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Antimicrobial Activity of Oligomer and Polymer Chitosan from Different Sources against Foodborne Pathogenic Bacteria

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ABSTRACT

The antibacterial activities of oligomer and polymer chitosan from different sources (shrimp, crab and squid) were examined against foodborne pathogenic bacteria. Sixteen tested foodborne microorganisms comprised of eight Gram-positive bacteria—namely, *Listeria monocytogenes* strains 101, 310, 108, Scott A and V7, *Staphylococcus aureus, Bacillus cereus* and *Bacillus subtilis* and eight Gram-negative bacteria—namely, *Vibrio parahaemolyticus* strains BCC 24339, FS 004, TDH 293, TDH 330 and FS 015, *Escherichia coli, Salmonella* Weltevreden and *Salmonella* Typhimurium. The minimum inhibitory concentration (MIC) of the chitosans was analyzed by an agar dilution method and the minimum bactericidal concentration (MBC) was analyzed by a broth dilution method. Polymer chitosan from crab showed the best antilisterial activity while polymer chitosan from squid showed the best efficiency for inhibiting *S. aureus*, *B. cereus* and *B. subtilis*. Oligomer chitosan from squid showed the strongest antibacterial activity on *V. parahaemolyticus*. However, *S.* Weltevreden was the most resistant to the chitosan groups (MIC = 0.15%v/v). In addition, chitosan with a degree of deacetylation (DD) 98.01% had the strongest antimicrobial activity against the growth of *L. monocytogenes* and *B. cereus*. The antibacterial activity against *B. cereus* was enhanced with increasing DD. Therefore, the antimicrobial activity of chitosan depends on the target microorganism, chitosan source, molecular size and DD. These observations suggested that the source, DD and molecular size of chitosan must be chosen selectively to control the target foodborne pathogens.

Keywords: chitosan, polymer, oligomer, shrimp, crab, squid, foodborne pathogens

INTRODUCTION

Nowadays, consumers recognize the importance of food safety and especially contamination by foodborne pathogenic microorganisms and the potential harm of various artificial food preservatives. The awareness of foodborne pathogenic bacteria contamination in food has arisen because it has been a major cause of morbidity and mortality worldwide (WHO, 2010) through such potent pathogenic bacteria as *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* spp. (CDC, 2005). Moreover, *Vibrio parahaemolyticus* is the most

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frequent pathogenic strain causing foodborne disease in Thailand (Bureau of Epidemiology, 2010).

Even though common food preservatives such as nitrite and sodium benzoate have a long history of safe use (Gould and Russell, 2003) and they play an important role in maintaining the quality of food and inhibiting the growth of various spoilage and pathogenic bacteria, the formation of potentially carcinogenic by-products (for example, nitrosamines from nitrite) and the potential effects of preservatives on health have raised concerns. Thus, it is desirable that new, nontoxic natural preservatives be studied and developed in order to improve the quality and extend the shelf life of foodstuffs without compromising the microbial safety or sensory properties so that consumers are reassured about food safety.

Chitosan, a linear polysaccharide consisting of (1,4)-linked 2-amino-deoxy-β-D-glucan (Dutta *et al.,* 2009), is derived by the deacetylation of chitin. Chitosan is a natural antimicrobial agent found in the shells of crustaceans, such as crab, shrimp, squid pen and crawfish (No *et al.,* 2002). The mechanism of chitosan as an antimicrobial compound is not well understood. However, when chitosan is dissolved in acid, it forms an ammonia group which is attached to the phosphoryl group of phospholipids at the bacterial cell membrane. In addition, it causes a hole in the cell membrane leading to cytoplasmic leakage and causing cell death (Raafat *et al.,* 2008). Furthermore, chitosan could attack the cell nucleus and then interrupt DNA transcription, and mRNA and protein synthesis, thus impairing multiplication (Zivanovic *et al.,* 2004).

