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A rapid, simple and sensitive fluorescence method for the assay of angiotensin-I converting enzyme

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Abstract

A fluorescent assay for angiotensin-I converting enzyme (ACE; EC 3.4.15.1) is described. It is based in the hydrolysis of the internally quenched fluorescent substrate *o*-aminobenzoylglycyl-*p*-nitrophenylalanylproline by the action of ACE. The fluorescence generated by the liberation of the product (the *o*-aminobenzoylglycine group) is read in a microtiter-plate multiscan fluorometer. The different conditions for the assay have been optimised for linearity, sensitivity and precision. Maximal enzyme activity was reached in the pH range 8.0–8.5 and 0.5–0.75 M NaCl concentration in the assay mixture. Kinetics of the enzyme reaction displayed a $K_m = 109$ μM, obtaining an optimal substrate concentration of 0.3 mM in the assay mixture. The assay was adequate for the study of ACE inhibition by captopril and several peptides and it also showed a very good correlation with a well-established method [Biochemical Pharmacology, 20(7) (1971) 1637]. The method has important advantages, being the availability of reagents, its simplicity and the capacity to process a high number of samples in a short time, most outstanding.

Keywords: Angiotensin-I converting enzyme; ACE; Hypertension; Fluorimetric assay; Enzyme assay

1. Introduction

Angiotensin-I converting enzyme (ACE; EC 3.4.15.1) is also known as peptidyl dipeptidase A because it removes C-terminal dipeptides from a wide variety of peptide substrates. One of its key actions is the regulation of blood pressure together with water and salt metabolism, since it cleaves angiotensin I into the potent vasopressor angiotensin II. It also inactivates bradykinin, which is an hypotensive peptide, by sequential removal of two C-terminal dipeptides. So, the result of ACE action is an elevation of blood pressure (Corvol, Eyries, & Soubrier, 2004). Apart from this, angiotensin-I converting enzyme has also been related with other physiological processes, and so abnormal levels of ACE activity in serum have been related with the apparition of some diseases (Beneteau-Burnat & Baudin, 1991; Lieberman, 1975). Thus, during the last three decades there has been great interest in the development of methods for accurate assay of ACE activity. In the food area, studies have been mainly focused on the identification of food components, principally peptides, able to inhibit ACE activity with the aim to control hypertension and then prevent cardiovascular diseases through diet. In fact, nutrition has been reported as one of the main factors influencing blood pressure (Houston, 2002). Several peptides from a wide variety of foods with ACE inhibitory activity have been recently reported (Jang & Lee, 2005; Kitts & Weiler, 2003; Yamamoto, Ejiri, & Mizuno, 2003; Yang, Marczak, Usui, Kawamura, & Yoshikawa, 2004). In most of these works, the assay used for ACE activity was based in the hydrolysis of the synthetic peptide hippuril–His–Leu as described by Cushman and Cheung (1971) or with some modifications

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(Arihara, Nakashima, Mukai, Ishikawa, & Itoh, 2001; Hernandez-Ledesma, Martin-Alvarez, & Pueyo, 2003; Kim, Kim, & Song, 2003). Although this assay has been very useful during decades, it has some limitations, such as the required extraction of the product from the reaction mixture with an organic solvent, which limits the number of samples that can be analysed per day and is also an additional source of error. This fact also makes unfeasible the continuous monitoring of the enzyme reaction.

In the present work, we describe the development of a rapid, simple and sensitive assay for ACE activity. The chosen substrate was the intramolecularly quenched fluorescent tripeptide *o*-aminobenzoylglycyl-*p*-nitrophenylalanylproline (Abz–Gly–Phe(NO_2)–Pro) developed by Carmel and Yaron (1978). Hydrolysis of this substrate by the action of ACE generates the fluorescent product *o*-aminobenzoylglycine (Abz–Gly). We have established the optimal conditions for the development of a continuous assay in a fluorescent microplate reader, using a single reagent in an automated assay (once the microplate is ready), very easy to carry on and with capacity to process a high number of samples.

2. Materials and methods

Reagents. ABz–Gly–Phe(NO_2)–Pro and Abz–Gly were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland). Captopril and $CoCl_2$ were from Fluka Chemie AG (Buchs, Switzerland). Angiotensin-I Converting Enzyme from rabbit lung, $ZnCl_2$ and the peptides employed in the inhibition studies were obtained from Sigma (St. Louis, MO, USA). Ethyl acetate was from Merck (Darmstadt, Germany). Buffer salts and general reagents were from PanReac Química S.A. (Montcada i Reixac, Barcelona, Spain).

2.1. Preparation of enzyme solutions

Stock solution. Commercial angiotensin-I converting enzyme (0.51 mg) was diluted in 3.334 mL of a 50% glycerinated solution with 0.15 M tris buffer, pH 8.3, containing 1 μM $ZnCl_2$. This solution was kept at −20 °C until use.

