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Original Article

Effect of Kitocohol[R] on Proliferation of Virulent Bacteria

Hideo Hayashi, Yoshie Manabe, Sanae Ko and Izumi Koujima*

Department of Human Nutrition, Faculty of Contemporary Life Science
Chugokugakuen University, Okayama 701-0197, Japan
**Shujitsu Junior College, Okayama 703-8516, Japan*

Antiseptic activity of Kitocohol, a commercially developed reagent intended for use as an antiseptic for foodstuffs, was examined in term of effect on the proliferation of virulent bacteria in the presence of the reagent in culture media. It was effective to inhibit proliferation of gram positive bacteria, *S. aureus, B. subtilis, E. faecalis* and *C. perfringens* at the concentration of 0.1% in liquid media as well as on solid media. The effect was bactericidal and the effectiveness lasted stably for 4 weeks in both liquid media and solid media if it was kept in a well sealed bag. Gram negative bacteria, *E. coli, V. parahemolyticus, S. enteritidis* and *P. aeruginosa* were less sensitive to the reagent even at the concentration of over 1% in the media. An antiseptic factor in the reagent could be chitosan, and the application for practical usage of Kitocohl was suggested.

Key Words: Kitocohol[R], chitosan, virulent bacteria, bactericidal effect, antiseptic.

Introduction

Kitocohol is commercially developed reagent (Jinro Distillers Co. LTD) intended for use as antiseptic for foodstuffs. The reagent is an acidic solution of chitosan containing: 2.98g of chitosan, 2.45g of glycerin, 1.0g of fermentative alcohol, 0.1g of Vitamin C and 1.0g of citric acid in 100ml of distilled water pH 4.2. Chitosan appears to be the major factor affections on microorganisms, and the other additives may help chitosan to dissolve in the water phase. Chitosan is a deacethylated product of chichin which is a fibrous polysaccharide (more than 5,000 molecules of β-1,4-polyglucosamin) extracted from shellfish [1]. Purified chitosan is a white fibrous aggregate that does not dissolve readly in water in solvents, but becomes soluble if it is deacethylated and decomposed to a smaller molecular weight of less than 10,000Da, which is called water soluble chitosan. Kitocohol may contain such small variable size molecule of chitosan (details are not available because of the commercial patent).

It would be a good idea if Kitocohol were applicable as a food preservative or antiseptic for food. Chitosan, a kind of wastely, is a natural product of chichin which is abundant in shellfish and has been proven to be non-toxic to human. If chitosan is effective on virulent organisms in reducing the number of contaminated pathogens or depressing the proliferation of such organisms in stored foods. Chitosan has been recognized to be an active biological agent that affects proliferation of epithelial cells, enforcement

Corresponding author.
Hideo Hayashi
Department of Human Nutrition, Faculty of Contemporary Life Science
Chugokugakuen University, Okayama 701-0197, Japan
Tel & FAX;

of immune system, inhibition of cancer cell proliferation, reduction of serum cholesterol content, inhibition of microorganism proliferation. Antibiotic activity of chitosan has been reported on fungi and some bacteria, such as *E. coli* or *S. aureus* [2]. The molecular mechanism of how chitosan acts on bacteria has not been well documented, but it has been suggested that poly-glucose-amine may be a major component that binds to the bacterial cell wall to cause aggregation of the organisms or lyses of the cells [3].

In this report, the effect of Kitocohol on pathogenic and opportunistic pathogenic bacteria is examined to determine if it can be applicable to disinfect virulent agents in foodstuffs or to protect fresh foods from decomposition caused by microbial proliferation.