The antimicrobial efficiency of chitosan varies depending on four factors: 1) microbial factors which relate to the species of microorganism and cell age; 2) the intrinsic factors of chitosan, involving positive charge density, molecular weight, concentration, hydrophilic/hydrophobic characteristics and chelating capacity; 3) the physical state, including the water-soluble and solid state of chitosan; and 4) environmental factors, including the ionic strength in the medium, pH, temperature and reactive time (Kong *et al*., 2010). There is an extensive amount of literature on studies of intrinsic factors that supports the essential importance of a polycationic structure in antimicrobial activity (Kong *et al*., 2008; Takahashi *et al*., 2008) and numerous studies on the correlation between the molecular weight of chitosan and its antimicrobial activity (Uchida *et al.,* 1989; Jeon *et al.,* 2001; No *et al*., 2002; Tikhonov *et al*., 2006). However, specific information is lacking on the antibacterial activity of chitosan from different sources. Therefore, the objective of this research was to examine the antimicrobial activity of polymer and oligomer chitosan from different sources of shrimp, crab and squid against foodborne bacteria.

MATERIALS AND METHODS

Sources of chitosans

The chitosans were characterized from three different sources (prepared from shrimp, crab crust and squid pen), with each source having two molecular sizes (oligomer and polymer). The molecular weight of the oligomer and polymer chitosans ranged from 700 to 1,700 KDa and from 25 to 80 KDa, respectively. The molecular weight and the degree of deacetylation (DD) of each chitosan are shown in Table 1. The DD was more than 95% in flake form except for the oligomer chitosan from squid in a powder form. All six chitosans were obtained from Ta Ming Enterprises Co., Ltd, Samut Sakhon, Thailand.

Tested microorganism

Sixteen bacterial strains were tested for the antibacterial activity of the chitosans. These bacteria consisted of eight gram positive bacteria—namely, *L. monocytogenes* strain 101, 310, 108, Scott A and V7, *S. aureus, B. cereus* and

Table 1 Sources of chitosans and their characteristics.

Sources	Molecular size	Form of chitin*	Molecular weight (KDa)	Degree of deacetylation (%)
Shrimp	Oligomer	α-chitin	30 – 50	97.15
	Polymer	α-chitin	1,000 – 1,500	95.78
Crab	Oligomer	α-chitin	25 – 40	96.66
	Polymer	α-chitin	700 – 1,000	97.38
Squid	Oligomer	β-chitin	50 – 80	95.70
	Polymer	β-chitin	1,200 – 1,700	98.01

* Source: Peniche *et al.* (2008)

B. subtilis obtained from the Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University and eight Gram-negative bacteria—namely, *V. parahaemolyticus* strain BCC 24339 (pathogenic *tdh*-gene positive, shrimp isolates) obtained from the National Center for Genetic Engineering and Biotechnology (BIOTEC) and strains FS 004, FS 015, TDH 293 and TDH 330 (patient isolates) obtained from the Faculty of Tropical Medicine, Mahidol University, *E. coli* 780, *S.* Weltevreden and *S.* Typhimurium ATCC 13311 were bought from the Department of Medical Sciences, Ministry of Public Health.

B. cereus and *B. subtilis* were grown in a trypticase soy broth (TSB; Merck Labolatories, Darmstadt, Germany) at 30 °C for 24 h for two successive times and kept 18 h before use. The same procedure using TSB supplemented with 0.6% yeast extract was used for *L. monocytogenes*. *S. aureus, E. coli, S.* Weltevreden and *S.* Typhimurium were grown in TSB and *V. parahaemolyticus* was grown in TSB supplemented with 3% NaCl at 37 °C for 24 h for two successive times and kept 18 h before use.

Minimum inhibitory concentration (MIC) determination

The antibacterial activity of the chitosans was assayed by preparing chitosan solutions at a concentration of 1% (w/v) in 1% (v/v) acetic acid. A number of test tubes each containing 5 mL of Müller-Hinton Agar (MHA; Merck) or MHA+NaCl (for *V. parahaemolyticus*) at double strength concentration was mixed with chitosan solution and distilled water at pH 6.3 (adjusted with 1N HCl and 1N NaOH) to give a final chitosan concentration in the range between 0.05 to 0.15% (v/v) into 10 mL. The pH of each concentration was approximately 5.8. After mixing, the solution was poured onto plates and left until the agar had set. Each bacterium culture which specified growth duration at 18 h was diluted with 0.1% peptone to get the cell concentration to about 7 log CFU/mL. One microliter of each bacterium culture was dropped on the agar in 5–6 circles (1 microliter/circle) by micropipette (so that the space on the agar allocated to each circle was equal) and allowed to dry. The beginning cell culture on the agar was approximately 4 log CFU/mL. Then, the agar was incubated at 37 °C. The growth of bacteria was determined at 24 h after incubation. The growth of bacteria from the colony density was compared to the control which consisted of the same bacterium cultured under the same conditions on MHA or MHA+NaCl without adding the chitosan solution. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antimicrobial concentration required to inhibit the visible growth of the bacterium after incubation for 24 h (Marques *et al.*, 2008). All experiments were repeated in triplicate and duplicated in each experiment.

