Characterization of a 27 kDa Fibrinolytic Enzyme from
Bacillus amyloliquefaciens CH86-1 Isolated from Cheonggukjang

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Bacillus amyloliquefaciens CH86-1 isolated from cheonggukjang was found to have strong fibrinolytic activity when grown on Luria-Bertani medium, and this activity increased sharply when the cells entered the stationary phase. The major fibrinolytic enzyme, AprE86-1, was purified from culture supernatant and identified by tandem mass spectrometry. The molecular weight of the mature enzyme was determined to be 27 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The optimum pH of partially purified AprE86-1 was 6.0-7.0 and it was stable at up to 45°C.

Key words: Bacillus amyloliquefaciens, cheonggukjang, fibrinolytic activity, proteases

Cheonggukjang is a traditional Korean fermented soy food that has important health promoting effects including the prevention of high blood pressure, hypcholesterolemic effects, fibrinolytic activity, antimutation effects, anticarcinogenic effects and antioxidative effects [Kim et al., 1999; Lee et al., 2005]. The fibrinolytic activity of cheonggukjang is produced by some proteases secreted by bacilli. Nattokinase, the most well-known fibrinolytic enzyme, is produced by Bacillus subtilis Natto and belongs to the serine type proteases, which have a high specificity to fibrin [Sumi et al., 1987]. Nattokinase is used to treat and prevent vascular diseases caused by fibrin clots. Other fibrinolytic enzymes with similar properties are also produced by bacilli including a Bacillus species that has been isolated from cheonggukjang [Kim et al., 1996], Bacillus amyloliquefaciens (B. amyloliquefaciens) from douchi [Peng et al., 2003], a Chinese traditional soy food, and Bacillus species from doenjang [Choi et al., 2004]. Fibrinolytic enzymes from bacilli are useful for the production of functional foods or medicines for the prevention of vascular diseases.

We previously isolated Bacillus strains with fibrinolytic activities from cheonggukjang prepared using traditional methods and identified them using various molecular biological methods [Kwon et al., 2009]. Among six isolates, B. amyloliquefaciens CH86-1 showed the highest fibrinolytic activity when assayed by the fibrin plate method [Jeong et al., 2007]. Considering its GRAS, generally recognized as safe, status and high fibrinolytic activity, B. amyloliquefaciens CH86-1 appears to be an ideal starter for the fermentation of cheonggukjang. The strain is also useful as a source of fibrinolytic enzyme genes, which can be utilized for the construction of Bacillus strains that overproduce fibrinolytic enzymes for use in basic and applied research. In this study, B. amyloliquefaciens CH86-1 was evaluated for its fibrinolytic activities during 100 h of growth in LB at 37°C. In addition, a 27 kDa fibrinolytic enzyme, AprE86-1, was purified from culture supernatant and its properties were investigated.

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Materials and Methods

Bacterial strain and culture conditions. *B. amyloliquefaciens* CH86-1 was previously isolated from cheonggukjang prepared using traditional methods in Sunchang, Jeollabukdo, Korea in 2006 [Kwon et al., 2009]. *B. amyloliquefaciens* CH86-1 was grown in LB (Bacto tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) broth at 37°C with aeration. BHI, NB and TSB (BD Difco, Sparks, MD) were used for the comparison of growth and fibrinolytic activities of *B. amyloliquefaciens* CH86-1.

Fibrinolytic activities. Cultures of *B. amyloliquefaciens* CH86-1 were centrifuged at 5,000×g for 20 min at 4°C, after which the supernatant was assayed for the fibrinolytic activity by the fibrin plate method [Jeong et al., 2007]. Briefly, 20 µL of the supernatant were dropped into a hole that had been made on a fibrin plate using a capillary glass tube, after which the plate was incubated at 37°C for 10 h. The size of the clear zone that formed was then converted into a plasmin unit by comparison with the clear zones formed by different concentrations of plasmin (1.5–30 mU). The protein concentration was determined according to the Bradford method using bovine serum albumin (BSA) as a standard.

