Antibacterial Activity of Heated Cabbage Juice, S-Methyl-L-Cysteine Sulfoxide and Methyl Methanethiosulfonate

KYU HANG KYUNG, DUCK CHUL HAN, and HENRY P. FLEMING

ABSTRACT

Autoclaved cabbage juice was inhibitory to growth of *Staphylococcus aureus*. S-Methyl-L-cysteine sulfoxide (SMCSO), autoclaved either together with or separately from nutrient broth, also inhibited the growth of *S. aureus*, but inhibition was greater when SMCSO was autoclaved separately. Methyl methanethiosulfonate ($MMTSO_2$), a thermal breakdown product of SMCSO, completely inhibited growth of *S. aureus* at 10 ppm. $MMTSO_2$ was formed in both autoclaved samples of cabbage juice and aqueous solution of SMCSO. Thus, evidence indicates that the bacterial inhibitory activity in autoclaved cabbage juice was due to heat-induced formation of $MMTSO_2$ from SMCSO.

Key Words: antibacterial activity, autoclaved cabbage, S-methyl-L-cysteine sulfoxide, methyl methanethiosulfonate, *Staphylococcus aureus*

INTRODUCTION

ANTIMICROBIAL ACTIVITY in cabbage juice has been the subject of many studies (Dickerman and Liberman, 1952; Kyung and Fleming, 1994a, b; Little and Grubaugh, 1946; Liu et al., 1986; Pederson and Fisher, 1944; Yildiz and Westhoff, 1981) following the initial demonstration of the activity by Sherman and Hodge (1936). Reports concerning inhibitory activity of cabbage have been conflicting. Sherman and Hodge (1936) and Pederson and Fisher (1944) reported that the antimicrobial substance was destroyed by heating. However, Yildiz and Westhoff (1981) reported that filter-sterilized fresh cabbage juice was a better growth medium for *Leuconostoc mesenteroides* C33 than autoclaved cabbage juice. We observed inhibitory activity in fresh, unheated juice of several cultivars of cabbage (Kyung and Fleming, 1994a) and identified methyl methanethiosulfinate (MMTSO) as the principal antibacterial compound in fresh cabbage (Kyung and Fleming, 1994b). Heating the cabbage before juice extraction prevented formation of the inhibitor(s) in some cultivars, but not others (Kyung and Fleming, 1994a).

MMTSO is formed enzymatically from SMCSO in cabbage juice (Fig. 1; Chin and Lindsay, 1994; Kyung and Fleming, 1994b; Marks et al., 1992). SMCSO, a nonprotein sulfur amino acid, has been reported in *Cruciferae*, including cabbage (Arnold and Thompson, 1962; Kyung and Fleming, 1994b; Mae et al., 1971; Marks et al., 1992; Morris and Thompson, 1956; Pederson and Albury, 1969; Synge and Wood, 1956). SMCSO was not growth-inhibitory (Kyung and Fleming, 1994b, 1996). MMTSO was inhibitory to growth of bacteria (Kyung and Fleming, 1994b; Small et al., 1949). MMTSO spontaneously disproportionates into DMDS and $MMTSO_2$ (Chin and Lindsay, 1994). $MMTSO_2$ thus formed was reported to be equally or slightly more inhibitory than MMTSO to growth of bacteria (Small et al., 1949). SMCSO is known to be hydrolyzed into dimethyl disulfide (DMDS) and $MMTSO_2$ on heating (Fig. 1, Ostermayer and Tarbell, 1960).

Thus, our objective was to test the hypothesis that the antibacterial activity of autoclaved cabbage juice was due to formation of $MMTSO_2$ from SMCSO.

Authors Kyung and Han are with the Dept. of Food Science, Sejong Univ., Kunja-dong, Sungdong-ku, Seoul 133-747, Korea. Author Fleming is with the Food Fermentation Laboratory, USDA-ARS and North Carolina Agricultural Research Service, Dept. of Food Science, North Carolina State Univ. Raleigh, NC 27695-7624.

