Research Article

Received: 16 May 2018 Revised: 16 October 2018 Accepted article published: 25 October 2018 Published online in Wiley Online Library: 18 December 2018

(wileyonlinelibrary.com) DOI 10.1002/jsfa.9449

Novel antioxidant peptides obtained by alcalase hydrolysis of *Erythrina edulis* (pajuro) protein

Arturo Intiquilla,[a] Karim Jiménez-Aliaga,[a] Fanny Guzmán,[b] Claudio A Alvarez,[c,d] Amparo I Zavaleta,[a] Víctor Izaguirre[a] and Blanca Hernández-Ledesma[e*]

Abstract

BACKGROUND: Oxidative reactions are responsible for the changes in quality during food processing and storage. Oxidative stress is also involved in multiple chronic diseases, such as cardiovascular and neurodegenerative disorders, diabetes, cancer, and aging. The consumption of dietary antioxidants has been demonstrated to help to reduce the oxidative damage in both the human body and food systems. In this study, the potential of *Erythrina edulis* (pajuro) protein as source of antioxidant peptides was evaluated.

RESULTS: Pajuro protein concentrate hydrolyzed by alcalase for 120 min showed potent $ABTS^{\cdot+}$ and peroxyl radical scavenging activity with Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) values of 1.37 ± 0.09 μmol TE mg^{-1} peptide and 2.83 ± 0.07 μmol TE mg^{-1} peptide, respectively. Fractionation of the hydrolyzate to small peptides resulted in increased antioxidant activity. *De novo* sequencing of most active fractions collected by chromatographic analysis enabled 30 novel peptides to be identified. Of these, ten were synthesized and their radical activity evaluated, demonstrating their relevant contribution to the antioxidant effects observed for pajuro protein hydrolyzate.

CONCLUSIONS: The sequences identified represent an important advance in the molecular characterization of the pajuro protein, demonstrating its potential as a source of antioxidant peptides for food and nutraceutical applications.
© 2018 Society of Chemical Industry

Keywords: *Erythrina edulis*; alcalase hydrolysis; bioactive peptides; antioxidant activity

INTRODUCTION

Free radicals are constantly generated as byproducts of physiological processes that occur in the body or as a consequence of external exposures such as contact with tobacco smoke, radiation, or pollution.[1] Although the controlled formation of free radicals is essential for many signaling pathways and normal functions, their accumulation due to excessive production or deficient removal by endogenous antioxidant systems induces damage in cell biomolecules leading to oxidative stress, a state involved in cardiovascular and neurodegenerative disorders, diabetes, cancer, and aging.[2] Oxidative reactions may also be responsible for the deterioration of the quality of foods during processing and storage. The consumption of dietary antioxidants has been demonstrated to help to reduce the oxidative damage both in the human body and in food systems,[3] thus increasing interest in searching for new antioxidant agents from natural sources.

Bioactive peptides are defined as specific protein fragments that, once released by *in vivo* digestion or *in vitro* processing, exert beneficial properties on one or more body functions beyond their nutritional value.[4] A wide variety of biological activities have been demonstrated for peptides derived from animal and plant food sources, although antioxidant and antihypertensive activities have been the most commonly studied.[5–7] In addition to their nutritional, biological, and functional attributes, antioxidant peptides have simpler structures than their parent proteins, with reduced molecular weight, which endows them with improved features like increased absorption and stability in response to proteases.[8] In recent years, the antioxidant peptides have gained interest due to consumer health concerns associated with synthetic food additives such as butylated hydroxyanisole (BHA) and

* *Correspondence to: B Hernández-Ledesma, Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM, CEI UAM+CSIC), Nicolás Cabrera, 9. 28049 Madrid, Spain. E-mail: b.hernandez@csic.es*

a *Laboratorio de Biología Molecular, Facultad de Farmacia y Bioquímica, Universidad Nacional Mayor de San Marcos, Lima, Peru*

b *Núcleo de Biotecnología Curauma (NBC), Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile*

c *Laboratorio de Fisiología y Genética Marina (FIGEMA), Centro de Estudios Avanzados en Zonas Áridas (CEAZA), Coquimbo, Chile*

d *Facultad de Ciencias del Mar, Universidad Católica del Norte, Coquimbo, Chile*

e *Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM, CEI UAM+CSIC), Madrid, Spain*

butylated hydroxytoluene (BHT), the toxic and harmful effects on human health of which have limited their use.[2] Although the first studies were focused on the release of antioxidant peptides from animal sources,[9–11] multiple plant-derived proteins, including soybean, canola, flaxseed, common beans, or amaranth have also been recognized as sources of antioxidant peptides.[12–14] These plant-protein-derived antioxidant hydrolyzates and the peptides that they contain are being used to prevent foods from undergoing deterioration and / or to develop functional foods or supplements for the management of diverse oxidative-stress associated health problems.[15] The antioxidant properties have been demonstrated to depend mainly on the native protein and the method applied to purify it, the specificity of the protease used for hydrolysis, the degree of hydrolysis (DH), structure, molecular weight, and amino acid composition of the released peptides.[15,16]

In the food industry, adding value to under-utilized plants to optimize their use for human consumption has become popular. New and alternative protein sources are currently required to provide nutritional, technological, and functional properties.[17] *Erythrina edulis* (pajuro or chachafruto) is a legume with a broad range of uses, from the human (seeds) and animal (forage) diet to the recovery of nitrogen content of the soil. This plant has also been used traditionally because of its health-promoting and nutritional properties. Pajuro has been demonstrated to exert different activities such as diuretic, hypotonic, and osteoporosis preventive activities.[18] This plant has a high protein content (18–25%) with high digestibility,[19] and a quality higher than that of other legumes and similar to the egg's protein quality.[18,20] However, the information available on the functional, nutritional, and antioxidant properties of pajuro protein hydrolyzates is still scarce.[21] The objectives of this work were therefore to produce an antioxidant *Erythrina edulis* (pajuro) protein hydrolyzate with alcalase, fractionate it into peptides of different molecular sizes, evaluate these samples for *in vitro* antioxidant properties, and identify the peptides potentially responsible for the observed effects. Preparation of antioxidant peptides from pajuro proteins could be one way of producing high-value ingredients from this underutilized legume, to be incorporated in new value-added food products.

