Inactivation of *Escherichia coli* O157:H7 by cinnamic aldehyde purified from *Cinnamomum cassia* shoot

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Abstract

*Escherichia coli* O157:H7 is a pathogen, which causes the hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura in humans. Control of the bacterial cells in foods is an important factor to reduce outbreaks of the foodborne diseases. In this study, cinnamic aldehyde possessing antimicrobial activity against the bacterial cells was purified from the extract of cinnamon (*Cinnamomum cassia* Blume) shoot by sequential fractionation with various solvents and silica gel column chromatography. When *E. coli* O157:H7 cells were incubated at 37°C for 12 h in the presence of 500 mg ml⁻¹ of the purified from cinnamic aldehyde, the viable counts decreased dramatically (from 4.9 × 10⁶ to 1.0 × 10² cfu ml⁻¹). In the presence of 1000 μg ml⁻¹ of the substance, most of the cells were killed after 2 h of incubation suggesting that the antimicrobial activity of cinnamic aldehyde is bacteriocidal in *E. coli*. Scanning electron microscopic observations revealed that the bacterial cells treated with the cinnamic aldehyde suffered from severe damages in their surface structure. Minimal inhibitory concentration of the cinnamic aldehyde was determined to be 250 μg ml⁻¹ against *E. coli* strains O157:H7 and O26 or 500 μg ml⁻¹ against strains ATCC11105 and O111.

1. Introduction

Strains of *Escherichia coli* reside in the intestine of humans as an integral part of the normal bacterial flora. These commensal strains may perform essential functions for the host, but a few strains are pathogenic, which cause distinct diarrhea syndromes. Among them are enteropathogenic, enteroinvasive, enterotoxigenic and enterohemorrhagic *E. coli* (EHEC). *E. coli* O157:H7, a strain of EHEC, was first recognized as a human pathogen in 1982 which causes the hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura in humans (Zottola and Smith, 1991). Although person-to-person transmission of the pathogenic *E. coli* has been reported in some daycare centers and nursing home outbreaks, its main transmission mode has been reported to be through foods. Many food-associated outbreaks caused by *E. coli* O157:H7 have occurred from apple cider, ground beef, poultry, milk, hamburger, ham and cheese sandwiches (Zottola and Smith, 1991). Therefore, there have been many studies on the detection and control methods of *E. coli* O157:H7 in foods (Onoue et al., 1999). Studies on the control of *E. coli* O157:H7 have been largely focused on the use of chemical additives (Conner and Kotrola, 1995; Zhao et al., 1993; Bari et al., 1999).

Various spices have been used for the purpose of food preservation and appetizer promotion as well as medicinal purposes. In particular, extracts from many kinds of oriental spice plants containing their essential oil and essence have been known to possess antimicrobial effects. Among them are cassia, clove, garlic, sage, oregano, pimento, thyme, rosemary, mint and allspice (Saleem and Ai-Delaimy, 1982; Shelef et al., 1980; Tassou et al., 2000; Yildirim et al., 2000, Zaika and Kissinger, 1981). Oils of cinnamon, clove, bay and thyme (Bullerman et al., 1977; Smith et al., 1998) as well as plant flavonones (Deng et al., 2000) have been reported to exhibit inhibitory effects against many...
foodborne pathogenic bacteria. Cassia has been used as a spice source for a long period of time in East Asian countries. Especially, its shoot has been known as an important medicinal source, which is effective to relieve fever and disturbed urination (Kim et al., 1998). The antimicrobial substance of cassia bark has been identified to be trans-cinnamic aldehyde, which showed insecticidal and fumigant activities against Mechoris ursulus (Park et al., 2000). However, the active principles of cassia shoot have not yet been reported thus far and little is known about its inhibitory effects on the bacteria.

In this study, cinnamic aldehyde was purified from cassia shoot and its antimicrobial effects were studied against foodborne pathogenic E. coli strains including E. coli O157:H7. It was also investigated whether cinnamic aldehyde possesses bacteriocidal effects against the bacteria.

2. Materials and methods

2.1. Samples, test organisms and media

Bark and shoot of Cinnamomum cassia Blume obtained from commercial sources in Korea were extracted with methanol to test their antimicrobial effects against various E. coli strains. The microorganisms used in this study were E. coli strains ATCC11105, O157:H7 (ATCC43888), O111 and O26 from the laboratory stock. Difco Mueller–Hinton agar or broth was used for antimicrobial activity test and LB medium was used to investigate the effect of cinnamic aldehyde on the viability of the bacterial cells (Davidson and Parish, 1989).

