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Modification of the furanacryloyl-L-phenylalanylglycylglycine assay for determination of angiotensin-I-converting enzyme inhibitory activity

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Abstract

Angiotensin-I-converting enzyme (ACE, EC 3.4.15.1) plays a central role in the regulation of blood pressure in man. The objective of this study was to evaluate and modify the furanacryloyl-L-phenylalanylglycylglycine (FAPGG) assay method for quantification of ACE activity. The fixed time conditions developed for assay of ACE activity were as follows: 0.8 mM FAPGG, 175 ± 10 units l^{-1} ACE, incubation at 37 °C for 30 min and enzyme inactivation with 100 mM ethylenediaminetetra-acetic acid (EDTA). Hydrolysis of FAPGG to FAP and GG was quantified by measuring the decrease in absorbance at 340 nm. It was shown that increasing the level ACE activity in the assay from 155 to 221 ± 15 units l^{-1} resulted in a corresponding increase in the apparent IC_{50} value for Captopril® from 9.10 to 39.40 nM. Similar trends in the apparent IC_{50} values for a whey protein hydrolysate were obtained. The results demonstrate the requirement for carefully controlling ACE activity levels in the assay in order to obtained comparable and reproducible values for the inhibitory potency of ACE inhibitors.

Keywords: Angiotensin-I-converting enzyme; FAPGG; IC_{50}; Captopril; Whey protein; ACE inhibition; Functional food ingredients

1. Introduction

Milk proteins, in addition to being a source of nitrogen and essential amino acids, also contain a range of bioactive peptides encrypted within their primary structures. Milk

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protein-derived bioactive peptides having numerous functions including opioid (agonist and antagonist), immunomodulatory, antithrombotic, antimicrobial, mineral binding, growth and muscle stimulating properties have been identified (for review see Refs. [1–4]). Potential antihypertensive peptides which inhibit angiotensin-I-converting enzyme (ACE) have also been identified in bovine caseins and whey proteins (for reviews see Refs. [5–8]).

ACE is a zinc metallocarboxydipeptidase that has also been shown to display endopeptidase activity with certain C-terminal amidated peptides [9,10]. ACE plays an important role in the control of blood pressure in humans by regulating the levels of endogenous regulatory peptides such as angiotensin II, which is a potent vasoconstrictor, and bradykinin, which is a potent vasodilator. Inhibition of ACE activity by food-protein derived peptides can lead to an overall antihypertensive effect [11,12].

Numerous methods have been used to quantify ACE activity. These include colorimetric, flourometric, radiochemical and liquid chromatographic procedures [13–17]. These assay methods may be used to obtain information on the inhibitory potency or IC_{50} value, i.e., the concentration of inhibitor needed to inhibit ACE activity by 50%, for different ACE inhibitory substances. The most widely used assays for determining ACE activity and inhibition in vitro employ hippuryl-L-histidyl-L-leucine (HHL) and furanacryloyl-L-phenylalanylglycylglycine (FAPGG) as substrates for ACE activity. The HHL assay is based on the hydrolysis of HHL to hippuric acid and HL. This is performed as a fixed time assay where the hippuric acid formed is generally extracted with ethyl acetate prior to its spectrophotometric quantification at 328 nm [13]. The FAPGG assay was originally developed to measure ACE activity in serum [18–20]. This continuous spectrophotometric assay is based on the blue shift in the absorption spectrum between 328 and 352 nm that occurs upon hydrolysis of FAPGG to furanacryloyl-L-phenylalanine (FAP) and glycylglycine (GG). Much interest has recently focused on isolation and identification of food protein-derived peptide inhibitors of ACE as these potentially represent functional food ingredients for the control of blood pressure [12]. The ACE inhibitory potency of milk protein-derived peptides and milk protein hydrolysates has generally been determined using HHL as a substrate for ACE activity [21–26]. Schlothauer et al. [27] and Vermeirssen et al. [28] recently used the continuous FAPGG assay to quantify ACE inhibitory potency of the peptides present in milk protein hydrolysates.