MATERIALS AND METHODS

Materials and reactives

Erythrina edulis (pajuro) seeds were collected in Otuzco (La Libertad, Peru), cleaned to remove foreign material, peeled, and cut in small pieces that were dried at 40 °C. Dried seeds were ground to powder in a domestic mill, and sieved using mesh number 60 to obtain a homogenous flour sample.

Alcalase from *Bacillus licheniformis* (2.4 activity units UA g^{-1}), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), bovine serum albumin (BSA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), and fluorescein disodium (FL) were purchased from Sigma-Aldrich (St Louis, MO, USA). Ortho-phthalaldehyde (OPA) was from Merck Millipore Corp. (Darmstadt, Germany). All other reagents were of analytical grade.

Preparation of pajuro protein concentrate

The pajuro protein concentrate (PC) was obtained following the procedure described by Betancur and coworkers,[22] with slight modifications. Pajuro flour was suspended in distilled water (ratio 1:6, v/v) at pH 11.0, stirred at 4 °C for 1 h, and homogenized using a conventional blender for 3 min. To eliminate fiber, the suspension was filtered through 80- and 100-mesh screens, and the solids on the screen were washed using distilled water (1:3, w/v). Once the retentate containing starch was eliminated, the pajuro proteins were precipitated after adjusting the pH of the supernatant to pH 4.5 with 1 mol L^{-1} HCl, and centrifuging at 1317×*g* for 20 min at 4 °C. Pajuro proteins were freeze-dried and stored at −20 °C for further analysis. The protein content of PC was analyzed by Kjeldahl[23].

Enzymatic hydrolysis of pajuro protein concentrate

A 40 g L^{-1} (w/v) PC suspension in water was subjected to hydrolysis using alcalase at an enzyme : substrate (E/S) ratio of 1:200 (v/v). The hydrolysis was carried out at 50 °C and pH 8.3 for 120 min under constant stirring. Aliquots were withdrawn at 0, 15, 30, 60, and 120 min, and the enzymes were inactivated by heating at 100 °C for 10 min. The resulting PC hydrolyzates (PCHs) were cooled rapidly and centrifuged at 10 000×*g* for 20 min at 4 °C. The supernatants were freeze-dried and stored at −20 °C until use. The DH value was determined following Nielsen *et al.* assay by measuring the free amino groups (h) by reaction with OPA.[24] The total number of amino groups (h_t) was determined in samples 100% hydrolyzed with 6 mol L^{-1} HCl and 1 g kg^{-1} phenol at 150 °C for 6 h. The DH was calculated using the formula:

$$\%DH = \left(h/h_t\right) \times 100$$

and expressed as mg L-serine mL^{-1}.

The PCHs were subjected to ultrafiltration through 3000 Da and 10 000 Da cutoff hydrophilic and low protein-binding Omega membranes (Macrosep Advance Centrifugal Devices, Pall Corporation, New York, NY, USA). Fractions UF1 (> 10 kDa), UF2 (3–10 kDa), and UF3 (< 3 kDa) were obtained, freeze-dried, and stored at −20 °C for further analyses. The protein content of PCHs and fractions was measured by the bicinchoninic acid method (BCA) (Pierce, Rockford, IL, USA) using BSA as standard protein.

Antioxidant activity

$ABTS^{\cdot+}$ scavenging activity

Antioxidant capacity was measured following the $ABTS^{\cdot+}$ method described by Re and coworkers.[25] Briefly, the radical $ABTS^{\cdot+}$ stock solution was generated by reacting 7 mmol L^{-1} ABTS and 2.45 mmol L^{-1} potassium persulfate in the dark at room temperature for 16 h. The $ABTS^{\cdot+}$ solution was obtained by dilution of the stock solution in 0.01 mol L^{-1} sodium phosphate buffer (pH 7.3) to an absorbance at 734 nm of 0.70 ± 0.02. After addition of 980 µL of $ABTS^{\cdot+}$ solution to 20 µL of sample or standard (Trolox) in PBS, the absorbance was recorded after 6 min in Jasco V-630 spectrophometer (JASCO Deutschland GmbH, Pfungstadt, Germany). The percentage inhibition was calculated and plotted as a function of the quantity of antioxidants (mg or µmol peptide) or standard (µmol Trolox). The Trolox equivalent antioxidant capacity (TEAC) value was calculated dividing the gradient of the plot for sample by the gradient of the plot for Trolox and expressed as µmol Trolox equivalent (TE) mg^{-1} or μmol^{-1} of peptide.

Oxygen radical absorbance capacity (ORAC) assay

The oxygen radical absorbance capacity (ORAC) was determined following the protocol described by Hernández-Ledesma *et al.*[26] The final assay mixture (200 µL) contained FL (30 nmol L^{-1}), AAPH (12 mmol L^{-1}), and antioxidant (Trolox (0–5 nmol L^{-1}) or sample

 © 2018 Society of Chemical Industry

(at different concentrations)) in 75 mmol L^{-1} phosphate buffer (pH 7.4). The reaction was carried out at 37 °C and the fluorescence was recorded every 2 min for 120 min in an Infinite M200 Pro plate reader (Tecan Group AG, Männendorf, Switzerland) at 485 and 520 nm of excitation and emission, respectively. The equipment was controlled by the Icontrol software version 1.11.10 for fluorescence measurement. Clear 96-well microplates (96F untreated, pure Grade™, Brand, Wertheim, Germany) were used. Three independent runs were performed for each sample. ORAC values were expressed as µmol TE mg^{-1} or µmol^{-1} of peptide.

