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Effects of Sulforaphane, Grapefruit Seed Extracts, and Reuterin on Virulence Gene Expression Using *hilA* and *invF* Fusion Strains of *Salmonella typhimurium*

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Abstract This study assessed the effects of the antimicrobial substances sulforaphane, grapefruit seed extracts (GSE), and reuterin on the expression of *Salmonella* HilA and InvF virulence gene using a LacZY assay (β-galactosidase assay) with *hilA:lacZY* and *invF:lacZY* fusion strains of *Salmonella typhimurium* SL1344. *Salmonella* was grown for 8 hr at 37°C in the presence of diluted antimicrobial substances (2 μg/mL sulforaphane, 20 μg/mL GSE, and 0.26 mM reuterin) at concentrations that did not inhibit the cellular growth of *Salmonella*. Sulforaphane inhibited the expression of HilA and InvF by 50-90 and 20-80%, respectively. GSE also inhibited the expression of both genes, but to a lesser degree. Among the 3 antimicrobial substances, reuterin showed the least inhibition, which was abolished after 3-4 hr. None of the antimicrobial substances inhibited the β-galactosidase enzyme activity of *S. typhimurium*. The assay used in this study represents a very sensitive method for screening bioactive substances that inhibit the expression of virulence genes in *Salmonella*.

Keywords: virulence expression, antimicrobial substance, *hilA*, *invF*

Introduction

Salmonella is a useful model system for studying fundamental mechanisms of bacterial pathogenesis. Enteric fever and gastroenteritis associated with *Salmonella* infection are due to virulence gene expression. *Salmonella* virulence requires the coordinated expression of complex arrays of virulence factors. These factors involve HilA and InvF (1, 2), 2 transcriptional regulatory proteins encoded within SPI (*Salmonella* pathogenic island)-1, which regulate the expression of invasion genes in response to a variety of environmental stimuli (3). Certain environmental factors such as slightly alkaline pH, low oxygen levels, low concentrations of nutrients, and high osmolarity are required for virulence gene expression in *Salmonella* (1).

We used 3 antimicrobial agents that exhibit distinctive features: grapefruit seed extract (GSE), reuterin, and sulforaphane. Among them, GSE has been used as an antimicrobial agent in various foods in Korea (4, 5). GSE exerts antimicrobial effects on bacteria and yeast (6), and commercial GSE comprises various flavonoids (7) and preservative agents (8-10). Reuterin is an antimicrobial compound produced by *Lactobacillus reuteri* detected in human feces (11) that exhibits broad-spectrum antimicrobial activity against prokaryotes (both Gram-positive and Gram-negative) and eukaryotes. Reuterin's chemical identity is 3-hydroxypropionaldehyde (3-HPA), which is at equilibrium with HPA-hydrate and HPA-dimer in aqueous solution (12). Sulforaphane, or 1-isothiocyanato-4-(methylsulfinyl)-butane, is present naturally in widely consumed vegetables, with a particularly high concentration in broccoli. This compound is a glucosinolate precursor that exhibits anticarcinogenic activity and also inhibits the growth of a variety of microorganisms, including some human pathogens (13, 14).

A simple experimental system for measuring the gene expression of *hilA* and *invF* was previously developed using a LacZY assay (β-galactosidase assay) on *hilA:lacZY* and *invF:lacZY* fusion strains of *Salmonella typhimurium* SL1344 (15), and for this study we used this system to assess the effects of sulforaphane, GSE, and reuterin on the expression of virulence genes HilA and InvF.

Materials and Methods

Bacterial strains and media The *S. typhimurium* SL1344 strains containing *hilA:lacZY* and *invF:lacZY* fusions used to determine virulence expression were originally obtained from professor Sangryul Ryu (Food Microbiology Technology Laboratory, Department of Food Science and Technology, Seoul National University, Seoul, Korea). *S. typhimurium* was taken from a plate streaked on Luria-Bertani (LB) agar containing 20 mg/mL tetracycline that is a selective media for these strains. After the plate was incubated overnight at 37°C, the *Salmonella* fusion strains formed white colonies on the plate. A single colony was used to inoculate 5 mL of LB broth (Hardy Diagnostics, Santa Maria, CA, USA) in a test tube, which was then incubated aerobically for 12 hr at 37°C. For the experiments, activated *Salmonella* was inoculated at 1%(v/v) in LB broth and incubated aerobically for 8 hr at 37°C.

