An exploration of angiotensin-converting enzyme (ACE) inhibitory peptides derived from gastrointestinal protease hydrolysate of milk using a modified bioassay-guided fractionation approach coupled with in silico analysis

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ABSTRACT

An improved bioassay-guided fractionation was performed to effectively screen angiotensin-I converting enzyme inhibitory (ACEI) peptides from milk protein hydrolysate. The aqueous normal phase liquid chromatography, namely hydrophilic interaction liquid chromatography (HILIC), was used as a format of solid-phase extraction (SPE) short column for the first fractionation, then the HILIC-SPE fraction with the best ACEI activity ($IC_{50} = 61.75 \pm 5.74 \mu\text{g/mL}$; IC_{50} = half-maximal inhibitory concentration) was obtained when eluted by 95% acetonitrile + 0.1% formic acid (fraction F1). The best HILIC-SPE fraction was further fractionated using reversed-phase (RP)-SPE short column. The best RP-SPE fraction was obtained when eluted by 20% acetonitrile + 0.1% formic acid (fraction P3) with an ACEI activity of $IC_{50} 36.22 \pm 1.18 \mu\text{g/mL}$. After the 2-step fractionation, the IC_{50} value of fraction P3 significantly decreased by 8.92-fold when compared with the crude hydrolysate. Several peptides were identified from fraction P3 using liquid chromatography-tandem mass spectrometry. The in silico analysis of these identified sequences based on the BIOPEP database predicted that HLPLPLL (HL-7) was the most active peptide against angiotensin-converting enzyme (ACE). The HL-7 derived from β -casein showed a potent ACEI activity (IC_{50} value is $16.87 \pm 0.3 \mu\text{M}$). The contents of HL-7 in the gastrointestinal protease hydrolysate and RP-SPE fraction originated from 1 mg of milk proteins were quantified using a multiple reaction monitoring mode upon liquid chromatography-tandem mass spectrometry analysis to give $19.86 \pm 1.14 \text{ pg}$ and $14,545.8 \pm 572.9 \text{ pg}$, respectively. Besides, the kinetic study indicated that HL-7 was a competitive inhibi-

tor and the result was rationalized using the docking simulation. The study demonstrated an efficient screening of ACEI peptides from commercially available milk powders using a simple SPE process instead of a sophisticated instrument such as HPLC. Moreover, the potent ACEI peptide HL-7 uncovered by this method could be a natural ACE inhibitor.

Key words: angiotensin-converting enzyme inhibitory peptide, gastrointestinal protease, milk, modified bioassay-guided fractionation

INTRODUCTION

Angiotensin-converting enzyme (ACE, EC 3.4.15.1) is an important factor in blood pressure regulation associated with the renin-angiotensin-aldosterone system, in which ACE have the ability to convert the inactive decapeptide called angiotensin I (DRVYIHPFHL) into the active vasoconstrictor octapeptide angiotensin II by removal of the C-terminal His-Leu and cleave the bradykinin to abolish its vasodilating activity (Acharya et al., 2003). Therefore, ACE activity should be inhibited to decrease the production of angiotensin II and increase bradykinin level, which will regulate blood pressure (FitzGerald et al., 2004; Ko et al., 2017). Several synthetic ACE inhibitors such as captopril, enalapril, and benazepril (Ondetti et al., 1977; Geng et al., 2016), presented certain side effects in long-term use such as dry cough, headache, insomnia, and fever (Atkinson and Robertson, 1979; Dicipinigitis, 2006). In recent years, food-derived ACE inhibitory (ACEI) peptides have gradually received attention and are considered to be gentler and safer alternatives for blood pressure control. Those active peptides derived from different food sources have been collectively reported in some previous reviews (Möller et al., 2008; Saleh et al., 2016; Daskaya-Dikmen et al., 2017). Among food various sources, food commercial products derived from milk, such as sour milk (Nakamura et al., 1995), kefir (Quirós et al., 2007), cheese (Gómez-Ruiz et al., 2002; Kajimoto

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et al., 2002; Ong et al., 2007), yogurt (Kajimoto et al., 2002; Korhonen, 2009), dahi (Ashar and Chand, 2004), skim milk (Quirós et al., 2007), and koumis (mare milk; Chen et al., 2010), were reported widely as promising sources of ACEI bioactive peptides to manage blood pressure (Fujita et al., 2000; Hartmann and Meisel, 2007; Khueychai et al., 2018). In addition, there are available commercial product derived from milk containing ACE inhibitor bioactive peptide such as Calpis that commercialized in Japan (Calpis Co. Ltd.) and Flora/Becel Pro-Active launched by Unilever, both of those products containing VPP and IPP were reported significant decrease systolic blood pressure in human body (Korhonen and Pihlanto, 2006; López-Fandiño et al., 2006).

Among food commercial products, milk plays a leading role as a source of ACE inhibitors (Vukic et al., 2017) and is a common edible protein sources from mammals (Madureira et al., 2007; Costa et al., 2014). It contains 3.5 to 6% protein consisting of 2 major families of protein, such as 80% casein (including β -CN, α_{S1} -CN, κ -CN, α_{S2} -CN) and 20% whey (including β -LG, α -LA; Madureira et al., 2007; Nielsen et al., 2017). Moreover, numerous well-known bioactive peptides derived from milk have been published in the milk bioactive peptide database (<http://mbpdb.nws.oregonstate.edu/>; Nielsen et al., 2017); thus, it can be easier to identify bioactive peptides. Although it has been widely investigated, ACEI peptides derived from milk remain an attractive option to be explored because they could be easily developed as an oral feeding product without drug consumption.

In this study, the release of bioactive peptide from the parent protein of intact milk was conducted by enzymatic hydrolysis using gastrointestinal protease (pepsin, trypsin, and chymotrypsin; López-Fandiño et al., 2006; Rai et al., 2017; Vukic et al., 2017). Each protease has a preferential cleavage site that could produce the unique sequence peptide (different size and sequence AA; Rai et al., 2017; Zhang et al., 2017). This process could simulate the conditions in the gastrointestinal tract (Ruiz et al., 2004) and anticipated the possibilities of decreasing in vitro ACEI activity compared with in vivo antihypertensive activity (Fujita et al., 2000; Yamada et al., 2002).

