Effectiveness of Some Natural Antimicrobial Compounds in Controlling Pathogen or Spoilage Bacteria in Lightly Fermented Chinese Cabbage

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ABSTRACT: This study was designed to evaluate the bactericidal or bacteriostatic effect of chitosan, an allyl isothiocyanate (AIT) product, and nisin for the artificially inoculated pathogenic bacteria (Escherichia coli, Salmonella Enteritidis, Staphylococcus aureus, and Listeria monocytogenes) or natural microflora of fermented Chinese cabbage. Addition of 0.1% chitosan decreased the population of pathogens from 0.7 to 1.7 log colony-forming units (CFU)/g after 4 d of storage at 10 °C. The bactericidal activity of chitosan was found to be stronger than that of nisin (0.05 mg/g). Addition of 0.2% of the AIT product (containing AIT and hop extract) exhibited a bacteriostatic effect. However, a combination of AIT product and chitosan enhanced bactericidal efficacy against L. monocytogenes. The addition of chitosan or AIT product was observed to suppress the populations of mesophilic and coliform bacteria during storage at 10 °C for 4 d. Moreover, the use of chitosan or the AIT product did not change the sensory quality of the lightly fermented vegetable. Therefore, these results suggest that chitosan or the AIT product could be useful to improve the microbial safety and quality of lightly fermented vegetable.

Keywords: lightly fermented cabbage, pathogens, chitosan, allyl isothiocyanate, beta-acid

Introduction

Fermented vegetables are an integral part of people’s diet in many countries (Steinkraus 2000). Normally, leafy vegetables harbor 2 to 7 log10 colony-forming units (CFU)/g of mesophilic bacteria including coliform or spoilage bacteria such as Pseudomonas, as their normal microflora (Daeschel and others 1987; Nguyen-The and Carlin 1994). Lactic acid bacteria (LAB) on the leaves play an important role in fermentation. In addition, vegetables can act as a vector in transporting pathogenic bacteria from the farm (Beuchat 1996; NACMCF 1999). Chinese cabbage is commonly used for the production of lightly fermented products, a very popular foodstuff in Japan. These products were traditionally produced in the home. Storing vegetables with salt (3% to 5%) and other ingredients under pressure produces the fermented products. It might be assumed that growth of pathogenic or spoilage bacteria would be suppressed by high salt concentration and/or development of inhibitory chemical compounds (such as organic acid and bacteriocin) produced by the coexisting LAB during fermentation (Adams and Nicolaides 1997; Lee 1997). Commercially prepared fermented cabbage has been gaining popularity with consumers because of its convenience, and in most cases, these foods are consumed raw within 2 to 4 d of preparation. Recently, several foodborne outbreaks with lightly fermented (salted) vegetables have been reported in Japan (Ozaki and others 2003; Inatsu and others 2004). The application of natural antimicrobial compounds for increasing food quality or hygiene has been increasing based on the consumer’s preference. For example, bacteriocins, small peptide compounds effective against Gram-positive bacteria, or bacteriocin-producing LAB have been applied for bacterial control in many foods, including some vegetable products (Choi and Park 2000; O’Sullivan and Hill 2002). Chitosan, a deacetylated form of chitin, consists of polymeric 1, 4-linked 2-amino-2-deoxy-β-D-glucose and has been shown to have antimicrobial activity against a range of foodborne bacterial pathogens, fungi, and yeast (Helander and others 2001; Devlieghere and others 2004). Allyl isothiocyanate (AIT), one of the major compounds responsible for pungent flavor of black mustard (Brassica nigra) and Japanese horseradish (Eutrema wasabi Maxim), exhibits antimicrobial activity against some pathogenic bacteria, yeasts, or molds (Ishike and others 1992; Weisinger and others 2001). The alpha or beta acid containing resin of hop (Humulus lupulus L.) inhibits the growth of some fungi and Gram-positive bacteria, except for some beer spoilage bacteria (Simpson and Smith 1992; Sakamoto and Konings 2003). Therefore, in this study we evaluated the efficacy of these natural antimicrobial compounds for controlling the population of pathogenic or spoilage microorganisms in lightly fermented cabbage.

