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RESEARCH NOTE

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Heterologous Gene Expression of *aprE2* Encoding a 29 kDa Fibrinolytic Enzyme from *Bacillus subtilis* in *Bacillus licheniformis* ATCC 10716

Gun-Hee Kwon[1], Woo-Ju Jeong[1], Ae Ran Lee[1], Jae-Yong Park[2], Jaeho Cha[3], Young-Sun Song[4], and Jeong Hwan Kim[1,2]*

[1]*Division of Applied Life Science (BK21 Program), Gyeongsang National University, Jinju, Gyeongnam 660-701, Korea*
[2]*Institute of Agriculture and Life Science, Gyeongsang National University, Jinju, Gyeongnam 660-701, Korea*
[3]*Department of Microbiology, Pusan National University, Busan 609-735, Korea*
[4]*School of Food and Life Science, Inje University, Gimhae, Gyeongnam 621-749, Korea*

Abstract The *aprE2* gene from *Bacillus subtilis* CH3-5 was expressed in *Bacillus licheniformis* ATCC 10716 using a *Bacillus-Escherichai coli* shuttle vector, pHY300PLK. The fibrinolytic activity of transformant (TF) increased significantly compared to *B. licheniformis* 10716 control cell. During the 100 hr incubation in Luria-Bertaini broth at 37°C, fibrinolytic activity of *B. licheniformis* TF increased rapidly at the late growth stage, after 52 hr of incubation, which was confirmed by zymography using a fibrin gel. pHY3-5 was stably maintained in *B. licheniformis* without tetracycline (Tc) in the media, 60.9% of cells still maintained pHY3-5 after 100 hr of cultivation.

Key words: *Bacillus licheniformis*, fibrinolytic enzyme, gene expression, transformation, *Bacillus subtilis*

Introduction

Bacillus licheniformis is one of the most common *Bacillus* species isolated from fermented soy foods such as *cheonggukjang* (1-3). Some *B. licheniformis* strains produce fibrinolytic enzymes and such organisms and their fibrinolytic enzymes can be useful sources for functional foods or medicines preventing or curing vascular diseases caused by fibrin accumulation (4,5). Nattokinase is the most well-known example and products containing nattokinase are available in the market (6). It is expected that other fibrinolytic enzymes as efficient as nattokinase will be characterized from other *Bacillus* species from fermented foods such as *cheonggukjang* and used for functional foods or drugs (7). In this work, *aprE2*, encoding a 29 kDa fibrinolytic enzyme from *B. subtilis* CH3-5, was overexpressed in *B. licheniformis* ATCC 10716, a strain lacking fibrinolytic activity. If the same or similar plasmid construct is introduced into *B. licheniformis* strains with inherent fibrinolytic activity, strains with higher fibrinolytic ability can be constructed. The ultimate goal will to construct of *Bacillus* strains with improved fibrinolytic activity by introducing various fibrinolytic enzyme genes. Such strains are obviously very useful for the production of fibrinolytic enzymes.

Materials and Methods

Strains and cultivation conditions *Bacillus licheniformis* ATCC 10716 was cultivated in Luria-Bertani (LB) broth at 37°C with shaking. pHY3-5, A pHY300PLK (Takara, Shiga, Japan) containing an 1.8 kb *Eco*RI fragment encompassing *aprE2*, was described previously (8). Cells harboring pHY3-5 was cultivated in LB broth containing tetracycline (Tc, 10 μg/mL).

Electroporation conditions pHY3-5 DNA was prepared from *Escherichia coli* DH5α harboring pHY3-5 using a plasmid mini prep kit (Qiagen, Valencia, CA, USA). Bacilli competent cell preparation and electroporation were done as described previously (8).

Fibrinolytic activity measurements *B. licheniformis* was cultivated in LB broth for 100 hr at 37°C. At intervals, culture supernatant was obtained by centrifugation of culture and filtered using a 0.45-μm syringe filter. Twenty μL was spotted on a fibrin plate and the plate incubated for 18 hr at 37°C. The fibrinolytic activity was calculated by comparing the area of the lysis zone with those of plasmin standards (7.5-60 mU). A fibrin plate was prepared according to the method described by Noh *et al.* (9).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fibrin zymography Filtered culture supernatant from *B. licheniformis* was freeze-dried and resuspended in sterile water. Cell pellet obtained by centrifugation of culture was resuspended in small volume of phosphate buffered saline (PBS, pH 7.0) and subjected to ultrasonication. Disrupted cells were centrifuged and the supernatant was used as the cellular protein sample containing soluble cytoplasmic proteins. Ten μg of each protein sample was applied for SDS-PAGE and zymography using a 15% acrylamide gel. SDS-PAGE and zymography were done according to the methods described previously (8).

