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Immobilization of Angiotensin-Converting Enzyme on Glyoxyl-Agarose

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The assay of angiotensin-converting enzyme (ACE) inhibition by food-derived peptides is usually carried out by using soluble ACE in a batch process. The purification of this enzyme from tissues is not an easy task, and the resulting preparation loses activity very fast. In addition, ACE commercial preparations are very expensive. In this work the immobilization of ACE, through lysine amino groups, to 4% beads cross-linked (4 BCL) glyoxyl-agarose is described. The amount of immobilized enzyme increased with increasing concentrations of enzyme and with incubation time until a saturation point was reached at 50 mg protein/mL gel and 3.5 hours, respectively. The IC_{50} values for a noncompetitive sunflower peptide inhibitor were similar for the soluble (30.56 μM) and immobilized (32.7 μM) enzymes. An immobilized derivative was obtained that was 60 times more stable than the soluble enzyme at 60 °C. This procedure yields a derivative that can be reused and has increased thermal stability compared to that of the soluble enzyme. Thus, ACE immobilization is a good alternative to using soluble freshly prepared or commercial preparations because of economical and practical reasons.

KEYWORDS: Immobilization; glyoxyl-agarose; angiotensin-converting enzyme; inhibitory peptides

1. INTRODUCTION

Food is not only a source of energy and basic components for the maintenance and growth of the body but also a source of bioactive compounds with beneficial health effects, including bioactive peptides (*1*). These are small amino acid sequences that have a certain biological activity after they are released during gastrointestinal digestion or by previous in vitro protein hydrolysis. For example, biopeptides with antihypertensive, inmunomodulatory, opioid, antioxidant, and hypocholesterolemic activity have been described (*2*, *3*). Peptides with two or more of these activities have been purified (*4*). The antihypertensive peptides have received a lot of attention (*5*−*7*). These peptides inhibit the angiotensin-converting enzyme (ACE), which is a dipeptidyl carboxypeptidase that is essential for blood pressure regulation. 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 (*8*, *9*) and plant proteins (*10*, *11*). Many antihypertensive peptides have been purified from milk (*12*, *13*) and derived products such as cheese (*14*), whey (*15*), and skimmed milk (*16*). The hypotensive activity of the ACE inhibitory peptides has also been demonstrated in vivo in spontaneously hypertensive rats (*17*) and human volunteers (*18*).

Although several approaches have been used in order to stabilize enzymes, including genetic and protein-engineering techniques (*19*, *20*), immobilization on porous solid carriers is probably the most popular strategy. Immobilization frequently involves the formation of covalent or ionic bonds but can also be based on physical adsorption. The immobilization of enzymes forms the basis of many biotechnological processes and analytical devices. Immobilization of proteins on solid supports results in an increased stability of the insolubilized proteins (*21*) due to rigidification of their three-dimensional structures, which results in a higher resistance to conformational changes induced by heat, organic solvents, or pH (*22*−*24*). In addition, immobilization per se involves the fixation of the individual molecules of enzyme, which reduces losses of activity by autolysis and increases the half-life of the enzymes.

Glyoxyl-agarose beads have been successfully employed for the immobilization-stabilization of enzymes, resulting in high stabilization factors and preservation of enzymatic activities. Immobilization is based on the reaction between aldehyde groups in glyoxyl-agarose and primary amino groups in the enzymes, which are mostly ϵ-amino groups in lysine residues but also terminal amino groups. Many enzymes have been stabilized using this technique, including trypsin and chymotrypsin (*25*), alcalase (*26*), carboxypeptidase A (*27*), catalases (*28*), and lipases from different sources (*29*).

The assay of ACE inhibition by food-derived peptides is usually carried out by using soluble ACE in a batch process (*30*, *31*). The purification of this enzyme from tissues is not an easy task, and the resulting preparation loses activity very fast. In addition, ACE commercial preparations are very expensive. The goal of this work was to develop a procedure for

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immobilization of ACE in order to increase its stability and ease the screening for ACE inhibitory peptides in food products.

