New ACE inhibitory peptides from hemp seed (*Cannabis sativa* L.) proteins

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Abstract

An hemp seed protein isolate, prepared from defatted hemp seed meals by alkaline solubilization/acid precipitation, was subjected to extensive chemical hydrolysis under acid conditions (6 M HCl). The resulting hydrolysate was fractionated by semipreparative RP-HPLC and the purified fractions were tested as inhibitors of angiotensin converting enzyme (ACE). Mono- and bi-dimensional NMR experiments and LC-MS/MS analyses led to the identification of four potential bioactive peptides, i.e. GVLY, IEE, LGV, and RVR. They were prepared by solid-phase synthesis, and tested for ACE-inhibitory activity. The IC_{50} values were GVLY 16 ± 1.5 µM, LGV 145 ± 13 µM, and RVR 526 ± 33 µM, confirming that hemp seed may be a valuable source of hypotensive peptides.

Keywords: ACE-inhibitors; bioactive peptides; *Cannabis sativa* L.; ~~chemical hydrolysis;~~ hemp seed protein hydrolysate; LC-MS/MS~~; ROESY; TOCSY~~

Introduction

Modifications of diet and lifestyle are recommended strategies for the prevention of hypertension, a main risk factor for cardiovascular disease. One of the main pathways involved in human blood pressure control is the renin-angiotensin system. Renin converts angiotensinogen to angiotensin I, while angiotensin I converting enzyme (ACE; EC 3.4.15.1) catalyses the conversion of the biologically inactive angiotensin I to the vasoconstrictor angiotensin II. ACE also inactivates the potent vasodilator bradykinin, causing an overall increase of blood pressure [1]. ACE-inhibitory peptides bind tightly to the ACE active site competing with angiotensin I for occupancy, inactivate ACE, and prevent blood pressure enhancement [1, 2].

There is now a great interest for bioactive peptides that are encrypted in food proteins and may be enzymatically released from their precursor proteins during food processing, gastrointestinal digestion or specific hydrolytic processes; they are often named as "cryptides" [3].

Cryptides showing ACE-inhibitory activity have been obtained from different sources such as animal products, marine organisms and plants, recently reviewed [3-6]. In particular vegetable proteins, such as rice, sunflower, soybean, pea, lupin, and lentil [7-13] had gained attention in this field.

Industrial hemp, the non-drug type of *Cannabis sativa* L., is a well-known plant of industrial importance, being a relevant source of fiber, food, and bioactive phytochemicals [14]. For some decades, hemp cultivation was prohibited in numerous countries due to its affinity with the Δ9-tetrahydrocannabinol (THC) rich varieties. In the last years, however, industrial hemp can be legally grown again in some countries and its global market is rapidly increasing, since low-THC cultivars are available [15].

The use of hemp seed as human food dates back to prehistory, together with the fiber utilization in textiles. The great current attention for hemp seed is related to its nutritional content: 35.5% oil, 24.8% protein, 20-30% carbohydrates, 27.6% total fiber (5.4% digestible and 22.2% non-digestible) and 5.6% ash in the whole seed [14]. Up-to-now, the main industrial interest is for the oil that has

numerous applications either in food or body care products, being rich in polyunsaturated fatty acids [16].

In parallel, there is an increasing attention for hemp seed proteins owing to their digestibility, satisfactory essential amino acid composition [17], and techno-functional properties [18]. The two main protein classes in hemp seed are the globulins and albumins. The formers are the most abundant, corresponding to 60-80% of total protein content. Edestin, the main globulin, is constituted by two main fractions Edestin 1 and 2, each characterized by several isoforms, i.e. Edestin 1A, 1B, 1C, 1D and Edestin 2A; 2B; 2C. Each isoform is composed by an acidic and a basic subunit linked by a disulphide bond [19].

Recent research, based either on *in vitro* or *in vivo* experiments, has shown that hydrolysates obtained by treating hemp seed protein with different enzymes such as pepsin, alcalase, papain, pancreatin, or other proteases showed ACE-inhibitory activity [17, 20-22]. The most promising mixtures were submitted to purification processes and structural determination of peptide composition obtaining specific bioactive peptides sequences [23]. In particular WVYT and WYT were identified as ACE inhibitors peptides in a pepsin+pancreatin hydrolysate [23].

In this context, the present investigation had the objective of identifying novel ACE-inhibitory cryptides from hemp seed protein. To achieve this goal, hemp seed protein was submitted to chemical hydrolysis under experimental conditions suitable to produce fragments containing 3-5 amino acid residues. After purification, LC-MS/MS and 1D and 2D NMR analyses were performed with the aim of identifying some ACE-inhibiting peptides, whose structures were confirmed by solid-phase synthesis of authentic samples.

