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Inactivation Mechanism of *Bacillus subtilis* Spores by Ethanol Extract of *Torilis japonica* Fruit

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Abstract To confirm the antimicobial mechanism of *Torilis japonica*, antimicrobial profile was observed on various spore conditions by combining 0.1% (3 mM) torilin with antimicrobial activity and 0.27% water fraction with germinants. A 75% ethanol extract of *T. japonica* fruit reduced *Bacillus subtilis* ATCC 6633 spore counts by 3 log cycles and reduced the vegetative cells to undetectable level (by about 6 log cycles) (both in terms of CFU/mL). Further fractionating the ethanol extract into *n*-hexane and water fractions revealed that the former reduced the spore count by 1 log cycle whereas the latter had no effect. The antimicrobial active compound was isolated and purified from the hexane layer, and identified as torilin ($C_{22}H_{32}O_5$). The water fraction of the ethanol layer did not show antimicrobial activity, whereas the antimicrobial effect of 0.1% (3 mM) torilin was significantly enhanced in the presence of the water fraction (0.27%). This result can be explained by synergistic effects of the water fraction containing considerable amounts of germinants such as L-alanine and K^+ ions that triggered germination.

Keywords: *Bacillus subtilis*, endospore, *Torilis japonica* fruit, torilin, germinant

Introduction

Spores, the dormant form of bacteria, are resistant to environmental stresses, including heat, radiation, and toxic chemicals. Some species of spores that cause foodborne illness and spoilage of foods present significant practical problems in food microbiology.

Extreme heating in a retort condition (121°C, 1.0-1.5 kg/cm^2) can sterilize spores, but retort-based processing can decompose nutrients and modify the fresh-like organoleptic qualities. Artificial chemicals, such as hydrogen peroxide, benzoic and sorbic acids, and aldehyde are used widely to extend food shelf life and to delay or inhibit the growth of pathogenic bacteria including spores. However, the questionable safety of some chemical food preservatives, such as due to allergic reactions in sensitive individuals and the formation of potentially carcinogenic by-products, and the increasing reluctance of consumers to accept their use in foods have stimulated efforts to develop alternatives based on natural substances. This has led to a resurgence of interest in antimicrobial compounds found in nature.

Some food grade antimicrobial agents, such as protamine, polylysine, sodium lactate, poly fatty acid ester, and essential oils, exhibit sporostatic and sporicidal activities (1-4). Protamine extracted from the spermary of fishes inhibits the functions of peptidoglycan, DNA, RNA, protein synthesis, and ATP related to the growth of spores (4,5) with this inactivation being enhanced when combined with heat treatment (4,5). Polylysine inhibits spore germination by acting as a surfactant (3,6). Sodium lactate, glycine, lysolecithin, poly fatty acid ester, L-phenylalanine, essential oils, and L-serine can also inhibit the growth of spores (7-9). *Bacillus subtilis* spores break their metabolic dormancy through a process called germination. Spore germination is triggered by specific molecules called germinants. In *B. subtilis*, L-alanine or a combination of L-asparagine, D-fructose, D-glucose, and K^+ ions acts as a germinant to initiate spore germination (20,21).

More than 1,300 plants are a potential source of antimicrobial agents. Antimicrobial botanicals that have the potential to be used as preservatives can be divided into numerous categories, including phenolics, polyphenols, guinones, flavones, flavonoids, flavonols, tannins, coumarins, terpenoids, alkaloids, lectins, and polypeptides. Many plant-derived antimicrobial compounds exhibit a wide spectrum of activity against bacteria, which has led to suggestions that they could be used as natural preservatives in foods (4,10,11). Nishina *et al.* (11) reported that tannin, polyphenol, theaflavin, and catechin in tea extract, and caffeic acid were effective inhibitors of the germination of *Bacillus* spores. This inhibition of germination was assumed to originate from eugenol, thymol, citral, cinnamaldehyde, camphor, limonene, carvone, cymene, and capsaicin, but the actual mechanism was not clarified (2,12).

Torilis japonica fruit has been used therapeutically for antimicrobial treatments in Korea and China since ancient times, but there is little information on the knowledge of its antimicrobial activity (13). The objectives of this study were to elucidate the characteristics and mechanism of spore inactivation of ethanol extract of *T. japonica* fruit against *B. subtilis* spores and vegetative cells, in order to facilitate the development of novel natural antimicrobial agent against spores.

