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ARTICLE

Lipase-Catalyzed Synthesis of Conjugated Linoleyl β-Sitosterol and Its Cholesterol-Lowering Properties in Mice

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Conjugated linoleyl β-sitosterol (CLS) was prepared from β-sitosterol and conjugated linoleic acid (CLA) via lipase-catalyzed synthesis in *n*-hexane in the presence of molecular sieves. Its plasma cholesterol-lowering properties were also studied. The optimal biosynthesis conditions were as follows: β-sitosterol concentration of 50 μmol/mL, the molar ratio of CLA to β-sitosterol of 1:1, the lipase concentration of 20 mg/mL, and 4 Å molecular sieve concentration of 60 mg/mL in *n*-hexane at 50 °C with vigorous shaking of 150 rpm for 72 h, and the highest yield of CLS reached 72.6%. The isolated CLS mixed with mice feed had good cholesterol-lowering properties. It decreased serum total cholesterol (TC), serum triacylglycerols (TAGs), serum low-density lipoprotein cholesterol (LDL-C), atherogenic index (AI), liver weight (LW), liver index (LI), liver TC, and TAGs of mice, which was shown that CLS could prevent the formation of atherosclerosis and moderate the fat pathologic changes of liver. However, the HDL-C was not decreased, which proved the advantage of CLS over the other cholesterol-lowering products.

KEYWORDS: Conjugated linoleyl β-sitosterol (CLS); immobilized lipase; cholesterol-lowering

INTRODUCTION

Plant sterols (phytosterols) are naturally occurring components of plants, which are a group of cholesterol analogues with different side-chain configurations and have been used successfully for lowering plasma cholesterol levels and shown to be safe for half a century. β-Sitosterol is the most abundant in plant sterol analogues (*1*). It is estimated that the intake of phytosterol in humans can reach 160−360 mg/day, and it has been suggested that the daily consumption of 2 g of phytosterols can effectively lower the cholesterol by 9−14% in humans (*2*). The mechanism for differential absorption of cholesterol and phytosterols in the intestine is partially known. First, phytosterols are largely prevented from being absorbed by intestine. Second, phytosterols are also inhibitors of intestinal cholesterol absorption and may cause an effective displacement of cholesterol from micellar binding in the intestine or affect cholesterol synthesis (*2*).

However, practical applications of free plant sterols in foods are limited because of their poor solubility and low bioavailability (*3*). The amount of plant and other sterols in diets is comparable to that of dietary cholesterol, but only 5% is absorbed (*2*). Therefore, it is needed to add some beneficial molecules to plant sterols to enhance their solubility and bioavailability. Esterification or transesterification of plant sterols with fatty acids or oils and fats can increase their lipid solubility and thus facilitates the incorporation into a variety of foods (*4*, *5*). Plant sterol fatty acid esters synthesized by chemical methods have been added to margarine, and the margarine-containing plant sterol ester entered the United States market in 1999 (*6*). The US Food and Drug Administration had reviewed thoroughly the safety of plant sterols and their esters and approved them to be used in foods in 2000 (*3*).

Conjugated linoleic acid (CLA) is a naturally occurring fatty acid and has an uncommon structure containing conjugated double bonds in the carbon skeleton. CLA is found mainly in dairy products and exhibits many physiological properties and has many benefits for human, such as anticarcinogenic properties (*7*), helping to normalize blood cholesterol level (*8*).

It has been known that, in the presence of organic solvent, lipase can catalyze the esterification reaction (*4*, *9*, *10*) and transesterification (*4*, *10*−*13*). However, lipase has a rigorous requirement for water content in the reaction of lipase-catalyzed synthesis in organic solvent, and the removal of the water by addition of a desiccant, i.e., molecular sieves, can shift the reaction toward the desired product (*13*). But up to now, no researcher has performed the synthesis of conjugated linoleyl β-sitosterol (CLS) with the addition of desiccant in lipase-catalyzed synthesis. Vu et al. used the lipase-catalyzed method to synthesize CLS, the molecular ratio of β-sitosterol to CLA was 1:3, the reaction temperate was 55 °C, and the reaction was conducted with a magnetic stirrer (175 rpm) using different lipases as catalysts. But the desiccant was not used during the reaction. The maximum yield of the ester was only 28.3% when the reaction was conducted for 48 h in the presence of *n*-hexane (*6*).