Materials and methods

Chemicals and sampling

All chemicals (reagents and solvents) were from Sigma-Aldrich (St. Louis, MO, USA), if not otherwise specified. Hemp seeds (*C. sativa* L., variety Futura) were obtained from the Institute of Agricultural Biology and Biotechnology (IBBA-CNR, Milan, Italy); they were stored in an air-tight container at 4 °C in the dark until use.

Preparation of hemp seed protein isolate (HPI)

Hemp seeds were finely ground in a coffee mill, defatted by stirring under *n*-hexane at room temperature (rt) (twice, 1:4 w/v for 3 h and 1:6 w/v overnight), and then air-dried under a fume hood for 12 h. Its protein content was determined by the Kjeldahl method, using 6.25 as protein conversion factor [24]. Hemp seed protein isolate (HPI) was prepared according to a literature method [18], with some modifications. Specifically, defatted hemp seed meal (12 g) was suspended in deionized water (160 mL) at rt under stirring, and the mixture was adjusted to pH 10.0 with 2 N NaOH. After 90 min of extraction, samples were centrifuged at 8000*g* for 30 min at rt. The pellet was discarded, the supernatant was adjusted to pH 5.0 with 2 N HCl, and the precipitate was collected by centrifugation (8000*g*, 30 min). The precipitate was then resuspended in deionized water, and the resulting suspension was freeze-dried. The efficiency of protein extraction was checked by SDS-PAGE analyses that were performed on a discontinuous buffered system according to the method of Laemmli [25] using 12% separating gel and 4% stacking gel. The proteins extracted in different concentration were directly mixed with fold volume of 0.0125 mol/L tris(hydroxymethyl)aminomethane-hydrochloric acid (tris-HCl) buffer containing 1% (w/v) Sodium Dodecyl Sulphate (SDS), 2% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol and 0.025% (w/v) bromophenol blue. The samples were then heated for 5 min in boiling water before electrophoresis. Every sample (20 µL) was applied to each lane. The gel was stained with 0.25% Coomassie brilliant blue (R-250) in 50% methanol, and destained in 7% acetic acid in methanolic solution (50%, v/v). Images of SDS PAGE gels were reported in Supporting Information (S1).

Preparation and RP-HPLC separation of hemp seed protein hydrolysate (HPH)

A mixture of HPI (5 g) in 6 M HCl (25 mL) was stirred at 110 °C for 6 h. After cooling down in an ice bath, the solution was treated with 4 M NaOH and then with 1 M Na_2CO_3 under stirring until pH 5.8 was reached, followed by freeze-drying.

An aliquot of 1 mL of HPH (80 mg/mL in solvent A) was filtered through Millex-HV syringe filter, 0.45 µm (Millipore, Billerica, MA, USA) and loaded on a semipreparative RP-HPLC AKTA Basic 100 instrument (GE Healthcare Life Science, AB, Sweden), using the following chromatographic conditions: column, Jupiter® 10 µm Proteo 90 Å (250 x 10 mm) (Phenomenex, Torrence, CA, USA); flow rate, 5 mL/min; UV detector, λ 226 nm; mobile phase, 0.1% trifluoroacetic acid (TFA) in water (solvent A) and acetonitrile (MeCN)/0.1% TFA in water (8:2) (solvent B), gradient elution from 5% to 40% B in 2 column volume (CV), to 80% B in 1 CV then to 100% B in 1 CV.

Four fractions named as PHPH1 to PHPH4 were collected for each injection; corresponding fractions from different replicate chromatography runs were pooled and analyzed by analytical RP-HPLC (column, Jupiter® 10 µm Proteo 90 Å C12, 250 x 4.6 mm (Phenomenex), 250 x 4.6 mm; flow rate, 0.5 mL/min; detection and eluent, as above) to check their composition. Removal of the solvent under reduced pressure followed by freeze-drying afforded four samples that were stored at -20 °C before further analysis.

NMR analysis

NMR experiments were performed at 298 K on a Bruker Avance Spectrometer (Bruker Corporation, Billerica, MA, USA) operating at 400.10 ^{1}H frequency and equipped with a z gradient coil probe. All NMR samples were prepared with a peptide concentration of *ca*. 20 mg/mL in DMSO-d_6. Chemical shifts (δ) are given in parts per million and were referenced to the solvent signals (δ_H 2.50 and δ_C 39.50 ppm for DMSO-d_6). All 1D and 2D NMR spectra were collected using the standard pulse sequences available with Bruker Topspin 1.3. Short mixing times (200 ms)

were used in the ROESY experiments to minimize spin-diffusion effects. Proton resonances were assigned using standard methods [26].