2. MATERIALS AND METHODS

2.1. Materials. Hippuryl-L-histidyl-L-leucine (HHL), 2,4,6-trichloro-S-triazine (TT), and phenyl-methylsulfonyl fluoride (PMSF) were purchased from Sigma (Tres Cantos, Madrid, Spain). 4 BCL agarose (cross-linked 4% beads) was provided by Iberagar (Coina, Barreiro, Portugal). All other chemicals were of analytical grade. The sunflower inhibitory peptide Phe-Val-Asn-Pro-Gln-Ala-Gly-Ser was synthesized using Fmoc chemistry at the protein facility of the Centro de Biología Molecular (CSIC, Madrid), using a 431A peptide synthesizer (Applied Biosystems Inc., Uberlingen, Germany).

2.2. Preparation of ACE. ACE was prepared using porcine lungs provided by MACUMASA (Cumbres Mayores, Huelva, Spain). Tissue was diced and homogenized in 10 vol 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. 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 (*10*).

2.3. Activation of Agarose Gels. Activation of the 4 BCL was done according to the procedure previously described by Guisán (*21*) 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 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. 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 μmoles/mL gel). The oxidation with $NaIO_4$ was allowed to proceed for 2−3 h under 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. With the use of 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.

2.4. Immobilization of ACE. Immobilization of the enzyme on activated glyoxyl-agarose gels was done according to procedures previously described with slight modifications (*21*, *27*). The gels and different amounts of ACE were suspended in 0.2 M sodium bicarbonate pH 10 1:10 (v/v) and gently stirred at room temperature for different times. 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 (*32*).

2.5. Assay of ACE. ACE activity was determined as described by Hayakari et al. (*33*) with modifications. This method uses HHL as a substrate for ACE and relies on the reaction of the resulting hippuric acid with 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 different amounts of enzymatic extract or agarose containing ACE. 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). The immobilized enzyme is centrifuged, and the supernatant is taken where the TT is added. Absorbance at 382 nm was determined in the supernatant obtained after centrifugation at 10 000*g* for 10 min. The IC_{50} value, defined as the concentration of peptide in μM required to produce 50% inhibition of ACE, was determined by regression analysis of ACE inhibition (%) versus peptide concentration.

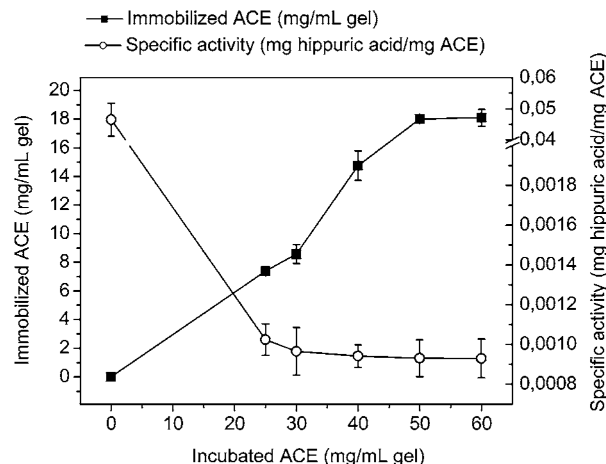

Figure 1. Effect of protein concentration on the amount of immobilized protein and specific activity of the derivatives. Different concentrations of ACE extract were incubated with the gels for 30 min at room temperature. Aliquots were taken in order to determine immobilized protein by amino acid analysis and specific activity as described in the Materials and Methods. Data represent the average ± standard deviation of three experiments.

2.6. Protein Determination. Soluble proteins were determined according to the method described by Bradford (*34*). Immobilized proteins were determined after amino acid analysis of derivatives according to the method of Alaiz et al. (*35*).