The objective of this study was to develop a fixed time assay for quantification of ACE activity using FAPGG as a substrate. Furthermore, the effect of the level of ACE activity in the assay on the apparent IC_{50} obtained for Captopril, a synthetic ACE inhibitory pharmacological agent, and a whey protein hydrolysate sample containing ACE inhibitory peptides was determined.

2. Materials and methods

2.1. Materials

Captopril, *N*-(3-[2-furyl]acryloyl)-L-phenylalanine (FAP), *N*-(3-[2-furyl]acryloyl)-L-phenylalanylglycylglycine (FAPGG), rabbit lung acetone powder and sodium tetraborate

decahydrate (borax) were from the Sigma (Poole, Dorset, UK). Ethylenediaminetetra-acetic acid (EDTA) disodium salt was from BDH Laboratory Supplies (Poole, UK). Hydrochloric acid (37%) was from Mallinckrodt Baker (Deventer, Holland). Deionised, distilled water was used throughout and was obtained from Alkem Chemicals (Little Island, Cork, Ireland). All other reagents were of analytical grade.

2.2. Extraction and preparation of ACE from rabbit lung acetone powder

A modification of the method of Cushman and Cheung [13] was used to extract ACE from rabbit lung acetone powder. Rabbit lung acetone powder (1 g) was mixed in 10 ml of 100 mM sodium borate buffer containing 5% (v/v) glycerol at pH 8.3 using gentle magnetic stirring at 4 °C overnight. The extract solution was then centrifuged at 40,000 × *g* for 30 min in a Sorvall RC5C Plus centrifuge (Sorvall, Centrifuges, Newtown, Connecticut, USA) at 4 °C and the clear wine red-coloured supernatant containing ACE activity was retained and stored at 4 °C. These extracts generally contained ~ 2000 units ACE activity L^{-1}.

2.3. Preparation of standard curves for quantification of the extent of hydrolysis of FAPGG to FAP and GG

The following concentrations of (a) FAPGG and (b) a solution containing equimolar concentrations of FAP and GG were prepared in assay buffer, i.e., 100 mM sodium borate buffer, containing 300 mM NaCl, pH 8.3: 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM. The absorbance at 340 nm of these solutions was determined in duplicate using an Ultrospec 2000 ultraviolet/visible spectrophotometer (Pharmacia Biotech, Cambridge, UK) blanked against deionised water. A range of solutions at an overall concentration of 1 mM were prepared in assay buffer containing decreasing concentrations of FAPGG (1.0–0.0 mM) and increasing concentrations of an equimolar mixture of FAP and GG (0.0–1.0 mM). The absorbance at 340 nm of these solutions was determined in duplicate as described above.

2.4. Determination of the effect of the level of enzyme activity on the initial rate of degradation of FAPGG

ACE activity was determined in duplicate incorporating increasing levels of ACE in the assay system. Prior to the assay, the ACE extract was diluted 10-fold in assay buffer. Each assay was carried out in 1.5-ml eppendorf tubes containing the following components: 500 μl of 1.6 mM FAPGG; 12.5, 25.0, 37.5, 50.0, 67.5, 75.0, 87.5, 100.0, 150.0 or 200.0 μl of the diluted ACE extract and the appropriate volume of assay buffer to bring the final volume to 900 μl. ACE extract was added last to initiate the reaction. Each assay was incubated at 37 °C for 60 min. The reaction was terminated by the addition of 100 μl of 100 mM EDTA. The EDTA was added immediately before the ACE extract in zero-time control assays. The decrease in absorbance at 340 nm was determined in duplicate over a 60-min incubation period. The mean initial rate of degradation of FAPGG was determined from the slope of the decrease in absorbance at 340 nm in each of the ACE assays in the first 30 min of the reaction. All subsequent assays of ACE activity were carried out under

the following assay conditions: 500 μl of 1.6 mM FAPGG, 325 μl of assay buffer and 75 μL of an appropriately diluted ACE extract.