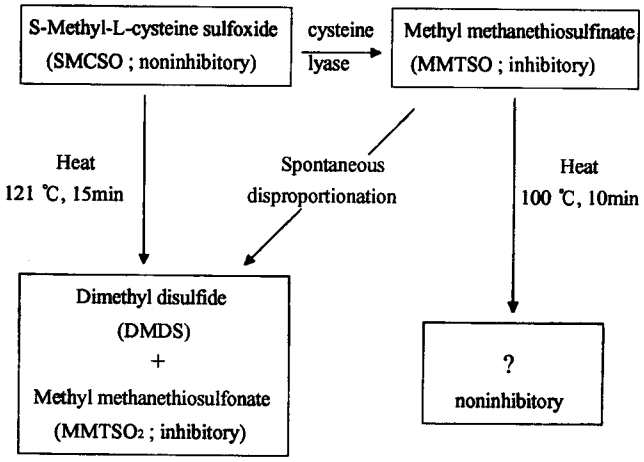

Fig. 1—Proposed degradation products of SMCSO in heated and unheated cabbage juice.

MATERIALS & METHODS

Materials

Methyl methanethiosulfonate ($MMTSO_2$), S-methyl-L-cysteine, and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO). DMDS was purchased from Aldrich Chemical Co. (Milwaukee, WI). Dimethyl trisulfide (DMTS; Eastman Kodak Co., Rochester, NY) was provided by Dr. R.C. Lindsay, Dept. of Food Science, Univ. of Wisconsin-Madison (Madison, WI). Cabbage juice prepared and previously described by Harris et al. (1992) was used to make an autoclaved cabbage juice. The juice was made by passing quartered cabbage (cv. Green Boy purchased from North Carolina State Farmer's Market, Raleigh, NC) through a Fitzmill (model D comminuting machine, the Fitzpatrick Co., Chicago, IL). The resulting pulp was pressed to extract juice which was then filtered through an ultrafiltration unit with a 1×10^6 Mw cartridge (Amicon Corp., Danvers, MA). The juice was frozen and stored at −20°C in glass containers, then was thawed at room temperature before use. Unheated cabbage juice was extracted by an electrical centrifuge-type juice extractor (Braun, Germany) as described by Kyung and Fleming (1994a). Boiled cabbage juice was made by quartering cabbage and steaming (≈ 100°C) it in an autoclave at atmospheric pressure for 10 min.

Bacterial strain and culture condition

Staphylococcus aureus B31 was obtained from the culture collection maintained by the Food Fermentation Laboratory, USDA-ARS, Raleigh, NC. It was stored at −64°C in trypticase soy (TS) broth (BBL Microbiology Systems, Cockeysville, MD) containing 16% glycerol. The frozen stock culture was streaked onto TS agar, and an isolated colony was transferred to nutrient broth (Difco Laboratories, Detroit, MI) before each experiment. A 10 μL (3.0×10^7 cells) aliquot of overnight statically grown culture of *S. aureus* in nutrient broth was inoculated into 10 mL of nutrient broth in 16 mm × 150 mm glass culture tubes. All growth experiments were done at 30°C. Viable cell numbers were determined as colony-forming units (CFU)/mL by spiral plating (Spiral Biotech, Bethesda, MD) onto plate count agar (Difco Laboratories) and incubating at 30°C for 36 hr.

Preparation of S-methyl-L-cysteine sulfoxide (SMCSO)

SMCSO was prepared by oxidizing S-methyl-L-cysteine with peroxide (Lepp and Dunn, 1955). SMCSO thus prepared was confirmed by

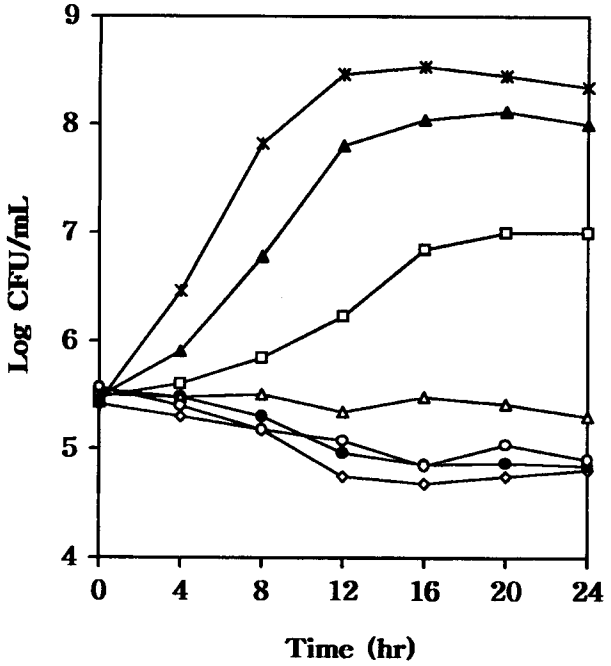

Fig. 2—Growth of *S. aureus* B31 in heated and unheated cabbage juice. Autoclaved cabbage juice (▲, 20; □, 40; △, 60; ●, 80; and ◇, 100%). ○, Unheated cabbage juice and ★, Boiled cabbage juice.