3. RESULTS

3.1. Effect of ACE Extract Concentration and Reaction Time on Immobilization. Increasing amounts of ACE tissue extract yielded increasing amounts of immobilized protein up to a maximum loading capacity of 18 mg protein/mL agarose (**Figure 1**). A similar loading capacity was previously reported for the immobilization of carboxypeptidase using the same support (*27*). Our data also shows that increasing amounts of immobilized ACE translate in decreasing specific activity of the derivatives (**Figure 1**). This is probably due to diffusion problems that limit access of the enzyme to the substrate. Diffusion problems are common when immobilizing enzymes so that a good immobilization procedure should provide a good loading while not impairing much specific activity.

Another factor that determines the amount of immobilized protein and the specific activity of the derivatives is the duration of the immobilization reaction. Thus, increasing incubation times translates into more immobilized protein up to a saturation point that is reached after incubation for 3.5 h (**Figure 2**). The specific activity keeps going down with longer incubation times. This is probably due to rigidification of the already immobilized enzyme by formation of new covalent bonds between the enzyme and the support. This rigidification will likely result in higher stability to environmental factors such as high temperatures.

3.2. Inhibition of Soluble and Immobilized ACE by a Sunflower Inhibitory Peptide. The inhibition of ACE activity by a sunflower inhibitory peptide was investigated in order to compare the soluble ACE preparation with the new ACE derivate in a real life situation. This ACE inhibitory peptide was discovered in a sunflower protein hydrolysate obtained by

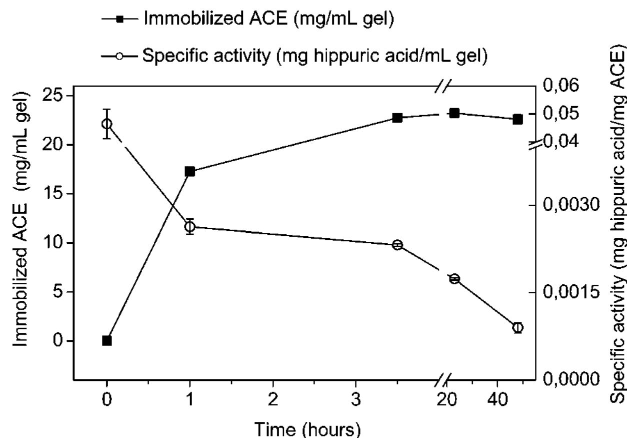

Figure 2. Effect of incubation time on the amount of immobilized protein and specific activity of the derivatives. ACE extracts (30 mg protein/mL gel) were incubated with the gel for different times. Aliquots were taken in order to determine the immobilized protein by amino acid analysis and specific activity as described in the Materials and Methods. Data represent the average ± standard deviation of three experiments.

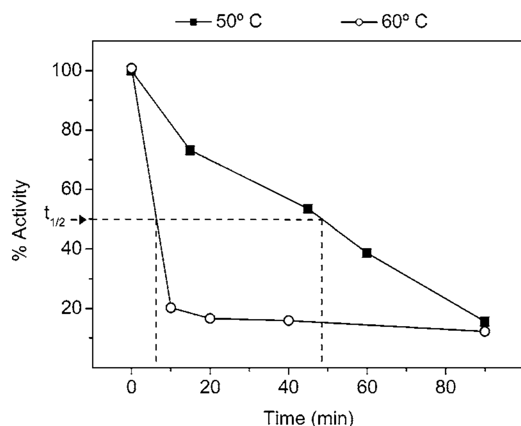

Figure 3. Thermal stability of soluble ACE at 50 and 60 °C. ACE extracts in 10 mM potassium phosphate buffer were incubated at 50 or 60 °C, and aliquots were taken at different times for assay of ACE activity as described in the Materials and Methods. The half-life ($t_{1/2}$) is the time needed to decrease the activity by half.

