RESEARCH LETTER

Characterization of an endolysin, LysBPS13, from a *Bacillus cereus* bacteriophage

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Abstract

Use of bacteriophages as biocontrol agents is a promising tool for controlling pathogenic bacteria including antibiotic-resistant bacteria. Not only bacteriophages but also endolysins, the peptidoglycan hydrolyzing enzymes encoded by bacteriophages, have high potential for applications as biocontrol agents against food-borne pathogens. In this study, a putative endolysin gene was identified in the genome of the bacteriophage BPS13, which infects *Bacillus cereus*. *In silico* analysis of this endolysin, designated LysBPS13, showed that it consists of an N-terminal catalytic domain (PGRP domain) and a C-terminal cell wall binding domain (SH3_5 domain). Further characterization of the purified LysBPS13 revealed that this endolysin is an *N*-acetylmuramyl-L-alanine amidase, the activity of which was not influenced by addition of EDTA. In addition, LysBPS13 demonstrated remarkable thermostability in the presence of glycerol, and it retained its lytic activity even after incubation at 100 °C for 30 min. Taken together, these results indicate that LysBPS13 can be considered a favorable candidate for a new antimicrobial agent to control *B. cereus*.

Introduction

Bacteriophages are viruses that invade bacterial cells. They are ubiquitous, obligate parasites that are highly specific to their hosts (Hermoso *et al.*, 2007). Because of their bactericidal activity, phages have been proposed as natural antimicrobial agents to prevent bacterial infection (Hanlon, 2007; Hermoso *et al.*, 2007). In addition to bacteriophages, endolysins have been successfully applied as alternative antimicrobial agents (Fischetti, 2005, 2008, 2010; Obeso *et al.*, 2008). Endolysins are phage-encoded enzymes that break down bacterial peptidoglycan at the terminal stage of the phage reproduction cycle (Fischetti, 2005; Borysowski *et al.*, 2006). Depending on their enzymatic specificity, endolysins are categorized into four classes: (1) *N*-acetylmuramidases (lysozymes or muramidases), which cleave 1,4-beta-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues; (2) endo-β-*N*-acetylglucosaminidases (glucosaminidases), which cleave the sugar moiety of peptidoglycan; (3) *N*-acetylmuramyl-L-alanine amidases (NAM-amidases), which cut the amide bond between *N*-acetylmuramic acid and L-alanine; and (4) endopeptidases, which cleave the peptide moiety (Loessner, 2005; Borysowski *et al.*, 2006). Endolysins are candidates for effective antibacterial agents, because they can be exogenously applied to lyse Gram-positive bacteria, they do not develop bacterial resistance, and they have a highly specific host range without disturbing the natural microbial communities of the host (Borysowski *et al.*, 2006).

Bacillus cereus is a Gram-positive spore-forming bacterium that can cause systemic and local infections (Bottone, 2010). It is widely distributed in the environment, mostly in soil from which it is easily spread to many types of foods, especially those of vegetable origin, as well as meat, eggs, milk, and dairy products. *Bacillus cereus* is one of the leading causes of food poisoning in the industrialized world, causing gastrointestinal disorders (Ceuppens *et al.*, 2011). However, eliminating or controlling *B. cereus* in foods is impractical, so preventing germination and multiplication of large bacterial populations has been suggested (Granum & Lund, 1997).

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In a previous study, the bacteriophage BPS13, a lytic phage that targets *B. cereus*, was isolated from food sewage (Shin *et al.* unpublished). BPS13 belongs to the *Myoviridae* family, and genomic DNA analysis (accession no. JN654439) revealed a 158 305 base pair (bp), double-stranded DNA genome with 282 open reading frames (ORFs). In this study, we identified a putative endolysin gene, *lysBPS13*, from the genome of the bacteriophage BPS13, and purified recombinant endolysin was characterized for its biochemical properties. LysBPS13 showed remarkably high thermostability in the presence of glycerol, suggesting that it can be used in industry to control *B. cereus*.

