Production and transepithelial transportation of angiotensin-I-converting enzyme (ACE)-inhibitory peptides from whey protein hydrolyzed by immobilized *Lactobacillus helveticus* proteinase

Yuxing Guo,¹ Xiaoxiao Jiang,¹ Binyi Xiong,¹ Tao Zhang,¹ Xiaoqun Zeng,² Zhen Wu,² Yangying Sun,² and Daodong Pan^{1,2*}

¹Department of Food Science and Technology, Ginling College, Nanjing Normal University, Nanjing 210097, Jiangsu, China

²Food Science and Technology Department, Marine Science School, Ningbo University, Ningbo 315211, Zhejiang, China

ABSTRACT

Lactobacillus helveticus LB 10 proteinases immobilized with sodium alginate were used to hydrolyze whey protein to produce angiotensin-I-converting enzyme (ACE)-inhibitory peptides. The generated hydrolysates were tested for ACE-inhibitory activity and for their ability to be transported across Caco-2 cell monolayers. Using a response surface method, we determined that a proteinase concentration of 7.55 mg/mL, sodium alginate concentration of 2.03 g/100 mL, and glutaraldehyde concentration of 0.39% were found to be the optimal immobilization conditions. Compared with free proteinase, the immobilized proteinase had significantly higher pH, thermal and storage stability, and reusability. Whey protein hydrolysates were fractionated by gel filtration chromatography and ACE-inhibitory peptide mixtures were transported across Caco-2 cell monolayers in a human intestinal-absorption model. The di- and tripeptides KA, EN, DIS, EVD, LF, AIV, and VFK (half-maximal inhibitory concentrations (mean $\pm$ standard deviation) of 1.24 ± 0.01 , 1.43 ± 0.04 , 1.59 ± 0.27 , 1.32 ± 0.05 , 1.60 ± 0.39 , 2.66 ± 0.02 , and 1.76 ± 0.09 mmol/L, respectively) were detected on the basolateral side of the Caco-2 cell monolayer using ultra-performance liquid chromatography-tandem mass spectrometry. These results highlight that ACE-inhibitory peptides are present on the basolateral side of the Caco-2 cell model after transportation of whey protein hydrolysate across the Caco-2 cell membrane.

Key words: angiotensin-I-converting enzyme (ACE)-inhibitory peptide, proteinase, *Lactobacillus helveticus*, Caco-2 cell, bioavailability

INTRODUCTION

Angiotensin-I-converting enzymes (ACE; EC 3.4.15.1) play an important role in modulating blood pressure: ACE converts inactive angiotensin I into angiotensin II (a potent octapeptide vasoconstrictor), which can increase blood pressure. Angiotensin-I-converting enzymes can also degrade the vasodilator peptide bradykinin (Stuknytė et al., 2015). Therefore, ACE-inhibitory peptides may be used as a nutraceutical to prevent or manage hypertension.

Lactobacillus helveticus is a thermophilic lactic acid bacterium widely used in the manufacture of dairy products such as cheese and fermented milk (Sadat-Mekmene et al., 2011). Some studies have found that milk fermented with *L. helveticus* exhibits ACE-inhibitory activity, which is due to the specific proteolytic activity of *L. helveticus* (Nakamura et al., 1995; Yamamoto et al., 1999). Cell-envelope proteinase (CEP), a key enzyme in the proteolytic system of *Lactobacillus*, hydrolyzes protein into different oligopeptides (Sadat-Mekmene et al., 2011). The hydrolysates can be transported across the cell membrane by AA transport systems, including oligopeptide and di- and tripeptide transporters. Then, the peptides are further hydrolyzed into AA or smaller peptides by intracellular peptidases, including endopeptidases, aminopeptidases, and X-prolyl-dipeptidyl aminopeptidases (Pan and Guo, 2010; Wakai et al., 2013). Wakai et al. (2013) reported that CEP affects the production of VPP [β -CN f(84–86)] and IPP [β -CN f(74–76)] from milk protein, and Guo et al. (2009) identified RLSFNP, a new ACE-inhibitory peptide from whey protein hydrolyzed by crude proteinases (cell-free extracts containing CEP and peptidases) of *L. helveticus* LB 10. However, ACE-inhibitory peptide production using free *L. helveticus* LB 10 proteinases is expensive because of the difficulty in extracting the proteinases. Therefore, in this study, we immobilized *L. helveticus* LB 10 proteinases to produce ACE-inhibitory peptides from whey protein.

Received April 9, 2018.

Accepted October 31, 2018.

*Corresponding author: daodongpan@163.com

One of the greatest challenges in developing milk peptides as antihypertensive food ingredients has been proving their *in vivo* efficacy (Hernández-Ledesma et al., 2014). To exert an antihypertensive effect after oral administration, ACE-inhibitory peptides must remain active after intestinal transport and at least until they reach the cardiovascular system (Fernández-Musoles et al., 2013). Most ACE-inhibitory peptides have between 2 and 12 AA (Stuknytė et al., 2015). Among ACE-inhibitory peptides, several di- and tripeptides, such as VPP and IPP, have been reported to be inhibitors that can be transported intact across the intestinal epithelium (Nakamura et al., 1995). However, long-chain ACE-inhibitory peptides may have difficulty crossing the intestinal epithelial cell barrier and thereby lose their antihypertensive capabilities *in vivo* (Vermeirssen et al., 2002). The antihypertensive peptide YAEERYPIL was completely hydrolyzed into YAEER and YP by peptidases in the intestinal brush-border membrane (Miguela et al., 2007). The ACE-inhibitory peptide RLSFNP was hydrolyzed into fragments (F, FNP, SFNP, and RLSF) when it crossed Caco-2 cell monolayers (Guo et al., 2018). The intestinal transport of ACE-inhibitory peptides is an important factor in the *in vitro* investigation of the maintenance of ACE-inhibitory activity through the oral pathway (Vermeirssen et al., 2002).

The Caco-2 cell model can be used to study absorption of ACE-inhibitory peptides and to screen transportable ACE-inhibitory peptides. The transport of milk-derived peptide mixtures across Caco-2 cell monolayers has been reported (Stevenson et al., 1999). Caco-2 cells, which are derived from human intestinal adenocarcinoma, exhibit spontaneous enterocyte-like differentiation, microvillus structure, and functional tight junctions between adjacent cells, as well as transport systems, cellular peptidases, and other characteristics that mimic human intestinal epithelial cell absorption (Sonnier et al., 2010; Picariello et al., 2013).

The bioavailability of milk-derived peptides is a major issue that may hamper their use as bioactive ingredients in functional dairy foods. The objective of the present research was to produce ACE-inhibitory peptides hydrolyzed from whey protein using immobilized *L. helveticus* proteinases and to study transepithelial transportation of ACE-inhibitory peptides in a Caco-2 cell model. Immobilized proteinases of *L. helveticus* were used as catalytic agents to hydrolyze whey protein into ACE-inhibitory peptides. The hydrolysate was fractionated using gel filtration chromatography and the low-molecular-mass fraction having ACE-inhibitory activity was then presented to Caco-2 monolayers to assess the ability of the peptides to cross the intestinal barrier.

The peptides present in the basolateral solution were identified using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The detected peptides were synthesized and their ACE-inhibitory activity was analyzed.