pHY3-5 stability During 100 hr cultivation of *B. licheniformis* TF in LB broth, aliquots were taken, serially

*Corresponding author: Tel: +82-55-751-5481; Fax: +82-55-753-4630
E-mail: jeonghkm@gsnu.ac.kr
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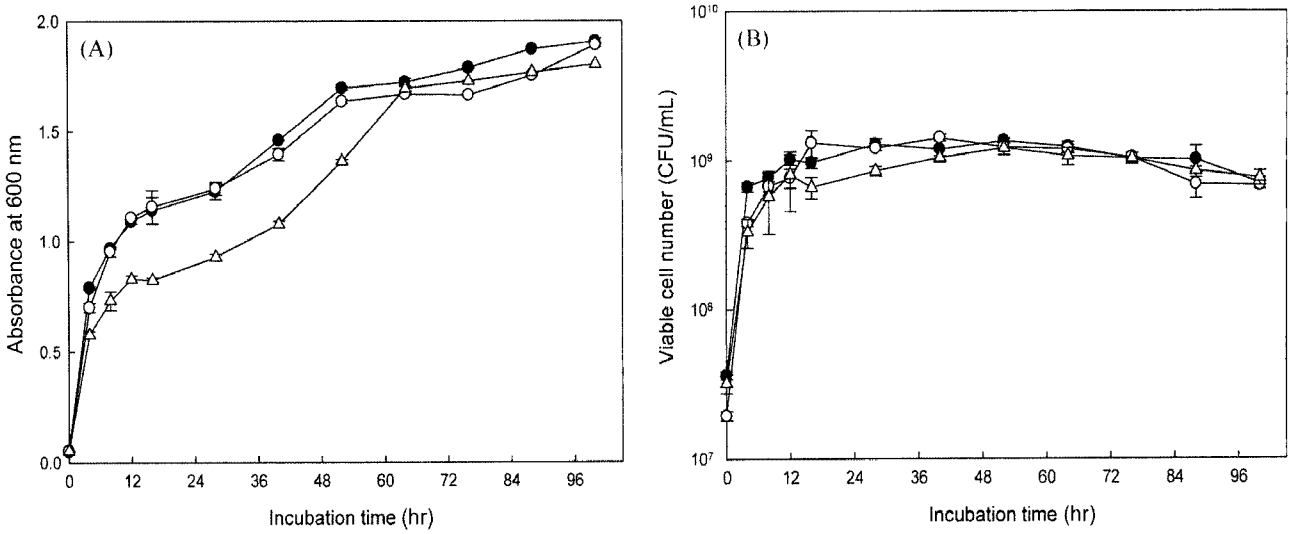

Fig. 1. Growth of *B. licheniformis* cells in LB broth. *B. licheniformis* was cultivated for 100 hr at 37°C. At intervals, absorbance at 600 nm (A) and viable cell number (B) of culture were measured. ●, 10716 in LB broth; ○, 10716[pHY3-5] in LB broth; △, 10716[pHY3-5] in LBTc (10 μg/mL).