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fibrin zymography. *B. amyloliquefaciens* CH86-1 was grown in LB at 37°C with aeration and the culture supernatant was obtained by centrifugation. Next, the culture supernatant was concentrated by ammonium sulfate (Amresco, Solon, OH) precipitation (80% saturation, w/v). The ammonium sulfate pellet was then resuspended in a small volume of 30 mM Tris (pH 7.4) and dialyzed against the same buffer for 24 h at 4°C. For SDS-PAGE, protein samples were loaded onto gels (15% acrylamide) after being boiled for 3 min in SDS sample buffer. A polyacryamide gel containing fibrin was prepared by mixing fibrinogen (0.12%, w/v, Sigma, St. Louis, MO), separating gel solution (15% acrylamide) and thrombin (100 NIH units/mL, Sigma) for fibrin zymography. After electrophoresis in a cold room at a constant current of 10 mA, the gel was incubated in 50 mM Tris (pH 7.4) buffer containing 2.5% Triton X-100 for 30 min at room temperature on a rotary shaker. The gel was then washed with distilled water for 30 min to remove the Triton X-100 and incubated in zymogram reaction buffer (30 mM Tris, pH 7.4, and NaN₃) for 12 h at 37°C. Finally, the gel was stained with coomassie blue R-250 for 1 h, after which it was destained and bands with fibrinolytic activities were visualized as the nonstained regions on the gel.

Purification of AprE86-1. *B. amyloliquefaciens* CH86-1 was cultivated in LB (3 L) for 48 h at 37°C with shaking, after which the culture supernatant was concentrated by ammonium sulfate precipitation (80% saturation, w/v). The ammonium sulfate pellet was then resuspended in 10 mL of 20 mM Tris at pH 7.0 (buffer A) and the solution was dialyzed against 20 volumes of buffer A for 24 h with three buffer changes at 4°C. The dialyzed solution was then applied onto a CM-Sephadex (Amersham Pharmacia Biotech, Uppsala, Sweden) column (2.5×8 cm). The proteins were eluted by sequential application of six 200 mL aliquots of buffer A with NaCl concentration starting at 0 and increasing to 1 M in 0.2 M increments. Next, 20 µL from each fraction (15 mL) were spotted onto a fibrin plate and fractions showing the fibrinolytic activities were pooled, dialyzed against buffer A, and lyophilized. Hydrophobic interaction chromatography using Phenyl Sepharose 6 Fast Flow (Amersham Pharmacia Biotech) was then used for further purification of the fibrinolytic proteins. Briefly, the column (2.5×12 cm) was washed and equilibrated with buffer A containing 1 M (NH₄)₂SO₄. Next, the proteins were eluted by sequential applications of six 200 mL aliquots of buffer A with (NH₄)₂SO₄ concentrations that decreased from 1 to 0 M in 0.2 M decrements. The active fractions (each 15 mL) were identified as above, pooled, dialyzed against buffer A and lyophilized prior to analysis by SDS-PAGE and zymography. For protein identification, the bands were excised from a coomassie blue stained SDS-gel and then subjected to trypsin digestion. Tandem mass spectrometry was conducted at Genomine (Pohang, Korea).

Stabilities of partially purified AprE86-1. Eight micrograms of AprE86-1 were incubated in either 0.1 M citrate-NaOH buffer (pH 3.0 to 5.0), 0.1 M sodium phosphate buffer (pH 6.0 to 7.0), 0.1 M Tris-HCl (pH 8.0 to 9.0) or 0.1 M glycine-NaOH buffer (pH 10.0) for 2 h at 37°C, after which the remaining fibrinolytic activities were measured using a fibrin plate. The heat stability of AprE86-1 was examined by incubating 8 µg of AprE86-1 in 0.1 M sodium phosphate buffer (pH 6.0) for 30 min at different temperatures (37-55°C) and then measuring the remaining activities. The effects of metal ions and inhibitors on the fibrinolytic activities were also examined. Briefly, 8 µg of AprE86-1 were incubated in 0.1 M sodium phosphate buffer (pH 6.0) containing a metal ion (KCl, MgCl₂, CoCl₂, CaCl₂, Ag₂SO₄, CuSO₄, or ZnCl₂) at a concentration of 5 mM or an inhibitor (PMSF, SDS, or EDTA) at a concentration of 1 mM for 1 h at 45°C, after which the remaining activities were measured.