Combined Effect of Hop Resins and Sodium Hexametaphosphate against Certain Strains of Escherichia coli

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MS 99-111: Received 27 April 1999/Accepted 17 December 1999

ABSTRACT

The combined antimicrobial effects of hop resins with sodium hexametaphosphate, glycerol moncaprate, and lysozyme were investigated aiming to make an effective agent against Escherichia coli. When they are used separately, the antimicrobial activity against E. coli was minimal. However, the combination of hop resins with sodium hexametaphosphate exhibited strong antimicrobial activity against E. coli, but no effect was found in combinations of hop resins with the other agents. The activity was strongest when the combination was added at the beginning of growth of the bacteria, resulting in a prolonged lag phase. However, when the antimicrobials were added during the log phase, growth was depressed considerably. By addition of these materials, cell components with absorbance near 260 nm were leaked out. This possibly may have resulted from damage to the cell membranes of the bacteria. The combined effect was also detected in model food systems such as mashed potatoes. The use of hop resins and sodium hexametaphosphate in combination may thus be useful for controlling E. coli.

The bitter resin components of hop (Humulus lupulus L.) have been used for brewing beer since ancient times and give beer a characteristic aroma and a refreshing bitter taste (1, 14, 15, 33), as well as protection from microbial spoilage (4, 5, 13, 17, 34, 35). The antimicrobial activity of hop resins has been studied extensively, and α- and β-acids of hop resins have shown activity against gram-positive bacteria such as Bacillus cereus, Staphylococcus aureus, but only little effect against gram-negative bacteria such as Escherichia coli (6–9, 11, 22, 24–27, 31). Some inhibitory activity has been reported against fungi, but no effect on yeast has been noted (18–21).

The combined use of antimicrobial agents is generally well known to have a wider antimicrobial spectrum than each used separately (12, 16). The antimicrobial effects of hop resins that are free of α-acids (main bitterness components) in combination with antibacterial agents (glycerol moncaprate, lysozyme, and sodium hexametaphosphate) against E. coli were examined in this study. Glycerol moncaprate is a glyceride that may be used as a surfactant, emulsifier, or an antibacterial agent (23). Lysozyme is a cell wall lytic enzyme against gram-positive bacteria (2). Sodium hexametaphosphate is a typical chelator that bacteriolyzes gram-positive bacteria (10). These agents have strong antimicrobial effects against gram-positive bacteria, but little is known about their antimicrobial activity against gram-negative bacteria including E. coli (2, 10, 23, 32). Some E. coli cause food poisoning and are significant food-borne pathogens. In Japan, E. coli is an indicator organism used in food sanitation but must not be allowed to remain present in food. Thus E. coli, including nonpathogenic strains, must be given serious consideration in food hygiene. The control of these bacteria should enhance food safety. The combined antimicrobial effects of hop resins and antibacterial agents have not been studied to date, and thus the usefulness of hop resins in combination with food-grade antibacterial agents was evaluated in the present study.

MATERIALS AND METHODS

Test materials, test strains, and cultivating medium. Hop resins were commercially obtained as Aromahop, from which α-acids were already removed with a super critical carbon dioxide extraction method (Culter Food Sci. Co., Milwaukee, Wis.). Sodium hexametaphosphate (Taiheikagaku Co., Osaka, Japan; hereafter referred to as SHMP), glycerol moncaprate (Taiyokagaku Co., Mie, Japan), and lysozyme (Kewpie Co., Tokyo, Japan) are food-grade additives. The concentrations used in this study were determined not to cause unacceptable sensory effects on kamaboko. Kamaboko is a traditional Japanese food made from fish pate, starch, salt, and seasonings, and it has a weak flavor and thus is suitable for determining concentrations that would not affect flavor or taste. The bacterial strains listed in Table 1 were used. Nutrient broth (Difco Laboratories, Detroit, Mich.) was used for cultivation of bacteria. A pH combination electrode (GST-5211C, Toa Electronics, Ltd., Tokyo, Japan) was used to determine the pH of the media.