Material and Method

Bacterial strains: Laboratory stocked strains of *Bacillus subtilis, Staphylococcus aureus, Clostridium perfringens, Enterococcus faecalis, Eschrichia coli, Salmonella enteritidis, Pseudomonas aeruginosa, Vibrio parahemolyticus* were subjected to the experiments. For the culture media, LB broth and LB agar (Nakarai Tesque, Kyoto), nutrient agar and nutrient broth (Nissui Co. Tokyo) were used for the cultures of all the strains except for *V. parahemolyticus* and *C. Perfringens. V. parahemolyticus* was cultured on TCBS (Nissui Co. Tokyo) agar or LB broth supplemented with 3% sodium chloride. *C. perfringens* was cultured on GAM medium (Nissui Co. Tokyo) in anaerobic condition using Gas-Pack (Eiken Co. Tokyo). All the strains were pre-cultured in the liquid medium over night and the bacterial concentration was adjusted to $1\sim5\times10^8$ CFU/ml with broth. Kitocohol (Jinro Distillers Co. LTD) was added to the liquid media at the adequate concentration as indicated. For the test on agar plate, the indicated concentration of Kitocohol solution was either sprayed by aerosol 0.1ml or the solution was spead evenly on the plate to coat the surface. 0.1ml of the cultures were inoculated onto the plates.

The number of viable bacteria was measured by conventional colony forming unit (CFU) assay [4] Namely, 0.5ml culture was taken from the cultured tubes and it was diluted serially ten-fold with broth, then 50 μl of the diluted culture was inoculated on agar plates and cultured at 37℃ for over night. The number of colony on the plates were counted.

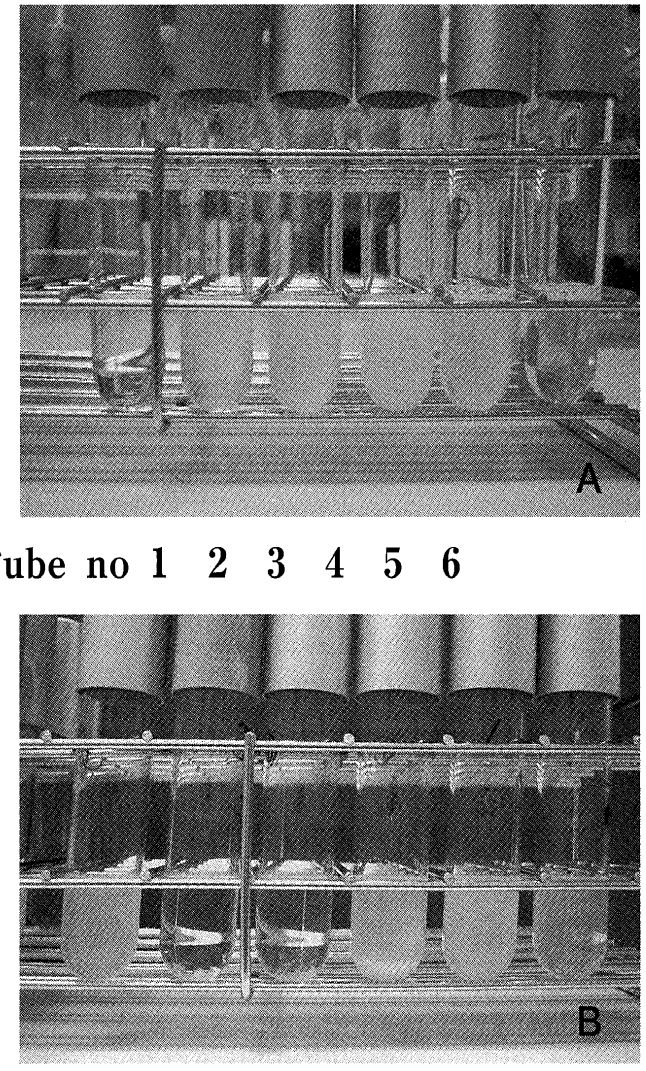

Fig. 1 Effect of Kitocohl in LB broth before and after incubation at 37℃ with and without bacterial inoculation.
Panel A,. tubes from left to right, tube No 1: none, No 2: 0.05%, No 3: 0.1%, No 4: 1.0%, No 5: 5% and No 6: 10% of Kitocohl was added, respectively. Panel B same order as panel A but after bacterial inoculation and cultured for 12h at 37 degree. Before incubation Kitocohl caused turbidity at the concentration 0.05 to 5% but at the 10% it was transparent. After incubation, the culture media became transparent at the concentration of 0.05% to 0.1% forming precipitation at the bottom on the tubes, 1% and 5% remained turbid with some precipitate, and 10% became turbid. No 1 of panel B (turbid) indicated bacterial proliferation in the medium (control).

