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Pisum Sativum protein hydrolysates: Production, Sensitive screening of nickel chelating peptides and evaluation of antioxidant properties

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ABSTRACT

Hydrolysates can be obtained by enzymatic treatments and present various bioactivities and functional properties such as antioxidant, metal-chelation *etc.*, enabling their industrial application. These activities depend on amino acid composition, peptides size profile, peptide structure, which are related to proteolysis conditions. Hence, pea protein hydrolysates were produced by either Alcalase® followed by Flavourzyme® (Alc + Flav≤1 kDa) or Protamex® followed by Flavourzyme® (Prot + Flav≤1 kDa). Physico-chemical characterization of these hydrolysates shows that Prot + Flav≤1 kDa hydrolysate has higher degree of hydrolysis and and thus, higher small-size peptides quantity compared to Alc + Flav≤1 kDa. Besides, switchSENSE® technology allowed to unveil the presence of Ni^{2+} chelating peptides, demonstrating the high potential of such innovative technology to screen metal-complexing peptides in hydrolysates. Moreover, the Prot + Flav≤1 kDa hydrolysate shows higher radical scavenging and reducing power, related to its higher degree of hydrolysis and small-size peptides quantity. The peptides identified in both hydrolysates by LC-MS/MS analysis contain high amount of aspartic acid, glutamic acid, proline, leucine, valine and glycine, which might be responsible of their antioxidant properties. The sequential proteolysis may increase metal chelating and antioxidant activities of hydrolysates.

1. Introduction

Thanks to their high nutritional value, food proteins can be used as functional food components. Leguminous, attracting the attention of researchers, are interesting sources of proteins, not expensive compared to animal proteins (Pownall et al., 2010). Dried yellow pea (*Pisum Sativum)*, one of various types of leguminous, contains high level of carbohydrates, proteins (21–25%), low fiber, digestible nutrients (86–87%) (Lazányi, 2002) and is allergens-free according to the Official Journal of European Union (García Arteaga et al., 2020). Yet, according to the Food and Agricultural Organization (FAO), France needs to valorise pea production since this is the 6th producer and the 4th exporting country of peas (Vikas Rawal & Dorian Kalamvrezos Navarro, 2019). However, pea proteins has a lower digestibility due to their large size and the presence of antinutritional factors like trypsin inhibitors (Aryee & Boye, 2016). Thus, proteins are extracted and their isolates are further broken down by enzymatic treatments to produce hydrolysates containing small-sized peptides, endowed with various bioactivities (*e.g.,* antioxidant, antihypertensive, anti-inflammatory, antimicrobial, free radical scavenging etc.) (Chalamaiah et al., 2019; Chao et al., 2013; El Hajj, Sepulveda-Rincon et al., 2021). In addition, peptide hydrolysates are also characterized by various functional properties such as emulsification, foaming and metal-chelation (Barac et al., 2012), enabling their use as potential additives in the food industry (Boye et al., 2010) and for

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medical applications (Contreras et al., 2019). The application of pea protein hydrolysates, various protein resources of metal-chelating peptides, the production and separation of metal-chelating peptides were discussed in our review article (Irankunda et al., 2022). All the properties of metal chelating peptides mentioned above depend on Amino Acid (AA) composition, peptide size, peptide sequence, protein/peptide conformation and structure, which are related to enzymatic treatment conditions (Boye et al., 2010).

Hence, this study aims to valorise pea proteins via enzymatic treatments to produce hydrolysates containing potential bioactive peptides, notably for their antioxidant and metal-chelation activities. To date, various enzymatic treatments were applied on pea protein mainly with Alcalase®, Flavourzyme®, Protamex®, pepsin, trypsin, chymotrypsin and papain (Barbana & Boye, 2010). In the present study, hydrolysates were produced by sequential enzymatic treatment carried out first with Alcalase® or Protamex® followed then by Flavourzyme® and finally submitted to 1 kDa ultrafiltration. Such sequential enzymatic hydrolysates were investigated in order to improve hydrolysis and thus biofunctional properties such as metal chelation and antioxidant properties. Indeed, metal chelation is one of the mechanisms that leads to antioxidant activity, for example metal ions that are prooxidant of lipid oxidation in food emulsions can be chelated by peptides, thus inhibiting lipid oxidation (Yesiltas et al., 2022).

According to the literature, pea hydrolysates contain peptides, which are potentially good metal chelators (Barac et al., 2012; Barbana & Boye, 2010; Pownall et al., 2010). To our knowledge, the investigation of metal chelation activities in pea hydrolysates produced by sequential enzymatic treatment using electrically switchable nanolevers has never been studied to date. Therefore, in this study, we aimed to screen the presence of metal-chelating peptides in such sequential hydrolysates using switchSENSE®, an innovative technique. This biophysical technology, works with electrically actuated Deoxyribonucleic Acid (DNA) nanolevers on chip-based gold microelectrodes. In switchSENSE®, one single strand DNA (ssDNA) is functionalized with a fluorophore, the second complementary strand (cDNA-NTA) is grafted with a capture molecule (Nitrilotriacetic acid, NTA), onto which, Ni^{2+} is loaded in excess, and real time-interaction occurring between metal ions and peptides is analyzed.

The physico-chemical properties of hydrolysates were investigated such as the degree of hydrolysis, the peptides size profile, and the amino acid composition in order to correlate them with their nickel chelating properties and other antioxidant properties such as radical scavenging activity, iron chelating activity and reducing activity.

2. Material and methods

2.1. Chemicals

The following chemicals were provided by Sigma–Aldrich (St Louis, MO, USA) notably: Alcalase® (Protease from *Bacillus licheniformis* - ≥ 2.4 U/g), Protamex® (Protease from Bacillus sp. - ≥ 1.5 U/g), Flavourzyme® (Protease from *Asperdillus oryzae* - ≥ 500 U/g), ammonium bicarbonate, O-phthaldialdehyde (OPA), N,N-dimethyl-2-mercaptoethyl ammonium, sodium tetraborate, sodium dodecyl sulfate, glycine, sodium nitrate ($NaNO_3$), sodium azide (NaN_3), ethylenediaminetetraacetic acid (EDTA), carnosine, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate 97% (ferrozine), ferrous chloride solution ($FeCl_2$), murexide, copper sulfate ($CuSO_4$), hexamine, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®), potassium persulfate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), Prussian blue, and ascorbic acid. Other analytical grade chemicals were purchased from VWR International (Atlanta, USA): potassium sulfate (K_2SO_4), sodium phosphate monobasic (NaH_2PO_4), and sodium phosphate dibasic (Na_2HPO_4).

All spectrophotometric experiments were carried out using a spectrophotometer MRX Multiskan Go (ThermoFicher Scientific, Vantaa, Finland) equipped with a microplate reader and using 96-well microplates (Nunclon™ delta surface, Thermo scientific, Roskilde, Denmark).

2.2. Proteolysis

Pea protein hydrolysates were produced from Pisane® C9, a yellow pea protein isolate with 86 ± 2 % protein w/w, Fat ~1.5 %, Carbohydrates ~ 3.4 %, ash ~ 6% (Group Warcoing S.A, Belgium) similarly to El Hajj et al. (2022). First, pea isolate was diluted at 2% w/v in 50 mM ammonium bicarbonate buffer (5 g qsf 250 mL) and preheated at 90 °C for 5 min to breakdown large proteins. On total, two hydrolysates were prepared using sequential enzymatic hydrolysis with either Alcalase® followed by Flavourzyme® (Alc + Flav) or Protamex® followed by Flavourzyme® (Prot + Flav). Whatever the enzyme (E), the enzyme-to-substrate ratio (E/S) was always set at 1% (v/v). The first enzymatic treatment was performed in thermostatically controlled reaction vessels at 55 °C by adding either Alcalase® for 1h at pH 8, or Protamex® for 1h at pH 7, followed by enzyme inactivation (95 °C, 15 min). After Alcalase® or Protamex® treatment, a second enzymatic hydrolysis was performed by Flavourzyme® for 2h at pH 7, which was then inactivated (95 °C, 15 min). All over proteolysis, the mixture was stirred while monitoring and maintaining the temperature and pH. At the end of the sequential treatment, the hydrolysate was centrifuged (*10 000 g*, 15 min; Hettich Universal 320 R, Tuttlingen, Germany) to precipitate and remove large unhydrolyzed proteins and enzymes. The whole hydrolysates were finally lyophilized (LABCONCO, Kansas City, USA).

For ultrafiltration step, each former lyophilized hydrolysate powder was dispersed in 200 mL MilliQ water (18 mΩ cm^{-1}) to reach a concentration of 20 g/L and was fractionated in 2 steps, under stirring using a 75 mL solvent-resistant stirred cell, with 47 mm disc filter (Millipore, Jaffrey, France). First, a 10 kDa Ultracel® ultrafiltration membrane (Millipore, Jaffrey, USA) was used. Then, the 10 kDa permeate was filtrated onto a 1 kDa Ultracel® ultrafiltration membrane (Millipore, Jaffrey, USA). These ≤1 kDa fractions were lyophilized to obtain final hydrolysates (Alc + Flav ≤ 1 kDa and Prot + Flav ≤ 1 kDa) and stored at −20 °C for a maximum of 52 weeks for further analysis.

2.3. Physico-chemical characterization of the produced hydrolysate

2.3.1. Determination of the degree of hydrolysis

The molar concentration of the whole pea protein hydrolysates and the corresponding ≤1 kDa fraction (Alc + Flav ≤1 kDa and Prot + Flav ≤1 kDa) was determined by OPA quantification according to Nielsen et al. (2001), adapted for microplates (Canabady-Rochelle et al., 2018). Briefly, The OPA quantification was performed by adding 200 μL of the OPA reagent (preparation described in supplementary data SD1) to 20 μL of sample or standard solution, prepared at 1 mg/mL in ultrapure water, and the absorbance value was measured at 340 nm. A calibration curve was performed with glycine (0–5 mM) diluted in ultrapure water. The results were expressed as mmol equivalent glycine per gram of sample (mmol Eq G/g).

