

## Cloning and Expression of a *bpr* Gene Encoding Bacillopeptidase F from *Bacillus amyloliquefaciens* CH86-1

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Received: October 27, 2010 / Revised: December 30, 2010 / Accepted: February 12, 2011

**A gene encoding bacillopeptidase F, *bpr86-1*, was cloned from *B. amyloliquefaciens* CH86-1 isolated from *cheonggukjang*. This gene could encode a preproenzyme of 1,431 amino acids. When *bpr86-1* was introduced into *B. subtilis* WB600 via pHY300PLK, an *E. coli*–*Bacillus* shuttle vector, the transformant showed fibrinolytic activity. During growth on LB, the fibrinolytic activity of cells increased sharply when they entered the stationary phase. The highest activity (761.4 mU/mg protein) was observed at 96 h of cultivation.**

**Keywords:** *Bacillus amyloliquefaciens* CH86-1, bacillopeptidase F, *bpr* gene, fibrinolytic enzymes

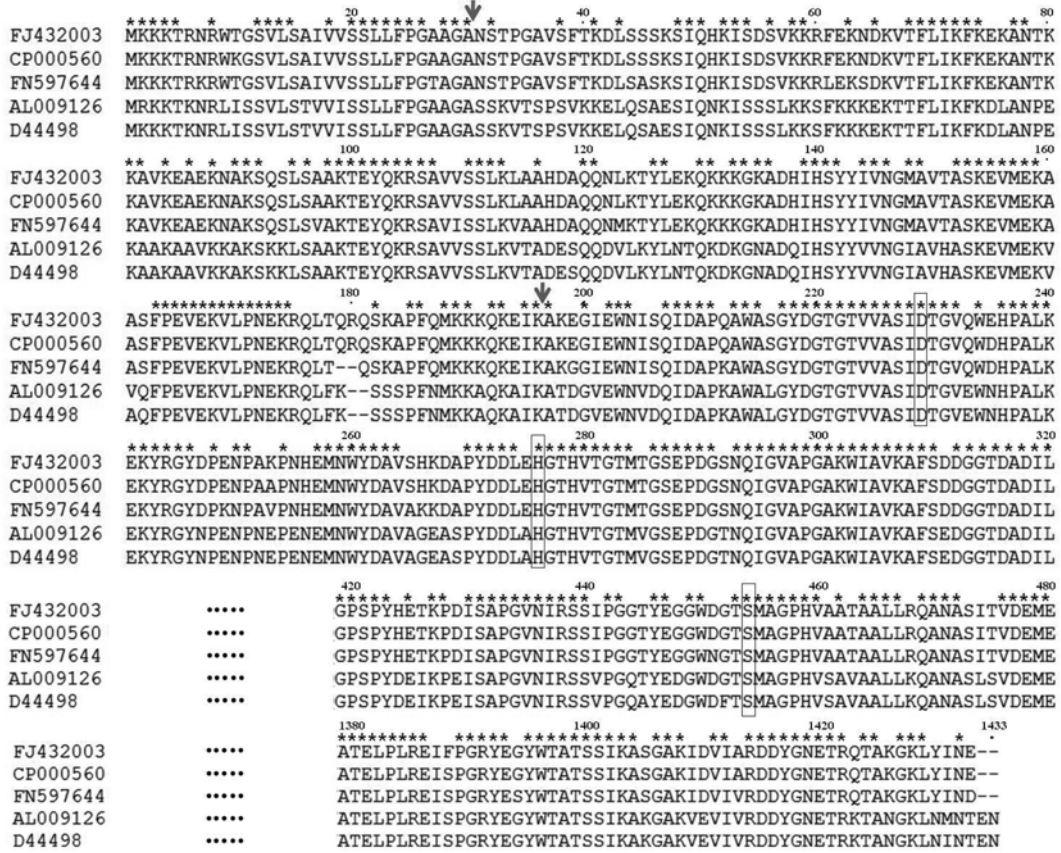
*Bacillus amyloliquefaciens* CH86-1, which was isolated from *cheonggukjang*, a traditional Korean fermented soyfood, secretes several proteases with fibrinolytic activity [4]. Among these proteases, AprE86-1, a 27-kDa enzyme in its mature form, is the main fibrinolytic enzyme and is encoded by *aprE86-1*, a homolog of *aprE* from *Bacillus subtilis* [6, 7]. This gene shares significant homology with nattokinase genes from *B. subtilis* and closely related species [6]. *B. amyloliquefaciens* CH86-1 secretes other minor fibrinolytic proteases, the presence of which can be demonstrated by fibrin zymography of culture supernatants [4] (see also Fig. 3). Bacillopeptidase F is one of the minor proteases secreted by *B. subtilis* and other species [2]. Wu *et al.* [11] reported that bacillopeptidase F of *B. subtilis* is synthesized as a preproenzyme, and that small proteins are