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Materials and Methods

***T. japonica* fruit and spore preparation** Dried *Torilis japonica* fruit was purchased at a local market in Korea and stored at 4°C. The *B. subtilis* ATCC 6633 strain used as a test microorganism in this study was obtained from the culture collection of the Food and Bioprocess Engineering Laboratory of Yonsei University in South Korea. *B. subtilis* ATCC 6633 was incubated on nutrient agar at 30°C for at least 1 week. The vegetative cells with endospores were suspended in sterile 0.85% NaCl solution, and then sonicated for 5 min (on for 15 sec, off for 15 sec) to destroy the vegetable cells so as to obtain the spores. The samples were centrifuged at 4,200×g for 20 min at 4°C, and the resulting cell pellet was resuspended in sterile 0.85% NaCl solution. The spore condition was examined by spore staining based on the Dorner method. A 1 mL portion of each spore suspension containing 10^6-10^7 spores/mL in a 1.5 mL plastic cryopreservation tube was stored at −70°C until used (10).

The spore's suspension was smeared on a glass slide and fixed with a Busen flame for microscopic observation. Slides were flooded with 5% aqueous malachite green (Fisher Scientific Co. Fair Lawn, NJ, USA). Slides were intermittently heated with a Bunsen flame for approximately 5 min to ensure that the dye remained hot but not boiling. Slides were rinsed with tap water, and then counterstained with 0.5% Safranin-O (Sigma-Aldrich, St. Louis, MO, USA) for 1 min. After drying, the slides were examined using a light microscope (BH-2; Olympus, Tokyo, Japan) (14).

Measurements of antimicrobial activity The following materials were tested for their antimicrobial activity to spores and vegetable cells of *B. subtils* ATCC 6633: ethanol extract, 4 fractions (*n*-hexane fraction, ethyl acetate fraction, *n*-butyl alcohol fraction, and water fraction of *T. japonica* fruit), and pure compound. The antimicrobial activity of medicinal plants extracts against *B. subtilis* spores was measured by colony counting on tryptic soy broth (TSB, Difco, Detroit, MI, USA) agar plate as follows: The materials were added at various final concentrations to the 1 mL spore suspension (10^6 spores/mL) in sterile 0.85% NaCl solution and 0.1% of Tween 80 (which is particularly suitable for insoluble materials). The tube was incubated for 2 hr at 30°C, and then the suspensions were washed 3 times by repeated centrifugation/resuspension with 1 mL of sterile 0.85% NaCl solution to avoid any effects of the extract residue. The final spore pellet was resuspended in 1 mL of sterile 0.85% NaCl solution. Each spore suspension was inoculated to 5 mL of TSB medium and incubated at 37°C for 18 hr. The number of viable spores was determined by the standard colony count method on TSB agar medium.

Extraction and fractionation procedure The dried *T. japonica* fruits were washed to remove extraneous substances and then ground into fine powders. Samples (400×g) of the powders were subsequently dispersed in 75% ethanol (2×4 L) for 2 days at room temperature with shaking. The solvent was exchanged every day, and the extracted solvents were combined, filtered through a Whatman No. 1 paper, and concentrated by vacuum evaporation (Eyela, Tokyo, Japan) at 40°C (15). The concentrated extracts were lyophilized at 40°C for 48 hr, and the lyophilized extracts were then dissolved in a small amount of 75% ethanol at the concentration of 2%. The ethanol extract of *T. japonica* fruit was partitioned sequentially in 3 solvents (*n*-hexane, ethyl acetate, and *n*-butyl alcohol) so as to fractionate the polar and nonpolar compounds. The aqueous suspension remaining after fractionation was prepared as the water fraction. The resulting 4 extracts were evaporated to dryness in a rotary vacuum evaporator, and then stored in the dark at 4°C until use (16).

Purification of torilin The active *n*-hexane fraction was subjected to column chromatography on a silica gel (Kieselgel 60, 70-230 mesh; Merck, Whitehouse Station, NJ, USA). The hexane fraction was dissolved in *n*-hexane-ethyl acetate (3:1, v/v) at a concentration of 50 mg/mL. The sample was applied to a column (1.7×20 cm) and eluted with *n*-hexane-ethyl acetate (3:1, v/v). An aliquot of each eluate was spotted on a silica gel plate with a solvent system of *n*-hexane-ethyl acetate (3:1, v/v). The spots were visualized by spraying the plates with 10% H_2SO_4 solution. The hexane fraction was divided into 4 subfractions (F1, F2, F3, and F4) according to yellow spots on the thin layer chromatography (TLC) plates. Subfraction F3 was applied to the column for further purification with *n*-hexane-ethyl acetate (2:1, v/v), and subfractions F3-A and F3-B were separated from subfraction F3. Each subfraction was concentrated by vacuum evaporation at 40°C, and its antimicrobial activity was determined by TLC bioautography (16). This protocol finally yielded compound F3-A, which exhibited antimicrobial activity against *B. subtilis* spores. To identify the components of compound F3-A, its ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR spectra were measured with an NMR spectrometer (Unity-Inova 500 MHz; Varian, Palo Alto, CA, USA). Elements were analyzed with an elemental analyzer (Flash EA 1112 series; CE Instruments, ThermoQuest, Italy), and mass spectra (FAB-MS) were also measured (JMS-700; Mstation, Jeol, Tokyo, Japan)