From OPA quantification, the degree of hydrolysis (DH) was determined for the non-ultrafiltrated hydrolysates (whole hydrolysate) solely. Indeed, the DH cannot be determined on an ultrafiltrated hydrolysate since this last fraction has not the same composition than the initial protein sample before hydrolysis. The DH was expressed as the ratio of free amino groups present in the hydrolysate over the total amino groups present in the completely hydrolyzed pea protein isolate (acidic hydrolysis conditions: 10 mg of sample were diluted in 10 mL 6 N HCl, the mixture was placed in a microwave digestion system at 130 °C for 24h), assuming the cleavage of all peptide bonds of the protein during acid digestion (Huang et al., 2018). The DH was calculated by Equation (1):

$$\text{Degree of hydrolysis } (\%) = \left[\frac{NH_2\ X - NH_2\ 0}{NH_2\ T - NH_20}\right] x\ 100 \quad (1)$$

Where: NH_2X is the number of free-NH_2 groups at final time of enzymatic hydrolysis, NH_20 is the number of free-NH_2 groups before hydrolysis, and NH_2T is free-NH_2 after complete acid hydrolysis.

2.3.2. Size profile determination

SDS-PAGE. The molecular weight (MW) profile of pea protein isolate (PPI) and pea protein hydrolysates (PPH, Alc + Flav ≤1 kDa, and Prot + Flav ≤1 kDa) was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), similarly to Laemmli (1970) using a Mini-Protean tetra cell electrophoresis system (Bio-Rad Laboratories, Hercules, USA) and determined in reference to a marker size.

The PPI and PPH were first solubilized at 20 mg/mL in ultrapure water and then dissolved at a ratio of 1:4 (v/v) with sample buffer. This sample buffer was composed of 0.5 M Tris-HCl, pH 6.8, and contained 10% (w/v) SDS, 50% (v/v) glycerol, and 0.05% (w/v) bromophenol blue in the presence of 0.5% (w/v) 2-mercaptoethanol as reducing agent. These mixtures were heated at 100 °C for 5 min to completely denature proteins. Then, 10 μL of each sample (*i.e.,* PPI and hydrolysates, containing around 40 μg of protein) or MW maker (Catalog N°: 26619, Thermo Scientific™, Vilnius, Lithuania; range: 10–250 kDa) were loaded onto the polyacrylamide gel (5% (w/v) stacking gel and a 7.5% (w/v) separation gel; Table SD2). The electrophoresis was conducted at a voltage of 120 V for 15 min and then 180 V for 1 h. After electrophoresis, the gel was stained for 1h with a solution containing 0.16% (w/v) Coomassie blue R-250, 7.5% (v/v) acetic acid and 5% (v/v) ethanol. Then, the gel was destained with a solution of 30% (v/v) ethanol and 7.5% (v/v) acetic acid for 1 h.

Gel Filtration Chromatography. GFC was used to determine the MW distribution between 0.2 and 10 kDa of PPH solely (*i.e.*, Alc + Flav ≤1 kDa and Prot + Flav ≤1 kDa), since according to SDS-PAGE profile, PPI does not contain any proteins with MW less than 10 kDa. The GFC system was composed of a degazer, a water 515 HPLC pump, two separation columns in series, and a refractive index detector (RID 10-A Shimadzu). The first column Phenomenex PolySep-GFC-P 2000 (7.8 x 35 mm - 8 μm–200 A-separation range 100 Da–10 kDa) was connected with a guard column (7.8 * 300 mm) and followed by a column Phenomenex PolySep-GFC-P 1000 (7.8 x 35 mm - 8 μm–100 A - separation range 20 Da–3 kDa). An aqueous buffer containing sodium nitrate ($NaNO_3$) at 0.1 M and sodium azide (NaN_3) at 0.2% (w/v) was used as mobile phase. The flow rate was set at 0.7 mL/min and each sample (200 μL, 5 mg/L) was injected and separated at room temperature (20 °C). The GFC was calibrated with Polyethylene Glycol (PEG) solutions (200, 600, 1000, 3000 and 8000 Da) injected in the same conditions as samples. Results were analyzed by ASTRA® software (Wyatt Technology).

2.3.3. Amino acid composition determination

Pea protein isolate and hydrolysates (10 mg of sample dissolved in 10 mL of 6 N HCl) were totally hydrolyzed using a microwave digestion system (Microwave Start D Milestone, Milan, Italy) at 130 °C during 2 h, according to Rutherfurd and colleagues (Rutherfurd & Gilani, 2009). For sulfur-containing amino acids (cysteine and methionine) determination, before total hydrolysis using 6 N HCl, the samples were submitted to the performic acid oxidation according to Gehrke and colleagues (Gehrke et al., 1985). For tryptophan determination, the samples were submitted to alkaline hydrolysis as described by La Cour, Jørgensen, & Schjoerring (2019).

Estimation of the amino acid content of samples was carried out on a UHPLC-MS system (ThermoFisher Scientific, San Jose, CA, USA) connected to a photodiode array detector (PDA) and a LTQXL mass spectrometer equipped with an atmospheric pressure ionization interface operating in electrospray positive mode (ESI+). MS data were processed using Xcalibur v.2.1 software (Thermo Scientific). For AA quantification, a homemade standard mixture of the twenty amino acids and cysteine (0.01, 0.02, 0.03, 0.04 and 0.05 mM) were injected before and after the samples, in order to ensure reliable external quantification. Concentration of each AA was given as the mean of the two values obtained from the calibration curves of the standard mixture carried out before and after samples injection, the standard deviation indicating the stability of the MS response along the sequence. The results were finally normalized and expressed in gram of amino acid per 100 g of sample. The details of this protocol and MS conditions are presented in supplementary data SD3.

2.4. Screening of metal-chelating peptides using time-resolved molecular dynamics measurement

2.4.1. Development of biosensor for MCPs detection

The switchSENSE® technology was used to study the ability of Alc + Flav ≤ 1 kDa and Prot + Flav ≤ 1 kDa hydrolysates to chelate nickel ions (Ni^{2+}) by real-time molecular interaction analysis, as MCPs biosensor (Fig. 1). Nickel chelation on the DNA nanolever is usually used for the immobilization of His-tagged proteins, but in our case, chelated nickel was used directly as a ligand, as described by El Hajj, Sepúlveda Rincón, et al. (2021).

First, the single-stranded ssDNA nanolever covalently fixed on the chip surface of the sample spots of the biosensor was functionalized by hybridization of a complementary cDNA nanolever containing NTA_3 (cDNA-NTA_3), leading to the formation of a double-stranded DNA nanolever (dsDNA) on the surface, and by nickel chelation (Fig. 1, step 1). Secondly, the association and dissociation kinetics as well as sizing analysis were performed (Fig. 1, step 2,3,4,5). Finally, the biosensor was regenerated by EDTA treatment for further analysis (Fig. 1, step 6).

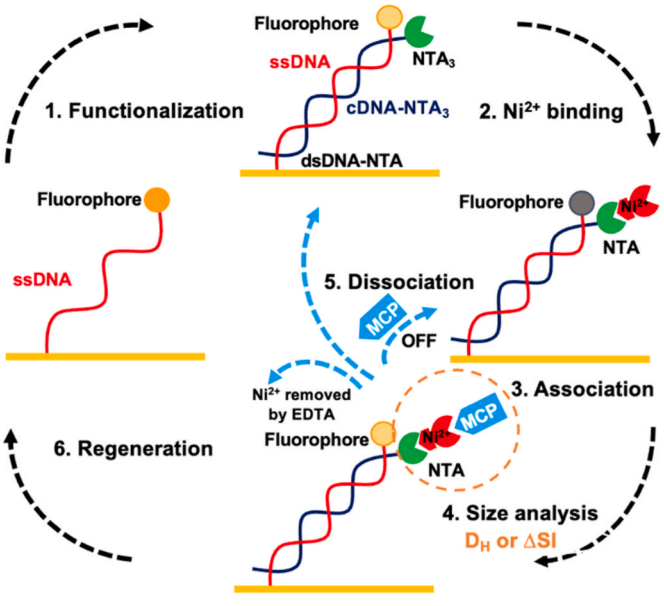


Fig. 1. Schematic overview of the switchSENSE® analysis set-up in this study. Steps: (1) hybridization and functionalization of ssDNA with cDNA-NTA_3 nanolever. (2) nickel ions binding. (3) association of metal-chelating peptides present in pea protein hydrolysates and real time monitoring of binding kinetics. This cycle continues until the highest concentration (200 μM) is reached. (4) Sizing analysis: schematic of a DNA nanolever with NTA, Ni^{2+} and peptide (D_H: Hydrodynamic diameter, ΔSI: size index), dashed lines indicate the 'Lollipop' silhouette used for the calculation. (5) dissociation of 200 μM using EDTA. (6) regeneration step: the sensor surface of the biochip regenerated yielding only ssDNA nanolever fixed with the fluorophore on the chip's surface.

2.4.2. Experimental setup

Experiments were performed on both ultrafiltrated PPH (Alc + Flav ≤1 kDa and Prot + Flav ≤1 kDa) using a biosensor analyzer DRX (switchSENSE® Dynamic Biosensors GmbH, Planegg, Germany) according to the method initiated by El Hajj, Sepúlveda Rincón, et al. (2021) with some modifications. This method was used in two modes, static and dynamic mode. The static mode, known as the fluorescence proximity sensing mode, detects the binding of molecules (*i.e.,* peptides onto Ni^{2+} ions) in real-time through changes in the fluorophore local environment in order to determine kinetics and affinity constants (k_{on}, k_{off}, K_D), whereas, in dynamic mode, based on hydrodynamic friction increasing, the binding of molecules is detected by following the DNA nanolever switching speed (dynamic response, DR), revealing information about size or conformational changes of ligands, interacting or not with analytes (Müller-Landau & Varela, 2021).