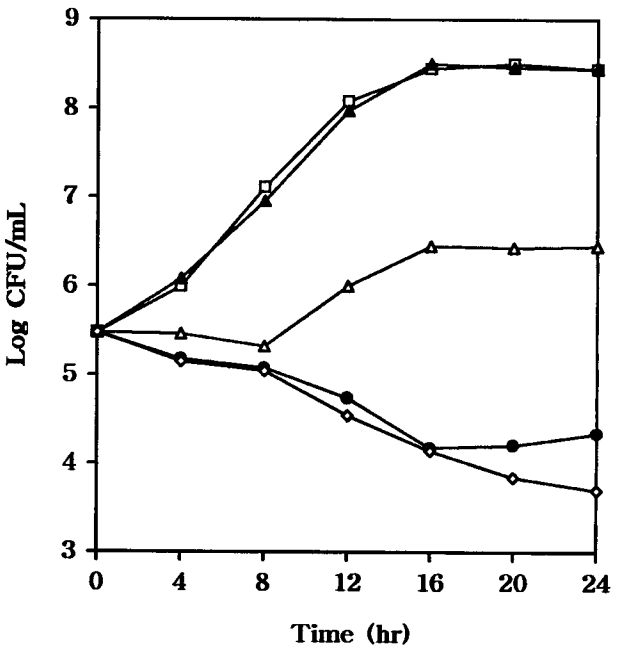

Fig. 3—Growth inhibition of *S. aureus* B31 when SMCSO was autoclaved in nutrient broth. Concentrations of added SMCSO were: ▲, 0; □, 0.025; △, 0.05; ●, 0.075; and ◇, 0.1%.

mass spectrometry (JOEL, HX-110) using fast bombardment with glycerol as the matrix.

Media for growth inhibition studies

Autoclaved (121°C/15 min) cabbage juice was used either undiluted or diluted to 80, 60, 40, 20% of full strength by using sterile distilled water. SMCSO was added to nutrient broth either before or after autoclaving. In the first instance, 0.1% SMCSO was prepared in nutrient broth, diluted using nutrient broth as necessary to yield concentrations of 0.025, 0.050, 0.075, 0.10%, dispensed into glass culture tubes, and autoclaved. In the second instance, 0.02% SMCSO in distilled water and 2X nutrient broth were separately prepared and autoclaved, then aseptically mixed to yield 0.025, 0.050, 0.075, and 0.10% SMCSO in 1X nutrient broth.

A stock solution of 100 ppm $MMTSO_2$ was prepared by dissolving the compound in autoclaved nutrient broth. The solution was filter-sterilized (0.2 μm, Costar bottle filter, Costar Corp., Cambridge, MA), and diluted to 0, 5, 10, 20, 50 ppm with autoclaved nutrient broth.

Separation and identification of $MMTSO_2$

Portions of autoclaved cabbage juice and an autoclaved SMCSO solution were extracted with an equal volume of methylene chloride (Ostermayer and Tarbell, 1960) by magnetic stirring the mixture for 22 hr. Methylene chloride extracts were dewatered over anhydrous sodium sulfate, filtered (Whatman #44, Whatman International LTD., Maidstone, England) and the volume was reduced 400-fold using rotary vacuum evaporator (Eyela, Tokyo Rikakikai Co., LTD., Japan) and flowing nitrogen. Concentrated methylene chloride extracts were stored at −20°C when held before analysis.