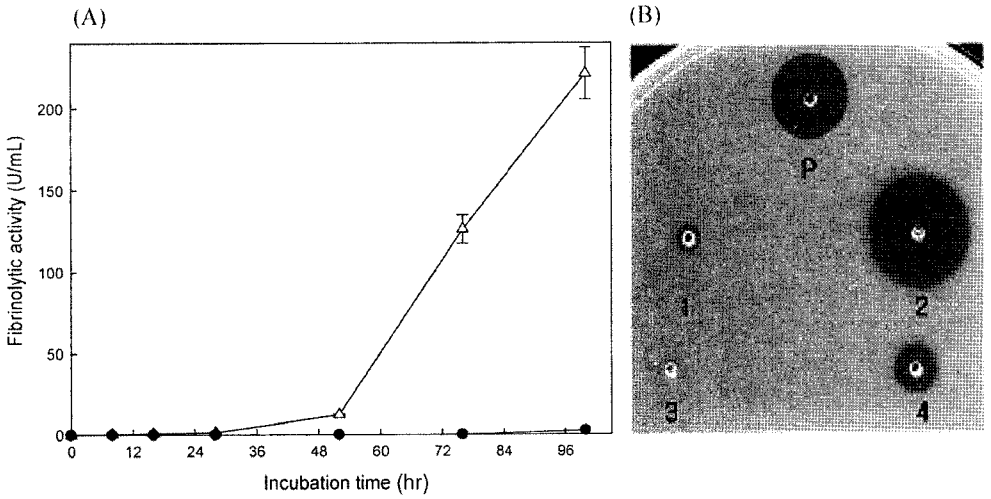

Fig. 2. Fibrinolytic activity of *B. licheniformis* TF. (A) 20 μL of culture supernatant at each time point was spotted on a fibrin plate and the fibrinolytic activity was calculated. ●, 10716; △, 10716[pHY3-5]. (B) Fibrin plate assay for *B. licheniformis* TF cultivated for 52 hr. Culture supernatant of 10716 control (1) and 10716 TF (2); cellular protein sample from 10716 control (3) and 10716 TF (4); P, plasmin (6 mU).

diluted, and then spread onto LB broth and LBTc plates. Plates were incubated for 48 hr at 37°C. The portion of cells maintaining pHY3-5 was calculated as follows: number of cells on LBTc plates/number of cells on LB plates×100%.

Results and Discussion

Introduction of *aprE2* into *B. licheniformis* cells To select a host strain for *aprE2* expression, 3 *B. licheniformis* (ATCC 21415, 10716, and 14580) and 2 *B. amyloliquefaciens* (ATCC 23350 and 23845) strains were examined for the fibrinolytic activity and the presence of plasmids. *B. licheniformis* ATCC 10716 was selected as the host because it lacked indigenous fibrinolytic activity and plasmids (results not shown). After electroporation, TFs appeared on LBTc plates around 24 hr at 37°C. *Eco*RI digestion of the plasmid preparation from the TFs confirmed the introduction of pHY3-5 into *B. licheniformis* 10716 (results not shown). The transformation efficiency, however, was low, 10^1-10^2 TFs/μg DNA. *B. licheniformis* 10716 harboring pHY3-5 was cultivated in LB broth up to 100 hr at 37°C with shaking (128 rpm, IS971-R; Jeio Tech., Seoul, Korea) and the $OD_{600\,nm}$, viable cell numbers, and fibrinolytic activity of the culture were measured. Figure 1 shows that cells harboring pHY3-5 grew at the same rate with 10716 control cells in LB broth. When Tc was included in the medium, TFs grew slowly until 60 hr but after 60 hr, all 3 cultures showed basically the same $OD_{600\,nm}$ values. Viable cell numbers of cultures changed in a similar way. From the initial population of ca. 3×10^7 cells/mL, the cell numbers reached the peak around 40 hr and then decreased gradually. At 100 hr, the viable cell number was around 7×10^8 CFU/mL, 20-fold increase from the initial number.