Purification of antioxidant peptides

The dried PCHs were reconstituted with water and passed through a DPA-6S polyamide solid-phase extraction (SPE) cartridge (Sigma-Aldrich) to remove polyphenols. The aqueous elution was applied onto a Sep-pak C18 Vac cartridge (Waters Corp., Milford, MA, USA) equilibrated in acidified water (0.5 mL L^{-1} trifluoroacetic acid (TFA) in ultra pure water). After washing with acidified water, the peptides were eluted at a flow-rate of 1 mL min^{-1} with 50, 100, 150, 200, 250, and 300 mL L^{-1} acetonitrile (ACN). The appropriate fractions were collected, and the ACN was evaporated on a speedvac centrifugal device. Most active fraction were further purified eluting peptides with 20, 40, 60, 80, and 100 mL L^{-1} ACN. The fractions were then analyzed by reversed-phase (RP)-HPLC on a Waters Corp XBridge™ BEH C18 column (100 × 4.6 mm, 3.5 µm, Waters Corp.) using 0–70% acetonitrile gradient, water containing 0.5 mL L^{-1} TFA as solvent A, and acetonitrile containing 0.5 mL L^{-1} TFA as solvent B, at a flow rate of 1 mL min^{-1} for 8 min. The acquisition of mass spectra of each ACN fraction was performed in a matrix-assisted laser desorption ionization-time of flight mass spectrometer (MALDI-TOF). Samples (ACN fractions) were mixed with α-cyano-4-hydroxycinnamic acid (CHCA) matrix (10 mg mL^{-1}) in a 1:1 ratio. Mass spectra were acquired in a Microflex MALDI-TOF instrument (Bruker Daltonics, Inc., Billerica, MA, USA) in positive and reflection mode. Before mass spectra acquisition, the instrument was externally calibrated using a peptide mix of a 1000–3000 mass range. Final spectra corresponded to 15 scans of 30 laser shots traced uniformly across the sample spot.

Peptides identification by *de novo* sequencing

The peptides' identification of the 40 mL L^{-1} (F4) and 60 mL L^{-1} (F6) ACN fraction were obtained by *de novo* sequencing. Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) analysis was performed on an Agilent 1100 HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) connected online to an electrospray (ESI)-TRAP Esquire 4000 ion-trap type mass spectrometer (Bruker Daltonik GmbH). The column used was a Luna C_{18} (2) column (150 × 2.0 mm) (Phenomenex Inc., Torrance, CA, USA). Peptides were eluted with a linear gradient of solvent B (ACN : formic acid, 1000:0.85, v/v) in A (water: formic acid, 1000:1, v/v) going from 5% to 80% in 15 min at a flow rate of 0.25 mL min^{-1}. The injection volume was 20 µL. The ionization process by electrospray was carried out at 3000 V using nitrogen as a nebulizer gas at 340 °C and as a dry gas at 37.5 psi and 7.5 L min^{-1}. Data were acquired in the positive polarity mode for interval of 20–1000 m/z. The m/z signal detection was carried out with APEX algorithm with the following parameters: width peak MS/MS, 0.01 m/z, signal / background threshold, 0; relative intensity threshold, 0%; absolute intensity threshold, 0. The m/z signals (precursors) were associated with their corresponding fragmentations by Compounds-AutoMSn mode. Finally, the processed data were exported as an mgf file (Mascot generic format) for identification and sequencing analysis *de novo*. DataAnalysis 3.2 software (Bruker Daltonik GmbH) was used for the analysis of chromatograms and mass spectra.

The *de novo* sequencing was done through the PepNovo construct 20 101 117 program (Ari Frank, Center for Computational Mass Spectrometry, University of California, San Diego, CA, USA).[27] The mgf files were processed using the following parameters: post-translational modifications, methionine oxidation (M + 16); precursor tolerance, 0.7 Da; fragmentation tolerance, 0.7 Da; interpretation model, CID-IT-TRYP (proteolysis, trypsin, fragmentation, CID collision cell in IT ion trap); proteolysis, non-specific; load correction, no; and number of solutions, 1.

Peptide synthesis

Peptides were synthesized by the conventional Fmoc solid-phase synthesis. The purity of synthetic peptides, determined by HPLC-MS analysis, was higher than 96.5%.

Statistical analysis

All data were analyzed in three independent experiments and the results were expressed as the mean ± standard deviation (SD). A one-way ANOVA was applied, followed by the

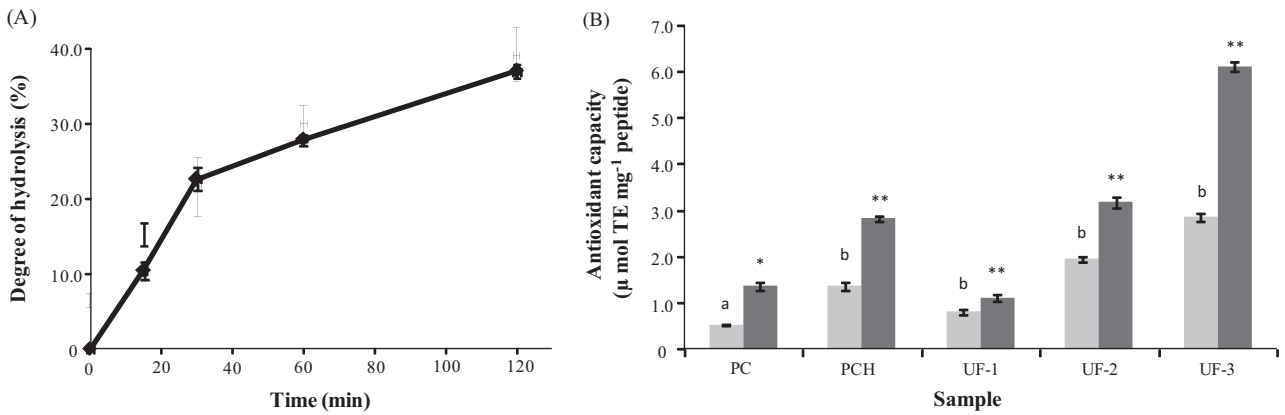

Figure 1. (A) Degree of hydrolysis (%) of the hydrolyzates from *Erythrina edulis* (pajuro) protein concentrate (PC) with alcalase at 15, 30, 60, and 120 min incubation. (B) (▒) ABTS and (■) peroxyl radical scavenging activity (expressed as µmol Trolox equivalents (TE) mg^{-1} peptide) of pajuro protein concentrate (PC), its hydrolyzate at 120 min (PCH) and peptidic fractions higher than 10 kDa (UF-1), between 3 and 10 kDa (UF-2) and lower than 3 kDa (UF-3).$^{a-b}$ and *–** indicate significant differences among samples ($P < 0.05$).

