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Purification of an ACE Inhibitory Peptide after Hydrolysis of Sunflower (*Helianthus annuus* L.) Protein Isolates

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Sunflower protein isolates and the proteases pepsin and pancreatin were used for the production of protein hydrolysates that inhibit angiotensin-I converting enzyme (ACE). Hydrolysates obtained after 3 h of incubation with pepsin and 3 h with pancreatin were studied. An ACE inhibitory peptide with the sequence Phe-Val-Asn-Pro-Gln-Ala-Gly-Ser was obtained by G-50 gel filtration chromatography and high-performance liquid chromatography C_{18} reverse phase chromatography. This peptide corresponds to a fragment of helianthinin, the 11S globulin from sunflower seeds, which is the main storage protein in sunflower. These results show that sunflower seed proteins are a potential source of ACE inhibitory peptides when hydrolyzed with pepsin and pancreatin.

KEYWORDS: Sunflower protein hydrolysate; ACE inhibitors; bioactive peptides; *Helianthus annuus* L.

INTRODUCTION

In recent years food has been considered not only a source of energy and basic components for the maintenance and growth of the body but also a source of bioactive compounds that may exert beneficial effects in humans. The presence of bioactive compounds in food is even more important for cells of the digestive system, because it is directly exposed to food and the products of its digestion. Bioactive peptides are among the many functional components identified in foods in recent years (*1*). These are small protein fragments that have biological effects once they are released during gastrointestinal digestion in the organism or by previous in vitro protein hydrolysis. For example, biopeptides with antihypertensive, immunomodulatory, opioid, antioxidant, or hypocholesterolemic activity have been described (*2*, *3*). Some of these peptides possess several bioactivities, and peptides with two or more activities have been purified (*4*).

Plant protein isolates and hydrolysates, especially soy isolates, are used in the elaboration of infant formulas, meat, soups, and bakery and dairy products (*5*). Sunflower (*Helianthus annuus* L.) constitutes an interesting raw material for the preparation of protein isolates and hydrolysates. It is one of the most important oilseed crops worldwide, representing the fifth largest source of edible oil (*6*). Defatted sunflower meal contains about 30% protein, which could potentially be used as a food ingredient instead of being wasted or being used for animal feed. Sunflower protein isolates have been used as substrates for the production of hydrolysates with better functional or nutritional properties than the original isolate (*7*). Sunflower protein hydrolysates may also represent a source of bioactive peptides, enhancing the value of the hydrolysates and the overall value of the starting material, i.e., protein isolates and defatted sunflower meal.

Among bioactive peptides, those with antihypertensive effects have been the most extensively studied (*8*−*10*). These peptides exert their antihypertensive activity by inhibition of the angiotensin-I converting enzyme (ACE). ACE activity leads to an increase in blood pressure by producing the vasoconstrictor peptide angiotensin II and by degrading the vasodilator peptide bradyquinin. Antihypertensive peptides with ACE inhibitory activity have been purified from animal (*11*, *12*) and plant proteins (*13*, *14*). Many antihypertensive peptides have been purified from milk (*15*, *16*) and derived products such as cheese (*17*), whey (*18*), or skimmed milk (*19*). Most peptides have been generated with microbial proteases, such as those involved in milk processing. In some cases, the release of antihypertensive peptides in in vitro models simulating gastrointestinal digestion has been studied (*20*, *21*). The hypotensive activity of ACE inhibitory peptides has also been demonstrated in vivo in spontaneously hypertensive rats (*22*) and human volunteers (*23*).

The in vitro digestion of plant proteins with pepsin and pancreatin may result in the production of bioactive peptides that could be used in the elaboration of foods. Because these enzymes are involved in gastrointestinal digestion in man, the results obtained will also provide information on the generation of bioactive peptides during the physiological digestion of these or similar proteins.

The production of ACE inhibitory peptides during digestion of sunflower protein isolates with pepsin plus pancreatin is described in the present paper. We show that an underutilized byproduct such as defatted sunflower meal may be useful in human food nutrition as a source of bioactive peptides with antihypertensive properties.

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MATERIALS AND METHODS

Materials. Hippuryl-L-histidyl-L-leucine (HHL), captopril, 2,4,6-trichloro-s-triazine (TT), trinitrobenzenesulfonic acid (TNBS), phenylmethylsulfonyl fluoride (PMSF), gastric porcine pepsin, and porcine pancreatin were purchased from Sigma. All other chemicals were of analytical grade. Defatted sunflower meal was supplied by MIGASA (Sevilla, Spain).