The metal ion binding experiments were carried out using an electro-switchable DNA chip MPC-48-2-R1-S containing tris-nitrilotriacetic acid, and Cy5 dye was used as fluorescent probe. The chip is composed of 6 spots related to electrodes, 2 of them are used as references and the 4 others as sample spots functionalized with Ni^{2+}-chelating DNA nanolevers (El Hajj, Sepúlveda Rincón et al., 2021). Briefly, each hydrolysate was diluted in T40 buffer (10 mM Tris-HCl containing 40 mM NaCl and 0.05% v/v Tween 20, pH 7.4) to investigate 3 concentrations (*i.e.*, 50, 100, and 200 μM eq. NH_2). Each diluted sample was injected in the microfluidic device at a flow rate of 30 μL min^{-1} for 4 min (association phase). Then, T40 buffer was injected at 50 μL min^{-1} for 4 min to release the analytes (dissociation phase). At the end of each investigated concentration, the immobilized Ni^{2+} ions were removed by 100 mM EDTA and followed by another Ni^{2+} re-binding phase before studying another concentration. Buffer blank was also investigated and subtracted to the signal to correct all the kinetic experiments from unspecific interactions (static mode measurements).

Between the association and the dissociation phases investigated during the static mode measurement, the flow was stopped and dynamic analysis (dynamic mode measurement) were carried out to determine the specificity of the peptide-metal ion interaction and the size analysis of the peptide bound onto Ni^{2+}. Dynamic measurements expressed as ΔDR (%) were calculated by the difference between the rate of nanolever motion of samples containing metal-chelating peptides ($DR_{peptide}$) and the rate of nanolever motion of the blank ($DR_{T40\ buffer}$), using equation (2).

$$\Delta DR\ (\%) = \frac{DR_{peptides} - DR_{T40buffer}}{DR_{T40buffer}} x\ 100 \quad (2)$$

Using the dynamic mode of the switchSENSE® technology, the hydrodynamic diameter (D_H) is usually determined for proteins immobilized on the nanolevers - interacting or not with an analyte - from the motion of the loaded nanolevers according to the Lollipop mathematical model (Vicente et al., 2020). In the present study, in the absence of any protein, the D_H variation calculated between the empty nickel ions and nickel ions carrying MCPs would correspond to a binding index (ΔSI) rather than to any change in hydrodynamic diameter.

The SwitchANALYSIS® software from Dynamic Biosensors was used to analyze the various curves by nonlinear fitting of single-exponential functions, the global fit error of all measurements corresponding to the errors on results. Each kinetics experiment was performed twice with different sample preparations. The ΔSI values were the mean of 8 measurements.

2.5. Antioxidant activity evaluation

Iron-chelating activity. The capacity of pea protein hydrolysates to chelate Fe^{2+} was determined by spectrophotometry measuring the formation of the Fe^{2+}-ferrozine complex as in Canabady-Rochelle et al. (2018) (more details in supplementary data SD3). Briefly, the EDTA (0–0.04 mg/mL) and carnosine (0–1 mg/mL) were used as positive controls. The hydrolysate solutions were investigated between 0 and 15 mg/mL in ultrapure water. The absorbance of controls or samples was measured at 562 nm. The iron-chelating activity of pea protein hydrolysates was calculated as described in SD4. The results were expressed as the efficient concentration of hydrolysates able to chelate 50% of iron (EC_{50}).

Reducing activity. The reducing activity was determined by spectrophotometry similarly to Canabady-Rochelle et al. (2015) in reference to ascorbic acid and carnosine as positive controls (more details in supplementary data SD5). Briefly, ascorbic acid (0–0.1 mg/mL), carnosine (0–10 mg/mL) and hydrolysate solutions (0–10 mg/mL) were prepared in phosphate buffer (0.2 M, pH 6.6). The reduction of Fe^{3+}/ferricyanide to Fe^{2+}/ferrocyanide produces Prussian blue, whose absorbance is determined at 700 nm after 10 min of incubation at 25 °C. The capacity of pea protein hydrolysates to reduce Fe^{3+} into Fe^{2+} was calculated in % as described in SD5. The results were expressed as the effective concentration of sample (hydrolysates or positive control) to reach 50% of the maximal reducing activity (EC_{50}).

Radical-scavenging activity. The ABTS radical scavenging activity was determined by spectrophotometry similarly to Canabady-Rochelle et al. (2015) (more details in supplementary data SD6). Briefly, the $ABTS^{\circ+}$ cationic radical solution was diluted in phosphate buffer (4 mM, pH 7.4) to reach an absorbance value of 0.70 ± 0.02 at 734 nm. The positive control Trolox® was investigated from 0 to 120 μM to plot a calibration curve. The samples (hydrolysates or carnosine) were prepared at various concentrations (0–1 mg/mL) in the 4 mM phosphate buffer. To determine the $ABTS^{\circ+}$ radical scavenging activity, 150 μL $ABTS^{\circ+}$ radical solution was mixed with 150 μL of sample or Trolox® solution, incubated at 30 °C for 10 min and the absorbance measured ($\lambda = 734$ nm). The $ABTS^{\circ+}$ radical scavenging activity was calculated as described in Table SD6. In addition, the Trolox® Equivalent Antioxidant Capacity (TEAC index) was calculated from calibration curve and results were expressed as μmol Trolox® Equivalent per gram of sample (μmol TE/g sample).

2.6. Mass spectrometry identification and sequences analysis

2.6.1. LC-MS/MS analysis

To identify the peptide sequences, present in the produced hydrolysates, the samples (1 mg/mL) were analyzed on a Thermo Scientific UHPLC-HRMS system composed of a Vanquish Flex™ liquid chromatography unit coupled to a photodiode array detector (PDA) and an Orbitrap ID-X™ Tribrid™ high-resolution mass spectrometer operating in positive electrospray ionization mode (ESI^+). Chromatographic conditions, MS parameter settings, thresholds for peptide identification and detailed protocol are provided in supplementary data (SD7).

2.6.2. Peptide sequences identification

The Thermo Xcalibur raw data resulting from LC-MS/MS analysis of pea protein hydrolysates were processed using free MaxQuantTM proteomics software (version 2.4.13.0) to automatically elucidate the amino acid sequences of the hydrolysis peptides. The peptide search was conducted using a restricted pea (*Pisum Sativum*) protein data base containing 36 entries obtained from UniProtKB (https://www.uniprot.org/, accessed on April 20, 2024, Supplementary data, SD8). The parameters of database search were adjusted as shown in Table SD9. Finally, the AAs composition of identified peptides present in pea protein hydrolysates were computed through ExPASy ProtParam server (https://web.expasy.org/protparam/).

2.7. Statistical analysis

The results were statistically analyzed by one-way analysis of variance (ANOVA). All the measurements of AAs composition, OPA quantification and biofunctional properties (Ni^{2+} binding ability, Fe^{2+}-chelating, reducing power and radical scavenging activities) were

triplicated and data presented as means ± standard deviation. Significant differences between means of samples were determined by Fisher's multiple range test. Differences at *p-value* < 0.05 were considered significant. Statistical calculations were conducted using the software Statgraphics centurion XVI version 16.1.03 (Statgraphics Technologies Inc, Virginia, USA).

3. Results and discussion

3.1. Physical-chemical characterization of pea hydrolysates

3.1.1. Peptide quantification and determination of the degree of hydrolysis

For pea protein isolate, the peptide concentration (OPA quantification) is equal to 0.66 ± 0.07 mM Eq Gly/g powder. After proteolysis, the peptide concentration is equal to 2.31 ± 0.04 mM Eq Gly/g and 2.71 ± 0.06 mM Eq Gly/g, for the whole Alc + Flav and Prot + Flav hydrolysates, respectively. Besides, after 1 kDa ultrafiltration, the peptide concentration is equal to 2.54 ± 0.07 mM Eq Gly/g and 3.55 ± 0.12 mM Eq Gly/g for the Alc + Flav ≤ 1 kDa and the Prot + Flav ≤ 1 kDa hydrolysates, respectively. Thus, in accordance with various studies, both an enzymatic treatment and ultrafiltration increase the small peptide concentration by a reactional mechanism and a separation process, respectively, which potentially leads to the improvement of biofunctionalities.

From OPA quantification, the degree of hydrolysis (DH) was calculated for the 2 whole hydrolysates solely. The DH is of 36.23 ± 0.85 % and 45.05 ± 1.23 % for the whole Alc + Flav and the whole Prot + Flav hydrolysates, respectively. The DH is higher when the Protamex® is first used, compared to Alcalase®. Indeed, Alcalase® is a mixture of endopeptidases while Protamex® is a mixture of both an endo and exopeptidases (Moroni Silva et al., 2014), increasing the cleavage sites and facilitating the second hydrolysis. Flavourzyme® used for second hydrolysis is an endo and exopeptidase as well, which increases cleavage sites thus leading to higher DH. Various researchers studied pea protein hydrolysates obtained by individual treatment of Alcalase®, Protamex®, and Flavourzyme® and they found lower DH (García Arteaga et al., 2020). Thus, the sequential enzymatic treatment using Alcalase® or Protamex® followed by Flavourzyme® may increase the yield of hydrolysis. Besides, Barac and colleagues (Barac et al., 2012) submitted pea protein isolate to papain enzymatic treatment, the DH varied from 12.34 % to 16.84 % which prove the efficacy of Alcalase®, Protamex® and Flavourzyme®. The DH of the produced hydrolysates is nonetheless affected by many other hydrolysis conditions. For example, Barbana and Boye (Barbana & Boye, 2010) determined a higher DH than ours (58.89 ± 0.24 %) for pea protein hydrolysates with the same enzymatic treatment (Alc + Flv) but different E/S ratio and hydrolysis duration (1/8 for Alcalase® in1 h then 1/10 for Flavourzyme in 1.5 h) used compared to our study (E/S ratio of 1/100 for both enzymes; 1 h for Alcalase and then 2 h for Flavourzyme).

3.1.2. Size distribution of pea isolate and hydrolysates

The protein MW profile in the pea protein isolates and in the ≤ 1 kDa hydrolysates (Alc + Flav ≤ 1 kDa and Prot + Flav ≤ 1 kDa) was investigated by SDS-PAGE in reference to a 10–250 kDa marker size (Fig. 2).