Analysis of germinants in the water fraction The germinants in the water fraction were analyzed by high-performance liquid chromatography (HPLC) (1100 Series, Hewlett Packard, Palo Alto, CA, USA) with a C18 column (4.6×250 mm and 5 μm, Waters, Milford, MA, USA). Each sample was filtered through 0.45-μm syringe filter and extracted with phenylisothiocyanate (PITC). The flow rate of the mobile phase was 1.0 mL/min, and a gradient was employed to perform the elution. Mobile phase A was 1.4 mM sodium acetate containing 6% acetonitrile and 0.1% triethylamine adjusted to pH 6.1 by acetic acid, and mobile phase B was 60% acetonitrile. The concentrations of mobile phases, A and B were varied from 0 to 100%, respectively, over a 50 min period. Minerals in the water fraction were analyzed by microwave digestive system (MLS, Milestone Ethos Plus; Milestone, Monroe, CT, USA), and inductively coupled plasma (ICP) (Optima 5300 DV; Perkin Elmer, Beaconsfield, UK).

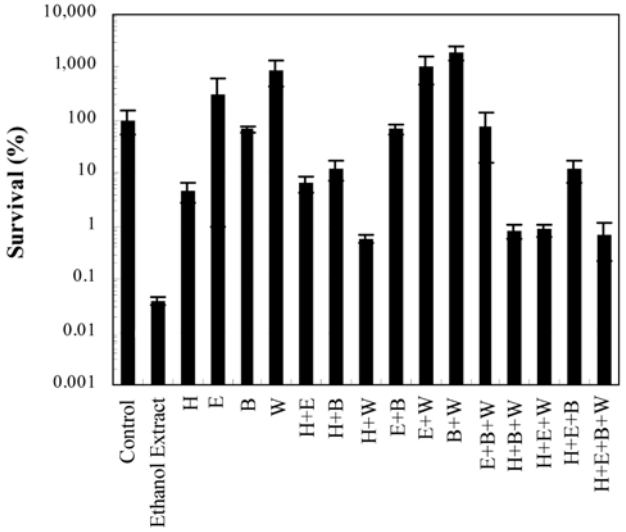


Fig. 1. Antimicrobial effect of mixed fractions from the 1% ethanol extract of *T. japonica* against *B. subtilis* spores. H, hexane fraction (0.42%); E, ethyl acetate fraction (0.14%); B, *n*-butyl alcohol fraction (0.1%); W, water fraction (0.27%). The survival % of *B. subtilis* spores is shown as the mean±SD.

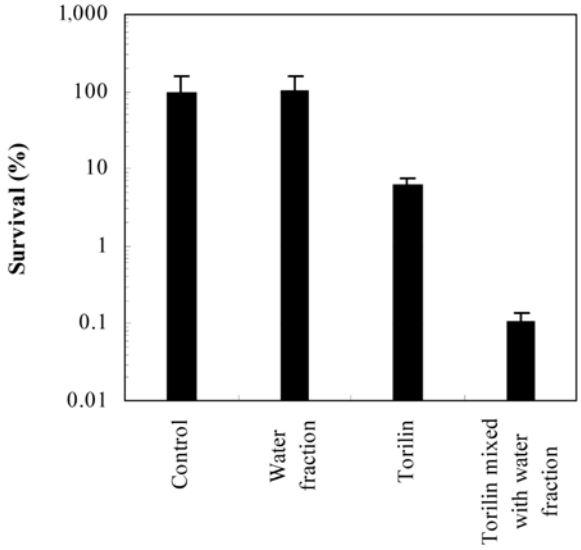


Fig. 2. Synergistic effect of the water fraction on antimicrobial activity of 0.1% torilin against *B. subtilis* spores. Concentration of the water fraction was 0.27%(w/v) of the spore suspension. The survival % of *B. subtilis* spores is shown as the mean±SD.

