PAPER

Identification, characterization and resistance to digestion assessment of hemp-derived dipeptidyl-peptidase IV inhibitory peptides

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Hemp seeds (*Cannabis sativa* L.) are an adequate source of protein. Food-derived peptides exert bioactivity depending on the sequences. In this work, enzymatic hydrolysis with Alcalase (subtilisin) and Flavourzyme was carried out to obtain hemp protein hydrolysates with increased dipeptidyl-peptidase IV (DPP-IV) inhibitory activity. In addition, the hydrolysates were subjected to digestion following the INFOGEST protocol. The serial hydrolysis with both proteases showed the highest inhibition compared to the hydrolysis only with Alcalase. The peptides sequences contained in the samples and the digested samples were identified and characterized employing different bioinformatics tools. The sequences TNGPQLIH (released after addition of Flavourzyme), and GKLDLVKPQ (from Alcalase-treated samples) were proposed as the most active peptides. The peptides were chemically synthesized, and TNGPQLIH showed an IC_{50} value of 1.70 mg mL^{-1}. The DPP-IV inhibitory activity was generally conserved or improved after digestion. These peptides could be employed as constituents in foods helping to prevent the development of diabetes.

1. Introduction

Bioactive peptides can be obtained from several food sources, ideally enriched in the protein content such as concentrates or isolates, by enzymatic hydrolysis or fermentation. Numerous reports, from *in vitro* to human studies have proved the benefits of these components in modulating different metabolic pathways (antihypertensive, antidiabetic, anti-inflammatory) in the organism, by targeting receptors or enzymes.[1–4] Their efficacy as bioactive agents is dependent on their molecular weight and amino acid profile, and consequently the treatment applied to the native protein (*e.g.*, enzyme, operating conditions of the reaction, *etc.*) are crucial in obtaining a bioactive pool of peptides.[5,6] In addition to that, the ONE health principles and the worldwide aim to achieve a sustainable world led to a neces-

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sity of introducing environmentally friendly sources into the food system, and ideally, capable to be used in foods to prevent some disorders due to the bioactivity.[7] One such interesting source of protein is hemp seeds, as it has been described that production of hemp could promote circular economy, being an interesting, environmental and cost-effective agent for phytoremediation, while its protein being of high quality and containing several bioactive peptides.[8–10]

Concerning antidiabetic peptides, there are several metabolic routes in which peptides can have a functional role, including the inhibition of the enzyme dipeptidyl-peptidase IV (DPP-IV). This DPP-IV is in charge of modulating the levels of incretins (*e.g.*, glucagon-like peptide-1, GLP-1), and inhibiting DPP-IV has as consequence, an increased half-life of this hormone, responsible for insulinotropic effects.[11] Existing drugs used as inhibitors, show undesirable effects in humans, such as hypoglycemia, pancreatitis or gastrointestinal issues.[12] As consequence, the use of food-derived peptides with bioactivity appears as a sustainable option for the prevention and pre-treatment of diabetes.[13]

The use of hemp protein hydrolysates (HPHs) as possible antidiabetic agents has been scarcely evaluated. In this regard, analysis of hemp-derived peptides has been described previously employing proteases such as Corolase L10, Promod 144MG and Protamex, leading to values of inhibition, as half-maximal inhibitory concentration (IC_{50}) values, ranging from 1.84 to 5.71 mg mL^{-1}.[14,15] hydrolysed proteins from *Cannabis*

sativa seeds with gastrointestinal enzymes and evaluated *in vitro*, *in situ* and *ex vivo* the activity towards DPP-IV in Caco-2 cell culture. In addition, hemp seed protein was hydrolysed with Alcalase, and more than 500 short peptides with multifunctional activity, including inhibition of DPP-IV were identified.[16] In addition to that, using bioinformatics,[17,18] identified 16 peptides by *in silico* enzymatic hydrolysis (with proteases such as trypsin, chymotrypsin, pepsin, proteinase K, and thermolysin) of several hemp seeds protein, and the *in vitro* DPP-IV inhibition assay yielded 8 peptides having IC_{50} values lower than 0.5 mM and cell assays demonstrated also increased insulin and GLP-1 concentrations. Nonetheless, it has been widely reported in several food sources, that the use of Flavourzyme is highly adequate in releasing DPP-IV inhibitory peptides.[19–21] To the authors' knowledge, no information employing Flavourzyme to obtain DPP-IV inhibitory peptides from hemp has been published so far, and the validation on whether this enzymatic treatment is adequate in releasing a highly bioactive protein hydrolysate is lacking. The hypothesis is that the use of Flavourzyme after the hydrolysis with Alcalase (subtilisin) will enhance the DPP-IV inhibitory potential of the sample.