Minimum inhibitory concentration (MBC) determination

The antibacterial activity of the chitosans was assayed by preparing chitosan solutions at a concentration of 1% (w/v) in 1% (v/v) acetic acid. Test tubes containing 5 mL of each bacterium (4–5 log CFU/mL) were diluted with 0.1% peptone and Müller-Hinton Broth (MHB; Merck) or MHB+NaCl (for *V. parahaemolyticus*) at double strength concentration. Then 5 mL of chitosan solution and distilled water at pH 6.3 (adjusted with 1N HCl and 1N NaOH) were mixed into a test tube to give a final concentration in the range from 0.025 to 0.15% (v/v). After mixing, the pH of each concentration was approximately 5.8 and the test tubes were incubated at 37 °C for 24 h with shaking at 100 rpm. The amounts of bacteria were examined using the spread plate method. *L. monocytogenes* was grown on trypticase soy agar (TSA; Merck) supplemented with 0.6% yeast. *B. cereus, B. subtilis, S. aureus, E.coli, S.* Weltevreden and *S.* Typhimurium were grown on TSA. *V. parahaemolyticus* was grown on TSA supplemented with 3% NaCl. The control set used the same bacteria grown in MHB under the same conditions without adding chitosan. The minimum bactericidal concentration (MBC) was the lowest concentration of antimicrobial required to diminish more than 99.99% of the initial bacterial amount at 24 h (Marques *et al.,* 2008). All experiments were repeated in triplicate and duplicated in each experiment.

RESULTS AND DISCUSSION

MIC value, sources of chitosan and molecular size

The MIC values ranged from 0.04 to 0.09% for the Gram-positive bacteria (Figure 1A) and from 0.03 to 0.15% for the Gram-negative bacteria (Figure 1B). Moreover, each type of bacteria was sensitive to different types of chitosan. *L. monocytogenes* was sensitive to polymer chitosan from crab (MIC = 0.05%). *S. aureus*, *B. subtilis* and *B. cereus* were sensitive to polymer chitosan from squid (MIC range from 0.05 to 0.06%). *V. parahaemolyticus* was sensitive to oligomer chitosan from squid (MIC range from 0.03 to 0.04%) as shown in Figure 1B. However, the inhibitory efficiency in various sources of chitosans on *S.* Welterveden did not differ (MIC = 0.15%). Among the bacteria tested, *V. parahaemolyticus* was the most sensitive to chitosans while *S.* Weltevreden was the most resistant to chitosans.

Even though the bacterial cell walls of Gram-negative and Gram-positive bacteria are different, antimicrobial modes of action for both begin with interaction at the cell surface, involving the cell wall or outer membrane at first. For Gram-positive bacteria, lipoteichoic acid which is anchored into the outer leaflet of the cytoplasmic membrane via a glycolipid could provide a molecular link to chitosan at the cell surface, allowing it to disturb membrane functions (Raafat *et al.*, 2008). For Gram-negative bacteria, lipopolysaccharide and proteins in the outer membrane are held together by electrostatic interactions with divalent cations that are required to stabilize the outer membrane. Under the studied conditions, where the pH of 5.8 was below the pKa of chitosan (6.3–6.5), polycations of chitosan may compete with divalent metals and cause disruption of the cell wall integrity (Kong *et al.*, 2010).