For pea protein isolates (PPI lane), some protein bands of high MW (15–250 kDa) are observed while almost no band is observed below 15 kDa. Specifically, some bands are observed as described hereafter: within 100–120 kDa, ~70 kDa, ~50–55 kDa, ~40 kDa, ~32–35 kDa, ~25 kDa, ~15–20 kDa. In agreement with the literature (García Arteaga et al., 2020), the bands identified on our SDS-PAGE in the PPI would probably correspond to the following proteins: convicilin (67–72 kDa), vicilin mature (47–52) kDa, vicilin $\alpha\beta$ (32 kDa), vicilin gamma (12–16 kDa). Besides Barbana and Boye (Barbana & Boye, 2010), who studied pea protein concentrate, also identified vicilin (48 kDa), convicilin (63 kDa) and an unkown band (14–30) kDa. The study of Ma and colleagues (Ma et al., 2017) on yellow pea protein evidenced three bands (*i.e.,* 50 kDa, 52 kDa, 32 kDa) all attributed to vicilin subunits and another band at 67 kDa, attributed to legumin subunit. Chang and colleagues (Chang et al., 2021) observed bands of convicilin (65 kDa), vicillin (45 kDa), legumin acidic unit (38 kDa) and legumin basic unit (19–22 kDa) in PPI. Albumin with its 2 polypeptides weighting around 25 kDa was additionally reported by Boye and colleagues (Boye et al., 2010). However, no band is observed whatever the ≤ 1 kDa hydrolysates since they were filtrated on a 1 kDa membrane. In addition to the previous OPA quantification and the DH determination, this result indirectly confirms the hydrolysis of pea protein isolate into small-sized peptides.

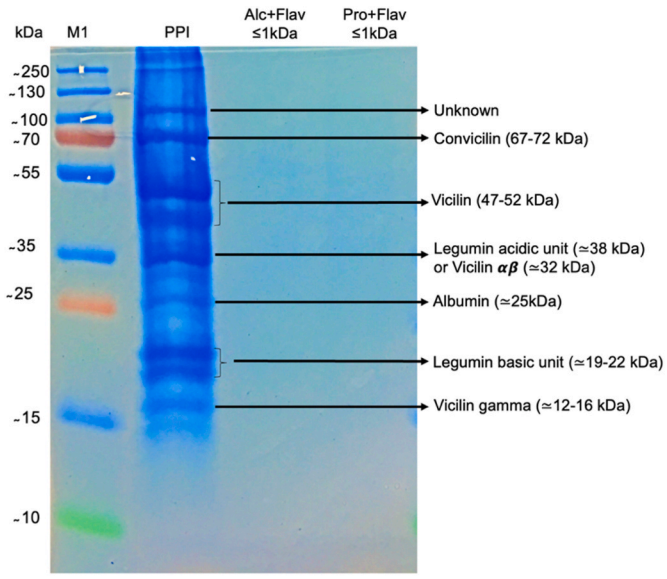


Fig. 2. SDS-PAGE of pea protein isolate and both hydrolysates (Alc + Flav≤1 kDa and Prot + Flav≤1 kDa). MW makers (Catalog N°: 26619, Thermo Scientific™, Vilnius, Lithuania; range: 10–250 kDa).

Meanwhile, the MW distribution profile of Alc + Flav ≤ 1 kDa and Prot + Flav ≤ 1 kDa hydrolysates was investigated with GFC using PEG standards. The standard mixture of MW of 8000, 3000, 1000, 600 and 200 Da was injected and peaks are defined at 11.2, 12.0, 14.0, 14.9, and 17.7 mL, respectively. According to the GFC principle, peptides with high MW are quickly eluted (*i.e.,* small elution volumes), while low MW peptides are eluted lately (large elution volumes).

According to Fig. 3, hydrolysates show several peaks at various elution volumes and no peptides were observed with MW above 1000 Da (arrow 1) since hydrolysates were filtered onto a 1 kDa membrane. Between 1000 and 600 Da (arrow 2), peptides are observed in both hydrolysates while at around 500 Da (15.9 mL) (arrow 3), peptides are only observed in Prot + Flav ≤ 1 kDa. Between 500 Da and 200 Da (16.6 mL, arrow 4), peptides are abundant in Alc + Flav ≤ 1 kDa hydrolysate compared to Prot + Flav ≤ 1 kDa hydrolysate while peptides with less than 200 Da MW (arrow 5) are more abundant in Prot + Flav ≤ 1 kDa hydrolysate. Taken altogether, Prot + Flav ≤ 1 kDa hydrolysate has a high quantity of low MW peptides compared to Alc + Flav ≤ 1 kDa hydrolysates, which is in agreement with their DH (45.05 % and 36.23 %, respectively).

3.1.3. Amino acid composition of hydrolysates

The amino acid (AA) composition of pea protein isolates and the two hydrolysates (Alc + Flav ≤ 1 kDa and Prot + Flav ≤ 1 kDa) was studied using LC-MS. The following AAs were quantified in pea proteins: serine, threonine alanine, proline, valine, tyrosine, isoleucine, leucine, histidine, lysine, phenylalanine, tryptophan, methionine, cysteine, glycine and arginine. Asparagine and glutamine can be oxidized into aspartic acid and glutamic acid, respectively; thus, each couple is analyzed together and AA concentrations are presented in Table 1.

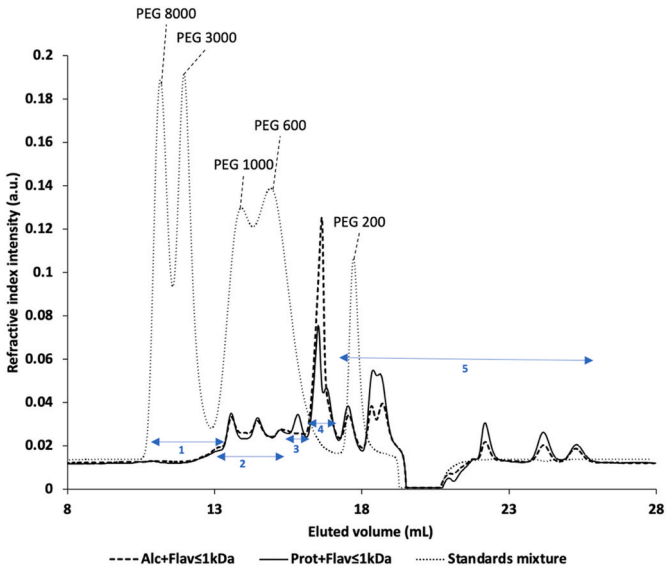


Fig. 3. GFC chromatograms of both hydrolysates (Alc + Flav≤1 kDa and Prot + Flav≤1 kDa). Polyethylene Glycol (PEG) solutions (200, 600, 1000, 3000 and 8000 Da). PPI was not investigated because MW range in SEC is too low for characterizing a protein isolate which does not contain peptides below 10 kDa according to SDS-PAGE.

Table 1
Amino-acid composition wt % (% g amino acid/g sample) grouped in 4 categories: AAA. Aromatic amino acids (phenylalanine, tryptophan, tyrosine); NCAA. Negatively charged amino acids (aspartic acid, glutamic acid); PCAA. Positively charged amino acids (arginine, histidine, lysine); SCAA. Sulfur containing amino acids (cysteine and methionine). Values are mean ± SD (n = 3). Values with different letters within the same row indicate significant differences (*p-value* < 0.05).

Amino acid	Pea isolate	Alc + Flav≤1 kDa	Prot + Flav≤1 kDa
Serine	0.47 ± 0.06^{a}	8.99 ± 0.61^{b}	11.35 ± 0.54^{c}
Aspartic acid	6.72 ± 0.61^{a}	11.18 ± 0.75^{b}	10.03 ± 0.63^{b}
Glycine	6.93 ± 0.34^{b}	6.61 ± 0.24^{ab}	6.39 ± 0.10^{a}
Glutamic acid	16.21 ± 2.57^{b}	14.26 ± 1.08^{ab}	11.50 ± 0.28^{a}
Threonine	1.57 ± 1.36^{a}	6.05 ± 0.47^{b}	5.75 ± 0.39^{b}
Alanine	6.09 ± 0.46^{a}	9.00 ± 0.21^{b}	8.67 ± 0.05^{b}
Proline	5.71 ± 0.27^{c}	4.46 ± 0.27^{b}	3.61 ± 0.21^{a}
Valine	6.95 ± 0.18^{b}	5.30 ± 0.26^{a}	5.64 ± 0.58^{a}
Cysteine	1.32 ± 0.14^{a}	4.93 ± 0.62^{b}	8.62 ± 1.06^{c}
Tyrosine	3.74 ± 0.29^{a}	4.20 ± 0.40^{a}	3.97 ± 0.37^{a}
Methionine	0.14 ± 0.01^{c}	0.06 ± 0.01^{b}	0.02 ± 0.01^{a}
Isoleucine	8.28 ± 0.04^{b}	4.67 ± 0.09^{a}	4.73 ± 0.06^{a}
Histidine	2.49 ± 0.01^{c}	1.77 ± 0.05^{b}	1.40 ± 0.02^{a}
Leucine	9.35 ± 0.28^{b}	6.08 ± 0.24^{a}	6.44 ± 0.29^{a}
Lysine	9.79 ± 0.38^{c}	5.62 ± 0.22^{b}	4.88 ± 0.21^{a}
Phenylalanine	6.46 ± 0.67^{b}	3.94 ± 0.40^{a}	3.85 ± 0.36^{a}
Arginine	7.08 ± 0.57^{b}	2.68 ± 0.35^{a}	3.08 ± 0.35^{a}
Tryptophan	0.69 ± 0.03^{c}	0.21 ± 0.01^{b}	0.04 ± 0.01^{a}
PCAA	19.36 ± 0.18^{c}	10.07 ± 0.08^{b}	9.36 ± 0.13^{a}
NCAA	22.93 ± 1.96^{ab}	25.44 ± 0.33^{b}	21.54 ± 0.91^{a}
AAA	10.89 ± 0.40^{c}	8.34 ± 0.01^{b}	7.87 ± 0.02^{a}
SCAA	1.46 ± 0.15^{a}	4.99 ± 0.62^{b}	8.64 ± 1.07^{c}

