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Running title: anti-hypertensive peptides from yogurt

Summary

This study investigated the effects of fermentation conditions on the production of angiotensin-converting enzyme inhibitory (ACE-I) peptide in yogurt by *Lactobacillus helveticus* 881315 (*L. helveticus*) in the presence or absence of Flavourzyme®, which is derived from a mold *Aspergillus oryzae* and used for protein hydrolysis in various industrial. Optimal conditions for peptides with the highest ACE-I activity were 4% (v/w) inoculum size for 8 h without Flavourzyme® supplementation, and 1% inoculum size for 12 h when combined with Flavourzyme®. The yogurt fermented by *L. helveticus* resulted in IC_{50} values (concentration of inhibitor required to inhibit 50% of ACE activity under the assayed conditions) of 1.47 ± 0.04 and 16.91 ± 0.25 mg/mL with and without Flavourzyme®, respectively. Seven fractions of ACE-I peptide from the yogurt incorporated with *L. helveticus* and Flavourzyme® were separated using the preparative high-performance liquid chromatography. Fraction (F3) showed the highest ACE-I activity with an IC_{50} of 35.75 ± 5.48 μg/mL. This study indicates that yogurt may be a valuable source of ACE-I peptides, which may explain the outcomes observed in the experimental and clinical studies and foresee the application of fermented milk proteins into functional foods or dietary supplements.

Keywords

Yogurt, Peptides, Angiotensin-converting enzyme, Flavourzyme®, *Lactobacillus helveticus*

Introduction

Yogurt is generally made from milk by mixed cultures, including two types of homo-fermentative bacteria, *Streptococcus thermophilus* (*S. thermophilus*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) (Radke-Mitchell & Sandine, 1986; De Brabandere & De Baerdemaeker, 1999). *Lactobacillus helveticus* (*L. helveticus*), is a lactic acid bacterium (LAB) and is used for the production of fermented milk beverages and some types of hard cheeses (Griffiths & Tellez, 2013). In previous studies, fermented milk with *L. helveticus* exhibited anti-hypertensive effect in both animal and clinical studies due to its angiotensin-converting enzyme inhibitory (ACE-I) activity (Chen *et al.*, 2014; Jauhiainen *et al.*, 2005; Aihara *et al.*, 2005; Griffiths & Tellez, 2013). Angiotensin-converting enzyme (ACE), as a part of the renin-angiotensin system, has an important role in the regulation of blood pressure by converting angiotensin-I into a potent vasoconstrictor, angiotensin-II. Angiotensin-II induces the release of aldosterone and therefore increases the sodium retention and blood pressure (Muro Urista *et al.*, 2011). Thus, inhibition of ACE can lower the blood pressure, and has potential health benefits to hypertensive patients (Tuomilehto *et al.*, 2004; Aihara *et al.*, 2005). The renin-angiotensin system has therefore become a key target for anti-hypertensive drugs (Miura *et al.*, 2011), however, conventional drugs targeting this system cause various adverse effects, such as headache, dizziness and cough (Soleimani *et al.*, 2015; Coulter D, 1987).

Anti-hypertensive peptides isolated from fermented dairy products such as fermented milk drink and cheese could represent a healthier and natural alternative for the ACE-I drugs as a non-pharmacological therapy to reduce the risk of hypertension (Hannu & Anne, 2006; Minervini *et al.*, 2003; Pan & Guo, 2010; Donkor *et al.*, 2007b). Previous studies showed that two tripeptides [Val-Pro-Pro (VPP), Ile-Pro-Pro (IPP)] released from fermented milk by

several types of *L. helveticus* strains exhibited anti-hypertensive peptide effects due to high ACE-I activity in both human (Tuomilehto *et al.*, 2004; Ishida *et al.*, 2011; Cicero *et al.*, 2013) and rats (Chen *et al.*, 2014; Jauhiainen *et al.*, 2010). However, little information is available on whether yogurt is a better source of ACE-I peptides when it is further fermented by *L. helveticus* strains.