Materials and methods

Bacterial strains, bacteriophage, and growth conditions

Bacillus cereus ATCC 10876 was used as the host of the bacteriophage, BPS13 (Shin *et al.* unpublished), as well as the target for evaluation of the lytic activity of the recombinant endolysin protein. *Escherichia coli* BL21 Star^{TM} (DE3) (Invitrogen) was used as the host for expression of the recombinant endolysin protein. All the bacteria were cultivated in Luria-Bertani broth or agar at 37 °C.

DNA manipulation, overexpression, and purification of the recombinant endolysin

The genomic DNA of the bacteriophage BPS13 was prepared by phenol extraction (Manfioletti & Schneider, 1988). The 834-bp-long putative endolysin gene was amplified using the following primers: BPS13ORF194_F (5′-GATGATTCACAT**ATG**AATATCAATACA-3′) and BPS13ORF194_R (5′-AACCCCGAAGGATCCTCTTAAT-3′). The resultant polymerase chain reaction (PCR) product was digested with NdeI and BamHI, followed by ligation into the expression vector pET15b (Novagen, Germany) containing a His-Tag at the N-terminus. Plasmid-expressing *E. coli* BL21 Star^{TM} (DE3) cells were grown until the optical density at 600 nm ($OD_{600\ nm}$) reached 0.5. Then, 1 mM isopropyl-β-D-thio-galactoside (IPTG) was added, followed by further incubation for 5 h at 30 °C. Cells were harvested, resuspended in lysis buffer (20 mM Tris–Cl, pH 8.0, and 300 mM NaCl), and lysed by sonication (Branson Ultrasonics). After centrifugation at 15 000 ***g*** for 15 min, the supernatant was added to Ni-NTA Superflow resin (Qiagen, Germany) and gently mixed in a column for 1 h at 4 °C. The resin was washed with lysis buffer four times and eluted with elution buffer (20 mM Tris–Cl, pH 8.0, 300 mM NaCl, and 170 mM imidazole). The buffer was changed to storage buffer [20 mM Tris–Cl, pH 8.0, 300 mM NaCl, and 30% (v/v) glycerol] by dialysis, and the purified protein was stored at −80 °C until use.

Lytic activity assay

The lytic activity of the endolysin was determined by measuring decreases in the optical density of the cell suspension after the addition of endolysin. Bacterial cells were grown to the exponential phase, harvested, washed twice, and resuspended in 50 mM glycine (pH 9.5) to adjust the $OD_{600\,nm}$ = 0.8–1.0, as described previously (Loessner *et al.*, 1997). To test the lysis of Gram-negative bacteria, harvested cells were incubated with 0.1 M EDTA for 5 min prior to the washing and resuspension steps. The endolysin solution (100 μL) was added to 900 μL of cell suspension. In control samples, one hundred microliter of resuspension buffer was added instead of the endolysin solution. Unless indicated otherwise, 5 μg of LysBPS13 was added per 1 mL reaction. The $OD_{600\,nm}$ was measured after incubation at room temperature for 5 min, and the lytic activity was calculated using the following equation: 100 × ($OD_{600\,nm}$ of control without enzyme − $OD_{600\,nm}$ of reaction mixture)/$OD_{600\,nm}$ of control without enzyme.

When determining the optimal pH for endolysin activity, the following buffers were used for cell suspension instead of the glycine buffer: 0.1% (w/v) Trifluoroacetic acid (TFA) for pH 2.0; 50 mM sodium acetate for pH 4.0 and 5.0; 50 mM MES for pH 6.0; 50 mM potassium phosphate for pH 7.0; 50 mM Tris–Cl for pH 7.5, 8.0, and 8.5; 50 mM glycine for pH 9.0 and 9.5; and 50 mM CAPS for pH 10.0 and 10.5. Different temperatures (4–55 °C) were applied to test the effect of temperature on the enzymatic activity of 0.1 μg LysBPS13. When necessary, EDTA (300 mM), NaCl (0–300 mM), or detergents (0.1%) were added.