2.2. Purification of antimicrobial substance

A mixture of 10 g of cassia bark or shoot powder and 100 ml of methanol was heated in a boiling water-bath for 8 h with reflux. After the extraction, the mixture was filtered and methanol was evaporated from the filtrates on an evaporator to obtain dry extracts. The extracts were resuspended in dimethyl sulfoxide (DMSO) and used for the antimicrobial activity test. For the purification of the antimicrobial substance, the extract from cassia shoot was sequentially fractionated with chloroform, ethyl acetate, butanol and water. The active chloroform fraction was applied into a silica gel column (35 mm × 500 mm) and eluted stepwise with n-hexane: ethyl acetate (5:1, v/v) and n-hexane: ethyl acetate (3:1, v/v) solvent. The most active fraction was rechromatographed on the silica gel column and eluted with n-hexane: ethyl acetate (7:1, v/v).

2.3. Determination of cinnamic aldehyde content

The content of cinnamic aldehyde was determined by the method of Okamura et al. (1999) using a high-performance liquid chromatograph (HPLC, Waters Corporation Model Waters 712, Milford, USA). For the operation of the HPLC, μ-bondapak C18 column (3.9 mm × 300 mm), solvent (60%, v/v methanol in water) and a UV detector (UV 254 nm) were used. The flow rate was 1.0 ml min⁻¹.

2.4. Antimicrobial activity test

Impregnated paper disc method (Davidson and Parish, 1989) was used for the antimicrobial activity test. Cells of test micro-organisms grown overnight in LB media were spread on the Mueller–Hinton agar surface, and 1.5 mg of samples resuspended in DMSO were absorbed onto a paper disc (8 mm in diameter) placed on the top of the agar plate. After the plates were incubated at 37°C for 24 h, the diameter of the clear zone around the paper disc resulted from each sample was measured to determine their antimicrobial activities. Equal amounts of DMSO and benzoic acid were used as negative and positive controls, respectively. Inhibitory effect of cinnamic aldehyde on the bacterial growth was investigated using LB media containing cinnamic aldehyde. The bacterial cells were grown in LB media to exponential phase were harvested and resuspended in LB containing 250, 500 and 1000 µg ml⁻¹ of cinnamic aldehyde. The resuspension was incubated at 37°C for 12 h. During the incubation, aliquots were taken and centrifuged to harvest cells, which were then, washed and resuspended in 0.9% NaCl solution to eliminate cinnamic aldehyde. The cell resuspension (0.1 ml) was spread on an LB agar plate after dilution with 0.9% NaCl solution when needed. Colonies formed were directly counted after incubation at 37°C for 24 h. The data used in the tables and figures indicate a representative of the data obtained from three independent trials.

2.5. Scanning electron microscope

In order to investigate the effect of cinnamic aldehyde on the surface structure of the bacteria, E. coli O157:H7 cells were obtained with the centrifugation of the culture grown to exponential phase in LB media. The cells were resuspended in LB media containing 500 µg ml⁻¹ of cinnamic aldehyde and the resuspension was incubated at 37°C for 2 h. Morphology of the bacterial cells was observed on a scanning electron microscope (Hitachi Co. HCP-2, Tokyo, Japan).
2.6. Determination of minimal inhibitory concentration (MIC)

MIC of the samples was determined by the broth dilution method using Mueller–Hinton media as described (Anonymous, 1994). The media containing 2000 µg ml⁻¹ of cinnamic aldehyde were serially twofold each with the media to give concentrations of 2000, 1000, 500, 250, 125 and 62.5 µg ml⁻¹. To the diluted solution, equal volume of Mueller–Hinton media inoculated with overnight culture of test strains at a concentration of 2 x 10⁶ cfu ml⁻¹ was added and mixed well. The mixture was incubated at 37°C for 24h to check the minimal concentration at which growth of the bacterial cells was fully inhibited. A similar experiment was carried out using DMSO instead of cinnamic aldehyde as a control. The experiments were performed in triplicate with three independent trials.

