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Release of Angiotensin I Converting Enzyme (ACE) Inhibitory Activity during in Vitro Gastrointestinal Digestion: from Batch Experiment to Semicontinuous Model

VANESSA VERMEIRSSEN,[†,‡] JOHN VAN CAMP,[‡] LIESBET DEVOS,[†] AND WILLY VERSTRAETE*[,†]

Laboratory of Microbial Ecology and Technology and Department of Food Technology and Nutrition, Faculty of Agricultural and Applied Biological Sciences, Ghent University, Coupure Links 653, 9000 Ghent, Belgium

Gastrointestinal digestion is of major importance in the bioavailability of angiotensin I converting enzyme (ACE) inhibitory peptides, bioactive peptides with possible antihypertensive effects. In this study, the conditions of in vitro gastrointestinal digestion leading to the formation and degradation of ACE inhibitory peptides were investigated for pea and whey protein. In batch experiments, the digestion simulating the physiological conditions sufficed to achieve the highest ACE inhibitory activity, with IC_{50} values of 0.076 mg/mL for pea and 0.048 mg/mL for whey protein. The degree of proteolysis did not correlate with the ACE inhibitory activity and was always higher for pea than whey. In a semicontinuous model of gastrointestinal digestion, response surface methodology studied the influence of temperature and incubation time in both the stomach and small intestine phases on the ACE inhibitory activity and degree of proteolysis. For pea protein, a linear model for the degree of proteolysis and a quadratic model for the ACE inhibitory activity could be constituted. Within the model, a maximal degree of proteolysis was observed at the highest temperature and the longest incubation time in the small intestine phase, while maximal ACE inhibitory activity was obtained at the longest incubation times in the stomach and small intestine phase. These results show that ACE inhibitory activity of pea and whey hydrolysates can be controlled by the conditions of in vitro gastrointestinal digestion.

KEYWORDS: ACE inhibitory peptides; pea protein; whey protein; response surface methodology

INTRODUCTION

Angiotensin I converting enzyme (ACE, EC 3.4.15.1) inhibitory peptides are bioactive peptides with possible antihypertensive effects in vivo (*1*). High blood pressure is a major risk for cardiovascular diseases, one of the most important causes of mortality in the developed world. In a recent study performed in five European countries, 34% of the adult population had a blood pressure higher than 140/90 mmHg, the treatment of which contributes substantially to health care costs (*2*). Recently, foods containing ACE inhibitory peptides have shown to be effective in both the prevention and the treatment of hypertension (*3*, *4*).

Research on ACE inhibitory peptides has mainly concentrated on milk proteins, but vegetable and other animal proteins have been studied as well (*5*). To our knowledge, pea protein has not yet been the subject of study for ACE inhibitory activity. In a previous paper, we observed a large impact of in vitro gastrointestinal digestion on the formation of ACE inhibitory activity from pea and whey protein (*6*). The scope of this paper is to further investigate the conditions of in vitro gastrointestinal digestion leading to the formation and/or degradation of ACE inhibitory peptides. First, this is of physiological importance, because, upon oral administration, these bioactive peptides have to reach the blood stream in an active form to exert an antihypertensive effect (*7*). Gastrointestinal digestion and transport are the major barriers in the bioavailability of ACE inhibitory peptides (*8*). Second, digestion by gastrointestinal proteases can be used as a production process for ACE inhibitory peptides, with the advantage that the formed peptides will resist the physiological digestion after oral intake (*9*).

Few studies have been performed on the conditions during digestion necessary to release ACE inhibitory activity. Some hydrolysis of the proteins is required in order to free the bioactive peptide sequences. At some point during hydrolysis, however, no further increase in ACE inhibitory activity is observed (*10*). Meisel et al. (*11*) reported that the ACE inhibitory activity in ripened cheese increases during cheese maturation but decreases when the proteolysis exceeds a certain level. At

* To whom correspondence should be addressed. Tel: +32 92 64 59 76. Fax: +32 92 64 62 48. E-mail: willy.verstraete@UGent.be.
† Laboratory of Microbial Ecology and Technology.
‡ Department of Food Technology and Nutrition.

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this point, the degradation of bioactive peptides starts to dominate the formation of new ones. Digestion of bovine skin gelatine independently by different proteases at incubation times up to 24 h also demonstrates an optimal incubation time for maximal ACE inhibitory activity (*12*). Hence, there seems to be an optimal and not always maximal hydrolysis for maximal ACE inhibitory activity. Moreover, the specificity of the enzymes plays a major role in the formation of ACE inhibitory peptides (*6*, *10*). ACE inhibitory peptides are most commonly produced by trypsin (EC 3.4.21.4) (*8*). This readily available enzyme forms peptides with a terminal lysine or arginine, the latter being reported in structure−activity studies of ACE inhibitory peptides (*1*). The digestion of α-lactalbumin and *β*-lactoglobulin by pepsin (EC 3.4.23.1), trypsin, α-chymotrypsin (EC 3.4.21.1), pancreatin, elastase (EC 3.4.21.36), or carboxypeptidase A (EC 3.4.17.1) and B (EC 3.4.17.2) alone and in combination revealed that trypsin is necessary to release high ACE inhibitory activity from whey protein (*13*). The gastrointestinal protease elastase, on the other hand, is associated with a low production of ACE inhibitory peptides from α-lactalbumin and *β*-lactoglobulin (*10*, *13*).

The physiological conditions of the gastrointestinal protein digestion are more or less known. The gastric pH is between 1.5 and 2 during fasting, and it can increase up to 5 after ingestion of a meal due to the diluting and buffering effect of the food components (*14*). Proteins are cleaved in the stomach by the endopeptidase pepsin with rather broad substrate specificity and an optimal pH in the range of 1−2 (*15*). The fasting pH of the overall small intestine is situated around 6.5, while for the postprandial pH this is around 5.5 (*14*). In the duodenum, endopeptidases such as trypsin, chymotrypsin, and elastase, and carboxypeptidases A and B continue the splicing of the polypeptide chain at a more alkaline pH with an optimal activity in the range of 7−8. At the brush border membrane, the oligopeptides are further cleaved by amino-, di-, and endopeptidases, resulting in a mixture of amino acids and small peptides, which can be absorbed by the enterocytes (*15*). The half-emptying time for the stomach is 0.5−3 h for fed conditions, while the residence time in the duodenum and jejunum is 2−2.75 h and in the ileum 5−7 h (*16*).