 © 2018 Society of Chemical Industry

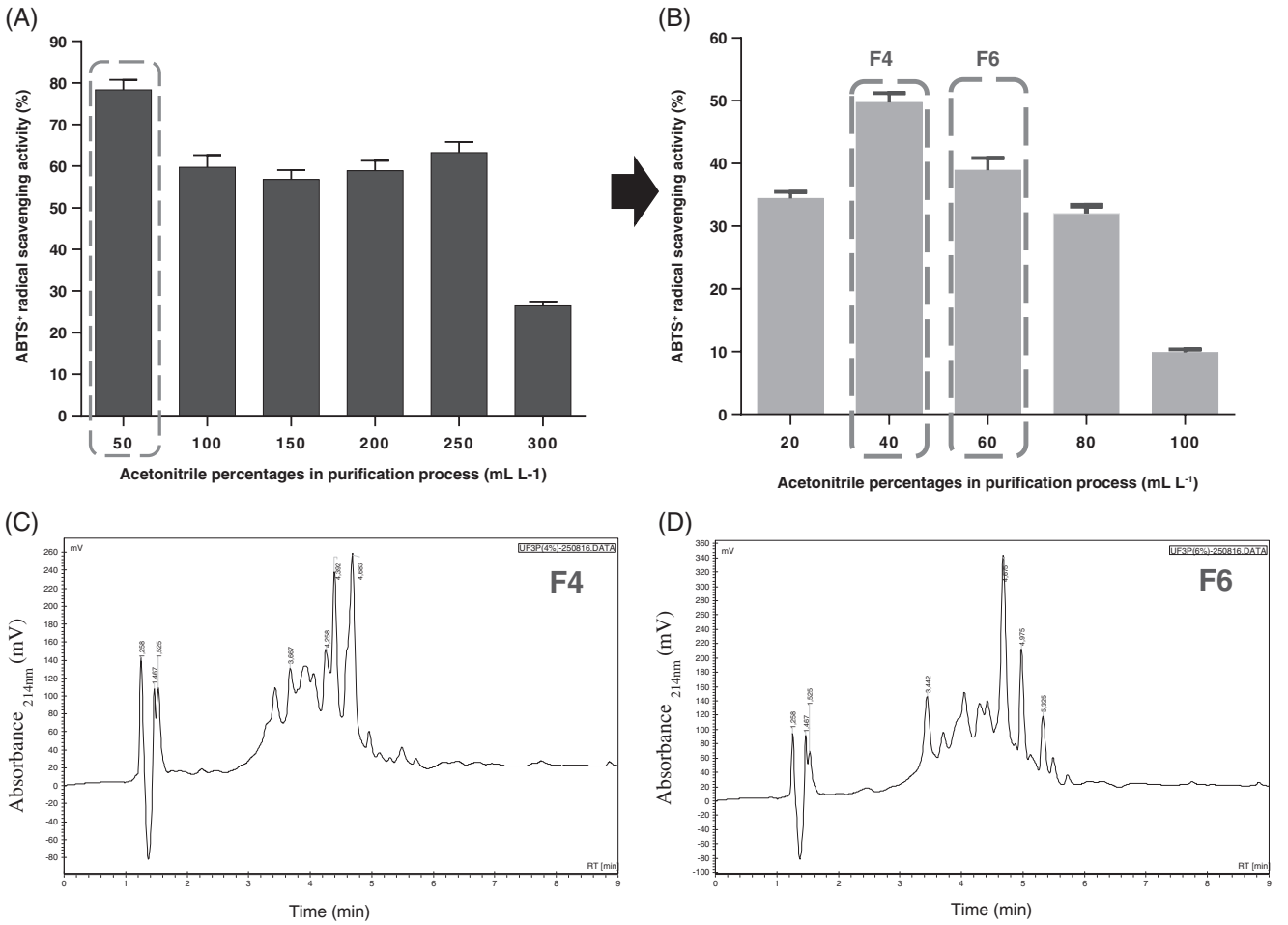

Figure 2. Antioxidant activity and chromatographic profile of acetonitrile fractions of enzymatically hydrolyzed *E. edulis* proteins. $ABTS^{\cdot+}$ scavenging activity of 50–300 mL L^{-1} acetonitrile fractions of pajuro hydrolyzates were evaluated (A). Then, the most active fraction (50 mL L^{-1}) was fractionated again with 20–100 mL L^{-1} of acetonitrile and the $ABTS^{+}$ scavenging activity was evaluated (B). The chromatographic profile of 40 mL L^{-1} (F4) and 60 mL L^{-1} (F6) acetonitrile fractions are shown (C and D respectively).

multiple comparison Student–Newman–Keuls test. All analyses were run with Sigma Plot 11.0 software (Systad Software Inc. San Jose, CA, USA). Statistical significance was defined as $P < 0.05$.

RESULTS AND DISCUSSION

Effect of alcalase on the release of antioxidant peptides

The PC was subjected to hydrolysis by alcalase under the optimal conditions for this microbial enzyme and PCHs were collected at the starting point of hydrolysis, and after 15, 30, 60, and 120 min incubation. Digestion efficiency was monitored by the estimation of the DH. As shown in Fig. 1(A), the DH increased during hydrolysis, reaching a final value of 37.03 ± 0.88% at the end of the enzymatic reaction. This value was higher than that reported for mung bean protein hydrolyzates (20.0%) produced with alcalase for 10 h[28] but lower than that described for by *Vigna unguiculata* protein hydrolyzed with that enzyme during 90 min at the same E/S ratio (53.0%).[29] The observed differences could be due to the protease specificity as alcalase is an industrial alkaline protease produced from *Bacillus licheniformis*, the main enzyme component (serine endopeptidase subtilisin Carlsberg) of which presents broad specificity and hydrolyzes most peptide bonds.[30]

