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Cloning and Characterization of the Major Extracellular Neutral Protease (NprM) from *Bacillus megaterium* ATCC 14945

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A gene, *npr*M, from *Bacillus megaterium* ATCC 14945 was obtained by PCR using primers synthesized based on two *npr*M sequences from two different strains, and cloned into *Escherichia coli*. The gene *npr*M encoded an extracellular neutral protease, and the molecular mass of the expressed enzyme was estimated to be approximately 36 kDa on a denaturating gel. The enzyme was activated by Ca^{2+}, and the optimum concentration of Ca^{2+} was 5 mM. The enzyme was inhibited by EDTA but not by PMSF. The optimal pH and temperature of the cloned enzyme were 50°C and pH 7.5-8.0, respectively, and were similar to those of the enzyme from the gene donor cell. The cloned NprM caused internal cleavage of the native endoglucanase of *B. subtilis* BSE616 as a model foreign protein, and resulted in a small truncated but still active endoglucanase.

Key words: *Bacillus megaterium, extracellular neutral protease, cloning, foreign protein cleavage.*

The ability of *Bacillus* species to secrete proteins into culture medium is very advatageous in an expression system. Because of this ability, *Bacillus subtilis* has become one of the most thoroughly studied bacterium with respect to its molecular biology and biotechnology. *B. megaterium* has also been considered as a suitable system for gene cloning and heterologous gene expression. In addition to the ability to secrete proteins, the organism supports stable maintenance of plasmids and efficient expression of cloned genes.[1,2]

However, *Bacillus* species have a critical drawback as an expression system in that they usually cleave expressed foreign proteins in culture medium and render them to lose some of their useful properties or to be totally inactive. For example, when an endoglucanase gene of *B. subtilis* BSE616 was cloned into *E. coli*,[3] and was transferred to *B. megaterium* ATCC 14945,[4] the native endoglucanase was internally cleaved into a truncated but still active form.[5] An extracellular protease purified from the supernatant of *B. megaterium* ATCC 14945 was found to be responsible for the cleavage.[6] The protease was suspected to be NprM, an extracellular protease encoded by *npr*M gene. *B. megaterium* has been known to produce a major extracellular protease NprM [7-9] and at least one minor extracellular serine protease.[10] The *npr*M gene, encoding the extracellular neutral protease in *B. megaterium*, has previously been cloned from two different strains, ATCC 14581[9] and DSM319.[11]

Here, we describe the cloning of the *npr*M gene from another strain of *B. megaterium*, ATCC 14945, and the characteristics of the purified gene product. The cleavage reaction of the gene product was also investigated using the endoglucanase from *B. subtilis* BSE616 as a model foreign protein.

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Abbreviations: endoglucanase, endo-β-1,4-glucanase; MUC, 4-methylumbelliferyl-β-D-cellobioside; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Materials and Methods

Bacterial strains, plasmids, and culture conditions. *B. megaterium* ATCC 14945 (KCTC 3071) and *E. coli* DH5α were grown aerobically in Luria-Bertani (LB) broth medium at 37°C as a protease gene source and a cloning host, respectively. When necessary, ampicillin (50 μg/m*l*) was added to the medium. Plasmid pUC19 and pGEM-T Easy system (Promega, USA) were used as cloning vectors.

Cloning of the NprM gene from *B. megaterium*. *B. megaterium* chromosomal DNA was prepared as described by Rodriguez and Tait.[12] Competent cells of *E. coli* DH5α were prepared by the method of Hanahan.[13] The standard procedures for cloning were done according to the techniques of Sambrook *et al.*,[14] or the manufacturer's recommendation. Primer sequences were designed to minimize primer-dimer production and to reduce mispriming. Oligonucleotide primers were synthesized by Bioneer Co. (Korea). PCR was carried out with a GeneAmp thermal cycler (Model 9600, Perkin Elmer, USA) in 50 μl of reaction volume. PCR mixtures containing 50 pmol of each

primer were subjected to 35 cycles of 1 min at 94°C, 2 min at 52°C, and 2 min at 72°C after 5 min preheating at 94°C. One additional cycle was performed as follows: 1 min at 94°C, 7 min at 72°C and cooling at 4°C. PCR mixtures were then separated on an 1.0% (w/v) agarose gel. The PCR product was eluted from the gel, ligated into pGEM-T Easy vector, and transformed into *E. coli* DH5α. *E. coli* transformants were primarily grown on LBS agar plates (LB medium containing 0.7% skim milk) supplemented with ampicillin for two days. The transformants were then screened by tooth-picking and streaking into approximately 1 cm in length (25 transformants per plate). The transformants were grown for two days, and a transformant showing a clear zone around its margin was selected. Nucleotide sequences of 5'- and 3'-ends of the cloned fragment were determined using the dideoxy-chain termination method.[15]

