Nutritional composition, ultrastructural characterization, and peptidome profile of antioxidant hemp protein hydrolysates

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ABSTRACT

Hemp is the term commonly used to refer to the variety of *Cannabis sativa* L. cultivated for industrial purposes. The seeds have gained interest in recent years as functional foods due to their nutritional composition and high content of protein and bioactive compounds. In this study, ten hemp protein hydrolysates (HPHs) were obtained by enzymatic hydrolysis with Alcalase and Flavourzyme from hemp protein isolate (HPI) and their antioxidant properties (DPPH radical scavenging activity, beta-carotene activity and ferric ion reducing antioxidant power (FRAP)) were evaluated. Shorter peptide sequences, mainly obtained with Flavourzyme, were found to react with free radicals more easily. The peptidome of all the hydrolysates was characterized, identifying, and quantifying the peptides. Furthermore, 19 unique peptides were assessed by *in silico* tools to hypothesize those that could be responsible of the bioactivity reported for the hydrolysates. From the identified peptides, based on the molecular features and the predictions, the peptides KNAIYTPH, EERPGHF, and KNGMMAPH, among others, are proposed to be highly contributing to the antioxidant activity of the hydrolysates.

1. Introduction

In recent years, the cultivation of industrial hemp has grown exponentially due, among other factors, to the nutritional composition and the high content of bioactive compounds in its seeds with beneficial properties for health (Aiello et al., 2016). Hemp seeds are made up of 35.5% fat, 24.8% protein, 20–30% carbohydrates, and 27.6% total fibre (dry weight). In addition, they have a high content of minerals (mainly phosphorus, potassium, calcium, and magnesium) and vitamins (E, B1, and B2) . The protein present in hemp seeds is considered to be of high quality since it mainly includes edestin and albumin, storage proteins that are easily digested and contain significant amounts of all the essential amino acids, notably arginine and sulphur-containing amino acids (Rivero-Pino et al., 2023; Rodriguez-Martin et al., 2019).

Plant proteins have recently gained interest as important raw materials for the food industry due to their potential applications as functional ingredients and their greater sustainability and lower production cost compared to proteins of animal origin. Defatted meals from oilseeds such as hemp are usually a by-product of the industry with low added value obtained after the extraction of oil from the seeds, but they represent a potential reservoir of plant protein. The interest in the protein content of this by-product has promoted the development of extraction and improvement processes for its use in other applications, such as human nutrition (Y. Xu, Bai, et al., 2022). Hemp products have several potential applications as functional ingredients in food, but there are still many challenges to be addressed in order to fully exploit and increase its use to promote health in humans (AL Ubeed et al., 2022). The structural characterization of unidentified hempseed components and their functionality, as well as the impact of hempseed inclusion on food items, potentially modifying physicochemical properties, sensory quality, shelf life, and health benefits, all require further study (J. Xu, Bai, et al., 2022).

Beyond their nutritional value, protein are a source of peptides that can be used for fortifying foodstuff, since these low molecular weight peptides released after the processing of the protein (for instance by enzymatic hydrolysis or fermentation) can exert certain bioactivities

(Rivero-Pino, 2023). For hemp peptides, bioactivities such as antidiabetic (Lammi et al., 2019) antihypertensive (Samaei et al., 2021), anti-inflammatory (Hong et al., 2022; Rodriguez-Martin et al., 2020), antioxidant (Mahbub et al., 2022), or neuroprotective (Rodriguez-Martin et al., 2019), have been described. However, there is still a gap in the research of hemp peptides concerning the identification of the peptides released because of the action of proteases during enzymatic hydrolysis and their correlation with the mentioned bioactivities.

The aim of this work is to describe the nutritional composition and ultrastructural characterization of hemp protein isolate, as well as the antioxidant activity and the peptidome of ten hemp protein hydrolysates, obtained by two commercially available food-grade proteases commonly employed in the food industry. In addition, from the peptides identified in the hydrolysate by mass spectrometry, bioinformatics tools were employed to predict the sequences responsible for the antioxidant activity reported for the hydrolysates *in vitro*.

2. Materials and methods

2.1. Chemicals and samples

Cannabis sativa L. seeds were provided by Sensi Seeds Bank. Alcalase 2.4 L and Flavourzyme (1000 L) were obtained from Novozymes (Bagsvaerd, Denmark). All the chemicals (reagents and solvents) were of analytical grade and provided by Sigma Chemical Co., Bachem AG (Bubendorf, CH, EU), and Gibco (Waltham, MA, USA).

2.2. Preparation of hemp protein isolate

Hemp protein isolate (HPI) was obtained using the method of Lqari et al. (2002). Briefly, hemp defatted flour was extracted employing 0.25% Na_2SO_3 (p/v) at a pH of 10.5 for 1 h. After centrifuging the extract at 7500 rpm for 15 min, the supernatant was recovered, and the pellet was extracted again. Both supernatants were adjusted to the isoelectric point (pI) of hemp protein, and the precipitate was washed with distilled water adjusted to pH 4.3. Subsequently, it was centrifuged to remove residual salts and other non-protein compounds and the protein precipitate obtained was freeze-dried and stored at room temperature.

2.3. Hydrolysis of hemp protein isolate

The hydrolysis was carried out in a jacketed reactor under continuous stirring at constant temperature (50 °C) and pH. At first, the HPI was resuspended in distilled water (10% w/v) and the enzyme Alcalase was added at 0.3 AU/g of protein at pH 8 and aliquots were taken at the 10 (HPH10A), 20 (HPH20A), 30 (HPH30A), 45 (HPH45A), and 60 min (HPH60A). After 60 min of hydrolysis with Alcalase, Flavourzyme was added at 60 LAPU/g of protein at pH 7 for 120 min, obtaining protein hydrolysates at the 15 (HPH60A + 15F), 30 (HPH60A + 30F), 60 (HPH60A + 60F), 90 (HPH60A + 90F), and 120 min (HPH60A + 120F). Enzyme deactivation for each sample was achieved by heating for 15 min at 85 °C.

2.4. Chemical characterization of hemp protein products

Protein concentration was determined by elemental microanalysis, using a LECO TRUSPEC MICRO analyzer (Leco Corporation, St. Joseph, MI, USA) and expressed as a percentage after multiplying the nitrogen content by a conversion factor of 6.25 (conversion factor for plants). Total dietary fiber was determined using the gravimetric method (Prosky et al., 1984). The ash content was determined according to the direct ignition method (550 °C for 36 h). Soluble polyphenols and sugars were measured by colorimetry using standard curves for chlorogenic acid and glucose, respectively, as described by Dubois et al. (1956) and Moores et al. (1948). Each sample was analysed in triplicate.