Preparation of ACE. ACE was prepared according to Hayakari et al. (*24*) with modifications. Porcine lungs purchased in a local market were used as the starting material. Tissue samples were diced and homogenized in 10 volumes of ice-cold 10 mM potassium phosphate buffer, pH 8.3, containing 100 μM pepstatin and 0.1 mM PMSF. The homogenate was centrifuged at 5000*g* for 10 min, and the resulting supernatant was used as the source of ACE. This supernatant possessed a specific activity around 20 U/mg protein.

To verify the absence of undesirable proteases such as carboxypeptidase in ACE extracts, the kinetics of HHL hydrolysis by ACE was followed in the presence (0.5 μM) or absence of captopril, a potent ACE inhibitor. The results indicated that the hydrolysis was due to ACE because hydrolysis of HHL in the presence of captopril is residual and similar to the blank (data not shown).

Assay of ACE. ACE activity was determined according to Hayakari et al. (*24*) with modifications. ACE hydrolyzes HHL to yield hippuric acid and His-Leu. This method relies on the colorimetric reaction of hippuric acid with TT, developed in a 0.5 mL incubation mixture containing 40 μmol of potassium phosphate buffer, pH 8.3, 300 μmol of sodium chloride, 1.5 μmol of HHL, 1 μg of enzyme extract, and different amounts of hydrolysate extracts. Incubation was carried out for 15 min at 37 °C. The reaction was terminated by addition of 1.5 mL of 3% TT in dioxane, followed by 3 mL of 0.2 M phosphate buffer, pH 8.3. After the reaction mixtures were centrifuged at 10 000*g* for 10 min, enzymatic activity was determined in the supernatant. The absorbance was read at 382 nm. The IC_{50} value was defined as the concentration of peptide in μg protein/mL required to produce 50% inhibition of ACE in the described conditions and was determined by regression analysis of ACE inhibition (%) vs peptide concentration.

Hydrolysis. Protein isolates, prepared as previously described (*25*), were hydrolyzed by sequential treatment with pepsin and pancreatin using a hydrolysis reactor. A digestion with pepsin for 180 min was followed by incubation with pancreatin for another 180 min. Hydrolysis parameters were used as follows: protein isolate concentration, 10% (w/v); enzyme/substrate ratio, 1:20 (w:w); pH 2 for pepsin and 7.5 for pancreatin; temperature, 37 °C. Hydrolysis was conducted in a 1000 mL reaction vessel equipped with a stirrer, thermometer, and pH electrode. Hydrolysis was stopped by heating at 80 °C for 20 min. Hydrolysates were clarified by ultrafiltration through 0.45 μm filters (Millipore, Bedford, MA) to remove insoluble substrate and lyophilized.

Degree of Hydrolysis. The degree of hydrolysis was calculated by determination of free amino groups by reaction with TNBS (*26*). The total number of amino groups was determined in a sample of protein isolate hydrolyzed by incubation with 6 N HCl at 120 °C for 24 h.

G-50 Gel Filtration Chromatography. After filtration through 5 kDa membranes (Millipore) in an Amicon cell system, 1 mL of sunflower protein hydrolysates (10 mg/mL) was injected into a Sephadex G-50 gel filtration column (2 cm × 55 cm) at a flow rate of 10 mL/h 50 mM ammonium bicarbonate, pH 9.1. Fractions selected for further purification of peptides were pooled and lyophilized before high-performance liquid chromatography (HPLC) C_{18}. Peptide molecular masses were determined by reference to a calibration curve created by running molecular mass markers on the Sephadex G-50 under identical running conditions to test samples. Molecular mass standards were cytochrome C (12 384 Da), bacitracin (1400 Da), Val^4-angiotensin (917 Da), Arg-Lys-Glu-Val-Tyr (693 Da), and Trp-Gly (261 Da). Protein elution was monitored at 280 nm.