2.5. Determination of the IC_{50} value for an ACE inhibitory compound

Each assay was carried out in 1.5-ml eppendorf tubes as outlined previously, where 50 μl of an ACE inhibitory compound was also present in the assay solution. The assay was carried out under the conditions already outlined and terminated by the addition of 100 μl of 100 mM EDTA after 30-min incubation at 37 °C. The activity of ACE in units l^{-1}, where 1 unit of ACE is that amount of enzyme that hydrolyses 1 μmol of FAPGG to FAP and GG in 1 min at 37 °C, is given as:

$$\text{ACE activity (units L}^{-1}) = \left(\frac{V_t \times 100}{\Delta\varepsilon \times V_s \times d}\right) \times \Delta A \text{ min}^{-1} \quad [18]$$

where V_t = the final assay volume (1000 μl); V_s = the volume of ACE present in the assay (75 μl); d = light path (cm); $\Delta\varepsilon$ = absorbance change at 340 nm obtained by the complete hydrolysis of 1 mM of FAPGG to FAP and GG; ΔA min^{-1} = decrease in absorbance at 340 nm per minute.

Estimated IC_{50} values were obtained from a plot of percentage ACE inhibition as a function of log inhibitor (Captopril and whey protein hydrolysate) concentration present in the assay [13]. Uninhibited or 100% ACE activity was the activity obtained in the absence of an inhibitory substance in the reaction mixture. All IC_{50} values quoted are the mean of independent duplicates, tested in triplicate.

2.6. Determination of the effect of the level of ACE activity in the assay on the IC_{50} for Captopril

Determination of the IC_{50} values for Captopril (tested at 2, 4, 6, 8, 10, 12, 14, 16 and 18 nM in the final assay mixture) were performed in assay solutions containing the following ACE activities: 155, 164, 175, 190, 221 ($\pm$ 15) units l^{-1}. The level of ACE activity added to the assay solution (75 μl) was pre-adjusted prior to addition to the assay by appropriately diluting the ACE extract in assay buffer.

2.7. Determination of the effect of the level of enzyme activity on the IC_{50} for a whey protein hydrolysate

Determination of IC_{50} values for a whey protein hydrolysate was performed in assay solutions containing the following ACE activities: 117, 145, 171, 195 and 234 ($\pm$ 15) units l^{-1}. The activity of the ACE extract was adjusted as previously described (see Section 2.6). Whey protein hydrolysate IC_{50} assays were carried out as previously described (see Section 2.5) adding 50 μl of a 0.025, 0.050, 0.100, 0.0250, 0.300, 0.400, 0.500, 0.700 and 1.000% (w/v) protein solution to the assay. The protein concentration in the whey protein hydrolysate was determined using the Kjeldahl procedure [29].

3. Results and discussion

ACE was extracted from rabbit lung acetone powder in 100 mM sodium borate buffer containing glycerol as a stabilising agent in order to maintain enzyme activity during subsequent storage [28,30]. Activity was stable for 4 weeks of storage at 4 °C under these conditions. This procedure differs from the extraction employed by Cushman and Cheung [13], who extracted ACE activity in 50 mM potassium phosphate buffer, pH 8.3, in the absence of glycerol.