Preparation of antimicrobial substances Sulforaphane was purchased from Sigma-Aldrich (St. Louis, MO, USA), GSE was purchased from Komipharm International (Siheung, Gyeonggi, Korea), and reuterin was produced

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from *L. reuteri* strain ATCC 53608 using a two-step fermentation process as follows (16): *L. reuteri* cells cultured for 15 hr at 37°C in MRS broth (Hardy Diagnostics) were harvested by centrifugation at 4,000×g for 10 min at 20°C, washed twice with 50 mM sodium phosphate buffer (pH 7.5), and resuspended up to 40 mL with 250 mM glycerol solution. The cell suspension was kept for 10 hr at 37°C. After a second fermentation, the suspension was centrifuged at 8,000×g for 25 min at 4°C. The supernatant was analyzed quantitatively according to the methods of Luthi-Peng *et al.* (17).

Inhibition of virulence gene expression *S. typhimurium* SL1344 was grown in the presence of serial dilutions of each antimicrobial substance. Each antimicrobial substance was used at the maximum concentration which did not inhibit the growth of *Salmonella*. Two hundred μL of the *S. typhimurium* inoculum was placed in a test tube containing 10 mL of LB broth, to which antimicrobial substances or negative controls were added. Sulforaphane (2 μg/mL) or control (PBS), GSE (20 μg/mL) or control (d.w.), and reuterin (0.26 mM) or control (250 mM glycerol) were added to test tubes, and then all test tubes were incubated for 8 hr at 37°C. Cell suspensions (1 mL) were collected at 0, 2, 3, 4, 6, and 8 hr of incubation and used for the assessment of the gene expressions of HilA and InvF in a β-galactosidase assay. All experiments were performed in triplicate independent runs. After 8 hr of incubation, 1 mL of the *Salmonella* culture was collected and the degree of survival was assessed.

β-Galactosidase assay The collected samples were centrifuged for 5 min at 1,100×g. After centrifugation, the cells were resuspended in 1 mL of Z buffer solution (60 mM $Na_2HPO_4 \cdot 7H_2O$, $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, and 1 mM 2-mercaptoethanol) and placed on ice. The optical density of the cell suspensions was measured at 600 nm (OD_{600}). Samples of the cell suspensions (0.1 mL) were added to 0.9 mL of Z buffer solution. The cells were permeabilized with one drop of 0.1% sodium dodecyl sulfate (SDS) and 2 drops of chloroform, vortexed for 10 to 15 sec, and then equilibrated for 15 min in a 30°C water bath. O-Nitrophenyl-β-D-galactopyranoside (ONPG, 0.2 mL) was used as the substrate in the assay, and vortexed for 5 sec. The tubes were placed in a 30°C water bath and the reaction time was measured. When a moderate yellow color had developed, the reaction was stopped by the addition of 0.5 mL of 1 M Na_2CO_3. After the tubes were centrifuged for 5 min at 1,100×g, the OD_{420} and OD_{550} values of the supernatants were determined using a spectrophotometer (Beckman DU 530, Beckman Coulter, Fullerton, CA, USA). Numeric Miller unit values of β-galactosidase activity were calculated as follows (18):

$$U = [1{,}000 \times \{OD_{420} - (1.75 \times OD_{550})\}]/(t \times v \times OD_{600})$$

Measurement of *Salmonella* survival The collected samples were diluted in sterile PBS buffer solution through a series of 10-fold dilutions, and spread on LB agar containing 20 mg/mL tetracycline. The plates were incubated for 12 hr at 37°C. The degree of survival of *Salmonella* was quantified as the number of colony-forming units per mL.