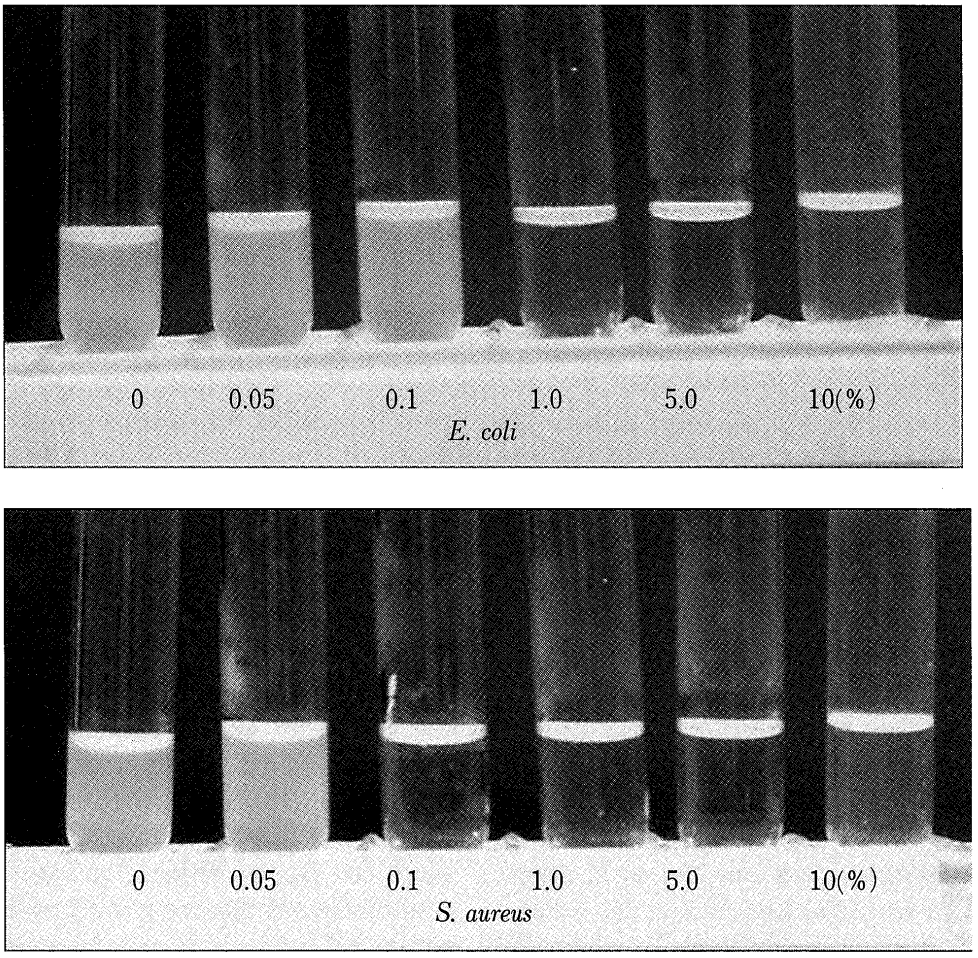

Fig. 2 Effect of Kitocohol in liquid media on the proliferation of *E. coli* and *S. aureus*. Proliferation of *S. aureus* was surpressed at the concentration 0.1% of Kitocohol, while that of *E. coli* was at 1%.

Result and Discussion

1. Inhibitory effect of Kitocohol on bacterial growth in liquid media

The liquid media (4.9ml) that contained the indicated serial concentration of Kitocohol were prepared and 0.1ml of bacterial culture containing $5x10^7$ CFU was added. The culture tubes were incubated at 37℃ for 12 hours. Bacterial growth was measured by the turbidity and the viability was confirmed by the CFU assay.

