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RESEARCH ARTICLE

Development of a Spectrophotometric Method for Monitoring Angiotensin-Converting Enzyme in Dairy Products

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ABSTRACT

The angiotensin-converting enzyme (ACE) regulates the levels of blood pressure through generation of angiotensin-II from angiotensin-I. It is of great importance to have a reliable and yet simple method for a quantitative determination ACE inhibitory peptides in whey of milk products. A rapid, simple, sensitive and accurate spectrophotometric kinetic method has been developed for determination of ACE inhibitory peptides, using competitive inhibition. Samples of dairy product from the market were used for the determination of ACE inhibitory peptides in whey. Holmquist's kinetic method was used for determining ACE inhibitory activity in blood serum and Ronca-Testoni method was used for the determination of ACE inhibitory activity in whey. Enzymatic inhibition activity was determined using 0.8 mmol/L FAPGG (N-[3-(Furyl) –Acryloyl]-L-Phenylalanyl Glycyl Glycyne) as the substrate in 50 mmol/L Tris buffer at pH 8.2 at 37°C and a standard serum containing ACE. First, a solution of whey was mixed in a 1 to 10 ratio with serum (elevation) containing high ACE activity. The enzymatic activity was determined by monitoring the decrease in absorbance at 340 nm as result of hydrolysis of the substrate. The concentration of ACE inhibitory peptides was determined from a standard curve of inhibitor concentration versus percent of ACE inhibition. The study suggests that the method possesses good reproducibility and accuracy. The linear range enabled determination of high enzymatic activity of ACE and all ACE inhibitory peptides from dairy products act as competitive inhibitors.

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INTRODUCTION

The angiotensin-converting enzyme (ACE) regulates the levels of blood pressure levels (Inoue *et al*., 2011) through generation of angiotensin II from angiotensin I. It has been considered as a target for research on antihypertensive agents and a therapeutic approach for blood pressure management.

Synthetic ACE inhibitors lead to development of adverse consequences, hence, low-cost, safer alternatives is being explored. ACE inhibitory activity have been reported for peptides from protein of animals' origin, including casein (Miguel *et al.*, 2009; Ayyash *et al*., 2012), whey (Mullally *et al*., 1997), egg (Miguel and Aleixandre, 2006), soybean (Lo and Li-Chan, 2005) and chicken breast muscle (Saiga *et al*., 2006). A research study (Lin *et al*., 2012) recommended ACE inhibitors derived from squid byproducts for developing anti-hypertensive functional foods. The hydrolysate (HSSG-III) was found as having a potent ACE inhibitory activity in vitro having an IC50 value of 0.33 mg/ml. After oral intake, the HSSG-III lowered the systolic and diastolic blood pressure in renovascular hypertensive rats.

These authors developed a rapid, reproducible evaluation of ACE inhibition in various foods and developed an assay to screen inhibitors of ACE using high-throughput LC/SID-ESI-MS/MS. Several spectro-photometric methods have been developed for determination of the enzymatic activity of the angiotensin-converting enzyme (ACE), and are regularly used because

of their availability, simplicity and the high level of accuracy. The first developed method uses the substrate N-α-hipuryl-L-histidyl-L-leucine, synthesized on the analogy of the natural substrate A-I introduced by Cushman and Cheung (1971). The same method with minor modifications was used by Liberman (1975). The method is based on the determination of the released hipuric acid. Hayakari *et al*. (1984) introduced into the reaction 2, 4, 6-trichloro-s-triazin in 1, 4-dioksan which in the reaction with the hipuric acid gives colored compound.

Holmquist *et al.* (1979) have synthesized new substrate N-[3-(2-furil)-akriloil]-L-fenilalanil-glycyl-glycine (FAPGG) and have introduced, for the first time, a new continued kinetic method for determining the ACE activity in serum. With hydrolysis, the resulting product has a lower absorbance than the substrate at 345 nm, thus the resulting absorbance is negative and there is a drop in the absorbance level. The substrate is specific to ACE and has faster hydrolysis process than other used substrates. There are several developed methods using this substrate, from which several are adapted for usage in automatic analisators. The same method is applied in determining ACE inhibition in whey and other dairy products.

