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Assessment of the Spectrophotometric Method for Determination of Angiotensin-Converting-Enzyme Activity: Influence of the Inhibition Type

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A set of in vitro assay conditions were selected for the determination of ACE-inhibitory activity, and the need was demonstrated to standardize this assay so that the results obtained by different authors may be comparable. The conditions selected were as follows: 10 mM HHL concentration in 0.2 M potassium phosphate buffer and 0.3 M NaCl and 26 mU of ACE/mL as reaction medium; incubation time, 80 min at 37 °C. The method was applied to the study of ACE-inhibitory activity of dairy product and wine samples. Of the samples assayed, it was infant formulae whey that produces the greatest ACE inhibition. Red wine also presents a high inhibition percentage. This latter sample has an important matrix effect that must be corrected in the calculation. ACE-inhibition type was also studied, using a yogurt whey and a Captropil solution as substrates. The whey produced noncompetitive inhibition and the Captropil competitive inhibition.

KEYWORDS: Angiotensin converting enzyme; spectrophotometric assay; inhibition type

INTRODUCTION

Hypertension is a multifactorial process and the main cause of illness in industrialized countries. One of the most important intermediary factors is the action of the angiotensin-converting enzyme (ACE). ACE is a dipeptidilpeptidase containing Zn in its composition. It acts by hydrolyzing the inactive decapeptide Angiotensin I and by releasing the octapeptide Angiotensin II (*1*), which exercises a powerful vasoconstrictive action and stimulates the secretion of aldosterone, which favors the retention of sodium and water and the consequent increase in artery pressure. ACE also acts on the kinin−kallikrein system, breaking down the bradykinins (*2*), compounds with a powerful vasodilatory action. Thus, ACE-inhibitor compounds will result in an antihypertensive action.

Different ACE-inhibitory peptides have been described originating from different food sources, released after hydrolytic (*3*) and/or fermentation processes (*4*). Thus, ACE-inhibitory peptides originating from vegetable proteins (*5, 6*) and from animal proteins (*7, 8*) have been isolated and characterized. In recent years, study has focused on the isolation of peptides released after the fermentation processes taking place during the dairy products (*9, 10*) and wines (*11*) elaboration.

All of the in vitro assays used to determine ACE-inhibitory activity are based on the method of Cushman and Cheung (*12*). However, different authors have developed different modifications to the method. These changes affect the buffer composition used in the reaction, the enzyme/substrate relation, and the reaction time (*5*−*7, 9, 10, 13*−*24*).

In vitro ACE-inhibitory activity is quantified by means of the hippuric acid formation by causing hippuryl−histidyl−leucine to react with the angiotensin-converting enzyme in the presence and absence of inhibitor, measuring the absorbances at 228 nm (λ maximum absorption of hippuric acid). The decrease in absorbance is proportional to the inhibition exercised by the assayed inhibitor. Any modification of the method may give rise to a variation in the hippuric acid formation, thus making it very difficult to compare the results obtained by the different authors.

The objective of this study is to select a set of in vitro assay conditions for the determination of ACE-inhibitory activity by progressively studying the influence of the different variables of the reaction, concentration and nature of the buffer used, enzyme/substrate relation, and reaction time, and to demonstrate the need to standardize this assay so that the results of the studies obtained by the different authors may be comparable.

MATERIALS AND METHODS

Reagents. Angiotensin-converting enzyme (ACE) of rabbit lung (EC 3.4.15.1), hippuryl−histidyl−leucine (HHL) and Captopril were purchased from Sigma Chemical Co. (St. Louis, MO).

Samples. The samples used in this study were purchased from Spanish markets.

There were two samples of wine, one white and one red, and wheys from two yogurts, one full-fat and one skimmed, and the infant formula whey. All the wheys were obtained by centrifuging the samples at 8000 g for 30 min at 5 °C and filtering the supernatant through Whatman

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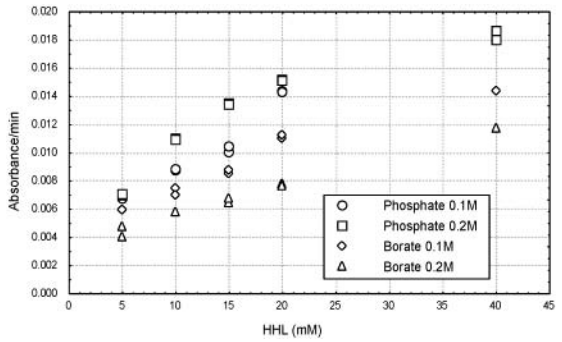

Figure 1. Reaction rate (absorbance/min) as a function of the HHL concentration.