To efficiently screen potent ACEI bioactive, some bioassay-guided fractionation approaches have been suggested, such as one-dimensional fractionation (Priyanto et al., 2015), 2-dimensional fractionation (Jang et al., 2011), and 2-orthogonal bioassay-guided fractionation (Pujiastuti et al., 2017; Sutopo et al., 2020). However, these approaches have some drawbacks; for instance, they are time-consuming, costly, and the separation

process is complex. To overcome these challenges, a modified bioassay-guided fractionation was conducted in the present study by solid-phase extraction (SPE) short column because it is a convenient (in regards to both cost and time), simple, and effective way that is promising to develop. The elution fraction that has the highest ACEI value was continued for peptide identification coupled with in silico analysis database (BIOPEP, PeptideRanker, and ToxinPred) to predict the most potent ACEI bioactive peptide. Afterward, the ACEI mechanism of the most potent peptide was elucidated by a Lineweaver-Burk plot and molecular docking simulation. The activity confirmations of the most potent peptide were conducted by the determination of half-maximal inhibitory concentration (IC_{50}), inhibitor type, peptide stability, and quantification of sequence peptide using multiple reaction monitoring (MRM) analysis. Hence, the aim of the present study was to explore the bioactive peptide from milk protein hydrolysate digested by gastrointestinal proteases responsible to ACEI activity through the modified bioassay-guided fractionation.

MATERIALS AND METHODS

Material and Chemical Reagents

Milk powder was obtained from Fonterra Co-operative Group, Kaohsiung, Taiwan. Pepsin (from porcine gastric mucosa), trypsin (from bovine pancreas), and α -chymotrypsin (from bovine pancreas) were purchased from Sigma Chemical Co. Sodium bicarbonate ($NaHCO_3$), hydrogen chloride (HCl), sodium chloride (NaCl), boric acid, formic acid (**FA**; HCO_2H), and acetonitrile (**ACN**) were purchased from J.T. Baker. The ACE (EC 3.3.4.15.1) from rabbit lungs, hippuryl-L-histidyl-L-leucine (**HHL**), ferulic acid, NaCl and sodium hydroxide (NaOH) were purchased from Sigma Chemical Co. Trifluoroacetic acid (**TFA**) was purchased from Alfa Aesar. Trifluoroacetic acid, 4-methylmorpholine (**NMM**), N-ethyl-19-diisopropylamine (**DIPEA**), O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyl hexafluorophosphate were obtained from Alfa Aesar. Piperidine was purchased from Cleo Salus. Methylene chloride was purchased from SeedChem; N,N-dimethylformamide (**DMF**) was purchased from Duksan. Ethyl ether, triisopropylsilane, and N,N'-diisopropylcarbodiimide (**DIC**) were purchased from Sigma Chemical Co. Oxymapure and F-moc were obtained from Novabiochem. The water was obtained from a Milli-Q (Millipore) water purification system. Other chemicals and solvents used in this experiment were of analytical grade.

Enzymatic Digestion of Milk Powder

Milk powder (50 mg) was hydrolyzed using 2-stage hydrolysis based on Alting et al. (1997) as described by Ruiz et al. (2004) with several modifications. The first stage of hydrolysis was performed using pepsin at 37°C for 16 h with an enzyme-to-protein ratio of 1:200 (wt/wt) and dissolved in 35 mM NaCl with pH adjusted to 1.9 using HCl. In the second stage of hydrolysis, the mixture was adjusted to a pH of 8.3 using NaOH, then hydrolysis continued using trypsin and α -chymotrypsin with the same ratio 1:200 (wt/wt) at 37°C for 16 h. Afterward, the reaction was quenched by boiled water for 10 min and centrifuged at $16,200 \times g$ at 4°C for 15 min using a centrifuge (Hitachi Koko Co.). The resulting supernatant was taken to continue into the molecular weight cut-off ultrafiltration membrane (<3 kDa; Millipore) and centrifuged at $18,700 \times g$ at 10°C for 15 min to obtain short-chain peptides.

Modified Bioassay-Guided Fractionation

The HILIC fractionation used in this study was performed using the Zwitterionic HILIC-SPE cartridge (ZIC-HILIC SPE, SeQuant), which separated peptides via weak electrostatic interaction between charged peptides and the neutral zwitterionic stationary phase, according to the manufacture's instruction. Briefly, the short-chain peptides (<3 kDa) dissolved in 95% acetonitrile (ACN) and 0.1% FA in deionized water at a concentration of 10 mg/mL were loaded onto ZIC-HILIC SPE (1 mL of column) and fractionated using the mobile phase was composed of solution A (95% ACN and 0.1% FA in deionized water) and solution B (5% ACN and 0.1% FA in deionized water). The hydrolysate was simply separated into 5 fractions: F1 (95% ACN+0.1% FA), F2 (50% ACN+0.1% FA), F3 (20% ACN+0.1% FA), F4 (10% ACN+0.1% FA), and F5 (0.1% FA in deionized water). All fractions were lyophilized and the ACEI activity of each fraction (0.16 mg/mL) was evaluated using in vitro ACEI assay. The fraction with the highest ACEI activity was further fractionated using the reversed-phase chromatography with the form of SPE (RP-SPE). The RP-SPE fractionation was carried out using the HyperSep Retain PEP 30 mg/1 mL (Thermo Scientific Inc.) according to the manufacture's instruction. The composition of the mobile phase for RP-SPE was the same as HILIC-SPE but with a reverse elution order. Briefly, the most active HILIC fraction dissolved in 5% ACN and 0.1% FA with a concentration of 10 mg/mL was separated into 6 fractions: P1 (0.1% FA in deionized water), P2 (5% ACN + 0.1% FA), P3 (20% ACN + 0.1% FA), P4 (50% ACN + 0.1% FA), P5 (80% ACN + 0.1% FA), and P6

(95% ACN + 0.1%). Similar to the first bioassay-guided fractionation, all elution fractions were lyophilized and the ACEI activity of each fraction (0.1667 mg/mL) was examined using in vitro ACEI assay. The fraction with the highest ACEI activity was further identified using LC-MS/MS coupled with the database-assisted peptide sequencing.

