Antimicrobial Effect of Chemical Preservatives on Enterohemorrhagic *Escherichia coli* O157:H7

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Three strains of enterohemorrhagic *Escherichia coli* O157:H7 derived from patients of outbreaks in Osaka, Japan 1996 were used to determine the antimicrobial effect of the chemical preservatives: sorbic acid, benzoic acid, *p*-hydroxybenzoic acid, and dehydroacetic acid. The pH of the media strictly limited the growth of the pathogenic strains; a pH of 5.5 or below completely inhibited growth of these strains. The minimum inhibitory concentration (MIC) value for sorbic acid, benzoic acid, and dehydroacetic acid was 4 mg/ml and that of *p*-hydroxybenzoic acid was 16 mg/ml for the three pathogenic strains. Under the MIC conditions, the action of sorbic acid was bactericidal, whereas the other three antimicrobial chemicals were bacteriostatic. In Japan, the maximum allowable dose level of sorbic acid in meat products is 2 mg/g, which corresponds to half the MIC concentration. To determine if this concentration resulted in an antimicrobial effect, homogenized hamburger beef was experimentally contaminated with *E. coli* O157:H7 at an initial level of 10^4 colony forming unit/g. After incubation for 7 days, this concentration of sorbic acid demonstrated a bacteriostatic effect in the meat.

Key words —— antimicrobial action, chemical preservatives, *Escherichia coli* O157:H7 growth

INTRODUCTION

Enterohemorrhagic *Escherichia coli* O157:H7 has been recognized as the causative agent of human diseases such as hemorrhagic colitis and hemolytic-uremic syndrome.[1-3] Outbreaks and sporadic cases due to infection with *E. coli* O157:H7 associated with food have been frequently reported in various places around the world.[4] In Japan during 1996, numerous large outbreaks and sporadic cases of *E. coli* O157:H7 occurred and over 9000 individuals was affected.[5] Various types of hygienic countermeasures, including pasteurization during processing and use of food additives, have been taken to prevent food-associated outbreaks. To preserve the quality or freshness of food products from the time of production to consumption, antimicrobial agents are added to eliminate or retard microbial growth.

This study assessed the effects on *E. coli* O157:H7 of four chemical preservatives allowed in Japan. Mueller Hinton broth and model meat products were experimentally contaminated with *E. coli* O157:H7 and the antimicrobial effects of sorbic acid, *p*-hydroxybenzoic acid, dehydroacetic acid, and benzoic acid were determined. Sorbic acid was found to effectively inhibit the growth of *E. coli* O157:H7 at half the MIC concentration, which is an acceptable concentration for meat products in Japan.

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ATERIALS AND METHODS

Bacterial Strains and Growth —— The three strains of enterohemorrhagic *Escherichia coli* O157:H7 used in this study were S-180, OCI-189, and F-41, all of which were isolated from fecal specimens of patients from outbreaks in Osaka, Japan, in 1996. *E. coli* K-12 was used as a control strain. Cells were grown in Mueller Hinton broth (Difco) at 37℃.

Chemical Preservatives and Antibiotics —— The following chemical preservatives were used: sorbic acid potassium salt and *p*-hydroxybenzoic acid sodium salt, both dissolved in distilled water, and dehydroacetic acid sodium salt and benzoic acid, which were dissolved in distilled dimethyl sulfoxide. The final concentrations were 100 mg/ml and they were stored in the dark. Fosfomycin (100 mg/ml)

(Fosmicin-S, Meiji Seika, Japan) was used as a control antibiotic.

Determination of Minimum Inhibitory Concentration (MIC) —— MIC was determined by the standard method for the dilution antimicrobial susceptibility test.[6] The pH of Mueller Hinton broth was adjusted to 6.5 with 50 mM phosphate buffer (MHB-P medium). Each well of the assay plate contained 0.1 ml of the MHB-P medium including serially diluted chemical preservatives and overnight culture of *E. coli* (approximately 10^4 colony forming units (CFU)/ml well). The plates were incubated at 37℃ overnight and the turbidity of each well was measured by a plate reader by optical density at 595 nm.

Viable Cell Count —— Overnight growing cultures of *E. coli* were added into the MHB-P medium containing various concentrations of chemical preservatives and incubated at 37℃ with shaking. The initial level of *E. coli* in the medium was adjusted to approximately 10^6 CFU/ml. At intervals, 0.1 ml of the samples were plated on desoxycholate hydrogen sulfide lactose (DHL) agar (Eiken) and incubated at 37℃ overnight. The viable cell numbers were determined by counting colonies.