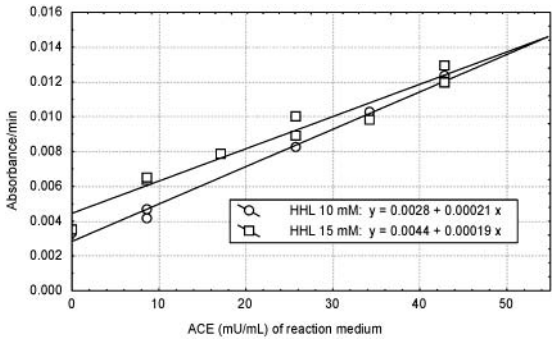

Figure 2. Reaction rate (absorbance/min) as a function of ACE concentration for the two HHL concentrations assayed and regression lines obtained.

no. 40 filters. The pH of the infant formulae was previously adjusted to 4.6 with HCl 1 N.

ACE Activity Assay. The following method is based on that described by Cushman and Cheung (*12*), with some modifications.

A 110 μL amount of substrate (HHL dissolved in a pH 8.3 buffer with 0.3 M NaCl) and 25 μL of ACE dissolved in glycerol at 50% were added to 15 μL of distilled water. The reaction solution was incubated at 37 °C. ACE activity was stopped by a decrease in pH by addition of 110 μL of 1 N HCl. The hippuric acid formed in the enzymatic process was extracted with 1 mL of ethyl acetate, shaken, and later centrifuged at 3000 g for 10 min. A 750 μL amount of the organic layer was taken and dried out at 95 °C for 10 min. The residue was redissolved in 1 mL of distilled water, and the absorbance was measured at 228 nm.

The reaction blank was prepared in the same way indicated above, changing the order in which the reagents were added, i.e., by adding the HCl before adding the enzyme. In this case, it is not necessary to perform the incubation.

ACE-Inhibitory Activity. The inhibition percentages of the yogurt wheys, infant formulae whey, and wines were determined using the conditions selected in this study, replacing the 15 μL of water with the same volume of the samples to be studied. The determinations were carried out in triplicate.

The sample blank was prepared in the same way that the reaction blank was prepared, replacing the volume of water by the sample evaluated.

Statistical Methods. The statistical methods used for analysis were as follows: linear regression analysis of reaction rate against enzyme concentration and absorbance versus reaction time; ANOVA to determine if there are significant differences between absorbance values in the study of the reaction time and between the inhibition percentages in the study of the type of inhibition.

STATISTICA (*25*) and STATGRAPHICS (*26*) programs were used for data processing. These programs were run on a Pentium personal computer.

RESULTS AND DISCUSSION

Selection of Reaction Buffer. Using 6 mU of ACE in 25 μL (corresponding to 40 mU/mL of reaction medium), HHL concentrations were assayed ranging from 5 to 40 mM. The buffers assayed were potassium phosphate and sodium borate at concentrations of 0.1 and 0.2 M (pH 8.3). Incubation time was 60 min. Different authors have used HHL concentrations ranging from 3.8 to 50 mM (*10*, *14*) and 0.1 M phosphate buffer (*19*), 0.1 M borate buffer (*20*), and 0.2 M borate (*22*). However, we have not found studies in which the reaction was carried out in a 0.2 M phosphate buffer medium.

Figure 1 shows the experimental reaction rate, expressed as absorbance per minute, as a function of HHL concentration for the different buffers assayed. At the HHL concentrations assayed (5−40 mM), no substrate inhibition was observed. At concentrations higher than 40 mM, HHL was insoluble. However, Cushman and Cheung (*12*) observed substrate inhibition when assaying HHL concentrations ranging from 0 to 20 mM. This fact could be due to the different enzyme activity used by them.

The 0.2 M potassium phosphate buffer was selected for the later studies because it provided a reaction medium in which the reaction rate of the enzyme on the substrate was faster. The HHL concentration used in the assay must supply appropriate hippuric acid absorbance values to get maximum differences between the reaction blank and enzymatic reaction without inhibitor. This fact would allow different substances with similar ACE-inhibitory activities. For later studies we selected HHL concentrations of 10 and 15 mM.

Selection of the Enzyme and Substrate Concentrations. ACE activity was studied by assaying variable quantities of the enzyme, ranging from 8 to 43 mU/mL of reaction medium. The HHL concentration was 10 and 15 mM in 0.2 M phosphate buffer. Incubation time was 60 min. The ACE quantity used in the different studies varied from 8 to 480 mU/mL of reaction medium (*14*, *18*).

Figure 2 shows the experimental reaction rate vs ACE concentration for the two HHL concentrations used and the two fitted lines. From the *F*-ratio test of lack of fit (*26*) the lineal model appears to be adequate for the observed data ($p > 0.10$). The values of the determination coefficients (R^2) of the lines were 0.99 using 10 mM HHL and 0.95 for 15 mM HHL. These lines do not pass through the origin because ethyl acetate was able to extract 1% of unhydrolyzed HHL (*12*), and λ of HHL and hippuric acid maximum absorbance is the same.