In our study, digestion was first simulated in batch, where the enzyme over substrate ratio was set at 1 over 250 (*17*). Nonoptimal, physiological, and prolonged optimal gastrointestinal digestion, varying in pH and residence time in the stomach and small intestine, were compared for the formation of ACE inhibitory activity and degree of proteolysis. Subsequently, a semicontinuous model based on the batch physiological digestion was developed. In this reactor, the influence of temperature and incubation time in the stomach and small intestine phase on the formation of ACE inhibitory activity and degree of proteolysis was investigated by means of an experimental design.

MATERIALS AND METHODS

Products. The pea protein isolate Pisane HD (90% protein on dry matter) and the whey protein isolate Lacprodan DI-9213 (min 90% protein on dry matter) were obtained from Cosucra SA (Fontenoy, Belgium) and Acatris Belgium NV (Londerzeel, Belgium), respectively. Pepsin (P 6887), trypsin (T 1426), α-chymotrypsin (C 4129), rat intestinal acetone powder (I 1630), trichloroacetic acid solution (490-10), ACE reagent (305-10), ACE control-E (A 7040), and 100% (w/w) trifluoroacetic acid solution (30 203-1) were purchased from Sigma-Aldrich (St. Louis, MO). Nonspecified products were analytical grade and came from VWR International (Zaventem, Belgium).

Batch Gastrointestinal Digestion. A 100 mL 4% (w/v) protein isolate solution was brought to the desired pH for the stomach digestion with 1 and 10 N HCl and NaOH under rigorous mixing. Pepsin was added in a ratio of enzyme and substrate of 1/250 (w/w) after which the incubation at 37 °C on a shaker started. After a defined residence time, the pH was set at the desired value for small intestine digestion and trypsin and α-chymotrypsin were supplemented both at a ratio of enzyme and substrate of 1/250 (w/w). Then, the solution was again incubated at 37 °C. When samples were taken at the start and the end of digestion, the pH was adjusted to 5. As this is a pH near the isoelectrical point for both proteins (pea, pH 4.5; whey, pH 4−5), a clear separation was obtained by subsequent centrifugation. Incubation time and pH in the stomach and small intestine phase varied in the three types of in vitro gastrointestinal digestion: nonoptimal, physiological, and prolonged optimal digestion (**Table 1**).

Table 1. pH and Incubation Time (h) in the Stomach and Small Intestine Phase for the Nonoptimal, Physiological, and Prolonged Optimal Digestion

	stomach		small intestine	
type of digestion	pH	incubation time	pH	incubation time
nonoptimal	4	0.5	5	0.5
physiological	2	2	6.5	2.5
prolonged optimal	2	4	7	4.5

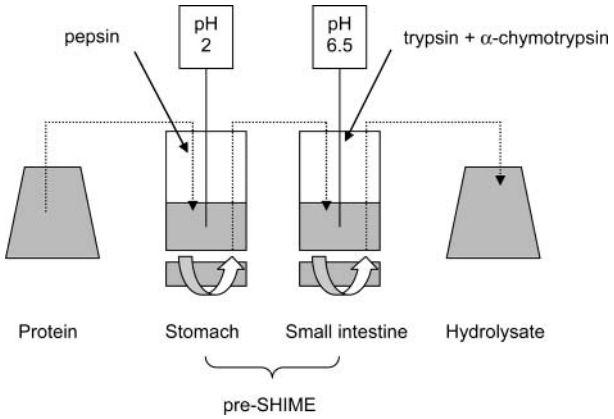

Figure 1. Experimental setup for the semicontinuous digestion.

Experimental Design in a Semicontinuous Model for Gastrointestinal Digestion. Figure 1 shows the reactor set up for the semicontinuous digestion. This model was called the pre-SHIME, because it can precede the SHIME, the simulator of the human intestinal microbial ecosystem (*18*). By means of double-jacketed vessels and a warm water bath, the content of the vessels was kept at a defined temperature. The pH in the two vessels was controlled by a pH stat (Consort R305), and the content was mixed by magnetic stirring. A peristaltic pump brought 200 mL of 4% (w/v) protein solution in the first reactor vessel that simulated the stomach. The pH was adjusted to 2 (lower and upper limits, 1.85 and 2.15) with 1 N HCl and NaOH. Pepsin was supplemented in a ratio of enzyme and substrate of 1/250 (w/w). After the stomach phase, the solution was pumped to the second reactor vessel, where the pH was set at 6.5 (lower and upper limits, 6.35 and 6.65). Subsequently, trypsin and α-chymotrypsin were added in a ratio of enzyme and substrate of 1/250 (w/w) and the solution was incubated for the small intestine phase. At the end of the in vitro digestion, the hydrolysate was pumped to an Erlenmeyer flask and adjusted pH to 5, and samples were taken out for the analyses.

The experimental design was created and analyzed by Design-Expert 6.0.3 (Stat-Ease, Minneapolis, MN). Response surface methodology linked the digestion parameters temperature, incubation time in the stomach phase, and incubation time in the small intestine phase (three factors) via a polynome to the degree of proteolysis and the 50% ACE inhibitory concentration (IC_{50}) (two responses). A central composite design was chosen, where the factors vary around a central point, here the conditions of the physiological digestion (**Table 1**). This design contains five levels for each digestion parameter, coded by −α, −1, 0,

Table 2. Different Factors of the Central Composite Design with Their Codes and Values

	code				
factor	−α	−1	0	+1	+α
temperature (°C)	18.5	26.0	37.0	48.0	55.5
incubation time stomach (min)	19	60	120	180	221
incubation time small intestine (min)	0	60	150	240	301

+1, and +α (**Table 2**). In this way, the factors are standardized and vary in the same range, which facilitated the interpretation of the effects and interactions. The design exists of three experiment points. First, at the central point, all of the factors are at level 0. Second, the factorial points have all combinations of the factors set at the levels −1 and +1 and indicate the range where the design is valuable. These points give information about the linear effects and the interaction effects. Third, for the axial points, all factors are set at level 0, except for one, which adopts the levels −α or +α, an extreme value. These points determine the estimation of the quadratic effects. The value of α was chosen so that the design was rotatable, which guaranteed that the precision of the estimated values (standard error) was only influenced by the distance to the central point and not by the direction. The final experimental design consisted of 20 experiments for both proteins: six repetitions of the central point, eight factorial points, and six axial points. On the basis of the repetitions of the central point, the experimental error was estimated.

Degree of Proteolysis. The degree of proteolysis was determined by the ratio of the nonprotein Kjeldahl nitrogen to the total Kjeldahl nitrogen. Samples for nonprotein nitrogen determination were treated with trichloroacetic acid solution to a final concentration of 6% (v/v), shaken for 5 min, and then centrifuged at 12000*g* for 10 min at 4 °C. This supernatant and a sample for total nitrogen determination were stored at −80 °C prior to analysis.

