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Antimicrobial activity of mustard essential oil against *Escherichia coli* O157:H7 and *Salmonella typhi*

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ABSTRACT

The aim of this study was to investigate how mustard essential oil (EO) affected the cell membrane of *Escherichia coli* O157:H7 and *Salmonella typhi*. Intracellular pH and ATP concentration and the release of cell constituents were measured when mustard EO was in contact with *E. coli* and *S. typhi* at its minimal inhibitory concentration (MIC) and maximal tolerated concentration (MTC). The treatment with mustard EO affected the membrane integrity of bacteria and induced a decrease of the intracellular ATP concentration. Also, the extracellular ATP concentration increased and a reduction of the intracellular pH was observed in both bacteria. A significantly ($P \leqslant 0.05$) higher release of cell constituent was observed when both bacteria cells were treated with mustard EO. Electronic microscopy observations showed that the cell membranes of both bacteria were apparently damaged by mustard EO. In conclusion, mustard EO affects the concentration of intracellular component, such as ATP in both bacteria and affects the pH suggesting that cytoplasmic membrane is involved in the antimicrobial action of mustard EO. Mustard EO can be used as an effective antimicrobial agent. We have demonstrated that mustard EO affected the cell membrane integrity, resulting in a loss of cell homeostasis.

1. Introduction

EOs are well-known antimicrobial agents that could be used to control food spoilage and foodborne pathogenic bacteria (Burt, 2004). It has been demonstrated that several spices, herbs and fruits containing EOs effectively inhibit microbial growth, although different results are observed depending on test conditions, microorganisms, and the source of the antimicrobial compound (Roller, 2003). One of the EOs from a plant-derived mustard (*Brassica* spp.), is generally recognized as safe (GRAS) for food application based on 21 CFR part 182.20 ([CFR] Code of Federal Regulations., 2003). It occurs naturally in a wide range in the seeds, stems, leaves, and roots of cruciferous plants such as mustard, broccoli, horseradish, cabbage, cauliflower, kale and turnips (Nielsen & Rios, 2000). The primary component of mustard EO is allyl isothiocyanate (AIT), and it is a non-phenolic volatile compound found in plants belonging to the *Crucifereae* family. Many studies have reported that AIT effectively inhibited a variety of pathogenic microorganisms even when used at low concentrations (Delaquis, Ward, Holley, Cliff, & Mazza, 1999; Isshiki, Tokuoka, Mori, & Chiba, 1992; Kanemaru & Miyamoto, 1990; Lin, Kim, Du, & Wei, 2000; Ohta, Takatani, & Kawakishi, 1995; Turgis, Borsa, et al., 2008). Kanemaru and Miyamoto (1990) compared the antimicrobial effect of mustard EO and purified AIT at equal concentrations. Interestingly, they found that mustard EO was more effective against *E. coli* than purified AIT. They reported that 0.1% mustard EO containing 9.4 ppm of AIT inhibited the growth of *E. coli* in culture medium, while 12.3 ppm of purified AIT was required to achieve the same inhibitory activity. Previous studies by Lacroix group also observed an effective antimicrobial activity of mustard EO against *E. coli* O157:H7 and *S. typhi* in ground beef (Turgis, Han, et al., 2008; Turgis, Borsa, et al., 2008).

The antibacterial activity of mustard EO is probably due to the ability of its components to disrupt the membranes of bacterial cells, causing lysing of the cell (Burt, 2004). According to Helander et al. (1998), the EOs could inhibit *E. coli* O157:H7 at a concentration between 1 and 3 mmol/l by disintegration outer membrane and by the release of outer membrane-associated materials from the cells to the external medium. However, it has not been documented in detail how such EOs would achieve the antimicrobial activity.

In this study, we have investigated the effect of mustard EO on cell membranes of *E. coli* O157:H7 and *S. typhi* by measuring: (i) the intra- and extracellular ATP concentrations, (ii) the intracellular pH (pH_{in}), (iii) the release of 260-nm-absorbing cellular components, and (iv) morphology of the cell membranes using an electron microscopy.

