Synergistic Antibacterial Effect and Antibacterial Action Mode of Chitosan–Ferulic Acid Conjugate against Methicillin-Resistant Staphylococcus aureus

Sung-Hwan Eom†, Shin-Kook Kang‡, Dae-Sung Lee†, Jeong-In Myeong†, Jinhwan Lee†, Hyun-Woo Kim‡, Kyoung-Ho Kim†, Jae-Young Je§, Won-Kyo Jung&&, and Young-Mog Kim*†

1Korea Food Research Institute, Sungnam 13539, Republic of Korea
2Department of Food Science and Technology, Pukyong National University, Busan 48513, Republic of Korea
3National Marine Biodiversity Institute of Korea, Seocheon 33662, Republic of Korea
4Aquaculture Management Division, National Institute of Fisheries Science, Busan 46083, Republic of Korea
5Department of Marine Biology, 6Department of Microbiology, 7Department of Marine-Bio Convergence Science, 8Department of Biomedical Engineering, 9Marine-Integrated Bionics Research Center, Pukyong National University, Busan 48513, Republic of Korea

Introduction

The improper use of antibiotics in the treatment of bacterial infections has resulted in the appearance of spreading resistant strains [12]. Among them, Staphylococcus aureus has been recognized as an important pathogen both in community-acquired and healthcare-associated infections. S. aureus has successfully become resistant to practically all antibiotics, and this is a serious clinical problem in the world today [10]. Methicillin-resistant S. aureus (MRSA) infections occur widely in both hospitals and communities. Of further concern, MRSA strains are now evolving additional resistance against standard types of antibiotics [5]. The resistance mechanism against methicillin is mediated via the mec operon, which is a part of the staphylococcal cassette chromosome mec (SCCmec) [8]. Therefore, MRSA exhibits resistance to β-lactam antibiotics by the acquisition of the mecA gene, which encodes penicillin binding protein 2a (PBP2a) [20]. The β-lactam groups of antibiotics are derived from a β-lactam structure. The major properties of β-lactam antibiotics inhibit several enzymes associated with the final step of peptidoglycan synthesis [6]. β-Lactam antibiotics preferentially bind to penicillin binding proteins in the cell wall and inactivate their transpeptidase and carboxypeptidase.

We evaluated the synergistic antibacterial effect in combination with the chitosan–ferulic acid conjugate (CFA) and β-lactam antibiotics, such as ampicillin, penicillin, and oxacillin, against methicillin-resistant Staphylococcus aureus (MRSA) using fractional inhibitory concentration (FIC) indices. CFA clearly reversed the antibacterial activity of ampicillin, penicillin, and oxacillin against MRSA in the combination mode. Among these antibiotics, the combination of oxacillin-CFA resulted in a ΣFICmin range of 0.250 and ΣFICmax of 0.563, suggesting that the oxacillin-CFA combination resulted in an antibacterial synergy effect against MRSA. In addition, we determined that CFA inhibited the mRNA expression of gene mecA and the production of PBP2a, which is a key determinant for β-lactam antibiotic resistance, in a dose-dependent manner. Thus, the results obtained in this study supported the idea on the antibacterial action mechanism that oxacillin will restore the antibacterial activity against MRSA through the suppression of PBP2a production by CFA.

Keywords: Antibacterial activity, chitosan–ferulic acid conjugate, fractional inhibitory concentration, mecA gene, methicillin-resistant Staphylococcus aureus, synergy effect
activities [6]. Since PBP2a has a lower affinity for binding to β-lactam antibiotics (penicillins, cephalosporins, and carbapenems), MRSA is resistant to all β-lactam agents. Despite an urgent need for new antibiotics, the number of newly approved drugs is decreasing continuously [7]. Thus, the development of new drugs or alternative therapies is truly needed to treat MRSA.

Chitosan is a naturally occurring mucopolysaccharide, and it has low toxicity as well as being biodegradable and biocompatible. Several bioactivities of chitosan and chitosan derivatives, such as antioxidant, anticancer, antimicrobial, and enzyme inhibitory effects, have also been reported, and its unique bioactivities have been leading to its applications in the pharmaceutical industry [2, 11, 13]. We recently evaluated the antibacterial activity of chitosan-hydroxycinnamic acid conjugates against food-borne pathogenic bacteria and MRSA [13]. However, there is no further information on the antibacterial mechanism of the conjugates against MRSA. In the present work, we reported the in vitro antibacterial activity of the chitosan–ferulic acid conjugate (CFA) in combination with antibiotics against MRSA. In addition, we investigated the effect of CFA on the expression of the meca gene and the production of PBP2a, which is a key determinant for β-lactam antibiotic resistance [20].

