



Inhibition Activities of Angiotensin Converting Enzyme and Amino Acid Kefir Whey Profile of Skim Milk Fermented by Kefir Grains



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Abstract

Bioactive peptides fermented milk is very potential as functional food products for health. The aim of this study is to analyze the inhibition activity of Angiotensin Converting Enzyme and kefir whey amino acid profile and IC₅₀. The design being applied is a comprehensive random design with five treatments (fermentation time 0, 3, 6, 9 and 12 days) and 3 replications. ACE inhibition indicators include ACE inhibition with IC₅₀, protein, peptide concentration, total proteolytic, and amino acid profiles. The results show that ACE inhibition activity ranges from 35.94 - 66.67% with peptide levels of 872.80 - 1084.74 mg/mL and IC₅₀ of 65.48 µg/mL, and contained hydrophobic amino acids which functioned for ACE inhibition. The conclusion of this study that the highest ACE inhibition is obtained at 0 day of fermentation with inhibition ability (IC₅₀) of 65.48 µg/mL and functioning as nutraceuticals food.

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1. Introduction

The finding of Angiotensin Converting Enzyme (ACE) inhibition of fermented milk peptides orally is very important in inhibiting hypertension. Inhibition of hypertension, usually using drugs that have side effects, coughing, taste disorders, and skin rashes (Qian *et al.*, 2007). Therefore ACE inhibition can be considered as a useful approach in dealing with blood pressure problems.

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Angiotensin Converting Enzyme (ACE) is an enzyme that catalyzes the conversion of angiotensin I decapeptide to angiotensin II octapeptide in the Renin-Angiotensin System (RAS) that regulates blood pressure (Riordan, 2003). ACE inhibition is an inhibition of ACE enzymes by bioactive compounds which can reduce blood pressure in humans. This bioactive compound is found in milk. But it has not been able to function optimally as a functional food. Therefore, the potential of functional food is very necessary to improve this functional property, one of which is caused by the help of microorganisms.

Kefir whey is a transparent yellow fermented milk drink, made by fermented milk with kefir seeds, then incubation at $25-27\text{ }^{\circ}\text{C} \pm 24$ hours (Ot'es and Cagindi, 2003) so that a transparent liquid and separate curd are formed separately. Fanworth (2005) reported that in kefir grains containing lactic acid bacteria (LAB) and *khamir* which work in a symbiotic mutualism. Liu *et al.*, (2010) reported that this bacteria is very important in the process of milk fermentation to increase the production of bioactive peptides which have the potential to lower blood pressure through inhibition of Angiotensin Converting Enzyme (ACE inhibitors). The same researcher also reported that the type of bacteria used as a starter will greatly affect the type of peptide produced.

Kefir whey is thought to have the same function as kefir because kefir whey is a liquid, so the components in kefir are dissolved in whey. This condition is in accordance with the explanation of LeBlanc *et al.* (2004) that bioactive peptides dissolve in water so that they are thought to be components of dissolved protein in the transparent kefir liquid. This is the reason that kefir whey still has high economic value. However, the potential of kefir whey as an ACE inhibitor during storage, little information is obtained, because the ACE inhibitory ability correlates with the number of LAB and metabolites produced. The purpose of this study is to analyze the ability of kefir whey, the highest ACE inhibitor with IC_{50} during fermentation. The success of this research is a benchmark for increasing food potential as nutraceuticals food.

2. Materials and Methods

Material

The main materials in this study are 15 liters of fresh cow skim milk and kefir seeds as a starter. The chemicals used in this study, the media Mann Rogosa Shape Agar (MRSA), skim Agar, Pepton Water Buffer (BPW), alcohol, *aquades*, HCL, and biuret solution. The main equipment in this study are ACE KIT-WST, micro Kjehldal, *centrifius*, spectrophotometer, microplate reader, incubator, petri dish, refrigerator, autoclave, oven, and pipette.

Metode

This study applies a Completely Randomized Design (CRD) with five storage treatments (0, 3, 6, 9 and 12 days) with 3 replications (Stell and Torrie, 1993).

Research Procedure

Preparation of the main tools, such as a petri dish, test tube, pipette, Erlenmeyer, measuring cup is by autoclave sterilizing at $121\text{ }^{\circ}\text{C} \pm 30$ minutes.

