

The characterization of bioactive peptides of goat milk fermented to activities as anti-hypercholesterolemia

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Abstract

Bioactive peptides of fermented goat milk had many benefits the body's regulatory system provides enough on health. This study aims to explore the fermented goat milk products as decreased malondialdehyde (MDA) and its influence on the expression of the accumulation of fat in a mouse hypercholesterolemia model. The characterization of bioactive peptides fermented goat milk products with a concentration of 3% and 5% using SDS PAGE and LC-MS-MS. Bioactive peptides fermented used as the lowering levels of MDA and the decreased expression of the accumulation of fat in a mouse model of hypercholesterolemia. The characterization of bioactive peptides (SDS-PAGE analyses) showed protein fraction Mr<3KDa. The analysis of LC-MS/ MS showed three types of bioactive peptide composed of 16 amino acids. Bioactive peptides had the ability to reduce the production MDA were significantly (P<0.01) and the reducing of expression of hepatic fat accumulation histopathology mouse model of hypercholesterolemia. The bioactive peptides of goat milk fermented had the ability to decrease as hypercholesterolemia.

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Keywords

Bioactive peptides,
Fermented goat milk,
Activities,
decrease malonydialdehyde,
Anti-hypercholesterolemia

Introduction

Food Protein is one food component of today to the attention by researchers and industries. Protein known have biological activities that to influence functional and body of both and whole proteins or the hydrolyzate. The hydrolyzate of food proteins that have a biological function has an important function on regulating body functions and bodies healthy are known as bioactive peptides. Bioactive peptides bounded on protein precursor of food and will be released through the hydrolysis process by proteolysis enzymes in the gastrointestinal tract, or maybe by in-vitro using proteolysis enzymes that isolated from the microorganism or the fermentation process (Walther and Sieber, 2011; Choi *et al.*, 2012). Several bioactive peptides had functions of known as an antioxidant, antimicrobial, anti-hypertensive, anti-cholesterol, cyto-modulator and immunomodulatory (Möller *et al.*, 2008; Sarmadi and Ismail, 2010; Sharma, S. *et al.*, 2011; Ngo *et al.*, 2012).

Milk is the primary source of bioactive peptides that have various biological functions when compared with other food ingredients (Korhonen, 2009) and some empirical fermented milk products have been trusted to give effect can improve and cure certain diseases. At the time biopeptides still bound to the protein, cause the biopeptides inactive.

Therefore, there are needed hydrolyze methods to release bioactive peptides from whole protein, so the bioactive peptides to be maximally useful for body health (Korhonen, 2009; Walther and Sieber, 2011; Choi *et al.*, 2012). Therefore, it was important to study the effective methods milk product with high bioactive content and availability.

The utilization of milk as a nutritional ingredient containing bioactive peptides can be increased levels of bioactive peptides (Korhonen, 2009; Ebringer *et al.*, 2008), so it was important to study effective methods to produce dairy products containing high enough from bioactive peptides. Goat's milk is a natural functional food had different milk with cow's chemical properties and it was many similarities with the mother's milk. Lactose and protein content of goat milk is similar to cow's milk, but there are differences in the structure of proteins and immunology. In addition to goat's milk contains medium chain fatty acids. Fat in goat milk is relatively small compared with cow milk fat (Eknæs *et al.*, 2009). Fermentation using lactic acid bacteria (LAB) as a famous method that is used to produce safe fermented milk is believed to give health benefits and cure several diseases (Donkor *et al.*, 2007). Several results of research showed that bioactive peptides from fermented milk have many benefits for body regulation system and the enhancing body

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healthy (Mejia, 2013). The optimization fermentation method of bioactive peptide product to be a focus of research in many states. Some research suggests that the bioactive peptides have activity as an anti-hypercholesterolemia through supplementation in rat models of hypercholesterolemia. Hypercholesterolemia is a condition cholesterol levels exceed the normal limits. Normal cholesterol levels in humans 120-240 mg/ dL, the dogs 50-300/ dL and white rat 40 to 130 mg/ DL (Ford *et al.*, 2010; Stapleton *et al.*, 2010). This study aims to explore the concentrations of lactic acid bacteria levels required in fermented goat milk products as bioactive peptides lowering malonyldialdehyd (MDA) and its effect on fat accumulation expression in a mouse model of hypercholesterolemia. We hope to find the optimum fermentation through this study will be used to develop fermented milk products with better value when compared to fresh milk