HPLC C_{18} Chromatography. Fractions collected from Sephadex G-50 column were redissolved in deionized water and injected in a preparative HPLC reverse phase column (C_{18} Hi-Pore RP-318, 250 mm × 10 mm BIO-RAD column). The injection volume was 100 μL, and the sample concentration was 20 mg/mL. Elution was achieved by a linear gradient of acetonitrile in water (0−30% in 50 min) containing 0.1% trifluoroacetic acid at a flow rate of 4 mL/min at 30° C. Elution was monitored at 215 nm, and five fractions were collected for assay of ACE inhibitory activity. One of these fractions (fraction V) was injected in an analytical HPLC reverse phase column (C_{18} Hi-Pore RP-318, 250 mm × 4.6 mm BIO-RAD column) for the analysis of ACE inhibitory activity in individual peaks. The injection volume was 50 μL, and the sample concentration was 2 mg/mL. Elution was achieved by a linear gradient of acetonitrile in water (10−30% in 50 min) containing 0.1% trifluoroacétic acid at a flow rate of 1 mL/min at 30 °C. Elution was monitored at 215 nm.

Electrophoresis. Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) was performed following the method of Schaggerr and von Jagow (*27*) with slight modifications. The separating gel consisted of 20% T, 6% C gel where the composition of the acrylamide mixtures was defined by the letters T (total percentage concentration of acrylamide and bisacrylamide) and C (percentage concentration of the cross-linker relative to the total concentration T) according to Hjerten (*28*). The stacking gel consisted of a 4% T, 3% C gel. The lengths of the separating and stacking gels were 6 and 2 cm, respectively, with a gel thickness of 1 mm. Electrophoresis was performed at a constant voltage of 60 V for stacking and 120 V for separation. Protein bands were fixed in a solution containing 20% methanol and 8% acetic acid for 15 min before they were stained with 0.25% coomassie brilliant blue G in 45% methanol and 10% acetic acid for 24 h. Destaining of the gel was performed in 10% acetic acid.

Peptide Sequencing. The sequence of the purified peptides was identified by sequence analysis with a Perkin-Elmer/Applied Biosystems Procise 494 microsequencer (Überlingen, Germany) running in pulsed liquid mode.

RESULTS AND DISCUSSION

Hydrolysis of Sunflower Protein Isolates with Pepsin and Pancreatin. Pepsin and pancreatin were used to obtain an extensively hydrolyzed sunflower protein hydrolysate. Pepsin is the main proteolytic enzyme generated in the stomach during food digestion. Pancreatin includes proteases, such as trypsin, chymotrypsin, and elastase that are released by the pancreas in the small bowel. Thus, the final sunflower protein hydrolysate obtained by hydrolysis using pepsin and pancreatin represents a pool of peptides resembling those generated during the digestion of sunflower proteins in the organism. These peptides, being resistant to pepsin and pancreatin, could be absorbed by digestive epithelial cells along the small intestine. **Figure 1** shows the kinetics of the hydrolysis of sunflower protein isolate by pepsin and pancreatin and the ACE inhibitory activity at different times of the hydrolysis. The rate of hydrolysis with pepsin is very high in the first 10 min, and after that hydrolysis proceeds more slowly. On the other hand, pancreatin activity is observed even after 3 h of incubation with the protease. Most likely, the sequential action of the different proteases included in the pancreatin extract extends the hydrolysis over a longer time.

A dramatic increase in the generation of ACE inhibitory peptides is observed at the beginning of the hydrolysis with pepsin. These initial batches of peptides are probably hydrolyzed as the reaction progresses, since ACE inhibitory activity decreases very quickly after that, but from 45 to 180 min, the ACE inhibitory activity increased again up to 45%. Digestion with pancreatin also leads to a maximum of ACE inhibition at the beginning of the hydrolysis.

Unlike pepsin, pancreatin acts in the small intestine, so that the peptides produced could be readily absorbed by the organism. The activity of ACE was inhibited around 25% inhibition during the digestion with pancreatin. However, the degree of hydrolysis increased progressively reaching 37% at the end of the hydrolysis. This degree of hydrolysis would correspond to a pool of peptides containing to two or three amino acids on average. Probably, a steady state between the generation and the degradation of inhibitory peptides was maintained during hydrolysis.

