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Functional analysis of antibacterial activity of *Bacillus amyloliquefaciens* phage endolysin against Gram-negative bacteria

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Abstract To analyze the antibacterial activity of *Bacillus amyloliquefaciens* phage endolysin, nine deletion derivatives of the endolysin were constructed. Each deletion mutant was overexpressed, purified and characterized. The catalytic domain was located on the N-terminal region and the C-terminus had an affinity with the bacterial envelope. The enzymatic activity remained in spite of the deletion of the C-terminal 116-amino acid region; however, the antibacterial activity was lost. These results indicate that antibacterial action requires both the C-terminal cell-binding and the N-terminal enzymatic activities.

Key words: *Bacillus* phage; Endolysin; Antibacterial activity; Cell-binding activity; Deletion derivative

1. Introduction

Release of the progeny phage, which is considered to be the last phenomenon of bacteriophage infection, is usually carried out by two proteins: a murein-degrading enzyme termed endolysin, essential in hydrolyzing the peptidoglycan, and a tunnel-like protein termed holin, which produces non-specific holes in the cytoplasmic membrane to facilitate the transport of endolysin to the periplasm. Previously, the antibacterial activity of *Bacillus amyloliquefaciens* (IAM1521) phage endolysin was characterized [1,2]. Generally, peptidoglycan-degrading enzymes are active against Gram-positive bacteria, but are limited in activity against Gram-negative bacteria, which have an outer membrane, a permeability barrier that inhibits the access of the enzymes to the peptidoglycan layer. However, the viability of the Gram-negative bacterium *Pseudomonas aeruginosa* PAO1, which is resistant to most available antibiotics and causes opportunistic infections, was drastically decreased by addition of endolysin. In addition, intracellular production of the endolysin led to cell lysis of *Escherichia coli* without holin conjugation. A relatively high homology was found between the N-terminal region of the endolysin and the entire region of T4 phage lysozyme consisting of 164 amino acids (aa), which is the catalytic domain of the endolysin probably located in the N-terminal region. The C-terminal region of the endolysin has two sequence repeats of 43 aa, which was also found in the lysis protein of *Bacillus subtilis* phage and lactococcal phage [3,4]. These repeats may be responsible for substrate binding, or allow the catalytic domain to reach its substrate [5].

In this study, to elucidate the function of the antibacterial action of the *B. amyloliquefaciens* phage endolysin against *P. aeruginosa* PAO1, its deletion derivatives were constructed and their enzymatic and cell-binding activities that are related to the antibacterial activity were analyzed.

2. Materials and methods

2.1. Construction of plasmids

To obtain the genes encoding the deletion derivatives, polymerase chain reaction (PCR) analysis was performed using *Pfu* DNA polymerase (Stratagene). Each amplified gene and the primer pair, constructed based on a DNA sequence alignment of *B. amyloliquefaciens* phage endolysin (DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AY030242), are shown in Fig. 1 and Table 1. For immunoblotting detection, the FLAG peptide, consisting of the following amino acids: DYKDDDDK, was fused to the C-terminus of the recombinant proteins. The gene with a nucleotide sequence of 5′-gactacaaggatgacgatgacaag-3′ that encoded the FLAG peptide was inserted into the region downstream of the *Xho*I site of pET21d(+) (Novagen). Each PCR fragment was replaced with a fragment corresponding to the *Nco*I–*Xho*I site of pET21d(+) harboring the FLAG sequence, and the resultant plasmids were used for the transformation of *E. coli* JM109(DE3).

2.2. Overexpression and purification of deletion mutants

The histidine hexamer (6-His)-tagged deletion derivatives of *B. amyloliquefaciens* phage endolysin were produced in *E. coli* and purified as follows: the transformed *E. coli* JM109(DE3) was grown in the M9G medium containing per liter: Na_2HPO_4, 7 g; KH_2PO_4, 3 g; NaCl, 5 g; NH_4Cl, 1 g; $CaCl_2{\cdot}2H_2O$, 0.015 g; $MgSO_4$, 0.25 g; glucose, 4 g; casamino acid, 10 g; and thiamine, 0.002 g, supplemented with 50 μg/ml of ampicillin, at 37°C overnight. The overnight culture of the transformed *E. coli* was diluted 100 times with the same medium and incubated at 37°C with shaking (150 rpm) until the optical density of the medium at $OD_{600\,nm}$ reached 1.0. Expression of the target gene was induced by addition of isopropyl-β-D-thiogalactopyranoside at a final concentration of 1 mM. After further incubation for 2 h, cells were harvested by centrifugation. The cell pellets were resuspended in 1:20 volume of a buffer containing 20 mM phosphate, 0.5 M NaCl, and 10 mM imidazole, then cell disruption was performed by ultrasonication. After centrifugation at 12 000×*g* for 20 min, the supernatant was filtered (0.45 μm pore size; Millipore) and loaded onto a pre-equilibrated HiTrap™ (Amersham Pharmacia Biotech).