MS analysis

An ESI-Q-Tof Micro-Waters mass spectrometer system (Waters Corporation, Milford, MA, USA) was used to perform MS analysis. Peptide samples were dissolved in a mixture of MeCN/0.1% TFA in water (8:2) at a concentration of 10 µg/mL and directly injected at 10 µL/min. Mass spectrum acquisition was performed in positive ion mode. In particular, the optimized Q-TOF operating conditions were as follows: capillary voltage, 3200 V; source temperature, 100°C; cone voltage, 45 V. The ESI gas was nitrogen, and the collision gas was argon. The time-of-flight analyzer collected data between *m/z* 100 and *m/z* 1000. The acquired MS spectrum was analyzed with MassLynx software (version 4.1, Waters).

Synthesis and characterization of identified peptides

The four identified peptides, namely GVLY, IEE, LGV, and RVR, were prepared by standard fluorenyl-9-methoxycarbonyl (Fmoc) solid-phase synthetic protocol on a Biotage Initiator + SP Wave Peptide Synthesizer (Biotage Sweden AB, Uppsala, Sweden) using a trityl chloride resin support (loading about 1.6 mmol/g). The functional groups of the amino-acid side chains were protected as follows: Glu(OtBu), Arg(Pbf), Tyr(tBu). HBTU/HOBT/DIPEA (VWR, Milan, Italy) were used as the coupling reagents. The peptides were side-chain deprotected and cleaved from the resin with a mixture of trifluoroacetic acid/phenol/H_2O/triisopropylsilane in the ratio 88:5:5:2. All crude peptides were purified by semipreparative HPLC using an AKTA Basic100 instrument (GE Healthcare Life Science, Italy) and the following chromatographic conditions: column, Jupiter® 10 µm Proteo 90 Å C12, 250 x 10 mm (Phenomenex); flow rate, 5 mL/min; detector, λ 226 nm; mobile phase, 0.1% TFA in water (solvent A) and MeCN/0.1% TFA in water (8:2) (solvent B), gradient elution from 5% to 40% B in 3 CV, to 70% B in 3 CV, then to 100% B in 2 CV min. Collected

fractions were lyophilized and their purity was shown to be >95% by analytical HPLC: column, Jupiter® 10 µm Proteo 90 Å C12, 250 x 4.6 mm (Phenomenex); flow rate, 1 mL/min; detection and eluent, as above). The peptide identity and molecular weight were confirmed by Q-Tof mass spectrometry (see above) (*m/z* 451.23 $[M+H^+]^+$, 390.16 $[M+H^+]^+$, 288.17 $[M+H^+]^+$, 430.27 $[M+H^+]^+$ for GVLY, IEE, LGV, and RVR, respectively).

ACE inhibition assay

Both mixtures and synthetic peptides were tested for their ACE-inhibitory activity as previously described [8, 27], evaluating hippuric acid (HA) formation from hippuryl-histidyl-leucine (HHL), a mimic substrate for angiotensin I [28]. Briefly, 100 µL of 2.5 mM HHL in 100 mM tris-formic acid (tris-HCOOH), 300 mM NaCl pH 8.3 (buffer 1) was mixed with 30 µL of ~~potential ACE inhibitor sample~~ peptide mixture or single peptide solution in buffer 1. In particular six different concentrations of sample were used; they were obtained by serially dilution of the most concentrate one (see Table 1) and each solution was tested twice. Samples were pre-incubated at 37 °C for 15 min, then 15 µL of ACE solution, in 100 mM tris-HCOOH, 300 nM NaCl, 10 µM $ZnCl_2$, pH 8.3, were added. Samples were incubated for 60 min at 37 °C, then the reaction was stopped with 125 µL of 0.1 M HCl. The aqueous solution was extracted twice with 600 µL of ethyl acetate; the solvent was evaporated, the residue was dissolved in 500 µL of buffer 1 and then analyzed by HPLC, in order to determine HA. HPLC analyses were performed with a HPLC 1200 Series (Agilent Technologies, Santa Clara, US) equipped with an autosampler using the following conditions: column, Lichrospher® 100 C18 (4.6 x 250 mm, 5 µm; Grace, Italy); flow rate, 0.5 mL/min; detector, λ 228 nm; mobile phase, water and MeCN, gradient elution from 5 to 60% MeCN in 10 min and 60% MeCN for 2 min, then back to 5% MeCN in 3 min; injection volume, 10 µL; R_t (HA), 4.2 min.