Measurement of antimicrobial activity. Antimicrobial activity was estimated by monitoring the turbidity of liquid cultures. A preculture of each strain was prepared as follows: one loopful of slant culture was inoculated into 10 ml of nutrient broth in a test tube and incubated at 30°C for 24 h on a shaker. Then, the culture was diluted 104-fold with 40 mM sterile phosphate buffer (pH 6.5) containing NaCl (0.9% wt/vol). An aliquot portion (0.01
TABLE 1. Antimicrobial activity of hop resins against bacterial strains used in these experiments

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain no. or source</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-positive</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>OUTb 8032</td>
<td>&lt;10</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>Custard cream</td>
<td>40</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>FDAc 209P</td>
<td>20</td>
</tr>
<tr>
<td>Micrococcus flavus</td>
<td>Unknown</td>
<td>50</td>
</tr>
<tr>
<td>Lactobacillus brevis</td>
<td>Bacon</td>
<td>20</td>
</tr>
<tr>
<td>Leuconostoc mesenteroides</td>
<td>Meatball</td>
<td>20</td>
</tr>
<tr>
<td><strong>Gram-negative</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>K-12 IFOd 3301e</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>E. coli</td>
<td>ATCCe 15224</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>E. coli</td>
<td>K-12 OUT 8401</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>E. coli var. communior</td>
<td>Oyster</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>A food factory</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>A food factory</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

a MIC (minimum inhibitory concentration) was measured by a broth dilution method at 30°C for 48 h.

b Osaka University, Technology.
c Food and Drug Administration.
d Institute of Fermentation, Osaka.
e Hereafter referred to as strain A.
f American Type Culture Collection.
g See the text.

ml of the diluted culture was inoculated into 9 ml of nutrient broth that was made in 40 mM phosphate buffer (pH 6.5). One milliliter of the test material solution was added to the final broth. For the control experiment, the solution was replaced with 1 ml of distilled water. Incubation was carried out at 30°C as above. At 48 h of incubation, absorbance at 650 nm was measured with a spectrophotometer (U-2000, Hitachi Co., Tokyo, Japan). The bacterial strains listed in Table 1 were used. The test material solution was prepared as follows. Hop resins were solubilized in water by melting at 60°C in a beaker and mixing with the same volume of Tween 80 (Nacalai Tesque Inc., Kyoto, Japan). The mixture was dissolved in distilled water. Glycerol moncaprate was dissolved in 10% (vol/vol) ethanol (Nacalai Tesque Inc., Kyoto, Japan). Other test materials were directly dissolved in water. These test material solutions were prepared to give a 10-fold concentration that was required. All samples were made in duplicate, and experiments were repeated three times.

Time course of test material addition. E. coli K-12 IFO3301 (hereafter referred to as strain A) was cultivated in the liquid nutrient medium as above. Influence of the addition time was investigated with the following methods. (i) In the case of the lag-phase addition: the test materials (0.01% hop resins + 1% SHMP, in the working concentrations) were added at 3 or 6 h of incubation time (the lag phase of the growth). Incubation and growth measurement were carried out at 30°C with an automatic growth measuring apparatus equipped with a monitoring system of the turbidity at 650 nm (Bio-scanner, Outakuseisakusho Co., Tokyo, Japan). The effects of the test materials on growth were compared with the control experiment. (ii) In the case of the log-phase addition: the test materials (the same concentration as shown above) were added at 10 h of incubation time (the early log phase). After the viable cell count had increased to 10⁴ CFU/ml by incubation at 30°C for 10 h, the test materials were added. The incubation was carried out on a shaker. The growth was monitored by enumerating the viable cells with employing the standard plate method (3). The standard plate (Nissui-seiyaku Co., Tokyo, Japan) was incubated at 30°C for 48 h. Incubation in the control experiment was carried out without the test materials. All samples were made in duplicate, and experiments were repeated three times.

Treatment of the washed (resting) cells with the test materials. The cells were prepared as follows: strain A was cultivated in nutrient broth at 30°C for 24 h and harvested by centrifugation at 10,000 × g for 15 min. The precipitate was washed twice with sterile phosphate buffer (40 mM, pH 6.5) with centrifugation. The washed cells were suspended in test material solution to produce a cell concentration of 10⁶ CFU/ml. The suspension was incubated at 30°C for 4 or 24 h and then centrifuged as above. The supernatant fluid was subjected to spectrophotometric analysis. The test material solution was prepared as follows: hop resins (0.01%), SHMP (1%), and a combination of both were used as test materials. These materials were dissolved in sterile phosphate buffer (40 mM, pH 6.5). All samples were made in duplicate, and experiments were repeated three times.