MATERIALS AND METHODS

Preparation of Cell-Free Proteinases from *L. helveticus*

Lactobacillus helveticus LB10 (conserved by the Dairy Biotechnology Key Laboratory, Nanjing Normal University, Nanjing, Jiangsu, China) was grown in liquid de Man, Rogosa, and Sharpe broth at 37°C for 24 h. The *L. helveticus* culture medium was centrifuged ($4,500 \times g$, 20 min, 4°C) to harvest the cells, which were then washed 3 times with Tris-HCl buffer (50 mM, pH 7.1). The washed cells were suspended in Tris-HCl buffer (50 mM, pH 7.1) at a final concentration of 0.2 g/mL, and then treated with ultrasonic waves (300 W) at 30-s intervals for 26 min at 4°C. To remove the cell debris, the samples were centrifuged at $4,500 \times g$ for 20 min at 4°C. The supernatant containing cell-free proteinases was collected.

Immobilization of *L. helveticus* Proteinases

Proteinase Immobilization. The proteinases were immobilized under sterile conditions. Sodium alginate was suspended in proteinase solution. The mixture was extruded dropwise through a syringe into CaCl_2 solution. The capsules were hardened for 4 h at 4°C and then washed with sterile distilled water. Glutaraldehyde was added to crosslink the proteinases in capsules for 6 h. The capsules were washed again with sterile distilled water and used as immobilized proteinases. The factors (sodium alginate, CaCl_2 , glutaraldehyde, and proteinase concentrations) were optimized using a single-factor experiment and response surface methodology (RSM). The activity of the immobilized proteinases was analyzed according to the method described below.

Single-Factor Experiment. The effect of sodium alginate on the activity of the immobilized proteinases was studied at concentrations of 1, 1.5, 2, 2.5, and 3 g/100 mL. The effect of CaCl_2 on immobilized proteinase activity was studied at concentrations of 0.05, 0.1, 0.15, 0.2, and 0.25 mol/L. The effect of glutaraldehyde on immobilized proteinase activity was conducted at concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0%. The effect of proteinase concentration on immobilized proteinase activity was conducted at concentrations of 4, 8, 12, 16, and 20 mg/mL.

Table 1. Experimental design used in the response surface method (RSM) studies with 3 independent variables showing observed specific immobilized proteinase activity (%; y)

Run	Variable ¹			Specific immobilized proteinase activity (U/mg)
	x_1	x_2	x_3	
1	4 (−1)	1.5 (−1)	0.4 (0)	1.95
2	12 (1)	1.5 (−1)	0.4 (0)	1.33
3	4 (−1)	2.5 (1)	0.4 (0)	1.95
4	12 (1)	2.5 (1)	0.4 (0)	1.90
5	4 (−1)	2 (0)	0.2 (−1)	1.98
6	12 (1)	2 (0)	0.2 (−1)	1.32
7	4 (−1)	2 (0)	0.6 (1)	2.04
8	12 (1)	2 (0)	0.6 (1)	1.20
9	8 (0)	1.5 (−1)	0.2 (−1)	2.06
10	8 (0)	2.5 (1)	0.2 (−1)	1.99
11	8 (0)	1.5 (−1)	0.6 (1)	1.33
12	8 (0)	2.5 (1)	0.6 (1)	1.91
13	8 (0)	2 (0)	0.4 (0)	4.15
14	8 (0)	2 (0)	0.4 (0)	3.73
15	8 (0)	2 (0)	0.4 (0)	3.90

¹The independent variables were concentrations of proteinase (x_1), sodium alginate (x_2) and glutaraldehyde (x_3). The independent variables contained 3 equidistant levels (4, 8, and 12 mg/mL for x_1 ; 1.5, 2.0, and 2.5 g/100 mL for x_2 ; and 0.2, 0.4, and 0.6 g/100 mL for x_3), which were coded (parenthetical values) as −1, 0, and 1.

RSM Experimental Design. An RSM experiment was conducted using Minitab 16 software (Minitab Inc., State College, PA). A Box-Behnken design was used to optimize the process parameters. The design involved 15 runs with 3 independent variables in 1 block (Van der Ven et al., 2002). The independent variables included the concentrations of proteinase (x_1), sodium alginate (x_2), and glutaraldehyde (x_3). The independent variables contained 3 equidistant levels (4, 8, and 12 mg/mL for x_1 ; 1.5, 2.0, and 2.5 g/100 mL for x_2 ; and 0.2, 0.4, and 0.6 g/100 mL for x_3), which were coded as −1, 0, and +1 (Table 1).

The average of the immobilized proteinase activity (all experiments were performed 3 times) was selected as the response (y). The system was explained by the polynomial equation

$$y = \beta_0 + \sum_{i=1}^3 \beta_i x_i + \sum_{i=1}^3 \beta_{ii} x_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} x_i x_j, \quad [1]$$

where y is the dependent variable, and β_0 , β_i , β_{ii} , and β_{ij} are coefficients estimated by the model; β_0 , β_i , β_{ii} , and β_{ij} represent, respectively, the constant, linear, quadratic, and cross effects of the factors (x_1 , x_2 , and x_3) on the response (y) (Pan and Guo, 2010).

Characteristics of Free and Immobilized Proteinases

Effect of pH and Temperature on Free and Immobilized Proteinases. The free and immobilized proteinases were incubated in 50 mM Tris-HCl buffer

(pH 5.0–9.0) at 37°C, and the activity of the proteinases was assayed according to the method described below. The free and immobilized proteinases were also incubated at different temperatures ranging from 29°C to 49°C in 50 mM Tris-HCl buffer (pH 7.0) and activity was assayed according to the method described below.

pH and Thermo Stability of Free and Immobilized Proteinases. The free and immobilized proteinases were incubated at different pH values (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0) for 30 min to measure pH stability. The free and immobilized proteinases were incubated between 30°C and 90°C (10°C increments) for 30 min in a water bath to measure thermo stability. The residual activity was determined under standard conditions according to the method described below.

Storage Stability and Reusability of Immobilized Proteinases. To evaluate their storage stability, the immobilized proteinases were stored at 4°C for 30 d; their activity was measured at 3, 7, and 30 d. The immobilized proteinases were analyzed for their reusability by recycling 5 times. The alginate beads were separated from substrate solution by filtration and washed with Tris-HCl buffer (50 mM, pH 7.0) after each proteinase activity measurement. Then, the next activity measurement was carried out and the first-run activity was defined as 100% (Zhou et al., 2010).