Total ion chromatograms (TIC) and mass spectra of volatiles were obtained using a Hewlett-Packard Model 5890 Series II Plus capillary gas chromatograph equipped with a model 5972 mass selective detector (Hewlett-Packard Co., Wilmington, DE). An HP-20 bonded phase, fused silica capillary column (30m × 0.25 mm i.d., df = 0.2 μm) was coupled directly to the MSD capillary interface. The oven temperature was programmed from 40°C to 100°C at 2.4°C/min increase and from 100°C to 200°C at 20°C/min with initial and final temperatures maintained for 1 min and 3 min, respectively. Samples were injected in the splitless mode. The injection port and detector were at 250°C and 280°C, respectively. Electron impact ionization (potential 70 eV) was used and the mass range scanned was 40–200 daltons. $MMTSO_2$, MMTSO, DMDS, DMTS and 1-cyano-2,3-epithiopropane were identified by both retention time and mass spectra. 1-Cyano-3-methylthiopropane was identified by comparing experimental with published mass spectra (Spencer and Daxenbichler, 1980).

RESULTS & DISCUSSION

Growth of *S. aureus* in autoclaved cabbage juice

Autoclaved cabbage juice undiluted or diluted with distilled water to 60% juice completely inhibited growth of *S. aureus* (Fig. 2). When the inhibitory autoclaved cabbage juice was diluted further (40 or 20% cabbage juice), *S. aureus* grew. Growth rates were faster and total cell populations were higher for *S. aureus* at lower concentrations of autoclaved juice (Fig. 2). *S. aureus* grew well in boiled juice but not in unheated juice. This indicated that the inhibitory compound (MMTSO) in unheated juice was destroyed during boiling and that an inhibitory compound (presumably $MMTSO_2$) was newly generated during autoclaving. The inhibition of bacterial growth in autoclaved cabbage juice was not due to lack of essential nutrients caused by thermal destruction, but due to the presence of an inhibitory compound(s) generated during autoclaving, since *S. aureus* grew well in diluted autoclaved cabbage juice. The inhibitory action of unheated fresh cabbage juice was shown to be destroyed on boiling (Pederson and Fisher, 1944; Sherman and Hodge, 1936). Yildiz and Westhoff (1981), however, reported that autoclaved cabbage juice was more inhibitory to the growth of *L. mesenteroides* C33 than unheated juice. We have observed that boiled cabbage juice, as well as unheated juice, was antimicrobial (Kyung and Fleming, 1994b).

Growth inhibition by autoclaved SMCSO

Growth of *S. aureus* was completely inhibited when SMCSO was autoclaved together with nutrient broth, and when its concentration was >0.075%. Autoclaved SMCSO at 0.025% in nutrient broth was not growth inhibitory. At 0.05%, autoclaved SMCSO in nutrient broth initially inhibited bacterial growth and later resulted in slight growth of *S. aureus* (Fig. 3).

When SMCSO and nutrient broth were separately autoclaved and mixed, the growth inhibition was greater than when they were autoclaved together (Fig. 3 and 4). In this case, 0.05% SMCSO completely inhibited growth of *S. aureus*. When

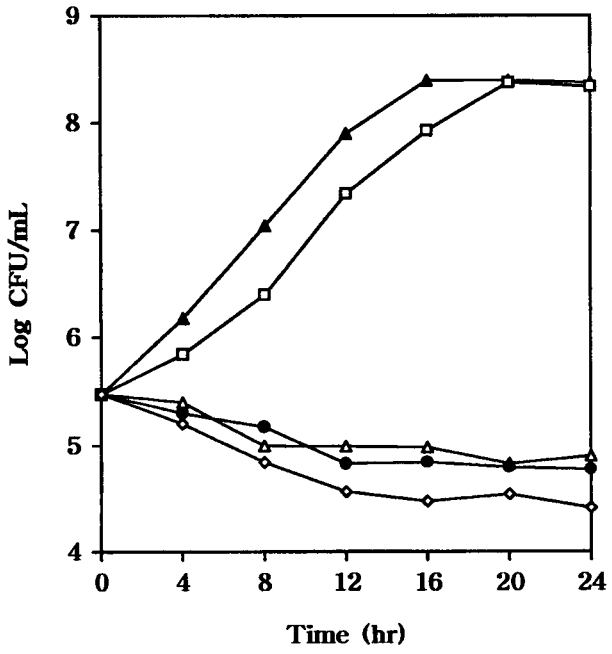

Fig. 4—Growth inhibition of *S. aureus* B31 when SMCSO was autoclaved separately and then added to nutrient broth. Concentrations of added SMCSO were: ▲, 0; □, 0.025; △, 0.05; ●, 0.075; and ◇, 0.1%.