Preparation of mashed potatoes. One hundred grams of mashed potato powder (ingredients: potato, skimmed milk powder, glyceride, sodium polyphosphate, citric acid; component analysis per 100 g: moisture 6.1 g, protein 6.5 g, lipid 0.8 g, carbohydrate 83.9 g, ash 2.7 g, potassium 1,300 mg, vitamin C 10 mg; Snow Brand Milk Co., Tokyo, Japan) was mixed with 400 ml of hot water (90°C). Then, the test materials (0.1% hop resins, 1% SHMP, and both) were added to the paste. It was sterilized at 121°C for 20 min. After cooling, 1 ml of cell suspension (10⁴ CFU/ml) of strain A was inoculated into the autoclaved paste. The system was then incubated at 20°C to examine growth of the bacterium. Growth was monitored by enumerating the viable cells by employing the standard plate method (3). The cell suspension was prepared by the same method described in the section on measurement of antimicrobial activity. Viable cell counts were carried out in duplicate, and experiments were repeated three times.

RESULTS AND DISCUSSION

Antimicrobial activity of hop resins. Antimicrobial activity of hop resins against bacterial strains is shown in Table 1. The hop resins inhibited the growth of the six gram-positive bacteria at concentrations <50 µg/ml. Specifically, the hop resins inhibited the growth of B. cereus below 10 µg/ml. Minimum inhibitory concentration values of the hop resins against B. subtilis and Micrococcus flavus were 40 and 50 µg/ml, respectively. The growth of other gram-positive bacterial strains was inhibited at a concentration of 20 µg/ml. In contrast, the growth of all gram-negative bacterial strains tested was not inhibited even at more than 10,000 µg/ml, as also noted previously (8, 9, 11, 22, 24–27, 31).

Antimicrobial activity by combined use against E. coli strains. Like hop resins, when either SHMP (1%), glycerol moncaprate (0.01%), or lysozyme (0.1%) was used separately, no antimicrobial activity against strain A was noted. The values shown in the brackets are the maximum concentration permitted in food use that was determined by organoleptic examination of kamaboko. The
TABLE 2. Antimicrobial activity by combined use of hop resins and sodium hexametaphosphate against strain A

<table>
<thead>
<tr>
<th>Hop resins (%)</th>
<th>Sodium hexametaphosphate (%)</th>
<th>24 h Growth (OD 660 nm)</th>
<th>48 h Growth (OD 660 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0025</td>
<td>0.5</td>
<td>0</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>0.005</td>
<td>0.5</td>
<td>0</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>0.0075</td>
<td>0.5</td>
<td>0</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>0.01</td>
<td>0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>0.01</td>
<td>0.25</td>
<td>0.2</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>0.01</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
<td>0.75</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
</tr>
</tbody>
</table>

\(^a\) Strain A was incubated in nutrient broth with materials at 30°C for 48 h.

above agents have strong antimicrobial activity against gram-positive bacteria at concentrations even below values shown in the brackets but little effect against gram-negative bacteria (2, 10, 23, 32).

A combination of hop resins (0.01%) with glycerol monopropionate (0.01%) or lysozyme (0.1%) had no activity against strain A, while that of hop resins (0.01%) with SHMP (1%) was found to have a strong antimicrobial activity against strain A. This combination completely inhibited the growth of the strain for 48 h at 30°C. Even after 7 days of incubation, no growth was observed. Initial pH of the media with and without test materials was 6.73 and 6.55, respectively. Tween 80 and ethanol failed to have any effect on growth of the strain at the tested concentrations (0.01 and 0.001%, respectively).

The effects of the above combination should be examined in greater detail. The concentration of hop resins was maintained at 0.01%, whereas that of SHMP varied from 0.25 to 1.0%. As shown in Table 2, there was strong antimicrobial activity at an SHMP concentration exceeding 0.5%. When the concentration of SHMP was kept constant at 0.5% and that of hop resins varied from 0.0025 to 0.01%, antimicrobial activity was noted at concentration as high as 0.0075%.

The combined effect was tested on the growth of five different strains of E. coli including strain A. As shown in Table 3, the two strains, E. coli ATCC 15224 and E. coli var. communor, were inhibited by combined use of hop resins and SHMP. The growth rate of two other strains, E. coli (isolated from oyster) and E. coli K-12 OUT 8401, was considerably decreased by the combined use of the test materials. The combined use of these materials thus results in a significant effect. Pathogenic strains of E. coli were not examined, as these strains cannot be dealt with in our laboratory.