3.1. Standard curves for quantification of the extent of hydrolysis of FAPGG to FAP and GG

The absorbance change observed during the degradation of FAPGG to FAP and GG is due to the presence of FAPGG and FAP, while GG does not absorb at 340 nm. Therefore, the combined absorbance of the substrate, FAPGG, and product, FAP, contributes to the final absorbance obtained during the assay. A standard curve for the degradation of FAPGG to FAP and GG was constructed where the concentration of both substrate and product was related to absorbance at 340 nm (Fig. 1a). From Fig. 1a it was calculated that the difference between the extinction coefficients ($\Delta\varepsilon$) of FAPGG ($\varepsilon = 2270\ M^{-1}\ cm^{-1}$) and FAP ($\varepsilon = 1512\ M^{-1}\ cm^{-1}$) was 758 $M^{-1}\ cm^{-1}$ at 340 nm. Therefore, the estimated change in absorbance on complete hydrolysis of a 1 mM FAPGG to FAP and GG was 0.758 absorbance units at 340 nm. These results compare well with those reported in literature. Lundberg et al. [19] showed similar standard curves for FAPGG and FAP at 340 nm in the presence of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES) buffer containing 300 mM NaCl, pH 8.35. Their reported $\Delta\varepsilon$ was 800 $M^{-1}\ cm^{-1}$. However, Harjanne [18] reported a change in absorbance at 340 nm of 0.58 units for the complete hydrolysis of 1 mM of FAPGG in the presence of 80 mM sodium borate buffer containing 300 mM NaCl at pH 8.2; this is equivalent to a $\Delta\varepsilon$ of 580 $M^{-1}\ cm^{-1}$. The wavelength of maximum absorbance (λ_{max}) and the λ at which the maximum difference in absorbance occurs between FAPGG and FAP are at 328 nm [14]. However, analysis of the degradation of FAPGG at 340 nm, as in the present study, allows for the inclusion of high [FAPGG]'s in the assay system, i.e., it allows the possibility to work at substrate concentrations in excess of the K_m for ACE [14].

A standard curve representing the breakdown of FAPGG to FAP and GG as related to absorbance at 340 nm where the cumulative concentration of FAPGG and FAP in all instances was 1 mM is shown in Fig. 1b. It is again clear from Fig. 1b that at any given point during the degradation of FAPGG to FAP and GG, the concentration of substrate and product present in the solution directly affects the absorbance obtained at 340 nm. These results are in agreement with those of Maguire and Price [20] who quantified the change in absorbance at 334 nm of equimolar solutions of FAPGG and FAP in the presence of 50 mM Tris containing 400 mM NaCl at pH 8.3. The reason for incorporating a final [FAPGG] of 0.8 mM in the assay system used in the present study was to allow for an increase in absorbance due to subsequent addition of inhibitory substances while maintaining the initial absorbance of the starting reaction at <2.5 absorbance units at 340 nm.