The evaluation of the inhibition of ACE activity was based on the comparison between the concentrations of HA in the presence or absence of the inhibitor (Inhibitor Blank). The phenomenon

of autolysis of HHL to give HA was evaluated by a reaction blank, *i.e.* a sample with the higher inhibitor concentration but without the enzyme. The percentage of ACE inhibition was computed considering the area of HA peak with the following formula:

$$\text{ACE-Inhibition}\,(\%) = \frac{A_{IB} - A_N}{A_{IB} - A_{RB}} \times 100$$

where A_{IB} is the area of HA in Inhibitor Blank (IB) sample (*i.e.* sample with enzyme but without inhibitor), A_N is the area of HA in the samples containing different inhibitor amounts and A_{RB} is the area of HA in the Reaction Blank (RB) sample (i.e. sample without enzyme and with inhibitor in the highest concentration). The percentages of ACE inhibition were plotted *vs.* Log_{10} inhibitor concentrations obtaining a sigmoid curve; IC_{50} was the inhibitor concentration needed to observe a 50% inhibition of the ACE activity and is expressed as mean value ± standard deviation of three independent assays.

Statistical analysis

Statistical analyses were performed with StatGraphics Plus (version 2.1 for Windows). The data were evaluated using one-way analysis of variance followed by Fisher's Least Significant Difference procedure; values with different letters are significantly different for $p < 0.05$.

Results

Preparation of HPI

Hemp seeds were ground and defatted obtaining a defatted meal with 65% w/w yield and a protein content of 35.8%. HPI was separated in 34% yield from defatted hemp seed meal by basic extraction followed by acidic precipitation. The obtained HPI had 94% of protein content.

Preparation and fractionation of HPH and evaluation of ACE-inhibitory activity

The hydrolysis of HPI was performed treating with 6 M HCl for 6 h at 110 °C monitoring the progress of hydrolysis by HPLC analysis. Hydrolysis was performed several times and HPLC chromatograms of obtained mixtures were characterized always by the same peaks; in Figure 1A an HPLC chromatogram as an example is reported.

Fractionation of raw HPH by semi-preparative RP-HPLC enabled the separation of four fractions named as PHPH1-PHPH4 having the retention times (R_t) 2-5 min, 5-7 min, 11-13 min and 14-19 min, respectively (Figure 1A), being PHPH3 the most abundant one (Figure1B).

Both HPH and the four collected fractions were tested for ACE-inhibitory activity. In Table 1 ACE inhibitory activity was reported as the average ± standard deviation of three different assays.

~~by an *in vitro* assay based on hippuryl-histidyl-leucine (HHL), as model peptide [28], and HPLC-DAD, as analytical method [8, 27].~~ HPH achieved a 44.8% ACE-inhibitory activity at 1.1 mg/mL. After fractionation procedure, PHPH3 showed an 84.9% ACE-inhibitory activity at 1.0 mg/mL, with an IC_{50} value of 180.1 ± 3 µg/mL, whereas all other fractions, i.e. PHPH1, PHPH2 and PHPH4, do not achieve 50% of ACE inhibition even at the highest tested concentration.

Identification of peptides in PHPH3 fraction

Fraction PHPH3 was submitted to NMR and MS analyses both suggesting the sample was not a pure peptide, but a mixture consisting of different components.

One- and two-dimensional ^{1}H NMR spectra (COSY, TOCSY, NOESY, and ROESY) enabled to identify the spin systems of Glycine (G), Valine (V), Leucine (L), Tyrosine (Y), Glutamic acid (E), Arginine (R), and Isoleucine (I) and to make proton resonance assignment of the individual residues [26] (see Supporting Information, S2). In addition, a number of sequential $H_{\alpha i}$-H_{Ni+1} connectivities together with cross peaks due to NOEs involving side chain protons observed in the ROESY spectrum (Supporting Information, S3) were indicative of the presence of the following couples of consecutive amino acid residues: GV, another GV, different from the previous one, LY, RV, VR, and IE (Table 2).

These data along with the *m/z* values in ESI-MS spectrum and of the fragment ions in MS/MS experiments suggested the amino acid sequences reported in Table 3 as potential peptides constituting the PHPH3 fraction. In Figure 2A the ESI-MS/MS spectrum of ion *m/z* 451.1 (GVLY) and in Figure 2B the ESI-MS spectrum of PHPH3 were reported.

Convincing evidence in favor of GVLY, LGV, RVR and IEE came from inspection of the sequence of edestin, the main protein in hemp seed, accounting for about 60-80% of the total protein content [19], in which such fragments (or the alternative ones IQQ, IEQ, and IQE, Table 3) are contained. The four peptides were synthesized using Fmoc chemistry, purified and their spectral data, in particular ESI-MS and MS/MS spectra, compared with those of PHPH3 fraction.