treatment with pepsin and pancreatin (*36*) and was synthesized for these studies as described in the Materials and Methods. It is an octapeptide with the sequence Phe-Val-Asn-Pro-Gln-Ala-Gly-Ser that behaves as a noncompetitive inhibitor of ACE (J. Vioque et al., unpublished results). The peptide had the same inhibitory effect on both the soluble and the immobilized ACE, as shown by the IC_{50} values that were obtained by regression analysis of ACE inhibition versus peptide concentration: the IC_{50}'s were 30.56 ± 1.53 and 32.7 ± 1.27 for the soluble and the immobilized enzyme, respectively (average ± standard deviation of three experiments, differences not significant $P < 0.05$).

3.3. Thermal Stability of Soluble and Immobilized ACE. Immobilization provides an increased resistance to denaturing conditions such as high temperatures. ACE in particular is rapidly inactivated by heat, as shown in **Figure 3** for incubation of soluble ACE at 50 and 60 °C. Increasing the incubation temperature from 50 to 60 °C reduced the half-life of the soluble enzyme from 48 to 6 min. In other words, soluble ACE loses its activity 7.6 times faster at 60 than at 50 °C.

The stability of soluble ACE was compared with the stability of several immobilized ACE derivatives by incubation at 60 °C for 3.5 h. Comparison is facilitated by taking the half-life times from the experimental inactivation time courses (**Figure 4**). The immobilized ACE derivatives were obtained by immobilization for 3.5, 24, and 48 h. Increasing immobilization times yield a higher number of covalent bonds between proteins and the support, resulting in a higher resistance to inactivation by high temperature. Thus, the immobilization for 3.5 h produced a derivative that was 4 times more stable than soluble ACE. Immobilization for 23 or 48 h produced derivatives 16 and 61 times more stable than soluble ACE, respectively. However, increasing number of covalent bonds between proteins and support also lead to rigidification of immobilized ACE, resulting in decreasing ACE activity. This inverse relationship is highlighted in **Figure 5**, where half-life values and activity versus incubation time are plotted.

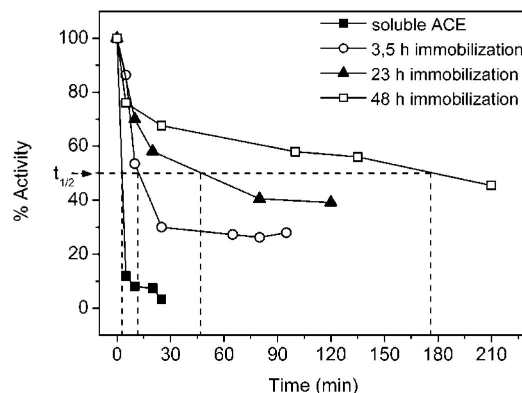

Figure 4. Thermal stability at 60 °C of soluble ACE and ACE derivatives obtained by immobilization for 3.5, 24, and 48 h (30 mg ACE/mL agarose). ACE extracts and derivatives were incubated in 10 mM potassium phosphate buffer in a heater block, and aliquots were taken at different times for assay of ACE activity.

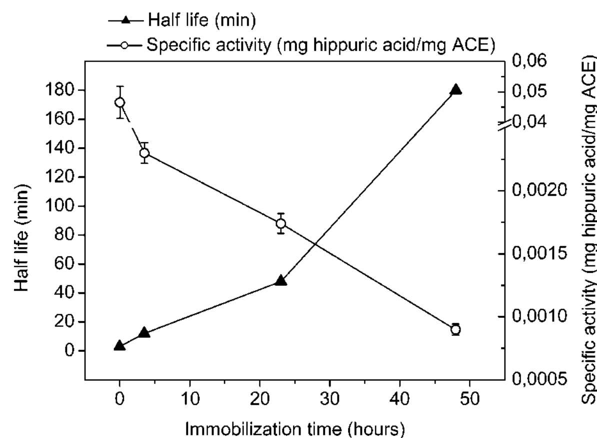

Figure 5. Half-life at 60 °C and specific activity of the derivatives obtained by immobilization for 3.5, 24, and 48 h (30 mg ACE/mL agarose). Data represent the average ± standard deviation of three experiments.

