Identification of Didecyldimethylammonium Salts and Salicylic Acid as Antimicrobial Compounds in Commercial Fermented Radish Kimchi

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ABSTRACT: Daikon radish (Raphanus sativus) fermented with lactic acid bacteria, especially Leuconostoc or Lactobacillus spp., can be used to make kimchi, a traditional Korean fermented vegetable. Commercial Leuconostoc/radish root ferment filtrates are claimed to have broad spectrum antimicrobial activity. Leuconostoc kimchii fermentation products are patented as preservatives for cosmetics, and certain strains of this organism are reported to produce antimicrobial peptides (bacteriocins). We examined the antimicrobial agents in commercial Leuconostoc/radish root ferment filtrates. Both activity-guided fractionation with Amberlite XAD-16 and direct extraction with ethyl acetate gave salicylic acid as the primary agent with activity against Gram-negative bacteria. Further analysis of the ethyl acetate extract revealed that a didecyldimethylammonium salt was responsible for the Gram-positive activity. The structures of these compounds were confirmed by a combination of 1H− and 13C NMR, high-performance liquid chromatography, high-resolution mass spectrometry, and tandem mass spectrometry analyses. Radiocarbon dating indicates that neither compound is a fermentation product. No antimicrobial peptides were detected.

KEYWORDS: antimicrobial peptides, salicylic acid, didecyldimethylammonium salts, bacteriocins, Raphanus sativus, Leuconostoc/radish root ferment filtrate

INTRODUCTION

Kimchi is a popular Korean dish made from fermented Chinese cabbage and/or other ingredients like winter radish, cucumber, and scallions. As with many fermented foods, it is the action of lactic acid bacteria that leads to the dish’s distinctive taste and prolonged shelf life. Although much of the food-preserving effects of lactic acid bacteria are due to the consumption of carbohydrates and production of organic acids, such bacteria may also prevent spoilage by ribosomal production of antimicrobial peptides, known as bacteriocins. Bacteriocins may be unmodified peptides or may have elaborate post-translational modifications that are key to their activity. Some bacteriocins of lactic acid bacteria are used commercially in partly purified form; for example, nisin A has been used to preserve a variety of foods for over 40 years.

The kimchi fermenter Leuconostoc kimchii is a potential source of antimicrobial peptides, and a mixture of L. kimchii fermentation products was recently patented as a cosmetics preservative. The patent claims that this mixture has activity against Gram-positive and Gram-negative bacteria, as well as fungi. Although a number of antimicrobial peptides have been isolated from kimchi fermentations and at least one strain of L. kimchii produces bacteriocins, no known antimicrobial peptide from L. kimchii displays such broad spectrum of activity. The antimicrobial preservatives widely used in cosmetics to extend shelf life and lower the risk of bacterial contamination often include simple aromatic derivatives such as salicylic acid. This compound is modestly antimicrobial against Gram-negative organisms, displays antifungal activity, and also has a number of useful effects on keratocytes. Stronger antimicrobial activity in cosmetics can be provided by quaternary ammonium compounds, a class of surfactants also used as a general surface disinfectant. Although salicylic acid is a natural product, both of these types of preservatives are produced on an industrial scale by chemical synthesis. In recent years many consumers have expressed a desire for preservatives completely derived from natural plant and bacterial sources. However, in some cases it has become evident that these biological preservatives have been adulterated with synthetic antimicrobial agents (e.g., benzethonium chloride was found in natural grapefruit seed extract). Commercial Leuconostoc/radish root ferment filtrates (LRRFF), ostensibly obtained from Daikon radish (Raphanus sativus) fermented with L. kimchii, are claimed to naturally have broad spectrum antimicrobial activity. It seemed reasonable that LRRFF could be used in food as well as in cosmetics, and that the activity could be due to antimicrobial peptides produced by the plant or its bacterial fermentation.

Our interest in antimicrobial peptides produced by bacteria, especially systems claimed to have activity against both Gram-negative and Gram-positive pathogens, led us...
to characterize the antimicrobial components of commercial Leuconostoc/radish root filtrates.

