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Penicillin-like mimotopes from autodisplayed Fv-antibody library inhibiting β-lactamase activity†

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A penicillin-like mimotope was screened from an Fv-antibody library which had the inhibition activity of β-lactamase. Fv-antibody indicated the variable region (V_H) of the immunoglobulin *G*, which includes three complementarity determining regions (CDRs). The Fv-antibody library was then prepared by randomizing the complementarity determining region 3 (CDR3), and it was expressed on the outer membrane of *E. coli*. The penicillin-like mimotopes were screened from the Fv-antibody library using magnetic beads with an immobilized monoclonal anti-penicillin antibody. The screened mimotopes were expressed as soluble Fv-antibodies and were also synthesized into peptides (11-mer). The binding affinity (K_D) of the expressed Fv-antibodies and synthesized peptides was estimated using SPR measurements. The β-lactamase inhibition activity of the Fv-antibodies and synthetic peptides was estimated using colorimetry based on the formation of penicilloic acid. The penicillin-like mimotopes of the expressed Fv-antibodies and synthesized peptides were demonstrated to have β-lactamase inhibition activity in the bacterial lysates. Finally, the docking analysis of β-lactamase and the screened CDR3 sequences demonstrated that the screened CDR3 sequences were specifically bound to the binding sites of β-lactamase.

1. Introduction

β-Lactamases are enzymes that deactivate the β-lactam antibiotics like penicillin, cephalosporin, and carbapenem through hydrolysis of the β-lactam ring.[1,2] β-Lactamases are usually classified according to the Ambler classification based on either the functional characteristics of the enzymes or their primary structure. The simplest classification is using protein sequence, wherein the β-lactamases are classified into four molecular classes, A, B, C, and D, based on the conserved and distinguishing amino acid motifs.[3,4] Classes A, C, and D include enzymes that hydrolyze their substrates by forming an acyl enzyme through an active site serine, whereas class B β-lactamases are metalloenzymes that utilize at least one active-site zinc ion to facilitate β-lactam hydrolysis.[5] Inhibitors for these β-lactamases have been developed using two kinds of major mechanisms. The first mechanism for the inhibition of β-lactamases has been the inhibition of the acylation step of the hydrolysis of the β-lactam ring.[6,7] Serine-based β-lactamases of Class A, C and D all rely on a key water molecule to hydrolyze and inactivate β-lactam antibiotics. This process involves two conserved catalytic steps. In the first acylation step, the β-lactam antibiotic forms an acyl–enzyme intermediate (ES*) containing a catalytic serine residue. In the second deacylation step, an activated water molecule serves as a nucleophile to attack ES* and release the inactivated β-lactam antibiotics. The inhibitors block the hydrolysis of β-lactam through the formation of another intermediate. The inhibitors avibactam and relebactam are known to deactivate β-lactamase through this mechanism.[8,9] The second mechanism is "suicide inhibitors", which permanently inactivate the enzyme through secondary chemical reactions at the active site. It covalently binds to a serine residue on the active site of β-lactamase, restructuring the molecule to create a highly reactive species that attacks another amino acid on the active site, permanently inactivating the enzyme.[10,11] The inhibitors called clavulanic acid deactivates β-lactamase through this mechanism.[12] In this study, the amino acid sequences that bind to the active site of β-lactamases are screened, which can inhibit the hydrolysis of the β-lactam ring. The mimotopes usually refer to molecules that have a chemical environment similar to that of the original molecule. Herein, penicillin-like mimotopes were screened from the Fv-antibody library, which were recognized as penicillin-like molecules by monoclonal anti-penicillin antibodies as well as β-lactamase.[13,14] As the active site of β-lactamase recognizes the

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β-lactam ring of antibiotics as a substrate, this enzyme is deactivated to hydrolyze the β-lactam ring by binding penicillin-like mimotopes.[15,16] The screening of penicillin-like mimotopes from the Fv-antibody library was performed to delineate the inhibitors of β-lactamase activity.

The Fv-antibody represented the variable region (V_H) at the heavy chain of immunoglobulin *G* (Ig*G*), which comprised three complementarity-determining regions (CDRs) and four frame regions (FRs).[17,18] As shown in Fig. 1(a), the amino acid sequence of the CDR3 region was randomized to prepare the Fv-antibody library. The randomization of the 11 amino acid sequences of the CDR3 region was performed by site-directed mutagenesis using the monoclonal anti-thyroid peroxidase (TPO) antibody sequence as a template.[19,20] As shown in Fig. 1(b), the Fv-antibody library was expressed on the outer membrane of *E. coli* using autodisplay technology based on the auto-transporter protein AIDA-1.[21,22] The prepared autodisplayed Fv-antibody library had a high surface density of $>10^5$ Fv-antibodies *E. coli* and a high diversity of $>10^6$ clones/libraries.[23] Owing to such properties, the Fv-antibody library can be used to screen target Fv-antibodies against specific antigens without repeated biopanning.[24] To screen enzyme inhibitors, monoclonal antibodies against substrate molecules were used as screening probes. The screened Fv-antibody (*i.e.*, the amino acid sequences of the CDR3 region) exhibited a specific binding affinity to the monoclonal antibodies. These sequences represent a chemical environment similar to that of an enzyme substrate. Recently, dopamine-like mimotopes were screened using an anti-dopamine antibody as a screening probe, and the screened CDR3 sequence inhibited monoamine oxidase (MAO) B, which oxidizes dopamine.[25,26] Serotonin-like mimotopes were also screened using monoclonal antibodies from the Fv-antibody library, and the screened CDR3 sequence inhibited MAO-A, which oxidizes serotonin.[13,27]

Herein, penicillin-like mimotopes were screened from the Fv-antibody library using magnetic beads with immobilized monoclonal anti-penicillin antibodies as a screening probe. The screened Fv-antibodies were expressed as soluble proteins and synthesized into peptides of the screened CDR3 sequences. The inhibition activity (IC_{50}) was estimated for the expressed Fv-antibodies and synthesized CDR3 peptides. The effective inhibition of the β-lactamase activity in bacterial lysates was demonstrated using the expressed Fv-antibodies and synthesized peptides.[7] Finally, docking analysis was performed to investigate the interaction between β-lactamase and the screened CDR3 sequence.

