Article

Enhancement effects of antimicrobial activities of β-lactam antibiotics by combination with persimmon tannin against β-lactamase-producing *Staphylococcus aureus*

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Background β-lactamase-producing *Staphylococcus aureus* is one of the most important bacterial pathogens. Combinations of β-lactam antibiotics with β-lactamase inhibitors such as sulbactam and tazobactam are useful therapeutic methods for combating infections of β-lactamase-producing bacteria. However, bacterial strains which have acquired inhibitor resistance have appeared so new therapeutic agents or new approaches are urgently needed for β-lactamase-producing bacteria.

Objective We investigated the antibacterial activity of persimmon tannin derived from the *Diospyros kaki* cultivar "mishirazu" against β-lactamase-producing *S. aureus* strains, and the enhancement effects of the antimicrobial activity of β-lactam antibiotics against those strains by combining them with persimmon tannin. The possibility of this combination as a new therapeutic agent against β-lactamase-producing bacteria was examined.

Methods The enhancement effects of the antimicrobial activities of β-lactam antibiotics in combination with persimmon tannin were tested by using an MBC (minimum bactericidal concentration) assay.

Results The antimicrobial activities of β-lactam antibiotics against β-lactamase-producing *S. aureus* strains were obviously enhanced by the combination with persimmon tannin. Furthermore, it was clarified that the enhancement effect by persimmon tannin was due to the decomposition control of β-lactam antibiotics by β-lactamase.

Conclusion The combined persimmon tannin/β-lactam antibiotic is expected to be a new therapeutic method and/or a new therapeutic agent against infectious diseases caused by microorganisms producing β-lactamase.

Key words β-lactamase-producing *Staphylococcus aureus*, persimmon tannin, *Diospyros kaki*, antimicrobial activity, *blaZ* gene

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Ⅰ. Introduction

Staphylococcus aureus is one of the most important bacterial pathogens (Eykyn *et al.*; 1990, Schaberg *et al.*; 1991). It causes skin infections, osteoarthritis, and respiratory tract infections in the community. Although β-lactam antibiotics (such as penicillin) are effective against the *S. aureus* infection; penicillin-resistant *S. aureus* strains were found to produce a β-lactamase (penicillinase) that inactivated the antibiotics (Kirby.;1944, Spink and Ferris.;1945). The β-*lactam*-ase-producing strains have a *blaZ* gene, which encodes the β-lactamase enzyme (Okamoto *et al.*: 1996), and the strains were universally present in hospitals by the early 1950s. The emergence and spread of β-lactamase-producing bacterial strains have diminished the usefulness of the β-lactam antibiotics (Medeiros.; 1984). Combinations of β-lactam antibiotics with sulbactam, tazobactam and clavulanic acid, which are β-lactamase inhibitors, are useful therapeutic methods for treating infections of β-lactamase-producing bacteria (Rizwi *et al.*; 1989, Maddux.; 1991). However, it was later reported that bacterial strains which had acquired inhibitor resistance had appeared (Blasquez *et al.*; 1993, Chaibi *et al.*; 1999). New therapeutic agents or new approaches are urgently needed for this antibiotic-resistant bacteria.

The scientific name of persimmons is *Diospyros kaki*. The genus *Diospyros* is widely distributed from tropical to temperature regions, mostly found in the humid tropics of Asia, Africa, and Central and South America (Whitmore.; 1978). They are especially well-known as a Japanese fruit. This fruit generally contains a large amount of tannin. Persimmon tannin has been used as a domestic medicine for burns, chilblains and stomach ulcers in Japan (Yoshimura.; 2002). Furthermore, the persimmon tannin is reported to have antibacterial activity (Inoue *et al.*; 1981, Nishiyama and Kozaki.;1984, Yoshioka *et al.*; 2005). It contains a condensed form of catechin gallate, gallocatechin gallate and catechin. A certain catechin, especially epigallocatechin gallate derived from green tea, is also well known to have antibacterial activity (Toda *et al.*; 1991, Ikigai *et al.*; 1993), and synergistically enhances the antimicrobial activity of β-lactam antibiotics (Yam *et al.*; 1998, Zhao *et al.*; 2001, 2002, Stapleton *et al.*; 2004, Horie *et al.*; 2009).

In this study, we investigated the antibacterial activity of persimmon tannin derived from the *D. kaki* cultivar "mishirazu" against β-lactamase-producing *S. aureus* strains, and the enhancement effects of the antimicrobial activity of β-lactam antibiotics against those strains by combining them with persimmon tannin.