2.5. Determination of amino acid composition

The amino acid composition was determined according to the method of Alaiz et al. (1992) with slight modifications. For this, the samples (4–6 mg of protein) were hydrolysed by incubation with 6 N HCl at 110 °C for 24 h in closed tubes and under a nitrogen atmosphere. Once the hydrolysis was complete, the samples were dried in a rotary evaporator and dissolved in 1 M sodium borate buffer pH 9 until a final volume of 10 mL was obtained. After derivatization with diethyl ethoxymethylenemalonate, the amino acids were determined in the acid hydrolysate by ultra-high performance liquid chromatography (uHPLC) (Acquity Arc, Waters, USA), using D,L-α-aminobutyric acid as standard internal and a 3 mm × 150 mm reverse phase column (XSelect HSS T3 XP, 2.5 μm; Waters). A binary gradient system was used with the solvents (A) 25 mM sodium acetate, 0.02% sodium azide (pH 6), and (B) acetonitrile. The flow rate was 0.8 mL/min and the elution gradient used was: time 0–1 min, elution with A:B 92:8, time 1–4.33, linear gradient from A:B 92:8 to A:B 86:14; time 4.33–7.32 min, elution with A:B 86:14; time 7.32–11.65 min, linear gradient from A:B 86:14 to A:B 72:28; time 11.65–13.31 min linear gradient from A:B 72:28 to A:B 65:35, time 13.31–15.64 linear gradient from A:B 65:35 to A:B 92:8, time 15.64–16 elution with A:B 92:8. The calibration curves for each amino acid were made using a mixture of amino acid standards (Merck, Spain) under the same hydrolysis conditions as the samples, and the resulting peaks were analysed with the EMPOWER software (Waters, USA). In addition, the tryptophan content was evaluated according to the method of Yust et al. (2004). Each sample was analysed in triplicate.

2.6. Ultrastructural characterization by scanning electron microscopy analysis

Ultrastructural characterization of the lyophilized hemp defatted flour, HPI, and two of the protein hydrolysates obtained (samples HPH20A and HPH60A + 15F) was performed using Scanning Electron Microscopy (SEM). A thin layer of each sample was mounted using sticky conductive carbon tape on an aluminium stub, removing any unattached particle. The specimen holder was then sputter-coated under vacuum with approximately 20 nm gold-palladium using a Leica EM ACE600 instrument (Leica Microsystems, Wetzlar, Germany). Images were obtained with a Zeiss Crossbeam 550 scanning electron microscope (Zeiss, Madrid, Spain) at 2.00 kV accelerating voltage and different magnifications(Martinez-Lopez et al., 2022).

2.7. Antioxidant activity

2.7.1. DPPH radical scavenging activity

The DPPH (2,2-diphenyl-1-picrylhydracil) radical scavenging effect of the samples was measured according to Awika et al., (2003) with some modifications. For this, a solution of butylhydroxytoluene (BHT) was used as a positive control and water as a negative control. Different concentrations of BHT and samples were prepared in order to calculate the half maximal inhibitory concentration (IC_{50}) value, 0.001–0.05 μg/μL and 0.1–5 μg/μL, respectively. The samples, positive control, and negative control (150 μL) together with DPPH 0.1 mM solution (150 μL) were arranged in 96-well plates. It was then incubated at room temperature for 30 min and the absorbance at 517 nm was measured in a model DU-640 spectrophotometer (Beckman Instruments, Fullerton, CA, USA). For each replicate of the extraction, 3 analysis were carried out in each laboratory assay.

2.7.2. Beta-carotene activity

The experiment was carried out according to the method of Pastor-Cavada et al. (2009) with some modifications. 20 μL of BHT and of the samples were prepared in order to calculate the IC_{50} at different concentrations 0.001–0.05 μg/μL and 0.1–4.55 μg/μL, respectively. Then, 200 μL of β-carotene reagent (1 mL of a 0.2 mg/mL solution of

β-carotene in chloroform) were added to a mixture of 20 mg of linoleic acid and 200 mg of Tween 20. After that, the chloroform was eliminated using a rotary evaporator and 50 mL of oxygen-rich water, obtained by bubbling oxygen for 25 min, are added. Finally, the absorbance at 492 nm was measured at 10, 20, 30, 40, 50, and 60 min. Each sample was analysed in triplicate.

2.7.3. Ferric ion reducing antioxidant power (FRAP)

The determination of the reducing power of the hydrolysates was carried out according to the method of Oyaizu (1988) with some modifications. 20 μL of BHT and of the samples were prepared in order to calculate the IC$_{50}$ at different concentrations 0.0012–0.0611 μg/μL and 0.011–2.5 μg/μL, respectively. The samples, BHT, and negative control or water (20 μL), were placed in 96-well plates together with 50 μL of K$_3$[Fe(CN)^6] 1% (w/v) and 50 μL 0.2 M phosphate buffer (PBS), pH 6. Next, it was incubated for 20 min at 50 °C and 50 μL of trichloroacetic acid 10% (w/v) and 10 μL of FeCl$_3$ 0.1% (w/v) were added. The reaction was incubated again for 10 min at 50 °C and the absorbance at 700 nm was measured. Each sample was analysed in triplicate.

2.8. Peptide extraction, purification, and sequence identification by LC-TIMS-MS/MS

Samples were acidified with 0.5% trifluoroacetic acid (v/v). Desalting and concentration step was performed with ZipTip C18 (Millipore) and the digested (with trypsin) samples were speed-vacuum dried. LC-TIMS-MS/MS was carried out using a nanoElute nanoflow ultrahigh-pressure LC system (Bruker Daltonics, Bremen, Germany) coupled to a timsTOF Pro 2 mass spectrometer, equipped with a CaptiveSpray nanoelectrospray ion source (Bruker Daltonics) according with the procedure described in the patent P202230873. Briefly, around 200 ng of peptide digest was loaded onto a Bruker FIFTEEN C18 capillary column (15 cm length, 75 μm ID, 1.9 μm particle size, 120 Å pore size; Bruker Daltonics). Peptides were separated at 30 °C using a 20 min gradient at a flow rate of 300 nL/min (mobile phase A (MPA): 0.1% FA; mobile phase B (MPB): 0.1% FA in acetonitrile). A step gradient from 0 to 35% MPB was applied over 13 min, followed by a 35–90% MPB step of 13–15 min, and finished with a 90% MPB wash for an additional 5 min for a further time. timsTOF Pro 2 was run in DDA-PASEF mode. Mass spectra for MS and MS/MS scans were recorded between 100 and 1700 m/z. Ion mobility resolution was set to 0.85–1.30 V s/cm^2 over a ramp time of 100 ms. Data-dependent acquisition was performed using 4 PASEF MS/MS scans per cycle with a duty cycle close to 100%. A polygonal filter was applied on the m/z space and ion mobility to exclude low m/z, mainly single-charged ions from the selection of PASEF precursors. An active exclusion time of 0.4 min was applied to precursors that reached 20,000 intensity units. The collision energy was increased stepwise as a function of the ion mobility ramp, from 27 to 45 eV. The raw data were analysed in PEAKS Studio ProX (Bioinformatics Solution Corp). The reference library is acquired from UniProt_proteome_Cannabis-sativa_Feb22. The raw data were analysed with parent mass error tolerance set to 15 ppm and a fragment mass error tolerance of 0.05 Da. To account for post-translational modifications and chemical labelling, the following settings were used: Carbamidomethylation of cysteine residues was set as fixed modification, methionine oxidation, and Acetylation (Protein N-term) was set as variable modification. Protein unique peptides was set to larger than 1 and a high confidence score of −10lgP >20 was applied to indicate an accurately identified protein.