Evaluation of ACE Inhibitory Activity and IC₅₀ Value

The ACE inhibitory assay was conducted to measure the ability of the sample to inhibit ACE. This assay was adopted from the method of Cushman and Cheung (1971) as described by Sutopo et al. (2020) with several modifications. Thirty microliters of 2.5 mM HHL was mixed with 10 μ L of ddH₂O as the blank, 10 μ L of 10 μ M captopril as the control and all of the samples (hydrolysate-peptide solution), respectively. Each sample was pre-incubated at 37°C for 5 min. Afterward, 20 μ L of ACE (0.05 mU/ μ L in 3 mM borate buffer) was added to the blank, control, and sample, individually. The reaction was incubated statically at 37°C for 30 min then incubated thermostatically using shaker incubation (200 rpm) at 37°C for 30 min. Sixty microliters of 1 M HCl was added to quench the reaction and added 10 μ L of 0.25 mM ferulic acid, as the internal standard for the downstream quantitation. The quantitative analysis of hippuric acid (HA), as the hydrolyzed product, was performed using RP-HPLC equipped with a C₁₈ column (4.6 mm $\times$ 250 mm; particle size of 5 μ m, Thermo Scientific Inc.) and detected by UV detector at 228 nm. The mixture was separated using an isocratic elution composed of 18% mobile phase A (5% ACN and 0.1% TFA in deionized water) at a constant flow rate of 1 mL/min for 25 min. The ACE inhibition (%) was determined according to the following equation:

$$\text{ACE inhibition (\%)} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100.$$

The A blank and A sample were the HA area under the peak in the mixture without and with the sample, respectively. The IC₅₀ was determined by plotting the ACE inhibitory activity (%) against at least 5 different concentrations of inhibitory samples. The logarithm of the inhibitor concentration was carried out using GraphPad 5 Software (GraphPad Software Inc.).

Peptide Identification Using LC-MS/MS Followed with Database-Assisted Matching

The most potent elution fraction of RP-SPE was lyophilized and redissolved in 5% ACN and 0.1% FA in

deionized water for peptide identification using LC-MS/MS. The mass spectrometric analysis was performed in electrospray ionization mode as described by Sutopo et al. (2020) with slight modifications. The sample was loaded into using Ultimate 3000 RSLC system (Dionex) coupled with the C₁₈ column (Acclaim PepMap RSLC, 75 μ m $\times$ 150 mm, Thermo Scientific) and analyzed with Thermo Q-Exactive mass spectrometer (Thermo Scientific). The sample was eluted with the mobile phase which was composed of solution A (5% ACN and 0.1% FA in deionized water) and solution B (95% ACN and 0.1% FA in deionized water) at a flow rate of 200 μ L/min. The gradient was performed as (1) isocratic elution with 5% solution B (0–3 min), (2) linear gradient from 5 to 20% solution B (3–10 min), (3) linear gradient from 20 to 80% solution B (10–20 min), and finally (4) isocratic elution maintained at 5% solution B (20–30 min). The scanning mass range was set in the range of m/z 100–1,500. The MS/MS raw data were processed into PEAKS X+ (Bioinformatics Solutions Inc.) Proteomic Mass Spectrometry software based on the protein database of *Bos taurus* in the National Center for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov/protein/?term=Bos+taurus>) with the following parameters: (1) enzyme: none; (2) instrument: Orbitrap (Orbi-Trap); (3) parent mass error tolerance: 10 ppm; and (4) fragment mass error tolerance: 0.5 Da.

Prediction of the Most Potent Sequence Peptide Using In Silico Analysis

In silico analysis was performed to predict the most potent peptide sequence by potential bioactive fragments. In this study, in silico analysis was applied in PeptideRanker database (<http://distilldeep.ucd.ie/PeptideRanker/>; Mooney et al., 2012), BIOPEP (<http://www.uwm.edu.pl/biochemia/index.php/en/biopep>; Minkiewicz et al., 2019), and ToxinPred (<http://crdd.osdd.net/raghava/toxinpred/>; Gupta et al., 2013). The peptide with highest BIOPEP score was regarded as the most potential ACEI peptide.

Synthesis and Purification of HL-7 Synthetic

The most potent peptide was synthesized using the microwave-assisted solid-phase peptide synthesizer (CEM Microwave Technology Ltd.) as described by Joel et al. (2018). The C-terminal AA was the first residue to attach the Wang resin. The Wang resin dissolved in DMF (5 mL) was added the first AA, DIC (0.5 mmol), DIPEA (1.0 mmol), and Oxyma Pure (0.5 mmol) and the mixture was stirred for overnight (more than 8 h) at rt. Afterward, the coupling step was presented to

attach the first AA on Wang resin using microwave irradiation (20 W) at 75°C for 5 min. DMF (3 mL), acetyl anhydride (1 mL), and 4.5% NMM (1 mL) were added to block the remaining hydroxyl groups of Wang resin to avoid any crosstalk reaction. The second AA was connected with the first AA by deprotection step (microwave irradiation 20 W at 75°C for 3 min) using 20% piperidine in DMF. Afterward, the reaction was continued by coupling with the pre-activated second AA (AA was dissolved in 4 mL of DMF, 1 mL of 4.5% NMM, and 0.3 mmol of HBTU). The coupling–deprotection cycle was returned until last AA was completed. The completed peptide was released from the resin by TFA cleavage. The synthetic peptide was further purified using RP-HPLC and characterized using LC-MS/MS. The retention time (t_R), the m/z value of precursor ion, and MS/MS fragments of the synthetic peptide was used to confirm the identity of the ACEI peptide identified from the most potent fraction of RP-SPE. The synthetic peptide was also applied to the determination of the peptide's inhibition (IC₅₀) and kinetics toward ACE.