Materials and Methods

The characterization of goat milk fermented

Starter preparation was done according instruction of starter product industry. 100 mL of fresh goat milk was pasteurized at 72°C for 5 minutes, and then cooled to 40°C–45°C. 0.5 g frozen starter was dissolved to 5 mL of pasteurized milk (from 100 mL of Pasteurized milk). Futhermore, the starter was added into 100 mL pasteurized milk, the homogenized and the incubated at 40°C to 45°C for 4 to 8 hours, until milk starter reached pH 4.0 – 4.5. The goat milk fermented preparation method was done 500 mL goat fresh milk was pasteurized at 72°C for 5 minute, then cooled at 40°C - 45°C. It was incubated with milk starter concentration solution 3% and 5% (according study of treatments), The homogenized and incubated at 40°C to 45°C for 4 to 8 hours respectively until pH value of goat milk fermented reach 4.5 to 5.0. The product of goat milk fermented was saved at 4°C to 5°C.

Hydrolysis of goat milk fermented was prepared by solving 100 mg of sample of dry frozen fermented product to 1 mL buffer ammonium bicarbonate solution (NH₄HCO₃ 50mM,; pH 8.5), then it was sonification (10 second, 40 Hz), and replicated 4 times. It was centrifuged (12,000 rpm, for 10 minutes at 4°C). The supernatant (500 µL) was entered through in ultra-filtration membrane 3 kDa molecular weight cut of (MWCO) and then was centrifuged for 15 minutes (12,000 rpm, 4°C), and 100 microliters buffer NH₄HCO₃ was added to avoid waste protein, it was then be centrifuged with cold centrifugation for 7 minutes (12,000 rpm, 4°C).The bottom of solution

phase was protein fraction ≤ 3 kDa was collected for profile peptides identification immediately by SDS-PAGE analyses. The solution of protein fraction was frizzed at -20°C for next laboratory analysis purpose (Javanovic *et al.*, 2007).

The dry sample of peptides sample was solved in 5% acetonitrile and 0.1% formic acid in deionization water for LC-MS/MS analysis. LC-MS/MS analysis method was done by LCQ Deka XP System MAX Thermo with ionization electrospray (ESI) Thermo Scientific InC. USA) used C-18 Bio-basic column, with 150 x 2.1 mm diameter, 5 µm particle size. LC-MS condition was gas flow rate 50 absorb prayer potential 4 kV, capillary potential 20v, capillary temperature of 300°C. Interval of MS Scan was between m/z 100 – m/z 1600 with flow rate 200 µL/min. The separation of peptides was used with gradient linier gradually from 5% B solution to 70% B solution (Formic acid 0.1% in acetonitrile) for 90 minutes. The mass spectra was reading by thermo–Xialibor TM program (Thermo Scientific USA). Data of MS/MS were calculated by using Format File MGF with Mascot Distiler 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 peptide sequence in Base data.

Effect of goat milk fermented (yoghurt) therapy to malondialdehyde levels (MDA) animal liver model rat (Rattus norvegicus) hypercholesterolemia

Method of hypercholesterolemia diet feed consists of cholic acid 0.1%, lard 10%, and poached quail egg yolks fresh 5%. All the ingredients are mixed and diluted with distilled water to a volume of 2 mL. Feed hypercholesterolemia diet was administered daily for 14 days beginning on day 8 with force feeding method with 3.02 g/ 2 mL. After that, the rats were given a drink and a standard feed SP 16.98 grams. Feed given as much as 20 g/ head/ day (Titis Nurmasitoh, T. and Pramaningtyas, M.D., 2015).

Goat milk Fermented is given after the rat induced hypercholesterolemia diet for 14 days. We have five groups. Group A (negative control), Group B (positive group of rat hypercholesterolemia), and groups of rat were given the therapy that is in group C, D, and E on dose of 300 mg/ kg, 600 mg/ kg, and 900 mg/ kg, respectively. The therapy was given orally by oral administration of 1.5 mL for 4 weeks (28 days) beginning on day 22.