During yogurt fermentation, bacteria growth and product synthesis depend on medium compositions and culture conditions such as temperature, pH, inoculum size and fermentation duration and so on (Agyei *et al.*, 2012). Inoculum size was an important factor for the growth rate of the bacterial culture. When the inoculum size is too low, it would take longer for bacteria to reach the logarithmic growth phase (Min *et al.*, 2013). In contrast, a greater inoculum size could result in a quick consumption of nutrients required by bacterial strains, thus fermentation could be interrupted (Min *et al.*, 2013). The optimisation of various fermentation parameters for maximising active peptide production by LAB is therefore a major research endeavour. Supplementation of some enzymes with strong proteolytic activity is alternative method to improve functional peptide production during yogurt fermentation (Donkor *et al.*, 2007a; Shabboo & Ahmad, 2011). Among of these enzymes, Flavourzyme® is an important contributor to hydrolyse milk protein for the production of ACE-I peptides (Tsai *et al.*, 2008).

Flavourzyme® contains enzymes that are generally recognized as safe for use in the food industry (Boschin *et al.*, 2014). It is derived from a mold *Aspergillus oryzae* and used for protein hydrolysis in various industrial and research applications due to its high endoprotease and exopeptidase activities (Merz *et al.*, 2015).. Ahtesh *et al*. (2016 a, b) reported that Flavourzyme® can be used to increase the hydrolysis of the milk protein into further small molecular weight ACE-I peptides in 12% of reconstituted skim milk (RSM). However, the role of Flavourzyme® in the production of bioactive peptides from yogurt is not well

reported. Therefore, the aim of this study was to determine the optimal fermentation conditions for the production of ACE-I peptides from yogurt fermented by *Lactobacillus* strains. Furthermore, we investigated whether the inclusion of Flavourzyme® is able to increase the release of ACE-I peptides in yogurt media.

Material and Methods

Culture medium and reagents

Glycerol de Man, Rogosa, Sharpe (MRS) broth was purchased from Oxoid, Ltd., West Heidelberg, Victoria, Australia. Reconstituted skim milk (RSM) powder (52% lactose, 37% protein, 8.6% ash, and 1.2% fat) was obtained from Woolworths Ltd, Australia. M17 broth was purchased from Oxoid, Ltd, Hampshire, England. Flavourzyme® (EC 3.4.11.1, an amino peptidase with an activity of 500 Leucine Amino-peptidase per gram), hippuryl-L-histidyl-L-leucine (HHL), ACE enzyme (from rabbit lung, 0.1 UN) and hippuric acid (HA) were purchased from Sigma-Aldrich Pty. Ltd., NSW Australia.

Bacteria storage and propagation

Lactobacillus helveticus ASCC 881315 (*L. helveticus*), *Streptococcus thermophiles* ASCC 1275 (*S. thermophiles*) and *Lactobacillus delbrueckii* subsq. *bulgaricus* 1466 (*L. bulgaricus*) were obtained from Dairy Innovation Australia Ltd, Werribee, Victoria, Australia. The *L. helveticus* strain was stored in MRS broth, while *S. thermophiles* and *L. bulgaricus* strains were kept in RSM at −80 °C. For activation of these strains, 100 µL of *L. helveticus* and *L. bulgaricus* strains were transferred separately into 9.9 mL of 40% MRS broth, whereas *S. thermophiles* was activated via transfer of 100 µL to 9.9 ml of 3.7% M17 broth, containing 0.5% lactose. All

media were autoclaved at 121 °C for 15 minutes before use. Following activation, all strains were incubated at 37 °C for 24 h

Yogurt preparation

Yogurt was prepared by dissolving skim milk powder (12%, w/w) in distilled water. RSM was heated to 90 °C for 30 min, then inoculated with 1% activated *S. thermophiles* and *L. bulgaricus* cultures (1: 1 of ratio) and fermented at 42 °C for 6 hours to produce yogurt. Then the fermentation process was stopped by cooling at 4 °C overnight.

Release of bioactive peptides from yogurt

L. helveticus 881315 strain was incorporated into yogurt with or without Flavourzyme® (0.14%). Specifically, the yogurt was re-incubated at 37 °C with stirring (200 rpm) for the purpose of a better homogenization between yogurt and Flavourzyme® during 16 h. After incubation for 4, 8, 12 and 16 h, with different inoculum sizes of *L. helveticus* (1%, 2%, 3% and 4% v/w), samples were taken for analysis. The fermentation process was terminated by heating the yogurt at 90 °C for 20 min to stop enzyme activity. Subsequently, 25 mL of yogurt sample was centrifuged at 4000 × g at 4 °C for 30 min to separate proteins. The supernatant containing soluble peptides was freeze-dried (Freeze-drier, John Morris Scientific Pty Ltd, Australia) for 72 h. The freeze-dried peptides powder was stored at -20 °C for analysis.