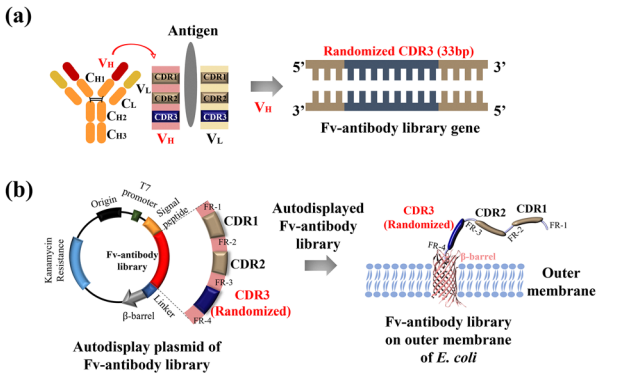

Fig. 1 Preparation of the Fv-antibody library. (a) Fv-antibody library was prepared through site-directed mutagenesis of the CDR3 region with 33 nucleotide sequences. (b) Expression of the Fv-antibody library on the outer membrane of the *E. coli via* transformation of an autodisplay vector.

2. Materials and methods

2.1 Materials

Monoclonal antibodies against penicillin (MIP0701), Dynabeads™ protein G, FastDigest *DpnI* and Phusion High-Fidelity DNA polymerase were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The β-lactamase, *Bacillus cereus* 569/H9, bovine serum albumin (BSA), penicillin-G sodium salt, neocuproine hydrochloride hydrate, copper(II) sulfate pentahydrate, sodium dodecyl sulfate (SDS), sodium acetate, and acetic acid were purchased from Sigma-Aldrich (Seoul, Korea). NEBuffer™ 2 and Klenow DNA polymerase were purchased from New England Biolabs (Ipswich, MA, USA). The primers were custom-synthesized by Bionics (Seoul, Korea). A PCR purification mini kit was obtained from Favorgen (Pingtug, Taiwan). Isopropyl β-D-1-thiogalactopyranoside (IPTG) and kanamycin were purchased from Kisan Bio (Seoul, Korea). Peptides were synthesized by Peptron Co. (Daejeon, Korea). An Amicon® Ultra 0.5 mL filter with cutoffs of 10 and 100 kDa was purchased from Millipore Co. (Darmstadt, Germany). Finally, phosphate buffered saline (PBS) (20×) was obtained from Curebio Co. (Seoul, Korea).

2.2 Autodisplay of the Fv-antibody library

The Fv-antibody library was prepared by site-directed mutagenesis, as previously reported.[14,28] To obtain a single-stranded DNA primer with a randomized CDR3 sequence, 40 μL were prepared by combining 2 μL of synthesized single-stranded primers containing random CDR3 oligonucleotide sequences with 2 μL of their corresponding reverse primers (Table S1, ESI†). The mixture included NEBuffer 2 (50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NaCl, 100 μg mL^{-1} BSA, pH 7.9, 4 μL), and the remaining volume was filled with DW (32 μL). After annealing (heating for 5 min at 95 °C and then cooling to 36 °C at a rate of −0.3 °C s^{-1}), a final volume of 200 μL was obtained through an extension reaction (37 °C, 15 min) involving Klenow (*exo*-) enzyme (3 μL), dNTPs (10 mM, 8 μL), NEBuffer 2 (16 μL), and DW (133 μL). After deactivating the enzyme reaction (75 °C, 20 min), PCR products containing random CDR3 sequences within double-stranded Fv-antibody library primers were purified using a NucleoSpin® Gel and PCR Clean-up kit. The Fv-antibody library plasmids were synthesized by adding a template plasmid (pST009, 150 ng), a mixture of double-stranded Fv-antibody library primers (150 ng), HF buffer (10 μL), 10 mM dNTPs (1 μL), Phusion high-fidelity DNA polymerase (0.5 μL), and DW to bring the final volume to 50 μL.

PCR cycling conditions were as follows: (1) initial denaturation at 98 °C for 1 min, (2) denaturation at 98 °C for 30 s, (3) annealing at 68 °C for 1 min, (4) elongation at 72 °C for 5 min, (5) repeated (2) to (4) for 30 cycles, and (6) final extension step at 72 °C for 10 min. After PCR, the template plasmids were digested with *DpnI* restriction enzyme (37 °C for 16 h), followed by filtration of the digested Fv-antibody library plasmids through a 100 K Amicon filter (Millipore, Billerica, MA, USA). The Fv-antibody library was autodisplayed on the outer membrane of *E. coli* BL21(DE3) cells by transforming the prepared library plasmid into competent *E. coli* BL21(DE3) cells using electroporation. After transformation, *E. coli* cells were grown in LB broth supplemented with 50 mg L^{-1} kanamycin at 37 °C for 16 h.

Subsequently, the cultured cells (100 μL) were transferred to 10 mL of fresh LB medium containing β-mercaptoethanol (10 mM), kanamycin (50 mg L^{-1}), and ethylenediaminetetraacetic acid (EDTA; 10 μM) and incubated at 37 °C with shaking (200 rpm) until reaching an optical density (O.D.) of 0.5 at 600 nm. To activate the expression of the Fv-antibody library, *E. coli* cells cultured in 10 mL were treated with 1 mM IPTG and incubated at 30 °C with shaking at 150 rpm for 3 h.

2.3 Screening and expression of the Fv-antibody library

Penicillin-like mimotopes were screened from the Fv antibody library using magnetic beads immobilized with monoclonal anti-penicillin antibodies, as previously reported.[14,29] The procedures involved the following steps: (1) the Fv-antibody library (100 μL, $OD_{600\,nm}$ = 1.0) was mixed with Dynabeads™ Protein G (5 μL) and coated with monoclonal anti-penicillin antibody (10 μg) for 1 h at 37 °C. (2) Monoclonal antibody-bound magnetic beads with the attached targeted *E. coli* cells were separated using an external magnet. Unbound *E. coli* cells were eliminated by washing the beads five times with 0.01% PBST and PBS. (3) The isolated magnetic beads containing *E. coli* cells bound to monoclonal anti-penicillin antibody were re-suspended in 100 μL of PBS. (4) The magnetic beads isolated in PBS were plated onto agar plates to obtain screened *E. coli* clones (Fig. 2(a)).

