JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Affinity Purification of Angiotensin Converting Enzyme Inhibitory Peptides Using Immobilized ACE

CRISTINA MEGÍAS, JUSTO PEDROCHE, MARÍA DEL MAR YUST, MANUEL ALAIZ, JULIO GIRÓN-CALLE, FRANCISCO MILLÁN, AND JAVIER VIOQUE*

Instituto de la Grasa (CSIC), Padre García Tejero 4, 41012-Sevilla, Spain

A lung extract rich in angiotensin converting enzyme (ACE) and pure ACE were immobilized by reaction with the activated support 4 BCL glyoxyl-agarose. These immobilized ACE derivatives were used for purification of ACE inhibitory peptides by affinity chromatography. The immobilized lung extract was used to purify inhibitory peptides from sunflower and rapeseed protein hydrolysates that had been obtained by treatment of protein isolates with alcalase. The ACE binding peptides that were retained by the derivatives were specifically released by treatment with the ACE inhibitor captopril and further purified by reverse-phase C_{18} HPLC chromatography. Inhibitory peptides with IC_{50} 50 and 150 times lower than those of the original sunflower and rapeseed hydrolysates, respectively, were obtained. The derivative prepared using pure ACE was used for purification of ACE inhibitory peptides from the same type of sunflower protein hydrolysate. ACE binding peptides were released from the ACE-agarose derivatives by treatment with 1 M NaCl and had an IC_{50} a little higher than those obtained using immobilized extract and elution with captopril. Affinity chromatography facilitated the purification of ACE inhibitory peptides and potentially other bioactive peptides present in food proteins.

KEYWORDS: Affinity purification; angiotensin converting enzyme; inhibitory peptides

1. INTRODUCTION

The search for functional components in foods has become a major area of research in recent years. These functional components include the bioactive peptides, which are small amino acid sequences in food proteins that have a beneficial biological activity after they are released during gastrointestinal digestion or by previous *in vitro* protein hydrolysis. For example, peptides with antihypertensive, immunomodulatory, opioid, antioxidant, and hypocholesterolemic activity have been described (*1*, *2*). The antihypertensive peptides exert their antihypertensive activity by inhibition of the angiotensin-I converting enzyme (ACE) (*3*, *4*). ACE activity leads to an increase in blood pressure by producing the vasoconstrictor peptide angiotensin II and by hydrolyzing the vasodilator peptide bradykinin. Antihypertensive peptides with ACE inhibitory activity have been purified from animal (*5*, *6*) and plant proteins (*7*, *8*). Many antihypertensive peptides have been purified from milk (*9*, *10*) and derived products such as cheese (*11*), whey (*12*), and skimmed milk (*13*).

Sunflower and rapeseed protein isolates, containing more than 90% protein (*14*, *15*), could be used for the production of protein hydrolysates with improved functional and nutritional properties. These protein hydrolysates could also be a source of bioactive peptides. Thus, it has been reported that sunflower and rapeseed protein hydrolysates contains ACE inhibitory peptides (*16*, *17*). ACE inhibitory peptides are usually purified using classical filtration and chromatographic methods. These may include enrichment in bioactive peptides by ultrafiltration followed by purification using gel filtration or ion exchange chromatography. Most often, HPLC reverse-phase chromatography is used in order to further purify the bioactive peptides (*18*−*20*).

Affinity chromatography is a powerful protein purification method that relies on the specific reversible complexes that are formed between the molecule to be purified and a ligand bound to a suitable polymer support such as agarose. After incubation of the affinity adsorbent with the mixture containing the molecule of interest and washing to remove unbound molecules, the molecules that are retained are eluted by using specific or nonspecific elution agents. Nonspecific elution procedures include changes in ionic strength, pH, or temperature. Specific elution agents are molecules that bind to the molecule of interest, including competitors, substrates, inhibitors, and cofactors.