Viability of *E.coli* in Meat Products Containing Chemicals —— Hamburger meat and poultry block obtained from a market were homogenized in distilled 0.85% saline to prepare 10% solution. Approximately 5 g (wet weight) of the homogenized meat samples were mixed with overnight culture of *E. coli* (about 10^4 CFU/g) and various concentrations of sorbic acid, and incubated at 37℃ with shaking. At intervals, supernatant solution was taken and serially diluted samples were plated on the DHL agar at 37℃ overnight and the colonies were counted.

RESULTS

Effect of pH on Growth of *E. coli* O157:H7

Unlike most food borne pathogens, *E. coli* O157:H7 is uniquely tolerant to acidic environments.[7,8] The effect of pH on growth of *E. coli* O157:H7 used in this work was examined to confirm the acidic tolerance and to optimize the conditions for further assays. The growth of *E. coli* was strictly limited when the Mueller Hinton broth had a low pH (Fig. 1). The growth of the strain S-180 was completely inhibited at 5.5 or below; there was limited growth at pH 5.8, and optimal growth was between pH 6.0 and 7.0. Acidic tolerance was also observed in the strains OCI-189 and F-41. Interestingly, the growth of the non-pathogenic *E. coli* K-12 was completely inhibited at a pH of 5.8 or below. These results suggest that enterohemorrhagic *E. coli* O157:H7 isolated from patients is more tolerant to acidic conditions than non-pathogenic *E. coli* K-12. From these results, the optimal pH of the antimicrobial assay was determined to be 6.5. The addition of antimicrobial chemicals at the concentrations used in this study did not change the pH of the media.

Determination of MIC

The MIC for chemical preservatives was determined and the results are shown in Table 1. MICs of sorbic acid, benzoic acid, and dehy-

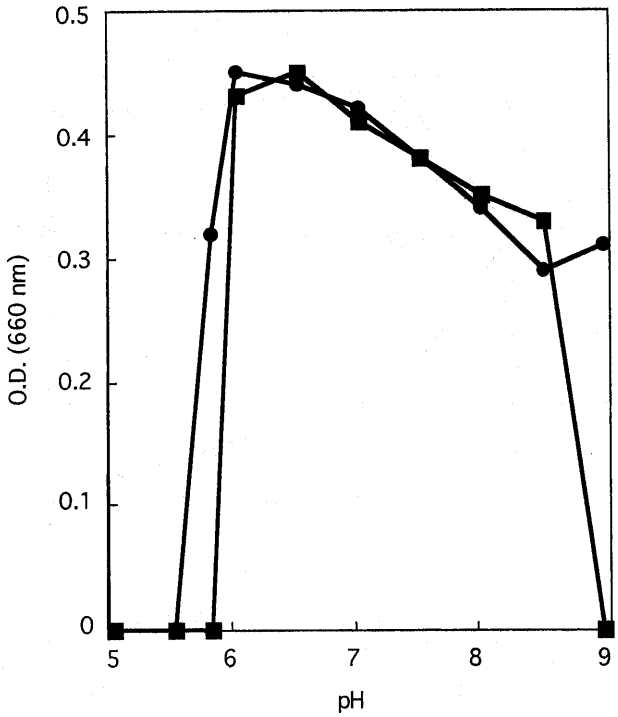

Fig. 1. Effect of pH on Growth of *E. coli*

Overnight growing cultures of *E. coli* O157:H7 strain S-180 (●) and *E. coli* K-12 (■) were added to Mueller Hinton broth (approximately 104 CFU/ml medium) at different pHs and the optical density at 660 nm was measured after incubation for 24 h at 37℃.