Preparation of the main chemicals, such as MRSA, is by means of weighing 52 grams, then adding 1000 mL of distilled water and heating it so that it is homogeneous. Furthermore, it was sterilized by autoclaving at a temperature of $121\text{ }^{\circ}\text{C} \pm 15$ minutes (Swanson *et al.*, 1993).

The procedure of making Kefir whey follows the Ot'es and Cagindi (2003) method by means of milk being pasteurized to $85\text{ }^{\circ}\text{C} \pm 30$ minutes then cooled and fermented with kefir grains at a temperature of $25 - 27\text{ }^{\circ}\text{C} \pm 24$ hours. When the kefir is formed, there are two layers: the curd and liquid. Then they are separated so that a transparent yellow liquid is obtained which is called kefir whey. Kefir whey was stored in a refrigerator at $4\text{ }^{\circ}\text{C}$ with storage times of 0, 3, 6, 9 and 12 days.

The variables in this study, ACE kefir whey activity was analyzed by ACE-WST KIT (Nakamura *et al.*, 1995), protein, following the AOAC (2005) method and peptide content with the Biuret method (Brunelli and Waino, 1949), total proteolytic LAB according to the method of Swanson *et al.*, (1993)

3. Results and Discussions

The results showed that the ACE inhibitory activity and the quality of kefir whey fermented for 0, 3, 6, 9 and 12 days can be seen in Table 1. Amino acid profile of kefir whey with the highest ACE inhibition on 0 days fermentation in Table 2

Table 1
Inhibition Activity of ACE and the quality of kefir whey skim milk which is fermented by kefir seed during its storage

Variable	Treatment (day)				
	0	3	6	9	12
Inhibition ACE (%)	66.67 ^a	54.84 ^b	35.49 ^c	39.79 ^c	38.79 ^c
Protein (%)	1.58 ^b	1.52 ^b	1.37 ^a	1.39 ^a	1.37 ^a
Total BAL	4.4x10 ³ ^a	3.1x10 ³ ^a	5.5x10 ³ ^a	7.2x10 ³ ^a	2.0x10 ¹ ^b
Proteolytic (cfu/mL)					
Peptide (μg/mL)	1084.74 ^a	995.11 ^b	872.88 ^c	1066.95 ^a	869.18 ^c

Description: different superscripts on the same line show a significant difference (P<0.05)

3.1 ACE Inhibition

ACE inhibition is an inhibition of the ACE enzyme that causes hypertension by blocking the change of angiotensin I to angiotensin II, which is a strong vasoconstrictor that causes hypertension (Liu *et al.*, 2010, Korhonen and Pihlanto 2006).

Fermentation duration of 0, 3 and 6 days (P <0.05) decreased ACE kefir whey inhibitory activity. However, 6 - 12 days of fermentation were no a significant difference (P> 0.05). The highest ACE inhibition is obtained on 0 fermentation days with IC50 is 65.49 μg/mL. The high ACE inhibition is due to the activity of *Lactobacillus* and *Leuconostoc* which a proteolytic LAB, hydrolyzing proteins into oligopeptides, peptides during fermentation (Fanworth, 2005). This occurs at the beginning of fermentation because LAB is in a position on the surface of kefir seeds, while *khamir* is on the inside (Graciela *et al.*, 2010) and peptides formed at 0 days are thought to be metabolites that have a function as ACE inhibitors. These results are similar to those reported by Febrisiantosa *et al.*, (2013) that ACE inhibition correlates with the number of peptides. However, ACE inhibition on fermentation duration of 6 - 12 days was lower (P> 0.05), although it was followed by an increase in the number of the proteolytic labs at 9 days. When viewed from the peptide concentration, there was an increase in 9 days, i.e. 1066.95 mg / mL but ACE inhibition was low, then at 12 days, there was a decrease (Table 1). Low ACE inhibition, presumably produced metabolites, only partly as a peptide that functions as a hypertensive inhibitor, because Korhonen and Pihlanto (2006) reported that peptides have different functions, namely as antihypertensive, opioid, antimicrobial and immune systems. Miller *et al.*, (2007) reported that whey still has bioactive components, especially from whey protein. Ebringer *et al.* (2008), a source of peptides from whey, namely β-lactoglobulin and α-lactalbumin have functioned as a source of antihypertensive peptides. ACE inhibition in this study is different from that produced by Febrisiantosa *et al.*, (2013) that is equal to 73.07% with raw ingredients of fermented whey cheese with kefir grains