The immobilization of ACE on glyoxyl-agarose supports and the use of these derivatives for the assay of ACE inhibitory peptides was described in a previous paper (*21*). Glyoxyl-agarose beads have been successfully used for the immobilization−stabilization of enzymes, resulting in high stabilization factors and preservation of enzymatic activities. Immobilization using glyoxyl-agarose is based on the reaction between aldehyde groups in the support and primary amino groups in the enzymes, which are mostly ϵ-amino groups in lysine residues but also

* Corresponding author. Tel: 954611550. Fax: 954616790. E-mail: jvioque@cica.es.

10.1021/jf061488b CCC: $33.50
Published on Web 08/18/2006

terminal amino groups. The use of these agarose derivatives for the affinity purification of sunflower and rapeseed ACE inhibitory peptides is described in this article. To our knowledge, this is the first time that ACE inhibitory peptides are purified by affinity chromatography using immobilized ACE.

2. MATERIALS AND METHODS

2.1. Materials. Hippuryl-L-histidyl-L-leucine (HHL), 2,4,6-trichloro-*s*-triazine (TT), phenyl-methylsulfonyl fluoride (PMSF), and rabbit lung ACE were purchased from Sigma (Tres Cantos, Madrid, Spain). 4 BCL agarose (4% beads cross-linked) was provided by Iberagar (Coina, Barreiro, Portugal). Alcalase 2.4 L was purchased from Novo-Nordisk (Bagsvaerd, Denmark). Alcalase 2.4 L is a microbial protease of *Bacillus licheniformis* with endopeptidase activity. A main component of the commercial preparation is subtilisin A. The specific activity of Alcalase 2.4 L is 2.4 Anson unit/g. One Anson unit is the amount of enzyme that digests hemoglobin to produce an amount of trichloroacetic acid soluble product which gives the same color with the Folin reagent as 1 mequiv of tyrosine released per minute. All other chemicals were of analytical grade.

2.2. Preparation of Sunflower and Rapeseed Protein Isolates. Sunflower or rapeseed flour (20 g) was extracted by stirring for 1 h in 200 mL of 0.2% NaOH pH 12. After centrifugation at 8000*g* for 20 min, two additional extractions were carried out with half the volume of alkaline solution. The pH of the supernatant was adjusted to the isoelectric point (pH 4.3 for sunflower and pH 5 for rapeseed), and the precipitate formed was recovered by centrifugation as described above. The precipitate was washed with distilled water adjusted to the isoelectric point and freeze-dried until further use.

2.3. Preparation of Sunflower and Rapeseed Protein Hydrolysates. Sunflower and rapeseed protein isolates were hydrolyzed with alcalase using a hydrolysis reactor vessel equipped with a stirrer, thermometer, and pH electrode. Sunflower and rapeseed proteins were digested for 5 and 30 min for preparation of protein hydrolysates with a degree of hydrolysis of 21.6% and 26.5%, respectively. Hydrolysis parameters were as follows: protein isolate concentration, 10% (w/v); enzyme/substrate ratio 0.3 Anson units g^{-1}; pH 8; temperature, 50 °C. 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 for storage at −20 °C.

2.4. Degree of Hydrolysis. The degree of hydrolysis was calculated by determination of free amino groups by reaction with TNBS (*22*). The total number of amino groups was determined in a sample of protein isolate hydrolyzed by treatment with 6 N HCl at 120 °C for 24 h.

2.5. Preparation of ACE. ACE was prepared using porcine lungs provided by MACUMASA (Cumbres Mayores, Huelva, Spain). Tissue was 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 units/mg of protein. In order to verify the absence of undesirable proteases such as carboxypeptidases in the extracts, the kinetics of HHL hydrolysis was followed in the presence (0.5 μM) or absence of captopril, a potent ACE inhibitor. The results indicated that the hydrolysis was due exclusively to ACE because hydrolysis of HHL in the presence of captopril is residual and similar to hydrolysis in the blank control (*7*).