Evaluation of the ACE-inhibitory activity of synthetic peptides

The synthetic peptides were then screened for their ACE-inhibitory activity. Figure 3, showing the ACE inhibition curves of the four peptides, clearly indicates that IEE was almost inactive, since it was able to inhibit ACE only by 20.5% even at the highest tested concentration. The activity of the other peptides, instead, enables the calculation of the IC_{50} values, which are reported in Table 1 together with the highest tested concentrations (µg/mL) and the percentages of ACE inhibition at these concentrations. GVLY was the most active peptide with an IC_{50} value equal to 16 ± 1.5 µM, LGV was the second with an IC_{50} equal to 145 ± 13 µM, and RVR the third, with an IC_{50} equal to 526 ± 33 µM. These values are significantly different for $p < 0.05$ (Table 1).

Discussion

Whereas the production of ACE-inhibitory peptides from proteins is usually performed by proteolytic enzymes, opting for a different approach, in this investigation we performed a drastic acidic hydrolysis of hemp seed proteins. The main reason for this choice was that a small molecular weight favors the stability of peptides towards stomach proteases and an efficient absorption at intestinal level [3, 29]. Moreover ACE-inhibitors peptides are generally short chained [9]. As expected, a

complex mixture of low molecular weight peptides was produced, from which four single peptides, i.e. GVLY, LGV, RVR and IEE, were ~~partially purified and~~ identified. As for our knowledge, these peptides were never cited before in literature as ACE-inhibitors or bioactive peptides. Searching in different antihypertensive peptides databases, such as ACE-pepDB, BIOPEP and EROP-Moscow database, no matches were found (last search on 19th September 2017).

These four peptides derive from edestin hydrolysis. The LGV sequence is present in the acidic subunit of edestin 1 (A, B, C, D); RVR is part of the acidic subunit of edestin 2 (A, B, C); GVLY is part of the acidic unit of both edestin 1 (A, B, C, D) and edestin 2 (A, B, C) [19]. The last sequence has been previously reported as part of the ACE-inhibitory peptide whose sequence is AAKGVLY, deriving from an *in silico* hydrolysis of a 11S globulin from *Amaranthus hypochondriacus* seed [13].

A recent paper [23] has investigated the ACE-inhibitory activity of different peptides purified by preparative HPLC from a pepsin + pancreatin hydrolysate from hemp seed protein. The most active ones were WYT (IC_{50} = 574 µM) and WVYY (27 µM). Although the structures are quite different, the range of activities are in very good agreement with the results obtained for RVR and GVLY, respectively.

Biological activity of peptides is related to chain length, amino acid composition and sequence [9]. Some literature evidence indicate that the most effective ACE-inhibitory peptides identified up-to-now contain 2-20 amino acids, must have a good hydrophobicity/hydrophilicity balance and some particular structural characteristic related to its sequence [4, 9].

Hydrophobic amino acids can improve the solubility of peptide in lipid based conditions such as in cell membrane, enabling to exert a greater antihypertensive effect [4, 30, 31], enhancing their interaction with target organs through hydrophobic associations with cell membrane lipid bilayer, needful to exert different biological activity.

One parameter related to peptide solubility is log P value, i.e. the logarithm (base 10) of the partition coefficient (P) defined as the ratio of the solubility of peptide organic-to- aqueous phase concentration. This parameter can be either measured experimentally or calculated *in silico*. Log P

calculation performed with ACD/ChemSketch© (Freeware 2016 2.2 Version) enable to obtain positive values for GVLY (+1.78) and LGV (+0.94), assessing the hydrophobic character of this peptides.

On the contrary RVR and IEE, that are less active, have an hydrophobic character being Log P -2.34 and -1.29 respectively. Obviously this is only one of requested features for a peptide to be an ACE inhibitor: other features may concur.

Also already known ACE-inhibitors peptides [23] exert positive values: +3.54 for WVYY and +1.34 for WYT, respectively, in agreement with Authors' statement that their ACE-inhibitors peptides contain about 80% hydrophobic amino acids.

Moreover experimental evidences show that residues with cyclic or aromatic rings, such as Tyrosine, Phenylalanine, Tryptophan, and Proline, at the C-terminal and hydrophobic amino acids, especially those with aliphatic chains such as Glycine, Isoleucine, Leucine, and Valine, at the N-terminal, are typical of ACE-inhibitor peptides [5, 9, 29].

Two out of these features are present in GVLY, the most active peptide (Y at the C-terminal and G at the N-terminal) and one in LGV, which is only moderately active (L in the N-terminal).

Other structure-activity data suggests that the positive charge of Lysine and Arginine as the C-terminal residue may contribute to the inhibitory activity [29]. Regarding RVR, Arginine at the C-terminal seems to give only a low contribute to ACE inhibitory activity, being RVR the least active peptide.

It is also important to underline that all active peptides isolated in this work contain a residue of Valine, a hydrophobic branched chain amino acid reported to have high affinity for the active site of ACE [23].

