractions; (C) Flavourzyme hydrolysed 2–3 kDa fractions.
(Note: Different letters on the graph (A-F) and (a-d) indicates significant differences ($p \leq 0.05$), with respect to concentration and hydrolysis time respectively, according to Duncan's Multiple Range Test).

good scavenging activity towards $ABTS^{\cdot+}$ radical. Ngoh and Gan (2016)) reported the ABTS activities of pinto bean protein hydrolysates. The 3–10 kDa fraction presented 2.61% activity while the <3 kDa fraction exhibited 42.18% activity. These results are comparable to results of lupin protein hydrolysates reported in this research.

3.3.3. OH• radical scavenging assay

Hydroxyl radicals are produced in the human body and are highly reactive species. It is therefore very important to test scavenging activities against these biologically relevant radicals. OH• radicals react readily with most of the biomolecules present in the body including proteins, nucleic acids, polyunsaturated fatty acids and are capable of producing severe damage to the human body. Prolonged presence of hydroxyl radicals in the body produces oxidative stress leading to serious diseases such as cancer and diabetes. Suitable OH• radical scavenging agents can eliminate such an oxidative stress and prevent the occurrence of these diseases (Zhu, Zhou, & Qian, 2006). The results of IC_{50} values of OH• scavenging activities of various lupin derived peptide fractions are presented in Table 1. Significant OH• radical scavenging activities were observed for the hydrolysates produced by the three enzymes studied. Pepsin produced the best activities for 5–10 kDa fractions (Fig. 3A) followed by <2 kDa fractions. The highest activities (~23% at 1 mg/mL) were observed for 5–10 kDa fraction obtained after 3 h hydrolysis (Fig. 3A). Pancreatin produced the best activities for <2 kDa fractions (Fig. 3B) followed by 5–10 kDa fractions. Overall, the <2 kDa fractions performed consistently well even at fairly low concentrations. Lower IC_{50} values (larger scavenging activities) were observed for the fractions isolated from 3 h Flavourzyme hydrolysates (<2 kDa) (Table 1). Flavourzyme produced consistently good results for all the molecular weight fractions. This observation is consistent with the fact that the degree of hydrolysis was high with this commercial enzyme. The 2–3 kDa fractions (Fig. 3C) showed the highest activities followed by <2 kDa fractions.

A previous study on pea protein hydrolysates produced by Thermolysin exhibited 17% scavenging activity at 1 mg/mL (Pownall et al., 2010). The results presented above demonstrate that lupin hydrolysates display much higher activities at the same concentration.

Significant OH• radical scavenging activities were observed for a majority of high molecular weight fractions (5–10 kDa) and average molecular weight fractions (2–3 kDa) produced by all enzymes studied (Table 1). Lower IC_{50} values (larger scavenging activities) were observed for 5–10 kDa fractions of pancreatin hydrolysates. However, best IC_{50} values were observed for most of the fractions isolated from 3 h and 4 h hydrolysates obtained from Flavourzyme (Table 1). Excellent scavenging activities against OH• radicals indicate that lupin derived bioactive peptides have great potential to be developed as antioxidants towards this biological relevant free radical. Lower molecular weight fractions obtained from Size Exclusion separation have displayed superior OH• radical scavenging activity (Fig. 5B & Table S2). In order to gain further insight into the beneficial applicability of peptide fractions from blue lupin, it is important to compare and contrast the antioxidant activities of these fractions with the activities of other legume derived peptides published in the literature. Soybean based infant formulas containing 90% soybean protein were hydrolysed by four different methods and ultrafiltered through MWCO membranes. These peptide fractions exhibited significant scavenging activities against DPPH, ABTS and hydroxyl radicals with 5–10 kDa fractions yielding best results (Puchalska et al., 2014). A comparison of the results presented in this paper with the results of soybean protein hydrolysates indicates that the peptide fractions derived from blue lupin protein exhibit superior radical scavenging activities. Pownall et al. (2010)) have studied the antioxidant activities of <3 kDa fractions obtained from RP-HPLC separation of yellow pea protein hydrolysates. The authors concluded that the HPLC fractionation improved the reducing power and scavenging abilities. It should be noted that the pea protein hydrolysates by flavourzyme exhibited lower DPPH• scavenging activities than the activities displayed by lupin protein hydrolysates presented in this paper at 1 mg/ml, indicating high potential of lupin protein hydrolysates. The fractions of chickpea protein hydrolysates separated by size exclusion chromatography were studied for DPPH•, superoxide and hydroxyl radical scavenging activities by Y. Li et al. (2008). The lowest molecular weight fraction displayed highest DPPH• and hydroxyl radical scavenging activity. It has been observed that the DPPH• radical scavenging activity of chickpea protein hydrolysate could be maximised by 60 min hydrolysis with Alcalase followed by Flavourzyme (Yust et al., 2012). A comparison of the results presented in this research (Fig. 5) with those reported by Li et al. (2008) indicate that both lupin protein and chickpea protein derived peptide fractions display comparable radical scavenging activities.

