

# Identification and Characterization of Bioactive Peptides of Fermented Goat Milk as a Sources of Antioxidant as a Therapeutic Natural Product

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**Abstract.** The increasing of functional food is rising in line with public awareness for healthy food consumption. Provision of functional food source is developed through enhanced bioactive that has a regulatory function for body. Bioactive peptides in milk is known have variety of beneficial function of the body such as immunomodulator, immunostimulatory, anti-hypertension, anti-hyper cholesterol, as well as a variety of other beneficial function. The aim of this study is to obtain fermentation methods to product functional dairy product contain bioactive peptides and beneficial of fermented goat milk. The result of this study showed that goat milk fermented using 3 % commercial starter able to produce the best yoghurt than using local yoghurt starter. Analysis of protein content showed that the fermentation processing increased the amount of protein in goat milk sample. Using SDS- PAGE showed that the breakdown of protein into fraction of fermented goat milk greater than unfermented goat milk. The result of fractional protein was analyzed by LC MS/MS and showed that there were three kind bioactive sequences of bioactive peptides. Each of which consist of 16 amino acids that safely protected from gastrointestinal animal model that fed by dietary treatment of hypercholesterolemia.

**Keywords:** *Food functional; bioactive peptides; fermented goat milk; anti-hyper cholesterol*

## 1. Introduction

Nowadays, food protein is one of food component which interest for researcher and industries because protein is a source of amino acids and known has biology activities that influence functional and body system, both on whole protein or hydrolysis product. Hydrolysis product of food protein that has biologist function on regulation body function and healthy is known as bioactive peptides [1]. Bioactive peptides bonded on food protein precursor and will be released through hydrolysis process by proteolysis enzyme in gastrointestinal tract, or maybe by in-vitro using proteolysis enzyme that isolated from plant or microorganism, or by fermentation processing [2]. Several biological functions of bioactive peptides that have known are as an antioxidant, antimicrobial, anti-hypertension, anti-cholesterol, cyto-modulator and immunomodulator [3].

Bioactive peptides have been isolated and identified from several of food sources, such as instant milk, egg, cattle meat, chicken, fermented milk product, fish and soybean [4]. Milk is the main sources of bioactive peptides and has several biological function compared with throw foodstuff [2]. However, bioactive peptides are inactive form in protein binding condition. Its require hydrolysis to release



bioactive peptides, so milk can be used as food sources with high content of bioactive peptide, thus milk can be used as food sources with high content of bioactive [4]. Hence, studying the effective methods to produce high bioactive content milk is important.

Fermentation using lactic acid bacteria as a famous method that is used to produce fermented milk is believed to give healthy benefits and cure several diseases ([2];[5] ).Several results of research showed that bioactive peptides from fermented milk have many benefits for body regulation system and enhance body healthy [6]. Therefore, the researcher focus on developing and optimization fermentation method to product bioactive peptides in many states.

Goat milk as one of natural functional food has chemical characteristic that different from cattle milk, but same as human milk. Lactose and protein contained in goat milk are almost equal to cattle milk, but there are some differences on structure and immunological protein. Beside, goat milk contains several middle chain fatty acid and lipid globular that are relatively small compared to cattle milk. Therefore, it is important to do research use goat milk as source of bioactive peptides.

Nowadays, goat milk industry in Indonesia is very fast, It was important to develop processing technology to receive milk product processing that has economical value than fresh milk. Foods products with basic source of goat milk have been develop as a food medicinal product or biomedicine that is useful for health. Thus it is very important and urgent to explore bioactive peptides from goat milk through fermentation processing method.

The purpose of this study is to know the level concentration of lactic acid bacteria that is needed and how long fermentation processing needed to produce bioactive of goat milk. We hope with to find the optimal of fermentation through this study, it will be used to develop milk fermentation product with have better value if be compared with fresh milk.