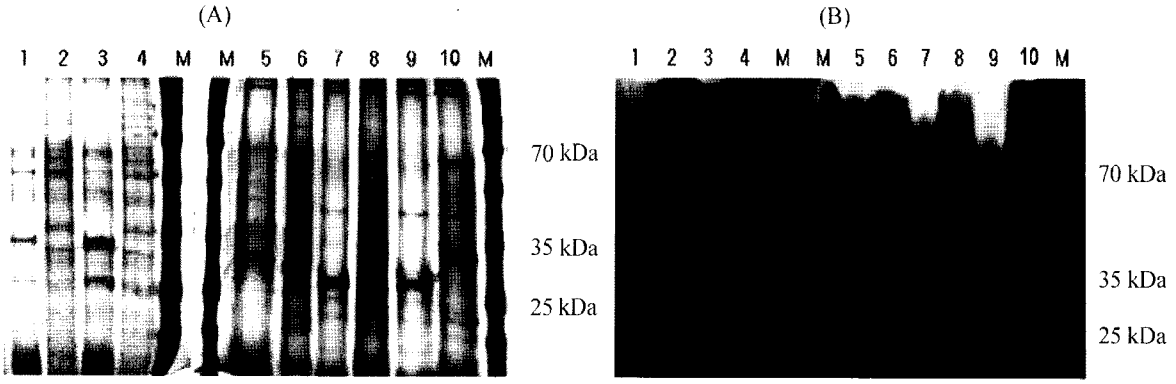

Fig. 3. SDS-PAGE and zymography. SDS-PAGE (A) and zymography (B) of culture supernatant from *B. licheniformis* control containing pHY300PLK (designated as C) and TF containing pHY3-5 (designated as S). A. Culture supernatant from C at 16 (1), 28 (3), 52 (5), 76 (7), and 100 hr (9) cultivation time. Culture supernatant from S at 16 (2), 28 (4), 52 (6), 76 (8), and 100 hr (10). After SDS-PAGE, the gel was silver stained. M, Protein size marker (ELPIS, Daejeon, Korea). B. Zymogram results. S at 16 (1), 28 (3), 52 (5), 76 (7), and 100 hr (9). C at 16 (2), 28 (4), 52 (6), 76 (8), and 100 hr (10).

Expression of *aprE2* in *B. licheniformis* ATCC 10716 Fibrinolytic activity of the *B. licheniformis* 10716 TF remained at low levels until ca 52 hr and after that increased sharply (Fig. 2). The fibrinolytic activity at 52 hr was 12.41 U/mL. Then the activity increased rapidly, reaching 221.2 U/mL at 100 hr. *B. licheniformis* control cells, however, did not show any significant activity during the same period. It is interesting why the fibrinolytic activity increased rapidly at the late growth stage. A protease less active in its original form might change into a more active form after degradation by itself or other protease. Another explanation is the induction of a protease which is synthesized specifically at late growth stages. Figure 2B shows the fibrin plate assay results of cells at 52 hr. Tewnty μL of each sample was applied onto a fibrin plate and the plate was incubated for 24 hr at 37°C. *B. licheniformis* TF produced a larger lysis zone than control cell. The lysis zone of culture supernatant was much larger than that of cytoplasmic fraction, indicating that 29 kDa AprE2 secreted into culture medium after synthesized in the cytoplasm. Prolonged cultivation of *B. licheniformis* in LB broth apparently caused the protein degradation and the changes seemed more significant in cells harboring pHY3-5 (Fig. 3A). Zymogram result confirmed that fibrinolytic activity of TF increased as cultivation time increased (Fig. 3B). The size of the big smear at the top of the fibrin gel, caused by overexpression of *aprE2*, increased as incubation time extended.

Stability of pHY3-5 in *B. licheniformis* Without Tc selection, 60.9% of *B. licheniformis* TF still maintained pHY3-5 after 100 hr (Fig. 4). During the first 40 hr, more than 80% of cells maintained pHY3-5. The result showed that pHY3-5 was reasonably stable in *B. licheniformis*. Plasmid vectors have instability problem in nature and absence of selection pressure, antibiotics in this case, accelerates plasmid loss from the host. Since antibiotic resistance markers are not allowed for food applications, food-grade plasmid vectors should be constructed if heterologous gene expression is necessary. Another approach is the integration of a fibrinolytic enzyme gene into chromosome of host *Bacillus*. In terms of gene stability, the latter strategy seems to be a better choice. In conclusion, *aprE2* from *B. subtilis* was successfully introduced into *B. licheniformis* ATCC 10716 and expressed, endowing host cells with fibrinolytic activity. The works presented here are the first steps for constructing recombinant *B. licheniformis* strains for the production of functional foods or medicines.

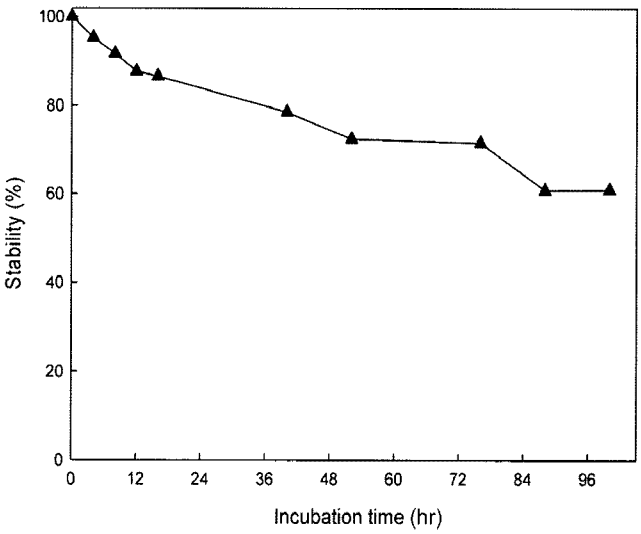

Fig. 4. Stability of pHY3-5 in *B. licheniformis* without antibiotic selection.

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