N-acetylmuramyl-L-alanine amidase assay and glycosidase assay

Peptidoglycan was prepared from *B. cereus* ATCC 10876, as described previously (Kuroda & Sekiguchi, 1990), and incubated with 5 μg LysBPS13 at 25 °C for 30 min. *N*-acetylmuramyl-L-alanine amidase activity was measured as described previously (Hadzija, 1974; Hazenberg & de Visser, 1992). Briefly, muramic acid was degraded to lactic acid by *N*-acetylmuramyl-L-alanine amidases, and the lactic acid product was degraded to acetaldehyde, which was determined colorimetrically with *p*-hydroxydiphenyl (PHD). Muramic acid was used as the standard. Glycosidase activity was assayed by quantifying the released reducing sugars from the extracted peptidoglycan, according to Pritchard *et al.* (2004).

Results and discussion

Identification of the phage lysin, LysBPS13

A putative endolysin gene was identified in the genome of the bacteriophage BPS13, which infects *B. cereus* (H Shin, J Park, and S Ryu, unpublished data). According to BLASTP analysis (Marchler-Bauer *et al.*, 2011), an 834-bp-long ORF (locus tag 0008) showed high similarity to the *N*-acetylmuramyl-L-alanine amidase of *Bacillus* phage TP21-L (CAA72267.1, E-value = 2×10^{-110}) and other amidases of *Bacillus* strains and *Bacillus*-infecting bacteriophages (ZP_03236042, E-value = 2×10^{-76}; YP_002154393, E-value = 6×10^{-74}). However, this ORF, termed *lysBPS13*, was not similar to the well-characterized *N*-acetylmuramyl-L-alanine amidases, such as PlyCA (AAP42310.2), Ply511 (CAA59368.1), T7 lysozyme (AAB32819.1), and PlyL (YP_002868169.1).

Searching for conserved domains in the Conserved Domain Database (Marchler-Bauer *et al.*, 2011) revealed that LysBPS13 consisted of an N-terminal catalytic domain and a C-terminal cell wall binding domain, similar to most endolysins from bacteriophages that infect Gram-positive bacteria (Fischetti, 2008) (Fig. 1a). The predicted N-terminal catalytic domain was the peptidoglycan recognition protein (PGRP; cd06583, E-value = 2.19×10^{-19}). As a subset of the PGRP family binds zinc (Zn^{2+}), which is coordinated by two His residues and a Cys or Asp residue (Cheng *et al.*, 1994; Dziarski & Gupta, 2006), LysBPS13 was found to contain the conserved motif of three zinc-binding residues (His29, His129, and Cys137) (Fig. 1a). This N-terminal catalytic domain was found in many *N*-acetylmuramyl-L-alanine amidases of *Bacillus* phages or *Bacillus* species and even in the genomes of many vertebrates (Dziarski & Gupta, 2006). In mammals, some PGRPs belong to *N*-acetylmuramyl-L-alanine amidases, which are involved in reducing proinflammatory acidity or in killing bacteria (Dziarski, 2004; Vollmer *et al.*, 2008). Among endolysins, PGRP domains correspond to catalytic domains of amidases such as Ply21 and mycobacteriophage Ms6 LysA (Loessner *et al.*, 1997; Catalao *et al.*, 2011). However, the PGRP domain was not well characterized with regard to peptidoglycan degradation, unlike the CHAP domain (PF05257) of other *N*-acetylmuramyl-L-alanine amidases such as PlyC and LytA (P24556) (Bateman & Rawlings, 2003; Nelson *et al.*, 2006).