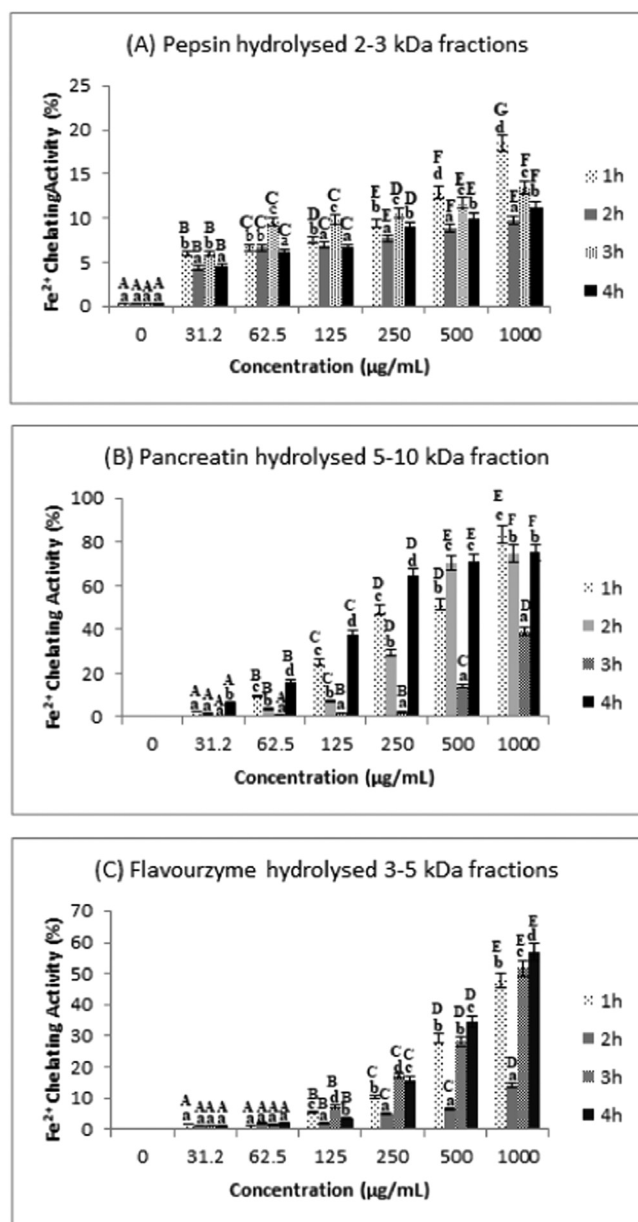

Fig. 4. Fe^{2+} chelating Activity of lupin protein hydrolysed by different enzymes: (A) Pepsin hydrolysed 2–3 kDa fractions; (B) Pancreatin hydrolysed 5–10 kDa fractions; (D) Flavourzyme hydrolysed 3–5 kDa fractions.
(Note: Different letters on the graph (A-F) and (a-d) indicates significant differences ($p \le 0.05$), with respect to concentration and hydrolysis time respectively, according to Duncan's Multiple Range Test).