Test strains

The strains studied and their sources were as follows: Enterohemorrhagic Escherichia coli O157:H7 strains CR-3, MN-28, and MY-29 were isolated from bovine feces. Salmonella Enteritidis strains SE-1, SE-3, and SE-4 were from chicken feces. SE-2 from bovine feces were provided by the Laboratory of Zoonosis, Natl. Inst. of Animal Health, Tsukuba, Japan. Staphylococcus aureus strains IFO 13276 (human lesion), JCM 2413 (clinical isolate), JCM2874 (wound), and JCM2151 (human lesion) were purchased from the Inst. for Fermentation (Osaka, Japan) and the Japan Collection of Microorganism Riken (Saitama, Japan). Strains of Listeria monocytogenes were ATCC 49594 (derived from L. monocytogenes strain Scott A), JCM7672 (salami sausage), and JCM7676 (roast beef). To minimize growth on enumeration media by microorganisms naturally present in the test vegetables, the following inhibitors were added to the enumeration media: 100 mg/liter of gentamicin (Sigma Chemicals) and 50 mg/liter of vancomycin (Sigma Chemicals) in the case of Escherichia coli; 100 mg/liter of polymyxin B (Sigma Chemicals) for Salmonella Enteritidis; 50 mg/liter of rifampin (Sigma Chemicals) for Staphylococcus aureus; 30 mg/liter of cefuroxime (Sigma Chemicals) for Listeria monocytogenes.
rally present on cabbage leaves, rifampicin-resistant mutants were spontaneously obtained from these strains and used in this study. All test strains were adapted to grow in tryptic soy broth (TSB, pH 7.3; Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with rifampicin (50 μg/mL). The mutant strains were compared with parent strains to determine that they had no phenotypic differences (Inatsu and others 2003).

Preparation of inocula

Each strain of E. coli O157:H7, S. Enteritidis, S. aureus, and L. monocytogenes was cultured at 37 °C in brain heart infusion (BHI) (Nissui Pharmaceutical Co.) medium (100 mL) containing 0.5% yeast extract supplemented with 50 μg/mL rifampicin (BHI/Rif). Cultures were transferred to BHI/Rif by loop at 3 successive 24-h intervals immediately before they were used as inocula. A “cocktail method” was used for inoculation. Cells of each strain were collected by centrifugation (3000 x g, 10 min, 20 °C) and resuspended in 10 mL of sterile phosphate buffered saline (PBS, pH 7.2). Equal volumes of cell suspensions (about 5.0 x 10^8 CFU/mL) of 3 or 4 strains of each pathogen were combined to give approximately equal populations of each strain. The inoculum (50 mL) with final concentration of approximately 8.0 log CFU/mL was maintained at 22 °C ± 1 °C and used within 30 min of preparation.

Preparation of lightly fermented cabbage

Chinese cabbages were purchased from local supermarkets and stored at 4 °C for a maximum of 2 d before being used in the experiments. For laboratory-scale preparation of lightly fermented Chinese cabbage, 1 whole Chinese cabbage (2.0 kg) was cut into small pieces (3 cm x 3 cm), which were washed twice with tap water for 5 min. The leaf pieces were soaked with salt (30 g/kg) and 7% sodium chloride water (300 mL/kg) for 6 h at 4 °C under pressure (20 g/cm²).

Preparation of antimicrobials

Three antimicrobial chemicals were used for the experiment: (1) Chitosan 10 (Wako Pure Chemical, Osaka, Japan) was dissolved in distilled water to give 5% (w/v) by adjusting the pH to 5.0 with HCl. (2) Wasaouro EXT® (designated as “AIT-Hop”) supplied by Mitsubishi-Kagaku Foods Co., Tokyo, Japan, was an emulsion liquid containing AIT and hop extract. (3) Commercial nisin (Sigma Chemical Co., St. Louis, Md., U.S.A.) was dissolved in distilled water with HCl to give 0.25 mg/mL of the product (pH 5.0). Chitosan, AIT-Hop, or nisin was added to each cup containing salted Chinese cabbage to give the final concentration of 0.1%, 0.2%, or 0.05 mg/mL, respectively. Five hundred microliters of each pathogen (about 5.0 x 10^5 to 10^6 CFU/mL) were inoculated into each cup containing salted Chinese cabbage, mixed well using a sterile glass rod, and stored at 10 °C for 4 d under pressure (20 g/cm²). Sterile distilled water was used as control.