ACE Inhibitory Activity. Samples for the determination of ACE inhibitory activity were centrifuged at 10 000*g* for 15 min at 4 °C, and the supernatant was frozen in liquid nitrogen and stored at −80 °C. Freezing in liquid nitrogen was preferred over heating at 98 °C for 10 min to inactivate the proteases and to maintain the bioactivity of the peptides. Next, the frozen samples were lyophilized to obtain a dry powder that was dissolved in demineralized water in 10 mg/mL and analyzed by an ACE inhibition assay using the substrate furanacryloyl-Phe-Gly-Gly (ACE reagent) and ACE control-E as the enzyme source (*6*). When ACE inhibitory activity exceeded 80%, dilution series were made to determine the IC_{50} value. The IC_{50} value was obtained by fitting dose−response data to a four parametric logistic model using the Marquardt−Levenberg algorithm (Sigmaplot 4.0, SPSS Inc., Chicago, IL).

Statistical Analysis. All values are reported as mean ± standard error of the mean ($n_{min} = 3$). For the comparison of the three digestions, significant differences in type of protein and type of digestion were assigned by means of the general linear model univariate analysis of variance (ANOVA) procedure (SPSS 11.0.1). To exclude the initial effect of the protein itself, the statistical analysis was performed on the changes in degree of proteolysis and ACE inhibitory activity during digestion. When there was a significant type of protein × type of digestion interaction for both proteins, one way ANOVA analysis was carried out by type of digestion. All data used in the variance analysis met the homogeneity of variance requirement assessed by Levene's test. Indication of subgroups in type of digestion was done by Tukey's post hoc test ($p < 0.05$). Differences between the digestion in the semicontinuous reactor model and the physiological digestion in batch were assigned by an independent samples *t*-test.

RESULTS AND DISCUSSION

Gastrointestinal digestion is of major importance in the bioavailability of ACE inhibitory peptides. After oral administration, gastrointestinal proteases may splice the ACE inhibitory peptides, thereby increasing or decreasing their activity. For example, the ACE inhibitory activity of KVLPVPQ, derived from β-casein, is increased by pancreatin digestion and the derivative KVLPVP lowers the blood pressure of spontaneously hypertensive rats (*19*). In the same study, an α_{s1}-casein derived peptide, YKVPQL, with strong ACE inhibitory activity, fails to exert an antihypertensive effect due to pancreatin degradation.

During a first set of experiments, the gastrointestinal digestion was simulated in batch, while for the experimental design a semicontinuous reactor model was developed. In the literature, different in vitro models are reported from simple batch experiments (*16*, *20*), dialysis bags (*17*), to more complex computer-controlled models such as artificial stomachs (*21*), TIM (*22*), SHIME (*18*), and models coupled to cell cultures (*23*). Although some of them use pancreatin as a small intestinal enzyme source, we preferred to use relatively pure trypsin and chymotrypsin.

Comparison of Three in Vitro Gastrointestinal Digestions. Before digestion, the pea protein solution showed a lower degree of proteolysis than whey, respectively, 2.4 ± 0.4 and 12.9 ± 0.7% ($p < 0.001$), while the ACE inhibitory activity of both proteins did not differ and was, respectively, 10 ± 0.7 and 15 ± 2% ($p = 0.083$). IC_{50} values were 16 mg/mL for pea and 18 mg/mL for whey protein.

To investigate the conditions during gastrointestinal digestion leading to the release of ACE inhibitory activity, we compared three in vitro digestions (**Table 1**). The nonoptimal digestion was carried out at nonoptimal pH in the stomach and small intestine phase for short incubation times, minimizing protein hydrolysis. The physiological digestion simulated the in vivo conditions of protein digestion. The prolonged optimal digestion combined optimal pH in the stomach and small intestine phase with long incubation times in order to get ideal conditions for protein hydrolysis.

The degree of proteolysis after the three in vitro digestions is depicted in **Figure 2a**. The statistical analysis, however, was performed on the change in degree of proteolysis during digestion, to exclude the initial degree of proteolysis of the protein itself. For both proteins, the increase in degree of proteolysis was significantly different between the three digestions (one way ANOVA, $p < 0.001$; Tukey, $p = 0.05$). From the nonoptimal over the physiological to the prolonged optimal digestion, protein hydrolysis increased due to a more optimal pH for the gastrointestinal proteases and longer incubation times. For all digestions, pea showed a higher increase in degree of proteolysis than whey. The major whey proteins α-lactalbumin and β-lactoglobulin partially resist gastrointestinal digestion. Pepsin degrades α-lactalbumin and only denaturated β-lactoglobulin, trypsin slowly cleaves α-lactalbumin and denaturated β-lactoglobulin, while α-chymotrypsin hydrolyzes both α-lactalbumin and β-lactoglobulin to a limited extent (*24*, *25*). The higher initial degree of proteolysis of the whey protein may also have resulted in more byproduct inhibition for proteolysis. For whey protein, the degree of proteolysis increased proportionally between the three digestion types, while for pea protein, the prolonged optimal digestion contributed only a little more to the degree of proteolysis than did the physiological digestion (type of protein × type of digestion, $p = 0.003$). In the physiological digests, the degree of proteolysis amounted to 72 ± 1% for pea and 58 ± 2% for whey.

The increase in percent ACE inhibitory activity did not vary significantly between the three in vitro gastrointestinal digestions ($p = 0.078$) and amounted to 89.8 ± 0.3% for pea and 83.8 ± 1.2% for whey digest, respectively ($p < 0.001$). As the maximum level of 100% ACE inhibitory activity was reached

