Isolation of Antimicrobial Substance from the Korean Traditional Leaf Mustard, *Brassica juncea* Coss.

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ABSTRACT

The antimicrobial effect of each fraction after fractionation of an ethanol extract of leaf mustard was examined in terms of nucleic acid, chloroform, ethylacetate, and butanol. The ethylacetate fraction, which showed the strongest level of antimicrobial effect among the different ethanol extract fractions of leaf mustard, was isolated and purified using silica gel column chromatography and HPLC, respectively, to obtain a single antimicrobial substance called KLM-1. The antimicrobial effect of this substance was 10 times higher than that of the ethylacetate fraction. A further study is on the way to confirm the structure of the antimicrobial substance KLM-1 through LC/Mass and NMR.

Key words : Antimicrobial activity, HPLC, isolation, silica gel column chromatography

INTRODUCTION

Spices have been used primarily as flavoring and seasoning agents in food and beverages, and they have also been used for food protection and prevention, as they possess significant antioxidant and antimicrovial activities(Fromtling and Bulmer, 1978; Farag *et al.*, 1989; Conner *et al.*, 1984).

Leaf mustard is a cruciferous vegetable whose seed in powder form is used as mustard spice in the US, Europ and Japan (Farrell, 1985). Originally from China, it is cultivated widely in Korea and Japan. The Korean variety, *Brassica juncea* Coss. is used not only as a spice but also as an ingredient of nappa cabbage *Kimchi*, or as the main ingredient of *Kimchi*, due to its unique taste and flavor (Kim and Kim, 1987). When used as a *Kimchi* ingredient, it is a good source of minerals including abundant Ca and K and is known to prolong the storage period of *Kimchi* due to its slow fermentation speed, and to maintain stable color during a long storage period. Leaf mustard contains the allylisothiocyanate (AIT) glucosinolate called sinigrin, which gives a unique hot flavor. Sulfur compounds and related compounds are produced by this glycoside due to myrosinase action (Morimoto *et al.*, 1983). Sulfur compounds which have a high reactivity change themselves due to other organic sulfur compounds and are reported to contain many physiologically active substances having antimicrobial, anti-fungal, and anti-coagulative effects (Eric, 1985; Bordia *et al.*, 1997).

Studies on leaf mustard include studies on antioxidative substances in leaf mustard and mustard (Han *et al.*, 1987), the purple color antocyanin in leaf mustard (Park, 1979a; Park, 1979b), nutrient contents in

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leaf mustard (Morimoto *et al.*, 1983; Saito and Iwasaki, 1980), volatile compounds in leaf mustard (Kameoka and Hashimoto, 1980), the types and content of glucosinolate (Curitis *et al.*, 1987; Diana *et. al.*, 1987; Shen, 1987), genetic analysis (Reddy *et al.*, 1988) and enzymes (Kumar and Gupta, 1987). Most of these studies on leaf mustard were on nutrition or food compounds, and studies are rare on antimicrobial effects in leaf mustard.

The authors reported in a previous paper (Kang, 2005) that leaf mustard tested for antimicrobial effects against some microorganisms. In this study, using ethylacetate fraction, which is the fraction having the strongest antimicrobial effect, the main antimicrobial substance was isolated and purified. The antimicrobial effect of this substance was examined, and results obtained.

MATERIALS AND METHODS

Plant materials

The Korean traditional leaf mustard, *Brassica juncea* Coss. was collected in Suncheon, in the south of Korea in 2002. The plants were washed in tap water, and milled after being dried in the dark.

Microbial strains and reagents used in experiments

Tabel 1 shows the list of strains used. The nutrient media was purchased from Difco Co. (U.S.A.).

Antimicrobial activity measurement

Each strain was grown in a nutrient broth at 30℃ for 18-24 hrs prior to testing, and subcultured three times for 18-24 hrs. The turbidity of the bacterial cell suspensions was adjusted with the same sterile broth to a 0.3 optical density (OD) unit at 660nm.