The ACE enzyme is metalopeptidase that depends on zinc, which severs the S-terminal peptides of different oligopeptides. This enzyme has a broad range of substrates and contains two terminals for connecting with the substrate with variable affinity towards the substrates and/or the inhibitors (Cushman *et al*., 1973). The peptides may react as ACE inhibitors trough multiple different mechanisms. As first, the peptides may bond to the active side of the ACE without being hydrolyzed by the enzyme. These peptides are called real inhibitors. As second, the inhibitory peptides may be substrates to the ACE. These peptides are hydrolyzed by ACE, releasing new peptides, which may be more or less active inhibitors than the initial peptide. The peptides which are more active inhibitors after the decomposition from the ACE, are called prodrug-type inhibitors (Cheung *et al*., 1980). The less active peptides are called substrate-type inhibitors. Examples for real inhibitors may include LKP, IWH and IKP. LKPNM and IWHHT are examples for prodrug-type inhibitors. A great number of protein hydrolysates and peptides derived from different nutrients are known to present their activity as ACE inhibitors. There are a large number of hydrolysates and peptides that manifest ACE inhibitory activity. Hydrolysates from fermented milk, casein and whey proteins have shown *in vitro* ACE inhibitory activity, and/or *in vivo* antihypertensive activity, and have different amino acid profile and derive from whey protein and casein peptides with significant ACE inhibition. Hydrolysates derived with different enzymes and different source of protein have shown ACE inhibitory activity. From that can be concluded that the ACE inhibition can be induced by different peptides or a combination of peptides. The ACE inhibitory activity depends on the origin of the protein or peptide, and the enzyme used in the proteolysis.

The most common factor for the ACE inhibitory activity is the small molecular mass of the ACE inhibitory peptides. The procedures implemented for *in vivo* studies for ACE inhibitory activity of peptides are not standardized. The quantity of ACE used in the procedures, and the type of ACE substrate are important for calculating the IC_{50} value (concentration of peptide required for lowering the ACE activity by 50%). Despite that, very often the concentration of ACE is not even mentioned. The difference in the conditions of the procedure may be a reason for the differences between IC_{50} values published for the same peptide.

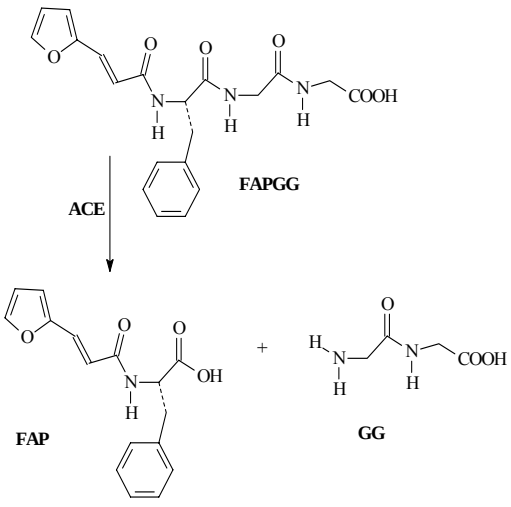
The objectives of this research were examination of the optimum conditions for application of the method for determining the activity of ACE in whey, examining the performance of the method in samples of standard serum, and determining *in vitro* ACE inhibition in milk samples from commercial dairy products.

MATERIALS AND METHODS

Random samples of dairy products were collected from retail shops for this study. The method for determining ACE inhibitory activity in serum as already described (Ronca-Testoni, 1983), was modified. The separation of the low molecular proteins and peptides from macromolecules proteins, casein and fat was conducted using centrifuge IEC (Micromax), having at 20000 g. Fermentation process was monitored using pH-meter (Hanna Instruments). Spectroscopic determination of total peptides and ACE inhibitory activity was conducted through spectrophotometer (Specol, 1200).

Determining the enzymatic activity of ACE in whey: Enzymatic activity of ACE in a serum samples was determined (Ronca-Testoni, 1983). The substrate used was N-[3-(2-furil)-acryloil]-L-phenylalanine-glycyl-glycine (FAPGG, $C_{20}H_{21}H_3O_6$, Sigma Cat # 7131). The determination is based on the difference in absorbance of furanacryloil blocked tripeptide FAPGG and the product of the enzyme-catalyzed hydrolysis FAP at wavelength of 340 nm. Gly-Gly is the result of hydrolyses of FAPGG and does not absorb light at these conditions which is evident from the following equation-1). As the hydrolysis product FAP has low absorbance at 340 nm the resulting change of the absorbance is negative:

FAPGG $\xrightarrow{ACE}$ FAP + Gly-Gly (Equation 1)



The following procedure was used for preparing buffer solution: a) tris buffer 0.2 mol/L (2 hydroxymethyl-1,3-propanediol, Merck No 108382 MM=121,14 g/mol) was mixed with 24.23 g/L boiled and cooled deionized water to prepare standard buffer solution; b) hydrochloric acid solution (0.1 mol/L) was prepared; c) the buffer solution was prepared using different ratios of a and b solutions; sodium chloride solution 300 mmol/L was added.