Several candidate clones from the agar plate were randomly selected, and their binding affinity to the monoclonal anti-penicillin antibody was evaluated using fluorescence-activated cell sorting (FACS) to identify the final clones. The FACS screening process was as follows: (1) the screened *E. coli* cells ($OD_{600\,nm}$ = 1.0, 100 μL) were mixed with FITC (λ_{Ex} = 488 nm, λ_{Em} = 525 nm)-labeled monoclonal anti-penicillin antibody (1 mg mL^{-1}, 5 μL) and incubated with shaking at 15 rpm for 1 h. (2) Unattached FITC-labeled monoclonal antibodies were removed by washing the samples five times with 0.01% PBST and PBS. (3) The FACS analysis of each selected clone was conducted using a FACSCalibur flow cytometer (Becton–Dickinson, Franklin Lakes, NJ, USA) with an excitation wavelength (λ_{Ex}) of 488 nm, emission wavelength (λ_{Em}) of 525 nm. (4) For each individual clone bound with the FITC-labeled monoclonal anti-penicillin antibody, approximately 50 000 *E. coli* cells were analyzed and sorted using the FACSCalibur flow cytometer.

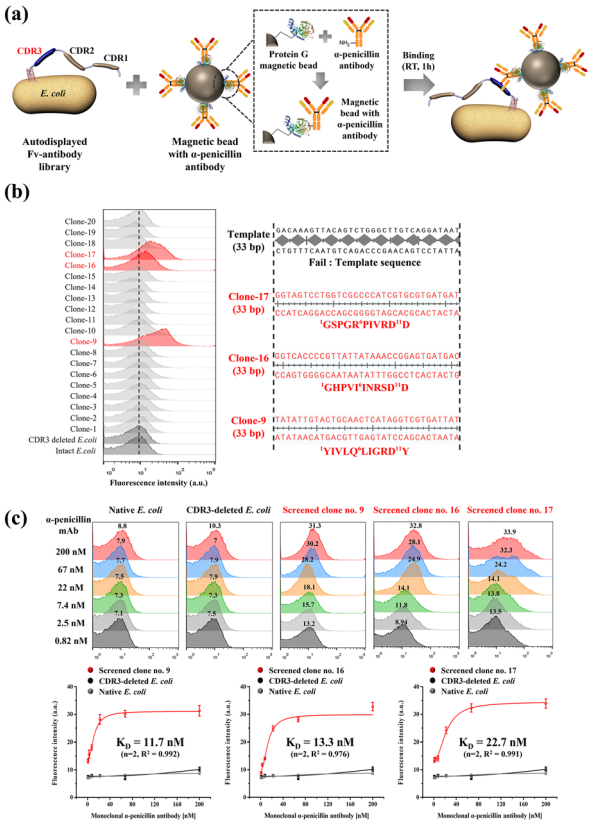

Fig. 2 Screening of the Fv-antibody library. (a) Screening procedure of the Fv-antibody library using magnetic beads with an immobilized monoclonal anti-penicillin antibody. (b) Binding assay of the screened clones from the Fv-antibody library and the oligonucleotide sequencing results of the CDR3 region. (c) Binding affinity of screened clones no. 9, no. 16, and no. 17 was determined using flow cytometry.

(5) The bound *E. coli* cells were plated and cultured on LB agar plates.

The screened Fv-antibodies were expressed as soluble fusion proteins with green fluorescent proteins (GFP) and his-tag. The plasmids (pHE001, pHE002, and pHE003) encoding Fv-antibodies and GFP were custom-synthesized by Cosmogenetech (Seoul, Korea). The amino acid sequences of the Fv antibodies, which include both CDRs and FRs, are summarized in Table S2 (ESI†). The Fv-antibodies were produced in *E. coli* cells by transforming custom plasmids into competent cells that were cultured in 20 mL of high-salt LB medium containing 1 mM IPTG and 60 μg mL^{-1} carbenicillin for 16 h at 30 °C.[30,31] The cultured *E. coli* cells were harvested by centrifugation (at 3000×*g*, 3 min) and then mixed with 20 mL of binding buffer (containing 5 mM Tris–HCl, 0.5 mM EDTA, and 1 M NaCl) with 3 M urea. The mixture was sonicated using an ultrasonic processor (Vibra cell VCX-130, Sonics, USA). The lysate underwent centrifugation at 25 000×*g* for 10 min. The Fv-antibodies from the resulting supernatant were purified using a His-tag column (Roche, Bassel, Switzerland) with an elution buffer containing 3 M urea and 200 mM imidazole.[32] The proteins were dialyzed for 16 h at 100 rpm and 4 °C to eliminate imidazole and urea.

2.4 SPR biosensor analysis

Binding affinity (K_D) between the monoclonal anti-penicillin antibody and both synthesized peptides, as well as expressed Fv-antibodies, was assessed using SPR analysis with the SPR biosensor provided by i-Cluebio (Seongnam, Korea).[33,34] The interaction was evaluated by immobilizing the monoclonal anti-penicillin antibody onto the gold-coated surface of the SPR chips. The SPR chips comprised a 2 nm Ti film and a 48 nm Au layer, both coated with BK-7 glass (1×1 cm^2). The SPR chip was modified to incorporate carboxylic acid using 1 mU MUA. Then, the monoclonal anti-penicillin antibody was covalently immobilized using the EDC/NHS reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide. As shown in Fig. S1 (ESI†), the SPR chips were incubated for 1 h with monoclonal anti-penicillin antibody (10 μg mL^{-1}). Subsequently, the chips were washed with PBS, followed by a 1 h incubation for non-specific blocking with BSA (1 mg mL^{-1}). Fv-antibodies (7.4–600 nM) were introduced into the flow cell for 15 min (at a rate of 15 μL min^{-1}) as the association step. In conclusion, PBS was introduced into the flow cell for 15 min (at a rate of 15 μL min^{-1}) as the dissociation step. Moreover, β-lactamase was first incubated on the SPR chip, followed by the flow of Fv-antibodies and peptides over the surface.