The suspensions were then used for the tests. For the Disc plate method, 0.1mℓ of the bacterial cell suspension was poured uniformly into the plate. The paper disks containing the extracts were carefully placed on the seeded petri dishes. The diameter of the inhibition zone was measured in millimeters after incubation at 30℃ for 24-48 hrs depending on the strains (Bauer *et al.*, 1966; Branch *et al.*, 1965; Piddock,

Table 1. List of microorganisms used

Gram positive bacteria	*Bacillus cereus* ATCC 27348 *Bacillus subtilis* ATCC 9372 *Bacillus natto* IFO 3009 *Streptococcus faecalis* IFO 3971
Gram negative bacteria	*Staphylococcus aureus* ATCC 13301 *Escherichia coli* ATCC 15489 *Salmonella typhimurium* ATCC 14028 *Pseudomonas fluorescens* ATCC 11250
Lactic acid bacteria	*Lactobacillus plantarum* ATCC 8014 *Lactobacillus brevis* IFO 13110 *Luconostoc mesenteroides* IFO 12060 *Pediococcus cerevisiae* ATCC 11250
Yeast	*Saccharomyces cerevisiae* IFO 1950 *Hansenula anomala* KCCM 11473 *Hansenula anomala* KCCM 11473

1990). For the minimum inhibitory concentration (MIC) determination, the broth dilution method was used. MIC was determined as the lowest concentration that completely inhibited bacterial growth (MacLowry and Jaqua, 1970, Lee *et al.*, 1989).

Ethanol extract, and fractionations of ethanol extract

1.0 Kg of leaf mustard dried and milled was extracted by stirring in 3L ethanol for 24 hrs at room temperature. The resulting extract was filtered on Whatman No.2 (1st extraction). Six liters of ethanol were added for the 2nd and 3rd extraction which were then treated by the same method as the first extraction. The extracts was evaporated by a rotary evaporator in 50℃ water bath until to be 100ml. One liter of distilled water was added to the above and mixed in well. The mixture was kept in a refrigerator at 5℃ for 24 hrs, centrifuged twice at 3500 rpm to remove resin and then evaporated at 50℃ with a rotary evaporator. This ethanol extract (160.9g of solid) was isolated and purified as shown in Fig. 1. After extraction and evaporation 3 times in a separatory funnel in 1L of hexane: methanol: water (10:1:9 v/v/v), 42.3g of a hexane fraction was obtained. Using the same procedure, the aqueous layer was solvent step fractionated to obtain chloroform, ethylacetate and n-butanol saturated with H_2O fractions at 0.8, 2.7 and 11.5g, respectively. The final water fraction was 96.4g.

Isolation of main antimicrobial substance

Silica gel column chromatography

The ethanol extract was successively fractionated with different solvents. Each fractionated material was tested for antimicrobial activity. The ethylacetate fraction showed the strongest antimicrobial activity, and was concentrated in vacuo till dry. The resulting dark-green gum was tested by silica gel column chromatography and eluted with the stepwise solvent mixtures : $CHCl_3$-MeOH (10:0→9:1→8:2→7:3→6:4→5:5→3:7→0:10). Each fraction was collected in 10㎖ amounts. Active fractions were monitored for antimicrobial activity against E. coli. They were pooled and then each active fraction was further purified by HPLC.

HPLC

After vacuum evaporation of the subfraction, confirmed to have antimicrobial activities by silica gel column chromatography, the subfraction was purified to obtain the final antimicrobial substance using HPLC (Waters Co., M224). As shown in Fig. 2, initial subfractions having antimicrobial activities were purified using a Radial Pak C_{18} (8 ㎜ × 10 ㎝, Waters Co.) column. At this time, the solvent used was 70% methanol at flow rate of 2.0 ㎖/min and the volume of injection was 30㎕. Eight subfractions were collected for 16 minutes at 2 minute fraction intervals. After these 8 subfractions were vacuum evaporated at 50℃ and diluted, the antimicrobial activity in each subfraction was confirmed. Then, these fractions were purified for a second time using a μ-Bonda Pak C_{18} (8 ㎜ × 30 ㎝, Waters Co.) column in 50% methanol (v/v) at a flow rate of 1.5 ㎖/min using a UV detector (214 nm). After the isolated peaks were fractioned and vacuum evaporated, the antimicrobial activities were confirmed. A single substance having an antimicrobial activity was finally isolated.