2.9. In silico analysis

The peptides chosen from the pool of peptides identified (criteria: from those with molecular weight <1000 Da; the first 5 more abundant from each hemp protein hydrolysates (relative content) were subjected to *in silico* analyses: a) The net charge of identified peptides at neutral pH

was calculated via Peptide Property Calculator (http://pepcalc.com/), where peptide solubility was also computed and estimated, according to their pI, the number of charged residues, and the peptide length; b) ToxinPred software was used to predict, for each peptide, its hydrophobicity, amphipathicity, steric hindrance, toxicity, and molecular weight (https://webs.iiitd.edu.in/raghava/toxinpred/design.php); c) PeptideRanker, to ranks the sequences by the predicted probability to be bioactive (http://bioware.ucd.ie/~compass/biowareweb) The assigned scores ranged from 0 to 1.0 at a threshold of 0.5; and d) AnOxPePred-1.0 was used for predict the antioxidant (quantified by free radical scavenging and ion chelating scores) properties of peptides using convolutional neural network (https://services.healthtech.dtu.dk/service.php?AnOxPePred-1.0).

2.10. Computational molecular stability

According to Li et al. (2022) the web server PASTA 2.0 (http://protein.bio.unipd.it/pasta2/) was used to compute the tendency of peptide self-aggregation and the predicted amyloid-like structure (parallel/anti-parallel aggregation) that were specific to the possible region at sequence. For peptide discrimination, the optimal thresholds were switched as Top = 1 and Energy < −5 PEU (1 PEU (Pasta Energy Unit) = 1.192 kcal/mol). The probability of intrinsic disorder and portion of estimated secondary structure that complement the aggregation data were also reported. For peptides of interest, the best energy within cross-sequence matrix were simulated to evaluate the likelihood of inherent propensity of peptide self-/co-aggregation (the

threshold of best energy for peptide-peptide pairing was set as zero).

2.11. Statistical analysis

All values are presented as the means ± standard deviations (SD). Data were evaluated using Graph Pad Prism version 9.1.2 (San Diego, CA, USA). To antioxidant activity, the statistical significance of any difference in parameters between the groups was assessed using two-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test. *P* values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Proximate composition of the hemp seed, HPI and the HPHs

The chemical composition of hemp seed, HPI, and HPHs are shown in Table 1. The protein content of HPI is greater than 96% in dry weight, a value similar to that obtained by other authors in previous studies on hemp (Ajibola & Aluko, 2022; Karabulut & Yemiş, 2022). The protein content after the isolation procedure was increased by 75% compared to the original hemp flour. In comparison, the HPHs have a lower percentage of protein, reaching 86%, and a higher ash content, which is due to the alkali necessary to keep the pH constant during the hydrolysis process. Differences are observed in the samples hydrolysed with Alcalase and Flavourzyme, since when the second enzyme is added, the hydrolysis continues in a larger extent. No major differences are observed in the samples hydrolysed during the hydrolysis time in each lot of hydrolysates, since the main change occurs at the beginning of the hydrolysis, since the cleavage of peptides (and consequent addition of alkali) is higher at the beginning of the reaction. However, this increase in ash content should not imply a disadvantage at nutritional level compared to the isolate.

3.2. Amino acid composition

Table 2 shows the amino acid analysis of the HPI and two HPHs, one obtained with Alcalase (HPH20A) and the other with Alcalase and Flavourzyme (HPH60A + 15F). Furthermore, a comparison with the

Table 1

Chemical composition of hemp seed, protein isolate (HPI), and hemp protein hydrolysates (HPH). The data is expressed as a percentage of dry weight and shows the mean ± standard deviation of three determinations.

Proximate composition (g/100 g of product)	Seed	HPI	HPH										
			10A	20A	30A	45A	60A	60A + 15F	60A + 30F	60A + 60F	60A + 90F	60A + 120F
Ash	5.0 ± 0.0	1.0 ± 0.1	6.1 ± 0.7	8.1 ± 0.2	8.7 ± 0.7	8.4 ± 0.1	9.2 ± 0.0	10.1 ± 0.3	10.5 ± 0.1	9.4 ± 0.7	10.1 ± 0.2	9.9 ± 0.7
Proteins	23.8 ± 1.0	96.4 ± 0.9	86.5 ± 0.7	83.0 ± 0.2	84.0 ± 0.2	83.5 ± 0.1	83.3 ± 0.2	82.3 ± 0.0	71.7[a] ± 0.8	74.3[a] ± 0.7	76.8[a] ± 0.3	77.8[a] ± 0.4
Fat	31.8 ± 0.5	0.2 ± 0.0	ND	ND	ND	ND	ND	ND	NA	NA	NA	NA
Fibre	29.3 ± 1.4	ND	ND	ND	ND	ND	ND	ND	NA	NA	NA	NA
Polyphenols	0.3 ± 0.0	4.0E-02 ± 0.0	ND	ND	ND	ND	ND	ND	NA	NA	NA	NA
Soluble sugars	5.1 ± 1.0	ND	ND	ND	ND	ND	ND	ND	NA	NA	NA	NA

ND: not determined. NA: not analysed.

[a] Concentration in mg protein/mL.

nutritional recommendations proposed by the FAO/WHO/UNU in adults was reported and it is observed that the samples evaluated meet the total nutritional requirements for essential amino acids. The full amino acid composition of all the hydrolysates can be found in the Supplementary Material 1. Overall, enzymatic hydrolysis does not substantially alter the nutritional value of the original HPI, though that some the content of some essential amino acids is increased (e.g. isoleucine and valine) or decreased (e.g. cysteine).

The amino acid content of the sample evaluated is comparable to the results reported for hemp protein isolates extracted at 0.1 M NaOH by Banskota et al. (2022) and to the amino acid composition of the globulin fraction from hemp since the protein isolation procedure employed extracts mainly globulin-type proteins.

The high content of arginine, glutamic acid, and aspartic acid is remarkable and interesting at nutritional level when considering hemp as food. For instance, the intake of arginine could be helpful in certain traumatic or inflammatory situations, where an overexpression of arginase and inducible nitric oxide synthase (iNOS) in myeloid cells and some granulocytes, deplete this amino acid and favour the dysfunction of T lymphocytes and macrophages and the generation of ROS, leading to an increase in inflammation (Adebayo et al., 2021). The oral administration of arginine could correct this deficiency could prevent the accumulation and activation of these immune cells, the positive regulation of arginase and promote the blockade of arginase activity and improve the resolution of the immune process (Popovic et al., 2007). Aspartate and glutamate, among many other functions, are involved in the electron donation mechanisms between ROS, thus, increasing the antioxidant activity of the sample, as it will be discussed in section 3.4. Overall, the presence of hydrophobic (such as valine, leucine and alanine) and aromatic amino acids (phenylalanine, tyrosine and tryptophan) can enhance the antioxidant activity of peptides (Gao et al., 2021).