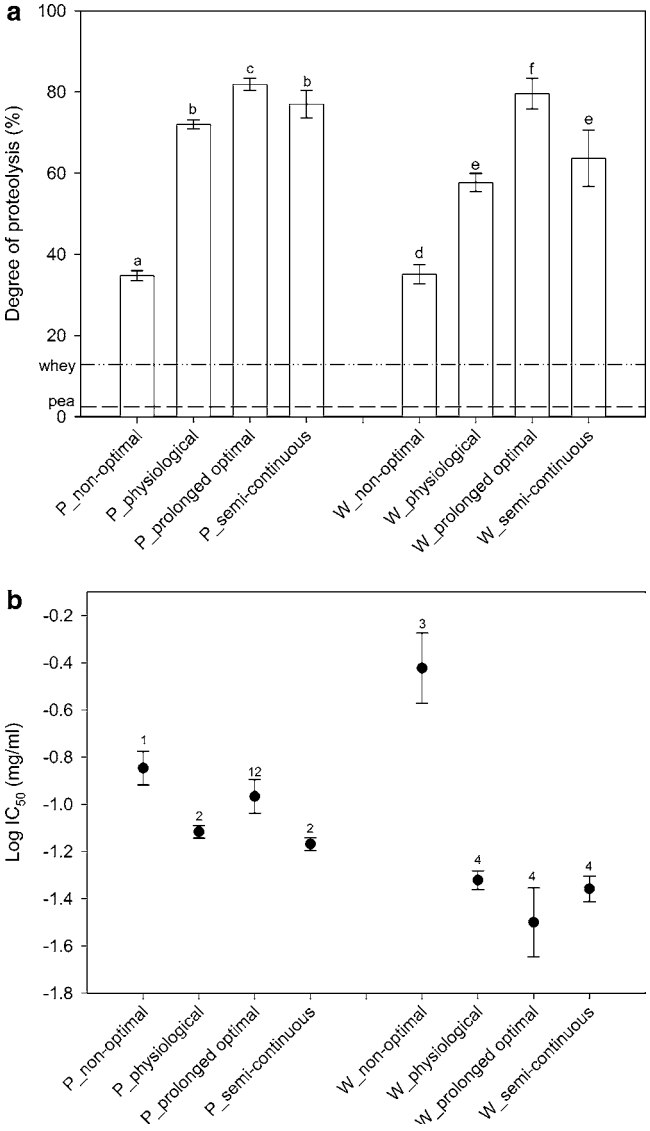

Figure 2. (**a**) Degree of proteolysis (%) (bars, a, b, ...) and (**b**) log IC_{50} (mg/mL) (bullets, 1, 2, ...) after the nonoptimal, physiological, and prolonged optimal digestion and the semicontinuous digestion of pea (P) and whey (W) protein ($n_{min} = 3$). Straight, dashed lines indicate the initial degree of proteolysis of pea and whey protein. Different letters and numbers indicate significant differences ($p < 0.05$). The semicontinuous digestion was only compared to the physiological digestion by an independent samples *t*-test.

after all digestions, the IC_{50} value or 50% inhibitory concentration was determined. A higher IC_{50} indicated a lower ACE inhibitory activity. The three digestions had a different effect on the log IC_{50} value for pea as compared with whey protein (type of protein × type of digestion, $p < 0.001$) (**Figure 2b**). For pea, the only significant difference was found between the nonoptimal and the physiological digestion, where the IC_{50} of the first was double the one of the latter (one way ANOVA, $p = 0.015$; Tukey, $p = 0.05$). For whey protein, on the other hand, a relatively high IC_{50} was obtained by nonoptimal digestion, which displayed a 10-fold difference from those of the physiological and prolonged optimal digestion (one way ANOVA, $p < 0.001$; Tukey, $p = 0.05$). Both pH and incubation time had an influence on the formation of ACE inhibitory activity. A physiological digestion sufficed for both proteins to obtain minimal IC_{50} and hence maximal ACE inhibitory activity. The formation of ACE inhibitory activity reached a saturation level, after which no major breakdown occurred or equilibrium existed between the formation and the degradation of ACE inhibitory peptides. Apparently, a certain degree of proteolysis was necessary to exert high ACE inhibitory activity, as the nonoptimal digestion resulted in lower ACE inhibitory activity than the other two digestions and this effect was larger for whey than for pea. This may be of physiological importance since a higher, nonoptimal pH in the stomach can occur in humans with increasing age (*26*). The higher ACE inhibitory activity of whey protein as compared to that of pea protein after a physiological or prolonged optimal digestion can be attributed to their amino acid sequences, which determine the presence of bioactive peptide sequences and enzyme splicing sites.

IC_{50} values as low as 0.076 mg/mL for the physiological digestion of pea and 0.032 mg/mL for the prolonged optimal digestion of whey protein were observed. The IC_{50} ranges of the physiological and prolonged optimal whey digests are lower than the IC_{50} ranges of 0.130−0.201 and 0.345−1.733 mg/mL reported, respectively, by Mullally et al. (*10*) and Pihlanto-Leppällä et al. (*13*) for whey protein digested by gastrointestinal proteases. However, differences in the ACE inhibition assay and the way of calculation of the ACE inhibitory activity complicate the comparison of IC_{50} values reported in the literature (*27*). No IC_{50} values for pea hydrolysates are reported in the literature.

Experimental Design in a Semicontinuous Reactor Model. A semicontinuous reactor model was developed with the aim to produce high ACE inhibitory activity from pea and whey protein by means of gastrointestinal proteases. As a result, the formed peptides resist the physiological digestion after oral administration (*9*). The process conditions of the semicontinuous reactor differed slightly from the batch experiments in the continuous and more rigorous pH and temperature control and a slight dilution effect by the pH control with only 1 N HCl and 1 N NaOH.

When the physiological digestion was simulated in the semicontinuous reactor, no significant differences in change in degree of proteolysis (pea, $p = 0.13$; whey, $p = 0.38$) and log IC_{50} value (pea, $p = 0.20$; whey, $p = 0.60$) were observed in comparison to the batch physiological digestion (**Figure 2**).

Preliminary experiments in batch did not find a significant influence of protein concentrations and enzyme-to-substrate ratio on the formation of ACE inhibitory activity from pea and whey protein (data not shown). It is expected that a protein concentration of 4% or higher is sufficient to provide substrate saturation conditions (*28*). For fish protein hydrolysates, an increase in enzyme concentration and/or hydrolysis time is shown to result in improved ACE inhibitory activity (*29*). A similar evolution is obtained during the digestion of blood plasma albumin by alkalase, while during digestion of casein by trypsin the high ACE inhibitory activity obtained the first hour decreases temporarily and then increases gradually with hydrolysis time and increasing enzyme concentration (*30*). The influence of the incubation time in the stomach and small intestine phase was already suggested in the previous experiments. In addition, it is known that pepsin A has an optimal activity at 42 °C (*31*), while for trypsin this is at 45 °C (*32*). The optimal hydrolysis temperature for α-chymotrypsin is 50 °C (*33*). Therefore, temperature and incubation time in the stomach and small intestine phase were chosen as parameters in the experimental design in the semicontinuous reactor. The batch physiological digestion sufficed to achieve maximal ACE inhibitory activity and consequently served as a central point in the experimental design. Hence, the digestion was performed at physiological

