Antimicrobial effect of a combination of herb extract and organic acid against *Bacillus subtilis* spores

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Received: 1 January 2017 / Revised: 6 May 2017 / Accepted: 8 May 2017 / Published online: 23 October 2017
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Abstract The sporicidal activities of the herbs were investigated to screen for novel antimicrobial substances against *Bacillus subtilis* spores. The bacterial inactivation effects of ethanol extracts of coriander, caraway, mace at concentrations of 0.5% (w/v) and 1.0–2.5% were about 10- and 100-fold respectively against spores. At pH 5, the antimicrobial activity was about 92%, but at pH 4 the sporicidal activity was particularly high, reducing the spore count by 99.99%. The 0.1–2.5% ethanol extract of herbs adjusted to pH 4–5 exhibited significantly marked deactivation effects, with 3–4 log CFU/mL reductions. The herb–acid combination exerted a further increase in sporicidal activity, with an additional 1–3 log CFU/mL reduction. The sporicidal mechanism was assumed to involve a two-step: (1) the hydrophobic binding of surfactants in the herbs onto the spore coat destroys its protein, and (2) the acid then penetrates into the interior, generating unstable growth conditions.

Keywords *Bacillus subtilis* spore · Sporicidal activity · Herbs · Organic acid · Coriander

Introduction

The genus *Bacillus* are rod-shaped, Gram-positive cells that when exposed to stressful environmental conditions, such as lack of nitrogen or a carbon source, can form stable dormant endospores intracellularly. Endospores may survive for a long period and are more resistant than vegetative cells to drying, heating, chemical treatments, and radiation [1–3]. The spore coat is composed of two layers of protein that provide the defense against chemical agents such as acids and alkalis. The spore coats are also resistant to high pressures and electrical energy [4–6]. The resistance of the spore is increased by many factors in addition to this proteinaceous coat, such as the dry conditions, high dipicolinic acid content, and the divalent cations in the core [1, 3, 7]. In particular, the peptidoglycan cortex of the spore represents an important barrier to the small hydrophilic molecules found in antimicrobial components [4]. The genus *Bacillus*, including *Bacillus cereus*, cause food poisoning in humans, and *Bacillus stearothermophilus* causes food spoilage. Therefore, treatments to inhibit the growth and to sterilize these organisms must be developed for application during the production of processed foods.

While heat-sterilization techniques using high temperatures, such as the retort process, are effective for inactivating heat-resistant spores in food ingredients, excessive heating is detrimental to the sensory qualities and nutrient components. Therefore, various studies have been conducted toward the development of natural antimicrobial agents for the sterilization of bacterial spores. From a safety point of view, natural antibacterial agents based on extracts of herbs and spices are preferred, and various studies on such agents are currently in progress. Thus far, the cinnamic aldehyde in cinnamon, the eugenol in cloves and allspice, and the thymol in

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oregano and thyme have been found to contain effective antimicrobial components [8–10].

The finding that the growth of microorganisms is inhibited or inactivated at a range of low pHs has also led to the application of a variety of organic acids as a food additives in processed foods. The organic acids used in foods can be divided into two kinds of natural materials: those derived from fruits (e.g., citric acid from citrus fruits, benzoic and sorbic acids from berries) and those generated as a result of microbial fermentation of a starter culture (e.g., acetic acid, lactic acid, and propionic acid). A pH lower than that necessary for minimum growth could prevent microbial growth due to its effects on energy production, enzyme activity, nutrient transport, and other microbial functions [11–13]. And low-pH also suppresses the germination of bacterial spores [13, 14]. The mechanism underlying this inhibition of spore germination involves the unstable growth environment generated by changes in the ion composition, such as the replacement of Ca^{2+} by H^+ that occurs at low pH [11, 14, 15].

The antimicrobial effects of organic acids increase in proportion to the amount of undissociated molecules, and the concentration and lipophilic property of the acid. The strength of the antimicrobial effect is thus greatest for acetic acid, followed in descending order by propionic acid, lactic acid, and citric acid. Citric acid has a particularly weak antimicrobial effect due to the presence of a special transport mechanism on the cell membrane involving a citric-acid-specific permeation enzyme, citrate permease [11, 12, 14, 15].