Bioavailability of hydrolysates through cell culture or *in vivo* models should be carried out in order to confirm that these protein hydrolysates and the amino acids contained in the peptide sequences are indeed reaching the systemic circulation without being digested by digestive proteases, since scarce information is currently available in published literature (Santos-Sanchez et al., 2022).

3.3. Ultrastructure characterization of hemp flour, HPI and HPHs

The surface morphology of the hemp flour, the HPI and two protein hydrolysates (HPH20A and HPH60A + 15F) observed by SEM is shown in Fig. 1. The hydrolysates shown in the figure were chosen in order to show one hydrolysed with Alcalase (reported to have higher antioxidant activity, HPH20A) and other one hydrolysed with Alcalase and

Flavourzyme, to see if the structural changes in the protein subjected to proteases with different specificities could be also observed at this level. From SEM images, it has observed that the protein presented in the original sample has degraded into small fragments after enzyme hydrolysis, increasing by reaction time, which led to reduction in particle size under the same SEM parameters (Mag = 90x and AV = 2.0 kV/Mag = 345x and AV = 2.0 kV). These findings are closely associated to recent studies with other protein sources such as kiwicha or turtlegrass, where the protein hydrolysates showed the same behaviour (release of small fragments and reduction of particle size reduction), potentially correlated with the increased solubility of the protein hydrolysates (Islam et al., 2021; Martinez-Lopez et al., 2022).

3.4. Antioxidant activity of HPHs

The antioxidant activity of the HPHs is shown in Table 3. It has to be considered that FRAP and DPPH analyses measure the direct quenching of the radical while b-carotene analysis measures the peptide's capacity to protect it against oxidation by oxygenated water. Regarding the DPPH radical scavenging activity, the activity of the HPH20A and the hydrolysates obtained by hydrolysis with Alcalase and Flavourzyme show a high antioxidant activity, which means that the peptides in these hydrolysates are capable of reacting with free radicals to convert them into more stable products. According to the results obtained, hydrolysis with Flavourzyme contributes notably to the DPPH scavenging activity of the hydrolysates. Among the different HPHs obtained with Flavourzyme, no significant differences were observed with respect to DPPH uptake, although the activity of the HPH60A + 60F is significantly higher than the others. The published literature reports different results depending on the protein and proteases used, but also by the amino acid sequence and composition. However, in this case, the amino acid composition of the different hydrolysates was found to be similar (Table 2), so the differences in the bioactivity are due to the presence of specific amino acid in certain positions in the sequences. Overall, shorter peptide sequences can more easily react with free radicals (Karimi et al., 2020), supporting the results obtained in this research where hydrolysates treated with Flavourzyme exert higher levels of bioactivity, as this exopeptidase can release short peptides from terminals. Hong et al. (2022) reported a decreased production of DPPH radicals by hempseed protein hydrolysates obtained by Alcalase and bromelain. The hydrolysate obtained with Alcalase showed a degree of hydrolysis of 62% and the highest inhibition reported was ca. 25% at 2 mg/mL, suggesting that extensive hydrolysis with Alcalase does not necessarily release more antioxidant peptides, as also reported in our results.

All HPHs showed ferric reduction power, FRAP, which indicates their ability to donate electrons and their potential antioxidant activity.

Table 2

Amino acid composition of hemp protein isolate (HPI) and two HPHs, one obtained with Alcalase (HPH20A) and the other with Alcalase and Flavourzyme (HPH60A + 15F). The full amino acid composition of all the hydrolysates can be found in the Supplementary Material 1. The data, expressed as milligrams of amino acids by grams of total protein, are the mean ± standard deviation of three determinations.

	HPI	HPH20A	HPH60A + 15F	2007 FAO/ WHO/UNU [a,b]
Essential amino acids (mg/g prot)				
Histidine	28.6 ± 0.2[a]	28.5 ± 0.1[a]	27.7 ± 0.1[b]	15
Isoleucine	23.8 ± 0.0[a]	41.0 ± 0.4[b]	39.2 ± 0.7[c]	30
Leucine	62.9 ± 0.4[a]	62.3 ± 0.5[a]	64.6 ± 0.9[b]	59
Lysine	29.2 ± 0.2[a]	33.1 ± 0.5[b]	33.4 ± 0.4[b]	45
Methionine + Cysteine	29.6 ± 6.6[a]	30.0 ± 0.3[a]	25.0 ± 0.7[a]	22
Methionine	17.9 ± 6.2[a]	24.2 ± 0.0[a]	18.5 ± 0.4[a]	16
Cysteine	11.7 ± 0.3[a]	5.8 ± 0.3[b]	6.6 ± 0.3[c]	6
Phenylalanine + Tyrosine	78.7 ± 0.7[a]	78.6 ± 0.7[a]	80.3 ± 1.2[a]	38
Threonine	34.1 ± 0.4[a]	32.1 ± 0.2[b]	34.4 ± 0.5[a]	23
Tryptophan	11.9 ± 0.2[a]	10.7 ± 0.0[b]	11.1 ± 0.5[b]	6
Valine	34.8 ± 1.1[a]	43.4 ± 0.5[b]	42.6 ± 1.0[b]	39
Total essential amino acids	363.2	389.5	383.3	**277**
Non-essential amino acids (mg/g prot)				
Aspartic acid	151.7 ± 0.8[a]	142.2 ± 2.3[b]	146.1 ± 1.7[c]	
Glutamic acid	194.2 ± 1.7[a]	191.9 ± 2.3[a]	189.8 ± 3.6[a]	
Serine	59.6 ± 0.4[a]	50.0 ± 0.4[b]	54.1 ± 0.8[c]	
Glycine	40.1 ± 0.2[a]	36.7 ± 0.4[b]	36.9 ± 1.0[b]	
Arginine	126.5 ± 0.9[a]	131.0 ± 1.2[b]	131.6 ± 1.6[b]	
Alanine	47.6 ± 0.4[a]	43.4 ± 0.5[b]	44.7 ± 1.0[b]	
Proline	58.6 ± 10.7[a]	45.4 ± 9.5[b]	28.8 ± 0.4[c]	
Total non-essential amino acids	678.2	640.5	632.1	

[a] FAO/WHO/UNU. Protein requirement mg/g score pattern in adults.
[b] FAO and FINUT 2017. Evaluation of the quality of dietary protein in human nutrition. FAO Food and Nutrition Document NO. 92.

Contrary to the results obtained in the DPPH assay, Flavourzyme does not seem to contribute much to the reducing power of the hydrolysates and the highest antioxidant activity correspond to the hydrolysates obtained with Alcalase in short reaction times (HPH10A and HPH20A). These results are in line with a study with horse mackerel protein no significant changes were found in the reducing power between the hydrolysates of subtilisin and Flavourzyme at degree of hydrolysis (DH) between 5 and 15%, however at higher DH, (ca. 25%), the subtilisin hydrolysates showed less capacity (Egerton et al., 2018). These results suggest that the cleavage of the protein at short reaction times promotes the reducing power of the hydrolysates, because of the sequences released, whereas further hydrolysis of these peptides does not increase the activity. This can be due to the structure that these higher molecular weight peptides would have, which can be degraded with the action of Flavourzyme, destabilising the compounds and leading to a reduced antioxidant activity.