Inhibition Type Determination of HL-7

The inhibition type was classified into competitive, noncompetitive, or uncompetitive, which was determined by the Lineweaver-Burk graph. The graph was created by plotting linear regression using the reciprocals of several substrate concentrations (HHL: 5; 2.5; 1.25; 0.625 mM) as the independent variable (x) and the reciprocals of the HA formation rate as the dependent variable (y) at 3 different concentrations (no inhibitor, 12 μ M, and 20 μ M). The x-intercept and y-intercept of the linear regression provided information about $1/K_m$ (mM^{-1}) and $1/V_{\text{max}}$ (maximum velocity of the reaction; min/mM) for the ACE inhibitory kinetics, respectively. The type of ACE inhibition was examined by comparing the reaction curves in the absence and presence of the inhibitor (Rawendra et al., 2013).

Molecular Docking Simulation Between ACE and HL-7

There are 2 isoforms of ACE transcribed by same gene, somatic ACE (sACE) and testis ACE (tACE). sACE has 2 domain C-domain and N-domain, whereas tACE only has C-domain; these 2 domains of sACE differ in substrate and inhibitor specificity, as well as chloride activation (Natesh et al., 2003; Pina and Roque, 2009). Even though both the N-domain and the C-domain are effective at cleaving angiotensin-I, the N-domain has no effect on blood pressure, indicating that the C-domain is the dominant angiotensin-converting

site. Moreover, C-domain of tACE has essentially identical to the C-domain of sACE that responsible to control blood pressure (Natesh et al., 2003; Pina and Roque, 2009; Masuyer et al., 2014). Therefore, PDB: 1O8A has represented the crystal structure of testicular human ACE (imported from the Protein Data Bank <https://www.rcsb.org/structure/1o8a>) used as a molecular model of human ACE. The most stable structure with the minimized energy for the ACEI peptide HL-7 was calculated using CHARMM (Chemistry at Harvard Macromolecular Mechanics). The cofactors Zn^{2+} and chloride ions were retained in the active site of the ACE model whereas all water molecular were removed before docking. The docking was carried out using Discovery Study Visualizer version 3.0 (Accelrys Software) via cavity detection with a radius 16 Å centered at the coordinates X: 39.073; Y: 38.287; Z: 50.139. The docking carried out in 3 runs. The molecular docking results (10 best poses) appeared in each run were based on the minimum interaction energy with a population size of 100 and a maximum interaction 10,000. The evaluation of the molecular docking was analyzed according to the docking energy value and the best pose of HL-7 interacted with ACE. Moreover, Accelrys Discovery Studio Visualizer version 3.0 was used to visualize hydrogen bonds, interactions, and hydrophobic interactions between peptides and ACE.

Peptide Stability of HL-7 Against ACE

The stability of HL-7 in the medium of ACE was examined by the pre-incubation of the potent ACEI peptide with ACE. This method was conducted to determine type of inhibitor and carried out based on the method described by Fujita and Yoshikawa (1999) with slight modifications. Several concentrations of HL-7 (8.3–100 μM) were pre-incubated with ACE for 3 h before the addition of substrate. Afterward, 2.5 mM HHL (30 μL), was added and incubated statically at 37°C for 30 min then incubated thermostatically using shaker incubation (200 rpm) at 37°C for another 30 min. The reaction was quenched by adding 60 μL of 1 N HCl and the inhibition degree of the peptide calculated as above. Meanwhile, the reaction mixture of the pre-incubation experiment was also analyzed using LC-MS/MS to monitor whether the degradation product was formed or not.

Peptide Stability of HL-7 Under Gastrointestinal Digestion

To examine stability and absorption of peptide inhibitors in the intestinal tract, simulated gastrointestinal digestion was conducted based on Priyanto et al.

(2015) with slight modifications. The peptide inhibitor was hydrolyzed using 2-step digestion using pepsin [ratio of enzyme to substrate (E/S) = 1:50 wt/wt] for 120 min at 37°C pH 1.8 then the pH was adjusted until it reached 8.3 using sodium hydroxide (NaOH). The second digestion was done by trypsin (E/S = 1:50 wt/wt) and α -chymotrypsin (E/S = 1:50 wt/wt) for 300 min at 37°C under a thermostatically controlled shaker incubator (200 rpm) before mixing. The reaction was quenched by heating 95°C for 10 min. Afterward, the peptide mixture was examined using LC-MS/MS to determine whether the degradation product was formed or not.

Quantification of HL-7 in the Hydrolysate and Fraction

The abundance of peptide inhibitors in gastrointestinal proteases digest derived from 1 mg of milk hydrolysate was determined using an MRM mode during LC-MS/MS analysis based on Pujiastuti et al. (2017) with slight modifications. Five standard peptide concentrations (0.01–2 ng/ μL) were used to inaugurate the standard calibration curve, which were constructed by plotting peak areas against corresponding concentrations. The slope, intercept, and correlation coefficient were obtained from least squares regression analysis. The milk hydrolysate was diluted and separated using ODS Hypersil column (150 $\times$ 2.1 mm, particle size 5 μm , Thermo Scientific) with a flow rate of 0.35 mL/min. The mobile phase consisted of solution A (95% ACN and 0.1% FA in deionized water) and solution B (5% ACN and 0.1% FA in deionized water). The gradient solvent was set as follows: 0 to 5 min, 5% mobile phase B; 5 to 25 min, 80% mobile phase B; and 25.10 to 30 min, 5% mobile phase B. The quantitative analysis was carried out under the SRM mode with a triple quadrupole mass analyzer (Quantum Access Max, Thermo Scientific). The ionization parameters were listed as follows: electrospray: positive mode; spray voltage: 3.5 kV; sheath gas pressure: 40 arbitrary units; auxiliary gas pressure: 5 arbitrary units; capillary temperature: 300°C; tube lens offset: 124 V. The scanning mass range was set at m/z 100 to 1,000. The SRM transition of HL-7 was m/z 802 > 342, and the collision-induced dissociation energy was set at 39 V.

