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Effect of Scutellariae Radix as a Novel Antibacterial Herb on the *ppk*(Polyphosphate Kinase) Mutant of *Salmonella typhimurium*

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Abstract The antibacterial effects of water extracts of Scutellariae Radix (a dried root of *Scutellaria baicalensis* GEORGI) and its major flavonoid components, Baicalin and Baicalein, on *Salmonella typhimurium*, a representative enteric pathogen, were studied. Through a Kirby-Bauer disc analysis, the growth-inhibition activity of Scutellariae Radix against *S. typhimurium* was found to be compatible with commercial antibiotics, such as ampicillin, chloramphenicol, and streptomycin. In contrast, the growth of a nonpathogenic *E. coli* strain was unaffected by Scutellariae Radix. To examine the effect of polyphosphate kinase (*ppk*), a putative virulence factor, on the antibacterial activity of Scutellariae Radix, the growth profile of a *ppk* mutant of *S. typhimurium* was investigated in a tryptic soy broth containing different concentrations of water extracts of Scutellariae Radix. The *ppk* mutant was able to grow in 6 mg/ml of water extracts of Scutellariae Radix, whereas the wild-type could not, implying that the inactivation of *ppk* made *S. typhimurium* more resistant to the antibacterial activity of Scutellariae Radix. No enhanced resistance was observed in a *ppk* mutant of *S. typhimurium* complemented with a *ppk* expression vector. The attenuation of the virulence by *ppk* inactivation was also observed in a virulence assay using BALB/c mice. Neither Baicalin nor Baicalein exhibited any growth-inhibition activity against *S. typhimurium*. The water extracts of Scutellariae Radix stimulated the transcription of *ppk*, especially in the early growth-stage of *S. typhimurium*.

Key words: Scutellariae Radix, polyphosphate kinase (*ppk*), *Salmonella typhimurium*, antibacterial activity

The use of natural herbs as a screening pool for novel antibiotics has several advantages related to safety, availability, and minimizing the risk of side-effects and addiction. Since the discovery of penicillin in 1928, much effort has been focused on developing new antibiotics with stronger activity, lower toxicity, and a broader spectrum. Moreover, because of the significant increase in the prevalence of resistance to antibiotics, the desire to limit the appearance of bacterial mutants exhibiting antibiotic resistance has increased in relation to preventing antimicrobial drugs from abuse [1]. Recently, several candidates, such as aminoacyl-tRNA synthetase (AARS) and sortase, have been proposed as novel targets for isolating new anti-infectives that do not exert selective pressure toward the development of bacterial resistance by the pathogen [2, 3, 4]. However, little attention has been paid to the selective action of antibiotics between pathogenic and nonpathogenic bacteria. Scutellariae Radix is a traditional medicinal herb used to treat a wide range of infectious diseases, including upper respiratory infections, scarlet fever, viral hepatitis, nephritis, and pelvitis in far-east Asia [5, 6]. While searching for new antibiotics from natural herbs, it was found that extracts of Scutellariae Radix exhibited a remarkable bacteriostatic activity that was highly selective to Gram (-) pathogenic bacteria, such as *S. typhimurium*, *Shigella sonnei*, *Klebsiella pneumoniae*, and *Proteus vulgaris*. Accordingly, this study investigated the antibacterial activity of water extracts of Scutellariae Radix on *S. typhimurium*, a representative enteric pathogen, and also proposed polyphosphate kinase (*ppk*) as a potential target for Scutellariae Radix.

Materials and Methods

Bacterial Strains, Plasmids, Media, and Growth Conditions

The wild type of *Salmonella enterica* serovar *typhimurium* (hereafter referred to as *S. typhimurium*) was purchased