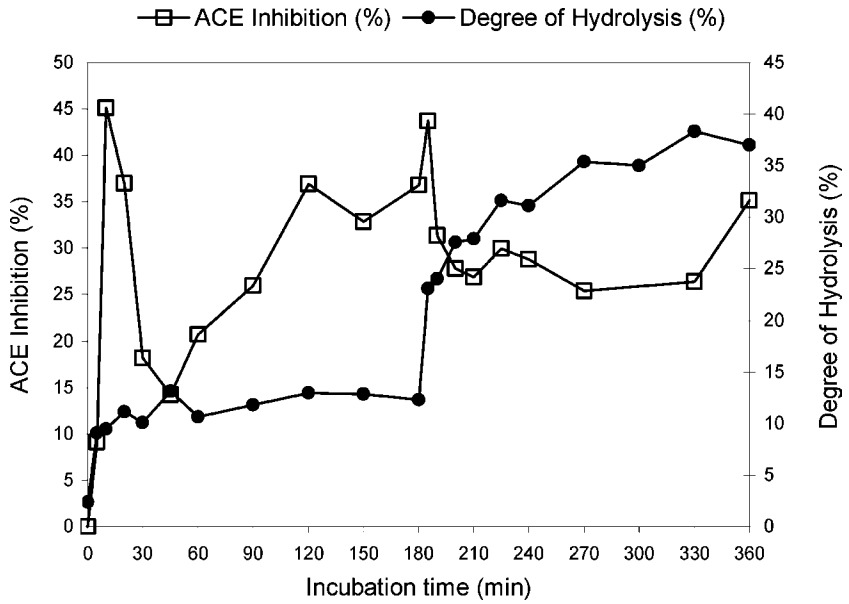

Figure 1. Enzymatic hydrolysis of sunflower protein isolate with pepsin (added at time 0) and pancreatin (added at 180 min) and percentage inhibition of ACE by the resulting hydrolysates. Ten micrograms of protein hydrolysate was used for the inhibition assay.

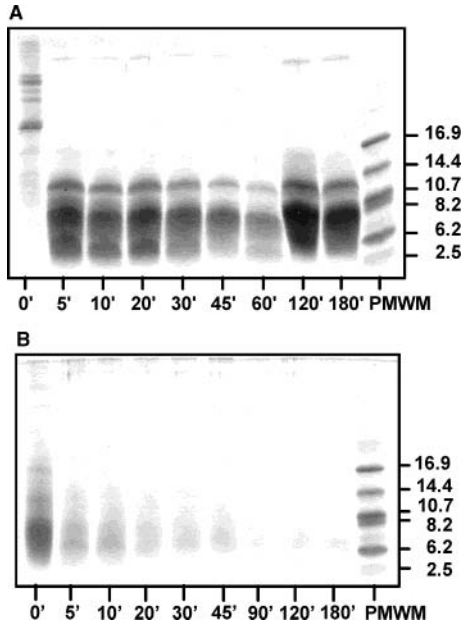

Figure 2. (**A**) SDS–PAGE (20% T, 6% C) profile of sunflower protein isolate (0 min) and sunflower protein hydrolysates obtained by treatment with pepsin for up to 180 min. PMWM, peptides molecular weight markers. (**B**) SDS–PAGE profile of sunflower protein hydrolysates obtained with pancreatin for up to 180 min. PMWM, peptides molecular weight markers.

SDS−PAGE of the pepsin hydrolysates (**Figure 2A**) showed that most bands corresponding to sunflower proteins had disappeared after 5 min of hydrolysis. This was in accordance with the increase in the degree of hydrolysis observed in the initial minutes of the incubation with pepsin. This profile was maintained for the duration of hydrolysis with bands of molecular masses 10.5 and 7.6 kDa resistant to pepsin activity observed at the end of the incubation. However, hydrolysis with pancreatin (**Figure 2B**) resulted in the disappearance of these bands yielding a profile with a smear of lower molecular mass peptides. As the incubation with pancreatin progressed, the staining became more faint due to the difficulty to stain small peptides with coomassie blue.

The final hydrolysate obtained after an extensive incubation with pepsin and pancreatin consisted of small peptides that might be readily absorbed by the organism. Peptides included in this hydrolysate might exert some biological activity locally at the intestinal epithelium. If they are absorbed, they could then reach the bloodstream and act peripherally.