4. DISCUSSION

Enzyme immobilization is a very useful tool in biotechnology because it greatly improves protein stability in adverse physical or chemical conditions such as those involving high temperatures or the presence of organic solvents. Additional advantages are that immobilized enzymes can be used in continuous reactors and are more resistant to autolysis than soluble enzymes. The immobilization of ACE on glyoxyl-agarose supports that is reported in this paper resulted in increased thermal stability. Nevertheless, the improved thermal stability was concomitant

with a decrease in specific activity which is probably due to several factors. These include diffusion problems that limit access of the substrate to the enzymes and rigidification of the enzyme molecules. Immobilization on glyoxyl-agarose is based on the formation of covalent bonds between protein amino groups, namely, lysine and terminal amino groups, and diols in the support. Lysine residues are not usually involved in the active site of enzymes. ACE in particular contains 29 lysine residues that could theoretically react with agarose (*37*). ACE contains two identical active sites with a HEXXH zinc-binding motif (*37*) and two chloride ions that in addition to the substrate are needed for activation of the enzyme. Although none of these 29 lysine residues are directly involved in the substrate- or chloride-binding sites, the immobilization of ACE may distort the three-dimensional structure of the protein. This could affect not only the accommodation of the substrate into the active site and the coordination sites for zinc but also the interaction with the chloride ions, critical for ACE activity. However, the sunflower inhibitory peptide tested possesses an IC_{50} value similar for soluble and immobilized ACE. This peptide is a noncompetitive inhibitor, i.e., it binds to ACE in a location other than the active site (data not shown). These results suggest that the site of interaction of the enzyme with the peptide is not affected by immobilization on glyoxyl-agarose.

Partially purified ACE preparations freshly obtained from animal tissues are frequently used for assaying ACE inhibitory peptides because the commercial enzyme is expensive. The preparation of fresh ACE extracts is time-consuming and provides extracts with specific activities that vary from batch to batch. Immobilization of ACE as described here is uncomplicated and can be readily performed in a few hours, yielding preparations that are stable at 4 °C for several months. In addition, the reagents that are needed are easily available and agarose supports with different characteristics can be obtained in bulk quantities. A variety of activated supports that can be directly used for ACE immobilization are commercially available.

Agarose derivatives are well-known supports for protein immobilization. These derivatives are gels that are very easily handled and are compatible with spectrophotometric determinations. Most importantly, agarose gels can be used in batch processes because they are compressible and resistant to mild stirring devices (*21*). Since the porosity of the agarose gels is inversely proportional to the agarose concentration, a low agarose concentration (4% beads cross-linked) was chosen as the solid phase for our experiments. A high porosity was desirable because a whole tissue extract was to be used as the source of ACE. However, if pure ACE is used for immobilization, using a higher agarose concentration (6%, 8%, or 10%) may be useful in order to increase the loading capacity of the derivatives. Cross-linking does not change the porosity of the gels and increases their strength and resistance to denaturing conditions as compared to those of uncross-linked agarose. By last, the activation of the gels with glycidol (see the Materials and Methods) produces reactive aldehyde groups that are well exposed on the gel surface, which reduces diffusion problems.

In conclusion, ACE immobilization is a good alternative to using soluble freshly prepared or commercial preparations because of economical and practical reasons. It provides a great improvement in enzyme stability and facilitates the implementation of protocols in which the enzymatic reaction does not need to be stopped by addition of any chemicals or high temperature because the immobilized enzyme can be removed mechanically by centrifugation or filtration. In addition, the successful immobilization of ACE opens the door to methodologies for purification of ACE inhibitory peptides by affinity chromatography (manuscript in preparation).

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