Hence, in this work, hemp protein was enzymatically hydrolysed employing an endopeptidase (subtilisin, of broad spectrum) at different reaction times, as well as to evaluate whether the addition of an exopeptidase (Flavourzyme) for 15 min increase the activity. The hydrolysates were characterized, and their DPP-IV inhibitory activity was analyzed. Then, the peptidome of the hydrolysates was determined by liquid chromatography – trapped ion mobility spectrometry – tandem mass spectrometry. In addition, the hydrolysates were subjected to simulated gastrointestinal digestion (SGID) following the INFOGEST protocol, and the peptidome of these samples was also characterized. *In silico* analysis aiming to characterize the physicochemical properties and the DPP-IV inhibitory activity of peptides were used to suggest the most active ones, whose bioactivity was validated *in vitro* with the chemically-synthesized pure peptide sequences.

2. Materials and methods

2.1. Materials

Cannabis sativa L. seeds were offered by Sensi Seeds Bank. The proteases employed in the hydrolysis reactions (*i.e.*, Alcalase 2.4 L and Flavourzyme (1000 L)) were obtained from Novozymes (Bagsvaerd, Denmark). All the chemicals (reagents and solvents) were of analytical grade, either from Sigma Chemical Co., Bachem AG (Bubendorf, CH, EU) or Gibco (Waltham, MA, USA).

2.2. Hydrolysis procedure

At first, a hemp protein isolate (HPI) was produced following the protocol of Montserrat de La Paz.[22] Then, a hydrolysis reaction was carried out at fixed temperature (50 °C) and pH, in a jacketed stirred vessel. To the HPI diluted in distilled water (10% w/v), Alcalase was used at 0.3 AU g^{-1} of protein at pH 8,

and the reaction lasted one hour, while aliquots were sample at 10 (HPH10A), 20 (HPH20A), 30 (HPH30A), 45 (HPH45A), and 60 min (HPH60A). After that, by adding Flavourzyme at 60 LAPU per g of protein at pH 7, leaving the enzyme act for 15 min, the sample HPH60A + 15F was obtained. Enzyme was deactivated with heat for 15 min at 85 °C.

2.3. *In vitro* DPP-IV inhibition assay

DPP-IV inhibitory activity analysis (of hydrolysates, digested and synthesized peptides) was carried out as described by Harnedy and Fitzgerald,[23] using Diprotin A™ at 5 μM as the positive control. Experiments were conducted in triplicate. The values were expressed as the mean half maximal inhibitory concentration (IC_{50}) ± standard deviation ($n = 3$).

2.4. *Peptide* identification

Peptides of both the hydrolysates and the digested samples analyses were prepared and analyzed in the Proteomics Facility at Research Support Central Service, University of Cordoba. Samples were acidified with 0.5% Trifluoroacetic acid. Desalting and concentration step was performed with ZipTip C18 (Millipore) and the digested samples were finally Speedvac dried and resuspended in 5 μl of mobile phase (2% CAN, 0.05% TFA, 200 ng per sample) for HPLC injection. Sequences were identified by liquid chromatography followed by high resolution trapped ion mobility spectrometry-mass spectrometry (LC-TIMS-MS/MS) following the methodology of Montserrat de La Paz *et al.*,[22] PEAKS Studio ProX (Bioinformatics Solutions Inc) was used to process the raw data, with the reference library of UniProt, with parent mass error tolerance set to 15 ppm and a fragment mass error tolerance of 0.05 Da. Protein unique peptides was set to larger than 1 and a high confidence score of $-10 \lg P > 15$ was applied to indicate an accurately identified protein.

2.5. *In silico* characterization of bioactive peptides

To identify the peptides most likely to be bioactive in the samples, various bioinformatic tools were utilized:

(a) Peptide Property Calculator (https://pepcalc.com/) was employed to determine the net charge of identified peptides at neutral pH, as well as solubility and isoelectric point.

(b) ToxinPred software, to estimate physicochemical properties;[24]

(c) PeptideRanker, to estimate the likelihood of being bioactive.

(e) Three different tools aiming to predict whether a peptide is able to inhibit DPP-IV were used: iDPPIV-SCM[25] (https://camt.pythonanywhere.com/iDPPIV-SCM), StackDPPIV[26] (https://pmlabstack.pythonanywhere.com/StackDPPIV) and AntiDMPpred[27] (https://i.uestc.edu.cn/AntiDMPpred/cgi-bin/AntiDMPpred.pl).

The peptides subjected to the analyses were the first five with the highest value of $-10 \lg P$, with a molecular weight <1000 Da, as DPP-IV inhibitory peptides have been highly reported as low-molecular weight peptides, and a lower molecular weight is correlated with increased bioavailability.

2.6. Simulated gastrointestinal digestion (SGID)

The simulated gastrointestinal digestion was conducted according to the INFOGEST protocol[28] with slight modifications, including the following proteases: α-amylase (EC 3.2.1.1), pepsin (EC 3.4.23.1), pancreatic lipase (EC 3.1.13) and bile salts (according to supplier's protocol). The digests obtained were also evaluated for their DPP-IV inhibitory activity as described in section 2.3.