Microbiological analysis

Twenty-five grams of cabbage leaves was placed in a stomacher bag, and 225 mL of PBS was added and homogenized for 90 s. Serial decimal dilutions were prepared with PBS (pH 7.2), and the diluted and undiluted samples were surface plated in quadruplicate on both selective and nonselective medium supplemented with 50 μg/mL rifampicin. Tryptic soy agar (TSA) (Nissui Pharmaceutical Co.) supplemented with 50 μg/mL rifampicin was used as nonselective medium for viable cell determination of all the pathogens tested. In addition, E. coli O157:H7 suspensions were surface plated onto sorbitol MacConkey agar (CT-SMAC) (Nissui Pharmaceutical Co.) (CT-selective supplement, Oxoid, Hampshire, U.K.) and CT-SMAC containing 50 μg/mL rifampicin. Samples containing Salmonella were surface plated onto DHL agar (Nissui Pharmaceutical Co.) supplemented with 50 μg/mL rifampicin. Suspensions of S. aureus were surface plated onto Mannitol salt agar (Eiken Chemical Co., Tokyo, Japan) supplemented with 50 μg/mL rifampicin. Diluted and undiluted suspensions of L. monocytogenes were surface plated onto modified oxford medium (Oxoid) supplemented with 50 μg/mL rifampicin.

Standard method agar (Nissui Pharmaceutical Co.) and desoxycholate agar (Eiken) were used for enumeration of aerobic microflora and coliforms, respectively, in the uninoculated samples. Lactic acid bacteria were enumerated on plate count agar containing bromocresol purple (Nissui Pharmaceutical Co.). Enumeration media were incubated at 37 °C for 24 to 28 h before presumptive colonies were counted. In each experiment, at least 5 presumptive colonies of E. coli O157:H7 were confirmed with an E. coli O157 direct immunoassay test kit (Universal Health Watch, Columbia, Md., U.S.A.). Salmonella confirmation was carried out by testing reactions on triple sugar iron (Nissui Pharmaceutical Co.) slants and LIM medium (Nissui Pharmaceutical Co.) and serological tests using Salmonella polyvalent O, A1 antisera (Denka Seiken, Tokyo, Japan). Randomly picked presumptive colonies of S. aureus and L. monocytogenes were confirmed with API Staph diagnostic kits and API Listeria diagnostic kits, respectively. All experiments were done 4 times with duplicate samples being analyzed at each sampling time.

Sensory evaluation

During storage at 10 °C, the products were sensorially evaluated for taste, odor color, texture, and overall acceptability by a panel consisting of 13 sensory experts. The panelists, who have considerable background knowledge in sensory evaluation, were selected from the staff, researchers, food co. personnel, and teachers from Univ. Evaluation was conducted in a well-equipped taste panel booth at Natl. Food Research Inst. The score given by the panel varied from 1 (dislike extremely) to 5 (like extremely). The sensory characteristics of odor, texture, and taste were assessed on each test sample. For each of the quality attributes, 3 packs of each treated and untreated uninoculated samples were examined and/or tested by the judges. A sample was considered as unacceptable for a sensory characteristic if the score was less than 2.5 (Delaquis and others 2000).

Statistical analyses

All experiments were done 4 times with duplicate samples being analyzed at each sampling time. Data were subjected to analysis of variance (ANOVA) using Microsoft Excel (Microsoft Corp., Redmond, Wash., U.S.A.). Significant differences in plate count data were established by least significant difference at the 5% level of significance. Sensory evaluation tables represented the mean values ± SD obtained from 3 individual trials.