2.6. Activation of Agarose Gels. Activation of the 4% beads cross-linked agarose gels (4 BCL) was done according to the procedure previously described by Guisán (*23*) with slight modifications. Agarose contains many hydroxyl groups and about 10% diol groups. Since immobilization requires free diol groups, activation involves etherification of hydroxyl to diol groups by reaction with glycidol. The reaction was carried out in a 2:1 (v/v) suspension of the gels in 1 M NaOH, 0.5 M $NaBH_4$ (0.7 g of swelling agarose is roughly equivalent to 1 mL). These reducing conditions prevent oxidation of the gel. While keeping this mixture on an ice bucket, glycidol was added dropwise in order to reach a 2 M final concentration, and the resulting suspension was gently stirred overnight at room temperature. In order to generate the aldehyde groups that will react with proteins, the diol groups in the activated gels were transformed into aldehyde groups by reaction with $NaIO_4$. The gels were washed with abundant distilled water adjusted at pH 7.0 and then suspended in water (300 mL) containing $NaIO_4$ (300 μmol/mL of gel). The oxidation with $NaIO_4$ was allowed to proceed for 2−3 h with stirring at room temperature. These conditions yield a very high concentration of aldehyde groups in the gel so that multipoint attachment of protein molecules to the gel is made possible. Using this procedure the glyceryl groups that are formed in the etherification reaction with glycidol are oxidized by periodate mol to mol. The resulting gels were washed with abundant distilled water (pH 7) and stored at 4 °C.

2.7. Immobilization of ACE. Immobilization of the enzyme on activated glyoxyl-agarose gels was done according to procedures previously described with slight modifications (*23*, *24*). The gel (1 mL) and 30 mg of pig lung ACE extract or 14 μg of pure rabbit ACE were suspended in 0.2 M sodium bicarbonate pH 10 and gently stirred at room temperature for 30 min. The reaction between aldehyde groups in the gel and amino groups in the proteins results in the formation of Schiff bases that are then reduced to stable secondary amine bonds by adding $NaBH_4$ (0.1% w/v) and stirring for 30 min at room temperature. Addition of $NaBH_4$ also results in the elimination of unreacted aldehyde groups that are reduced to inactive hydroxyl groups. Residual $NaBH_4$ was eliminated by washing with distilled water (*25*).

2.8. Assay of ACE. ACE activity was determined as described by Hayakari et al. (*26*) with modifications. This method uses HHL as substrate for ACE and relies on the reaction of the resulting hippuric acid with 2,4,6-trichloro-*s*-triazine (TT). The assay mixture (0.5 mL) consisted of potassium phosphate buffer pH 8.3 (40 μmol), sodium chloride (300 μmol), HHL (1.5 μmol), and 1 μg of pig lung ACE extract. Incubation was carried out at 37 °C for 15 min and was terminated by addition of TT in dioxane (1.5 mL, 3% w/v), followed by 0.2 M phosphate buffer pH 8.3 (3 mL). Absorbance at 382 nm was determined in the supernatant obtained after centrifugation at 10000*g* for 10 min. The IC_{50} value, defined as the concentration of peptide in μg of protein/mL required to produce 50% inhibition of ACE, was determined by regression analysis of ACE inhibition (%) versus peptide concentration. Captopril assayed in the same conditions yielded an IC_{50} of 0.009.

2.9. Purification of ACE Inhibitory Peptides. *Purification Using Immobilized Pig Lung ACE Extract.* Different amounts of sunflower or rapeseed protein hydrolysate were mixed with immobilized lung extract and stirred at 37 °C in 100 mM ammonium bicarbonate 300 mM NaCl pH 8.3 for different times. This buffer was used because ammonium bicarbonate will be later eliminated during the lyophilization and vacuum desiccation of the final peptide preparations. After incubation with the hydrolysates, the immobilized extract was recovered by filtration through a porous glass plate and washed several times with ammonium bicarbonate buffer until absorbance of the filtrates at 214 nm reached a baseline. Bound peptides were then released by incubation with different amounts of captopril in the same buffer, and separated from the immobilized extract by filtration as above. The filtrates were concentrated and applied to a preparative HPLC reverse-phase column (C_{18} Hi-Pore RP-318, 250 mm × 10 mm BIO-RAD) for elimination of captopril and separation of the peptides into different fractions. Injection volume and sample concentration were 100 μL and 20 mg of peptide/mL, respectively. A linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid (0−30% in 50 min) at a flow rate of 4 mL/min at 30 °C was used. Elution was monitored at 215 nm. Captopril eluted at 26.5 min, and the eluate from 24 to 29 min was not collected to prevent contamination of bioactive peptides with captopril (data not shown). The fractions collected from HPLC were lyophilized for further assay of ACE inhibitory activity. Because it was important to make sure that the collected fractions did not carry any residual captopril, a control was carried out by injection of captopril alone and collecting fractions as above. Fractions collected before 24 min and after 29 min did not show any ACE inhibitory activity, indicating that no captopril was present.