**MATERIALS AND METHODS**

**Materials.** Leuconostoc/radish root ferment filtrate (LRRFF) was obtained from retail stores in Lincolnton, NC and Olga, WA, via their Web sites. The samples were produced by Active Micro Technologies (Lincolnton, NC), and were marketed as Leucilid Liquid. Lot numbers were given as FSS130415, FSSI111019-1, and 2923P-5139. *Raphanus sativus* (daikon/winter radish) was purchased at Planet Organic Market (Edmonton, Canada). Strong anion exchange resin AG 1-X8 (chloride form, 50–100 mesh) was purchased from Bio-Rad (Hercules, CA). Before use, the resin was pretreated with 6 M HCl for 20 min then washed with deionized water until pH 7.

**Chemicals.** Salicylic acid was purchased from Sigma-Aldrich (St. Louis, MO). Didecyldimethylammonium chloride was purchased from Lonza Inc. (Basel, Switzerland). High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were purchased from Caledon Laboratory Chemicals (Georgetown, Canada) and filtered through a 0.22 μm membrane before use. All other solvents were of American Chemical Society (ACS) grade and purchased from Fisher Scientific (Hampton, NH). Deionized water was obtained from a Milli-Q reagent water system (Millipore Co., Milford, MA). Protease XIV was obtained from Sigma-Aldrich (St. Louis, MO) and used according to the manufacturer’s instructions.

**Instrumental Analysis.** Analytical high-performance liquid chromatography was done on a Varian ProStar equipped with model 210 pump heads, a model 325 dual wavelength detector, and a 1 mL injection valve. A gradient of (A) methanol and (B) water, each containing 0.1% trifluoroacetic acid (TFA). A gradient HPLC method was used, consisting of 5 min hold at 5% A; 15 min decrease from 5% to 0% A; 5 min hold at 0% A; 5 min decrease from 0% to 5% A; and 5 min hold at 5% A, with a flow rate of 1 mL/min and ultra violet (UV) detection at 220 nm.

Nuclear magnetic resonance (NMR) spectra were recorded from samples in CDCl3 on a Varian Unity 500 MHz or Varian Inova 600 MHz spectrometer at ambient temperature. Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane and are corrected to the solvent residual peak (7.26 ppm).

High-resolution electrospray ionization (ESI) mass spectra were acquired by liquid injection analysis and mass spectral conditions was methanol.

Fractionation of Leuconostoc/Radish Root Ferment Filtrate by Hydrophobic Interaction Resin. Amberlite XAD-16 resin (40 g) was swelled by gentle stirring in isopropanol (IPA) for 30 min, followed by three rinses with deionized H2O. To this resin was added a 1:1 mixture of LRRFF (100 mL) from the 1100 mL bottle purchased from Formulator Sample Shop and deionized H2O (100 mL). The resulting slurry was gently shaken for 20 min then loaded into a 2.5 × 0.6 cm fritted column. The flow through was collected, and the column was washed with three bed-volumes of deionized H2O, then two bed-volumes each of 20% IPA, 40% IPA, 60% IPA, 80% IPA, and finally 80% IPA containing 0.1% TFA. All solutions were collected, concentrated to remove isopropanol, and evaluated for biological activity using a spot-on-lawn assay.

**Extractive Analysis of LRRFF.** A 50 mL portion of Leuconostoc/radish root ferment filtrate was mixed with 40 mL of deionized water and extracted with 4 × 70 mL of ethyl acetate (EtOAc). The organic extracts were combined and dried over Na2SO4, and then concentrated in vacuo. The resulting white residue was analyzed by NMR spectrometry, HPLC, and elemental analysis. This residue could be further purified by dissolving in approximately 20 mL of EtOAc and extracting into water with 5% NaOH (3 × 10 mL). After the aqueous layers were combined and acidified with 6 M HCl to pH 2, a white solid (salicylic acid) crystallized from the solution. This solid was recrystallized from water, then dissolved in a 1:1 mixture of methanol (MeOH) and water for the spot-on-lawn assay.