Purification of cloned enzyme. *E. coli* (pBK49) was aerobically cultured in 2 L of LB broth (10 × 200 m*l* in 1-L Erlenmeyer flasks) containing ampicillin (50 μg/m*l*) at 37°C and harvested at the stationary phase by centrifugation at 6,000 × g for 10 min. After the cells were washed with 500 m*l* of 50 mM TRIS/HCl buffer (pH 8.0) containing 1 mM $CaCl_2$, they were resuspended in 50 m*l* of the same buffer, disrupted two times with a sonicator (Model VCX600, Sonics & Materials, USA) for 1 min at a pulse-mode in an ice bath, and centrifuged at 15,000 × g for 20 min. The crude supernatant was fractionated by precipitation with solid ammonium sulfate (30-60%). The precipitate was collected by centrifugation at 15,000 × g for 20 min, dissolved in 3 m*l* of 10 mM TRIS/HCl (pH 8.0) containing 5 mM $CaCl_2$, and dialyzed against the same buffer overnight. The dialysate was applied to an Econo Pac High-S column (BioRad, 5 m*l*), and the adsorbed proteins were eluted with a linear gradient of NaCl (0-1.0 M) in the same buffer at 1.5 m*l*/min for 40 min using the BioLogic LP System (BioRad, USA). Active fractions (1.5 m*l* each) were collected, dialyzed against 50 mM TRIS/HCl buffer (pH 8.0) containing 5 mM $CaCl_2$ and 0.1 M KCl, and separated on a Sephadex G-75 column (2.5 × 75 cm) with the same buffer. Active fractions were lyophilyzed and stored at -20°C until use.

Determination of protease activity. Protease activity was measured by a modified method of Sarath *et al.*[16] The reaction mixture (200 μ*l*) contained 1.25% azocasein (Sigma Chemical Co., USA) and 10 mM $CaCl_2$ in 50 mM TRIS/HCl (pH 7.5). After incubation at 50°C for 30 min, the reaction was stopped by the addition of 1.2 m*l* of 10% trichloroacetic acid, and the undigested azocasein was pelleted by centrifugation for 5 min at 15,000 × g. Subsequently, 1.4 m*l* of 1.0 N NaOH was added to 1.2 m*l* of the supernatant, and the absorbance was determined at 440 nm. Each assay was done at least in duplicate. One unit of the enzyme activity was defined as the amount of the enzyme that produced a A_{440} change of 1.0 in 30 min at 50°C. PMSF (Sigma) and other inhibitors were preincubated with the enzyme for 15 min at 37°C before the substrate was added.

Protein analysis. Protein concentration was determined using either the method of Lowry *et al.*[17] or that of Bradford.[18] The molecular mass of the enzyme was determined by SDS-PAGE on an 11.5% gel according to the method of Laemmli.[19] Low-molecular weight standard protein markers for SDS-PAGE were obtained from Bio-Rad. The native *B. subtilis* BSE616 endoglucanase was prepared from *B. megaterium* (pCK98) as described previously[20] and used as a substrate for the cleavage reaction of the cloned NprM. After incubation with the NprM, the reaction mixtures were then separated on a denaturating polyacrylamide gel, and the cleaved product was identified by activity staining with 1 mM MUC.[21]

Results and Discussion

Cloning of the nprM gene from *B. megaterium*. The entire *npr*M gene of *B. megaterium* ATCC 14945 was obtained by PCR using a primer set synthesized based on the two *npr*M gene sequence from *B. megaterium* ATCC 14581[9] and DSM319.[11] The first base of the forward primer corresponded to the 175th-bp upstream from the initiation codon, and the last base of the reverse primer corresponded to the 70th-bp downstream from the termination codon (Table 1). With this primer set, an 1.7-kb fragment was amplified from the chromosomal DNA of *B. megaterium* ATCC 14945. The fragment was inserted into pGEM-T Easy vector and transformed into *E. coli* DH5α. A transformant showing a clear zone around its margin was selected (Fig. 1),

Table 1. Primers used in the PCR amplification of the *npr*M gene from *B. megaterium* ATCC 14945.