## **2. Method**

### *2.1 Starter Preparation*

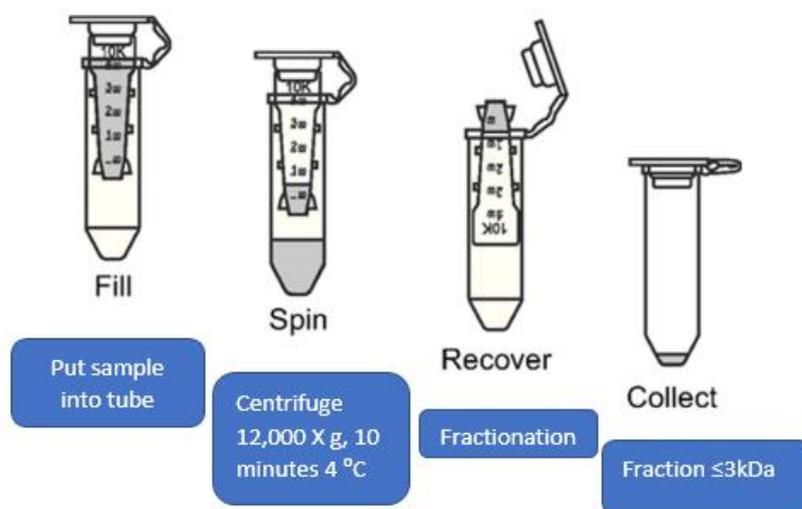
Starter was prepared according to a previously protocol by [www.yogourmet.com](http://www.yogourmet.com): 100 mL of fresh goat milk was pasteurized at 72°C for 5 minute, then cooled to 40 °C – 45 °C. 0.5 gram frozen starter was solved in 5 mL of pasteurized milk (from 100 mL of Pasteurized milk). The starter was added into 100 mL pasteurized milk, then homogenized and incubated at 40°C – 45°C for 4 to 8 hours respectively until milk starter reached pH of 4.0 – 4.5.

### *2.2 Yogurt Preparation*

The yogurt preparation method was conduct according to previously protocol by Position et al. (2005). 500 mL of goat fresh milk was pasteurized at 72°C for 5 minutes then cooled at 40°C – 45°C. After that, it was incubated with 3% and 5% milk starter solution (according to previously study), later homogenized and incubated at 40°C – 45°C for 4 to 8 hours until pH value of yogurt reaches 4.5 to 5.0 respectively. The product of yogurt was keep at 4 °C to 5 °C for the next analysis purpose

### *2.3 Protein Yogurt $\leq 3kD$ preparation*

Hydrolyzed product of yogurt protein was prepared by solving 100 mg of dry sample frozen yogurt into 1 mL buffer ammonium bicarbonate solution ( 50 mM; pH 8.5), then it was sonicated (10 second, 40 Hz), and replicated 4 times. It was centrifuged of cold centrifugation (12,000 Xg; for 10 minutes at 4 °C). The supernatant (500  $\mu$ L) was poured through in ultrafiltration membrane 3 kDa molecular weight cut of (MWCO). It was centrifuged in cold centrifugation for 15 minutes (12.,000 x g; 4 °C), and 100  $\mu$ L buffer ammonium carbonate was added to avoid waste protein. The mixture was centrifuged with cold centrifugation for 7 mins (12,000 x g; 4 °C). The precipitation of solution considered as protein fraction  $\leq 3$  kDa, was collected for profile peptides identification immediately. The solution of protein fraction was frizzed at -20 °C for next laboratory analysis purpose.



**Figure 1.** Hydrolysis result of protein fractionation processing ( $<3$  kDa) used ultrafiltration method

#### 2.4 Protein Determination by Kjehdahl Method

0.5 g of sample was weighted in Kjehdahl flask. The sample was transfer into the Installed digest tube using 10 mL concentrated  $\text{H}_2\text{SO}_4$ . Switch the digestion unit off and let the stand with the digestion tubes cool down in  $400^\circ\text{C}$ . Destruction processing will be running as a programming. After raised color change from black to green color, the processing of digestion was stopped. The tube was waited for a moment (until cold room temperature reached), and continued to distillation and titration processing.

#### 2.5 Peptides Identified by LC-MS/MS

The peptides dry sample was solved in 5 % of acetonitrile and 0,1 % of formic acid in deionized water for LC-MS/MS analysis. LC- MS/MS analysis method was determined using LCQ DeKa XP System MAX Thermo with Electrospray Ionization (ESI) (Thermo Scientific Inc. USA) (C 18 Bio-basic column, with  $150 \times 2,1$  mm diameter,  $5 \mu\text{m}$  particle size. LC- MS condition was gas flow rate 50 absorb prayer potential 4 kV, capillary potential 20 V, capillary temperature  $300^\circ\text{C}$ . Interval of MS Scan was between  $m/z$  100 –  $m/z$  1600 with flow rate  $200 \mu\text{L}/\text{min}$ . Peptides separation was used with gradient linier gradually from 5% B solution to 70 B solution (Formic acid 0,1 % in acetonitrile) for 90 mins. Massa spectra data reading was used by Thermo– Xialibor/TM program (Thermo Scientific USA). Data of MS/MS were calculated by using Format File MGF with Mascot Distiller V2.3.2.0. (Matrix Saints, London United Kingdom), and continued with Blast by MGF file to Mascot search engine V 2.3 (Matrix Science, UK. Sequence peptides were identified by Based of peptides sequence in database.