The results obtained from the SPR signals were subjected to fitting using Hill's equation:[35,36]

$$y = \frac{a - d}{1 + (x/c)^b} + d$$

where "*a*" represents the maximum signal, "*d*" represents the minimum signal, "*c*" stands for the concentration of the solution protein, and "*b*" signifies the Hill's slope of the curve.[37–39]

2.5 Measurement of the inhibitory activity of β-lactamase

β-Lactamase IC_{50} was measured using a colorimetric assay.[40,41] The colorimetric test was performed following previous protocols.[42,43] Solution A contained 40 mg of neocuproine-HCl and 500 mg of SDS dissolved in 50 mL of 0.2 M acetate buffer at pH 4.8. Solution B was prepared by dissolving 100 mg of $CuSO_4 \cdot 5H_2O$ and 500 mg of SDS in 50 mL of 0.2 M acetate buffer at pH 4.8. The reaction buffer was created by combining solutions A and B in equal volumes at a ratio of 1 : 1 (v : v). The IC_{50} of β-lactamase was measured by reacting β-lactamase with both Fv-antibodies and the synthesized peptide at concentrations ranging from 1.6 nM to 5 μM, followed by the addition of penicillin to measure the hydrolysis product, penicilloic acid (PA), at 454 nm. This procedure was performed on β-lactamase using bacteria resistant to β-lactam antibiotics, which were processed by sonication, syringe filtration, and Amicon filtration to remove bacterial debris. As β-lactamase is localized in the periplasmic space,[44,45] the assay of inhibitory activity (IC_{50}) of penicillin-like mimotopes was determined using bacterial lysates. Additionally, *E. coli* UT5600 bacteria carrying the pET-Z-18-3 plasmid were used as antibiotic-resistant strains.[46] The final results of the β-lactamase assay were combined with the reaction buffer to obtain a total volume of 260 μL. After a 20 min incubation period at room temperature, the absorbance was measured at 454 nm using a Glomax Discover microplate reader from Promega (Sunnyvale, CA, USA).[47] The positive control (100% inhibition) for the colorimetry analysis was performed using only penicillin and the bacterial lysate of UT5600.

3. Results and discussion

3.1 Screening of penicillin-like mimotopes from the Fv-antibody library

The Fv-antibody represents the V_H domain of Ig*G*, which comprises three CDRs and four FRs.[48,49] The Fv-antibody library was prepared by site-directed mutagenesis to generate a random amino acid sequence of CDR3.[50,51] This study aimed to inhibit β-lactamase activity through the direct interaction of mimotopes with the active site of β-lactamase. In detail, the targeted active site pocket of class A β-lactamases has been reported in the dimension of approximately 300 to 450 Å^3.[52] Therefore, the effective access and interaction at the active site, the inhibitors were aimed to have the corresponding size to enhance the specific interaction with the active site. For this reason, the CDR3 region with an 11 amino acid sequence was considered to be an effective candidate for the screening of β-lactamase inhibitors, and it was randomized to produce an Fv-antibody library.

As shown in Fig. 1(a), the CDR3 template from TPO was randomized to have 11 amino acid residues through site-directed mutagenesis.[20] As shown in Fig. 1(b), the Fv-library was expressed on the outer membrane of *E. coli* using auto-display technology, and the library had an expression yield of $>10^5$ Fv-antibodies/*E. coli*[23,53] and a diversity of $>10^6$ clones/library.[54] Herein, penicillin-like mimotopes were screened from the Fv-antibody library, which was recognized as penicillin by monoclonal anti-penicillin antibody and β-lactamase.[13,14] The screening of penicillin-like mimotopes selection was carried out in two sequential steps. First, the clones have specific binding affinity to the monoclonal anti-penicillin antibody. The binding affinity (K_D) of selected clones to the monoclonal antibody, as well as β-lactamase evaluated using surface plasmon resonance (SPR). As the next step, the inhibitory activity (IC_{50}) of expressed Fv-antibodies and synthesized mimotope-peptides with the screened CDR3 sequences was estimated using a colorimetric assay of β-lactamase activity.

The screening of penicillin-like mimotopes was performed using magnetic beads from the Fv-antibody library. To screen the Fv-antibody library, a monoclonal anti-penicillin antibody was immobilized on the magnetic beads through covalent bonding between the sulfonic acid groups on the surface of the magnetic beads and the primary amine groups of the monoclonal antibodies (Fig. 2(a)). The Fv-antibody library was reacted with antibody-immobilized magnetic beads, and then the magnetic beads were isolated using an external magnet. The isolated magnetic beads were spread on agar plates, and the bound *E. coli* cells were cultured and grown into clones. The grown clones were cultured again, and the binding activity

Table 1 Screened nucleotide and amino acid sequences

Screened CDR3		Sequences
Mimotope-9 (clone no. 9)	Oligo-nucleotide Amino acid sequences	5′-^{1}TATAT^{6}TGTAC^{11}TGCAA^{16}CTCAT^{21}AGGTC^{26}GTGAT^{31}TA^{33}T-3′ ^{1}Y I V L Q ^{6}L I G R D ^{11}Y
Mimotope-16 (clone no. 16)	Oligo-nucleotide Amino acid sequences	5′-^{1}GGTCA^{6}CCCCG^{11}TTATT^{16}ATAAA^{21}CCGGA^{26}GTGAT^{31}GA^{33}C-3′ ^{1}G H P V I ^{6}I N R S D ^{11}D
Mimotope-17 (clone no. 17)	Oligo-nucleotide Amino acid sequences	5′-^{1}GGTAG^{6}TCCTG^{11}GTCGC^{16}CCCAT^{21}CGTGC^{26}GTGAT^{31}GA^{33}T-3′ ^{1}G S P G R ^{6}P I V R D ^{11}D