Primer		Sequence
Entire gene primers	Forward primer (24-mer)	TTTAAATAGGTAAAAAAGACTTAT
	Reverse primer (30-mer)	ATTGGTAGTAAGGTAGTATAGAGAGTCGTA
Degenerate primers	Forward primer (17-mer)	GCAGGAGTAGACGCACA C G CG G CT G C C C C T T T T
	Reverse primer (21-mer)	AAACATCATCGCCGACAAGCC G TG C A A TTCC C T T T C

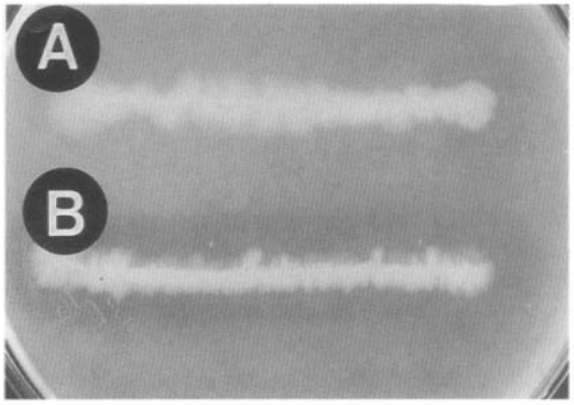

Fig. 1. Proteolytic activity of the cloned NprM. A, *E. coli* DH5 α (pUC19); B, *E. coli* DH5α (pBK49). The cells were grown on a Luria-Bertani agar medium containing 0.7% skim milk.

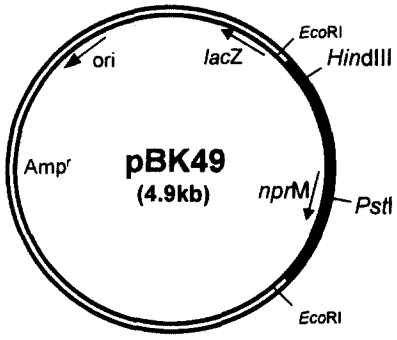

Fig. 2. Restriction map of the plasmid pBK49. The plasmid consisted of the pGEM-T Easy vector (open line) and the PCR-amplified fragment (filled line) from the chromosomal DNA of *B. megaterium* ATCC 14945.

and the recombinant plasmid was named pBK49 (Fig. 2). The restriction pattern of the insert DNA of pBK49 was found to be the same as the structural DNA of pSK44 which contains the entire *npr*M gene of *B. megaterium* ATCC 14581 (data not shown).[9]

An attempt to clone the *npr*M gene from *B. megaterium* ATCC 14945 by PCR using degenerate primers was unsuccessful. The degenerate primers were designed based on the nucleotide sequence of the most highly conserved region of the genes of extracellular neutral proteases from *Bacillus* species (Table 1). *Bacillus* species used in finding the conserved region were *B. megaterium* ATCC 14581,[9] and DSM319,[11] *B. amyloliquefaciens*,[22] *B. caldolyticus*,[23] *B. polymyxa*,[24] *B. stearothermophilus*,[25] and *B. subtilis*.[26] A PCR amplification of the chromosomal DNA from *B. megaterium* ATCC 14945 with the degenerate primers resulted in a 370-bp fragment along with minor fragments. When the amplified 370-bp fragment was used as a probe, the *npr*M gene could not be found from the *B. megaterium* ATCC 14945 gene library (data not shown).

Characteristics of the cloned NprM. The cloned protease produced by *E. coli* (pBK49) was purified and

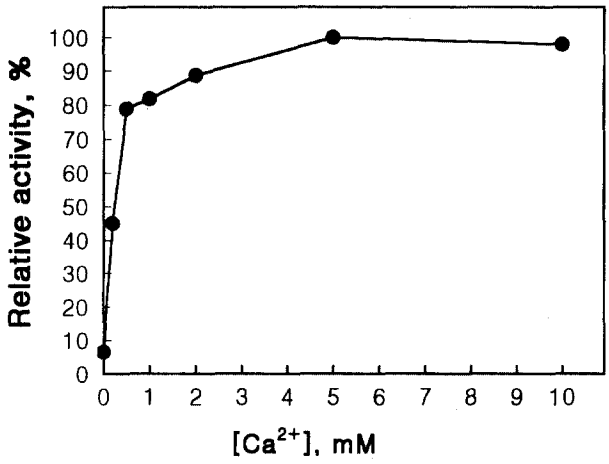

Fig. 3. Calcium activation of the cloned NprM.