2.7. *In silico* digestion of bioactive peptides

The tool BIOPEP, which can be found https://biochemia.uwm.edu.pl/biopep/rec_pro1.php?x=72&y=0 [29] was used to subject the peptides to an *in silico* gastrointestinal digestion, to forecast the resulting peptides after the cleavages. Then, these fragments were screened whether they are DPP-IV inhibitory sequences according to the database.

2.8. Chemical synthesis of peptides

The chemical synthesis of the TNGPQLIH and GKLDLVKPQ peptides from hemp protein hydrolysates (released after addition of Flavourzyme and Alcalase-treated samples respectively), have been performed by the ICTS "NANBIOSIS", more specifically, by the Synthesis of Peptides Unit at Institute of Advanced Chemistry of Catalonia from Consejo Superior de Investigaciones Científicas (IQAC-CSIC), at >95% purity, measured by HPLC–PAD at 220 nm, using a XBridge® C18 3.5 μm, 4.6 × 100 mm column and a gradient of 5% to 100% of B in 8 min at 30 °C (1 mL min^{-1}). These peptides were subjected to *in vitro* DPP-IV inhibitory activity as described in section 2.3

2.9. 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 DPP-IV inhibitory activity, the statistics between the groups was done using two-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test or independent sample *t*-test, where applicable. *P* values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. DPP-IV inhibitory activity from hydrolysates

The protein content of hempseed usually ranges from 20 to 25%, whereas this content is maximized up to a value of 90–95% after isolation. Among the different proteins identified in hemp seed (>180), 60–80% accounts for edestin (legumin-type globulin), 13% of albumin (globular-type) and in minor proportion, β-conglycinin.[17,30,31] The characteristics of the hydrolysates have been previously reported, including protein content (around 80–85%, dry weight) and amino acid profile (high content of arginine, glutamic acid, and aspartic acid).[22]

In this study, hydrolysates exhibited a dose-dependent DPP-IV inhibition behavior, allowing to determine the IC$_{50}$ values of each sample (Fig. 1). In the case of HPI, an IC$_{50}$ value

could not be determined (>5 mg mL^{-1}). The hydrolysates obtained exclusively with Alcalase showed an IC$_{50}$ value ranging from 2.29 to 2.54 mg mL^{-1}, whereas after the action of Flavourzyme, the value was lowered up to 1.59 mg mL^{-1}. According to the results obtained, the hypothesis was confirmed. The statistically significant differences among the samples obtained by Alcalase were not relevant, as overall, all the samples could be grouped under the same range of inhibition. After addition of Flavourzyme, the value was highly lowered, leading to statistically significant differences. The cleavage of the protein during the Alcalase hydrolysis most likely have changed the conformational structure of the protein, allowing Flavourzyme to exert the activity. Flavourzyme is usually employed along with, or after an endo-peptidase, in order to fully take advantage of its potential as protease. The accessibility of N-terminal sites is higher after the hydrolysis with Alcalase 2.4 L because of its *endo*-peptidase high-spectrum activity,[32] paving the way to an efficient hydrolysis by Flavourzyme, which would change the profile of peptides in the samples based on its ability to cleave low molecular weight peptides.[33,34]

The values obtained are in the same order as values obtained in other sources with similar enzymatic treatments, such as boarfish,[35] *Porphyra dioica*[19] or other vegetables,[36] with values ranging from 0.5 to 5 mg mL^{-1} generally. The relevance of hemp protein as antidiabetic has assessed up to human studies. In this regard,[37] carried out two acute randomized repeated-measures crossover studies in which the subjects ingested hemp protein (doses of 20 or 40 g), soy protein (20 or 50 g) or carbohydrates as control. The objective was to measure and compare glycemic response (glucose and insulin levels) and satiety following the different ingestions. According to the authors, both test items could decrease postprandial blood glucose response. A nutritional intervention with hemp peptides, more digestible and potentially with improved DPP-IV inhibitory activity might result in a more biologically relevant result in terms of antidiabetic potential.

To sum up, the significance of Flavourzyme in the liberation of DPP-IV inhibitory peptides has been extensively reported related to its exopeptidase activity, able to release small peptides. However, a proper identification of the sequences responsible for the bioactivity demonstrated *in vitro* is needed.