MDA (malondialdehyde) standard kit stock solution with a concentration of 1, 2, 3, 4, 5, 6, 7 and 8 mg/ mL are taken from each of 100 mL The

solution was putting into a different reaction tube and add 550 ml distilled water and 100 mL trichloroacetic acid (TCA) 100% then homogenized vortex excited. The solution was added to 250 mL HCl (Hydrogen Chloride) 1N and 100 mL Na-thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$) 1% into the tube and homogeneous by centrifugation at a speed of 500 rpm for 10 minutes. Thereafter, to heated for 30 minutes at 100°C. The solution was allowed to stand at room temperature and the supernatant was taken and the standard solutions were reading at a maximum wavelength using a spectrophotometer. The Absorbance of MDA standard solution and make the curve.

Rat prepared beforehand by cervical dislocation. Rat positioned on a surgical board using the pin. Rat dissected from stomach and liver were taken and separated. The carefully cleaned of fat still attached and washed with NaCl (sodium chloride) 0.9%, then put in a solution sodium-azide phosphate buffer (Na-azide-PBS). 1 gram of liver then crushed with a mortar until smooth cool then adds 1 mL NaCl 0.9% and homogenized. Homogenates were taken and microtube. The centrifugation tube was moved to do with the speed of 8000 rpm for 20 minutes and the supernatant was taken. 100 mL supernatant incorporated into a new microtube and then added 550 mL distilled water and homogenized. After that, 100 mL TCA 100% was added and homogenized. The solution was added 100 mL HCL 1N and 100 mL Na-thiosulphate 1% and homogeneous back. The centrifugation performed at 500 rpm for 10 minutes and the supernatant was collected. Thereafter, it was heated for 30 minutes at 100°C. The supernatant taken standard solution is then read at a wavelength of maximum using a spectrophotometer.

The process of making preparations histopathology consisted of fixation, dehydration and infiltration, purification, paraffin infiltration, embedding, sectioning, pasting on a glass object, and staining. Fixation is done to prevent damage to the tissue, stop the process of metabolism and harden soft material so that the network can be colored. Fixation is done by the network put in solution of 4% PFA (paraformaldehyde).

Hematoxylin eosin (HE) staining is done to look at the morphology of liver tissue. Staining begins with de-paraffined and rehydration preparations

using xylol and alcohol-rise 95%, 90%, 80%, and 70%. The prepartate were washing with distilled water. The process of staining was performed using Hematoxylin for 1 minute and eosin dye for 5 minutes. After the preparations colored, made of dehydrated with alcohol 70%, 80%, 90% and 95% and to continue with absolute alcohol I, II and III respectively 5 minutes. Once that is done clearing process with xylol I and II for 5 minutes, preparations wind dried and covered with cover glass.

The histopathological observations of liver tissue preparations using a light microscope Olympus BX51 started weak magnification (40x) to powerful magnification (1000x) for 5 fields of vision to see the changes in the structure and shape of the heart tissue. This histopathological picture liver tissue magnification 100x, 400x and 1000x analyzed descriptively. The calculation of changes in the liver tissue of the controls is converted in to a percentage using the formula of the average percentage of area treatment groups was reduced by an average percentage of are groups with an average normal be divided control multiplied by 100%.

Results

The characterization of goat milk fermented

Good quality of goat milk fermented was the milk fermented was able to produce good goat milk fermented with good viscosity. In this research shows the differences of the quality of fermented goat milk produced by two types of starter with a concentration of 3% and 5%, as shown in Table 1. The result of protein contents of fresh goat and goat milk fermented was 20.81% and 22.80% (dry contents), respectively. The high quality product is the produced was commercail goat milk fermented 3%. The goat milk fermented 3% is used for protein analysis, as shown Figure 1. In Figure 1 [C] showed that a dominant peak at 4603 (A) is selected ion chromatogram at retention time 46.03; 46.32; 46.03 (B, C, D) showed dominant peak have m/z 891.37; 891.49 and 866.48. The result of blast using mascot distiller™ showed peak identity come from Beta casein Capra hercus with sequent LYQEPVLGPVVRGPFPI, YQEPVLGPVVRGFPIL, and VQSWMHQPPQPLSPT, as shown in Figure 1 [D].