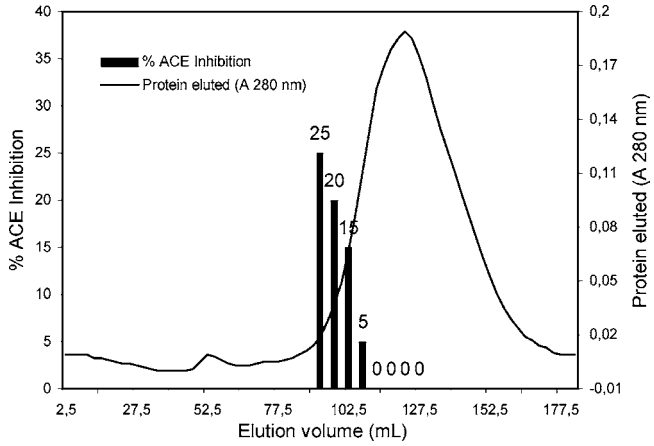

Figure 3. Elution profile of a sunflower hydrolysate obtained by incubation with pepsin and pancreatin in a Sephadex G-50 gel filtration column. Protein elution was monitored at 280 nm. ACE inhibitory activity was assayed in the eluted fractions. Five micrograms of protein hydrolysates was used for the ACE assay.

Gel Filtration Chromatography of Protein Hydrolysate Obtained with Pepsin Plus Pancreatin. The final hydrolysate obtained by sequential incubation with pepsin and pancreatin was used for purification of peptidic fractions and peptides with ACE inhibitory activity. This hydrolysate was filtered through a 5 kDa membrane to remove high molecular mass peptides and residual proteins and then applied to a G-50 gel filtration column (**Figure 3**). The profile that was obtained is typical of a protein hydrolysate formed by a pool of peptides of gradually decreasing molecular masses. Elution volumes between 75 and 175 mL included free amino acids and peptides with molecular masses up to 8.5 kDa. ACE inhibitory activity was determined in fractions with a higher absorbance at 280 nm. Fractions with an elution volume of 135 mL or higher were not analyzed since they included mostly free amino acids. As observed in **Figure 3**, the highest ACE inhibitory activity was found in the fractions including the peptides with higher molecular masses, with

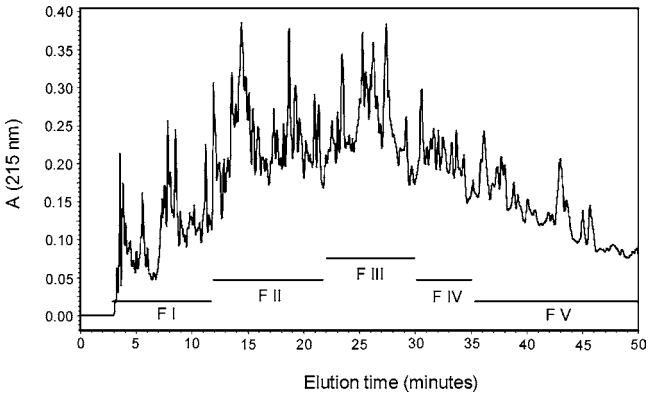

Figure 4. Preparative RP-HPLC C_{18} chromatography of the fractions obtained by gel filtration. Fractions I–V were collected for ACE assay as indicated.

Table 1. ACE Inhibition from Fractions Purified by HPLC Reverse Phase Chromatography[a]

fraction	elution time (min)	ACE inhibition (%)
I	3–12	8.5 ± 0.8
II	12–22	13.6 ± 0.5
III	22–30	19. 2 ± 2.6
IV	30–35	12. 8 ± 1.4
V	35–50	30.0 ± 1.8

[a] Five micrograms of the hydrolysates was used for the incubation assays. Results are the average ± SD of two independent experiments.

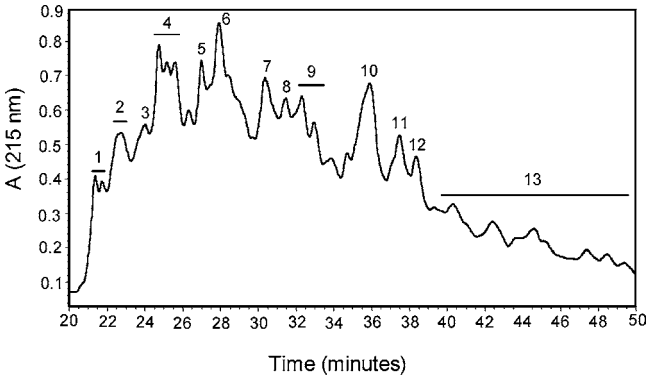

Figure 5. Analytical RP-HPLC C_{18} analysis of fraction V from **Figure 4**.

elution volumes around 95 mL. Peptides corresponding to elution volumes between 75 and 105 mL were pooled and concentrated by lyophilization for further analyses by RP-HPLC chromatography.