was estimated by sequential treatment with monoclonal anti-penicillin antibody and fluorescence-labeled secondary antibody. *E. coli* cells with high fluorescence were isolated as candidate clones using flow cytometry. After oligonucleotide sequencing of CDR3, the final clones were selected to have an oligonucleotide sequence different from the template CDR3 sequence (before site-directed mutagenesis). As shown in Fig. 2(b), three clones with binding affinity to monoclonal anti-penicillin antibodies were determined to be the final clone. When the final clone (clone no. 9) was reacted with different concentrations of monoclonal anti-penicillin antibody, the fluorescence increased quantitatively (Fig. 2(c)). In the case of native *E. coli* without any autodisplayed and control strains with autodisplayed Fv-antibodies, except for those with CDR1 and CDR2 (without CDR3), the fluorescence signal was maintained at the baseline level. Such results were also observed for the other two final clones (clone no. 16 and 17; Fig. 2(c)). From the analysis of flow cytograms, K_D was calculated to be 11.7 nM for clone no. 9, 13.3 nM for clone no. 16, and 22.7 nM for clone no. 17 (n = 3). These results showed that CDR3 of these clones exhibited specific binding activity with monoclonal antibodies. The oligonucleotide and amino acid sequences are summarized in Table 1.

The screened Fv-antibodies were expressed as soluble proteins and GFP was co-expressed. The co-expressed GFP was employed for the following two purposes: (1) the Fv-antibody exhibited limited solubility, whereas the fusion protein with GFP demonstrated significantly enhanced solubility.[55,56] (2) GFP proved effective in immobilizing onto the metal surface of bio-sensors, displaying comparable immobilization efficiency to that of BSA, a commonly used blocking protein in immunoassays. As shown in Fig. 3(a), the expression of the Fv-antibodies comprises three CDRs and GFP with a molecular weight of 40.3 kDa. As shown in Fig. 3(b), the K_D of the expressed Fv-antibodies to β-lactamase was estimated using the SPR biosensor with the immobilized β-lactamase on Au-Chip. The K_D was calculated to be 82.6 nM for Fv-9 (from clone no. 9), 93.8 nM for Fv-16 (from clone no. 16), and 101.0 nM for Fv-17 (from clone no. 17). Additionally, the K_D of the synthesized peptides to β-lactamase was confirmed using SPR. As shown in Fig. 3(c), the K_D of the synthesized CDR3 peptides to β-lactamase was estimated to be 667.7 nM for peptide-9 (from clone no. 9), 732.8 nM for peptide-16 (from clone no. 16), and 732.2 nM for peptide-17 (from clone no. 17).

Considering the binding affinity of antibiotics or other inhibitors to β-lactamase (Table 2), these results indicated that the binding of screened Fv-antibodies strongly inhibited

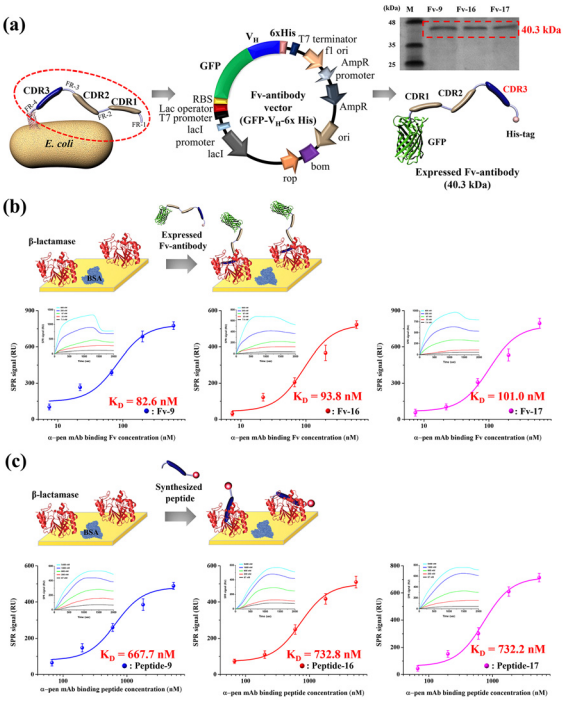

Fig. 3 Expression of the screened Fv-antibodies. (a) Expression of the screened Fv-antibodies as a soluble protein fused with green fluorescence protein (GFP) with a molecular weight of 40.3 kDa (SDS-PAGE). (b) Schematics of the binding assay and analysis of the binding affinity (K_D) of the expressed Fv-antibodies to β-lactamase using an SPR biosensor and dose–response curves. (c) Schematics of the binding assay and analysis of the binding affinity (K_D) of the synthesized peptide (screened CDR3 sequences) to β-lactamase using an SPR biosensor and dose–response curves.

Table 2 Binding affinity of antibiotics or other inhibitors to β-lactamase

Antibiotics or β-lactamase inhibitors	Binding affinity to β-lactamase (Type of enzyme)
Imipenem	1240.0 μM (KPC-2)[57]
QPX7728	0.06 nM–28.0 nM (Class A)[58]
Clavulanic acid	140.0 nM (SHV-1)[59]
Tazobactam	70.0 nM (SHV-1)[59]
L-Captopril	2.2 μM (NDM-1)[60]

β-lactamase. The K_D of the expressed Fv-antibodies to monoclonal anti-penicillin antibodies was estimated using an SPR biosensor with the immobilized monoclonal anti-penicillin

antibody (Fig. S1(a), ESI†). As shown in Fig. S1(b) (ESI†), the K_D of the expressed Fv-antibodies to monoclonal anti-penicillin antibody was calculated as 71 ± 7.1 nM for Fv-9 (from clone no. 9), 87 ± 3.4 nM for Fv-16 (from clone no. 16), and 95 ± 4.4 nM for Fv-17 (from clone no. 17). These results show that the expressed Fv-antibodies and synthesized peptides can be used as penicillin-like mimotopes, which are bound to monoclonal anti-penicillin antibody as well as β-lactamase.