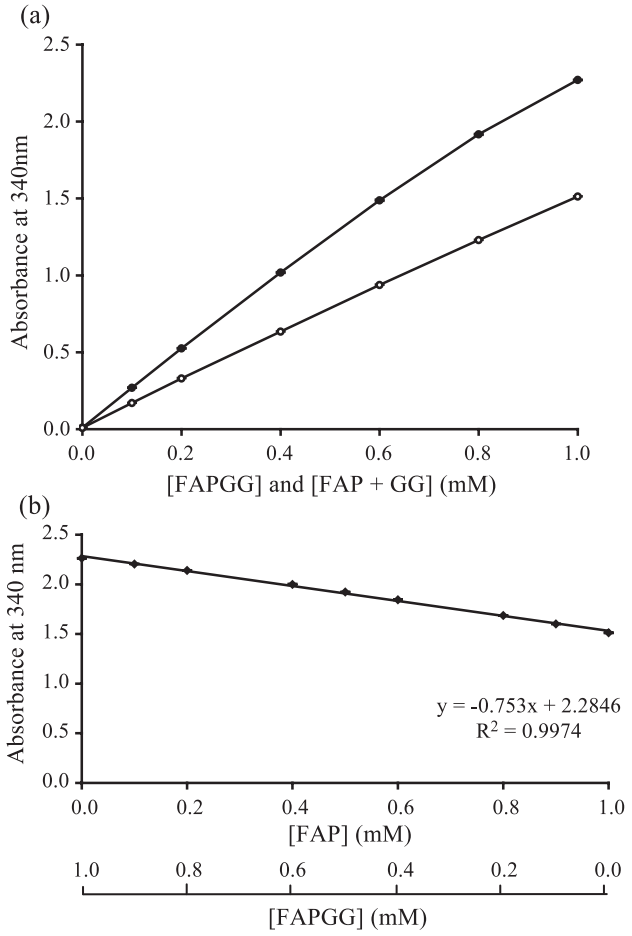

Fig. 1. (a) Standard curve of [*N*-(3-[2-furyl]acryloyl)-L-phenylalanylglycylglycine] (FAPGG, ●) and [*N*-(3-[2-furyl]acryloyl)-L-phenylalanine] (FAP)+[glycylglycine] (GG) (○) versus absorbance at 340 nm. Data points plotted are means of independent duplicate determinations; error bars represent standard deviations. (b) Standard curve for the theoretical breakdown of *N*-(3-[2-furyl]acryloyl)-L-phenylalanylglycylglycine (FAPGG) to *N*-(3-[2-furyl]acryloyl)-L-phenylalanine (FAP) + glycylglycine (GG), where the cumulative concentration of FAPGG and FAP in all instances was 1 mM, versus absorbance at 340 nm. Data points plotted are means of independent duplicate determinations; error bars represent standard deviations.

3.2. Effect of the level of ACE activity present in the assay on the initial rate of degradation of FAPGG

Fig. 2 shows the effect of increasing the level of ACE activity on the rate of reaction for the hydrolysis of FAPGG to FAP and GG. As expected, increasing the level of ACE activity resulted in faster rates of hydrolysis. The initial rate of reaction (V_o) was linear up to 30-min incubation at 37 °C, after which time the rate begins to

