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SHORT COMMUNICATION

Inhibitory effect of soy saponins on the activity of β-lactamases, including New Delhi metallo-β-lactamase 1

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Abstract β-Lactamase-producing bacteria encode enzymes that inactivate β-lactam antibiotics by catalyzing the hydrolysis of the β-lactam ring. Crude soy saponins were observed to have synergistic effects on the antimicrobial activity of β-lactam antibiotics against β-lactamase-producing *Staphylococcus aureus* strains. Furthermore, the activities of β-lactamases derived from *Enterobacter cloacae*, *Escherichia coli*, and *S. aureus* were decreased significantly in the presence of crude soy saponins. This inhibitory effect was also observed against the New Delhi metallo-β-lactamase 1 (NDM-1), an enzyme whose activity is not inhibited by the current β-lactamase inhibitors. The synergistic effect on the antimicrobial activity of β-lactam antibiotics by crude soy saponins was thought to result from the inhibition the β-lactamase activity. The components of crude soy saponins include several kinds of soyasaponins and soyasapogenols. It was revealed that soyasaponin V has the highest inhibitory activity against NDM-1. The combined use of soy saponins with β-lactam antibiotics is expected to serve as a new therapeutic modality, potentially enhancing the effectiveness of β-lactam antibiotics against infectious diseases caused by β-lactamase-producing bacteria, including those encoding NDM-1.

Keywords Soybean · Soy saponins · Soyasaponin V · β-Lactamase · NDM-1

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Introduction

β-Lactamases catalyze the hydrolysis of the β-lactam ring, thereby inactivating β-lactam antibiotics (Bush 1988). The spread of β-lactamase-producing bacterial strains has diminished the usefulness of β-lactam antibiotics. The combinations of β-lactam antibiotics with β-lactamase inhibitors (such as sulbactam, tazobactam, or clavulanic acid) is a useful therapeutic method for treating infections caused by β-lactamase-producing bacteria (Drawz and Bonomo 2010; Sood 2013). However, some metallo-β-lactamase-producing bacterial strains appear to have acquired resistance to β-lactamase inhibitors and to almost all β-lactam antibiotics, with the exception of monobactams (Palzkill 2013). Furthermore, a strain harboring the recently identified New Delhi metallo-β-lactamase 1 (NDM-1) has been reported to show resistance not only to β-lactam antibiotics and β-lactamase inhibitors but also to aminoglycoside and fluoroquinolone antibiotics (Yong et al. 2009; Rolain et al. 2010). Drug resistance in pathogenic bacteria is a serious global problem because the antibiotics available to treat infectious diseases are becoming increasingly limited. In fact, it has recently been reported that NDM-1-producing bacterial infections are resistant to virtually all currently available antibiotics (Poirel et al. 2014; Karabay et al. 2016). New therapeutic agents or new approaches are urgently needed for the treatment of drug-resistant bacteria.

It has previously been reported that epigallocatechin gallate derived from green tea synergistically enhances the antimicrobial activity of β-lactam antibiotics against methicillin-resistant *Staphylococcus aureus* (Zhao et al. 2001) while also inhibiting the activity of penicillinase (Zhao et al. 2002). We screened the components of multiple herbal medicines for β-lactamase-inhibitory activities

and crude soy saponins were found to exhibit an inhibitory effect against β-lactamase. Soy saponins are amphiphilic compounds present in soybeans. Soy saponins (soyasaponins) are mainly divided into group A, with soyasapogenol A as the aglycone, and group B, with soyasapogenol B as the aglycone. Group A soyasaponins (including soyasaponins A1, A2, A3, A4, A5) are glycosylated at the C-3 and C-22 positions of soyasapogenol A, and group B soyasaponins (including soyasaponins I, II, III, IV, V) are glycosylated at the C-3 position of soyasapogenol B (Berhow et al. 2006; Kamo et al. 2014). Recently, it has been reported that soy saponins have anticolon cancer activity (Tsai et al. 2010) and soy saponin (soyasaponin I) can contribute to reduced blood pressure (Takahashi et al. 2008; Hiwatashi et al. 2010), neuroprotection and neuronal regeneration in memory-deficient rats (Hong et al. 2013). Thus, soy saponins have promising potential applications in the health and pharmaceutical fields. However, there have been no previous reports on the potential effects of soy saponins on antimicrobial activity or the inhibition of β-lactamase activity.