ACE working solution. Stock solution was diluted 1/10 with 0.15 M tris buffer, pH 8.3 having a concentration of 14.4 μg mL^{-1} of ACE corresponding to 7.1 mU mL^{-1} of enzyme activity in the final reaction solution.

2.2. Assay of angiotensin-I converting enzyme

Fluorimetric assay. The assay was optimised until reaching the following standard method: 50 μl of ACE working solution were added to each microtiter-plate well, then adjusted to 100 μl by adding either bidistilled water to controls or samples in inhibition studies. The enzyme reaction was initiated by the addition of 200 μl of 0.45 mM Abz–Gly–Phe(NO_2)–Pro dissolved in 150 mM Tris-base buffer, pH 8.3, containing 1.125 M NaCl that are immediately mixed and incubated at 37 °C. For the optimisation, each variable was assayed in different ranges of values. The generated fluorescence was measured at 15 min intervals till 1 h using a multiscan microplate fluorometer (Fluoroskan Ascent, Labsystems, Finland). Ninetysix-well microplates were used. Excitation and emission wavelengths were 355 and 405 nm, respectively.

Spectrophotometric assay. As reference, ACE activity was also measured spectrophotometrically ($\lambda = 228$ nm) according to the method of Cushman and Cheung (1971). This method is based on the release of hippuric acid from the synthetic peptide hippuril–His–Leu (Hip–His–Leu).

One unit of ACE activity (U) was defined as the amount of enzyme hydrolysing 1 μmol of Abz–Gly–Phe(NO_2)–Pro per minute at 37 °C. Three measures were done for each experimental point.

Precision of the assay. Three different concentrations (7.2, 14.4 and 28.7 μg mL^{-1}) of pure rabbit lung ACE were used to calculate both intra and inter-assay variabilities of the ACE assay described in the present paper. A total of 10 repetitions were done for each determination.

Inhibition studies. Inhibitory activity of captopril (0.1–100 nM) and the peptides Ala–Ala, Ala–Ala–Ala, Arg–Ala, Gly–Pro–Ala, Tyr–Ala, Ala–Tyr and Phe–Gly–Gly–Phe was determined at different concentrations (0.5–100 μM) by using the optimised fluorimetric assay previously described, adding 50 μl of each compound to the enzyme solution before the addition of the substrate. All measures were carried out by triplicate.

3. Results and discussion

The methodology presented in this work is based on the hydrolysis of the intramolecularly quenched fluorogenic substrate Abz–Gly–Phe(NO_2)–Pro by angiotensin-I converting enzyme, resulting in the release of free *o*-aminobenzoylglycine (Abz–Gly), which generates fluorescence that can be detected in a fluorimeter, reaching maximal emission at 405–415 nm. As usual for fluorometric determinations, it is necessary to accurately determine optimal conditions for linearity and reproducibility.

3.1. Effect of pH and salt concentration

The assay of angiotensin-I converting enzyme from rabbit lung showed maximal activity at pH 8.3

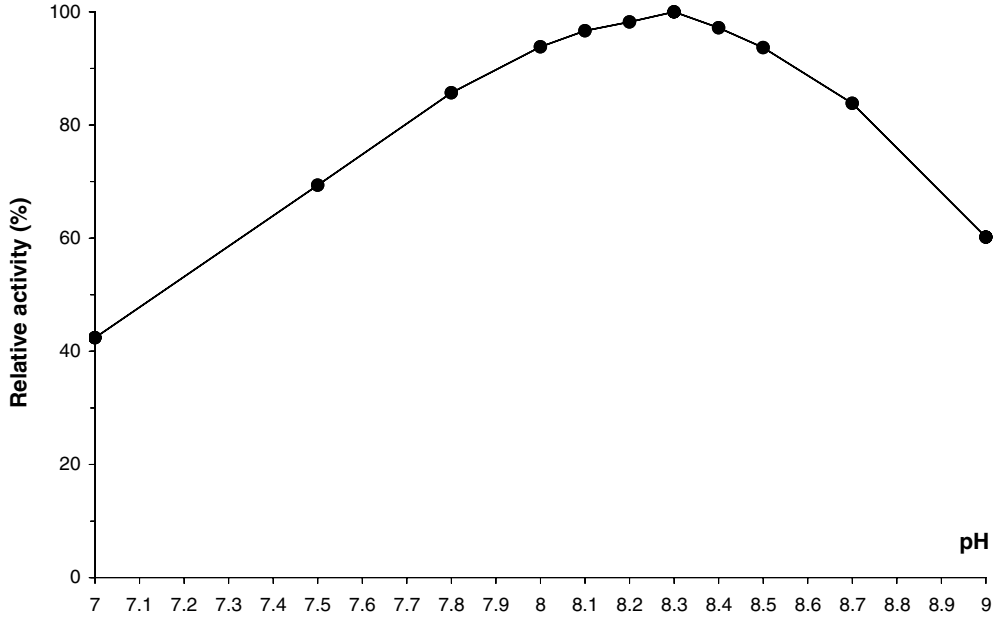

Fig. 1. pH dependence of ACE activity in the hydrolysis of Abz–Gly–Phe(NO_2)–Pro. Assay at various pH values following the standard assay.