The mechanism of ACE inhibition by peptide whey kefir as an inhibitor, due to the peptide produced by LAB, will inhibit the ACE enzyme as a precursor that converts Angiotensin I to II Angiotension, which is a strong constructor that triggers aldosterone synthesis and release, which causes increased blood pressure, narrowing of blood vessels (Liu *et al*, 2010). ACE enzyme inhibition by inhibitory peptides is initiated by the binding of ACE-inhibiting peptides to the three active sides of the ACE enzyme. Wijesekara and Kim (2010) this binding peptide changes the composition of the protein so that there is no interaction between angiotensin I and the active side of the ACE enzyme. It was also reported that by binding to the peptides on the three active sides of the ACE enzyme,

namely C-terminal, plays an important role in ACE inhibitors, which binds to hydrophobic amino acids. Nakamura *et al.* (1995), Korhonen and Pihlanto (2006) report that the functional properties of ACE inhibitors are largely determined by the amino acid composition of bioactive peptides derived from milk protein namely Val-Pro-Pro or Ileu-Pro-Pro. However, the amino acid sequence in the C-terminal is only a control of the inhibiting peptide, because this sequence is still learned. Besides that, Madureira *et al.* (2010) stated the type of BAL used as a starter will determine the type of peptide.

3.2 Protein level

The highest protein levels were found in the fermentation period of 0 days which was 1.58% and the lowest was at 12 days, which was 1.37%. The low protein content in all treatments was caused, the sample used in this study was kefir whey, in the form of *transparat liquid*, the result of separation with curd kefir (Figure 2.) so that part of the biomass was still bound to the kefir grain matrix or polysaccharide (Graciela *et al.*, 2010). However, LeBlanc *et al.*, (2004) reported that kefir granules

However, LeBlanc *et al.*, (2004) reported were dissolved in water, so that in kefir whey still contain kefir protein content in curd kefir consumption was consumption during storage up to 12 days was 4.74- 4.08%. Compared to SNI, it was still in standard. BSN-SNI (2009) that the minimum limit of kefir protein is 1%.



Figure 1. Kefir Whey

In the process of fermentation of milk by kefir grains, Fanworth (2005) reported that the activity of LAB (*Lactobacillus* and *Leuconostoc*), begins with the degradation of lactose into lactic acid. Lactic acid is formed, coagulating proteins so that curd occurs. Then this bacterium with its proteolytic enzyme (intracellular peptidase enzyme) hydrolyzes proteins into oligopeptides, tri-di peptides, and amino acids. Pihlanto *et al.*, (2000) reported that the results of this hydrolysis were first used as nutrients by the LAB and the remaining potential as a functional food for health. This situation stimulates *Streptococcus* growth. The presence of *Streptococcus*, causes the atmosphere to become acidic and can inhibit its growth. However, it actually stimulates *Lactobacillus* to regrow so that the total proteolytic LAB is currently low.

The high levels of this protein are also caused by the total proteolytic BAL that forms a clear zone in its growth in *petri* dishes (Figure 2). This indicates a higher level of protein biodegradation (Table 1), thus causing an increase in biomass from metabolic LAB results. Acidity, a decrease in protein levels was followed by a total proteolytic BAL, although it was not statistically significant ($P < 0.05$), because Farnworth (2005), states that bioactive component in kefir is kefir seed microbes, its metabolites (polysaccharides, peptides) which have potential health.



Figure 2. Zona of LAB proteolitik on skim agar

3.3 Total BAL Proteolytic

The results of variance showed that the amount of proteolytic LAB with fermentation duration of 0 - 9 days was not significant ($P > 0.05$), but in the 12th fermentation was significantly ($P < 0.05$) decreased. This indicates that the fermentation duration of 0 - 9 days has not shown an increase in the number of proteolytic bacteria, assumed to enter the stationary phase. [Mainvifle \(2006\)](#) reported that in the stationary phase there was a decrease in the number of *Streptococcus*, due to the acid produced (the higher the fermentation time, 2.52 - 3.77), but *Lactobacillus* growth occurred. While Lund and Eklund (2000) reported that LAB grew optimally at pH 4.82 - 4.39. However, there was a decrease in the amount of proteolytic BAL in the 12-day fermentation period, due to the growth of molds.