In conclusion, NMR and mass spectrometry enabled the identification of three peptides with interesting ACE-inhibitory activity, confirming that hemp seed may be a valuable source of hypotensive peptides. Of course, this is only a first step, since other studies are necessary in order to establish their stability, i.e. the capability to survive to the gastrointestinal digestion, as well as their

bioavailability, i.e. the capacity to reach the target organ in the active form. Moreover, we have evaluated their ability to inhibit the ACE activity *in vitro*; the hypotensive activity should be also assessed by *in vitro* tests on cell model systems and, afterwards, *in vivo* by using suitable animal models, such as the spontaneously hypertensive rats. ~~(SHR).~~ Nevertheless, our experimental results may be very useful also for encouraging structure-activity studies that may provide templates for the development of future peptidomimetics.

Abbreviations

ACE, angiotensin I converting enzyme; COSY, correlation spectroscopy; DIPEA, *N*-ethyldiisopropylamine; Fmoc, fluorenyl-9-methoxycarbonyl; HA, hippuric acid; HBTU, *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate; HCOOH, formic acid; HHL, hippuryl-histidyl-leucine; HOBT, 1-hydroxybenzotriazole hydrate; HPH, hemp protein hydrolysate; HPI, hemp protein isolate; MeCN, acetonitrile; NOESY, nuclear Overhauser spectroscopy; PHPH, purified hemp protein hydrolysate; ROESY, rotating-frame overhauser spectroscopy; SDS-PAGE, sodium dodecyl sulphate-polycrylamide gel electrophoresis; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy; Tris, tris(hydroxymethyl)aminomethane.

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Supporting Information

S1 (Supporting 1). SDS PAGE gel of HPI.

S2 (Supporting 2). ^{1}H chemical shifts for the amino acid residues identified in PHPH3 sample (ppm, DMSO-*d6*) at 298 K.

S3 (Supporting 3). Finger print region of ROESY spectrum of PHPH3 sample (DMSO-*d6*) at 298 K.

References da sistemare alla fine

1. Skeggs, L. T., Jr.; Kahn, J. R.; Shumway, N. P., Preparation and function of the hypertensin-converting enzyme. *J Exp Med* **1956,** *103*, 295-9.
2. Yang, H.-Y. T.; Erdos, E. G.; Levin, Y., Dipeptidyl carboxypeptidase that converts angiotensin I and inactivates bradykinin. *Biochim Biophys Acta Protein Struct* **1970,** *214*, 374-376.
3. Dhaval, A.; Yadav, N.; Purwar, S., Potential Applications of Food Derived Bioactive Peptides in Management of Health. *Int. J. Pept. Res. Ther.* **2016,** *22* (3), 377-398.
4. Lee, S. Y.; Hur, S. J., Antihypertensive peptides from animal products, marine organisms, and plants. *Food Chem.* **2017,** *228*, 506-517.
5. Iwaniak, A.; Minkiewicz, P.; Darewicz, M., Food-Originating ACE Inhibitors, Including Antihypertensive Peptides, as Preventive Food Components in Blood Pressure Reduction. *Compr. Rev. Food Sci. Food Saf.* **2014,** *13* (2), 114-134.
6. Rudolph, S.; Lunow, D.; Kaiser, S.; Henle, T., Identification and quantification of ACE-inhibiting peptides in enzymatic hydrolysates of plant proteins. *Food Chem.* **2017,** *224*, 19-25.
7. Wu, J.; Ding, X., Characterization of inhibition and stability of soy-protein-derived angiotensin I-converting enzyme inhibitory peptides. *Food Res Int* **2002,** *35*, 367-375.
8. Boschin, G.; Scigliuolo, G. M.; Resta, D.; Arnoldi, A., ACE-inhibitory activity of enzymatic protein hydrolysates from lupin and other legumes. *Food Chemistry* **2014,** *145*, 34-40.
9. Daskaya-Dikmen, C.; Yucetepe, A.; Karbancioglu-Guler, F.; Ozcelik, B.; Daskaya, H., Angiotensin-I-Converting Enzyme (ACE)-Inhibitory Peptides from Plants. *Nutrients* **2017,** *9* (4).
10. Guang, C.; Phillips, R. D., Plant Food-Derived Angiotensin I Converting Enzyme Inhibitory Peptides. *J Agric Food Chem* **2009,** *57* (12), 5113-5120.
11. Li, H.; Aluko, R. E., Identification and Inhibitory Properties of Multifunctional Peptides from Pea Protein Hydrolysate. *J Agric Food Chem* **2010**, 11471-11476.
12. Roy, F.; Boye, J. I.; Simpson, B. K., Bioactive proteins and peptides in pulse crops: Pea, chickpea and lentil. *Food Res Int* **2010,** *43*, 432-442.
13. Montoya-Rodriguez, A.; Gomez-Favela, M. A.; Reyes-Moreno, C.; Milan-Carrillo, J.; Gonzalez de Mejia, E., Identification of Bioactive Peptide Sequences from Amaranth (Amaranthus hypochondriacus) Seed Proteins and Their Potential Role in the Prevention of Chronic Diseases. *Compr. Rev. Food Sci. Food Saf.* **2015,** *14* (2), 139-158.
14. Callaway, J. C., Hempseed as a nutritional resource: An overview. *Euphytica* **2004,** *140* (1), 65-72.
15. Oomah, B. D.; Busson, M.; Godfrey, D. V.; Drover, J. C. G., Characteristics of hemp (Cannabis sativa L.) seed oil. *Food Chem.* **2001,** *76* (1), 33-43.
16. Torres-Salas, P.; Pedrali, A.; Bavaro, T.; Ambrosini, S.; Marrubini, G.; Pappalardo, V. M.; Massolini, G.; Terreni, M.; Ubiali, D., Preparation of PUFA concentrates as acylglycerols via enzymatic hydrolysis of hempseed oil (Cannabis sativa L.) in a homogeneous low-water medium. *Eur. J. Lipid Sci. Technol.* **2014,** *116* (11), 1496-1504.
17. Malomo, S. A.; He, R.; Aluko, R. E., Structural and Functional Properties of Hemp Seed Protein Products. *J. Food Sci.* **2014,** *79* (8), C1512-C1521.
18. Tang, C.-H.; Ten, Z.; Wang, X.-S.; Yang, X.-Q., Physicochemical and Functional Properties of Hemp (Cannabis sativa L.) Protein Isolate. *J. Agric. Food Chem.* **2006,** *54* (23), 8945-8950.