Determination of ACE-Inhibitory activity

20 mg of the freeze-dried peptide powder from normal yogurt with starter culture or yogurt incorporated with *L. helveticus* without Flavourzyme® was dissolved in 1 mL of Tris buffer (50 mM, pH 8.3) containing 300 mM sodium chloride, respectively. However for the peptides from yogurt incorporated with *L. helveticus* and Flavourzyme®, 2.5 mg/mL of powder was

dissolved in the same Tris buffer, as these peptides showed 100% ACE-I activity, when the concentration was 20 mg/mL; therefore, lower concentration (2.5 mg/mL) was used to assess the production of ACE-I peptides under different fermentation conditions.

To determine the peptide fractions from yogurt in the presence of Flavourzyme®, freeze-dried powder (2.5 mg) was dissolved in Tris buffer (50 mM, pH 8.3) containing 300 mM sodium chloride to prepare the sample solutions of 62.5, 125, 250, and 500 µg/mL concentrations to calculate IC_{50} value of different fractions. The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of ACE activity under the assayed conditions.

Evaluation of ACE-I activity was assayed using a reversed-phase HPLC system (RP-HPLC, from Varian Analytical Instruments, Santa Clara, CA, USA) previously described by Donkor et al 2007a. It is based on the hydrolysis HHL by ACE to hippuric acid (HA) and histidyl-leucine (HL) as products. The HA released from HHL is directly related to the ACE activity. ACE enzyme and HHL was prepared in Tris buffer. Briefly, the assay consisted of 300 µL of 3.0 mM HHL, 300 µL of 3.0 mU ACE enzyme, and 300 µL of peptide solution. The mixture was placed in a glass tube and then incubated at 37 °C in a water bath for 0.5 h, mixed for 1 min, then returned to the water bath for another 0.5 h. The reaction was stopped by heating the mixture in an 85 °C water bath for 10 min in order to inactivate enzymes. The reaction mixture was stored at -20 °C before further analysis of released hippuric acid by RP- HPLC.

HA standard curve was prepared in five different concentrations (5, 10, 15, 20 and 25 µg/mL). The isocratic mobile phase composition was optimized to 12.5% acetonitrile (Merck) in MilliQ water (v/v) containing 0.1% trifluoroacetic acid (TFA), which was filtered (0.45 µm) prior to running through the column. The temperature of the column was kept at room temperature (~22 °C). An aliquot (10 µL) of the mixture was injected onto a VyDAC® C18 300 Å (250 mm x 4.6 mm, 5 µm) column (Grace Vydac, Hesperia CA, USA) using a Varian HPLC equipped

with an auto sampler. The flow rate was set at 0.6 mL/min. The quantitative estimation of HA present was determined using a calibration curve of standard HA using a UV detector set at 228 nm.

The percent of ACE inhibition was calculated as follows:

$$\text{ACE inhibition (\%)} = \frac{\text{C (control)} - \text{C (sample)}}{\text{C (control)}} \times 100$$

Where: C (control) was the concentration of HA without the tested samples (active peptides), which contained 300 µL of HHL, 300 µL of ACE enzyme and 300 µL of Tris buffer, and C (sample) was the concentration of HA with the tested samples, which contained 300 µL of HHL, 300 µL of ACE enzyme and 300 µL of peptide solution.

Peptide profile of water-soluble extract

The RP-HPLC assay was developed for the profile of water-soluble peptides extracted from yogurt as control, and yogurt containing *L. helveticus* strain with or without Flavourzyme®, respectively (Nielsen *et al.*, 2009). The freeze-dried peptide powder (20 mg) was dissolved in 1 mL of 0.1% TFA in distilled water. All the supernatants thus obtained were filtered through a 0.45 µm membrane filter and stored at –20 °C until assayed. Water soluble peptides were profiled by a RP- HPLC (SHIMADZU Corporation, Japan) using C-18 monomeric column (5 µm, 300 Å, 250 mm x 4.6 mm; Grace Vydac, Hesperia CA, USA). The injection volume was 10 µL. Solvent A was a mixture of water with 0.1% TFA (v/v) and solvent B contained acetonitrile with 0.1% TFA (v/v). Active peptides were eluted with a linear gradient of solvents B in A at concentrations from 0 to 100% over 30 min at a flow rate of 0.75 mL/min. The elution profile was monitored at 215 nm by UV-Vis detector at room temperature (~22 °C).