Enzyme (β-galactosidase) activity inhibition test *S. typhimurium hilA* and *invF* fusion strains were incubated in 100 mL of LB broth in a 500-mL flask. After incubation, 1 mL of the cells was collected, washed once in PBS buffer solution, and the OD_{600} value was determined. *S. typhimurium* was centrifuged for 25 min at 1,600×g and the supernatant was discarded. The cells were resuspended in 5 mL of PBS buffer solution and sonicated. The broken cell suspension was added to PBS buffer to a final volume of 50 mL. The cell extracts (1 mL) were placed in a 1.5-mL Eppendorf tube, to which antimicrobial substances or controls of the same volume were added as in the virulence gene expression assay. Samples were taken at 2, 4, 6, and 8 hr during incubation for 8 hr at 37 °C. The samples (15 μL) were added to 985 μL of Z buffer solution and the β-galactosidase assay was performed. The subsequent procedures were the same as those described above.

Results and Discussion

The *S. typhimurium* fusion strains reached the stationary phase at 9-10 hr (data not shown). The expression of HilA and InvF virulence gene was the highest in the early exponential phase, and decreased thereafter. Each antimicrobial substance was tested at various concentrations to determine the maximum concentration under which the growth of *Salmonella* was not affected (data not shown). These concentrations turned out to be 2 μg/mL for sulforaphane, 20 μg/mL for GSE, and 0.26 mM for reuterin. The minimal inhibitory concentration (MICs) of each antimicrobial substances toward *S. typhimurium* were 100 μg/mL for sulforaphane, 100 μg/mL for GSE, and 5.2 mM for reuterin, respectively.

As shown in Fig. 1, the number of cells did not differ between control *Salmonella* samples and experimental *Salmonella* samples grown in the presence of antimicrobial substances. This indicated that the concentration of each antimicrobial substance was suitable for evaluating the expression of the virulence genes. Therefore we decided to use each antimicrobial substance at 5-50 fold lower concentrations than antibiotic concentrations to evaluate if they could inhibit the expression of virulence genes in *Salmonella* at nonlethal concentrations.

To check that the observed decrease in β-galactosidase was not due to the inhibition of the enzyme activity of β-galactosidase, the β-galactosidase enzyme activity of *Salmonella* extracts (sonicated cells) was determined in the presence of the antimicrobial substances at the same experimental concentrations as those used for the gene expression assays. None of the 3 antimicrobial substances affected the enzyme activity of β-galactosidase (Fig. 2) at the concentrations used, hence their effects on the decrease in β-galactosidase was attributed to the inhibition of virulence gene expression.

The control samples of *S. typhimurium* showed active expression of HilA from 2 hr, which gradually increased up to 3-4 hr, and decreased thereafter. Among the 3 antimicrobial substances, sulforaphane produced the highest inhibition of HilA expression (Fig. 3). At 4 and 8 hr, sulforaphane inhibited the expression of HilA by 90 and 58% compared to the control, respectively. GSE

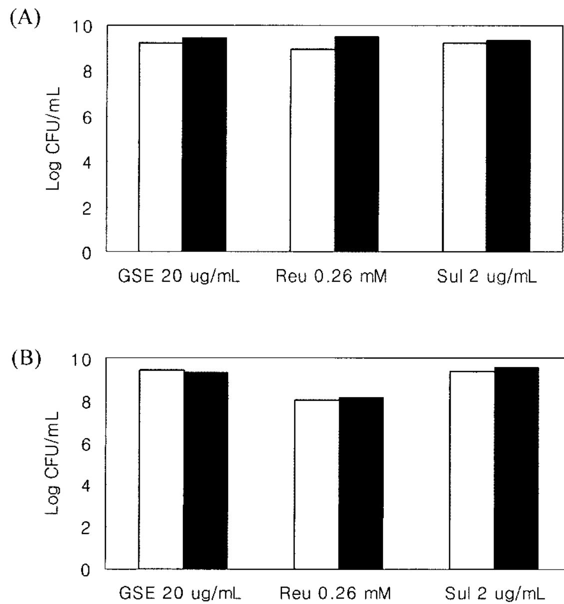

Fig. 1. Effects of antimicrobial substances on the survival of *Salmonella*. Sul, sulforaphane; Reu, reuterin; GSE, grapefruit seed extracts; □, anti-microbial substances; ■, control. (A) *Salmonella typhimurium* SL1344 *hilA:lacZY*; (B) *S. typhimurium* SL1344 *inv F:lacZY*.