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Abbreviations: aa, amino acid; CEWL, chicken egg white lysozyme; PBS, phosphate-buffered saline

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2.3. Lysozyme assay

Activities of the purified 6-His-tagged proteins towards lyophilized *Micrococcus luteus* cells (0.2 mg/ml, Sigma) were measured [6]. The activity of chicken egg white lysozyme (CEWL, Wako) was also measured in the same manner. The activity was determined from the first-minute linear decrease in absorbance at 450 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyzes a decrease in absorbance of 0.001 min^{-1}.

2.4. Antibacterial assay against P. aeruginosa

In vitro antibacterial assay was performed for each purified deletion mutant and CEWL. The concentration of each mutant was determined by the Lowry method [7] using bovine serum albumin as standard. *P. aeruginosa* PAO1 was grown in Luria–Bertani (LB) medium. An overnight bacterial culture (25 μl) was mixed with enzyme diluted in phosphate-buffered saline (PBS) (enzyme concentration, 80 μg/ml) as needed to make a final volume of 50 μl (final concentration, 40 μg/ml). The experiment of the control condition was performed with PBS. The mixture was incubated in an Eppendorf tube at 37°C for 10 min. Then, the mixture was diluted with PBS and overlaid on a LB agar plate. Antibacterial activity was defined as the common logarithm of the ratio of the number of colony-forming units under the control condition (N_0) to that under the test condition (N_1). The equation of antibacterial activity is expressed as log (N_0/N_1). The assays were conducted in triplicates.

2.5. Assay of cell binding to P. aeruginosa

An overnight culture of *P. aeruginosa* PAO1 (150 μl) was mixed with the protein solution at 100 μg/ml (150 μl) in the Eppendorf tube, and incubated at 4°C for 10 min. 100 μl of this mixture was collected as the whole-cell sample, and the remaining solution was centrifuged at 12 000×*g* for 5 min. After removal of the supernatant, the cell pellet was resuspended in 20 μl PBS. The whole-cell samples and the cell pellet were subjected to ultrasonication. The three resultant samples (whole-cell, supernatant and cell pellet) were run on an SDS–PAGE gel and electroblotted onto an Immobilon-P transfer membrane (Millipore). An anti-FLAG M2 antibody (Sigma) was used as the primary antibody; an anti-mouse Ig, horseradish peroxidase-linked whole antibody (Amersham Pharmacia Biotech) was used as the secondary antibody. An ELC Western blotting detection reagent (Amersham Pharmacia Biotech) was used for light-emitting detection of FLAG-containing proteins.

Table 1
Oligonucleotide primers for PCR

Primer designation	Nucleotide sequence
DEL+0	5′-CATgCCATggCCATgCAACTTTCACAAgCAggC-3′
DEL−0	5′-CCgCTCgAggCTTAgTCTAATTgTTTgACC-3′
DEL+1	5′-CATgCCATggCCCAAgTTATCACTgCTAAACA-3′
DEL−1	5′-CCgCTCgAggCTACCCgTAACATTAATTgT-3′
DEL+2	5′-CATgCCATggCCTTTAATAgTggAACACCTAAA-3′
DEL−2	5′-CCgCTCgAgCAAggCTTgCTCTTgTgC-3′
DEL+3	5′-CATgCCATgggCggCAAAACACATAAg-3′
DEL−3	5′-CCgCTCgAgTCTAAgTgAAACATCAgAACC-3′

3. Results

3.1. Purification and enzymatic activity of deletion mutants

According to the predicted hydrophobic/hydrophilic property of the endolysin [8], 10 deletion derivatives were constructed and named D1–D10 (Fig. 1). Each deletion mutant was purified using the HiTrap™ except for D6 and D7, which could not be purified because of their insolubility. The purified proteins were assayed for their enzymatic activity (Table 2). D1, D2 and D3 had lysozyme activities, with D2 having the highest activity ($1.2 \pm 0.3 \times 10^5$ U/mg). The activity of D2 was almost the same as that of the purchased CEWL ($1.6 \pm 0.01 \times 10^5$ U/mg). However, the activity of D1, the complete endolysin, was $2.3 \pm 0.1 \times 10^4$ U/mg, about one fifth of the D2 activity. The increase in the lysozyme activity as a result of the deletion remains to be elucidated. On the other hand, the activity of D3 was $2.4 \pm 0.1 \times 10^2$ U/mg, which was equivalent to 1/100 of the D1 activity. No activity was detected in the other deletion mutants. From the results mentioned above, the N-terminal 142-aa region is needed for the enzymatic activity and the catalytic domain is located in this region.