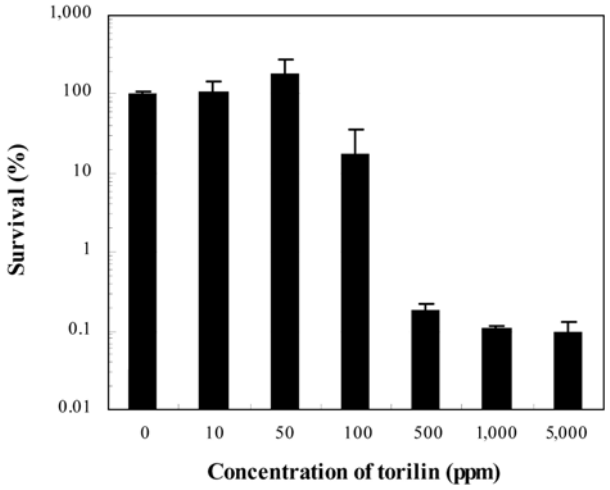


Fig. 3. Effect of concentrations of torilin on antimicrobial activity against *B. subtilis* spores in the presence of 0.27% water fraction. The survival % of *B. subtilis* spores is shown as the mean±SD.

Statistical analysis The responses of *B. subtilis* spores to treatment with medicinal herbs were analyzed using analysis of variance to assess differences between treatments that were at the $p<0.05$ significance level.

Results and Discussion

Synergistic effects of hexane and water fractions obtained from the ethanol extract of *T. japonica* fruit The antimicrobial activity was highest for the ethanol extract of *T. japonica* fruit than for the other fraction, from which we speculated that there are synergic effects between certain fractions. In order to confirm whether such synergistic effects were present, the antimicrobial activities of mixtures of each of the other fractions with the ethanol extract were examined. It is interesting that although the water fraction slightly enhanced the cell population, all mixtures that included both the hexane and water fractions showed higher activities than the other mixtures (Fig. 1). These results suggest that the hexane fraction exerted direct antimicrobial effects and that the water fraction enhanced its activity against spores.

The possible synergistic effect of mixing the hexane fraction with the water fraction was further confirmed with torilin ($C_{22}H_{32}O_5$), the active compound purified from the hexane fraction. Pure torilin reduced the activity, in terms of colony-forming units (CFU)/mL, by about 1 log cycle at a concentration of 0.1%(w/v, 3 mM) (Fig. 2). However, the antimicrobial activity of the torilin increased markedly in the presence of 0.27%(w/v) water fraction, and increased with the concentration of torilin (Fig. 3). No antimicrobial activity of torilin was observed at concentrations below 0.005%(w/v, 50 ppm). Therefore, its minimum inhibitory concentration (MIC) was estimated to be 0.01%(w/v, 100 ppm) in the presence of the water fraction at 0.27%(w/v).

Torilin is a guaiane-type sesquiterpene compound. Torilin was first isolated from a methanol extract of the fruits of *T. japonica*, and its structure was determined in 1969 (19). The NMR assignment of torilin was finalized in 1984 by Kang *et al.* (17) and Ryu and Jeong (19). Kim *et al.* (18) reported that torilin exhibited antitumorigenic activity and was a potent inhibitor of angiogenesis. It was also reported that torilin exhibited analgesic and anti-inflammatory activities, and strongly inhibited 5-α-reductase (17-19). However, its antimicrobial activity on *Bacillus* spores has been not reported previously.

The antimicrobial activity of torilin at a concentration of 0.1%(w/v, 3 mM) was also tested with various concentrations of the water fraction. The presence of the water fraction at concentrations above 0.5%(w/v, 5,000 ppm) consistently reduced the activity by about 3 log cycles (Fig. 4), with the activity also decreasing markedly when the water fraction

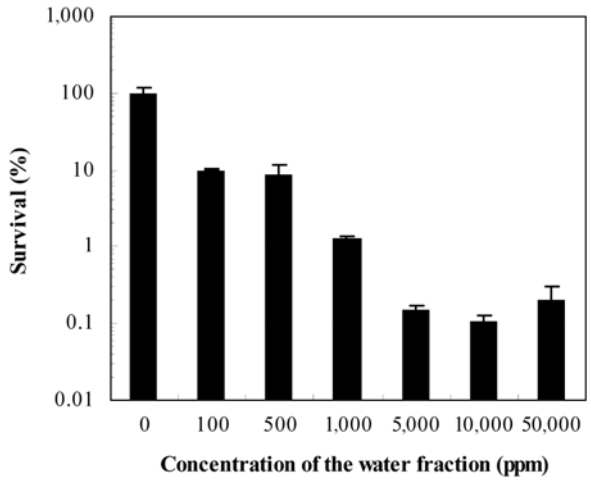


Fig. 4. Effect of concentration of the water fraction on antimicrobial activity against *B. subtilis* spores in the presence of 0.1%(w/v) torilin. The survival % of *B. subtilis* spores is shown as the mean±SD.