3.3.4. Iron (Fe^{2+}) chelating activity

As can be seen from Fig. 4A–C, significant iron (Fe^{2+}) chelating activities are observed for the hydrolysates produced by the three enzymes studied. Pepsin produced the best activities for 2–3 kDa fractions (Fig. 4A) followed by 5–10 kDa fractions. However, the highest activities (~28% at 1 mg/mL) (lower IC_{50} values) were observed for 5–10 kDa fraction obtained after 4 h hydrolysis (Table 1). Pancreatin produced the best activities for 5–10 kDa fractions (Fig. 4B) followed by <2 kDa fractions. Overall, the 5–10 kDa fractions performed consistently well at medium to high concentrations. Flavourzyme has produced relatively low activities for all the molecular weight fractions. The 3–5 kDa fractions (Fig. 4C) showed the highest activities followed by 5–10 kDa fractions. This observation indicates that the degree of hydrolysis is high with this commercial enzyme and may inversely affect the ability of the

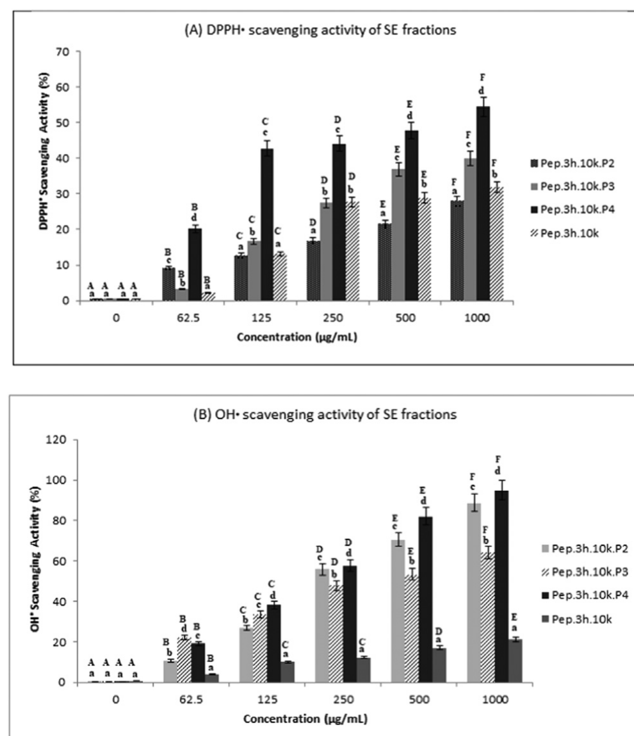

Fig. 5. Radical Scavenging activities of size exclusion fractions of 3 h Pepsin hydrolysed MWCO fraction (5–10 kDa); P2, P3, P4 are size exclusion fractions in decreasing order of their sizes: (A) $DPPH^{\bullet}$ Scavenging Activity; (B) $OH^{\bullet}$ Scavenging Activity.
(Note: Different letters on the graph (A-F) and (a-d) indicates significant differences ($p \le 0.05$), with respect to concentration and size exclusion fractions respectively, according to Duncan's Multiple Range Test).