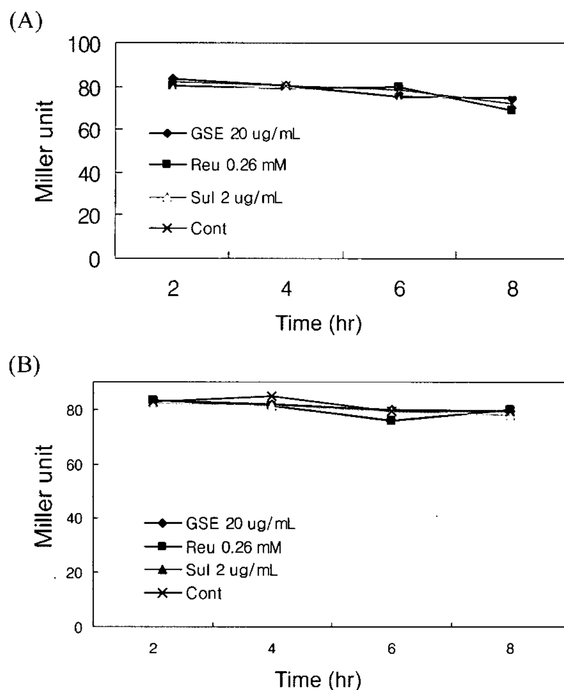

Fig. 2. Effects of antimicrobial substances on enzyme (β-galactosidase) activity. Sul, sulforaphane; Reu, reuterin; GSE, grapefruit seed extracts; cont, control. (A) *S. typhimurium* SL1344 *hilA: lacZY*; (B) *S. typhimurium* SL1344 *invF:lacZY*.

inhibited the expression of HilA by 22-50% compared to the control, and reuterin inhibited the expression of HilA until 4 hr of incubation (by 11-30%), but had no effect thereafter.

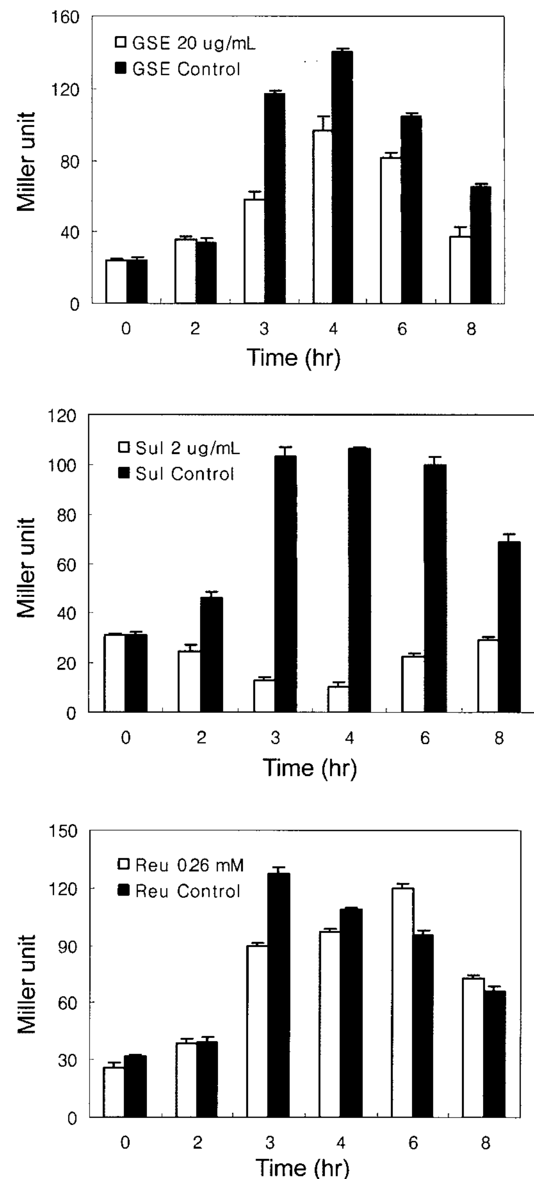

Fig. 3. Inhibitory effects of antimicrobial substances on HilA expression. Sul, sulforaphane; Reu, reuterin; GSE, grapefruit seed extracts.