Purification Using Immobilized Commercial Rabbit Lung ACE. Purification of ACE inhibitory peptides using commercial rabbit lung

Table 1. Effect of the Captopril to Protein Ratio on the Release of ACE Binding Peptides[a]

captopril /immobilized protein ratio (w/w)	eluted peptides (% of total loaded peptides)
435/1	0.17 ± 0.02
10/1	0.065 ± 0.006
1/1	0.03 ± 0.012

[a] Results are the average ± standard deviation of three determinations.

ACE was carried out as described above, with the exception of using 1 M NaCl instead of captopril for elution of peptides bound to immobilized ACE. Purification of the peptides by HPLC as above was not carried out. NaCl concentration in the filtrates containing the bioactive peptides was adjusted to the optimum for ACE activity by dilution with water.

2.10. Protein Determination. Soluble proteins were determined according to the method described by Bradford (*27*). Immobilized proteins and soluble peptides were determined after amino acid analysis according to the method of Alaiz et al. (*28*).

3. RESULTS

3.1. Purification of Sunflower and Rapeseed ACE Binding Peptides Using Immobilized Pig Lung Extracts. Pig lung extracts immobilized on 4% BCL glyoxil-agarose were used for affinity purification of ACE inhibitory peptides in sunflower and rapeseed protein hydrolysates. Covalent binding to agarose resulted in immobilization not only of ACE but also of other proteins and molecules carrying a reactive amino group that are present in the extracts. In addition, the sunflower and rapeseed protein hydrolysates are a complex mixture of different peptides (*14*, *15*) that might bind not only to ACE but also to other immobilized molecules. Therefore, it is necessary to use a specific elution method in order to recover the ACE binding peptides without releasing the peptides that bind to any other immobilized molecules. This was accomplished by using captopril for elution of the peptides bound to immobilized ACE. Captopril is a specific ACE competitive inhibitor that is used in the treatment of high blood pressure (*29*),

Different amounts of captopril were assayed for elution of ACE binding peptides from the agarose matrix, starting with a 10 mM concentration that corresponds to a captopril to protein ratio of 435 to 1 (w/w) (**Table 1**). Captopril coelutes in reverse-phase HPLC with some of the released peptides. Larger concentrations of captopril provided higher recovery of ACE binding peptides, but also led to a larger overlapping of the peak corresponding to captopril with peaks corresponding to peptides (not shown). Although recovery using a 1/1 ratio was the lowest (Table 1), this concentration of captopril was chosen as the best because it greatly reduced coelution of captopril with peptides, resulting in the highest overall recovery (not shown).

It has been reported that soluble ACE activity is optimal in the presence of 300 mM NaCl (*30*). The ACE activity in our immobilized preparation was 7.5 times higher in the presence of 300 mM NaCl than in the absence of NaCl. In addition, the peptide binding capacity also increased in the presence of NaCl. Thus, peptide recovery increased from 0.03 ± 0.0012% to 0.306 ± 0.013% of initial peptides (average of three determinations ± standard deviation). The length of the incubation of the hydrolysates with the immobilized extracts and the length of the subsequent incubation with captopril also had a large effect on the recovery of ACE binding peptides. Thus, incubation of the extract with the hydrolysates for 1 h and with captopril for 30 min provided a recovery of 0.03% ± 0.0012, while running both incubations for 2 h improved the recovery to 0.513% ± 0.017 (average ± standard deviation of three experiments). Finally, the effect of the concentration of sunflower hydrolysate during the incubation with the immobilized extract on the recovery was also studied. As shown in **Figure 1**, there was a concentration dependent increase of the recovery with protein concentration in the hydrolysates up to a saturation point at a ratio of protein hydrolysate to immobilized protein (w/w) of 10/1.