Peptide fractions

Bioactive peptide fractions from yogurt with *L. helveticus* and Flavourzyme® were collected using a column (Prep Nova-Pack HR C18, 60 Å, 250 mm x 10 mm, 10 µm, Phenomenex, Pty Ltd, Australia) by preparative RP-HPLC. Solvent A was a mixture of water and TFA (1000:1, v/v), and solvent B contained acetonitrile and TFA (1000:1, v/v). The injection volume was 1 mL with 20 mg/mL of yogurt peptides. The peptides were eluted with a linear gradient of solvent B in A ranged from 0% to 40% over 90 min, at a flow rate 4 mL/min. Detection was carried out at 215 nm by UV-Vis detector. According to the retention time of peaks, 7 fractions were collected at 10 min for fraction F2, F3, F4 and F7, 12 min for F1, and 14 min for F5 and F6, respectively. All fractions were frozen dried under vacuum. IC_{50} of ACE activity was determined for each fraction.

Statistical analysis

All results are expressed as mean ± standard deviation for each measurement (n = 3), including yogurt fermentation, the pH, determination of ACE-I activity and calculation of the IC_{50} value. The yogurt fermentation was carried out in two separate procedures. The first was focused on the four fermentation times (4, 8, 12 and 16 h) with 1% inoculum sizes of *L. helveticus*. The second was performed with different inoculum sizes of *L. helveticus* (1%, 2%, 3% and 4% v/w), after the optimal fermentation time was determined. One-way ANOVA was performed using software SPSS version 22 (IBM Chicago, IL, USA) to analyse the significant differences in the treatments, which were fermentation time, inoculum sizes and presence or absence of Flavourzyme®. Fisher's (least significant difference; LSD) test was used to differentiate significant differences among the treatments. $P < 0.05$ was considered as significant.

Results and Discussion

Effects of *L. helveticus* strains and Flavourzyme® on the pH of yogurt

The pH of yogurt containing *L. helveticus* strain with or without Flavourzyme® with different fermentation time and inoculation volume is shown in Figure 1. The pH of yogurt fermented by *S. thermophiles* and *L. bulgaricus* was 4.71 ± 0.04. The pH was decreased to 4.45 ± 0.04 when *L. helveticus* strains were incorporated alone into the yogurt at 37 °C for 16 h ($P < 0.05$; Figure 1A). The pH was further decreased to 4.06 ± 0.03 when Flavourzyme® and *L. helveticus* strains were added in the yogurt for 16 h of fermentation ($P < 0.01$; Figure 1A). The decrease of the pH could be due to the production of lactic acid by lactic acid bacteria strains (Donkor *et al.*, 2007a). The pH of yogurt in the presence of Flavourzyme® was significantly lower than that without Flavourzyme® for the same fermentation time ($P < 0.01$; Figure 1A). The presence of Flavourzyme® could result in higher proteolysis and the release of more peptides, which was further supported by the experimental data on the fermentation time and peptides of ACE-I due to further hydrolysis of milk protein by Flavourzyme® to small size molecular weight of peptides (Fatah *et al.*, 2016a; Fatah *et al.*, 2016b). Previous research has demonstrated the same trends using these *L. helveticus* strains in the 12 h fermentation of skim milk and the pH of the fermented product was 3.4 when Flavourzyme® was added compared with 5.0 without addition of Flavourzyme® (Fatah *et al.*, 2016b). In the same study, it was also reported that the growth of all strains of *L. helveticus*, when mixed with Flavourzyme®, was increased significantly at 8 h and declined after 8 h of fermentation at pH 3.4. This was possibly due to the low pH and heat treatment reducing available nutrients for growth (Fatah *et al.*, 2016b; Dissanayake *et al.*, 2013). The reason for the higher pH (4.15) in the present study may be attributed to the type of media. In this study, yogurt was used as a media while RSM was used in the study by Ahtesh *et al* (2016b).