The putative cell wall binding domain at the C-terminus of LysBPS13 was of the SH3_5 superfamily (pfam08460, E-value = 1.67×10^{-15}) (Fig. 1a). This C-terminal domain was also found in the protein of *B. cereus* AH676 (ZP 0419059), the *Bacillus* phages TP21-L (Ply21, CAA72267) and bg1 (LysBG1, ABX56141), and the *Lactobacillus* phage LL-Ku (AAV30211). However, this domain was not fully characterized.

Recombinant LysBPS13 was cloned and expressed in *E. coli* and purified. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed a single band of the purified endolysin (Fig. 1b). As expected, the purified, recombinant LysBPS13 showed lytic activity

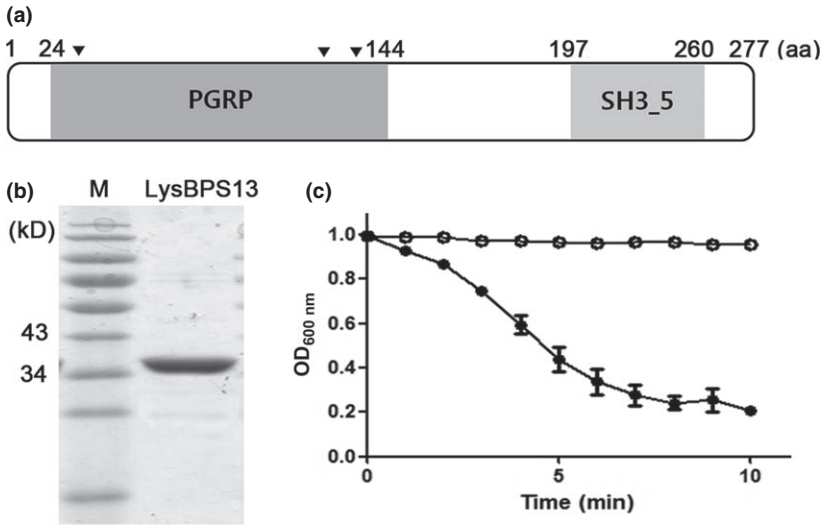

Fig. 1. Recombinant LysBPS13 demonstrates high lytic activity against *Bacillus cereus*. (a) LysBPS13 consists of a PGRP domain and an SH3_5 superfamily domain. The predicted Zn^{2+}-binding residues in the PGRP are indicated by inverted triangles. (b) SDS-PAGE analysis of the purified, recombinant LysBPS13. (c) Lysis of *B. cereus* ATCC 10876 by the purified LysBPS13 protein. *Bacillus cereus* ATCC 10876 was treated with 5 μg of LysBPS13 (closed circles) or the storage buffer only (open circles). Data are presented as the mean ± standard deviation of triplicate assays.

against *B. cereus* cells. As little as 5 μg mL^{-1} of LysBPS13 effectively lysed *B. cereus* cells within 10 min (Fig. 1c). Viable cell counting showed that there was approximately 3-log reduction by 5 μg of LysBPS13 under this reaction condition after 5 min (data not shown).

N-acetylmuramyl-L-alanine amidase activity of LysBPS13

Because BLASTP analyses showed that LysBPS13 had high similarity to a number of *N*-acetylmuramyl-L-alanine amidases, the amidase activity of LysBPS13 was evaluated by measuring free *N*-acetylmuramic acid liberated from peptidoglycan (Hadzija, 1974). When peptidoglycan of *B. cereus* was treated with LysBPS13 for 30 min, a significant increase in free muramic acid was detected resulting from cleavage of the bond between *N*-acetylmuramic acid and L-alanine (Fig. 2). This demonstrates that LysBPS13 has *N*-acetylmuramyl-L-alanine amidase activity. Because the glycosidase assay revealed that free reducing sugars were not generated from peptidoglycan after LysBPS13 treatment, this enzyme is not a glucosaminidase or a muramidase.