From comparison of the regression lines (*26*) there was not a statistically significance difference ($p > 0.10$) between the slopes for the two HHL concentrations used. Nevertheless, there were statistically significant differences ($p < 0.10$) between the intercept for the two HHL concentration. The absorbance of the reaction blank was least for a HHL concentration of 10 mM, thus, this concentration was selected for later studies. The ACE concentration selected was 26 mU/mL of reaction medium. With this ACE concentration it was possible to obtain reaction rate values that showed suitable sensitivity to measurement and the reduction of ACE consumption.

Study of the Reaction Time. With the buffer, substrate, and enzyme conditions selected, a study was carried out of the enzymatic reaction at different incubation times, ranging from 30 to 120 min. The incubation time was extended to observe whether the sensitivity of the method improved. Different authors have incubated the reaction for a period ranging from 30 to 60 min (*5*, *22*).

Figure 3 shows the absorbance values as a function of reaction time. At incubation times close to 100 min, a balance was attained with values that were not significantly different

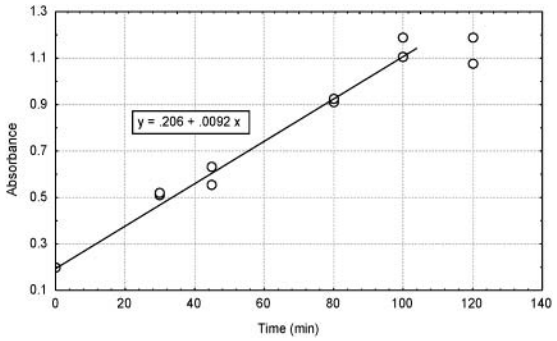

Figure 3. Absorbance values observed as a function of reaction time and regression line obtained.

from those obtained at 120 min ($p > 0.05$) (*25*). **Figure 3** shows a lineal adjustment of the experimental data corresponding to incubation times of at least or equal to 100 min ($R^2 = 0.99$) (*26*). The increase of the incubation time to 80 min leads to an important increase in absorbance.

Study of the Matrix Effect of the Inhibitor. A study was done on the possible presence in the samples of compounds that could be extracted by ethyl acetate and absorb at 228 nm. These compounds, therefore, would be responsible for interferences in measurement.

The absorbance values of the sample blanks at 228 nm, using the yogurt and infant formulae wheys, were not significantly different from those obtained in the reaction blanks. This is due to the absence of substances that can be extracted with ethyl acetate in an acid medium and with absorbance at the wavelength of the study. Similar results were obtained when studying the white wine sample blank. However, the red wine sample blank showed a high absorbance (0.41 mean value), compared to the absorbance of the reaction blank (0.23 mean value). This different behavior between the white wine and the red wine could be due to the different composition of phenol compounds of the two wines, a fundamental difference between them.

ACE-inhibition percentage in the yogurt wheys, the infant formulae whey and the white wine were calculated in accordance with eq 1

$$\%\text{IACE} = 100(A - C)/(A - \text{B}) \qquad (1)$$

where *A* represents absorbance in the presence of ACE, *B* absorbance of the reaction blank, and *C* absorbance in the presence of ACE and inhibitor.

For samples in which there are substances responsible for interferences in the analysis, such as the red wine, ACE-inhibition percentage is calculated in accordance with eq 2

$$\%\text{IACE} = 100[(A - B) - (C - D)]/(A - B) \qquad (2)$$

where *A* represents absorbance in the presence of ACE, *B* absorbance of the reaction blank, *C* absorbance in the presence of ACE and inhibitor, and *D* absorbance of the sample blank.

Thus, it is necessary to know, prior to the analysis, the composition of the sample whose ACE-inhibitory activity is to be determined. The existence of substances that could interfere in the analysis will be taken into account.

The conditions established in this study to determine ACE activity in vitro were as follows: 110 μL of 10 mM HHL, dissolved in a pH 8.3 buffer (0.2 M phosphate and 0.3 M NaCl), 26 mU ACE (dissolved in glycerol at 50%) per milliliter of reaction medium. The total reaction volume was 150 μL. Incubation time was 80 min at 37 °C. The enzyme was inactivated by the addition of 110 μL of 1 N HCl. The reaction product was extracted with 1 mL of ethyl acetate, taking 750 μL of the organic layer, which was dried out. The hippuric acid was redissolved in 1 mL of distilled water, and the absorbance value was determined at 228 nm. In those cases where the sample to be studied contains substances responsible for interferences, it is necessary to determine the absorbance of these substances and apply the formula provided in this study.