Table 3. Average ± Standard Error and Range of All Points in Comparison to the Central Points for the Degree of Proteolysis (%) and the log IC_{50} (mg/mL) in the Central Composite Design

		degree of proteolysis (%)		log IC_{50} (mg/mL)	
		all points	central points	all points	central points
pea	av	73 ± 9	77 ± 3	–1.24 ± 0.04	–1.19 ± 0.01
	range	49–86	65–85	–0.90 to –1.66	–1.16 to –1.23
whey	av	53 ± 4	64 ± 4	–1.32 ± 0.06	–1.42 ± 0.04
	range	32–82	40–81	–0.37 to –1.66	–1.23 to –1.66

Table 4. Coefficients in Terms of Actual and Coded Factors and Their *p* Values, and Validation Parameters of the Linear and Quadratic Model Equations of, Respectively, the Degree of Proteolysis (%) and the log IC_{50} Value (mg/mL) as a Function of Temperature (°C) (A), Incubation Time in the Stomach (min) (B), and Incubation Time in the Small Intestine Phase (min) (C)

	degree of proteolysis (%)			log IC_{50} (mg/mL)		
model	linear ($p = 0.0232$)			quadratic ($p < 0.0001$)		
factors	actual	coded	*p* value	actual	coded	*p* value
constant	43.441	73.2		–2.438	–1.19	
A	0.536	5.90	0.0124	0.039	–0.017	0.1494
B	0.023	1.39	0.5152	0.010	–0.096	<0.0001
C	0.047	4.27	0.0589	1.7×10^{-3}	–0.094	<0.0001
A^2				-1.4×10^{-4}	–0.017	0.1286
B^2				-1.8×10^{-5}	–0.066	0.0002
C^2				4.1×10^{-6}	0.033	0.0120
AB				-1.6×10^{-4}	–0.100	0.0001
AC				-7.4×10^{-5}	–0.073	0.0014
BC				-1.1×10^{-5}	–0.057	0.0056
lack of fit		0.65			0.20	
adjusted R^2		0.33			0.95	
predicted R^2		0.16			0.80	
adequate precision		6.68			27.05	
PRESS		1442			0.08	

pH, which is close to the optimal pH for the gastrointestinal proteases (*15*).

The aim of this design was to establish, for both pea and whey protein, a relation between the responses, the degree of proteolysis, and the ACE inhibitory activity, expressed as log IC_{50} value, and the variables, temperature (from 26 to 48 °C), incubation time in the stomach phase (from 1 to 3 h), and incubation time in the small intestine phase (from 1 to 4 h) within a certain degree of freedom around the physiological digestion parameters (37 °C, 2 and 2.5 h) (**Table 2**). As the IC_{50} was calculated by means of the four parametric logistic model, only the log IC_{50} is normally distributed. Moreover, the Box Cox plot for power transformation in the Design Expert analysis program indicated that a log transformation for the IC_{50} data was necessary.

The statistic properties of the data points in the experimental design were investigated for the two responses for both proteins (**Table 3**). Both for the degree of proteolysis and the log IC_{50} value of the pea digests, the range of all data points included the range of the central points, the only replicates in the design. Moreover, the standard error on the average of the central points was smaller than the one on the average of all data points. This indicated a good reproducibility of the semicontinuous digestion of pea protein. Hence, the large standard error on the average of all pea digests and the large range were probably not due to experimental error but to variation in the process parameters. This justified the creation of a response surface model for pea protein in the two responses. For whey protein, however, the

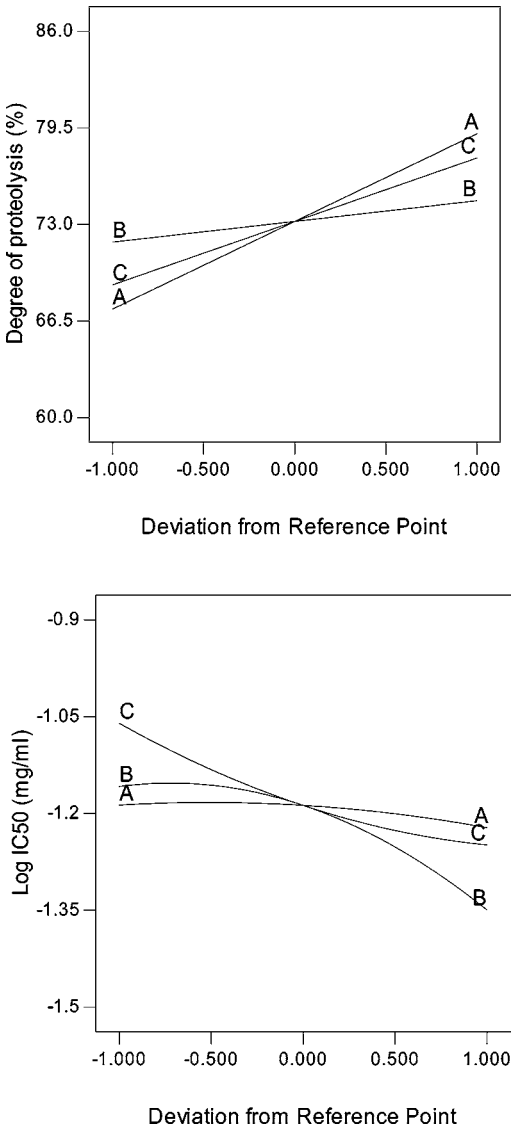

Figure 3. Perturbation plots for degree of proteolysis (%) and log IC_{50} (mg/mL) as a function of temperature (**A**), incubation time in the stomach (**B**), and incubation time in the small intestine phase (**C**).

central points showed a rather large variation for both responses, which indicated a poor reproducibility of the semicontinuous digestion of whey protein. Because the standard error on the average of all samples was about as large as the one on the average of the central points, it could not be explained by a variation in process parameters and was merely due to experimental error. As a result, neither for the degree of proteolysis nor for the log IC_{50} of the whey, a significant model could be established. The higher variability of whey protein in comparison to pea protein is also observed in higher standard errors for the degree of proteolysis and the log IC_{50} in the comparison of the three batch digestions. This can partially be explained by the lower susceptibility to hydrolysis, resulting in higher variations when slightly different conditions are applied.