Antimicrobial spectrum of LysBPS13

Four genera of Gram-positive bacteria, including *Bacillus* sp., and five genera of Gram-negative bacteria were examined for their susceptibility to LysBPS13 (Fig. 3). LysBPS13 exhibited the strongest activity against *B. cereus* ATCC 10876, and it could lyse all of the tested *Bacillus* species, including pathogenic *B. cereus* and *Bacillus thuringiensis*. However, other tested Gram-positive bacteria, such as *Listeria monocytogenes*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*, were not lysed by LysBPS13. Among the tested Gram-negative bacteria, LysBPS13 was active against *Salmonella*, *E. coli, Cronobacter sakazakii*, and *Shigella* strains, when these bacteria were treated with EDTA (data not shown). The relative lytic activity against these bacteria was as strong as it was against Gram-positive bacteria (74 ~84%). However, the endolysin did not show lytic activity against these Gram-negative bacteria in the absence of EDTA treatment.

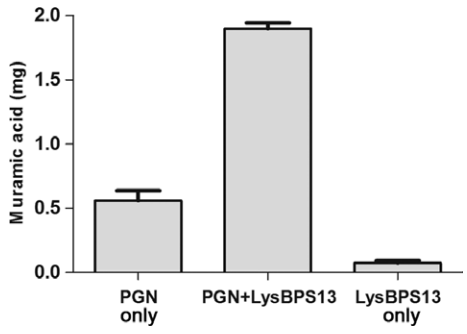

Fig. 2. LysBPS13 has *N*-acetylmuramyl-L-alanine amidase activity. Petidoglycan extracted from *Bacillus cereus* ATCC 10876 ($OD_{550\,nm}$ = 5) was incubated with or without 5 μg of purified LysBPS13. The level of released muramic acid was measured after a 30-min incubation. Each column represents the mean ± standard deviation of triplicate assays.

Biochemical properties of LysBPS13

Effects of pH, temperature, and ionic strength

To determine the optimal conditions for LysBPS13 function, the exogenous lytic activity of LysBPS13 was examined under different conditions. Lytic activity was highest at pH 9.5 and significantly decreased at pH > 10.5 and < 7.5 (Fig. 4a). The optimal temperature for lytic activity was 42–45 °C (Fig. 4b).

The effect of ionic strength on the lytic activity of LysBPS13 was assessed with different concentrations of NaCl (Fig. 4c). The highest lytic activity was observed in the presences of 250 mM NaCl, similar to that seen with many other lysins (Vasala *et al.*, 1995; Loeffler *et al.*, 2003; Schmelcher *et al.*, 2012). This may be an advantage of this endolysin, as these ionic conditions correspond to the salt concentration of many food products.

Effects of EDTA and detergents

LysBPS13 seems to need no metal ions for its lytic activity, because the addition of EDTA (300 mM) did not affect its lytic activity (Fig. 4d), nor did the presence of metal ions (1 mM $MgCl_2$, $CaCl_2$, $ZnCl_2$, or $MnCl_2$) (data not shown). This result was unexpected because the three Zn^{2+}-binding residues in the PGRP domain were completely conserved in LysBPS13. While T7 lysozyme that belongs to the PGRP family has Zn^{2+}-dependent amidase activity (Gelius *et al.*, 2003; Kim *et al.*, 2003), another report found a Zn^{2+}-independent amidase (ORF9) in the *E. faecalis* bacteriophage EF24C (Uchiyama *et al.*, 2011). Like LysBPS13, *E. faecalis* ORF9 has a PGRP domain at its N-terminus, and BLASTP analysis indicated Zn^{2+}-binding sites, but Zn^{2+} did not seem to be essential for its activity. Yet, we cannot rule out the possibility that Zn^{2+} or other metal cofactors are bound to LysBPS13 too tightly to be removed by EDTA. Therefore, further study is necessary to elucidate the structure of the PGRP domain in endolysins, particularly the Zn^{2+}-binding site.