Since the AA composition is responsible of biological activities, they have been grouped into categories to make a correlation between AA composition and bioactivities (Ajibola et al., 2011). Results show that the quantity of aromatic AA (AAA) (Tyr, Phe, Trp) does not vary a lot within isolate and hydrolysates but they have been reported to increase antioxidant activities (Girgih et al., 2014). AA like histidine and tryptophan were reported many times to be responsible of Ni^{2+} and Cu^{2+} chelation due to the presence of aromatic nitrogen (Bernaudat & Bülow, 2005; Caetano-Silva et al., 2020), but their quantity is also almost similar in all samples. Besides, the quantity of negatively charged amino acids (NCAA) (Asp, Glu) of Alc + Flav ≤ 1 kDa, Prot + Flav ≤ 1 kDa and PPI are almost similar, these AA contains the carboxylate group, which favorize the chelation of metals such as Ca^{2+} and Fe^{3+} (Guo et al., 2013; Lv et al., 2017). Meanwhile, the AA composition obtained in pea protein isolate and Alc or Prot + Flav ≤ 1 kDa hydrolysates are in agreement with the one reported for pea protein concentrate and whole pancreatic trypsin hydrolysates (Felix et al., 2017), pea protein isolate and thermolysin <3 kDa hydrolysates (Pownall et al., 2010). Slight differences were observed between these studies and our results, which means that sequential enzymatic treatments might not modify a lot the AA composition.

3.2. Screening of MCPs in pea hydrolysates using SwitchSENSE® technology

3.2.1. Static mode measurement

The static mode measurement, based on fluorescence proximity sensing (FPS) was used to unveil potential MCPs in pea protein hydrolysates and to investigate the kinetic of interaction between nickel ions and MCPs in the two hydrolysates (Alc + Flav ≤ 1 kDa and Prot + Flav ≤ 1 kDa). For each hydrolysate, three concentrations (50, 100, and 200 μM eq. NH_2) were loaded into the microfluidic device.

Upon association phase, in both hydrolysates, the fluorescence increases for 100 μM and 200 μM eq. NH_2 concentrations while it increases slightly for a 50 μM eq. NH_2 concentration until reaching equilibrium (Fig. 4A and C, respectively). This fluorescence increase means that MCPs present in hydrolysates do bind to nickel ions, which are no longer able to quench the fluorescent probe due to a steric hindrance of peptide, which moves both dsDNA away from each other, and thus increases the fluorescence intensity.

On the other hand, before studying the dissociation phase, T40 buffer was infused through the fluidic device at a higher flow rate (50 μL min^{-1}) compared to the one used for association phase (30 μL min^{-1}) to avoid a re-binding phenomenon, as the metal-peptide exchanges are fast at the equilibrium. The dissociation kinetics are only recorded for the highest concentration (200 μM eq. NH_2) for each hydrolysate since it is independent of the sample concentration (Müller-Landau & Varela, 2021). Upon dissociation, the fluorescence decreases for Alc + Flav ≤ 1 kDa and Prot + Flav ≤ 1 kDa hydrolysates (Fig. 4B and D, respectively). This is due to the MCPs release, inducing the disappearance of the steric hindrance effect related to their binding onto Ni^{2+} and the reappearance of Ni^{2+} quenching effect on the fluorophore. Dissociation phases are observed for both hydrolysates with similar behaviour (fast dissociation), suggesting that similar diffusion after dissociation occurs.

Global fitting of the resulting association and dissociation curves of the two hydrolysates has to be done in order to determine the association rate (k_{on}) and dissociation rate (k_{off}) constants, and the dissociation constant at equilibrium (K_D=k_{off}/k_{on}). In our study, no dissociation rate (k_{off}) was obtained with any models, which may be due to the fast dissociation observed (Fig. 4D and F), thus, K_D could not be determined. Due to this, the observed constant rate ($k_{obs} = k_{on}. C + k_{off}$) - which depends on hydrolysates concentration C - was calculated and results are shown in Table 2.

Similar results were also found by El Hajj and colleagues (El Hajj, Sepúlveda Rincón et al., 2021), where the presence of MCPs able to bind nickel ions in soy and tilapia protein hydrolysates was evidenced by the k_{obs}, since the constants of dissociation (K_D) could not be determined.

The observed constant rates (k_{obs}) found for Prot + Flav ≤ 1 kDa hydrolysate shows a higher value compared to Alc + Flav ≤ 1 kDa hydrolysate, suggesting that Prot + Flav ≤ 1 kDa hydrolysate possess more MCPs able to complex nickel ion. The difference in affinity toward nickel ions observed between the two hydrolysates might be due to the higher amount of small-sized peptides present in Prot + Flav ≤ 1 kDa and to AA composition. Indeed, AA like histidine, glutamic acid, aspartic acid (Elias et al., 2008), tryptophan and cysteine are known to be metal

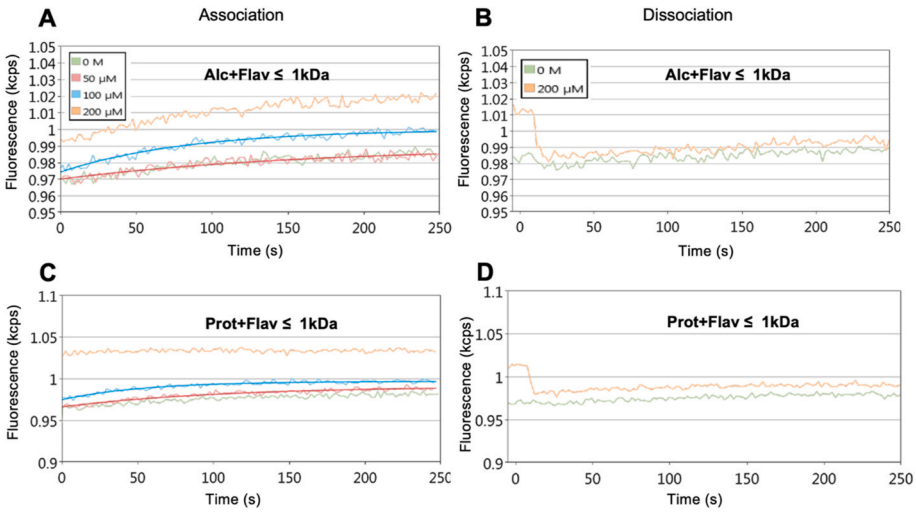


Fig. 4. Kinetics measurement of real-time molecular association and dissociation between metal-chelating peptides present in ≤1 kDa pea protein hydrolysates and nickel ions using switchSENSE® technology. Observed association rate (k_{obs}) of Alc + Flav≤1 kDa, Prot + Flav≤1 kDa pea protein hydrolysates interacting with metal ions, respectively.

Table 2
Biophysical parameters determined using switchSENSE® technology in static and dynamic mode.

Sample	Static mode		Dynamic mode	
	k_{on} (x10^2 M^{-1}.s^{-1})	k_{obs} (s^{-1})[a]	ΔSI (nm)	R^2
NTA			0.27 ± 0.12^a	0.99
NTA-Ni^{2+}			0.73 ± 0.17^b	0.99
Alc + Flav ≤ 1 kDa	1.19 ± 0.09^a	23.80 ± 1.8^a	1.80 ± 0.22^c	0.99
Prot + Flav ≤ 1 kDa	1.84 ± 0.11^b	36.80 ± 2.2^b	1.97 ± 0.19^c	0.99

Results are presented as the mean ± standard deviation (n = 3). Values with different letters within the same column indicate significant differences ($p < 0.05$).

[a] Kobs determined at 200 µM eq. NH_2 (static mode). ΔSI: size index (n = 8, dynamic mode).

chelators. Thus, both hydrolysates contain almost the same quantity of glutamic acid, aspartic acid but Prot + Flav ≤ 1 kDa hydrolysate has higher quantity of cysteine compared to Alc + Flav ≤ 1 kDa hydrolysate, which may probably explain the good metal chelating activity of Prot + Flav ≤ 1 kDa hydrolysate.

3.2.2. Dynamic mode measurement

A complementary analysis using the dynamic mode was performed to investigate metal chelation by peptides. In this mode, the measurement was carried out in the intermediary phase of the static mode, between the association and dissociation phase. In the dynamic mode, the switching speed decreases upon the peptide binding onto Ni^{2+} since the hydrodynamic friction of the nanolevers becoming heavier is increased.

In both hydrolysates, the metal-chelating peptides have negligible effect on the motion of nanolevers of reference spots (E1-E2) and sample spots (E3-E6), since the dynamic response for sample spots is less than 1% in all the cases (results not shown). This can be attributed either to negligeable unspecific interactions between the nanolevers and the hydrolysates (E1-E2), or to the fact that the motion change is not detected due to low molecular mass peptides in both hydrolysates, as observed in GFC analysis (section 3.1.2) or both.

To resolve this ambiguity, we used the sizing analysis mode of the switchSENSE® technology. Actually, the dynamic mode enables to determine the hydrodynamic diameter D_H of proteins immobilized on the nanolevers. In the present study, as no protein was used as ligand, the D_H variations for both hydrolysates correspond to indexes (size index variation, ΔSI) allowing one to unveil the binding of MCPs onto the nickel ions. It is not possible to determine the diameter of small peptides attached to the end of nanolevers as can be done for proteins assimilated to more or less spherical particles (Vicente et al., 2020). Table 2 shows that the ΔSI values of NTA and NTA-Ni^{2+} are very low (obviously, no protein is immobilized). In the presence of each of the pea protein hydrolysates, the ΔSI values increase significantly ($p < 0.05$), which definitively unveils the presence of peptides able to bind nickel ions. However, the ΔSI determined in the absence and in the presence of MCPs does not provide information on the nature of the attached peptides.