Table 1. ACE-Inhibitory Activity of Yogurt Whey and Wine Samples[a]

sample	% IACE	RSD (%)[b]
full-fat yogurt whey	43.4	3.4
skimmed yogurt whey	39.5	3.5
infant formulas whey	87.2	4.4
white wine	13.1	8.9
red wine	79.7	5.0

[a] ACE-inhibitory activity is expressed as the percent inhibition. Results are expressed as an average ($n = 3$). [b] Relative standard deviation (RSD) expressed as percent.

ACE-Inhibitory Activity of the Samples Studied. The mean values and standard relative deviation of ACE-inhibition percentages of the wheys and wines samples are shown in **Table 1**. ACE-inhibitory activity values ranged from 13.1% to 87.2%. ACE-inhibitory activity was similar for the two yogurt whey samples. The fat content had no effect on the ACE-inhibitory activity of these wheys. The high ACE-inhibitory activity values could be due to the peptides released after the action of the proteolytic enzymes from the bacterial strains, responsible for the fermentation process, on the yogurt proteins. The greatest ACE-inhibitory activity was obtained for the infant formulae whey. The hydrolysis of the seroproteins used in the infant formulae manufacture leads to peptides release with ACE-inhibitory action. Both enzymatic hydrolysis and fermentation processes have been described as being responsible for the ACE-inhibitory peptides released from proteins of different origins (*27*).

The white wine showed low ACE-inhibitory activity values. This could be due to the low proportion of peptides existing in the wines (*28*) compared to the fermented or hydrolyzed dairy products. However, the red wine showed an ACE-inhibitory activity of 80%. Phenolic compounds are found in red wines in larger quantities than those found in white wines (*29*). It could be these compounds that are responsible for this activity (*30*, *31*). Numerous beneficial properties have been cited for these compounds (*32*), among them the ability to reduce arterial pressure. Their mechanism of action is not well-known at present. ACE-inhibitory activity determined in this study could contribute to this antihypertensive action.

Study of ACE-Inhibition Type. Maintaining the quantity of enzyme constant at 26 mU/mL and varying the concentration of HHL from 5 to 40 mM, ACE-inhibitory activity of the full-fat yogurt whey and of a 1.3 mg/L Captropil solution was determined. This made it possible to compare the type of inhibition exercised by each one. Captropil is the most widely used antihypertensive drug at present (*33*). Different authors have used Captopril as a control in their ACE-inhibition studies (*34*).

Table 2 shows ACE-inhibition percentages for the full-fat yogurt whey as a function of the HHL concentration used. The percentages obtained were not significantly different ($p > 0.05$) (*25*). This behavior is characteristic of noncompetitive inhibitors. These inhibitors bind to the enzyme in an area other than the active center, deform the enzyme, and hinder binding to the substrate (*35*). This inhibition type usually occurs for enzymes

Table 2. ACE-Inhibitory Activity of Full-Fat Yogurt Whey and Captopril Solution[a]

	% IACE[b]	
HHL (mM)	full-fat yogurt whey	Captopril
5	46.6	63.8
10	45.1	60.6
20	48.8	40.1
40	45.9	35.0

[a] ACE-inhibitory activity is expressed as the percent inhibition. Results are expressed as the average ($n = 3$). [b] Percent inhibition measures the percent inhibition of ACE activity as a function of HHL concentration.

containing a functional group necessary to maintain its catalytically active three-dimensional form. ACE is a zinc−metalloenzyme, and so the binding of an inhibitor to this ion could lead to a decrease in the enzyme activity.

Table 2 also shows ACE-inhibition percentages for a Captopril solution as a function of the HHL concentration used. ACE-inhibitory activity exercised by Captopril decreased when the HHL concentration increased ($p < 0.05$) (*25*). This behavior is typical of competitive inhibitors. These types of inhibitors compete with the substrate for the active center of the enzyme. The increase in the substrate concentration results in a reduction of the inhibitor's capacity to compete with the substrate (*35*).

Existing studies in the literature have been carried out using specific analysis conditions for each sample studied. However, comparison of ACE-inhibition percentage values is only possible if the conditions of the method are identical or for those samples that inhibit ACE in a noncompetitive way. For this reason it is extremely important to unify the conditions of the study and to know the composition and characteristics of the sample to be analyzed.

A set of analysis conditions is proposed for the in vitro evaluation of ACE-inhibitory activity and the need to correct the formula for the calculation of ACE-inhibition percentages for those samples that contain substances that interfere in the analysis.

The study of ACE-inhibition type demonstrated the existence of compounds that act as competitive inhibitors, such as Captopril, where the analytical conditions have an effect on the inhibition percentage. It is necessary, therefore, to unify these conditions, and our method makes it possible for the different studies in the literature to be comparable.

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