The antioxidant activity of PC and PCHs obtained at different incubation times was measured using the $ABTS^{\cdot+}$ and peroxyl (ORAC) radical scavenging assays (Fig. 1(B)). The spectrophotometric $ABTS^{\cdot+}$ assay quantifies the suppressive capacity of an antioxidant against the radical cation $ABTS^{\cdot+}$ based on single-electron reduction of the relatively stable radical cation $ABTS^{\cdot+}$ formed previously by an oxidation reaction. When added to PBS medium containing $ABTS^{\cdot+}$, the peptides in the hydrolyzates act as electron donors, transforming this radical cation into the non-radical ABTS. In the case of the ORAC assay, ORAC is based on a hydrogen atom transfer mechanism (the hydrogen of the peptides neutralizes the radicals formed) and therefore measures the capacity of antioxidant peptide to break the chain reactions of thermally generated peroxyl radicals from AAPH. The PCHs showed higher $ABTS^{\cdot+}$ and peroxyl radical scavenging activity than the non-hydrolyzed protein (Fig. 1(B)). When the DH was increased from 0 to 37.03%, the TEAC and ORAC values increased from 0.53 ± 0.02 and 0.79 ± 0.06 μmol TE mg^{-1} peptide (non-hydrolyzed protein) to 1.37 ± 0.09 μmol TE mg^{-1} peptide and 2.83 ± 0.07 μmol TE mg^{-1} peptide (120 min-hydrolyzate), representing an increase of 258.5% and 358.2%, respectively (Fig. 1(B)). These results indicated that antioxidant peptides were released from protein during alcalase hydrolysis. Similarly, the potential of this microbial enzyme to release potent radical scavenging peptides has been reported for other plant proteins such as canola meal, pinto bean, or flaxseed protein.[31–33] The efficiency

 © 2018 Society of Chemical Industry

Table 1. Peptides identified using *de novo* sequencing in the fractions eluted with 40 mL L^{-1} (F4) and 60 mL L^{-1} acetonitrile (F6) from permeate < 3 kDa of the pajuro protein concentrate hydrolyzed with alcalase for 120 min

Fraction	MALDI-MS $[M+H]^+$	LC-ESI-MS/MS $[M+H]^+$	Sequence	Amino acid modification	Bioactive peptide[a]	Protein source	Biological activity[a]
F4	389.187	389.8	WAL	–	–	–	–
	410.196	410.9	PGTH	–	–	–	–
	416.200	416.3	DLGL	–	DLGLGLPGAH	Marine rotifer (*Brachionus rotundiformis*)	Antioxidant
	425.167	425.1	VVAH	–	–	–	–
	439.236	439.5	HAVL	–	AVL	Several food proteins	ACE inhibitor
	530.298	530.4	GNLGGL	–	–	–	–
	572.328	572.6	SPQLQ	–	–	–	–
	589.329	589.6	AALWE	–	LWE	Synthetic peptide	Antioxidant
	615.367	615.5	GAVASPL	–	–	–	–
	620.322	619.5	EVDGNS	–	–		–
	635.374	634.6	MWPW	Oxidation (M)	MWP	Synthetic peptide	DPP-IV inhibitor
	645.362	644.4	ETPAGAV	–	–	–	–
	663.372	662.5	DFFMS	Oxidation (M)	–	–	–
	664.380	664.5	EEENGS	–	EEE	Synthetic peptide	Stimulating vasoactive substance release
	665.377	665.6	YLLTR	–	YLL YLLF	Synthetic peptide β-lactoglobulin	Antioxidant ACE inhibitor, opioid, Osteoanabolic
	680.372	680.6	SKDAPY	–	–	–	–
	687.443	686.6	DGLGYY	–	DGL LGY GYY	Synthetic peptide Casein Gluten	ACE inhibitor ACE inhibitor, opioid, immunostimulating Antioxidant, ACE inhibitor, opioid
	709.411	708.8	ELGMGDS	–	–	–	–
	717.413	716.6	MFTGPY	–	TGP	Synthetic peptide	ACE inhibitor
	722.426	721.5	HVHEPC	–	HVH	Synthetic peptide	Antioxidant
	723.425	723.3	CCGDYY	–	DYY	Synthetic peptide	Antioxidant
	737.422	737.7	GESWCR	–	–	–	–
	765.413	765.1	RLEGFGS	–	–	–	–
	766.437	766.5	YDLHGY	–	LHG	Synthetic peptide	Antioxidant
	792.462	793.2	GSYHDSK	–	–	–	–
	828.506	828.0	AVAVPSING	–	VAV, AVP	Casein	ACE inhibitor
F6	573.330	572.5	APGTVK	–	APG	Several protein sources	ACE and DPP-IV inhibitor Antioxidant
	687.389	687.6	SQLPGW	–	LPG	Several protein sources	ACE and DPP-IV inhibitor Antioxidant
	722.420	722.5	NMGQADS	–	–	–	–
	792.442	793.1	MFLTHK	Oxidation (M)	–	–	–

[a] Data accessed from BIOPEP, available at http://www.uwm.edu.pl/biochemia/index.php/pl/biopep on April 2018.

of this enzyme has been associated with its broad specificity and its preferential breakdown of hydrophobic amino acids that act as hydrogen donors.[34]

To study the contribution of the molecular weight to the antioxidant activity of peptides contained in the PCH obtained at the end of hydrolysis, it was subjected to ultrafiltration to separate three fractions, higher than 10 kDa (UF-1) between 3 and 10 kDa (UF-2), and lower than 3 kDa peptide (UF-3) fractions. The ORAC and $ABTS^{+}$ radical scavenging activity of these fractions was determined (Fig. 1(B)). The TEAC value of UF-1 was lower (0.81 µmol TE mg^{-1} peptide) when compared to the whole PCH (1.37 µmol TE mg^{-1} peptide). However, low molecular-weight peptides contained in UF-2 and UF-3 showed high TEAC values of 1.96 and 2.87 µmol TE mg^{-1} peptide, respectively. A similar trend was observed for peroxyl radical scavenging activity. The ORAC value of UF-1 was 1.12 µmol TE mg^{-1} peptide that was lower than that shown by the PCH (2.83 µmol TE mg^{-1} peptide) whereas the UF-2 and UF-3 fractions showed higher ORAC values of 3.19 and 6.11 µmol TE mg^{-1} peptide, respectively. As previously reported for hydrolyzates derived from rapeseed and canola meal protein,[31,35] fractionation of *Erythrina edulis* hydrolyzate to small peptides resulted in an increase in antioxidant activity. This behavior may be due to the enhanced accessibility of small peptides to the redox reaction system.[36]

Identification of antioxidant peptides

Fraction UF-3, containing peptides < 3 kDa, was selected for further purification. The different fractions collected by elution