peptides produced to chelate the iron (Fe^{2+}) ions. The results showed that hydrolysis of lupin protein with pancreatin could produce peptides with Fe^{2+} chelating ability slightly lower than the Fe^{2+} chelating ability of some food protein hydrolysates reported in the literature (Tang and Zhuang (2014)). It is reported the Fe^{2+} chelating ability of zein protein hydrolysate <3 kDa purified fraction was 95.4%. The results obtained in this study, nonetheless, indicated the ability of the enzymes employed to produce bioactive peptides with significantly high Fe^{2+} chelating ability that can be used to minimize the effect of this radical-generating ion in biological systems. Results presented in this paper demonstrate that many of the lupin derived peptide fractions have displayed excellent radical scavenging abilities ($IC_{50} \le 150$ μg/mL) and good iron chelating abilities indicating their potential for nutraceutical applications. Antioxidant properties of lupin protein hydrolysates together with other biological activities investigated in the authors' laboratory suggest that they are highly desirable ingredients for developing functional foods and nutraceuticals. An important undesirable characteristic hindering the utilization of lupin derived bioactive peptides for human consumption is the possibility of their allergenicity (Garmidolova et al., 2022). A thorough investigation of this feature will provide confidence to extensively use lupin peptides to develop functional foods and nutraceuticals for human consumption. The food/nutraceutical industry will then be expected boost the production of lupin based products with easing of scientifically backed approval processes.

3.4. Identification of most active peptides using mass spectrometry results

Raw LC-MS/MS data of peptide fractions was searched against databases to aid with identification of active peptides. The databases UniProt and BLAST from National centre for Biotechnology Informa-

tion (NCBI) were used for sequence identification. By considering the antioxidant analyses results, the pepsin, 3 h hydrolysate, 5–10 kDa fraction was purified by size exclusion chromatography and separated 6 peaks, from which peaks 2 and 3 were employed to mass spectrometry studies. The MS spectrometry identified 2 pure sequences as TEINEGALLLPH and EAGTIETWNPN that might be responsible for antioxidant activity. The MS yielded a strong peak with *m/z* value of about 653. This corresponds to TEINEGALLLPH sequence (Fig S2). The sequence TEINEGALLLPH exists in the conglutin beta and EAGTIETWNPN exists in the conglutin alpha fraction of lupin protein. The sequences TEINEGALLLPH and EAGTIETWNPN contain hydrophobic amino acid residues Proline (P), Histidine (H), Tyrosine (Y) or Tryptophan (W); that are favourable for antioxidant activity (Pihlanto, 2006). The electron rich aromatic rings of Phenylalanine (F), Tyrosine (Y) and Tryptophan (W) facilitate the pro-oxidant metal ion chelating reaction, that are responsible for the antioxidant activity of sequence (Udenigwe & Aluko, 2012). It is reported that the sequences with 5–16 amino acid residues with hydrophobic amino acids valine (V) or leucine (L) at the N-terminal positions, and also contain proline (P), histidine (H) or tyrosine (Y) in the sequences exhibit potent antioxidant activity. At C-terminal hydrophilic residues (Q, N, T and S) and polar residues (E, D, H, K and R) are favourable for antioxidant activity (Kamran et al., 2016; Li et al., 2011). The presence of hydrogen donating residues next to C-terminal facilitates free radical scavenging and chelating property and hence display improved antioxidant activity. Therefore, the sequences containing a hydrophobic residue at the N-terminal and a polar residue at the C-terminal display greater antioxidant activities (Kamran et al., 2016; Li et al., 2011). A study (Chen et al., 1996) estimated antioxidant activities of 28 synthetic peptides, based on a natural peptide sequence LLPHH from soybean protein hydrolysate. The removal of a histidine residue in the synthesized sequence, LLPH lowers the antioxidant activity as compared to the activity of the original sequence. The sequence LLPH displays high antioxidant activity because of the presence of hydrophobic amino acid (L) at the N-terminal and polar residue (H) at the C-terminal and hence is identified as antioxidant sequence (Chen et al., 1996). The lupin peptide sequence TEINEGALLLPH identified in this research also has same residues at the C-terminal that may be responsible for its activity, making it a potential for nutraceutical applications. Chickpea isolated antioxidant peptide sequence TETWNPNHPEL discovered by Torres-Fuentes et al. (2015) also displayed extent antioxidant activity. Faba bean also contains the same peptide sequence exhibiting antioxidant activity (Samaei et al., 2020). The sequence contains Threonine (T) and Tryptophan (W) residues at N-3 and N-4 positions which are responsible for the antioxidant activity. The residues ETWNPN are also available in lupin peptide sequence EAGTIETWNPN that exhibit good antioxidant activity. A multifunctional peptide sequence LTFPGSAED, extracted from conglutin-β lupin protein hydrolysate is reported to have hypocholesterolemic and anti-diabetic properties (Lammi et al., 2019). In this research the pepsin, 3 h hydrolysate, 5–10 kDa fraction contains a very similar sequence LIFPGSAED, that might have similar multifunctional properties.