3.3. Evaluation of the antioxidant properties

After the screening of metal chelating peptides in Alc + Flav≤1 kDa and Prot + Flav≤1 kDa hydrolysates, their antioxidant properties related to their Iron (Fe^{2+})-chelating activity and radical scavenging activity have been investigated, results are illustrated on Fig. 5 and are summarized in supplementary data SD10. Indeed, transition metals - although essential for cellular life - are catalysts (prooxidative species) and their excess can provoke the oxidation phenomenon in various systems, thus the chelation of these metals leads to antioxidant activity (Elias et al., 2008).

3.3.1. Iron chelating activity

Iron chelation is promising therapeutic approach against oxidative stress driven diseases. Indeed, excess iron ions ($Fe^{2+}$and Fe^{3+}) are known to catalyse the Fenton reaction that leads to the production of reactive oxygen species and thus to the cellular deaths (Pownall et al., 2010). Iron chelation activity of pea protein hydrolysates (Alc + Flav ≤1 kDa and Prot + Flav ≤1 kDa), carnosine and EDTA was studied (Fig. 5A). The efficient concentration to chelate 50% of the quantity of iron (EC_{50}) is 0.016 ± 0.001 mg/mL, 0.072 ± 0.004 mg/mL, 14.991 ± 1.799 mg/mL,14.570 ± 0.613 mg/mL for EDTA, carnosine, Alc + Flav≤1 kDa and Prot + Flav≤1 kDa hydrolysates, respectively. Iron chelating activities of both hydrolysates are almost similar (EC_{50} = 14.991 mg/mL vs. 14.570 mg/mL), which can be explained by the fact that the quantity of aromatic AA and histidine, involved in metal chelation is almost the same in both hydrolysates. In comparison with the control, the EC_{50} of our hydrolysates are ≃ 900 times higher compared to the one of EDTA, known to be a strong synthetic complexing agent, thus hydrolysates are less efficient than EDTA. However, according to the Metal Chelating Activity (MCA) calculated for a hydrolysate concentration of 1 mg/mL, MCA of Alc + Flav≤1 kDa and Prot + Flav≤1

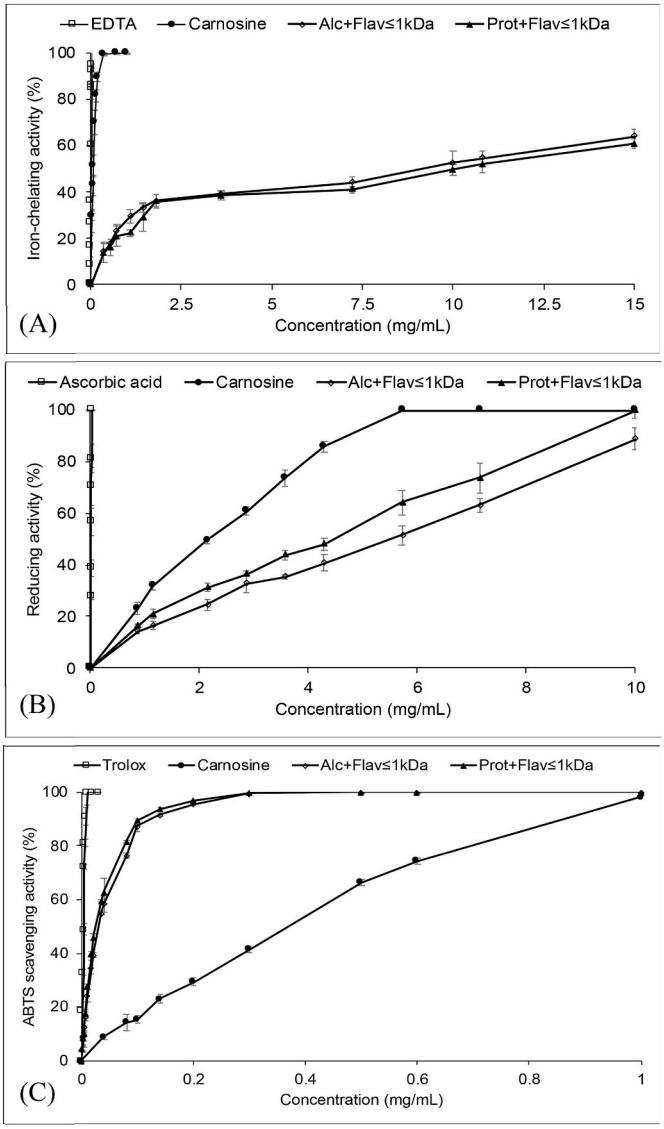


Fig. 5. Antioxidant activities of both hydrolysates (Alc + Flav≤1 kDa and Prot + Flav≤1 kDa). (A) Iron-chelating activity. (B) Reducing activity. (C) ABTS radical scavenging activity.

kDa are 29 % and 22%, respectively and these results are promising compared to the one reported by Girgih and colleagues (Girgih et al., 2015). Indeed, in this former study, MCA values for Fe^{2+} were reported of 3 % and 12 %, for pea protein (1 mg/mL) either submitted to Alcalase® alone or to 200 MPa pressure followed by Alcalase®, respectively. Thus, the sequential enzymatic treatment Alc + Flav≤1 kDa applied in our study may increases the MCA compared to the Alcalase® treatment alone.

3.3.2. Reducing activity

The reducing activity of pea protein hydrolysates (Alc + Flav≤1 kDa and Prot + Flav≤1 kDa), carnosine and ascorbic acid as references was studied (Fig. 5B). Indeed, hydrolysates participate in reducing Fe^{3+} into Fe^{2+} by donating an electron (Pownall et al., 2010). The EC_{50} of ascorbic acid, carnosine, Prot + Flav ≤ 1 kDa hydrolysates and Alc + Flav ≤ 1 kDa hydrolysates are 0.01 ± 0.00 mg/mL, 2.23 ± 0.05 mg/mL, 4.24 ± 0.24 mg/mL and 5.62 ± 0.22 mg/mL, respectively. The better reducing activity of Prot + Flav ≤ 1 kDa is in agreement with its higher DH, higher concentration in peptides according to OPA and higher quantity of low molecular weight peptides according to SEC, compared to the one of Alc + Flav ≤ 1 kDa hydrolysates ($P < 0.05$). In addition, a better reducing activity of Prot + Flav ≤ 1 kDa hydrolysate compared to Alc + Flav ≤ 1 kDa hydrolysate might be due to the presence of peptide sequences with better electron transfer properties, containing AA residues such as hydrophobic AA and aromatic AA. Besides, in our study the reducing power are 16% and 21% for Alc + Flav ≤ 1 kDa and Prot + Flav ≤ 1 kDa respectively, at 1 mg/mL concentration while the reducing activity was 30.3% for 25 mg/mL in the study of Torres-Fuentes and colleagues on chick-pea hydrolysates (pepsin followed by pancreatin, DH 27.03%) (Torres-Fuentes et al., 2015). Thus, reducing activity in our study (pea hydrolysates) is better compared to reducing activity of chick-pea hydrolysates determined in Torres-Fuentes and colleague's study since they used a much higher concentration in hydrolysates (1 mg/mL in our study vs. 25 mg/mL), which means that pea hydrolysates could give better results compared to chickpea hydrolysates if a high concentration were studied.

3.3.3. Radical-scavenging activity

ABTS radical scavenging activity of pea protein hydrolysates (Alc + Flav≤1 kDa and Prot + Flav≤1 kDa) was studied, with Trolox® and carnosine as references (Fig. 5C). The $ABTS^{\circ+}$ radical accepts a hydrogen from an antioxidant to form a stable complex, thus it was used to evaluate antioxidant activity of biomolecules. The EC_{50} of Trolox®, carnosine, Alc + Flav≤1 kDa, Prot + Flav≤1 kDa is 0.004 ± 0.00 mg/mL, 0.434 ± 0.039 mg/mL, 0.045 ± 0.00 mg/mL, 0.041 ± 0.00 mg/mL, respectively. The ABTS radical scavenging activity is 30.12 ± 1.09, 335.22 ± 15.07, and 387.51 ± 23.75 μmol TE/g sample for carnosine, Alc + Flav≤1 kDa and Prot + Flav≤1 kDa, respectively, suggesting that Prot + Flav≤1 kDa hydrolysate has slightly higher radical scavenging activity (*i.e* higher μmol TE/g sample and lower EC_{50}) compared to Alc + Flav≤1 kDa hydrolysate ($P < 0.05$). The good radical scavenging activity of Prot + Flav≤1 kDa can be attributed to its initial higher DH and higher quantity of low MW peptides compared to Alc + Flav≤1 kDa. Indeed, higher DH and low MW peptides were reported to increase radical scavenging activity (Karamać et al., 2016; Wen et al., 2020). In addition, according to the literature, imidazole group of histidine, indolyl group of tryptophan and phenolic hydroxyl of tyrosine can act as hydrogen donors to catch free radicals thus leading to radical scavenging effect (Wen et al., 2020). However, in our study, aromatic AA quantity is almost the same in both hydrolysates, suggesting that the radical scavenging activity depends not only on the AA composition and small-sized peptides but also on the sequences of peptides in hydrolysates. Meanwhile, the study of Karamać and colleagues (Karamać et al., 2016) showed that Alcalase® hydrolysates from flaxseed had a Trolox® equivalent activity of ≃220 μmol TE/g sample, while 225 μmol TE/g sample was determined for Flavourzyme® hydrolysates. The higher radical scavenging activity in our study (*i.e* higher values of μmol TE/g sample) compared to the former study of Karamać and colleagues can be due to the difference in protein source (pea protein vs. flaxseed protein) but also to the sequential treatment (Alc + Flav or Prot + Flav) we set up. In Ambigaipalan and colleagues study (Ambigaipalan et al., 2015), the sequential treatment Alc + Flav of date seed protein (DH: 11.29 %) showed a ABTS radical scavenging activity of 800 μmol TE/g sample while a value of 820 μmol TE/g sample was obtained for hydrolysates treated by Alc + Flav + thermolysin (DH: 13.08 %). Their higher radical scavenging activities compared to our results can be attributed to the difference in protein sources (date seed protein vs. pea protein). Besides, it proves the relevance of the sequential treatments, as a high radical scavenging activity was observed for Alc + Flav + thermolysin compared to Alc + Flav data seed hydrolysates.