 © 2018 Society of Chemical Industry

with ACN from 50 to 300 mL L^{-1} were evaluated for their $ABTS^{\cdot+}$ radical scavenging activity. The inhibitory percentages determined for fractions (0.04 mg protein) varied from 26.48 ± 0.31% (300 mL L^{-1}-ACN fraction) to 78.35 ± 0.20% (50 mL L^{-1}-ACN fraction) (Fig. 2(A)). The most potent fraction was subjected to an additional purification step by using ACN at percentages from 20 to 100 mL L^{-1}. The antioxidant activity of the collected fractions is shown in Fig. 2(B). The lowest activity was determined for the fraction eluted with 100 mL L^{-1} ACN (9.94 ± 0.62%) whereas fractions eluted with 40 mL L^{-1}- and 60 mL L^{-1}-ACN inhibited $ABTS^{\cdot+}$ radical up to 49.71 ± 1.52% and 38.67 ± 1.55%, respectively, at 0.04 mg protein. These two fractions, named F4 and F6, the chromatographic profiles of which are shown in Fig. 2(C) and (D), were selected to identify the peptides potentially responsible for the antioxidant effects. The identification of peptides was carried out using the PepNovo peptide sequencing software for the spectral data of each MS/MS. A total of 88 and 144 sequences was observed for F4 and F6 fractions, respectively, of which 25 and 5 peptides were identified. Table 1 lists the amino acid sequences of identified peptides, the observed and calculated peptide masses, and the previously bioactive peptide sequences available in the BIOPEP database with their corresponding activities.[37] As an example, peptide DLGL identified in pajuro protein hydrolyzate corresponds to N-terminal of marine rotifier peptide DLGLGLPGAH with demonstrated antioxidant properties.[38] Pajuro-derived peptide YLLTR contains the sequence YLL, which is also present in β-lactorphin (YLLF), in which ACE inhibitory, opioid, and osteoanabolic properties have been reported.[39–41]

Antioxidant activity of synthetic peptides

Of 30 identified peptides, ten were selected for chemical synthesis and use in *in vitro* bioassays to confirm their contribution to the antioxidant effects observed in the PCH. The peptides were chosen based on current evidence concerning the known attributes of $ABTS^{\cdot+}$ and peroxyl radical scavengers. Studies carried out with peptides from different protein sources have reported that antioxidant activity mainly depends on the bioactive amino acids acting as electron or hydrogen donors,[42] their sequence position, and their hydrophobicity.[43] The radical scavenging activity of peptides can be enhanced by the presence of amino acids such as methionine and cysteine containing a sulfur group that has nucleophilic character. The aromatic amino acids phenylalanine, tyrosine and tryptophan have also been described as mainly responsible for the antioxidant activity of peptides in the $ABTS^{\cdot+}$ and ORAC models,[26,44] principally by the special capability of phenolic and indol groups to serve as hydrogen donors. Hydrophobic amino acids valine, leucine and isoleucine could also improve solubility in lipids, facilitating contact with radical species produced in the hydrophobic phase.[34] In the present work all selected peptides contained one or two hydrophobic amino acids and one or two aromatic residues that could explain their antioxidant activity. The TEAC values obtained for pajuro peptides were from 0.23 to 1.18 μmol TE $μmol^{-1}$ peptide (Table 2). These values are within the range (0.45–4.8 μmol TE $μmol^{-1}$ peptide) reported for synthetic Trp-, Tyr-, and Cys-peptides.[45] Similarly, TEAC values between 0.15 and 4.46 μmol TE $μmol^{-1}$ peptide have been calculated for synthetic peptides identified in casein hydrolyzates.[46] In our study, the highest TEAC values were exhibited for the peptides with aromatic residues along with cysteine or methionine residues. The presence of tyrosine at the C-terminal extreme of peptide seems to be essential for the $ABTS^{\cdot+}$ radical scavenging activity of vegetable proteins-derived peptides.[47] Peptide CCGDYY with the highest TEAC value (1.18 μmol TE $μmol^{-1}$ peptide) contains two cysteine residues at N-terminus and two tyrosine residues in their C term, which could contribute to its antioxidant activity because the role of these amino acids has been widely recognized.[48]

Table 2. Antioxidant activity of synthetic peptides identified in the fractions eluted with 40 mL L^{-1} (F4) and 60 mL L^{-1} acetonitrile (F6) from permeate < 3 kDa of the pajuro protein concentrate hydrolyzed with alcalase for 120 min

Peptide sequence	ABTS radical (μmol TE $μmol^{-1}$ peptide)	ORAC (μmol TE $μmol^{-1}$ peptide)
DGLGYY	0.63 ± 0.04	3.83 ± 0.19
MFTGPY	0.94 ± 0.01	2.44 ± 0.02
WAL	0.58 ± 0.03	3.38 ± 0.09
YDLHGY	0.64 ± 0.05	3.59 ± 0.46
GESWCR	1.12 ± 0.02	2.43 ± 0.01
CCGDYY	1.18 ± 0.03	3.61 ± 0.00
GSYHDSK	0.23 ± 0.02	1.74 ± 0.07
YLLTR	0.40 ± 0.01	1.04 ± 0.07
SQLPGW	0.53 ± 0.01	2.95 ± 0.24
SKDAPY	0.43 ± 0.04	1.06 ± 0.13

Pajuro-derived peptides showed ORAC values ranging from 1.04 to 3.83 μmol TE $μmol^{-1}$ peptide (Table 2). The three highest values were determined for peptides DGLGYY and CCGDYY, which contain two tyrosine residues at the C-terminus, and peptide YDLHGY, which contains one tyrosine residue in the front of the sequence and another one in the terminal. The presence of peroxyl radical scavenging amino acids as C- and N-terminal residues has been described as determining the antioxidant activity of peptides.[49] Thus, tryptophan at N-terminus was considered to play an essential contribution to the antioxidant activity of β-lactoglobulin-peptides WYSL and WYS which ORAC values were 4.51 and 4.45 μmol Trolox $μmol^{-1}$ of peptide, respectively.[50] In our study, peptides WAL and SQLPGW, containing tryptophan at N- and C-terminus, respectively, showed higher ORAC values, 3.38 and 2.95 μmol TE $μmol^{-1}$ peptide, than peptide GESWCR, containing tryptophan in the middle of the sequence. Seven of 10 selected peptides released from pajuro protein showed similar or higher antioxidant activity than that shown by BHA (ORAC value of 2.43 μmol Trolox $μmol^{-1}$ BHA).[26] Although BHA has traditionally been used in the food industry as a synthetic antioxidant, in recent years its demonstrated adverse effects have stimulated its substitution by new natural antioxidants derived from dietary sources.