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from American Type Culture Collection (Manassas, VA, U.S.A.) and its *ppk* null mutant (*ppk::Sm/Spc*) was constructed by site-directed insertion and deletion mutagenesis using pGP704, a wide-host-range suicide vector, and pHP45W for the Sm^r/Spc^r segment [7, 8]. The *ppk* gene fragment (2,067 bp) of *S. typhimurium* (NCBI Accession number: AF085682) was amplified by a PCR and cloned into pGP704 using the *Xba*I and *Eco*RI sites. The Sm^r/Spc^r fragment of pHP45W was then inserted into the *Sma*I site in the central region of *ppk* to inactivate the gene. The final construct was used to transform *S. typhimurium* cells using a MicroPulser™ (Bio-Rad Laboratories, CA, U.S.A.). After the primary screening of the mutant on an LB agar plate including streptomycin, the insertional mutation of the chromosomes was verified by a genomic PCR. The biochemical verification of the mutant was performed by assaying the loss of PPK activity [9, 10]. The expression vector, pQE30 (Qiagen Co., Germany), harboring the *ppk* gene of *E. coli*, was used for the PPK expression in *S. typhimurium*. All plasmids used for the expression in *S. typhimurium* were prepared in *S. typhimurium* SF586 (JR501) for the appropriate genetic modification [11]. *E. coli* DH5α was used as the host strain for the plasmid preparations. An LB and Tryptic Soy Broth (TSB) (BD Diagnostic Systems, MD, U.S.A.) were used to cultivate the *E. coli* and *S. typhimurium*, respectively. Most cultivations were performed at 37°C and 200 rpm in a shaking incubator. The bacterial growth was monitored by measuring the increase in optical density (O.D.) at 580 nm using a spectrophotometer, HP8453 (Hewlett-Packard Co., CA, U.S.A.).

Preparation of Water Extracts of Scutellariae Radix

The air-dried roots (1 kg) of *Scutellariae baicalensis* GEORGI were chopped into small chips and boiled for 3 h, while avoiding evaporation through the use of a condenser. The boiled solution was filtered and evaporated under a vacuum to give a concentrated extract. The extract was then freeze-dried and milled to a powder. A fixed amount of the powder was weighed, mixed with water, and vigorously vortexed to become solubilized. After centrifugation for 30 min at 12,000 rpm, the supernatant was used for further study.

Electroporation of *Salmonella typhimurium*

The *S. typhimurium* cells were cultivated with vigorous shaking to an O.D. of approximately 0.5–0.7 and harvested by centrifugation. The pellets were washed 3–4 times with ice-cold 10% glycerol while taking care not to lyse them. In a cold 1.5 ml eppendorf tube, 50 µl of the cell suspension at a cell concentration of $1-3\times10^{10}$ cells/ml was mixed with 1–2 µl of the plasmid DNA. The mixture was transferred to a cold electroporation cuvette (0.2 cm) and pulsed once at 2.4 kV using a MicroPulser™. One ml of LB was then added. The final broth was incubated without shaking at 37°C for 1 h and spread on LB agar plates supplemented with ampicillin. After overnight incubation at 37°C, the colonies exhibiting substantial growth were selected for further study.

Kirby-Bauer Antibacterial Susceptibility Testing

The bacteriostatic activity of the extract of Scutellariae Radix was assayed as described earlier [12]. Approximately 500 µl of the diluted cell suspension (1×10^6 cells/ml) was evenly spread on TSB agar plates and dried at 37°C in an incubator. Small paper disks with diameter of 5 mm were soaked with each volume of the extract and commercial antibiotics and then placed on an agar plate with a lawn of bacterial cells. After incubation at 37°C for 8–12 h, the zone of inhibition around the disk was measured.

PCR and RT-PCR

The total RNA was isolated from the cell pellets with an RNeasy-Kit (Qiagen Co., Germany). Prior to the RT-PCR, any contaminating DNA was removed from the RNA samples by treatment with RNase-free Dnase (Promega Co., WI, U.S.A.). Each sample was checked for the absence of DNA using a PCR. Twenty µl of the RT reaction mixture containing 4 µl of a 5× first strand buffer (Life Technologies, NY, U.S.A.), 4 µl of 10 mM dNTP, 0.5 µg of a random hexamer, 2 µl of 100 mM DTT, 1 µl of denatured RNA, and 18 U of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) was incubated at 37°C for 1 h and finally heated at 95°C for 10 min. The PCR was performed using a Programmable Thermal Controller (PTC-100, MJ Research, U.S.A.) under the following conditions: denaturation at 94°C for 5 min, 25 cycles of denaturation (94°C, 30 sec), annealing (65°C, 30 sec) and primer extension (72°C, 30 sec), followed by a final extension at 72°C for 10 min. The primer sequences were (forward) 5'-cta caa agt gcg ttt cgc tg-3', (reverse) 5'-tct cca ccg cca gat agg t-3' (345 bp) for the *ppk*, and (forward) 5'-tgg gga gca aac agg att ag-3', (reverse) 5'-gct ggc aac aaa gga taa gg-3' (361 bp) for the 16S rRNA of *S. typhimurium*.