Results and Discussion

The initial mesophilic bacterial count of Chinese cabbage just before fermentation was recorded as 6.38 log CFU/g. Addition of chitosan, AIT-Hop, and other combinations did not decrease the population of mesophilic bacteria, coliforms, or LAB at day 0 (Figure 1). However, addition of chitosan, AIT-Hop, and other combinations did reduce mesophilic and coliform bacteria nearly 0.5 log CFU/g after 1 day of storage. These counts remained constant at day 2 with slight increases or decreases at days 3 and 4. On the other hand, the population of mesophilic or coliform bacteria in control samples increased gradually between 10-fold to 100-fold after 4 d.
of incubation. The combination of chitosan and AIT-Hop exhibited a slightly greater bactericidal effect against mesophilic and coliform bacteria compared with chitosan or AIT-Hop alone after 4 d of storage. The combination of chitosan and AIT-Hop suppressed growth of these bacteria by approximately 2.0 log CFU/g after 4 d of storage compared with control. There was little difference observed in samples with chitosan and AIT-Hop alone.

LAB or coliform are considered spoilage microorganisms because they could increase the turbidity of the seasoning liquid and reduce the quality of the fermented vegetables (Miyao 1997). The initial counts of LAB were 2.85 log CFU/g, and the pH of the sample was 6.4. The viable cell counts of LAB in the control sample increase gradually up to 5.20 log CFU/g at day 4 without any change in the final product pH. In the sample containing chitosan, LAB counts were lower than that of control sample after 2 d of storage, although the count increased slowly up to day 4. The addition of AIT-Hop suppressed the growth of the LAB by 2.0 log CFU/g compared with the control sample after 4 d of storage. However, the combination of chitosan and AIT-Hop did not enhance the efficacy. Addition of chitosan, AIT-Hop, or a combination did not reduce the initial pH (6.4 to 6.6) at day 0, and at day 4, a slight drop of pH (6.2 to 6.4) was observed.

Savard and others (2002) reported that 10 g/L of chitosan did not suppress the growth of LAB (Lactobacillus plantarum, Pediococcus acidilactici, and Leuconostoc mesenteroides) in vegetable juice. In our experiment, we also found that chitosan did not suppress the growth of LAB in fermented Chinese cabbage. The population of LAB was suppressed to its initial level by the addition of AIT-Hop.

AIT activity is believed to involve respiratory inhibition and thus suppressed the growth of aerobic or facultative anaerobic bacteria (Kojima and Ogawa 1971). However, the inhibitory activity of AIT on LAB or other strictly anaerobic bacteria tends to be less than that of other bacteria (Tokouka and Ishihiki 1994). On the other hand, alpha- or beta-acid of hop extract act as protonophores and dissipate the trans-membrane pH gradient, resulting in the growth inhibition of LAB or other Gram-positive bacteria (Sakamoto and Konings 2003). Therefore, the bacteriostatic effect of AIT-Hop on LAB was believed to be derived from hop extract.

Antimicrobial activity was also analyzed in the lightly fermented Chinese cabbage with spiked samples. There was no significant change in viable counts observed for both the Gram-negative strains of E. coli and S. Enteritidis in the control and AIT-Hop treated samples. But the addition of chitosan alone or with AIT-Hop reduced the viable counts by 1.2 and 0.7 log CFU/g for E. coli and S. Enteritidis, respectively, by day 4. Wang (1992) reported that the addition of 0.5% of chitosan could reduce inoculated S. aureus, E. coli, Y. enterocolitica, and L. monocytogenes by 1 to 2 log CFU/g in 4 d at pH 5.5 in nutrient broth, but was less effective against S. Typhimurium. Our results (Figure 2a) are consistent, since the combination of chitosan and AIT-Hop slightly reduced the bacterial count of E. coli but not of S. Enteritidis when compared with the chitosan only (Figure 2a).

Both Gram-positive strains grew gradually to give 0.5 (S. aureus) and 1.3 (L. monocytogenes) log CFU/g of increase in 4 d of storage in control (Figure 2b). The addition of AIT-Hop, however, retained the viable counts in both strains during storage. But chitosan exhibited bactericidal activity against these strains. The larger reduction of viable cell counts 1.3 (S. aureus) and 0.9 log CFU/g (L. monocytogenes) was observed on day 1 (Figure 2b). Thereafter, S. aureus retained its viable counts, but the growth of L. monocytogenes started after 3 d of storage. The growth of L. monocytogenes could not be suppressed completely by the addition of chitosan or nisin. The addition of nisin exhibited similar bacteriostatic effect to the AIT-Hop treatment for both Gram-positive strains, although the growth of L. monocytogenes started after 2 d of storage. There were no significant differences of the effectiveness of nisin and AIT-Hop for both strains. Similarly with Gram-negative strains, bactericidal efficacy of chitosan against S. aureus partially reduced in combination with AIT-Hop. In contrast, synergistic effect was observed against L. monocytogenes (Figure 2b).