Measuring Activity of Free and Immobilized Proteinases

One milliliter of free proteinase (0.1 g/mL) or 1 g of immobilized proteinase and 0.05 mL of 6.4 mM substrate

methoxy-succinyl-arginyl-prolyl-tyrosyl-*p*-nitroanilide (MeOsuc-Arg-Pro-Tyr-pNA; MP Biomedicals, Solon, OH) dissolved in methanol were added to 2.85 mL of 50 mM Tris-HCl buffer (pH 7.0), and the mixtures were incubated at 37°C for 60 min. Then, 0.5 mL of 30% (vol/vol) acetic acid was added to the mixtures to stop the reactions. The absorbance (A) of the liberated paranitroaniline was measured at 410 nm. Proteinase activity (U) of proteinase activity was defined as the amount of enzyme that produced an increase of 0.01 unit of A_{410nm} per hour at 37°C:

$$\text{Specific immobilized proteinase activity} \\ (\text{U/mg}) = \frac{\text{immobilized proteinase activity (U)}}{\text{weight of free proteinases (mg) contained} \\ \text{in 1 g of immobilized proteinase.}} \quad [2]$$

Production of Whey Hydrolysates

Whey protein concentrate (WPC)-80 (Beijing Milky Way Trade Corp., Beijing, China) had the following composition: whey protein (80%), fat (6%), moisture (5%), lactose (4.7%), and minerals (2.8%). An aqueous WPC-80 solution (5% wt/vol, protein basis) was heated at 65°C for 20 min and then cooled to room temperature. Immobilized proteinase of *L. helveticus* LB 10 was added to the WPC-80 solution at a final concentration of 15% (wt/vol) and incubated at 37°C for 12 h. To observe production of ACE-inhibitory peptides in the process of fermentation for 12 h, 1-mL aliquots were taken at 1-h intervals over 12 h and centrifuged at 7,000 × *g* for 10 min to remove immobilized proteinases, which were washed with sterilized water for reuse. The supernatant, composed of crude ACE-inhibitory peptides, was adjusted to pH 3.8 with 50% lactic acid solution (Pan and Guo, 2010). After centrifugation at 7,000 × *g* for 10 min, the supernatant was collected and the pH was readjusted to 7.0. Then, the ACE-inhibitory activity of the supernatant was measured. The whey hydrolysate supernatant was collected after at 8 h of centrifugation (7,000 × *g*, 10 min). The peptide concentration of the supernatant was analyzed and the supernatant freeze-dried for further use. Peptide concentration was determined according to the biuret reaction test (Pan et al., 2011).

Fractionation of Whey Hydrolysates by Gel Filtration

Whey hydrolysates were applied to a Sephadex G-15 column (1.0 × 40 cm, Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with distilled water.

Peptides were eluted with distilled water at a flow rate of 25 mL/h. Elution curves were obtained by measuring absorbance at 220 nm. The fractions were collected at 5-min intervals. The experiments were conducted at room temperature using MA99-2A Protein Chromatography Equipment (Shanghai Huxi Analysis Instrument Factory, Shanghai, China). All fractions under each peak were pooled separately and concentrated by freeze-drying; then, their ACE-inhibitory activity was determined. The fraction with the highest ACE-inhibitory activity was then applied to a Sephadex G-10 column (1.0 × 40 cm, Amersham Pharmacia Biotech) equilibrated with distilled water. Peptides were eluted under the same conditions as for the Sephadex G-15 column. The fractions were pooled, concentrated by freeze-drying, and assayed for ACE-inhibitory activity.

Transport of Peptide Mixtures Across the Caco-2 Cell Monolayer

Establishment of Caco-2 Cell Monolayer

Model. Caco-2 cells (Shanghai Institute of Cell Biology, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco Life Technologies) and 1% penicillin-streptomycin solution in an incubator with a 5% CO₂ atmosphere at 37°C. The Caco-2 cell monolayer was incubated in Transwell Permeable Supports (1.12 cm², 0.4-μm pore size) (Corning Costar, Cambridge, MA) at 2 × 10⁵ cells/cm². The medium was changed every day until a monolayer formed and then the monolayer was allowed to grow for 21 d until it took shape. The integrity of the monolayer was observed using a JEM-1400 electron microscope (JEOL, Tokyo, Japan) and was also evaluated by measuring the transepithelial electrical resistance (TEER) using a Millicell ERS-2 V ohm meter (Millipore, Bedford, MA; Srinivasan et al., 2015). Lucifer yellow and alkaline phosphatase activity were analyzed and used to measure the integrity and differentiation of the monolayer (Mineo et al., 2002; Dihal et al., 2006).

Peptide Transport Across the Caco-2 Cell Monolayer

The Transwell inserts were incubated with Hanks' balanced salt solution (HBSS) buffer (pH 7.4) at 37°C for 30 min. The peptide fraction from the Sephadex G-10 column separation in 0.5 mL of HBSS buffer (pH 7.4) at a concentration of 1 mg/mL was added to the apical chamber. An aliquot of 1.5 mL of HBSS buffer (pH 7.4) was added to the basolateral (BL) side of each well. The same volume of HBSS buffer (pH 7.4) without sample (blank control) was added to the apical and BL sides. After 1 h of incubation, BL

solutions were collected and analyzed using UPLC-MS/MS.

Identification of ACE-Inhibitory Peptides Using UPLC-MS/MS Analysis

Samples before and after transport were analyzed using a Waters MALDI (matrix-assisted laser desorption/ionization) Synapt Q-TOF (quadrupole-time-of-flight) MS (Waters, Milford, MA). The UPLC was performed on a Waters Acquity UPLC system with an Acquity UPLC BEH 130 C18 column (2.1 × 150 mm, 1.7 μm; Waters). An 8-μL aliquot of sample was injected. Elution was performed with solvent A (100% acetonitrile) and B (0.1% methanoic acid in H₂O). Peptides were eluted with a linear gradient of solvent A from 2 to 40% in 10 min, to 100% in 17 min, and to 2% in the next 5 min, and solvent B from 98 to 60% in 10 min, to 0% in 17 min, and to 98% in the next 5 min. The flow rate was 0.3 mL/min.

The ion source was ESI+, the capillary and cone voltages were 3.5 kV and 30 V, respectively, and the source block temperature was 100°C. The desolvation temperature was 300°C, and the desolvation and cone gas flows were 500 and 50 L/h, respectively. The low collision energy was 6 V and high collision energy was 35 V. Low collision energy was used to measure peptide accurate mass and intensity (abundance) and high collision energy was used to generate MS/MS spectra and identify peptide fragments (Foltz et al., 2015). The mass-to-charge range was 100 to 1,500 *m/z*. The detector voltage was 1,600 to 1,700 V.

MassLynx v4.1 (Waters) and GPMAW 8.0 (GPMAW, Odense, Denmark) software were used to identify the peptide sequences. Detected peptides were compared against fragments of known whey proteins including β-LG, α-LA, glycomacropeptide, immunoglobulins, BSA, lactoferrin, and lactoperoxidase using the National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>). The peptides detected by UPLC-MS/MS, KA, EN, DIS, EVD, LF, AIV, and VFK, from the BL side of the Caco-2 cell model were synthesized at ≥90% purity by Shanghai Peptide Co. (Shanghai, China), and their ACE-inhibitory activity was measured.

Measurement of ACE-Inhibitory Activity

A 100-μL sample of peptides at different concentrations (0.05, 0.5, 1, 2, 3 and 4 mM) was mixed with 200 μL of hippuryl-L-histidyl-L-leucine (HHL) substrate solution (6.7 mM, pH 8.3; Sigma, St. Louis, MO) and incubated at 37°C for 5 min. Then, ACE from rab-

bit lung (30 μL, 0.33 U/mL; Sigma) was added to the mixture and incubated for 30 min at 37°C. The ACE-inhibitory activity was measured according to the method described by Cushman and Cheung (1971), according to the following equation:

$$\text{ACE-inhibitory activity (\%)} = 100\% \times [(A - B) - (C - D)] / (A - B), \quad [3]$$

where *A* is the absorbance of the solution containing ACE without the peptide sample, *B* is the absorbance of the solution with ACE before inactivation by adding HCl without the sample, *C* is the absorbance in the presence of ACE and sample, and *D* is the absorbance in the presence of ACE and the sample, with ACE measured before inactivation by HCl (Toopcham et al., 2015).