Virulence Assay in BALB/c Mice

The wild-type of *S. typhimurium* and its *ppk* mutant were tested for their ability to cause lethal infection in 6-week-old female BALB/c mice. The bacterial cultures were started from a single colony which had been previously streaked on a TSB agar. After overnight incubation at 37°C, the cultures were diluted into a fresh medium and allowed to resume growth until an O.D. of 0.5. The cells were harvested and resuspended in PBS (phosphate-bufferred saline, pH 7.2) to a concentration of approximately 10^{10} cells/ml. Suitably diluted aliquots (0.3 ml) of this suspension were then used for the mouse inoculation. All mice were

orally administered with a dose of $1-8\times10^8$ cfu, which was equal to approximately 1,000 times the oral LD_{50} (fifty percent lethal doses) for the wild-type strain by oral gavage [13]. The control mice received only PBS. The morbidity and mortality were scored daily for 10 days.

RESULTS AND DISCUSSION

Antibacterial Activity of Scutellariae Radix by Kirby-Bauer Testing

As a fast and reliable method for determining the effectiveness of antibacterial activity, Kirby-Bauer disk diffusion was used to test the water extract of Scutellariae Radix along with other commercial antibiotics, including ampicillin, chloramphenicol, and streptomycin. As shown in Fig. 1, the clear zone around the disk where an amount of the extract of Scutellariae Radix had been dropped was distinct, even though a high concentration of the extract was required to make a comparable size of zone with those of the other antibiotics. This might have been due to the low purity of the bioactive compound in the Scutellariae Radix powder, since the powder was made by crude extraction, as described in Materials and Methods, and also slightly soluble in water. The water extracts of Scutellariae Radix were also found to be effective with other Gram (-) pathogenic bacteria, such as *Shigella sonnei*, *Klebsiella pneumoniae*, and *Proteus vulgaris*, yet not with nonpathogenic *E. coli* strains. The *E. coli* cells did not exhibit any growth defect with a concentration of more than 30 mg/ml of Scutellariae Radix (data not shown), indicating that the antibacterial activity of the extract was highly specific to Gram (-) pathogenic bacteria, although the mechanism was not characterized in detail. Baicalin and Baicalein, the representative pharmaceutical components of Scutellariae Radix, were also tested for their antibacterial activities. However, neither exhibited any growth-inhibition activity against *S. typhimurium* (data not shown).

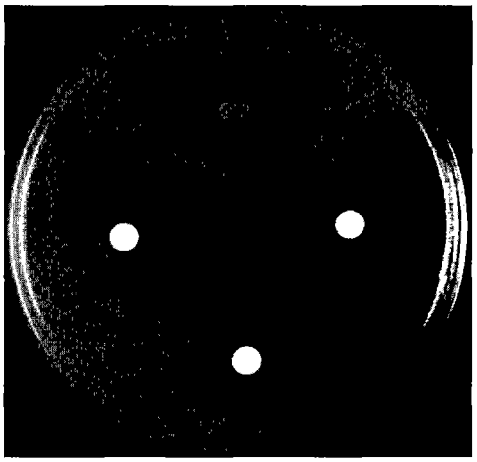

Fig. 1. Kirby-Bauer disc assay showing antibacterial activity of Scutellariae Radix compared with commercial antibiotics.
S, Scutellariae Radix (250 mg); A, ampicillin (50 μg); C, chloramphenicol (50 μg); St, streptomycin (50 μg).