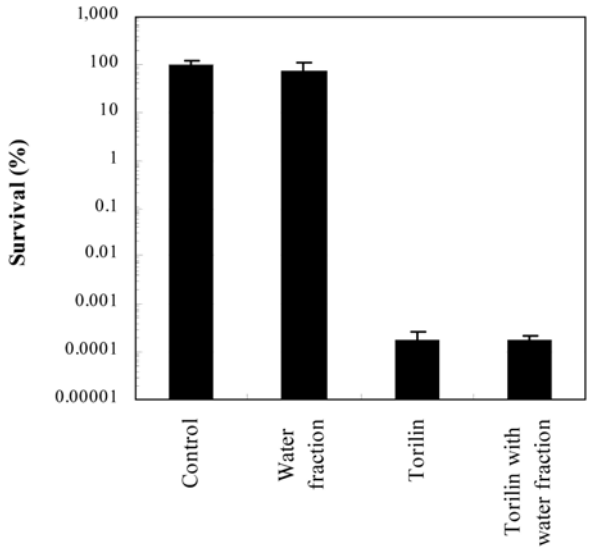


Fig. 5. Effect of water fraction on antimicrobial activity of torilin against *B. subtilis* vegetative cells. The survival % of *B. subtilis* spores is shown as the mean±SD.

was present at concentrations below 0.1%(w/v, 1,000 ppm). Therefore, the most effective combination for the inactivation of spores was assumed to be torilin at 0.1% (w/v, 3 mM) and the water fraction at 0.5%(w/v). By contrast with the spores, the vegetable cells of *B. subtilis* were effectively inactivated by torilin with or without the water fraction (Fig. 5).

Germination of *B. subtilis* spores by the water fraction obtained from the ethanol extract of *T. japonica* fruit

The effect of the water fraction on the spores was observed using microscopy. Spores added to the 0.27%(w/v) water fraction germinated within 1 hr, similar to the germination in TSB medium (data not shown). This was further confirmed by a staining method (14), in which spores suspended in deionized water were stained blue, indicating that none of the spores germinated (Fig. 6). When the

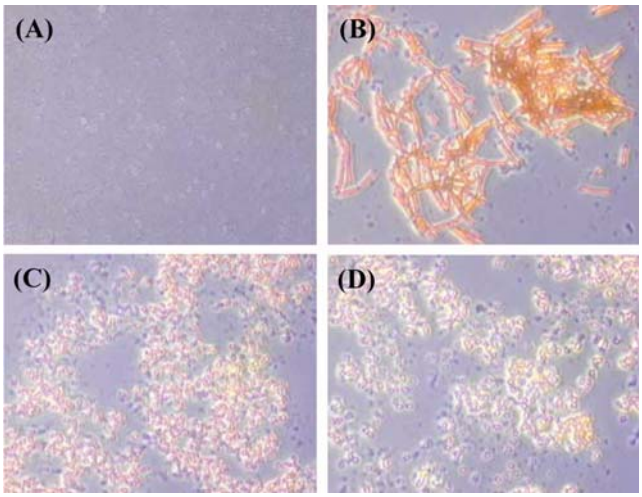


Fig. 6. Microscopic observation of germination of *B. subtilis* spores suspended in deionized H_2O (A), TSB medium (B), 0.27% the water fraction (C), and 10 mM L-alanine solution (D). The germinating suspensions were exposured at room temperature for 1 hr and then stained.

spores were suspended in TSB medium, pink bacilli were observed due to complete outgrowth to vegetative cells. However, when the water fraction was added to the spore suspension in deionized water, the spores were stained pink, which meant that germination was initiated without outgrowth to vegetative cells. *B. subtilis* spores break their metabolic dormancy through a process called germination, which is triggered by specific molecules called germinants. In *B. subtilis*, L-alanine or a combination of L-asparagine, D-fructose, D-glucose, and K^+ ions acts as a germinant to initiate spore germination (20,21). As described above, the water fraction triggered germination.

To identify germinants in the water fraction, amino acids and carbohydrates were analyzed using HPLC and a biological liquid chromatography (bio-LC) system. The concentration of L-alanine in the 0.27%(w/v) water fraction was calculated to be 256.0 μM, which was sufficient for germination. In addition to L-alanine and L-valine, small amounts of amino acids such as L-isoleucine, L-cystein, and L-glutamine were detected (Table 1). As shown in Table 1, carbohydrate germinants such as D-glucose and D-fructose were not detected, but very small amounts of mannose and sorbitol were present.