The half-maximal inhibitory concentration (IC₅₀) was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity under the assay conditions. The IC₅₀ was calculated by Probit analysis using IBM Statistical Package for the Social Sciences (SPSS) Statistics 20 software (IBM Corp., Armonk, NY).

Measurement of Protein Concentration

The protein concentration of proteinase was determined according to the Bradford assay using BSA as a standard (Bradford, 1976).

Statistical Analyses

All analyses were conducted in triplicate and the results were expressed as mean values ± standard deviations. Analysis of variance was used to quantify significant differences between levels. Significant differences were determined by one-way ANOVA followed by a Tukey honestly significant difference (HSD) test for multiple comparisons. A probability value of *P* < 0.05 was recognized as statistically significant. Statistical analyses were performed using a Minitab 16 system (Minitab Inc.).

RESULTS AND DISCUSSION

Immobilization of Proteinases

Figure 1A shows the activity of the immobilized proteinases at different concentrations of sodium alginate. Increasing the sodium alginate concentration increased the thickness of the alginate-bead membrane. Substrate

has difficulty diffusing through alginate beads at high concentrations of sodium alginate (Idris and Suzana, 2006). We found that beads of 2.0 g/100 mL of sodium alginate were hard enough and were optimal, because they provided the highest level of immobilized proteinase activity. Therefore, 2.0 g/100 mL was chosen as the center point of the RSM, with 0.5 g/100 mL as the step change (Table 1). Calcium chloride is used as a cross linking agent, and its concentration is directly related to bead intensity and stability (Ganaie et al., 2014). Concentrations of CaCl_2 of 0.1 mol/L (Ganaie et al., 2014) and 0.2 mol/L (Nawaz et al., 2015) were reported to perform well for cross linkage of enzyme-entrapped beads. Concentrations of CaCl_2 in the range of 0.05 to 0.25 mol/L were chosen to study the effects on activity of the immobilized proteinases in the current study. As can be seen in Figure 1B, we observed no significant difference in the activity of immobilized proteinases at different CaCl_2 concentrations from 0.05 to 0.25 mol/L. This is consistent with a previous report that a CaCl_2 concentration of 0.05 to 0.2 mol/L had slight effects on immobilized lipase yield and activity (Jeamjounkhaw et al., 2007). Therefore, CaCl_2 was not chosen as a center point of the RSM. Figure 1C shows the activity of immobilized proteinases at different glutaraldehyde concentrations (0.2 to 1.0%). Activity increased with glutaraldehyde concentrations from 0.2 to 0.4% and then decreased from 0.4 to 1.0%. Therefore, 0.4% was chosen as the center point with 0.2% as the step change (Table 1). Figure 1D shows the activity of immobilized proteinases at different proteinase concentrations (4 to 20 mg/mL); activity increased from 4 to 8 mg/mL and then decreased from 8 to 20 mg/mL. Therefore, 8 mg/mL was chosen as the center point with 4 mg/mL as the step change (Table 1). From this experiment, the sodium alginate, glutaraldehyde and proteinase concentrations were chosen as factors for the RSM experiment. Alginate has been widely used to immobilize many enzymes via entrapment (Pal and Khanum, 2011). Alginate is composed of α -L-guluronic and β -D-mannuronic acid. Proteinases can be embedded in alginate beads with cross-linking between α -L-guluronic acid and calcium ions (Zhou et al., 2010). As a hardening agent, glutaraldehyde was used to increase the rigidity and mechanical strength of immobilized enzymes (Zhou et al., 2010). Pal and Khanum (2011) reported that an enzyme attached to the surface of alginate beads through glutaraldehyde could enhance the accessibility of substrates to the enzyme trapped in the beads and improve the outward diffusion efficiency of hydrolytic products.

Table 1 shows the design and results of the RSM experiment, and Tables 2 and 3 show the statistical

analysis of results. The effect of each factor on specific immobilized proteinase activity was identified by their *t*-test and *P*-values. Factors x_1 (proteinase concentration), x_2 (sodium alginate concentration), and x_3 (glutaraldehyde concentration) had significant effects ($P < 0.05$) on specific immobilized proteinase activity (Tables 2 and 3). The square terms $x_1 \times x_1$, $x_2 \times x_2$, and $x_3 \times x_3$ also had significant effects ($P < 0.05$) on specific immobilized proteinase activity (Tables 2 and 3). Equation [4] shows the specific immobilized proteinase activity in relation to the 3 factors. Multiple regression analysis of the experimental data was used to obtain the parameters of the equation. The experimental data were explained by the following quadratic model:

$$y = -18.4133 + 0.9780 \times x_1 + 14.7033 \times x_2 + 19.1729 \times x_3 \\ + 0.0712 \times x_1 \times x_2 - 0.0562 \times x_1 \times x_3 + 1.6250 \times x_2 \times x_3 \\ - 0.0729 \times x_1^2 - 3.9133 \times x_2^2 - 28.1458 \times x_3^2, \quad [4]$$

where y was the predicted response, and x_1 , x_2 , and x_3 were the uncoded values of the variable proteinase, sodium alginate and glutaraldehyde concentrations, respectively.

The adjusted coefficient of determination (R^2 -adj) was used to check the fit of the model (Eq. [4]). A high R^2 -adj value indicated that the model was well adapted to the response. The R^2 -adj was 0.946, which indicated that 94.6% of the variability in the response in relation to the activity of the specific immobilized proteinases could be explained by the model (Table 2). Table 3 shows a nonsignificant ($P > 0.05$) lack of fit, which indicates that the proposed model (Eq. [4]) fits well. The response (specific immobilized proteinase activity) on the z -axis against any 2 independent variables was plotted, whereas the other variables were maintained at their optimal levels. Therefore, 3-dimensional response surface plots were constructed (Figure 1E, 1F, and 1G). The activity of specific immobilized proteinases increased until the proteinase, sodium alginate, and glutaraldehyde concentrations reached an optimum point and then decreased as the concentrations increased. The optimal conditions of the variables were a proteinase concentration of 7.55 mg/mL, a sodium alginate concentration of 2.03 g/100 mL, and a glutaraldehyde concentration of 0.39%. The predicted activity of specific immobilized proteinases was 3.95 U/mg under these conditions. An experiment was run 3 times under the optimal conditions, and the activity was 3.80 ± 0.16 U/mg. Thus, we confirmed that the conditions analyzed by Minitab 16 (Minitab Inc.) were optimal to immobilize the proteinases. We found that RSM was an

Figure 1. Effect of sodium alginate (A; % mass/volume), calcium chloride (B; mol/L), glutaraldehyde (C; % mass/volume), and proteinase (D; mg/mL) concentrations on specific immobilized proteinase (SIP) activity. Data are the mean $\pm$ SE ($n = 3$) of at least 3 independent experiments. Response surface plots for the effect of variables on SIP activity: (E) proteinase (x_1) and sodium alginate (x_2) concentrations; (F) proteinase (x_1) and glutaraldehyde (x_3) concentrations; (G) sodium alginate (x_2) and glutaraldehyde (x_3) concentrations. Different letters indicate significant differences among groups ($P < 0.05$).