Ⅱ. Materials and Methods

1) Bacterial strains

The bacterial strains used in this study are listed in Table 2. The *Staphylococcus aureus* NBRC12732, NBRC14462, *Streptococcus mutans* NBRC13955, *Bacillus cereus* NBRC13494, *Escherichia coli* NBRC 14237, *Salmonella enterica* serovar Typhimurium (S. Typhimurium) NBRC13245 and *Pseudomonas aeruginosa* NBRC12582 strains were obtained from the National Institute of Technology and Evaluation Biological Research Center, Chiba, Japan. The *S. aureus* SA-22 and SA-24 strains were isolated from healthy adult volunteer.

2) Persimmon tannin, antibiotics and susceptibility testing

Purified persimmon tannin was kindly supplied from Ms. Yuko Goto, Aizu-Wakamatsu Technical Support Centre, Fukushima Technology Center, Fukushima, Japan. Preparation of purified persimmon tannin was performed based on the standard protocol (Kojima *et al.*; 2006). Briefly, immature persimmon fruits, *Diospyros kaki* cultivar "mishirazu", were squeezed by a juicer. The obtained juice was heated at 75° C for 15 min, and then centrifuged at 4,700g for 20 min. The tannin was purified by using the ion exchange resin DAIAION HP20 (Mitsubishi Chemistry Co., Ltd.). The tannin fractions were concentrated with an evaporator, and the products were dissolved in distilled water. The amount of tannin obtained was measured by the Folin-Denis standard method (Tsushida.; 2000).

Oxacillin (MPIPC) was obtained from Wako Pure Chemical Industries, Ltd. Benzil penicillin (PCG) and ampicillin (AMP) were obtained from Nacalai Tesque, Inc.

MIC (minimum inhibitory concentration) was determined by a liquid microdilution method in 96-well microtiter plates according to the protocol recommended by the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards; 1997). However, in this study, an accurate measurement by the MIC method was difficult because of turbidity from the extraction of tannin. Therefore, the MBC (minimum bactericidal concentration) was determined based on the MIC method as follows. Two-fold serially diluted antibiotics or persimmon tannin were prepared by using the Sensitivity Test broth (ST-broth, Nissui Pharmaceutical Co., Ltd.) and approximately 5×10^4 CFU bacteria were inoculated. When the enhancement effects of the antimicrobial activity of the antibiotics used in combination with persimmon tannin were investigated, ST-broths which contained persimmon tannin at 56, 112 or 223 μg/mL were used for the preparation of two-fold serially diluted antibiotics. After cultivation at 35℃ for 24h under an aerobic condition, each 2 μL of culture supernatant was inoculated in other 96-well plates containing the ST-broth. Cultivation was performed at 35℃ for 8 h and for 24h under an aerobic condition. The MBC was determined as the lowest concentration of antibiotic at which the bacteria were not able to grow.

3) PCR

PCR primers for the *blaZ* gene (Okamoto *et al.*: 1996) are described in Table 1. The PCR was performed using a DNA thermal cycler, model TP600 (Takara Bio Inc.), with 30 cycles of denaturation for 30 s at 95℃, annealing for 30 s at 62℃, and extension for 30 s at 72℃. The PCR products were analyzed on 1.2% agarose gels and visualized by CYBR Safe DNA gel staining (Invitrogen). A 325-base-pair fragment of the *blaZ* gene was amplified by using the primers described above.

4) Inhibition assay of persimmon tannin against β-lactamase

Approximately 5×10^4 CFU penicillin-susceptible *S. aureus* NBRC12732 or NBRC14462 strains were inoculated in 96-well microtiter plates which contained 0.006U/mL of β-lactamase (Calbiochem, EMD Bioscience, Inc.) and 56, 112 or 223 μg/mL of persimmon tannin in the presence of two-fold serial dilutions of PCG. After cultivation at 35℃ for 24h, each 2 μL of culture supernatant was inoculated in other 96-well plates containing the ST-broth. After inoculation at 35℃ for 8 h and for 24h, the MBC was determined.

Ⅲ. Results

1) PCR analysis of *blaZ* gene in *S. aureus*

A PCR assay of the *blaZ* gene employing the primer pair described in Table 1 produced a DNA product of the predicted DNA size (Fig.1). DNA fragments of 325 bp of the *blaZ* gene were amplified from the S. aureus SA-22 and SA-24 strains. It was estimated that both strains would produce the β-lactamase enzyme and be resistant to β-lactam antibiotics. On the other hand, the DNA fragment derived from the *blaZ* gene was not amplified from the *S. aureus* NBRC12732 and NBRC14462 strains. Both strains were estimated to be susceptible to β-lactam antibiotics.