In the fermentation of yogurt, the pH decreases steadily in correspondence with the milk acidification (two homofermentative bacteria transform fermented (milk) sugar into lactic acid) that underlies the fermentation process (De Brabandere & De Baerdemaeker, 1999). Many

factors including starter culture, heat treatment, fermentation time, inoculum size and incubation temperature, affect the pH in yogurt fermentation. In this study, the decrease in pH was observed in yogurt combined with different inoculum size of *L. helveticus* ($P < 0.01$; Figure 1B). After fermentation, the pH of yogurt decreased to 3.91 ± 0.04 without Flavourzyme®, and 4.00 ± 0.03 with Flavourzyme® ($P < 0.05$; Figure 1B). The pH was lower with 1% inoculum of *L. helveticus* with Flavourzyme® than those without Flavourzyme® in the same inoculum size of *L. helveticus* ($P < 0.01$; Figure 1B). Then there were no significant changes with the increase of inoculum size of *L. helveticus* in the presence of Flavourzyme®. However, the pH declined with the increase of inoculum size of *L. helveticus* in absence of Flavourzyme®.

Effect of fermentation time on ACE-I activity with or without Flavourzyme®

A time course of peptide production showed that ACE-I activity of peptides was significant difference between the groups with and without Flavourzyme® at the same fermentation time ($P < 0.01$; Figure 2A). The results demonstrate that ACE-I activity increased gradually with the fermentation time, when Flavourzyme® was supplemented during yogurt fermentation. Specifically, ACE-I activity in the absence of Flavourzyme® group increased significantly from 13.0 ± 1.8 % to 50.8 ± 3.3% (20 mg/mL) during 8 h fermentation, then mildly decreased at 12 h fermentation (46.0 ± 0.9%, $P = 0.12$; Figure 2A), but increased again with by 16 h fermentation back to similar levels seen at 8 h fermentation (51.4 ± 0.2%, $P = 0.81$; Figure 2A). These may be associated with the hydrolysis of ACE-I peptides, and the hydrolysed peptides can then be transported across the cell membrane of bacteria by several transporters (Law & Haandrikman, 1997). Similar results were reported by Pan & Gao (2010) who showed

that the optimal fermentation time to produce ACE-I activity from fermented sour milk was 8 h (Pan & Guo, 2010).

A similar trend in ACE-I activity dependence to fermentation time was noted in the presence of Flavourzyme®, compared with the absence of Flavourzyme® (Figure 2A). The results showed that in the presence of Flavourzyme®, ACE-I activity increased gradually and reached the highest level in 12 h fermentation (86.5 ± 0.5% at concentration of 2.5 mg/mL) ($P < 0.05$; Figure 2A). It then decreased after an additional 4 h fermentation ($P < 0.05$; Figure 2A). These results suggest that 12 h incubation was the optimal fermentation time to produce anti-hypertensive peptides from yogurt, in combination with Flavourzyme®. These studies indicate that Flavourzyme® allows reaching high concentrations in ACE-I peptides in a short fermentation time, contrarily to formulation without Flavourzyme®. This was also confirmed by the change of pH levels in the presence or absence of Flavourzyme® (4.06 ± 0.03 and 4.42 ± 0.02, respectively, Figure 1). In agreement with this result, a previous study showed that ACE-I reached the highest level (approximate 70%) at 12 h fermentation of RSM by *L. helveticus* strain 881315 without Flavourzyme® addition; however, more than 70% ACE-I activity was obtained after only 4 h fermentation by the same strain with the supplementation of Flavourzyme® (Fatah *et al.*, 2016b).

Effect of inoculum size on ACE-I activity with or without Flavourzyme®

Figure 2B shows the effects of inoculum size of *L.* helveticus strains on ACE-I activity with or without Flavourzyme® in yogurt. Generally, the results showed a significant difference between the groups with and without Flavourzyme® in the same level of inoculum size ($P < 0.01$; Figure 2B). ACE-I activity increased gradually with increased inoculum size without Flavourzyme®. *L. helveticus* strains (4%) in the yogurt exhibited the highest ACE-I activity, with 59.2 ± 0.9% at 20 mg/mL of the hydrolysate, for optimal fermentation time of 12 h ($P <$

0.05; Figure 2B). It has been shown that 4% of inoculum size produced ACE-I activity in sour milk fermented by *L. helveticus* LB10, possibly due to the high cell-envelope proteinase activity (Pan & Guo, 2010), which may have also occurred in the present study.