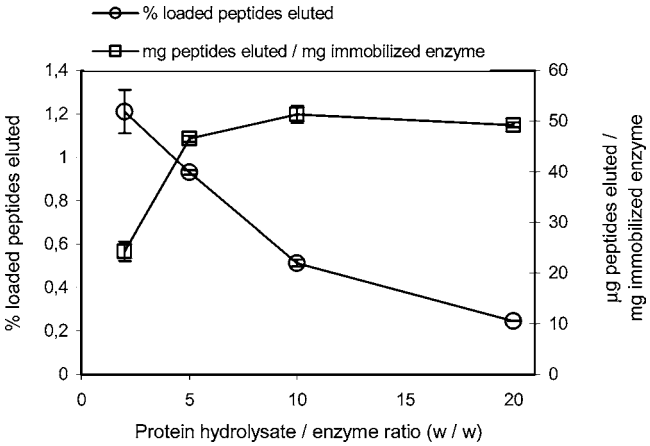

Figure 1. Effect of the hydrolyzed protein to immobilized protein ratio on the recovery of ACE binding peptides. Lung extracts were immobilized on agarose and used at different hydrolyzed protein to immobilized protein ratios for purification of ACE binding peptides.

Table 2. IC_{50} (μg/mL) of Sunflower and Rapeseed Protein Hydrolysates and the Peptides Purified from These Hydrolysates[a]

	sunflower	rapeseed
protein hydrolysate	62.18 ± 0.98	37.97 ± 0.29
purified peptides	1.18 ± 0.04	0.25 ± 0.02

[a] Results are the average of three determinations ± standar deviation.

All these preliminary assays provided optimized conditions for the affinity purification of ACE binding peptides. These conditions are as follows: a hydrolyzed protein to immobilized protein ratio of 10/1 (w/w) with an incubation time of 2 h for binding of peptides to the immobilized extract, and a captopril to immobilized protein ratio of 1/1 (w/w) with an incubation time of 2 h for release of ACE binding peptides. The buffer to be used for both incubations is 100 mM ammoniun bicarbonate 300 mM NaCl pH 8.3. These conditions were applied to sunflower and rapeseed hydrolysates obtained by treatment with alcalase that have ACE inhibitory activity (*19*−*20*). Although not only ACE inhibitory peptides can potentially bind immobilized ACE, enrichment of ACE binding peptides by affinity chromatography as described above should enrich the hydrolysates in ACE inhibitory peptides. As shown in **Table 2**, the IC_{50} of the pool of sunflower and rapeseed ACE binding peptides purified by affinity purification was 50 and 150 times lower, respectively, than the IC_{50} of the original hydrolysates. The yield of this purification using affinity chromatography was much higher than the yields that were obtained using other chromatographic methods such as gel filtration ionic exchange or reverse-phase chromatography (*21*−*23*). Reverse-phase chromatography of the original sunflower and rapeseed hydrolysates and the ACE binding peptides is shown in **Figure 2**. The chromatograms of the original and purified peptides are quite different, but some of the prominent peptides of the original hydrolysates are kept in the ACE binding fraction.