3.2. Identification of peptides in the hydrolysates and *in silico* characterization

The peptidome for the six hydrolysates was fully characterized, and the amount of peptides ranged from 1185 (HPH60A + 15F) to 2113 (HPH10A). A more detailed description and characterization of the peptidome can be found elsewhere. Overall, in the samples enzymatically hydrolyzed exclusively with Alcalase, peptides had higher molecular weight than in the sample obtained with Flavourzyme.[22]

In the scope of this study, the peptides with higher quality of the match in the spectrum having a molecular weight below <1000 Da were selected and reported. Among the +200 peptides
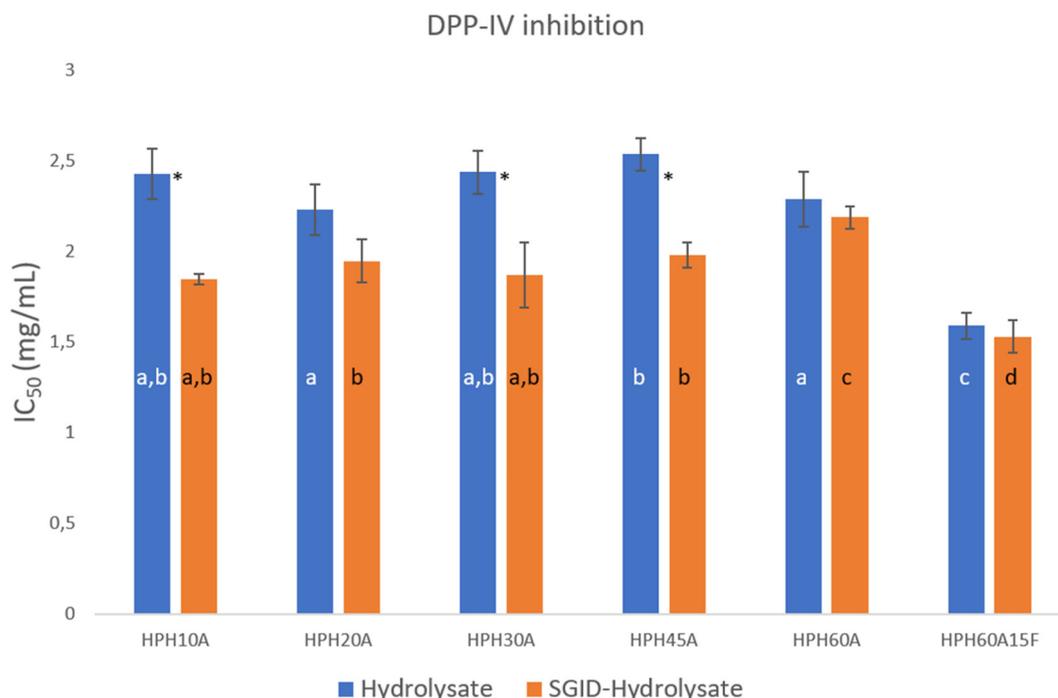
DPP-IV inhibition

Fig. 1 *In vitro* dipeptidyl peptidase-IV (DPP-IV) inhibitory of hemp protein hydrolysates (HPHs) pre- and post-simulated gastrointestinal digestion (SGID-Hydrolysate). Values are presented as the mean of three replicates ± standard deviation. Different letters within the same lot of samples (before and after) denotes significant differences among hydrolysates. The presence of * denotes significant difference of the same sample before and after digestion.

assessed by,[38] around 90% had a molecular weight inferior to 1000 Da, and in more than half of them, the value was less than 500 Da. Based on this hypothesis, the *in silico* analyses to determine and propose the active sequences were exclusively performed to the lower molecular weight peptides (Table 1).

In Table 2, the physical–chemical characterization, the likelihood to be bioactive and the DPP-IV inhibition score according to three different tools of each peptide are shown. In addition, the results from the *in silico* digestion are also depicted, discussed in section 3.4.

The analysis conducted with the Toxin Pred software indicated that none of the peptides were toxic. No correlation has been described linking the pI, solubility, and charge of peptides with the potential DPP-IV inhibitory activity.[39–41]

A score >0.5 from PeptideRanker would firmly indicate that the sequence is bioactive.[42] Consequently, the peptides that

could be considered potentially bioactive according to this *in silico* tool would only be the sequences SAERGFLY, RNIFKGF and ADIFNPR, found in the sample hydrolysed with Flavourzyme. This *in silico* prediction is in line with the results obtained *in vitro*, since this sample was the one with the lowest IC_{50} value obtained.