Based on the source of the chitosan, chitosan from squid (both oligomer and polymer) had the best antimicrobial activity against *V. parahaemolyticus*, particularly strains BCC24339 and TDH 330 (Figure 1B). This observation related to the differences in the crystallinity of the polymorphs of chitosan, as the chitosan from squid was prepared from β-chitin which is commonly found in squid pen. Chitosan from shrimp and crab was prepared from α-chitin which is the most stable and ubiquitous form, with its molecular

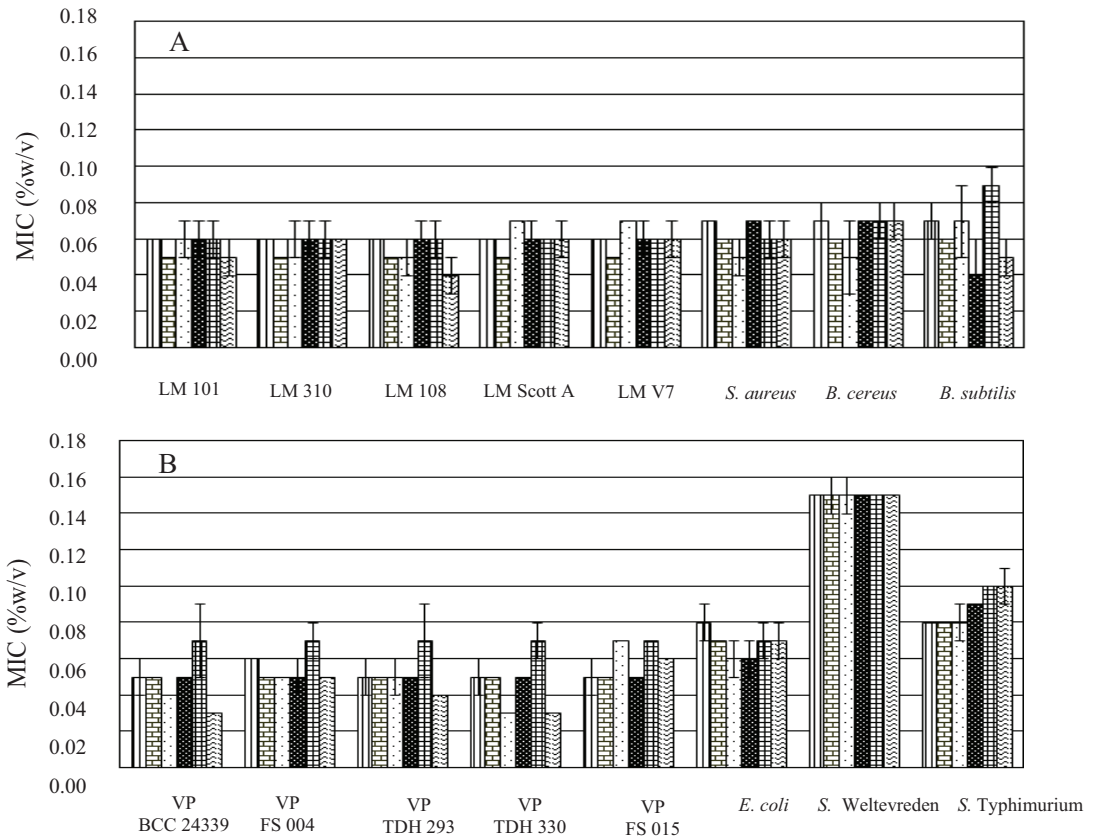

Figure 1 MIC values of chitosans from various sources against: (A) Gram-positive; and (B) Gram-negative bacteria. Error bars represent ± standard deviation of triplicate sample.

Shrimp polymer Crab polymer Squid polymer

Shrimp oligomer Crab oligomer Squid oligomer

LM = *Listeria monocytogenes*; VP = *Vibrio parahaemolyticus*.

chains in an antiparallel arrangement, while β-chitin chains are arranged in a parallel structure (Peniche *et al*., 2008). These two different forms may affect the electrostatic interaction among bacterial cell walls in different ways. Their mode of action should be further investigated.

In addition, chitosans from the same source with different molecular size had different inhibitory effects against Gram-positive and Gram-negative bacteria. However, the source and molecular size of chitosan had no effect on *S*. Weltevreden (Figure 1B). Recent studies on the antimicrobial activity of oligomer and polymer chitosan have revealed that polymer chitosan was more effective in inhibiting the growth of bacteria than oligomer chitosan (Uchida *et al*., 1989; Jeon *et al*., 2001; No *et al*., 2002; Tikhonov *et al*., 2006) due to chitosan acting as an outer membrane disruptor rather than as a penetrating material (Raafat *et al*., 2008).