In the present study, we investigated the synergistic effects of soy saponins on the antimicrobial activity of β-lactam antibiotics against β-lactamase-producing *S. aureus* strains, because the inhibitory effect of soy saponins on β-lactamase activity is thought to enhance the antibacterial activity of β-lactam antibiotics. Furthermore, we investigated the inhibitory effect of soy saponins on various β-lactamases including NDM-1. The results reported here indicate the potential for soy saponins to be used in the development of new therapeutic agents or strategies to treat infectious diseases caused by β-lactamase-producing bacteria.

Materials and methods

MIC assay

β-Lactam antibiotics; benzylpenicillin (PCG), oxacillin (MPIPC), and ampicillin (ABPC) were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan and piperacillin (PIPC) was obtained from Sigma-Aldrich Japan K.K., Tokyo, Japan. The minimum inhibitory concentrations (MICs) of these antibiotics against *S. aureus* strains were determined by a liquid microdilution method in 96-well microtiter plates. Two-fold serially diluted antibiotics were prepared using sensitivity test broth (ST-broth, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and inoculated with approximately 5×10^4 CFU bacteria per well. For assessing the synergistic effects of soy saponins on the antimicrobial activities of β-lactam antibiotics, ST-broth containing crude soy saponins at 1000 μg/mL was used for the preparation of the two-fold serially diluted antibiotics. Crude soy saponins (≥ 80% saponins from soybean) were obtained from Wako Pure Chemical Industries, Ltd. Stock solutions of crude soy saponins were prepared by adding a small amount of sodium hydroxide solution to dissolve. Cultivation was performed at 37 °C for 20–24 h under aerobic conditions.

Synergism was evaluated by a fractional inhibitory concentration (FIC) index. The FICs were calculated as the MIC of the antibiotic + the crude soy saponin combination divided by the MIC of the antibiotic alone, and the concentration of the crude soy saponins when used in combination divided by the MIC of the crude soy saponins alone. The FIC index was obtained by adding the FICs. If the FIC index was ≤ 0.5, the combination was defined as synergistic (Zhao et al. 2001). MIC measurements were performed five times, and the average values are shown. The FIC index was calculated from this average value.

Bacterial strains

Five strains of penicillin-resistant *S. aureus* (SA-24, SA-69, SA-78, SA-85, and SA-91) were isolated from a healthy adult volunteer. These strains were screened by PCR analysis for the presence of the *blaZ* gene (data not shown); PCR employed the primer pair described in a previous report (Okamoto et al. 1996). Standard *S. aureus* strain, SA-12732 (NBRC12732) was obtained from the National Institute of Technology and Evaluation Biological Research Center, Chiba, Japan.

Inhibition assay against β-lactamases

Inhibition assays were performed against NDM-1 (recombinant) (RayBiotech, Inc., Georgia, USA) and β-lactamases derived from *Enterobacter cloacae* (Sigma-Aldrich Japan K.K.), *Escherichia coli* (ProSpec-Tany TechnoGene, Ltd., Ness Ziona, Israel), and *S. aureus* (obtained by lysostaphin treatment of approximately 2×10^9 CFU β-lactamase-producing strain SA-69 according to the procedure of a previous report (Tabata et al. 2003), and the preparation of 1 mL of crude β-lactamase solution). These inhibition assays tested the effects of using crude soy saponins, soyasaponin I and V (group A soyasaponins), and soyasapogenol A and B (both aglycone soyasapogenols). The compounds without crude soy saponins were obtained from Tokiwa Phytochemical Co., Ltd., Chiba, Japan. Each β-lactamase (NDM-1 at 2 μg/mL; enzymes from *E. cloacae* at 1 μg/mL, *E. coli* at 0.1 μg/mL, or *S. aureus* SA-69 as a fourfold dilution of crude β-lactamase solution) was preincubated at 37 °C for 2 h in 200 μL of ST-broth with 1000 μg/mL of crude soy saponin or 200 μg/mL of soyasaponin I, V, or soyasapogenol A, B. Stock solutions of

soyasaponin I, V, and soyasapogenol A, B were dissolved in dimethyl sulfoxide (DMSO, Nacalai Tesque, Inc., Kyoto, Japan). Nitrocefin (Oxoid Limited, Hampshire, UK) was used as a substrate for, and indicator of, β-lactamase activity; this compound changes color from light yellow to dark red upon cleavage by β-lactamase. A color change was detected spectrophotometrically at an absorbance of 492 nm. The test was performed three times independently and the data were analyzed using the Student's *t* test. Values of $P < 0.05$ were considered statistically significant.