Table 1. MIC of Chemical Preservatives for *E. coli* O157:H7

Chemical preservatives	MIC			
	S-180	OCI-189	F-41	K-12
Sorbic acid	4(mg/ml)	4(mg/ml)	4(mg/ml)	4(mg/ml)
Benzoic acid	4(mg/ml)	4(mg/ml)	4(mg/ml)	4(mg/ml)
p-Hydroxybenzoic acid	16(mg/ml)	16(mg/ml)	16(mg/ml)	16(mg/ml)
Dehydroacetic acid	4(mg/ml)	4(mg/ml)	4(mg/ml)	4(mg/ml)
Fosfomycin	32(μg/ml)	32(μg/ml)	32(μg/ml)	16(μg/ml)

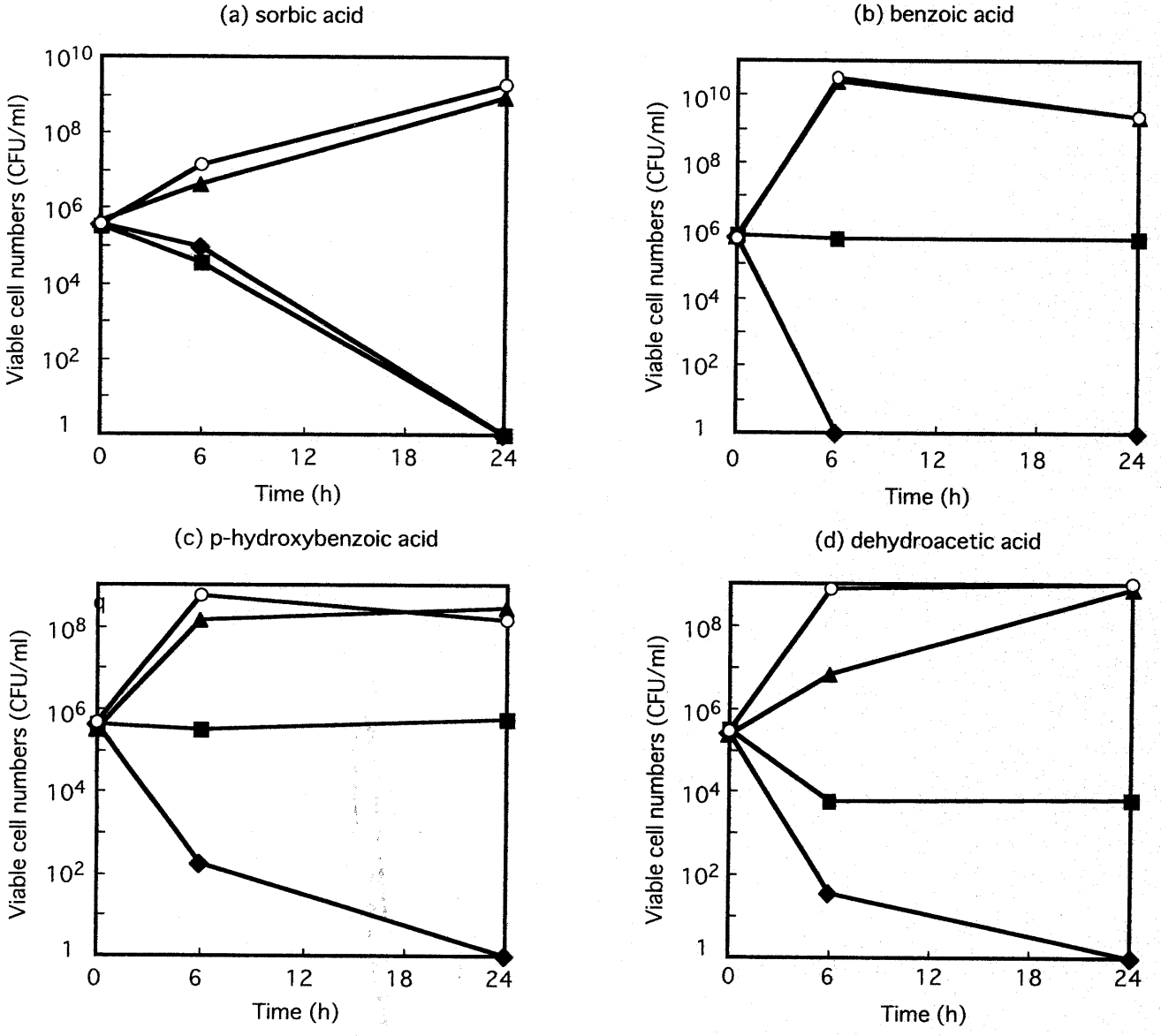

Fig. 2. Effect of Chemical Preservatives on Survival of *E.coli* O157:H7

Overnight cultures of strain S-180 were incubated in MHB-P medium with various concentrations of sorbic acid (a), benzoic acid (b), *p*-hydroxybenzoic acid (c), and dehydroacetic acid (d) at 37℃ as described in Materials and Methods. The concentrations of chemicals are as follows: ◆, 4×MIC; ■, 1×MIC; ▲, 1/4×MIC; ○, without chemical. Viable cell numbers were counted after incubation on DHL agar plates overnight at 37℃.

droacetic acid were 4 mg/ml, and that of *p*-hydroxybenzoic acid was 16—32 mg/ml. The MIC of fosfomycin for the pathogenic strains was 32 μg/ml.