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Table 1. Formulation of Synthetic Diet

basal feed (%)		hyperlipidemic feed (%)		hyperlipidemic feed supplemented with CLS
corn powder	26	basal feed	57.5	0.4 g of CLS mixed with 1 kg of hyperlipidemic feed
flour	34	cholesterol	2	
soybean residue	24.7	bile salt	0.5	
fish powder	5	milk powder	20	
soybean oil	2.3	yolk powder	10	
clover fodder	3	pork oil	10	
premix	5			

In the present study, we optimized the reaction conditions of CLS by condensation of β-sitosterol and CLA in organic solvent using immobilized lipase, Chirazyme L-2 c.-f. C2 (from *Candida antarctica*), molecular sieves were used to remove waters as the byproduct, and a high yield of CLS was achieved. The prepared CLS was isolated by column chromatography and identified by thin-layer chromatography (TLC) and mass spectrometry (MS). Furthermore, its plasma cholesterol-lowering properties were detected using a mouse test.

MATERIALS AND METHODS

Materials. Immobilized lipase from *C. antarctica*, Chirazyme L-2 c.-f. C2, was purchased from Roche Molecular Biochemicals (Mannheim, Germany). β-Sitosterol was purchased from Fluka (Taufkirchen, Germany). Commercial conjugated linoleic acid was purchased from Dongying Dazhongnan Yellow Delta Industrial Co., Ltd. (Dongying, China). The 3 Å 1/16 and 4 Å 1/16 molecular sieves were purchased from Shanghai UOP (Shanghai, China). Total cholesterol (TC) enzymatic kits, triacylglycerol (TAG) enzymatic kits, high-density lipoprotein cholesterol (HDL-C) kits, and low-density lipoprotein cholesterol (LDL-C) kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Lipase-Catalyzed Synthesis of CLS. The reaction mixture consisted of β-sitosterol (0.125−0.75 mmol), CLA (0.125−1.25 mmol), the immobilized lipase (100 mg), 3 or 4 Å activated molecular sieves (activated by heating at 180 °C for 12 h in a drying oven, 100−500 mg), and 5 mL of solvent. The reaction mixture was put into a tightly sealed vial and incubated at 50 °C with vigorous shaking (150 rpm). The solvents were dehydrated with 4 Å 1/16 activated molecular sieves for at least 24 h. An aliquot sample (5 μL) was removed periodically, and the product concentration was analyzed by an HPLC system (Agilent 1100 Series, Santa Clara, CA) equipped with a Si-60 normal phase column (4.6 mm × 250 mm; Hewlett-Packard, Palo Alto, CA) and an evaporative light scattering detector (ELSD) 200ES (Alltech, Deerfield, IL). The drift-tube temperature and nitrogen carrier gas flow rate of the ELSD were set at 60 °C and 1.7 L/min, respectively. The eluent was a mixture of *n*-hexane and isopropyl alcohol by a linear programming, and the flow rate of the eluent was 0.8 mL/min. The elution gradients were 100% *n*-hexane for 0−10 min, *n*-hexane/isopropyl alcohol (90:10 v/v) for 10−20 min, *n*-hexane/isopropyl alcohol (30:70 v/v) for 20−25 min, and 100% isopropyl alcohol for 25−30 min. The column temperature was 30 °C. The calibration curves were prepared using the purified products. The yield of CLS was calculated by the molar ratio of CLS to β-sitosterol at the beginning of the reaction.

Isolation, Purification, and Identification of CLS. β-Sitosterol (6 mmol), CLA (6 mmol), immobilized lipase (2.4 g), and activated 4 Å molecular sieves (7.2 g) were mixed with *n*-hexane (120 mL), and the condensation was carried out at 50 °C with vigorous shaking (150 rpm) for 72 h.

All reaction mixture was filtered (Whatman No. 1), and the filtrate was then rotary-evaporated (RE-52; Yarong Biochemical Instrument Plant, Shanghai, China). The residue was deacidified with 200 mL of 2% aqueous sodium bicarbonate solution, followed by centrifugation (Avanti J-HC; Beckman Coulter Inc., CA) at 1006*g* for 10 min. The mixture was filtered. The filter cake was mixed with 200 mL of water, followed by centrifugation at 1006*g* for 10 min to remove the unesterified acid as sodium salts. The deacidification process was repeated three times. The deacidified solid containing the desired products was dried in a vacuum at 50 °C for 12 h.