At the concentration of Kitocohol between 0.5% and 5%, the mixed liquid media appeared flocculent, and above 5% containing medium became turbid suspension. Kitocohol might be aggregated with some components in the medium, but it did not disturb further experiments. The turbid media containing over

Table 1 Efect of Kitocohol on the proliferation of virulent bacteria in liquid media

Bacterial species	Concentration of Kitokohl (%)					
	0	0.1	0.5	1	5	10
B. subtilis	+++	−	−	−	−	−
B. cereus	+++	−	−	−	−	−
S. aureus	+++	−	−	−	−	−
E. faecalis	+++	−	−	−	−	−
C. perfringens	+++	−	−	−	−	−
E. coli	+++	+	+	+	+	−
S. enteritidis	+++	++	++	+	+	
P. aeruginosa	+++	+++	+++	+++	++	−
V. parahemoplyticus	+++	+++	+++	+++	++	−

Flock precipitation appeared after the addition of Kitokohl at a concentration between 0.5% and 5%. Above 5% concentration homogeneous turbidity inceased.
+~+++ indicates grown bacterial population measured by the turbidity, −indicates no growth.

Table 2 Change of pH of media before and after the culture

	Concentration (%) of Kitocohl in the media											
	0		0.05		0.1		1		5		10	
Bacterial species	before	after	before	after	before	after	before	after	before	after	before	after
B. subtilis	7.15	7.8	7.15	7.65	7.02	7.2	6.68	6.95	4.92	6.05	4.55	5.25
S. aureus	7.15	7.53	7.15	7.45	7.02	7.38	6.68	7.15	4.92	6.77	4.55	5.15
E. faecalis	7.15	7.42	7.15	7.2	7.02	7	6.68	6.9	4.92	6.52	4.55	5.5
E. coli	7.15	8.21	7.15	8.12	7.02	8.13	6.68	7.9	4.92	7.8	4.55	6.25
S. enteritidis	7.15	7.74	7.15	7.51	7.02	7.78	6.68	6.99	4.92	6.68	4.55	5.3
P. aeruginosa	7.15	8.17	7.15	8.17	7.02	8.12	6.68	7.99	4.92	7.41	4.55	5.12
V. parahemolyticus	7.15	8.16	7.15	8.12	7.02	7.06	6.68	7.01	4.92	5.6	4.55	5.24

pH of the original Kitocohol was 4.05. Media was LB broth. After: after 18 hours culture. The numers are the average of experiments which were repeated three times.

1% Kitocohol became clear over night with a culture forming a white precipitate at the bottom of the tube as shown in Fig. 1.

The effect of Kitocohol on bacterial proliferation in liquid media was shown in Table 1 and Fig 2. Kotocohol at the concentration of 0.1 % inhibited the growth of *B. subtilis, S. aureus, E. faecalis.* The same effect was observed with anaerobic bacteria *C. perfringens.* The effect was not so remarkable with *E. coli* and *S. enteritidis*, and less effective on *P. aeruginosa* and *V. parahemolyticus.* Kitocohol at the concentration of 0.1% in liquid media seems to inhibit the growth of Gram positive bacteria but not of Gram negative bacteria. At a concentration of over 5%, Kitokohol tended to depress the proliferation of *P. aeruginosa* and *V. parahemolyticus* as well.

Since the pH of Kitokohol is acidic, pH of the culture media can be a factor in the growth inhibition. The change of pH of Kitocohl containing media was monitored before and after the incubation as shown in Table 2. One percent of containing media were not so low as to inhibit the growth of bacteria. Over 5% of Kitocohol, the pH became lower than 5, which can depress bacterial growth in general. The result indicated that the growth inhibition factor was not the pH but could be chitosan itself.