RESULTS AND DISCUSSION

Antimicrobial activities of leaf mustard ethanol extract fractions

Table 3 shows the results of the antimicrobial activities of hexane, chloroform, ethyl acetate, butanol (saturated with water), and water fractions successively obtained, after being fractionated with different solvents, from leaf mustard ethanol extract. These

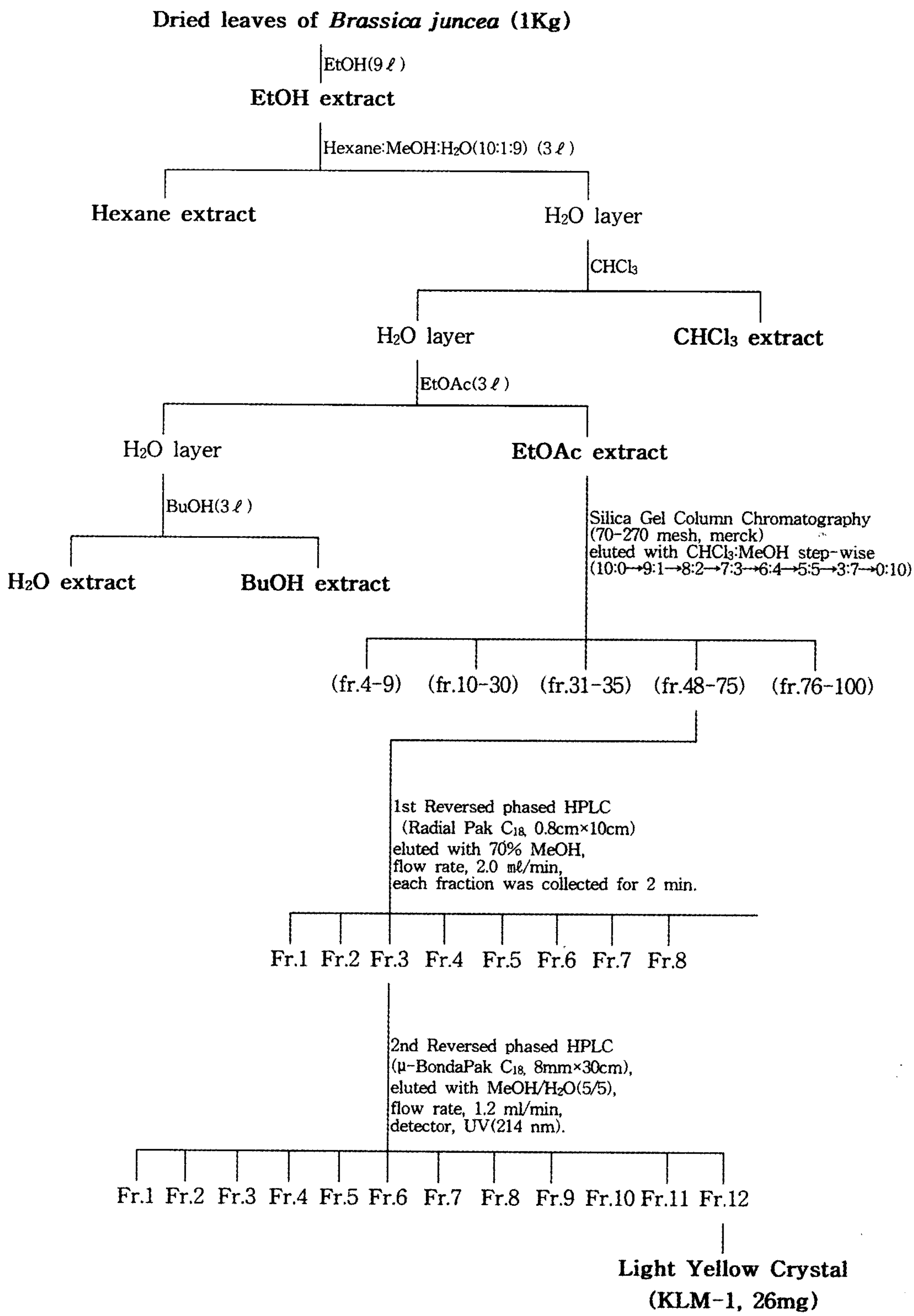

Fig. 1. Purification procedure of antimicrobial substance from Korean traditional leaf mustard (*Brassica juncea* Coss.).

substances having antimicrobial effect are measured according to the disc plate method by measuring the inhibition zone of growth.

A growth inhibitory effect was seen significantly in ethylacetate and butanol fractions acting on all gram positive and negative bacteria, and by chloroform and water fractions although less significantly than in the former two fractions. Then nucleic acid fraction showed less significant antimicrobial effect on *S. aureus, S. typhimurium* and *P. fluorescens* compared with other microorganisms. Especially, when the growth inhibitory effect by ethylacetate fraction on *S. aureus* and *P. fluorescens* was examined, high antimicrobial activities were seen at 18㎜ and 15㎜, respectively. On the other hand, other than the ethylacetate and butanol fractions showed weak antimicrobial activities, the fractions showed almost no antimicrobial activities in lactic acid bacteria. Hexane and chloroform fractions which showed almost no antimicrobial activities in yeasts compared with bacteria and ethylacetate, butanol and water fractions showed weak antimicrobial activities between 9~10㎜.

Hong *et al.* (1990) reported that a butanol fraction from *Ulmus pumila* L. methanol fraction showed a growth inhibitory effect on gram positive bacteria including *S. aureus, S. faecalis, P. aeruginosa* and *B. sp.* and had no effect on the gram negative bacteria E. coli and the fungus *Candida albicans*. However, leaf mustard extract showed an antimicrobial effect on the gram negative bacteria, *E. coli, S. typhimurium* and *P. fluorescens*.