A common method of increasing the antimicrobial effect of an organic acid is to use a mixture of, for example, acetic acid, lactic acid, and propionic acid, or else by combining it with other antimicrobial substances such as nisin and surfactants [11]. The objectives of the present study were to measure the antimicrobial activity of herb extract–organic acid combinations and to screen for novel antimicrobial substances against *B. subtilis* spores.

Materials and methods

Plant materials

The following dried herbs powders tested in this study were purchased at a local market in Seoul, Korea: celery, coriander, caraway, marjoram, oregano, sage, thyme, and mace. The samples were stored at 4 °C in a refrigerator to minimize any changes in quality.

Spore suspension

The *B. subtilis* ATCC 6633 strain used in this study was incubated on nutrient agar at 30 °C for 1 week, and the cultured vegetative cells containing endospores were suspended in sterile 0.85% NaCl solution. The suspension was sonicated for 5 min (with a 15-s:15-s on:off cycle) to destroy the vegetative cells, allowing the spores to be collected by centrifugation at a speed of $12,000\times g$ for 2 min [16–18]. The harvested spores were then washed by repeated centrifugation/resuspension in sterile distilled water at 4 °C. Spore pellets were resuspended in sterile 0.85% NaCl solution after two washes and the spore condition was confirmed using modified Wirtz-Conklin spore stain [16]. Plastic cryopreservation tubes (1.5 mL) containing 1 mL of spore suspension (7–8 log CFU/mL) were stored at –70 °C until used.

Measurements of sporicidal activity

The antimicrobial activity of 8 kinds of the herb extracts known to exert antibacterial effects and organic acids against *B. subtilis* spores was measured by colony counting on tryptic soy broth agar (TSA, Difco, Detroit, MI, USA) plates. Five hundred microliters of spore suspension (4–5 log CFU/mL) and 500 μL of various concentrations of ethanol extract of each herb with and without lactic acid or sodium bicarbonate (Sigma-Aldrich, Saint Louis, MO, USA) to adjust the pH were mixed in a test tube and incubated in a shaker at 30 °C for 2 h. The suspensions were then washed three times by repeated centrifugation/resuspension with 1 mL of sterile 0.85% NaCl solution to halt the effects of the extract residue and acid. The final spore pellet was resuspended in 1 mL of sterile 0.85% NaCl solution, and each spore suspension was inoculated into 5 mL of TSB (Difco, Detroit, MI, USA) medium and incubated at 37 °C for 18 h. The number of viable spores was then counted using a standard colony-counting method in TSA medium [16, 18].

Extraction procedure

The dried herbs were washed to remove extraneous substance as contaminant. The washed herbs to be tested were ground into fine powders, and then 400 g of each were dispersed separately in 75% ethanol (2 $\times$ 4 L) for 2 days at room temperature with shaking. The ethanol was refreshed on a daily basis. The combined supernatants as extracted solvents were filtered through Whatman No. 1 paper and concentrated using rotary vacuum evaporation (EYELA, Tokyo, Japan) at 40 °C. The concentrated extracts were lyophilized at 40 °C for 48 h and then dissolved in a small amount of 75% ethanol to produce a concentration of 2%

[17]. The antimicrobial effect of each of these herb extracts on spore condition was assessed to screen effective sporicidal substances.

Statistical analysis

All of the data are expressed as mean $\pm$ SD values of five replicate measurements. The statistical analysis was conducted using Minitab (MTB13, Minitab Inc., State College, PA, USA). In order to verify to significant differences between the samples, analysis of variance and Duncan's multiple range test were carried out ($p < 0.05$) [18].