3.2 Inhibition activity (IC_{50}) of penicillin-like mimotopes

β-Lactamase activity was estimated using colorimetry analysis based on the quantification of penicilloic acid (PA), which is produced by the reaction of β-lactamase with penicillin.[15,61] As shown in Fig. 4(a), penicillin was hybridized into PA by β-lactamase, and then the blue-colored PA was reduced by Cu^{2+} and it produced an orange-colored neocuproine copper complex (ε = 14 500 M^{-1} cm^{-1} at 454 nm).[42] Therefore, β-lactamase activity was estimated using the decrease in the absorption according to the amount of produced PA (100%, positive control). When inhibitors were treated with β-lactamase, the amount of reaction product (PA) decreased; the final volume was lower than that of the positive control (without inhibitor treatment). Herein, β-lactamase activity from the colorimetry was correlated with the inhibitory activity of Fv-antibodies and the synthesized CDR3 peptides. Colorimetry analysis was conducted using both kinds of inhibitors in a concentration range of 1.6–5000 nM with 2.5 mU mL^{-1} β-lactamase and 100 μM benzylpenicillin as substrate and the IC_{50} was calculated. As shown in Fig. 4(b), the conventional β-lactamase inhibitor called avibactam, with a known IC_{50} of 0.6–3100 nM, was used for the comparison of its inhibition activity.[62] β-Lactamase activity without inhibitor treatment was considered 100%, and the IC_{50} was estimated to be 45.5 nM from an inhibitor concentration of 50% of β-lactamase activity.[63,64] As shown in Fig. 4(c), the IC_{50} values of the expressed Fv-antibodies were estimated to be 69.7 nM for Fv-9 (from clone no. 9), 109.8 nM for Fv-16 (from clone no. 16), and 101.4 nM for Fv-17 (from clone no. 17). The IC_{50} values of the synthetic CDR3 peptides were also determined using the same colorimetric method. As shown in Fig. 4(d), the IC_{50} values of the synthesized peptides were estimated to be 72.4 nM for peptide-1 (from clone no. 1), 117.2 nM for peptide-16 (from clone no. 16), and 108.3 nM for peptide-17 (from clone no. 17). The IC_{50} of the commercial inhibitors were avibactam (45.5 nM), vaborbactam (110 nM), clavulanic acid (920 nM), and relebactam (34–330 nM). These results show that the expressed Fv-antibodies and synthesized peptides can be used as penicillin-like mimotopes for effective inhibition of β-lactamase.

The applicability of inhibitors (the expressed Fv-antibodies and synthesized peptides) was estimated for β-lactamase using bacteria with resistance to β-lactam antibiotics. The bacterial lysate was prepared using an *E. coli* UT5600 bacterial strain transformed with the pET-Z-18-3 plasmid. The pET-Z-18-3 plasmid carries the *bla* gene, which encodes β-lactamase degradation of ampicillin (Fig. 5(a)). The colorimetry analysis was conducted with a fixed number of bacteria of 5 × 10^6 ea and the benzylpenicillin at a concentration of

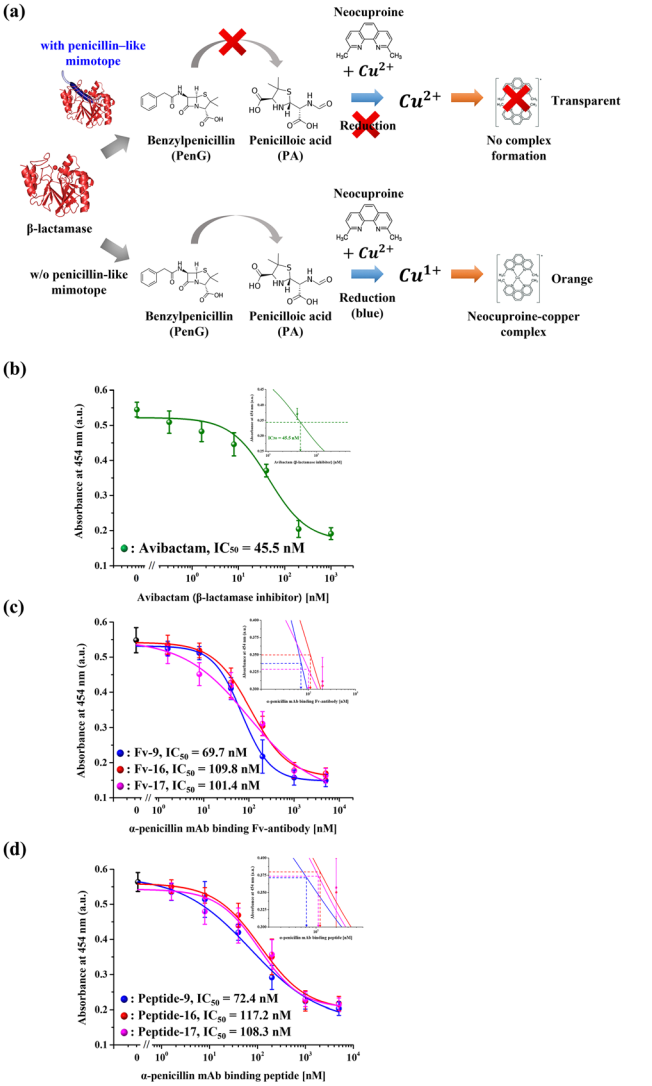

Fig. 4 Analysis of the inhibition activity of penicillin-like mimotopes using a colorimetric assay. (a) Schematic of the inhibition activity (IC_{50}) of penicillin-like mimotopes using a colorimetric assay. Analysis of the inhibition activity and evaluation of the IC_{50} value of (b) avibactam, (c) Fv-9, Fv-16, and Fv-17, and (d) peptide-9, peptide-16, and peptide-17.

100 μM. The bacterial lysate of UT5600 without the pET-Z-18-3 plasmid was used as a positive control (100% inhibition) for colorimetry.