Table 1. Result of Qualitative analyzed of Goat Milk Fermented

Treatment	pH	Viscosity consistency	Taste	Aroma
A 3%	5.2	Less	Acid less	Milk
A 5%	5.0	Less	Acid less	Milk
B 3%	4.3	Viscous	Yoghurt	Yoghurt
B 5%	3.8	Viscous	Acid	Acid

Note: A : Local Goat Milk Fermented

B : Commercial Goat Milk Fermented

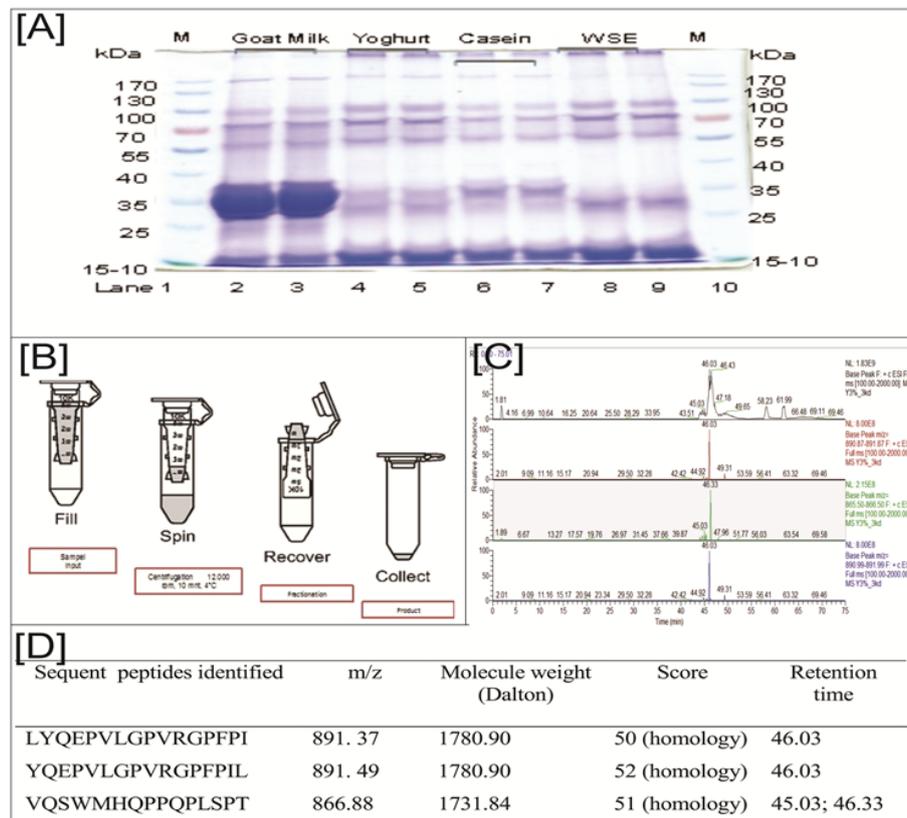


Figure 1. [A] Biopeptide bands of Goat Milk Fermented (Yoghurt) and Fresh Goat Milk by SDS PAGE; [B] Protein hydrolyses fractionation processing (<3 kDa) used ultra-filtration Method; [C] LC-MS/MS Chromatogram of Goat Milk Fermented sample <3 kDa; and [D] Sequent peptides identified of Goat Milk Fermented (Yoghurt) sample peptides <3kDa by Mascot Distiller and NCBI base data.