3.4 Kadar Peptide

The level of kefir whey peptide is the result of biodegradation of BAL, an especially proteolytic group so that all processes during fermentation are affected by its activity and nutrient content in the product. Peptides obtained in this study were significant ($P < 0.05$) influenced by fermentation time (Table 1). The high levels of peptide were found in the fermentation period 0 day. This is due to the process of fermentation of milk by proteolytic lactic acid bacteria causing an acidic atmosphere. Acidic conditions besides causing coagulation of proteins, further hydrolysis of proteins occurs by proteolytic enzymes to oligopeptide and peptide.

In the fermentation period of 3 - 9 days there was a decrease in peptide levels, due to a decrease in proteolytic lactic acid bacteria (*Streptococcus*), while at day 9 there was an increase in peptide levels ($P < 0.05$). It is caused by the increase of *Lactobacillus* ([Farnworth, 2005](#)). Meanwhile on the 12 days, the decrease of peptide level occurs, because it is dominated by the growth of molds. [Nurhayati et al., \(2013\)](#) stated that the acidity causes a positive ion exchange with negative ions in the acid, so that the breakdown of the protein structure results in the breakdown of the peptide bond chain into peptides with shorter chain amino acids.

3.5 Amino Kefir Whey Acid Profile

The amino acid profile shown is the amino acid from whey kefir with the highest ACE inhibition on 0 days of fermentation and also displayed 3 days of fermentation time and milk can be seen in Table 2.

Table 2
Profile of Amino Acid Milk, Kefir Whey with Fermentation Length
0 and 3 days

Amino Acid	Milk	Whey 0 day	Whey 3 days
Serine	1607.22	-	<830.17
Glutamic acid	6559.33	834.75	702.92
Phenylalanine	1486.66	191.57	173.60
Isoleucine	1537.70	223.80	208.65
Valine	1723.38	222.74	203.83
Alanine	1026.18	373.33	369.55
Arginine	907.54	<153.15	<153.15
Glycine	618.36	<402.00	<402.00
Lysine	2909.85	393.11	359.22
Aspartate acid	2220.37	<399.37	<399.37
Leucine	2894.32	381.18	339.39
Tyrosin	1242.26	<222.88	<222.88
Proline	2616.84	352.30	274.68
Threonine	1385.21	294.74	250.63
Histidine	736.58	<136.06	<136.06
Metionine	712.70	87.17	61.02
Sistine	161.24	-	-
Tryptopan	258.56	<56.14	<56.14

The results of analysis of the content of amino acid kefir whey with fermentation time of 0, 3 days and milk, with HPLC, consists of hydrophobic amino acids (Isoleucine, Alanine, Leucine, Proline, Valine, Glycine) and hydrophilic (Serine, Glutamic Acid, Aspartate Acid, Threonine, Methionine,). However, it differs in the ability to inhibit ACE. This difference is thought to be caused by the short and length of the peptide chain. [Wikandari and Yuanita \(2014\)](#) reported that peptides have the potential as antihypertensive agents are short-chain with 2 - 12 amino acid residues and are strongly influenced by fermentation duration. The content of amino acids is very closely related to ACE inhibition. [Qian et al., \(2007\)](#), reported that ACE inhibition occurs, if there is a hydrophobic amino acid peptide inhibiting bond with three active ACE enzymes in C-terminal. Meanwhile, the hydrophilic amino acid, which has ACE inhibition, is very weak because it is not compatible with the active side of the ACE enzyme. Abubakar (2014) reported the results of his research that ACE inhibition occurs if there are several proline amino acid residues in the peptide chain,

4. Conclusion

The conclusion of this study that:

- Inhibition activity of Angiotensin Converting Enzyme kefir whey is obtained on 0 fermentation days with IC_{50} is 65.48 μ g/mL
- Profile of amino acids that function to inhibit ACE including hydrophobic
- Amino acids with peptide levels 1084.74 mg/mL.
- Kefir whey functions as nutraceuticals food.

Conflict of interest statement and funding sources

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Statement of authorship

The authors have a responsibility for the conception and design of the study. The authors have approved the final article.

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