A second compound (didecyldimethylammonium salt) was isolated as follows: LRRFF (50 mL) was mixed with 5% NaOH (20 mL) to a pH of 9–10 and then extracted with dichloromethane (4 × 70 mL). The organic extracts were pooled, dried with Na2SO4, and the solvent was removed in vacuo. The resulting residue was resuspended in a 1:1 mixture of MeOH:H2O (2 mL) and then combined with a slurry of AG 1-X8 anion exchange resin (2 g. Cl− form). This mixture was incubated at room temperature for 20 min with occasional swirling, followed by gravity filtration to remove the resin. The flow-through was collected, concentrated to dryness in vacuo, and subjected to NMR analysis.

**Characterization Data.** Salicylic Acid. FTIR (neat, cm−1, ν): 3236, 3004, 2858, 2598, 1669, 1612, 1444. 1H NMR (600 MHz, CDCl3, δ): 7.94 (m, 1H, Ph-H), 7.56 (m, 1H, Ph-H), 7.03 (m, 1H, Ph-H), 6.95 (m, 1H, Ph-H). 13C NMR (125.7 MHz, CDCl3, δ): 174.7, 162.2, 137.0, 130.9, 119.6, 117.9, 111.3. Anal. Calcd. for C7H6O3: C , H, O. Found: C, H, O.

Didecyldimethylammonium Chloride. 1H NMR (600 MHz, CDCl3, δ): 3.51 (m, 4H, CH2N3), 3.40 (s, 6H, CH3N3), 1.70 (br, s, 1H, CH2N3), 1.35 (m, 28H, CH2−CH2−CH2−CH2−N), 0.88 (t, 6H, CH3), 1.15 ppm (4H). 13C NMR (125.7 MHz, CDCl3, δ): 63.9, 51.6, 31.8, 29.7, 29.4, 29.2, 26.4, 22.9, 22.7, 14.1. ESI-HRMS m/z calc. for C23H37N3: 326.3781 [M + H]+. Found: 326.3784.

**RESULTS AND DISCUSSION**

### Results

In a spot-on-lawn assay, the commercial LRRFF inhibited the growth of *E. coli* DH5α, *E. coli* 25922, and *L. lactis* subsp. *cremonis* HP (Table 1). Antimicrobial activity was retained following treatment with Protease Type XIV or exposure to 100 °C for 60 min, conditions under which peptides typically degrade. Fractionating the liquid with Amberlite XAD-16 localized activity against both *E. coli* and *L. lactis* strains to the 60% isopropanol and 80% isopropanol, 0.1% TFA fractions. Trifluoroacetic acid was included in the final wash to protonate any potential amines which might otherwise be too hydrophobic to elute from the XAD-16 resin.

When the XAD-16 fractions were left at room temperature overnight, a white needle-like solid precipitated from the 60% IPA and 80% IPA with 0.1% TFA fractions. This solid was collected and characterized by Fourier transform infrared (FTIR), NMR, elemental analysis, and mass spectrometry (EI) analysis. The solid was found to have a mass of 138 g/mol.
consistent with a molecular formula of C$_7$H$_6$O$_3$. The mass, FTIR, $^1$H NMR, and $^{13}$C NMR data and chromatographic behavior were in accord with that of salicylic acid, with a minor alkyl impurity visible by $^1$H NMR (Figure 1).

Salicylic acid is a natural product, produced by both lactic acid bacteria and plants, and is present in fermented radish brine. It inhibits the growth of some Gram-negative bacteria and fungi but is not generally active against Gram-positive bacteria. This was confirmed with a spot-on-lawn assay using commercial salicylic acid, dissolved in 1:1 MeOH:H$_2$O, which inhibited the growth of E. coli DH5$\alpha$ and not L. lactis subsp. cremoris HP. The solid which was isolated from LRRFF following XAD-16 fractionation was active against both organisms (Figure 2). High-resolution ESI-MS analysis of the solid showed only one signal in the positive mode, with a m/z value of 326.3776. This corresponded to a chemical formula of C$_{22}$H$_{48}$N$, suggesting that at least part of the salicylate had a saturated alkyl amine counterion. This was verified via direct extraction of LRRFF with ethyl acetate, followed by washing with sodium hydroxide and acidification with hydrochloric acid. Upon acidification, salicylic acid precipitated out of solution and was collected via filtration. This sample was identical to a commercial sample by HPLC and did not inhibit the growth of L. lactis subsp. cremoris HP (Figures 2 and 3, Table 2). MS analysis no longer showed a peak at 326.3776 in the positive mode.