Substrate solution (2 mmol/L) was prepared by dissolving 0.07988 g FAPGG (MM=399.4 g/mol in 100 ml buffer solution. The primary solution was diluted with the buffer solution. Control serums for ACE (Sigma) were measured using the spectroscopic kinetic method (Specol 1200) at 340 nm and 37°C.

The buffer solution comprised of: Tris buffer 50 mmol/L pH 8,2 (37°S), 300 mmol/L chlorides: 25 ml tris buffer solution 0.2 mol/L, 20 ml 0.1 mol/L HCl and 10 ml 3 mol/L NaCl solution. The measuring flask was filled up to 100 ml with deionized water. The substrate solution comprised of 0.8 mmol/L FAPGG: 0.032 g in 100 ml 50 mmol/L Tris buffer solution pH 8.2.

Determining serum ACE activity: Buffered substrate was added (2000 µL) along with 200 µL serum sample, into a 10 ml cuvette. The mixture was incubated for 5 min. at 37°C. The initial absorbance was read at 340 nm. Than after exactly 5 min. the drop in the absorbance level was recorded and the difference was calculated using equation 2.

$$ACE\,(U/L) = \Delta A \cdot \frac{V_t}{V_s} \cdot \frac{1}{t} \cdot \frac{1}{\Delta\varepsilon} \cdot 1000 \qquad \text{(Equation 2)}$$

ΔA- change in the absorbance after 5 min.;
Vt -total volume of the reaction mixture
Vs - volume of the serum sample
t - interval of measuring the change of the absorbance
Δε - differential molar absorbing coefficient FAPGG / FAP at 340 nm.

The reaction mixture comprises of 0.8 mol/L FAPGG, 50 mmol/L tris buffer, 8.2 (37°C) pH, chlorides 300 mmol/L, with addition of 100 µL serum in 2000 µL reaction mixture. The determinations were made at 340 nm, 37°C, and serum reagent ratio of 1:10, time of incubation 5 min, measuring interval 5 min, and calculations made with calibration factor. Commercial solutions for ACE (Sigma) were used.

The kinetic curve of the dependence of the speed of the enzymatic reaction for hydrolysis of FAPGG catalyzed by the serum ACE, from the concentration of the substrate (Michaelis-Menten reaction) is presented in Fig.1 where the calculated Michaelis constant is Km=0.376 mmol/L. The enzymatic kinetic constants were calculated using software GraFit v.4 (Eritchacus software Limited 2002), and the statistical processing of the obtained data was made with Statistica (data analysis software system, v.6 StatSoft, Inc. 2001).

Accuracy of the test: Several tests were conducted to test the accuracy, the precision and the linearity of the method. The precision of the kinetic method was determined with serum samples with normal and high ACE activity. The enzyme activity for whey samples was determined by the kinetic method with substrate FAPGG and commercial standard serum containing ACE.

Procedure of the serum test is described as follows: a) in a 2 ml cuvette we poured 1 ml ACE reagent (substrate and Tris buffer) and 0.1 ml standard solution of serum containing ACE and 0.1 ml water; b) the solutions were stirred by flipping the cuvette several times; c) the cuvette was placed in an incubator at 37°C for 45 min; d) after 5 min, the absorbance was measured and the values for time and absorbance were plotted to construct a calibration curve for time/absorbance.

The ACE inhibitory activity of the whey samples was determined as follows: In a 2 ml cuvette pour 1 ml ACE reagent (substrate and Tris buffer) and 0.1 ml standard solution of serum which contained ACE and 0.1 ml water. The solutions were stirred by flipping the cuvette several times. Then the cuvette was placed in a thermostat at 37°C for 45 min and at an interval of 5 min the value of the absorbance was measured. With the values for time and absorbance, a calibration curve (time/absorbance) was constructed. The ACE inhibitory activity (in %) was determined from the calibration curve and calculated by the following equation:

$$\%\ \text{inhibition} = \frac{\text{Uninhibited activity-inhibited activity}}{\text{Uninhibited activity}} * 100$$

(Equation 3)

RESULTS AND DISCUSSION

pH influence and ionic concentration on ACE activity: The influence of pH of the buffer solution, concentration of chloride ions and the concentration of the substrate affecting the activity of ACE in serum, were determined. The determination was made by the ACE activity in control serum for the range of the Tris buffer from 7.05-8.95 pH, presented in Table 1. The optimal pH level was found to be 8.2.