(Fig. 1), and this value was chosen for the microplate standard enzyme assay. Under our assay conditions, more than 93% of maximal activity was retained in the pH range 8.0–8.5, in accordance with results obtained previously by other authors either using the same substrate with different enzyme sources (Carmel, Ehrlich-Rogozinsky, & Yaron, 1979; Maguire & Price, 1984; Neels, Scharpe, Fonteyne, Yaron, & van Sande, 1984a) or using different substrates (Cushman & Cheung, 1971; Hayakari & Kondo, 1977; Johansen, Marstein, & Aas, 1987; Ronca-Testoni, 1983). On the other hand, optimal pH values around 7.5 have also been described (Persson & Wilson, 1977; Piquilloud, Reinharz, & Roth, 1970; Yang, Erdos, & Levin, 1971) for ACE from human and hog samples.

The effect of NaCl concentration on the hydrolysis of Abz–Gly–Phe(NO_2)–Pro by ACE was assayed at pH 8.3 using both Tris or phosphate buffer (see Fig. 2). The chloride requirement for ACE activity against most substrates is extensively reported in the literature (Corvol et al., 2004; Ryan, 1988; Stewart, Weare, & Erdos, 1981). Maximal activity is reached within the interval 0.5–0.75 M NaCl, that is in agreement with results of other works using the same substrate (Carmel & Yaron, 1978; Maguire & Price, 1984). If we compare total enzyme activity, 50% lower activity was obtained when using phosphate buffer instead of Tris (Fig. 2). This behaviour has been reported by other authors with different substrates (Bunning, Holmquist, & Riordan, 1983; Oliveira, Santos, & Krieger, 2000; Persson & Wilson, 1977; Yang et al., 1971). However, Neels et al. (1984a) did not observe any inhibition due to phosphate buffer using the same substrate like us. When using the spectrophotometric assay of angiotensin-I converting enzyme with Hip–His–Leu as substrate, which is the method most frequently used in food-related works to determine ACE activity, 40% lower activity was reported when Tris buffer instead of phosphate was used (Cushman & Cheung, 1971). Some authors have observed an increase of the enzyme activity when Na_2SO_4 was also present in the reaction mixture (Neels et al., 1984a; Ryan, 1988). In our case this increase was lower than 15% (results not shown), making unnecessary the addition of this salt in the final reaction mixture.

3.2. Linearity and sensitivity

Kinetics of the enzyme at different incubation times are shown in Fig. 3. At short incubation times (15 min), a linear dependence of the enzyme reaction was observed for the whole concentration range. At longer incubation times, linearity was progressively lost towards slower reaction rates for the highest enzyme concentrations. Thus, the continuous ACE assay using Abz–Gly–Phe(NO_2)–Pro as substrate in multi-well plate readers remains linear for the different enzyme concentrations and incubation times up to 20% of substrate hydrolysis. For the standard assay conditions, an enzyme concentration of 2.4 μg mL^{-1} in the final mixture and incubation time of 30 min were selected. Under these conditions, the generated fluorescence response was adequate for the study of potential inhibitory actions of some food components on ACE activity.