HPLC Reverse Phase Chromatography of Fractions Pooled from G-50 Gel Filtration Chromatography. Figure 4 shows RP-HPLC analysis of fractions purified from gel filtration chromatography. The eluate was divided into five fractions, as indicated in the figure, to assay ACE inhibitory activity (**Table 1**). Fraction V provided the highest inhibition of ACE. As previously observed with chickpea protein hydrolysates (*29*), the peptides that eluted last from the reverse phase column had the highest ACE inhibitory activity. It has been suggested that ACE inhibitory peptides are rich in hydrophobic amino acids, which results in a higher retention in hydrophobic chromatography column (*30*).

To further purify ACE inhibitory peptides, fraction V was applied to an analytical C_{18} reverse phase column (**Figure 5**). Eluted peptides were divided in 13 fractions, and their ACE inhibitory activity was studied. **Table 2** shows % ACE inhibition of the 13 fractions as well as the IC_{50} of the most active

Table 2. ACE Inhibition from Fractions Purified by a Second Step of HPLC Reverse Phase Chromatography and for Captopril[a]

fractions	ACE inhibition (%)	IC_{50} (μg/mL)	IC_{50} (μM)
1	10.8 ± 7.4		
2	13.0 ± 4.4		
3	3.8 ± 0.5		
4	10.4 ± 5.3		
5	10.9 ± 2.0		
6	20.2 ± 3.9	13.7	
7	20.0 ± 4.3	20.8	
8	19.8 ± 2.9	15	
9	28.5 ± 2.4	10	
10	17.8 ± 0.4		
11	53.0 ± 3.7	5.7	6.9
12	47.8 ± 2.8	2.4	
13	28.0 ± 2.3		
captopril		0.0089	0.041

[a] Two micrograms of peptides was used for the inhibition assay. Results are the average ± SD of two independent experiments.

fractions, except for number 13 that was made of a complex peptide mixture. Again, peptides eluting later, represented by fractions 9, 11, 12, and 13, were the most active. Fractions 11 and 12, with the highest % ACE inhibition and consequently lowest IC_{50} value, were submitted for sequencing, although only number 11 yielded a single sequence, corresponding to the octapeptide FVNPQAGS. A sequence search revealed that this sequence corresponds to a fragment of helianthinin, the 11S globulin of sunflower seed (*31*). This is the main storage protein in this crop. Thus, helianthinin is the source of an ACE inhibitory peptide upon hydrolysis with pepsin and pancreatin. It is not known in which moment of the hydrolysis this peptide was generated, but it should be highly resistant to pepsin and pancreatin since it has remained intact after the hydrolysis with both proteases. Furthermore, in its sequence, there are no targets for the action of the most common digestive enzymes pepsin, trypsin, and chymotrypsin.

There is no clear relationship between structure and activity in ACE inhibitory peptides. An increasing number of sequences reported in recent years have revealed a variety of structures with inhibitory activity, and some common structure patterns have emerged from these studies. Thus, peptides rich in hydrophobic amino acids (aromatic or branched chains) in the C-terminal region (*32*) and peptides rich in proline (*33*) are frequently found among ACE inhibitors. To our knowledge, the bioactive sequence that we have found in helianthinin has not been described before in any source of protein. However, a similarity has been observed with the sequences of others peptides. For example, the sequence FVXP of the N-terminal region of the peptide is also observed in peptides generated from α_{s1}-casein with trypsin such as FFVAPFPEVFGK (*34*) or FFVAP (*35*). Also, the domain PQ is observed in ACE inhibitory peptides such as KVLPVPQ from β-casein (*36*) or YKVPQL from α_{s1}-casein (*36*).

Inhibitory potency may be very variable between peptides (*37*). The IC_{50} (6.9 μM) for the peptide released from helianthinin is in the same order of magnitude than the IC_{50} reported for many other natural ACE inhibitory peptides. Nevertheless, this IC_{50} value is still far from the IC_{50} of the synthethic ACE inhibitor captopril (**Table 2**).