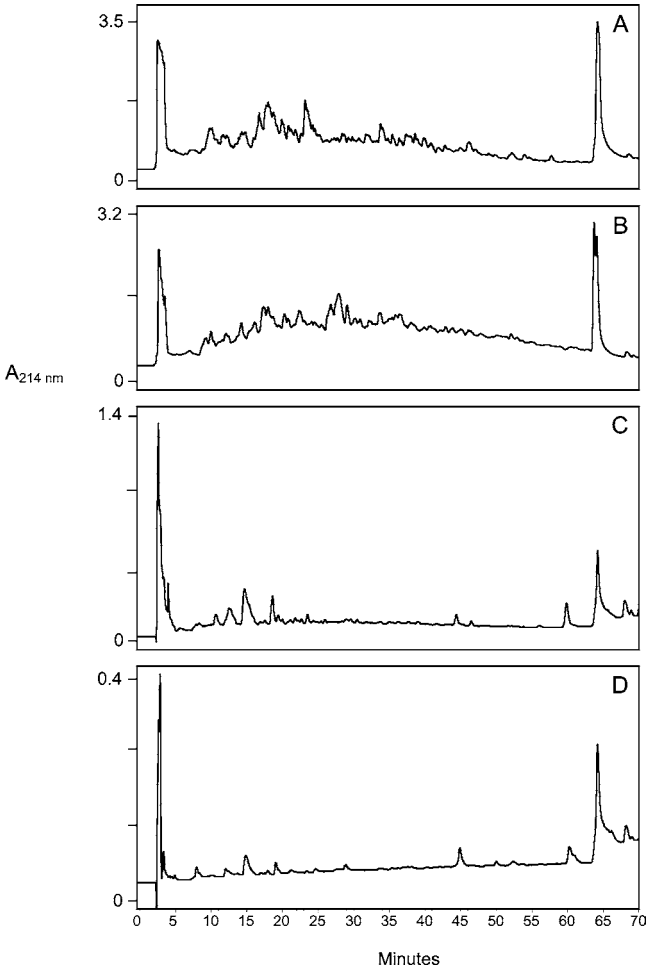

Figure 2. C_{18} reverse-phase HPLC profile of sunflower and rapeseed protein hydrolysates before and after affinity chromatography for purification of ACE binding peptides. Lung tissue extracts were immobilized on agarose, and specific elution of ACE binding peptides was carried out using captopril. (**A**) Sunflower hydrolysate. (**B**) Rapeseed hydrolysate. (**C**) Sunflower ACE binding peptides. (**D**) Rapeseed ACE binding peptides.

3.2. Purification of Sunflower ACE Binding Peptides by Affinity Chromatography Using Immobilized Commercial Rabbit Lung ACE. A commercial preparation of rabbit lung ACE was also immobilized on agarose for affinity purification of ACE binding peptides as described above. The use of pure ACE has the advantage over the lung tissue extract that a nonspecific method, namely, washing the immobilized protein with a high ionic strength buffer as shown in **Figure 3**, can be used for elution of the ACE binding peptides. Most of the peptides, which showed ACE inhibitory activity, were released in the second and third washes. These peptides had an IC_{50} of 2.31 ± 0.16 μg of peptides/mL, which is significantly higher than the IC_{50} of the peptidic fraction that was purified using immobilized lung extract and elution with captopril (1.18 ± 0.04 μg of peptides/mL) (average $\pm$ standard deviation of three experiments, differences significant $P < 0.05$).

4. DISCUSSION

Many ACE inhibitory peptides derived from food proteins have been purified in the past, usually by using several chromatographic steps that include size exclusion, ionic exchange, and/or HPLC-RP chromatography. Affinity chromatography is a powerful method for the purification of biomolecules. It is based on the use of specific ligands to absorb the

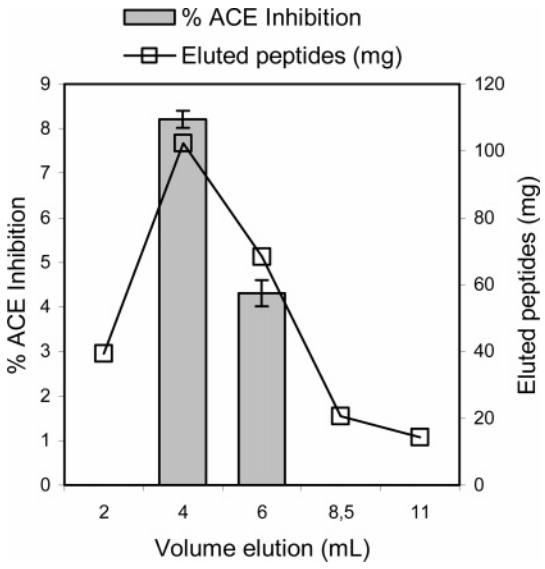

Figure 3. Elution profile and ACE inhibitory activity of the sunflower peptides purified using immobilized ACE. Rabbit lung ACE was immobilized on agarose and incubated with sunflower seed protein hydrolysates. The peptides that were retained by the immobilized protein were eluted by washing with NaCl 1 M.