Statistical Analysis

The results were expressed in triplicate and carried out as the mean $\pm$ standard error. The analysis was accomplished by using ANOVA in SPSS 16.0 (SPSS Inc.). Graphic data and IC_{50} were established using GraphPad Prism 6.0 (GraphPad Software Inc.) by a

nonlinear regression. To identify significant differences among treatments, the statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

IC₅₀ Determination of Small Peptides (<3 kDa) Derived from Milk Protein Hydrolysate

Milk powder was hydrolyzed using pepsin then followed by trypsin and chymotrypsin, commonly referred to the simulated gastrointestinal digestion. Enzymatic hydrolysis is one of the principal approaches for releasing bioactive peptides from the parent protein of milk (Rai et al., 2017). The 2-stage hydrolysis using gastrointestinal proteases is to simulate the condition of the gastrointestinal tract (Ruiz et al., 2004). The condition of the simulated gastrointestinal digestion (enzyme preparation, temperature, pH, and incubation time) highly encourages the degree of proteolysis and the resultant ACEI activity (Vermeirssen et al., 2003). The utilization of gastrointestinal proteases for releasing bioactive peptides from parent proteins is an appropriate method for generating bioactive peptides which can retain their active form during digestion (Ruiz et al., 2004). It was also previously reported that ACEI sequence peptides were identified in hydrolysates of several samples of human milk and infant formulas after digestion using gastrointestinal protease (Hernandez-Ledesma et al., 2007).

According to previous studies, short-chain peptides typically show higher ACEI activity (Chen et al., 2012; Li et al., 2016, 2018). Therefore, milk protein hydrolysate was loaded onto the molecular weight cut-off ultrafiltration membrane (3 kDa) to separate short-chain peptides (<3kDa) from larger peptides and proteins/proteases (>3 kDa) based on their molecular weight. The ACE inhibition activity (%) of short-chain peptides (<3 kDa) derived from milk protein was examined and its IC_{50} value was determined by 5 different concentrations to give an IC_{50} value of $323.1 \pm 8.72 \mu\text{g/mL}$ (Figure 1).

Modified Bioassay-Guided Fractionation

To efficiently screen the ACEI bioactive peptide from gastrointestinal hydrolysate of milk, a modified bioassay-guided fractionation was conducted. Previously, several fractionation approaches had been widely used such as one-dimensional fractionation (Priyanto et al., 2015), 2-dimensional fractionation (Jang et al., 2011), and 2-orthogonal bioassay-guided fractionation (Pujiastuti et al., 2017; Sutopo et al., 2020). However, all of those approaches have some drawbacks such as

insufficiency to distinguish peptide in one fraction, insufficient amount for the subsequent ACEI assay, time-consuming due to complex process separation, and costly. To overcome drawbacks, we used a modified bioassay-guided fractionation, which is expected to be simpler, more convenient and cost effective. The modified bioassay-guided fractionation consists of HILIC-SPE and RP-SPE short column, which are carried out continuously to increase the separation efficiency.

Hydrophilic interaction liquid chromatography provides alternative ways to effectively separate polar compounds on polar stationary phase and had been widely used to analyze peptides, carbohydrates, and polar pharmaceuticals (Buszewski and Noga, 2012). A less polar organic solution was used for the initial gradient elution then followed by increasing the polar aqueous content (Cubbon et al., 2007; Buszewski and Noga, 2012). The milk protein hydrolysate was fractionalized into 5 elution fractions (fractions F1–F5). The HILIC-SPE fraction with the highest ACEI activity was obtained when eluted by 95% ACN + 0.1% FA (fraction F1; Figure 2a). Fraction F1 showed significantly high ACEI activity (73.33%) when compared with the others elution fractions. The IC_{50} of fraction F1 was determined as $61.75 \pm 5.74 \mu\text{g/mL}$ by 6 different concentrations (Figure 2b). Fraction F1 has the best ACEI activity that might due to contain hydrophobic peptide components, which appeared in the initial elution fraction. This result agreed with the study of Buszewski and Noga (2012) which revealed that the retention increases as the polarity of the mobile phase decreases, and thus polar analytes are more strongly retained than nonpolar ones.

Figure 1. Half-maximal inhibitory concentration (IC_{50}) of milk protein hydrolysate after molecular weight cut-off ultrafiltration membrane (<3 kDa) using concentration between 1 and 5 mg/mL. ACE = angiotensin-converting enzyme. Error bars indicate SD.

Fraction F1 was further separated using RP-SPE for the second bioassay-guided fractionation. The fractionation using RP-SPE aimed to emphasize the screening process of bioactive peptide that responsible to ACEI activity of each fraction. Fraction F1 was fractionalized into 6 elution fractions (P1–P6). Because the elution conditions of RP-SPE are opposite to those of HILIC-SPE, the elution step of RP-SPE begins from the polar mobile phase to the less polar solution. The ACEI activity of each fraction derived from RP-SPE was examined as shown in Figure 3. The highest ACEI activity (85.65%) was obtained in elution fraction P3, which was eluted by 20% ACN+0.1% FA (Figure 3a). The IC_{50} of P3 was $36.22 \pm 1.18 \mu\text{g/mL}$ determined by ACEI assay using 5 different concentrations (Figure

Figure 2. (a) Angiotensin-converting enzyme (ACE) inhibitory activity and (b) half-maximal inhibitory concentration (IC_{50}) of hydrophilic interaction liquid chromatography-solid-phase extraction (HILIC-SPE): F1 elution fraction. FT = flow through. The milk protein hydrolysate that passed from the MWCO M3 kDa was loaded into reverse phase-solid-phase extraction (RP-SPE) and collected. Error bars indicate SD. Different letters indicate significance ($P < 0.05$).

Figure 3. (a) Angiotensin-converting enzyme (ACE) inhibitory activity and (b) half-maximal inhibitory concentration (IC_{50}) of reverse phase-solid-phase extraction (RP-SPE): P3 elution fraction. FT = flow through. The best fraction from hydrophilic interaction liquid chromatography-solid-phase extraction (HILIC-SPE) that loaded into RP-SPE was then collected. Error bar values (SD) were obtained from triplicate experiments. Different letters indicate significance ($P < 0.05$).

3b). After the 2-step fractionation, the IC_{50} value of elution fraction P3 dramatically decreased by 8.92-fold when compared with the crude hydrolysate, which revealed that the modified bioassay-guided fractionation is effective to uncover ACEI peptides. The peptides which contributed the ACEI activity in P3 were further identified using LC-MS/MS.