Nonetheless, it must be noticed that the sequence TNGPQLIH, showing a score of 0.23, is the only one showing inhibition of DPP-IV activity according to the StackDPPIV tool, thus, validation of its activity is needed. The structure of this peptide can be seen in Fig. 2, retrieved from USCF Chimera.[43] According to the StackDPPIV tool, the other sequences identified would not exert the bioactivity claimed. According to this iDPPIV-SCM score, also the peptides KNGMMAPH, PQNFAVVK, PQNHAVVK, PQLVYIVK, TDHYLPIH and TNGPQLIH would exert DPP-IV inhibitory activity, as the score obtained is higher than 300, being the threshold established by the tool developers of 294. The values obtained are promising and are in line with values obtained in peptides derived from other vegetable source such as DLLGLW from soy or RMGPL from quinoa.[44]

Concerning the AntiDMPpred tool, the peptides in which the score was higher than the threshold were only three sequences: GKLDLVKPQ, TNGPQLIH and SAERGFLY. There are differences found in the *in silico* prediction outcomes, which are mainly due to the negative and positive controls that the authors have used, considering that for instance, 60% of the peptides employed to develop the iDPPIV-SCM tool had a

Table 1 Characterization of the hemp-derived peptidome according to the treatment employed

Sample	Number of peptides identified	% of <1 kDa peptides
HPI	3353	3.9
HPH10A	2113	6.6
HPH20A	2707	8.4
HPH30A	1704	9.6
HPH45A	1632	10.2
HPH60A	1525	11.6
HPH60A + 15F	1185	14.5

Table 2 *In silico* characterization and digestion of the selected peptides sequences (<1000 Da) identified in the six hemp protein hydrolysate (HPH), a total of sixteen unique sequences

Peptide sequence	Sample (HPH)	−10 lg P	Res. Length[a]	Molec. Weight[a]	Calculated net charge[a]	Predicted pI[a]	Estimated solubility in pure water[a]	Hydro-phobicity[b]	Steric hindrance[b]	Amphi-pathicity[b]	Self-aggregation-prone region & Amyloids[c]	Disorder Probability (%)[c]	α-helix	β-strand	coil	Peptide Ranker score[d]	iDPPIV-SCM[e]	Stack-DPPIV[f]	Anti-DMPpred[g]	Digested	Active fragments	Sequence in digested HPH[h]
KNGMMAPH	10A / 30A	52.09 / 43.80	8	884.39	1.1	9.91	Good	−0.16	0.57	0.64	2–5 [NI]	100	0	0	100	0.443	318	0.39	0.41	K–N–GM–M–APH	No	YES / NO
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	252.88	0.45	0.61	GK–L–DL–AVK–PQ	PQ VK	YES
PQNFAVVK	10A / 20A / 30A / 45A / 60A	48.28 / 49.07 / 48.13 / 45.37 / 47.51	8	901.50	1	10.57	Poor	−0.07	0.64	0.61	4–7 [NI]	100	0	25	75	0.318	309.14	0.39	0.35	PQN–F–AVVK	No	YES / NO / NO / NO / NO
PQNHAVVK	10A / 20A	46.15 / 47.14	8	891.49	1.1	10.57	Good	−0.20	0.55	0.80	4–7 [NI]	100	0	0	100	0.154	311.86	0.23	0.47	PQN–H–AVVK	No	NO / YES
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	266.25	0.13	0.39	AM–R–N–PL–AGK	PL	NO
PQLVYIVK	20A / 30A / 45A / 60A	51.08 / 47.84 / 44.58 / 45.32	8	958.59	1	10.09	Poor	0.06	0.63	0.61	3–7 (PA)	100	0	62.5	37.5	0.206	310	0.24	0.46	PQL–VY–IVK	VY	NO / NO / NO / NO
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	276.43	0.36	0.45	VK–EPVF–SF	SF VK	NO
KNAIYTPH	20A / 30A / 45A / 60A	47.36 / 58.92 / 59.54 / 58.67	8	942.49	1.1	9.55	Poor	−0.17	0.53	0.64	3–6 [NI]	100	0	0	100	0.241	295.29	0.09	0.31	K–N–AIY–TPH	No	YES / YES / YES / YES
MRNPLAGK	30A / 45A / 60A	41.73 / 46.52 / 44.12	8	885.49	2	11.39	Good	−0.3	0.62	0.77	5–8 [NI]	100	0	0	100	0.39	273.43	0.28	0.35	M–R–N–PL–AGK	PL	NO
DDRNSIIR	45A / 60A	44.52	8	987.51	0	6.83	Good	−0.55	0.7	0.61	4–7 [NI]	100	0	0	100	0.323	197.29	0.3	0.33	DDR–N–SIIR	No	NO
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	332.29	0.35	0.48	TDH–Y–L–PIH	No	NO
TNGPQLIH	60A + 15F	43.2	8	878.46	0.1	7.5	Poor	−0.07	0.53	0.34	5–8 [NI]	100	0	0	100	0.23	334.71	0.53	0.51	TN–GPQL–IH	IH TN	YES
SAERGFIY	60A + 15F	33.46	8	941.46	0	6.58	Good	−0.13	0.63	0.47	4–8 [NI]	100	0	0	100	0.615	233	0.17	0.56	SAER–GF–L–Y	GF	NO
DDNGRNVF	60A + 15F	31.19	8	935.41	−1	3.71	Good	−0.4	0.73	0.31	5–8 [NI]	100	0	0	100	0.424	258.29	0.45	0.27	DDN–GR–N–VF	VF	NO
RNIFKGF	60A + 15F	28.14	7	880.49	2	11.39	Good	−0.2	0.7	0.87	1–4 [NI]	100	0	0	100	0.798	219.67	0.26	0.15	R–N–IF–K–GF	GF	NO
ADIFNPR	60A + 15F	27.18	7	831.42	0	6.71	Good	−0.23	0.64	0.35	2–5 [NI]	100	0	0	100	0.798	296	0.13	0.3	ADIF–N–PR	No	NO