The influence of the concentration of chloride ions on the activity of ACE were determined using control serum, for range of concentrations between 0-0.7 mol/L. The results indicated that the optimal concentration of chloride ions was 0.30 mol/L. The initial absorbance (A) and ΔA/min were determined for range of concentrations of substrate FAPGG between 0.1-2.0 mol/L, and using control serum. The results are presented in Table 2.

The data presented in figure 1, shows that all ACE inhibitory peptides from milk whey as competitive inhibitors, directly binds to the active site of enzymes and are characterized by unchanged maximal rate of reaction. Precision data obtained through testing within and between series of samples with normal and high activity of ACE, is expressed trough the coefficient of variation (Table 3).

The accuracy of the method was tested trough determination of the ACE activity in serum samples (92 U/L and 31 U/L), mixed in different ratios. Results were calculated as average three determinations. Table 4 reports the accuracy of the method for determining the ACE activity in serum samples as compared with the calculated values.

The linearity of the method for determining the enzymatic activity of ACE in serum samples was tested in series of dilutions of control serum with high ACE activity.

Table 1: The effect of Tris buffer pH level on the hydrolysis of 0.8 mmol/L FAPGG catalyzed by ACE (340 nm, 37°C)

Dependant variable	pH level							
	7.05	7.52	7.90	8.18	8.27	8.48	8.60	8.95
ACE (U/L)*	20	40	50	55	52	46	40	23

* angiotensin-converting enzyme

Table 2: Effect of the concentration of substrate FAPGG on the initial absorbance and ΔA/min of control serum for ACE (Tris buffer, pH 8, 2; 300 mmol/L chloride ions)

Absorbance	Substrate (mmol/L)						
	0.1	0.2	0.5	0.8	1.0	1.5	2.0
Initial A	0.2248	0.3390	0.6338	0.8809	0.8804	1.3270	1.4497
ΔA/min	0.0006	0.0009	0.0014	0.0018	0.0018	0.0020	0.0021

Table 3: Precision data of the method for ACE activity in serum with kinetic method with FAPGG substrate

Parameter	In series		Between series	
N	10	10	15	15
X (U/L)	62	112	58	121
±SD	20	1.8	2.8	4.8
CV (%)	3.2	1.8	4.8	4.0

Table 4: Accuracy of the method for determining the ACE activity (U/L) in serum samples determined by the test of revealing the theoretically calculated data

Calculated	Determined	% Revealing
61.5	63±1.4	102±2.2
51	52±0.9	102±1.8
72	72±1.4	100±1.0

In determining the activity of the enzyme with the method, the linear accuracy was to 175 U/L (Fig. 2).

Whey samples derived from different types of dairy products of cows, sheep and mixture of the both in different ratios were studied. Total samples were 70 (27 yogurt, 9 sour cream, 14 cheese and 20 white cheeses). The mean values were worked out. ACE inhibitory activity was reflected in % and IC50 (μL) (Table 5). The yogurt and white cheese samples presented the highest ACE inhibition, as seen descriptive statistics of ACE inhibition in dairy products (Table 6). In a pervious study the most effective inhibitors were EDTA; CdBr2; angiotensin II; bradykinin; and a pentapeptide, L-pyroglutamyl-L-lysyl-L-tryptophyl-L-alanyl-L-proline, a component of Bothrops jararaca venom (Cushman and Cheung, 1971).

It has been noted that all ACE inhibitory peptides from dairy products act as competetive inhibitors. There was no known correlation between total peptides in whey and *in vitro* ACE inhibitory activity. This notation is in compliance with the linear data.