In the present study, 10 novel peptides with *in vitro* antioxidant effects were identified from pajuro protein hydrolyzate with alcalase. Three of these peptides, DGLGYY, CCGDYY, and YDLHGY, showed potent $ABTS^{\cdot+}$ and peroxyl radical scavenging activity. The results presented here indicate that pajuro protein hydrolyzate, containing a complex mixture of peptides, might be a good source of antioxidant peptides for food and nutraceutical applications. Further studies confirming the *in vivo* antioxidant capacity of these peptides as well as their bioavailability should be conducted.

ACKNOWLEDGEMENTS

This work has received financial support from projects 186-FINCyT-IA-2013 (Programa Nacional de Innovación

 © 2018 Society of Chemical Industry

para la Competividad y Productividad) (Innovate-Peru), N° 007-FONDECYT-2014 (Fondo Nacional de Desarrollo Científico, Tecnológico y de Innovación Tecnológica-Peru), and AGL2015-66886-R (Ministry of Economy and Competitiveness, MINECO, Spain).

REFERENCES

1 Wu D and Cederbaum AI, Alcohol, oxidative stress, and free radical damage. *Alcohol Res Health* **27**:277–284 (2003).
2 Sarmadi BH and Ismail A, Antioxidative peptides from food proteins: a review. *Peptides* **31**:1949–1956 (2010).
3 Halliwell B, The antioxidant paradox: less paradoxical now? *Brit J Clin Pharmacol* **75**:637–644 (2013).
4 Hartmann R and Meisel H, Food-derived peptides with biological activity: from research to food applications. *Curr Opin Biotechnol* **18**:163–169 (2007).
5 Hernández-Ledesma B, García-Nebot MJ, Fernández-Tomé S, Amigo L and Recio I, Dairy protein hydrolysates: peptides for health benefits. *Int Dairy J* **38**:82–100 (2014).
6 Cicero AFG, Fogacci F and Colletti A, Potential role of bioactive peptides in prevention and treatment of chronic diseases: a narrative review. *Brit J Pharmacol* **174**:1378–1394 (2017).
7 Lee SY and Hur SJ, Antihypertensive peptides from animal products, marine organisms, and plants. *Food Chem* **228**:506–517 (2017).
8 Xie J, Huang J, Xu X and Jin Z, Antioxidant activity of peptides isolated from alfalfa leaf protein hydrolysate. *Food Chem* **111**:370–376 (2008).
9 Chalamaiah M, Dinesh kumar B, Hemalatha R and Jyothirmayi T, Fish protein hydrolysates: proximate composition, amino acid composition, antioxidant activities and applications: a review. *Food Chem* **135**:3020–3038 (2012).
10 Power O, Jakeman P and FitzGerald RJ, Antioxidative peptides: enzymatic production, *in vitro* and *in vivo* antioxidant activity and potential applications of milk-derived antioxidative peptides. *Amino Acids* **44**:797–820 (2013).
11 Nimalaratne C, Bandara N and Wu J, Purification and characterization of antioxidant peptides from enzymatically hydrolyzed chicken egg white. *Food Chem* **188**:467–472 (2015).
12 García MC, Puchalska P, Esteve C and Marina ML, Vegetable foods: a cheap source of proteins and peptides with antihypertensive, antioxidant, and other less occurrence bioactivities. *Talanta* **106**:328–349 (2013).
13 Maestri E, Marmiroli M and Marmiroli N, Bioactive peptides in plant-derived foodstuffs. *J Proteomics* **147**:140–155 (2016).
14 Rizzello CG, Tagliazucchi D, Babini E, Rutella GS, Taneyo Saa DL and Gianotti A, Bioactive peptides from vegetable food matrices: research trends and novel biotechnology for synthesis and recovery. *J Funct Foods* **27**:549–569 (2016).
15 Saadi S, Saari N, Anwar F, Abdul Hamid A and Ghazali HM, Recent advances in food biopeptides: production, biological functionalities and therapeutic applications. *Biotechnol Adv* **33**:80–116 (2015).
16 Udenigwe CC and Aluko RE, Food protein-derived bioactive peptides: production, processing, and potential health benefits. *J Food Sci* **77**:R11–R24 (2012).
17 Pihlanto A, Mattila P, Mäkinena S and Pajari A-M, Bioactivities of alternative protein sources and their potential helath benefits. *Food Funct* **8**:3443–3458 (2017).
18 Acero Duarte LE, *Guía para el cultivo y aprovechamiento del Chachafruto o Balú (Erythrina edulis)*. Convenio Andrés Bello, Spain (2002).
19 Cárdenas SE and El p, (*Erythrina edulis*) alimento andino en extinción. *Inv Soc* **16**:16–20 (2012).
20 Arango Bedoya O, Bolaños Patiño V, Ricaurte García D, Caicedo M and Guerrero Y, Obtaining a protein extract from chachafruto flour (*Erythrina edulis*. *Revista Universidad y Salud* **14**:161–167 (2012).
21 Intiquilla A, Jiménez-Aliaga K, Zavaleta AI, Arnao I, Peña C, Chávez-Hidalgo EL *et al.*, *Erythrina edulis* (Pajuro) seed protein: a new source of antioxidant peptides. *Nat Prod Commun* **11**:781–786 (2016).
22 Betancur D, Gallegos S and Chel L, Wet-fractionation of *Phaseolus lunatus* seeds: partial characterization of starch and protein. *J Sci Food Agric* **84**:1193–1201 (2004).
23 IDF. Milk: determination of nitrogen content: inspection by attributes. IDF Standard 20B. (1993).
24 Nielsen PM, Petersen D and Dambmann C, Improved method for determining food protein degree of hydrolysis. *J Food Sci* **66**:642–646 (2011).
25 Re R, Pellegrini N, Proteggente A, Pannala A, Yang M and Rice-Evans C, Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad Biol Med* **26**:1231–1237 (1999).
26 Hernández-Ledesma B, Dávalos A, Bartolomé B and Amigo L, Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *J Agric Food Chem* **53**:588–593 (2005).
27 Frank A and Pevzner P, PepNovo: *de novo* peptide sequencing via probabilistic network modeling. *Anal Chem* **77**:964–973 (2005).
28 Hong LG, Wei L, Liu H and Hui SY, Mung-bean protein hydrolysates obtained with alcalase exhibit angiotensin I-converting enzyme inhibitory activity. *Food Sci Technol Int* **11**:281–287 (2005).
29 Segura Campos MR, Chel Guerrero LA and Betancur Ancona DA, Angiotensin-I converting enzyme inhibitory and antioxidant activities of peptide fractions extracted by ultrafiltration of cowpea *Vigna unguiculata* hydrolysates. *J Sci Food Agric* **90**:2512–2518 (2010).
30 Doucet D, Otter DE, Gauthier SF and Foegeding EA, Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: peptide identification and determination of enzyme specificity. *J Agric Food Chem* **51**:6300–6308 (2003).
31 Alashi AM, Blanchard CL, Mailer RJ, Agboola SO, Mawson AJ, He R *et al.*, Antioxidant properties of Australian canola meal protein hydrolysates. *Food Chem* **146**:500–506 (2014).
32 Garcia-Mora P, Frias F, Peñas E, Zielinski H, Giménez-Bastida JA, Wiczkowski W *et al.*, Simultaneous release of peptides and phenolics with antioxidant, ACE-inhibitory and anti-inflammatory activities from pinto bean (*Phaseolus vulgaris* L. var. pinto) proteins by subtilisins. *J Funct Foods* **18**:319–332 (2015).
33 Karamac M, Kosinska-Cagnazzo A and Kulczyk A, Use of different proteases to obtain flaxseed protein hydrolysates with antioxidant activity. *Int J Mol Sci* **17**:1–13 (2016).
34 Samaranayaka AGP and Li-Chan ECY, Food-derived peptidic antioxidants: a review of their production, assessment, and potential applications. *J Funct Foods* **3**:229–254 (2011).
35 He R, Alashi A, Malomo SA, Girgih AT, Chao D, Ju X *et al.*, Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein hydrolysates. *Food Chem* **141**:153–159 (2013).
36 Dávalos A, Gómez-Cordovés C and Bartolomé B, Extending applicability of the oxygen radical absorbance capacity (ORAC-Fluorescein). *J Agric Food Chem* **52**:48–54 (2004).
37 Minkiewicz P, Dziuba J, Iwaniak A, Dziub M and Darewicz M, BIOPEP database and other programs for processing bioactive peptide sequences. *J AOAC Int* **91**:965–980 (2008).
38 Byun H-G, Lee JK, Park HG, Jeon J-K and Kim S-K, Antioxidant peptides from the marine rotifier *Brachionus rotundiformis*. *Process Biochem* **44**:842–846 (2009).
39 Mullally MM, Meisel H and Fitzgerald RJ, Synthetic peptides corresponding to alpha-lactalbumin and beta-lactoglobulin sequences with angiotensin-I-converting enzyme inhibitory activity. *Biol Chem Hoppe Seyler* **377**:259–260 (1996).
40 Martínez- Maqueda D, Miralles B, Ramos M and Recio I, Effect of β-lactoglobulin hydrolysate and β-lactorphin on intestinal mucin secretion and gene expression in human goblet cells. *Food Res Int* **54**:1287–1291 (2013).
41 Pandey M, Kapila R and Kapila S, Osteoanabolic activity of whey-derived anti-oxidative (MHIRL and YVEEL) and angiotensin-converting enzyme inhibitory (YLLF, ALPMHIR, IPA and WLAHK) bioactive peptides. *Peptides* **99**:1–7 (2018).
42 Sila A and Bougatef A, Antioxidant peptides from marine by-products: isolation, identification and application in food systems. A review. *J Funct Foods* **21**:10–26 (2016).
43 Wattanasiritham L, Theerakulkait C, Wickramasekara S, Maier CS and Stevens JF, Isolation and identification of antioxidant peptides