3.4. Peptide sequences identification by LC-MS/MS

Peptides in pea protein hydrolysates were analyzed by LC-MS/MS (Table 3). From Table 3, the MS/MS spectra of the 12 most scored ions were loaded into the MaxQuant Viewer and are shown in Fig. 6A–L.

Table 3
Peptides sequences identified from Alc + Flav ≤1 kDa and Prot + Flav ≤1 kDa pea protein hydrolysates. Alc + Flav ≤1 kDa: Alcalase® followed by Flavourzyme® hydrolysate; Prot + Flav ≤1 kDa: Protamex® followed by Flavourzyme® hydrolysate; n.i: Sequence not identified.

Sequence	Intensity (x 10^5)		Length	Mass (Da)	Score	Entry
	Alc + Flav ≤1 k Da	Prot + Flav ≤1 kDa				
ADF	64.88	48.64	3	351.14	25.95	D3VND7
AGTI	85.85	17.07	4	360.20	41.72	D3VND7
ALE	63.20	40.24	3	331.17	30.28	P05692
APQE	n.i	121.92	4	443.20	**60.94**	P02857
DDEEDLR	n.i	21.77	7	890.36	11.64	P13915
DDNEELR	n.i	31.09	7	889.38	20.45	P13918
DEEDLR	n.i	26.62	6	775.33	15.77	P13915
DPL	31.86	93.00	3	343.17	30.28	P02856
DVVIIP	5.75	59.47	6	654.40	32.45	P13915
DVVIIPA	n.i	12.33	7	725.43	14.58	P13915
EEL	n.i	45.95	3	389.18	30.28	P13918
ELTP	122.40	n.i	4	458.24	**64.61**	P02856
EPFN	n.i	345.93	4	505.22	52.96	P13918
GDEPLVA	n.i	38.95	7	699.34	6.35	P05692
GDFE	68.74	95.26	4	466.17	33.91	P13918
GDIIA	n.i	106.33	5	487.26	22.12	P05692
GDTIK	n.i	36.87	5	532.29	22.12	P13918
GDVF	140.55	343.02	4	436.20	29.93	P13918
GELE	29.03	35.80	4	446.20	24.30	P02857
GEVL	52.25	96.31	4	416.23	29.93	O24294
GGLIE	n.i	255.69	5	487.26	33.91	P02857
GPF	104.09	276.75	3	319.15	22.01	P02856
GPLVQ	n.i	27.19	5	512.30	45.11	O24294
GPTPVRDGF	n.i	40.22	9	944.47	7.29	P08688
GQIEE	n.i	37.95	5	574.26	32.45	P13918
GSDDNVISQ	n.i	13.98	9	933.40	7.29	P13915
IEFP	22.51	5.06	4	504.26	29.93	O24294
IENPVK	72.27	12.21	6	698.40	36.47	P13915
IENPVKE	10.92	437.28	7	827.44	36.13	P13915
IPVN	n.i	151.77	4	441.26	49.86	P13915
IPY	98.61	120.34	3	391.21	36.83	P05692
LDL	61.87	60.09	3	359.21	25.95	P13918
LDY	323.53	196.86	3	409.18	38.72	P08688
LEEL	4.79	44.84	4	502.26	26.86	P02856
LPAGT	n.i	86.93	5	457.25	33.91	P13918
LPQ	15.87	73.07	3	356.21	**55.25**	P13918
LPV	735.17	1238.50	3	327.22	36.83	P05692
LSSIL	n.i	4891.70	5	531.33	**83.38**	Q9M3X6
LSSVL	n.i	114.00	5	517.31	37.87	P13915
NEGKGDF	15.15	139.83	7	765.33	14.58	P13918
NEGKGDFE	n.i	40.68	8	894.37	11.64	P13918
NILE	130.02	255.69	4	487.26	24.30	P13918
NVISQ	n.i	184.39	5	559.30	**75.13**	P13918
PGDVF	421.02	1121.10	5	533.25	33.91	D3VND7
PGDVVIIP	n.i	38.17	8	808.47	11.64	P13915
PGDVVIIPA	n.i	12.28	9	879.51	6.72	P13915
PLY	155.60	30.08	3	391.21	36.83	P02856
PNY	13.03	249.26	3	392.17	**55.25**	P02856
QDTPVIA	13.48	89.35	7	742.39	36.04	P02857
QLEE	27.50	71.58	4	517.24	82.17	P02856
QLEEL	11.03	93.37	5	630.32	**77.20**	P02856
REQIEE	n.i	18.17	6	802.38	13.51	D3VND9
SDLFE	13.33	78.25	5	609.26	20.22	P13915
SDLN	43.52	225.83	4	447.20	33.91	D3VND7
SEPFN	n.i	138.56	5	592.25	**52.33**	P13918
SGDVF	340.51	573.13	5	523.23	48.67	P13918
SGF	347.07	545.36	3	309.13	30.28	P13918
SGPFN	n.i	123.14	5	520.23	13.33	P02856
SLLSL	n.i	342.47	5	531.33	22.12	P05692
SLSLL	n.i	4891.70	5	531.33	**74.89**	P05692
SPDIY	79.09	398.26	5	593.27	**52.96**	P02857
SPGDVF	57.14	70.30	6	620.28	32.45	D3VND7
SPGDVVIIP	2.30	91.73	9	895.50	39.96	P13915
SPL	25.64	207.19	3	315.18	30.28	P13915
SPPEK	n.i	186.73	5	556.29	32.45	P02857
TDIRS	n.i	82.16	5	590.30	22.45	P02857
TDYEE	n.i	30.80	5	655.23	14.67	P13918
TEGKGDF	n.i	60.20	7	752.33	13.51	D3VND7
TEGKGDFE	n.i	20.42	8	881.38	7.91	D3VND7
TIDPN	21.43	n.i	5	558.26	20.22	P05692
TPGEVL	26.67	140.33	6	614.33	**59.81**	O24294
TPI	55.48	160.14	3	329.20	30.28	D3VND7
TPV	n.i	161.37	3	315.18	30.28	P02857
TPVIA	17.36	385.77	5	499.30	**52.96**	P02857
VDLK	64.87	35.04	4	473.28	26.86	P02856
VDLKE	66.29	704.21	5	602.33	22.45	P02856
VDLVIP	9.39	85.05	6	654.40	32.45	Q9M3X6
VEDGLH	7.18	49.55	6	668.31	8.64	O24294
VEI	39.27	115.62	3	359.21	30.28	P13918
VEIK	298.71	437.20	4	487.30	41.19	P13918
VEIKE	58.68	515.62	5	616.34	45.75	P13918
VEIKEG	n.i	72.61	6	673.36	13.51	P13918
VIIP	n.i	7.68	4	440.30	25.95	P13915
VPQN	n.i	55.38	4	456.23	29.93	P05692
VPT	9.69	161.37	3	315.18	30.28	P02857
VVIIP	n.i	15.77	5	539.37	29.93	P13915
YEEIE	n.i	16.46	5	681.29	20.22	P13918

A total of 48 and 85 peptides were identified for Alc + Flav ≤1 kDa and Prot + Flav ≤1 kDa, respectively. Note that there are 46 identical peptide sequences present in both previous hydrolysates, while Prot + Flav ≤1 kDa hydrolysate contains 39 other peptide sequences. This observation strongly supports the higher efficiency of Prot + Flav treatment to generate peptides compared with the Alc + Flav treatment as observed in the DH analysis. Whatever the 1 kDa hydrolysate, the molecular weight range of identified peptides varies between 309.13 and 944.47 Da (Table 3) and consists of 3–9 AAs (peptide length Fig. 7A). In agreement with the MW distribution determined by SEC, the Alc + Flav ≤1 kDa hydrolysate contains a high number of peptides comprising between 3 and 4 AAs residues, whereas the Prot + Flav ≤1 kDa hydrolysate shows a high number of peptides constituted of around 5 AAs residues (500 Da). Antioxidant peptides identified from food protein hydrolysates have been reported in the literature to be constituted between 2 and 20 AA residues, thus having a molecular weight comprised within 200 and 1800 Da (2–20 AAs)(Wen et al., 2020).

In terms of amino acid composition (analyzed by the ProtParam tool https://web.expasy.org/protparam/), Fig. 7B reveals that the peptides identified in both hydrolysates contain high amount of aspartic acid, glutamic acid, proline, leucine, valine and glycine in their sequences, which might be responsible of the antioxidant properties of the studied hydrolysates (Guo et al., 2013; Lv et al., 2017). Regarding the composition of AAs considered as a group of AAs, the major components of the identified peptides in both hydrolysates were essential (EAA) and hydrophobic (HAA) amino acids (Fig. 7C). Particularly, HAA could contribute to radical scavenging properties of hydrolysates due to an increase of peptide solubility in lipid phase which enhances the interaction with radical species (Wen et al., 2020).

Particularly, the Prot + Flav ≤1 kDa contains 4 peptide sequences rich in Asp at the N-terminus *(i.e.,* DDEEDLR, DDNEELR, DEEDLR and DVVIIPA; Table 3) which are absent in Alc + Flav ≤1 kDa. These peptides could explain the high metal-chelating properties as it was observed in DDDNLVLPEVYDQD peptide from potato proteins (Yesiltas et al., 2022). In addition, the presence of a His-containing peptide (VEDGLH) in both hydrolysates could explain their high metal-chelating and antioxidant activities strongly associated with its imidazole group as observed in SwitchSENSE® and ferrozine analysis. Similar to our study, several His-containing peptides (*i.e.,* FVPH and SAEHGSLH) were reported to be implicated in the antioxidant activity of chickpea protein hydrolysates (Torres-Fuentes et al., 2015).