**Materials and Methods**

**Preparation of the Chitosan–Ferulic Acid Conjugate**

Chitosan (average molecular mass 310 kDa and 90% degree of deacetylation) was kindly donated by Kitto Life Co. (Seoul, Korea). The CFA, which exhibited the highest anti-MRSA activity, was prepared according to our previous method [13] (Fig. 1). The molar ratio of chitosan residue to ferulic acid is 1:0.1. After the preparation of CFA, 1H nuclear magnetic resonance and differential scanning calorimetry analysis were conducted and compared with the results of the previous report by Lee et al. [13].

The ferulic acid content in the CFA was determined by using the Folin-Ciocalteau method. The CFA contains 7.08 ± 0.14 mg of ferulic acid/g CFA, and the average molecular mass of the CFA is 312 kDa, which is calculated by the content of ferulic acid in the CFA. The CFA is well soluble in water up to 10 mg/ml.

**Bacterial Strains and Medium**

The methicillin-susceptible S. aureus (MSSA) standard bacterial strain KCTC 1927 (ATCC 6538P, penicillin-resistant strain) was obtained from the Korea Collection of Type Culture (Daejeon, Korea) to assure reliability of the research results. Two standard MRSA strains, KCCM 40510 (ATCC 33591) and KCCM 40511 (ATCC 33593), were purchased from the Korea Culture Center of Microorganisms (KCCM, Seoul, Korea). These strains were grown aerobically at 37°C in Mueller-Hinton broth (MHB; Difco, Detroit, USA) and were subsequently used in experiments to measure the antibacterial activity.

**Synergy Test by Combination with Commercial Antibiotics**

The interactions between CFA and β-lactam antibiotics, including ampicillin, penicillin, and oxacillin, against MRSA were tested by the checkerboard method [15]. The synergy effect between CFA and the antibiotics was evaluated as a fractional inhibitory concentration (FIC) index [15]. The FIC was calculated as the minimum inhibitory concentration (MIC) of an antibiotic or CFA in combination divided by the MIC of the antibiotic or CFA alone.

**RNA Isolation and RT-PCR Analysis**

The effect of CFA on expression of the meca gene in MRSA KCCM 40511 cells was determined using the reverse transcriptase polymerase chain reaction (RT-PCR). To investigate the mechanism of anti-MRSA activity of CFA, MRSA cells were grown aerobically at 37°C in MHB, in the presence of CFA at the concentrations of 4, 8, 16, and 32 µg/ml for 16 h. After harvesting, total RNA was isolated with zirconia beads and an RNAwiz kit (Ambion Inc., Austin, USA), according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from the isolated RNA (1 µg) through a reaction catalyzed by SuperScript II reverse transcriptase (Life Technologies Inc., Gaithersburg, USA) with random hexanucleotides, according to the manufacturer’s instructions. The synthesized cDNA was qualified and quantified by a VERSA Max microplate reader, and the cDNA (200 ng) was used as template for PCR. Sequence-specific primers of each gene were designed from a previous result: meca (527 bp PCR product: annealing temperature 54.4°C) [14]. The cycling conditions (33 cycles) using a Thermal cycler (Takara Bio Inc., Shiga, Japan) were

![Fig. 1. Preparation of the chitosan–ferulic acid conjugate.](image-url)
as follows: denaturation at 94°C for 30 sec, annealing at the indicated temperature of each primer for 30 sec, and extension at 72°C for 30 sec. The amplified PCR products were analyzed electrophoretically on 1.5% agarose gels and visualized with ethidium bromide.

**Western Blot Analysis**

The western blot technique was performed according to the standard procedures to measure the translated protein level [19]. Briefly, harvested cells were suspended in lysis buffer (20 mM Tris-HCl, 2 mM ethyleneglycoltetraacetic acid, 2 mM ethylenediaminetetraacetic acid, and 0.25 M sucrose, pH 7.5) and sonicated three times at 95 µA for 30 sec each time with an ultrasonicator (Ultrasonic Ltd., Hants, UK). Following 10 min of centrifugation at 13,000 × g, the supernatant was obtained as the cell lysate. Protein concentration was measured with the Bradford protein assay method [1]. Cell lysates (10 µg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech., Rainham, UK), blocked with 10% skim milk, and hybridized with MRSA PBP2a monoclonal antibody (diluted 1:1,000; Abnova, Taipei, Taiwan). After incubation with horseradish peroxidase-conjugated anti-mouse immunoglobulin G secondary antibody (diluted 1:1,000; Santa Cruz Biotechnology, Santa Cruz, USA) at room temperature, immunoreactive proteins were detected using a chemiluminescent ECL assay kit (Amersham Pharmacia Biotech.) according to the manufacturer’s instructions. Western blot bands were visualized using a LAS3000 Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan).