Results and discussion

Results revealed the synergistic effects on antimicrobial activity of the combination of crude soy saponins and β-lactam antibiotics against β-lactamase-producing bacteria. The antimicrobial activities were tested against five distinct strains of β-lactamase-producing *S. aureus* (SA-24, SA-69, SA-78, SA-85, and SA-91). The MICs of β-lactam antibiotics against the *S. aureus* strains are shown in Table 1. Three kinds of β-lactam antibiotics, PCG, ABPC, and PIPC, showed little or no activity against these strains, exhibiting MICs ranging from 13 to 90 μg/mL. By contrast, these strains were highly susceptible to MPIPC, a β-lactam antibiotic that is not cleaved by β-lactamase. Crude soy saponins (at 1000 μg/mL) demonstrated synergistic effects against *S. aureus* when combined with the cleavable β-lactams (PCG, ABPC and PIPC) (Table 1). The β-lactam + 1000 μg/mL saponin combinations yielded MIC ranges for the three β-lactams of 0.45–2.8 μg/mL. Notably, crude soy saponins alone (at 1000 μg/mL) did not inhibit the proliferation of *S. aureus*; the MIC of crude soy saponins alone was approximately 5000 μg/mL or more (data not shown). The synergistic effects were evaluated using an FIC index (Table 1; see "Materials and methods" section). The resulting FIC index values for the three cleavable β-lactams ranged from 0.22 to 0.28, below the cut-off value of 0.5. Thus, these data indicated that crude soy saponins had a synergistic effect on the antimicrobial activity of these β-lactam antibiotics against β-lactamase-producing *S. aureus* strains. These synergistic effects were observed at 500 μg/mL of crude soy saponins, but not at 200 μg/mL of saponins (data not shown).

Then, we investigated the effect of crude soy saponins on β-lactamase activity using nitrocefin. Recombinant NDM-1 and the β-lactamases derived from *E. cloacae*, *E. coli*, and *S. aureus* were demonstrated to have high enzyme activity (Fig. 1), with the β-lactamase activity being proportional to the absorbance (Zhao et al. 2002). By contrast, the activities were significantly ($P < 0.01$) decreased in the presence of 1000 μg/mL of crude soy saponins against all β-lactamases including NDM-1 (Fig. 1). In the case of sulbactam, the current β-lactamase inhibitor of choice, 2 μg/mL inhibited the β-lactamase activities of enzymes derived from *E. cloacae*, *E. coli*, and *S. aureus*, but this compound at this concentration did not inhibit the activity of NDM-1 (data not shown). The synergistic effect of the crude soy saponins on the antimicrobial activity of β-lactam antibiotics against β-lactamase-producing *S. aureus* strains was thought to be the result of inhibition of the β-lactamase activity.

The components of crude soy saponins contain several kinds of soyasaponins and soyasapogenols. We investigated which of these constituents exhibited inhibitory effects against β-lactamase. The inhibitory effects of soyasaponin I and V, and soyasapogenol A and B against recombinant NDM-1 are shown in Fig. 2. These are representative saponins in soybeans. The activity of NDM-1 was significantly ($P < 0.01$) decreased in the presence of 200 μg/mL of soyasaponin V, and a weak inhibitory effect was observed in the presence of 200 μg/mL of soyasaponin I or soyasapogenol B (Fig. 2). The inhibitory effect was not observed with 100 μg/mL soyasaponin V (data not shown). Although the potential mechanism of inhibitory action by

Table 1 Synergistic effects of the combination of crude soy saponins with β-lactam antibiotics on the antimicrobial activity against β-lactamase-producing *S. aureus*

S. aureus	MIC of β-lactam antibiotics (U, μg/mL)							FIC index		
	Without soy saponins				With soy saponins					
	PCG	MPIPC	ABPC	PIPC	PCG	ABPC	PIPC	PCG	ABPC	PIPC
SA-24	22	0.13	22	90	0.45	0.70	2.8	0.22	0.23	0.23
SA-69	26	0.13	13	48	0.45	0.70	2.0	0.22	0.25	0.24
SA-78	16	0.13	18	51	0.45	0.70	2.2	0.23	0.24	0.24
SA-85	16	0.13	14	51	0.45	1.1	2.4	0.23	0.28	0.25
SA-91	16	0.13	14	45	0.83	0.90	2.6	0.25	0.26	0.26
SA-12732	0.20	0.13	0.50	1.5	nd	nd	nd			