Table 1 PCR primers used for detection of *blaZ* genes

Gene	Primer name	Primer sequence	Positions
blaZ	BlaF (sense)	5'-ACT CTT TGG CAT GTG AAC TG-3'	5458-5477
	BlaR (antisense)	5'-AAT CCT GCA AGA AGA GTT AG-3'	5172-5153

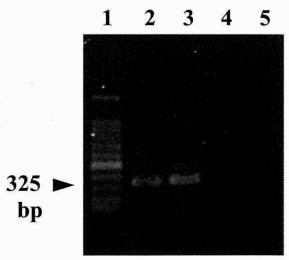

Fig. 1 PCR analysis of *blaZ* gene in *S. aureus*. Lane 1, 100-bp DNA ladder (molecular weight marker); lane 2, *S. aureus* SA-22; lane 3, *S. aureus* SA-24; lane 4, *S. aureus* NBRC12732; lane 5, *S. aureus* NBRC14462. Expected size of PCR products (325 bp) is shown by arrows.

2) MBCs of persimmon tannin against Gram-positive and Gram-negative bacteria

The MBCs of persimmon tannin were measured to confirm the antimicrobial activities of the tannin against Gram-positive and Gram-negative bacteria (Table 2). Persimmon tannin exhibited antimicrobial activities against Gram-positive bacteria *S. aureus*, *S. mutans* and *B. cereus* (each MBC: 445 μg/mL). However, antimicrobial activities against Gram-negative bacteria *E. coli*, *S.* Typhimurium and *P. aeruginosa* were hardly observed (MBC: 3,560 μg/mL or more). Persimmon tannin was shown to have high specificity to bacteria species in its antimicrobial activity.

Table 2 MBC of persimmon tannin against Gram-positive and Gram-negative bacteria

	Bacteria	Strain	MBC (μg/mL)
Gram-positive	*S. aureus*	NBRC12732	445
	S. aureus	NBRC14462	445
	S. aureus	SA-22	445
	S. aureus	SA-24	445
	S. mutans	NBRC13955	445
	B. cereus	NBRC13494	445
Gram-negative	*E. coli*	NBRC14237	>7130
	S. Typhimurium	NBRC13245	7130
	P. aeruginosa	NBRC12582	3560

3) Enhancement of antimicrobial activity of the antibiotics by persimmon tannin

The MBCs of β-lactam antibiotics against the *S. aureus* SA-22, SA-24, NBRC12732 and NBRC14462 strains are shown in Table 3. Three kinds of β-lactam antibiotics, PCG, AMP and MPIPC, showed high antimicrobial activities against the NBRC12732 and NBRC14462 strains. However, PCG and AMP hardly showed any activity against the SA-22 and SA-24 strains which were detected in the *blaZ* gene described in Fig. 1. Since the strains were highly susceptible to MPIPC (MPIPC is not decomposed by β-lactamase), it was expected that both strains would produce the β-lactamase enzyme.

Table 3 MBC of β-lactam antibiotics against *S. aureus*

S. aureus	MBC (U, μg/mL)		
	PCG	AMP	MPIPC
SA-22	32	32	0.5
SA-24	>128	>128	1
NBRC12732	2	4	0.25
NBRC14462	<0.125	0.5	<0.125

PCG, benzil penicillin (U/mL); AMP, ampicillin (μg/mL); MPIPC, oxacillin (μg/mL)

The enhancement effects of the antimicrobial activity of the β-lactam antibiotics against *S. aureus* by combining them with persimmon tannin are shown in Table 4. Persimmon tannin was used in a concentration by which the proliferation of *S. aureus* was not inhibited (223, 112 or 56 μg/mL, half, quarter or 1/8 of the MBC). The antimicrobial activities of two β-lactams (PCG and AMP) were hardly observed to work against the *S. aureus* SA-22 and SA-24 strains during the 24h bacteria cultivation period. On the other hand, the antimicrobial activities of the β-lactam antibiotics against both strains were obviously enhanced in combination with 223 μg/mL of persimmon tannin (Table 4). Also, in combination with 112 μg/mL of persimmon tannin, the enhancement effects of the antimicrobial activity of the β-lactam antibiotics were shown, especially in the 8 h bacteria cultivation period. However, the enhancement effect was hardly observed with 56 μg/mL of persimmon tannin.