For pea protein, the best fitting model equation of the degree of proteolysis and log IC_{50} was, respectively, calculated as a linear and quadratic polynomial equation of temperature, incubation time in the stomach, and incubation time in the small intestine phase. **Table 4** shows the coefficients of the obtained response equations and the corresponding model validation parameters. For a good model, lack of fit should be nonsignificant, R^2 values should be high (predicted R^2 in reasonable

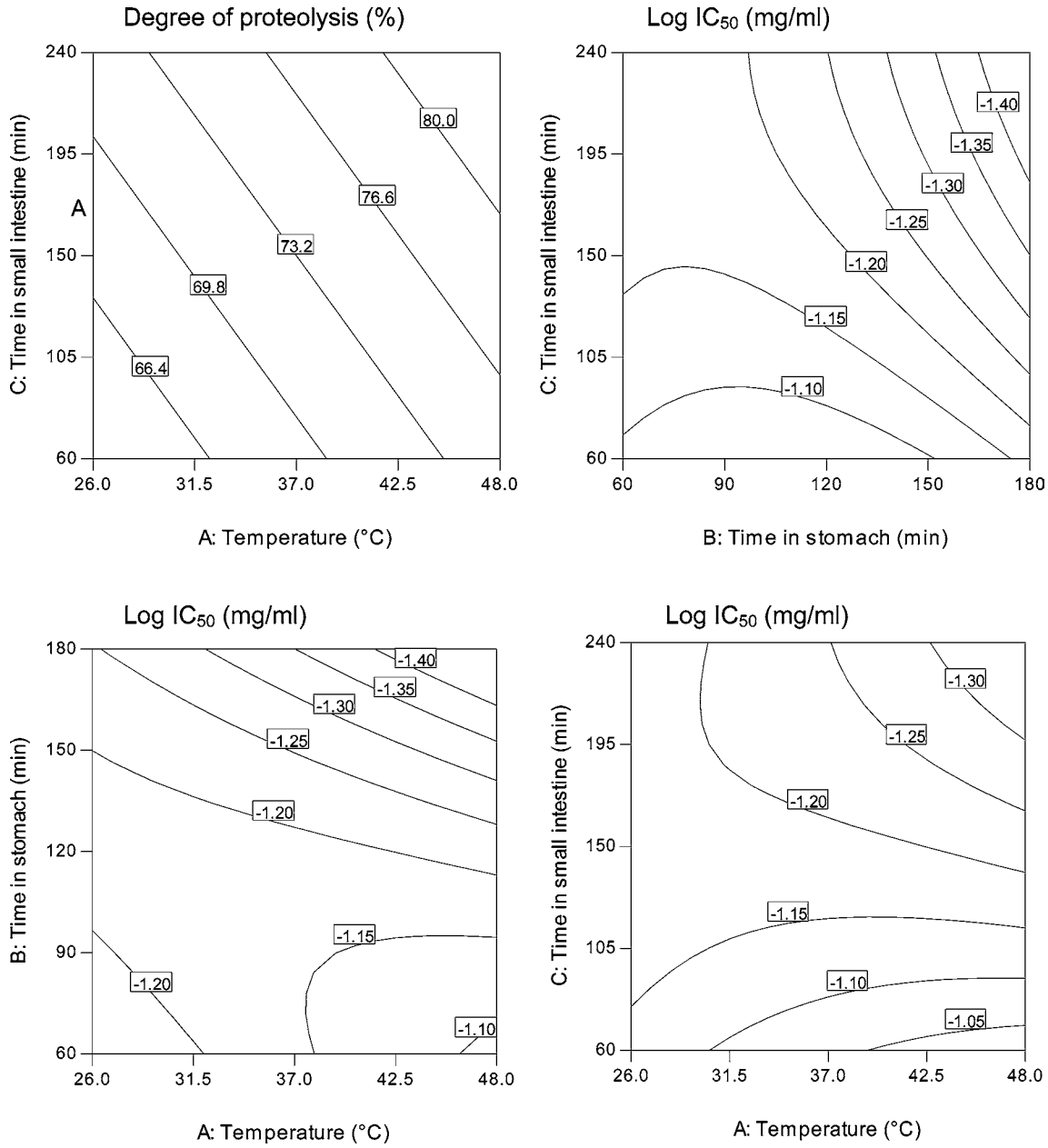

Figure 4. Two-dimensional contour plots for the degree of proteolysis (%) and log IC_{50} (mg/mL). The nondisplayed parameter is set at the central point.

agreement with adjusted R^2), adequate precision should be above 4, and PRESS values should be low. The adequate precision value measures the signal over noise ratio, while the PRESS statistic points to the lack of predictive value of the model. Reduced models could be obtained by omitting the nonsignificant model terms ($p > 0.1$), taking into account the hierarchy of the model, but responses and validation parameters did not change much (data not shown). Both the linear model for the degree of proteolysis and the quadratic model for the log IC_{50} were significant. Furthermore, the model validation parameters demonstrated that the equations were representative for the observed data and useful to navigate the design space. Two different models obtained for the two responses indicate that no clear relationship exists between the (log) IC_{50} and the degree of proteolysis during digestion, which is also observed for the comparison of the three batch digestions and is already reported in the literature (*10*).

For the linear model of the degree of proteolysis, the incubation time in the stomach did not contribute significantly. The equation in terms of coded factors revealed that the effects of temperature and incubation time in small intestine on the degree of proteolysis were about the same. The degree of proteolysis of a casein hydrolysate increases with longer incubation times in the presence of pancreatin, while a short or long preincubation with pepsin has the same effect (*17*). A higher temperature exerted a major effect, which can be attributed to the optimal temperatures of the enzymes, which are above 37 °C (*31*−*33*). Quadratic terms of the parameters were important for the description of the log IC_{50}. Two experiments out of 20 were discarded to increase the R^2 values and the similarity between the predicted R^2 and the adjusted R^2. Here, the temperature and its quadratic term were nonsignificant, but it could not be omitted in the model because of the hierarchy and the significant interaction terms. From the equation in terms of coded factors, it was seen that incubation time in the stomach, incubation time in the small intestine, and the interaction between temperature and incubation time in the stomach exerted major effects of similar impact.