### 3. Result and Discussion

#### 3.1 Optimum Concentration, pH, and Fermentation Time of Both Local and Commercial Yoghurt

**Table 1** Result of qualitative analyzed of yoghurt

	Treatment			
	A 3 %	A 5 %	B 3 %	B 5 %
pH	5.2	5,0	4.3	3.8
Viscosity	Less	Less	Viscous	Viscous
consistency				
Taste	Acid less	Acid less	Yoghurt	Acid
Aroma	Milk	Milk	Yoghurt	Acid

A : Local yoghurt starter

B : Commercial yoghurt starter

Good quality yoghurt starter was able to produce with good viscosity needed. Table 1 shows that there are different of yoghurt quality produced by two kind of yoghurt starter in both 3 % and 5 %

concentration. Based on Table 1, showed that 3 % concentration of commercial yoghurt starter with pH 5.2 over 8 hours fermentation has a good quality. This results agree well with the previous experimental results that the best starter concentration was 2-3% ([2];[7]). Therefore, yoghurt starter with 3 % concentration was used for protein analysis.

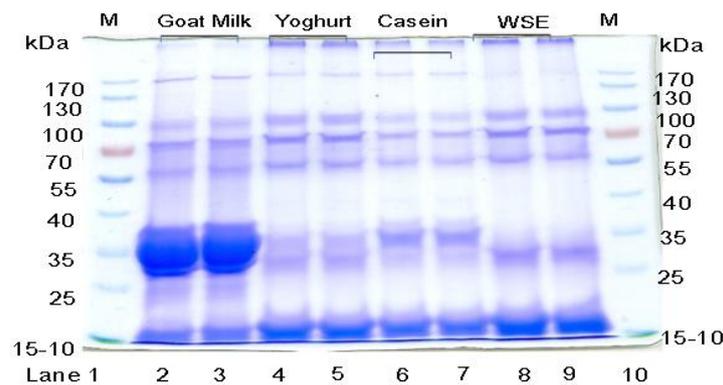
### 3.2 Protein analysis

**Table 2** Protein analysis of yoghurt of 3% concentration commercial starter

Sample	Protein content (% dry matter)
Milk	20.81
Yoghurt	22.80

Protein content analysis on goat milk and yoghurt were produced from 3% of starter yoghurt commercial showed to produce yoghurt with high quality, compared to yoghurt produced by local yoghurt starter. The purpose of protein content analysis was to investigated protein content in yoghurt and fresh goat milk. Protein content of yoghurt was 22.80 %, higher than protein content in fresh goat milk (unfermented goat milk) that was 20.81 % (Table 2). Many milk proteins possess specific biological properties that make potential components ingredients for healthy food. The increasing attention is being focused to physiological active peptides derived from milk protein. These peptides are inactive within the sequence of the source protein molecule and can be released by milk fermentation with proteolytic starter culture [2]. For this reason, it can be concluded that yoghurt of goat milk is potential to be developed as drinking product as a functional food.