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2. Materials and methods

2.1. Preparation of bacterial cultures

E. coli O157:H7 (EDL 933) was obtained from Prof. Charles Dozois (INRS-Institut Armand-Frappier, Laval, QC, Canada). *Salmonella enterica* subsp. *enterica* serotype Typhi strain ATCC 19430 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained at −80 °C in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD, USA) containing glycerol 10% (v/v). Before each experiment, stock cultures were propagated through two consecutive 24 h growth cycles in TSB at 35 °C and washed twice in saline solution 0.85% (w/v) to obtain working cultures containing approximately 10^7 CFU/ml.

2.2. Essential oil dispersion

Mustard EO was provided by Hilltech (Vankleek Hill, ON, Canada) and stored at 4 °C until used. Mustard EO was dispersed in sterile 10% (v/v) modified food starch (Purity Gum BE, National Starch & Chemical Co., Boucherville, QC, Canada) solution at room temperature for 2 min using an Ultra-Turrax homogenizator (model TP18/1059, Janke & Funkel, Staufen, Germany) at 20,000 rpm to obtain a colloidal suspension (10%, v/v) (Oussalah, Caillet, Salmieri, Saucier, & Lacroix, 2004). The presence of modified starch improved suspension stability and antimicrobial properties (Burt & Reinders, 2003). The starch-oil suspension was added to the experimental medium at the MIC and MTC. The MIC and MTC of mustard EO were determined using an agar dilution method (Robert-Dernuet, 1995). Petri plates of BHI agar containing various concentrations (0.003%, 0.006%, 0.013%, 0.025%, 0.05%, 0.1%, 0.2%, 0.4% and 0.8%, v/v) of mustard EO were inoculated with *E. coli* O157:H7 or *S. typhi*. Each working culture (2×10^7 CFU/ml) was diluted to obtain 10^{-4} and 10^{-5} in peptone water (0.1%, w/v) and 40 μl of each diluted culture was individually spread on the surface of the solidified agar plates. The positive control consisted of BHI agar without mustard EO, inoculated with the diluted medium culture. Uninoculated plates containing mustard EO served as negative control. Test and control plates were then incubated at 35 ± 1 °C. Plates were evaluated for the presence or the absence of colonies after 48 h of incubation. For each treatment, the absence of colony on all plates tested was considered as an inhibitory effect. The lowest concentration of mustard EO required to completely inhibit the growth of the tested bacterium was defined as the MIC. The MIC of mustard EO was 0.2% (v/v) for both *E. coli* O157:H7 and *S. typhi*. On the other hand, the MTC was the highest concentration of EO, which did not affect the bacterial growth. The MTC of mustard EO for both bacteria was same as 0.1% (v/v).