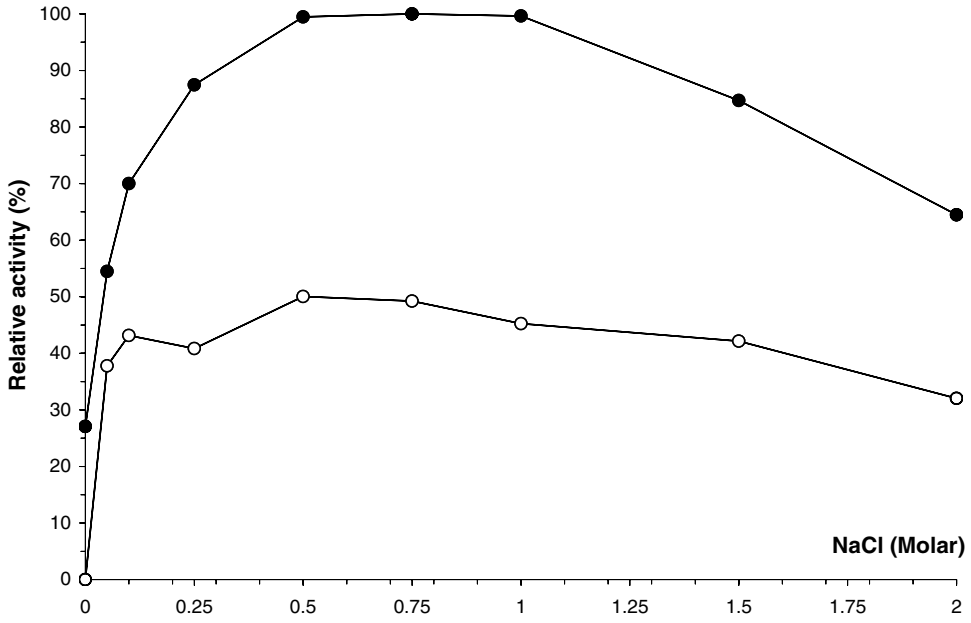

Fig. 2. Effect of sodium chloride concentration on ACE activity at pH 8.3 in (●) 0.15 M Tris buffer and (○) 0.15 M phosphate buffer.

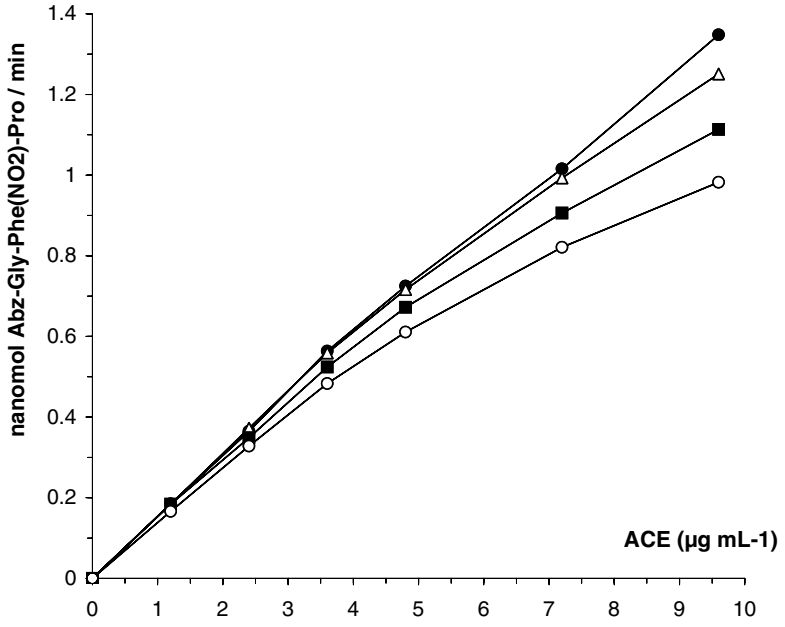

Fig. 3. Hydrolysis rate of Abz–Gly–Phe(NO_2)–Pro by ACE at different incubation times: (●) 15 min; (Δ) 30 min; (■) 45 min and (○) 60 min.

3.3. Calibration curve

In order to correlate the fluorescence obtained when reading the microtiter plate wells with the amount of product generated during the hydrolysis of Abz–Gly–Phe(NO_2)–Pro by the action of angiotensin I-converting enzyme, a standard curve was made with increasing concentrations of the product (Abz–Gly) ranging from 0 to 100 μM. As reported by Maguire and Price (1984) working with this same substrate, or by authors working with other intramolecularly quenched fluorescent substrates (Araujo et al., 1999; Persson & Wilson, 1977; Russo, Persson, & Wilson, 1978), the fluorescence of the Abz–Gly group decreased proportionally to the concentration of the substrate (see Fig. 4). This can be due to the fact that not only the product of the reaction (Abz–Gly), but also the substrate, the Abz–Gly–Phe(NO_2)–Pro molecule, absorbs the exciting light at $\lambda = 355$ nm. Another possibility is to consider a reduction in fluorescence intensity due to a partial quenching