### 3.3 Protein profile



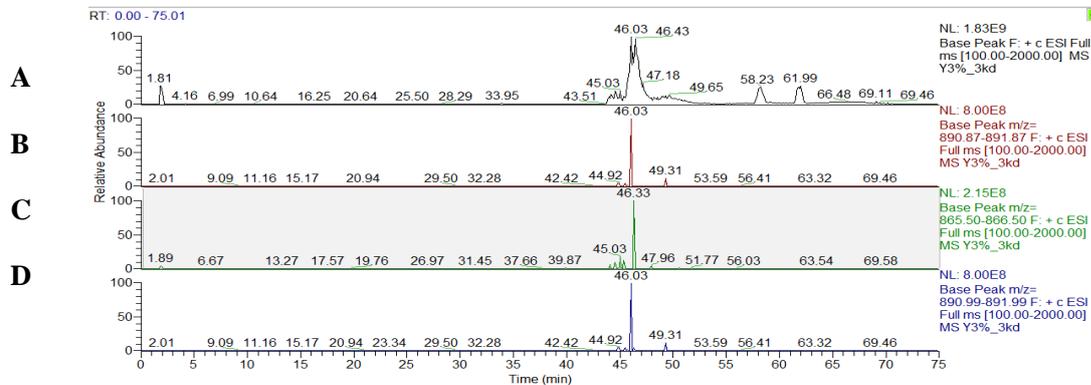
**Figure 2.** Figure of protein bands of yoghurt and fresh milk of goat

As can be seen in Figure 2, the fermentation treatment processing cause peptides of protein milk became hydrolyzed and denaturated. It was known from total band of peptides protein formed at SDS-PAGE. The electrophoresis processing showed the total of peptides protein bands of fermented milk more than peptides protein band of fresh milk. The hydrolyzed or denaturated of peptides protein fermented milk was reached optimally because of the presence of lactic acid bacteria. According to Korhonen and Pihlanto (2006) [2], that peptides are inactive within the sequence of the source protein molecule and can be released by (1) gastrointestinal digestion of milk, (2) fermentation of milk with proteolytic starter cultures or (3) hydrolysis by proteolytic enzymes.

Since there is difference variation of peptides bands total in fermented goat milk or yoghurt, the next research is suggested to determine peptides character that is useful to decrease cholesterol in blood. The peptides characterization is done by protein hydrolyzed fractionation method, that have molecule weight less than 3 kDa, that has potential of both as an antioxidant and anti hypercholesterol.

The functional properties of bioactive peptides are determined by the amino acid composition of the bioactive peptide, e.g peptides with the amino acid composition Val-Lys-Glu-Ala-Meta-Pro-Lys have antioxidant functions [1]. In general, the functional properties of bioactive peptides derived from milk can be divided into four groups that are the functional properties associated with the circulatory system

(cardiovascular system), the nervous system (nervous system), the gastrointestinal system (immune system) and immune system (immune system) [2].



**Figure 3.** LC-MS/MS chromatogram of fermented milk yoghurt sample <3 kDa (A) LC-MS/MS chromatogram of fermented milk yoghurt (B) LC-MS/MS chromatogram of LYQEPVLGPVVRGPFPI sequence (C) LC-MS/MS chromatogram of YQEPVLGPVVRGPFIL sequence (D) LC-MS/MS chromatogram of VQSWMHQPPQPLSPT sequence

The results of LC/MS chromatogram were showed at Figure 3. The fermented milk yoghurt was known by chromatogram A. The derivative sequence of fermented milk yoghurt amino acid from  $\beta$ -casein Capra hercus was showed by chromatogram B (LYQEPVLGPVVRGPFPI), C (YQEPVLGPVVRGPFIL), and D (VQSWMHQPPQPLSPT). These chromatograms had the highest peak in retention time 46.03; 46.33, and 46.03 respectively. This result in line with chromatogram A, suggesting that the three of sequence is derivate of amino acid from fermented milk yoghurt. Based on data in Table 3, it showed these three sequence had a low molecular mass. Hence it were keep from protease activities of gastrointestinal.

**Table 3** Result of yoghurt sample peptides <3kDa identification sing Mascot Distiller and NCBI databse

LC-MS/MS Chromatogram	Identified Peptide Sequence	m/z	Molecule weight (Dalton)	Score	Retention time (min)
B	LYQEPVLGPVVRGPFPI	891.37	1780.90	50 (homology)	46.03
C	YQEPVLGPVVRGPFIL	891.49	1780.90	52 (homology)	46.33
D	VQSWMHQPPQPLSPT	866.88	1731.84	51 (homology)	46.03

#### 4. Conclusion

The best quality of yoghurt was produced by Canadian commercial yoghurt starter by 3 % concentration pH 5.2 over 8 h of fermentation. The content of dry matter protein fermented goat milk was 22,80 % higher than protein content of fresh goat milk. The result of isolation and characterization of peptides bioactive showed that there were 3 kinds of bioactive peptides sequences, each 16 amino acids protected from protease gastrointestinal enzymes. These peptide sequences had a function as antioxidant potential.

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