Finally, the hydrolysates HPH10A, HPH20A, and HPH30A are the ones that have shown the greatest β-carotene activity and hydrolysis

with Flavourzyme does not increase the antioxidant power of the HPHs in this case either. Hemp peptides obtained from the simulated gastro-intestinal digestion of HPI showed that low molecular weight peptides exhibited the highest radical scavenging activity and high molecular weight peptides, higher metal chelation and ferric reducing power (Girgih et al., 2011), in line with our results reported for the hemp treated with Alcalase and Flavourzyme.

3.5. Peptidome profile

The peptidome for the HPI and the ten hydrolysates was fully characterized by LC-TIMS-MS/MS. As shown in Table 4, the number of peptides identified in each sample was ranging from 1134 (HPH60A + 60F) to 3353 (HPI). Furthermore, the relative abundance of each peptide was calculated. In the HPI, the non-hydrolysed sample, the number of high molecular peptides was higher compared to low molecular weight. Sequences up to 50 amino acids were identified. The average length of the 20 most abundant peptides was 20 residues, it is > 2 kDa. Only around 4% of the sample corresponded to <1 kDa peptides. Due to the technique employed for the identification of peptides, proteins such as edestin would not be included in the outcome of this analysis, but it only encompasses the peptide up to a certain amino acid length. The enzymatic hydrolysis of the HPI led to a decrease of high molecular weight peptides, as it was observed in the peptidome obtained for all the hydrolysates. In the less-hydrolysed sample, HPH10A, the peptide with the highest molecular weight corresponds to a length of 36 amino acids (3.8 kDa), followed by a peptide with 31 residues (3.4 kDa), which also has a very low relative abundance compared to the rest of the peptides. In this sample, the average length of the 20 most abundant peptides (PCL: peptide chain length) was 17 residues, whereas around 6.6% of the sample corresponded to <1 kDa peptides, slightly increased compared to the HPI, indicating the hydrolysis of the previously reported sequences. A summary of the number of peptides, the average chain length of the 20 most abundant peptides and the percentage of peptides having a molecular weight below 1 kDa is shown in Table 4. Overall, the tendency of the results shows the increase in the degree of hydrolysis, as expected, based on longer reaction times. The activity of the proteases is maintained at the conditions established. In all the samples analysed with Alcalase, at least one peptide of 30 residues could be identified, whereas once the Flavourzyme is added, in sample HPH60 + 15F, the longest peptide contains 21 residues, due to the exopeptidase activity of the protease added. As it can be observed in the table, the samples hydrolysed with Alcalase and Flavourzyme presented a higher content of <1 kDa peptides and the most abundant peptides had lower molecular weight that HPI and samples hydrolysed only with Alcalase.

In HPHs, 50 peptides identified in each of the ten hydrolysates evaluated within this research are reported. Considering the high amount of peptides identified in each sample, the criteria was to select the first 10 peptides with highest relative abundance (determined by −10lgP) with a molecular weight below 1000 Da, as it is expected that these peptides are more easily absorbed and can exert the activity in the target cells.

From the 50 peptides selected (5 peptides x 10 samples), some of the peptides were found in more than one samples. Nineteen unique sequences were retrieved from this selection and were subjected to characterization (section 3.6, Table 5). From the peptides identified in each hydrolysate, it is really difficult to draw global conclusions since the content reported is in relative amount and the number of peptides identified in each sample is more than 1600 on average. This highlights the need, in addition to the improvement of analytical techniques, that research must have updated databases capable of storing this information in a coherent and orderly manner, to compare results between different sources of hydrolysed proteins with specific proteases, in order to determine which treatment is suitable for the release of specific sequences in an optimized abundance.

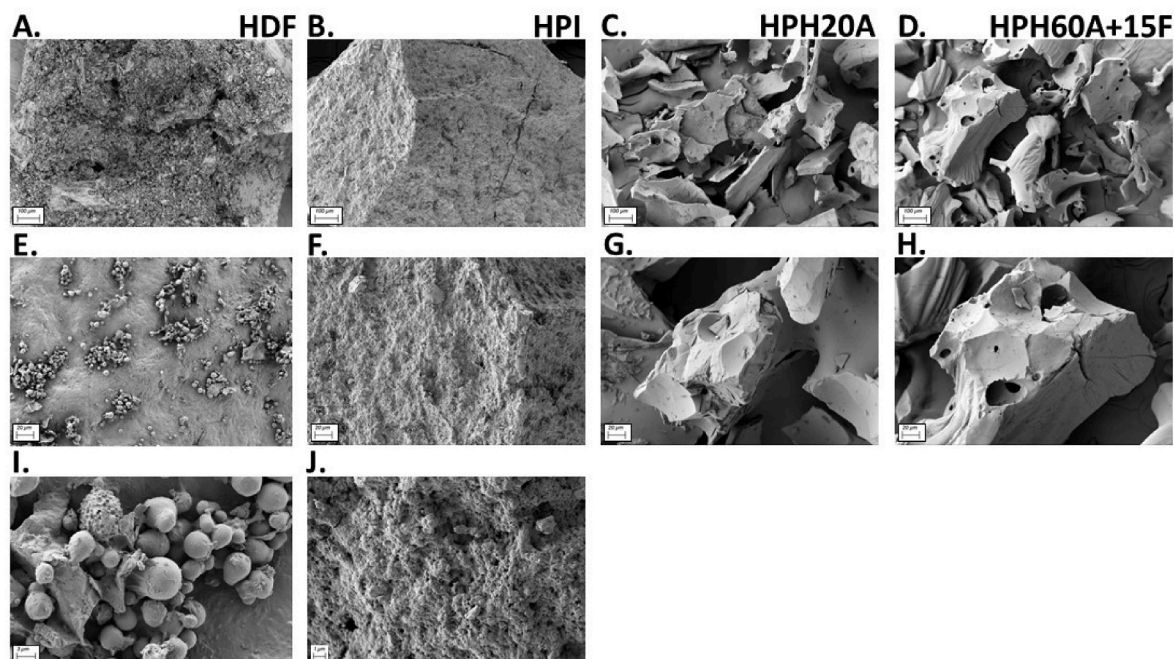Alcalase is a broad-spectrum endo-peptidase enzyme that mostly

Fig. 1. Surface characteristics of hemp defatted flour (HDF), HPI, and hemp protein hydrolysates (HPH20A and HPH60A + 15F) by Scanning Electron Microscopy (SEM) at two magnifications (Mag.). Upper SEM images (A–D) were taken at Mag = 90x and AV = 2.0 kV. Lower SEM images (E–H) were taken at Mag = 345x and AV = 2.0 kV. Finally, SEM images (J and I) were taken at Mag = 2.5kx and AV = 2.0 kV and at Mag = 5kx and AV = 2.0 kV, respectively. Scale marks: 100 μm (upper raw), 20 μm (middle raw), 3 μm (image I), and 1 μm (image J).