From the above results, it could be concluded that the antimicrobial substance in leaf mustard ethanol extract was not dissolved in a certain solvent but dissolved in other solvents as well; thus, many substances rather than one substance are involved in complex antimicrobial functions. Furthermore, many of the antimicrobial substances in leaf mustard ethanol extract were transferred to the two fractions including ethylacetate and butanol fractions.

Isolation of antimicrobial substances

Silica gel column chromatography

For the purposes of determining the active ingredient in the fractions showing high antimicrobial activities, 2.7g of ethylacetate fraction was fractioned using silica gel column chromatography and its antimicrobial activities on S. *aureus* measured (Fig. 2). The results showed that among 100 fractions passed through the column, a weak antimicrobial activity was seen in fraction numbers 3 to 10 and fraction numbers 30 to 40. A strong antimicrobial activity was seen in fraction numbers 51 to 74. Therefore, the major antimicrobial substance in leaf mustard ethanol extract was mainly present in the ethylacetate fraction. This result is similar to the study by Choi *et al.* who examined active substances involved in drug metabolism in wild edible plants and reported that the ethylacetate fraction contained a significantly higher level of antimicrobial substance compared with the chloroform and butanol fractions of *Allium tuberosum*.

HPLC

After silica gel column chromatography, the evaporated fraction with confirmed antimicrobial activities derived from the ethylacetate fraction was used to perform HPLC to isolate and purify a single substance. The fraction was initially separated through a Radial Pak C18 (8 ㎜ × 10 ㎝) column and the antimicrobial activity of each subfraction was measured according to the paper disc method (Table 4). The results showed that the antimicrobial activity against E. *coli* and S. *aureus* was highest in the four of 8 subfractions obtained using HPLC.

