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Twofold enhanced dispersin B activity by N-terminal fusion to silver-binding peptide for biofilm eradication

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ABSTRACT

Dispersin B (DspB) has shown a great potential for the hydrolysis of polymeric β-1,6-*N*-acetyl-D-glucosamine (PNAG) to disperse the biofilms formed by various bacteria but with no killing activity. Here we have investigated whether a silver-binding peptide (AgBP) fused to DspB can induce the *in situ* formation of silver nanoparticles (AgNP) and conjugated to the structure of DspB so that the bacteria cells released from the dispersed biofilm will be killed by the conjugated AgNP. However, the desired conjugate could be obtained because of the silver ions itself was found to precipitate DspB. But, the fusion of AgBP2 to DspB (AgBP2-DspB) could generate at least 2 fold higher activity against soluble substrate 4-nitrophenyl *N*-acetyl-β-D-glucosaminide (NP-GlcNAc). By applying to a preformed *Staphylococcus epidermidis* biofilm, AgBP2-DspB could clear 69% of the biofilm while only 37% could be cleared by DspB as observed by fluorescent microscope. As measured by crystal violet staining, biofilm could be eradicated to the same extent by loading AgBP2-DspB activity level approximately 20 fold lower than that of DspB. The biofilm formation could be prevented on a AgBP2-DspB immobilized surface as observed by confocal laser microscope.

1. Introduction

Many microorganisms in nature tend to grow on surfaces or interface and develop into biofilms to aid their survival during starvation, desiccation, attack from antimicrobial agents and host immune system [1–3]. Biofilms exist on the surfaces of implanted biomedical devices usually will cause serious infection and inflammation. For example, biofilms of *Staphylococcus epidermidis* and *Escherichia coli* commonly cause intravascular catheter-associated infection, and biofilm of *Staphylococcus aureus* is usually associated with infection caused by metallic implants [4, 5]. In the structure of biofilms, bacterial cells are well-protected by a 3-D extracellular polymeric substance (EPS) matrix composed of proteins, polysaccharides and extracellular DNA secreted by the embedded cells. In the pathogenic *E. coli* and staphylococci biofilms, commonly prevailed on indwelling medical devices, cell-bound polymeric β-1,6-*N*-acetyl-D-glucosamine (PNAG) has been proved to be the polysaccharide required for biofilm formation [6, 7]. Recently, biological methods using enzymes such as proteases, deoxyribonuclease and glycosidases to hydrolyze EPS for disrupting the existing biofilms have gained a lot of attentions [8] because without EPS protection the bacteria cells are easily accessible to antimicrobial agents and efficiently killed.

Dispersin B (DspB), a protein homologous to the catalytic domain of the family 20 β-hexosaminidase, found by Kaplan et al. [9] in *Aggregatibacter actinomycetemcomitans* can hydrolyze the glycosidic linkage of PNAG in the EPS of *S. epidermidis* biofilm. Recombinant DspB containing an N-terminal His-tag has been expressed in *E. coli* and purified to confirm its depolymerization activity against PNAG for biofilm disruption [10]. The enhanced production of recombinant DspB in *E. coli* has also been achieved by engineering dspB to transcribe mRNAs devoid of the trinucleotide ACA [11]. Recently, synthetic gene encoding DspB has been expressed in *E. coli* for the eradication of biofilms preformed by various microorganisms [12]. Instead of using purified recombinant DspB, a genetically engineered *E. coli* strain has also been constructed and used as biofilms disruptor by secreting DspB to hydrolyze the PNAG of biofilms [13]. In addition to eradication of the preformed biofilms, immobilization of DspB on various surfaces has been reported could prevent the surfaces from forming biofilms [14–16]. Due to its very effective biofilm eradication activity, recombinant DspB has been used in combination with silver and peptide antimicrobial agent in wound spray and gel, respectively against chronic wound infection associated bacteria [17, 18].

Silver ions released from silver nanoparticles (AgNP) and AgNP itself have been demonstrated to have broad antimicrobial activity, active against Gram-positive and negative bacteria, and antibiotic resistant bacteria strains [19]. Besides, antimicrobial applications of AgNP have also been realized in many commercial products [20]. Silver binding peptides (AgBP2 and Ag4) screened by phage-display technique have

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a very strong affinity toward silver [21]. AgNP formation can be induced by silver binding peptides (AgBP) when in contact with silver ions [22]. Several proteins have been fused with AgBP and surface immobilized to induce AgNP formation on the surface for antimicrobial coating and antibiofilms [23–25].

DspB has known to be an effective enzyme for the disruption of biofilms by hydrolyzing the PNAG of biofilms. However, it has no antimicrobial activity [10], the embedded bacteria cells released from disrupted EPS matrix are still alive and will reinitiate the formation of biofilm on the surface. To this end, if DspB could be fused with AgBP that AgNP is likely to be generated *in situ* on the structure of DspB. The AgNP-DspB conjugate is expected to not only disrupt biofilm by hydrolyzing PNAG but also kill the released planktonic cells simultaneously by AgNP. In this work, AgBP2 silver binding peptide was fused with dispersin B (DspB) of *Aggregatibacter actinomycetemcomitans* at the N-terminus and expressed in *E. coli*. The purified recombinant AgBP2-DspB fusion protein was studied for its effectiveness on AgNP formation. *Staphylococcus epidermidis*, an opportunistic pathogen involved in numerous nosocomial infections including those related to skin wounds and implanted medical devices, was therefore employed as a model biofilm forming strain to investigate the effect of AgBP2-DspB on eradication of its biofilm. In addition, AgBP2-DspB surface immobilization has also been carried out to protect the surface from forming biofilm of *S. epidermidis*.