Table 3
DPPH radical scavenging activity, β-carotene activity, and ferric ion reducing antioxidant power (FRAP) of hemp protein hydrolysates (HPHs) and BHT as positive control. Values, expressed as IC$_{50}$ mg/mL, are the mean ± standard deviation of three determinations.

	DPPH	β-carotene	FRAP
BHT	0.01 ± 0.01[c]	5.2E-08 ± 8.58E-0.8[c]	1.18E-02 ± 1.04E-04[a]
HPH10A	5.19 ± 0.61[c]	1.02E-03 ± 4.01E-04[c]	0.88 ± 0.07[c]
HPH20A	1.04 ± 0.18[c]	8.99E-03 ± 9.4E-05[b,c]	0.79 ± 0.12[b]
HPH30A	57.04 ± 5.45[b]	3.75E-03 ± 5.73E-04[b,c]	1.29 ± 0.13[d]
HPH45A	99.67 ± 53.14[a]	0.14 ± 0.09[a,b]	1.48 ± 0.16[d]
HPH60A	41.43 ± 6.56[c]	0.11 ± 0.02[a,b,c]	1.56 ± 0.28[d]
HPH60A + 15F	1.82 ± 0.21[c]	0.11 ± 0.04[a,b,c]	1.29 ± 0.15[d]
HPH60A + 30F	1.81 ± 0.08[c]	0.04 ± 0.01[a,b,c]	1.49 ± 0.10[d]
HPH60A + 60F	1.42 ± 0.04[c]	0.16 ± 0.11[a]	1.18 ± 0.09[c,d]
HPH60A + 90F	1.73 ± 0.09[c]	0.14 ± 0.03[a,b]	1.22 ± 0.04[c,d]
HPH60A + 120F	1.83 ± 0.17[c]	0.13 ± 0.03[a,b,c]	1.24 ± 0.1[c,d]

Table 4
Summary of the peptidome characterization of HPI and the HPH obtained by the hydrolysis with Alcalase and with Alcalase and Flavourzyme.

	Number of peptides identified	Mean PCL of the 20 most abundant peptides[a]	% of < 1 kDa peptides
HPI	3353	20	3.9
HPH10A	2113	17	6.6
HPH20A	2707	18	8.4
HPH30A	1704	16	9.6
HPH45A	1632	16	10.2
HPH60A	1525	15	11.6
HPH60A + 15F	1185	13	14.5
HPH60A + 30F	1221	14	13.1
HPH60A + 60F	1134	14	12.7
HPH60A + 90F	1163	14	12.7
HPH60A + 120F	1200	15	13

[a] Value rounded; PCL: peptide chain length.

binds to hydrophobic amino acids, increasing the amount of N-terminal locations available. Samaei et al. (2021) processed proteins from hemp bran with Alcalase, and separated by membrane ultrafiltration to concentrate peptides. Antioxidant properties were evaluated and 239 peptides were identified, among which 12 (di and tripeptides and free amino acids) exhibited structural features to exert antioxidant activity. Some of the molecular features found in the peptides described by these authors are in line with the features found in our peptides, such as the presence of the motifs LY or PQ, which are highly reported in the peptides found in the samples analysed in this report and by Samaei et al. (2021).

The peptide profile of the hydrolysates would vary if Flavourzyme is used as secondary enzyme. Flavourzyme is a complicated blend of endo- and exopeptidases that may release free amino acids and very tiny peptides from lineal chains. The release of shorter peptides by the action

of Flavourzyme would have an effect in the antioxidant properties of peptides, depending on the mechanism by which the sequence exert the activity, as discussed previously (Table 3).

In terms of highest relative abundance, the peptide KNAIYTPH was found to be most abundant in 4 samples (HPH30A, HPH45A, HPH60A + 30F and HPH60A + 60F) and among the most abundant in two other samples, including those hydrolysed with Alcalase and with Flavourzyme. This suggest that the peptide was released by the action of Alcalase and it is not especially prone to be cleaved by the action of Flavourzyme. Similarly, the peptide PQNFAVVK is found in 9 of the 10 hydrolysates evaluated, among the most abundant peptides. The sample not having this peptide among the most abundant ones is HPH60A + 15F, whose most abundant peptides accounts for 4 unique sequences (not among the most abundant in any of the other samples). This can be explained by the peptidase action of Flavourzyme, highly active at the

Table 5
Characterization of the highest relative abundance (determined by −10lgP) peptide sequences (<1000 Da) identified in hemp protein hydrolysate (HPH) based on *in silico* analyses.

PEPTIDOME					Physical and chemical characteristics						Self-aggregation-prone region & Amyloids[c]	Disorder Probability (%)[c]	Probability in secondary structure (%)[c]			Bioactivity		
Peptide Sequence	HPH	Rel. abundance (−10lgP)	Res. Length	Molec. Weight	Calculated net charge[a]	Predicted pI[a]	Estimated solubility in pure water[a]	Hydrophobicity[b]	Steric hindrance[b]	Amphipathicity[b]			α-helix	β-strand	coil	PeptideRanker score[d]	Free radical scavenger score[e]	Chelation score[e]
KNGMMAPH	10A	52.09	8	884.39	1.1	9.91	Good	−0.16	0.57	0.64	2-5 (NI)	100	0	0	100	0.443	0.440	0.227
	30A	43.80																
GKLDLVKPQ	10A	48.68	9	996.60	1	9.93	Good	−0.21	0.62	0.95	3-6 (NI)	100	0	0	100	0.196	0.337	0.229
PQNFAVVK	10A	48.28	8	901.50	1	10.57	Poor	−0.07	0.64	0.61	4-7 (NI)	100	0	25	75	0.318	0.341	0.203
	20A	49.07																
	30A	48.13																
	45A	45.37																
	60A	47.51																
	60A + 30F	46.23																
	60A + 60F	50.45																
	60A + 90F	47.50																
	60A + 120F	43.59																
PQNHAVVK	10A	46.15	8	891.49	1.1	10.57	Good	−0.20	0.55	0.80	4-7 (NI)	100	0	0	100	0.154	0.409	0.222
	20A	47.14																
AMRNPLAGK	10A	45.99	9	956.52	2	11.42	Good	−0.24	0.61	0.68	1-4 (NI)	100	0	0	100	0.469	0.393	0.235
PQLVYIVK	20A	51.08	8	958.59	1	10.09	Poor	0.06	0.63	0.61	3-7 (PA)	100	0	62.5	37.5	0.206	0.441	0.191
	30A	47.84																
	45A	44.58																
	60A	45.32																
	60A + 60F	44.03																
	60A + 90F	46.95																
	60A + 120F	46,86																
VKEPVFSF	20A	47,78	8	951,51	0	6,82	Good	0,03	0,63	0,62	5-8 (NI)	100	0	0	100	0,442	0354	0,204
KNAIYTPH	20A	47,36	8	942,49	1,1	9,55	Poor	−0,17	0,53	0,64	3-6 (NI)	100	0	0	100	0,241	0447	0,205
	30A	58,92																
	45A	59,54																
	60A	58,67																
	60A + 30F	58,77																
	60A + 60F	50,55																