Twelve different substances with confirmed antimicrobial activity (Fig. 3) were isolated when a μ-BondaPak C18 (8 ㎜ × 30 ㎜) column was used to isolate the final antimicrobial substance from the

Table 3. Antimicrobial activities against several microorganisms of fractions from ethanol extract of Korean traditional leaf mustard

Strains	Clear zone on plate (mm)[a]				
	n-Hexane extract (3.0 mg/disc)	Chloroform extract (1.5 mg/disc)	Ethylacetate extract (1.0 mg/disc)	Butanol extract (3.0 mg/disc)	Water extract (3.0 mg/disc)
B. cereus	-[b]	13	14	13	10
B. subtilis	-	12	13	12	10
B. natto	-	12	14	12	10
S. faecalis	-	10	13	12	9
S. aureus	10	15	19	16	9
L. plantarum	-	-	10	9	-
L .brevis	-	-	10	9	-
L. mesenteroides	-	-	10	9	-
P. cerevisiae	-	-	10	10	-
E. coli	-	-	16	14	10
S. typhimurium	11	12	15	14	12
P. fluorescens	11	13	14	13	11
S. cerevisiae	-	-	10	9	9
S. coreanus	-	-	11	9	9
H. anomala	-	-	10	9	9

a) Diameter ; b) No inhibitory zone was formed.

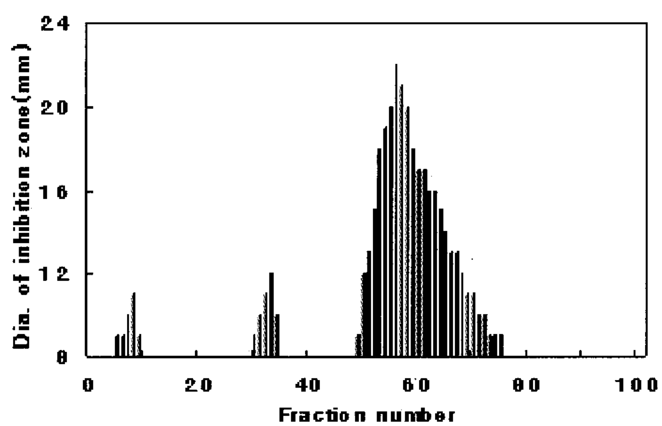

Fig. 2. Antimicrobial activities against *S. aureus* of the fraction of the ethylacetate extract fractionated by the silica gel column chromatography.

Table 4. Antimicrobial activity of fractions of the ethanol extract separated by the HPLC

Fraction No.	Clear zone on plate (㎜)[a)]	
	E. coli	*S. aureus*
1	-[b]	-
2	-	-
3	-	-
4	14	16
5	-	-
6	-	-
7	-	-
8	-	-

a) Diameter; b) No inhibitory zone was formed.

Column, Radial Pak C_{18}(0.8㎝ × 10㎝) ; Solvent, 70% MeOH; Flow rate, 2.0 ㎖/min

Each fraction was collected for 2 min.

Table 5. Antimicrobial activity of each peak of the HLPC fractions separated from the ethanol extract in Fig. 3

Peak No.	Clear zone on plate (㎜)[a)]	
	E. coli	*S. aureus*
3	-[b]	-
11	-	-
12	-	-
13	-	-
14	-	-
15	-	-
16	17	19
17	-	-
18	-	-

a) Diameter ; b) No inhibitory zone was formed.

Column, μ-BondaPak C_{18} column (Φ 8㎜ × 30㎝); Mobile phase, MeOH/H_2O(5/5); Flow rate, 1.2 ㎖/min; Detector, UV(214 ㎚).

Table 6. Antimicrobial activity against *S. aureus* and *E. coli* of ethylacetate fraction and KLM-1

Strains	Clear zone on plate (㎜)[a)]	
	Ethylacetatefraction (1.0 ㎎/disc)	KLM-1 (0.1 ㎎/disc)
E. coli	16	20
S. aureus	16	21

subfraction number 4. After each separated peak was fractionated and vacuum evaporated, the antimicrobial activities were measured using the paper disc method, and Table 5 shows the results. The results of measuring the antimicrobial activities of these 9 substances on *E. coli* and *S. aureus* showed that the antimicrobial effect against these two strains was seen in the peak number 16, with the growth inhibitory zone at 17 ㎜ and 19 ㎜, respectively. Consequently, 26㎎ of light yellow powder was obtained after the peak number 16 was again fractionated and vacuum evaporated.