Table 5: Average values of ACE inhibition in the tested dairy products

Product	Inhibition%	Inhibition IC50
Yogurt (cow's milk)	36.6	323.7
Sour cream (cow's milk)	28.0	1199.3
Sour cream (sheep's milk)	11.4	734.1
White cheese (cow's milk)	38.7	162.9
White cheese (sheep's milk)	26.9	190.0
White cheese (goat's milk)	23.5	212.7
White cheese (cow's+sheep's milk)	18.9	271.5
Cheese (cow's milk)	20.8	316.1

In a study, all probiotic yoghurts showed appreciable ACE-I activity during initial stages of storage which deseased ($P<0.05$) afterwards (Donkor *et al*., 2007). The ACE-I activity ranged from IC50 of 103.30–27.79 mg mL^{-1} with the greatest ACE inhibition achieved during first and third week of storage. The *in vitro* ACE-I activity could be related to the peptide liberation via degradation of caseins. In total, 8 ACE-I peptides were characterized originating from αs2-casein, k-casein and β-casein.

The ACE inhibitory activity was determined in 70 comercially available samples of dairy products. There was a significant variation in the inhibition in different products (Fig. 3). This may be the result of origin of samples from different production technologies. It was noted that ACE inhibitory peptides are most common in whey from yogurt and white cheese.

Table 6: Descriptive statistics of ACE inhibition in dairy products

Type of product	Average value	Standard deviation	Maximum value
Yogurt	36.63	50.5	273
Sour cream	24.3	11.66	46.6
White cheese	29.9	14.96	76.4
Cheese	20.8	12.83	55.8

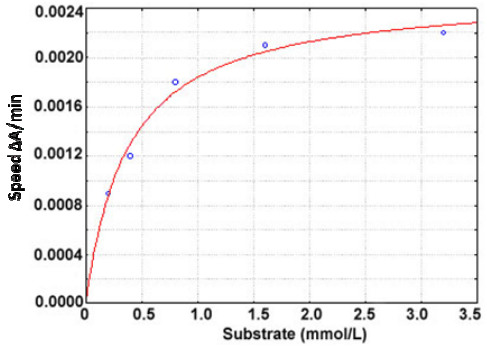


Fig. 1: Michaelis-Menten dependence curve of reaction speed of FAPGG hydrolysis / concentration of substrate (0.1-3.2 mmol/L; tris buffer 50 mmol/L, pH 8.2, t = 37°C)

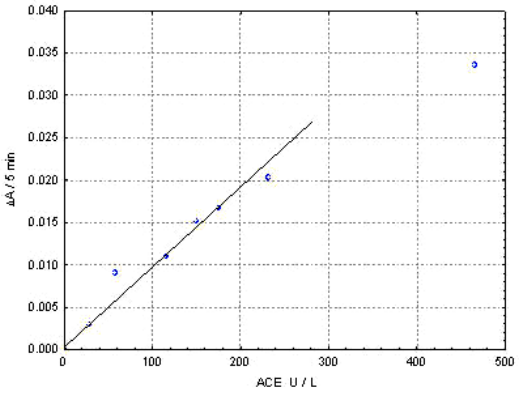


Fig. 2: Linearity of the determination of ACE activity in serum samples with the kinetic method and FAPGG substrate.

Chen *et al*. (2012) reported a single Val-Ala-Pro (VAP) tripeptide in grass carp protein. The tripeptide with excellent ACE-inhibitory activity (IC50 value of 0.00534 mg/mL) was a competitive ACE inhibitor and stable against both ACE and gastrointestinal enzymes of pepsin

and chymotrypsin. The identified unique biochemical properties of VAP may enable the application of grass carp protein hydrolysates as a functional food for treatments of hypertension.

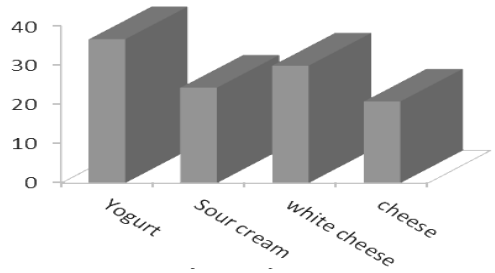


Fig. 3: Maximal peaks for ACE inhibition (%) in dairy products.

Other animal products posses antihypertensive properties. Jang and Lee (2005), Saiga *et al*. (2006) and Miguel *et al*. (2007) reported ACE inhibitory peptides in eggs, beef and chicken, respectively.

A phylogenetic tree constructed with actin sequences of both vertebrate and invertebrate species indicated a high homology (Vercruysse *et al*., 2009). *Bombyx mor* was found as a high-potential source of ACE inhibitory peptides and this was supported by the ACE inhibitory activity of the partially purified actin preparation. It was suggested that in food science, in silico analysis can be used as fast initial screening tool to look for high-potential sources of ACE inhibitory peptides and other peptidic bioactivities.