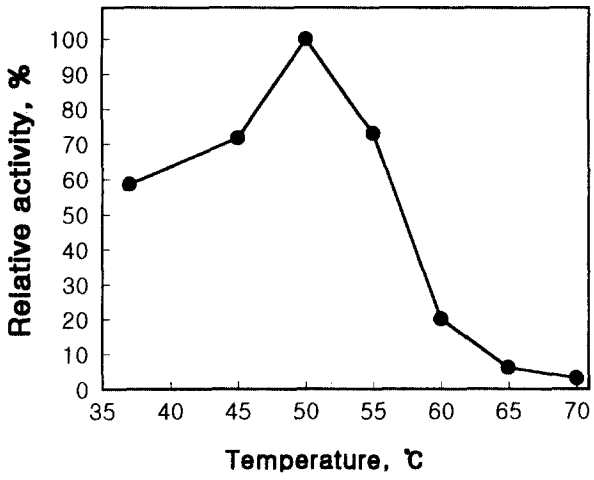

Fig. 4. Effect of temperature on the activity of the cloned NprM.

characterized to confirm the cloning of the neutral protease gene *npr*M. The cloned protease was purified by ammonium sulfate, High-S, and Sephadex G-75 chromatographies. The protease was bound in the cation exchange chromatography and was eluted with 0.1 M NaCl (data not shown). The purified protease displayed a major protein band corresponding to a molecular mass of approximately 36 kDa (data not shown). The molecular mass of the putative mature NprM from *B. megaterium* ATCC 14581 was estimated to be approximately 35 kDa based on its deduced amino acid sequence (317 amino acids).[9] The extracellular neutral protease from the gene donor *B. megaterium* ATCC 14945 was reported to have a molecular mass of 38 kDa.[6] The protease activity was dependent on calcium ion. Only negligible amount of the protease activity was observed in the absence of Ca^{2+} (Fig. 3). The protease activity was almost completely inhibited by 5 mM EDTA but not by 5 mM PMSF. The optimal pH and temperature for the protease activity were in the range of 7.5-8.0 and 50°C, respectively (Fig. 4). These characteristics of the cloned protease were

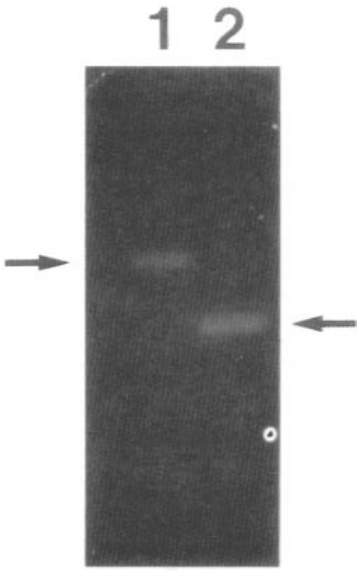

Fig. 5. Cleavage of the native endoglucanse of *B. subtilis* BSE616 by the cloned NprM. The native (lane 1) and truncated (lane 2) enzymes were separated on a denaturing gel and activity stained with 1 mM MUC. The arrows indicate the positions of the activity bands.

basically the same as those of the purified extracellular protease from the gene donor *B. megaterium* ATCC 14945[6] and those of the cloned *npr*M gene product of *B. megaterium* ATCC 14581.[9]

Cleavage reaction of NprM for the native endoglucanase from *B. subtilis* BSE616. It has previously been reported that the *B. subtilis* BSE616 endoglucanase was produced as smaller truncated forms in *E. coli* and *B. megaterium* transformants and that this cleavage was caused by the protease NprM.[5,6] In order to confirm the ability of the cloned NprM from *B. megaterium* ATCC 14945 to cleave a foreign protein, the native endoglucanase from *B. subtilis* BSE616 was incubated with the purified cloned NprM. After the reaction, the native endoglucanase was cleaved into a smaller truncated form (Fig. 5). The restriction pattern of the insert in plasmid pBK49, and the enzymatic characteristics and the cleavage reaction pattern of the protease strongly indicated that the cloned protease is the neutral protease NprM.

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