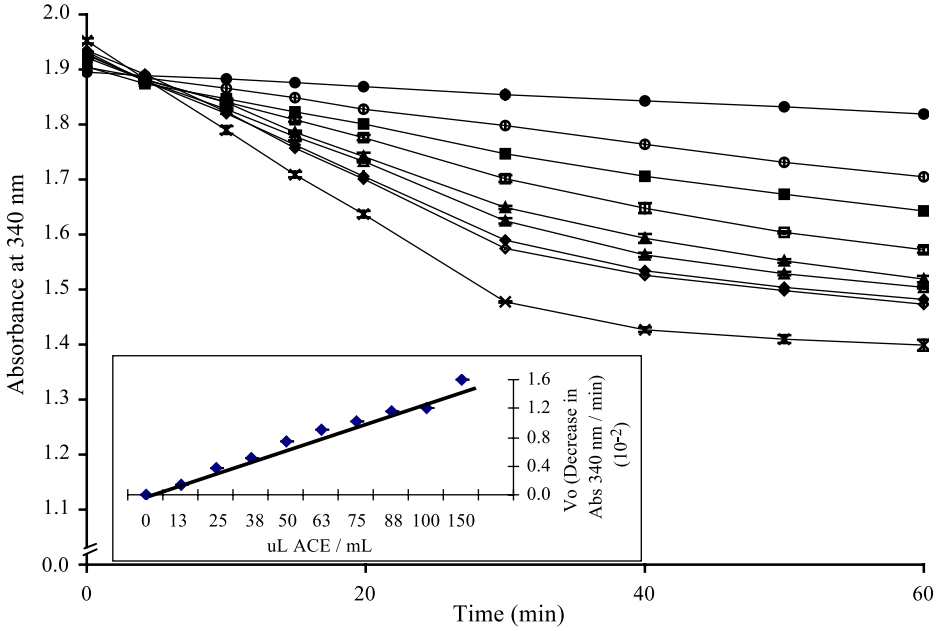

Fig. 2. Plot of the absorbance at 340 nm as a function of time (min) for the hydrolysis of 0.8 mM *N*-(3-[2-furyl]acryloyl)-L-phenylalanylglycylglycine (FAPGG) with increasing levels of angiotensin-I-converting enzyme (ACE) extract (~ 2000 units l^{-1}) ml^{-1} assay solution [12.5 (●), 25.0 (○), 37.5 (■), 50.0 (□), 62.5 (▲), 75.0 (△), 87.5 (◆), 100 (◇), 150 (×) μl ACE extract ml^{-1}]. Inset: plot of the initial rate (V_o) of hydrolysis FAPGG by ACE at 37 °C during the first 30 min of the reaction as a function of the level of ACE present in the assay. Data points plotted are means of independent duplicate determinations, error bars represent standard deviations.

slow down. The plot of V_o for the degradation of FAPGG by ACE at 37 °C for 30 min as a function of the level of ACE activity present in the assay is shown in Fig. 2 (inset). The V_o for an assay containing 200 μl ACE/ml assay was only linear up to 20 min (data not shown). Fig. 2 also demonstrates that the overall decrease in absorbance at 340 nm in a 30-min incubation period increased with increasing level of ACE activity in the assay. Therefore, by carefully choosing the level of ACE activity used in the reaction, it is possible to maximise the decrease in absorbance due to hydrolysis of FAPGG and thereby potentially increase the accuracy of the determinations. Holmquist et al. [14], using a continuous assay approach, also reported an increase in the rate of degradation of FAPGG over a 5-min period with increasing level of ACE activity. Based on the fact that ACE is a zinc metalloenzyme, EDTA was added in the present study to terminate the reaction. To date, assay of ACE activity using FAPGG has been on the basis of a continuous assay approach. In the HHL assay, HCl is usually used to inactivate ACE activity [13]. From the data shown in Fig. 2, the quantification of ACE activity using FAPGG was modified to a fixed time assay where substrate was incubated with ACE for 30 min at 37 °C, during which time it has been shown that a linear rate of reaction takes place under the assay conditions specified.

3.3. Effect of the level of ACE activity present in the assay on the estimated IC_{50} value for Captopril

Using the assay conditions previously outlined (Section 3.2), the effect of the level of ACE activity present in the assay on the IC_{50} values obtained for a known ACE inhibitor, such as Captopril, was examined. The ACE IC_{50} value reported for Captopril ranges from 5 to 23 nM using HHL and ranges from 1.61 to 8.91 nM when using FAPGG for assay of ACE activity [23,27,31–33]. The main reason for these reported variations in IC_{50} values may, in part, be attributed to variations in the assay conditions per se and in the level of ACE activity used in the assay. Fig. 3 clearly shows that, as expected, the apparent ACE IC_{50} for Captopril ranged from 9.10 to 39.40 nM when the level of ACE activity in the assay ranged from 155 to 221 units l^{-1}. This plot shows that as the ACE activity increases, there is a corresponding increase in the apparent IC_{50} value obtained for Captopril. Therefore, the level of ACE activity present in the assay must be carefully controlled when trying to obtain meaningful and reproducible IC_{50} values for different ACE inhibitory substance.

3.4. Effect of the level of ACE activity present in the assay on the estimated IC_{50} value for a whey protein hydrolysate sample

A plot of apparent IC_{50} value for a whey protein hydrolysate sample as a function of the level of ACE activity present in the assay is shown in Fig. 4. Apparent IC_{50} values for the hydrolysate ranged from 52.30 to 124.28 mg protein l^{-1} when the ACE activity level in the assay ranged from 117 to 234 units l^{-1}. Again, as already demonstrated using Captopril, the level of ACE activity present in the assay system determines the apparent IC_{50} value obtained for the whey protein hydrolysate sample. The results presented herein demonstrate