Degree of deacetylation (DD)

In the present experiment, oligomer chitosan from crab with the highest DD (DD = 98.01%) had the strongest antimicrobial efficiency against the growth of Gram-positive bacteria

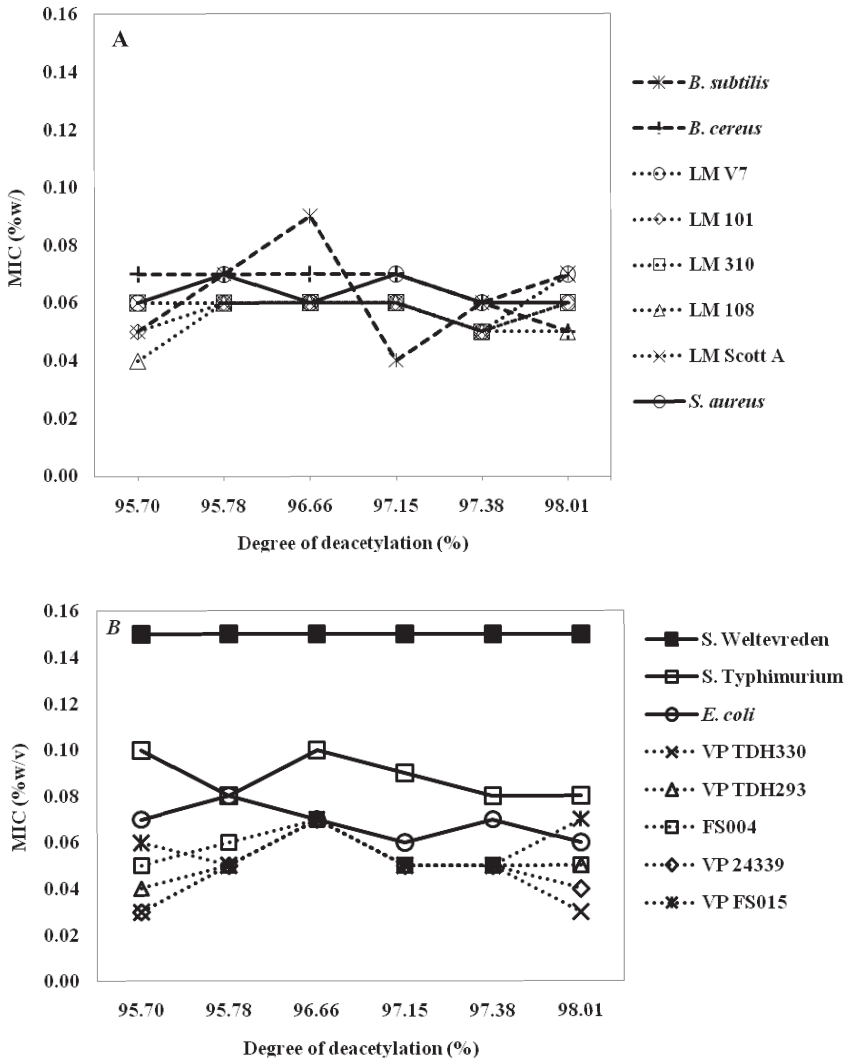

Figure 2 MICs of chitosans with degree of deacetylation against: (A) Gram-positive; and (B) Gram-negative bacteria. LM = *Listeria monocytogenes*; VP = *Vibrio parahaemolyticus*.

(*L. monocytogenes* and *B. cereus*) as shown in Figure 2A. In particular, in *B. cereus*, the antimicrobial activity was enhanced with an increase in the DD. According to the results of Takahashi *et al*. (2008), chitosan with a higher DD successfully inhibited the growth of *S. aureus*. Similarly, Sekiguchi *et al*. (1994) reported that highly deacetylated chitosans showed more antimicrobial effect than lower deacetylated chitosan, due to the increased solubility and higher positive charge density that led to a strong electrostatic interaction. Therefore, the source, DD and molecular size of chitosan must be chosen selectively to control the target foodborne pathogens.