Results and discussion

Sporicidal activity of the herbs

The sporicidal activities of eight different kinds of herbs that are known to possess natural antibiotic properties—celery, coriander, caraway, marjoram, oregano, sage, thyme, and mace—are shown on Table 1. The ethanol extracts of most of the herbs exerted inactivation effects against *B. subtilis* spores, as assessed by a plate-count method. At a 1% (w/v), only three of the ethanol extracts exhibited antimicrobial activity against *B. subtilis* spores, causing a reduction in the spore count of at least 90%: crude extracts of mace, caraway, and coriander exhibited effective sporicidal activities, reducing the spore count by 91–97% (Table 1). In this result, the celery also showed effective antimicrobial activity as 87.44% reduction against *Bacillus subtilis* spore, but the celery was excluded because the final samples were predefined such as herbs with more than 90% reduction.

Table 1 Comparison of the inactivation effects of ethanol extracts of various herbs against *Bacillus subtilis* spores

Herb name	<i>B. subtilis</i> spores (CFU/mL)	Inactivation efficacy (%)
Control	$2.62 \times 10^3 \pm 5.90 \times 10^2$ ^a	0
Celery	$3.29 \times 10^2 \pm 5.90 \times 10^1$ ^c	87.44
Coriander	$6.90 \times 10^1 \pm 1.60 \times 10^1$ ^d	97.37
Caraway	$1.05 \times 10^2 \pm 2.40 \times 10^1$ ^d	95.99
Marjoram	$7.65 \times 10^2 \pm 1.37 \times 10^2$ ^b	70.80
Oregano	$8.10 \times 10^2 \pm 1.46 \times 10^2$ ^b	69.08
Sage	$5.92 \times 10^2 \pm 1.33 \times 10^2$ ^b	77.40
Thyme	$7.47 \times 10^2 \pm 6.70 \times 10^1$ ^b	71.49
Mace	$2.83 \times 10^2 \pm 4.30 \times 10^1$ ^c	90.92

Values marked above the bar with different letters are significantly different by ANOVA with Duncan's multiple range test at $p < 0.05$. All data are mean $\pm$ standard deviation values for five replicate measurements

The spore-inactivation properties of ethanol extracts of coriander, caraway, and mace at concentrations of 0.5% (w/v) and 1.0–2.5% were about 10- and 100-fold of the control value (i.e., spore count reductions of 90 and 99%, respectively). The minimum concentration at which spore inactivation occurred was estimated to be 0.1% (w/v) on ethanol extracts of coriander and mace, and 0.25% on caraway considering the level of spore reduction with statistical significance ($p < 0.05$), respectively (Fig. 1). The chemical constituents of coriander include many essential oils, such as linalool, α -pinene, β -pinene, dipentene, and p -cymene, which can kill or inhibit the growth of *B. subtilis* spores [9, 10, 18]. Caraway and mace contain various antimicrobial components, such as carvone, limonene, myristicin, α -pinene, β -pinene, geraniol, terpineol, eugenol, limonene, and p -cymene [9, 10, 18, 19].

Our previous study have shown that surfactants with hydrophilic and hydrophobic properties, such as β -pinene and p -cymene, have effective antimicrobial properties against intact *Bacillus subtilis* spores [20]. This surfactants compounds with emulsifying activity caused meaningful modifications of the cell surface structure by direct interaction with the hydrophobic regions of membrane proteins in the microbial cell [9, 18, 20].

In our previous and the present study, mace, caraway, and coriander, which was estimated to have similar components, destroyed the spore structure by acting as surfactants [18, 20]. The sporicidal mechanism by surfactants although not yet clearly revealed, researchers speculate that the surfactants damage the protective protein coats of spores and allow the antimicrobial components attack the genetic material inside [1, 4, 9, 18, 21]. The structure of protein on the spore coat appears to be disorganized by these components due to binding between the polar groups in the coat and the hydrophobic ($-\text{CH}_3$ and $=\text{CH}-$) groups of surfactant components such as β -pinene and p -cymene (Table 2) [10, 18, 20]. And the sporicidal effect of surfactant components increased depending on the hydrophile–lipophile balance (HLB) [9, 20]. The hydrophobic surfactants were more effective at inactivating spores than their hydrophilic counterparts due to the hydrophobic nature of the spores and their coating [10, 20].