2.3. Measurement of intra- and extracellular ATP concentrations

The intra- and extracellular ATP concentrations were measured according to the method described by Lee, Kim, and Shin (2002). The working culture of *E. coli* O157:H7 or *S. typhi* containing approximately 10^7 CFU/ml was centrifuged for 10 min at 1000 *g*, and the supernatant was removed. The cell pellets were washed three times with 0.1 mol l^{-1} of sodium phosphate buffer (pH 7) and then cells were collected by centrifugation under the same conditions. A cell suspension (10^7 CFU/ml) was prepared with 9 ml of sodium phosphate buffer (0.1 mol/l, pH 7) and 0.5 ml of cell solution was taken into the eppendorf tube for mustard EO treatment. Then, the different concentrations (0 (control), MIC, and MTC) of mustard EO suspensions were added to the cell solution. Samples were maintained at room temperature for 30 min according to Ultee, Kets, and Smid (1999), centrifuged for 5 min at 2000*g*, and incubated in ice to prevent ATP loss until measurement. The extracellular (upper layer) and the intracellular (lower layer) ATP concentrations were measured using an ATP assay kit (Calbiochem, EMD Biosciences Inc., San Diego, CA, USA) which comprised nucleotide releasing buffer, ATP monitoring enzyme, enzyme reconstitution buffer and ATP. The ATP concentration of the supernatants, which represents the extracellular concentration, was determined using a luminometer (EG&G Berthold Lumat model LB 9507-2, Mandel Scientific Co. Inc., Guelph, ON, Canada) after the addition of 100 μl of nucleotide releasing buffer to 10 μl of supernatant and 1 μl of luciferase (1 mg/ml). To determine the ATP concentrations representing intracellular content, cell pellets were washed with 0.85% (w/v) sodium chloride solution and centrifuged for 5 min at 1000 *g*, and the supernatant was removed. The resulting cell pellets were maintained in 20 ml of 53 passive lysis buffer (Promega, Madison, WI, USA) at room temperature for 15 min to disrupt the microorganisms, and then centrifuged directly at 1200 *g* for 1 min. Subsequently, 1 μl of luciferase and 100 μl of nucleotide releasing buffer were added to 10 μl of resulting supernatants, and the ATP concentrations were measured for 1 min using a luminometer. To calculate the intra- and extracellular ATP concentrations, a standard ATP curve ranging from 10^{-1} to 10^{-9} ng/ml portion was used to obtain a linear relationship between ATP concentration (ng/ml) and the relative light unit which resulted in an r^2 (coefficient of determination) value of 0.98.

2.4. pH_{in} measurements

The pH_{in} was determined according to the fluorometric method described by Breeuwer, Drocourt, Rombouts, and Abee (1996). Working cultures of *E. coli* O157:H7 and *S. typhi* containing approximately 10^7 CFU/ml were treated separately with mustard EO at three different concentrations (0 (control), MIC, and MTC). The treated and untreated (control) cells (9 ml) were incubated at 35 ± 1 °C for 1 h, harvested by centrifugation (15 min at 1000 *g*), and then washed three times with 5 mmol/l of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 8.0) containing 50 mmol/l of EDTA. Subsequently, cells were resuspended in 8.8 ml of carboxyfluorescein diacetate succinimidyl ester (CFDASE, 1.5 μmol/l) solubilized in phosphate buffer (50 mmol/l) and incubated for 10 min at 30 ± 1 °C. CFDASE is hydrolysed by esterase to carboxyfluorescein succinimidyl ester (CFSE) in the cell and subsequently conjugated to aliphatic amines. Cells were washed with 50 mmol/l potassium phosphate buffer (pH 5.8) and incubated in the presence of 10 mmol/l glucose for 30 min at 30 ± 1 °C to eliminate nonconjugated CFSE. The cells containing fluorescent probe were then washed twice and resuspended in 50 mmol/l potassium phosphate, centrifuged at 1000*g* for 15 min, and kept on ice until measurement. The cell suspension was transferred into a 3-ml glass quartz cuvette, placed in the spectrofluorometer with a thermostated cuvette holder (Varian Canada Inc., Mississauga, ON, Canada), and stirred continuously. Fluorescence intensities were measured with two different excitation wavelengths (440 nm and 490 nm) by rapidly alternating the monochromator between both wavelengths. The emission was collected at 525 nm, and the excitation and emission slit widths were set as 5 and 10 nm, respectively. The pH_{in} of bacteria was determined from the ratio of the fluorescence signal at the pH-sensitive wavelength (490 nm) and the fluorescence signal at the pH-insensitive wavelength (440 nm). A calibration curves for bacteria were determined in buffers with pH values ranging from 4 to 8. Different pH buffers were prepared as 50 mmol/l citric acid (pH 4 and 5), 50 mmol/l Na_2HPO_4 (pH 6 and 7), and 50 mmol/l Tris – HCl (pH 8). The pH_{in} was equilibrated by addition of 1 μmol/l valinomycin and 1 μmol/l nigericin.