Growth-Inhibition of *ppk* Mutant of *S. typhimurium* by Scutellariae Radix

Polyphosphate kinase (PPK) is the principal enzyme that synthesizes polyphosphate (poly-P) from ATP and has been found to be highly conserved in many bacteria including some of the major pathogens, such as *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Vibrio cholerae*, *Bordetella pertussis*, and *Neiserria meningitidis* [14]. In prokaryotes, PPK regulates the starvation response through controlling the expression of *rpoS* (a sigma factor for starvation gene expression) and most of the virulence factors are expressed in the stationary phase, thereby implying that PPK is dependent on the virulence factors of pathogenic bacteria and that *ppk* may be an important target for antibacterial drugs [14, 15]. Therefore, MIC (minimum inhibitory concentration at which growth was inhibited) of the wild-type strain and *ppk* mutant of *S. typhimurium* was determined to analyze their susceptibility to the water extract of Scutellariae Radix in TSB. It was found that the MIC of the *ppk* mutant (7 mg/ml) was higher than that of the parental strain (6 mg/ml), indicating that the inactivation of *ppk* made *S. typhimurium* more resistant to the antibacterial activity of Scutellariae Radix (Fig. 2). The *E. coli ppk* gene cloned into a pQE vector was used to transform the *ppk* mutant of *S. typhimurium* to complement the intracellular PPK. Next, the growth patterns of the recombinant strain, *ppk*/*ppk*$^+$, were compared with other strains, such as the wild-type and *ppk* mutant, that had

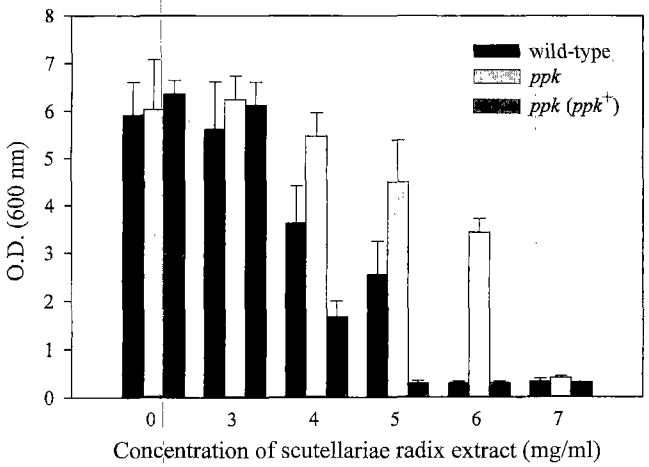

Fig. 2. Growth-inhibition of *S. typhimurium* wild-type, its *ppk* mutant, and *ppk*(*ppk*$^+$) recombinant strain with various concentrations of Scutellariae Radix extract.
The cell density was measured after a 20 h-cultivation in a TSB medium. *ppk*(*ppk*$^+$) indicates *ppk* mutant harboring *ppk* expression vector.

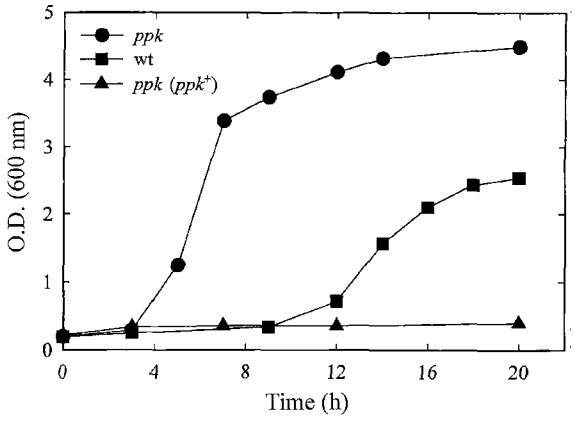

Fig. 3. Effects of Scutellariae Radix extract (5 mg/ml) on growth of *S. typhimurium* wild-type, its *ppk* mutant, and *ppk*(*ppk*$^+$) recombinant strain.

been previously transformed with the same vector free of the gene to exclude any possibility of growth inhibition by the addition of antibiotics (Figs. 2 and 3). As expected, the recombinant strain was unable to grow in the presence of 5 mg/ml of the water extract of Scutellariae Radix. The higher susceptibility of the recombinant strain seemed to be attributed to the high expression level of intracellular PPK due to the high copy number of the gene. In conclusion, the *ppk* gene exhibited a significant effect on the susceptibility of *S. typhimurium* to Scutellariae Radix, thereby confirming the feasibility of *ppk* or *ppk*(poly-P)-regulated genes as a target for the antibacterial herb, Scutellariae Radix.