The crude powder of CLS obtained was applied to a silica gel column (60−100 mesh, 12 × 600 mm) and then eluted with cyclohexane/ethanol (19:1 v/v). The flow rate was 15 mL/h, and the eluent, 1 tube/10 min, was collected and then detected by thin-layer chromatography (TLC). The optimum conditions for TLC were confirmed: 3 μL samples were applied to silica gel G plates, developed by cyclohexane/diethyl ether (19:1 v/v) and then detected by spraying with 5% sulfuric acid in ethanol and heated at 120 °C for 15 min. The fractions containing the desired products were collected and analyzed by the mass spectrometry.

Mass spectrometry was employed to identify the isolated product. The sample was diluted 1:50 in *n*-hexane, and 1 μL of the dilution was analyzed on Platform ZMD 4000 (Waters Corp., Milford, MA). MS conditions: ionization type APCI+, corona 3.19 kV, cone 30 V, source block temperature 100 °C, APCI probe temperature 400 °C, multiplier 700 V, desol gas flow 4.2 L/h, and mass scan range 200−800 amu. The protonated molecular ion $[M + H]^+$ was at m/z 677.8, and the product was identified to be CLS.

Animal Treatments. Eight-week-old male Kun Ming species mice (18−22 g) were obtained from th Medical School of Suzhou University (Suzhou, China). The mice were housed in polypropylene cages in a room maintained at 25 ± 1 °C and 60% humidity with 12 h light and 12 h dark cycle and were acclimated for 3 days before initiating studies. All of the animal research complied with the International Guiding Principles for Biomedical Research Involving Animals (1985), and the protocols were approved by the Animal Use and Care Committee of Jiangnan University.

Formulation of synthetic diet is shown in **Table 1**. Mice were randomly divided into three groups of 20 animals each: normal group (NG), fed with basal feed for 4 weeks consecutively; hyperlipidemic group (HG), fed with hyperlipidemic feed for 2 weeks consecutively and then fed with basal feed for 2 weeks consecutively; hyperlipidemic and CLS group (HCG), fed with hyperlipidemic feed for 2 weeks consecutively and then fed with hyperlipidemic supplemented CLS feed for 2 weeks consecutively. Every mouse intakes about 5 g of feed every day. At the end of the second and fourth week, 10 mice from each group were kept fasting overnight and sacrificed for biochemical analysis, respectively. Blood and tissues were collected for various tests.

Sample Preparation. Blood was collected from the retroorbital sinus at the beginning and at the end of the second and fourth weeks of the feeding trial, then placed in sterile tubes, and centrifuged at 4000*g* for 20 min. The obtained serum samples were analyzed for TC, HDL-C, LDL-C, and TAGs by the commerical kits. The atherogenic index (AI, (TC-HDL-C)/HDL-C) was also calculated.

The livers were excised, cleaned of adhering matter, blotted on filter paper, weighed and rinsed with normal saline solution (1:10 w/v), and then homogenized using a tissue homogenizer with a Teflon pestle. The homogenate was centrifuged at 12000*g* for 20 min, and the supernatant samples (serum) were analyzed for TC and TAGs by the kits. The liver index (LI, liver weight/body weight) was also calculated.

Statistical Analysis. The data were statistically analyzed using the SPSS 13.0 software package (SPSS, Shanghai, China) and reported as mean ± SD. The differences among the experimental groups were identified by one-way analysis of variance (ANOVA) using Duncan's multipic range test, and statistical significance was considered at $P < 0.01$.