Effect of Chemical Preservatives on Enterohemorrhagic *E. coli* Strains

After incubation with various concentrations of the 4 chemicals, the surviving *E. coli* S-180 was determined by colony counting. As shown in Fig. 2a, after a 24-hour incubation in the MHB-P medium, the viable cell number was completely reduced to undetectable amounts after incubation with sorbic acid at the MIC concentration (1× MIC) and four times the MIC concentration (4× MIC). Furthermore, cells incubated in the presence of one-fourth the MIC concentration (1/4× MIC) grew as well as the control. A 4×MIC, benzoic acid (Fig. 2b), *p*-hydroxybenzoic acid (Fig. 2c), and dehydroacetic acid (Fig. 2d) each demonstrated the same killing effect as sorbic acid on strain S-180. Among the four chemicals, benzoic acid was the most effective in killing strain S-180. At 1×MIC, benzoic acid, *p*-hydroxybenzoic acid, and dehydroacetic acid completely prevented the growth of strain S-180

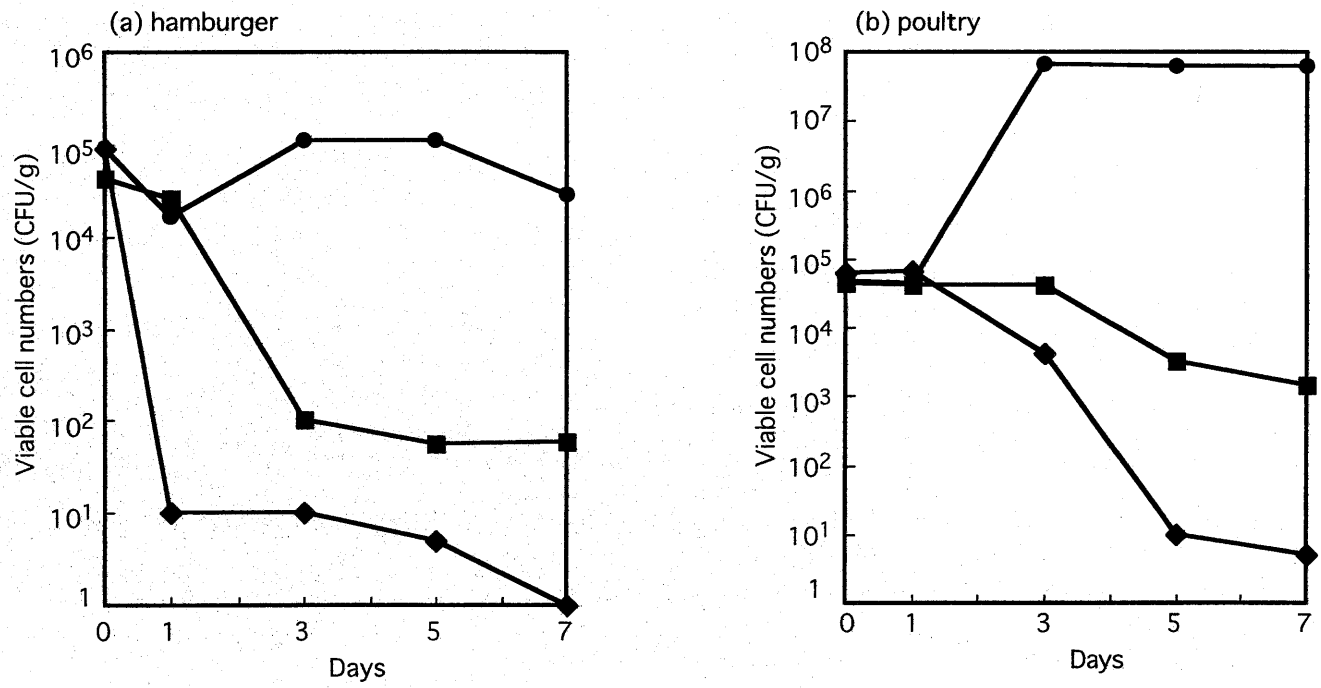

Fig. 3. Viability of *E. coli* O157:H7 in the Meat Products in the Presence of Sorbic Acid