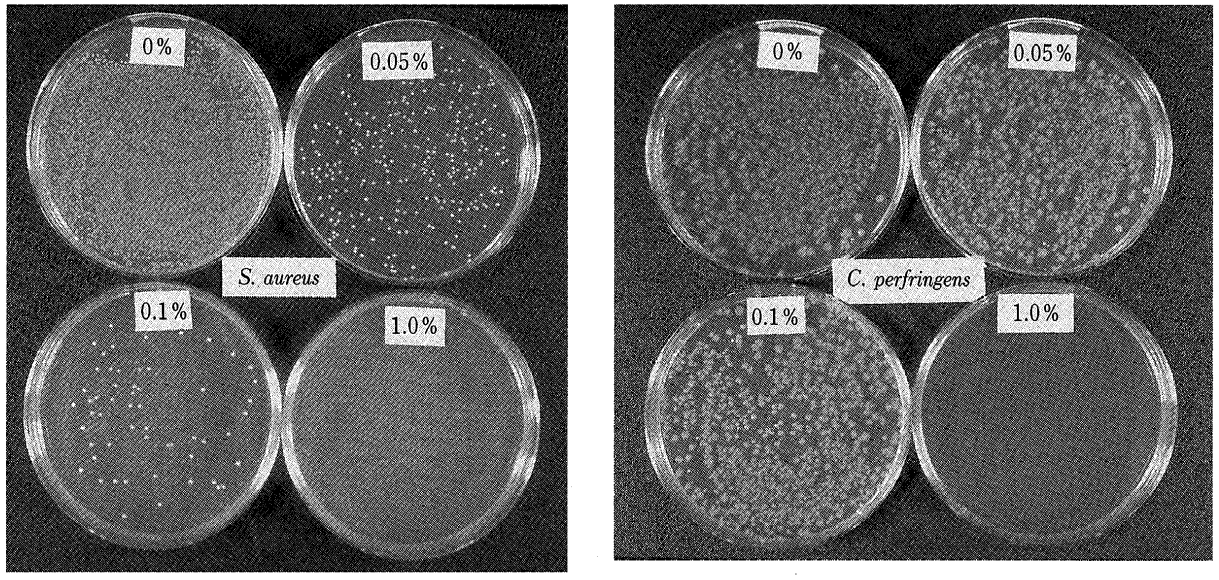

Fig. 3 Effect of Kitocohol on solid media on the proliferation of S. aureus (on LB agar) and *C. perfringens* (on GAM agar and anerobically cultured). Kitocohol was sprayed on the plates prior to the inoculation of the organisms.

Table 3 Effect of Kitocohol on the proliferation of virulent bacteria on the plates which were pre-coated with Kitokohl

Bacterial species	Concentration of Kitokohl (%)					
	0	0.1	0.5	1	5	10
B. subtilis	+++	−	−	−	−	−
B. cereus	+++	−	−	−	−	−
S. aureus	+++	−	−	−	−	−
E. faecalis	+++	++	−	−	−	−
C. perfringens	+++	+	−	−	−	−
E. coli	+++	+++	++	+	+	+
S. enteritidis	+++	+++	++	+	+	+
P. aeruginosa	+++	+++	+++	+++	++	++
V. parahemoplyticus	+++	+++	+++	+++	+++	++

Each agar culture plate was coated with a concentration of Kitokohl solution and kept for 4 hours at room temperature to allow the solution to penetrate into the agar. Then 10^7 cfu organism were inoculated and incubated at 37℃ over night.

Table 4 Effect of Kitocohol on the proliferation of bacteria on the agr plates which were aerosol-sprayed with Kitocohl before bacterial inoculation

Bacterial species	Concentration of Kitokohl (%)					
	0	0.1	0.5	1	5	10
B. subtilis	+++	−	−	−	−	−
B. cereus	+++	−	−	−	−	−
S. aureus	+++	−	−	−	−	−
E. faecalis	+++	++	−	−	−	−
C. perfringens	+++	+	−	−	−	−
E. coli	+++	+++	++	+	−	−
S. enteritidis	+++	++	++	+	+	−
P. aeruginosa	+++	+++	++	++	++	++
V. parahemoplyticus	+++	+++	+++	+++	+++	++

The culture agar plates were air-sprayed with each concentration of Kitocohl solution and kept for 4 hours at room temperature to allow the solution to penetrate into the agar. Then 10^7 cfu organism were inoculated and incubated at 37℃ over night.

2. *Inhibitory effect of Kitocohol on bacterial growth on solid media.*

The effect of Kitocohol on the solid media was examined. One tenth of milliliter of Kitocohol solution, at the concentration indicated, was applied to the plate and spread evenly on the surface, then the pre-cultured organism was inoculated as described in the method and material. The result is shown in Table 3 and Fig. 3. The result indicats that the reagent was effective on Gram positive bacteria at a concentration of 0.1%, while it needed more than 5 % inhibit the proliferation in Gram negative enteric bacteria. It was almost ineffective on *V. parahemolyticus* and on *P. eruginosa* which are routinely isolated from natural water environment.