RESULTS AND DISCUSSION

Effect of Different Organic Solvents on the Yield of CLS. β-Sitosterol (60.0%) containing campesterol and a little stigmasterol was used as a starting substrate. β-Sitosterol (0.25 mmol) was esterified with CLA (0.50 mmol) with 300 mg of 3 Å molecular sieves and 100 mg of lipase using either *n*-hexane or acetone (5 mL) as reaction medium at 50 °C with vigorous

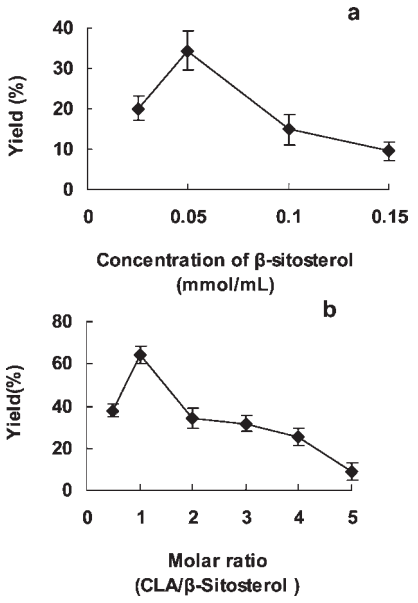

Figure 1. Effect of β-sitosterol concentration (**a**) and molar ratio of CLA to β-sitosterol (**b**) on the yield of CLS. Reaction conditions: (**a**) molar ratio of CLA to β-sitosterol (2:1), 300 mg of 3 Å molecular sieves, and 100 mg of lipase in 5 mL of *n*-hexane, 50 °C, 150 rpm, 72 h; (**b**) 0.25 mmol of β-sitosterol, 300 mg of 3 Å molecular sieves, and 100 mg of lipase in 5 mL of *n*-hexane, 50 °C, 150 rpm, 72 h.

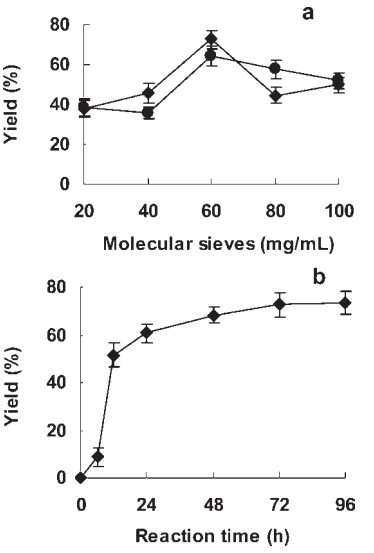

Figure 2. Effect of molecular sieve type and amount (**a**) and time course (**b**) on the yield of CLS. Reaction conditions: (**a**) 0.25 mmol of β-sitosterol, molar ratio of CLA to β-sitosterol (1:1), and 100 mg of lipase in 5 mL of *n*-hexane, 50 °C, 150 rpm, 72 h [4 Å (◆), 3 Å (●)]; (**b**) 0.25 mmol of β-sitosterol, molar ratio of CLA to β-sitosterol (1:1), 300 mg of 4 Å molecular sieves, and 100 mg of lipase in 5 mL of *n*-hexane, 50 °C, 150 rpm.

shaking (150 rpm) for 48 h. The yields of 32.38% and 25.53% were obtained in *n*-hexane and acetone, respectively. Zaks and Klibanov (*14*) reported that the polarity of the solvent determines the esterifying activity of lipase in the reaction system. Generally, all immobilized lipases exhibit high yield in nonpolar solvents. When a polar organic solvent is used, significantly lower yield is detected. In such a medium, the solvent may alter the native conformation of the enzyme by disrupting hydrogen-bonding and hydrophobic interactions, thereby leading to very low yield (*15*).

Only limited organic solvents are used in the food industry. Acetone and *n*-hexane are the solvents which are approved in many countries, and immobilized lipase, Chirazyme L-2 c.-f. C2, is active in them. The reaction in *n*-hexane showed higher yield than that in acetone. Therefore, *n*-hexane was selected as the proper reaction medium of the condensation of β-sitosterol and CLA.

Effect of β-Sitosterol Concentration on the Yield of CLS. The yield of CLS in *n*-hexane at different β-sitosterol concentrations is shown in **Figure 1a**. When β-sitosterol concentration was 50 μmol/mL, the yield of CLS was the highest. With the increase of the concentration of β-sitosterol the yield of CLS decreased. Most of the β-sitosterol remained undissolved in the solvent, especially at higher concentration, which might be the reason for the lower yield at higher β-sitosterol concentration. So the best β-sitosterol concentration was selected as 50 μmol/mL.