As shown in Fig. 5(b), the IC_{50} of avibactam, a conventional inhibitor, was also estimated for comparison.[62,65] β-Lactamase activity without inhibitor treatment was considered 100%, and the IC_{50} was estimated to be 59.8 nM.[41,66] As shown in Fig. 5(c), the IC_{50} values of the expressed Fv-antibodies were estimated to be 79.8 nM for Fv-9 (clone no. 9), 102.7 nM for Fv-16 (clone no. 16), and 98.0 nM for Fv-17 (clone no. 17). As shown in Fig. 5(d), the IC_{50} values of the synthesized peptides were estimated to be 138.3 nM for peptide-9 (clone no. 9), 210.4 nM for peptide-16 (clone no. 16), and 151.3 nM for peptide-17 (clone no. 17). In comparison with the assay result from the previous enzyme solution, the IC_{50} was slightly increased. These results

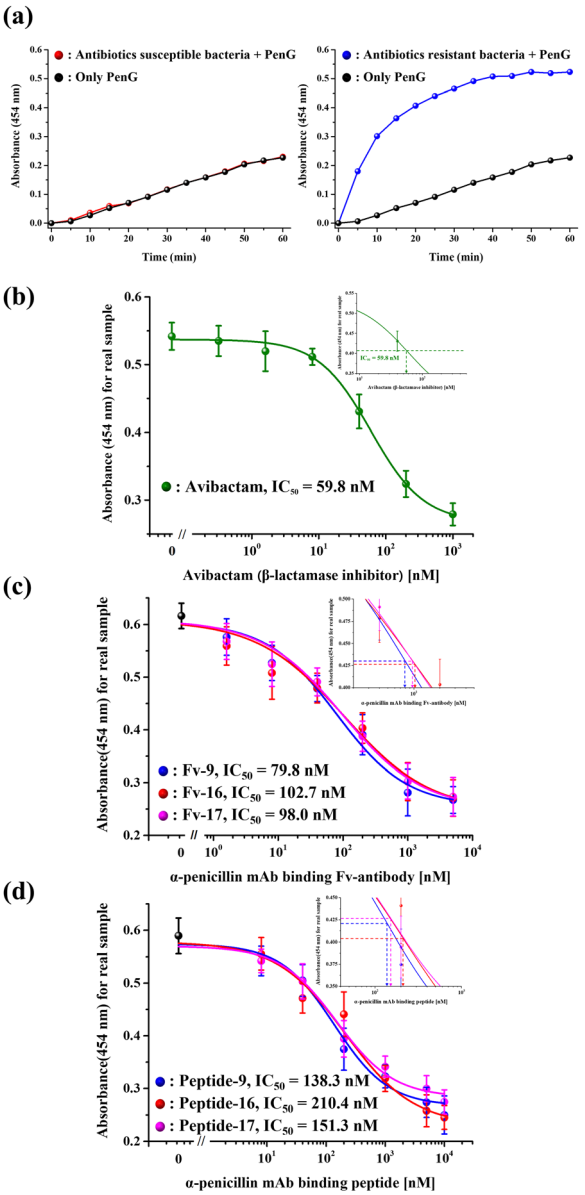

Fig. 5 Analysis of the inhibition activity of penicillin-like mimotopes using a colorimetric assay with bacterial resistance to β-lactam antibiotics. (a) Comparison of the antibiotics susceptible bacteria (left) and the antibiotics resistance bacteria (right) using a colorimetric assay. Analysis of the inhibition activity and evaluation of the IC_{50} value of (b) avibactam, (c) Fv-9, Fv-16, and Fv-17, and (d) peptide-9, peptide-16, and peptide-17.

show that the expressed Fv-antibodies and synthesized peptides are feasible penicillin-like mimotopes for the effective inhibition of β-lactamase activity in bacterial lysates.

3.3 Docking analysis of β-lactamase and penicillin-like mimotopes

The interactions between β-lactamase and inhibitors (expressed Fv-antibodies and synthesized peptides) were analyzed by computer-aided docking simulation using AutoDock Vina software from Scripps Research.[67–69] Because the specific interaction between β-lactamase and inhibitors predominantly occurs

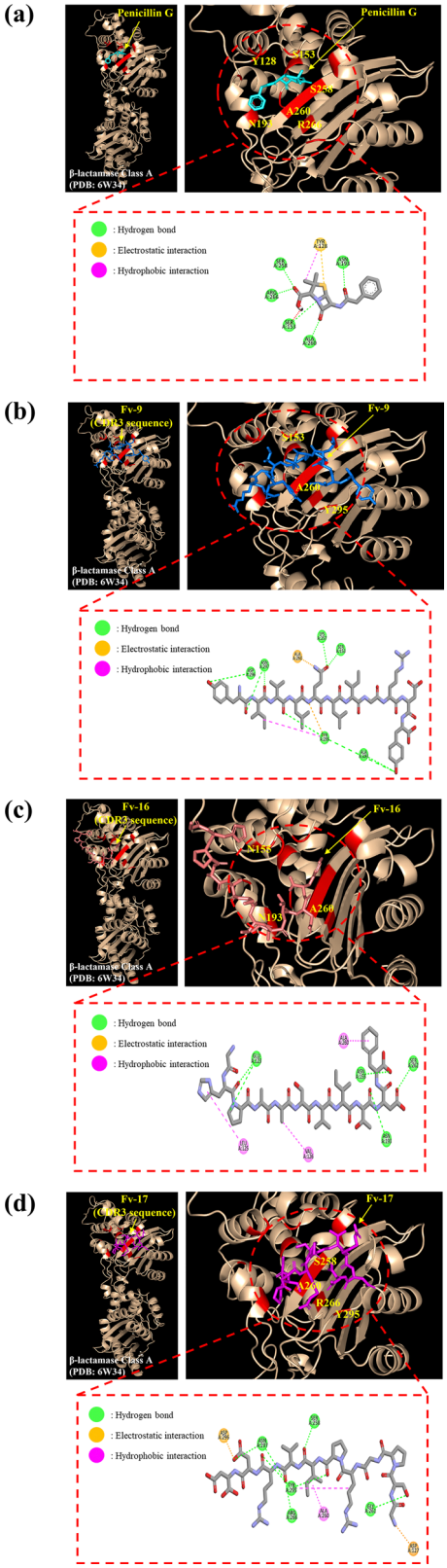

Fig. 6 Docking analysis of β-lactamase with benzylpenicillin and the three Fv-antibodies. Interactions between β-lactamase and (a) benzylpenicillin, (b) Fv-9, (c) Fv-16, and (d) Fv-17.