Table 2. Estimated uncoded regression coefficients for specific immobilized proteinase activity (U/mg)

Term	Coeff. ¹	SE Coeff.	<i>t</i> -value	<i>P</i> -value
Constant	−18.4133	2.38279	−7.728	0.001**
<i>x</i> ₁	0.9780	0.17059	5.733	0.002**
<i>x</i> ₂	14.7033	1.95745	7.511	0.001**
<i>x</i> ₃	19.1729	3.41181	5.620	0.002**
<i>x</i> ₁ × <i>x</i> ₁	−0.0729	0.00722	−10.097	0.000**
<i>x</i> ₂ × <i>x</i> ₂	−3.9133	0.46187	−8.473	0.000**
<i>x</i> ₃ × <i>x</i> ₃	−28.1458	2.88668	−9.750	0.000**
<i>x</i> ₁ × <i>x</i> ₂	0.0712	0.05547	1.285	0.255
<i>x</i> ₁ × <i>x</i> ₃	−0.0562	0.13867	−0.406	0.702
<i>x</i> ₂ × <i>x</i> ₃	1.6250	1.10937	1.465	0.203
SE		0.221875		
PRESS ²		2.71085		
R ²		98.07%		
R ² -adj ²		94.60%		

¹Coeff. = coded regression coefficient for specific immobilized proteinase activity (U/mg).

²PRESS = predicted residual sum of squares.

³The adjusted R² (R²-adj) value is high, indicating that the models were well adapted to the responses. It was calculated to be 0.946, indicating that the model can explain 94.6% of the variability in the response on specific immobilized proteinase activity (U/mg) (Eq. [1]). The use of R²-adj is an attempt to take account of the phenomenon of the R² automatically and spuriously increasing when extra explanatory variables are added to the model.

**Indicates an extremely significant effect (*P* < 0.01) on specific immobilized proteinase activity (U/mg).

efficient statistical tool to optimize the immobilization conditions for the proteinases.

Characteristics of Immobilized Proteinases of *L. helveticus* LB10

Effect of pH and Temperature. Enzymes have ionic groups on their active sites and these ionic groups must be in a suitable form to function. Changes in pH affect the ionic form of the active site and change the activity of the enzyme (Tümtürk et al., 2007). In our study, the activity of the free and immobilized proteinases exhibited maximal activity at pH 7.0 (Figure 2A). A similar observation has been made in other studies, in which immobilization of an enzyme did not change its optimum pH (Nawaz et al., 2015). The optimum

temperatures for free and immobilized proteinases were 37°C and 41°C, respectively (Figure 2B). Some researchers have reported that the optimal temperature of the immobilized enzyme shifted to a higher value than that of the free enzyme (Tümtürk et al., 2007). Immobilized enzymes have the potential to change the microenvironment surrounding enzyme molecules, thereby improving the stability of the enzyme (Zhao et al., 2017).

pH and Thermo Stability. The immobilized proteinases displayed good pH and thermo stability. Immobilized proteinases retained more than 60% of their activity, whereas the free enzyme retained only 10% at pH 9.0 (Figure 2C). The activities of free and immobilized proteinases were stable up to 50°C (Figure 2D). When heated to 60°C, the immobilized protein-

Table 3. Analysis of variance for specific immobilized proteinase activity (U/mg)

Source	df	Sequential sums of squares	Adjusted sums of squares	Adjusted mean square	<i>F</i> -value	<i>P</i> -value
Regression	9	12.5078	12.5078	1.38975	28.23	0.001**
Linear	3	0.8290	4.4106	1.47021	29.87	0.001**
Square	3	11.4838	11.4838	3.82793	77.76	0.000**
Interaction	3	0.1949	0.1949	0.06498	1.32	0.203
Residual error	5	0.2461	0.2461	0.04923		
Lack of fit ¹	3	0.1569	0.1569	0.05229	1.17	0.491
Pure error	2	0.0893	0.0893	0.04463		
Total	14	12.7539				

¹The *P*-value of 0.491 indicates a nonsignificant (*P* > 0.05) lack of fit, which further validates the model.

**Indicates an extremely significant effect (*P* < 0.01) on specific immobilized proteinase activity (U/mg).

Figure 2. Effect of (A) pH and (B) temperature on free and immobilized proteinase activity; (C) pH (C) and thermal (D) stability of free and immobilized proteinases; storage stability (E) of immobilized proteinases over 30 d; and (F) reusability of immobilized proteinases for 5 cycles. Data are the mean $\pm$ SE ($n = 3$) of at least 3 independent experiments.

ases retained 70% of their control activity, whereas free enzyme activity was only 30%. The greater stability of the immobilized proteinases in relation to pH and heating may be related to covalent linkages and secondary interactions (e.g., ionic and polar stabilization, hydrogen bonding) between the enzyme and the matrix (LeTien et al., 2004).

Storage Stability and Reusability. Storage stability is one of the key factors that affects the evaluation and selection for use of any industrially important enzyme (Kumar et al., 2017). The storage stability of the immobilized proteinases in this study was high. The residual activity was 95.3, 86.2, and 68.5% after 3, 7, and 30 d, respectively (Figure 2E). The residual

activity of the immobilized proteinases was 60.4% of the original activity after 5 cycles (Figure 2F). The improved storage stability and reusability could be due to strong covalent linkage between the enzyme and matrix or structural rigidity (Pal and Khanum, 2011; Kumar et al., 2017).

Production and Purification of Whey Hydrolysates

Whey was hydrolyzed into mixtures of peptides by using immobilized proteinases of *L. helveticus* as a catalytic agent. As shown in Figure 3A, the ACE-inhibitory activity of the hydrolysate increased from 2 to 8 h. After 8 h, the ACE-inhibitory activity of the hydrolysate decreased. The same phenomenon was reported by Toopcham et al. (2017), who found that further digestion resulted in a decrease in ACE-inhibitory activity. This might be explained by the substrate being exhausted and peptides with ACE-inhibitory activity being further hydrolyzed and losing their activity (Guo et al., 2009). The whey protein hydrolysate was collected at 8 h and showed ACE-inhibitory activity of $43.00 \pm 1.50\%$; the peptide concentration was 1.80 ± 0.11 mg/mL.

The hydrolysate was separated using Sephadex G-15 gel filtration chromatography with an exclusion limit of 1,500 Da and 2 fractions were obtained (Figure 3B). High-molecular-weight substances, which are excluded from the gel, are eluted first. The ACE-inhibitory activity of fractions 1 and 2 was $32.28 \pm 1.32\%$ and $62.82 \pm 2.21\%$, respectively. The second fraction, with greater ACE-inhibitory activity, was further separated using Sephadex G-10 gel filtration chromatography with an exclusion limit of 700 Da. Only one fraction was obtained, which exhibited ACE-inhibitory activity of $67.80 \pm 1.81\%$, (Figure 3C).