**Statistical Analysis**

Analyses were performed in triplicates, and data were averaged over the three measurements. The standard deviation was also calculated. Multiple comparisons were evaluated by two-way analysis of variance using SPSS ver. 12.0 statistical software (SPSS Inc., Chicago, IL, USA). Significant differences between means were determined using Duncan’s multiple range test. A \( p < 0.05 \) was considered significant.

**Results**

**Synergy Effects between CFA and β-Lactams against MRSA**

In this experiment, we evaluated the combined effects of CFA and β-lactam antibiotics, such as ampicillin, penicillin, and oxacillin, against MRSA. The MIC values of ampicillin, penicillin, and oxacillin against MRSA strains ranged from 128 to 256 µg/ml (Table 1). These results imply that β-lactam antibiotics (ampicillin, penicillin, and oxacillin) are no longer effective against the resistant bacteria [16]. However, the MICs of ampicillin against two standard MRSA strains (KCCM 40510 and KCCM 40511) were reduced from 128 to 32 µg/ml, when it was administered in combination with 32 µg/ml of CFA (KCCM 40510) and with 16 µg/ml of CFA (KCCM 40511), respectively (Table 1). The MICs of penicillin against MRSA strains were also reduced from 128 to 32 µg/ml, when it was administered in combination with 32 µg/ml of CFA (KCCM 40510) and with 16 µg/ml of CFA (KCCM 40511), respectively. In addition, the MIC value of oxacillin was considerably reduced from 256 to 32 µg/ml against MRSA (KCCM 40510) and from 128 to 16 µg/ml against MRSA (KCCM 40511), when it was in combination with CFA. In order to evaluate a synergy effect of β-lactam antibiotics in combination with CFA against MRSA, we performed the analysis using fractional inhibitory concentration indices (Table 1) [15]. A combination of CFA-ampicillin resulted in a median \( \Sigma \text{FIC} \) range of 0.500 against the two MRSA strains, suggesting that the combination would cause an additive antibacterial effect against MRSA strains tested in this study (Table 2). An additive antibacterial effect of CFA-penicillin combination against the MRSA

| Table 1. Minimum inhibitory concentrations (MIC) and fractional inhibitory concentration (FIC) indices of the CFA (chitosan-ferulic acid) in combination with ampicillin against MSSA and MRSA strains. |
|----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Strains        | Test compound   | MIC (µg/ml)     | \( \Sigma \text{FIC} \) | \( \Sigma \text{FIC}_{\text{min}} \) | \( \Sigma \text{FIC}_{\text{max}} \) | Minimum concentration for observing synergy |
| MSSA (KCTC 1927) | CFA             | 64              | 1.125           | 1.500           | 0.625           | 16              |
| MRSA (KCCM 40510) | Ampicillin      | 2               | 0.500           | 0.675           | 0.375           | 32              |
| MRSA (KCCM 40511) | CFA             | 128             | 0.500           | 1.125           | 0.375           | 16              |
| MRSA (KCCM 40511) | Ampicillin      | 128             | 0.500           | 1.125           | 0.375           | 32              |

\( \Sigma \text{FIC} \), the sum of FICs; \( \Sigma \text{FIC}_{\text{min}} \), minimum FIC; \( \Sigma \text{FIC}_{\text{max}} \), maximum FIC; The FIC index indicated; synergistic, <0.5; additive, 0.5 to <1.0; indifferent, >1.0 to <2.0; antagonistic, >2.0. \( \Sigma \text{FIC} \) was calculated for each well with the equation \( \Sigma \text{FIC} = \text{FIC}_A + \text{FIC}_B = (C_A/MIC_A) + (C_B/MIC_B) \), where MIC\(_A\) and MIC\(_B\) are the MICs of drugs A and B alone, respectively, and C\(_A\) and C\(_B\) are the concentrations of the drugs in combination, respectively.
strains was also observed. Moreover, we observed a synergistic antibacterial activity in the combination of oxacillin and CFA, with a \( \Sigma FIC_{\text{min}} \) range of 0.250 against MRSA strains.