Analysis of the ions contents in the water fraction using an inductively coupled plasma-atomic emission spectrometer (ICP-AES) system revealed a high content of K^+ ions (Table 1), which suggest that these ions in the water fraction trigger the germination of *B. subtilis* spores.

To assess the contribution of these germinants to the antimicrobial activity of the hexane fraction, the activities of various mixtures were evaluated (Fig. 7). A 0.6% hexane fraction (3 mM torilin) in the presence of the 0.27% water fraction reduced the CFU concentration by 3 log cycles, which was very similar to the activity of the ethanol extract. However, a mixture of the hexane fraction and L-alanine reduced the concentration of CFU by only 2 log cycles. The reaction mixture was prepared by mixing 0.6% hexane fraction and 0.03%(w/v) L-alanine so that the levels

Table 1. Analysis of amino acids, carbohydrates, and minerals in the water fraction of ethanol extract of *Torilis japonica* fruit

Amino acid & Carbohydrate	Concentration[1)] (μM)	Ion	Concentration (ppm)
Amino acid		Cu	21
L-Alanine	256.02	Si	207
L-Valine	130.90	Na	1,215
L-Isoleusine	47.75	Pb	0
L-Cystein	21.59	Ni	5
L-Glutamine	5.90	Mg	4,620
		Mn	10
		As	1
Carbohydrate		Se	0
Glucose	N/A	Zn	53
Mannose	0.0593	Al	0
Xylose	N/A	P	20
Rhamnose	N/A	Sn	1
Sucrose	N/A	Fe	15
Mannitol	N/A	Cd	0
Sorbitol	0.0306	K	102,376
Xylitol	0.0107	Ca	1,356
		Co	1
		Cr	0

[1)]N/A, below 0.001 μM.

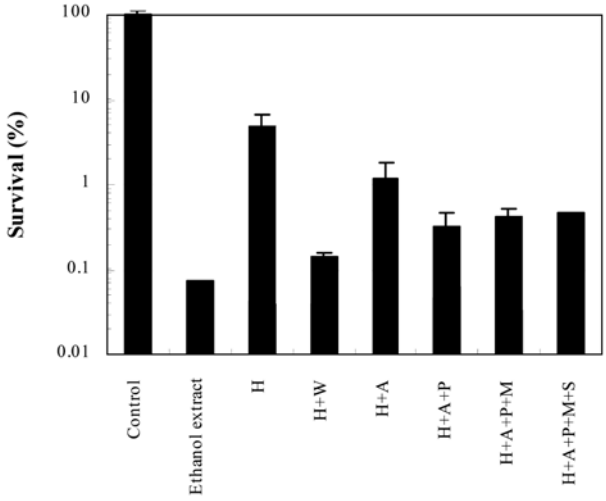
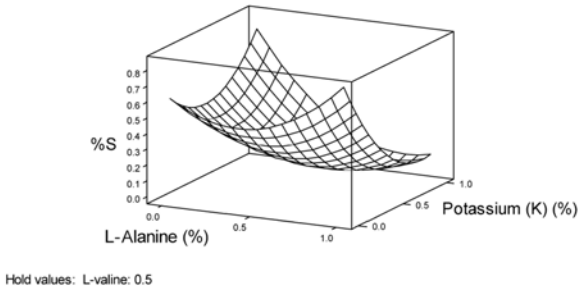
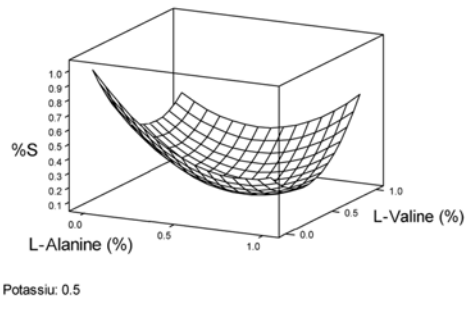


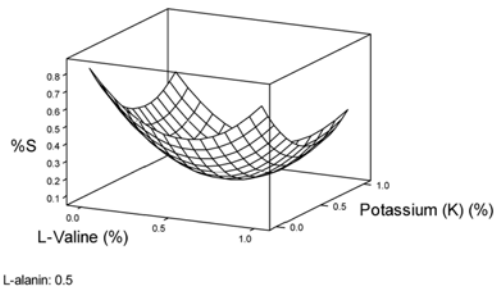
Fig. 7. Antimicrobial activity of mixtures with the hexane fraction and germinants in the water fraction of ethanol extracts of *T. japonica* fruit against *B. subtilis* spores. H, hexane fraction (0.6%); W, water fraction (0.27%); A, L-alanine (0.03%); P, potassium (0.05%); M, mannose (0.01%); S, sorbitol (0.01%). The survival % of *B. subtilis* spores is shown as the mean±SD.

of torilin and L-alanine in the mixture were the same as in the ethanol extract. The antimicrobial effect of the reaction mixture was further enhanced by adding 0.05% K^+ ions. However, the addition of carbohydrate germinants such as mannose and sorbitol to the reaction mixture had no effect on the antimicrobial activity. The above results were optimized with response surface methodology (Fig. 8).