desired substances on solid supports and/or to elute them from the support. The immobilization of ACE on glyoxyl-agarose supports and the high stability of the resulting ACE-agarose derivatives were described in a previous paper (*21*). These derivatives were proposed for the assay of ACE inhibitory peptides. Here, the use of these derivatives for affinity chromatography purification of ACE inhibitory peptides is described. The process is described using either pig lung extract or pure ACE as ligands for the purification of ACE binding peptides that had a high ACE inhibitory activity. While using a tissue extract is inexpensive, it requires the use of a specific ACE ligand for the recovery of the ACE binding peptides. The specific ligand that has been used, captopril, was removed by reverse-phase HPLC. This cleanup chromatography was not necessary when pure ACE was used as the ligand for affinity chromatography because elution of the ACE binding peptides in this case does not require the use of a specific ligand such as captopril.

The IC_{50} of the peptidic fraction purified using pure ACE and a nonspecific elution method was significantly higher than the IC_{50} of the fraction obtained using immobilized lung extract and a specific elution method, probably indicating that more peptides other than those that are ACE inhibitors are released in the first case than in the second. These could be peptides that bind to ACE but are not inhibitors of ACE, or peptides that bind to proteins contaminating the ACE preparation and are released by the nonspecific elution method. It is also possible that the differences are due to the different origin of the immobilized ACE protein, pig or rabbit lung.

Immobilized ACE is very stable (*21*), so that the ACE derivatives can be easily reused and the process could be scaled up for the purification of ACE inhibitory peptides in higher quantities. These peptides could be used as functional ingredients for the elaboration of functional foods with antihypertensive-like activity. The high specificity of affinity chromatography has facilitated the development of these two methods for purification of ACE inhibitory peptides that represent a qualitative advance in the purification of these peptides from food sources, and could also be applied for the purification of other

bioactive peptides. For example, by immobilizing opioid receptors, agonist and antagonist opioid peptides derived from food proteins that bind to these receptors could be purified.

ACKNOWLEDGMENT

Thanks are due to MACUMASA and to Rogelio Orquín from NICBSL for providing pig lungs.

LITERATURE CITED

(1) Smacchi, E.; Gobbetti, M. Bioactive peptides in dairy products: synthesis and interaction with proteolytic enzymes. *Food Microbiol.* **2000**, *17*, 129−141.

(2) Vioque, J.; Sánchez-Vioque, R.; Clemente, A.; Pedroche, J.; Yust, M.M.; Millán, F. Péptidos bioactivos en proteínas de reserva. *Grasas Aceites* **2000**, *51*, 361−365.

(3) Yamamoto, N. Antihypertensive peptides derived from food proteins. *Biopolymers* **1997**, *43*, 129−134.

(4) Takano, T. Milk derived peptides and hypertension reduction. *Int. Dairy J.* **1998**, *8*, 375−381.

(5) 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.

(6) 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.

(7) 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.

(8) 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.

(9) Pihlanto-Leppala, A.; Rokka, T.; Korhonen, H. Angiotensin I-converting enzyme inhibitory peptides derived from bovine milk proteins. *Int. Dairy J.* **1998**, *8*, 325−331.

(10) 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.

(11) 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.

(12) Pihlanto-Leppala, 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.

(13) 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.

(14) Villanueva, A.; Vioque, J.; Sánchez-Vioque, R.; Clemente, A.; Bautista, A.; Millán, F. Production of an extensive sunflower protein hydrolysate by sequential hydrolysis with endo- and exoproteases. *Grasas Aceites* **1999**, *50*, 472−476.

(15) Vioque, J.; Sánchez-Vioque, R.; Clemente, A.; Pedroche, J.; Bautista, J.; Millán, F. Production and characterization of an extensive rapeseed protein hydrolysate. *J. Am. Oil Chem. Soc.* **1999**, *76*, 819−823.