Overnight cultures of strain S-180 were incubated in homogenized hamburger (a) or poultry samples (b) in the presence of various concentrations of sorbic acid as described in Materials and Methods. Viable cell numbers were counted on DHL agar plates. The symbols in the figure are as follows: ◆, 4×MIC; ■, 1×MIC; ●, 0.5×MIC.

throughout 24-hour incubation in the MHB-P medium, but did not reduce the viable cell numbers. These results suggest that 1×MIC, the effect of sorbic acid is bactericidal, whereas the other three chemicals are bacteriostatic.

Effect of Chemicals on the Viability of *E. coli* O157:H7 in the Meat Products

Many confirmed human *E. coli* O157:H7 outbreaks have been associated with the consumption of undercooked ground beef. Undercooked beef and chickens have been readily colonized following administration of a small population of *E. coli* O157:H7,[9] which suggests that these meat products can serve as a source of *E. coli* O157:H7 contamination.[9,10] To assess the effect of chemical preservatives on the growth of *E. coli* O157:H7 in meat products, homogenized hamburger and poultry block were experimentally contaminated with the bacteria. Sorbic acid is one of the allowed antimicrobial agents for meat products in Japan. In accordance with the assay conditions used in the determination of MIC, the effect of sorbic acid on the pathogenic strains was measured after contamination with 10^4 CFU/g of *E. coli* O157:H7. Before contamination, the initial level of bacteria found in the meat samples was about 5×10^2 CFU/g when plated on nutrient agar plates (non-selective medium). When the same samples were plated on DHL agar plates, which are selectively countable for *E. coli*, no colonies were detected (data not shown). The results in Fig. 3a showed that the viable cell numbers of S-180 in hamburger were reduced from the initial contamination level to 10 CFU/g during a 24 h co-incubation with 4×MIC of sorbic acid. Similar bactericidal action was observed with 1×MIC of sorbic acid. At half the MIC concentration (0.5×MIC, 2 mg/ml), which corresponds to the maximum allowed dose level in meat products in Japan, a bacteriostatic action throughout the 7 day incubation period was noted. As shown in Fig. 3b, sorbic acid in the homogenized poultry revealed a bactericidal effect only at 4×MIC. The 1×MIC concentration produced a bacteriostatic action in the homogenized poultry. On the contrary, 0.5×MIC of sorbic acid showed only a bacteriostatic action on strain S-180 during the first day's of incubation and failed to prevent bacterial growth during further incubation. The same antimicrobial results were observed in the pathogenic strains, OCI-189 and F-41 (data not shown).

DISCUSSION

Enterohemorrhagic *E. coli* O157:H7 is considered one of the emerging infectious diseases associated with food and has recently caused numerous outbreaks. The most effective method to maintain food safety is to control the level of microbial contamination in foods; chemical preservatives are widely used for such a purpose. Incubation studies have revealed that *E. coli* O157:H7 can survive the fermentation, drying, and storage processes of producing sausage (pH 4.5)[11] since the optimal pH for *E. coli* growth is reported to be between 5 and 9.6.[12] In consideration of these results, the effect of pH on the growth of *E. coli* O157:H7 was examined. The pathogenic strains showed strict acidic limitation on growth in Mueller Hinton broth at pH 5.5 or below, which is agreement with the results observed previously in the incubation with vinegar and acidic foods.[13]

According to the MIC determined in Mueller Hinton broth in this work, the effectiveness of sorbic acid was examined in the model foods, homogenized hamburger and poultry, which were experimentally contaminated with *E. coli* O157:H7 at the level of 10^4 CFU/g. Examining 0.5× MIC of sorbic acid, which corresponds to the maximum allowable dose level in these meat products, revealed a bacteriostatic action in the hamburger sample, but not in the poultry sample. The cause for this difference in effect in these foods is uncertain, but one possibility is that there is an intrinsic property of the samples that stimulates or diminishes the antimicrobial action of sorbic acid. It has been reported that even a small amount of *E. coli* O157:H7 (less than 100 CFU) causes enterohemorrhagic pathogen when contaminated in the food.[13] The addition of allowable levels of sorbic acid into the meat products seems to be effective to control the contamination by *E. coli* O157:H7, except in the case of a relatively high level of contamination.

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