Effect of the Molar Ratio of CLA to β-Sitosterol on the Yield of CLS. The molar ratio of CLA to β-sitosterol also influenced the yield of CLS (**Figure 1b**). At a fixed β-sitosterol concentration of 50 μmol/mL, the highest yield was achieved when the molar ratio was 1:1. The yield of CLS started to decline when the molar ratio surpassed 1:1. The reason could be that with the increase of CLA the viscosity of reaction system increased, which led to the decrease in the efficiency of esterification.

Effect of Molecular Sieve Type and Amount on the Yield of CLS. Lipase-catalyzed esterification in organic solvents is a reaction where water plays a key role. A minimal amount of water is necessary for the enzyme to ensure its optimal conformation and then to become optimally active. But, an excess of water decreases the enzyme catalytic activity from both kinetic and thermodynamic points of view (*16*). There are many methods for water removal in the lipase-catalyzed reaction in organic solvent (*16*, *17*), and the adsorption method is most widely used. In this study, activated molecular sieves were used to remove the water produced during the reaction, which has the advantage of lower cost and is easy to be separated and regenerated.

Figure 2a shows the effect of 3 and 4 Å molecular sieve amount on the yield of CLS. The highest yield of CLS was achieved at 60 mg/mL for both 3 and 4 Å molecular sieves, while the yield of CLS using 4 Å molecular sieves was higher than that with 3 Å molecular sieves at 60 mg/mL in *n*-hexane. Thus, 4 Å molecular sieves were more fit for this condensation reaction, and its optimum concentration was 60 mg/mL. The effect of reaction medium and substrate on the dehydration ability of molecular sieve is complicated (*18*), so the reason for the effect of molecular sieves on the yield in such a system is not very clear. The lower yield with 3 Å molecular sieves can be attributed to their strong water removal ability which may reduce the lipase activity.

Effect of Time Course on the Synthesis of CLS. Figure 2b shows the time course of condensation of β-sitosterol with CLA. The reaction was catalyzed in *n*-hexane for 96 h. The results showed that CLS was rapidly produced within the first 24 h of the 96 h reaction. The reaction yield tended to gradually increase until 72 h; therefore, the optimum reaction time was taken as 72 h, and the yield was 72.6%.

Effects of CLS on Plasma Cholesterol-Lowering Properties. Tables 2 and **3** show blood lipid index and liver weight (LW), LI, liver TC, and TAGs of mice at different intervals, respectively. It could be seen that at 0 week there were no differences of blood lipid index and LW, LI, liver TC, and TAGs among the three groups.

LDL provides cholesterol to the tissues, and it is positively associated with the risk of atherosclerosis (AS). HDL removes excessive cholesterol from peripheral tissues back to the liver and

Table 2. Blood Lipid Index of Mice at Different Intervals (Mean ± SD, $n = 10$)[a]

time (week)	group	TC (mmol/L)	TAGs (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	AI (mmol/L)
0	NG	2.05 ± 0.23	0.64 ± 0.06	1.13 ± 0.06	0.69 ± 0.25	0.80 ± 0.18
	HG					
	HCG					
2	NG	2.87 ± 0.09	0.66 ± 0.10	1.48 ± 0.06	1.09 ± 0.11	0.95 ± 0.12
	HG	5.53 ± 0.16[b]	0.86 ± 0.08[b]	1.37 ± 0.08[b]	3.77 ± 0.20[b]	3.05 ± 0.34[b]
	HCG	5.55 ± 0.09[b]	0.84 ± 0.07[b]	1.36 ± 0.08[b]	3.81 ± 0.11[b]	3.10 ± 0.23[b]
4	NG	2.88 ± 0.07	0.68 ± 0.08	1.46 ± 0.07	1.15 ± 0.16	0.98 ± 0.12
	HG	5.51 ± 0.09[b]	0.85 ± 0.07[b]	1.35 ± 0.06[b]	3.78 ± 0.10[b]	3.10 ± 0.19[b]
	HCG	3.41 ± 0.05[c]	0.76 ± 0.09[c]	1.44 ± 0.09[c]	1.63 ± 0.11[c]	1.38 ± 0.15[c]

[a] Results are mean ± SD of triplicate measurement. *n*: the number of mice used for the statistical analysis in each different time interval. [b] Values are significantly different from those of NG at $P < 0.01$ within the same interval. [c] Values are significantly different from those of HG at $P < 0.01$ within the same interval.