generated through processings at both the amino and carboxyl termini [11]. Yamagata *et al.* [13] found that a 90-kDa serine protease, secreted by *B. subtilis* (natto) No. 16, might be a degradation product from the 133-kDa mature enzyme derived from a preproenzyme (1,433 amino acids). Although proteases are generally believed to help cells survive in adverse environments by providing peptides and amino acids from denatured or unnecessary proteins [8, 10], the exact role and contribution of each enzyme is largely unknown. We cloned a gene encoding bacillopeptidase F from the chromosome of *B. amyloliquefaciens* CH86-1 by PCR. Primers were designed based on the *bpr* sequence of *B. amyloliquefaciens* FZB42 for which the genome sequence is available. PCR was performed with *Ex-Taq* polymerase (Takara, Shiga, Japan) using an MJ Mini PCR system (BioRad, Hercules, CA, USA) and the primers *bprF* (5'-GCGGATCCATGATGCCCTCGACATT-3', *Bam*HI site underlined) and *bprR* (5'-CGTCTAGACATCAGAAGCACAATGGCTG-3', *Xba*I site underlined). PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 74°C for 2 min, and a final 4 min extension at 74°C. The 4,988 bp fragment thus amplified was cloned into pHY300PLK (Takara), a *Bacillus*–*E. coli* shuttle vector, resulting in pHY**bpr86-1**. DNA sequencing confirmed that the fragment contained *bpr*, and the gene was named *bpr86-1* accordingly. The ORF of *bpr86-1* begins at nucleotide (nt) 327 and ends at nt 4,622. It could encode a protein of 1,431 amino acids, designated Bpr86-1, and the pI and molecular mass of which were calculated to be 5.95 and 154,512.71 Da, respectively. The first 30 amino acids seem to constitute a signal peptide, as predicted by the SignalP 3.0 Server program, and comparison with other bacillopeptidase F