It was found that, with the same inoculum size, ACE-I can be improved significantly when combined with Flavourzyme® ($P < 0.01$; Figure 2B). The highest ACE-I (86.5% at concentration of 2.5 mg/mL) was obtained with 1% of *L. helveticus* plus Flavourzyme® following a 12 h fermentation ($P < 0.01$; Figure 2B). Then, ACE-I activity showed slight decrease with increasing inoculum size. ACE-I activity of peptides reduced to 82% with 4% of *L. helveticus* combined with Flavourzyme®, however this is still higher than those in the absence of Flavourzyme® (59.17 ± 0.88%).

ACE-inhibition activity

There were significant differences in ACE-I activity between yogurt with starter culture and yogurt with addition of *L. helveticus* and/or Flavourzyme®. Peptides from yogurt showed the lowest activity of ACE-I, which was only 33.1 ± 2.7% at the concentration of 20 mg/mL ($P < 0.01$; Figure 3). In contrast, ACE-I activity was increased to 59.2 ± 0.9%, when *L. helveticus* strain was added to the yogurt at 37 °C for 12 h ($P < 0.01$; Figure 3). Furthermore, a 100% inhibition was observed when both *L. helveticus* strain and Flavourzyme® were added to the yogurt ® (Figure 3).

The IC_{50} value is an important indicator to assess the ACE-I potential. It can be used to classify individual bioactive peptides from different fermentation products based on their ACE-I capacity *in vitro*. In this study, the IC_{50} value of yogurt only fermented by starter cultures (30.45 ± 2.32 mg/mL) showed the lowest effect on ACE-I ($P < 0.01$; Figure 3). The hydrolysate extracted yogurt incorporated with *L. helveticus* strains demonstrated lower IC_{50} (16.91 ± 0.25

mg/mL), compared with yogurt without these strains ($P < 0.01$; Figure 3), which supported that *L. helveticus* has a potential to hydrolyse protein from yogurt and produce anti-hypertensive peptides. In yogurt supplemented with Flavourzyme®, significantly more ACE-I peptides were released with an IC_{50} of 1.47 ± 0.04 mg/mL, which was 20-fold higher than yogurt without *L. helveticus* strains, and 12-fold higher than yogurt without Flavourzyme® ($P < 0.01$; Figure 3). In general, the effect of peptides on ACE-I depends on several factors, such as the type of bacteria strains, media used, fermentation conditions and enzymes used. Tsai *et al*. (2008) reported that, in milk fermented by lactic bacteria with Flavourzyme®, more active peptides were produced to exhibit higher ACE-I activity, with an IC_{50} value of 0.226 mg/mL in Flavourzyme-facilitated fermentation in contrast to an IC_{50} value of 0.515 mg/mL in the fermentation without Flavourzyme®. The different media and fermentation condition could possibly contribute to the higher IC_{50} values found in study of Tsai et al. (2008), which used the mixture of 4.5% (w/v) skimmed milk powder, 5.5% (w/v) whole milk powder and 7% (w/v) sucrose as a medium that was fermented by 0.1% (w/v) of lactic acid bacteria powder. By contrast, 12% skimmed milk powder was used as a medium and it was fermented with 1% of activated lactic acid bacteria in the current study.