**Inhibitory Activity of Chitosan–Ferulic Acid on the Expression of Gene mecA and the Production of PBP2a Related to Drug Resistance**

As shown in Fig. 2A, the expression of gene mecA was inhibited by CFA in a dose-dependent manner. This result indicated that CFA inhibited the mRNA expression of the key antibiotic-resistant gene, mecA. To further elucidate the inhibitory effect of CFA on the production of PBP2a, western blot analysis was performed and its efficiency was compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The presence of the PBP2a band was detected in the western blot for MRSA cultures that were grown at sub-inhibitory concentrations of CFA (1/2, 1/4, 1/8, and 1/16× MIC). As shown in Fig. 2B, the PBP2a production was gradually attenuated or inhibited in a dose-dependent manner.

**Discussion**

Our group has previously reported about chitosan–hydroxycinnamic acid (caffeic acid, ferulic acid, and sinapic acid) conjugates, which have a high antioxidant and antimicrobial activity compared with unmodified chitosan [13]. The MIC patterns of the conjugates were similar to the previous results. Among these, CFA showed the highest

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**Table 2.** Minimum inhibitory concentrations (MIC) and fractional inhibitory concentration (FIC) indices of the CFA (chitosan–ferulic acid) in combination with penicillin against MSSA and MRSA strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Test compound</th>
<th>MIC (µg/ml)</th>
<th>Median ( \Sigma FIC )</th>
<th>( \Sigma FIC_{\text{min}} )</th>
<th>( \Sigma FIC_{\text{max}} )</th>
<th>Minimum concentration for observing synergy</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSSA (KCTC 1927)</td>
<td>CFA</td>
<td>64</td>
<td>1.250</td>
<td>1.500</td>
<td>1.000</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Penicillin</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>MRSA (KCCM 40510)</td>
<td>CFA</td>
<td>128</td>
<td>0.563</td>
<td>0.750</td>
<td>0.375</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Penicillin</td>
<td>128</td>
<td></td>
<td></td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>MRSA (KCCM 40511)</td>
<td>CFA</td>
<td>64</td>
<td>0.500</td>
<td>0.750</td>
<td>0.375</td>
<td>16</td>
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<tr>
<td></td>
<td>Penicillin</td>
<td>128</td>
<td></td>
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<td>32</td>
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</table>

\( \Sigma FIC, \) the sum of FICs; \( \Sigma FIC_{\text{min}} \), minimum \( \Sigma FIC; \) \( \Sigma FIC_{\text{max}} \), maximum \( \Sigma FIC; \) The FIC index indicated: synergistic, <0.5; additive, 0.5 to <1.0; indifferent, >1.0 to <2.0; antagonistic, >2.0. \( \Sigma FIC \) was calculated for each well with the equation \( \Sigma FIC = FIC_{A} + FIC_{B} = \left( \frac{C_{A}}{MIC_{A}} \right) + \left( \frac{C_{B}}{MIC_{B}} \right) \), where MIC\(_{A}\) and MIC\(_{B}\) are the MICs of drugs A and B alone, respectively, and C\(_{A}\) and C\(_{B}\) are the concentrations of the drugs in combination, respectively.

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Fig. 2. Effect of the chitosan–ferulic acid conjugate (CFA) on the mRNA expression of the mecA gene (A) and on the expression of penicillin binding protein 2a (PBP2a) against an MRSA strain (B).

Methicillin-resistant *Staphylococcus aureus* (MRSA) KCCM 40511 was treated with the indicated concentrations of CFA.
Table 3. Minimum inhibitory concentrations (MIC) and fractional inhibitory concentration (FIC) indices of the CFA (chitosan–ferulic acid) in combination with oxacillin against MSSA and MRSA strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Test compound</th>
<th>MIC (µg/ml)</th>
<th>Median ΣFIC</th>
<th>ΣFIC&lt;sub&gt;b&lt;/sub&gt;</th>
<th>ΣFIC&lt;sub&gt;min&lt;/sub&gt;</th>
<th>Minimum concentration for observing synergy</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSSA (KCTC 1927)</td>
<td>CFA</td>
<td>64</td>
<td>1.125</td>
<td>1.250</td>
<td>0.500</td>
<td>32</td>
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<tr>
<td></td>
<td>Oxacillin</td>
<td>128</td>
<td></td>
<td></td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>MRSA (KCCM 40510)</td>
<td>CFA</td>
<td>128</td>
<td>0.375</td>
<td>0.563</td>
<td>0.250</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Oxacillin</td>
<td>256</td>
<td></td>
<td></td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>MRSA (KCCM 40511)</td>
<td>CFA</td>
<td>64</td>
<td>0.375</td>
<td>0.563</td>
<td>0.250</td>
<td>16</td>
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<tr>
<td></td>
<td>Oxacillin</td>
<td>128</td>
<td></td>
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<td></td>
<td>16</td>
</tr>
</tbody>
</table>