A single peak was shown when the finally crystals were obtained, and this peak was confirmed using HPLC (Fig. 4). A single peak was shown even when HPLC used a different column and solvent, confirming the fact that a pure substance of a purity higher than 99% had been obtained. This final crystal isolate having antimicrobial activities was named, KLM-1.

When the antimicrobial activities of KLM-1 were confirmed using the ethylacetate fraction, and *S. aureus* and *E. coli*, which are all the strains in which the ethylacetate fraction had an antimicrobial effect, the results showed that the ethylacetate fraction (1.0 ㎎/disc) from leaf mustard ethanol extract showed the growth inhibitory level at 16mm and the antimicrobial substance KLM-1 (0.1 ㎎/disc) at 20 mm. Hence, the antimicrobial substance KLM-1 has a 10 times higher antimicrobial activity compared with the ethylacetate fraction. A further study is being conducted to analyze the structure of the antimicrobial substance KLM-1 using LC/Mass and NMR.

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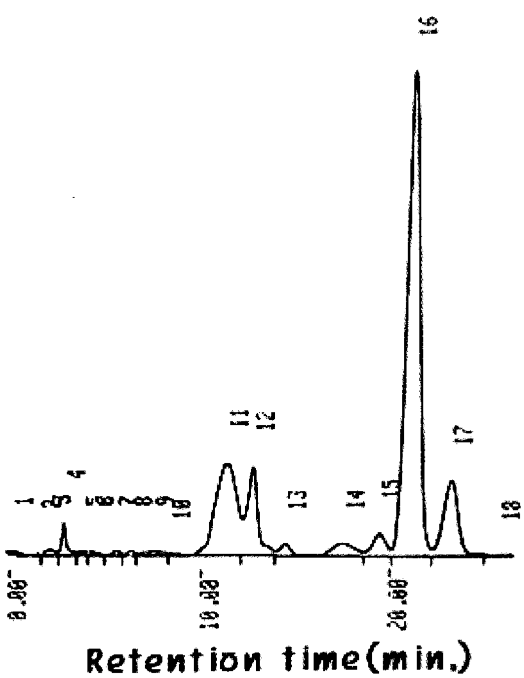

Fig. 3. HPLC chromatogram of fraction No.4 in Table 4 monitored by UV detector.
Column, μ-BondaPak C18 column (Φ 8 ㎜ x 30 ㎝); Mobile phase, MeOH/H2O (5/5); Flow rate, 1.2 ㎖/min; Detector, UV (214 ㎚).

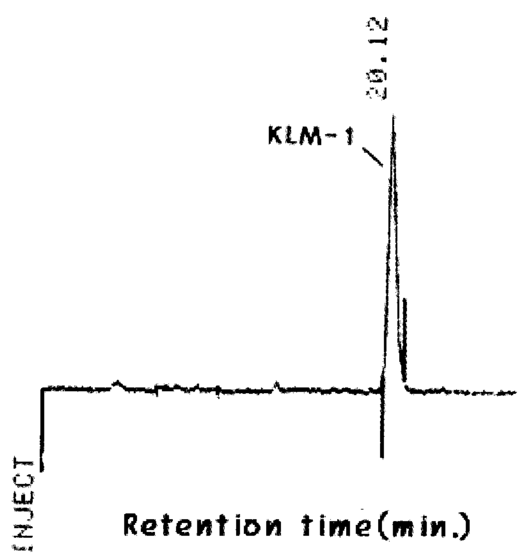

Fig. 4. HPLC chromatogram of KLM-1.
Column, μ-BondaPak C18 column (Φ 8 ㎜ x 30 ㎝); Mobile phase, MeOH/H2O (5/5); Flow rate, 1.2㎖/min; Detector, UV (214 ㎚).

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