 © 2018 Society of Chemical Industry

from enzymatically hydrolyzed rice bran protein. *Food Chem* **192**:156–162 (2016).
44 Hernández-Ledesma B, Miralles B, Amigo L, Ramos M and Recio I, Identification of antioxidant and ACE-inhibitory peptides in fermented milk. *J Sci Food Agric* **85**:1041–1048 (2005).
45 Zheng L, Zhao Y, Dong H, Su G and Zhao M, Structure–activity relationship of antioxidant dipeptides: dominant role of Tyr, Trp, Cys and met residues. *J Funct Foods* **21**:485–496 (2016).
46 Shanmugam VP, Kapila S, Sonfack TK and Kapila R, Antioxidative peptide derived from enzymatic digestion of buffalo casein. *Int Dairy J* **42**:1–5 (2015).
47 Babini E, Tagliazucchi D, Martini S, Dei Più L and Gianotti A, LC-ESI-QTOF-MS identification of novel antioxidant peptides obtained by enzymatic and microbial hydrolysis of vegetable proteins. *Food Chem* **228**:186–196 (2017).
48 Zheng L, Zhao M, Xiao C, Zhao Q and Su G, Practical problems when using ABTS assay to assess the radical-scavenging activity of peptides: importance of controlling reaction pH and time. *Food Chem* **192**:288–294 (2016).
49 Zhang D, Wang Y, Xu M, Ding L, Zhang T and Liu J, Antioxidant synergetic effect between the peptides derived from the egg white pentapeptide Trp-Asn-Trp-ala-asp. *Int J Pept Res Ther* **23**:509–518 (2017).
50 Hernández-Ledesma B, Amigo L, Recio I and Bartolomé B, ACE-inhibitory and radical-scavenging activity of peptides derived from α-Lactoglobulin f(19-25). Interactions with ascorbic acid. *J Agric Food Chem* **55**:3392–3397 (2007).

 © 2018 Society of Chemical Industry