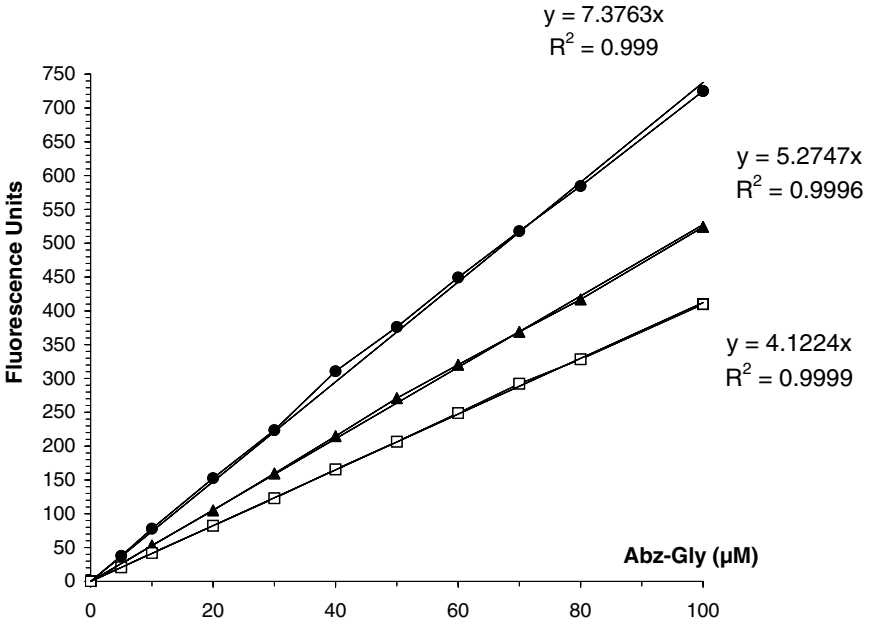

Fig. 4. Relationship between the amount of Abz–Gly in the reaction mixture and the fluorescence for the following conditions: (●) Only Abz–Gly in the reaction mixture; (▲) Abz–Gly incubated in the presence of 0.3 mM of substrate and (□) Abz–Gly in the presence of 0.3 mM of substrate and 2.4 μg mL^{-1} of ACE.

of the emission light by the substrate molecules, similar to the phenomenon reported by Volle, Dutaud, and Ouali (1999) when assaying different fluorescent probes in the presence of myoglobin. In addition, the concentration of angiotensin I-converting enzyme in the reaction mixture also decreased the fluorescence intensity of the Abz group. In any case, the linear dependence of fluorescence intensity with product concentration is maintained in all cases. The effect of the presence of substrate and enzyme in the reaction mixture is just a decrease in the slope, being data from the different curves proportionally related by a numerical factor. The curve with the lowest slope in Fig. 4 is representative of the conditions found in the reaction mixture for our standard assay procedure.

3.4. Effect of the presence of Zn^{2+} and Co^{2+} on ACE activity

The effect of the divalent cations Zn^{2+} and Co^{2+} on ACE activity under the conditions developed for the multi-well plate assay presented here was assayed. Angiotensin I-converting enzyme is classified as a metallopeptidase, requiring Zn^{2+} at the active site for stability and development of its activity (Corvol et al., 2004). So, ACE stock solution was made in 50% glycerol with tris buffer, pH 8.3, containing 1 μM $ZnCl_2$. As shown in Table 1, further addition of $ZnCl_2$ to the reaction mixture not treated previously with EDTA, had no effect on the enzyme activity, as previously reported (Maguire & Price, 1984).

Table 1
Effect of the divalent cations Zn^{2+} and Co^{2+} on ACE activity

Cation	0.01 mM	0.04 mM	0.4 mM
Zn^{2+}	99.8	98.6	91.7
Co^{2+}	71	70	71.3

The activity with no cation added was taken as 100%.

Depending on the nature of the substrate, the effect of divalent cations and other compounds seem to be different. In our case (see Table 1), cobalt exerted lower inhibition than that reported by other authors (Maguire & Price, 1984; Ronca-Testoni, 1983). In contrast, Cushman and Cheung (1971) reported an enhancement of activity with Co^{2+} using Hip–His–Leu as substrate in the spectrophotometric assay taken as reference in the present work. Similarly, in a fluorimetric assay using also Hip–His–Leu as substrate, Friedland and Silverstein (1976) obtained an enhanced ACE activity, previously inhibited by EDTA, in the presence of cobalt.

3.5. Enzyme kinetics

Fig. 5 shows the change in the enzyme reaction rates at different substrate concentrations, ranging from 0 to 0.8 mM. Experimental data fit well with a Michaelis–Menten kinetics except for the two highest assayed concentrations (0.6 and 0.8 mM), where the reaction rate decreases considerably. This can be attributed most probably to a reduction in fluorescence intensity of the product due to the absorption of either the exciting or emitting light by the substrate at high concentrations,