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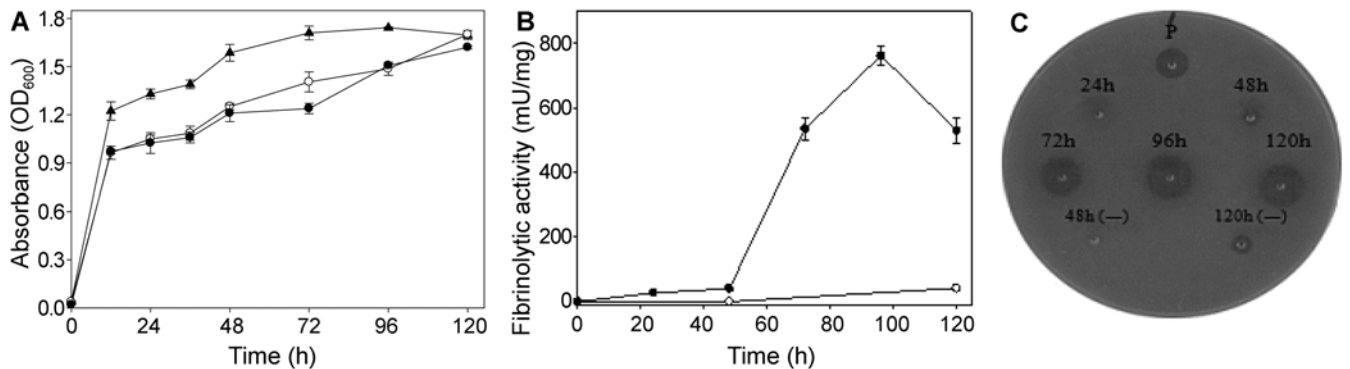
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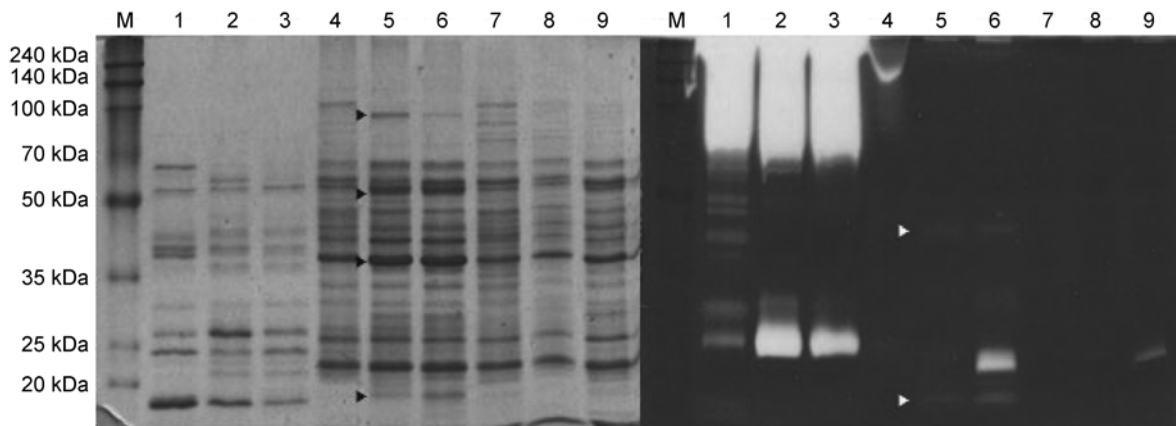
**Fig. 1.** Alignment of the first 320 amino acids of Bpr86-1 with other bacillopeptidase F sequences. FJ432003 (*B. amyloliquefaciens* CH86-1); CP000560 (*B. amyloliquefaciens* FZB42); FN597644 (*B. amyloliquefaciens* DSM7); AL009126 (*B. subtilis* 168); D44498 [*B. subtilis* (natto) No. 16]. \* indicates conserved amino acids among five sequences. Amino acids constituting the catalytic triad are marked with a rectangle. The presumed cleavage points for the pre- and pro-sequences are marked with an arrow. Other portions of the enzymes are shown (near the serine residue of the catalytic triad and the C-terminus).

sequences suggests that the next 166 amino acids might be a pro-sequence [13]. The first 320 amino acids of Bpr86-1 and portions of other regions translated from the DNA sequence are shown together with other bacillopeptidases

in Fig. 1. Bpr86-1 shows 99%, 94%, 71%, and 70% homology to the enzymes from *B. amyloliquefaciens* FZB42, *B. amyloliquefaciens* DSM7, *B. subtilis* 168, and *B. subtilis* (natto) strain 16, respectively. The pI and the molecular



**Fig. 2.** Growth and fibrinolytic activities of bacilli. **A.** -▲-, *B. amyloliquefaciens* CH86-1; -●-, *B. subtilis* WB600 [pHYbpr86-1]; -○-, and *B. subtilis* WB600 [pHY300PLK] (control). **C.** A fibrin plate showing fibrinolytic activity of cells. P denotes plasmin (Sigma) at 1.5 mU. Numbers indicate incubation time in h and (-) denotes the control. A standard curve was obtained at different plasmin concentrations (0.75–12 mU). Ten  $\mu$ l (20  $\mu$ g) was spotted and the plate was incubated for 16 h at 37°C. All the measurements were repeated three times and the average values are shown.



**Fig. 3.** SDS-PAGE (left) and fibrin zymogram (right) of culture supernatant.

M, protein size marker (DokDo-MARK, Elpisbio, Daejeon, Korea); *B. amyloliquefaciens* CH86-1 at 48 h (lane 1), 96 h (2), and 120 h (3); *B. subtilis* WB600 [pHYbpr86-1] at 48 h (4), 96 h (5), and 120 h (6); *B. subtilis* WB600 [pHY300PLK] at 48 h (7), 96 h (8), and 120 h (9). Fifteen  $\mu$ g was applied to a 10% acrylamide gel. Bands marked on the left are absent in the control.