Influence of the addition time of the combined materials. The effects of hop resins and SHMP in combination were studied as a function of addition time. To clarify the characteristics of combined use, strain A, the most sensitive, was used. The results are shown in Figures 1 and 2. When materials were added at the beginning of incubation, strain A growth was completely inhibited. But with both added after 3 h incubation, the lag-phase stage was extended by 44 h. With this addition at 6 h incubation, this phase

TABLE 3. Antimicrobial activity by combined use of hop resins and sodium hexametaphosphate against Escherichia coli

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Hop resins (%)</th>
<th>0</th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
<th>3.0</th>
<th>4.0</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0 0.01</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0 0.01</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>C</td>
<td>0 0.01</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>0.80</td>
<td>0.55</td>
</tr>
<tr>
<td>D</td>
<td>0 0.01</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>0.96</td>
<td>0.12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>0 0.01</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
<td>&gt;1.0</td>
</tr>
</tbody>
</table>

\(^a\) A. E. coli K-12 IFO3301 (strain A); B. E. coli ATCC 15224; C. E. coli var. communor (isolated from a food factory); D. E. coli (isolated from oyster); E. E. coli K-12 OUT8401.
stage was extended by 17 h (Fig. 1). When added at an earlier stage during the lag time stage, the lag phase was longer than when the addition was made at a later time. These reagents thus appear more effective when added to the medium prior to growth.

The addition of materials in combination during the log-phase stage caused growth to cease (Fig. 2). No bacteriolysis was observed in strain A. Hop resins or SHMP added above failed to decrease growth. The antimicrobial effect of their combination on *E. coli* may thus possibly be bacteriostatic.

**Leakage of cell components from the treated cells.**

Absorption spectra of supernatant fluids, prepared from strain A cell suspension treated with test materials, are shown in Fig. 3 (I–V). (i) Nontreated cells: no peaks appeared (Fig. 3-I). (ii) Treated cells with hop resins only: the peaks at 230 and 330 nm appeared with the cells treated as shown in Figure 3-II. (iii) Treated cells with SHMP only: as shown in Figure 3-III, the peaks from 210 to 300 nm became higher as compared with those in Figure 3-I. (iv) Treated cells with the mixture of hop resins and SHMP: the peak near 260 nm appeared and became higher with the increase in the treatment time (Fig. 3-IV). (v) Control experiment. *B. cereus* was treated with hop resins only: as shown in Figure 3-V, the similar absorption patterns were obtained with those shown in Figure 3-IV. Also, the similar patterns were obtained with the cells of *B. cereus* treated with SHMP only.

When gram-positive bacteria were treated with SHMP, there was leakage of cell components. Isshki et al. (10) reported *B. subtilis*, treated with SHMP, to give on absorption spectrum with a maximum of 258 nm due to leakage of nucleic acid-based substances from *B. subtilis*. Suda et al. (28, 29) and Tamaki and Matsuhashi (30) previously reported such leakage not to occur with gram-negative bacteria. The outer membrane of *E. coli* may possibly ward off the effects of SHMP. In this study, the supernatant fluid of the cell suspension of *E. coli* treated with test materials showed greater absorbance at 260 nm, suggesting the combined use of hop resins and SHMP to possibly damage the outer membrane of *E. coli*. But leakage was considered to be too little to kill the bacteria. The bacteria were actually shown to have survived even at 24 h following test materials addition. As reported previously, SHMP might have an effect on the membrane transport of hydrophobic materials (28).

**Effect of the test materials on the growth of strain A in mashed potatoes.** Figure 4 shows the growth of strain...
A in mashed potatoes supplemented with hop resins or SHMP or both. Hop resins (0.1%) or SHMP (1%) above had virtually no inhibitory effect on growth. But together, they suppressed the growth of strain A to 1/10^6. The antimicrobial effect is thus demonstrated for a model food system such as mashed potatoes.

Hop resins have been used for brewing beer since ancient times, therefore there are few problems in terms of its safety. Hop resins have not been used for food preservation as fully as possible, because hop resins do not have antimicrobial activity against gram-negative bacteria. With the combined use of hop resins and SHMP, a new antimicrobial effect was observed against \textit{E. coli}, and effects against other gram-negative bacteria are now being studied. Although we must wait for the final results, some antimicrobial effect has already been noted against other gram-negative bacteria (unpublished). More detailed study of hop resins should indicate new applications.

**ACKNOWLEDGMENTS**

The authors thank Dr. Toshiyuki H. and Suekazu O., Nippon-Shinyaku Co., for their valuable suggestions.

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