Results are displayed as perturbation plots of the nonreduced models and contour plots (**Figures 3** and **4**). A perturbation

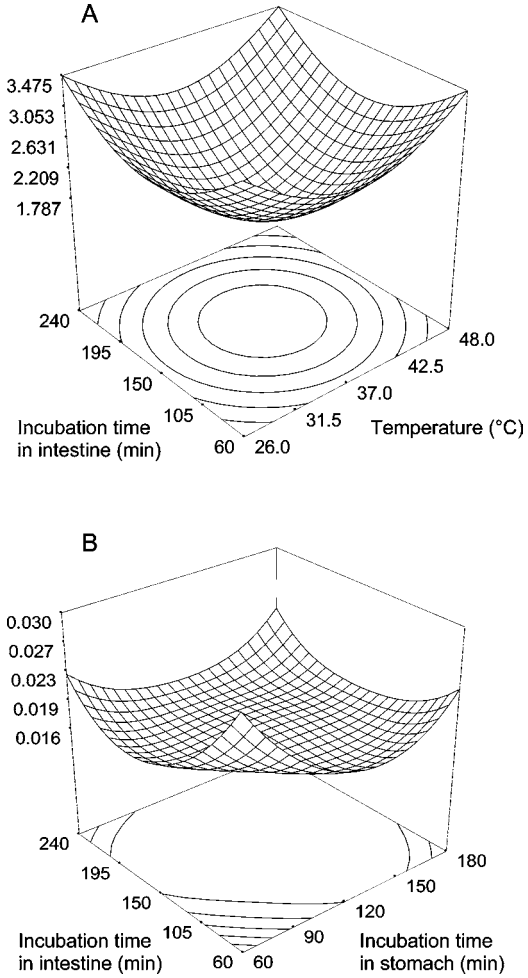

Figure 5. Three-dimensional plot of the standard error of the degree of proteolysis (%) (**A**) and the standard error of the log IC_{50} (mg/mL) (**B**) in function of the most significant parameters of the model, while the other parameter was set at the central point.

plot shows how the response changes as each factor, expressed as coded value, moves from the central point of the design toward the borders, while all other factors are held constant at the central point. The degree of proteolysis showed the major deviation from the central point when the temperature varied, while the log IC_{50} changed substantially from the central point when the incubation time in the stomach increased and the incubation time in the small intestine phase decreased. A contour plot is a two-dimensional representation of the response for selected factors. The contour plot for the degree of proteolysis indicates that this response increased as pea protein was digested at higher temperatures and with longer incubation times in the small intestine phase, when the incubation time in the stomach was set at 120 min. At a temperature of 37 °C, the log IC_{50} lowered when the incubation time in both the stomach and the small intestine phase increased. This is somewhat contradictory to the results obtained for the batch prolonged optimal and physiological digestion for pea, but there, the lower ACE inhibitory activity for longer incubation times may be due to the lack of pH control. In the contour plots for the log IC_{50}, where the temperature is displayed at the *X*-axis, the rather horizontal lines point to the limited importance of the temperature.

At the central point, the observed degree of proteolysis and log IC_{50} value were, respectively, 77 ± 3% and −1.19 ± 0.01 mg/mL (IC_{50} = 0.065 mg/mL), while their predicted values were, respectively, 73% and −1.19 mg/mL. From the three-dimensional plot of the standard errors, it can be found that the model for the log IC_{50} is more reliable than the one for the degree of proteolysis: the central composite design provided relatively precise predictions over a broader area around the central point (**Figure 5**). The circular contours confirmed the rotatability of the design.

The central composite design was used to optimize toward a maximal degree of proteolysis on one hand and a minimal log IC_{50} on the other hand, when all of the parameters were in the design space. For both criteria, this resulted in maximal parameters: maximal temperature (48 °C), maximal incubation time in the stomach (3 h), and maximal incubation time in the small intestine phase (4 h). The maximal degree of proteolysis amounted to 85%, and the minimal log IC_{50} was −1.66 mg/mL (IC_{50} = 0.022 mg/mL). As this maximum was situated at the edge of the design space, it was associated with a relatively large standard error. Hence, the optimization results have to be interpreted with caution. Because of the nonsignificance of the factor incubation time in the stomach in the model of the degree of proteolysis, the maximal degree of proteolysis was also attained when only the temperature and the incubation time in the small intestine phase were maximized in the model. When the temperature, the least important factor in the model of the log IC_{50}, was set at 37 °C, a slightly lower maximal ACE inhibitory activity (log IC_{50} = −1.47 mg/mL, IC_{50} = 0.034 mg/mL) was obtained for maximal incubation time in the stomach and maximal incubation time in the small intestine phase.

During the reaction of this paper, one very recent study appeared on the optimization of ACE inhibition by response surface methodology (*34*). In this paper, a quadratic model for the IC_{50} of a pancreatic whey digest is obtained. Whereas we studied both the stomach and the small intestine phase of the gastrointestinal digestion, this study focuses only on the small intestine phase as a production process for ACE inhibitory peptides. Moreover, the authors included pH, enzyme over substrate ratio, and pretreatment temperature of the protein as process parameters, in addition to temperature and hydrolysis time. They found maximal ACE inhibitory activity at a rather low pretreatment temperature of the protein and for hydrolysis at a high enzyme over substrate ratio, a long hydrolysis time (optimally 5 h), pH 8−9, and a temperature of 45 °C. Hence, their findings on hydrolysis time and temperature are in agreement with our results for the pea digest.

In conclusion, gastrointestinal digestion is physiologically important after oral administration of ACE inhibitory peptides. The normal physiological process resulted in vitro in maximal ACE inhibitory activity release from pea and whey protein. When performed in a semicontinuous reactor model, it could be used as a production process for ACE inhibitory peptides from pea and whey protein. For pea, a response surface model indicated the process parameters necessary to release maximal ACE inhibitory activity.

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LITERATURE CITED

(1) Fitzgerald, R. J.; Meisel, H. Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *Br. J. Nutr.* **2000**, *84*, S33−S37.