Identification of ACE-Inhibitory Peptides Transported Across a Caco-2 Cell Monolayer

Figure 3D and 3E show total ion chromatograms (TIC) of the whey peptide mixtures before and after transport across Caco-2 cell monolayers. The fractions in Figure 3D are complex and it is difficult to analyze the peptide sequences of all fractions. By comparing Figure 3D and 3E, it can be seen that many of whey protein hydrolysate fractions could not cross the intestinal epithelium. Seven peptides were identified after transport, at retention times of 9.55, 9.93, 11.12, 11.57, 11.75, 11.89, and 12.21 min, and were confirmed as Lys-Ala (KA), Glu-Asn (EN), Asp-Ile-Ser (DIS), Glu-Val-Asp (EVD), Leu-Phe (LF), Ala-Ile-Val (AIV), and Val-Phe-Lys (VFK) from MS/MS spectrum (Figure 4).

We were not able to identify other peaks because of the low concentration or poor MS/MS spectrum, such as the peak at retention time 1.03 min. The tripeptides DIS, EVD, and VFK correspond to the 44–46, 143–145, and 97–99 fragments of β -LG, respectively. The tripeptide AIV corresponds to α -LA f(59–61) (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-14899>). The dipeptides KA, EN, and LF are found in fragments of β -LG, α -LA, IgG, BSA, lactoferrin, and lactoperoxidase (Supplemental Table S1) and their exact source could not be confirmed.

The IC_{50} of synthesized peptides KA, EN, DIS, EVD, LF, AIV, and VFK were measured and found to be 1.24 ± 0.01 , 1.43 ± 0.04 , 1.59 ± 0.27 , 1.32 ± 0.05 , 1.60 ± 0.39 , 2.66 ± 0.02 , and 1.76 ± 0.09 mmol/L, respectively (Table 4). In previous reports, the IC_{50} value of the ACE-inhibitory peptides IPP [β -CN f(74–76)] and VPP [β -CN f(84–86)] were 5.0 and 9.0 μ M under measurement conditions of 200 μ L of 5 mM HHL, 20 μ L of ACE (0.1 U/mL), and 80 μ L of peptide sample (Nakamura et al., 1995). Under measurement conditions of 20 μ L of 12.5 mM HHL, 30 μ L of peptide sample, and 25 mU of ACE in 2 μ L of glycerol, the IC_{50} of VYP [β -CN f(59–61)] was 288 μ M (Abubakar et al., 1998). The IC_{50} of ALPM [β -LG f(142–145)] was 928 μ M when 75 μ g or 150 μ L of peptide sample solution was mixed with borate buffer containing 12.5 mM HHL and 5 mU of ACE (Murakami et al., 2004). The IC_{50} of KVLPVPQ [β -CN f(169–175)] was 1,000 μ M under the conditions of 200 μ L of 3.8 mM HHL, 20 μ L of peptide sample, and 2 mU of ACE (Maeno et al., 1996). It has been reported that ACE prefer an inhibitor containing hydrophobic (aromatic or branched side chains) AA residues and a positively charged AA at the C-terminal (Meisel, 1997; Hernández-Ledesma et al., 2011). The presence of Tyr, Phe, Trp, Pro, Ile, Val, Lys, or Arg at the C-terminal has been reported to contribute to ACE-inhibitory activity (Fu et al., 2016). In the present study, DIS and AIV have Ile and Lys, which are branched side-chain AA, at the C-terminal. The dipeptide LF has Phe, an aromatic AA, at the C-terminal; VFK has Lys, which is a positively charged AA, at the C-terminal; KA has Ala, which is a hydrophobic AA, at the C-terminal. The other 2 peptides, EN and EVD, do not have any reported specific AA at the C-terminal. The other characteristics may be related to the ACE-inhibitory activity.

The characteristics of 7 detected ACE-inhibitory peptides including molecular weight, grand average of hydropathy (GRAVY), and electrostatic charge of the ACE-inhibitory peptides are listed in Table 4. Size of the peptides could be a critical factor influencing intestinal absorption (Raikos and Dassios, 2014). Long-

Figure 3. (A) Effect of hydrolysis time on angiotensin-I-converting enzyme (ACE)-inhibitory peptide production using immobilized proteinases. Data are the mean $\pm$ SE ($n = 3$) of at least 3 independent experiments. (B) Sephadex-15 (Amersham Pharmacia Biotech, Uppsala, Sweden) chromatography of whey protein hydrolysate (measured as absorbance, A, at 220 nm); (C) Sephadex G-10 chromatography of fraction 2 obtained from Sephadex G-15; total ion chromatograms of fraction 1' obtained from Sephadex G-10 before (D) and after (E) transport across Caco-2 cell monolayers.

Figure 4. Tandem mass spectra of peptides KA [retention time (RT) 9.55 min; A], EN (RT 9.93 min; B), DIS (RT 11.12 min; C), EVD (RT 11.57 min; D), LF (RT 11.75 min; E), AIV (RT 11.89 min; F), and VFK (RT 12.21 min; G).

chain peptides are probably degraded by brush-border or cellular peptidases across the intestinal brush-border membrane (Zhu et al., 2008). The 7 transported ACE-inhibitory activity peptides may be the products of long-chain whey hydrolysates degraded by brush-border or cellular peptidases when crossing the Caco-2 cell monolayer. These transported di- and tripeptides with ACE-inhibitory activity would be “true” ACE inhibitors and physiologically important. The peptide

LHLPLP was hydrolyzed by cellular peptidases to HLPLP before transport across the intestinal epithelium and HLPLP was the active form and responsible for the antihypertensive effect (Quirós et al., 2008). Thus, the Caco-2 cell monolayer may be an efficient way to select deliverable peptides from complex protein hydrolysates. Toopcham et al. (2017) also used Caco-2 cell monolayers to screen 6 identified ACE inhibitory peptides, including LLP, AHL, PQP, MCS, GTY, and

Table 4. Characteristics of angiotensin-I-converting enzyme (ACE)-inhibitory peptides hydrolyzed from whey protein¹

RT (min)	Peptide sequence	M_r (Da)	$[M+H]^+ m/z$	GRAVY	Electrostatic charge (pH = 7.0)	IC ₅₀ (mmol/L)
9.55	KA	217.27	218.3	-1.05	0.86	1.24 ± 0.01
9.93	EN	261.23	262.31	-3.5	-0.92	1.43 ± 0.04
11.12	DIS	333.34	334.39	0.67	-1.23	1.59 ± 0.27
11.57	EVD	361.35	362.42	-0.93	-2.15	1.32 ± 0.05
11.75	LF	278.35	279.17	3.3	0.31	1.60 ± 0.39
11.89	AIV	301.39	302.39	3.5	0.31	2.66 ± 0.02
12.21	VFK	392.50	393.19	1.03	0.92	1.76 ± 0.09

¹RT = retention time; M_r = molecular weight; GRAVY (grand average of hydropathy) was calculated from www.gravy-calculator.de (positive value represents hydrophobic; negative value represents hydrophilic); electrostatic charge was calculated from <http://www.innovagen.se/index.asp>; and IC₅₀ = half-maximal inhibitory concentration. Data are the mean ± SE (n = 3) of at least 3 independent experiments.

ALSC, from tilapia muscle fractions hydrolyzed by *Virgibacillus halodenitrificans* SK1-3-7 proteinases.