When LysBPS13 was tested in combination with various detergents (Fig. 4d), LysBPS13 showed full or higher activity in the presence of zwitterionic (CHAPS) and

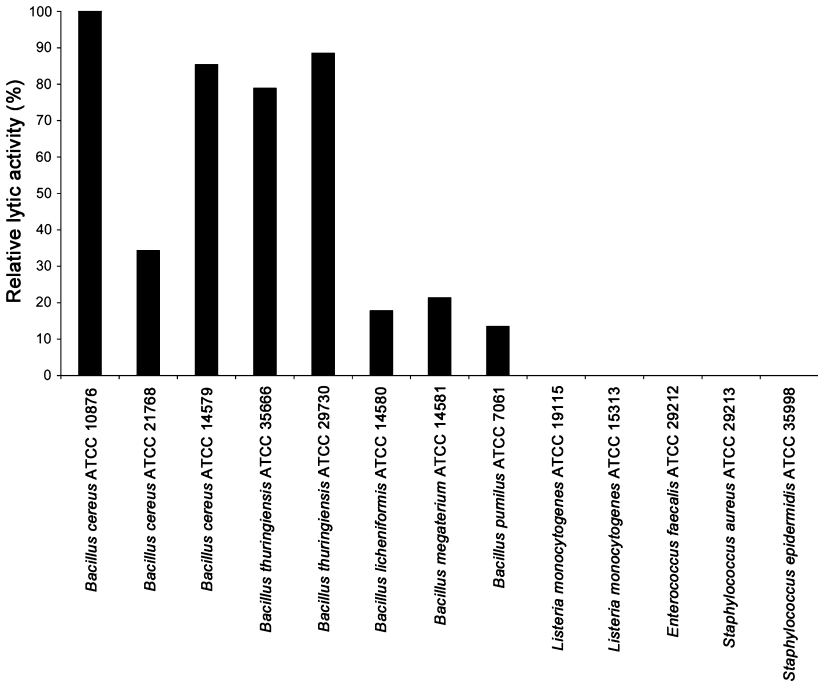

Fig. 3. Antibacterial spectrum of LysBPS13. Five microliter of LysBPS13 was added to 1 mL of reaction mixture of each bacterial strain. The relative lytic activity (%) was defined as the lytic activity against each strain divided by the lytic activity against the reference strain, *Bacillus cereus* ATCC 10876.