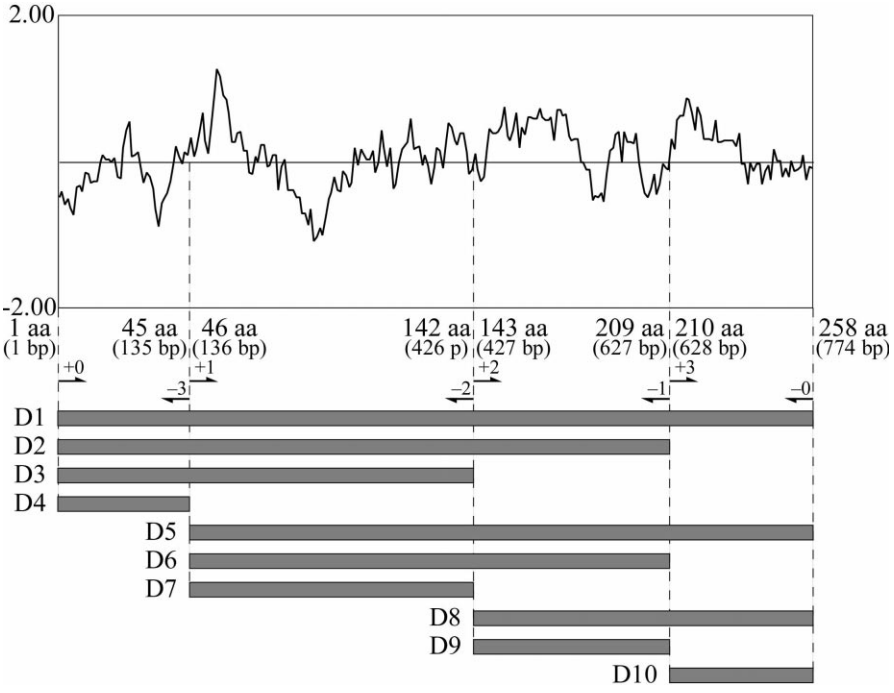

Fig. 1. Hydrophilic (+)/hydrophobic (−) profile of the endolysin and construction of deletion derivatives. Arrows indicate the oligonucleotide primers.

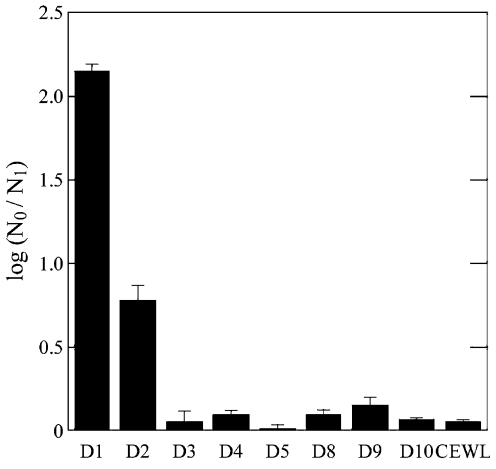

Fig. 2. Antibacterial activity of each deletion derivative and CEWL against *P. aeruginosa* PAO1. The activity was evaluated by the common logarithm of the ratio of the number of colony-forming units under the control condition (N_0) to that under the test condition (N_1).

3.2. Antibacterial activity of deletion mutants

In the previous study, the antibacterial spectrum of endolysin was analyzed [2]. Since endolysin showed strong antibacterial action against *P. aeruginosa* PAO1, a Gram-negative bacterium, *P. aeruginosa* PAO1 was used for the antibacterial assay in this study (Fig. 2). Each deletion mutant was added to the bacterial culture and their activity was measured. The viability of *P. aeruginosa* PAO1 decreased to less than 1% following the D1 addition, thus the antibacterial activity was more than 2. While D2 showed a higher enzymatic activity than D1 did, the antibacterial action of D2 was less effective. The deletion of the C-terminal 49-aa region reduced the antibacterial activity. In addition, all the other deletion mutants, including D3, with lysozyme activity did not exhibit significant activity. Therefore, the N-terminus of endolysin is responsible for the antibacterial action against Gram-negative bacteria.