Table 5 (*continued*)

PEPTIDOME					Physical and chemical characteristics						Self-aggregation-prone region & Amyloids[c]	Disorder Probability (%)[c]	Probability in secondary structure (%)[c]			Bioactivity		
Peptide Sequence	HPH	Rel. abundance (−10lgP)	Res. Length	Molec. Weight	Calculated net charge[a]	Predicted pI[a]	Estimated solubility in pure water[a]	Hydrophobicity[b]	Steric hindrance[b]	Amphipathicity[b]			α-helix	β-strand	coil	PeptideRanker score[d]	Free radical scavenger score[e]	Chelation score[e]
MRNPLAGK	30A	41,73	8	885,49	2	11,39	Good	−0,30	0,62	0,77	5-8 (NI)	100	0	0	100	0,390	0384	0,263
	45A	46,52																
DDRNSIIR	45A	44,52	8	987,51	0	6,83	Good	−0,55	0,70	0,61	4-7 (NI)	100	0	0	100	0,323	0246	0,238
	60A	44,12																
	60A	47,45																
	+ 30F																	
	60A + 60F	49,92																
	60A + 90F	45,00																
	60A + 120F	46,30																
TDHYLPIH	60A	61,15	8	994,49	−0,8	6,04	Poor	−0,06	0,45	0,36	3-8 (NI)	100	0	0	100	0,447	0548	0,271
TNGPQLIH	60A	43,20	8	878,46	0,1	7,50	Poor	−0,07	0,53	0,34	5-8 (NI)	100	0	0	100	0,230	0424	0,253
	+ 15F																	
	60A + 30F	49,32																
	60A + 60F	48,28																
	60A + 120F	43,72																
SAERGFLY	60A	33,46	8	941,46	0	6,58	Good	−0,13	0,63	0,47	4-8 (NI)	100	0	0	100	0,615	0423	0,205
	+ 15F																	
DDNGRNVF	60A	31,19	8	935,41	−1	3,71	Good	−0,40	0,73	0,31	5-8 (NI)	100	0	0	100	0,424	0376	0,245
	+ 15F																	
RNIFKGF	60A	28,14	7	880,49	2	11,39	Good	−0,20	0,70	0,87	1-4 (NI)	100	0	0	100	0,798	0343	0,225
	+ 15F																	
ADIFNPR	60A	27,18	7	831,42	0	6,71	Good	−0,23	0,64	0,35	2-5 (NI)	100	0	0	100	0,798	0368	0,266
	+ 15F																	
HWNVNAH	60A	44,48	7	876,40	0,2	7,72	Poor	−0,13	0,46	0,41	2-5 (NI)	100	0	0	100	0,276	0417	0,256
	+ 30F																	
	60A + 90F	50,40																
SDEQHQKV	60A	44,28	8	969,45	−0,9	5,17	Good	−0,49	0,59	1,11	3-7 (NI)	100	25	0	75	0,129	0437	0,282
	+ 90F																	

8

Table 5 (*continued*)

PEPTIDOME				Physical and chemical characteristics										Bioactivity		
Peptide Sequence	HPH	Rel. abundance (−10lgP)	Res. Length	Molec. Weight	Calculated net charge[a]	Predicted pI[a]	Estimated solubility in pure water[a]	Hydrophobicity[b]	Steric hindrance[b]	Amphipathicity[b]	Self-aggregation-prone region & Amyloids[c]	Disorder Probability region (%)[c]	Probability in secondary structure (%)[c] α-helix / β-strand / coil	PeptideRanker score[d]	Free radical scavenger score[e]	Chelation score[e]
EERPGHF	60A + 120F	42,97	7	870,40	−0,9	5,26	Good	−0,39	0,54	0,92	4-7 (NI)	100	0 / 0 / 100	0,393	0536	0,265

[a] Peptides were subjected to calculation via http://pepcalc.com/, where the net charge at neutral pH was calculated. Meanwhile, peptide solubility in pure water was estimated on this web server based on the combined result of isoelectric point (pI), the number of charged residues and the peptide length.

[b] Peptides were subjected to calculation via https://webs.iiitd.edu.in/raghava/toxinpred/design.php/, where the hydrophobicity, steric hinderance, and amphipathicity was calculated.

[c] The web server PASTA 2.0 (http://protein.bio.unipd.it/pasta2/) was implicated to compute the tendency of peptide self-aggregation specific to the possible region at sequence (with the recorded number starting from N-terminus). For peptide discrimination, the optimal thresholds were switched as Top = 1 and Energy < - 5 PEU (1 PEU (Pasta Energy Unit) = 1.192 kcal/mol). NI: no amyloid predicted; PA: parallel aggregation computed. The probability of intrinsic disorder and portion of estimated secondary structure that complement the aggregation data were also reported.

[d] The likelihood for the peptides as bioactive was evaluated by PeptideRanker (http://bioware.ucd.ie/~compass/biowareweb/), a server to predict bioactive peptides based on a novel N-to-1 neural network, by giving scores ranging from 0 to 1. Higher score indicated the greater the likelihood of the peptide being bioactive.

[e] AnOxPePred tool (http://services.bioinformatics.dtu.dk/service.php?AnOxPePred-1.0) uses deep learning to predict the antioxidant properties (quantified by free radical scavenging and ion chelating scores) of peptides by giving scores ranging from 0 to 1.

beginning of the reaction, thus, cleaving peptidic bonds more effectively than Alcalase. However, it is likely that with longer reaction times with both enzymes hydrolysing simultaneously results in a higher activity of Alcalase, which keeps on hydrolysing peptides with specific molecular features that Flavourzyme is not capable of. On the other hand, the peptide TNGPQLIH, which was among the most abundant in 4 of the 5 samples hydrolysed with Flavourzyme, is likely to have been released to the action of the exopeptidase. Girgih et al. (2014) identified 23 peptides from an antioxidant hemp hydrolysate obtained by simulated gastrointestinal digestion, and these authors indicated that the sequences WVYY and PSLPA were the most active. As indicated previously, the characterization of hemp peptides is still a gap in research, since the identification of bioactive peptides is not widely reported in literature. Considering that protein hydrolysates contain a large number of components (free amino acids, small-/medium-size peptides, polypeptides, oligomers, undigested proteins, etc.), a complete and accurate identification of every single peptide is a challenge and has certain limitations. For instance, it is difficult to distinguish short molecular weight peptides (4 amino acids in length), which are often those responsible for the bioactivity, from high molecular weight compounds when they are present. In this sense, bioinformatics analysis are crucial for identifying compounds that are biologically active.