In conclusion, our results showed that a complex pattern of peptides with ACE inhibitory activity could be obtained by treating deffated sunflower meal with pepsin and pancreatin. A single peptide was purified and sequenced, which corresponded to a fragment in the sequence of the main sunflower seed storage globulin, helianthinin. Following this procedure, deffated sunflower meal, an inexpensive byproduct or waste of sunflower

oil production, could be used for producing a value-added food component with health promoting characteristics.

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LITERATURE CITED

(1) Kitts, D. D. Bioactive substances in food: identification and potential uses. *Can. J. Physiol. Pharm.* **1993**, *72*, 423−434.
(2) Smacchi, E.; Gobbetti, M. Bioactive peptides in dairy products: synthesis and interaction with proteolytic enzymes. *Food Microbiol.* **2000**, *17*, 129−141.
(3) Schlimme, E.; Meisel, H. Bioactive peptides derived from milk proteins. Structural, physiological and analytical aspects. *Nahrung* **1995**, *39*, 568−587.
(4) Meisel, H. Overview on milk protein-derived peptides. *Int. Dairy J.* **1998**, *8*, 363−373.
(5) Endres, J. G. *Soy Protein Products. Characteristics, Nutritional Aspects and Utilization*; AOCS Press: Champaign, Illinois, 2001.
(6) FAO/WHO/UNU. *Agricultural Bulletin Board on Data Collection, Disemination and Quality of Statistics*; World Health Organization: Geneva, 2002.
(7) Villanueva, A.; Vioque, J.; Sánchez-Vioque, R.; Clemente, A.; Pedroche, J.; Bautista, J.; Millán, F. Peptide characteristics of sunflower protein hydrolysates. *J. Am. Oil Chem. Soc.* **1999**, *76*, 1455−1460.
(8) Yamamoto, N. Antihypertensive peptides derived from food proteins. *Biopolymers* **1997**, *43*, 129−134.
(9) Takano, T. Milk derived peptides and hypertension reduction. *Int. Dairy J.* **1998**, *8*, 375−381.
(10) Pihlanto-Leppälä, A. Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. *Trends. Food Sci. Technol.* **2001**, *11*, 347−356.
(11) Oshima, G.; Shimabukuro, H.; Nagasawa, K. Peptide inhibitors of angiotensin I-converting enzyme in digests of gelatin by bacterial collagenase. *Biochem. Biophys. Acta* **1979**, *566*, 128−137.
(12) Yokokawa, K.; Chiba, H.; Yoshikawa, M. Peptide inhibitors for angiotensin I-converting enzyme from thermolysin digest of dried bonito. *Biosci., Biotechnol., Biochem.* **1992**, *56*, 1541−1545.
(13) Gobbetti, M.; Smacchi, E.; Corsetti, A.; Bellucci, M. Inhibition of proteolytic enzymes from *Pseudomonas fluorescens* ATCC 948 and angiotensin-I converting enzyme by peptdes from zein, hordein and gluten hydrolysates. *J. Food Prot.* **1997**, *60*, 499−504.
(14) Yano, S.; Suzuki, K.; Funatsu, G. Isolation from a-zein of thermolysin peptides with angiotensin-I converting enzyme inhibitory activity. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 661−663.
(15) Pihlanto-Leppälä, A.; Rokka, T.; Korhonen, H. Angiotensin I-converting enzyme inhibitory peptides derived from bovine milk proteins. *Int. Dairy J.* **1998**, *8*, 325−331.
(16) Mullally, M. M.; Meisel, H.; FitzGerald, R. J. Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine β-lactoglobulin. *FEBS Lett.* **1997**, *402*, 99−101.
(17) Smacchi, E.; Gobbetti, M. Peptides from several Italian cheeses inhibitory to proteolytic enzymes of lactic acid bacteria, *Pseudomonas fluorescens* ATCC 948 and to the angiotensin-I converting enzyme. *Enzyme Microb. Technol.* **1998**, *22*, 687−694.
(18) Pihlanto-Leppälä, A.; Koskinen, P.; Piilola, K.; Tupasela, T.; Korhonen, H. Angiotensin I-converting enzyme inhibitory properties of whey protein digest: concentration and characterization of active peptides. *J. Dairy Res.* **2000**, *67*, 53−64.