Table 3 Analysis of the interactions of β-lactamase class A obtained from *B. cereus* with PenG and the expressed Fv-antibodies

	Binding site of β-lactamase class A			Binding affinity (kcal mol^{-1})
	Hydrogen bond	Hydrophobic interaction	Electrostatic interaction	
Benzylpenicillin (PenG)	S_{153}, N_{193}, S_{258}, A_{260}, R_{266}	Y_{128}	Y_{128}	−7.8
Fv-9	S_{153}, K_{257}, A_{293}, Y_{295}, D_{296}, N_{297}	Y_{295}	A_{260}	−8.1
Fv-16	E_{123}, N_{155}, N_{193}, S_{262}	L_{125}, V_{126}, A_{260}	—	−7.7
Fv-17	S_{258}, G_{261}, R_{266}, Y_{295}, N_{297}	A_{260}, Y_{295}	D_{127}, D_{296}	−7.7

through the screened CDR3 region, the docking simulation was performed between β-lactamase and the amino acid sequence of CDR3 with 11 amino acid residues. The results of the docking analysis were analyzed using the PyMOL Molecular Graphics System, Version 2.0, developed by Schrödinger from DeLano Scientific LLC (South San Francisco, CA, USA) and Discovery Studio Visualizer version 21.1.0.20298 (Dassault Systèmes BIOVIA, San Diego, CA, USA). For the docking simulation, the structure of β-lactamase (class A, PDB ID: 6W34) was obtained from the Protein Data Bank (**https://www.rcsb.org**). Docking analysis was performed using the structure of benzylpenicillin and the three Fv-antibodies. β-Lactamase has 13 sites, including five binding sites for the benzylpenicillin interaction.[70] Four binding sites (E_{189}, N_{193}, S_{258}, R_{266}) were involved in hydrogen bonding, while the other nine binding sites (A_{94}, K_{98}, Y_{128}, S_{153}, N_{155}, T_{239}, G_{259}, A_{260}, Y_{295}) were associated with electrostatic interactions. As shown in Fig. 6(a), interactions between β-lactamase were made *via* the partial binding of benzylpenicillin to the binding site of β-lactamase by docking simulation. The interaction between benzylpenicillin and β-lactamase involved hydrophobic and electrostatic interactions with amino acid Y_{128}, while the other amino acids S_{153}, N_{193}, S_{258}, A_{260}, and R_{266} formed hydrogen bonds with benzylpenicillin. The binding affinity (ΔG) was calculated to be −7.8 kcal mol^{-1}, which represented the spontaneous binding of β-lactamase and benzylpenicillin.[67–69] The docking analysis between Fv-9 and β-lactamase is shown in Fig. 6(b). Y_{295} of β-lactamase class A engaged in hydrophobic interactions with Fv-9, and S_{153}, K_{257}, A_{293}, Y_{295}, D_{296}, and N_{297} of β-lactamase formed hydrogen bonds, and A_{260} of β-lactamase participated in electrostatic interactions. The ΔG was calculated to be −8.1 kcal mol^{-1}, which represents the spontaneous binding between β-lactamase and Fv-9. The docking analysis between Fv-16 and β-lactamase is illustrated in Fig. 6(c). The analysis revealed hydrogen bonding with E_{123}, N_{155}, N_{193}, and S_{262} of β-lactamase, and L_{125}, V_{126}, and A_{260} of β-lactamase was involved in hydrophobic interactions with Fv-16, and the ΔG was calculated as −7.7 kcal mol^{-1}. The docking results between Fv-17 and β-lactamase class A are shown in Fig. 6(d). The analysis revealed hydrogen bonding at sites S_{258}, G_{261}, R_{266}, Y_{295}, and N_{297} of β-lactamase and hydrophobic interactions at A_{260} and Y_{295}, and electrostatic interactions at D_{127} and D_{296}. The ΔG was calculated as −7.7 kcal mol^{-1}. The docking analysis of β-lactamase and screened CDR3 sequences showed that the binding of screened CDR3 sequences was spontaneous at the known binding sites of β-lactamase spontaneously with negative ΔG. Table 3 provides a summary of the interactions observed between β-lactamase class A and the three Fv-antibodies.

4. Conclusions

Penicillin-like mimotopes were screened from the Fv-antibody library and recognized as penicillin by monoclonal anti-penicillin antibody and β-lactamase. The screening of penicillin-like mimotopes was conducted using magnetic beads with immobilized monoclonal anti-penicillin antibody on the surface. Three clones were screened from the Fv-antibody library with specific binding activity to the monoclonal antibody. The screened Fv-antibodies were expressed as soluble proteins co-expressed with GFP and were synthesized into peptides of the screened CDR3 sequences. The K_D values determined from SPR measurements were estimated to be 83–101 nM for Fv-antibodies and 667–733 nM for synthesized peptides. These results showed that the expressed Fv-antibodies and synthesized peptides could be used as penicillin-like mimotopes and bound to monoclonal anti-penicillin antibody and β-lactamase. β-Lactamase activity was estimated using colorimetry based on PA quantification, which was produced by the β-lactamase reaction, and was correlated to the IC_{50} of Fv-antibodies and CDR3 peptides. The IC_{50} values were estimated to be 69–109 nM for Fv-antibodies and 72–117 nM for synthesized peptides. The IC_{50} indicated that the expressed Fv-antibodies and synthesized peptides could be used as penicillin-like mimotopes for the effective inhibition of β-lactamase activity. The effective inhibition of β-lactamase activity in bacterial lysates was also demonstrated using the expressed Fv-antibodies and synthesized peptides. Finally, docking analysis of β-lactamase and screened CDR3 sequences showed that the binding of screened CDR3 sequences was spontaneous at the known binding sites of β-lactamase.

Data availability

All data supporting the findings of this study are available within the article and its ESI† files.

Conflicts of interest

There are no conflicts to declare.

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