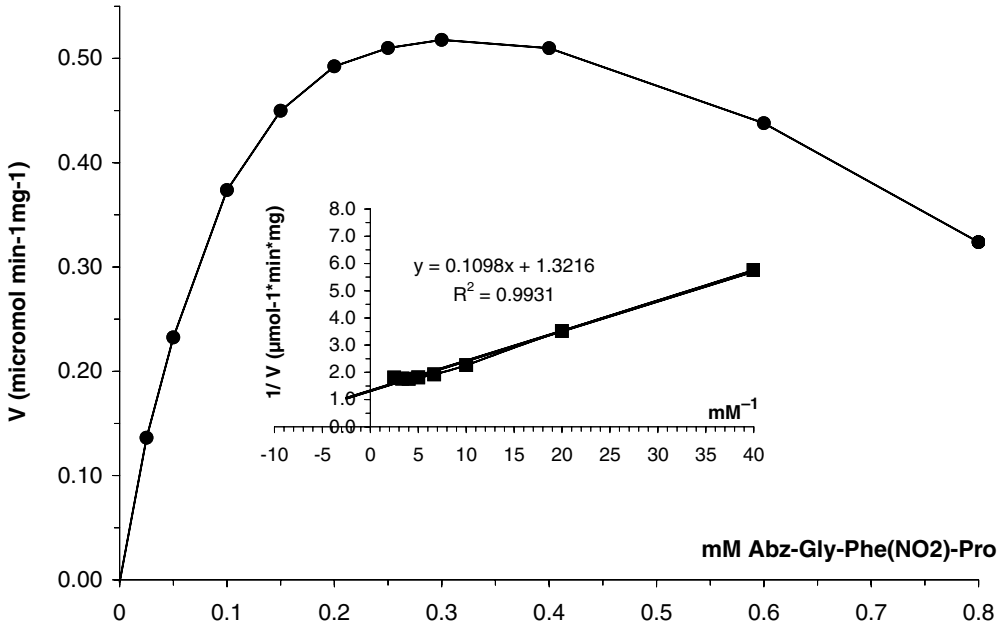

Fig. 5. Kinetics of the hydrolysis of Abz–Gly–Phe(NO_2)–Pro by ACE, following the standard assay with 30 min incubation time.

as previously discussed. Maximal hydrolysis was achieved at 0.3–0.4 mM and then, 0.3 mM concentration of Abz–Gly–Phe(NO_2)–Pro in the final assay mixture was chosen for our standard assay protocol, giving a reaction rate of 0.52 μmol min^{-1} mg^{-1}. The K_m value obtained from Lineweaver–Burk plot (Fig. 5, inset) was 109 μM for rabbit lung ACE, similar to that obtained by Carmel et al. (1979) using this substrate and human serum as enzyme source. The same authors obtained a lower K_m value (32 μM) in the case of ACE from Guinea pig serum. Maguire and Price (1984) obtained, however, a notably higher K_m value (400 μM) working also with human serum. Carmel and Yaron (1978) working with ACE from calf lung obtained also a higher K_m value of 210 μM. The good affinity of ACE by Abz–Gly–Phe(NO_2)–Pro is reflected in the notably lower K_m value obtained in comparison to those reported for Hip–His–Leu (Cushman & Cheung, 1971; Friedland & Silverstein, 1976; Hayakari & Kondo, 1977; Kapiloff, Strittmatter, Fricker, & Snyder, 1984) and also for other synthetic substrates (Bunning et al., 1983; Cheviron et al., 2000; Ronca-Testoni, 1983). We have obtained a V_{max} value of 0.74 U mg^{-1}. In practical terms, this allows appreciable hydrolysis rates at relatively short incubation times (15 min or even shorter, depending on the enzyme concentration in the assay mixture). This makes possible the rapid processing of an elevated number of samples (one microplate, 96 samples, in about hour), much more samples than with previous reported assays for determining ACE activity, even with those involving only one-step reagent (Buttery & Stuart, 1993; Maguire & Price, 1984; Neels, Scharpe, van Sande, Verkerk, & Van Acker, 1982; Neels, Scharpe, van Sande, & Fonteyne, 1984b; van der Linden, van Twisk, & Kok, 1985).

3.5.1. Precision of the assay and correlation with an established method

As shown in Table 2, the reproducibility was good in view of the small coefficients of variation obtained, comparable to data obtained previously for other methods (Johansen et al., 1987; Neels et al., 1982; Ronca-Testoni, 1983; van der Linden et al., 1985).

Activity of ACE at different enzyme concentrations was measured with both the optimised standard assay described in the present paper and that of Cushman and Cheung (1971), used as reference as it is the most common reported assay for the study of ACE inhibitors in foods. Linear regression analysis of data represented in Fig. 6 shows a good correlation ($r = 0.9925$) between both methods, obtaining a higher hydrolysis rate of Abz–Gly–Pro(NO_2)–Pro with respect to hydrolysis of Hip–His–Leu at a given enzyme concentration.

Table 2
Precision data of the assay for angiotensin-I converting enzyme using Abz–Gly–Phe(NO_2)–Pro as substrate and a multiscan fluorometer

	Final activity (mU mL^{-1})	SD (mU mL^{-1})	CV (%)
Within-run (n = 10)			
A	3.0	0.09	3.0
B	7.1	0.25	3.4
C	13.4	0.31	2.3
Between-run (n = 10)			
A	2.8	0.2	6.8
B	6.4	0.4	6.4
C	12.7	0.6	5.1

Three different concentrations for ACE, prepared from stock solution, were used as enzyme solution: (A) 7.2 μg mL^{-1}; (B) 14.4 μg mL^{-1} and (C) 28.7 μg mL^{-1}.