Peptide profile of water-soluble extracts

The degree of hydrolysis at the higher ACE-I activity was determined by analysis of peptides from different yogurts using RP-HPLC. The peptide profiles (20 mg/mL of the concentration) showed similar characteristics between yogurt with starter culture and yogurt incorporated with *L. helveticus* with or without Flavourzyme® (Figure 4). In particular, four major peaks appeared in the first eight minutes when the substantial hydrolysis took place. It was then followed by several minor peaks. Although the number of the proteolytic peptides in yogurt fermented by *S. thermophiles* and *L. bulgaricus* was almost the same as those in yogurt added with *L.*

helveticus based on the peptide profiles (Figure 4A, 4B), ACE-I activity in yogurt without *L. helveticus* was lower than that in yogurt with *L. helveticus* (Figure 3). Many anti-hypertensive milk peptides have been isolated and identified (Hannu & Anne, 2006). However, only specific peptides, especially those containing proline in the amino acid sequence at the C-terminal, such as VPP, IPP and LPP, have high ACE-I activity from fermented dairy products (Butikofer *et al.*, 2008). In fermented milk, high amounts of VPP and IPP were obtained in most cases after fermentation with *L. helveticus* due to their higher proteolytic activity and their special capability to degrade the milk protein into these tripeptides, and also accumulate these tripeptides in the dairy products (Hu *et al.*, 2011).

Yogurt supplemented with *L. helveticus* and Flavourzyme® showed different peptide peaks (Figure 4C). More peaks were detected with the retention times between 3 and 18 min due to the increase in milk protein hydrolyses by Flavourzyme®, which led to the release of peptides with small molecular weight. These results were consistent with the recent study by Ahtesh *et al.* (2016b) using the same strains in the fermentation of 12% of RSM (Fatah *et al,.* 2016b. Tsai *et al.* (2008) indicated that Flavourzyme® has a potency to increase the content of soluble protein (10-fold) and peptides (5-fold) in milk whey fermented by lactic acid bacteria (Tsai *et al.*, 2008).

ACE-I activity of fractions

In order to pinpoint the peptides bearing a potential ACE-I activity, peptides from yogurt supplemented with Flavourzyme® were separated using preparative RP-HPLC, and seven fractions (F1- F7) were collected according to the elution time. Their ACE-I activity was then determined (Figure 5). The results implied that fractions F3- F6 were primarily responsible for ACE-I activities of peptides. Among them, fraction F3 exhibited the highest activity, corresponding to an IC_{50} value of 35.75 ± 5.48 μg/mL, which was almost 42-fold higher than

that found in the total hydrolysate (1470 ± 40 μg/mL) ($P < 0.01$; Figure 5). Fraction F7 showed the lowest inhibitory effect on ACE, with an IC_{50} value of 2211.22 ± 213.41 μg/mL. These findings implied that some small peptides separated and collected from yogurt showed functional accumulation or synergism on ACE-I activities.

Six fractions were separated and collected based on the molecular weight of peptides extracted from fermented skim milk with *L. bulgaricus* LB340 in the study by Qian *et al* (2011), which revealed that fraction F2 showed the highest ACE-I activity (66.4%) at concentration of 100 mg/mL, with an IC_{50} value of 67.71 ± 7.62 mg/mL, which was lower compared with those in this study (Qian *et al.*, 2011). The differences observed in ACE-I effect of peptides may be attributed to the variation in proteolytic activity of bacteria stains (*L. bulgaricus* LB340 versus *L. helveticus* 881315) and cultures (skim milk versus yogurt), and therefore the variety of peptides present in the hydrolysates. The synthetic bioactive peptides, based on purification, characterization and identification of their amino acid sequence could be potentially used to develop functional foods that are beneficial to human health. Further work is required to identify the amino acid sequence of the ACE-I peptides extracted from yogurt.

Conclusion

The bioactive peptides released from the aqueous extracts of yogurt have ACE-I activity. The optimal fermentation conditions for producing ACE-I peptides were 1% inoculum size of *L. helveticus* strain 881315 for 12 h combined with Flavourzyme®. The IC_{50} value was 1.47 ± 0.04 mg/mL, which was higher than yogurt or yogurt supplemented with *L. helveticus* strain without Flavourzyme®. The observation of peptide profiles also confirmed different quantities

of peptides in different yogurts, and yogurt with or without Flavourzyme®. Among seven fractions of peptides, fraction F3 displayed the highest ACE-I effect. Therefore, yogurts provide a good source for the generation of bioactive peptides with ACE-I activity in fermented dairy products. Although further *in vivo* study is required to confirm the ACE-I activity of yogurt peptides and their potential anti-hypertensive properties, the outcomes of this study hold a promising potential as a complementary therapy for hypertension. It is necessary to identify the specific peptides in the yogurt for future development of potential functional foods.

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