Adequate concentration of Kitocohol solution was sprayed on the plate by aerosol sprayer, instead of spreading 0.1ml on the surface, then inoculing the cultures. The result is shown in Table 4. The application method, pre-coating with spray or dropspreading of the agent onto the agar plate, did not make any measurable difference in the effectiveness. In the application process of the reagent on solid media, the application of Kitocohol solution on the plates, followed by the inoculation did not result in any difference from inoculation of the culture first, then application of the solution.

This suggests that the reagent can be applied by a handy spray bottle onto the food surface to inhibit the gram positive bacterial proliferation.

3. *Minimum inhibitory concentration*

Since the reagent showed effectiveness on the gram positive bacteria, the minimum inhibitory concentration (MIC) for the bacteria was examined and the result was shown in Table 4. The MIC was estimated to be 0.05% of Kitocohol against the gram positive bacteria and 0.7% for S. enteritidis. (Table 5)

Whether the inhibitory effect is bactericidal or bacteristatic action was examined by CFU assay of

Table 5 Minimal Inhibitory Concentration of Kitocohl on *B. subtilis*, *S. aureus*, and *S. enteritidis*

	Kitocohl concentration (final % in the culture medium)												
	0.01	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1	5
B. subtilis	+		−	−	−	−	−	−	−	−	−	−	−
S. aureus	+	−	−	−	−	−	−	−	−	−	−	−	−
S. enteritidis	+++	+++	+++	+++	++	++	++	+	−	−	−	−	−

Condition of the media and the growth parameters are same as described in Table 1.

the cultures. Each of 50 μl of the culture of *S. aureus* and *B. subtilis* (tube number from 2 to 13 on Table 5) were cultured on agar plates. No colony grew colony on the plates, indicating that the effect of Kitocohol was bactericidal, not bactroistatic.

4. Duration of the effectiveness on agar plates

Agar plates were sptayed with a 0.1% Kitokohol solution and kept in a sealed bag at room temperature for 4 weeks. Every other day, the plates were taken out and inoculated with bacteria to check if the solution remained effective. Even after 4 weeks, if the plates were kept in well sealed bag, *B subtilis, S. aureus* and *E. fecalis*, did not grow on the plates, indicating the effectiveness was durable for weeks. This was same for the liquid media, indicating the anti-bacterial effectiveness was stable and durable for weeks.

5. Speculative mode of action and suggestion for usage in practice

It was reported that when bacteria were exposed to chitosan solution for a certain period of time, the viability of *S. aureus* and *E. faecalis* were decreased in 60min, but that of *E. coli* and *P aeruginosa* were not so effective. In this report, Kitocohol, chitosan solution, was mixed with culture media and examined to determine if it affects bacterial growth. The effect of Kitocohl on bacterial growth was in good agreement with the previous report [3] suggesting the effective factor in Kitocohl could be chitosan, because the other component did not have any effect on bacterial growth in/on the culture media The antibiotic activity of chitosan was reported to depend on the molecular size; short chain had a stronger effect than long chain, but detailed mechanism has not been made clear. Kitocohl should contain variable molecular sizes of soluble chitosan, possibly less than 10,000 Da which plays the bactericidal function of the solution.

A certain length of N-acetyl-glucose amine polymer may bind to the cell wall component of gram positive bacteria and hinder the synthesis of peptideglycan at the surface by competing with the murein molecule, that results in cell lysis.

The effectiveness of Kitocohl when sprayed on solid media was maintained for four weeks if it was kept in well sealed bag to prevent dryness. It may be applied to keep vegetables, and sliced vegetable and meats fresh during storage. We did a preliminary examination using white cabbage, radish, spinach, sliced meat and fish to determine if Kitocohl could keep them fresh and reduce or inhibit bacterial proliferation on the surface by spreading the reagent. Unfortunately it was not effective (data is not shown), and it also has a slight odor of chitosan that may cause some discomfort. It may not be appropriate to use it as a preservative in fresh food, but may be good for use in pickled foods that are served after washing out it with water.

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