Identification of ACEI Peptide Candidates Using LC-MS/MS Followed by In Silico Analysis

The peptides existed in the fraction P3 were identified using LC-MS/MS coupled with database-assisted sequencing using PEAKS X+ (Bioinformatics Solutions Inc.) proteomic mass spectrometry software. The bio-

Figure 4. Liquid chromatography-mass spectrometry chromatogram of reversed-phase solid-phase extraction fraction P3 (20% acetonitrile + 0.1% formic acid).

logical activities of identified peptides were predicted in silico using BIOPEP, PeptideRanker, and ToxinPred to screen the most potential peptide. As a result, 8 major peptides were identified as potential candidates of ACEI peptides after identified by PEAKS X+ based on the *Bos taurus* database and de novo sequencing. The LC-MS chromatogram of the fraction P3 was shown in Figure 4. The sequence assignment of the 8 peptides further analyzed using in silico analysis and HLPLPLL (HL-7) was regarded as the most potential one due to its highest BIOPEP and PeptideRanker values and nontoxic sequence were shown in Table 1. HL-7 was

derived from β -CN, one of the major proteins in milk (Costa et al., 2014). The heptapeptide was identified based on the m/z 401.76 of $[M+2H]^{2+}$ ion at t_R 34.00 min, and the matched b and y ions series in the MS/MS spectrum as shown in Supplemental Figure S1 (<https://doi.org/10.17632/gztrg9dghp.1>; Ningrum et al., 2021). The identity of HL-7 was further confirmed using the synthetic peptide with the sequence of HLPLPLL, by comparing the retention time and MS/MS spectrum between identified and synthetic peptides as shown in Supplemental Figure S2 (<https://doi.org/10.17632/gztrg9dghp.1>; Ningrum et al., 2021).

Table 1. In silico analysis of bioactive peptide prediction using PeptideRanker, BIOPEP, and ToxinPred

No.	Sequence	m/z	t_R^1 (min)	Area	PeptideRanker ²	BIOPEP ³		ToxinPred ⁴
						A	B	
1	HLPLPLL	401.76	34	3.31E+09	0.826017	1	0.274	Nontoxic
2	PGPLMLVYPN	550.79	36.67	2.37E+09	0.673603	0.9	0.07	Nontoxic
3	APGVPLVY	457.77	34.04	6.69E+06	0.396431	0.66	0.035	Nontoxic
4	APLVF	273.66	31.87	2.03E+07	0.76009	0.6	0.023	Nontoxic
5	EPVVPF	376.18	32.29	1.84E+08	0.734184	0.5	0.024	Nontoxic
6	NFVVPVKLPPE	619.85	40.84	4.94E+08	0.271237	0.5	0.02	Nontoxic
7	DYYPLRT	464.23	36.83	4.83E+07	0.619065	0.57	0.003	Nontoxic
8	FLTTWV	383.7	39.64	7.81E+06	0.422096	0.16	0.273	Nontoxic

¹ t_R = retention time.

²PeptideRanker = potential bioactive peptide score.

³BIOPEP = database for angiotensin-converting enzyme inhibitor. A: the frequency of the occurrence of bioactive fragments in a protein. B: the potential biological activity of protein.

⁴ToxinPred = prediction of toxicity of peptide.

IC₅₀ of HL-7 Against ACE

To estimate the concentration of HL-7 that could inhibit ACE's activity by 50%, 7 different concentrations of HL-7 between 0.625 and 100 μM were used to determine its IC₅₀ value against ACE. The IC₅₀ value of HL-7 was carried out by nonlinear regression to give $16.87 \pm 0.3 \mu\text{M}$ (Figure 5), this value was carried out by triplicate and become references to determine its kinetic study. The sequence of HL-7 was proved as HLPLPLL with molecular weight 802.02 Da, the low molecular weight peptides (<1,000 Da) were shown to be able to exhibit high ACE inhibitory activity (Du et al., 2013; Liu et al., 2013; P. Li et al., 2014). The ACEI activity of a peptide was dominated by AA sequence (Khueychai et al., 2018). The peptide HL-7 contained an aromatic residue His at the N-terminal and a hydrophobic residue Leu at the C-terminal, which may contribute to its potent ACEI activity. According to previous studies, the peptides with an aromatic residue (e.g., Tyr, Phe, Trp, His, or Pro) or a hydrophobic residue (e.g., Ile, Val, or Leu) at N-terminal position had been reported to have a significant ACEI activity (Cheung et al., 1980; Pihlanto-Leppälä, 2000; Wu et al., 2006; Kong et al., 2007; Iwaniak et al., 2014). The peptide HPHPHLSF, derived from κ -CN in milk, containing an N-terminal His had been demonstrated its potent ACEI activity (IC₅₀ = 28.9 μM) as well as significant antihypertensive effect (Cheung et al., 1980; Gómez-Ruiz et al., 2007; Miguel et al., 2010).

In addition, the importance of Leu as a C-terminal residue on ACEI activity was demonstrated by comparing the ACEI activity of VPSERY and VPSERYL (Ruiz et al., 2004). The latter's IC₅₀ value was 249.5 μM lowered than VPSERY (706.1 μM); KKYNVPQL

Figure 5. Half-maximal inhibitory concentration (IC₅₀) of HLPLPLL (HL-7).

Figure 6. Half-maximal inhibitory concentration (IC₅₀) of HL-7 after pre-incubation experiment. ACE = angiotensin-converting enzyme.

(IC₅₀ = 77.1 μM) showed superior ACEI activity than KKYNVPQ (IC₅₀ = 716.9 μM ; (Ruiz et al., 2004). Another study also reported the presence of Leu at C-terminus of the peptide GPL increased the ACEI activity by 2-fold when compared with GPV (Kim et al., 2001). Moreover, the presence of proline in the HLPLPLL (HL-7) may improve the binding with the enzyme and contribute the enhanced ACEI activity. Several proline-containing peptides derived from milk produced by gastrointestinal proteases, such as IAK (IC₅₀ = 15.7 μM), YAKPVA (IC₅₀ = 14.3 μM), WQV-LPNAVPAK (IC₅₀ = 10.1 μM), and HPHPHLSF (IC₅₀ = 28.9 μM), showed potent ACEI activities (Gómez-Ruiz et al., 2007; Miguel et al., 2010).