The expression of InvF in the controls was the highest at 3 hr of incubation and decreased at 4 hr and then increased again at 6 hr (Fig. 4). Sulforaphane inhibited InvF by 20-80% compared to the control. GSE and reuterin inhibited the expression of InvF until 4 hr of incubation (by maximums of 32 and 42%, respectively), but thereafter no difference was noted between the experimental and control groups. The expression patterns of InvF and HilA were similar, but the antimicrobial substances had smaller effects on InvF. These observations were attributed to the functional relationship between HilA and InvF. That is, HilA is upstream of InvF in the regulatory cascade, whereas both genes affect the expression of various sets of SPI-1-associated genes (19).

Sulforaphane and reuterin produced the highest and lowest inhibition of virulence expression, respectively,

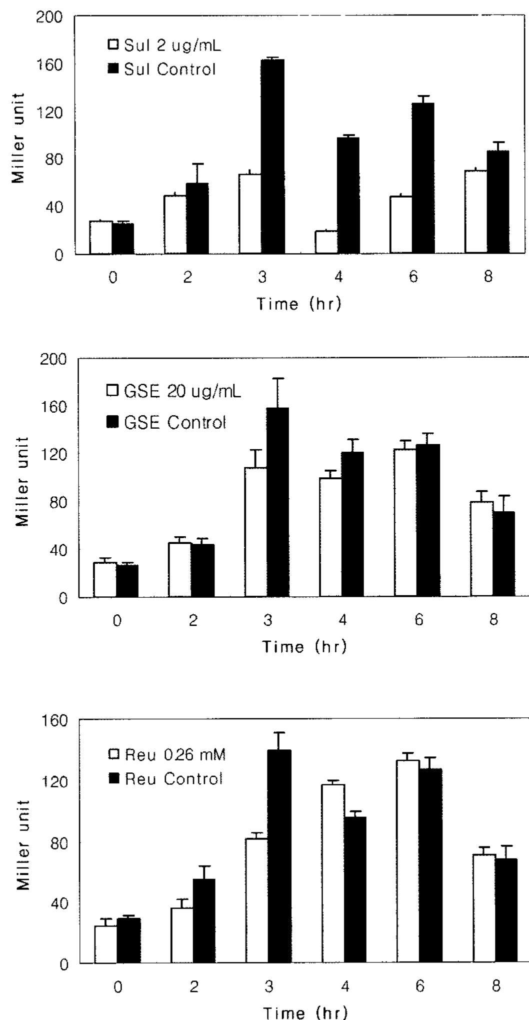

Fig. 4. Inhibitory effects of antimicrobial substances on InvF expression. Sul, sulforaphane; Reu, reuterin; GSE, grapefruit seed extracts.

which is probably attributable to variations in their mechanisms of action. Sulforaphane is an isothiocyanate whose uptake is rapid with high accumulation in various cell types (20). Isothiocyanate can act at the DNA level (21), and hence sulforaphane might act directly on the expression of the *Salmonella* virulence gene. GSE is composed of thousands of natural substances, and causes cell death by disruption of the bacterial cell membrane (22). A recent study demonstrated that GSE can affect gene expression (23). Reuterin (3-HPA) is a small molecule that contains an aldehyde functional group. Therefore reuterin might be less available after a certain period of incubation since it forms a covalent Schiff base with various molecules through its amino group. This might explain why the decrease in the rate of inhibitory activity was greatest for reuterin during incubation.

In conclusion, we have shown that antimicrobial substances can inhibit the expression of virulence genes in our model system, even at concentrations that do not affect the growth of *Salmonella*. This assay represents a very sensitive method for screening bioactive substances that inhibit the expression of virulence genes in *Salmonella*.

Acknowledgments

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