Column groups: **Physical and chemical characteristics** (Res. Length … Disorder Probability, incl. *Probability in secondary structure (%)*: α-helix, β-strand, coil); **Prediction of bioactivity** (Peptide Ranker score … Anti-DMPpred); **BIOPEP SGID[h]** (Digested, Active fragments).

a Peptides were subjected to calculation via https://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 (https://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^{-1}). 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 (https://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* Scores obtained by the DPP-IV inhibitory peptides predictor tool: iDPPIV-SCM (values higher than 294 are considered as positive result). *f* Scores obtained by the DPP-IV inhibitory peptides predictor tool: StackDPPIV (threshold = 0.5). *g* Scores obtained by the DPP-IV inhibitory peptides predictor tool: AntiDMPpred (threshold = 0.5). *h* Outcomes from the *in silico* analyses carried out with BIOPEP (digestion refers to the fragments indicated in the database). Digestion with pepsin, trypsin and chymotrypsin; active fragments are those among the products originated, which are considered DPP-IV inhibitory fragments according to the information contained in the database.

peptide length of 5 residues or smaller, adding uncertainty to the predictions carried out to longer peptides.

The *in silico* tools are useful in screening the activity of peptides, although researchers have to take into consideration the limitations and the small weight of evidence that these bioinformatic tools provide when used alone.[45] *In silico* prediction tools are valuable as a first-pass screening method to identify potential bioactive peptides, but the outcomes are highly dependent on the algorithms and training datasets applied, which may lead to discrepancies across different platforms. For this reason, computational results should be interpreted as preliminary, guiding the selection of promising candidates rather than serving as conclusive evidence.

The ability of a peptide to inhibit DPP-IV activity depends on the residues within its sequence and their interactions with the enzyme. According to Mora *et al.*,[46] low steric hindrance values and high amphiphilicity has been correlated with a more bioactive peptide, based on a better stabilization of the interaction with the enzyme conformation. The sequence with the highest amphiphilic nature was GKLDLVKPQ (value of 0.95), followed by PQNHAVVK and RNIFKGF. Among these peptides, the one with the lowest steric hindrance are PQNHAVVK and GKLDLVKPQ.

According to Berraquero-Garcia *et al.*,[47] out of 230 sequences identified as DPP-IV inhibitory peptides to which an experimental IC$_{50}$ value was calculated, the average length of the peptides of the 40 most active was 6, being more than 70% of this length or smaller. It has been reported the importance of the amino acid proline (P) in these type of peptides in the N-terminal, specifically in the second position gaining more importance.[48] This statement is in line with several of the peptides hereby identified such as PQNFAVVK, PQNHAVVK and PQLVYIVK, having P in the first position. VKEPVFSF and TNGPQLIH on the other hand, have the residue on the fourth position, being the latter one the only one found in the Flavourzyme-treated samples. Similarly, a higher bioactivity in inhibiting DPP-IV has been correlated with the presence of alanine (A) in the first two positions of the N-terminal, as found in the peptide AMRNPLAGK, from Alcalase-treated samples and ADIFNPR, potentially released due to Flavourzyme.

Concerning the C-terminal, it has been suggested the importance of aromatic amino acids in the last two positions, such as phenylalanine (F), tryptophan (W) and tyrosine (Y) in Ct in peptides capable of inhibit DPP-IV.[49] In this regard, the peptide VKEPVFSF appears as promising active peptide, as well as DDNGRNVF and RNIFKGF, although these two do not possess the N-terminal with P or A, as it does VKEPVFSF. Some similarities of specific fragments in the peptides hereby identified with already demonstrated DPP-IV peptides were found, such as the fragments DL and KP, found in the hemp peptide GKLDLVKPQ as well as in the whey peptides LKPTPEGDLE, having an IC$_{50}$ value of 42 μM;[50] or also PQ from this sequence, which is also found in LPQPPQE from Velvet aqueous extract[51] or VPYPQ from casein, among others.[52] One of the peptides hereby considered as highly bioactive based on
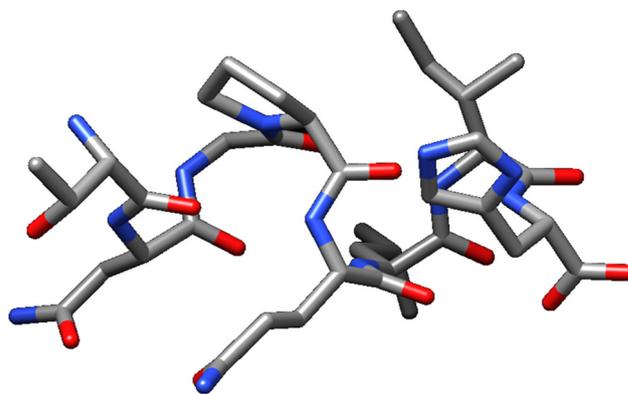
Fig. 2 3D structure of the peptide TNGPQLIH obtained from USCF Chimera.