There are 3 kinds of inhibition types such as true inhibitor, substrate inhibitor and prodrug inhibitor that classified based on the IC₅₀ value after pre-incubation experiment (Fujita and Yoshikawa, 1999). As illustrated in the Figure 6, the IC₅₀ value of HL-7 slightly increased from $16.87 \pm 0.3 \mu\text{M}$ to $26.04 \pm 1.48 \mu\text{M}$ after the pre-incubation experiment, indicating HL-7 was a substrate-type inhibitor. The decreasing inhibition activity may be due to the loss of C-terminal dipeptide hydrolyzed by ACE during the pre-incubation. Indeed, HLPLP (HP-5), the ACE hydrolysis product of HL-7, has been reported as an ACEI peptide with IC₅₀ value of 41 μM (Kohmura et al., 1989).

The IC₅₀ values from each purification step were summarized in Table 2. The IC₅₀ value significantly decreased by 8.92-fold after the third purification step, indicating 8.92 times of purity improvement of ACEI peptides. The HILIC-SPE purification step also showed high purification performance, with a decrease of IC₅₀ value by 5.23-fold. Hence, a modified bioassay-guided fractionation could be an alternative approach to enrich ACEI peptides from the complex hydrolysate.

Table 2. Half-maximal inhibitory concentration (IC_{50}) and purification folds summary in each purification step

No.	Purification step ¹	Fraction	$IC_{50} \pm SD$ ($\mu g/mL$)	Purification fold
1	Milk hydrolysate	CP ²	323.1 ± 8.72	1
2	HILIC-SPE	F1	61.75 ± 5.74	5.23
3	RP-SPE	P3	36.22 ± 1.18	8.92

¹HILIC-SPE = hydrophilic interaction liquid chromatography-solid-phase extraction. RP-SPE = reverse phase-solid phase extraction.

²After molecular weight cut-off ultrafiltration (<3 kDa).

Inhibitory Kinetics of HL-7

The inhibition mode of HL-7 was further examined by its kinetics study against ACE. The Lineweaver-Burk plot of HL-7 indicated that it is a competitive inhibitor due to the 3 lines (0, 12 μM , and 20 μM HL-7) with the different slopes converging to the same y-axis value ($1/V_{max}$; Figure 7). The V_{max} values are stable and the K_m values are increased according to the increasing concentration of HL-7 (Table 3). The constant V_{max} value and the increased K_m value suggested that the inhibitor can interact as well as the substrate with key residues in the active site of ACE and can compete with the substrate for accessing the binding site (Priyanto et al., 2015). In addition, the dissociation constant (K_i) of HL-7 was found approximately 0.8 μM that obtained from the secondary plot of the slopes of the 3 lines (no inhibitor, 12 μM , and 20 μM of HL-7, respectively).

Molecular Docking Analysis

Molecular docking analysis was conducted to simulate the interaction between HL-7 and ACE using Discovery Studio Visualizer (Accelrys Software). The structure of HLPLPLL was generated with Discovery Studio and the energy was minimized with the CHARMM program. The best docking pose between HL-7 with ACE (Figure 8) selected based on the docking energy, -101.058 kJ/mol (lowest energy). Three pockets including S1, S2, and S1' are located in the active site of ACE. The pocket S1 included Ala354, Glu384 and Tyr523, S2 included Gln281, His353, Lys511, His513, and Tyr520, and S1' contained the residue Glu162. Further, ACE has a zinc ion $[Zn(II)]$ in its active site that coordinates with His383, His387, and Glu411 (Pina and Roque, 2009).

The interaction bond and distance between HL-7 and ACE were stabilized by hydrogen interactions, charge interactions and pi-pi interactions with 8 total interactions toward ACE residues. HL-7 is a competitive inhibitor due to the 2 last sequences (Leucyl-Leu) from C-terminal bind with the active site of ACE. There

were 3 of 9 interactions with pocket ACE, such as hydrogen interaction Ala354 (S1), Gln281 (S2'), Lys511 (S2'), and charge interaction Lys511 (S2'); however, there was not a bind directly on the catalytic Zn (II) as shown in Table 4. In addition, referring to Table 5 about the comparison of the number of hydrogen bonds between lisinopril, captopril, HL-7 to ACE showed that HLPLPLL has fewer hydrogen bonds to the key ACE residues (3 hydrogen bonds) than lisinopril (9 hydrogen bonds) and captopril (5 hydrogen bonds). However, the H-bonds of HL-7 with ACE residues, such as Ala 354 (2.5 Å), Lys 511 (1.8 Å), Gln 281 (2.1 Å) are shorter than those reported by Rawendra et al. (2013) and Girgih et al. (2014) for lisinopril and captopril. A shorter distance will strongly raise the interaction between ACE residues and HL-7 and the correlation number of H-bond between bioactive peptide with ACE also affected its inhibition activity, these agreed with Girgih et al. (2014) which could have contributed to the ACEI activity.

Stability of HL-7 Against ACE and Gastrointestinal Digestion

The pre-incubation experiment was conducted to examine the stability of HL-7 against ACE. After pre-incubation, the whole mixture was monitored using LC-MS. As illustrated in Figure 9, HL-7 was partially hydrolyzed by ACE after pre-incubation for 4 h. The dipeptide (Leucyl-Leu) was cut from C-terminus by ACE gave the pentapeptide HLPLP (HP-5; m/z 576.33). Nevertheless, the degree of the HP-5 produced from

Table 3. The kinetic parameter comparison between no inhibitor and HL-7 using concentrations of 12 and 20 μM

Parameter ¹	No inhibitor	HL-7 (12 μM)	HL-7 (20 μM)
V_{max} ($\mu M/min$)	18.8	18.7	18.7
K_m (μM)	5.5	13.1	18.4

¹ V_{max} = maximum velocity of the reaction; K_m = Michaelis-Menten constant or enzyme affinity.

HL-7 during ACE incubation (4 h) is relatively low (<10%), which indicated that HL-7 can almost tolerate the hydrolysis of dipeptidase ACE.