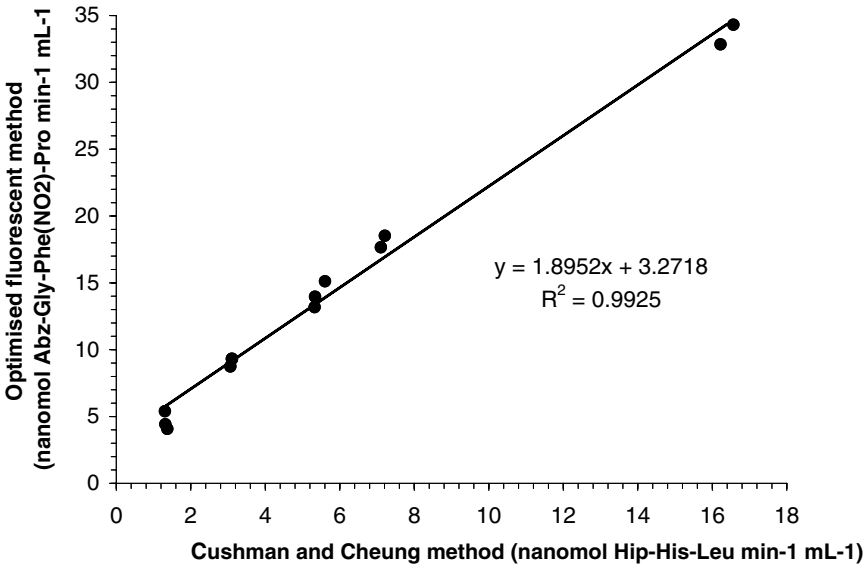

Fig. 6. Correlation between the optimised fluorimetric assay using Abz–Gly–Phe(NO_2)–Pro as substrate (ordinate) with that of Cushman and Cheung (abscissa).

3.6. Inhibition by several peptides and captopril

In order to evaluate the efficacy of our assay in the study of the inhibition exerted by some compounds (like those peptides contained in foodstuffs) on the activity of angiotensin-I converting enzyme, rabbit lung ACE was incubated with different concentrations of captopril and some peptides described in the literature as effective ACE inhibitors. As can be observed in Table 3, the selected peptides effectively exerted an inhibition of ACE activity on the hydrolysis of Abz–Gly–Phe(NO_2)–Pro, which is reflected in a clear decrease of the generated fluorescence with respect to wells without added peptide. Gly–Pro–Ala and Phe–Gly–Gly–Phe exerted moderate inhibition, not exceeding 30% of initial activity at 100 μM. Tri- and tetra-alanine, together with Arg–Ala and Tyr–Ala exerted stronger inhibition, reaching 50% of total activity, similar to results obtained by Soffer (1976). The inverse sequence of the latter, Ala–Tyr, proved to be a potent ACE inhibitor since half of total activity was inhibited with 25 μM of this peptide, and more than 80% of initial activity was suppressed at 100 μM peptide concentration (Table 3). Ala–Tyr was also assayed by Cheung, Wang, Ondetti, Sabo, and Cushman (1980) regarding the inhibition of ACE by series of dipeptides. Our results agree with their conclusions about the increased inhibitory activity of peptides containing aromatic amino acids in COOH- terminal position. However, Ala–Tyr was slightly less inhibitory of ACE activity in the hydrolysis of Hip–His–Leu (Cheung et al., 1980). Similarly, in the design of novel potent ACE inhibitors, Chen, Peng, and Yang (1992) reported that the most potent compound was found to be an Ala–Tyr derivative, *N*-(1-benzoyl-1-carboxymethyl)-L-Alanyl-L-Tyrosine. Other dipeptides derived from food proteins containing Tyr in COOH- ultimate position have also been described to be strong ACE inhibitors (Cheung et al., 1980; Kitts & Weiler, 2003; Yamamoto et al., 2003). In summary, our inhibition studies on ACE activity using Abz–Gly–Phe(NO_2)–Pro as substrate are coherent with data reported previously in the literature for the inhibition of ACE activity following the hydrolysis of Hip–His–Leu and other substrates (Cheung et al., 1980; Soffer, 1976), demonstrating the

Table 3
Effect of several synthetic peptides, described as ACE inhibitors, on the hydrolysis of Abz–Gly–Phe(NO_2)–Pro by ACE at pH 8.3

Peptide	0.5 μM	5 μM	25 μM	50 μM	100 μM
Ala–Ala	94.5	87.7	88.2	69.2	54
Ala–Ala-Ala	94.5	84.4	78.7	67	54.6
Arg–Ala	99.6	89.6	84.1	69.7	51.1
Gly–Pro–Ala	97.1	93.7	92.6	86.9	76.6
Phe–Gly–Gly–Phe	98.7	89.7	90.1	81.8	70.1
Tyr–Ala	102	101	83.3	69.2	47.6
Ala–Tyr	87.5	73	52.9	36.9	18.3

Activity in the absence of added peptide was taken as 100%.