Lin and others (2000) showed that the population of each pathogen (Salmonella Montevideo, Escherichia coli O157:H7, and Listeria monocytogenes) was reduced by 4 log CFU/g on the surface of fresh produces with gaseous AIT in 4 d. However, in our study, AIT-Hop
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Gaseous AIT has been reported to exhibit stronger antimicrobial activity than emulsion mixed directly in foods (Inoue and others 1983). Although AIT-Hop emulsion did not exhibit bactericidal effect, it enhanced the bactericidal activity of chitosan (Figure 2a, 2b).

The quality of uninoculated salted Chinese cabbage was evaluated by a panel consisting of 13 sensory experts (9 female and 4 male) were selected from the staff and researchers of Natl. Food Research Inst., Food Co. personnel, and teachers from the university. No significant influence on the color, taste, texture, odor, and overall acceptability was observed by the addition of chitosan or AIT-Hop (Table 1). The sensory evaluation was done after 1 d of fermentation because lightly fermented cabbage was a very popular food item and representative of commercial product in Japan. However, several research reports showed that there are no significant changes of appearance, color, odor, taste, and texture after 4 d of fermentation (Anonymous 1988; Kadowaki, and others 1988).

Conclusions

AIT-Hop, chitosan, or their combination could effectively suppress mesophilic or coliform bacteria, which is recognized as an indicator of the shelf life. In addition, AIT-Hop or its combination with chitosan could suppress the growth of LAB (Figure 1), which increased the turbidity of the soaking water and consequently caused the loss of commercial value of the product. The addition of these food additives did not change the taste or other sensory parameters (Table 1). Therefore, the combination of chitosan and AIT-Hop can be useful for controlling the microbial population in fermented cabbage products. A large-scale series of experiments using this method must be done to determine the reproducibility of the results obtained in the studies reported here.

Acknowledgments

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References

Devlieghere F, Vermeulen A, Debevere J. 2004. Chitosan: antimicrobial activity, emulsion during fermentation only suppressed the growth of pathogens (Figure 2a, 2b).

Table 1—Sensory evaluation of lightly fermented cabbage*  
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Chitosan</th>
<th>AIT</th>
<th>Chitosan + AIT</th>
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<tr>
<td>Color</td>
<td>3.5 ± 0.5</td>
<td>3.3 ± 0.5</td>
<td>3.3 ± 0.6</td>
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</tr>
<tr>
<td>Odor</td>
<td>3.3 ± 0.8</td>
<td>3.3 ± 0.6</td>
<td>3.2 ± 0.7</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>Taste</td>
<td>3.2 ± 1.1</td>
<td>3.2 ± 0.9</td>
<td>3.0 ± 1.0</td>
<td>3.2 ± 0.8</td>
</tr>
<tr>
<td>Texture</td>
<td>3.5 ± 0.5</td>
<td>3.4 ± 0.7</td>
<td>3.3 ± 0.6</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>Overall</td>
<td>3.4 ± 1.3</td>
<td>3.2 ± 0.8</td>
<td>3.1 ± 0.8</td>
<td>3.4 ± 0.9</td>
</tr>
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*The mean values ± SD obtained from 3 individual trials. The evaluation was based on a 5-point hedonic scale (1 = dislike extremely; 2 = moderately dislike; 3 = neither like nor dislike; 4 = moderately like; 5 = like extremely). Average (n = 3) and SD were evaluated from 13 panels.

Figure 2—(a) The change of viable cell counts of Gram-negative pathogens in lightly fermented Chinese cabbage. The samples were incubated at 10 °C with 3% salt for fermentation for 4 d. Symbol represents the same as shown in Figure 1. (b) The change of viable cell counts of Gram-positive pathogens in lightly fermented Chinese cabbage. Experimental condition and symbol represents the same as shown in Figure 1.


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