The potent ACEI peptide HL-7 was generated by simulated gastrointestinal digestion. Theoretically, HL-7 will not be further hydrolyzed by the same gastrointestinal proteases. To simulate its stability under gastrointestinal digestion in human body, HL-7 was incubated with pepsin at 37°C (pH 1.8) for 2 h and followed by trypsin and chymotrypsin (pH 8.3) for 5 h. After a total incubation time of 7 h, the hydrolysis reaction was monitored using LC-MS to give the result as shown in Supplemental Figure S3 (<https://doi.org/10.17632/gztrg9dghp.1>; Ningrum et al., 2021). Indeed, HL-7 was not hydrolyzed during incubating with gastrointestinal protease. As reported before, Amorim et al. (2019) revealed that some of bioactive peptide that seems resistance during gastrointestinal digestion (Supplemental Figure S4; <https://doi.org/10.17632/gztrg9dghp.1>; Ningrum et al., 2021). The reason why HL-7 has the ability to resist its active form was described by Yang and Russell (1992), the presence of proline can increase peptide resistance to hydrolysis by digestive enzymes. Besides that, HL-7 contains 2 proline residues that likely survive longer in the digestive system and more likely exert in vivo bioactivity (Nielsen et al., 2017). Therefore, HL-7 is the most potent that could be an alternative natural ACE inhibitor for controlling blood pressure and functional food development.

Quantification of HL-7 Using MRM Analysis

The potential ACEI bioactive peptide HL-7 obtained from enzymatic hydrolysis using gastrointestinal proteases, the quantification of HL-7 in the milk gastrointestinal hydrolysate, and elution fraction should be conducted to provide an overview of opportunities or support the development of product applications. In this study, 2-stage hydrolysis using gastrointestinal proteases not only was carried out to simulate the process conditions in the intestinal tract but also can provide more comprehensive hydrolysis and give higher yield than one-stage hydrolysis (Rawendra et al., 2014). Enzyme-to-substrate ratio (E/S; 1:200), time and temperature have been comprehensively considered to obtain HL-7 in the hydrolysate and elution fraction. The quantitative determination of HLPLPLL content was carried out under MRM mode in a triple quadrupole mass analyzer (Thermo Scientific). The MRM transition of HLPLPLL was m/z 802.02 > 342.1 under a collision-induced dissociation energy of 39V as shown in the Supplemental Figure S5 (<https://doi.org/10.17632/gztrg9dghp.1>; Ningrum et al., 2021), which can produce the most product ions. It used as a reference for making a standard calibration curve of HLPLPLL with the regression linear equation ($y = 934850x - 15408$) and correlation coefficient ($R^2 = 0.9997$) which are established by 5 different concentrations of HLPLPLL (0.01–2 ng/ μ L) as shown in Supplemental Figure S6

Figure 7. Lineweaver-Burk plot of HL-7. V = initial rate; S = substrate concentration.

Figure 8. Two-dimensional docking simulation between HL-7 and angiotensin-converting enzyme (ACE).

(<https://doi.org/10.17632/gztrg9dghp.1>; Ningrum et al., 2021). The milk protein hydrolysate derived from gastrointestinal protease and RP-SPE: P3 elution fraction with the concentration 1 mg/mL analyzed under MRM were seen in Supplemental Figure S7 (<https://doi.org/10.17632/gztrg9dghp.1>; Ningrum et al.,

2021). The quantification abundance of HL-7 derived from β -CN which was calculated based on the linear regression of the calibration curve and the peak area of HLPLPLL in each MRM chromatogram. The MRM experiment, 19.86 ± 1.14 pg and $14,545.8 \pm 572.9$ pg of HLPLPLL was obtained in the milk protein hydro-

Table 4. The interaction bond and distance between HL-7 and angiotensin-converting enzyme (ACE)

Sequence	ACE residue	Pocket	Interaction	Distance (Å)
His	TYR:360	Nonactive site	H-Bond (side chain)	1.8
	ARG:402	Nonactive site	H-Bond (main chain)	2
	PHE:391	Nonactive site	Pi-Pi	5.1
Leu	—	—	—	—
Pro	—	—	—	—
Leu	ALA:356	Nonactive site	H-Bond (main chain)	2.5
Pro	—	—	—	—
Leu	ALA:354	Active site-S1'	H-Bond (main chain)	2.5
Leu	GLN:281	Active site-S2'	H-Bond (side chain)	2.1
	LYS:511	Active site-S2'	H-Bond (side chain)	1.8
	LYS:511	Active site-S2'	Charge interaction	2.5

ACE residue in H-bond	Lisinopril	Distance (Å)	Captopril	Distance (Å)	HLPLPLL	Distance (Å)
Glu 162	✓	3.4	x	—	x	—
His 353	✓	2.8	✓	2.5	x	—
Ala 354	✓	2.9	x	—	✓	2.5
Glu 384	✓	2.7	✓	3.1	x	—
Lys 511	✓	2.9	✓	2.7	✓	1.8
His 513	✓	3.1	✓	2.7	x	—
Tyr 520	✓	2.6	✓	2.7	x	—
Tyr 523	✓	2.8	x	—	x	—
Gln 281	✓	2.2	x	—	✓	2.1
Total	9 hydrogen bonds		5 hydrogen bonds		3 hydrogen bonds

lysate and RP-SPE: P3 elution fraction derived from 1 mg/mL of milk protein, respectively. The yield was reasonable due to the molecular weight of HL-7 (401.76 Da) smaller than β -CN (24 kDa; Huppertz et al., 2018).

CONCLUSIONS

A modified bioassay-guided fractionation was performed to efficiently screen ACEI peptides milk protein hydrolysate derived from gastrointestinal protease. The peptide HL-7 was successfully screened from the milk protein hydrolysate that derived by β -CN with IC_{50} $16.87 \pm 0.3 \mu M$. The IC_{50} value significantly decreased by 8.92-fold after 3 purification steps. Furthermore, the kinetic study indicated that HL-7 was a competitive inhibitor as well as a substrate-type inhibitor, which was consistent with the simulation results of molecular docking. These findings revealed that HL-7 could be a natural ACE inhibitor bioactive peptide. Further investigation such as in vivo antihypertensive effect in spontaneously hypertensive rats is suggested to examine the efficacy.

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