3.6. In silico analyses of identified peptides

In Table 5, the physical-chemical properties including net charge, pI, hydrophobicity, steric hindrance, and amphipathicity (evaluated by PepCalc and ToxinPred), the likelihood to be bioactive and the free radical scavenger and chelation score of each peptide are shown. Based on the analysis carried out with the Toxin Pred software, all the peptides were predicted as non-toxic. Overall, a peptide is considered to more likely be bioactive according to the hydrophobicity. A score >0.5 would strongly suggest that the peptide is bioactive (Mooney et al., 2012), based on its molecular characteristics. Therefore, in order of highest to lowest bioactivity reported in the experimental assay, the peptides that could be considered as highly active would be ADIFNPR, RNIFKGF, and SAERGFLY. However, it must be noted that the peptide TDHYLPIH, showing a score of 0.45, is the one showing the highest free radical scavenger score, which is specific to predict antioxidant peptides. According to this Free Radical Scavenging score, also the peptides EERPGHF, KNAIYTPH, PQLVYIVK, and KNGMMAPH would exert a strong free radical scavenger activity. Concerning the chelation score, the values obtained for the peptides analysed ranged from 0.191 for SDEQHQKV to 0.282 for GKLDLVKPQ. The mechanisms by which a peptide can exert antioxidant activity depends on the residues on the sequences and how they interact consequently with the reactive oxygen species.

According to Lu et al. (2010) the amino acid valine located at the C-terminal highly contributes to the antioxidant activity of peptides (feature reported in the identified peptide SDEQHQKV), whereas tyrosine at any of the terminal is also relevant in enhancing antioxidant activity of peptides, as occurs in the peptide SAERGFLY. Furthermore, the sequences YGRDEISV and LDLVKPQ were reported as antioxidant peptides obtained by enzymatic hydrolysis of hemp with Protamex. The sequences GR and DE from the above-mentioned peptides were also detected in the peptides identified (DDNGRNVF and SDEQHQKV) in the present study, which may play a positive role in the antioxidant activity of hemp seed protein-derived peptides. Furthermore, the peptide GKLDLVKPQ identified in the present study contains in the sequence the peptide LDLVKPQ reported by the authors, as highly antioxidant(Gao et al., 2021), thus, supporting the hypothesis of being a strong antioxidant peptide. In fact, this peptide presents a relative high amphipathicity and a great score in the antioxidant peptide prediction tool. Other peptides previously identified are SVYT, PSLPA, and HVRETALV (Girgih et al., 2014; Lu et al., 2010). Similarly, some of the dipeptides contained in these sequences are also contained in the peptides identified in this

research, supporting the potential of these identified peptides as anti-oxidant agents.

In addition to these tools predicting the bioactivity of peptides, low steric hindrance values and high amphipathicity have been proposed as peptide characteristics that would increase the likelihood that they will exert their activity by stabilizing the interaction of the peptide with the target compound (Mora et al., 2020). The peptide with the highest amphipathicity was SDEQHQKV (value of 1.11), followed by GKLDLVKPQ, EERPGHF, RNIFKGF, and PQNHAVVK. Among these peptides, the one with the lowest steric hindrance are EERPGHF and PQNHAVVK. Similar results were reported for antidiabetic and anti-lipidemic plant-derived peptides (Alnuaimi et al., 2023; Rivero-Pino et al., 2021), suggesting the high potential and applicability that *in silico* tools can have on the discovery and verification of bioactivity characterization of peptides.

Taking into account all the parameters discussed, the peptide KNAIYTPH, previously indicated as one of the most abundant in the hydrolysates, show promising results in terms of potential antioxidant activity, having a free radical scavenger score of 0.447. The peptide EERPGHF, even when only reported among the most abundant in one sample, was also found in other samples analysed, and based on the *in silico* analysis as previously described, can be a good candidate to be exerting adequate antioxidant activity. In addition, considering that methionine is an essential component in the oxidative stress response because it can act as a reactive oxygen species scavenger, the sequence KNGMMAPH, which also contains histidine (scavenger of highly active singlet oxygen), is a promising candidate of antioxidant hemp peptide. In fact, the *in silico* parameters obtained for this sequence (mostly anti-oxidant scores and amphipacity) support the hypothesis. Apart from these factors related to primary structure of peptides, the advanced conformation such as secondary structure might also play a role in antioxidant functionality (Li et al., 2022). Herein, as computed by PASTA 2.0 server (Table 5), 16 of 19 peptide sequences (84.21% in quantity) derived from HPHs had entire probability to render random coil without any contribution to neither α-helix and β-strand, and only 1 (SDEQHQKV) of 19 sequences (5.26% in quantity) enabled the formation of α-helix in simulation. In addition, only 2 (PQNFAVVK and PQLVYIVK) of 19 sequences (10.52% in quantity) enabled the formation of β-strand in simulation. Overall, through our computation, the amyloids and self-aggregation were not substantially favoured by these peptide monomers of interest regardless of their hydrophobicity.

To the author's knowledge, these identified peptides proposed as antioxidant peptides stemming from hemp protein hydrolysates have not been identified previously. It must be considered that the beneficial effect of protein hydrolysates is not due to a specific peptide but to the consumption of this pool of peptides in its entirety. The following research steps should include the evaluation of the resistance of these peptides subjected to gastrointestinal digestion conditions, though it is reported that the presence of the amino acid arginine at the terminal of the sequence contributes to increase the resistance of the peptide to be cleaved during the proteolytic action of digestive enzymes. These molecular features were found in RNIFKGF, DDRNSIIR, and ADIFNPR. In addition, their bioactivity evaluation employing synthetic peptides, and their efficacy in real food matrices under specific processing and storage conditions should be carried out, as well as studies in animal models to unravel the underlying mechanisms by which these peptides exert *in vivo* activity, and human studies to propose health attributes to the peptides.

4. Conclusion

Recently, a plethora of factors (such as health risks, environmental concerns, manufacturing costs, etc.) have caused the food industry to place a higher priority on plant-based materials than on animal sources. A complete nutritional evaluation of hemp protein as isolate and as hydrolysates was carried out, including proximate analysis and amino acid composition, showing that hemp protein would not be nutritionally

disadvantageous for human consumption. Enzymatic hydrolysis was used to increase the bioactivity of hemp protein with the purpose of creating a functional ingredient. The antioxidant activity of these HPI and HPHs measured by three different *in vitro* methods highlighted their potential to be used as functional ingredient to promote healthy ageing, by alleviating oxidative stress and lipid peroxidation caused by free radicals, and consequently, could be used to maintain human health and food safety and quality. The full characterization of the proteome profile contained in each sample was carried out, and some peptides were found highly abundant in different samples. Based on molecular features and relative abundance in the samples, nineteen peptides were selected and subjected to *in silico* analysis, to physic-chemically characterize them and predict the antioxidant activity that could be attributed to each of them. These analyses suggested that the peptides KNAIYTPH, EERPGHF and KNGMMAPH, among others, could be highly contributing to the antioxidant potential of the HPHs obtained.

Author statement

Conceptualization, S.M.-d.l.P, M.C.M.-L.; methodology, A.V., R.T.; formal analysis, S.M.-d.l.P., F.R.-P.; investigation, A.V., R.T; resources, S.M.-d.l.P, M.C.M.-L.; writing—original draft preparation, F.R.-P.; writing—review and editing, S.M.-d.l.P; supervision, S.M.-d.l.P, M.C.M.-L.; funding acquisition, S.M.-d.l.P., M.C.M-L. All authors have read and agreed to the published version of the manuscript. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102561.

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