Aside from molecular weight, hydrophobicity or hydrophilicity also influences susceptibility to peptidases and intestinal absorption (Fernández-Musoles et al., 2013). Hydrophobic (KA, EN, and EVD) and hydrophilic (DIS, LF, AIV, and VFK) peptides were found in BL solution of the Caco-2 cell line (Table 4). It is known that hydrophobic peptides can transport easily across the intestinal epithelium because of the interaction between the peptides and the membrane. Hydrophilic drugs can also be transported across the intestine via the paracellular and transcellular routes (Artursson et al., 1996).

Negatively (EN, DIS, and EVD) and positively (KA, LF, AIV, and VFK) charged peptides were present in the transportation solution of the Caco-2 cell line (Table 4). Whether electrostatic charge influences the permeation of ACE-inhibitory peptides across Caco-2 cells is debatable. Pauletti et al. (1997) reported that permeation across the intestinal mucosa of a peptide sample with the same length was charge-dependent (negative < positive ≤ neutral), whereas Fernández-Musoles et al. (2013) reported that WQ (neutral charge) had a higher transepithelial flux value across the Caco-2 monolayer than positively charged tripeptides. However, Rubasa et al. (1993) reported that permeation across the cell monolayer of peptide RGD analogs (charges ranging from -3 to +1) with negative charges of -1 and -2 was higher than that of analogs with positive charges.

It is generally accepted that di- and tripeptides are easily absorbed in the intestine and transported via the proton-dependent PepT1 transport system (Satake et al., 2002; Vermeirssen et al., 2004). Apart from the PepT1 transport system, peptides can be absorbed intact across the intestinal mucosa via other mechanisms, including paracellular transport and the transcytosis route (Vermeirssen et al., 2002). Paracellular transport may be the main mechanism for the transport of intact VPP across the Caco-2 cell monolayer (Satake et al.,

2002). Further experiments are required to study the transportation ability and route of di- and tripeptides discovered in our study.

CONCLUSIONS

Angiotensin-I-converting enzyme-inhibitory peptides can be successfully produced from whey protein hydrolyzed by immobilized *L. helveticus* LB 10 proteinases. Compared with free proteinases, the immobilized proteinases had significantly higher pH, thermal, and storage stabilities. After peptide mixtures were transported across Caco-2 cell monolayers, 7 ACE-inhibitory peptides (KA, EN, DIS, EVD, LF, AIV, and VFK) were identified in the basolateral solution of Caco-2 cell model. This study provides a research basis for the development of bioactive peptide ingredients applied to the functional food market.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31571852, 31471598, 31671869, and 31601487), Natural Science Foundation of Jiangsu Province (BK20151544), and the “333 Project” Foundation of Jiangsu Province (BRA2017450). The authors do not have any conflicts of interest.

REFERENCES

- Abubakar, A., T. Saito, H. Kitazawa, Y. Kawai, and T. Itoh. 1998. Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. *J. Dairy Sci.* 81:3131–3138.
- Artursson, P., K. Palm, and K. Luthman. 1996. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Deliv. Rev.* 22:67–84.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation on microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- Cushman, D. W., and H. S. Cheung. 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol.* 20:1637–1648.