19. Docimo, T.; Caruso, I.; Ponzoni, E.; Mattana, M.; Galasso, I., Molecular characterization of edestin gene family in Cannabis sativa L. *Plant Physiol. Biochem. (Issy-les-Moulineaux, Fr.)* **2014,** *84*, 142-148.
20. Girgih, A. T.; Udenigwe, C. C.; Li, H.; Adebiyi, A. P.; Aluko, R. E., Kinetics of Enzyme Inhibition and Antihypertensive Effects of Hemp Seed (Cannabis sativa L.) Protein Hydrolysates. *J. Am. Oil Chem. Soc.* **2011,** *88* (11), 1767-1774.
21. Teh, S.-S.; Bekhit, A. E.-D. A.; Carne, A.; Birch, J., Antioxidant and ACE-inhibitory activities of hemp (Cannabis sativa L.) protein hydrolysates produced by the proteases AFP, HT, Pro-G, actinidin and zingibain. *Food Chem.* **2016,** *203*, 199-206.
22. Malomo, S. A.; Onuh, J. O.; Girgih, A. T.; Aluko, R. E., Structural and antihypertensive properties of enzymatic hemp seed protein hydrolysates. *Nutrients* **2015,** *7* (9), 7616-7632.
23. Girgih, A. T.; He, R.; Malomo, S.; Offengenden, M.; Wu, J.; Aluko, R. E., Structural and functional characterization of hemp seed (Cannabis sativa L.) protein-derived antioxidant and antihypertensive peptides. *J. Funct. Foods* **2014,** *6*, 384-394.
24. AOAC, Official Methods of Analysis. 15 th ed.15th ed.; Association of Official Analytical Chemists: Arlington, VA, 1990.
25. Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London, U. K.)* **1970,** *227* (5259), 680-685.
26. Wuthrich, K., *NMR of Proteins and Nucleic Acids*. John Wiley and Sons: 1986.
27. Boschin, G.; Scigliuolo, G. M.; Resta, D.; Arnoldi, A., Optimization of the Enzymatic Hydrolysis of Lupin (Lupinus) Proteins for Producing ACE-Inhibitory Peptides. *Journal of Agricultural and Food Chemistry* **2014,** *62* (8), 1846-1851.
28. Cushman, D. W.; Cheung, H. S., Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem Pharmacol* **1971,** *20*, 1637-48.
29. Pihlanto, A.; Makinen, S. In *Antihypertensive properties of plant protein derived peptides*, InTech: 2013; pp 145-182.
30. Kobayashi, Y.; Yamauchi, T.; Katsuda, T.; Yamaji, H.; Katoh, S., Angiotensin-I converting enzyme (ACE) inhibitory mechanism of tripeptides containing aromatic residues. *J. Biosci. Bioeng.* **2008,** *106* (3), 310-312.
31. Martinez-Maqueda, D.; Miralles, B.; Recio, I.; Hernandez-Ledesma, B., Antihypertensive peptides from food proteins: a review. *Food Funct.* **2012,** *3* (4), 350-361.