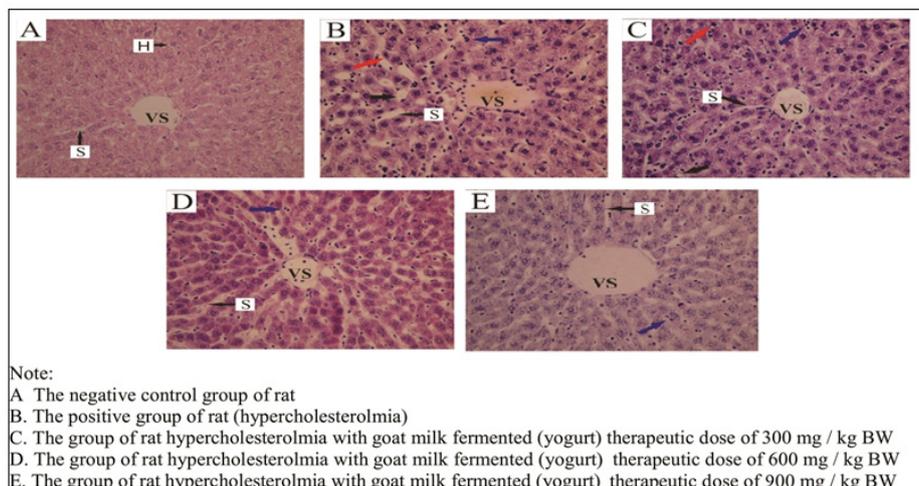


Figure 2. The Histopathological picture liver of *Rattus norvegicus* models hypercholesterolemia treated goat milk fermented (yogurt) with a magnification of 400X

Table 2. The average liver MDA levels in the treatment groups

Groups	Average level MDA ($\mu\text{g/ml}$)	Decreasing (%)
A	2.44 \pm 0.24 ^a	100
B	6.07 \pm 0.56 ^c	0
C	4.79 \pm 0.36 ^b	21.09
D	3.33 \pm 0.59 ^a	44.43
E	2.50 \pm 0.51 ^a	58.81

Description: The difference notation band show a highly significant difference ($p < 0.01$) between the treatment groups.

Note:

A. The negative control group of rat.

B. The positive group of rat (hypercholesterolemia)

C. The group of rat hypercholesterolemia with goat milk fermented (yogurt) therapeutic dose of 300 mg / kg BW.

D. The group of rat hypercholesterolemia with goat milk fermented (yogurt) therapeutic dose of 600 mg / kg BW.

E. The group of rat hypercholesterolemia with goat milk fermented (yogurt) therapeutic dose of 900 mg / kg BW.

Effect of goat milk fermented (yoghurt) therapy to malondialdehyde levels (MDA) animal liver model rat (Rattus norvegicus) hypercholesterolemia

The average MDA levels in the treatment group as presented in Table 2. The Histopathological picture liver rat models (*Rattus norvegicus*) hypercholesterolemia treated goat milk fermented (yogurt) with a magnification of 400X for 5 field of vision to see the changes in the structure and shape of the heart tissue, as presented on Figure 2.

Discussion

The purpose of protein analysis was to know protein content of goat milk fermented and fresh goat milk. Protein content of goat milk fermented is 22.80%, it was higher than protein content in fresh goat milk (20.81%). Many milk proteins possess specific biological properties that make components potential ingredients of health- promoting food. It is being focused to physiologically active peptides derived from milk protein. These peptides are inactive within the sequence of the parent protein molecule and can be liberated by fermentation of milk with proteolytic starter culture (Korhonen and Pihlanto, 2006). For this reason, the product fermented can be potential to be developed as drinking product as a functional food. In Figure 1 section [A], the fermentation processing treatment peptides bonding of protein milk became hydrolyzed and we were breakdown. It was known total band of peptides protein formed at SDS-PAGE electrophoresis processing the total of peptides protein bands more than peptides protein band of fresh milk. The hydrolyzed or breakdown of peptide protein fermented milk was reached optimally, because there were presenting of lactic acid bacteria. It was according to Korhonen and Pihlanto (2006), the peptides are inactive within the sequence of the parent protein molecule and can be liberated by (1) gastrointestinal digestion of milk,

(2) fermentation of milk with proteolytic starter cultures or (3) hydrolysis by proteolytic enzymes (Mejia, 2013). There is variation difference of total amount of peptides bands in fermented goat milk or yoghurt, so 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 with weight less than 3 kDa, that is known has potential anti hyper-cholesterol. In Figure 1 section [D] showed that result of isolation characterization of bioactive peptides of each was 16 amino acids were saved from protease activities of gastrointestinal.