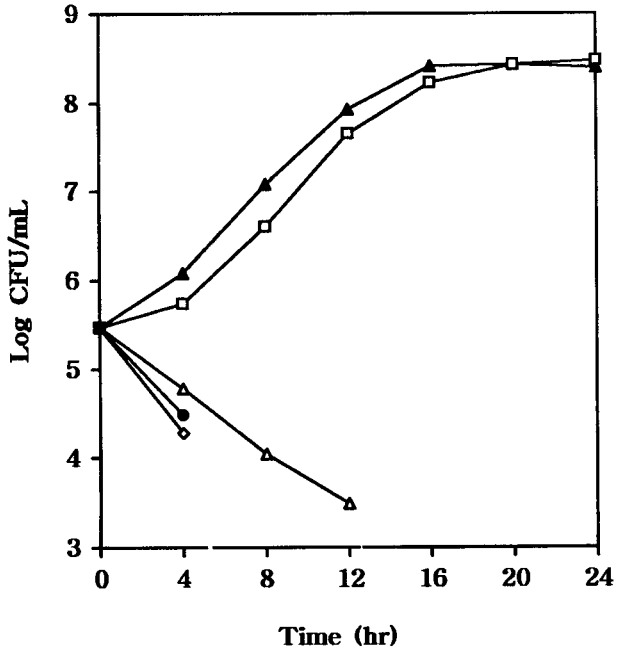

Fig. 5—Effect of $MMTSO_2$ on the growth of *S. aureus* B31 in nutrient broth. Concentrations of added $MMTSO_2$ were: ▲, 0; □, 5; △, 10; ●, 20; and ◇, 50 ppm.

SMCSO was added before autoclaving to TS broth which contained more nutrients than nutrient broth, 0.2% SMCSO was necessary to completely inhibit the growth of *S. aureus* (data not shown). We therefore assumed that SMCSO reacted with substances in nutrient broth or TS broth either before or after it was degraded into growth inhibitory compounds. It could also be that TS broth contained substances which inhibit the reaction that results in formation of $MMTSO_2$, so a higher concentration of SMCSO was needed to provide enough product for notable growth inhibitory effect.

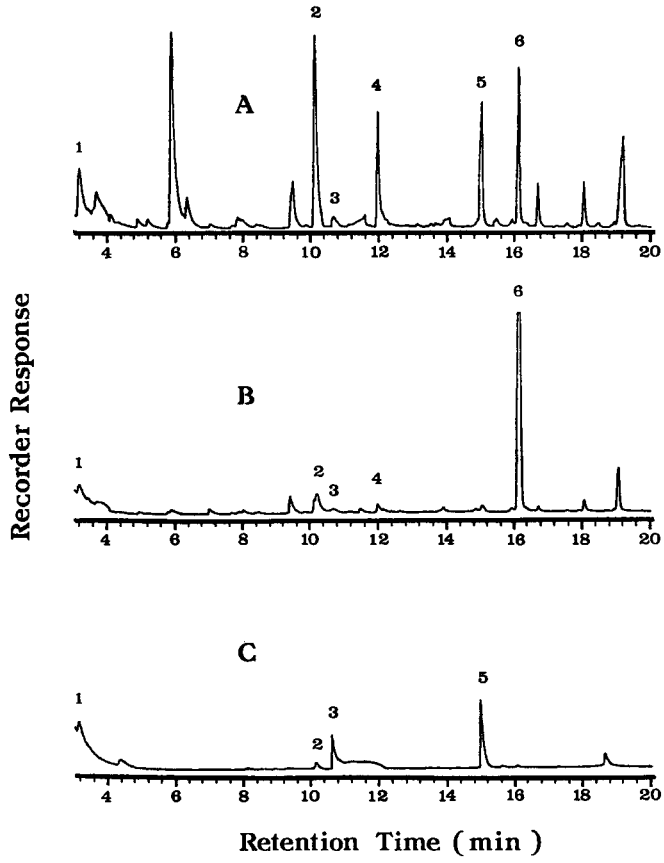

Fig. 6—(A) TIC of autoclaved cabbage juice: (A) juice shown to be growth inhibitory; (B) juice shown not to be growth inhibitory; and (C) an autoclaved 0.05% SMCSO solution 1 (DMDS), 2 (DMTS), 3 (MMTSO), and 5 ($MMTSO_2$) are SMCSO degradation products; 4 (1-cyano-2,3-epithiopropane) and 6 (1-cyano-3-methylthiopropane) are glucosinolate degradation products.