the previous discussion, TNGPQLIH, also has the GPQ fragment from GPAGPQGPR, which *in vitro* possess an IC$_{50}$ value of 0.51 μM, also extracted from the Velvet aqueous extract or in GPGA from Salmon.[53] In addition, recently,[54] reported the discovery of a hemp seed protein hydrolysate-derived peptide as highly inhibitory towards DPP-IV. The peptide VAMP has been reported to interact with DPP-IV, leading to enhanced glucose metabolism by increasing active GLP-1 levels in obese mouse models, as well as promotes an improvement of the intestinal barrier function and insulin resistance.

The similarity of fragments in the sequences indicate the similarity of the structure that these peptides might have, and consequently, correlation structure and activity, the activity is more likely to be similar. Overall, some of the di and tri-peptides contained in previously reported as DPP-IV inhibitory sequences are also inside the sequences of the hemp-derived peptides hereby identified.

Taking into account all the parameters discussed, the most active sequences proposed as responsible for the high bioactivity were proposed to be VKEPVFSF, GKLDLVKPQ and TDHYLPIH in the Alcalase-treatment samples and TNGPQLIH and SAERGFLY in the Flavourzyme-treated hydrolysate, based on the scores obtained in some of the tools employed and the molecular features of the sequences. TNGPQLIH (derived from 60A + 15F) and GKLDLVKPQ (derived from HPH20A) were chosen to be synthesized chemically and subjected to validation of their activity (section 3.4) because of the outcomes of the *in silico* analyses, and because they were gastro-resistant (sequences were identified in the corresponding digested sample of each of the hydrolysates).

Different recently developed prediction tools were employed in order to rank and compare the likelihood of the sequences to be responsible for the DPP-IV inhibitory activity of the protein hydrolysates evaluated *in vitro*. To the author's knowledge, these hemp peptides proposed as antidiabetic (capable of inhibiting the enzyme DPP-IV) have not been identified before. It is relevant to mention that the beneficial properties of protein hydrolysates arise from consuming the complete pool of peptides, not just one particular peptide. It has been

reported that the presence of specific amino acids, such as arginine at the sequence terminal, helps enhance the peptide's resistance to cleavage during the proteolytic activity of digestive enzymes, but this resistance should be evaluated.

3.3. DPP-IV inhibitory activity from digested hydrolysates

The action of the enzymes during the gastrointestinal digestion might be responsible for the further cleavage of peptides. This cleavage is specific sites according to the specificity of the enzymes (*e.g.*, trypsin, mainly in residues or arginine or lysine) would release new sequences which can be equally, more or less active, and as consequence, the activity of the hydrolysates after SGID might be different.

As observed in Fig. 1, the DPP-IV inhibitory activity of the hydrolysates after being subjected to SGID ranged from 1.53 to 2.19 mg mL^{-1}. In addition, the HPI, which originally was reported not to exert DPP-IV inhibitory activity on the conditions assayed, displayed activity with an IC$_{50}$ value of 1.74 ± 0.03 mg mL^{-1}. The samples HPH10A, 20A, 30A and 45A displayed a similar inhibition value, based on the statistical analysis, whereas the HPH60A displayed lower activity compared to these, not significantly different from its original hydrolysate. In the case of the sample obtained with Flavourzyme, the value was not significantly different from its original, indicating maintenance of the activity in an adequate range compared to other sources. In some of these digested, there were differences compared to the hydrolysates, which are likely to occur because more active peptides have been released due to the action of the digestive proteases.