From these results, it is clear that synergistic effect of the water fraction occurs via the initiation of germination by germinants such as L-alanine and K^+ ion, which makes the spores susceptible to antimicrobial compounds. However, the antimicrobial effect of the mixture of the hexane fraction, L-alanine, and K^+ ions showed lower activity than the mixture of the hexane and water fractions. This difference might be attributable to unknown factors in the water fraction that facilitate germination and thereby accelerate the activity.







Term	Coef	SE Coef	T	P
Constant	0.1300	0.09730	1.336	0.230
L-alanin	-0.1125	0.05959	-1.888	0.108
L-valine	-0.0475	0.05959	-0.797	0.456
Potassiu	-0.1325	0.05959	-2.224	0.068
L-alanin*L-alanin	0.2312	0.08771	2.637	0.039
L-valine*L-valine	0.2912	0.08771	3.321	0.016
Potassiu*Potassiu	0.2213	0.08771	2.523	0.045
L-alanin*L-valine	0.1825	0.08427	2.166	0.073
L-alanin*Potassiu	-0.2175	0.08427	-2.581	0.042

S = 0.1685 R-Sq = 87.4% R-Sq(adj) = 70.6%

Fig. 8. The response surface methodology (RSM) analysis to investigate an optimal condition for antimicrobial effect of various germinants in water fraction against *B. subtilis* spores.

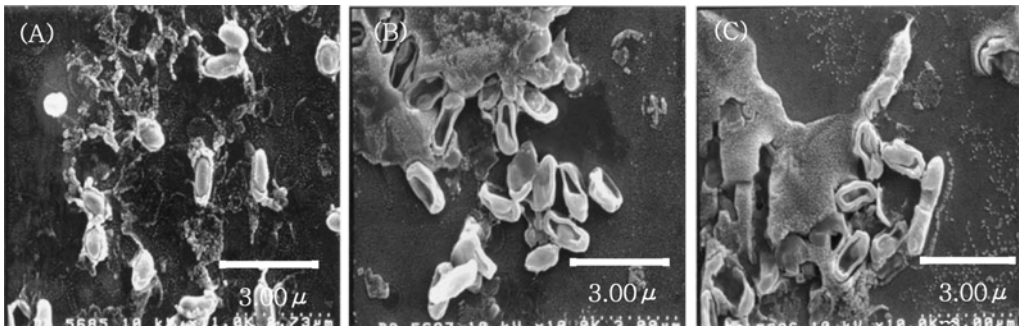


Fig. 9. SEM micrograph of *B. subtilis* spores on glass coupon. An aqueous solution of spores was placed on the SEM sample holder and allowed to air dry. A, suspended in saline (control); B, suspended in saline with the ethanol extract; C, suspended in saline with the water fraction of the ethanol extract of *T. japonica*.