MBC value

The MBC value in Gram-positive bacteria ranged from 0.07 to 0.09% and from 0.04 to 0.12% for Gram-negative bacteria (Figures 3A and 3B). The MBC value was higher than the MIC value for each bacterium because the MBC value

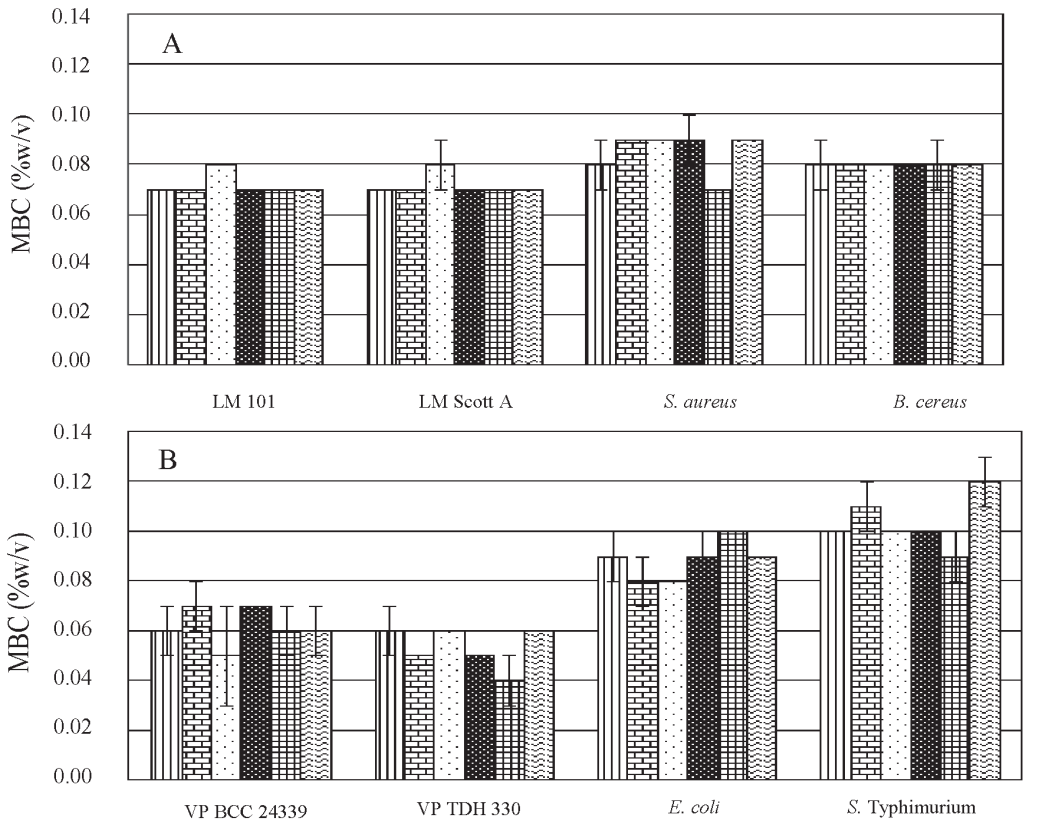

Figure 3 MBC values of chitosans from various sources against: (A) Gram-positive; and (B) Gram-negative bacteria. Error bars represent ± standard deviation of triplicate samples.

Shrimp polymer Crab polymer Squid polymer

Shrimp oligomer Crab oligomer Squid oligomer

LM = *Listeria monocytogene*; VP = *Vibrio parahaemolyticus*.

indicates the concentration to destroy the bacterium while the MIC value indicates the concentration to only inhibit the growth of the bacterium.

CONCLUSION

Polymer chitosan from crab showed the best antilisterial activity while polymer chitosan from squid showed the best efficiency for inhibiting *S. aureus*, *B. cereus* and *B. subtilis*. Moreover, oligomer chitosan from squid showed the strongest antibacterial activity on *V. parahaemolyticus*. Based on these results, the source, DD and molecular size of chitosan must be chosen selectively to control the target foodborne pathogens. Furthermore, the MIC and MBC values of the chitosan sources investigated can be applied in the food industry for various purposes such as a natural food preservative to extend the shelf life of food.

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