PCG, benzylpenicillin (U/mL); *ABPC*, ampicillin (μg/mL); *MPIPC*, oxacillin (μg/mL); *PIPC*, piperacillin (μg/mL); *nd*, not determined

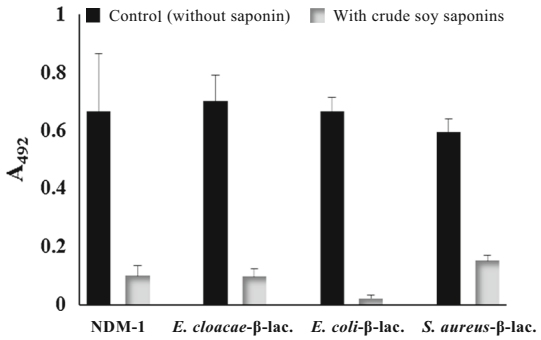

Fig. 1 Inhibitory effect of crude soy saponins against recombinant NDM-1 and β-lactamases derived from *E. cloacae*, *E. coli*, and *S. aureus*. Data are presented as the means ± standard deviations (n = 3 independent experiments). The activities of each of the tested β-lactamases were inhibited significantly ($P < 0.01$ compared with control) in the presence of 1000 μg/mL of crude soy saponins. *β-lac.*, β-lactamase

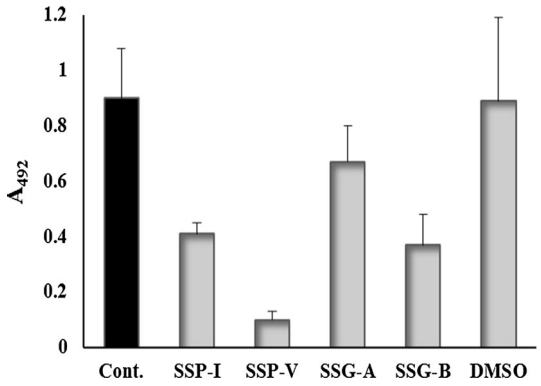

Fig. 2 Inhibitory effect of soyasaponins and soyasapogenols against recombinant NDM-1. Data are presented as the means ± standard deviations (n = 3 independent experiments). The activity of NDM-1 was inhibited significantly ($P < 0.01$ compared with control) in the presence of 200 μg/mL of soyasaponin V. *SSP*, Soyasaponin, *SSG*, soyasapogenol, *Cont.*, control (without saponin). DMSO was the solvent used to prepare the stock solution of soyasaponin and soyasapogenol

the soy saponins remains unclear, it was revealed that soyasaponin V is the component with the highest inhibitory activity against β-lactamase.

Recently, there has been a study on the interaction between β-lactamase and β-lactamase inhibitors (Danishuddin and Khan 2011), and it has been reported that certain plant extracts show inhibition of NDM-1 and synergistic effects with antibiotics against NDM-1-producing *E. coli* (Chandar et al. 2017). In the near future, inhibitors against NDM-1 may be developed based on the findings of these and other related studies. Whether the soy saponins used in our study will be effective against NDM-1-producing bacteria requires further investigation, because the NDM-1 used in our study is a recombinant enzyme, not a native type. Moreover, before soy saponins could be employed clinically as new β-lactamase inhibitors, their human safety profiles would need to be determined experimentally. However, since soybeans constitute part of a daily diet, it is likely that the compounds derived from soybeans will be safe for administration in humans. NDM-1 is not inhibited by current β-lactamase inhibitors and therefore the identification of an inhibitor with activity against this enzyme is especially promising. The combination of soy saponins and β-lactam antibiotics is expected to represent a new therapeutic modality and potentially a more effective strategy for the treatment of infectious diseases caused by bacteria that produce β-lactamases, including those that encode NDM-1.

Conclusion

Crude soy saponins were observed to have synergistic effects on the antimicrobial activity of β-lactam antibiotics against β-lactamase-producing *S. aureus* strains. The activities of β-lactamases including NDM-1 were decreased significantly in the presence of crude soy saponins. The observed synergistic effect on the antimicrobial activity of β-lactam antibiotics by crude soy saponins was thought to result from inhibition of the β-lactamase activity. Furthermore, it was revealed that soyasaponin V was the component of soy saponins that has the highest inhibitory activity against NDM-1. The combined use of soy saponins with β-lactam antibiotics may increase the effectiveness of β-lactam antibiotics against infectious diseases caused by bacteria that produce β-lactamases, including those that encode NDM-1.

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