Sporicidal activity of acid

The sporicidal activity of acid was investigated by using lactic acid to adjust the pH of the culture environment. The major reason for selecting lactic acid among the various organic acids was that it has the least effect on sour taste when applied to various processed foods. At a pH 5, the *B. subtilis* spore count was reduced by about 92%, but pH 4 was particularly sporicidal, reducing the spore count by about 99.99% (Fig. 2A). However, pH 6–8 had no

Fig. 1 Comparison of the sporicidal effects of ethanol extracts of (A) mace, (B) caraway, and (C) coriander at different concentrations against *B. subtilis* spores. Each vertical bar indicates the standard

deviation. Values marked above the bar with different letters are significantly different by ANOVA with Duncan's multiple range test at $p < 0.05$

Table 2 The surfactant components of ethanol extract of herbs with sporicidal activity against *Bacillus subtilis* spores

Component	β -pinene	p -cymene
Spore killing	89.1%, CH ₃ group, double bond	97.0%, CH ₃ group, double bond
Chemical structure

sporicidal effect. The inactivation mechanism was traditionally assumed that undissociated organic acids could penetrate the lipid membrane of the bacteria, and then internalized into the neutral pH of the cell cytoplasm dissociate into anions and protons. Generation of excess protons requires consumption of cellular adenosine triphosphate (ATP) and may result in depletion of cellular energy related to cell growth [11, 14, 15, 22].

The killing effect against intact spores on low pH condition was considered to be caused by the unstable growth environment due to changes in the ion composition, and basic mechanism was similar to the inactivation of bacteria [10, 13, 15, 22]. These findings indicate that the organic acids such as lactic acid are suitable for developing a novel antimicrobial substance for inactivating *B. subtilis* spores. When various organic acids such as acetic acid, citric acid

Fig. 2 Comparison of the sporicidal effects of (A) pH (achieved using different concentrations of lactic acid) alone, and ethanol extracts of (B) mace, (C) caraway, and (D) coriander herbs at various concentrations and at different pH levels (achieved by combining them with lactic acid; anpH 4.0; black diamond, pH 5.0; acid) alone,

and ethanol extr. *B. subtilis* spores. Each vertical bar indicates the standard deviation. Values marked above the bar with different letters are significantly different by ANOVA with Duncan's multiple range test at $p < 0.05$

and malic acid besides lactic acid were used singly or in combination, the antimicrobial effect against spore microorganisms was expected to be enhanced by the influence of the pH adjustment characteristics of each organic acid. We plan to verify the effect through future reinforcement experiments.

Combination effect of herb and acid

The combination effect of herb and acid was assessed by mixing crude ethanol extracts of mace, caraway, and coriander (which have proven effective sporicidal activities; Table 1) with lactic acid or sodium bicarbonate to adjust the pH. The ethanol extract of each of the herbs at a concentration of 0.1–2.5% (w/v) adjusted to pH 4–5 exhibited significant ($p < 0.05$) and remarkable deactivation effects, with 3–4 log CFU/mL reductions in the spore count (Fig. 2B–D). The sporicidal activity of the herb–acid

combination caused a additional reduction in the spore count of 1–3 log CFU/mL compared to either the herb or acid alone.

Although the antibacterial mechanism for surfactant components and organic acids are not fully understood. The sporicidal activity of this combination was interpreted to involve a two-step interaction between the sporicidal components and the spore structure by analysis of related research literature. First step, the hydrophobic and hydrophilic binding of the surfactants as β -pinene and p -cymene in the herbs to the surface of the outer spore coat first destroys the protein structure of the coat and protein complex, and then the components penetrate into the interior, continuously decomposing the cortex area [9–11, 14, 18]. And second step, the acids then penetrate into the interior of the spore, generating dissipation of proton-motive force and inability to maintain internal pH followed by denaturation of acid-sensitive proteins and DNA [14, 23–26].

The present study has demonstrated that these surfactants and acids can kill bacillus spores, and may be developed further to produce effective natural antimicrobial agents for the deactivation of spores. And the combination of herb including the surfactants and organic acid developed in this study, can be applied to a variety of HMR (Home Meal Replacement) foods composed of sauces and raw material solids requiring microbial safety by antimicrobial effect as growth inhibition and inactivation to spores.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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