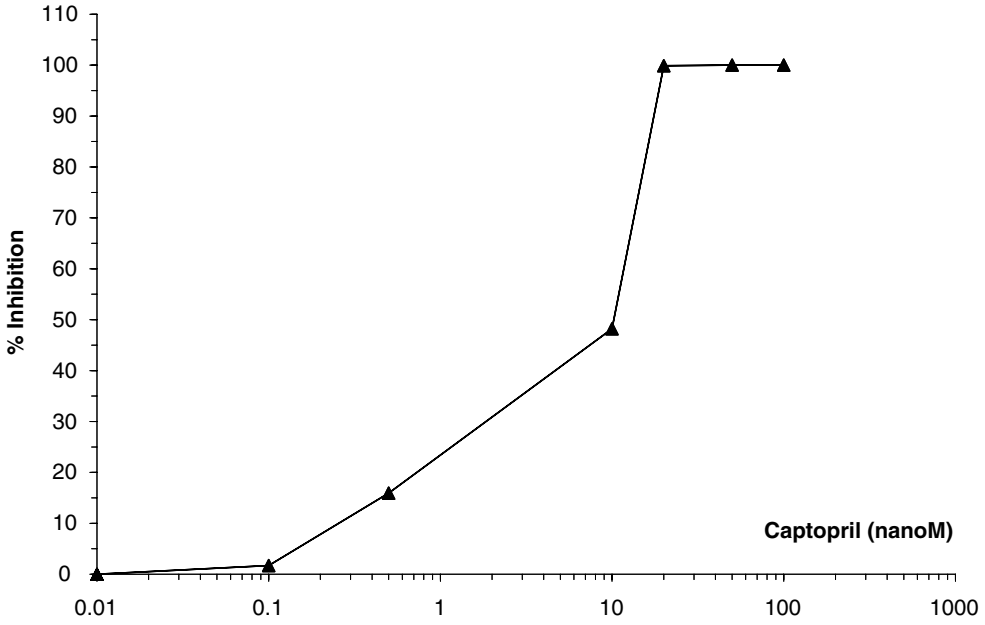

Fig. 7. Inhibition of ACE activity by captopril on the hydrolysis of Abz–Gly–Phe(NO_2)–Pro, following the standard assay. Activity without addition of captopril to the reaction mixture was taken as 100%.

utility of this assay in the study of functional foods related to ACE inhibition and hypertension.

Hydrolysis of Abz–Gly–Phe(NO_2)–Pro by the action of ACE was inhibited by captopril, as shown in Fig. 7. The high sensitivity and precision of the fluorimetric assay in microtiter plates described here allowed us to detect a remarkable inhibition of the enzyme activity with as low as 0.5 nM of captopril in the reaction mixture. Half of total enzyme activity was inhibited with 10 nM, whereas complete inhibition was achieved at 20 nM of captopril, similar to results obtained by Reneland and Lithell (1994) using Hip–His–Leu as substrate and fluorescence detection of the product.

4. Conclusion

In the present work we have described the development of a fluorimetric assay for measuring the activity of angiotensin-I converting enzyme by means of a fluorescence 96-well microplate reader, with the aim to provide newer, modern and powerful tools which can constitute a solid alternative in the study of the functional properties of foodstuffs in the regulation of blood pressure. The assay has many advantages with respect to assays most commonly used actually. Measurement of Abz–Gly–Phe(NO_2)–Pro hydrolysis in multi-well plates allows continuous monitoring of ACE activity, making possible to perform kinetic analysis easily. It involves only one-step reagent, avoiding neither further derivatization (Oliveira et al., 2000), extraction with organic solvents (Hernandez-Ledesma et al., 2003; Kim et al., 2003) nor chromatographic separations of the reaction products (Cheviron et al., 2000). This simplicity in the reaction steps also makes the assay easy to perform by non-specialised staff. Fluorescence detection of the reaction products results in high sensitivity and precision and the fact that all reagents are commercially available is a major advantage for an easy introduction of the assay in a laboratory. Multi-well plate fluorimeters are currently employed for many purposes in both research and analytical work. But the main advantage of the present method with respect to others currently in use is the processing of a considerable higher number of samples in short time, allowing rapid screening of fractions resulting from chromatographic separations of protein/food hydrolysates, or the study of many different food samples having different processing conditions.

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