Table 1—Concentration of $MMTSO_2$ in methylene chloride extracts of autoclaved cabbage juices

Cabbage juice	Cultivar	$MMTSO_2$ (ppm)	Antibacterial activity[a]
A	Unknown	0.31	Not inhibitory
B	Unknown	0.47	Not inhibitory
C	Unknown	0.59	Not inhibitory
D	Green Boy	5.40	Inhibitory

[a] Growth or non-growth of *S. aureus* in undiluted, autoclaved cabbage juice at 30°C for 24 hr.

Growth inhibition of $MMTSO_2$

Ten ppm (0.001%) $MMTSO_2$ in nutrient broth completely inhibited growth of *S. aureus* (Fig. 5). In that case, the number of surviving bacteria was reduced to an undetectable level between 8 and 16 hr incubation. Five ppm $MMTSO_2$ slightly inhibited growth of *S. aureus*. Reported minimum inhibitory concentrations (MIC) of $MMTSO_2$ for *S. aureus* were 5 ppm in beef extract broth (15 hr at 37°C; Small et al., 1949) and 50 ppm in TS broth (48 hr at 30°C; Kyung and Fleming, 1996). Since the MIC in our experiment was 10 ppm in nutrient broth, its antibacterial activity seems to be very much dependent on the test medium.

Presence of $MMTSO_2$ in autoclaved cabbage juice and in SMCSO solution

Both autoclaved cabbage juice (antibacterial and non-antibacterial) and autoclaved SMCSO solution contain $MMTSO_2$ (Fig.

6). $MMTSO_2$ had a retention time of 15.04 min. The mass spectrum of $MMTSO_2$ from cabbage juice matched well the published spectra and that of the standard compound. The proportions (%) of individual ion fragments for authentic $MMTSO_2$ were: 45 (85), 47 (100), 63 (87), 79 (70), 81 (91), 126 (44).

Methylene chloride extracts of cabbage juice with strong antibacterial activity showed a higher concentration of $MMTSO_2$ than that with no inhibitory activity (Table 1, Fig. 6A,B). $MMTSO_2$ was the major degradation product of SMCSO in aqueous solution (Fig. 6C). Other secondary degradation products were: MMTSO, dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and other unidentified compounds. DMDS and DMTS inhibited growth of *S. aureus* B31 very slightly, with an MIC of >500 ppm (Kyung and Fleming, 1996). MMTSO, the principal antibacterial compound in unheated cabbage, was found in small quantities both in growth inhibitory and uninhibitory heated cabbage juice. The MIC of both MMTSO and $MMTSO_2$ for *S. aureus* was 50 ppm in TS broth (Kyung and Fleming, 1996). Those compounds other than $MMTSO_2$ found in cabbage juice may exert inhibitory action on bacterial growth. 1-Cyano-2,3-epithiopropane (peak 4 of Fig. 6A), a sinigrin degradation product, was not antimicrobial (Kyung and Fleming, unpublished data). However, we believe that $MMTSO_2$, a major thermal degradation product of SMCSO, is the most important antibacterial compound in autoclaved cabbage juice.

CONCLUSIONS

THE ANTIBACTERIAL ACTIVITY of autoclaved cabbage juice is hypothesized to be due to $MMTSO_2$, thermally generated from SMCSO, a naturally occurring amino acid in *Cruciferae*. The presence of $MMTSO_2$ in autoclaved cabbage juices and autoclaved SMCSO solution was demonstrated. Growth inhibitory cabbage juice had more $MMTSO_2$ than uninhibitory cabbage juice, suggesting more SMCSO was present in inhibitory than in uninhibitory juice. SMCSO appears to be the precursor of the antibacterial compound in autoclaved cabbage juice ($MMTSO_2$), as was previously shown to be the case with unheated cabbage juice (MMTSO).

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