2. Materials and methods

2.1. Materials

All materials and chemicals used were analytical grade. DNA fragment of DspB (Gene Bank accession no: AAP31025.1) containing *Nde*I and *Sal*I restriction sites was codon-optimized and synthesized by Yao-Hong Biotechnology Inc. (Taipei, Taiwan). Silver nitrate, 4-nitrophenyl *N*-acetyl-β-D-glucosaminide (NP-GlcNAc), lysozyme and tris(2-carboxyethyl)phosphine (TCEP) were purchased from Sigma-Aldrich. Glutaraldehyde (25 wt% solution in water) was purchased from Acros Organics. Sharp Protein Markers III was obtained from Yeastern Biotech Co., Ltd. (Taipei, Taiwan). Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. IMAC Sepharose™ 6 Fast Flow and Vivaspin 500 ultrafiltration devices with 30 kDa molecular weight cut-off (MWCO) were obtained from Sartorius Ltd.

2.2. Plasmid construction

To construct the DspB expression vector, the DspB gene fragment were inserted into pET-24a after digested with *Nde*I and *Sal*I to generate pET-24a-DspB (Fig. 1a). Silver binding peptides, EQLGVRKELRGV (AgBP2) was fused to the N-terminus of DspB by carrying out 2 sequential PCR. Two forward primers encoding a $(GGGGS)_2$ linker peptide were designed to be followed by AgBP2, respectively. In the 1st PCR, pET-24a-DspB plasmid was used as a template using a forward primer 5′-CGTGGTGTTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTAACTGCTGCG TGAAGGGC-3′ and a reverse primer 5′-GCTTGTCGACCTCA TCGCC-3′. The 1st PCR product was used as template for the 2nd PCR and a forward primer 5′-TATACATATGGAACAGCTGGGTGTTCGTAAAGAACTGCGTG GTGTTGG TGGTGGTG-3′ and a reverse primer 5′-GCTTGTCGACCTCATC GCC-3′ were employed. The AgBP2-DspB gene fragment was inserted into pET-24a after digested with *Nde*I and *Sal*I to generate pET-24a-AgBP2-DspB (Fig. 1b) for the expression of recombinant AgBP2-DspB fusion protein. Both DspB and AgBP2-DspB were designed to have a C-terminal 6xHis-tag for their easy isolation and purification by immobilized metal affinity chromatography (IMAC).

2.3. Expression and purification

For the expression of DspB and AgBP2-DspB in *E. coli* BL21 (DE3), all strains were grown overnight at 37 °C in 3 mL of Luria-Bertani (LB) medium as initial cultures. Two milliliters of initial cultures were added to 200 mL of LB medium supplemented with 50 μg/mL kanamycin in a 1-L flask. The *E. coli* cultures were incubated at 37 °C with shaking speed of 200 rpm until the absorbance at 600 nm reached 0.4–0.6. The target proteins expression was then induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.4 mM with temperature lowered to 30 °C and incubated with shaking for 6 h. The cells were harvested by centrifugation and washed with 20 mM Tris/HCl buffer (pH 7.5). The washed cells were resuspended in the same buffer and disrupted using an ultrasonicator (QSONICA Q700). The cells suspension was ice-bathed and sonicated in short bursts in order to avoid overheating the cells suspension. The obtained cells lysate was centrifuged at 14,000 rpm for 5 min using a Hettich Mikro 200R Centrifuge. The proteins in the supernatant were considered as soluble fraction and used for protein purification.

The purification was performed on a Ni^{2+} column (1.0 mL of IMAC Sepharose chelating column charged with 0.1 M $NiSO_4$). After the crude protein was loaded onto a 1.0 mL of column pre-equilibrated with binding/wash buffer (20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole, pH 7.4), the IMAC Sepharose column was washed 10 times using 1 mL aliquots of binding/wash buffer. The protein was then eluted from the adsorption column using 2 mL of elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4). After purification, the protein sample was dialyzed with Vivaspin 500 ultrafiltration devices against 0.1 M Tris/HCl buffer (pH 7.5).

2.4. Analysis of Dispersin B

The protein concentration was determined by Bradford protein assay (Bio-Rad) using bovine serum albumin as a standard. The assay

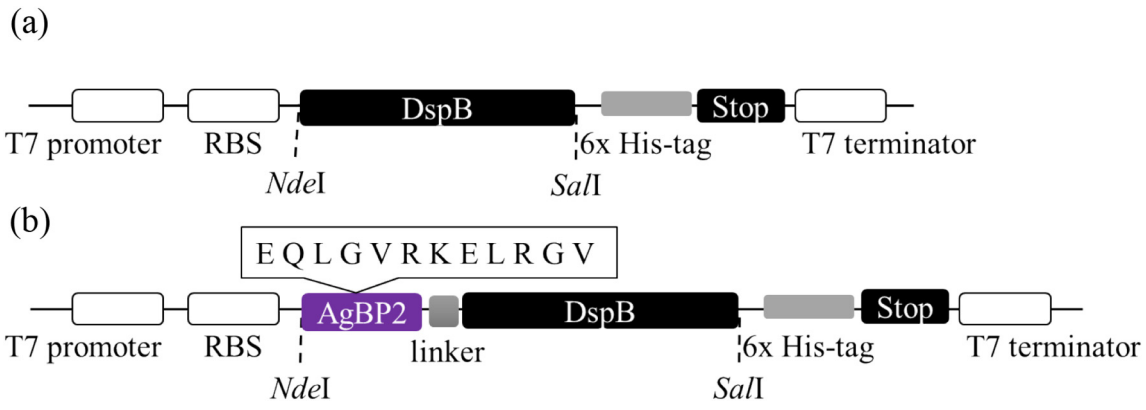

Fig. 1. Plasmid construction for (a) DspB and (b) AgBP2-DspB fusion protein expression in T7 expression system.