3. Results and discussion

3.1. Identification of antimicrobial substance in cassia shoot

Antimicrobial activities of methanol extracts from cassia bark and shoot were investigated against various *E. coli* strains (Table 1). The extract from cassia shoot showed higher yield and stronger antimicrobial activity than those from cassia bark against all the *E. coli* strains. Its antimicrobial activity was as high as that of benzoic acid used as a control. It is interesting to notice that the cassia shoot showed higher activity than the cassia bark. Cassia shoot has been used as a medicinal agent rather than as a spice and its price is generally lower than that of cassia bark. Therefore, cassia shoot is thought to have a great potential as a natural preservative in flavor, food, beverage and cosmetic industries.

<table>
<thead>
<tr>
<th>Parts used</th>
<th>Yield (%)</th>
<th>Inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATCC 1105</td>
<td>O157:H7</td>
</tr>
<tr>
<td>Bark</td>
<td>7.1</td>
<td>11</td>
</tr>
<tr>
<td>Shoot</td>
<td>8.6</td>
<td>13</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
</table>

*After the bacteria were grown at 37°C for 24h on Mueller–Hinton agar plates, diameter of the clear zone around a paper disc (Ø 8 mm) containing 1.5 mg of each methanol extract was measured to determine its antimicrobial activity.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Content (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cassia shoot</td>
<td>2.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Methanol extract</td>
<td>26.9</td>
<td>11.7</td>
</tr>
<tr>
<td>Chloroform fraction</td>
<td>43.8</td>
<td>19.0</td>
</tr>
<tr>
<td>First silica gel column chromatography</td>
<td>86.9</td>
<td>37.8</td>
</tr>
<tr>
<td>Second silica gel column chromatography</td>
<td>96.5</td>
<td>42.0</td>
</tr>
</tbody>
</table>

When the antimicrobial activities of chloroform, ethyl acetate, butanol and water fractions of methanol extract from cassia shoot were tested, the chloroform fraction showed the strongest activity among the fractions, which was even higher than that of benzoic acid. No significant activity was observed in the other fractions (data not shown). The active compound was further purified from the chloroform fraction by silica gel column chromatography. Analysis of the purified fraction by using an HPLC showed a single peak at a retention time of 5.43 min. Various instrumental analyses including mass spectrometry, Fourier transform infrared spectrometry (FT–IR) and nuclear magnetic resonance suggest the purified substance to be trans-cinnamic aldehyde. The FT–IR spectrum of the purified substance was exactly the same as that of standard trans-cinnamic aldehyde (data not shown). The content of the trans-cinnamic aldehyde was 2.3% in the cassia shoot. Its content in the purified fraction was 96.5%, suggesting that the substance was purified about 42 fold (Table 2).

Cinnamon is one of the world’s oldest spices that have been used in food, beverage and cosmetic industry. Its oil has been reported to inhibit the growth and subsequent toxin production of *Aspergillus paraticus* at 200–250 µg ml⁻¹ (Bullerman et al., 1977). Cinnamic aldehyde, which was identified in the oil, is an effective inhibitor of the growth of yeasts, bacteria and molds as well as toxin production by micro-organisms. It can completely inhibit the growth of a number of bacteria such as *Staphylococcus* sp., *Micrococcus* sp., *Bacillus* sp. and *Enterobacter* sp. at 500 µg ml⁻¹ (Masuda et al., 1998). It has been reported that application of 1.5 mg disc⁻¹ of cinnamic aldehyde revealed potent antimicrobial effects against *Clostridium perfringens*, *Bacteroides fragilis* and *Bifidobacterium bifidus* (Lee and Ahn, 1998).

3.2. Antimicrobial effect of cinnamic aldehyde against *E. coli* O157:H7

To investigate whether the growth-inhibiting effect of cinnamic aldehyde on *E. coli* O157:H7 is attributable to
bacteriostatic or bacteriocidal activity, viable cells were counted during the culture in LB media containing the purified cinnamic aldehyde (Fig. 1). In the absence of cinnamic aldehyde, viable counts increased from $4.9 \times 10^6$ cfu ml$^{-1}$ of the initial counts to $4.7 \times 10^9$ cfu ml$^{-1}$ at $37^\circ$C for 12 h of cultivation. No significant changes in the viable counts were observed in the presence of 250 $\mu$g ml$^{-1}$ of the substance during the culture time. However, addition of 500 $\mu$g ml$^{-1}$ of the substance in the media resulted in a dramatic decrease in the viable counts, which was $1.0 \times 10^2$ cfu ml$^{-1}$ after 12 h of incubation. In the presence of 1000 $\mu$g ml$^{-1}$, all the cells were killed after 2 h of incubation. These results suggest that the antimicrobial activity of cinnamic aldehyde is bacteriocidal. The cells treated with 1000 $\mu$g ml$^{-1}$ of the purified cinnamic aldehyde at $37^\circ$C for 2 h were observed on a scanning electron microscope (Fig. 2). Non-treated cells showed a smooth surface but the treated cells were severely damaged and destructed in the surface structure. Antimicrobial mechanisms of natural compounds found in herbs or spices have been discussed (Brul and Coote, 1999). Thymol and carvacrol have been reported to possess inhibitory effects against the growth of enteric bacteria ($E. coli$ O157:H7 and $Salmonella typhimurium$) at a similar concentration as shown above. They were proposed to have prominent outer membrane disintegration and increased the permeability of ATP through cytoplasmic membrane. However, trans-cinnamic aldehyde exhibited neither outer membrane disintegrating activity nor depletion of intracellular ATP (Helander et al., 1998).