Table 4 Effect of persimmon tannin in sensitizing β-lactamase producing *S. aureus* to β-lactam antibiotics

		MBC of β-lactam antibiotics (U, μg/mL)							
		Combination with persimmon tannin (μg/mL)							
		0		223		112		56	
	S. aureus	PCG	AMP	PCG	AMP	PCG	AMP	PCG	AMP
24h	SA-22	32	32	8	4	8	32	32	32
	SA-24	>128	>128	1	2	64	>128	>128	>128
8h	SA-22	16	32	1	4	2	2	2	8
	SA-24	>128	>128	1	0.25	32	64	>128	>128

PCG, benzil penicillin (U/mL); AMP, ampicillin (μg/mL)
24h, 8h, Cultivation period after inoculation of bacteria

4) Inhibition effect of persimmon tannin against β-lactamase activity

To elucidate the mechanism in the enhancement effects of the antimicrobial activity of the β-lactam antibiotics by persimmon tannin, the inhibition effect of persimmon tannin against β-lactamase activity was examined. The MBC of PCG for the *S. aureus* NBRC12732 and NBRC14462 strains which were PCG-susceptible rose remarkably from 2, <0.125 U/mL to >128, >128 U/mL, respectively, by using an ST-broth containing 0.006 U/mL of

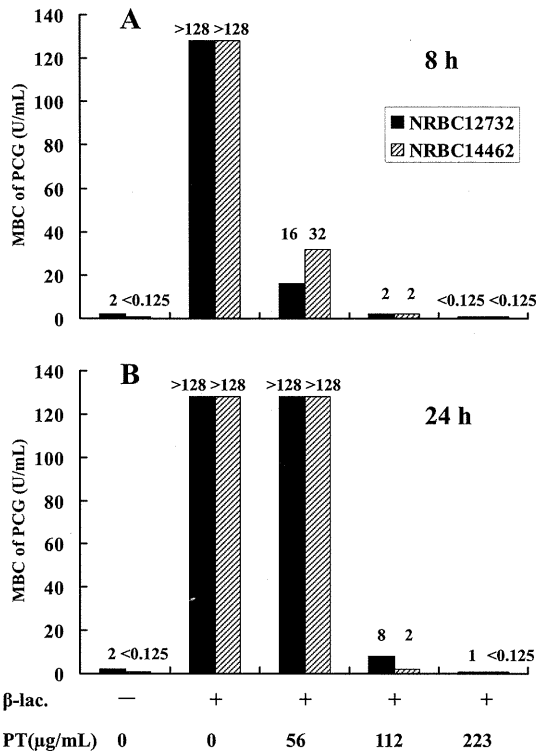

Fig. 2 Inhibition effect of persimmon tannin against β-lactamase activity. Approximately 5 x 10^4CFU penicillin-susceptible *S. aureus* MRBC12732 or MRBC14462 strains were inoculated in 96-well microtiter plates containing 0.006U/mL of β-lactamase and 56, 112 or 223 μg/mL of persimmon tannin in the presence of two-fold serial dilutions of benzil penicillin. After cultivation at 35° C for 24h, each 2 μL of culture supernatant was inoculated in other 96-well plates containing ST-broths. After inoculation at 35° C for 8 h (A) and 24h (B), the MBC was determined. β-lac.: β-lactamase, PT: persimmon tannin

β-lactamase (Fig. 2A, B). However, persimmon tannin blocked the β-lactamase activity in a dose-dependent manner. Even with 56 μg/mL of persimmon tannin, which is the lowest amount showing an inhibition effect against β-lactamase activity in the 8 h bacteria cultivation period, the MBCs of PCG were restored from >128 U/mL to 16 U/mL (MRBC12732) and to 32 U/mL (MRBC14462) (Fig. 2A).

Ⅳ. Discussion

In this study, the antimicrobial activity of persimmon tannin was demonstrated against Gram-positive bacteria (*S. aureus, S. mutans and B. cereus*), but was hardly observed to Gram-negative bacteria (Table 2). Although it was not clear that persimmon tannin showed Gram-positive-specific antimicrobial activity, this is possibly related to the difference in the structure of the cell wall between Gram-positive and Gram-negative bacteria because the epigallocatechin gallate, which is one of the tannin derived from green tea, enhanced the antimicrobial activity of β-lactam antibiotics against methicillin-resistant *S. aureus* (Yam *et al.*; 1998, Stapleton *et al.*; 2004, Horie *et al.*; 2009), and inhibits the synthesis of peptidoglycan on the cell wall of bacteria (Zhao *et al.*; 2001). It is thought that persimmon tannin has inhibition activity as well as green tea tannin.