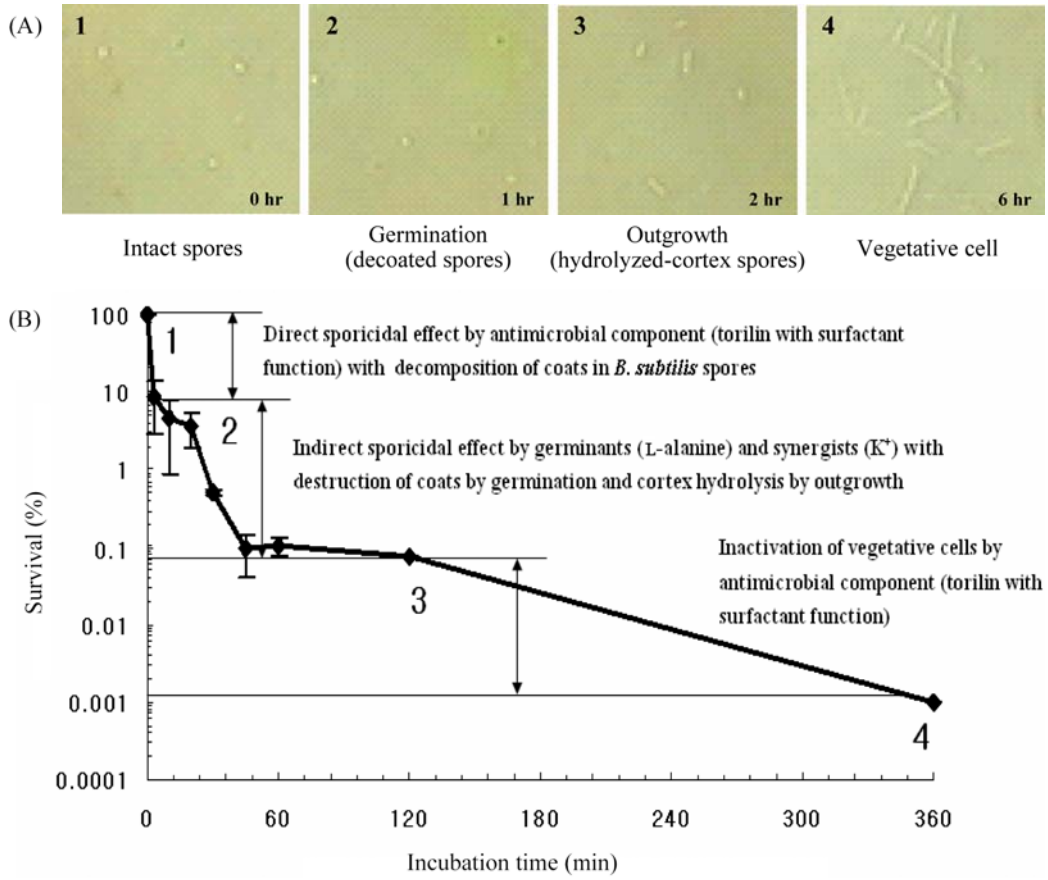


Fig. 10. Changes in spore conditions according to cultivation time of *Bacillus subtilis* ATCC 6633 (A) and antimicrobial mechanism of *T. japonica* against *B. subtilis* spores was interpreted by 3 stages (B). The antimicrobial profile was observed under various spore conditions by combining 0.1% (3 mM) torilin (for antimicrobial activity) and the 0.27% water fraction (for germination).

SEM examination of *B. subtilis* spores treated with the ethanol extract revealed release of the spore core, swelling, and partially disruption by synergistic effect of the water fraction occurs via the initiation of germination by germinants such as L-alanine and K^+ ion, which makes the spores susceptible to antimicrobial compounds (Fig 9B). However, SEM observations of *B. subtilis* spores suspended in saline with the water fraction revealed germination without damage of to the spore cortex and conversion to the vegetative cells (Fig. 9C).

Antimicrobial mechanism of the ethanol extract of *T. japonica* fruit Torilin, an effective antimicrobial compound in *T. japonica* fruit, is a guaian-type sequiterpene compound. The antimicrobial effects by torilin appear to start almost immediately, and result in 90% inactivation of the spores. In the second stage, various germinants trigger the initiation of germination, leaving the spore susceptible to disruption by torilin. Germination within 60 min further enhanced the activity of torilin, and reduced the spore counts by 3 log cycles. The last stage appears to be the inactivation of

vegetable cells. The microscopy studies showed disruption of the spore coat and cortex after these treatments.

To confirm the antimicrobial mechanism of *T. japonica*, the antimicrobial profile was observed under various spore conditions by combining 0.1% (3 mM) torilin (for antimicrobial activity) and the 0.27% water fraction (for germination). The resulting spore conditions were divided 4 groups: (i) intact spores, (ii) early germination spores with a decoated condition, (iii) outgrowth spores with hydrolyzed cortexes, and (iv) vegetative cells (Fig. 10A). The cultivation of the tested strains was tested under various growth conditions on a broth of 0.27%(w/v) water fraction with germinants. An investigation of the antimicrobial effects under each condition (Fig. 10B) revealed that the antimicrobial profile was similar to the inactivation profile of *B. subtilis* spores by the ethanol extract of *T. japonica* fruit.

Consequently, the inactivation mechanism of the ethanol extract of *T. japonica* against *B. subtilis* spores can be explained in three stages: (i) direct inactivation of the spores by antimicrobial compounds on intact spores (surfactant action of torilin), (ii) synergistic effects due to disruption of the spore coat after initiation of germination and cortex hydrolysis by germinants such L-alanine and potassium (acceleration of spore inactivation), and (iii) inactivation of vegetative cells.

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