- Dihal, A. A., R. A. Woutersen, B. van Ommen, I. M. Rietjens, and R. H. Stierum. 2006. Modulatory effects of quercetin on proliferation and differentiation of the human colorectal cell line Caco-2. *Cancer Lett.* 238:248–259.
- Fernández-Musoles, R., J. B. Salom, M. Castelló-Ruiz, M. M. Contreras, I. Recio, and P. Manzanera. 2013. Bioavailability of antihypertensive lactoferricin B-derived peptides: Transepithelial transport and resistance to intestinal and plasma peptidases. *Int. Dairy J.* 32:169–174.
- Foltz, E., S. Azad, M. L. Everett, Z. E. Holzknecht, N. L. Sanders, J. W. Thompson, L. G. Dubois, W. Parker, S. Keshavjee, S. M. Palmer, R. D. Davis, and S. S. Lin. 2015. An assessment of human gastric fluid composition as a function of PPI usage. *Physiol. Rep.* 3:e12269.
- Fu, Y., J. F. Young, M. M. Løkke, R. Lametsch, R. E. Aluko, and M. Therkildsen. 2016. Revalorisation of bovine collagen as a potential precursor of angiotensin I-converting enzyme (ACE) inhibitory peptides based on in silico and in vitro protein digestions. *J. Funct. Foods* 24:196–206.
- Ganaie, M. A., H. K. Rawat, O. A. Wani, U. S. Gupta, and N. Kango. 2014. Immobilization of fructosyltransferase by chitosan and alginate for efficient production of fructooligosaccharides. *Process Biochem.* 49:840–844.
- Guo, Y., J. Gan, Q. Zhu, X. Zeng, Y. Sun, Z. Wu, and D. Pan. 2018. Transepithelial transport of milk-derived angiotensin-I converting enzyme inhibitory peptide with the RLSFNP sequence. *J. Sci. Food Agric.* 98:976–983.
- Guo, Y., D. Pan, and M. Tanokura. 2009. Optimisation of hydrolysis conditions for the production of the angiotensin-I converting enzyme (ACE) inhibitory peptides from whey protein using response surface methodology. *Food Chem.* 114:328–333.
- Hernández-Ledesma, B., M. J. García-Nebot, S. Fernández-Tomé, L. Amigo, and I. Recio. 2014. Dairy protein hydrolysates: Peptides for health benefits. *Int. Dairy J.* 38:82–100.
- Hernández-Ledesma, B., M. del Mar Contreras, and I. Recio. 2011. Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Adv. Colloid Interface Sci.* 165:23–35.
- Idris, A., and W. Suzana. 2006. Effect of sodium alginate concentration, bead diameter, initial pH and temperature on lactic acid production from pineapple waste using immobilized *Lactobacillus delbrueckii*. *Process Biochem.* 41:1117–1123.
- Jeamjounkhan, P., A. H-kittikun, and B. Cheirsilp. 2007. Optimization of lipase entrapment in alginate gel bead for palm olein hydrolysis. *Songklanakarin J. Sci. Technol.* 29(Suppl. 2):3520–3522.
- Kumar, S., I. Haq, J. Prakash, and A. Raj. 2017. Improved enzyme properties upon glutaraldehyde cross-linking of alginate entrapped xylanase from *Bacillus licheniformis*. *Int. J. Biol. Macromol.* 98:24–33.
- LeTien, C., M. Millette, M. Lacroix, and M.-A. Mateescu. 2004. Modified alginate matrices for the immobilization of bioactive agents. *Biotechnol. Appl. Biochem.* 39(Pt. 2):189–198.
- Maeno, M., N. Yamamoto, and T. Takano. 1996. Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP790. *J. Dairy Sci.* 79:1316–1321.
- Meisel, H. 1997. Biochemical properties of bioactive peptides derived from milk proteins: potential nutraceuticals for food and pharmaceutical applications. *Livest. Prod. Sci.* 50:125–138.
- Miguela, M., M. J. Alonso, M. Salaices, A. Aleixandre, and R. López-Fandiño. 2007. ACE-inhibitory and vasodilator properties of an egg white hydrolysate: Effect of a simulated intestinal digestion. *Food Chem.* 104:163–168.
- Mineo, H., H. Hara, N. Shigematsu, Y. Okuhara, and F. Tomita. 2002. Melibiose, difructose anhydride III and difructose anhydride IV enhance net calcium absorption in rat small and large intestinal epithelium by increasing the passage of tight junctions in vitro. *J. Nutr.* 132:3394–3399.
- Murakami, M., H. Tonouchi, R. Takahashi, H. Kitazawa, Y. Kawai, H. Negishi, and T. Saito. 2004. Structural analysis of a new antihypertensive peptide (β -Lactosin B) isolated from a commercial whey product. *J. Dairy Sci.* 87:1967–1974.
- Nakamura, Y., N. Yamamoto, K. Sakai, A. Okubo, S. Yamazaki, and T. Takano. 1995. Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *J. Dairy Sci.* 78:777–783.
- Nawaz, M. A., H. U. Rehman, Z. Bibi, A. Aman, and S. A. Ul Qader. 2015. Continuous degradation of maltose by enzyme entrapment technology using calcium alginate beads as a matrix. *Biochem. Biophys. Rep.* 4:250–256.
- Pal, A., and F. Khanum. 2011. Covalent immobilization of xylanase on glutaraldehyde activated alginate beads using response surface methodology: Characterization of immobilized enzyme. *Process Biochem.* 46:1315–1322.
- Pan, D., and Y. Guo. 2010. Optimization of sour milk fermentation for the production of ACE-inhibitory peptides and purification of a novel peptide from whey protein hydrolysate. *Int. Dairy J.* 20:472–479.
- Pan, D., Y. Guo, and X. Jiang. 2011. Anti-fatigue and antioxidative activities of peptides isolated from milk proteins. *J. Food Biochem.* 35:1130–1144.
- Pauletti, G. M., F. W. Okumu, and R. T. Borchardt. 1997. Effect of size and charge on the passive diffusion of peptides across Caco-2 cell monolayers via the paracellular pathway. *Pharm. Res.* 14:164–168.
- Picariello, G., G. Mamone, C. Nitride, F. Addeo, and P. Ferranti. 2013. Protein digestomics: Integrated platforms to study food-protein digestion and derived functional and active peptides. *Trends Anal. Chem.* 52:120–134.
- Quirós, A., A. Dávalos, M. A. Lasunción, M. Ramos, and I. Recio. 2008. Bioavailability of the antihypertensive peptide LHLPLP: Transepithelial flux of HLPLP. *Int. Dairy J.* 18:279–286.
- Raikos, V., and T. Dassios. 2014. Health-promoting properties of bioactive peptides derived from milk proteins in infant food: A review. *Dairy Sci. Technol.* 94:91–101.
- Rubasa, W., M. Cromwell, T. Gadega, D. Narindraya, and R. Mrsny. 1993. Structural elements which govern the resistance of intestinal tissues to compound transport. *MRS Online Proc. Library Archive* 331:179.
- Sadat-Mekmene, L., M. Genay, D. Atlan, S. Lortal, and V. Gagnaire. 2011. Original features of cell-envelope proteinases of *Lactobacillus helveticus*. A review. *Int. J. Food Microbiol.* 146:1–13.
- Satake, M., M. Enjoh, Y. Nakamura, T. Takano, Y. Kawamura, S. Arai, and M. Shimizu. 2002. Transepithelial transport of the bioactive tripeptide, Val-Pro-Pro, in human intestinal Caco-2 cell monolayers. *Biosci. Biotechnol. Biochem.* 66:378–384.
- Sonnier, D. I., S. R. Bailey, R. M. Schuster, A. B. Lentsch, and T. A. Pritts. 2010. TNF-alpha induces vectorial secretion of IL-8 in Caco-2 cells. *J. Gastrointest. Surg.* 14:1592–1599.
- Srinivasan, B., A. R. Kolli, M. B. Esch, H. E. Abaci, M. L. Shuler, and J. J. Hickman. 2015. TEER measurement techniques for in vitro barrier model systems. *J. Lab. Autom.* 20:107–126.
- Stevenson, C. L., P. F. Augustijns, and R. W. Hendren. 1999. Use of Caco-2 cells and LC/MS/MS to screen a peptide combinatorial library for permeable structures. *Int. J. Pharm.* 177:103–115.
- Stuknytė, M., S. Cattaneo, F. Masotti, and I. De Noni. 2015. Occurrence and fate of ACE-inhibitor peptides in cheeses and in their digestates following in vitro static gastrointestinal digestion. *Food Chem.* 168:27–33.
- Toopcham, T., J. J. Mes, H. J. Wichers, S. Roytrakul, and J. Yongsawatdigul. 2017. Bioavailability of angiotensin I-converting enzyme (ACE) inhibitory peptides derived from *Virgibacillus halodennitrificans* SK1-3-7 proteinases hydrolyzed tilapia muscle proteins. *Food Chem.* 220:190–197.
- Toopcham, T., S. Roytrakul, and J. Yongsawatdigul. 2015. Characterization and identification of angiotensin I-converting enzyme (ACE) inhibitory peptides derived from tilapia using *Virgibacillus halodennitrificans* SK1-3-7 proteinases. *J. Funct. Foods* 14:435–444. (J).
- Tümtürk, H., F. Sahin, and G. Demirel. 2007. A new method for immobilization of acetylcholinesterase. *Bioprocess Biosyst. Eng.* 30:141–145.

- Van der Ven, C., H. Gruppen, D. B. A. De Bont, and A. G. J. Voragen. 2002. Optimisation of the angiotensin converting enzyme inhibition by whey protein hydrolysates using response surface methodology. *Int. Dairy J.* 12:813–820.
- Vermeirssen, V., J. Van Camp, and W. Verstraete. 2004. Bioavailability of angiotensin I converting enzyme inhibitory peptides. *Br. J. Nutr.* 92:357–366.
- Vermeirssen, V., B. Deplancke, K. A. Tappenden, J. Van Camp, H. R. Gaskins, and W. Verstraete. 2002. Intestinal transport of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg through a Caco-2 Bbe monolayer. *J. Pept. Sci.* 8:95–100.
- Wakai, T., T. Shinoda, N. Uchida, M. Hattori, Y. Nakamura, T. Beresford, R. P. Ross, and N. Yamamoto. 2013. Comparative analysis of proteolytic enzymes need for processing of antihypertensive peptides between *Lactobacillus helveticus* CM4 and DPC4571. *J. Biosci. Bioeng.* 115:246–252.
- Yamamoto, N., M. Maeno, and T. Takano. 1999. Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by *Lactobacillus helveticus* CPN4. *J. Dairy Sci.* 82:1388–1393.
- Zhao, F., Q. Wang, J. Dong, M. Xian, J. Yu, H. Yin, Z. Chang, X. Mu, T. Hou, and J. Wang. 2017. Enzyme-inorganic nanoflowers/alginate microbeads: An enzyme immobilization system and its potential application. *Process Biochem.* 57:87–94.
- Zhou, Z. D., G. Y. Li, and Y. J. Li. 2010. Immobilization of *Saccharomyces cerevisiae* alcohol dehydrogenase on hybrid alginate-chitosan beads. *Int. J. Biol. Macromol.* 47:21–26.
- Zhu, X. L., K. Watanabe, K. Shiraishi, T. Ueki, Y. Noda, T. Matsui, and K. Matsumoto. 2008. Identification of ACE-inhibitory peptides in salt-free soy sauce that are transportable across caco-2 cell monolayers. *Peptides* 29:338–344.