For instance, Tenenbaun *et al.*,[55] evaluated the antidiabetic activity of whey protein (isolate and hydrolysate) after SGID using the INFOGEST protocol. As expected, the digested samples were more hydrolyzed and as consequence, the amount of low molecular weight peptides was increased. In addition to that, after the SGID, the hydrolysate induced a greater secretion of GLP-1 in the enteroendocrine STC-1 cell line compared to the original sample. These results support the advantages of hydrolyzing proteins to obtain a more bioactive pool of peptides. Similarly, an increased in the DPP-IV inhibitory activity was reported in the duodenal digest of oat kernels, having an IC$_{50}$ value of 0.51 mg mL^{-1} compared to 10.82 mg mL^{-1} of the intact protein.[56]

This challenge of potential loss of bioactivity that the field of bioactive peptides faces during gastrointestinal digestion, due to the activity of the proteases have been investigated in order to ensure the efficacy of these protein hydrolysates as bioactive agents. Among the different possibilities to overcome this issue, the encapsulation of the protein hydrolysates appears as a promising strategy contributing not to loss functionality while improving bioaccessibility.[3]

3.4. Peptidome of digested samples and *in silico* digestion of identified peptides

The identified peptides were subjected, in addition to the *in vitro* SGID previously described, to an *in silico* digestion of the sequences identified as most bioactive from the results dis-

cussed in section 3.2. In addition, to compare with the experimental data, the peptidome of the digested samples was also analyzed (SI Table S1). The results obtained from the hydrolysis with the digestive proteases: pepsin, trypsin and chymotrypsin can be found in Table 2 (column BIOPEP-SGID), together with information on whether the sequence in each hydrolysate was found (or not) in their corresponding digested sample analysed by LC-MS (last column in Table 2). The tool is limited by the lack of amylase or bile salts, which are not included, although in the scope of peptides, only the action of proteases is of relevance.

The peptidome of the digested samples revealed that most of the identified sequences in the hydrolysates were not maintained after the digestion, since most of them were not identified in the digested sample (compared to their hydrolysate). In some cases, such as KNGMMAPH or PQNFAVVK, identified in the HPH10A and the digested HPH10A, and identified in other HPHs (30A, 45A, 60A), but not in the digested samples of these samples. The fact that a sequence is digested in a specific hydrolysate, but not in others can be attributed to differences in peptide accessibility and structural context resulting from the varying degrees of hydrolysis. Longer hydrolysis times modify the peptide profile, affecting sequence length, terminal residues, and exposure of cleavage sites to gastrointestinal enzymes. Consequently, some peptides may become more susceptible to digestion, while others are stabilized or protected within peptide aggregates or resistant motifs.

On the contrary, the sequence KNAIYTPH, found in all the Alcalase hydrolysates, was not digested, as it was also identified in all the digested samples. This reveals that this sequence is highly resistant to the activity of the digestive proteases, which makes it a suitable candidate for functional foods, since it could potentially reach the target organs.

However, it must be noted that these analyses are not accounting for the amount of peptide sequences in the sample, which is also a limitation in drawing conclusions. However, for the patterns of how the sequences are digested, and which amino acids, di or tri-peptides are released, the BIOPEP tool was used, since the mass spectrometry analyses is unable to identiy <4 aa sequences.

As it can be observed, for the *in silico* prediction, some of the fragments which are hypothetically released after the action of the proteases, have been also reported as DPP-IV inhibitory sequences, thus, would potentially exert the activity after being orally ingested. In addition, these dipeptides are likely to be absorbed through the PepT1 transporter, based on the features, and consequently, would be bioavailable as such.[57] For instance, from the peptide TNGPQLIH, the fragments IH and TN have been indicated as DPP-IV inhibitory sequences.[58] In the same line, other fragments such as GF, PL, VG or PQ have been reported as DPP-IV inhibitory peptides, for instance as aforementioned PQ as part of the fragment GPQ, already reported in other peptides as well. Nonetheless, for some of the peptides, no information on the potential DPP-IV inhibitory potential of the fragments was retrieved from the BIOPEP database. This outcome does not necessarily

TNGPQLIH and SAERGFLY in the Flavourzyme-treated hydrolysate, based on the prediction tools and the physicochemical characterization. The hydrolysates were also subjected to simulated gastrointestinal digestion *in vitro* (INFOGEST protocol) and *in silico* (BioPEP database). The inhibition of DPP-IV (IC_{50} value) ranged from 1.53 to 2.19 mg mL^{-1}, generally improved or maintained compared to the non-digested samples, while the *in silico* hydrolysis showed that some of the fragments released are also considered as DPP-IV inhibitors. The activity of the sequences TNGPQLIH and GKLDLVKPQ was confirmed using chemically synthesized peptides. The inhibition of DPP-IV was substantial for TNGPQLIH, supporting its role as a key contributor to the activity of the hydrolysate. In contrast, GKLDLVKPQ showed negligible inhibition, indicating that *in silico* predictions do not always translate into experimental activity. These results highlight the value of computational screening for guiding peptide discovery, but also the need for experimental confirmation to identify truly bioactive sequences. This is the first publication, to the authors' knowledge, in which hemp protein hydrolysates obtained with Alcalase and Flavourzyme and peptides' sequences have been characterized as capable to inhibit DPP-IV.

Author contributions

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

Conflicts of interest

The authors declare no conflict of interest.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: https://doi.org/10.1039/d5fo02421h

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