Table 3. LW, LI, Liver TC, and TAGs of Mice at Different Intervals (Mean ± SD, $n = 10$)[a]

time (week)	group	LW (g)	LI	TC (mg/100 g)	TAGs (mg/100 g)
0	NG	1.27 ± 0.07	4.96 ± 0.41	5.28 ± 0.41	5.25 ± 0.11
	HG				
	HCG				
2	NG	1.41 ± 0.09	4.85 ± 0.22	5.31 ± 0.33	5.29 ± 0.08
	HG	2.08 ± 0.14[b]	5.72 ± 0.09[b]	11.06 ± 1.85[b]	11.67 ± 2.03[b]
	HCG	2.10 ± 0.15[b]	5.77 ± 0.10[b]	11.04 ± 1.91[b]	11.75 ± 1.98[b]
4	NG	1.43 ± 0.07	4.86 ± 0.09	5.33 ± 0.42	5.30 ± 0.13
	HG	2.07 ± 0.13[b]	5.73 ± 0.08[b]	11.05 ± 1.86[b]	11.64 ± 2.01[b]
	HCG	1.46 ± 0.06[c]	4.91 ± 0.09[c]	6.18 ± 0.39[c]	6.95 ± 0.43[c]

[a] Results are mean ± SD of triplicate measurement. *n*: the number of mice used for the statistical analysis in each different time interval. [b] Values are significantly different from those of NG at $P < 0.01$ within the same interval. [c] Values are significantly different from those of HG at $P < 0.01$ within the same interval.

plays a great role in keeping cholesterol homeostasis in the plasma (*2*), and hence, HDL can prevent AS. After 2 weeks the hyperlipidemia mouse model was established, and then there was an abnormal increase in the levels of serum TC, serum TAGs, serum LDL-C, AI, LW, LI, liver TC, and liver TAGs of the HG group and HCG group, which were significantly higher than those of the NG group ($P < 0.01$).

Hyperlipidemia is the primary risk factor of AS and one of the important inducing factors of cardiovascular disease. The increase of serum TC, serum TAGs, and serum LDL-C and decrease of serum HDL-C can lead to the damnification of artery endothelial cells, accelerate the sedimentation of LDL-C in the endothelium, and promote the formation of AS. Hence, the primary measure for preventing AS is to regulate lipid metabolism. After 4 weeks, serum TC, serum TAGs, serum LDL-C, and AI of the HCG group were decreased, which were significantly lower than those of the HG group ($P < 0.01$), while HDL-C was not significantly different from that of the NG group, which is its advantage over the other cholesterol-lowering products such as HMG-CoA reductase inhibitors (*2*). The results showed that CLS may decrease serum AI of the HCG group, regulate the metabolism of blood lipids, and may prevent the formation of AS. The LW, LI, liver TC, and TAGs of the HG group were higher than those of the NG group ($P < 0.01$), while those of the HCG group were significantly lower than those of the HG group ($P < 0.01$), and had no differences from those of the NG group. It showed that CLS could moderate the fat pathologic changes of liver.

In conclusion, our work provides a method for the synthesis of CLS by lipase-catalyzed reaction in organic solvent using molecular sieves as a dehydrating agent. The reaction conditions were optimized, and high yields of CLS were achieved. Also, the cholesterol-lowering properties of CLS were investigated, which showed that CLS had a good effect for lowering plasma cholesterol level.

ABBREVIATIONS USED

AI, atherogenic index; CLA, conjugated linoleic acid; CLS, conjugated linoleyl β-sitosterol; ELSD, evaporative light scattering detector; HDL-C, high-density lipoprotein cholesterol; HCG, hyperlipidemic and CLS group; HG, hyperlipidemic group; HPLC, high-performance liquid chromatography; LDL-C, low-density lipoprotein cholesterol; LI, liver index; MS, mass spectrometry; NG, normal group; TAGs, triacylglycerols; TC, total cholesterol; TLC, thin-layer chromatography.

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