ΣFIC, the sum of FICs; ΣFIC<sub>max</sub>, minimum ΣFIC; ΣFIC<sub>min</sub>, maximum ΣFIC. The FIC index indicated: synergistic, <0.5; additive, 0.5 to <1.0; indifferent, >1.0 to <2.0; antagonistic, >2.0. ΣFIC was calculated for each well with the equation ΣFIC = FIC<sub>A</sub> + FIC<sub>B</sub> = (C<sub>A</sub>/MIC<sub>A</sub>) + (C<sub>B</sub>/MIC<sub>B</sub>), where MIC<sub>A</sub> and MIC<sub>B</sub> are the MICs of drugs A and B alone, respectively, and C<sub>A</sub> and C<sub>B</sub> are the concentrations of the drugs in combination, respectively.

antibacterial activity against the MRSA strains (Table 1). Therefore, as a part of our ongoing investigation on the alternative ways of overcoming MRSA, we investigated the anti-MRSA mechanism of CFA against MRSA.

The combination of CFA and oxacillin was more effective against the MRSA strains than the combination of CFA with ampicillin or penicillin. The analysis of FIC indices revealed that the antibacterial activity against MRSA is restored when using the old-fashioned β-lactams (ampicillin, penicillin, and oxacillin) in combination with CFA, suggesting that CFA might have potential to be used as an adjunct in the treatment for antibiotic-resistant bacteria. These results are in accordance with the study by Lee and Je [11] that the combination of gallic acid-grafted-chitosan conjugates with ampicillin, penicillin, and oxacillin restored the antibacterial activity of β-lactams against MRSA.

The FIC indices in Table 2 suggested that oxacillin can synergistically restore the antibacterial activity against MRSA, when it is combined with CFA. The susceptibility of MRSA to β-lactam antibiotics will be restored if the PBP2a production is suppressed. Therefore, we investigated the inhibitory effect of CFA on the expression of the mecA gene and the production of PBP2a in MRSA. Among the two MRSA strains, KCCM 40511 was selected for further study since the strain demonstrated less MIC values against CFA and a higher synergy effect in combination with β-lactams, compared with those of KCCM 40510. The inhibitory effect of CFA on the expression of mecA was monitored by RT-PCR.

The mecA gene encodes the 78 kDa protein called PBP2a [3]. The mecA gene is located on a mobile element, SCCmec, which is horizontally transferable among staphylococcal species. Resistance to the β-lactam antibiotics, including ampicillin, penicillin, and oxacillin, is principally mediated by the production of PBP2a encoded by the mecA gene [6, 12]. Whereas the PCR result consistently showed the band for the mec regulatory gene, mecI, between the MRSA KCCM 40510 (ATCC 33591) and 40511 (ATCC 33591) strains, the mecI gene was not detected in MRSA KCCM 40510 by PCR [5, 8]. In addition, the SCCmec element in MRSA KCCM 40510 was identified as SCCmec type I, whereas MRSA KCCM 40511 has SCCmec type II [5, 18]. According to Dutton et al. [4], the nucA gene is expressed in both S. aureus and MRSA. Therefore, in this present research, the nucA was used for the detection of S. aureus and MRSA, which served as an internal control [9].

Thus, CFA inhibited the mRNA expression of the mecA gene and eventually led to the reduction or inhibition of the resistance protein production, PBP2a, in MRSA cells. The inhibition or suppression of mecA gene expression and PBP2a production is a promising approach to restore the susceptibility of MRSA to old-fashioned β-lactam antibiotics [17].

In conclusion, we demonstrated an antibacterial synergy effect with the combination of CFA and β-lactams in this research. The antibacterial activity of β-lactams against MRSA was restored when they were combined with CFA, which led to the restoration of susceptibility of MRSA to old-fashioned β-lactams (ampicillin, penicillin, and oxacillin). In addition, CFA could suppress the expression of the mecA gene and the production of PBP2a related to β-lactam antibiotics resistance in a dose-dependent manner. However, there still remain some issues about whether CFA can directly inactivate PBP2a. Since a limited amount of information is available on the ability of plant and herbal extracts to regulate drug-resistant properties at the molecular level, these results suggest that CFA mediates the suppressive effects on the methicillin resistance-associated genes, showing its tremendous potential as a promising candidate for a...
variety of therapeutic applications.

Acknowledgments

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