Conclusion: The *in vitro* activity of angiotensin-converting enzyme was determined with FAPGG substrate in whey samples. The method was found as fast, simple, specific, free of any interference, having no problems regarding the stability of the reagent. The method is characterized by good reproducibility and accuracy, and also the range of linearity permits detection of high ACE activity values.

REFERENCES

Ayyash MM, F Sherkat and NP Shah, 2012. The effect of NaCl substitution with KCl on Akawi cheese: Chemical composition, proteolysis, angiotensin-converting enzyme-inhibitory activity, probiotic survival, texture profile, and sensory properties**.** J Dairy Sci, 95: 4747-4759.

Chen J, Y Wang, Q Zhong, Y Wu and W Xia, 2012. Purification and characterization of a novel angiotensin-I converting enzyme (ACE) inhibitory peptide derived from enzymatic hydrolysate of grass carp protein. Peptides, 33: 52–58.

Cheung HS, FL Wang and MA Ondetti, 1980. Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. J Biol Chem, 255: 401-407.

Cushman DW and HS Cheung, 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem Pharmacol, 20: 1637-1648.

Cushman DW, J Pluščec, NJ Williams, ER Weaver, EF Sabo, O Kocy, HS Cheung and MA Ondetti, 1973. Inhibition of angiotensin-converting enzyme by analogs of peptides from Bothrops jararaca venom. Cell Mol Life Sci, 29: 1032-1035.

Donkor ON, A Henriksson, TK Singh, T Vasiljevic and NP Shah, 2007. ACE-inhibitory activity of probiotic yoghurt. Int Dairy J, 17: 1321–1331.

Holmquist P, Bunning and JF Riordan, 1979. A continuous spectrophotometric assay for angiotensin-converting enzyme. Anal Biochem, 95: 540-548.

Hayakari M, R Scito, A Furugori, Y Hashimoto and S Murakami, 1984. An improved colorimetric assay of angiotensin-converting enzyme in serum. Clin Chim Acta, 144: 71-75.

Inoue K, M Kitade, T Hino and H Oka, 2011. Screening assay of angiotensin-converting enzyme inhibitory activity from complex natural colorants and foods using high-throughput LC-MS/MS. Food Chem, 126: 1909-1915.

Jang A and M Lee, 2005. Purification and identification of angiotensin converting enzyme inhibitory peptides from beef hydrolysates. Meat Sci, 69: 653–561.

Liberman J, 1975. Elevation of serum Angiotensin- converting enzyme (ACE) level in sarcoidosis. Am J Med, 59: 365-372.

Lin L, S Lv and B Li, 2012. Angiotensin-I-converting enzyme (ACE)-inhibitory and antihypertensive properties of squid skin gelatin hydrolysates. Food Chem, 131: 225–230.

Lo WMY and ECY Li-Chan, 2005. Angiotensin I converting enzyme inhibitory peptides from in vitro pepsin-pancreatin digestion of soy proteins. J Agric Food Chem, 53: 3369–3376.

Miguel M, MJ Alonso, M Salaices, A Aleixandre and R López-Fandiño 2007. Antihypertensive ACE-inhibitory and vasodilator properties of an egg white hydrolysate: effect of a simulated intestinal digestion. Food Chem, 104: 163–168.

Miguel M and A Aleixandre, 2006. Antihypertensive peptides derived from egg proteins. J Nutr, 136: 1457–1460.

Miguel M, MM Contreras, I Recio and A Aleixandre, 2009. ACE-inhibitory and antihypertensive properties of a bovine casein hydrolysate. Food Chem, 112: 211–214.

Mullally MM, H Meisel and RJ FitzGerald, 1997. Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey protein. Int Dairy J, 7: 299-303.

Saiga A, T Okumura, T Makihara, SI Katsuda, F Morimatsu and T Nishimura, 2006. Action mechanism of an angiotensin I-converting enzyme inhibitory peptide derived from chicken breast muscle. J Agric Food Chem, 54: 942–945.

Ronca-Testoni S, 1983. Direct spectrophotometric assay for angiotensin-converting enzyme in serum. Clin Chem, 29: 1093-1096.

Vercruysse L, G Smagghe, A Bent, A Amerongen, M Ongenaert and JV Camp, 2009. Critical evaluation of the use of bioinformatics as a theoretical tool to find high-potential sources of ACE inhibitory peptides. Peptides, 30: 575–582.