ANOVA test for MDA (malondialdehyde) in rats treated showed a highly significant difference ($p < 0.01$), as presented in Table 2. The MDA analyses known that group of control rat (A) has an average of the lowest levels of liver MDA is 2.44 \pm 0.24 $\mu\text{g/ml}$, while a rat groups hypercholesterolemia (B) has an average of the highest levels of liver MDA is 6.07 \pm 0.56 $\mu\text{g/ml}$. Goat's milk fermented (yoghurt) therapy at a dose of 300 mg/ kg, 600 mg / kg, and 900 mg kg body weight can reduce levels of hypercholesterol rat MDA 21.09%; 44.43%; and 58.81%, respectively. A group of rats had the lowest levels of MDA because only given normal feed so that the levels of cholesterol in the blood were still in the normal range is 10-54 mg/ dL. Cholesterol would trigger the formation of free radicals, but the number of free radicals in A-rat group does not exceed the amount of antioxidants found in the body so that the antioxidant is able to capture free radicals and inhibiting lipid peroxidation. In rat group B has the highest MDA levels because rats fed a diet containing cholesterol hypercholesterolemia 240.50 mg cholesterol levels significantly different to the condition of the body will try to normal balance so cholesterol to bile acid synthesis. The side effects of bile acid synthesis in the form free radicals to trigger lipid peroxidation. Lipid

peroxidation will form MDA as product of reactive metabolites, biomarkers for assessing oxidative stress. Total cholesterol is more and more in the body will increase the synthesis of bile acids so that more and more free radicals are produced. Increased free radicals will result in more MDA generated from lipid peroxidation process. These results are consistent with studies conducted Fki *et al.* that the group of rat were fed a rich cholesterol diet will increase MDA in hearts compared with the normal group (Atmaca *et al.*, 2008).

Goat milk fermented can provide a very real effect on rat hypercholesterolemia. Goat's milk fermented contains antioxidant compounds that can capture free radicals produced as a side effect of bile acid synthesis. Antioxidant functions as a catcher of free radicals and inhibiting lipid peroxidation process so that it can help reduce levels of free radicals as a result of the provision of hypercholesterol. Peroxidation inhibitor process dietary lipids by capturing free radicals and donate a hydrogen atom to form stable compounds that able to stop the chain reaction of lipid peroxidation.

Milk has bio-peptides active content that can be generated through hydrolysis by the digestive enzymes and enzymatic processes by microorganisms. Lactic acid bacteria in yogurt can ferment milk to produce active bio-peptides, such as lactoferrin. Lactoferrin can be able to repair cell damage by inhibiting the production of ROS in the cell membrane and works with vitamin E in limiting membrane lipid oxidation by ROS. Lactoferrin is an iron binding extracellular. Intracellular iron called heme and stored in the form of ferritin, extracellular bound by transferrin or lactoferrin protein. Iron that is not bound will catalyze the production of ROS, then through bio-peptides extracellular iron lactoferrin will be bound so that inhibited ROS generation (Eipper *et al.*, 2016),.

Goat Milk fermented also contains lactic acid bacteria have enzymes Bile Salt Hydrolyses (BSH) so as to de-conjugate bile salts to produce bile salts that poorly absorbed by the small intestine. Bile salts return to the liver to be reduced so that the body uses cholesterol as a precursor to balance the amount of bile salts that decrease the amount of cholesterol. Decrease in cholesterol levels resulted in a decrease in the amount of lipids which are exposed to free radicals is reduced so that the process is reduced lipid peroxidation accompanied by a decrease in the sheer number of levels of MDA in the liver of animal models of rats (*Rattus norvegicus*) hypercholesterolemia.

Conclusion

The best quality of goat milk fermented was produced commercial goat milk fermented 3% with protein contents 22.80%. The characterization of peptides bioactive were found three kinds of bioactive peptides sequences each switch 16 amino acids protected from protease gastrointestinal enzymes. Goat milk fermented can reduce levels of MDA and decreased fats accumulation of animal model of hypercholesterolemia. Goat's milk yoghurt therapy at a dose of 600 mg/ kg has been able to provide the best therapeutic effect in lowering levels of MDA and therapeutic dose of 900 mg/ kg give the best therapeutic effect in reducing the expression fat accumulation liver rat animal model of hypercholesterolemia.

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