3.3. Cell-binding activity of deletion mutants

The microscopic observation revealed that rod-shaped bacteria became spheroid after the treatment with the endolysin (data not shown). Thus, the endolysin may permeate the bacterial outer membrane and degrade the peptidoglycan layer, which is responsible for maintaining the cell structure. To determine the domain that has an affinity with the bacterial surface, the cell-binding ability of deletion mutants was investigated. After electrophoresis, proteins were transferred onto a polyvinylidene fluoride membrane and subjected to immunoblotting (Fig. 3). In the D5 and D8 samples, degradation of the FLAG peptide fusion protein was observed and the degradation products were detected as low-molecular-weight protein bands. All the deletion derivatives except D4 were detected in the cell pellet sample; D4 had no affinity with the cell surface of *P. aeruginosa* PAO1. In the D1, D2 and D5 samples, the band of the cell pellet was thicker than that of the supernatant, so that most of proteins were bound to the cell surface of *P. aeruginosa* PAO1. However, the other samples (D3, D8, D9 and D10) showed a lower affinity, because a fraction of the cell pellet had a small amount of the deletion mutant. These results indicate that the affinity with the bacteria increased with the increase in the number of C-terminal peptide residues.

Table 2
Enzymatic activity of deletion derivatives

Protein	Enzymatic activity (U/mg)
D1	$2.3 \pm 0.1 \times 10^4$
D2	$1.2 \pm 0.3 \times 10^5$
D3	$2.4 \pm 0.1 \times 10^2$
D4	N.D.
D5	N.D.
D8	N.D.
D9	N.D.
D10	N.D.
CEWL	$1.6 \pm 0.01 \times 10^5$

N.D.: not detected.

4. Discussion

Lysozyme is an antibacterial enzyme that hydrolyzes the β-1,4-glycosidic linkage between *N*-acetylglucosamine and muramic acid of the peptidoglycan in the bacterial cell wall. However, the antibacterial action of lysozyme is limited only to some Gram-positive bacteria. Gram-negative bacteria are resistant to lysozyme due to the effective permeability barrier function of their outer membrane. The outer membrane is impermeable to macromolecules and allows only limited diffusion of hydrophobic substances through its lipopolysaccharide-covered surface [9]. Enhancement of lysozyme penetration through the outer membrane was attempted using permeabilizers such as chelating agents [10] and the attach-

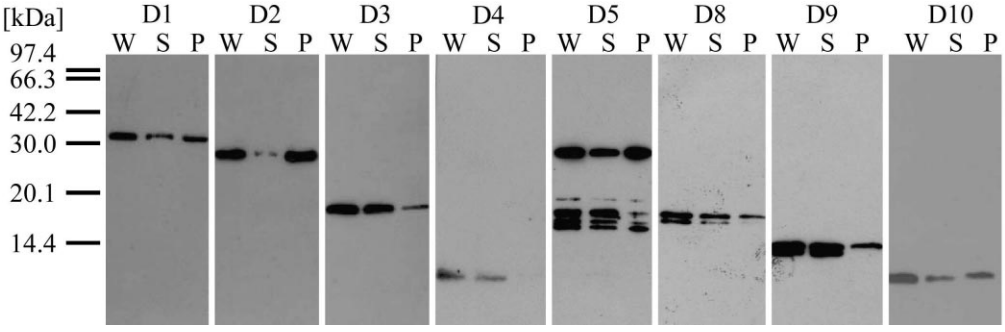

Fig. 3. Analysis of the cell-binding affinity of deletion derivatives. Lanes in each compartment are as follows: W: whole-cell, S: supernatant and P: cell pellet. The molecular mass standard is shown on the left.

ment of hydrophobic peptides to lysozyme [11–13]. It was found that helix-forming amphipathic peptides containing basic amino acid residues seem to interact with negatively charged membrane elements, i.e. lipopolysaccharide in Gram-negative bacteria [14]. Based on the predicted secondary structure [15], there are two helical peptides in the C-terminus of endolysin: one exists in the D9 region (aa 171–177) and the other in the D10 region (aa 212–216). These peptides may bind to the lipopolysaccharide of *P. aeruginosa* PAO1. Moreover, it is suggested that the C-terminal hydrophobic region of D3 penetrates into the outer membrane since D1, D2 and D5, which embed two helical peptides containing several basic amino acids and the hydrophobic region, show a high affinity with the *P. aeruginosa* PAO1 exogenously. However, the antibacterial activity was observed only in D1 and D2. Since the enzymatic activity of D5 was completely lost, the antibacterial action may be performed by the combination of the enzymatic activity and cell-binding ability. For example, D3 had a low cell-binding affinity and low enzymatic activity; thus, D3 did not act as an antibacterial agent. The mechanism of antibacterial function of endolysin seems to be as follows: the C-terminal region interacts or penetrates the cell envelope; subsequently, the N-terminus of endolysin, which may harbor the catalytic domain, approaches the peptidoglycan layer causing the lysis of the bacterial cell, and the conformation of the intact endolysin might be necessary for the effective function.

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