Founding sources

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Figure captions

Figure 1. HPLC chromatograms of A) HPH; B) PHPH3 fraction, after purification.

Figure 2. A) ESI-MS/MS spectrum of ion *m/z* 451.1, for clarity, only b, y and a ions are labeled; B) ESI-MS spectrum of PHPH3. By manual calculation, the sequence of GVLY is displayed with the fragment ions observed in the spectrum.

Figure 3. Diagram reporting % ACE inhibition *vs.* concentration (µmol/mL) for the four synthetic peptides GVLY (♦), IEE (■), LGV (▲), RVR (●).

Table 1. Highest tested concentration (µg/mL), highest ACE inhibition percentage and IC_{50} value for HPH, fractions PHPH1-PHPH4, and the four synthetic peptides. Values are reported as mean value ± standard deviation of three independent experiments.

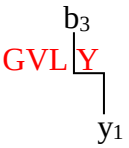
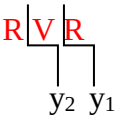
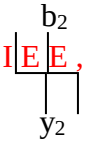
Sample	Max conc. (µg/mL)	Max ACE inhibition (%)	IC_{50} (µg/mL)	IC_{50} (µM) [a]
HPH	1118	44.8 ± 3.0	/	/
PHPH1	1035	14.5 ± 4.0	/	/
PHPH2	1008	26.4 ± 3.4	/	/
PHPH3	1036	84.9 ± 4.2	180 ± 3.1	/
PHPH4	990	29.4 ± 2.8	/	/
GVLY	862	95.1 ± 0.88	7.27 ± 0.7	16 ± 1.5a
IEE	787	20.5 ± 2.31	/	/
LGV	1000	95.2 ± 0.85	41.5 ± 3.8	145 ± 13b
RVR	1077	93.3 ± 1.78	226 ± 14	526 ± 33c

[a] values with different letters are significantly different ($p < 0.05$).

Table 2. Sequential ROEs correlations observed for PHPH3 sample.

Peptide	ROEs correlations
GV	NH (V) at 8.39 ppm → α (G) at 3.75 ppm
GV	NH (V) at 8.47 ppm → α1,α2 (G) at 3.93, 3.79 ppm
LY	NH (Y) at 8.66 ppm → α,β,γ (L) at 4.32, 1.54, 0.92 ppm
RV	NH (V) at 8.39 ppm → γ,δ (R) at 1.55, 3.09 ppm
VR	NH (R) at 8.33 ppm → β (V) at 2.00 ppm
IE	NH (E) at 8.65 ppm → β,γ,δ (I) at 1.82, 1.27-1.04 (CH_3), 0.93 ppm

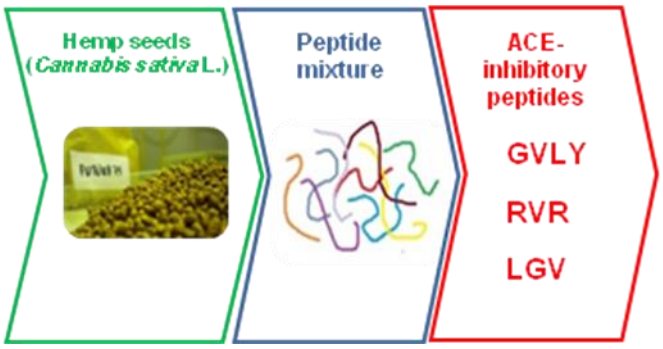
Table 3. Potential peptides identified in fraction PHPH3: observed mass $[M+H]^+$ (*m/z*), charge (*z*), calculated mass (*m/z*), MS/MS fragmentation and suggested sequence.

Obs. mass (*m/z*)	***z***	**Calc. mass (*m/z*)**	**MS/MS fragmentation (*m/z*)**	**Suggested sequence**
451.1	1	450.6	270.3 (b_3), 182.1 (y_1)	
288.1	1	287.1	175.08 (y_2), 171.09 (b_2)	 , IGV*
430.2	1	411.2	274.16 (y_2), 175.1 (y_1)	
390.1	1	389.1	277.03 (y_2), 243.05 (b_2)	 , IQQ**, IEQ**, IQE*

* fragments not present in the edestin sequence [19]

** fragments present in the edestin sequence [19], but not detectable due to the chemical hydrolysis of Glutamine in Glutamic acid.

TOC graphic


Hemp seeds
(Cannabis sativa L.)
Peptide
mixture
ACE-
inhibitory
peptides
GVLY
RVR
LGV