4. Conclusion

As can be seen from the results on radical scavenging activities presented in this paper, several peptide fractions obtained from lupin protein have displayed potent scavenging activities against all the radicals tested. Some of the active MWCO fractions were subjected to Size exclusion chromatographic separation that led to isolation and identification of active peptide sequences, TEINEGALLLPH and EAGTIETWNPN.

A 2–3 kDa fraction obtained by 4 h hydrolysis from pancreatin gave excellent scavenging activity against all the radicals tested. Many fractions have displayed good scavenging abilities ($IC_{50} \leq 150$ µg/mL) against all the three radicals tested. These fractions are expected to be potential candidates for production of antioxidant nutraceuticals from blue lupin protein and also are of great interest for further bioactivity studies.

Overall, flavourzyme appears to be a favourable enzyme to commercially produce nutraceutical peptides from blue lupin protein, as it has produced excellent number of active fractions that displayed significant scavenging activity against all the radicals tested. Also, the results indicate that the human digestive system is expected to produce significant quantities of antioxidant peptides from lupin protein as pepsin and pancreatin (human digestive enzymes) have produced high number of fractions with good scavenging abilities.

MWCO fractions obtained from pancreatin and flavourzyme hydrolysates displayed high scavenging abilities and a fewer number of pepsin hydrolysates showed good scavenging activities. Results presented in this paper indicate that a lower molecular weight is a favourable factor for antioxidant capacity which is consistent with the literature findings. The findings of this research also suggest the contribution from specific nature of amino acid residues and peptide structures to antioxidant activity in addition to molecular weights.

It is worth noting that a significant number of lupin peptide fractions displayed good scavenging activities against biologically relevant $OH^{\cdot}$ radicals indicating that lupin derived peptides have great potential to develop nutraceuticals for human consumption. On the other hand, the antioxidant capacities of pepsin and pancreatin (human digestive enzymes) hydrolysates indicate that lupin protein has great potential to be used directly as functional food. Antioxidant properties of lupin protein hydrolysates together with other biological activities investigated in the authors' laboratory suggest that they are highly desirable ingredients for developing functional foods and nutraceuticals. A thorough investigation of this feature is expected to boost the development of lupin based functional foods and nutraceuticals for human consumption. Food/nutraceutical industry and the consumers will be the beneficiaries of such scientific advancement which ultimately benefit the primary produces of lupin (Fig. 1).

Significance statement

This research is extremely significant for the development of lupin based functional foods and nutraceuticals. Peptides with strong antioxidant activities have been isolated from lupin flour and their chemical structures identified. Promising results presented in this paper are strong indicators that the primary producers, food/nutraceutical industry and consumers will all benefit from this research.

Credit author statement

Currently Food Chemistry Advances is offering free publication. Hence, there is no need of "Credit Author Statement".

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

Fozia Kamran acknowledges WSU Postgraduate Research Scholarship from the Western Sydney University and the School of Science during her PhD candidature. The authors also would like to thank ongoing support from the School of Science, Western Sydney University for their research on bioactive peptides. Finally, the WSU Mass Spectrometry Facility is acknowledged for access to instrumentation and informatics capabilities.

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