Effect of *ppk* on Virulence of *S. typhimurium*

Since the natural infection caused by *S. typhimurium* is generally associated with enteritis and capable of inducing severe systemic disease in mice, the *ppk* mutants of *S. typhimurium* were tested for their pathogenesis in BALB/c mice in comparison with the wild-type strains. As shown in Table 1, only 37% of the mice infected with 1×10^9 cfu of the *ppk* mutants died within 9 days, while 75% of the mice infected with the wild-type strains died within 10 days at a dosage of 1/10 cfu of the *ppk* strains (1×10^8 cfu). The noninfected control mice remained healthy. This means that *ppk* appeared to play a crucial role in the expression of virulence in *S. typhimurium*, thus confirming that the inactivation of *ppk* induced the attenuation of the virulence in *S. typhimurium*.

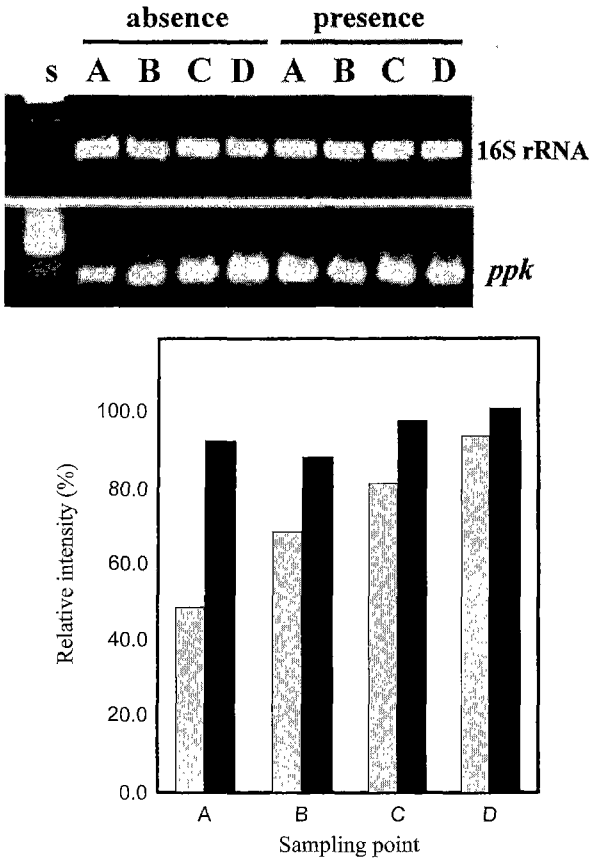

Fig. 4. RT-PCR analysis of transcription of the *ppk* gene in *S. typhimurium*, grown in TSB in the presence (■) and absence (▩) of Scutellariae Radix extract (5 mg/ml).
The relative intensity was the ratio of *ppk* to 16S rRNA. The samplings were executed at the same growth phase: A, early-exponential; B, mid-exponential; C, late-exponential; D, stationary.

RT-PCR Analysis of *ppk* Gene Expression in *S. typhimurium*

It was recently reported that intracellular poly-P (*ppk*) is involved in the strict regulation of the expression of several stress-inducible genes and SOS-regulated genes [15, 16]. Poly-P would seem to play an important role in the promoter selectivity in *E. coli* under high osmolarity by activating the transcription by σ^{38}-containing RNA

Table 1. Virulence assay of *S. typhimurium* wild-type and its *ppk* mutant in BALB/c mice.

Salmonella strains	Dose ([a]cfu/ml)	No. of surviving mice/ No. of inoculated mice	Day of mortality
Wild-type	1×10^8	2/8	6,8,8,9,9,10
ppk	1×10^9	5/8	7,8,9
Negative control	0	5/5	-

[a]colony forming unit.

polymerase and inhibiting σ^{70}-containing RNA polymerase. In order to identify how the water extracts of Scutellariae Radix affected *ppk* expression at the transcription level, the mRNA expression of *ppk* was analyzed in the presence and absence of Scutellariae Radix. As shown in Fig. 4, the expression level of *ppk* gradually increased when the cells entered the stationary phase in a TSB medium without the herbal extract. In contrast, the water extract of Scutellariae Radix highly stimulated *ppk* expression, especially in the early-exponential phase (A), thereby implying consecutive changes in the expression levels of *ppk*-regulated genes including the virulence factors. Accordingly, it was concluded that a *ppk*-regulated gene on a virulence pathway, which was regulated by *ppk* in *S. typhimurium*, was a target for the antibacterial action of the extracts of Scutellariae Radix, although the detailed mechanism of how the extracts of Scutellariae Radix become resistant through the inactivation of *ppk* remains unclear.

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