(2) Hansson, L.; Lloyd, A.; Anderson, P.; Kopp, Z. Excess morbidity and cost of failure to achieve targets for blood pressure control in Europe. *Blood Pressure* **2002**, *11*, 35−45.
(3) Sipola, M.; Finckenberg, P.; Korpela, R.; Vapaatalo, H.; Nurminen, M. L. Effect of long-term intake of milk products on blood pressure in hypertensive rats. *J. Dairy Res.* **2002**, *69*, 103−111.
(4) Takano, T. Milk derived peptides and hypertension reduction. *Int. Dairy J.* **1998**, *8*, 375−381.
(5) Yamamoto, N. Antihypertensive peptides derived from food proteins. *Biopolymers* **1997**, *43*, 129−134.
(6) Vermeirssen, V.; Van Camp, J.; Decroos, K.; Van Wijmelbeke, L.; Verstraete, W. The impact of fermentation and in vitro digestion on the formation of ACE inhibitory activity from pea and whey protein. *J. Dairy Sci.* **2003**, *86*, 429−438.
(7) Masuda, O.; Nakamura, Y.; Takano, T. Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides to spontaneously hypertensive rats. *J. Nutr.* **1996**, *126*, 3063−3068.
(8) Pihlanto-Leppälä, A. Bioactive peptides derived from bovine whey proteins: opioid and ACE-inhibitory peptides. *Trends Food Sci. Technol.* **2001**, *11*, 347−356.
(9) Matsui, T.; Yukiyoshi, A.; Doi, S.; Sugimoto, H.; Yamada, H.; Matsumoto, K. Gastrointestinal enzyme production of bioactive peptides from royal jelly protein and their antihypertensive ability in SHR. *J. Nutr. Biochem.* **2002**, *13*, 80−86.
(10) Mullally, M.; Meisel, H.; Fitzgerald, R. J. Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey proteins. *Int. Dairy J.* **1997**, *7*, 299−303.
(11) Meisel, H.; Goepfert, A.; Günther, S. ACE-inhibitory activities in milk products. *Milchwissenschaft* **1997**, *52*, 307−311.
(12) Kim, S. K.; Byun, H. G.; Park, P. J.; Shahidi, F. Angiotensin I converting enzyme inhibitory peptides purified from bovine skin gelatin hydrolysate. *J. Agric. Food Chem.* **2001**, *49*, 2992−2997.
(13) Pihlanto-Leppälä, A.; Koskinen, P.; Piilola, K.; Tupasela, T.; Korhonen, H. Angiotensin I-converting enzyme inhibitory properties of whey protein digests: concentration and characterization of active peptides. *J. Dairy Res.* **2000**, *67*, 53−64.
(14) Charman, W. N.; Porter, C. J. H.; Mithani, S.; Dressman, J. B. Physicochemical and physiological mechanisms for the effects of food on drug absorption: the role of lipids and pH. *J. Pharm. Sci.* **1997**, *86*, 269−282.
(15) Ganapathy, V.; Leibach, F. H. Protein digestion and assimilation. In *Textbook of Gastroenterology*; Yamada, T., Ed.; Lippincott Williams & Wilkins: Piladelphia, 1999; pp 456−467.
(16) Oomen, A. G.; Hack, A.; Minekus, M.; Zeijdner, E.; Cornelis, C.; Schoeters, G.; Verstraete, W.; Van de Wiele, T.; Wragg, J.; Rompelberg, C. J. M.; Sips, A.; Van Wijnen, J. H. Comparison of five in vitro digestion models to study the bioaccessibility of soil contaminants. *Environ. Sci. Technol.* **2002**, *36*, 3326−3334.
(17) Gauthier, S. F.; Vachon, C.; Savoie, L. Enzymatic conditions of an in vitro method to study protein digestion. *J. Food Sci.* **1986**, *51*, 960−964.
(18) Molly, K.; Van de Woestyne, M.; Verstraete, W. Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. *Appl. Microbiol. Biotechnol.* **1993**, *39*, 254−258.
(19) Maeno, M.; Yamamoto, N.; Takano, T. Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP790. *J. Dairy Sci.* **1996**, *79*, 1316−1321.
(20) Pihlanto-Leppällä, A.; Rokka, T.; Korhonen, H. Angiotensin I-converting enzyme inhibitory peptides from bovine milk proteins. *Int. Dairy J.* **1998**, *8*, 325−331.
(21) Yvon, M.; Beucher, S.; Scanff, P.; Thirouin, S.; Pelissier, J. P. In vitro simulation of gastric digestion of milk proteins−Comparison between in vitro and in vivo data. *J. Agric. Food Chem.* **1992**, *40*, 239−244.
(22) Minekus, M.; Marteau, P.; Havenaar, R.; Intveld, J. H. J. Development of a computer-controlled in vitro model of the gastrointestinal tract. *Gastroenterology* **1993**, *104*, A553−A553.
(23) Glahn, R. P.; Wortley, G. M.; South, P. K.; Miller, D. D. Inhibition of iron uptake by phytic acid, tannic acid, and $ZnCl_2$: Studies using an in vitro digestion/Caco-2 cell model. *J. Agric. Food Chem.* **2002**, *50*, 390−395.
(24) Schmidt, D. G.; van Markwijk, B. W. Enzymatic hydrolysis of whey proteins. Influence of heat treatment of α-lactalbumin and β-lactoglobulin on their proteolysis by pepsin and papain. *Neth. Milk Dairy J.* **1993**, *47*, 15−22.
(25) Guo, M. R.; Fox, P. F.; Flynn, A.; Kindstedt, P. S. Susceptibility of β-lactoglobulin and sodium caseinate to proteolysis by pepsin and trypsin. *J. Dairy Sci.* **1995**, *78*, 2336−2344.
(26) Morihara, M.; Aoyagi, N.; Kaniwa, N.; Kojima, S.; Ogata, H. Assessment of gastric acidity of Japanese subjects over the last 15 years. *Biol. Pharm. Bull.* **2001**, *24*, 313−315.
(27) Ariyoshi, J. Angiotensin-converting enzyme inhibitors derived from food proteins. *Trends Food Sci. Technol.* **1993**, *4*, 139−144.
(28) Mutilangi, W. A. M.; Panyam, D.; Kilara, A. Hydrolysates from proteolysis of heat-denatured whey proteins. *J. Food Sci.* **1995**, *60*, 1104−1110.
(29) Matsui, T.; Matsufuji, H.; Seki, E.; Osajima, K.; Nakashima, M.; Osajima, Y. Inhibition of angiotensin I-converting enzyme by *Bacillus licheniformis* alkaline protease hydrolysates derived from sardine muscle. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 922−925.
(30) Hyun, C. K.; Shin, H. K. Utilization of bovine blood plasma proteins for the production of angiotensin I converting enzyme inhibitory peptides. *Proc. Biochem.* **2000**, *36*, 65−71.
(31) Ryle, A. P. Pepsins, gastricins and their zymogens. *Methods Enzymol.* **1984**, *5*, 228−238.
(32) Venkatesk, R.; Sundaram, P. V. Modulation of the stability properties of bovine trypsin after in vitro structural changes with a variety of chemical modifiers. *Protein Eng.* **1998**, *11*, 691−698.
(33) Angelo, I. A.; Shanani, K. M.; Kilara, A. Effect of different proteases on milk proteins. *Milchwissenschaft* **1982**, *37*, 212−215.
(34) van der Ven, C.; Gruppen, H.; de Bont, D. B. A.; Voragen, A. G. J. Optimisation of the angiotensin converting enzyme inhibition by whey protein hydrolysates using response surface methodology. *Int. Dairy J.* **2002**, *12*, 813−820.

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