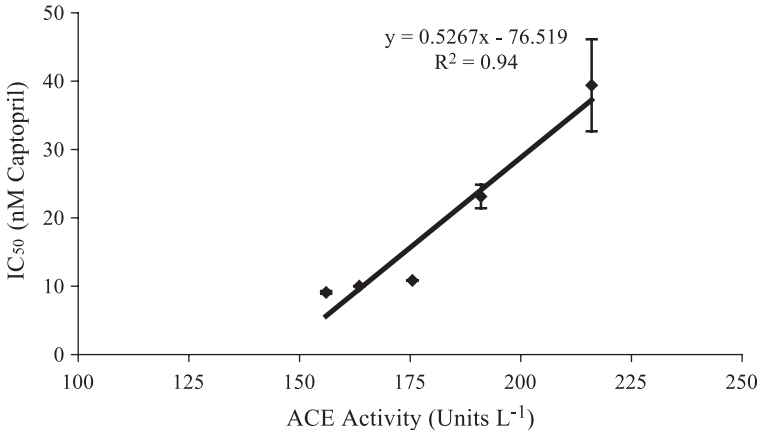

Fig. 3. Plot of angiotensin-I-converting enzyme (ACE) inhibitory potency (IC_{50}, nM) for Captopril as a function of the level of ACE activity (units l^{-1}) present in the assay. Data points are the mean of independent duplicates, tested in triplicate, and error bars show standard deviation.

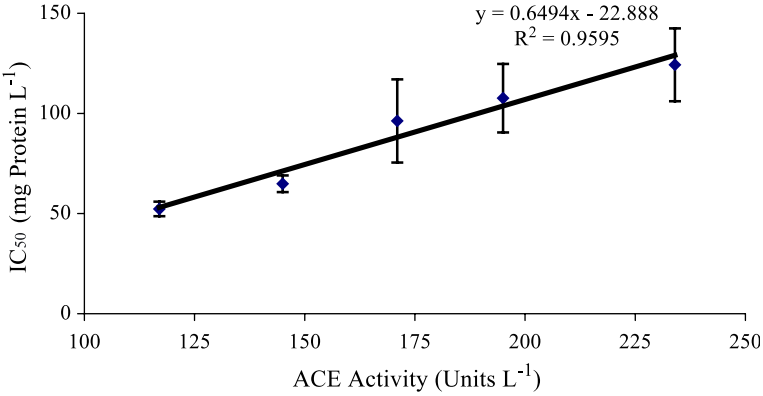

Fig. 4. Plot of angiotensin-I-converting enzyme (ACE) inhibitory potency (IC_{50}, mg protein l^{-1}) for a whey protein hydrolysate as a function of the level of ACE activity (units l^{-1}) present in the assay. Data points are the mean of independent duplicates, tested in triplicate, and error bars show standard deviation.

that the acquisition of comparable and reproducible ACE IC_{50} values requires the use of identical ACE activity levels in the assay solution. In this case, an ACE activity level of 175 ± 10 units l^{-1} in the assay was chosen as an appropriate level of ACE activity when quantifying future IC_{50} values. The IC_{50} value obtained for Captopril under the newly outlined assay conditions incorporating the ACE activity level specified above was 10.86 nM. This value corresponds well with values recently reported by Vermeirssen et al. [28].

4. Simplified description of the method and its (future) applications

We have outlined a detailed set of conditions for a fixed time assay in the determination of ACE inhibitory activity using FAPGG as a substrate for ACE. Previous manifestations of this assay have been in continuous assay mode. We have clearly demonstrated that the level of ACE activity in the assay determines the apparent IC_{50} value obtained for Captopril and a whey protein hydrolysate sample. Therefore, it is necessary to detail the number of enzyme units used in all ACE inhibition analyses for comparative purposes. Finally, always reporting an IC_{50} value for an ACE inhibitory standard such as Captopril should also make comparison of IC_{50} values for different ACE inhibitory substances more meaningful.

Acknowledgements

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