(16) Megías, C.; Yust, M. M.; Pedroche, J.; Lquari, H.; Girón-Calle, J.; Alaiz, M.; Millán, F.; Vioque, J. Purification of an ACE inhibitory peptide after hydrolysis of sunflower (*Helianthus annuus* L.) protein isolates. *J. Agric. Food Chem.* **2004**, 1928−1932.

(17) Pedroche, J.; Yust, M. M.; Megías, C.; Lqari, H.; Alaiz, M.; Girón-Calle, J.; Millán, F.; Vioque, J. Utilisation of rapeseed protein isolates for production of peptides with angiotensin I-converting enzyme (ACE)-inhibitory activity. *Grasas Aceites* **2004**, 354−358.

(18) Saiga, A.; Okumura, T.; Makihara, T.; Katsuta, S.; Shimizu, T.; Yamada, R.; Nishimura, T. Angiotensin I-Converting Enzyme Inhibitory Peptides in a Hydrolyzed Chicken Breast Muscle Extract. *J. Agric. Food Chem.* **2003**, *51*, 1741−1745.

(19) Shin, Z.-I.; Yu, R.; Park, S.-A.; Chung, D. K.; Ahn, C.-W.; Nam, H.-S.; Kim, K.-S.; Lee, H. J. His-His-Leu, an Angiotensin I Converting Enzyme Inhibitory Peptide Derived from Korean Soybean Paste, Exerts Antihypertensive Activity in Vivo. *J. Agric. Food Chem.* **2001**, *49*, 3004−3009.

(20) Choi, H. S.; Cho, H. Y.; Yang, H. C.; Ra, K. S.; Suh, H. J. Angiotensin I-converting enzyme inhibitor from *Grifola frondosa. Food Res. Int.* **2001**, *34*, 177−182.

(21) Megías, C.; Pedroche, J.; Yust, M. M.; Alaiz, M.; Girón-Calle, J.; Millán, F.; Vioque, J. Immobilization of angiotensin-converting enzyme on glyoxyl-agarose. *J. Agric. Food Chem.* **2006**, *54*, 4641−4645.

(22) Adler-Nissen, J. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *J. Agric. Food Chem.* **1979**, *27*, 1256−1262.

(23) Guisán, J. M. Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes. *Enzyme Microb. Technol.* **1988**, *10*, 375−382.

(24) Pedroche, J.; Yust, M. M.; Girón-Calle, J.; Vioque, J.; Alaiz, M.; Mateo, C.; Guisán, J. M.; Millán, F. Stabilization-immobilization of carboxypeptidase A to aldehyde-agarose gels. A practical example in the hydrolysis of casein. *Enzyme Microb. Technol.* **2002**, *31*, 711−718.

(25) Blanco, R. M.; Guisán, J. M. Stabilization of enzymes by multipoint covalent attachment to agarose-aldehyde gels. Borohydride reduction of trypsin-agarose derivatives. *Enzyme Microb. Technol.* **1989**, *11*, 360−366.

(26) Hayakari, M.; Kondo, Y.; Izumi, H. A rapid and simple spectrophotometric assay of angiotensin-converting enzyme. *Anal. Biochem.* **1978**, *84*, 361−369.

(27) Bradford, M. M. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(28) Alaiz, M.; Navarro, J. L.; Giron, J.; Vioque, E. Amino acid analysis by high-performance liquid chromatography after derivatization with diethylethoxymethylenemalonate. *J. Chromatogr.* **1992**, *591*, 181−186.

(29) Atkinson, A. B.; Robertson, J. I. S. Captopril in the treatment of clinical hypertension and cardiac failure. *Lancet* **1979**, *2*, 836−839.

(30) Cushman, D. W.; Cheung, H. S. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol.* **1971**, *20*, 1637−1648.

Received for review May 26, 2006. Revised manuscript received July 3, 2006. Accepted July 13, 2006. This work was supported by research grants AGL 2004-03930 (F.M.) and AGL 2005-01120 (J.G.-C.) from the Spanish Ministry of Education and Science.

JF061488B