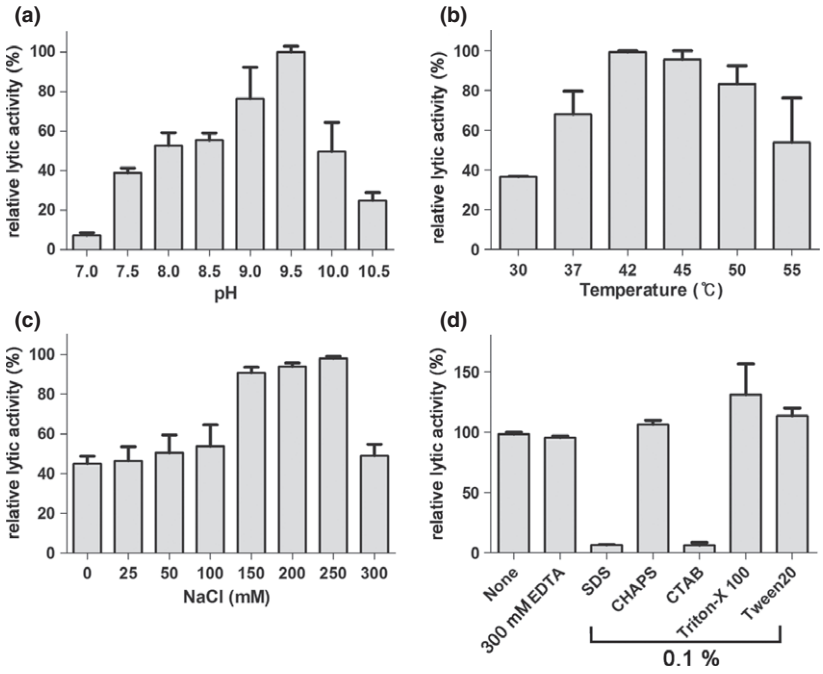

Fig. 4. Optimal conditions for the lytic activity of LysBPS13. (a) 5 μg, (b) 100 ng, (c) 2.5 μg, or (d) 5 μg LysBPS13 was added to *Bacillus cereus* ATCC 10876 cells at various pH, temperatures, concentrations of NaCl, and in the presence of various additives. Each column indicates the mean ± standard deviation of triplicate assays.

nonionic detergents (Triton X-100, Tween-20). However, both anionic (SDS) and cationic (CTAB) detergents inactivated LysBPS13.

The effect of glycerol on LysBPS13 thermostability

Thermostability of phage endolysins would be advantageous for applications as biocontrol agents that undergo heat treatment. *B. cereus* food poisoning is often associated with cooked rice products, because *B. cereus* spores are able to endure high temperatures and germinate when cooling down (Stenfors Arnesen *et al.*, 2008). Most endolysins are labile to heat (Lavigne *et al.*, 2004). However, to date, only a few lysins have been reported to be thermostable, including Gp36 from the *Pseudomonas aeruginosa* bacteriophage ϕKMV (Lavigne *et al.*, 2004); the lysins HPL118, HPL511,

and HPLP35 from *Listeria* bacteriophages (Schmelcher *et al.*, 2012); and the GVE2 lysin (EF079891) from *Geobacilllus* phage GVE2 (Ye & Zhang, 2008). Gp36 has extremely high thermostability, retaining 21% of its activity after autoclaving at 121 °C for 20 min; other lysins have milder thermostability (Lavigne *et al.*, 2004; Ye & Zhang, 2008).

LysBPS13 appeared to be highly stable, as the protein retained full lytic activity after a week-long incubation in storage buffer at room temperature. The thermostability of LysBPS13 was further assessed after pre-incubation of the enzyme at temperatures between 4 and 100 °C (Fig. 5). LysBPS13 demonstrated lytic activity after incubation for 30 min at all tested temperatures. Between 4 and 70 °C, the lytic activity of LysBPS13 did not decrease, compared with the non-heat-treated control (Fig. 5a). The lytic activity of the endolysin was not completely inactivated despite incubating at 100 °C for 30 min, with > 15% of its activity remaining compared with the non-heat-treated control (Fig. 5b). In contrast, autoclaving for 15 min at 121 °C completely inactivated LysBPS13. Taken together, these results indicate that LysBPS13 has exceptionally high thermostability.

We found that the high thermostability of LysBPS13 was dependent on the presence of glycerol in the storage buffer. Without glycerol, LysBPS13 still had higher thermostability than similar endolysins, such as T7 lysozyme, which is inactivated after a 5-min incubation at 50 °C (Kleppe *et al.*, 1977). However, addition of glycerol up to 30% (v/v) enhanced the thermostability of LysBPS13 dramatically (Fig. 5c). It has been reported that polyols, such as glycerol, preferentially hydrate protein molecules and, consequently, stabilize the native structure against thermal denaturation (Paciaroni *et al.*, 2002; Spinozzi *et al.*, 2008; Esposito *et al.*, 2009), but the effect of glycerol on thermostability is not universal to all enzymes. In the case of LysB4, another endolysin from a *B. cereus* bacteriophage, glycerol did not affect its thermostability at all (Son *et al.* 2012). Moreover, 30% glycerol did not influence the lytic activity of LysBPS13 (data not shown). Therefore, the ability of glycerol to support the high thermostability of LysBPS13 is an asset for its use in molecular biology as well as in industry.