To determine the structure of the substance responsible for the activity against L. lactis, we concentrated the base-washed ethyl acetate layer and examined the resulting solid by $^1$H NMR. While generally consistent with didecyldimethylammonium bromide, the spectra contained a small salicylate impurity. To remove this, the mixture was dissolved in water and passed through a column of anion exchange resin AG 1-X8 (Cl$^-$. Once the sample was concentrated to dryness and reconstituted in CDCl$_3$, NMR analysis showed no trace of salicylate (Figure 4), and all of the $^1$H and $^{13}$C NMR spectra were identical to that of didecyldimethylammonium bromide.

In mass spectral analyses, the molecular formula of the positive ion obtained from didecyldimethylammonium bromide matched that obtained by high-resolution mass spectrometry (HRMS), and tandem mass spectrometry analysis of our sample showed only one fragmentation peak, with a molecular formula of C$_{12}$H$_{28}$N$. This corresponds to loss of C$_{10}$H$_{20}$, one of the two decyl chains.

Didecyldimethylammonium chloride is known to inhibit the growth of several Gram-positive bacteria, including Staphylococcus aureus. Both the isolated salt and a commercial

<table>
<thead>
<tr>
<th>entry</th>
<th>sample</th>
<th>E. coli DH5$\alpha$</th>
<th>E. coli 25922</th>
<th>L. lactis subsp. cremoris HP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Leuconostoc/radish root ferment filtrate</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>following Protease treatment</td>
<td>n/a</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>following heating to 100 °C for 60 min</td>
<td>n/a</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>flowthrough</td>
<td>+</td>
<td>n/a</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>wash (0% IPA)</td>
<td>−</td>
<td>n/a</td>
<td>−</td>
</tr>
<tr>
<td>6</td>
<td>20% IPA</td>
<td>−</td>
<td>n/a</td>
<td>−</td>
</tr>
<tr>
<td>7</td>
<td>40% IPA</td>
<td>−</td>
<td>n/a</td>
<td>−</td>
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<tr>
<td>8</td>
<td>60% IPA</td>
<td>+</td>
<td>n/a</td>
<td>++</td>
</tr>
<tr>
<td>9</td>
<td>80% IPA with 0.1% TFA</td>
<td>+</td>
<td>n/a</td>
<td>++</td>
</tr>
</tbody>
</table>

Entries 4–9 represent various stages of Amberlite XAD-16 fractionation tested as 10 μL aliquots. ++ means that the sample was active; + means the sample was weakly active; − means the sample was inactive; n/a means the sample was not tested against that organism.

Table 1. Results of Spot-on-Lawn Assays of Leuconostoc/Radish Root Ferment Filtrate against Indicator Organisms

A: Commercial salicylic acid

B: White solid obtained after hydrophobic fractionation of Leuconococcus-fermented root filtrate

C: White solid extracted from Leuconococcus-fermented root filtrate

Figure 1. $^1$H NMR spectra of (A) commercial salicylic acid, (B) the white solid obtained after fractionation using Amberlite XAD-16 resin, and (C) the white solid obtained from EtOAc extraction of Leuconostoc/radish root ferment filtrate. The solvent peak of deuterated chloroform appears at 7.26 ppm.
sample strongly inhibited the growth of *S. aureus* ATCC 6538 and *L. lactis* subsp. *cremonis* HP at 8 \( \mu \text{g/mL} \) in our spot-on-lawn assay.