mass of the expected mature enzyme are 5.06 and 132,658.36 Da, respectively. In most fibrinolytic serine proteases, three amino acids constituting the catalytic triad are conserved [9]. These residues (Asp33, His80, Ser258) were located in Bpr86-1, by comparison with other bacillopeptidase F sequences, and are marked in Fig. 1. The entire sequence of *bpr86-1* was deposited in GenBank under the accession number FJ432003.

pHYbpr86-1 was introduced into *B. subtilis* WB600, a strain lacking six protease activities [12], by electroporation. *Bacillus* competent cell preparation and electroporation procedures were performed as previously described [3]. *B. subtilis* TF (cells harboring pHYbpr86-1) and a *B. subtilis* control (cells harboring pHY300PLK) were grown in LBTc (tetracycline, 10  $\mu$ g/ml) up to 120 h at 37°C with aeration (Fig. 2). *B. amyloliquefaciens* CH86-1 was grown in LB. At intervals, culture supernatant (70 ml) was obtained by centrifugation, and then filtered and precipitated with ammonium sulfate [80% (w/v)]. The pellet thus obtained was resuspended in 20 mM Tris-HCl (pH 8.0), dialyzed against the same buffer at 4°C overnight with four buffer changes, freeze-dried, and resuspended in a small volume of 20 mM Tris-HCl (pH 8.0). The protein concentration was determined by the Bradford method [1] and fibrinolytic activity was measured using a fibrin plate as previously described [3, 7]. The fibrinolytic activity of *B. subtilis* TF increased significantly during the stationary phase and reached the highest point at 96 h (Fig. 2). The control did not show any activity during the same period. TF showed a basal level of activity until 48 h, after which it increased sharply, reaching 533.8 (mU/mg protein) at 72 h, 761.4 at 96 h, and 528.9 at 120 h (Fig. 2). The control showed only 41.9 mU/mg protein at 120 h, which was less than 10% of the activity of TF. PCR-amplified *bpr86-1* contains its own promoter; expression of *bpr86-1* seemed to occur under

the control of this promoter. The results showed that the synthesis of bacillopeptidase F was greatly induced when cells entered the stationary phase. The increase in bacillopeptidase F activity was parallel to the increase in total fibrinolytic activity of *B. amyloliquefaciens* CH86-1 cells during the stationary phase [7]. Thus, it is likely that the expression of all fibrinolytic proteases is induced when cells enter the stationary phase. SDS-PAGE and fibrin zymography were performed on the culture supernatant from TF and control cells as previously described [3, 5]. Bands corresponding to peptides of 90, 55, and 40 kDa, observed on an SDS-polyacrylamide gel of *B. subtilis* TF, were most likely derived from bacillopeptidase F (Fig. 3). On the fibrin zymogram, bands of approximately 45 and 17 kDa were observed in CH86-1 and *B. subtilis* TF, but were absent in the control. Larger derivatives of Bpr86-1 were difficult to locate in *B. amyloliquefaciens* CH86-1. This result may be due to the fact that Bpr86-1 is one of the minor proteases in *B. amyloliquefaciens* CH86-1 and its derivatives are unstable. It has been reported that smaller fragments are generated from bacillopeptidase F as the results of processing at either the N- or C-terminus or both [11, 13]. When 2D gel fibrin zymography was performed on the culture supernatant of *B. amyloliquefaciens* CH86-1, synthesis of proteases larger than 70 kDa (pI around 7.0) was strongly induced at the stationary phase, and the band intensity was higher at 100 h than at 67 h (results not shown). Bacillopeptidase F and its derivatives were probably responsible for enhancing activity at least partially. Enhanced bacillopeptidase F activity might be advantageous for host cells in that it enables them to adjust to adverse growth conditions in which available nutrients including proteins and peptides are limited. Detailed studies on bacillopeptidase F and its processed derivatives will be helpful in understanding its function and role in the overall fibrinolytic capability of

*B. amyloliquefaciens* CH86-1. The results will help us to develop industrial applications of bacillopeptidase F and identify producing organisms.

## Acknowledgments

This work was supported by a research grant from the Biofoods Research Program of the Korea Science and Engineering Foundation (KOSEF), and G.-H. Kwon was supported by the 2<sup>nd</sup> stage of the Brain Korea 21 Program of the Ministry of Education, Science and Technology (MEST, Korea).

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