Bacterial strains producing β-lactamase acquire resistance to many β-lactam antibiotics used to treat infectious diseases caused by *S. aureus* or other microorganisms. In this study, the *S. aureus* SA-22 and SA-24 strains showed properties of resistance to β-lactam antibiotics (Table 3), and these strains had the *blaZ* gene, which encodes β-lactamase (Fig.1). The antimicrobial activities of the β-lactam antibiotics PCG and AMP used in combination with persimmon tannin were demonstrated to be enhanced against both strains. Furthermore, one of the mechanisms in the enhancement effect of the antimicrobial activity of persimmon tannin is elucidated. Persimmon tannin blocked the β-lactamase activity in a dose-dependent manner. The enhancement effect of the antimicrobial activity was due to the decomposition control of β-lactam antibiotics by β-lactamase. Interestingly, the β-lactamase (Calbiochem, EMD Bioscience, Inc.) used in this assay is derived from *B. cereus* strain (569/H 9), therefore, the inhibition effect of persimmon tannin against β-lactamase activity might be shown not only for *S. aureus* but also for other bacteria which produce β-lactamase.

However, there is another possibility that the enhancement effect of the antimicrobial activity by persimmon tannin is due to the synergistic effect of β-lactam antibiotics and persimmon tannin, since it is thought that the target of the β-lactams and persimmon tannin is the peptidoglycan on the bacterial cell wall. Further analyses of the specificity

to antibiotics and to bacteria species are necessary to clarify the mechanism of the enhancement effect in combination with persimmon tannin.

Persimmon tannin is the main component of *Diospyros kaki*. The combined persimmon tannin/ β-lactam antibiotic is expected to be a new therapeutic method which possesses high safety against infectious diseases caused by β-lactamase-producing bacteria. Moreover, it is expected that a new therapeutic agent which shows an inhibition effect against β-lactamase activity may be developed based on the above findings.

V. Conclusion

The antimicrobial activity of persimmon tannin was demonstrated against Gram-positive bacteria, but was hardly observed against Gram-negative bacteria. Moreover, the antimicrobial activities of the β-lactam antibiotics against β-lactamase-producing *S. aureus* strains were obviously enhanced by the combination with persimmon tannin. The enhancement effect of the antimicrobial activity by persimmon tannin was due to the decomposition control of β-lactam antibiotics by β-lactamase. The combined persimmon tannin/ β-lactam antibiotic is expected to be a new therapeutic method and/or a new therapeutic agent against infectious diseases caused by microorganisms producing β-lactamase.

VI. Acknowledgement

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(Summary)

柿由来タンニンによるβ－ラクタマーゼ産生黄色ブドウ球菌に対するペニシリン系抗生物質の抗菌活性増強効果

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背景　β-ラクタマーゼ産生黄色ブドウ球菌は、多くのβ-ラクタム系抗生物質に対して耐性である。更にこの細菌においては、スルバクタムやタゾバクタムのようなβ-ラクタマーゼインヒビターと、β-ラクタム系抗生物質を組み合わせて用いても、効果を示さないインヒビター耐性菌も出現している。β-ラクタマーゼ産生細菌による感染症に対し、新しい治療薬や治療方法の開発が急務となっている。

目的　柿由来タンニンが、β-ラクタマーゼ産生細菌に対する新しい医薬品となる可能性について検討を行うため、同タンニンのβ-ラクタマーゼ産生黄色ブドウ球菌に対する抗菌活性、並びに同タンニンの同細菌に対する、β-ラクタム系抗生物質の抗菌活性を増強する効果について解析を行った。

方法　柿由来タンニンの抗菌活性およびβ-ラクタム系抗生物質の抗菌活性増強効果は、MIC（minimum inhibitory concentration）法を基にしたMBC（minimum bactericidal concentration）法で試験した。また、柿由来タンニンのβ-ラクタマーゼに対する活性阻害効果に関しても、同様にMBC法で試験した。

結果　β-ラクタマーゼ産生黄色ブドウ球菌に対してほとんど抗菌活性を示さなかったβ-ラクタム系抗生物質が、柿由来タンニンと併用することで顕著な抗菌活性を示した。この効果は、同タンニンによるβ-ラクタマーゼの活性阻害効果によるものであることが、強く示唆された。

結論　柿由来タンニンは、単独あるいはβ-ラクタム系抗生物質と組み合わせて用いることにより、β-ラクタマーゼ産生黄色ブドウ球菌による感染症に対して、高い安全性と有効性をもった新しい医薬品や治療方法の開発に結びつく可能性が期待される。

キーワード　β-ラクタマーゼ産生黄色ブドウ球菌、柿由来タンニン、*Diospyros kaki*、抗菌活性、*blaZ*遺伝子