(19) Roy, M. K.; Watanabe, Y.; Tamai, Y. Yeast protease B-digested skimmed milk inhibits angiotensin-I-converting enzyme activity. *Biotechnol. Appl. Biochem.* **2000**, *31*, 95−100.
(20) Mullally, M. M.; Meisel, H.; Fitzgerald, R. J. Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey proteins. *Int. Dairy J.* **1997**, *7*, 299−303.
(21) Matsui, T.; Yukiyoshi, A.; Doi, S.; Sugimoto, H.; Yamada, H.; Matsumoto, K. Gastrointestinal enzyme production of bioactive peptides from royal jelly protein and their antihypertensive ability in SHR. *J. Nutr. Biochem.* **2002**, *13*, 80−86.
(22) Yamamoto, N.; Akino, A.; Takano, T. Antihypertensive effects of different kinds of fermented milk in spontaneously hypertensive rats. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 776−778.
(23) Sekiya, S.; Kobayashi, Y.; Kita, E.; Imamura, Y.; Toyama, S. Antihypertensive effects of tryptic hydrolysate of casein on normotensive and hypertensive volunteers. *J. Jpn. Soc. Nutr. Food Sci.* **1992**, *45*, 513−517.
(24) Hayakari, M.; Kondo, Y.; Izumi, H. A rapad and simple spectrophotometric assay of angiotensin-converting enzyme. *Anal. Biochem.* **1978**, *84*, 361−369.
(25) Villanueva, A.; Vioque, J.; Sánchez-Vioque, R.; Clemente, A.; Bautista, J.; Millán, F. Production of an extensive sunflower protein hydrolysates by sequential hydrolysis with endo- and exoproteases. *Grasas Aceites* **1999**, *50*, 472−476.
(26) Adler-Nissen, J. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *J. Agric. Food Chem.* **1979**, *27*, 1256−1262.
(27) Schägger, H.; von Jagow, G. Tricine- sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **1987**, *166*, 368−379.
(28) Hjerten, S. Chromatographic separation according to size of macromolecular and cell particles on columns of agarose suspensions. *Arch. Biochem. Biophys.* **1962**, *99*, 466−467.
(29) Pedroche, J.; Yust, M. M.; Girón-Calle, J.; Alaiz, M.; Millán, F.; Vioque, J. Utilisation of chickpea protein isolates for production of peptides with angiotensin I-converting enzyme (ACE)-inhibitory activity. *J. Sci. Food Agric.* **2002**, *82*, 960−965.
(30) Yust, M. M.; Pedroche, J.; Girón-Calle, J.; Alaiz, M.; Millán, F.; Vioque, J. Production of ace inhibitory peptides by digestion of chickpea legumin with alcalase. *Food Chem.* **2003**, *81*, 363−369.
(31) Vonder Haar, R. A.; Allen, R. D.; Cohen, E. A.; Nessler, C. L.; Thomas, T. L. Organization of the sunflower 11S storage protein gene family. *Gene* **1988**, *74*, 433−443.
(32) Clare, D. A.; Swaisgood, H. E. Bioactive milk peptides: a prospectus. *J. Dairy Sci.* **2000**, *83*, 1187−1195.
(33) Meisel, H. Biochemical properties of regulatory peptides derived from milk proteins. *Biopolymers* **1997**, *43*, 119−128.
(34) Maruyama, S.; Suzuki, H. A peptide inhibitor of angiotensin-I converting enzyme in the tryptic hydrolysate of casein. *Agric. Biol. Chem.* **1982**, *46*, 1393−1394.
(35) Maruyama, S.; Nakagomi, K.; Tomizuka, N.; Suzuki, H. Angiotensin-converting enzyme inhibitor derived from an enzymatic hydrolysate of casein. II. Isolation and bradykinin-potentiating activity on the uterus and the ileum of rats. *Agric. Biol. Chem.* **1985**, *49*, 1405−1409.
(36) Maeno, M.; Yamamoto, N.; Takano, T. Identification of antihypertensive peptides from casein hydrolysate produced by a proteinase from Lactobacillus helveticus CP790. *J. Dairy Sci.* **1996**, *73*, 1316−1321.
(37) Ariyoshi, Y. Angiotensin-converting enzyme inhibitors derived from food proteins. *Trends. Food Sci. Technol.* **1993**, *4*, 139−144.

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