3.3. MIC of the purified cinnamic aldehyde against various pathogenic $E. coli$ strains

To assay the antimicrobial activity of the cinnamic aldehyde purified from cassia shoot more carefully, its MIC was determined on various $E. coli$ strains. $E. coli$ O157:H7 and O111 showed higher sensitivity than $E. coli$ ATCC11105 and O26 to cinnamic aldehyde. To benzoic acid, sorbic acid and dehydroacetic acid used as a control, the strains O111 and O26 showed higher sensitivity than the strains ATCC11105 and O157:H7. The MIC of the substance was 250 $\mu$g ml$^{-1}$ against $E. coli$ strains O157:H7 and O26 or 500 $\mu$g ml$^{-1}$ against strains ATCC11105 and O111. Against $E. coli$ O157:H7, the MIC of cinnamic aldehyde (250 $\mu$g ml$^{-1}$) was found to be lower than those of sorbic acid and dehydroacetic
Table 3
Comparison in the minimal inhibitory concentration of cinnamic aldehyde purified from cassia shoot and synthetic preservatives against various \( E. coli \) strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (( \mu \text{g mL}^{-1} ))</th>
<th>Cinnamic aldehyde</th>
<th>Benzoic acid</th>
<th>Sorbic acid</th>
<th>Dehydroacetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC11105</td>
<td>500</td>
<td>200</td>
<td>1000</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>O157:H7</td>
<td>250</td>
<td>200</td>
<td>1000</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>O111</td>
<td>250</td>
<td>1000</td>
<td>500</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>O26</td>
<td>500</td>
<td>1000</td>
<td>500</td>
<td>1000</td>
<td></td>
</tr>
</tbody>
</table>

Acid (500 \( \mu \text{g mL}^{-1} \)) or benzoic acid (1000 \( \mu \text{g mL}^{-1} \)) (Table 3). \( E. coli \) O157:H7 has become a pathogen of concern in various food industries since it was reported to be associated with many serious outbreaks of foodborne illness. Other strains used in this study are also known as foodborne pathogens because of their vero-toxins in foods (Karmali et al., 1985). Recently, consumers have growing concern about the food safety related with hormones, food additives, food preservatives, etc. (Brewer et al., 1994). For this reason, the need for safe antimicrobial preservatives has been increased as an alternative of the chemical preservative in foods. And, many studies on the antimicrobial substances of spice against foodborne pathogens have been reported. Recently, renewed interests in the use of spice plants as a source of antimicrobial substance in foods are evident. When safety of synthetic additives is questioned, natural substances of plant origin may appeal to the public. In addition, reduction of salt and sugar in foods for dietary reasons tends to enhance the use of seasonings (Shelef, 1983). Cinnamic aldehyde, purified from cassia shoot, showed similar inhibitory effect against \( E. coli \) O157:H7 to that against mold growth and aflatoxin production reported by Mahmoud (1994), who described its MIC is as low as 200 \( \mu \text{g mL}^{-1} \). It is well known that most spices are more active against Gram-positive bacteria than Gram-negative bacteria (Smith et al., 1998; Kim et al., 1998). However, the cinnamic aldehyde purified from cassia shoot was effective for several \( E. coli \) strains as well, and its MIC was at a range of 250–500 \( \mu \text{g mL}^{-1} \) (Table 3).

References


Yildirim, A., Mavi, A., Oktay, M., Kara, A.A., Algur, O.F., Bilaloglu, V., 2000. Comparison of antioxidant and antimicrobial activities of tilia (\( Tilia argentea \) Desf Ex DC), sage (\( Salvia triloba \) L.), and...