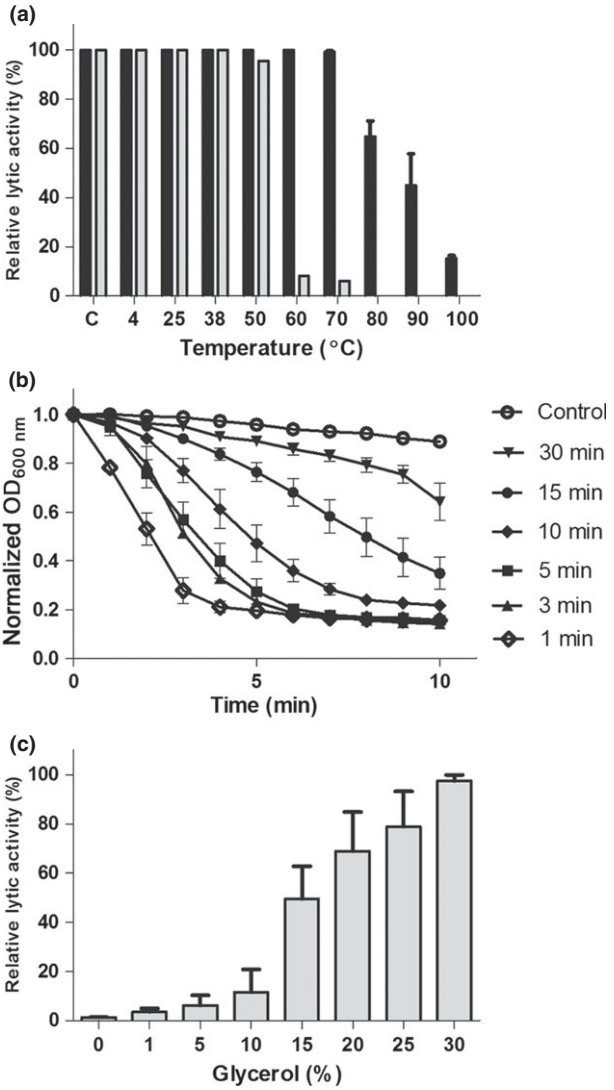

Fig. 5. The effect of glycerol on the thermostability of LysBPS13. Aliquots of the endolysin (5 μg) were incubated (a) at different temperatures in enzyme storage buffer (black bar) or enzyme activation buffer (gray bar) for 30 min, or (b) at 100 °C for 1–30 min in enzyme storage buffer (b). Lytic activity is relative to that of the non-heat-treated endolysin control. (c) Aliquots of endolysin (5 μg) were incubated at 70 °C in different glycerol concentrations. Data are presented as the mean ± standard deviation of triplicate assays.

Implications

In this study, a putative endolysin gene was identified in the genome of the *B. cereus* bacteriophage BPS13. This enzyme consisted of a catalytic domain and a cell-binding domain and was determined to be an *N*-acetylmuramyl-L-alanine amidase, active against *Bacillus* species and EDTA-treated Gram-negative bacteria.

Biochemical characterization showed that LysBPS13 possesses several advantageous features for industrial applications. LysBPS13 retained lytic activity under various conditions, including a broad range of temperatures and ionic strengths. Addition of detergents, such as Tween20, Triton X-100, and CHAPS, did not reduce the lytic activity of the endolysin, which supports its potential to serve as a detergent additive or disinfectant. In addition, it showed activity against some Gram-negative pathogens, and EDTA did not affect its lytic activity, suggesting that it could be easily applied to target Gram-negative pathogens when using EDTA as the permeabiliz-

ing agent. Furthermore, LysBPS13 has high thermostability and lytic activity in the presence of glycerol. Because glycerol is widely used in food, pharmaceutical, and personal care applications, this feature is favorable for applications in these fields. In conclusion, LysBPS13 is a competitive candidate as a biocontrol agent.

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