To estimate the amount of salicylic and didecyldimethylammonium salt in commercial LRRFF, 50 mL aliquots were drawn from three different samples obtained from two different suppliers. The results were performed without duplication, and are summarized in Table 3. We also blended a 150 g sample of daikon/winter radish (*Raphanus sativus*), and extracted the resulting aqueous mixture with EtOAc, but were unable to detect salicylic acid or didecyldimethylammonium salts by mass spectrometry. While salicylic acid is one of the organic acids produced by lactic acid bacteria and is known to occur in fermented radish brine, to date didecyldimethylammonium salts have been produced only through anthropogenic chemical synthesis.

To determine the origin of the salicylic acid and didecyldimethylammonium salts we isolated from LRRFF, samples were submitted for carbon dating. On the basis of the amount of \(^{14}\text{C} \) present, these compounds were dated to 52 000 \( \pm 2900 \) and 21 140 \( \pm 100 \) years old, respectively. This clearly indicates that the salicylic acid and the didecyldimethylammonium chloride are largely derived from petroleum-based precursors and that neither is the product of a recent fermentation of plant material. The didecyldimethylammonium salts, which are used as detergent biocides and wood preservatives, have toxicity to aquatic organisms and can also affect human health. They are known to enhance permeability of salicylic acid through

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**Table 2. Antimicrobial Activity of the Isolated Solid or Residue from *Leuconostoc*/Radish Root Ferment Filtrate**

<table>
<thead>
<tr>
<th>entry</th>
<th>sample (dissolved in MeOH:H(_2)O/1:1)</th>
<th><em>E. coli</em> DH5(\alpha)</th>
<th><em>L. lactis</em> subsp. <em>cremonis</em> HP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MeOH:H(_2)O/1:1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>commercial salicylic acid</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>solid obtained from fractionation by 60%, 80% IPA on XAD resin</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>solid obtained by EtOAc extract</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>residue obtained after the base wash of EtOAc extract</td>
<td>–</td>
<td>++</td>
</tr>
</tbody>
</table>

*Samples were dissolved at 1 mg/mL concentration and a 10 \( \mu \text{L} \) aliquot was tested. ++ means that the sample was active; + means the sample was weakly active; – means the sample was inactive.*
animal skin, and can cause skin allergic effects, asthma, and lung problems, as well as eye irritation. In Europe, the acceptable daily intake of didecyl dimethylammonium chloride has been set to 0.1 mg/kg body weight per day. As indicated in Table 3, a 50 mL batch of LRRFF would exceed this level for an adult human male.

In summary, the antimicrobial activity of commercial Leuconostoc/radish root ferment filtrates (LRRFF) are attributed to salicylic acid and didecyl dimethylammonium salts. Moreover, these two compounds are too deficient in 14C to be the product of recent fermentation, suggesting that they are derived from petroleum feedstock. We were unable to detect antimicrobial peptides in any sample of fermented radish root filtrate.

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**Funding**
This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC), Griffith Laboratories Limited, Alberta Innovates Health Solutions, and the Canada Research Chair in Bioorganic and Medicinal Chemistry.

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**Table 3. Amount of Purified Salicylic Acid and Didecyl Dimethylammonium Salts Obtained from Leuconostoc/Radish Root Ferment Filtrate Purchased from Two Companies**

<table>
<thead>
<tr>
<th>sample</th>
<th>batch 1 (8 oz)</th>
<th>batch 2 (1 kg)</th>
<th>batch 3 (8 oz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>isolated and purified salicylic acid (g/50 mL)</td>
<td>0.31</td>
<td>0.12</td>
<td>0.19</td>
</tr>
<tr>
<td>didecyl dimethylammonium salts as chloride (mg/50 mL)</td>
<td>8</td>
<td>16</td>
<td>10</td>
</tr>
</tbody>
</table>

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**Notes**

The authors declare the following competing financial interest(s): Griffith Laboratories produces and markets food ingredient systems for enhancement of food safety and flavor.

**ACKNOWLEDGMENTS**

We thank Dr. Randy Whittal (University of Alberta) for assistance with mass spectrometric analysis.

**REFERENCES**