Our results show that the peptides identified from Prot + Flav ≤1 kDa hydrolysate could possess interesting antioxidant properties due to

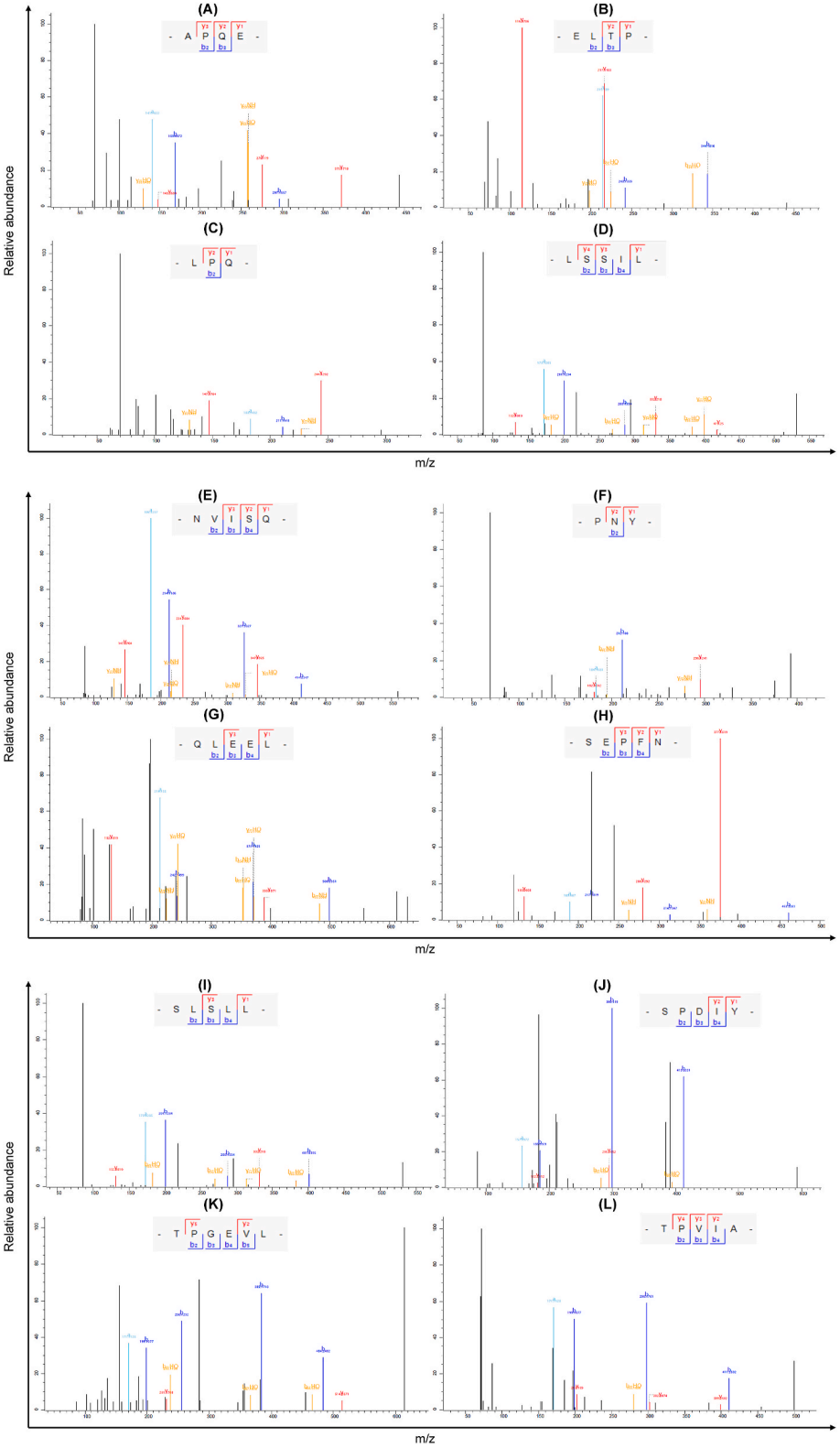


Fig. 6. Sequenced mass spectra of 12 most scored peptides obtained from pea protein hydrolysates and processes by MaxQuant software. (A) APQE, (B) ELTP, (C) LPQ, (D) LSSIL, (E) NVISQ, (F) PNY, (G), QLEEL, (H) SEPFN, (I) SLSLL, (J) SPDIY, (K) TPGEVL, (L)TPVIA.

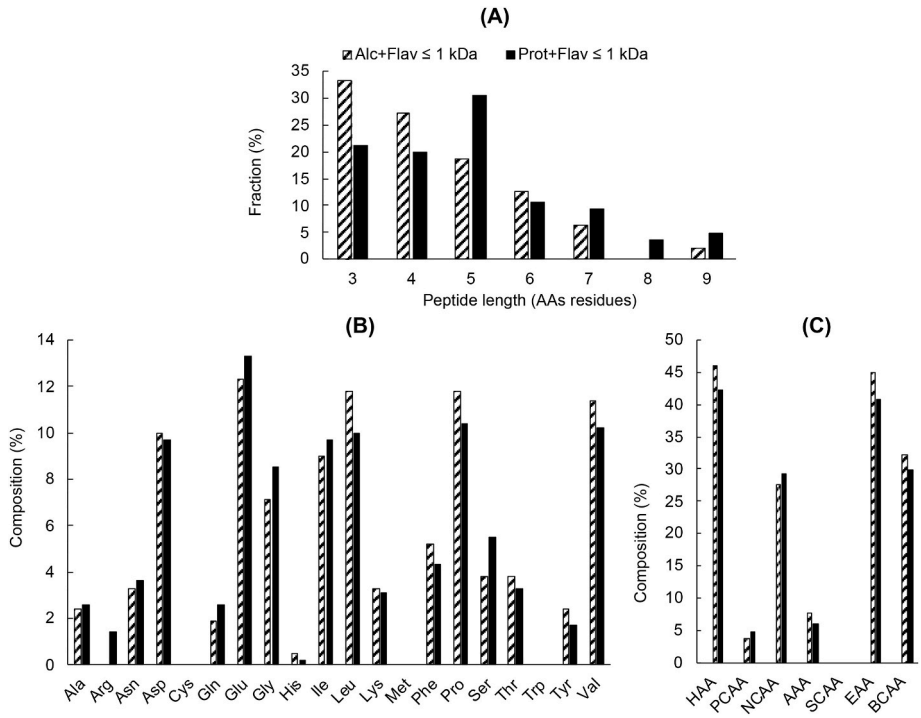


Fig. 7. Results obtained from the LC-MS/MS identification of peptides present in pea protein hydrolysates. (A) Peptide length distribution, (B) and (C) amino acid composition. Alc + Flav ≤1 kDa: Alcalase® followed by Flavourzyme® hydrolysate; Prot + Flav ≤1 kDa: Protamex® followed by Flavourzyme® hydrolysate. AAA: Aromatic amino acids = phenylalanine, tryptophan, tyrosine; NCAA: Negatively charged amino acids = aspartic acid, glutamic acid, asparagine, glutamine PCAA: Positively charged amino acids = arginine, histidine, lysine; HAA: Total Hydrophobic amino acids =alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine; SCAA: Sulfuric containing AA; EAA: Essential = tryptophan, lysine, histidine, phenylamine, leucine, isoleucine, methionine, valine, threonine; BCAA: Branch chain = isoleucine, leucine, valine.

their lower molecular weight and their 2 to 10 AAs residues composition, including Asp and His residues (Wen et al., 2020).

4. Conclusion

The physico-chemical characterization of two pea protein hydrolysates obtained by sequential treatments showed that Prot + Flav≤1 kDa hydrolysate had higher degree of hydrolysis and low MW peptides quantity compared to Alc + Flav≤1 kDa one. Besides, by the static and dynamic mode studies, switchSENSE® technology allowed to evidence the presence of small-size peptides able to chelate Ni^{2+} in pea protein hydrolysates, demonstrating the high potential of such technique to screen metal-chelating peptides in protein hydrolysates. Meanwhile, Iron chelating activity study showed that both hydrolysates were able to chelate iron. The Prot + Flav≤1 kDa hydrolysate showed higher radical scavenging and higher reducing power, which was related to its higher degree of hydrolysate and low MW peptides quantity. In addition, peptides identification in both hydrolysates showed that Prot + Flav≤1 kDa hydrolysate contained more peptide sequences compared to Alc + Flav≤1 kDa. As perspectives, the antioxidant activity of Alc + Flav≤1 kDa and Prot + Flav≤1 kDa hydrolysates could be evaluated for their ability to avoid lipid oxidation in systems like cosmetic and food emulsions. Other types of enzymes and their combination could also be investigated to obtain higher antioxidant activities and higher quantity of metal chelating peptides.

CRediT authorship contribution statement

Rachel Irankunda: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Jairo Andrés Camaño Echavarría:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Sarah El Hajj:** Conceptualization. **Cédric Paris:** Writing – review & editing, Formal analysis, Conceptualization. **Céline Cakir-Kiefer:** Conceptualization. **Jean-Michel Girardet:** Writing – review & editing, Conceptualization. **Philippe Arnoux:** Conceptualization. **Laurence Muhr:** Review - editing. **Laetitia Canabady-Rochelle:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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.

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Abbreviations

AA	Amino acid
NTA	Nitrilotriacetic acid
DNA	Deoxyribonucleic acid
OPA	O-phthaldialdehyde
EDTA	Ethylenediaminetetraacetic acid
ABTS	2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)

diammonium salt
DPPH 2,2-diphenyl-1-picrylhydrazyl
TCA trichloroacetic acid
Alc + Flav ≤ 1 kDa hydrolysate obtained by Alcalase® followed by Flavourzyme® treatment, ultrafiltrated on 1 kDa membrane
Prot + Flav ≤ 1 kDa hydrolysate obtained by Protamex® followed by Flavourzyme® treatment, ultrafiltrated on 1 kDa membrane
DH degree of hydrolysis
MW molecular weight
MCPs Metal-chelating peptides
PPI pea protein isolate
PPH pea protein hydrolysate
SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis
GFC Gel Filtration Chromatography
PEG Polyethylene Glycol
DR dynamic response
TEAC Trolox® Equivalent Antioxidant Capacity
MCA Metal Chelating Activity

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2025.106001.

Data availability

Data will be made available on request.

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